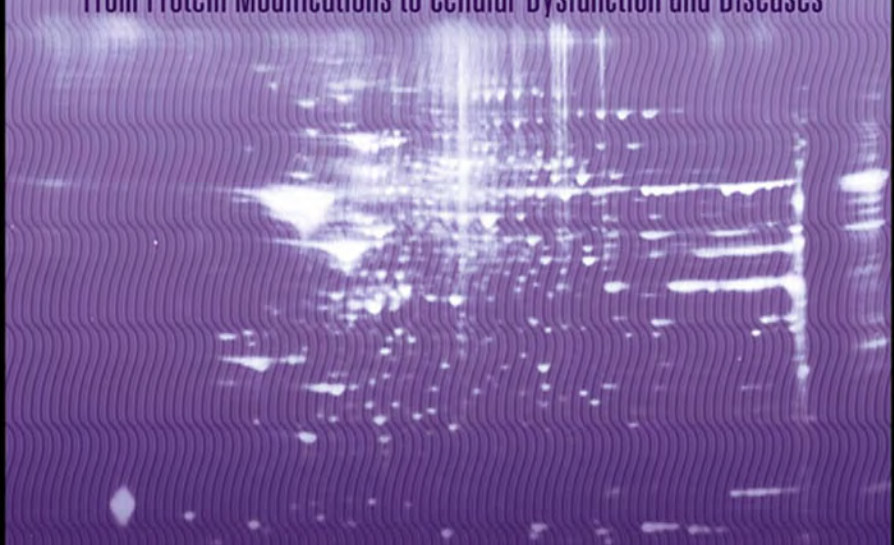
Wiley-Interscience Series on Mass Spectrometry
Dominic M. Desiderio and Nico M. Nibbering, Series Editors

WILEY

REDOX PROTEOMICS

From Protein Modifications to Cellular Dysfunction and Diseases



Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

REDOX PROTEOMICS

WILEY-INTERSCIENCE SERIES IN MASS SPECTROMETRY

Series Editors

Dominic M. Desiderio
Departments of Neurology and Biochemistry
University of Tennessee Health Science Center

Nico M. M. Nibbering
Vrije Universiteit Amsterdam, The Netherlands

John R. de Laeter • *Applications of Inorganic Mass Spectrometry*
Michael Kinter and Nicholas E. Sherman • *Protein Sequencing and Identification Using Tandem Mass Spectrometry*
Chhabil Dass, *Principles and Practice of Biological Mass Spectrometry*
Mike S. Lee • *LC/MS Applications in Drug Development*
Jerzy Silberring and Rolf Eckman • *Mass Spectrometry and Hyphenated Techniques in Neuropeptide Research*
J. Wayne Rabalais • *Principles and Applications of Ion Scattering Spectrometry: Surface Chemical and Structural Analysis*
Mahmoud Hamdan and Pier Giorgio Righetti • *Proteomics Today: Protein Assessment and Biomarkers Using Mass Spectrometry, 2D Electrophoresis, and Microarray Technology*
Igor A. Kaltashov and Stephen J. Eyles • *Mass Spectrometry in Biophysics: Confirmation and Dynamics of Biomolecules*
Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield • *Redox Proteomics: From Protein Modifications to Cellular Dysfunction and Diseases*

REDOX PROTEOMICS

From Protein Modifications to Cellular Dysfunction and Diseases

Edited by

ISABELLA DALLE-DONNE, ANDREA SCALONI, AND D. ALLAN BUTTERFIELD

A JOHN WILEY & SONS, INC., PUBLICATION

Published by John Wiley & Sons, Inc., Hoboken, New Jersey
Published simultaneously in Canada.

Library of Congress Cataloging-in-Publication Data:

Redox proteomics : from protein modifications to cellular dysfunction and diseases /
edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield.
p. ; cm.
Includes bibliographical references and index.
ISBN 0-471-72345-2 (cloth : alk. paper)
1. Proteomics. 2. Oxidation-reduction reaction. 3. Pathology, Cellular. 4. Proteins. 5. Genomics. I. Dalle-Donne, Isabella, 1964- II. Scaloni, Andrea. III. Butterfield, D. Allan.
[DNLM: 1. Proteomics–methods. 2. Cells–pathology. 3. Oxidation-Reduction. QU 58.5 R319 2006]
QP551 R2775 2006
612.3′98—dc22

2005057461

Printed in the United States of America

10 9 8 7 6 5 4 3 2 1

To Dr. Earl R. Stadtman of the National Institutes of Health, USA, for his pioneering research on oxidative modifications of proteins.

CONTENTS

PREFACE

The completion of the Human Genome Project has provided invaluable information for researchers and physicians on the fundamental nature of cells and, ultimately, human beings (Rogers, 2003). However, it is important to recognize that genes do not perform the work of cells; proteins do. Consequently, knowledge of protein identity and function is essential in order to better understand cellular physiology, both normal and aberrant. Proteomics provides the means to achieve these identifications.

In a number of disease states and in normal aging, oxidative modification of proteins occurs (Butterfield and Stadtman, 1997). While some biochemical pathways require free radical participation and transient oxidative modification of cellular components, including proteins, in aging and in some disease states, oxidative stress leads to a more long-lasting oxidative modification of proteins (Butterfield and Stadtman, 1997). Among others, the principal oxidative stress-mediated indexes of protein modification include protein carbonyls, nitration of tyrosine residues, addition of reactive alkenals such as 4-hydroxy-2-nonenal and glutathionylation (Dalle-Donne et al., 2005; Butterfield et al., 2005).

In general, oxidative modification of proteins leads to diminution, if not loss, of function (Butterfield and Stadtman, 1997; Butterfield et al., 2005). Hence, knowledge of the identity of oxidatively modified proteins would, in principle, provide insight into molecular mechanisms of cellular dysfunction present in various disease states. This is the realm of "redox proteomics," that branch of proteomics that identifies oxidatively modified proteins in order to gain insight into the effects of disease, metabolism, pharmacological agents, and aging, among other aspects of the cell.

This book, *Redox Proteomics*, is composed of a series of refereed chapters from world-renowned experts in oxidative stress and the applications of redox proteomics. In Part I of the book, experts describe processes involved in the oxidative modification of proteins and their consequences on cellular physiology. In addition, a background on proteomic technologies is provided. In Part II, chapters related to redox proteomics in normal cellular physiology and pharmacology are presented. In Part III, experts present applications of redox proteomics to disease states.

We believe that this comprehensive, refereed book, whose chapters also include dozens to hundreds of references, will provide researchers and clinicians with new insights into normal and altered physiology, into molecular mechanisms of disease states, and into a new application of the rapidly evolving techniques of proteomics.

Redox Proteomics, we believe, also will serve as a reference text for experts in cellular physiology and its alterations in disease states, as well as for experts in proteomics. In addition, it is our opinion that this book could serve as a basis for graduate-level coursework in proteomics and its applications.

The three co-editors have closely collaborated on this book. We greatly appreciate the generosity of time and expertise of those who have contributed chapters in this book. Expert referees from throughout the world also are heartily thanked by us for their skillful and insightful comments to each author on ways to improve their contributions. In our diligence to strive for uniformity of presentation of each chapter, we were assisted greatly by Ms. Heather Bergman of John Wiley & Sons, Inc. Last, we wish to express our thanks to professor Dominic Desiderio, co-editor of the Wiley book-series on mass spectrometry, for providing the opportunity to prepare this book and for his continual encouragement and helpful suggestions during the development of the book.

We commend to you this year-long effort to bring redox proteomics to the forefront of important techniques needed to gain new understanding into the molecular basis of cellular physiology and its alterations in disease states.

ISABELLA DALLE-DONNE, Ph.D.

Department of Biology
University of Milan
Milan, Italy

ANDREA SCALONI, Ph.D.

Proteomics and Mass Spectrometry Laboratory
ISPAAM National Research Council
Naples, Italy

D. ALLAN BUTTERFIELD, Ph.D.

Department of Chemistry, Center of Membrane Sciences,
and Sanders-Brown Center on Aging
University of Kentucky
Lexington

REFERENCES

Butterfield D.A., Stadtman E.R. (1997). Protein oxidation processes in aging brain. *Adv Cell Aging Gerontol* 2: 161–191.

Butterfield D.A., Boyd-Kimball D., Castegna A. (2003). Proteomics in Alzheimer's disease: Insights into mechanisms of neurodegeneration. *J Neurochem* 86: 1313–1327.

Dalle-Donne I., Scaloni A., Giustarini D., Cavarra E., Tell G., Lungarella G., Colombo R., Rossi R., Milzani A. (2005). Proteins as biomarkers of oxidative/nitrosative stress in diseases: The contribution of redox proteomics. *Mass Spectrom Rev* 24: 55–99.

Rogers J. (2003). The finished genome sequence of *Homo sapiens*. *Cold Spring Harbor Symp Quant Biol* 68: 1–11.

CONTRIBUTORS

Giancarlo Aldini, Istituto Chimico Farmaceutico Tossicologico, University of Milan, Milan, Italy

Mutay Aslan, Department of Biochemistry, Akdeniz University Medical School, Antalya, Turkey

Laura M. S. Baker, Departments of Anesthesiology, Biochemistry, and Molecular Genetics, and the Center for Free Radical Biology, Anesthesiology Research Division, The University of Alabama at Birmingham, Birmingham, AL 35294, USA

Elisa Basso, CNR, Istituto di Scienze e Tecnologie Molecolari, Padova, Italy

James W. Baty, Department of Pathology, Christchurch School of Medicine & Health Sciences, Christchurch, New Zealand

Valentina Bonetto, Assistant Telethon Scientist, Dulbecco Telethon Institute, Istituto di Ricerche Farmacologiche "Mario Negri", Milan, Italy

Peter F. Bove, Department of Pathology, College of Medicine, University of Vermont, Burlington, VT 05405, USA

Jonathan P. Brennan, Department of Cardiology, Cardiovascular Division, The Rayne Institute, St. Thomas' Hospital, King's College London, London, UK

D. Allan Butterfield, Department of Chemistry, Center of Membrane Sciences, and Sanders-Brown Center on Aging University of Kentucky, Lexington, KY 40506, USA

Elisa Cabiscol, Department de Ciències Mèdiques Bàsiques, Facultat de Medicina, Universitat de Lleida, Lleida, Spain

Marina Carini, Istituto Chimico Farmaceutico Tossicologico, University of Milan, Milan, Italy

Fabrizio Ceciliani, Department of Veterinary Pathology, Hygiene and Public Health, University of Milan, Milan, Italy

Karina Ckless, Department of Pathology, College of Medicine, University of Vermont, Burlington, VT 05405, USA

Richard A. Cohen, Vascular Biology Unit and Cardiovascular Proteomics Center, Boston University School of Medicine, Boston, MA 02118, USA

Roberto Colombo, Department of Biology, University of Milan, Milan, Italy

Catherine E. Costello, Cardiovascular Proteomics Center and Mass Spectrometry Resource, Boston University School of Medicine, Boston, MA 02118, USA

Isabella Dalle-Donne, Department of Biology, University of Milan, Milan, Italy

Victor M. Darley-Usmar, Center for Free Radical Biology and Department of Pathology, University of Alabama at Birmingham, AL 35294, USA

Christianne J. de Groot, Department of Obstetrics and Gynaecology, Erasmus University Medical Centre Rotterdam, The Netherlands

Dale A. Dickinson, Department of Environmental Health Sciences and Center for Free Radical Biology, University of Alabama at Birmingham, AL 35294, USA

Sonia Donzelli, Tumor Biology Section, Radiation Biology Branch, National Cancer Institute, NIH, Bethesda, MD 20892, USA

Philip Eaton, Department of Cardiology, Cardiovascular Division, The Rayne Institute, St. Thomas' Hospital, King's College London, London, UK

Michael Graham Espey, Tumor Biology Section, Radiation Biology Branch, National Cancer Institute, NIH, Bethesda, MD 20892, USA

Roberto Maffei Facino, Istituto Chimico Farmaceutico Tossicologico, University of Milan, Milan, Italy

Martin Feelisch, Whitaker Cardiovascular Institute, Boston University School of Medicine, 650 Albany Street, Boston, MA 02118, USA

Bruce A. Freeman, Department of Pharmacology, University of Pittsburgh School of Medicine, E1346 Biomedical Science Tower, Pittsburgh, PA 15261, USA

Pietro Ghezzi, Istituto di Ricerche Farmacologiche "Mario Negri", Milan, Italy

Daniela Giustarini, Department of Neuroscience, University of Siena, Siena, Italy

Anne-Katrin Greul, Department of Biological Chemistry and Nutrition, University of Hohenheim, Stuttgart, Germany

Tilman Grune, Research Institute of Environmental Medicine, Heinrich Heine University, Düsseldorf, Germany

Mark B. Hampton, Department of Pathology, Christchurch School of Medicine & Health Sciences, Christchurch, New Zealand

Tyler Heibeck, Vascular Biology Unit and Cardiovascular Proteomics Center, Boston University School of Medicine, Boston, MA 02118, USA

Jay W. Heinecke, Division of Metabolism, Endocrinology and Nutrition, University of Washington School of Medicine, Seattle, WA 98195, USA

Neil Hogg, Department of Biophysics, Medical College of Wisconsin, Milwaukee, WI 53226, USA

Milena Hristova, Department of Pathology, University of Vermont, Burlington, VT 05405, USA

Hua Huang, Cardiovascular Proteomics Center, Boston University School of Medicine, Boston, MA 02118, USA

Sequin Huang, Cardiovascular Proteomics Center, Boston University School of Medicine, Boston, MA 02118, USA

Jeffery S. Isenberg, Biochemical Pathology Section, Laboratory of Pathology, National Cancer Institute, NIH, Bethesda, MD 20892, USA

Yvonne M. Janssen-Heininger, Department of Pathology, University of Vermont, Burlington, VT 05405, USA

Aimee Landar, Center for Free Radical Biology and Department of Pathology, University of Alabama at Birmingham, AL 35294, USA

Annunziata Lapolla, Dipartimento di Scienze Mediche e Chirurgiche, Cattedra di Malattie del Metabolismo, Università di Padova, Padova, Italy

Rodney L. Levine, Laboratory of Biochemistry, National Heart, Lung and Blood Institute, Bethesda, MD, USA

Bing-Wen Lu, Department of Computational and Molecular Biology, University of Southern California, Los Angeles, CA 90089, USA

Daniele Mancardi, Tumor Biology Section, Radiation Biology Branch, National Cancer Institute, NIH, Bethesda, MD 20892, USA

Mark E. McComb, Cardiovascular Proteomics Center, Boston University School of Medicine, Boston, MA 02118, USA

Aldo Milzani, Department of Biology, University of Milan, Milan, Italy

Toshio Miyata, Tokai University School of Medicine, Kanagawa, Japan

Tatiana Nikolskaya, Genego, Inc., St. Joseph, MI 49085, USA

Yuzi Nikolesky, Chemical Diversity Labs., Inc., San Diego, CA 92121, USA

Toshimitsu Niwa, Nagoya University Hospital, Department of Clinical Preventive Medicine, Nagoya, Japan

Thomas Nyström, Department of Cell and Molecular Biology—Microbiology, Göteborg University, Göteborg, Sweden

Wilbert H. M. Peters, Department of Gastroenterology, University Medical Centre Nijmegen, The Netherlands

H. Fai Poon, Department of Chemistry, University of Kentucky, Lexington, KY 40506, USA

Maarten T. M. Raijmakers, Department of Clinical Chemistry, Maxima Medisch Centrum, Veldhoven, The Netherlands

Niki L. Reynaert, Department of Pathology, University of Vermont, Burlington, VT 05405, USA

Lisa Ridnour, Tumor Biology Section, Radiation Biology Branch, National Cancer Institute, NIH, Bethesda, MD 20892, USA

David D. Roberts, Biochemical Pathology Section, Laboratory of Pathology, National Cancer Institute, NIH, Bethesda, MD 20892, USA

Joaquim Ros, Department de Ciències Mèdiques Bàsiques, Facultat de Medicina, Universitat de Lleida, Lleida, Spain

Ranieri Rossi, Department of Neuroscience, University of Siena, Siena, Italy

Andrea Scaloni, Proteomics and Mass Spectrometry Laboratory, ISPAAM, National Research Council, Naples, Italy

Mahadevan Sethuraman, Vascular Biology Unit and Cardiovascular Proteomics Center, Boston University School of Medicine, Boston, MA 02118, USA

Earl R. Stadtman, Laboratory of Biochemistry, National Heart, Lung and Blood Institute, Bethesda, MD, USA

Eric A. P. Steegers, Department of Obstetrics and Gynaecology, Erasmus University Medical Centre Rotterdam, The Netherlands

Rukhsana Sultana, Department of Chemistry, University of Kentucky, Lexington, KY 40506, USA

Gianluca Tell, Department of Biomedical Sciences and Technologies, University of Udine, Italy

Douglas D. Thomas, Tumor Biology Section, Radiation Biology Branch, National Cancer Institute, NIH, Bethesda, MD 20892, USA

Stefani N. Thomas, Department of Pharmaceutical Sciences, University of Southern California, Los Angeles, CA 90089, USA

Paul J. Thornalley, Department of Biological Sciences, University of Essex, Colchester, Essex C04 3SQ, UK

Pietro Traldi, CNR, Istituto di Scienze e Tecnologie Molecolari, Padova, Italy

Dimitrios Tsikas, Institute of Clinical Pharmacology, Hannover Medical School, Hannover, Germany

Tomas Vaisar, Division of Metabolism, Endocrinology and Nutrition, University of Washington School of Medicine, Seattle, WA 98195, USA

Albert van der Vliet, Department of Pathology, College of Medicine, University of Vermont, Burlington, VT 05405, USA

Peter Voss, Research Institute of Environmental Medicine, Heinrich Heine University, Düsseldorf, Germany

David A. Wink, Tumor Biology Section, Radiation Biology Branch, National Cancer Institute, NIH, Bethesda, MD 20892, USA

Christine C. Winterbourn, Department of Pathology, Christchurch School of Medicine & Health Sciences, Christchurch, New Zealand

Austin J. Yang, Department of Pharmaceutical Sciences, University of Southern California, Los Angeles, CA 90089, USA

Yanhong Zhang, Department of Biophysics, Medical College of Wisconsin, Milwaukee, WI 53226, USA

PART I

OXIDATIVELY MODIFIED PROTEINS AND PROTEOMIC TECHNOLOGIES

1

CHEMICAL MODIFICATION OF PROTEINS BY REACTIVE OXYGEN SPECIES

EARL R. STADTMAN AND RODNEY L. LEVINE

1.1 INTRODUCTION

Proteins are highly sensitive to oxidative modifications by reactive oxygen species (ROS) and reactive nitrogen species (RNS). These include a number of primary radical species ($^{\bullet}OH$, $O_2^{\bullet-}$, $CO_2^{\bullet-}$, $NO^{\bullet}$), several nonradical species (H_2O_2, HOCl, O^3, ONO_2^-, $ONOCO_2^-$, CO, N_2O_2, NO_2, $^1O_2$), and also free radicals ($^{\bullet}C$, $RS^{\bullet}$, $RSO^{\bullet}$, $RSOO^{\bullet}$, $RSSR^{\bullet-}$, $R^{\bullet}$, $RO^{\bullet}$, $ROO^{\bullet}$) produced in secondary reactions of these reactive oxygen species with proteins, lipids, and nucleic acids. In addition native proteins can be modified by highly reactive aldehydes and ketones produced during ROS-mediated oxidation of lipids (Schuenstein and Esterbauer, 1979; Esterbauer et al., 1991; Uchida and Stadtman, 1993) and glycated proteins (Monnier, 1990; Monnier et al., 1995). (For reviews, see Baynes, 1991; Kristal and Yu, 1992.) Basic chemical mechanisms involved in free radical-mediated oxidation of proteins were elucidated by pioneering studies of Swallow (1960), Garrison et al. (1962), Garrison (1987), and Schuessler and Schilling (1984), and Kopoldova and Liebsier (1963), who exposed aqueous solutions of proteins to ionizing radiation (X rays, gamma rays) under conditions where only $^{\bullet}OH$ and/or $O_2^{\bullet-}$ was formed. They demonstrated that these conditions lead to oxidation of amino acid residue side chains, fragmentation of the polypeptide chain, and the formation of protein–protein cross-linked aggregates. Although most proteins are not normally subjected to ionizing

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield
Published 2006 by John Wiley & Sons, Inc.

radiation, basic principles established in these pioneering studies apply also under more physiological situations where metal-ion-catalyzed reactions mimic the effects of ionizing radiation (Garrison et al., 1970; Borg and Schaich, 1988). (For reviews, see Stadtman and Berlett, 1997; Stadtman, 1998a; Butterfield and Stadtman, 1997.)

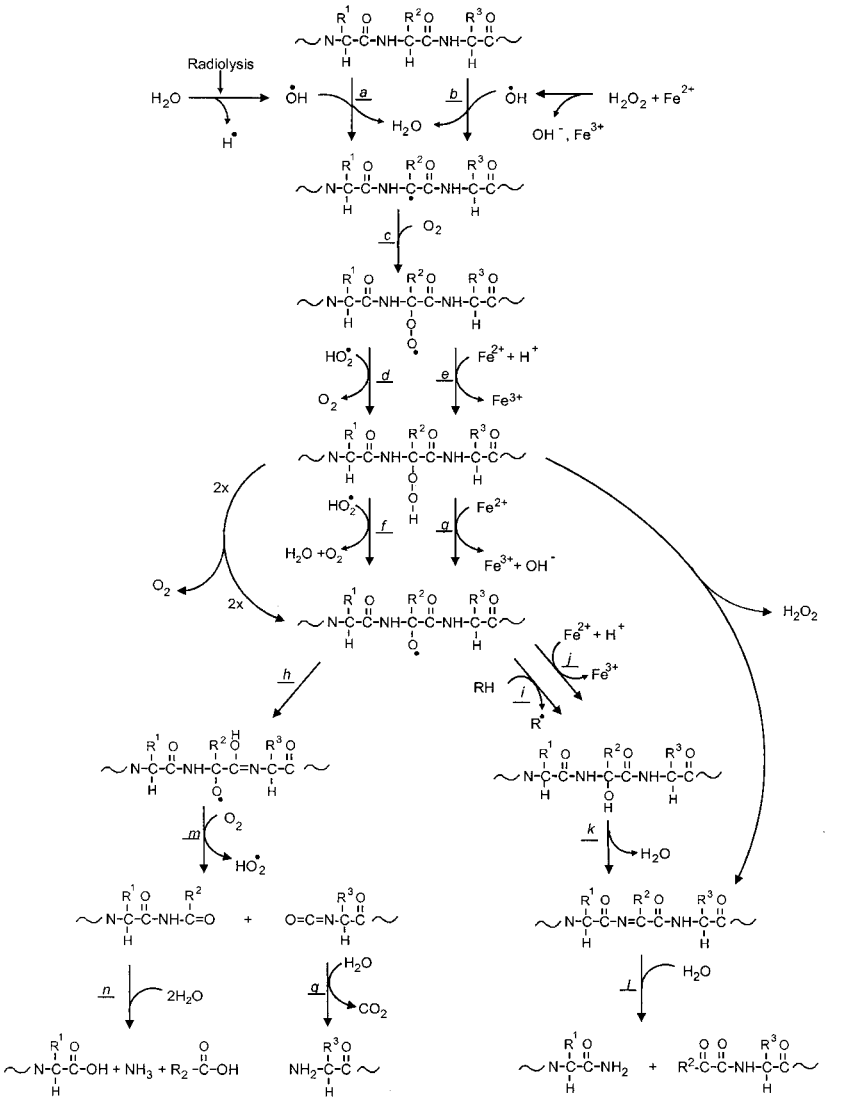


FIGURE 1.1 Role of reactive oxygen species in oxidation and cleavage of the protein backbone.

1.2 PEPTIDE BOND CLEAVAGE

Hydroxyl radicals (•OH) formed during exposure to ionizing radiation, or by ferrous ion-catalyzed cleavage of H_2O_2, are able to abstract the α-hydrogen atom of any amino acid residue of a protein, leading to formation of a carbon-centered radical (Fig. 1.1, reaction *a* or *b*). These carbon-centered radicals undergo rapid addition of O_2 to form peroxy radical derivatives (Fig. 1.1, reaction *c*) that are readily converted to the peroxide and subsequently to the alkoxyl derivatives by reaction with either $HO_2^{\bullet}$ or Fe^{2+} (Fig. 1.1, reactions *d*, *e* and *f*, *g*). This sets the stage for peptide bond cleavage by either of two different pathways, namely by the *diamide and* α*-amidation pathways* (Garrison, 1987). In the *diamide* pathway (Fig. 1.1, reactions *h*, *m*), the *C*-terminal amino acid of the peptide fragment derived from the *N*-terminal portion of the protein is present as a diamide derivative, and the *N*-terminal amino acid of the fragment derived from the *C*-terminal portion of the protein is present as an isocyanate derivative. In the α*-amidation* pathway (Fig. 1.1, reactions *j*, *i*, *k*), the *C*-terminal amino acid residue of the fragment derived from the *N*-terminal portion of the protein exists as an amide, and the *N*-terminal amino acid of the fragment derived from the *C*-terminal portion of the protein exists as an α-ketoacyl derivative. Significantly, cleavage by the α*-amidation* pathway provides a mechanism for the introduction of a carbonyl group into a peptide.

In addition to the peptide bond cleavage by the pathways illustrated in Figure 1.1, cleavage can occur also as a consequence of the •OH-dependent

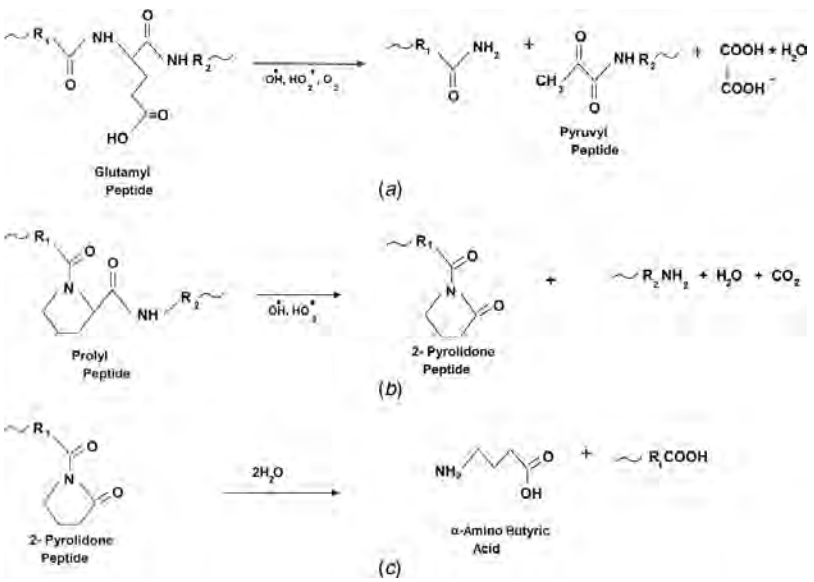


FIGURE 1.2 Cleavage of the protein backbone by oxidation of proline and glutamic acid side chains.

abstraction of a hydrogen atom from the side chain of glutamyl residues (Garrison, 1987) and also of prolyl residues (Uchida et al., 1990) of proteins according to the overall reactions *a* and *b*, respectively (Fig. 1.2).

Moreover, upon acid hydrolysis, the 2-pyrrolidone formed in reaction b is converted to 4-aminobutyric acid (reaction *c*, Fig. 1.2). The presence of 4-aminobutyric acid in acid hydrolysates of proteins is therefore presumptive evidence for peptide cleavage by the 2-pyrrolidone pathway.

1.3 β-SCISSION

In addition to the reactions illustrated in Figures 1.1 and 1.2, it was established that exposure of proteins to ionizing radiation leads to β-scission of amino acid

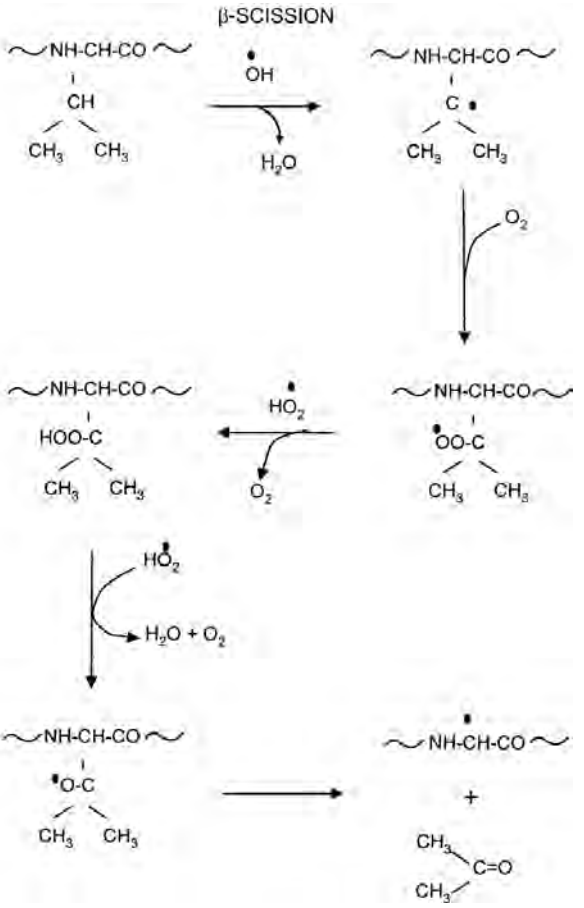


FIGURE 1.3 Beta-scission of protein amino acid side chains.

side chains (Dean et al., 1997; Headlam et al., 2000; Headlam and Davies, 2002). Thus β-scission of alanine, valine, leucine, and aspartic acid protein side chains leads to the formation of low molecular carbonyl compounds, including free formaldehyde, acetone, isobutyraldehyde, and glyoxylic acid, respectively, and in each case the side chain cleavage leads to the generation of a carbon-centered radical (~NH•CHCO~) in the polypeptide chain, as occurs when glycine residues undergo •OH-dependent α-hydrogen abstraction. The mechanism involved in all of these reactions is like that observed for the β-scission of a valine residue that, as shown in Figure 1.3, leads to the formation of acetone (Headlam and Davies, 2002).

1.4 OXIDATION OF AMINO ACID RESIDUE SIDE CHAINS

1.4.1 Oxidation of Aromatic and Heterocyclic Amino Acid Residues

The aromatic amino acids are very susceptible to oxidation by various forms of ROS (reviewed by Stadtman, 1998b; Davies et al., 1999; Dean et al., 1997). As is illustrated in Table 1.1, oxidation of phenylalanine residues yields 2-, 3-, and 4-mono-hydroxy derivatives and the 2,3-dihydroxy derivative. Hydroxy radical-mediated oxidation of tyrosine residues gives rise to dityrosine (2,2′-biphenyl-derivatives) DOPA. Reactions with RNS lead to formation of 3-nitrotyrosine, and reactions with HOCl lead to the generation of 3-chlorotyrosine and 3,5-dichlorotyrosine derivatives. Oxidation of tryptophan residues leads to *N*-formyl-kynurenine, 3-hydroxy-kynurenine, kynurenine, and 2-, 4-, 5-, 6-, or 7-hydroxy-tryptophans. Histidine residues of proteins are major targets of oxidation by metal-catalyzed oxidation systems (Creeth et al., 1983). Upon oxidation, histidine residues are converted to 2-oxo-histidine, 4-hydroxy glutamate, asparagine, and aspartate. As noted in Table 1.1, proline residues are oxidized to glutamic semialdehye, pyroglutamic acid, 2-pyrrolidone, and 4- and 5-hydroxy derivatives, and lysine residues are oxidized to α-aminoadiphylsemialdehyde and N^{ε}-(carboxymethyl)lysine derivatives.

1.4.2 Methionine Oxidation

Methionine (Met) residues of proteins are readily oxidized to methionine sulfoxide (MetO) by many different forms of ROS/RNS (Brot and Weissbach, 1983; Pryor et al., 1994; Pryor and Uppu, 1993; Vogt, 1995), as illustrated by reaction (1) below in which H_2O_2 represents the ROS. However, in contrast to the oxidation of other amino acid residues (except cysteine residues), the oxidation of Met residues to MetO is reversible. Most organisms contain MetO reductases (Msr's) that catalyze the thioredoxin [$Th(SH)_2$]-dependent reduction of MetO back to methionine (reaction 2). Moreover cells contain thioredoxin reductases that catalyze the NADPH-dependent reduction of oxidized thioredoxin [Th(SS)] back to $Th(SH)_2$ (reaction 3). Accordingly the coupling of reactions (1), (2), and (3) is described by reaction (4), and coupling provides a biological mechanism

TABLE 1.1 Oxidation of Amino Acid Residue Side Chains

Amino Acid	Products	References
Arginine	Glutamic semialdehyde	Amici et al., 1989
Cysteine	CyS-SCy; CyS-SG; CySOH; CySOOH; $CySO_2H$	Garrison, 1987; Swallow, 1960; Brodie and Reed, 1990; Takahashi and Goto, 1990; Zhou and Gafni, 1991
Glutamic acid	Oxalic acid; pyruvate adducts	Garrison, 1987
Histidine	2-Oxohistidine; 4-OH-glutamate; aspartic acid; asparagine	Garrison, 1987; Kopoldova and Liebsier, 1963; Uchida and Kawakishi, 1993
Leucine	3-OH-leucine; 4-OH leucine; 5-OH-leucine	Garrison, 1987
Lysine	α-aminoadipylsemialdehyde; N^{ε}-(carboxymethyl)lysine	Amici et al., 1989; Reddy et al., 1995; Daneshvar et al., 1997; Requena et al., 2001; Pietzsch and Bergmann, 2004
Methionine	Methionine sulfoxide; methionine sulfone	Garrison et al., 1962; Pryor et al., 1994; Vogt, 1995; Berlett and Stadtman, 1996; Berlett et al., 1996, 1998
Phenylalanine	2-, 3-, and 4-Hydroxyphenylalanine; 2,3-dihydroxyphenylalanine	Fletcher and Okada, 1961; Davies et al., 1987; Solar, 1985; Maskos et al., 1992a, b; Beckman et al., 1992; Gieseg et al., 1993
Proline	Glutamylsemialdehyde; 2-pyrrolidone, 4- and 5-OH-proline; pyroglutamic acid	Creeth et al., 1983; Poston, 1988; Amici et al., 1989; Uchida et al., 1990; Kato et al., 1992
Tryptophan	2-, 4-, 5-, 6-, 7-Hydroxytryptophan; formylkynurenine; 3-OH-kynurenine; nitrotryptophan	Armstrong and Swallow, 1969; Winchester and Lynn, 1970; Maskos et al., 1992a; Guptasarma et al., 1992; Pryor and Uppu, 1993; Kikugawa et al., 1994
Tyrosine	3,4-Dihydroxyphenylalanine; tyr-tyr cross-links; 3-nitrotyrosine; 3-chlorotyrosine; 3,5-dichlorotyrosine	Fletcher and Okada, 1961; Maskos et al., 1992a; Beckman et al., 1992; Giulivi and Davies, 1993; Heinecke et al., 1993; Dean et al., 1993; Huggins et al., 1993, van der Vliet et al., 1995; Ischiropoulos and Al-Medi, 1995; Domigan et al., 1995; Kettle, 1996; Berlett et al., 1996, 1998; Berlett and Stadtman, 1996; Fu et al., 2000; Buss et al., 2003

for the scavenging of H_2O_2 and other forms of ROS/RNS when they replace H_2O_2 in reaction (1).

$$\text{Met} + H_2O_2 \rightarrow \text{MetO} + H_2O \quad (1)$$

$$\text{MetO} + \text{Th(SH)}_2 \rightarrow \text{Met} + \text{Th(SS)} + H_2O \quad (2)$$

$$\text{NADPH} + H^+ + \text{Th(SS)} \rightarrow \text{NADP}^+ + \text{Th(SH)}_2 \quad (3)$$

$$\text{Sum:} \quad \text{NADPH} + H^+ + H_2O_2 \rightarrow \text{NADP}^+ + 2H_2O \quad (4)$$

Significantly the oxidation of methionine leads to a mixture of the R- and S-isomers of methionine sulfoxide (Schoneich et al., 1993). One of these enzymes (referred to as MsrA) contains a cysteine residue at the catalytic site and is stereo-specific for reduction of the S-isomer of MetO, whereas in most organisms, including mammals, the other reductase (MsrB) contains a selenocysteine at the catalytic site and is specific for reduction of the R-isomer of MetO (Krukov et al., 2002; Moskovitz et al., 1996, 2000, 2001, 2002; Kumar et al., 2002). Substitution of serine for cysteine in MsrA leads to loss of activity (Moskovitz et al., 2000), and substitution of cysteine for the selenocysteine residue in MsrB leads to a considerable decrease in its activity (Bar-Noy and Moskovitz, 2002).

Based on the consideration that cyclic oxidation-reduction of Met residues of proteins leads to consumption of ROS (reaction 4), it was proposed that this process may serve an important antioxidant function to protect cells from oxidative damage (Levine et al., 1996). This concept is supported by results of studies showing that mutations leading to a decrease in Msr activities in bacteria, yeast, and mice are associated with decreases in the resistance to oxidative stress and to elevations in the levels of oxidized proteins (Moskovitz et al., 1995, 1997, 1998, 2001; St. John et al., 2001), whereas overexpression of Msr in yeast, bacteria, and *Drosophila* leads to increases in their resistance to oxidative stress (Moskovitz et al., 1995, 1997, 1998, 2001; St. John et al., 2001; Ruan et al., 2002). Interestingly mutations in mice leading to a loss in the MsrA level lead also to a 40% decrease in the maximal life span (Moskovitz et al., 2001), and overexpression of MsrA in *Drosophila* leads to a nearly twofold increase in the maximal life span (Ruan et al., 2002). Oxidation of cysteine residues is the subject of another chapter in this book and will not be discussed here.

1.4.3 Protein Carbonylation

Whereas all amino acid residues of proteins are subject to oxidation by ROS (Dean et al., 1997; Davies et al., 1999), radiation-induced oxidation (Garrison et al., 1962) and metal ion-catalyzed oxidation (Levine, 1983; Levine et al., 1994) of some residues lead to the formation of protein carbonyl derivatives. As noted above, oxidation of glutamyl residues by the α-amidation pathway leads to peptide bond cleavage and formation of a peptide that is *N*-acylated by a pyruvyl group (Fig. 1.1, reaction *l*). Moreover metal-catalyzed oxidation of proline and arginine residues of proteins leads to formation of

glutamic semialdehyde derivatives of the protein, and oxidation of lysine residues leads to the formation of adipic semialdehyde derivatives (Oliver et al., 1984, 1985; Amici et al., 1989; Daneshvar et al., 1997; Requena et al., 2001). Threonine residues are oxidized to 2-amino-3-keto-butyric acid derivatives (Taborsky, 1973). In view of the fact that the formation of protein carbonyl groups is orders of magnitude greater than other oxidative modifications, the level of protein carbonyl groups has become the most widely used marker of protein oxidation during oxidative stress, aging, and diseases. (For reviews, see Stadtman, 1988, 1998b; Stadtman and Berlett, 1997; Butterfield and Stadtman, 1997; Dean et al., 1997, 1999; Levine and Stadtman, 2001.)

1.4.4 Protein–Protein Cross-Linkage

ROS-mediated oxidation reactions can lead to formation of protein-protein cross-linkages by several different mechanisms, illustrated in Figure 1.4. (1) Reaction of a carbonyl group in one protein with the N^{ε}-amino group of a lysine residue in another protein leads to formation of a Schiff-base cross-link (Fig. 1.4, reaction *a*). (2) Oxidation of cysteine residues in two different proteins can lead to formation of inter-molecular cross-linked disulfide derivatives (Swallow, 1960; Garrison, 1987) (Fig. 1.4, reaction *b*). (3) Interaction of carbon-centered radicals in two different proteins will lead to carbon–carbon cross-linked derivatives (Garrison, 1987) (Fig. 1.4, reaction *c*). (4) Michael addition of either a histidine, lysine, or cysteine residue of one protein with an α-β-unsaturated aldehyde, such as 4-hydroxynonenal (HNE), formed during the oxidation of poly-unsaturated fatty acids, gives rise to an active aldehyde derivative that can interact with the N^{ε}-lysine amino group of another protein to form a Schiff-base cross-linked derivative (Schuenstein and Esterbauer, 1979; Uchida and Stadtman, 1993; Friguet et al., 1994a) (Fig. 1.4, reaction *d*). (5) Protein–protein cross linkages are also formed by interaction of N^{ε}-lysine residues of two different proteins with the carbonyl groups of malondialdehyde generated in the oxidation of polyunsaturated fatty acids (Burcham and Kuhan, 1996) (Fig. 1.4, reaction *e*). (6) Cross-linkages are also formed by combination of two tyrosine residues of proteins following one electron oxidation of the aromatic rings (Heinecke et al., 1993; Huggins et al., 1993; Giulivi and Davies, 1993; Dean et al., 1993). Cross-links are also formed by interactions of lysine or arginine residues with Schiff-base or Amadori products generated during glycation-glycoxidation of proteins (Monnier, 1990; Wells-Knecht et al., 1993; Cerami et al., 1987) (Fig. 1.5, reactions *a* and *b*). For reactions described in Figures 1.4 and 1.5, P^1 and P^2 refer to different proteins leading to intermolecular cross-links; however, similar reactions can occur within the same protein to form intramolecular cross-links.

1.4.5 Protein Modification by Reactive Nitrogen Species

Nitric oxide ($NO^{\bullet}$), produced in the metabolism of arginine, plays an important role in a number of cellular processes, including smooth muscle relaxation, neurotransmission, autoimmune inflammatory states, and demyelination (reviewed

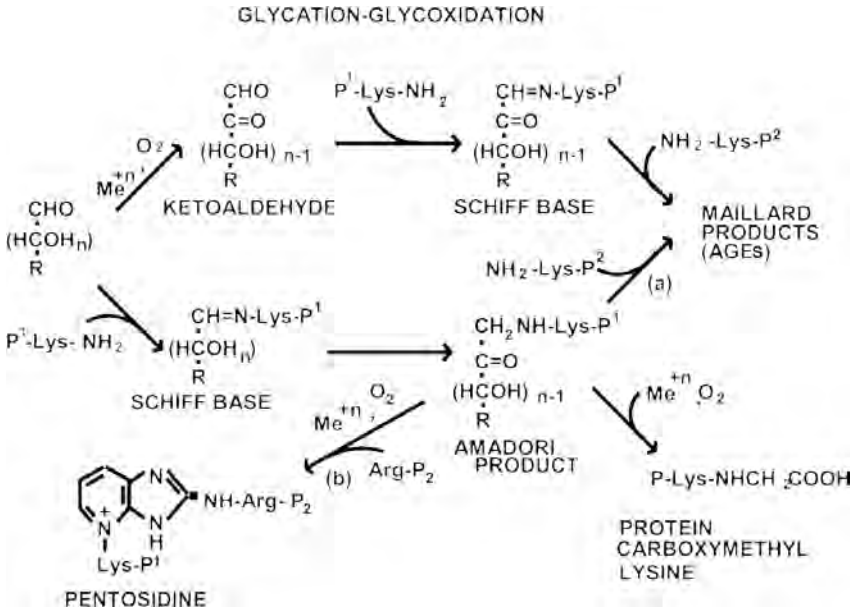


FIGURE 1.4 Glycation/glycoxidation-mediated generation of protein-protein cross-linked derivatives. P^1-LysNH$_2$ and P^2-Lys-NH$_2$ refer to epsilon amino groups of two different proteins (P^1 and P^2).

by Weinberg et al., 1998; Smith et al., 1999). However, NO$^\bullet$ reacts rapidly with $O_2^{\bullet -}$ to form the highly reactive peroxynitrite (PN) that is able to nitrate tyrosine residues (Beckman et al., 1992; Ischiropoulos et al., 1992; Ischiropoulos and Al-Medi, 1995) and to oxidize methionine residues (Pryor et al., 1994; Pryor and Squadrito, 1995; Berlett et al., 1998) and cysteine residues (Gatti et al., 1994) of proteins. The ability of PN to nitrate tyrosine residues and oxidize methionine residues is strongly affected by the presence of CO_2. Carbon dioxide stimulates the PN-dependent nitration of tyrosine residues (Lymar and Hurst, 1995; Denicola et al., 1996; Uppu et al., 1996) but inhibits the PN-dependent oxidation of methionine residues (Berlett and Stadtman, 1996; Denicola et al., 1996; Tien et al., 1999).

1.4.6 Chlorination Reactions

Activation of neutrophils leads to the release of myeloperoxidase and generation of hydrogen peroxide, which, in the presence of chloride ions, facilitates the generation of hypochlorous acid (reaction 5):

$$H_2O_2 + Cl^- \rightarrow HOCl + OH^- \qquad (5)$$

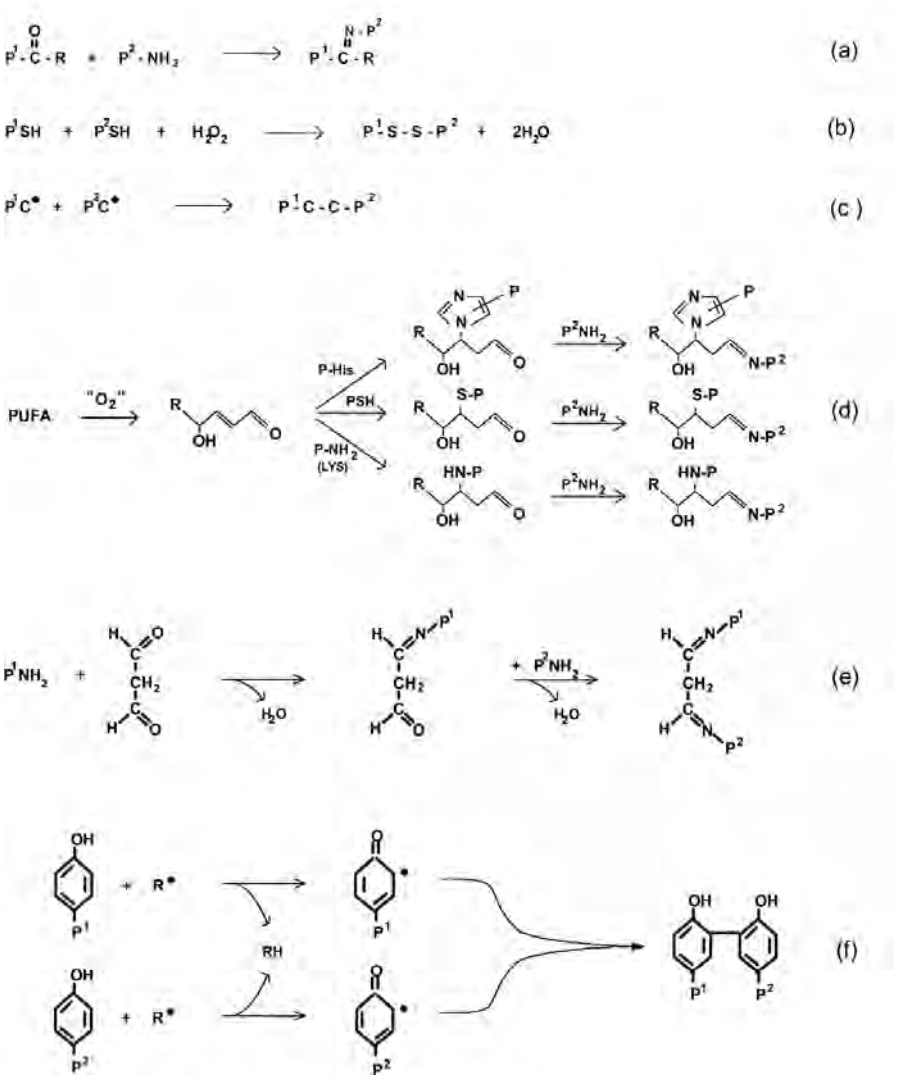


FIGURE 1.5 Generation of protein-protein cross-linkages. Reactions *a*, *b*, *c*, *d*, *e*, and refer to formation of cross-linked derivatives as described in the test. P^1 and P^2 refer to two different proteins. PUFA, polyunsaturated fatty acids.

Hypochlorous acid is a highly reactive compound that undergoes reactions with sulfur-containing amino acid residues (Folkes et al., 1995; Peskin and Winterbourn, 2001; Armesto et al., 2000). Reaction with cysteine residues leads to formation of –SCl derivatives, which may react with other cysteine residues to

form disulfides or undergo further reactions to form sulfenic, sulfinic, and sulfonic acid derivatives. The reaction with methionine residues leads to formation of methionine sulfoxide. Hypochlorous acid reacts with superoxide anion to form $^{\bullet}OH$ (reaction 6) (Folkes et al., 1995; Candias et al., 1993), and with *N*-terminal amino acid residues and the epsilon amino group of lysine residues to form the chloramine derivatives (Hawkins and Davies, 1998, 1999; Hawkins et al., 2002; Chapman et al., 2003) that upon decomposition can give rise to nitrogen-centered radicals, protein aggregation, and protein fragmentation by as yet ill-defined mechanisms. Interestingly treatment of cultured human Burkett's lymphoma cells with HOCl induces apoptosis by a choloramine-mediated pathway, whereas exposure to high concentrations of H_2O_2 leads to death by pyknosis/necrosis (Englert and Shacter, 2002):

$$HOCl + O^{\bullet -} \rightarrow O_2 + Cl^- + {}^{\bullet}OH \tag{6}$$

Furthermore HOCl reacts with tyrosine residues of proteins to form 3-chlorotyrosine (Kettle, 1996; Domigan et al., 1995; Buss et al., 2003) and also 3,5-dichlorotyrosine (Fu et al., 2000). In the latter study it was shown that oxidation of free tyrosine by HOCl gives rise to 3-chloro- and 3,5-dichloro-4-hydroxyphenylaldehydes. Other studies by Eiserich et al. (1996) showed that reaction of nitrite with HOCl yields reactive oxygen intermediates that can nitrate and chlorinate tyrosine residues.

1.4.7 Accumulation of Oxidized Proteins

It is well established that the level of oxidized proteins increases with animal age and in the development of a number of diseases (for reviews, see Oliver et al., 1984; Takahashi and Goto, 1990; Levine and Stadtman, 1992; Stadtman, 1992, 1998b, 2002; Agarwal and Sohal, 1994; Butterfield and Stadtman, 1997; Stadtman and Berlett, 1997, 1998; Smith et al., 1999; Halliwell and Gutteridge, 1999; Levine, 2002). However, because the cellular levels of oxidized proteins are dependent on many variables, the mechanisms responsible for accumulation of oxidatively modified proteins under one condition may be very different from those in another condition. Thus ROS and RNS generation varies, depending on the kind and length of exposure to a multiplicity of oxidative stress conditions, including irradiation by X, gamma, or UV rays, inflammation initiated by activation of neutrophils and/or macrophages, alteration of regulatory pathways involved in the conversion of arginine to nitric oxide, auto-oxidation of electron transport carriers, variations in the levels of toxic atmospheric pollutants, activation of oxidases, and mobilization of metal ions that are involved in metal-catalyzed oxidation processes. However, the cellular levels of these ROS/RNS are subject to control by a multiplicity of antioxidant enzymes/proteins (superoxide dismutases, catalases, peroxidases, glutathione transferases, Msr's, glutaredoxin

reductase, thioredoxin/thioredoxin reductase, ceruloplasmin, ferritins), metabolites (bilirubin, uric acid, NADPH/NADP, GSH/GSSG), and vitamins (vitamins C, E, and A) that are able, directly or indirectly, to neutralize their deleterious effects. Moreover oxidation of proteins predisposes them to proteolytic degradation (Oliver et al., 1980, 1982; Levine et al., 1981; Rivett, 1985a, b, c; Davies, 1986; Wolf et al., 1986; Davies and Goldberg, 1987; Davies et al., 1987; Davies and Delsignore, 1987; Roseman and Levine, 1987; Grune et al., 1995, 1996, 1997). (For reviews, see Davies, 1988a, b; Rivett, 1986; Rivett et al., 1985; Davies et al., 1990; Pacifici and Davies, 1990; Dean et al., 1994; Szweda et al., 2002; Shringarpure and Davies, 2002; Dunlop et al., 2002; Drake et al., 2002.) Accordingly the cellular levels of oxidized proteins are dependent on several protease (lysosomal, cathepsin, 20S, and 26S proteasome) activities. In view of the multitude of factors that govern the generation of ROS/RNS and the antioxidant systems that neutralize their effects, and also the fact that oxidized proteins are targets for proteolytic degradation, it is obvious that the intracellular accumulation of oxidized proteins reflects the balance between all of these processes. Therefore the accumulation of oxidized proteins as occurs during aging and in some diseases may reflect an increase in the rates of ROS/RNS formation, a loss in antioxidant capacity, or a decrease in the ability of proteases to degrade oxidized proteins. With regard to the latter it has been shown that the age-dependent increase in the level of oxidized protein is associated with an age-dependent loss of the 20S proteasome, which represents a major enzyme for the degradation of oxidized proteins (Starke-Reed and Oliver, 1989; Berlett and Stadtman, 1997; Carney et al., 1991; Petropoulos et al., 2000; Davies, 2001; Grune et al., 2001; Bulteau et al., 2002; Dunlop et al., 2002; Shringarpure and Davies, 2002; Szweda et al., 2002). Some workers failed to observe an age-related decline in the level of the alkaline proteasome but did observe an age-associated decline in the lysosomal proteolytic system (Vittorini et al., 1999). Results of recent studies have shown that whereas mild oxidation of some proteins facilitates their degradation by the 20S proteasome, prolonged oxidation converts them to forms that are not only resistant to degradation but are able to inhibit the ability of the proteasome to degrade other oxidized proteins (Rivett, 1986; Sitte et al., 2000; Davies, 2001). Moreover cross-linked derivatives of proteins formed by interactions with HNE (Friguet et al., 1994a, 1994b) and lipofuscin/ceroid (Sitte et al., 2000) inhibit proteolytic degradation of oxidized proteins. It is therefore evident that the inhibition of protease activities by overoxidation of proteins has a role in the accumulation of oxidized protein during aging and in some diseases.

LIST OF ABBREVIATIONS

Met(O), methionine sulfoxide
Msr's, methionine sulfoxide reductase
ROS, reactive oxygen species
RNS, reactive nitrogen species

REFERENCES

Agarwal S., Sohal R.S. (1994). Aging and protein damage. *Mech Aging Dev* 75: 11–19.

Amici A., Levine R.L., Tsai L., Stadtman E.R. (1989). Conversion of amino acid residues in proteins and amino acid homopolymers to carbonyl derivatives by metal-catalyzed reactions. *J Biol Chem* 264: 3341–3346.

Armesto X.L., Canle L.M., Fernandez M.I., Garcia M.V., Santaballa J.A. (2000). First steps in the oxidation of sulfur-containing amino acids by hypohalogenation: Very fast generation of intermediate sulfenyl halides and halosulfonium cations. *Tetrahedron* 56: 1103–1109.

Armstrong R.C., Swallow A.J. (1969). Pulse- and gamma-radiolysis of aqueous solutions of tryptophan. *Radiat Res* 41: 563–579.

Bar-Noy S., Moskovitz J. (2002). Mouse methionine mulfoxide reductase B: Effect of selenocysteine incorporation on its activity and expression of the seleno-containing enzyme in bacterial and mammalian cells. *Biochem Biophys Res Commun* 297: 956–961.

Baynes J.W. (1991). Role of oxidative stress in development of complications in diabetes. *Diabetes* 40: 405–412.

Beckman J.S., Ischiropoulos H., Zhu L., van der Woerd M., Smith C., Chen J., Harrison J., Martin J.C., Tsai M. (1992). Kinetics of superoxide dismutase- and iron-catalyzed nitration of phenolics by peroxynitrite. *Arch Biochem Biophys* 298: 438–445.

Berlett B.S., Levine R.L., Stadtman E.R. (1996). Comparison of the effects of ozone on the modification of amino acid residues in glutamine synthetase and bovine serum albumin. *J Biol Chem* 271: 4177–4182.

Berlett B.S., Levine R.L., Stadtman E.R. (1998). Carbon dioxide stimulates peroxynitrite-mediated nitration of tyrosine residues and inhibits oxidation of methionine residues of glutamine synthetase: Both modifications mimic effects of adenylylation. *Proc Natl Acad Sci USA* 95: 2784–2789.

Berlett B.S., Stadtman E.R. (1996). Carbon dioxide stimulates nitration of tyrosine residues and inhibits oxidation of methionine residues in *Escherichia coli* by peroxynitrite. *FASEB J* 10: Abstract 585.

Berlett B.S., Stadtman E.R. (1997). Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem* 272: 20313–20316.

Borg D.C., Schaich K.M. (1988). Iron and iron-derived radicals. In: Halliwell B. (ed.), *Proceedings of an Upjohn Symposium, Federation of American Societies for Experimental Biology*, Bethesda, MD, pp. 20–26.

Brot N., Weissbach H. (1983). Biochemistry and physiological role of methionine sulfoxide reductase in proteins. *Arch Biochem Biophys* 233: 271–288.

Bulteau A.-L., Szweda L.I., Friguet B. (2002). Age-dependent declines in proteasome activity in the heart. *Arch Biochem Biophys* 397: 298–304.

Burcham P.C., Kuhan Y.T. (1996). Introduction of carbonyl groups into proteins by the lipid peroxidation product, malondialdehye. *Biochem Biophys Res Commun* 220: 996–1001.

Buss I.H., Senthilmohan R., Darlow B.A., Mogridge N., Kettle A.J., Winterbourn C.C. (2003). 3-Chlorotyrosine as a marker of protein damage by myeloperoxidase in tracheal aspirates from preterm infants: Association with adverse respiratory outcome. *Pediatric Res* 53: 455–462.

Butterfield D.A., Stadtman E.R. (1997). Protein oxidation processes in aging brain. In: Timiras P.S., Bittar E.E. (eds.), *Advances in Cell Aging and Gerontology*, Vol. 2, JAI Press, pp. 161–191.

Candias L.P., Patel K.B., Stratford M.R.L., Wardman P. (1993). Free hydroxyl radicals are formed on reaction between the neutrophil-derived species superoxide anion and hypochlorous acid. *FEBS Lett* 333: 151–153.

Carney J.M., Starke-Reed P.E., Oliver C.N., Landum M.S., Cheng. M.S., Wu J.F. (1991). Reversal of age-related increase in brain protein oxidation, decrease in enzyme activity, and loss in temporal and spatial memory by chronic administration of the spin-trapping compound *N-tert*-butyl-α-phenylalanine. *Proc Natl Acad Sci USA* 88: 3633–3636.

Cerami A., Vlassara H., Brownlee M. (1987). Glucose and aging. *Sci Am* 256: 90–96.

Chapman A., Winterbourn C.C., Brennan S.O., Jordan T.W., Kettle A.J. (2003). Characterization of non-covalent oligomers of proteins treated with hypochlorous acid. *Biochem J* 375: 33–40.

Creeth J.M., Cooper B., Donald A.S.R., Clamp J.R. (1983). Studies of the limited degradation of mucous glycoproteins. *Biochem J* 211: 323–332.

Daneshvar B., Frandsen H., Autrup H., Dragsted L.O. (1997). γ-Glutamyl semialdehyde and 2-amino-adipic semialdehyde: Biomarkers of oxidative damage to proteins. *Biomarkers* 2: 117–123.

Davies K.J.A. (1986). Intramolecular proteolytic systems may function as secondary defenses: A Hypothesis. *Free Radic Biol Med* 2: 155–173.

Davies K.J.A. (1988a). Proteolytic systems as secondary antioxidant defenses. In: Chow C.K. (ed.), *Cellular Antioxidant Defense Mechanisms*, Vol. 2, CRC Press, Boca Raton, FL, pp. 25–67.

Davies K.J.A. (1988b). Oxidative stress causes protein Degradation and lipid peroxidation by different mechanisms in red blood cells. In: Sevanian A. (ed.), *Lipid Peroxidation in Biological Systems*, American Oil Chemists' Society, Champaign, IL, pp. 100–116.

Davies K.J.A. (2001). Degradation of oxidized proteins by the 20S proteasome. *Biochimie* 83: 301–310.

Davies K.J.A., Delsignore M.E. (1987). Protein damage and degradation by oxygen radicals. III. Modification of secondary and tertiary Structure. *J Biol Chem* 262: 9908–9913.

Davies K.J.A., Delsignore M.E., Lin S.W. (1987). Protein damage by oxygen radicals. II. Modification of amino acids. *J Biol Chem* 262: 9902–9907.

Davies K.J.A., Goldber A.L. (1987). Proteins damaged by oxygen radicals are rapidly degraded in extracts of red blood cells. *J Biol Chem* 262: 8227–8234.

Davies K.J.A., Wiese A.G., Sevanian A., Kim E.H. (1990). Repair systems in oxidative stress. In: Finch C.E., Johnson T.E. (eds.), *Molecular Biology of Aging*, Wiley-Liss, New York, pp. 123–141.

Davies M.J., Fu S., Wang H., Dean R.T. (1999). Stable markers of oxidant damage to proteins and their application in the study of human disease. *Free Radic Biol Med* 27: 1151–1163.

Dean R.T., Armstrong S., Fu S., Jessup W. (1994). Oxidized proteins and their enzymatic proteolysis in eucaryotic cells: A critical appraisal. In: Nohl H., Esterbauer H., Rice-Evans C. (eds.), *Free Radicals in the Environment, Medicine, and Toxicology*, Richelieu Press, London, pp. 47–79.

Dean R.T., Fu S., Stocker R., Davies M.J. (1997). Biochemistry and pathology of radical-mediated protein oxidation. *Biochem J* 324: 1–18.

Dean R.T., Gieseg S., Davies M.J. (1993). Reactive species and their accumulation on radical-damaged proteins. *Trends Biochem Sci* 18: 437–441.

Denicola A., Freeman B.A., Trujillo M., Radi R. (1996). Peroxynitrite reaction with carbon dioxide/bicarbonate: Kinetics and influence on peroxynitrite-mediated oxidations. *Arch Biochem Biophys* 333: 49–58.

Domigan N.M., Charlton T.S., Duncan M.W., Winterbourn C.C. (1995). Chlorination of tyrosyl residues in peptides by myeloperoxidase and human neutrophils. *J Biol Chem* 270: 16542–16548.

Drake S.K., Bourdon E., Wehr N.B., Levine RL., Backlund P.S., Yergey A.L., Rouault T.A. (2002). Numerous proteins in mammalian cells are prone to iron-dependent oxidation and proteasomal degradation. *Dev Neurosci* 24: 114–124.

Dunlop R.A., Rodgers K.J., Dean R.T. (2002). Recent developments in the intracellular degradation of oxidized proteins. *Free Radic Biol Med* 33: 894–906.

Eiserich J.P., Cross C.E., Jones D., Halliwell B. (1996). Formation of nitrating and chlorinating species by reaction of nitrite with hypochlorous acid. *J Biol Chem* 271: 19199–19206.

Englert R.P., Shacter E. (2002). Distinct modes of cell death induced by different reactive oxygen species. *J Biol Chem* 277: 20518–20526.

Esterbauer H., Schaur R.J., Zoller H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde, and related aldehydes. *Free Radic Biol Med* 11: 81–128.

Fletcher G.L., Okada S. (1961). Radiation-induced formation of dihydroxy phenylalanine from tyrosine and tyrosine-containing peptides in aqueous solution. *Radiat Res* 15: 349–351.

Folkes L.K., Candias L.P., Wardman P. (1995). Kinetics and mechanisms of hypochlorous acid reactions. *Arch Biochem Biophys* 323: 120–126.

Friguet B., Stadtman E.R., Szweda L.I. (1994a). Modification of glucose-6-phosphate dehydrogenase by 4-hydroxy-2-nonenal. *J Biol Chem* 269: 21639–21643.

Friguet B., Szweda L.I., Stadtman E.R. (1994b). Susceptibility of glucose-6-phosphate dehydrogenease modified by 4-hydroxy-2-nonenal and metal-catalyzed oxidation to proteolysis by the multicatalytic protease. *Arch Biochem Biophys* 311: 168–173.

Fu S., Wang H., Davies M., Dean R. (2000). Reactions of hypochlorous acid with tyrosine and peptidyl-tyrosyl residues give dichlorinated and aldehydic products in addition to 3-clorotyrosine. *J Biol Chem* 275: 10851–10858.

Garrison W.M. (1987). Reaction mechanisms in the radiolysis of peptides, polypeptides, and proteins. *Chem Rev* 87: 381–398.

Garrison W.M., Jayko M.E., Bennett W. (1962). Radiation-induced oxidation of protein in aqueous solution. *Radiation Res* 16: 483–502.

Garrison W.M., Kland-English M., Sokol H.A., Jayko M.E. (1970). Radiolytic degradation of peptide main chain in dilute aqueous solution containing oxygen. *J Physiol Chem* 74: 4506–4509.

Gatti R.M., Radi R., Agusto O. (1994). Peroxynitrite-mediated oxidation of albumin to the protein-thiyl free radical. *FEBS Lett* 348: 287–290.

Gieseg S.P., Simpson J.A., Charlton T.S., Duncan M.W., Dean R.T. (1993). Protein-bound 3,4-dihydroxyphenylalanine is a major product formed during hydroxyl radical damage to proteins. *Biochemistry* 32: 4780–4786.

Giulivi C., Davies K.J.A. (1993). Dityrosine and tyrosine oxidation products are endogenous markers for the selective proteolysis of oxidatively modified red blood cell hemoglobin by the 19S proteasome. *J Biol Chem* 268: 8752–8759.

Grune T., Reinheckel T., Davies K.J.A. (1996). Degradation of oxidized proteins in K562 human hematopoietic cells by proteasome. *J Biol Chem* 271: 15504–15509.

Grune T., Reinheckel T., Davies K.J.A. (1997). Degradation of oxidized proteins in mammalian cells. *FASEB J* 11: 526–534.

Grune T., Reinheckel T., Joshi M., Davies K.J.A. (1995). Protein degradation in cultured liver cells during oxidative stress. *J Biol Chem* 270: 2344–2351.

Grune T., Shringarpure R., Sitte N., Davies K. (2001). Age-related changes in protein oxidation and proteolysis in mammalian cells. *J Gerontol* 56: B459–B467.

Guptasarma P., Balasubramanian D., Matsugo S., Sito I. (1992). Hydroxyl radical-mediated damage to proteins, with special reference to the crystallins. *Biochemistry* 31: 4296–4302.

Halliwell B., Gutteridge J.M.C. (1999). Aging, nutrition, disease, and therapy: A role for antioxidants. In: Halliwell B., Gutteridge J.M.C. (eds.), *Free Radicals in Biology and Medicine*, Oxford University Press, Oxford, pp. 784–859.

Hawkins C., Davies M.J. (1998). Hypochlorite-induced damage to proteins: Formation of nitrogen-centered radicals from lysine residues and their role in protein fragmentation. *Biochem J* 332: 617–625.

Hawkins C.L., Davies M.J. (1999). Hypochlorite-induced oxidation of proteins in plasma: Formation of chloramines and nitrogen-centered radicals and their role in protein fragmentation. *Biochem J* 340: 539–548.

Hawkins C.L., Pattison D.I., Davies M.J. (2002). Reaction of protein chloramines with DNA and nucleosides: Evidence for formation of radicals, protein–DNA cross-links, and DNA fragmentation. *Biochem J* 365: 605–615.

Headlam H.A., Davies M.J. (2002). Beta-scission of side-chain alkoxyl radicals on peptides and proteins results in the loss of side-chains as aldehydes and ketones. *Free Radic Biol Med* 32: 1171–1184.

Headlam H.A., Mortimer A., Easton C.J., Davies M.J. (2000). Beta-Scission of C-3 (beta-carbon) alkoxyl radicals on peptides and proteins: a novel pathway which results in the formation of alpha-carbon radicals and the loss of amino acid side chains. *Chem Res Toxicol* 13: 1087–1095.

Heinecke J.W., Li W., Daehnke III H.L., Goldstein J.A. (1993). Dityrosine, a specific marker of oxidation is synthesized by the myeloperoxidase-hydrogen peroxide system of human neutrophils and macrophages. *J Biol Chem* 268: 4069–4077.

Huggins T.G., Wells-Knecht M.C., Detorie N.A., Baynes J.W., Thorpe S.R. (1993). Formation of O-tyrosine and dityrosine in proteins during radiolytic and metal-catalyzed oxidation. *J Biol Chem* 268: 12341–12347.

Ischiropoulos H., Al-Medi A.B. (1995). Peroxynitrite-mediated oxidative protein modifications. *FEBS Lett* 364: 279–282.

Ischiropoulos H., Zhu L., Beckman J.S. (1992). Peroxynitrite formation from macrophage-derived nitric oxide. *Arch Biochem Biophys* 298: 446–451.

Kato Y., Uchida K., Kawakishi S. (1992). Oxidative fragmentation of collagen and prolyl peptide by Cu(II)/H_2O_2 conversion of proline residue to 2- pyrrolidone. *J Biol Chem* 267: 23646–23651.

Kettle A.J. (1996). Neutrophils convert tyrosyl residues in albumin to chlorotyrosine. *FEBS Lett* 379: 103–106.

Kikugawa K., Kato T., Okamoto Y. (1994). Damage of amino acids and proteins induced by nitrogen dioxide, free radical toxin in air. *Free Radic Biol Med* 16: 373–382.

Kopoldova J., Liebsier J. (1963). The mechanism of radiation chemical degradation of amino acids. V. *Intern J Appl Radiation Isotopes* 14: 493–498.

Kristal B.S, Yu B.P. (1992). An emerging hypothesis: Synergistic induction of aging by free radicals and Maillard reactions. *J Gerontol* 47: B104–B107.

Krukov G.V., Kumar R.A., Koc A., Sun Z., Gladyshev V.N. (2002). Selenoprotein R is a zinc-containing stereo-specific methionine sulfoxide reductase. *Proc Natl Acad Sci USA* 99: 4245–4250.

Kumar R.A., Koc A, Cerny R.L., Gladyshev V.N. (2002). Reaction mechanism, evolutionary analysis, and role of zinc in *Drosophila* methionine-R-sulfoxide reductase. *J Biol Chem* 277: 37527–37535.

Levine R.L. (1983). Oxidative modifications of glutamine synthetase. *J Biol Chem* 258: 11823–11827.

Levine R.L. (2002). Carbonyl-modified proteins in cellular regulation, aging, and disease. *Free Radic Biol Med* 32: 790–796.

Levine R.L. Mosoni L., Berlett B.S., Stadtman E.R. (1996). Methionine residues as endogenous antioxidants in protein. *Proc Natl Acad Sci USA* 93: 15036–15040.

Levine R.L., Oliver C.N., Fulks R.M., Stadtman E.R. (1981). Turnover of bacterial glutamine synthetase: Oxidative inactivation precedes proteolysis. *Proc Natl Acad Sci USA* 78: 2120–2124.

Levine R.L., Stadtman E.R. (1992). Oxidation of proteins during aging. *Generations* 14: 39–42.

Levine R.L., Stadtman E.R. (2001). Oxidative modification of proteins during aging. *Exp Gerontol* 36: 1495–1502.

Levine R.L., Williams J.A., Stadtman E.R., Shacter E. (1994). Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol* 233: 346–357.

Lymar S.V., Hurst J.K. (1995). Rapid reaction between peroxynitrite ion and carbon dioxide: Implications for biological activity. *J Am Chem Soc* 117: 8867–8868.

Maskos Z., Rush J.C., Koppenol W.H. (1992a). The hydroxylation of phenylalanine and tyrosine: A comparison with salicylate and tryptophan. *Arch Biochem Biophys* 292: 521–529.

Maskos Z., Rush J.D., Koppenol W.H. (1992b). The hydroxylation of tryptophan. *Arch Biochem Biophys* 296: 514–520.

Monnier V. (1990). Nonenzymatic glycosylation, the Maillard reaction and the aging process. *J Gerontol* 45: B106–B111.

Monnier V., Gerhardinger C., Marion M.S., Tanaka S. (1995). Novel approaches toward inhibition of the Maillard reaction in vivo: Search, isolation, and characterization of prokaryotic enzymes which degrade glycated substrates. In: Cutler R.G., Packer L., Bertram J., Mori A., (eds.), *Oxidative Stress and Aging*, Berkhauser Verlag, Basel, pp. 141–149.

Moskovitz J., Bar-Noy S., Williams W.M., Requena J., Berlett B.S., Stadtman E.R. (2001). Methionine sulfoxide reductase (MsrA) is a regulator of antioxidant defense and lifespan in mammals. *Proc Natl Acad Sci USA* 98: 12920–12925.

Moskovitz J., Berlett B.S., Poston J.M., Stadtman E.R. (1997). The yeast peptide-methionine sulfoxide reductase functions as an antioxidant in vitro. *Proc Natl Acad Sci USA* 94: 9585–9589.

Moskovitz J., Flescher E., Berlett B.S., Azare J., Poston J.M., Stadtman E.R. (1998). Overexpression of peptide-methionine sulfoxide reductase in *Saccharomyes cerevisiae* and human T cells provides them with high resistance to oxidative stress. *Proc Natl Acad Sci USA* 95: 14071–14075.

Moskovitz J., Poston J.M., Berlett B.S., Nosworthy N.J., Szczepanowski R., Stadtman E.R. (2000). Identification and characterization of a putative active site for peptide methionine sulfoxide reductase (MsrA) and its substrate stereospecificity. *J Biol Chem* 275: 14167–14172.

Moskovitz J., Rahman M.A., Strassman J., Yancey S.O., Kushner S.R., Brot N., Weissbach H. (1995). *Escherichia coli* peptide methionine sulfoxide reductase gene: regulation of expression and role in protecting against oxidative damage. *J Bacteriol* 177: 502–507.

Moskovitz J., Singh F.K., Requena J., Wilkinson B.J., Jayaswal R.K., Stadtman E.R. (2002). Purification and characterization of methionine sulfoxide reductases from mouse and *Staphylococcus aureus* and their substrate stereospecificity. *Biochem Biophys Res Commun* 290: 62–65.

Moskovitz J., Weissbach H., Brot N. (1996). Cloning and expression of a mammalian gene that is involved in methionine sulfoxide reduction in proteins. *Proc Natl Acad Sci USA* 93: 2095–2099.

Oliver C.N., Ahn B., Wittenberger M.E., Levine R.L., Stadtman E.R. (1985). Age-related alterations of enzymes may involve mixed-function oxidation reactions. In: Adleman R.C., Dekker E.E. (eds.), *Modifications of Proteins during Aging*, Alan R. Liss., pp. 39–52.

Oliver C.N., Fulks R., Levine R.L., Fucci L., Rivett A.J., Roseman J.E., Stadtman E.R. (1984). Oxidative inactivation of key metabolic during aging. In: Roy A.K., Chatterjee B. (eds.), *Molecular Biology of Aging*, Academic Press, New York, pp. 235–260.

Oliver C.N., Levine R.L., Stadtman E.R. (1980). Regulation of glutamine synthetase degradation. In: Holzer H. (ed.), *Metabolic Interconversion of Enzymes*, Springer-Verlag, Berlin, pp. 259–267.

Oliver C.N., Levine R.L., Stadtman E.R. (1982). Regulation of glutamine synthetase degradation. In: Ornston L.N., Sligar S.G. (eds.), *Experiences in Biochemical Perception*, Academic Press, New York, pp. 233–249.

Pacifici R.E., Davies K.J.A. (1990). Protein degradation as an index of oxidative stress. *Methods Enzymol* 1886: 485–502.

Pietzsch J., Bergmann R. (2004). Analysis of 6-hydroxy-2-aminocaproic acid (HACA) as a specific marker of protein oxidation: The use of *N(O,S)*-ethoxycarbonyl trifluoroethyl ester derivatives and gas chromatography/mass spectrometry. *Amino Acids* 26: 45–51.

Peskin A.V., Winterbourn C.C. (2001). Kinetics of the reactions of hypochlorous acid and amino acid chloramines with thiols, methionine, and ascorbate. *Free Radic Biol Med* 30: 572–579.

Petropoulos I., Conconi M., Wang X., Hoenel B., Brégégère F., Milner Y., Friguet B. (2000). Increase of oxidatively modified protein is associated with a decrease of proteasome activity and content in aging epidermal cells. *J Gerontol Biol Sci* 55A: B220–B227.

Poston J.M. (1988). Detection of oxidized amino acid residues, using P-aminobenzoic acid adducts. *Fed Proc* (Abstract) 46: 1979.

Pryor W.A., Jen X., Squadrito G.L. (1994). One- and two-electron oxidations of methionine by peroxynitrite. *Proc Natl Acad Sci USA* 91: 11173–11177.

Pryor W.A., Squadrito G.L. (1995). The chemistry of peroxynitrite: A product from the reaction of nitric oxide with superoxide. *Am J Physiol* 268: L699–L722.

Pryor W.A., Uppu R.M. (1993). A kinetic model for the competitive reactions of ozone with amino acid residues in proteins in reverse micelles. *J Biol Chem* 268: 3120–3126.

Reddy S., Bichler J., Wells-Knecht K.J., Thorpe S.R., Baynes J.W. (1995). N^{ε}-(carboxymethyl) lysine is a dominant advanced glycation end product (AGE) antigen in tissue proteins. *Biochemistry* 34(34): 10872–10878.

Requena J., Chao C.C., Stadtman E.R. (2001). Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins. *Proc Natl Acad Sci USA* 98: 69–64.

Rivett A.J. (1985a). The effect of mixed-function oxidation of enzymes on their susceptibility to degradation by the nonlysosomal cysteine protease. *Arch Biochem Biophys* 247: 624–632.

Rivett A.J. (1985b). Preferential degradation of the oxidatively modified form of glutamine synthetase by intracellular mammalian proteases. *J Biol Chem* 260: 300–305.

Rivett A.J. (1985c). Purification of a liver alkaline protease which degrades oxidatively modified glutamine synthetase: Characterization as a high molecular weight cysteine protease. *J Biol Chem* 260: 12600–12606.

Rivett A.J. (1986). Regulation of intracellular protein turnover: Covalent modification as a mechanism of marking proteins for degradation. *Curr Top Cell Reg* 28: 291–337.

Rivett A.J., Roseman J.E., Oliver C.N., Levine R.L., Stadtman E.R. (1985). Covalent modification of proteins by mixed-function oxidation: Recognition by intracellular proteases. In: Khairallah E.A., Bond J.S. (eds.), *Intracellular Protein Catabolism*, Alan R. Liss, New York, pp. 317–328.

Roseman J.E., Levine R.L. (1987). Purification of a protease from *Escherichia coli* with specificity for oxidized glutamine synthetase. *J Biol Chem* 262: 2101–2110.

Ruan H., Tang X.D., Chen M.-L., Joiner M.A., Sun G., Brot N., Weissbach H., Heineman S.H., Iverson L., Wu C.-F., Hoshi T. (2002). High quality life extension by the enzyme peptide methionine sulfoxide reductase. *Proc Natl Acad Sci USA* 99: 2748–2753.

Schoneich C., Zhao F., Wilson G.S., Borchardt R.T. (1993). Iron-thiolate induced oxidation of methionine to methionine sulfoxide in small model peptides. Intramolecular catalysis by histidine. *Biochem Biophys Acta* 1158: 307–322.

Schuenstein E., Esterbauer H. (1979). Formation and properties of reactive aldehydes. In: *Submolecular Biology of Cancer, CIBA Foundation Series 67, Excerpta Medica*, Elsevier, Amsterdam, pp. 225–244.

Schuessler H., Schilling K. (1984). Oxygen effect in radiolysis of proteins. Part 2. Bovine serum albumin. *Int J Radiat Biol* 45: 267–281.

Shringarpure R., Davies K.J.A. (2002). Protein turnover by the proteasome in aging and disease. *Free Radic Biol Med* 32: 1084–1089.

Sitte N., Huber M., Grune T., Ladhoff A. Doecke W-D., Zglinicki T., Davies K.J.A. (2000). Proteasome inhibition by lipofuscin/ceroid during postmitotic aging of fibroblasts. *FASEB J* 14: 14490–14498.

Smith C.D., Carney J.M., Starke-Reed P.E., Oliver C.N., Stadtman E.R., Floyd R.A. (1991). Excess brain protein oxidation and enzyme dysfunction in normal and Alzheimer's disease. *Proc Natl Acad Sci USA* 88: 10540–10543.

Smith K.J., Kapoor R., Felts P.A. (1999). Demyelination: The role of reactive oxygen and nitrogen species. *Brain Pathol* 9: 69–92.

Solar S. (1985). Reactions of OH with phenylalanine in neutral aqueous solution. *Radiat Phys Chem* 26: 103–108.

St. John G., Brot N., Ruan J., Erdjument-Bromage H., Tempst R., Weissbach H., Nathan C. (2001). Peptide methionine sulfoxide reductase from *Escherichia coli* and *Mycobacterium tuberculosis* protects bacteria against oxidative damage from reactive nitrogen intermediates. *Proc Natl Acad Sci USA* 98: 9901–9906.

Stadtman E.R. (1988). Biomarkers of Aging. *Exp Gerontol* 10: 327–347.

Stadtman E.R. (1992). Protein oxidation and aging. *Science* 257: 1220–1224.

Stadtman E.R. (1998a). Free radical-mediated oxidation of proteins. In: Ozben T. (ed.), *Free Radicals, Oxidative Stress, and Antioxidants: Pathological and Physiological Significance*, NATO ASI Series, Series A: Life Sciences, Vol. 296, Plenum Press, New York, pp. 51–143.

Stadtman E.R. (1998b). Free radical-mediated oxidation of proteins. In: Ozben T. (ed.), *Free Radicals, Oxidative Stress, and Antioxidants: Pathological and Physiological Significance*, NATO ASI Series, Series A: Life Sciences,. Vol. 296, Plenum Press, New York, pp. 161–191.

Stadtman E.R. (2002). Importance of individuality in oxidative stress and aging. *Free Radic Biol Med* 33: 597–604.

Stadtman E.R., Berlett B.S. (1997). Free radical-mediated modification of proteins. In: Wallace K.B. (ed.), *Free Radical Toxicology*, Taylor and Francis, Washington, D.C., pp 71–87.

Stadtman E.R., Berlett B.S. (1998). Reactive oxygen-mediated protein oxidation in aging and disease. *Drug Metab Rev* 30: 225–243.

Starke-Reed P.E., Oliver C.N. (1989). Protein oxidation and proteolysis during aging and oxidative stress. *Arch Biochem Biophys* 275: 559–567.

Swallow A.J. (1960). *Effect of ionizing radiation on proteins, RCO groups, peptide bond cleavage Inactivation, –SH Oxidation*. In: Swallow A.J. (ed.), Pergamon Press, New York, pp. 211–224.

Szweda P.A., Friguet B., Szweda L.I. (2002). Proteolysis, free radicals, and aging. *Free Radic Biol Med* 33: 29–36.

Taborsky G. (1973). Oxidative modification of proteins in the presence of ferrous iron and air. Effect of ionic constituents of the reaction medium on the nature of the oxidation products. *Biochemistry* 12: 1341–1348.

Takahashi R., Goto S. (1990). Alteration of aminoacyl-tRNA synthetase with age: Heat labilization of the enzyme by oxidative damage. *Arch Biochem Biophys* 277: 228–233.

Tien M., Berlett B.S., Levine R.L., Chock P.B., Stadtman E.R (1999). Peroxynitrite-mediated modification of proteins at physiological carbon dioxide concentration: pH dependence of carbonyl formation, tyrosine nitration, and methionine oxidation. *Proc Natl Acad Sci USA* 96: 7809–7814.

Uchida K., Kato Y., Kawakishi S. (1990). A novel mechanism for oxidative damage of prolyl peptides induced by hydroxyl radicals. *Biochem Biophys Res Commun* 169: 265–271.

Uchida K., Kawakishi S. (1993). 2-Oxohistidine as a novel biomarker for oxidative modification of proteins. *FEBS Lett* 332: 208–210.

Uchida K., Stadtman E.R. (1993). Covalent attachment of 4-hydroxynonenal to glyceraldehyde-3-phosphate dehydrogenase: A possible involvement of intra- and intermolecular cross-linking reaction. *J Biol Chem* 268: 6388–6393.

Uppu R.M., Squadrito G.L., Pryor W.A. (1996). Acceleration of peroxynitrite oxidations by carbon dioxide. *Arch Biochem Biophys* 327: 335–342.

van der Vliet A., Eiserich J.P., O'Neill C.A., Halliwell B., Cross C.E. (1995). Tyrosine modification by reactive nitrogen species: A closer look. *Arch Biochem Biophys* 319: 341–349.

Vittorini S., Paradiso C, Donati A., Cavallini G., Masini M. Gori Z., Pollera M., Bergamini E. (1999). The age-related accumulation of protein carbonyl in rat liver correlates with the age-related decline in liver proteolytic activities. *J Gerontol A Biol Sci Med Sci* 54: B318–B323.

Vogt W. (1995). Oxidation of methionine residues in proteins: Tools, targets, and reversal. *Free Radic Biol Med* 18: 93–105.

Weinberg J.B., Gilkeson G.S., Mason R.P., Chamulitrat W. (1998). Nitrosylation of blood hemoglobin and renal nonheme proteins in autoimmune MRL-*lpr/lpr* mice. *Free Radic Biol Med* 24: 191–196.

Wells-Knecht M.C., Huggins T.G., Dyer G., Thorpe S.R., Baynes J.W. (1993). Oxidized amino acids in lens protein with age. *J Biol Chem* 268: 12348–12352.

Winchester R.V., Lynn K.R. (1970). X- and γ-radiolysis of some tryptophan dipeptides. *Int J Radiat Biol* 17: 541–549.

Wolf S.P., Garner A., Dean R.T. (1986). Free radicals, lipids, and protein degradation. *Trends Biochem Sci* 11: 27–31.

Zhou J.Q., Gafni A. (1991). Exposure of rat muscle phosphoglycerate kinase to a nonenzymatic MFO system generates the old form of enzyme. *J Gerontol* 46: B217–B222.

2

THE CHEMISTRY OF PROTEIN MODIFICATIONS ELICITED BY NITRIC OXIDE AND RELATED NITROGEN OXIDES

Douglas D. Thomas, Lisa Ridnour, Sonia Donzelli, Michael Graham Espey, Daniele Mancardi, Jeffery S. Isenberg, Martin Feelisch, David D. Roberts, and David A. Wink

2.1 INTRODUCTION

Our understanding of the physiological and pathophysiological mechanisms of nitric oxide (NO) and reactive nitrogen oxide species (RNOS) has rapidly expanded over the past decade. While NO regulates many normal cellular processes in health, it has also been associated with toxicity. Because of this dichotomous nature, different conclusions regarding its action have been made. Physiological functions of NO include the modulation of signaling cascades and immune responses (Moncada et al., 1991). Nitric oxide directs wound healing and angiogenesis by regulating adhesion and proliferation of endothelial and smooth muscle cells (Maulik and Das, 2002; Schwentker et al., 2002). In contrast, NO has also been implicated in tissue injury and progression of various inflammatory diseases (Ischiropoulos, 1998; Wink and Mitchell, 1998; Wink et al., 1998b). These divergent observations illustrate the dichotomous behavior of NO, which can be rationalized by considering the specific experimental conditions under which these results were obtained and the biochemistry involved.

The complex nature of NO can be understood by examining the chemical/biochemical environment in which NO and RNOS are formed. The concentration

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield
Published 2006 by John Wiley & Sons, Inc.

of and the duration of exposure to NO are important determinants of its action. Intracellular location and/or compartmentalization of NO formation are additional key factors that dictate the impact of NO on select molecular targets and signaling cascades, all of which are cell or tissue specific. The elucidation of these processes over the last two decades has lead to the development of specific biomarkers for NO. Methodologies that allow to identify nitrosated and nitrated reaction products have been instrumental in the assessment of the redox biology associated with the metabolism of NO donors in vivo and have provided insight into the local NO chemistry prevailing in tissues (Rassaf et al., 2003a; Bryan et al., 2004; Janero et al., 2004). The most commonly evaluated metabolites of NO are the products of oxidation, nitrite (NO_2^-) and nitrate (NO_3^-) (Granger et al., 1996; Grisham et al., 1996; Miranda et al., 2005). In addition, *S*-nitrosothiols have been identified in some tissues (Gaston et al., 1993; Welch et al., 1996; Gladwin et al., 2000). Nitration of tyrosine also provides a sensitive marker of NO-related redox stress in tissue (Greenacre and Ischiropoulos, 2001; Tarpey et al., 2004; Pignatelli et al., 2001; Pfeiffer et al., 2001a). Further development of these techniques has culminated in a comprehensive approach for the elucidation of an NO metabolic signature identifying NO_2^-/NO_3^-, *S*-nitrosation, *N*-nitrosation, and metal nitrosyl adducts in tissue. This metabolic signature serves as a unique footprint of the biological chemistry of RNOS and additionally reflects the specific redox environment prevailing (Gladwin et al., 2000; Bryan et al., 2004).

To correctly interpret the mechanism(s) of formation of NO-related species the chemical environment must be taken into consideration. Two-dimensional electrophoresis shows that under inflammatory conditions nitration of specific proteins occurs in cells (Aulak et al., 2004). Likewise techniques that allow examination of thiol nitrosation patterns show specific protein thiols to be modified (Jaffrey et al., 2002). These experiments indicate that adduct formation and nitrosative and nitrative protein modifications do not occur at random. Several important questions need to be addressed to understand the origin of these markers. What is the chemical and biochemical genesis of these adducts? Do they represent fingerprints of NO formation? Can we correlate these molecular changes with specific redox conditions? Can these molecular fingerprints be useful in unraveling disease mechanisms, developing novel pharmacological interventions or providing new diagnostic markers of potential clinical utility? In the following we will discuss the potential chemical reactions that lead to these modifications, attempt to address what these signatures might mean, how and why they might be specific, and where to look for them.

2.2 CHEMICAL BIOLOGY OF NO

One of the major problems in deciphering the pertinent chemical reactions of NO in biological systems is the vast body of literature pertaining to the chemistry as well as the biology of nitrogen oxides. Therefore, identifying the relevant chemical reactions applicable to specific biological conditions can be a daunting task.

Our approach to these reactions has been the development of a simplified conceptual framework: the *chemical biology* of NO and RNOS. This paradigm describes the relevant chemistry of nitrogen oxides as it pertains to specific redox conditions and concentrations and the possible and probable biological outcomes (Wink and Mitchell, 1998; Grisham et al., 1999; Espey et al., 2000, 2002b; Mancardi et al., 2004; Ridnour et al., 2004).

The chemical biology of NO divides the reactions into two categories: direct and indirect (Fig. 2.1). The "direct effects" include all those reactions in which NO as such directly interacts with its biological target to exert its effect. The best example of this kind of reaction is the formation of nitrosyl complexes following reaction of NO with either ferrous heme complexes or radicals. Conversely, "indirect effects" are those that involve reactive nitrogen oxide species (RNOS) such as nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$), and dinitrogen trioxide (N_2O_3). These species with a higher oxidation state of nitrogen are more oxidizing and are stronger electrophiles compared to NO itself. These reactions are defined as indirect because they involve the formation of RNOS by NO before they result in nitrosation, nitration, or oxidation of proteins.

The resultant chemistry of NO with other molecules is thought to be the genesis of these protein modifications by RNOS. However, alternative chemistries may also be involved. For example, nitrite (NO_2^-), an oxidative byproduct of NO decomposition, can be oxidized to yield NO_2 or acidified to give NO_2/N_2O_3. Although not fully understood at present, these potentially NO-independent sources of RNOS may exert a protective effect against ischemic tissue injury under certain conditions. Nitroxyl (HNO), the one-electron reduction product of NO, is another potential source of protein modification. Nitroxyl has unique chemical

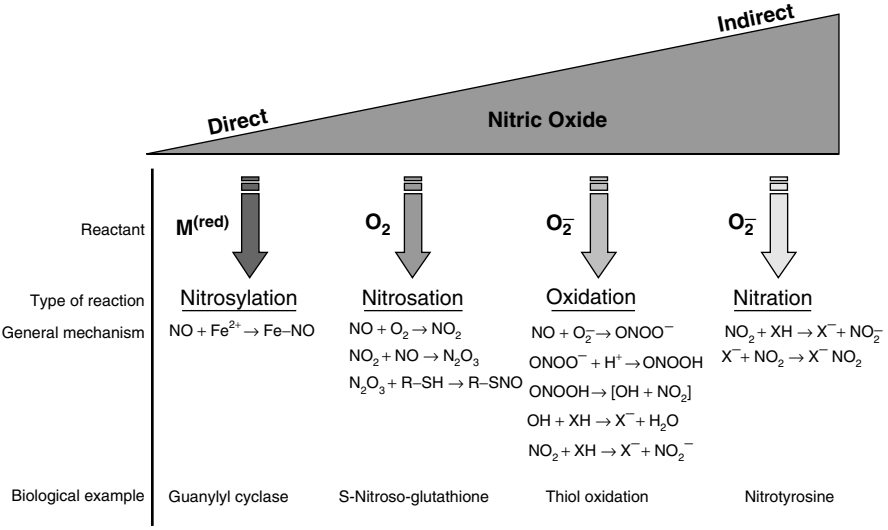


FIGURE 2.1 Chemical biology of nitric oxide; direct and indirect effects.

properties and may be produced physiologically under conditions where NO is not favored (Miranda et al., 2003a, 2003b; Fukuto et al., 2005).

Any of the chemistries above may be involved in the formation of protein modifications. So, how can we determine what chemistry is responsible for which adduct formation? Specifically, under what conditions is one adduct favored over another? Do certain products indicate specific environments? The recent use of a nitrosative in vivo signature is an important start in the attempt to unravel the significance of the biological chemistry of NO (Bryan et al., 2004; Janero et al., 2004). To understand the potential meaning of these biomarkers and what they may be able to tell us about the redox environment in which they have been generated, we will have to consider the possible chemistry involved, the probability with which these reactions may occur in vivo, and the probability of their detection using the analytical techniques currently available.

2.2.1 Direct Effects

NO Interaction with Metals The formation of heme nitrosyl complexes is one of the most important reactions in the biology of NO (Ford and Lorkovic, 2002). The activation of soluble guanylyl cyclase is a result of the rapid formation of a complex with the ferrous form of this hemeprotein ($k = 8 \times 10^7$ M^{-1} s^{-1}) (Makino et al., 1999; Zhao et al., 1999). This causes an allosteric change that facilitates the conversion of guanosine triphosphate to the second messenger, cyclic guanosine monophoshate (cGMP) (Koesling et al., 2004). In vascular tissue the EC_{50} for NO is typically in the range of 10 to 50 nM NO for this enzyme, thus low enough to be relevant for physiological signaling. However, it appears that the sensitivity to activation can be modified by translocation of sGC to the membrane as well as its proximity to NOS (Pyriochou and Papapetropoulos, 2005). Since NO is hydrophobic, translocation to the membrane would decrease the EC_{50}, making this enzyme even more sensitive to stimulation by NO.

Another important NO–heme reaction is the reversible inhibition of cytochrome c oxidase. The binding of NO to this respiratory chain complex (complex IV) prevents its reaction with oxygen, thus inhibiting mitochondrial respiration (Cleeter et al., 1994; Brown, 1995). This reaction is competitive in that the local concentrations of both NO and O_2 determine the actual degree of inhibition. Since NO has a higher affinity for this site than O_2 itself, at ambient O_2 concentrations (corresponding to $\sim$200 μM O_2), 50 to 100 nM NO are required to fully inhibit respiration. However, the partial pressure of O_2 in the intact tissue is considerably lower than that during experiments carried out in ambient air. Thus much lower concentrations of NO are required to competitively inhibit respiration in vivo. Other heme-containing proteins form stable nitrosyl adducts, generally in the ferrous rather than the ferric state. For instance, ferrous myoglobin and hemoglobin (deoxymyoglobin and deoxyhemoglobin) react with NO to form a ferrous nitrosyl complex (reaction 1, k_{on} 3×10^7 M^{-1} s^{-1}) (Ford and Lorkovic, 2002).

$$\text{MbFe(II)} + \text{NO} \rightarrow \text{MbFe(II)–NO} \quad (1)$$

Although the oxidized forms of these proteins (MetMb and MetHb) also forms nitrosyl comlexes, these adducts are relatively unstable and therefore probably of less significance in biological mechanisms (Hoshino et al., 1993; Ford and Lorkovic, 2002). However, even the ferrous nitrosyl adducts of Mb or Hb are somewhat unstable under aerobic conditions. This is due to the dissociation of NO followed by oxygen binding forming $Fe(II)O_2$ (at ambient oxygen levels their half-life is $\sim$20 min at 37°C) (Doyle and Hoekstra, 1981). As Doyle showed, NO reacts with $Fe(O_2)$ to form MetHb and nitrate (Doyle and Hoekstra, 1981). Thus, metal nitrosyl complexes of globins are considerably more stable under reduced oxygen tension.

Similar to Hb and Mb, peroxidases contain an axial histidine ligand and can form a stable nitrosyl species in the active ferric rather than the ferrous state (Garavito and Mulichak, 2003). Other peroxidases, such as cyclo-oxygenase, form nitrosyl adducts in the inactive ferrous state (Karthein et al., 1987; Kanner et al., 1992). These are predicted to be very stable, thus providing a mechanism for inhibition. In contrast, cytochrome P450 contains a cysteine moiety as axial ligand and can form the nitrosyl in both ferric and ferrous states due to the ability of sulfur to donate electron density to the metal (O'Keeffe et al., 1978, Tsubaki et al., 1987). This essentially softens the ferric ion and allows a stable nitrosyl to form, preventing the binding of oxygen and limiting its catalytic action. Generally, NO-mediated inhibition of cytochrome P450 occurs above 100 nM at ambient oxygen concentrations. This is supported by in vivo studies showing that under septic conditions hepatic cytochromes P450s as well as cyclo-oxygenase are inhibited (Stadler et al., 1993b, 1994).

Non-heme iron proteins are another possible source of stable metal-nitrosyl adducts. One of the first detections of NO involved an EPR signal at $g = 2.04$, in the presence of activated macrophages, that had been assigned to dinitrosyl-FeS complexes (Lancaster and Hibbs, 1990). Other activated cells with high NO levels were observed to exhibit a similar signal (Stadler et al., 1993a; Yoshimura et al., 1996). The same signal has been further observed in murine tumors in vivo, indicating that there is a high NO output under these conditions (Bastian et al., 1994; Doi et al., 1996). Aconitase, an enzyme critical to the citric acid cycle, is inhibited by the formation of a nitrosyl complex (Drapier and Hibbs, 1986). Cellular regulation of iron regulatory elements involves IRBs that contain aconitase cofactors to sense the iron status in the cell. Nitrosyl formation of these complexes stimulates iron uptake through increased transferrin synthesis (Klausner et al., 1993; Bouton and Drapier, 2003). While several of the above-described nitrosylation reactions may be of limited relevance for the physiological concentration range of NO (1–100 nM), it is important to keep in mind that concentrations > 0.3 μM NO can be sustained in the microenvironment of activated macrophages (Thomas et al., 2002a, 2004; Hofseth et al., 2003; Wang et al., 2003).

Consumption Mechanisms To understand the mechanisms of protein adduct formation, one must also define the consumption mechanisms of NO (Fig. 2.2). Nitric oxide is consumed very rapidly in tissue by two processes. The first is the

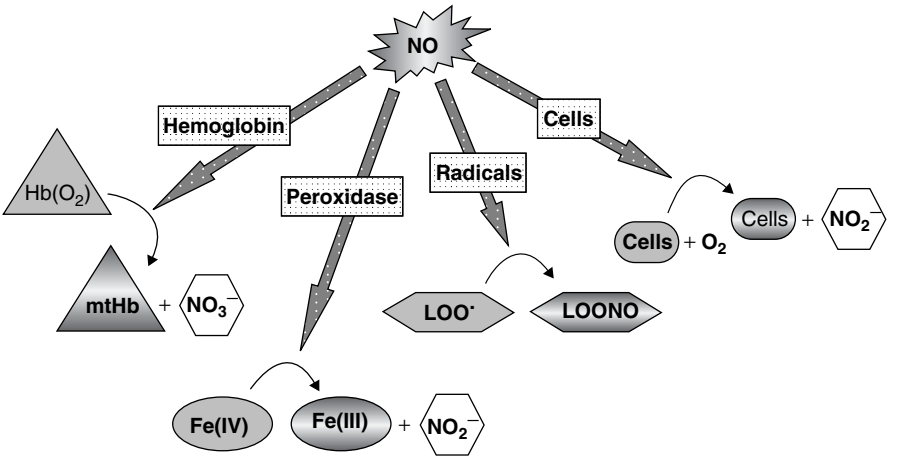


FIGURE 2.2 Mechanisms of nitric oxide consumption.

reaction within erythrocytes between NO and HbO_2, which occurs at 3.2×10^7 M^{-1} s^{-1} forming nitrate (Lancaster, 1994; Eich et al., 1996):

$$HbO_2 + NO \rightarrow MetHb + NO_3^- \tag{2}$$

However, with respect to tissue the erythrocyte membrane provides a barrier, which lowers the apparent rate from extracellular sources of NO by 10^3 (Liu et al., 1998a; Han et al., 2002). This increases the lifetime of NO in tissues considerably, allowing NO to interact more effectively with other targets. Thus, a concentration of 10 μM HbO_2 mimics the scavenging effect of an erythrocyte whose Hb concentration corresponds to about 2 mM.

The second process of consumption is via an oxygen-dependent process by a yet to be determined mechanism in cells (Thomas et al., 2001). This intracellular consumption mechanism sets up an important interaction between NO and O_2 involved in the reciprocal regulation of each other's concentrations. Whereas NO increases O_2 concentration (by inhibiting respiration), increasing concentrations of O_2 increase the consumption of NO. The intracellular consumption regulates NO levels and oxygen tension in different tissue (Thomas et al., 2001). These are important processes that keep the effects of NO confined to specific regions and limit its migration to neighboring cells or tissue. Hence processes triggered or mediated by NO must be competitive with these consumptive pathways to play an important role in biology.

Another important consumption reaction is that by proteins containing hypervalent metals engaged in Fenton-type reactions (Abu-Soud and Hazen, 2000; Thomas et al., 2003). These are reactions that occur at near-diffusion control and can play many roles in the biology and pathophysiology of NO (reaction 3) (Gorbunov et al., 1995; Abu-Soud and Hazen, 2000; Galijasevic et al.,

2003, 2004):

$$\mathrm{Fe(IV)} + \mathrm{NO} \xrightarrow{\mathrm{H_2O_2}} \mathrm{NO_2^-} + \mathrm{Fe(III)} \quad (3)$$

One important consequence of this type of reaction is that NO can serve as a powerful antioxidant (Goss et al., 1999; Darley-Usmar et al., 2000; Wink et al., 2001). Thus, despite its bad reputation, NO can prevent cell and tissue injury mediated by peroxides. In the cardiovascular system, NO is a critical component involved in the abatement of oxidative stress induced by reactive oxygen species (ROS) (Wink et al., 2001). The latter mediates much of the redox-related damage that occurs under both acute and chronic pathophysiological conditions.

2.2.2 Indirect Mechanisms

Chemistry of RNOS As the concentration of NO increases, so does the participation of RNOS in adduct formation (Fig. 2.1). At lower concentrations, most of the consumptive mechanisms for NO and simple diffusion prevent the formation of RNOS. However, at higher concentrations, proteins can be altered by the nitrosation and nitration of moieties via reaction with different RNOS derived from the reaction of NO with oxygen and ROS (Wink and Mitchell, 1998). This may result in the generation of species such as *N*-nitrosamines and *S*-nitrosothiols as well in the nitration of aromatic residues. Formation of such products are thought to indicate that toxic NO levels were reached, although the formation of such modifications at low abundance in normal physiology cannot be excluded. Below we discuss the chemistry and consecutive cytotoxicity of RNOS.

The NO/O_2 Reaction One of the most common mechanisms in *solution* chemistry for the disappearance of NO is its autoxidation reaction (Ford et al., 1993; Ignarro et al., 1993). Whether in a test tube or a Petri dish, the treatment of aqueous samples with NO results in this reaction, which yields exclusively nitrite rather than nitrate (reaction 4):

$$4\mathrm{NO} + \mathrm{O_2} \rightarrow 4\ \mathrm{HNO_2} \quad (4)$$

During the NO autoxidation reaction there is generation of RNOS that can oxidize, nitrate, or nitrosate depending on the medium and conditions (Wink et al., 1996b).

Despite the formation of these intermediates, exposure of NO to cells is not appreciably cytotoxic and usually even protects against an oxidant stress caused by hydrogen peroxide or superoxide. There appear to be two reasons for this: the particular kinetics of the autoxidation reaction and the fact that the RNOS intermediates generated in this reaction are not as potent oxidants as those formed from the reaction of NO with ROS.

The kinetics of the reaction follow the rate equation of $-d[\mathrm{NO}]/dt = k[\mathrm{NO}]^2[\mathrm{O_2}]$ (Ford et al., 1993). This third order equation provides some insight into the NO behavior in vivo. The second order dependence on NO dictates that the

lifetime of the NO is proportional to its concentration. For example, 1 μM NO has a half-life of 800 seconds whereas the half-life of 100 μM is less than 8 seconds. Therefore in vivo at nM concentrations of NO the rate of autoxidation is slow, and NO is long-lived with respect to this reaction, allowing much faster reactions in vivo to proceed relatively unaffected by the presence of oxygen. Thus, physiological NO signaling and other in vivo process can occur with little or no interference by the autoxidation reaction or generation of RNOS that can alter protein function. The conditions in vivo that might give rise to formation of RNOS via this reaction are limited to the hydrophobic region in the vicinity of cells that express high levels of iNOS (Liu et al., 1998b; Espey et al., 2000, 2001). It is in these areas that one expects nitrosative and nitrated protein products to result from this reaction.

An important factor to consider for the NO/O_2 reaction is that regions of higher NO concentration will increase oxygen tension through inhibition of respiration. However, the rate of cellular consumption of NO also increases via an O_2-dependent process (Thomas et al., 2001; Liu et al., 2004). Therefore it is difficult to determine which mechanism is more important in vivo. This reciprocal regulation of cellular consumption and oxygen concentration is important not only for RNOS formation but also for the regulation of NO signaling.

The decomposition of NO and subsequent RNOS formation depends on the hydrophobicity of the medium. Although the rate constants for the reactions in hydrophobic and hydrophilic solvents are the same, the solubility of NO and O_2 are considerably higher in hydrophobic environments. The rate of decomposition can therefore be increased by virtue of the increased solubility by up to 1500 times. In hydrophobic regions of cells, and possibly proteins, also the autoxidation reaction can be increased (Liu et al., 1998b). Thus an enhanced level of nitrosation, and to a lesser degree nitration of proteins, is predicted to occur in the hydrophobic region of cells and tissues (Espey et al., 2001).

The intermediates of the NO/O_2 reaction that are formed in the gas phase as well as in hydrophobic media initially form nitrogen dioxide (NO_2) (Schwartz and White, 1983). This is the brown colored gas that is associated with air pollution in our urban areas (reaction 5). Nitrogen dioxide can then react to form N_2O_3 (reaction 6) (Schwartz and White, 1983). In aqueous solution it appears that NO_2 is not an intermediate in the formation of N_2O_3 (reaction 8; Fig. 2.3) (Wink et al., 1993b).

Gas Phase/Hydrophobic Media

$$2\ NO + O_2 \rightarrow 2\ NO_2 \tag{5}$$

$$NO_2 + NO \rightarrow N_2O_3 \tag{6}$$

$$N_2O_3 + H_2O \rightarrow 2\ HNO_2 \tag{7}$$

Aqueous Phase

$$2\ NO + O_2 \rightarrow [ONOONO] \xrightarrow{+2NO} N_2O_3 \tag{8}$$

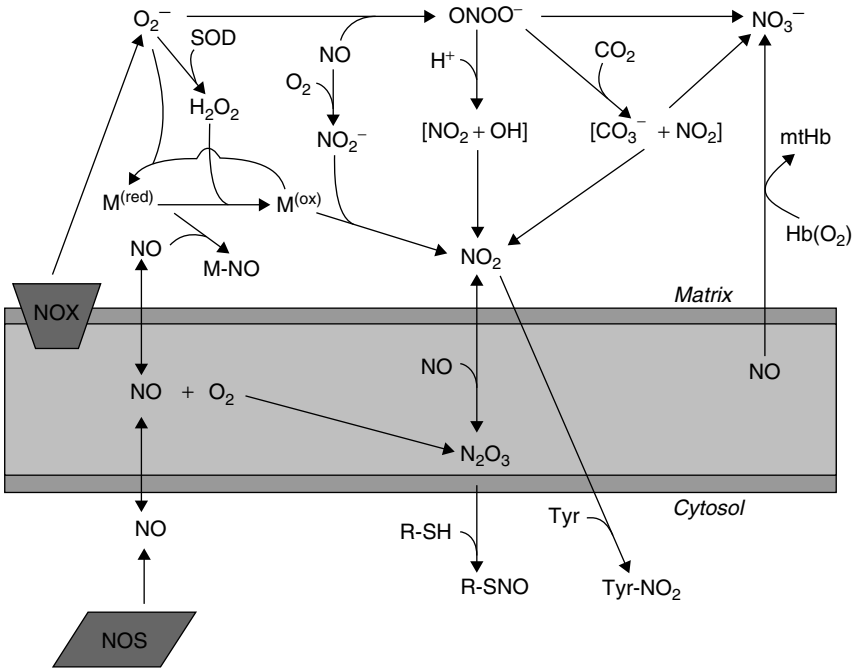


FIGURE 2.3 Biological fate of nitric oxide as dictated by concurrent oxidation, nitration, and nitrosation reactions.

Whereas NO_2 is an oxidant (~1.0 V) and can nitrate proteins, N_2O_3 is milder oxidant and nitrosates its substrates (Wink et al., 1993b; Grisham and Miles, 1994). In the aqueous phase at NO concentrations above 100 nM, the only observable intermediate is N_2O_3 and its associated chemistry (Espey et al., 2001). NO autoxidation reactions within the biological context may primarily occur in cell membranes rather than the cytoplasm or otherwise aqueous/charged regions of the tissue (Espey et al., 2001).

The NO/O_2^- Reaction The NO/O_2^- reaction has received much attention over the last decade. This reaction between these two radicals has been postulated to be deleterious due to formation of ONOO$^-$ (reaction 9), which generates powerful oxidants proposed to be the reactive hydroxyl radical (OH$^\bullet$) and nitrogen dioxide (NO_2) (Fig. 2.3) (reaction 10) (Pryor and Squadrito, 1996; Coddington et al., 2001):

$$NO + O_2^- \rightarrow ONOO^- \tag{9}$$

$$ONOO^- + H^+ \rightarrow HOONO \rightarrow OH^\bullet / NO_2 \rightarrow NO_3^- \tag{10}$$

Despite the formation of these potentially deleterious species, reaction (10) can protect cells from oxidative damage through the scavenging of O_2^-, thereby

abating the generation of ferrous ions (essential to oxidant formation in the Fenton reaction) and consecutive Haber-Weiss reaction chemistry (Wink et al., 2001).

$$\text{Fe(III)} + \text{O}_{2^-} \rightarrow \text{Fe(II)} + \text{O}_2 \tag{11}$$

$$\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{OH}^{\bullet} + \text{H}_2\text{O} + \text{Fe(III)} \text{ or } \text{Fe(V)O} + \text{H}_2\text{O} \tag{12}$$

However, despite the focus on peroxynitrite, reaction (12) is more diverse resulting in a variety of different reactions that can influence biology (Miles et al., 1996; Wink et al., 1997; Espey et al., 2002c, 2002d). There are three types of chemistry that have to be considered in order to gain an understanding of this reaction in biology. The first is whether O_2^- and NO react with each other or with other substrates in vivo. Although the reaction between these two species has been reported to be close to the diffusion limit of 10^{10} M^{-1} s^{-1} (Huie and Padmaja, 1993; Goldstein and Czapski, 1995), certain concentrations of other substrates can make alternative reactions more probable in biological situations. Since the concentration of NO is less than 0.1 μM and O_2^- is likely to be pM, the kinetics of either NO reactions with HbO_2 or O_2^- with SOD can out-compete peroxynitrite formation in cells and tissues (Kelm et al., 1997) SOD concentrations range from 2 to 10 μM in cells and are probably much higher in organelles such as the mitochondria (Nikano et al., 1990). Thus, NO concentrations need to be 0.1 to 1 μM to be competitive enough to form significant amounts of peroxynitrite.

In blood, NO sequestration by erythrocytes dominates its reactivity. As discussed above, the rate of NO scavenging by erythrocytes in blood has a rate similar to that of 10 μM HbO_2 because of the membrane barrier. Kelm et al. examined the NO/O_2^- reaction in the presence 5 to 20 μM HbO_2 and ferricytochrome c (Kelm et al., 1997). Remarkably it was found that NO would react 100% with HbO_2 despite the presence of O_2^-. Conversely, HbO_2 preserved the reaction between O_2^- and ferricytochrome c. Analysis of similar NO fluxes exposed to cells shows that this level of NO would stimulate HIF-1α in some cells (Thomas et al., 2004). This is notably the flux of O_2^-, which is expected to be generated by activated macrophages or neutrophils (Miles et al., 1995). Thus, peroxynitrite formation may be limited under conditions of concomitant NO and O_2^- consumption by, for example, hemeproteins and SOD.

If the radicals do react to form $ONOO^-$, the anion itself can react directly with a variety of complexes prior to the formation of oxidants. For example, peroxynitrite will react with GSH (rate = 10^4 M^{-1} s^{-1}) (Radi et al., 1991), selenium-containing proteins such as glutathione peroxidase (10^6 M^{-1} s^{-1}) (Sies et al., 1997; Fu et al., 2001), and heme proteins with rate constants ranging from 10^5 to 10^7 M^{-1} s^{-1} (Floris et al., 1993; Herold and Shivashankar, 2003; Boccini and Herold, 2004). These kinetics suggest that peroxynitrite will usually interact with other substances in the cell prior to reaction to form the oxidants.

The decomposition of $ONOO^-$ through HOONO is relatively slow compared to other processes in vivo. This suggests that these other reactions, namely those with GSH, would most likely be predominant with no appreciable generation of

oxidant such as $OH^{\bullet}$ and NO_2. The first-order rate varies with pH with a pK_a of 6.8 (Pryor and Squadrito, 1996). Thus, at neutral pH, the half-life for the decomposition through HOONO is 1 second. If we examine targets in the cytoplasm where GSH is 2 to 10 mM, it can be predicted that $ONOO^-$ will quantitatively react with the thiol prior to formation of oxidants (Radi et al., 1991). Thus, intracellular formation of these oxidants such as $OH^{\bullet}$ in the cytoplasm becomes severely limited. Even if some oxidants do form, their diffusion-controlled reaction with GSH will limit any further reactivity. Thus, the original hypothesis of hydroxyl radical formation from peroxynitrite and subsequent increased cytotoxicity has considerably limited value in a biological context except as noted below.

Peroxynitrite will react with CO_2 to form the oxidants carbonate radical ($CO_3^{-\bullet}$) and NO_2, representing a mechanism by which peroxynitrite formation can effectively generate reactive species (reaction 13) (Lymar and Hurst, 1995). The reaction between CO_2 and $ONOO^-$ is 1×10^4 M^{-1} s^{-1} to form CO_2OONO^- (reaction 13; Fig. 2.3) (Coddington et al., 2001):

$$ONOO^- + CO_2 \rightarrow CO_2OONO^- \rightarrow CO_3^{-\bullet}/NO_2 \rightarrow NO_3^- + CO_2 \qquad (13)$$

These processes make the formation of the oxidants competitive with other consumptive mechanisms in the cell. Physiological levels of carbonate (25 mM) translate into concentrations of nearly 1 mM CO_2. Since the rate constant for reaction (13) is similar to that with GSH, the reaction is competitive in allowing some formation of CO_3^- and NO_2. This effect can be referred to as "the RNOS shunt." Peroxynitrite can react with the selenium protein glutathione peroxidase at 10^7 M^{-1} s^{-1} and heme proteins, such as hemoglobin, peroxidase, and myoglobin at 10^{5-7} M^{-1} s^{-1}, which out-competes the oxidant formation via HOONO decomposition. However, the CO_2 reaction with $ONOO^-$ to generate oxidants is competitive with these other rates. In some cells the location of $ONOO^-$ formation will determine its reactivity. With CO_2 levels of about 1 mM, selenium, thiol, and heme proteins have to be present at concentrations of 10 to 100 μM to be competitive. Thus the oxidants that are relevant to $ONOO^-$ in vivo are $CO_3^{-\bullet}$ and NO_2, not $OH^{\bullet}$ (Beckman et al., 1990).

Even if the radicals $CO_3^{-\bullet}$ and NO_2 are formed, further constraints on their reactivity must be considered. The carbonate radical is an anion with a predicted short lifetime that will not readily migrate through membranes. The cytotoxicity of bolus peroxynitrite is nearly abated in the presence of bicarbonate because of formation of the anion species rather than ONOOH (Zhu et al., 1992; Hurst and Lymar, 1997). However, it has been shown that NO_2 produced in this reaction can migrate into cells, resulting in oxidation (Espey et al., 2002c, d). This may be nevertheless limited to the hydrophobic regions and GSH and ascorbate-depleted areas, since they react at 10^7 M^{-1} s^{-1}. Urate found in blood is an effective scavenger of NO_2 and $CO_3^{-\bullet}$ as well, thereby limiting the participation of these radicals in blood or the endothelial lining of blood vessels (Kooy et al., 1994; Reiter et al., 2000; Miranda et al., 2002). Thus, the oxidation and nitration reaction will be confined to a small area directed by the site of O_2^- formation (Espey et al., 2002c).

Although powerful oxidants are formed, these proposed radical species will react with NO and O_2^- at near diffusion-controlled rates. In the presence of excess O_2^-, the oxidation is quenched, presumably through reactions represented in reactions (14) and (15):

$$O_2^- + CO_3^{-\bullet} \rightarrow CO_3^{2-} + O_2 \quad (14)$$

$$O_2^- + NO_2 \rightarrow NO_2OO^- \rightarrow NO_3^- + O_2 \quad (15)$$

Goldstein and Czapski proposed a NO_2OO^- species (Olson et al., 2003). However, this oxidant would be less powerful under these conditions than $ONOO^-$. This is probably because the rapid decomposition reaction makes the reaction more of a detoxification mechanism than a reaction to generate oxidants.

When NO is in excess, $CO_3^{-\bullet}$ reacts with NO via a rate constant of 10^9 M^{-1} s^{-1}, resulting in NO_2^- and CO_3^{2-} (reaction 16) (Neta et al., 1988):

$$CO_3^{-\bullet} + NO + H_2O \rightarrow CO_3^{2-} + NO_2^- + 2H^+ \quad (16)$$

This leaves NO_2 as the major reactive species to oxidize protein substrates. However, NO_2 also reacts with NO to form N_2O_3, which mediates nitrosation reactions and leads to the formation of NO_2^-. Thus nitrosation, and not oxidation/nitration, is the primary chemistry of the NO/O_2^- reaction. These secondary reactions of NO and O_2^- show how the radical fluxes fine-tune the environment, changing from oxidative to nitrosative profiles (Grisham et al., 1999; Jourd'heuil et al., 1999; Espey et al., 2002c).

The NO/O_2^- reaction can be understood if the location of where and when these radicals interact in vivo is considered. As pointed out above, there are considerable constraints placed on the formation of peroxynitrite such as competitive reactions of oxyHb and SOD in vivo. The largest production O_2^- is from NADPH oxidase (NOX-2) (gp91), and for NO it is from iNOS under inflammatory conditions (Vignais, 2002). These conditions favor the modification of proteins from this reaction. Extracellular production of these radicals has to be timed perfectly in order to have appreciable nitrative chemistry from peroxynitrite. The induction of iNOS and the oxidative burst do not necessarily occur simultaneously during the course of an inflammatory reaction. It has been shown in murine macrophages that interferon and LPS stimulation results in a ROS burst initially followed by NO production 4 to 6 hours later (Pfeiffer et al., 2001b).

Enzyme location is also an important consideration. It should be noted that NOX-2 primarily produces O_2^- extracellularly, suggesting that $ONOO^-$ and other RNOS have to migrate to intracellular compartments. The best models for testing this reaction are extracellular O_2^- generation in the presence of an NO generator (Thomas et al., 2002b). The reaction of CO_2 with peroxynitrite limits its migration as well as excludes $CO_3^{-\bullet}$ from migrating across membranes. However, NO_2 can migrate into the cell where, depending on the flux of NO, NO_2 can facilitate oxidation (1 V) or nitrosation from a further reaction of NO to form N_2O_3 (Espey et al., 2002c). These less powerful oxidants may explain why the

NO/O_2^- reaction is not toxic (Wink et al., 1993a). This reaction illustrates how NO in the presence of ROS abates the species that have the potential to mediate cellular damage. NO is therefore an antioxidant and the formation of $ONOO^-$ is an intermediate in this pathway (Fig. 2.3). However, as discussed below, this reaction may lead to nitrosative rather than nitrative chemistry because of kinetic constraints.

Nitrite as Source of RNOS Nitrite is a stable end-product of NO autoxidation (Ignarro et al., 1993). While this statement may be true in the context of a simple cell culture system, recent evidence suggests nitrite in vivo can be further metabolized to active chemical species. Nitrite can either be oxidized to form NO_2 or reduced to form NO (summarized in Wink, 2003). The key determinant of these chemical outcomes is the oxidation state of competent metallo-enzymes or transition metals within the tissue.

As will be discussed below, nitrite oxidation via peroxidase/H_2O_2 oxidation has been suggested as a mechanism by which nitration occurs (reaction 17) (Eiserich et al., 1998; Thomas et al., 2002a):

$$\text{Fe(III) or Fe(II)} + H_2O_2 \rightarrow \text{Fe(IV) or Fe(V)} + NO_2^- \rightarrow \text{Fe(III)} + NO_2 \quad (17)$$

It has been found that oxidation of nitrite readily forms NO_2, which can facilitate intracellular protein modifications such as nitration of the green fluorescent protein (GFP) (Espey et al., 2002d). This reaction has been proposed as a mechanism by which nitrite imposes antibacterial properties (Klebanoff, 1993). Furthermore, other oxidants produced in Fenton-type reactions also result in nitrite to NO_2 conversion. Nitrogen dioxide formed in the absence of NO is a mild oxidant that can readily migrate through membranes to oxidize thiols and induce lipid peroxidation (Pryor, 1982; Hogg et al., 1993). This is why nitrite enhances the cytotoxicity of peroxides (Wink et al., 1996a). Thus, under oxidative conditions nitrite can be oxidized to NO_2, leading to oxidation and nitration of different proteins.

Oxygen is an obligate substrate for the oxidation of L-arginine by NOS to form NO. Recent reports show that there are oxygen-independent mechanisms of NO formation in vivo (Wink, 2003; Dejam et al., 2004; Gladwin and Schechter, 2004). Reduction of nitrite to NO has been shown to occur under hypoxic conditions, causing vascular relaxation (Cosby et al., 2003). It has also been demonstrated that tissues contain reservoirs of nitrite that are lost when exposed to brief periods of hypoxia (Bryan et al., 2004). These findings indicate that nitrite may be an important source of NO under oxygen-depleted conditions, serving as a hypoxic buffer (Wink, 2003). This presents a new mechanism to generate NO that does not require NOS. Where NO production from NOS is an oxygen-dependent process, nitrite is probably reduced to NO through the interaction with ferrous heme proteins, at least under hypoxic conditions (Gladwin et al., 2004). These findings suggest that NO can be generated in hypoxic regions of tissue, thus explaining why increasing local tissue nitrite levels may be beneficial. This

could result in the formation of metal–NO complexes that may be a source for these adducts in vivo.

It has been reported that xanthine oxidase (XO) has nitrite reductase activity (Li et al., 2005). We have found that this reaction in the presence of physiologic levels of nitrite is greatly enhanced under anaerobic conditions and in the presence of reductant (e.g., ascorbate, cysteine, NADH). Since XO is a major constituent of the peroxisome, a link between peroxisome proliferating activating receptor (PPAR) signaling and nitrite chemistry could be important.

HNO Chemistry The importance of nitrogen to biology has long been understood to originate from its capacity to exist in all oxidation states from +5 to −3. Reduced nitrogen oxides, such as HNO (+1) and hydroxylamine (NH_2OH) (−1), are known to be significant in prokaryotic chemistry, such as during conversion of NO_3^- (+5) to NH_3 (−3) by bacterial reductases (Zumft, 1993; Wasser et al., 2002). These reduced species have often been ignored in mammalian physiology due to the central role of NO and the oxidizing environment of mammalian cells. As a result there has been limited interest in reduced nitrogen oxides, particularly HNO, even in the original studies attempting to determine the identity of the EDRF (Murphy and Sies, 1991; Fukuto et al., 1992a, b, 1993; Dierks and Burstyn, 1996; Ellis et al., 2000). In fact investigations by several laboratories have suggested that oxidation of L-arginine by NOS initially produces HNO, which is then converted to NO either by NOS itself or by other metalloproteins (Fukuto et al., 1992b, c; Hobbs et al., 1994; Pufahl et al., 1995; Schmidt et al., 1996; Clague et al., 1997; Adak et al., 2000). Although release of HNO by NOS has yet to be observed in vivo, these preliminary investigations have led to several in vitro, in vivo, and ex vivo comparisons of the pharmacological effects of donors of NO and HNO.

A comparison of different agents in clonogenic assay systems showed that HNO donors (LD_{50} of 2 mM to fibroblasts; Wink et al., 1998a) are several orders of magnitude more toxic than NO donors. Furthermore, simultaneous exposure to HNO and peroxide decreased clonogenic cell survival, while NO confers protection against an oxidative insult (Ma et al., 1999). This analysis provided the first indication that HNO and NO exhibit distinct biological properties. Moreover the cytotoxicity of HNO, induced in part by double-strand DNA breaks and base oxidation, required an aerobic environment and was dramatically enhanced by chemical depletion of intercellular glutathione (GSH) (Wink et al., 1996b, 1998a; Ohshima et al., 1998, 1999b; Chazotte-Aubert et al., 1999; Miranda et al., 2002). This suggested the existence of specific molecular targets and reactive nitrogen oxide progeny for HNO in vivo, distinct from those of NO.

These results prompted us to begin to examine the chemical biology of HNO and to collaboratively perform more complex in vitro and in vivo comparisons of HNO and NO donors. Investigation of HNO migration and reactivity within cells under aerobic conditions revealed that it was distinct from that of NO (Espey et al., 2002a). Analogous to the consequences of in vitro exposure, infusion of HNO increased myocardial damage in a rabbit model of ischemia/reperfusion

injury, in direct contrast to the protective effects of NO (Ma et al., 1999; Vidwans et al., 1999). Since nitrogen oxides are known to influence neuronal function by affecting calcium channel function (Lipton and Stamler, 1994; Gbadegesin et al., 1999; Kim et al., 1999; Vidwans et al., 1999), we compared the effects of NO and HNO donors on *N*-methyl-D-aspartate (NMDA) receptor/channel response (Colton et al., 2001). Short-term, pulsed aerobic infusion of NO donors potentiated glutamate-stimulated calcium influx, with an enhanced effect under hypoxia. Substitution of an HNO donor in the perfusate produced similar augmentation aerobically; however, under hypoxia HNO attenuated channel response. These findings suggested an intriguing possibility for condition-specific control of channel flow by modulation of the nitrogen oxide product of NOS.

Perhaps even more important, it has been shown that HNO donors exhibit unique cardiovascular properties (Paolocci et al., 2001). In 2001 we showed that HNO donors can increase myocardial contractility and specifically dilate the venous side of the circulation, cardiovascular properties that appear to be ideal for the treatment of congestive heart failure (Paolocci et al., 2003). In addition it was found that HNO donors can function as chemical preconditioning agents, providing protection against myocardial infarction (Pagliaro et al., 2003). An intriguing aspect to these studies is that NO and HNO often induce opposite pharmacological effects, suggesting distinct functional mechanisms as a result of dissimilar chemical interactions (Wink et al., 2003).

The discrete chemistry of HNO and NO is exemplified by our measurement of elevated plasma levels of cGMP only following exposure to NO donors (Miranda et al., 2003b) and of the neuropeptide calcitonin gene-related peptide (CGRP) only in response to HNO exposure (Paolocci et al., 2001). That ferrous soluble guanylyl cyclase is activated to produce cGMP at low concentrations of NO ($<$100 nM; Morley et al., 1993) suggests that NO and HNO do not interconvert in blood (Miranda et al., 2003b). However, the literature prior to 2000 suggested that these species should readily interchange in biological environment (Bonner and Stedman, 1996). This discrepancy prompted a reexamination of the fundamental aqueous chemistry of HNO and related species.

In 1970 Gratzel and co-workers (Gratzel et al., 1970) examined the chemistry of NO^- via the reduction of NO by the hydrated electron generated during pulse radiolysis and reported a pK_a for HNO of 4.7 (spin states not specified). Based on this value, the standard potentials of the NO/$^3NO^-$ and NO/$^1NO^-$ couples were derived to be 0.39 and -0.35 V (1 M vs. NHE), respectively (Stanbury, 1989). These values suggest both that NO^- is the predominant species under physiological conditions and that NO should readily be oxidized to NO^-. However, that NO clearly functions as a physiological signal and the observations of distinct effects of NO and HNO donors in biological systems suggested a potential much lower than 0.39 V. In a collaborative reexamination we determined that in fact the NO/$^3NO^-$ standard potential is below -0.7 V and correspondingly that the pK_a of HNO exceeds 11 (Bartberger et al., 2002). These values support earlier, largely disregarded experimental analyses of the NO reduction potential (Ehman and Sawyer, 1968; Benderskii et al., 1989) and are now in perfect

agreement with the pharmacological differences observed between HNO and NO donors.

Since these studies the aqueous chemistry of HNO has been refined further. Of highest importance is the unconventional acid–base relationship between HNO and NO^- due to different ground state spins. The requirement for a spin-forbidden proton transfer essentially inhibits the establishment of an equilibrium such that HNO chemistry alone will be observed at physiological conditions (Shafirovich and Lymar, 2002). Furthermore, HNO predominantly functions as an electrophile while NO^- is a strong reductant (Bartberger et al., 2002). By comparing the relative rates of the reactions of an HNO donor with a variety of biological substrates and then utilizing the known rate constant for HNO dimerization (Shafirovich and Lymar, 2002), the absolute rate constants for these reactions were estimated (Liochev and Fridovich, 2003; Miranda et al., 2003b). This has provided for the first time a quantitative picture of the reactivity of HNO: the high reactivity of HNO with thiols and oxidized metals is likely to be responsible for the majority of the unique biological effects of HNO.

2.3 CHEMISTRY OF METABOLITE FORMATION

2.3.1 Nitrite and Nitrate Formation

The formation of nitrite and nitrate are important indicators of the biology of NO. Where NO_3^- is generally considered inert, being in the highest valence state of nitrogen, nitrite, as discussed above, can be converted into either NO or NO_2, depending on the redox environment. Therefore in eukaryotic organisms nitrate may be considered a final NO end-product, while nitrite can still be considered an active substrate. It should be noted, however, that many prokaryotic organisms readily convert nitrate to nitrite and further to NO and ammonia (Zumft, 1993; Wasser et al., 2002). This is also known to occur within the oral and gut flora. Nitrite is formed from nitrate by the reductive activity of commensal bacteria in the oral cavity to end up via swallowed saliva as acidified nitrite in the stomach (Correa et al., 1975; Duncan et al., 1995; McKnight et al., 1997; Yeomans et al., 1995). It has been proposed that this nitrite has antimicrobial properties (McKnight et al., 1999). When nitrite is infused into blood, it is rapidly converted to nitrate (Rassaf et al., 2003b). This oxidation reaction probably serves to control the nitrite chemistry in vivo.

Nitrate can be derived from various reactions. The predominant source of nitrate in vivo is either the reaction between NO and HbO_2 or other metal–O_2 species. Nitrite can be oxidized through a complex series of reactions to nitrate via oxyhemoglobin (reaction 1). Another route for NO_3^- formation is from dimerization of NO_2 and hydrolysis, to give equimolar NO_3^- and NO_2^- (reaction 18):

$$2NO_2 \rightarrow N_2O_4 \xrightarrow{H_2O} NO_2^- + NO_3^- \qquad (18)$$

This would occur under conditions where only NO_2 was produced and in very high concentrations in the absence of other substrate, so as to simply convert NO_2 to NO_2^-. If NO is formed in the presence of NO_2, N_2O_3 formation will predominate, yielding exclusively NO_2^-.

There are number of potential mechanisms by which nitrite can be derived. Under cell culture conditions nitrite is most easily formed via reactions of the autoxidation reaction. As discussed above, kinetic analyses indicate that this mechanism is only relevant in tissue under limited conditions where NO levels are very high. Kinetically one of the primary sources of nitrite formation is the production of NO in the presence of ROS. In Fenton chemistry the oxidants convert NO to NO_2^- (reaction 2). It should be noted, however, that under oxidative conditions nitrite may be immediately oxidized further to form nitrate. This has been proposed as a mechanism to consume and control NO. Although the NO/O_2^- yields peroxynitrite, which can generate nitrate, the primary product is nitrite due to subsequent reactions either with excess NO or O_2^-. Only when the fluxes of both radicals are exactly 1:1 is NO_3^- formed (Pfeiffer et al., 1997). The decomposition of HNO under aerobic conditions can give NO_3^- and NO_2^- (Miranda et al., 2001).

Thus, when determined in tissues and biofluids such as plasma and cerebrospinal fluid, measurement of the nitrate to nitrite ratio can provide important insight into the redox environment (Rassaf et al., 2003b; Bryan et al., 2004).

2.3.2 Metal Nitrosyl Formation

The metal–NO signal in heme proteins detected by chemiluminescence and by EPR has provided a powerful indication for the presence of NO (Maples et al., 1991; Stadler et al., 1993a; Gladwin et al., 2000; Rassaf et al., 2003a; Bryan et al., 2004). The sensitivity of the chemiluminescent techniques is nM while ESR methods are less sensitive, allowing one to measure EPR signals of metal-nitrosyls only under inflammatory conditions. The heme and nonheme iron signals can be readily distinguished by their EPR signature (Chamulitrat et al., 1995). However, under acidic conditions employed in the chemiluminescent method, Fe(S) decomposes rapidly. Only the heme signature can be expected to be stable under conditions for the chemiluminscence (Gladwin et al., 2000; Rassaf et al., 2003b; Bryan et al., 2004).

2.3.3 Nitrosation

Nitrosated protein moieties are some of the most commonly observed indicators of NO-related chemistry in vivo. The amount of different products depends on the amount of NO and correlates with the amount of nitrite and nitrate produced. As discussed above, the nitrosation of substrates can occur at thiol, amine, and hydroxy functionalities. Each can have a unique mechanism for its formation; it can form either in a metal-catalyzed fashion, via nitrosonium ions, or oxidative nitrosylation.

It has long been known that nitrosamines can be formed in vivo, in particular, under inflammatory conditions (Wishnok et al., 1993). If secondary amines could be derived from dietary sources, there was concern that these could conceivably represent precursors of nitrosamines. More recently it has been found that nitrosation of different amino acids does occur, suggesting that proline, lysine, and tryptophan can generate their corresponding nitrosamine (Liu et al., 1992; Wishnok et al., 1993; Bryan et al., 2004). There are two mechanisms by which amines can be nitrosated. One is oxidative nitrosylation, and the other is nitrosation by N_2O_3 or derivative X–NO. The latter occurs through N_2O_3 and donation of NO^+ equivalents. Tryptophan and proline, as well as unprotonated lysine, are not likely to undergo oxidative nitrosylation reactions. Although tryptophan and proline form stable secondary nitrosoamines, lysine forms a primary nitrosamine that rapidly deaminates to form homoserine. The detection of nitrosoproline or nitrosotryptophan may be a good indication of the presence of N_2O_3. These adducts have been proposed to represent part of the mercury-insensitive signal seen in tissues under physiological conditions (Bryan et al., 2004). It also has been found that inflammatory conditions generate nitrosoproline. This may be taken as evidence that N_2O_3 or nitrosation from X–NO adducts does occur in tissue under inflammatory conditions.

The requirement for oxidative nitrosation is that the oxidant be powerful enough to oxidize the amine. In the case of tryptophan and proline, the oxidation potential is >1.6 V (data not shown). Since only species such as Fe-oxo and $OH^{\bullet}$ radical have the sufficient oxidation potential, NO_2 and even $CO_3^{-\bullet}$ from $ONOO^-$ should not be able to oxidize these residues. Therefore, formation of these adducts in vivo should indicate that N_2O_3 formation has occurred.

S-Nitrosation is an important marker of nitrogen oxide chemistry. In addition to being an intermediate in the metabolism of nitroglycerin (GTN) and other nitrovasodilators (Ignarro et al., 1980), its endogenous formation has been proposed to modify cellular responses and regulate cell death. As discussed above, there are a number of different reactions that should be considered in the formation of RSNOs. Metal-mediated nitrosation reactions, oxidative nitrosylation reactions, and nitrosation via N_2O_3 can all potentially nitrosate thiols and amines. In the case of RSNO formation, all three reactions have been proposed to account for the formation of *S*-nitrosothiols in biological systems.

The reactions involved in the formation of RSNO are too diverse and complex to be readily assigned to a specific chemical reaction. Although N_2O_3 results in the generation of RSNOs, RSNOs can also be formed from metal-catalyzed reactions. It has been proposed that heme proteins can transfer NO^+ to form proteinous RSNOs (Wade and Castro, 1990; Vanin, 1995). However, this is believed not to occur to any great extent. Yet other heme proteins such as nitrophorins may generate RSNOs via a unique chemistry involving thiols and metals (Weichsel et al., 2005). More important, RSNO can be formed from non-heme complexes such as Fe(S) complexes and cobalamin, which can generate RSNO from NO (Vanin, 1995; Brouwer et al., 1996). The latter reactions have been much less studied but may provide important sources of RSNO in vivo.

If reduced glutathione (GSH) reacts with NO in the presence of O_2 in aqueous solution, *S*-nitrosoglutathione is the exclusive product with no GSSG formation. However, under low fluxes of NO and in low oxygen in hydrophobic regions of tissue, it is possible for radical-radical coupling of the thiyl radical and NO to lead to RSNO formation (Jourd'heuil et al., 2003). NO_2 reacts with GSH to form thiyl radical, which can undergo different reactions (reaction 19, $k = 3 \times 10^7\ M^{-1}\ s^{-1}$). The resulting thiyl radical can react with GS^- to give $GSSG^-$ (reaction 21, $k = 8 \times 10^8\ M^{-1}\ s^{-1}$), which reacts with O_2 to form superoxide (reaction 22, $k = 5 \times 10^8\ M^{-1}\ s^{-1}$) (Halliwell and Gutteridge, 1999).

$$NO_2 + GSH \rightarrow NO_2^- + GS \tag{19}$$

$$GSH \rightleftharpoons GS^- + H^+ \qquad (pK_a = 9.2) \tag{20}$$

$$GS^\bullet + GS^- \rightarrow GSSG^{-\bullet} \tag{21}$$

$$GSSG^{-\bullet} + O_2 \rightarrow GSSG + O_2^- \tag{22}$$

Alternatively, the thiyl radical can react with O_2 to form GSO_2H (reaction 23, $k_{22} = 3 \times 10^7\ M^{-1}\ s^{-1}$), which decomposes to GSSH and GSOH (Halliwell and Gutteridge, 1999):

$$GS^\bullet + O_2 \rightleftharpoons GSOO^\bullet \tag{23}$$

These radical reactions may be important in NO/O_2^- chemistry, and they demonstrate why GSNO is not readily formed in this reaction. Thus, it not likely that a significant amount of RSNO can be derived under normoxic conditions via oxidative nitrosylation reactions.

2.3.4 Nitration

The nitration of tyrosine and other aromatic amino acids has been thought to represent an important footprint for the production of nitric oxide under inflammatory conditions. While the anti-nitrotyrosine antibody has been one of the most employed tools to evaluate the presence of NO and RNOS in biology, the results obtained with this tool have also been subject to considerable misinterpretation of experimental results. A long-standing area of controversy over which chemistries may primarily account for nitration reactions in vivo and what the accompanying redox conditions may be is now over. There are a number of reactions that can lead to the nitration of tyrosine residues and phenolic compounds. Of note, the acidification of nitrite and nitrate was one of the first methods employed to generate nitrotyrosine (NT), and it is the commercial method of choice to generate NT (Giese and Riordan, 1975). Although these results are typically obtained at pH 1, small amounts of NT can also be generated at a more physiologically relevant pH.

There are several reactions that have been proposed as a source of nitrotyrosine at neutral pH. Peroxynitrite as a bolus can nitrate tyrosine (Beckman et al., 1992). The HNO/O_2 reaction can generate nitrotyrosine in proteins, but it does

not nitrate phenolic compounds or amino acid complexes to any appreciable extent (Ohshima et al., 1999a). The NO/O_2 reaction can generate nitration either from NO_2 (in hydrophobic regions) or from the nitrosation of tyrosine, which oxidatively decomposes to form NT (Wink et al., 1994; Simon et al., 1996). Finally, the oxidation of nitrite by peroxidases and Fenton-type reactions to give NT may well account for the majority of NT detected in biological samples (Eiserich et al., 1998; Sampson et al., 1998; Hazen et al., 1999; Espey et al., 2002d; Thomas et al., 2002a).

Peroxynitrite has often been correlated with the formation of NT (Viera et al., 1999). Although synthetic peroxynitrite delivered as a bolus will nitrate tyrosine residues, the yield of this reaction is rather low (1–2% at 1 mM; Pryor and Squadrito, 1996). Even if CO_2 does could double the yield of NT (Gow et al., 1996), the efficacy of the nitration reaction is subject to the flux of concomitantly generated NO and O_2^- (Pfeiffer and Mayer, 1998). It was shown that NO/O_2^- resulted in nitration only when the flux was about 1:1, the same as the constraints for oxidation and the formation of nitrate discussed above. The yield of nitration via the simultaneous formation of NO and O_2^- is even lower than that with bolus peroxynitrite, suggesting that this reaction pathway is not likely to play a significant role in biology (Pfeiffer and Mayer, 1998; Espey et al., 2002d; Thomas et al., 2002a).

The NO autoxidation reaction can also generate NT. When tyrosine is exposed to NO/O_2 in solution where N_2O_3 is predominant, nitrosotyrosine is formed. This occurs with free amino acids and with proteins. In both cases, after several hours, this adduct rearranges to form nitrotyrosine (Wink et al., 1994; Goldstein and Czapski, 1996; Simon et al., 1996). This suggests that detection of NT is not necessarily an indicator of nitrative chemistry alone but involves nitrosation reactions as well.

Whether or not endogenous formation of HNO occurs, it does form an oxidant similar to peroxynitrite. However, it appears that at neutral pH there is no associated radical type oxidation. For instance, peroxynitrite causes HPA to dimerize while HNO does not. Furthermore, where peroxynitrite nitrates HPA and tyrosine, the HNO/O_2 reaction does not. However, HNO/O_2 will nitrate proteins such as bovine serum albumin with similar efficiency as the NO/O_2^- generator SIN-1 (Miranda et al., 2002). Thus it is possible to generate nitration products via HNO-related chemistry. The kinetics of the reaction of HNO with O_2 are very slow compared to the reactions with thiols and heme proteins. With the exception of membrane regions, this reaction therefore seems to be an unlikely source of NT.

In the late 1990s it was shown that peroxidase-mediated oxidation of nitrite could result in the nitration of tyrosine. It has been debated whether this mechanism is the primary source of NT (Baldus et al., 2002). Although reports show that MPO knockouts still have nitration (Ichimori et al., 2003), it has been demonstrated that numerous heme and transition metal complexes can generate NT (Thomas et al., 2002a). A comparison of NT at more physiologically relevant fluxes of NO and O_2^- clearly demonstrates that nitration really occurs only when

the fluxes are 1:1 (Espey et al., 2002c, 2002d). Importantly, the presence of a heme catalyst eliminates this constraint increasing the yield and ubiquity (Thomas et al., 2002a), thus providing a source of NT without the constraints of the classical NO/O_2^-, NO/O_2, and HNO/O_2 reactions. These metal-mediated nitration reactions also offer an explanation for the apparent specificity of NT observed under inflammatory conditions.

ACKNOWLEDGMENTS

This research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

LIST OF ABBREVIATIONS

BSA, bovine serum albumin
CGRP, calcitonin gene-related peptide
CNS, central nervous system
CO_2, carbon dioxide
$CO_3^{-\bullet}$, carbonate radical
EC50, effective concentration, 50%
EDRF, endothelium-derived relaxant factor
EPR, electron paramagnetic resonance
GC, guanylyl cyclase
GFP, green fluorescent protein
GSH, glutathione
GSSG, oxidized glutathione
GTN, nitroglycerin
H_2O_2, hydrogen peroxide
Hb, hemoglobin
HbO_2, oxyhemoglobin
HIF-1α, hypoxia inducible factor
HNO/NO^-, nitroxyl
INOS, inducible nitric oxide synthase
IRBs, iron regulatory binding protein
LD50, lethal dose, 50%
LPS, lipopolysaccharide
Mb, myoglobin
MPO, myleoperoxidase
MtHb, methemoglobin

N_2O_3, dinitrogen trioxide
NH_2OH, hydroxylamine
NHE, Nerst hydrogen electrode
NO, nitric oxide
$NO_2^{\bullet}$, nitrogen dioxide
NO_2^-, nitrite
NO_3^-, nitrate
NOS, nitric oxide synthase
NOX-2 (gp91), NADPH oxidase
NMDA receptor/channel, *N*-methyl-D-aspartate receptor/channel
NT, nitrotyrosine
$OH^{\bullet}$, hydroxyl radical
$ONOO^-$, peroxynitrite
PPAR, peroxisome proliferating activating receptor
RNOS, reactive nitrogen oxide species
RSNO, *S*-nitrosothiol(s)
SOD, superoxide oxide dismutase
XO, xanthine oxidase

REFERENCES

Abu-Soud H.M., Hazen S.L. (2000). Nitric oxide is a physiological substrate for mammalian peroxidases. *J Biol Chem* 275: 37524–37532.

Adak S., Wang Q., Stuehr D.J. (2000). Arginine conversion to nitroxide by tetrahydrobiopterin-free neuronal nitric-oxide synthase. Implications for mechanism. *J Biol Chem* 275: 33554–33561.

Aulak K.S., Koeck T., Crabb J.W., Stuehr D.J. (2004). Proteomic method for identification of tyrosine-nitrated proteins. *Methods Mol Biol* 279: 151–165.

Baldus S., Eiserich J.P., Brennan M.L., Jackson R.M., Alexander C.B., Freeman B.A. (2002). Spatial mapping of pulmonary and vascular nitrotyrosine reveals the pivotal role of myeloperoxidase as a catalyst for tyrosine nitration in inflammatory diseases. *Free Radic Biol Med* 33: 1010.

Bartberger M.D., Liu W., Ford E., Miranda K.M., Switzer C., Fukuto J.M., Farmer P.J., Wink D.A., Houk K.N. (2002). The reduction potential of nitric oxide (NO) and its importance to NO biochemistry. *Proc Natl Acad Sci USA* 99: 10958–10963.

Bastian N.R., Yim C.Y., Hibbs J.B.J., Samlowski W.E. (1994). Induction of iron-derived EPR signals in murine cancers by nitric oxide: Evidence for multiple intracellular targets. *J Biol Chem* 269: 5127–5131.

Beckman J.S., Beckman T.W., Chen J., Marshall P.H., Freeman B.A. (1990). Apparent hydroxyl radical production by peroxylnitrites: Implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 87: 1620–1624.

Beckman J.S., Ischiropoulos H., Zhu L., van der Woerd M., Smith C., Chen J., Harrison J., Martin J.C., Tsai M. (1992). Kinetics of superoxide dismutase- and iron-catalyzed nitration of phenolics by peroxynitrite. *Arch Biochem Biophys* 298: 438–445.

Benderskii V.A., Krivenko A.G., Ponomarev E.A. (1989). Homogeneous and electrode-reactions of NO_2^- ions. *Soviet Electrochem* 25: 154–161.

Boccini F., Herold S. (2004). Mechanistic studies of the oxidation of oxyhemoglobin by peroxynitrite. *Biochemistry* 43: 16393–16404.

Bonner F.T., Stedman G. (1996). The chemistry of nitric oxide and redox-related species. In: *Methods in Nitric Oxide Research*, Wiley, New York, pp. 1–18.

Bouton C., Drapier J.C. (2003). Iron regulatory proteins as NO signal transducers. *Sci STKE* 2003: pp. 1–4 pe17.

Brouwer M., Chamulitrat W., Ferruzzi G., Sauls D.L., Weinberg J.B. (1996). Nitric oxide interactions with cobalamins: Biochemical and functional consequences. *Blood* 88: 1857–1864.

Brown G.C. (1995) Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase. *FEBS Lett* 369: 136–139.

Bryan N.S., Rassaf T., Maloney R.E., Rodriguez C.M., Saijo F., Rodriguez J.R., Feelisch M. (2004). Cellular targets and mechanisms of nitros(yl)ation: An insight into their nature and kinetics in vivo. *Proc Natl Acad Sci USA* 101: 4308–4313.

Chamulitrat W., Jordan S.J., Mason R.P., Litton A.L., Wilson J.G., Wood E.R., Wolberg G., Molina y Vedia L. (1995). Targets of nitric oxide in a mouse model of liver inflammation by *Corynebacterium parvum*. *Arch Biochem Biophys* 316: 30–37.

Chazotte-Aubert L., Oikawa S., Gilibert I., Bianchini F., Kawanishi S., Ohshima H. (1999). Cytotoxicity and site-specific DNA damage induced by nitroxyl anion (NO^-) in the presence of hydrogen peroxide: Implications for various pathophysiological conditions. *J Biol Chem* 274: 20909–20915.

Clague M.J., Wishnok J.S., Marletta M.A. (1997). Formation of N delta-cyanoornithine from NG-hydroxy-L-arginine and hydrogen peroxide by neuronal nitric oxide synthase: Implications for mechanism. *Biochemistry* 36: 14465–14473.

Cleeter M.W., Cooper J.M., Darley-Usmar V.M., Moncada S., Schapira A.H. (1994). Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide: Implications for neurodegenerative diseases. *FEBS Lett.* 345: 50–54.

Coddington J.W., Wherland S., Hurst J.K. (2001). Pressure dependence of peroxynitrite reactions: Support for a radical mechanism. *Inorg Chem* 40: 528–532.

Colton C.A., Gbadegesin M., Wink D.A., Miranda K.M., Espey M.G., Vicini S. (2001). Nitroxyl anion regulation of the NMDA receptor. *J Neurochem* 78: 1126–1134.

Correa P., Haenszel W., Cuello C., Tannenbaum S., Archer M. (1975). A model for gastric cancer epidemiology. *Lancet* 2: 58–60.

Cosby K., Partovi K.S., Crawford J.H., Patel R.P., Reiter C.D., Martyr S., Yang B.K., Waclawiw M.A., Zalos G., Xu X., Huang K.T., Shields H., Kim-Shapiro D.B., Schechter A.N., Cannon R.O. 3rd, Gladwin M.T. (2003). Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med* 9: 1498–1505.

Darley-Usmar V.M., Patel R.P., O'Donnel V.B., Freeman B.A. (2000). Antioxidant actions of nitric oxide. oxide. In: *Nitric Oxide*, Academic Press, San Diego, pp. 265–276.

Dejam A., Hunter C.J., Schechter A.N., Gladwin M.T. (2004). Emerging role of nitrite in human biology. *Blood Cells Mol Dis* 32: 423–429.

Dierks E.A., Burstyn J.N. (1996). Nitric oxide (NO), the only nitrogen monoxide redox form capable of activating soluble guanylyl cyclase. *Biochem Pharmacol* 51: 1593–1600.

Doi K., Akaike T., Horie H., Noguchi Y., Fujii S., Beppu T., Ogawa M., Maeda H. (1996). Excessive production of nitric oxide in rat solid tumor and its implication in rapid tumor growth. *Cancer* 77: 1598–1604.

Doyle M.P., Hoekstra J.W. (1981). Oxidation of nitrogen oxides by bound dioxygen in hemoproteins. *J Inorg Biochem* 14: 351–356.

Drapier J.C., Hibbs J.B.J. (1986). Murine cytotoxic activated macrophages inhibit aconitase in tumor cells. Inhibition involves the iron-sulfur prosthetic group and is reversible. *J Clin Invest* 78: 790–797.

Duncan C., Dougall H., Johnston P., Green S., Brogan R., Leifert C., Smith L., Golden M., Benjamin N. (1995). Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. *Nat Med* 1: 546–551.

Ehman D.L., Sawyer D.T. (1968). *J Electroanal Chem* 16: 541–549.

Eich R.H., Li T., Lemon D.D., Doherty D.H., Curry S.R., Aitken J.F., Mathews A.J., Johnson K.A., Smith R.D., Phillips G.N.J., Olson J.S. (1996). Mechanism of NO-induced oxidation of myoglobin and hemoglobin. *Biochemistry* 35: 6976–6983.

Eiserich J.P., Hristova M., Cross C.E., Jones A.D., Freeman B.A., Halliwell B., van der Vliet A. (1998). Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 391: 393–397.

Ellis A., Li C.G., Rand M.J. (2000). Differential actions of L-cysteine on responses to nitric oxide, nitroxyl anions and EDRF in the rat aorta. *Br J Pharmacol* 129: 315–322.

Espey M.G., Miranda K.M., Pluta R.M., Wink D.A. (2000). Nitrosative capacity of macrophages is dependent on nitric-oxide synthase induction signals. *J Biol Chem* 275: 11341–11347.

Espey M.G., Miranda K.M., Thomas D.D., Wink D.A. (2001). Distinction between nitrosating mechanisms within human cells and aqueous solution. *J Biol Chem* 276: 30085–30091.

Espey M.G., Miranda K.M., Thomas D.D., Wink D.A. (2002a). Ingress and reactive chemistry of nitroxyl-derived species within human cells. *Free Radic Biol Med* 33: 827–834.

Espey M.G., Miranda K.M., Thomas D.D., Xavier S., Citrin D., Vitek M.P., Wink D.A. (2002b). A chemical perspective on the interplay between NO, reactive oxygen species, and reactive nitrogen oxide species. *Ann NY Acad Sci* 962: 195–206.

Espey M.G., Thomas D.D., Miranda K.M., Wink D.A. (2002c). Focusing of nitric oxide mediated nitrosation and oxidative nitrosylation as a consequence of reaction with superoxide. *Proc Natl Acad Sci USA* 99: 11127–11132.

Espey M.G., Xavier S., Thomas D.D., Miranda K.M., Wink D.A. (2002d). Direct real-time evaluation of nitration with green fluorescent protein in solution and within human cells reveals the impact of nitrogen dioxide vs. peroxynitrite mechanisms. *Proc Natl Acad Sci USA* 99: 3481–3486.

Espey M.G., Miranda K.M., Feelisch M., Fukuto J., Grisham M.B., Vitek M.P., Wink D.A. (2000). Mechanisms of cell death governed by the balance between nitrosative and oxidative stress. *NY Acad Sci* 899: 209–221.

Floris R., Piersma S.R., Yang G., Jones P., Wever R. (1993). Interaction of myeloperoxidase with peroxynitrite. A comparison with lactoperoxidase, horse radish peroxidase and catalase. *Eur J Biochem* 215: 767–775.

Ford P.C., Lorkovic I.M. (2002). Mechanistic aspects of the reactions of nitric oxide with transition-metal complexes. *Chem Rev* 102: 993–1018.

Ford P.C., Wink D.A., Stanbury D.M. (1993). Autooxidation kinetics of aqueous nitric oxide. *FEBS Lett* 326: 1–3.

Fu Y., Porres J.M., Lei X.G. (2001). Comparative impacts of glutathione peroxidase-1 gene knockout on oxidative stress induced by reactive oxygen and nitrogen species in mouse hepatocytes. *Biochem J* 359: 687–695.

Fukuto J.M., Chiang K., Hszieh R., Wong P., Chaudhuri G. (1992a). The pharmacological activity of nitroxyl: A potent vasodilator with activity similar to nitric oxide and/or endothelium-derived relaxing factor. *J Pharmacol Exp Ther* 263: 546–551.

Fukuto J.M., Hobbs A.J., Ignarro L.J. (1993). Conversion of nitroxyl (NO^-) in biological systems: The role of physiological and relevance to the biological activity of HNO. *Biochem Biophys Res Commun* 196: 707–713.

Fukuto J.M., Hszieh R., Gulati P., Chiang K.T., Nagasawa H.T. (1992b). *N*,*O*-diacylated-*N*-hydroxyarylsulfonamides: Nitroxyl precursors with potent smooth muscle relaxant properties. *Biochem Biophys Res Commun* 187: 1367–1373.

Fukuto J.M., Switzer C.H., Miranda K.M., Wink D.A. (2005). Nitroxyl (HNO): Chemistry, biochemistry, and pharmacology. *Ann Rev Pharmacol Toxicol* 45: 335–355.

Fukuto J.M., Wallace G.C., Hszieh R., Chaudhuri G. (1992c). Chemical oxidation of N-hydroxyguanidine compounds: Release of nitric oxide, nitroxyl and possible relationship to the mechanism of biological nitric oxide generation. *Biochem Pharmacol* 43: 607–613.

Galijasevic S., Saed G.M., Diamond M.P., Abu-Soud H.M. (2003). Myeloperoxidase upregulates the catalytic activity of inducible nitric oxide synthase by preventing nitric oxide feedback inhibition. *Proc Natl Acad Sci USA* 100: 14766–14771.

Galijasevic S., Saed G.M., Diamond M.P., Abu-Soud H.M. (2004). High dissociation rate constant of ferrous-dioxy complex linked to the catalase-like activity in lactoperoxidase. *J Biol Chem* 279: 39465–39470.

Garavito R.M., Mulichak A.M. (2003). The structure of mammalian cyclooxygenases. *Ann Rev Biophys Biomol Struct* 32: 183–206.

Gaston B., Reilly J., Drazen J.M., Fackler J., Ramdev P., Arnelle D., Mullins M.E., Sugarbaker D.J., Chee C., Singel D.J., Loscalzo J., Stamler J.S. (1993). Endogenous nitrogen oxides and bronchodilator *S*-nitosothiols in human airways. *Proc Natl Acad Sci USA* 90: 10957–10961.

Gbadegesin M., Vicini S., Hewett S.J., Wink D.A., Espey M., Pluta R.M., Colton C.A. (1999). Hypoxia modulates nitric oxide-induced regulation of NMDA receptor currents and neuronal cell death. *Am J Physiol* 277: C673–C683.

Giese R., Riordan J.F. (1975). Nitrotyrosine internal standard for amino acid analysis. *Anal Biochem* 64: 588–592.

Gladwin M.T., Crawford J.H., Patel R.P. (2004). The biochemistry of nitric oxide, nitrite, and hemoglobin: role in blood flow regulation. *Free Radic Biol Med* 36: 707–717.

Gladwin M.T., Schechter A.N. (2004). NO contest: nitrite versus *S*-nitroso-hemoglobin. *Circ Res* 94: 851–855.

Gladwin M.T., Shelhamer J.H., Schechter A.N., Pease-Fye M.E., Waclawiw M.A., Panza J.A., Ognibene F.P., Cannon R.O. 3rd (2000). Role of circulating nitrite and *S*-nitrosohemoglobin in the regulation of regional blood flow in humans. *Proc Natl Acad Sci USA* 97: 11482–11487.

Goldstein S., Czapski G. (1995). The reaction of $NO^{\bullet}$ with $O_2^{\bullet-}$ and $HO_2^{\bullet-}$: A pulse radiolysis study. *Free Radic Biol Med* 19: 505–510.

Goldstein S., Czapski G. (1996). Mechanism of the nitrosation of thiols and amines by oxygenated NO solution: The nature of the nitrosating species. *J Am Chem Soc* 118: 3419–3425.

Gorbunov N.V., Osipov A.N., Day B.W., Zayas-Rivera B., Kagan V.E., Elsayed N.M. (1995). Reduction of ferrylmyoglobin and ferrylhemoglobin by nitric oxide: A protective mechanism against ferryl hemoprotein-induced oxidations. *Biochemistry* 34: 6689–6699.

Goss S.P., Kalyanaraman B., Hogg N. (1999). Antioxidant effects of nitric oxide and nitric oxide donor compounds on low-density lipoprotein oxidation. *Methods Enzymol* 301: 444–453.

Gow A., Duran D., Thom S.R., Ischiropoulos H. (1996). Carbon dioxide enhancement of peroxynitrite-mediated protein tyrosine nitration. *Arch Biochem Biophys* 333: 42–48.

Granger D.L., Taintor R.R., Boockvar K.S., Hibbs J.B. (1996). Measurement of nitrate and nitrite in biological system using nitrate reductase and greiss reaction. *Methods Enzymol* 268: 142–151.

Gratzel M., Taniguchi S., Henglein A. (1970). Pulsradiolytische Untersunchung Kurzlebiger zwischenprodukte der NO-reduktion in Wassriger Losung. *Ber Bunsen-Ges Phys* 74: 292–297.

Greenacre S.A., Ischiropoulos H. (2001). Tyrosine nitration: Localisation, quantification, consequences for protein function and signal transduction. *Free Radic Res* 34: 541–581.

Grisham M.B., Johnson G.G., Lancaster J.R. (1996). Quantitation of nitrate and nitrite in extracellular fluids. *Methods Enzymol* 268: 237–246.

Grisham M.B., Jourd'Heuil D., Wink D.A. (1999). Nitric oxide. I. Physiological chemistry of nitric oxide and its metabolites:implications in inflammation. *Am J Physiol* 276: G315–G321.

Grisham M.B., Miles A.M. (1994). Effects of aminosalicylates and immunosuppressive agents on nitric oxide dependent *N*-nitrosation reactions. *Biochem Pharmacol* 47: 1897–1902.

Halliwell B., Gutteridge J.M.C. (1999). *Free Radicals in Biology and Medicine*, Oxford University Press, New York.

Han T.H., Hyduke D.R., Vaughn M.W., Fukuto J.M., Liao J.C. (2002). Nitric oxide reaction with red blood cells and hemoglobin under heterogeneous conditions. *Proc Natl Acad Sci USA* 99: 7763–7768.

Hazen S.L., Zhang R., Shen Z., Wu W., Podrez E.A., MacPherson J., Schmitt D., Mitra S.N., Mukhopadhyay C., Chen Y., Cohen P.A., Hoff H.F., Abu-Soud H.M. (1999). Formation of nitric oxide-derived oxidants by myeloperoxidase in monocytes: pathways for monocyte-mediated protein nitration and lipid peroxidation in vivo. *Circ Res* 85: 950–958.

Herold S., Shivashankar K. (2003). Metmyoglobin and methemoglobin catalyze the isomerization of peroxynitrite to nitrate. *Biochemistry* 42: 14036–14046.

Hobbs A.J., Fukuto J.M., Ignarro L.J. (1994). Formation of free nitric oxide from L-arginine by nitric oxide aynthase: Direct enhancement of generation by superoxide dismutase. *Proc Natl Acad Sci USA* 91: 10992–10996.

Hofseth L.J., Saito S., Hussain S.P., Espey M.G., Miranda K.M., Araki Y., Jhappan C., Higashimoto Y., He P., Linke S.P., Quezado M.M., Zurer I., Rotter V., Wink D.A., Appella E., Harris C.C. (2003). Nitric oxide-induced cellular stress and p53 activation in chronic inflammation. *Proc Natl Acad Sci USA* 100: 143–148.

Hogg N., Darley-Usmar V.M., Wilson M.T., Moncada S. (1993). The oxidation of a in human low-density lipoprotein by the simultaneous generation of superoxide and nitric oxide. *FEBS Lett* 326: 199–203.

Hoshino M., Ozawa K., Seki H., Ford P.C. (1993). Photochemistry of nitric oxide adducts of water-soluble iron(III) porphyrin and ferrihemoproteins studied by nanosecond laser photolysis. *J Am Chem Soc* 115: 9568–9575.

Huie R.E., Padmaja S. (1993). The reaction of NO with superoxide. *Free Radic Res Commun* 18: 195–199.

Hurst J.K., Lymar S.V. (1997). Toxicity of peroxynitrite and related reactive nitrogen species toward *Escherichia coli*. *Chem Res Toxicol* 10: 802–810.

Ichimori K., Fukuyama N., Nakazawa H., Aratani Y., Koyama H., Takizawa S., Kameoka Y., Ishida-Okawara A., Kohi F., Suzuki K. (2003). Myeloperoxidase has directly-opposed effects on nitration reaction—Study on myeloperoxidase-deficient patient and myeloperoxidase-knockout mice. *Free Radic Res* 37: 481–489.

Ignarro L.J., Edwards J.C., Gruetter D.Y., Barry B.K., Gruetter C.A. (1980). Possible involvement of *S*-nitrosothiols activation of guanylate cyclase nitroso compounds. *FEBS Lett* 110: 275–278.

Ignarro L.J., Fukuto J.M., Griscavage J.M., Rogers N.E., Byrns R.E. (1993). Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: Comparison with enzymatically formed nitric oxide from L-arginine. *Proc Natl Acad Sci USA* 90: 8103–8107.

Ischiropoulos H. (1998). Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. *Arch Biochem Biophys* 356: 1–11.

Jaffrey S.R., Fang M., Snyder S.H. (2002). Nitrosopeptide mapping: a novel methodology reveals S-nitrosylation of dexras1 on a single cysteine residue. *Chem Biol* 9: 1329–1335.

Janero D.R., Bryan N.S., Saijo F., Dhawan V., Schwalb D.J., Warren M.C., Feelisch M. (2004). Differential nitros(yl)ation of blood and tissue constituents during glyceryl trinitrate biotransformation in vivo. *Proc Natl Acad Sci USA* 101: 16958–16963.

Jourd'heuil D., Jourd'heuil F.L., Feelisch M. (2003). Oxidation and nitrosation of thiols at low micromolar exposure to nitric oxide. Evidence for a free radical mechanism. *J Biol Chem* 278: 15720–15726.

Jourd'heuil D., Miranda K.M., Kim S.M., Espey M.G., Vodovotz Y., Laroux S., Mai C.T., Miles A.M., Grisham M.B., Wink D.A. (1999). The oxidative and nitrosative chemistry of the nitric oxide/superoxide reaction in the presence of bicarbonate. *Arch Biochem Biophys* 365: 92–100.

Kanner J., Harel S., Granit R. (1992). Nitric oxide, an inhibitor of lipid oxidation by lipoxygenase, cyclooxygenase and hemoglobin. *Lipids* 27: 46–49.

Karthein R., Nastainczyk W., Ruf H.H. (1987). EPR study of ferric native prostaglandin H synthase and its ferrous NO derivative. *Eur J Biochem* 166: 173–180.

Kelm M., Dahmann R., Wink D.A., Feelisch M. (1997). A new method for the simultaneous evaluation of rates of NO and O_2^- formation. Insights into the NO/O_2^- chemistry. *J Biol Chem* 272: 9922–9932.

Kim W.K., Choi Y.B., Rayudu P.V., Das P., Asaad W., Arnelle D.R., Stamler J.S., Lipton S.A. (1999). Attenuation of NMDA receptor activity and neurotoxicity by nitroxyl anion, NO^-. *Neuron* 24: 461–469.

Klausner R.D., Rouault T.A., Harford J.B. (1993). Regulating the fate of mRNA: The control of cellular iron metabolism. *Cell* 72: 19–28.

Klebanoff S.J. (1993). Reactive nitrogen intermediates and antimicrobial activity: Role of nitrite. *Free Radic Biol Med* 14: 351–360.

Koesling D., Russwurm M., Mergia E., Mullershausen F., Friebe A. (2004). Nitric oxide-sensitive guanylyl cyclase: Structure and regulation. *Neurochem Int* 45: 813–819.

Kooy N.W., Royall J.A., Ischiropoulos H., Beckman J.S. (1994). Peroxynitrite-mediated oxidation of dihydrorhodamine 123. *Free Radic Biol Med* 16: 149–156.

Lancaster J. (1994). Simulation of the diffusion and reaction of endogenously produced nitric oxide. *Proc Natl Acad Sci USA* 91: 8137–8141.

Lancaster J.R.J., Hibbs J.B.J. (1990). EPR demonstration of iron-nitrosyl complex formation by cytotoxic activated macrophages. *Proc Natl Acad Sci USA* 87: 1223–1227.

Li H., Cui H., Liu X., Zweier J.L. (2005). Xanthine oxidase catalyzes anaerobic transformation of organic nitrates to nitric oxide and nitrosothiols: Characterization of this mechanism and the link between organic nitrate and guanylyl cyclase activation. *J Biol Chem* 280: 16594–16600.

Liochev S.I., Fridovich I. (2003). The mode of decomposition of Angeli's salt ($Na_2N_2O_3$) and the effects thereon of oxygen, nitrite, superoxide dismutase, and glutathione. *Free Radic Biol Med* 34: 1399–1404.

Lipton S.A., Stamler J.S. (1994). Actions of redox-related congeners of nitric oxide at the NMDA receptor: *Neuropharmacology* 33: 1229–1233.

Liu R.H., Jacob J.R., Tennant B.D., Hotchkiss J.H. (1992). Nitrite and nitrosamine synthesis by hepatocytes isloated from normal woodchucks (*Marmota monax*) and woodchucks chronically infected with woodchuck hepatitis virus. *Cancer Res* 52: 4139–4143.

Liu X., Cheng C., Zorko N., Cronin S., Chen Y.R., Zweier J.L. (2004). Biphasic modulation of vascular nitric oxide catabolism by oxygen. *Am J Physiol Heart Circ Physiol* 287: H2421–2426.

Liu X., Miller M.J., Joshi M.S., Sadowska-Krowicka H., Clark D.A., Lancaster J.R.J. (1998a). Diffusion-limited reaction of free nitric oxide with erythrocytes. *J Biol Chem* 273: 18709–18713.

Liu X., Miller M.J.S., Joshi M.S., Thomas D.D., Lancaster J.R.J. (1998b). Accelerated reaction of nitric oxide with O_2 within the hydrophobic interior of biological membranes. *Proc Natl Acad Sci USA* 95: 2175–2179.

Lymar S.V., Hurst K. (1995). Rapid reaction between peroxynitrite ion and cabon dioxide: Implications for biological activity. *J Am Chem Soc* 290: 52–57.

Ma X.L., Gao F., Liu G.L., Lopez B.L., Christopher T.A., Fukuto J.M., Wink D.A., Feelisch M. (1999). Opposite effects of nitric oxide and nitroxyl on postischemic myocardial injury. *Proc Natl Acad Sci USA* 96: 14617–14622.

Makino R., Matsuda H., Obayashi E., Shiro Y., Iizuka T., Hori H. (1999). EPR characterization of axial bond in metal center of native and cobalt-substituted guanylate cyclase. *J Biol Chem* 274: 7714–7723.

Mancardi D., Ridnour L.A., Thomas D.D., Katori T., Tocchetti C.G., Espey M.G., Miranda K.M., Paolocci N., Wink D.A. (2004). The chemical dynamics of NO and reactive nitrogen oxides: a practical guide. *Curr Mol Med* 4: 723–740.

Maples K.R., Sandstrom T., Su Y.F., Henderson R.F. (1991). The nitric oxide/heme protein complex as a biologic marker of exposure to nitrogen dioxide in humans, rats, and in vitro models. *Am J Respir Cell Mol Biol* 4: 538–543.

Maulik N., Das D.K. (2002). Redox signaling in vascular angiogenesis. *Free Radic Biol Med* 33: 1047–1060.

McKnight G.M., Duncan C.W., Leifert C., Golden M.H. (1999). Dietary nitrate in man: Friend or foe? *Br J Nutr* 81: 349–358.

McKnight G.M., Smith L.M., Drummond R.S., Duncan C.W., Golden M., Benjamin N. (1997). Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans. *Gut* 40: 211–214.

Miles A.M., Bohle D.S., Glassbrenner P.A., Hansert B., Wink D.A., Grisham M.B. (1996). Modulation of superoxide-dependent oxidation and hydroxylation reactions by nitric oxide. *J Biol Chem* 271: 40–47.

Miles A.M., Gibson M., Krishna M., Cook J.C., Pacelli R., Wink D.A., Grisham M.B. (1995). Effects of superoxide on nitric oxide-dependent *N*-nitrosation reactions. *Free Radic Res* 233: 379–390.

Miranda K.M., Dutton A.S., Ridnour L.A., Foreman C.A., Ford E., Paolocci N., Katori T., Tocchetti C.G., Mancardi D., Thomas D.D., Espey M.G., Houk K.N., Fukuto J.M., Wink D.A. (2005). Mechanism of aerobic decomposition of Angeli's salt (sodium trioxodinitrate) at physiological pH. *J Am Chem Soc* 127: 722–731.

Miranda K.M., Nims R.W., Thomas D.D., Espey M.G., Citrin D., Bartberger M.D., Paolocci N., Fukuto J.M., Feelisch M., Wink D.A. (2003a). Comparison of the reactivity of nitric oxide and nitroxyl with heme proteins: A chemical discussion of the differential biological effects of these redox related products of NOS. *J Inorg Biochem* 93: 52–60.

Miranda K.M., Paolocci N., Katori T., Thomas D.D., Ford E., Bartberger M.D., Espey M.G., Kass D.A., Feelisch M., Fukuto J.M., Wink D.A. (2003b). A biochemical rationale for the discrete behavior of nitroxyl and nitric oxide in the cardiovascular system. *Proc Natl Acad Sci USA* 100: 9196–9201.

Miranda K.M., Yamada K., Espey M.G., Thomas D.D., DeGraff W., Mitchell J.B., Krishna M.C., Colton C.A., Wink D.A. (2002). Further evidence for distinct reactive intermediates from nitroxyl and peroxynitrite: effects of buffer composition on the chemistry of Angeli's salt and synthetic peroxynitrite. *Arch Biochem Biophys* 401: 134–144.

Miranda K.M., Espey M.G., Yamada K., Krishna M., Ludwick N., Kim S., Jourd'heuil D., Grisham M.B., Feelisch M., Fukuto J.M., Wink D.A. (2001). Unique oxidative mechanisms for the reactive nitrogen oxide species, nitroxyl anion. *J Biol Chem* 276: 1720–1727.

Moncada S., Palmer R.M.J., Higgs E.A. (1991). Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 43: 109–142.

Morley D.M., Maragos C.M., Zhang X.Y., Boignon M., Wink D.A., Keefer L.K. (1993). Mechanism of vascular relaxation induced by the NO/nucleophile complexes, a new class of nitric oxide-based vasodilators. *J Cardiovasc Pharm* 21: 670–676.

Murphy M.E., Sies H. (1991). Reversible conversion of nitroxyl anion to nitric oxide by superoxide dismutase. *Proc Natl Acad Sci USA* 88: 10860–10864.

Neta P., Huie R.E., Ross A.B. (1988). Rate constants for reactions of inorganic radicals in aqueous solution. *J Phys Chem Ref Data* 17: 1112–1115.

Nikano M., Kimura H., Hara M., Kuroiwa M., Kato M., Totsune K., Yoshikawa T. (1990). A highly sensitive method for determining both Mn–and Cu–Zn superoxide dismutase activities in tissue and blood cells. *Anal Biochem* 187: 277–280.

O'Keeffe D.H., Ebel R.E., Peterson J.A. (1978). Studies of the oxygen binding site of cytochrome P-450: Nitric oxide as a spin-label probe. *J Biol Chem* 253: 3509–3516.

Ohshima H., Celan I., Chazotte L., Pignatelli B., Mower H.F. (1999a). Analysis of 3-nitrotyrosine in biological fluids and protein hydrolyzates by high-performance liquid chromatography using a postseparation, on-line reduction column and electrochemical detection: Results with various nitrating agents. *Nitric Oxide* 3: 132–141.

Ohshima H., Gilibert I., Bianchini F. (1999b). Induction of DNA strand breakage and base oxidation by nitroxyl anion through hydroxyl radical production. *Free Radic Biol Med* 26: 1305–1313.

Ohshima H., Yoshie Y., Auriol S., Gilibert I. (1998). Antioxidant and pro-oxidant actions of flavonoids: Effects on DNA damage induced by nitric oxide, peroxynitrite and nitroxyl anion. *Free Radic Biol Med* 25: 1057–1065.

Olson L.P., Bartberger M.D., Houk K.N. (2003). Peroxynitrate and peroxynitrite: a complete basis set investigation of similarities and differences between these NOx species. *J Am Chem Soc* 125: 3999–4006.

Pagliaro P., Mancardi D., Rastaldo R., Penna C., Gattullo D., Miranda K.M., Feelisch M., Wink D.A., Kass D.A., Paolocci N. (2003). Nitroxyl affords thiol-sensitive myocardial protective effects akin to early preconditioning. *Free Radic Biol Med* 34: 33–43.

Paolocci N., Katori T., Champion H.C., St John M.E., Miranda K.M., Fukuto J.M., Wink D.A., Kass D.A. (2003). Positive inotropic and lusitropic effects of HNO/NO^- in failing hearts: Independence from beta-adrenergic signaling. *Proc Natl Acad Sci USA* 100: 5537–5542.

Paolocci N., Saavedra W.F., Miranda K.M., Martignani C., Isoda T., Hare J.M., Espey M.G., Fukuto J.M., Feelisch M., Wink D.A., Kass D.A. (2001). Nitroxyl anion exerts redox-sensitive positive cardiac inotropy in vivo by calcitonin gene-related peptide signaling. *Proc Natl Acad Sci USA* 98: 10463–10468.

Pfeiffer S., Gorren A.C.F., Schmidt K., Werner E.R., Hansert B., Bohle D.S., Mayer B. (1997). Metabolic fate of peroxynitrite in aqueous solution: Reaction with nitric oxide and pH-dependent decomposition to nitrite and oxygen in a 2:1 stoichiometry. *J Biol Chem* 272: 3465–3470.

Pfeiffer S., Lass A., Schmidt K., Mayer B. (2001a). Protein tyrosine nitration in mouse peritoneal macrophages activated in vitro and in vivo: Evidence against an essential role of peroxynitrite. *FASEB J* 15: 2355–2364.

Pfeiffer S., Mayer B. (1998). Lack of tyrosine nitration by peroxynitrite generated at physiological pH. *J Biol Chem* 273: 27280–27285.

Pfeiffer S., Lass A., Schmidt K., Mayer B. (2001b). Protein tyrosine nitration in cytokine-activated murine macrophages: Involvement of a peroxidase/nitrite pathway rather than peroxynitrite. *J Biol Chem* 276: 34051–34058.

Pignatelli B., Li C.Q., Boffetta P., Chen Q., Ahrens W., Nyberg F., Mukeria A., Bruske-Hohlfeld I., Fortes C., Constantinescu V., Ischiropoulos H., Ohshima H. (2001). Nitrated and oxidized plasma proteins in smokers and lung cancer patients. *Cancer Res* 61: 778–784.

Pryor W.A. (1982). Lipid peroxides in biology and medicine. In: Yagi K. (ed.), *Free Radicals in Biology*, Academic Press, New York, pp. 1–22.

Pryor W.A., Squadrito G.L. (1995). The chemistry of peroxynitrite: A product from the reaction of nitric oxide with superoxide. *Am J Phys* 268: L699–722.

Pufahl R.A., Wishnok J.S., Marletta M.A. (1995). Hydrogen peroxide-supported oxidation of NG-hydroxy-L-arginine by nitric oxide synthase. *Biochemistry* 34: 1930–1941.

Pyriochou A., Papapetropoulos A. (2005). Soluble guanylyl cyclase: More secrets revealed. *Cell Signal* 17: 407–413.

Radi R., Beckman J.S., Bush K.M., Freeman B.A. (1991). Peoxynitrite oxidation of sulfhydryls: The cytotoxic potential of superoxide and nitric oxide. *J Biol Chem* 266: 4244–4250.

Rassaf T., Bryan N.S., Maloney R.E., Specian V., Kelm M., Kalyanaraman B., Rodriguez J., Feelisch M. (2003a). NO adducts in mammalian red blood cells: too much or too little? *Nat Med* 9: 481–482.

Rassaf T., Bryan N.S., Kelm M., Feelisch M. (2003b). Concomitant presence of *N*-nitroso and *S*-nitroso proteins in human plasma. *Free Radic Biol Med* 34: 283.

Reiter C.D., Teng R.J., Beckman J.S. (2000). Superoxide reacts with nitric oxide to nitrate tyrosine at physiological pH via peroxynitrite. *J Biol Chem* 275: 32460–32466.

Ridnour L.A., Thomas D.D., Mancardi D., Espey M.G., Miranda K.M., Paolocci N., Feelisch M., Fukuto J., Wink D.A. (2004). The chemistry of nitrosative stress induced by nitric oxide and reactive nitrogen oxide species: Putting perspective on stressful biological situations. *Biol Chem* 385: 1–10.

Sampson J.B., Ye Y., Rosen H., Beckman J.S. (1998). Myeloperoxidase and horseradish peroxidase catalyze tyrosine nitration in proteins from nitrite and hydrogen peroxide. *Arch Biochem Biophys* 356: 207–213.

Schmidt H.H., Hofmann H., Schindler U., Shutenko Z.S., Cunningham D.D., Feelisch M. (1996). No • NO from NO synthase. *Proc Natl Acad Sci USA* 93: 14492–14497.

Schwartz S.E., White W.H. (1983). *Trace Atmospheric Constituents: Properties, Transformation and Fates*, Wiley, New York.

Schwentker A., Vodovotz Y., Weller R., Billiar T.R. (2002). Nitric oxide and wound repair: Role of cytokines? *Nitric Oxide* 7: 1–10.

Shafirovich V., Lymar S.V. (2002). Nitroxyl and its anion in aqueous solutions: Spin states, protic equilibria, and reactivities toward oxygen and nitric oxide. *Proc Natl Acad Sci USA* 99: 7340–7345.

Sies H., Sharov V.S., Klotz L.O., Briviba K. (1997). Glutathione peroxidase protects against peroxynitrite-mediated oxidations: A new function for selenoproteins as peroxynitrite reductase. *J Biol Chem* 272: 27812–27817.

Simon D.I., Mullins M.E., Jia L., Gaston B., Singel D.J., Stamler J.S. (1996). Polynitrosylated proteins: Characterization, bioactivity, and functional consequences. *Proc Natl Acad Sci USA* 93: 4736–4741.

Stadler J., Bergonia H.A., Di Silvio M., Sweetland M.A., Billiar T.R., Simmons R.L., Lancaster J.R.J. (1993a). Nonheme iron-nitrosyl complex formation in rat hepatocytes: Detection by electron paramagnetic resonance spectroscopy. *Arch Biochem Biophys* 302: 4–11.

Stadler J., Harbrecht B.G., Di Silvio M., Curran R.D., Jordan M.L., Simmons R.L., Billiar T.R. (1993b). Endogenous nitric oxide inhibits the synthesis of cyclooxygenase products and interleukin-6 by rat Kupffer cells. *J Leukoc Biol* 53: 165–172.

Stadler J., Trockfeld J., Schmalix W.A., Brill T., Siewert J.R., Greim H., Doehmer J. (1994). Inhibition of cytochromes P4501A by nitric oxide. *Proc Natl Acad Sci USA* 91: 3559–3563.

Stanbury D.M. (1989). Reduction potentials involving inorganic free radicals in aqueous solution. *Adv Inorg Chem* 33: 69–136.

Tarpey M.M., Wink D.A., Grisham M.B. (2004). Methods for detection of reactive metabolites of oxygen and nitrogen: In vitro and in vivo considerations. *Am J Physiol Regul Integr Comp Physiol* 286: R431–R444.

Thomas D.D., Espey M.G., Ridnour L.A., Hofseth L.J., Mancardi D., Harris C.C., Wink D.A. (2004). Hypoxic inducible factor 1alpha, extracellular signal-regulated kinase, and p53 are regulated by distinct threshold concentrations of nitric oxide. *Proc Natl Acad Sci USA* 101: 8894–8899.

Thomas D.D., Espey M.G., Vitek M.P., Miranda K.M., Wink D.A. (2002a). Protein nitration is mediated by heme and free metals through Fenton-type chemistry: An alternative to the NO/O_2^- reaction. *Proc Natl Acad Sci USA* 99: 12691–12696.

Thoma, D.D., Liu X., Kantrow S.P., Lancaster J.R. (2001). The biological lifetime of nitric oxide: Implications for the perivascular dynamics of NO and O_2. *Proc Natl Acad Sci USA* 98: 355–360.

Thomas D.D., Miranda K.M., Espey M.G., Citrin D., Jourd'heuil D., Paolocci N., Hewett S.J., Colton C.A., Grisham M.B., Feelisch M., Wink D.A. (2002b). Guide for the use of nitric oxide (NO) donors as probes of the chemistry of NO and related redox species in biological systems. *Methods Enzymol* 359: 84–105.

Thomas D.D., Miranda K.M., Colton C.A., Citrin D., Espey M.G., Wink D.A. (2003). Heme proteins and nitric oxide (NO): The neglected, eloquent chemistry in NO redox signaling and regulation. *Antioxid Redox Signal* 5: 307–317.

Tsubaki M., Hiwatashi A., Ichikawa Y., Hori H. (1987). Electron paramagnetic resonance study of ferrous cytochrome P-450scc-nitric oxide complexes: effects of cholesterol and its analogues. *Biochemistry* 26: 4527–4534.

Vanin A.F. (1995). Roles of iron ions and cystiene in formation and decomposition of *S*-nitrosocysteine and *S*-nitrosoglutathione. *Biochemistry (Moscow)* 80: 441–447.

Vidwans A.S., Kim S., Coffin D.O., Wink D.A., Hewett S.J. (1999). Analysis of the neuroprotective effects of various nitric oxide donor compounds in murine mixed cortical cell culture. *J Neurochem* 72: 1843–1852.

Viera L., Ye Y.Z., Estevez A.G., Beckman J.S. (1999). Immunohistochemical methods to detect nitrotyrosine. *Methods Enzymol* 301: 373–381.

Vignais P.V. (2002). The superoxide-generating NADPH oxidase: Structural aspects and activation mechanism. *Cell Mol Life Sci* 59: 1428–1459.

Wade R.S., Castro C.E. (1990). Redox reactivity of iron(III) porphyrins and heme proteins with nitric oxide: Nitrosyl transfer to carbon, oxygen, nitrogen, and sulfur. *Chem Res Toxicol* 3: 289–291.

Wang C., Trudel L.J., Wogan G.N., Deen W.M. (2003). Thresholds of nitric oxide-mediated toxicity in human lymphoblastoid cells. *Chem Res Toxicol* 16: 1004–1013.

Wasser I.M., de Vries S., Moenne-Loccoz P., Schroder I., Karlin K.D. (2002). Nitric oxide in biological denitrification: Fe/Cu metalloenzyme and metal complex NO(x) redox chemistry. *Chem Rev* 102: 1201–1234.

Weichsel A., Maes E.M., Andersen J.F., Valenzuela J.G., Shokhireva T.K., Walker F.A., Montfort W.R. (2005). Heme-assisted *S*-nitrosation of a proximal thiolate in a nitric oxide transport protein. *Proc Natl Acad Sci USA* 102: 594–599.

Welch G.N., Upchurch G.R.J., Loscalzo J. (1996). *S*-nitrosothiol detection. *Methods Enzymol* 268: 293–298.

Wink D.A., Hanbauer I., Krishna M.C., DeGraff W., Gamson J., Mitchell J.B. (1993a). Nitric oxide protects against cellular damage and cytotoxicity from reactive oxygen species. *Proc Natl Acad Sci USA* 90: 9813–9817.

Wink D.A. (2003). Ion implicated in blood pact. *Nat Med* 9: 1460–1461.

Wink D.A., Cook J., Pacelli R., DeGraff W., Gamson J., Liebmann J., Krishna M., Mitchell J.B. (1996a). Effect of various nitric oxide-donor agents on peroxide mediated toxicity: A direct correlation between nitric oxide formation and protection. *Arch Biochem Biophys* 331: 241–248.

Wink D.A., Cook J.A., Kim S., Vodovotz Y., Pacelli R., Kirshna M.C., Russo A., Mitchell J.B., Jourd'heuil D., Miles A.M., Grisham M.B. (1997). Superoxide modulates the oxidation and nitrosation of thiols by nitric oxide derived reactive intermediates. *J Biol Chem* 272: 11147–11151.

Wink D.A., Darbyshire J.F., Nims R.W., Saveedra J.E., Ford P.C. (1993b). Reactions of the bioregulatory agent nitric oxide in oxygenated aqueous media: determination of the kinetics for oxidation and nitrosation by intermediates generated in the NO/O_2 reaction. *Chem Res Toxicol* 6: 23–27.

Wink D.A., Feelisch M., Fukuto J., Chistodoulou D., Jourd'heuil D., Grisham M.B., Vodovotz Y., Cook J.A., Krishna M., DeGraff W.G., Kim S., Gamson J., Mitchell J.B. (1998a). The cytotoxicity of nitroxyl: Possible implications for the pathophysiological role of NO. *Arch Biochem Biophys* 351: 66–74.

Wink D.A., Grisham M., Mitchell J.B., Ford P.C. (1996b). Direct and indirect effects of nitric oxide: Biologically relevant chemical reactions in biology of NO. *Methods Enzymol* 268: 12–31.

Wink D.A., Miranda K.M., Espey M.G., Pluta R.M., Hewett S.J., Colton C., Vitek M., Feelisch M., Grisham M.B. (2001). Mechanisms of the antioxidant effects of nitric oxide. *Antioxid Redox Signal* 3: 203–213.

Wink D.A., Miranda K.M., Katori T., Mancardi D., Thomas D.D., Ridnour L., Espey M.G., Feelisch M., Colton C.A., Fukuto J.M., Pagliaro P., Kass D.A., Paolocci N. (2003). Orthogonal properties of the redox siblings nitroxyl and nitric oxide in the cardiovascular system: A novel redox paradigm. *Am J Physiol Heart Circ Physiol* 285: H2264–2276.

Wink D.A., Mitchell J.B. (1998). The chemical biology of nitric oxide: Insights into regulatory, cytotoxic and cytoprotective mechanisms of nitric oxide. *Free Radic Biol Med* 25: 434–456.

Wink D.A., Nims R.W., Darbyshire J.F., Christodoulou D., Hanbauer I., Cox G.W., Laval F., Laval J., Cook J.A., Krishna M.C., DeGraff W., Mitchell J.B. (1994). Reaction kinetics for nitrosation of cysteine and glutathione in aerobic nitric oxide solutions at neutral pH: Insights into the fate and physiological effects of intermediates generated in the NO/O_2 reaction. *Chem Res Toxicol* 7: 519–525.

Wink D.A., Vodovotz Y., Laval J., Laval F., Dewhirst M.W., Mitchell J.B. (1998b). The multifaceted roles of nitric oxide in cancer. *Carcinogenesis* 19: 711–721.

Wishnok J.S., Tannenbaum S.R., Stillwell W.G., Glogowski J.A., Leaf C.D. (1993). Urinary markers for exposures to alkylating or nitrosating agents. *Environ Health Perspect* 99: 155–159.

Yeomans N.D., Brimblecombe R.W., Elder J., Heatley R.V., Misiewicz J.J., Northfield T.C., Pottage A. (1995). Effects of acid suppression on microbial flora of upper gut. *Dig Dis Sci* 40: 81S–95S.

Yoshimura T., Yokoyama H., Fujii S., Takayama F., Oikawa K., Kamada H. (1996). In vivo EPR detection and imaging of endogenous nitric oxide in lipopolysaccharide-treated mice. *Nat Biotechnol* 14: 992–994.

Zhao Y., Brandish P.E., Ballou D.P., Marletta M.A. (1999). A molecular basis for nitric oxide sensing by soluble guanylate cyclase. *Proc Natl Acad Sci USA* 96: 14753–14758.

Zhu L., Gunn C., Beckman J.S. (1992). Bactericidal activity of peroxynitrite. *Arch Biochem Biophys* 298: 452–457.

Zumft W.G. (1993). The biological of nitric oxide in bacteria. *Arch Microbiol* 160: 253–264.

3

MASS SPECTROMETRY APPROACHES FOR THE MOLECULAR CHARACTERIZATION OF OXIDATIVELY/NITROSATIVELY MODIFIED PROTEINS

ANDREA SCALONI

3.1 INTRODUCTION

Under physiological conditions, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated at a low amount within cells (Mikkelsen and Wardman, 2003). Their moderate levels play an integral role in modulation of several cellular functions, including gene expression, signal transduction, vasorelaxation, and defense against invading pathogens (Kunsch and Medford, 1999; Stamler et al., 2001; Georgiou, 2002). However, interference with electron transport or redox environmental challenges may dramatically increase their concentrations, contributing to generate additional reactive species by various metabolic pathways. Almost 10 different ROS ($O_2^{\bullet -}$, $HO^{\bullet}$, $RO^{\bullet}$, $RO_2^{\bullet}$, $HO_2^{\bullet}$, H_2O_2, HOCl, HOBr, O_3, and $^1O_2$) have been identified within the cell (Winterbourn et al., 2000; Dalle-Donne et al., 2005); similarly, almost 14 RNS ($^{\bullet}NO$, $^{\bullet}NO_2$, HNO_2, NO^+, NO^-, N_2O_4, N_2O_3, $ONOO^-$, ONOOH, ROONO, $ONO_2CO_2^-$, NO_2^+, and $NOCl_2$) have also been detected (Mikkelsen and Wardman, 2003). Unregulated levels of these metabolites can cause extensive cellular damage (Mikkelsen and Wardman, 2003). A balance between physiological functions and damage is accomplished by their relative rates of formation and degradation. Under normal conditions these

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

species are removed rapidly before they cause cell dysfunction and, ultimately, cellular death via apoptosis or necrosis (Hensley et al., 2000). Naturally occurring enzymatic and nonenzymatic systems exist to protect cells against the continuous production of ROS/RNS. These include antioxidant enzymes such as catalases, superoxide dismutases, peroxiredoxins, glutathione peroxidases, ferritin, and ceruloplasmin. Other widespread antioxidants are low molecular weight compounds such as vitamin E, vitamin C, and glutathione (GSH). However, when ROS/RNS levels exceed the antioxidant capacity, a deleterious condition known as oxidative/nitrosative stress occurs. It describes a status in which cellular antioxidant defenses are insufficient to keep the ROS/RNS levels below a toxic threshold. This may be due to excessive production of ROS/RNS, loss of antioxidant defenses in the cell, or both (Dalle-Donne et al., 2003a). Thus, oxidative/nitrosative stress can strongly affect major cellular components, including lipids, proteins, carbohydrates, and DNA. The significance of oxidative/nitrosative stress has become increasingly recognized to the point that it is now considered to be a component of virtually every disease (Smith et al., 1996; Davies and Dean, 1997; Baynes and Thorpe, 2000; Dweik et al., 2001; Butterfield et al., 2001; Upston et al., 2002; Beal, 2002; Sandhu et al., 2003). Accumulating evidence demonstrates many interrelated mechanisms during pathogenesis that increase production of ROS/RNS or decrease antioxidant protection against oxidative/nitrosative insult, although the exact contribution of such mechanisms is not entirely clear. Information regarding the nature of ROS/RNS, as well as the localization and the effects of oxidative/nitrosative stress, may be gleaned from the analysis of discrete biomarkers isolated from tissues and biological fluids. However, the presence of oxidatively/nitrosatively damaged molecules could simply reflect secondary epiphenomena rather than having a causal role (Halliwell and Gutteridge, 1999; Dalle-Donne et al., 2003a). A clear delineation of the causal connections cannot be given at present, but a growing body of evidence indicates that high levels of ROS/RNS induce distinct pathological consequences that greatly amplify and propagate injury, leading to irreversible cell and tissue degeneration.

To investigate the role of oxidative/nitrosative stress and ROS/RNS in progression of diseases, optimization of appropriate analytical procedures is necessary. Sensitive techniques for the analysis of ROS/RNS are now available; measurements of $NO^{\bullet}$, $O_2^{\bullet -}$, H_2O_2, and $ONOO^-$ have recently been reviewed (Tarpey and Fridovich, 2001). However, direct determination of ROS/RNS is difficult because they are generally too reactive and have a too brief half-life for a direct measurement in tissues or body fluids. Since molecular products from oxidative/nitrosative stress are generally more stable than oxidants and nitrosants themselves—that is, oxidized proteins (e.g., carbonyl-labeled amino acid residues) or nucleic acids (e.g., 8-oxo-2′-deoxyguanosine) are more stable than the reactive species that effected their modification—ROS/RNS measurements often involve determining levels of their oxidation target products (Pryor, 2001; Griffiths et al., 2002). Accordingly, a variety of biological markers are available for determination of oxidative/nitrosative stress and have been discussed in a number of

publications (e.g., Davies et al., 1999; Halliwell and Gutteridge, 1999; Pryor, 2001; Griffiths et al., 2002; Dalle-Donne et al., 2003b).

There is no doubt that proteins are major targets for radicals and other oxidants when these are formed in vivo either in intra- or extra-cellular environments. On the basis of published rate constants and abundance of these macromolecules within cells, it has been estimated that proteins can scavenge 50% to 75% of reactive radicals (Davies et al., 1999). Furthermore, proteins have the potential not only to determine the extent of oxidative injury but also to identify the nature of the oxidant itself. For instance, $^{\bullet}$OH specifically converts protein Phe residues to the unnatural amino acid isomer *o*-tyrosine (*o*-Tyr). The Tyrosyl radical (the oxidizing intermediate generated by peroxidases) forms *o*,*o*′-dityrosine (di-Tyr) as the major product; in contrast, $ONOO^-$ generates 3-nitrotyrosine (NO_2-Tyr). This contrasts with lipid peroxidation, where propagation reactions involving the initial lipid oxidation products result in information loss about the initial oxidative insult. Moreover, amino acid oxidation products are superior to lipid oxidation products in terms of stability during sample storage and artifactual formation during analysis. Thus, stable species like 3-chlorotyrosine (Cl-Tyr) and NO_2-Tyr, whose concentration is not affected during prolonged storage in a freezer, can be considered as ideal markers of oxidative/nitrosative stress in vivo. Such data, together with the knowledge that some proteins have long half-lives and hence are likely to accumulate oxidative "hits," suggest that modified residues on proteins may be considered as most sensitive markers for oxidative damage in mammalian cells (Dean et al., 1997; Davies et al., 1999). Finally, protein markers will be extremely useful to monitor oxidative stress in vivo when they are directly present or are degradated into analytes measurable into body fluids (e.g., blood, urine). In order to assess the potential utility of oxidation products in humans, is extremely important to determine the fate of oxidized amino acids and other oxidation products in vivo (Bhattacharjee et al., 2001).

3.2 MASS SPECTROMETRY ANALYSIS OF OXIDATIVELY/NITROSATIVELY MODIFIED PROTEINS

Oxidative damage to proteins is induced either directly by ROS/RNS or indirectly by reaction of oxidative/nitrosative stress secondary products. The damage can occur via different mechanisms, resulting in polypeptide chain cleavage, protein inter-residues cross-linking, and addition of chemical moieties to amino acid side chains (Berlett and Stadtman, 1997; Dean et al., 1997; Dalle-Donne et al., 2003a). These reactions can lead to diverse functional consequences such as inhibition of enzymic and binding activities, increased susceptibility to aggregation and proteolysis, increased or decreased uptake by cells, and altered immunogenicity. These phenomena are now tentatively associated to injury and disease amplification/propagation. However, not all proteins are equally sensitive to oxidative damage, and oxidation susceptibility depends on protein structure (e.g., sequence motifs, residues exposed on the molecular surface, bound metal atoms).

Oxidative/nitrosative stress may cause reversible and/or irreversible modifications on sensitive proteins (Stadtman and Berlett, 1998). ROS/RNS leading to protein oxidation include both radical and nonradical species (Dalle-Donne et al., 2005). Reversible oxidation and nitrosation, usually at Cys and Met residues, may have a dual role of protection from irreversible damage and modulation of protein function (redox regulation). Often, they are reverted by dedicated enzymatic systems (Stadtman and Berlett, 1998; Levine et al., 2000a; Budanov et al., 2004). On the contrary, irreversible modifications induced by ROS/RNS, such as polypeptide cleavage, protein cross-linking (at Tyr, Lys, and Arg), carbonylation (at Lys and Arg), nitration (at Tyr, His, and Trp), halogenation (at Tyr), hydroxylation (at Tyr and Phe), and oxidation (at Met, Cys, His, and Trp), may be associated with permanent loss of function and may lead to either the degradation of the damaged proteins (Berlett and Stadtman, 1997; Dean et al., 1997; Grune et al., 2003) or their progressive accumulation into cytoplasmic inclusions, as observed in age-related neurodegenerative disorders (Giasson et al., 2000, 2002; Butterfield and Lauderback, 2002).

A series of analytical strategies based on mass spectrometry (MS) techniques has been reported in the literature for the detection of oxidation products in isolated proteins. In general, all methodologies are based on the observation that these reactions, causing a covalent modification of amino acids, determine a specific molecular mass variation in the products, which is easily detectable by mass spectrometric measurements. Depending on adduct nature and stability, different MS approaches have been developed for the detection of oxidatively/nitrosatively modified species in intact proteins, in peptide mixtures generated by digestion with proteases or reagents with high specificity, or in amino acid hydrolysates produced by extensive enzymic or chemical hydrolysis. In all cases, specific experimental conditions have to be carefully chosen for protein manipulation and/or hydrolysis with the aim to preserve the stability of the modified amino acids. Although all strategies can provide quantitative information on the modification extent, only mass spectrometric analysis of the modified peptides can be uniquely used for the assignment of the modification to specific amino acid residues. With the exception of polypeptide cleavage reaction, briefly discussed below, each type of amino acid modification will be discussed in a dedicated section containing also a detailed description of the dedicated MS methodologies reported for its detection.

A number of oxidative reactions determining the cleavage of the polypeptide backbone have been elucidated (Berlett and Stadtman, 1997; Dean et al., 1997). Although the cleavage can be easily detected with isolated proteins (e.g., using sodium dodecylsulfate polyacrylamide gel electrophoresis, SDS-PAGE, or high-performance liquid chromatography, HPLC), its use as a marker of protein oxidation in vivo is very limited because of the occurrence of other proteins in complex systems and the potential role of proteases in polypeptide hydrolysis. Thus, backbone fragmentation is rarely used to quantify protein oxidation in complex systems.

3.2.1 Analysis of Oxidized/Nitrosated Products of Protein Thiols

Thiol groups are easily oxidized by many ROS/RNS, their susceptibility being inversely influenced by their pK_a value. Protein thiol group modifications can have different physiological effects, depending on their reversible or irreversible nature. Mild oxidation of cysteines can generate sulfenic and sulfinic acid (P-SOH and P-SO_2H), inter- or intra-molecular disulfides, protein mixed disulfides with low molecular weight thiols (e.g., GSH), and *S*-nitrosothiols. It is general opinion that these reversible modifications could be part of the regulatory processes of protein function, in which cysteines cycle between the oxidized and reduced state. On the contrary, Cys residues can be irreversibly oxidized by strong oxidative insults to sulfonic acids (P-SO_3H) that cannot be reversed by metabolic processes and can cause loss of protein function.

While protein disulfide generation can be hampered by steric hindrance, protein SH groups can easily react with low molecular weight compounds in response to an oxidative insult, producing mixed disulfides. GSH is the dominant ligand in this reaction due to its high concentration (0.5–10 mM) in mammalian cells (Halliwell and Gutteridge, 1999). Different mechanisms have been deduced for protein *S*-glutathionylation (Cotgreave and Gerdes, 1998; Klatt and Lamas, 2000; Okamoto et al., 2001). In general, *S*-glutathionylation has been proposed as a means of storing GSH during oxidative stress, and in being reversible, it has been regarded as a protective mechanism guarding against irreversible protein thiol oxidation (Klatt and Lamas, 2000; Schafer and Buettner, 2001). Various enzymatic systems such as thioredoxin, glutaredoxin, and protein disulfide isomerase are able to reduce these disulfide bonds (Arner and Holmgren, 2000; Schwaller et al., 2003). Similarly, a dedicated enzymatic machinery has been recently proposed for rapid reduction of the catalytically inactive sulfinic form of peroxiredoxin generated during H_2O_2 exposure (Budanov et al., 2004).

Formation of inter-molecular disulfides following oxidative/nitrosative insult generates macroscopic variation of protein molecular mass; on this basis it has usually been detected by low-resolution techniques as SDS-PAGE under not reducing conditions. In contrast, the occurrence of mixed disulfides with low molecular weight compounds or intramolecular disulfides, determining limited variation in molecular mass of intact proteins, has been revealed by conventional MS procedures. In the case of *S*-glutathionylated, *S*-cysteinylglycinylated, *S*-cysteinylated, and *S*-sulfonated proteins, the occurrence of *S*-conjugated species has been ascertained by direct electrospray ionization (ESI) measurements of intact proteins, detecting the corresponding adducts presenting a mass difference of +305 Da, +176 Da, +119 Da, and +80 Da, respectively (Hanson et al., 1999; Naito et al., 2000; Lim et al., 2003). As expected, the mass spectra of species containing mixed disulfides were totally affected by reducing agent treatment. In the case of intra-molecular disulfides, the limited variation in molecular mass of intact proteins compared with not stressed species ($\Delta m = -2$ Da for each S–S bond) determined a need of additional measurements (Caselli et al., 1998). For this reason, a modification of the MS strategy conventionally used for the titration of free thiols in proteins has been applied for the detection of oxidized

cysteines. Simply in comparing the molecular mass value of the intact protein in its native and stressed state, before and following extensive alkylation with iodoacetamide under denaturing not reducing conditions, the number of the Cys residues involved in oxidative/nitrosative insult and the nature of the modification can be inferred (Vilardo et al., 2001; Cecconi et al., 2002). In fact, cysteines involved in disulfides will not react with iodoacetamide, and thus not generate the corresponding mass increase ($\Delta m = +57$ Da for each available SH), easily detectable by ESI measurements. If a comparable ionization tendency is assumed for all of the different species obtained following alkylation, this procedure can be successfully applied to evaluate the quantitative extent of the oxidative insult. This approach has been used for the molecular characterization of the products generated from the oxidative modification of bovine lens aldose reductase induced by copper ions or intermediates of GSH turnover, as illustrated in Figure 3.1 (Vilardo et al., 2001; Cecconi et al., 2002).

Mixed disulfide assignment to specific Cys residues in protein can be obtained by mass mapping experiments on peptide mixtures generated from carboxamidomethylated species following alkylation under denaturing, not reducing, conditions. A careful evaluation of experimental conditions suitable to avoid scrambling phenomena during protein hydrolysis is strongly recommended. Identification of the modified residues has been obtained by liquid chromatography–electrospray ionization (LC-ESI) or matrix-assisted laser desorption ionization (MALDI) mapping experiments by detecting the peptides bearing a mass difference of +305 Da (*S*-glutathionylated), +176 Da (*S*-cysteinyl-glycinylated), +119 Da (*S*-cysteinylated), and +80 Da (*S*-sulfonated), and eventually confirmed by collision-induced dissociation (CID) measurements (Vilardo et al., 2001; Lim et al., 2003). Similarly, cysteine pairing identification in species containing intra-molecular disulfides as a result of oxidative/nitrosative insult are derived by mass mapping and the tandem mass spectrometry approaches conventionally used for the assignment of disulfides in native polypeptide species. This approach has been used for characterization of disulfide-containing Pax8 and OxyR transcription factors, nucleoside diphosphate kinase, and aldose reductase (Tell et al., 1998; Zheng et al., 1998; Song et al., 2000; Vilardo et al., 2001; Cecconi et al., 2002).

On the other hand, oxidation of cysteines to sulfinic and sulfonic acids has been determined in proteins by measuring the occurrence of adducts with $\Delta m = +32$ Da and +48 Da, respectively, in the ESI-MS spectrum of the intact molecules (Mirza et al., 1995; Yang et al., 2002; Woo et al., 2003). These species are stable and unsensitive to treatment with reducing agents. The selectivity of this modification toward Cys and not Met residues was verified following specific labeling with iodoacetamide. Identification of the modified cysteines was obtained by MALDI or LC-ESI mass mapping experiments on protein digests, specifically revealing peptides bearing these mass increases, and confirmed by tandem mass spectrometry analysis. This approach has been successfully used for characterization of sulfinic and sulfonic acid-containing matrix metalloprotease 9, chaperone GroEL, tyrosine phosphatase, and peroxiredoxin I, II, and III (Gu et al., 2002;

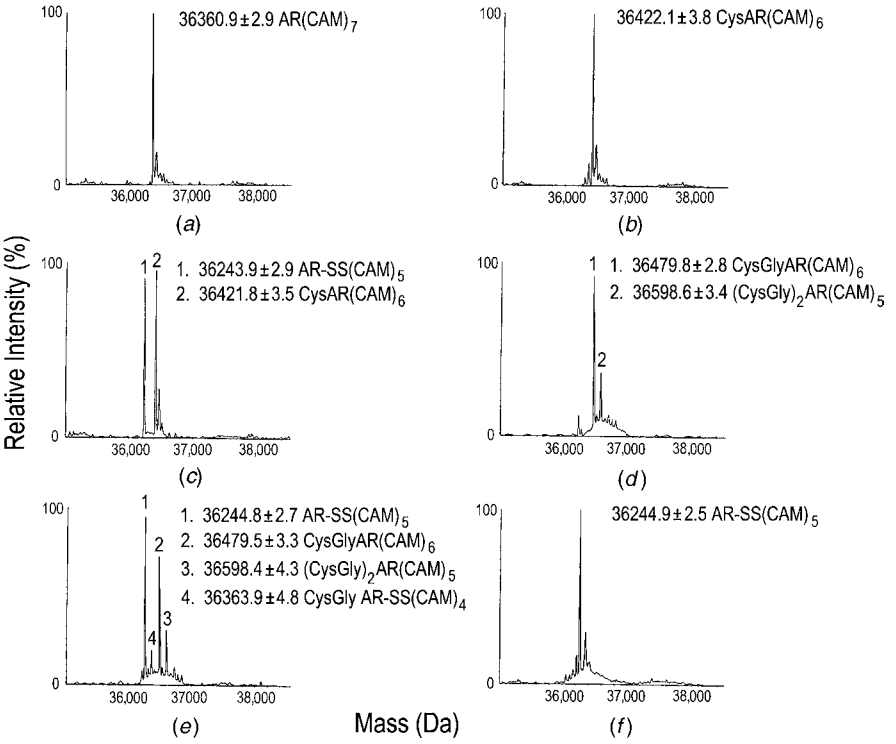


FIGURE 3.1 Electrospray mass spectrometric analysis of aldose reductase (AR) products generated from the reaction of the enzyme with intermediates of GSH turnover or copper ions. Native AR (*a*), AR with 0.4 mM cystine at 25°C (*b*), AR with 0.4 mM cystine at 37°C (*c*), AR with 0.4 mM CysGly disulfide at 25°C (*d*), AR with 0.4 mM CysGly disulfide at 37°C (*e*), and AR with 7 mM $CuCl_2$ at 25°C (*f*). All reactions were performed in 100 mM phosphate buffer, for 5 h, at pH 6.8. Samples were analyzed following alkylation with 1.1 M iodoacetamide in 0.25 M Tris-HCl, 1.25 mM EDTA, 6 M guanidinium chloride, pH 7, under not reducing conditions, for 1 min, at 25°C. Samples were desalted by reversed phase HPLC. CAM, carboxamidomethyl group; CysAR, Cys-AR mixed disulfide; CysGlyAR, CysGly-AR mixed disulfide; AR-SS, AR containing an intra-molecular disulfide (Dalle-Donne et al., 2005).

Yang et al., 2002; van Montfort et al., 2002; Rabilloud et al., 2002; Wagner et al., 2002; Khor et al., 2004). Frequently, fragments containing cysteic acid are entirely suppressed; this effect significantly facilitates peptide sequence determination (Sedo et al., 2004).

As a consequence of $NO^{\bullet}$ metabolism, protein cysteines can also undergo nitrosative modifications. Nitric oxide can form adducts with SH groups producing *S*-nitrosothiols. These molecules are thought to be intermediates in the storage and delivery of $NO^{\bullet}$, showing the biological properties of $NO^{\bullet}$ itself (e.g., vasodilation). The direct reaction between $NO^{\bullet}$ and SH groups does not yield

S-nitrosothiols. *S*-nitrosothiols are generated by reaction with metabolites formed during $NO^{\bullet}$ autooxidation ion, namely N_2O_3 and $ONOO^-$ (Hogg, 2002). Furthermore, equilibrium reactions named transnitrosations, in which the NO group is exchanged from a low molecular weight *S*-nitrosothiol to a thiol, may reversibly transfer the NO^+ moiety to protein SH groups (Hogg, 2002). The reversible nature of *S*-nitrosation is due to *S*-nitrosothiol susceptibility to catalytic decomposition by metal-induced hemolytic cleavage or to reduction by ascorbic acid, GSH, and thioredoxin.

The occurrence of protein *S*-nitrosation has been detected by ESI-MS measurement, as reported in the case of p21, hemoglobin, caspase 3 subunits, Ca-ATPase, and calbindin (Mirza et al., 1995; Ferranti et al., 1997; Zech et al., 1999; Viner et al., 1999; Tao and English, 2003). The occurrence in the spectra of adducts presenting a $\Delta m = +29$ Da was indicative of $NO^{\bullet}$ addiction to intact molecular species. In general, acid conditions are strongly recommended for the purification, digestion, and analysis of *S*-nitrosated species, as a result of their well-known instability (Ferranti et al., 1997; Viner et al., 1999). For this reason, peptide mixtures for mass mapping experiments are conventionally generated by pepsin hydrolysis, and their analysis is performed using soft ionization techniques (LC-ESI). Confirmation of signal assignment to *S*-nitrosated peptides is inferred by selective fragmentation at their S–NO bond upon increasing the cone voltage or temperature (Mirza et al., 1995; Ferranti et al., 1997). These notices allowed the identification of the unique *S*-nitrosated cysteine in hemoglobin following treatment with NOS or nitroso-cysteine.

3.2.2 Analysis of Oxidized/Nitrated Products of Tyrosine

A powerful strategy for understanding the underlying in vivo mechanisms of tyrosine oxidative injury is to identify stable end-products of protein oxidation produced by different reaction pathways (Heinecke, 1999a,b). In particular, specific tyrosine derivatives produced by oxidation have been characterized for myeloperoxidase-catalyzed reaction pathways through the action of HOCl (Cl-Tyr, and 3,5-dichlorotyrosine, di-Cl-Tyr) (Domigan et al., 1995; Podrez et al., 2000; Fu et al., 2000), for eosinophil peroxidase-catalyzed reaction pathways through the action of hypobromous acid (3-bromotyrosine, Br-Tyr, and 3,5-dibromotyrosine, di-Br-Tyr) (Wu et al., 1999), for free radical pathways (*o*-Tyr, *m*-Tyr, and di-Tyr), and for RNS-catalyzed pathways (NO_2-Tyr, di-Tyr, 3,4-dihydroxy phenylalanine, and the corresponding quinone).

Peroxynitrite is the principal mediator of tyrosine nitration (Mallozzi et al., 2001; Minetti et al., 2002). However, myeloperoxidase and other metalloproteins capable of peroxidase activity can catalyze Tyr nitration in the vascular compartment during inflammation by reaction with $NO^{\bullet}$, NO_2^-, and hydroperoxides (Eiserich et al., 1998; van der Vliet et al., 1999; Baldus et al., 2002; Gaut et al., 2002a). Accordingly, this modification is considered as a marker of oxidative/nitrosative injury during inflammatory conditions, frequently linked to altered protein function (MacMillan-Crow et al., 1998; Eiserich et al., 1999;

Aulak et al., 2001; Aslan et al., 2003). In fact, changing the chemical properties of the phenolic group that affect the capacity of this amino acid to function in electron-transfer reactions, and also the maintenance of protein conformation, can strongly affect enzymic activity (Eiserich et al., 1999; Zhu et al., 2000; Gole et al., 2000; Greenacre and Ischiropoulos, 2001). Nitration can be removed or reduced by either enzymatic or nonenzymatic mechanisms (Kamisaki et al., 1998; Balabanli et al., 1999; Davis et al., 2001). Reversibility of NO_2-Tyr formation implies that tyrosine nitration may not only represent a marker of RNS formation, as recently demonstrated for actin in both human sickle cell disease tissues and a murine model of sickle cell disease (Aslan et al., 2003), but may modulate protein function similar to other cell signaling events (Berlett et al., 1998). It is important to note that RNS, although yielding mainly NO_2-Tyr, can also concurrently induce di-Tyr formation and oxidative modification of even more RNS-susceptible protein targets (e.g., Cys, FeS, or ZnS complexes), determining impaired protein function (van der Vliet et al., 1999).

Depending on different oxidative/nitrosative pathways and active radicals involved in Tyr modification, a series of stable modification end-products have been identified through in vitro and in vivo studies in specific tissues or isolated proteins. Therefore Cl-Tyr, di-Cl-Tyr, Br-Tyr, di-Br-Tyr, NO_2-Tyr, di-Tyr, and the unnatural isomers *m*-Tyr and *o*-Tyr (derived from protein-Phe residues following reaction with hydroxyl radicals) have been selected as amino acid products stable to acid hydrolysis, making them useful markers for protein oxidation/nitration studies (Heinecke, 1999b; Heinecke et al., 1999). Thus, specific procedures for the detection of these derivatives in amino acid hydrolysates or biological fluids, following precolumn derivatization, have been optimized by using direct HPLC quantification (Shigenaga et al., 1997) and gas chromatography (GC)–or liquid chromatography (LC)–MS analysis (Heinecke, 1999b; Heinecke et al., 1999; Wu et al., 1999). However, when compared with the UV- or fluorescence-measurement counterpart, MS-based approaches provided structural information, thereby reducing chromatographic artifacts, and allowing researchers to perform selective ion monitoring (SIM) experiments for quantification of trace quantities. In addition, MS-based analyses permitted the use of stable, isotopically labeled internal standards essential for correction associated with analyte loss during processing and precision of quantitative measurements (Heinecke, 1999b; Heinecke et al., 1999). Accordingly, GC-MS analysis of *n*-propyl-heptafluorobutiryl-amino acid derivatives has successfully been used for the study of the oxidative pathways associated with Parkinson's disease and atherosclerosis (Pennathur et al., 1999; Heinecke, 1999b; Heinecke et al., 1999). Similarly, LC-MS procedures have been used for the characterization of the halogenated products generated by HOCl treatment or activated eosinophils (Wu et al., 1999; Fu et al., 2000). Recently, different GC- or LC-tandem mass spectrometry-based approaches have been proposed for the very accurate quantification of basal levels of NO_2-Tyr, di-Tyr, *o*-Tyr, and NO_2-Tyr-containing proteins in plasma and tissues (Yi et al., 2000; Frost et al., 2000; Gaut et al., 2002b; Tsikas et al., 2003; Marvin et al., 2003; Soderling et al., 2003; Tsikas and

Caidahl, 2005). These studies highlighted the possibility of artifactual formation of nitrated tyrosine during sample extraction and derivatization.

Contrarily to the above-mentioned procedures directed to ascertain the occurrence of Tyr-directed oxidative/nitrosative insults by GC- or LC-MS analysis of the modified amino acids recovered in protein hydrolysates, methodologies for the detection of these modifications by direct ESI- or MALDI-MS analysis of intact proteins or their peptide digests have found a positive application only in the case of NO_2-Tyr-, and di-Tyr-containing proteins and Tyr radical species (Minetti et al., 2000; Petersson et al., 2001; Sarver et al., 2001). Before the introduction of dedicated MS procedures, proteins containing modified tyrosine residues were detected by measuring its specific absorbance at 365 nm (NO_2-Tyr), fluorescence at 410 nm (excitation 315 nm) (di-Tyr), and ESR absorbance in the presence of spin-trapping compounds (Tyr radicals).

In the case of NO_2-Tyr-containing proteins, the occurrence of nitration events has been ascertained by direct measurements of intact molecules, detecting the corresponding adducts presenting a mass difference of +45 Da. However, when a comparative analysis of polypeptides containing NO_2-Tyr was performed by MALDI- and ESI-MS techniques, it was evident that MALDI measurements yielded unexpected significant underestimation of modification extent, as a result of a prompt fragmentation involving the nitro group (Petersson et al., 2001; Sarver et al., 2001). This phenomenon has been associated with a series of photodecomposition reactions determining the formation of 3NO-Tyr, 3NHOH-Tyr, and 3 NH_2-Tyr adducts, respectively. The effect of laser shots, laser power, and peptide concentration on the formation of these photodecomposition fragments was evaluated. These investigations ascertained that fragmentation of NO_2-Tyr cannot be controlled, thus highlighting the unreliability of this methodology for the sensitive detection of nitration products. On the contrary, ESI-MS measurements did not show this phenomenon, allowing a complete evaluation of the protein modification extent. Moreover, site-specific identification of NO_2-Tyr in proteins has been reported by LC-ESI or MALDI mapping experiments, detecting peptide species bearing the expected mass difference (Minetti et al., 2000; Kuhn et al., 2002; Schmidt et al., 2003). However, on the basis of the considerations reported above, most of the applications reported in the literature used ESI sources for the analysis of the nitrated peptides. Unequivocal assignment of NO_2-Tyr in various proteins has been determined by ESI-MS-MS experiments (Fig. 3.2) (MacMillan-Crow et al., 1998; Petersson et al., 2001; Miyagi et al., 2002; Haqqani et al., 2002; Aslan et al., 2003; Murray et al., 2003; Walcher et al., 2003; Koeck et al., 2004a). The use of precursor ion scanning for the specific immonium ion at m/z 181.06 has found a broader application in the identification of nitrated peptides during LC-ESI-MS/MS analysis of protein digests (Petersson et al., 2001). Very recently an integrated LC-ESI-MS/MS approach was used for the simultaneous assignment of nitration and chlorination sites on Apolipoprotein A-I (Zheng et al., 2005). This study was the first example of a quantitative peptide mapping investigation applied to the global analysis of in vitro and in vivo myeloperoxidase-catalyzed reactions affecting enzymatic functions in patient with

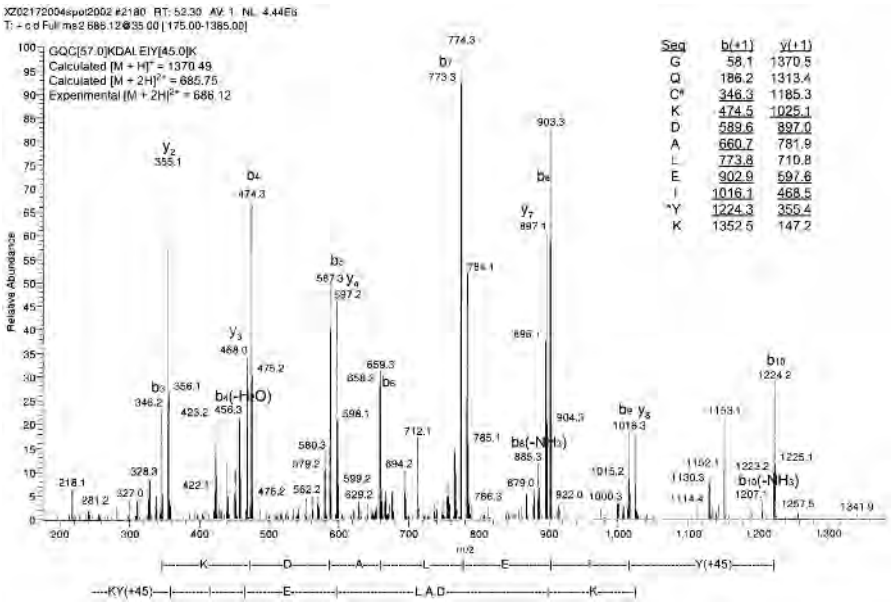


FIGURE 3.2 Tandem mass spectrometry analysis of the NO_2-Tyr-containing undecapeptide ^{228}GQCKDALEI*YK^{238} from synaptosomal-associated protein with the doubly charged precursor ion at $m/z = 686.12$ (Zhan and Desiderio, 2004).

cardiovascular diseases. In this case, detection of Cl-Tyr-containing peptides was achieved by specifically revealing species presenting a $\Delta m = +34/36$ Da, with typical halogen isotopic distribution, and confirmed by tandem mass spectrometry.

The occurrence of inter-molecular cross-linked di-Tyr residues in proteins following oxidative/nitrosative insult, determining macroscopic variation of the protein molecular mass, has been usually detected by low-resolution techniques such as SDS-PAGE under reducing conditions (MacMillan-Crow et al., 1998; Lardinois et al., 1999). In fact, contrarily to disulfide cross-linked polypeptides, these species are not sensitive to incubation with reducing agents. The occurrence of globin dimers following peroxynitrite treatment has been ascertained also by ESI measurements (Minetti et al., 2000). Cross-linking assignment to specific Tyr residues has been obtained by mass mapping experiments on peptide digests using either MALDI- or ESI-MS procedures (Lardinois et al., 1999; Minetti et al., 2000) and confirmed by CID experiments. In some cases, a selective isolation of the di-Tyr-containing peptides by a preliminary HPLC purification step has been performed.

The generation of tyrosyl radicals following oxidative insult of heme-containing proteins has been detected by trapping these species with specific spin-trapping agents and measuring the addition of the modifying moiety to the intact molecules by direct ESI or MALDI analysis (Gunther et al., 1998; Lardinois

et al., 1999; Zhang et al., 2002). Depending on the nature of the compound used, a mass increase of +344 Da (3,5-dibromo-4-nitrosobenzenesulfonic acid), +113 Da (5,5-dimethyl-1-pyrroline *N*-oxide), or +72 Da (2-methyl-2-nitrosopropane) was observed for the corresponding adducts. Investigation on the nature of the trapped derivatives obtained under different experimental conditions allowed researchers to elucidate the mechanism of interaction with heme group for different oxidative/nitrosative agents (Gunther et al., 1998; Pietraforte et al., 2002). Also in this case, identification of the tyrosine residues subjected to spin-trapping agent addition was obtained by LC-ESI-MS or MALDI mapping experiments on protein digests and confirmed by ESI-MS/MS or post-source decay (PSD) analysis of the modified peptides (Lardinois et al., 1999; Zhang et al., 2002).

3.2.3 Analysis of Oxidized Products of Methionine

Methionine residues are highly susceptible to oxidation by almost all ROS/RNS (Vogt, 1995). Mild oxidizing conditions determine the generation of methionine sulfoxide (MetO), which can be further oxidized to methionine sulfone ($MetO_2$) under stronger oxidizing conditions (Vogt, 1995; Levine et al., 2000a). Methionine oxidation has been associated with loss of protein function, as in the cases of α-1 protease inhibitor, α-chymotrypsin, ribonuclease, subtilisin, phosphoglucomutase, actin, and human immunodeficiency virus-2 protease as well as in several peptide hormones (Vogt, 1995; Davis et al., 2000; Dalle-Donne et al., 2002). Methionine sulfoxide can be reduced by NAD(P)H-dependent MetO reductases (Stadtman and Berlett, 1998; Levine et al., 2000a; Moskovitz et al., 2001). Since methionine oxidation is enzymatically reversible, it has been proposed that Met residues may serve an endogenous antioxidant defense, protecting target proteins from extensive irreversible damage at other essential amino acids (Levine et al., 1996, 2000a). This hypothesis is consistent with the observation that unlike other amino acid modifications, Met oxidation has little or no effect on the susceptibility of proteins to proteolytic degradation (Levine et al., 1996; Stadtman et al., 2002).

Determination of methionine oxidation products in protein hydrolysates by conventional chromatographic procedures has been discouraged as a result of the reducing conditions used during acid hydrolysis, which determine the spontaneous conversion of the oxidized products back to Met. A series of methodologies has been proposed to limit the extent of these side reactions, although a robust procedure for the determination of methionine redox state has not been developed (Sochaski et al., 2001). Oxidation of methionine thioether group to the corresponding sulfoxide and sulfone derivatives can be easily detected in the ESI or MALDI mass spectra of intact molecular species, by revealing the corresponding adducts with a mass difference of +16 Da and +32 Da, respectively (Hanson et al., 1999, 2000). In general, a numerical evaluation of the oxygen atoms introduced into a protein can be determined by counting the multiple additions of 16 mass units compared with the unmodified species. The selectivity of this modification toward Met and not Cys residues can be easily verified following mass

measurement of the alkylation products obtained with thiol-specific reagents. Moreover, the occurrence of MetO residues in oxidized proteins has also been verified by assaying the limited succeptibility of this species to cleavage by cyanogen bromide (Milzani et al., 2000).

Identification of modified Met residues has been obtained in different proteins by LC-ESI or MALDI mass mapping experiments, by detecting the peptides specifically bearing a mass difference of +16 and +32 Da, respectively (Hanson et al., 1999, 2000; Taggart et al., 2000; Griffiths and Cooney, 2002; Richardson et al., 2003; Khor et al., 2004; Requena et al., 2004). In most cases, it has been observed that the oxidized peptides usually elute earlier than the unmodified ones in a reversed phase chromatography separation. Very recently, a solid-phase isolation procedure for the selective enrichment of protein digests in MetO-containing peptides has been proposed (Grunert et al., 2003). In general, the occurrence of oxidized components presenting MetO or $MetO_2$ at specific positions was easily verified by low-energy CID experiments, revealing the characteristic loss of methanesulfenic acid (−64 Da or −32 Da for singly or doubly protonated ions, respectively) or methanesulfonic acid (−80 Da or −40 Da for singly or doubly protonated ions, respectively) from the side chain of oxidized Met derivatives (Lagerwerf et al., 1996; Guan et al., 2003; Reid et al., 2004). The correct assignment of the modification to a specific Met residue has easily been obtained by database search routines, after the necessary adjustment in the parameter file to account for the mass shift associated with the modification.

However, methionine oxidation in peptides and proteins occurs in vivo or may be an artifact resulting from sample manipulation during analytical characterization. To solve these difficulties, a dedicated procedure based on protein *N*-terminal acetylation, selective hydrolysis at Met residues by CNBr, and specific labeling of the newly generated amino groups with a bromine-containing compound has been proposed (Hollemeyer et al., 2002). This procedure allows the unequivocal localization of oxidized methionines even in complex peptide mixtures.

3.2.4 Analysis of Protein Carbonylation Products

Protein carbonylation is an irreversible oxidative modification. Carbonylated proteins are not repaired. They are removed by proteolytic degradation or they accumulate as damaged or unfolded proteins (Stadtman and Berlett, 1998). The number of carbonyl groups observed within a protein perfectly correlates with protein damage caused by oxidative stress (Shacter et al., 1994). Thus, protein carbonylation has been considered as a good indicator for the extent of oxidative damage of proteins in various human diseases (Levine et al., 2000b; Dalle-Donne et al., 2003a). Carbonyl groups (aldehydes and ketones) may be introduced in the protein at different sites and by different mechanisms (Stadtman and Berlett, 1998). Carbonyl moieties are produced on amino acid side chains (mainly at Pro, Arg, Lys, and Thr) when these residues are oxidized into ketone or aldehyde derivatives (Berlett and Stadtman, 1997). In parallel, protein carbonyl groups

can also be generated through oxidative cleavage of proteins by either the α-amidation pathway or oxidation of glutamyl side chains, leading to formation of a peptide in which the *N*-terminal amino acid is blocked by an α-ketoacyl derivative (Berlett and Stadtman, 1997). Protein carbonylation can also occur by reaction with various reactive products generated during lipid peroxidation, 4-hydroxynonenal (4-HNE), 2-propenal (acrolein), and malondialdehyde (MDA). It involves Michael addition of these reactive aldehydes to the nucleophilic side chain of Cys, His, or Lys residues, determining the incorporation of aldehyde/carbonyl group into the peptide chain. Finally, reactive carbonyl groups (ketoamines, ketoaldehydes, deoxyosones) can also be generated by secondary reactions of the primary amino group of Lys residues with reducing sugars or their oxidation products (glycation/glycoxidation) (Stadtman and Berlett, 1998). The occurrence of these carbonyl moieties may alter polypeptide chain conformation, thus determining the partial or total inactivation of numerous proteins.

Depending on the nature of the generated derivatives and their relative stability to drastic hydrolysis conditions, carbonylated adducts have been revealed either in protein and fluid hydrolysates by GC- and LC-ESI-MS analysis or by direct ESI- or MALDI-MS measurements on intact proteins or their peptide digests. The occurrence of Pro, Arg, and Lys residues in close proximity to the highly reactive $^{\bullet}$OH can directly convert these amino acids in carbonyl-containing derivatives (Requena et al., 2001a). The generated glutamic semialdehyde (Pro and Arg) and aminoadipic semialdehyde (Lys) products occurring in the polypeptide chain are usually detected by GC-MS measurement of the 5-hydroxy-2-aminovaleric acid (HAVA) and 6-hydroxy-2-caproic acid (HACA) obtained following reductive stabilization and extensive acid hydrolysis (Requena et al., 2001a). Amino acids are usually converted to their *N*,*O*-trifluoroacetyl methyl esters or *N*(*O*)-ethoxycarbonyl ethyl esters by precolumn derivatization. The general approach described by Stadtman and co-workers allowed SIM experiments to be performed for the quantification of trace quantities in biological samples as well as allowed the use of deuterated internal standards for analyte loss correction and precise quantitative measurements. Thus, the quantification of HAVA and HACA was obtained for a series of model proteins and mammalian tissues under normal and stressing conditions.

Nonenzymatic glycation of proteins (also designated as Maillard reaction) is initiated by the reaction of reducing carbohydrates with Lys or *N*-terminal residues, yielding Amadori compounds (aminoketoses) as primary products. These products have been detected by ESI or MALDI measurements of intact proteins, revealing the corresponding adducts presenting a mass difference of +162 Da (glucose and fructose) (Peterson et al., 1998; Saraswathi et al., 1999; Lapolla et al., 2000, 2005). Glycation assignment to specific Lys residues has been obtained by mass mapping experiments on peptide digests by either MALDI- or ESI-MS experiments (Miyata et al., 1994; Takahashi et al., 1995; McKillop et al., 2003), using a strategy similar to that used for the assignment of lactosylation

sites in milk proteins (Scaloni et al., 2002). Under strong oxidizing conditions, Amadori compounds are slowly degradated in complex reaction pathways, via dicarbonyl intermediates (3-deoxyglucosone, dideoxysones, methylglyoxal and glyoxal), to a plethora of linear or cross-linked derivatives (advanced glycation end products) as pyrralines, imidazolones, hydroimidazolones, crosslines, *N*-carboxymethyl-lysine (CML), *N*-carboxyethyl-lysine (CEL), 6-[1-(5-ammonio-6-oxido-6-oxohexyl)imidazolium-3-yl]-L-norleucinate (GOLD), 6-[1-(5-ammonio-6-oxido-6-oxohexyl)-4-methylimidazolium-3-yl]-L-norleucinate (MOLD), N^6-[2-(4-ammonio-5-oxido-5-oxopentyl)amino]-5-(2,3,4-trihydroxybutyl)-3,5-dihydro -4H-imidazol-4-ylidene]-L-lysinate (DOGDIC), N^6-[2-(4-ammonio-5-oxido-5-oxopentyl)amino]-5-(2,3,4-dihydroxypropyl)-3,5-dihydro-4H-imidazol-4-ylidene] -L-lysinate (DOPDIC), N^6-glycoloyl-lisine (GALA), N^6-[2-(5-ammino-5-carboxypentyl)amino]-2-oxoethyl-lysine (GOLA), and others. Contrarily to Amadori compounds, these derivatives have been routinely detected in vitro or in vivo following extensive protein/tissue acid or enzymic hydrolysis by dedicated GC-MS or LC-ESI-MS analytical procedures (Biemel et al., 2001, 2002; Glomb and Pfahler, 2001; Thornalley et al., 2003). Also in this case, the synthesis of ^{13}C-containing internal standards and the possibility to perform SIM experiments allowed an accurate evaluation of trace quantities in human serum albumin and lens proteins. Very recently, the direct characterization and assignment of these heterogeneous modifications to specific Lys or Arg residues was obtained by mass mapping experiments on protein digests by either MALDI- or ESI-MS techniques, using a strategy similar to that used for the assignment of other oxidative/nitrosative modifications and dedicated peptide-searching algorithms (Humeny et al., 2002; Brock et al., 2003; Marotta et al., 2003; Cocklin et al., 2003; Bidasee et al., 2004).

Protein modification by 4-HNE proceeds primarily via a Michael addition to Lys, His, and Cys residues. This reaction can be monitored for intact proteins by ESI- or MALDI-MS analysis, detecting the corresponding adducts with a mass increase of +156 Da (Bennaars-Eiden et al., 2002; Crabb et al., 2002; Alderton et al., 2003). The identification of modified residues has been obtained in different proteins by LC-ESI or MALDI mass mapping experiments, detecting the peptides specifically bearing this mass difference (Bennaars-Eiden et al., 2002; Crabb et al., 2002; Alderton et al., 2003). The nature of the modified amino acid was definitively ascertained by MS/MS-based investigations. Alternatively, the extent of lysine modification has been evaluated by GC-MS analysis, reavealing the 3-(N^ε-lysino)-4-hydroxynonal-1-ol generated in protein exaustive hydrolysates following reduction with $NaBH_4$ (Requena et al., 1997). The use of deuterated internal standards allowed an accurate measurement. This approach was also applied to the quantification of the modified Lys and Arg in proteins following MDA treatment. This reaction proceeds via a Schiff-base adduct formation. In this case, the quantification of 3-(N^ε-lysino)propan-1-ol, 1,3-di(N^ε-lysino)propane and 2-ornithinyl-4-methyl(1ε-lysyl)1,3-midazole led to an evaluation of the cross-linked adducts (Requena et al., 1997; Slatter et al., 2004).

3.2.5 Analysis of Oxidatively/Nitr(os)atively Products of Tryptophan and Histidine

Metal-catalyzed oxidation of proteins involves reduction of Fe(III) or Cu(II) by a suitable electron donor such as NADH, NADPH, ascorbate, or mercaptane. Fe(II) or Cu(I) ions bound to specific metal-binding sites on proteins react with oxidants to generate radicals (Berlett and Stadtman, 1997) that immediately oxidize neighboring amino acid residues. In general, protein metal-catalyzed oxidation is a highly selective reaction that occurs at sites with transition metal-binding capability. Thus, in addition to the Met, Cys, and Tyr oxidation already described, a close proximity of His and Trp residues to heme or Cu(II) binding sites can determine specific amino acid modifications. Thus, 2-oxo-histidine and 4- or 5-hydroxy-2-oxo-histidine are generated from His oxidation; similarly, hydroxytryptophan, *N*-formylkynurenine, kynurenine, and 3OH-kynurenine are Trp oxidation products. Examples of protein metal-catalyzed oxidation have been reported for myoglobin (Gunther et al., 1998; Hara et al., 2001), β-amyloid peptide (Schoneich and Williams, 2002), Cu,Zn-SOD (Kurahashi et al., 2001), and recombinant prion protein (Requena et al., 2001b). Moreover, the occurrence of proteins in close proximity to a general source of ROS, as normally respiring tissues or UV radiation, can determine His or Trp modification. In fact, *N*-formylkynurenine generation has been reported in normal conditions for lens proteins (Finley et al., 1998) and a series of cardiac mitochondrial proteins (Taylor et al., 2003). These reactions may result in structural alterations and loss of enzymatic activity implicated in a variety of diseases, including several neurodegenerative ailments as well as in aging. Specific nitration of tryptophan residues has also been reported (Wendt et al., 2003; Suzuki et al., 2004; Sala et al., 2004). This protein modification reaction has been recently demonstrated for different proteins following reaction with peroxynitrite or a series of metalloproteins capable of peroxidase activity.

The oxidation of tryptophan has been known for decades, since the inactivation of lysozyme by oxidants modifying a critical Trp residue was reported (Previero et al., 1967). These early reports relied on identification of tryptophan oxidation products only by characteristic electronic absorbance spectra. Few years ago, the first complete MS characterization of a protein from bovine lens, namely α-crystallin, presenting oxidized Trp residues as a result of exposition to oxidative Fenton insult was described. The occurrence of hydroxytryptophan, *N*-formylkynurenine, kynurenine, and 3OH-kynurenine in reaction products was ascertained by direct ESI measurements, detecting the corresponding adducts with a mass increase of +16 Da, +32 Da, +4 Da, and +20 Da, respectively (Finley et al., 1998). The identification of the modified residues was obtained by MALDI mass mapping experiments combined with ESI-MS/MS analysis of the oxidized peptides. Although it could not be demonstrated unequivocally whether oxidation occurred during sample handling or in vivo, the same Trp residues were found oxidized during the 2D-LC-MS/MS analysis of human cataract lens digests (MacCoss et al., 2002). Later on, it has been reported the oxidation of a critical

Trp residue in the chloroplast photosystem II protein CP43, providing the first example of this selective modification in vivo (Anderson et al., 2002). Recently, a massive analysis of tryptophan oxidation in cardiac mitochondrial proteins has been reported. The ESI-MS/MS detection of *N*-formylkynerenine in a series of target proteins suggested that Trp modification is obtained in vivo as a result of the close proximity of these polypeptides to a source of ROS (Taylor et al., 2003).

Heme-assisted oxidation of tryptophan in myoglobin mutants to 2,6-dihydro-2,6-dioxoindole and 2,6-dihydro-2-imino-6-oxoindole derivatives has been reported by a combined MALDI mass mapping, PSD analysis, and $^{1}H/^{13}C$ nuclear magnetic resonance spectroscopy approach. The oxidized peptides occurring in the digest presented a selective mass difference of +30 Da compared with the unmodified residue (Hara et al., 2001).

Very recently, the occurrence of nitrosation and nitration events affecting Trp residues has been ascertained by direct MS measurements. The occurrence of NO-Trp, 1-NO_2-Trp, and 6-NO_2-Trp has been demonstrated by detecting the corresponding adducts having a mass difference of +29 and +45 Da, respectively (Wendt et al., 2003; Suzuki et al., 2004; Sala et al., 2004). Under physiological conditions 6-NO_2-Trp was stable, whereas NO-Trp, 1-NO_2-Trp decomposed with half-lives of 1.5 and 18 hours, respectively. However, when peptides mapping experiments on modified creatine kinase isoenzymes were performed by MALDI-MS, it was evident that this ionization technique yielded photodecomposition of the nitro group to 3NHOH-Trp (Wendt et al., 2003), as already observed during analysis of NO_2-Tyr-containing peptides (Petersson et al., 2001; Sarver et al., 2001).

Histidine modification as a result of metal-catalyzed protein oxidation has been revealed by detecting 2-oxo-His and 4- or 5-hydroxy-2-oxo-His as the main reaction products (Schey and Finley, 2000; Requena et al., 2001b). Although the occurrence of 2-oxo-histidine in protein hydrolysates has been detected by conventional amino acid analysis procedures (Requena et al., 2001b), the presence of oxidized His derivatives has been also ascertained by direct ESI or MALDI analysis of intact molecules, detecting the corresponding adducts with a mass increase of +16 Da and +32 Da, respectively (Kurahashi et al., 2001; Schoneich and Williams, 2002). Assignment of the modified residues in the polypeptide sequence can be obtained by MALDI or ESI mass mapping experiments, revealing the peptides specifically bearing this mass difference or ESI-MS/MS precursor ion scanning for the specific immonium ion at m/z 126 or 142, respectively. The nature of the modified amino acid can be definitively ascertained by ESI tandem mass spectrometry analysis. These experiments led to the identification of 2-oxo-histidine and 4- or 5-hydroxy-2-oxo-histidine in the oxidation products of β-amyloid peptide, Cu,Zn-SOD, and recombinant prion protein, allowing a general scheme of reaction to be drawn (Kurahashi et al., 2001; Requena et al., 2001b; Schoneich and Williams, 2002). In these works, the detection of the oxidation products for other amino acids in close proximity to His in sites with transition metal-binding capability was also reported.

3.3 PROTEOMIC STRATEGIES FOR THE IDENTIFICATION OF ROS/RNS PROTEIN TARGETS IN BIOLOGICAL MATRICES

Oxidative/nitrosative damage by reactive radical species appears central to the pathogenesis of many disorders. Specific modified proteins are generated following stressing insults and accumulate in different degenerated tissues and fluids, determining in some cases altered organ functionalities. Proteome analysis, providing reaserchers with a global approach to describe all protein components at specific cellular moments, is an ideal choice for revealing all the polypeptide modifications due to a particular stressing condition or disease. Therefore, to obtain a comprehensive description of oxidative insults, different proteomic approaches have been developed and used for the detection and identification of ROS/RNS protein targets among all the species present in a biological sample (Ghezzi and Bonetto, 2003). These investigations have recently been ascribed to the general term of redox proteomics.

Whether the goal is the identification of differently modified or expressed proteins in oxidatively stressed or disease samples, accurate resolution of thousands of proteins is an absolute requirement. For this reason two-dimensional (2D) gel electrophoresis approaches have widely been used to separate proteins according to their pI and mass value. Detection of differently modified/expressed proteins is achieved by comparing 2D electrophoretic maps of samples subjected to oxidative/nitrosative insult with the corresponding controls (differential proteomics). Figure 3.3 reports the example of human epithelial cells following treatment with H_2O_2 (D'Elia et al., 2003). Adequate image analysis has been essential to properly map modified protein spots. Specialized image analysis softwares are now available to deal with the complexity of protein spot patterns, fulfilling protein spot detection and quantification, multiple image alignments, and image comparisons. Furthermore, massive screening and selective detection of oxidatively/nitrosatively modified proteins separated by 2D electrophoresis have been facilitated by the availability of specific staining compounds or commercial antibodies allowing their immunodetection (Fig. 3.4). Samples are separated on different 2D gels that are stained in parallel with derivatives specific for ROS/RNS-modified species and with aspecific staining chemicals. Again, adequate image analysis is essential to properly detecting modified protein spots and associating them with spots aspecifically stained. Similarly, the incorporation of radioactivity by using specific radioactive precursors of modifying groups has been used to detect ROS/RNS-modified proteins. Nowadays, this general methodology has been widely applicated in the description of ROS/RNS-related stressing events or diseases, demonstrating that, in a specific tissue or fluid, selected proteins are modified to a much greater extent than others. Alternatively, approaches based on specific immunoaffinity enrichment or purification before electrophoretic separation have been used to selectively bind possible ROS/RNS targets to derivatized beads (Lind et al., 2002; Eaton et al., 2003). Also in this case, the detection of the oxidatively modified proteins has been obtained by specific staining or Western blotting procedures.

On this basis, protein carbonyls have been detected and quantified on 2D gels, measuring the incorporation of tritium, following reduction with [^{3}H]-borohydride (Yan and Sohal, 1998) or following derivatization with fluorescein hydrazide (Ahn et al., 1987) or 2,4-dinitrophenylhydrazine (DNPH) (Levine et al., 1990). The latter procedure, based on the pioneering analysis of stable 2,4-dinitrophenyl (DNP) hydrazone products by Levine, Stadtman, and co-workers, has become the most widely used methodology for the measurement of protein oxidation in several human diseases (Dalle-Donne et al., 2003a, 2003b), experimental models (Powell et al., 2001), and purified proteins (Milzani et al., 2000; Dalle-Donne et al., 2001). The detection of carbonylated proteins has also been facilitated by the recent introduction of anti-DNP antibodies allowing carbonyl detection by Western blotting analysis (Oxyblot analysis) (Castegna et al., 2002a,

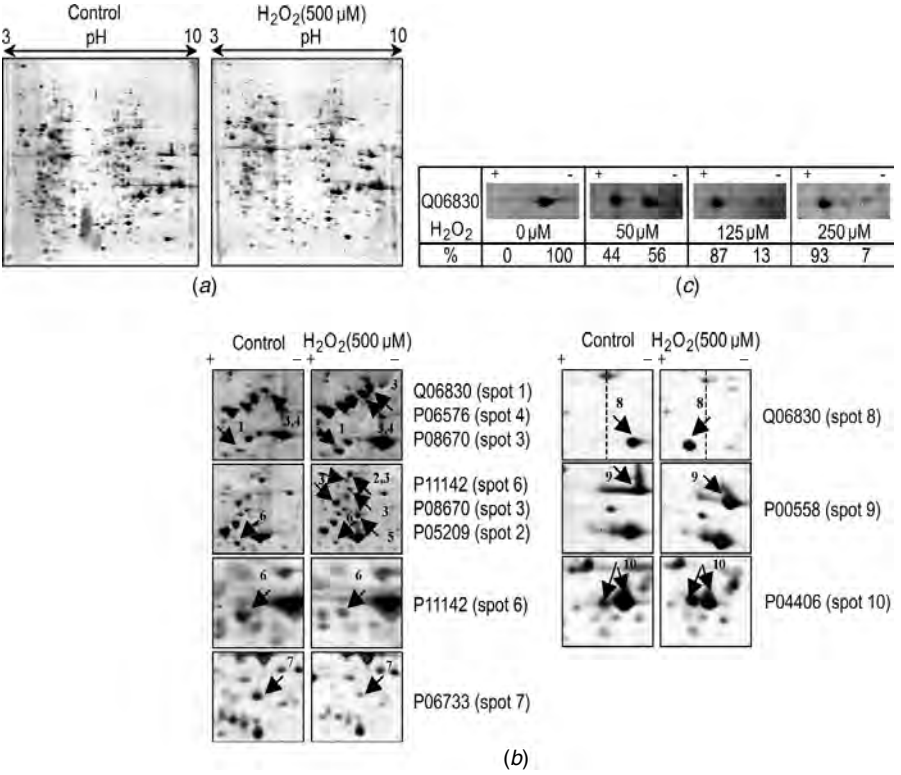


FIGURE 3.3 Proteomic analysis of human epithelial lens cells in response to H_2O_2. CD5A cells were incubated with and without 500 µM H_2O_2 for 30 minutes, at 37°C. Cells were harvested and lysed, and intracellular proteins were analyzed by 2D electrophoresis with subsequent silver staining (*a*). Regions comprising statistical significant differences were cropped (*b*). Quantitative analysis of reduced (*right*) and oxidized (*left*) peroxyredoxin I species detected following exposition to indicated doses of H_2O_2 is reported (*c*) (D'Elia et al., 2003).

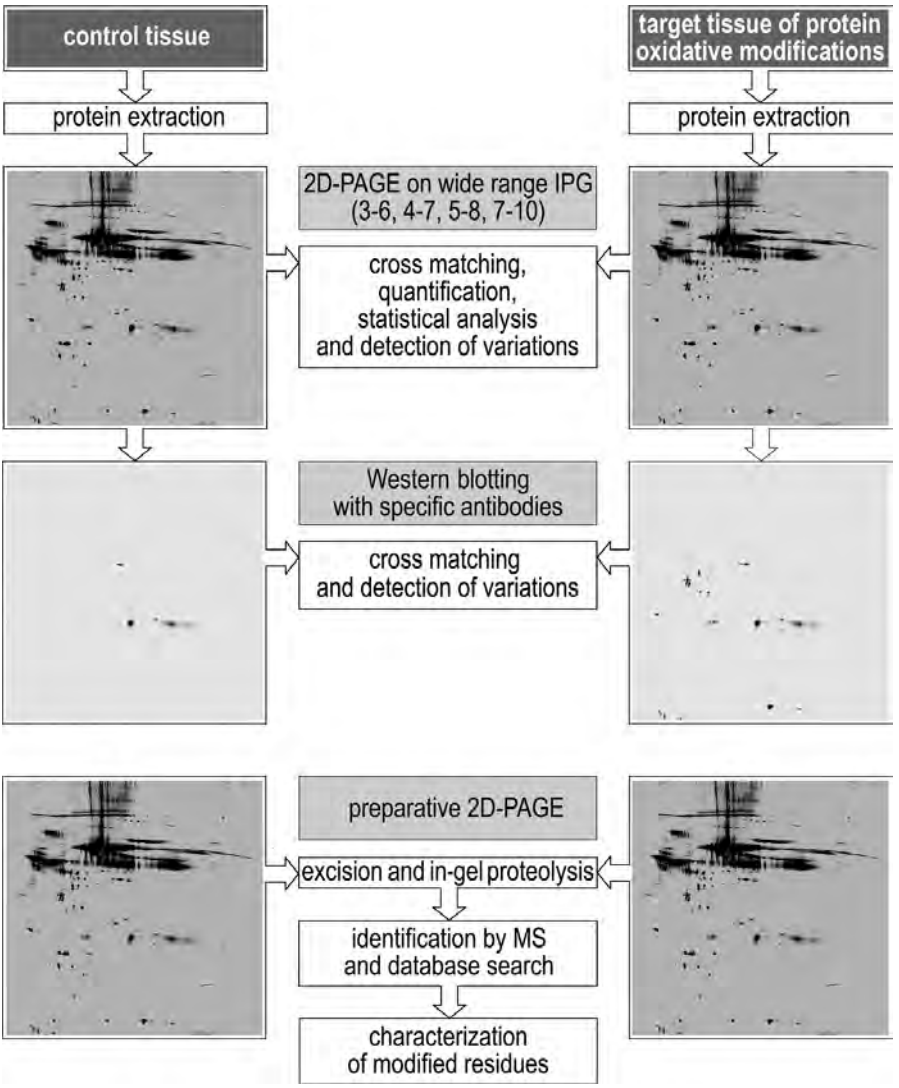


FIGURE 3.4 A general strategy for the proteomic analysis of oxidatively/nitrosatively stressed tissues/body fluids by 2D electrophoresis and specific immunodetection. Samples are separated on different 2D gels that are stained in parallel with derivatives or commercial antibodies specific for ROS/RNS-modified proteins and with aspecific staining chemicals. Spots from gels subjected to aspecific staining are analyzed by peptide mass fingerprint or tandem mass spectrometry analysis. Images are compared by dedicated analysis softwares (Dalle-Donne et al., 2005).

2002b). Similarly, an antibody raised against glycated RNAse has been used for immunodetection of advanced glycation end-products (Poggioli et al., 2002).

Analogous methodologies have been developed for detecting NO_2-Tyr in complex protein mixtures as a marker of nitrosative insult and irreversible protein damage. All these methodologies are based on the relative stability of this modification. A first approach uses immunochemical techniques for the specific detection of NO_2-Tyr-containing proteins following 2D-gel electrophoresis by anti-nitrotyrosine antibodies (Ye et al., 1996). False immunopositive spots are easily recognized by preventive reduction of NO_2-Tyr to NH_2-Tyr after dithionite treatment (Miyagi et al., 2002). A modification of this procedure has been recently developed (Nikov et al., 2003). It uses immunoprecipitation of NO_2-Tyr-containing proteins by anti-nitrotyrosine antibodies, dithionite reduction of NO_2-Tyr, and derivatization of resulting NH_2-Tyr with cleavable biotin tags. Finally, biotinylated proteins are purified on a streptavidin affinity column. The second approach is based on the release of the modified Tyr in protein hydrolysates and its combined detection by LC-UV, LC-ECD, GC, and GC-MS techniques (Herce-Pagliai et al., 1998).

Proteomics methods for the detection of *S*-glutathionylated proteins have been similarly devised, either using [^{35}S]-labeled GSH for the specific detection of [^{35}S] incorporation into proteins resolved on 2D gels (Fratelli et al., 2002) or biotinylated GSH ester for the selective immunoaffinity purification of *S*-glutathionylated proteins (Sullivan et al., 2000; Eaton et al., 2002a, 2002b, 2003). In the first case, *S*-glutathionylated proteins are identified by incubation of cells with radiolabeled Cys in the absence of protein synthesis, exposure to oxidants, isolation, and detection of radiolabeled (i.e., glutathionylated) proteins (Fratelli et al., 2002). This methodology applied to the study of stressed T lymphocytes has revealed that numerous proteins are targets for redox-dependent modification. Several of the enzymes identified were inactivated during in vitro assays, implying that *S*-glutathionylation of proteins upon alteration of the cellular redox status is a potential mechanism for regulating the activity of many targets. In the second approach, the amino terminus of cysteine is tagged with biotin and loaded into cells or tissues (Laragione et al., 2003). When oxidizing changes occur, formation of a disulfide bond between redox-sensitive protein cysteines and biotin-cysteine is induced. These oxidized proteins carrying a biotin tag are detected using nonreducing Western blotting and streptavidin-HRP or purified by streptavidin-agarose affinity chromatography. The efficiency of purification can be improved with the use of gel-filtration chromatography for separating proteins from free biotin-cysteine in homogenates, which otherwise competes with *S*-thiolated proteins for column binding. These proteins are then separated by SDS-PAGE and stained by aspecific compounds.

Detection of proteins undergoing regulatory formation of inter-molecular protein disulfide following different redox challenges has been achieved by 2D sequential nonreducing/reducing SDS-PAGE (Brennan et al., 2004). Protein targets are clearly visualized as spots running off of the diagonal after a careful comparison with a control. On the other hand, disulfide-containing protein targets

of thioredoxin have been detected by two complementary proteome approaches (Yano et al., 2002; Wong et al., 2004). The first one uses 2D electrophoresis following reduction of the extracts by the NADP/thioredoxin system and labeling the newly generated SH groups with monobromobimane. The second one uses 2D electrophoresis to resolve covalently interacting proteins trapped on affinity columns prepared with mutant thioredoxin in which one of the active site Cys has been replaced by Ser. In both cases, a careful evaluation of the false positive candidates is an essential prerequisite for optimal protein identification.

An elegant approach was reported for the isolation of *S*-nitrosated proteins (Jaffrey et al., 2001). Free SH groups were blocked with methyl methanethiosulfonate by this method. Protein-*S*-nitrosocysteine groups were successively reduced to free thiols by treatment with ascorbate. The newly released protein thiols were derivatized with a cleavable biotin-containing reagent, affinity purified using streptavidin agarose beads, and released for SDS-PAGE separation. This methodology was validated by confirmation that many targets of exogenous nitrosating agents are in vivo targets, as revealed by the lack of labeling in brain lysates from mice deficient in nNOS (Jaffrey et al., 2001).

Accurate global labeling and quantitation of protein cysteine residues often has been hampered by the lack of reagents suitable for in vivo experiments on intact organelles or cells. To overcome these difficulties a novel compound, namely (4-iodobutyl)-triphenylphosphonium, has been developed and applied to proteomic investigations (Lin et al., 2002). Its lipophilic cationic character allowed selective uptake into cellular organelles having large membrane potential across inner membranes; its specific reaction with cysteines to yield stable thioether adduct-containing proteins, easily detectable by specific monoclonal antibodies. This reagent has been successfully used in differential studies, providing results comparable to those obtained on liver mitochondria for *N*-(biotinoyl)-*N′*-(iodoacetyl)ethylendiamine. Proteins labeled by this latter compound present a biotin tag easily recognized and quantified by streptavidin-based immunostaining (Shiva et al., 2004).

In all the cases reported above, protein spots or bands presenting increased radioactivity or positive staining by specific chemicals or antibodies have to be further analyzed to identify their nature. Accordingly, protein species are excised, *in-gel* digested, and their peptide digests analyzed by MALDI-MS or LC-ESI-MS procedures. As a result of the dramatic technological developments affecting sensitivity, resolution, and accuracy properties, MS has increasingly become the method of choice for the analysis of separated protein components. Peptide-mass fingerprint and tandem mass spectrometry-based peptide sequencing approaches have been used extensively for the identification of the oxidatively/nitrosatively modified proteins as well as for assignment of the modification to specific amino acid residues (Pandey and Mann, 2000). The achievement of this second issue is strictly related to the possibility to obtain a detailed structural characterization of the entire protein primary structure, including modifications, even using the poor amounts recovered from gels or beads. In this sense, all technological improvements for sample manipulation and miniaturization of the

chromatographic devices coupled to mass spectrometers have been essential for an extensive protein characterization. Examination of the measured mass and fragmentation spectra obtained for a single protein via manual or computer-assisted interpretation led to the assignment of oxidized/nitrosated/nitrated amino acids using the criteria described above.

The integrated use of the above-mentioned methodologies has recently been applied to the global examination of the oxidatively/nitrosatively modified proteins in different tissues and body fluids. Differential proteomic investigations on samples deriving from different redox challenging conditions were performed by revealing spots presenting quantitative modification differences, following specific staining or Western blotting. This approach was used to study (1) oxidatively induced proteomic changes in stressed human epithelial cells (D'Elia et al., 2003), liver mitochondria (Lin et al., 2002; Shiva et al., 2004), muscle tissues (Stagsted et al., 2004), brain tissues during Parkinson's and Alzheimer's diseases (Choi et al., 2004), and ovarian epithelial cells during oncogenic transformation (Young et al., 2004); (2) *S*-glutathionylation in oxidatively stressed human T lymphocytes (Fratelli et al., 2002) and in heart and kidney tissues following ischemia and reperfusion (Eaton et al., 2003); (3) inter- and intra-molecular protein disulfide formation during cardiac oxidative stress (Brennan et al., 2004); (4) *S*-nitrosation in brain lysates and endothelial cells (Jaffrey et al., 2001; Yang and Loscalzo, 2005); (5) protein carbonylation in endoplasmic reticulum (England and Cotter, 2004), brain tissues during Alzheimer's disease or amyloid deposition (Castegna et al., 2002a,b; Choi et al., 2002, 2003; Korolainen et al., 2002; Yu et al., 2003; Shin et al., 2004), and aged liver mitochondria (Rabek et al., 2003); and (6) Tyr nitration in skin fibroblats and pituitary tissues (Koeck et al., 2004a; Zhan and Desiderio, 2004), heart and skeletal muscle tissues during aging or diabetes (Kanski et al., 2003, 2005; Turku et al., 2003), retina tissues following light irradiation (Miyagi et al., 2002), cellular lines exposed to particulate pollutants (Xiao et al., 2005), liver mitochondria exposed to hypoxia-anoxia and reoxygenation (Koeck et al., 2004b), brain, liver, lung, and kidney tissues during Alzheimer's, inflammation, and sickle cell disease (Aulak et al., 2001; Aslan et al., 2003; Castegna et al., 2003). Most protein targets of ROS/RNS insult have been identified by these high-throughput methodologies; sometimes, the assignment of modification to specific amino acids was achieved by peptide mass mapping and tandem mass spectrometry experiments, as reported in the previous sections (Aslan et al., 2003; D'Elia et al., 2003; Kanski et al., 2003, 2005; Zhan and Desiderio, 2004). The example in Figure 3.5 reports the ascertained modification of peroxiredoxin I Cys52 to cysteic acid in oxidatively stressed human epithelial cells (D'Elia et al., 2003).

Recently, attempts were made to define protein modifications on a proteome-wide scale without the use of 2D gel electrophoresis. Given the difficulties of identifying all modifications even in a single protein, at present it is clear that scanning for proteome-wide modifications is not comprehensive. Nevertheless, a large amount of biological useful information can, in principle, be generated

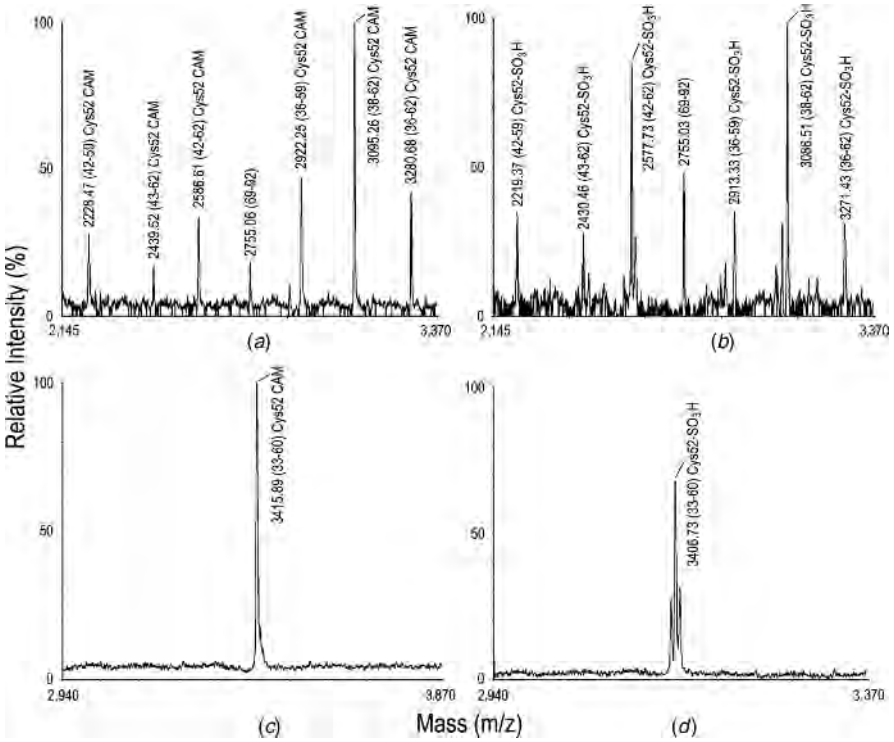


FIGURE 3.5 MALDI-TOF mass spectrometry analysis of the reduced and oxidized forms of PrxI reported in Figure 3.3, following carboxamidomethylation and trypsin or endoprotease AspN hydrolysis. The figure reports the spectrum of the tryptic digest fraction eluted from μZipTip devices with 40% acetonitrile for reduced (*a*) and oxidized PrxI (*b*) as well as the endoprotease AspN digest fraction eluted with 50% acetonitrile for reduced (*c*) and oxidized PrxI (*d*). CAM, carboxamidomethyl (D'Elia et al., 2003).

by this approach. One of the strategies used is essentially an extension of the approach used to analyze protein mixtures by 2D LC-ESI-MS/MS procedures (MacCoss et al., 2002). Instead of searching the database only for nonmodified peptides, the database search algoritm is instructed to also match potentially modified peptides. To avoid a "combinatorial explosion" resulting from the need to consider all the possible modifications for all the peptides in the database, the experiment is usually divided into identification of a set of proteins on the basis of nonmodified peptides, followed by searching only those proteins for modified peptides. An a priori oriented strategy focuses on the search for one selected type of modification on all the proteins present in a sample. This approach has successfully been used for the examination of the occurrence of *N*-formylkynurenine, a product of Trp oxidation, through the mitochondrial proteome of normal human heart tissues (Taylor et al., 2003). Thirty-seven different proteins were identified as "hot spots" for oxidation in close proximity to a source of ROS.

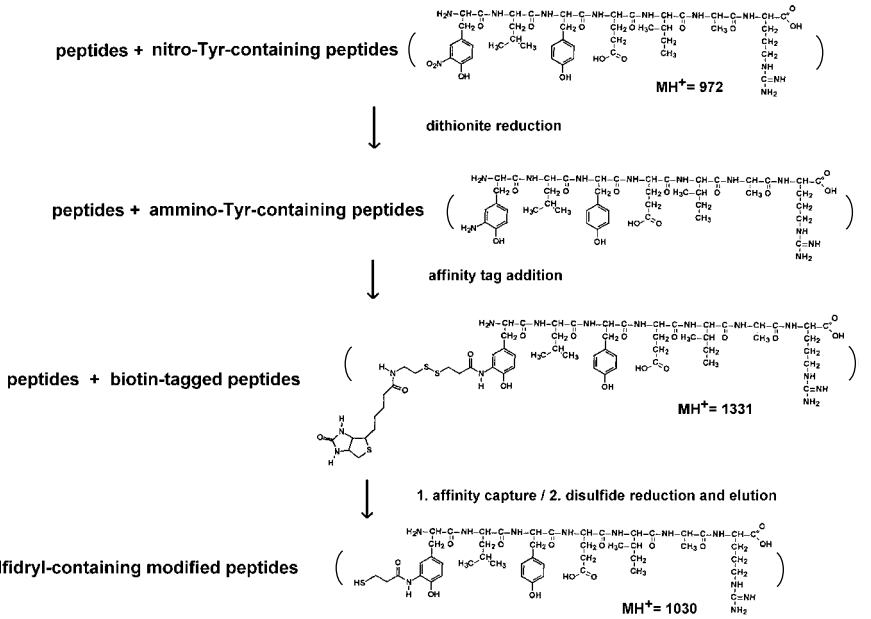


FIGURE 3.6 Selective enrichment of NO_2-Tyr-containing peptides from whole cellular lysates. Nitrotyrosine-containing proteins obtained by immunoprecipitation with anti-nitrotyrosine antibodies are proteolitically digested and resulting peptides are reduced with dithionite. Reduction of NO_2-Tyr and derivatization of resulting NH_2-Tyr with cleavable biotin tags allows selective purification of biotinylated peptides on streptavidin affinity column. Biotin tag removal by disulfide reduction precedes MALDI or LC-ESI-MS/MS analysis of resulting peptide mixtures for structural characterization (Nikov et al., 2003).

To reduce complexity of whole cellular protein digests, alternative approaches have been developed to enrich mixtures into peptides bearing specific oxidative/nitrosative modifications. A procedure has been developed that is dedicated to the identification of NO_2-Tyr-containing peptides. It involves immunoprecipitation of NO_2-Tyr-containing proteins by anti-nitrotyrosine antibodies, dithionite reduction of NO_2-Tyr, derivatization of resulting NH_2-Tyr with cleavable biotin tags, protein digestion, selective purification of biotinylated peptides on streptavidin affinity column, final tag removal before LC-ESI-MS/MS, and bioinformatic analysis (Fig. 3.6) (Nikov et al., 2003). A similar strategy has been used to identify and quantify oxidant-sensitive thiol-containing proteins in differential experiments by isotope-coded affinity tag (ICAT) reagents (Sethuraman et al., 2004). In this case, whole cellular extracts containing proteins with a different Cys-reducing state are separately labeled with light and heavy ICAT reagents and independently digested. Digests are mixed, desalted, enriched in biotinylated peptides by streptavidin affinity column elution and analyzed by LC-ESI-MS/MS analysis and database search. A quick comparison of the relative MS peak intensity for each light and heavy ICAT-labeled peptide allows identification and

quantitation of polypeptide species whose Cys redox state is variated under the different experimental conditions investigated; proteins containing these peptides are quickly identified with sequence information obtained by tandem mass spectrometry analysis.

3.4 CONCLUSIONS

MS-based technologies have been propitious to the development of molecular medicine, especially in the discovery of diagnostic biomarkers of oxidative/nitrosative stress, enabling early detection of diseases. Their contribution in enabling the identification and characterization of oxidatively/nitrosatively modified proteins in human diseases has just begun. Although redox proteomics is currently a nascent field of research, the opportunities for identification of proteins involved in diseases strictly linked to oxidative/nitrosative stress are clear and compelling. Since proteins are involved in virtually every cellular function, proteome dictates the functional phenotype in every tissue and organ. This phenotype varies continuously under normal conditions (depending on differentiation, age, and cell cycle stage), and it can change as a result of oxidative/nitrosative stress, thus initiate and/or advance acute or chronic diseases. Usually, acute insults lead to rapid post-translational modifications of proteins, but in chronic diseases co-translational (isoform switching) and post-translational protein modifications occur in concert with altered gene expression, leading to varied protein levels. For specific proteins, disease-induced modifications will substantially affect function, which in turn has the potential to affect other proteins. The result is a dynamic, ongoing process of protein expression and modification.

Already the oxidatively/nitrosatively modified proteins in a few human diseases have been identified. New proteomic tools, in the next future, will facilitate the identification of protein markers still unknown, allowing their complete characterization in a given pathophysiological condition. These findings should establish relationships between the pathological hallmarks of a disease and the protein functional and/or structural alterations. Furthermore, comparison of the molecular fingerprints obtained in cells or body fluids with those produced by various in vitro oxidation/nitrosation/nitration systems should indicate the biochemical pathways creating the damage in vivo. All these data should help decipher the potential roles played by ROS/RNS-induced modifications in human disorders. In this sense, redox proteomics is opening new ways to gain insight into the molecular mechanisms involved in human pathologies. This research should establish new hypotheses for disease-induced cell and tissue alterations. Hierarchies of altered proteins (i.e., which protein alteration is the primary event and which are secondary phenomena) will have to be established. Other approaches (e.g., animal models with overexpressed, knocked-in, or knocked-out proteins identified as potentially important in a certain disease) will have to be selected to complement MS-based redox proteomic investigations. As for any laboratory-based assay that is used in epidemiological research, methodological issues such as reproducibility, inter- versus intra-person variability, sensitivity, and specificity, will have to

be discussed. The key issues will have to be raised on the potential for artifacts in the estimation of modified protein baseline levels, as are associated with incorrect sample handling, processing, and analysis. Previous investigations have been plagued by these problems during the analysis of different biomarkers of protein oxidation. Quantitative proteomics will have a leading role in the solution of this problem as well as in the evaluation of all other issues.

As described in this chapter, a battery of markers of protein oxidation/nitrosation/ nitration is available or is under development. The challenge researchers face is sorting out the markers and combinations of markers that are predictive of human diseases associated with oxidative/nitrosative stress. At the center is a question still unresolved: Does the formation of oxidized proteins have a significant, direct physiological or pathological impact, or is it a secondary phenomenon? Most evidence points to ROS/RNS-induced protein modification having physiological significance, and in some pathologies to its being not solely a secondary phenomenon. Consequently, we do believe that redox proteomics will have a central role in future definition of redox molecular mechanisms associated with human pathologies.

ACKNOWLEDGMENTS

This work has been partially supported by FIRB 2001 grants (contract RBAU01T97W_003 and RBAU01PRLA_002).

LIST OF ABBREVIATIONS

Br-Tyr, 3-bromotyrosine
CID, collision-induced dissociation
Cl-Tyr, 3-chlorotyrosine
2D, two-dimensional
di-Br-Tyr, 3,5-dibromotyrosine
di-Cl-Tyr, 3,5-dichlorotyrosine
di-Tyr, *o*,*o*′-dityrosine
DNP, 2,4-dinitrophenyl
DNPH, 2,4-dinitrophenylhydrazine
ESI, electrospray ionization
GC, gas chromatography
GSH, glutathione
HACA, 6-hydroxy-2-caproic acid
HAVA, 5-hydroxy-2-aminovaleric acid
4-HNE, 4-hydroxynonenal
HPLC, high-performance liquid chromatography

LC, liquid chromatography
LC-ESI, liquid chromatography–electrospray ionization
MALDI, matrix-assisted laser desorption ionization
MDA, malondialdehyde
MetO, methionine sulfoxide
$MetO_2$, methionine sulfone
MS, mass spectrometry
NADPH, reduced nicotinamide adenine dinucleotide phosphate
NO_2-Tyr, 3-nitrotyrosine
o-Tyr, *o*-tyrosine
PSD, post-source decay
RNS, reactive nitrogen species
ROS, reactive oxygen species
SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis
SIM, selective ion monitoring
SH, sulfhydryl

REFERENCES

Ahn B., Rhee S.G., Stadtman E.R. (1987). Use of fluorescein hydrazide and fluorescein thiosemicarbazide reagents for the fluorometric determination of protein carbonyl groups and for the detection of oxidized protein on polyacrylamide gels. *Anal Biochem* 161: 245–257.

Alderton A.L., Faustman C., Liebler D.C., Hill D.W. (2003). Induction of redox instability of bovine myoglobin by adduction with 4-hydroxy-2-nonenal. *Biochemistry* 42: 4398–4405.

Anderson L.B., Maderia M., Ouellette A.J., Putnam-Evans C., Higgins L., Krick T., MacCoss M.J., Lim H., Yates J.R. III, Barry B.A. (2002). Post-translational modifications in the CP43 subunit of photosystem II. *Proc Natl Acad Sci USA* 99: 14676–14681.

Arner E.S., Holmgren A. (2000). Physiological functions of thioredoxin and thioredoxin reductase. *Eur J Biochem* 267: 6102–6109.

Aslan M., Ryan T.M., Townes T.M., Coward L., Kirk M.C., Barnes S., Alexander C.B., Rosenfeld S.S., Freeman B.A. (2003). Nitric oxide-dependent generation of reactive species in sickle cell disease: Actin tyrosine nitration induces defective cytoskeletal polymerization. *J Biol Chem* 278: 4194–4204.

Aulak K.S., Miyagi M., Yan L., West K.A., Massillon D., Crabb J.W., Stuehr D.J. (2001). Proteomic method identifies proteins nitrated in vivo during inflammatory challenge. *Proc Natl Acad Sci USA* 98: 12056–12061.

Balabanli B., Kamisaki Y., Martin E., Murad F. (1999). Requirements for heme and thiols for the nonenzymatic modification of nitrotyrosine. *Proc Natl Acad Sci USA* 96: 13136–13141.

Baldus S., Eiserich J.P., Brennan M.-L., Jackson R.M., Alexander C.B., Freeman B.A. (2002). Spatial mapping of pulmonary and vascular nitrotyrosine reveals the pivotal

role of myeloperoxidase as a catalyst for tyrosine nitration in inflammatory diseases. *Free Radic Biol Med* 33: 1010–1019.

Baynes J.W., Thorpe S.R. (2000). Glycoxidation and lipoxidation in atherogenesis. *Free Radic Biol Med* 28: 1708–1716.

Beal M.F. (2002). Oxidatively modified proteins in aging and disease. *Free Radic Biol Med* 32: 797–803.

Bennaars-Eiden A., Higgins L., Hertzel A.V., Kapphahn R.J., Ferrington D.A., Bernlohr D.A. (2002). Covalent modification of epithelial fatty acid-binding protein by 4-hydroxynonenal in vitroand in vivo: Evidence for a role in antioxidant biology. *J Biol Chem* 277: 50693–50702.

Berlett B.S., Levine R.L., Stadtman E.R. (1998). Carbon dioxide stimulates peroxynitrite-mediated nitration of tyrosine residues and inhibits oxidation of methionine residues of glutamine synthetase: Both modifications mimic effects of adenylylation. *Proc Natl Acad Sci USA* 95: 2784–2789.

Berlett B.S., Stadtman E.R. (1997). Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem* 272: 20313–20316.

Bhattacharjee S., Pennathur S., Byun J., Crowley J., Mueller D., Gischler J., Hotchkiss R.S., Heinecke J.W. (2001). NADPH oxidase of neutrophils elevates *o*,*o*′-dityrosine cross-links in proteins and urine during inflammation. *Arch Biochem Biophys* 395: 69–77.

Bidasee K.R., Zhang Y., Shao C.H., Wang M., Patel K.P., Dincer D., Besch H.R. (2004). Diabetes increases formation of advanced glycation end products on sarco(endo)plasmic reticulum ATPase. *Diabetes* 53: 463–473.

Biemel K.M., Friedl D.A., Lederer M.O. (2002). Identification and quantification of major maillard cross-links in human serum albumin and lens protein: Evidence for glucosepane as the dominant compound. *J Biol Chem* 277: 24907–24915.

Biemel K.M., Reihl O., Conrad J., Lederer M.O. (2001). Formation pathways for lysine-arginine cross-links derived from hexoses and pentoses by Maillard processes: Unraveling the structure of a pentosidine precursor. *J Biol Chem* 276: 23405–23412.

Brennan J.P., Wait R., Begum S., Bell J.R., Dunn M.J., Eaton P. (2004). Detection and mapping of widespread intermolecular protein disulfide formation during cardiac oxidative stress using proteomics with diagonal electrophoresis. *J Biol Chem* 279: 41352–41360.

Brock J., Hinton D., Cotham W.E., Metz T.O., Thorpe S., Baynes J., Ames J.M. (2003). Proteomic analysis of the site specificity of glycation and carboxymethylation of ribonuclease. *J Prot Res* 2: 506–513.

Budanov A., Sablina A., Feinstein E., Koonin E., Chumakov P.M. (2004). Regeneration of peroxiredoxins by p53-regulated sestrins, homologs of bacterial AhpD. *Science* 304: 596–600.

Butterfield D.A., Drake J., Pocernich C., Castegna A. (2001). Evidence of oxidative damage in Alzheimer's disease brain: Central role for amyloid beta-peptide. *Trends Mol Med* 7: 548–554.

Butterfield D.A., Lauderback C.M. (2002). Lipid peroxidation and protein oxidation in Alzheimer's disease brain: Potential causes and consequences involving amyloid beta-peptide-associated free radical oxidative stress. *Free Radic Biol Med* 32: 1050–1060.

Caselli A., Marzocchini R., Camici G., Manao G., Moneti G., Pieraccini G., Ramponi G. (1998). The inactivation mechanism of low molecular weight phosphotyrosine-protein phosphatase by H_2O_2. *J Biol Chem* 273: 32554–32560.

Castegna A., Aksenov M., Aksenova M., Thongboonkerd V., Klein J., Pierce W., Booze R., Markesbery W., Butterfield D. (2002a). Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part I: Creatine kinase BB, glutamine synthase, and ubiquitin carboxy-terminal hydrolase L-1. *Free Radic Biol Med* 33: 562–571.

Castegna A., Aksenov M., Thongboonkerd V., Klein J.B., Pierce W.M., Booze R., Markesbery W.R., Butterfield D.A. (2002b). Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part II: Dihydropyrimidinase-related protein 2, α-enolase and heat shock cognate 71. *J Neurochem* 82: 1524–1532.

Castegna A., Thongboonkerd V., Klein J.B., Lynn B., Markesbery W.R., Butterfield D.A. (2003). Proteomic identification of nitrated proteins in Alzheimer's disease brain. *J Neurochem* 85: 1394–1401.

Cecconi I., Scaloni A., Rastelli G., Moroni M., Vilardo P.G., Costantino L., Cappiello M., Garland D., Carper D., Petrash J.M., Del Corso A., Mura U. (2002). Oxidative modification of aldose reductase induced by copper ion. Definition of the metal–protein interaction mechanism. *J Biol Chem* 277: 42017–42027.

Choi J., Levey A.I., Weintraub S.T., Rees H.D., Gearing M., Chin L., Li L. (2004). Oxidative modifications and don-regulation of ubiquitin *C*-terminal hydrolase L1 associated with idiopathic Parkinson's and Alzheimer's diseases. *J Biol Chem* 279: 13256–13264.

Choi J., Malakowsky C.A., Talent J.M., Conrad C.C., Gracy R.W. (2002). Identification of oxidized plasma proteins in Alzheimer's disease. *Biochem Biophys Res Commun* 293: 1566–1570.

Choi J., Malakowsky C.A., Talenta J.M., Conrad C.C., Carroll C.A., Weintraub S.T., Gracy R.W. (2003). Anti-apoptotic proteins are oxidized by $A\beta_{25-35}$ in Alzheimer's fibroblasts. *Biochim Biophys Acta* 1637: 135–141.

Cocklin R.R., Zhang Y., O'Neill K.D., Chen N.X., Moe S.M., Bidasee K.R., Wang M. (2003). Identify and localization of advanced glycation products on human β2-microglobulin using MALDI-TOF-MS. *Anal Biochem* 314: 322–325.

Cotgreave I.A., Gerdes R.G. (1998). Recent trends in glutathione biochemistry-glutathione-protein interactions: A molecular link between oxidative stress and cell proliferation? *Biochem Biophys Res Commun* 242: 1–9.

Crabb J.W., O'Neil J., Miyagi M., West K., Hoff H.F. (2002). Hydroxynonenal inactivates cathepsin B by forming Michael adducts with active site residues. *Protein Sci* 11: 831–840.

Dalle-Donne I., Giustarini D., Colombo R., Rossi R., Milzani A. (2003a). Protein carbonylation in human diseases. *Trends Mol Med* 9: 169–176.

Dalle-Donne I., Rossi R., Giustarini D., Gagliano N., Di Simplicio P., Colombo R., Milzani A. (2002). Methionine oxidation as a major cause of the functional impairment of oxidized actin. *Free Radic Biol Med* 32: 927–937.

Dalle-Donne I., Rossi R., Giustarini D., Gagliano N., Lusini L., Milzani A., Di Simplicio P., Colombo R. (2001). Actin carbonylation: From a simple marker of protein oxidation to relevant signs of severe functional impairment. *Free Radic Biol Med* 31: 1075–1083.

Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. 2003b. Protein carbonyl groups as biomarkers of oxidative stress. *Clin Chim Acta* 329: 23–38.

Dalle-Donne I., Scaloni A., Giustarini D., Cavarra E., Tell G., Lungarella G., Colombo R., Rossi R., Milzani A. (2005). Proteins as biomarkers of oxidative/nitrosative stress in diseases: the contribution of redox proteomics. *Mass Spectrom Rev* 24: 55–99.

Davies M.J., Dean R.T. (1997). *Radical-Mediated Protein Oxidation: From Chemistry to Medicine, the Pathology of Protein Oxidation*, Oxford University Press, New York.

Davies M.J., Fu S., Wang H., Dean R.T. (1999). Stable markers of oxidant damage to proteins and their application in study of human disease. *Free Radic Biol Med* 27: 1151–1161.

Davis D.A., Newcomb F.M., Moskovitz J., Wingfield P.T., Stahl S.J., Kaufman J., Fales H.M., Levine R.L., Yarchoan R. (2000). HIV-2 protease is inactivated after oxidation at the dimer interface and activity can be partly restored with methionine solfoxide reductase. *Biochem J* 346: 305–311.

Davis K.L., Martin E., Turko I.V., Murad F. (2001). Novel effects of nitric oxide. *Ann Rev Pharmacol Toxicol* 41: 203–236.

D'Elia A., Paron I., D'Ambrosio C., Scaloni A., D'Aurizio F., Prescott A., Damante G., Tell G. (2003). A proteomic approach to identify early molecular targets of oxidative stress in human epithelial lens cells. *Biochem J* 378: 929–937.

Dean R.T., Fu S., Stocker R., Davies M.J. (1997). Biochemistry and pathology of radical-mediated protein oxidation. *Biochem J* 324: 1–18.

Domigan N.M., Charlton T.S., Duncan M.W., Winterbourn C.C., Kettle A.J. (1995). Chlorination of tyrosyl residues in peptides by myeloperoxidase and human neutrophils. *J Biol Chem* 270: 16542–16548.

Dweik R.A., Comhair S.A., Gaston B., Thunnissen F.B., Farver C., Thomassen M.J., Kavuru M., Hammel J., Abu-Soud H.M., Erzurum S.C. (2001). NO chemical events in the human airway during the immediate and late antigen-induced asthmatic response. *Proc Natl Acad Sci USA* 98: 2622–2627.

Eaton P., Byers H.L., Leeds N., Ward M.A., Shattock MJ. (2002a). Detection, quantitation, purification, and identification of cardiac proteins *S*-thiolated during ischemia and reperfusion. *J Biol Chem* 277: 9806–9811.

Eaton P., Fuller W., Shattock M.J. (2002b). *S*-Thiolation of HSP27 regulates its multimeric aggregate size independently of phosphorylation. *J Biol Chem* 277: 21189–21196.

Eaton P., Jones M.E., McGregor E., Dunn M.J., Leeds N., Byers H.L., Leung K.-I., Ward M.A., Pratt J.R., Shattock M.J. (2003). Reversible cysteine-targeted oxidation of proteins during renal oxidative stress. *J Am Soc Nephrol* 14: S290–S296.

Eiserich J.P., Estevez A.G., Bamberg T.V., Ye Y.Z., Chumley P.H., Beckman J.S., Freeman B.A. (1999). Microtubule dysfunction by post-translational nitrotyrosination of alpha-tubulin: a nitric oxide-dependent mechanism of cellular injury. *Proc Natl Acad Sci USA* 96: 6365–6370.

Eiserich J.P., Hristova M., Cross C.E., Jones A.D., Freeman B.A., Halliwell B., van der Vliet A. (1998). Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 391: 393–397.

England K., Cotter T. (2004). Identification of carbonylated proteins by MALDI-TOF mass spectrometry reveals susceptibility of ER. *Biochem Biophys Res Commun* 320: 123–130.

Ferranti P., Malorni A., Mamone G., Sannolo N., Marino G. (1997). Characterisation of *S*-nitroso-haemoglobin by mass spectrometry. *FEBS Lett* 400: 19–24.

Finley E.L., Dillon J., Crouch R.K., Schey K.L. (1998). Identification of tryptophan oxidation products in bovine alpha-crystallin. *Protein Sci* 7: 2391–2397.

Fratelli M., Demol H., Puype M., Casagrande S., Eberini I., Salmona M., Bonetto V., Mengozzi M., Duffieux F., Miclet E., Bachi A., Vandekerckhove J., Gianazza E., Ghezzi P. (2002). Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. *Proc Natl Acad Sci USA* 99: 3505–3510.

Frost M.T., Halliwell B., Moore K.P. (2000). Analysis of free and protein-bound nitrotyrosine in human plasma by a gas chromatography/mass spectrometry method that avoids nitration artifacts. *Biochem J* 345: 453–458.

Fu S., Wang H., Davies M., Dean R. (2000). Reactions of hypochlorous acid with tyrosine and peptidyl-tyrosyl residues give dichlorinated and aldehydic products in addition to 3-chlorotyrosine. *J Biol Chem* 275: 10851–10858.

Gaut J.P., Byun J., Tran H.D., Heinecke J.W. (2002b). Artifact-free quantification of free 3-chlorotyrosine, 3-bromotyrosine, and 3-nitrotyrosine in human plasma by electron capture-negative chemical ionisation gas chromatography mass spectrometry and liquid chromatography-electrospray ionization tandem mass spectrometry. *Anal Biochem* 300: 252–259.

Gaut J.P., Byun J., Tran H.D., Lauber W.M., Carroll J.A., Hotchkiss R.S., Belaaouaj A., Heinecke J.W. (2002a). Myeloperoxidase produces nitrating oxidants in vivo. *J Clin Invest* 109: 1311–1319.

Georgiou G. (2002). How to flip the (redox) switch. *Cell* 111: 607–610.

Ghezzi P., Bonetto V. (2003). Redox proteomics: Identification of oxidatively modified proteins. *Proteomics* 3: 1145–1153.

Giasson B.I., Duda J.E., Murray I.V., Chen Q., Souza J.M., Hurtig H.I., Ischiropoulos H., Trojanowski J.Q., Lee V.M. (2000). Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. *Science* 290: 985–989.

Giasson B.I., Ischiropoulos H., Lee V.M., Trojanowski J.Q. (2002). The relationship between oxidative/nitrative stress and pathological inclusions in Alzheimer's and Parkinson's diseases. *Free Radic Biol Med* 32: 1264–1275.

Glomb M.A., Pfahler C. (2001). Amides are novel protein modifications formed by physiological sugars. *J Biol Chem* 276: 41638–41647.

Gole M.D., Souza J.M., Choi I., Hertkorn C., Malcolm S., Foust R.F., Finkel B., Lanken P.N., Ischiropoulos H. (2000). Plasma proteins modified by tyrosine nitration in acute respiratory distress syndrome. *Am J Physiol Lung Cell Mol Physiol* 278: L961–967.

Greenacre S.A., Ischiropoulos H. (2001). Tyrosine nitration: Localisation, quantification, consequences for protein function and signal transduction. *Free Radic Res* 34: 541–581.

Griffiths H.R., Moller L., Bartosz G., Bast A., Bertoni-Freddari C., Collins A., Cooke M., Coolen S., Haenen G., Hoberg A.M., Loft S., Lunec J., Olinski R., Parry J., Pompella A., Poulsen H., Verhagen H., Astley S.B. (2002). Biomarkers. *Mol Aspects Med* 23: 101–208.

Griffiths S.W., Cooney C.L. (2002). Development of a peptide mapping procedure to identify and quantify methionine oxidation in recombinant human antitrypsin. *J Chromatogr A* 942: 133–143.

Grune T., Merker K., Sandig G., Davies K.J. (2003). Selective degradation of oxidatively modified protein substrates by the proteasome. *Biochem Biophys Res Commun* 305: 709–718.

Grunert T., Pock K., Buchacher A., Allmaier G. (2003). Selective solid-phase isolation of methionine-containing peptides and subsequent matrix-assisted laser desorption/ionisation mass spectrometric detection of methionine- and of methionine-sulfoxide-containing peptides. *Rapid Commun Mass Spectrom* 17: 1815–1824.

Gu Z., Kaul M., Yan B., Kridel S.J., Cui J., Strongin A., Smith J.W., Liddington R., Lipton S.A. (2002). *S*-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. *Science* 297: 1186–1190.

Guan Z., Yates N.A., Bakhtiar R. (2003). Detection and characterization of methionine oxidation in peptides by collision-induced dissociation and electron capture dissociation. *J Am Soc Mass Spectrom* 14: 605–613.

Gunther M.R., Tschirret-Guth R.A., Witkowska H.E., Fann Y.C., Barr D.P., Ortiz De Montellano P.R., Mason R.P. (1998). Site-specific spin trapping of tyrosine radicals in the oxidation of metmyoglobin by hydrogen peroxide. *Biochem J* 330: 1293–1299.

Halliwell B., Gutteridge J.M.C. (1999). *Free Radicals in Biology and Medicine*, Oxford University Press, New York.

Hanson S.R., Chen A.A., Smith J.B., Lou M.F. (1999). Thiolation of the gamma B-crystallins in intact bovine lens exposed to hydrogen peroxide. *J Biol Chem* 274: 4735–4742.

Hanson S.R., Hasan A., Smith D.L., Smith J.B. (2000). The major in vivo modifications of the human water-insoluble lens crystallins are disulfide bonds, deamidation, methionine oxidation and backbone cleavage. *Exp Eye Res* 71: 195–207.

Haqqani A.S., Kelly J.F., Birnboim H.C. (2002). Selective nitration of histone tyrosine residues in vivo in mutatect tumors. *J Biol Chem* 277: 3614–3621.

Hara I., Ueno T., Ozaki S., Itoh S., Lee K., Ueyama N., Watanabe Y. (2001). Oxidative modification of tryptophan 43 in the heme vicinity of the F43W/H64L myoglobin mutant. *J Biol Chem* 276: 36067–36070.

Heinecke J.W., Hsu F.F., Crowley J.R., Hazen S.L., Leeuwenburgh C., Mueller D.M., Rasmussen J.E., Turk J. (1999). Detecting oxidative modification of biomolecules with isotope dilution mass spectrometry: Sensitive and quantitative assays for oxidized amino acids in proteins and tissues. *Methods Enzymol* 300: 124–144.

Heinecke J.W. (1999a). Mechanisms of oxidative damage by myeloperoxidase in atherosclerosis and other inflammatory disorders. *J Lab Clin Med* 133: 321–325.

Heinecke J.W. (1999b). Mass spectrometric quantification of amino acid oxidation products in proteins: Insights into pathways that promote LDL oxidation in the human artery wall. *FASEB J* 13: 1113–1120.

Hensley K., Robinson K.A., Gabbita S.P., Salsman S., Floyd R.A. (2000). Reactive oxygen species, cell signaling, and cell injury. *Free Radic Biol Med* 28: 1456–1462.

Herce-Pagliai C., Kotecha S., Shuker D.E. (1998). Analytical methods for 3-nitrotyrosine as a marker of exposure to reactive nitrogen species: A review. *Nitric Oxide* 2: 324–336.

Hogg N. (2002). The biochemistry and physiology of *S*-nitrosothiols. *Annu Rev Pharmacol Toxicol* 42: 585–600.

Hollemeyer K., Heinzle E., Tholey A. (2002). Identification of oxidized methionine residues in peptides containing two methionine residues by derivatization and matrix-assisted laser desorption/ionization mass spectrometry. *Proteomics* 2: 1524–1531.

Humeny A., Kislinger T., Becker C., Pischetrieder M. (2002). Qualitative determination of specific protein glycation products by MALDI-MS peptide mapping. *J Agric Food Chem* 50: 2153–2160.

Jaffrey S.R., Erdjument-Bromage H., Ferris C.D., Tempst P., Snyder S.H. (2001). Protein *S*-nitrosylation: A physiological signal for neuronal nitric oxide. *Nat Cell Biol* 3: 193–197.

Kamisaki Y., Wada K., Bian K., Balabanli B., Davis K., Martin E., Behbod F., Lee Y.C., Murad F. (1998). An activity in rat tissues that modifies nitrotyrosine-containing proteins. *Proc Natl Acad Sci USA* 95: 11584–11589.

Kanski J., Alterman M.A., Sconeich C. (2003). Proteomic analysis of age-dependent protein nitration in rat skeletal muscle. *Free Radic Biol Med* 35: 1229–1239.

Kanski J., Behring A., Pelling J., Sconeich C. (2005). Proteomic identification of 3-nitrotyrosine-containing rat cardiac proteins: Effects of biological aging. *Am J Physiol Heart Circ Physiol* 288: H371–381.

Khan J., Brennand D.M., Bradley N., Gao B., Bruckdorfer R., Jacobs M., Brennan D.M. (1998). 3-Nitrotyrosine in the proteins of human plasma determined by an ELISA method. *Biochem J* 330: 795–801.

Khor H., Fisher M.T., Schoneich C. (2004). Potential role of methionine sulfoxide in the inactivation of the chaperone GroEL by hypochlorous acid and peroxynitrite. *J Biol Chem* 279: 19486–19493.

Klatt P., Lamas S. (2000). Regulation of protein function by *S*-glutathiolation in response to oxidative and nitrosative stress. *Eur J Biochem* 267: 4928–4944.

Koek T., Levison B., Hazen S.L., Crabb J.W., Stuehr D.J., Aulak K.S. (2004a). Tyrosine nitration impairs mammalian aldolase A activity. *Mol Cell Proteomics* 3: 548–557.

Koek T., Fu X., Hazen S.L., Crabb J.W., Stuehr D.J., Aulak K.S. (2004b). Rapid and selective oxygen-regulated protein tyrosine denitration and nitration in mitochondria. *J Biol Chem* 279: 27257–27262.

Korolainen M.A., Goldsteins G., Alafuzoff I., Koistinaho J., Pirttila T. (2002). Proteomic analysis of protein oxidation in Alzheimer's disease brain. *Electrophoresis* 23: 3428–3433.

Kuhn D.M., Sadidi M., Liu X., Kreipke C., Geddes T., Borges C., Watson J.T. (2002). Peroxynitrite-induced nitration of tyrosine hydroxylase. *J Biol Chem* 277: 14336–14342.

Kunsch C., Medford R.M. (1999). Oxidative stress as a regulator of gene expression in the vasculature. *Circ Res* 85: 753–766.

Kurahashi T., Miyazaki A., Suwan S., Isobe M. (2001). Extensive investigations on oxidized amino acid residues in H_2O_2-treated Cu,Zn-SOd protein with LC-ESI-Q-TOF-MS, MS/MS for the determination of the copper-binding site. *J Am Chem Soc* 123: 9268–9278.

Lagerwerf F.M., van de Weert M., Heerma W., Haverkamp J. (1996). Identification of oxidized methionine in peptides. *Rapid Commun Mass Spectrom* 10: 1905–1910.

Lapolla A., Fedele D., Traldi P. (2000). The role of mass spectrometry in the study of nonenzymatic protein glycation in diabetes. *Mass Spectrom Rev* 19: 279–304.

Laragione T., Bonetto V., Casoni F., Massignan T., Bianchi G., Gianazza E., Ghezzi P. (2003). Redox regulation of surface protein thiols: identification of integrin α-4 as a molecular target by using redox proteomics. *Proc Natl Acad Sci USA* 100: 14737–14741.

Lardinois O.M., Medzihradszky K.F., Ortiz de Montellano P.R. (1999). Spin trapping and protein cross-linking of the lactoperoxidase protein radical. *J Biol Chem* 274: 35441–35448.

Levine R.L., Garland D., Oliver C.N., Amici A., Climent I., Lenz A., Ahn B., Shaltiel S., Stadtman E.R. (1990). Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* 186: 464–478.

Levine R.L., Moskovitz J., Stadtman E.R. (2000a). Oxidation of methionine in proteins: roles in antioxidant defense and cellular regulation. *IUBMB Life* 50: 301–307.

Levine R.L., Mosoni L., Berlett B.S., Stadtman E.R. (1996). Methionine residues as endogenous antioxidants in proteins. *Proc Natl Acad Sci USA* 93: 15036–15040.

Levine R.L., Wehr N., Williams J.A., Stadtman E.R., Shacter E. (2000b). Determination of carbonyl groups in oxidized proteins. *Methods Mol Biol* 99: 15–24.

Lim A., Prokaeva T., McComb M.E., Connors L.H., Skinner M., Costello C.E. (2003). Identification of *S*-sulfonation and *S*-thiolation of a novel transthyretin Phe33Cys variant from a patient diagnosed with familial transthyretin amyloidosis. *Protein Sci* 12: 1775–1785.

Lin T., Hughes G., Muratovska A., Blaikie F.H., Brookes P.S., Darley-Usmar V., Smith R.A., Murphy M.P. (2002). Specific modification of mitochondrial protein thiols in response to oxidative stress. *J Biol Chem* 277: 17048–17056.

Lind C., Gerdes R., Hamnell Y., Schuppe-Koistinen I., von Lowenhielm H.B., Holmgren A., Cotgreave I.A. (2002). Identification of *S*-glutathionylated cellular proteins during oxidative stress and constitutive metabolism by affinity purification and proteomic analysis. *Arch Biochem Biophys* 406: 229–240.

MacCoss M.J., McDonald W.H., Saraf A., Sadygov R., Clark J.M., Tasto J.J., Gould K.L., Wolters D., Washburn M., Weiss A., Clark J.I., Yates J.R. III. (2002). Shotgun identification of protein modifications from protein complexes and lens tissue. *Proc Natl Acad Sci USA* 99: 7900–7905.

MacMillan-Crow L.A., Crow J.P., Thompson J.A. (1998). Peroxynitrite-mediated inactivation of manganese superoxide dismutase involves nitration and oxidation of critical tyrosine residues. *Biochemistry* 37: 1613–1622.

Mallozzi C., Di Stasi M.A., Minetti M. (2001). Peroxynitrite-dependent activation of src tyrosine kinases lyn and hck in erythrocytes is under mechanistically different pathways of redox control. *Free Radic Biol Med* 30: 1108–1117.

Marotta E., Lapolla A., Fedele D., Senesi A., Reitano R., Witt M., Seraglia R., Traldi P. (2003). Accurate mass measurements by Fourier transform mass spectrometry in the study of advanced glycation end products/peptides. *J Mass Spectrom* 38: 196–205.

Marvin L.F., Delatour T., Tavazzi I., Fay L.B., Cupp C., Guy P.A. (2003). Quantification of *o*,*o*′-dityrosine, o-nitrotyrosine, and o-tyrosine in cat urine samples by LC/electrospray ionization–MS/MS using isotope dilution. *Anal Chem* 75: 261–267.

McKillop A.M., Meade A., Flatt P.R., O'Harte F.P. (2003). Evaluation of the site(s) of glycation in human proinsulin by ion-trap LCQ electrospray ionization mass spectrometry. *Regul Pept* 113: 1–8.

Mikkelsen R.B., Wardman P. (2003). Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms. *Oncogene* 22: 5734–5754.

Milzani A., Rossi R., Di Simplicio P., Giustarini D., Colombo R., Dalle-Donne I. (2000). The oxidation produced by hydrogen peroxide on Ca-ATP-G-actin. *Protein Sci* 9: 1774–1782.

Minetti M., Mallozzi C., Di Stasi A.M. (2002). Peroxynitrite activates kinases of the src family and upregulates tyrosine phosphorylation signaling. *Free Radic Biol Med* 33: 744–754.

Minetti M., Pietraforte D., Carbone V., Salzano A.M., Scorza G., Marino G. (2000). Scavenging of peroxynitrite by oxyhemoglobin and identification of modified globin residues. *Biochemistry* 39: 6689–6697.

Mirza U., Chait B., Lander H.M. (1995). Monitoring reactions of nitric oxide with peptides and proteins by electrospray ionization mass spectrometry. *J Biol Chem* 270: 17185–17188.

Miyagi M., Sakaguchi H., Darrow R.M., Yan L., West K., Aulak K.S., Stuehr D.J., Hollyfield J.G., Organisciak D.T., Crabb J.W. (2002). Evidence that light modulates protein nitration in rat retina. *Mol Cell Proteomics* 1: 293–303.

Miyata T., Inagi R., Wada Y., Ueda Y., Iida Y., Takahashi M., Taniguchi N., Maeda K. (1994). Glycation of human beta 2-microglobulin in patients with hemodialysis-associated amyloidosis: Identification of the glycated sites. *Biochemistry* 33: 12215–12221.

Moskovitz J., Bar-Noy S., Williams W.M., Requena J., Berlett B.S., Stadtman E.R. (2001). Methionine sulfoxide reductase (MsrA) is a regulator of antioxidant defense and lifespan in mammals. *Proc Natl Acad Sci USA* 98: 12920–12925.

Murray J., Taylor S.W., Zhang B., Ghosh S.S., Capaldi R.A. (2003). Oxidative damage to mitochondrial complex I due to peroxynitrite: Identification of reactive tyrosines by mass spectrometry. *J Biol Chem* 278: 37223–37230.

Naito C., Niwa T. (2000). Analysis of glutathionyl hemoglobin levels in diabetic patients by electrospray ionization liquid chromatography-mass spectrometry: effect of vitamin E administration. *J Chromatogr B Biomed Sci Appl* 746: 91–94.

Nikov G., Bhat V., Wishnok J.S., Tannenbaum S.R. (2003). Analysis of nitrated proteins by nitrotyrosine-specific affinity probes and mass spectrometry. *Anal Biochem* 320: 214–222.

Okamoto T., Akaike T., Sawa T., Miyamoto Y., van der Vliet A., Maeda H. (2001). Activation of matrix metalloproteinases by peroxynitrite-induced protein *S*-glutathiolation via disulfide *S*-oxide formation. *J Biol Chem* 276: 29596–29602.

Pandey A., Mann M. (2000). Proteomics to study genes and genomes. *Nature* 405: 837–846.

Pennathur S., Jackson-Lewis V., Przedborski S., Heinecke J.W. (1999). Mass spectrometric quantification of 3-nitrotyrosine, ortho-tyrosine, and *o*,*o*′-dityrosine in brain tissue of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice, a model of oxidative stress in Parkinson's disease. *J Biol Chem* 274: 34621–34628.

Peterson K.P., Pavlovich J.G., Goldstein D., Little R., England J., Peterson C.M. (1998). What is hemoglobin A1c? An analysis of glycated hemoglobins by electrospray ionization mass spectrometry. *Clin Chem* 44: 1951–1958.

Petersson A.S., Steen H., Kalume D.E., Caidahl K., Roepstorff P. (2001). Investigation of tyrosine nitration in proteins by mass spectrometry. *J Mass Spectrom* 36: 616–625.

Pietraforte D., Salzano A.M., Scorza G., Marino G., Minetti M. (2002). Mechanism of peroxynitrite interaction with ferric hemoglobin and identification of nitrated tyrosine residues: CO_2 inhibits heme-catalyzed scavenging and isomerization. *Biochemistry* 40: 15300–15309.

Podrez E.A., Abu-Soud H.M., Hazen S.L. (2000). Myeloperoxidase-generated oxidants and atherosclerosis. *Free Radic Biol Med* 28: 1717–1725.

Poggioli S., Bakala H., Friguet B. (2002). Age-related increase of protein glycation in peripheral blood lymphocytes is restricted to preferential target proteins. *Exp Gerontol* 37: 1207–1215.

Powell S.A., Gurzenda E.M., Wahezi S.E. (2001). Actin is oxidized during myocardial ischemia. *Free Radic Biol Med* 30: 1171–1176.

Previero A., Coletti-Previero M.A., Jolles P. (1967). Localization of non-essential tryptophan residues for the biological activity of lysozyme. *J Mol Biol* 24: 261–268.

Pryor W.A. (2001). *Bio-assays for Oxidative Stress Status (BOSS),* Elsevier Science, Amsterdam.

Rabek J.P., Boylston W.H. 3rd, Papaconstantinou J. (2003). Carbonylation of ER chaperone proteins in aged mouse liver. *Biochem Biophys Res Commun* 305: 566–572.

Rabilloud T., Heller M., Gasnier F., Luche S., Rey C., Aebersold R., Benahmed M., Louisot P., Lunardi J. (2002). Proteomics analysis of cellular response to oxidative stress: Evidence for in vivo overoxidation of peroxiredoxins at their active site. *J Biol Chem* 277: 19396–19401.

Reid G.E., Roberts K.D., Kapp E.A., Simpson R.J. (2004). Statistical and mechanistic approaches to understanding the gas-phase fragmentation behavior of methionin sulfoxide containing peptides. *J Prot Res* 3: 751–759.

Requena J.R., Chao C.C., Levine R.L., Stadtman E.R. (2001a). Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins. *Proc Natl Acad Sci USA* 98: 69–74.

Requena J.R., Fu M.X., Ahmed M.U., Jenkins A.J., Lyons T.J., Baynes J.W., Thorpe S.R. (1997). Quantification of malondialdehyde and 4-hydroxynonenal adducts to lysine residues in native and oxidized human low-density lipoprotein. *Biochem J* 322: 317–325.

Requena J.R., Groth D., Legname G., Stadtman E.R., Prusiner S.B., Levine R.L. (2001b). Copper-catalyzed oxidation of the recombinant SHa(29–231) prion protein. *Proc Natl Acad Sci USA* 98: 7170–7175.

Requena J.R., Dimitrova M., Legname G., Teijera S., Prusiner S.B., Levine R.L. (2004). Oxidation of methionine residues in the prion protein by hydrogen peroxide. *Arch Biochem Biophys* 432: 188–195.

Richardson D.E., Regino C.A., Yao H., Johnson J.V. (2003). Methionine oxidation by peroxymonocarbonate, a reactive oxygen species formed from CO_2/bicarbonate and hydrogen peroxide. *Free Rad Biol Med* 35: 1538–1550.

Sala A., Nicolis S., Roncone R., Casella L., Monzani E. (2004). Peroxidase catalyzed nitration of tryptophan derivatives. *Eur J Biochem* 271: 2841–2852.

Sandhu J.K., Robertson S., Birnboim H.C., Goldstein R. (2003). Distribution of protein nitrotyrosine in synovial tissues of patients with rheumatoid arthritis and osteoarthritis. *J Rheumatol* 30: 1173–1181.

Saraswathi M., Nakanishi T., Shimizu A. (1999). Relative quantification of glycated Cu-Zn superoxide dismutase in erythrocytes by electrospray ionization mass spectrometry. *Biochim Biophys Acta* 1426: 483–490.

Sarver A., Scheffler N.K., Shetlar M.D., Gibson B.W. (2001). Analysis of peptides and proteins containing nitrotyrosine by matrix-assisted laser desorption/ionization mass spectrometry. *J Am Soc Mass Spectrom* 12: 439–448.

Scaloni A., Perillo V., Franco P., Fedele E., Froio R., Ferrara L., Bergamo P. (2002). Characterization of heat-induced lactosylation products in caseins by immunoenzymatic and mass spectrometric methodologies. *Biochim Biophys Acta* 1598: 30–39.

Schafer F.Q., Buettner G.R. (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med* 30: 1191–1212.

Schey K.L., Finley E.L. (2000). Identification of peptide oxidation by tandem mass spectrometry. *Acc Chem Res* 33: 299–306.

Schmidt P., Youhnovski N., Daiber A., Balan A., Arsic M., Bachschmid M., Przybylski M., Ullrich V. (2003). Specific nitration at Tyr430 revealed by high resolution mass spectrometry as basis for redox regulation of bovine prostacyclin synthase. *J Biol Chem* 2003: 12813–12819.

Schoneich C., Williams T.D. (2002). Cu(II)-catalyzed oxidation of beta-amyloid peptide targets His13 and His14 over His6: Detection of 2-Oxo-histidine by HPLC-MS/MS. *Chem Res Toxicol* 15: 717–722.

Schwaller M., Wilkinson B., Gilbert H.F. (2003). Reduction-reoxidation cycles contribute to catalysis of disulfide isomerization by protein-disulfide isomerase. *J Biol Chem* 278: 7154–7159.

Sedo O., Novotna K., Havel J. (2004). Advantages of derivatization by osmium tetroxide and 2,2′-bipyridine for MALDI-PSD fragment ion analysis of peptides. *Rapid Commun Mass Spectrom* 18: 339–344.

Sethuraman M., McComb M.E., Heibeck T., Costello C.E., Cohen R.A. (2004). ICAT approach to identify and quantify oxidant-sensitive protein thiols. *Mol Cell Proteomics* 3: 273–278.

Shacter E., Williams J.A., Lim M., Levine R.L. (1994). Differential susceptibility of plasma proteins to oxidative modification: Examination by Western blot immunoassay. *Free Radic Biol Med* 17: 429–437.

Shigenaga M.K., Lee H.H., Blount B.C., Christen S., Shigeno E.T., Yip H., Ames B.N. (1997). Inflammation and NO(X)-induced nitration: Assay for 3-nitrotyrosine by HPLC with electrochemical detection. *Proc Natl Acad Sci USA* 94: 3211–3216.

Shin S.J., Lee S.E., Boo J.Y., Kim M., Yoon Y.D., Kim S., Mook-Jung I. (2004). Profiling proteins related to amyloid deposited brain of Tg2576 mice. *Proteomics* 4: 3359–3368.

Shiva S., Crawford J.H., Ramachandran A., Ceaser E.K., Hillson T., Brookes P.S., Patel R.P., Darley-Usmar V.M. (2004). Mechanisms of the interaction of nitroxyl with mitochondria. *Biochem J* 379: 359–366.

Slatter D.A., Avery N.C., Bailey A.J. (2004). Identification of a new cross-link and unique histidine adduct from bovine serum albumin incubated with malondialdeheyde. *J Biol Chem* 279: 61–69.

Smith M.A., Perry G., Richey P.L., Sayre L.M., Anderson U.E., Beal M.F., Kowall N. (1996). Oxidative damage in Alzheimer's. *Nature* 382: 120–121.

Sochaski M.A., Jenkins A.J., Lyons T.J., Thorpe S.R., Baynes J.W. (2001). Isotope dilution gas chromatography/mass spectrometry method for the determination of methionine sulfoxide in protein. *Anal Chem* 73: 4662–4667.

Soderling A., Ryberg H., Gabrielsson A., Larstad M., Toren K., Niari S., Caidahl K. (2003). A derivatization assay using GC negative chemical ionization tandem mass

spectrometry to quantify 3-nitrotyrosine in human plasma. *J Mass Spectrom* 38: 1187–1196.

Song E.J., Kim Y.S., Chung J.Y., Kim E., Chae S.K, Lee K.J. (2000). Oxidative modification of nucleoside diphosphate kinase and its identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Biochemistry* 39: 10090–10097.

Stadtman E.R., Berlett B.S. (1998). Reactive oxygen-mediated protein oxidation in aging and disease. *Drug Metab Rev* 30: 225–243.

Stadtman E.R., Moskovitz J., Berlett B.S., Levine R.L. (2002). Cyclic oxidation and reduction of protein methionine residues is an important antioxidant mechanism. *Mol Cell Biochem* 234–235: 3–9.

Stagsted J., Bendixen E., Andersen H.J. (2004). Identification of specific oxidatively modified proteins in chicken muscles using a combined immunologic and proteomic approach. *J Agric Food Chem* 52: 3967–3974.

Stamler J.S., Lamas S., Fang F.C. (2001). Nitrosylation: The prototypic redox-based signaling mechanism. *Cell* 106: 675–683.

Sullivan D.M., Wehr N.B., Fergusson M.M., Levine R.L., Finkel T. (2000). Identification of oxidant-sensitive proteins: TNF-α induces protein glutathiolation. *Biochemistry* 39: 11121–11128.

Suzuki T., Mower H.F., Friesen M.D., Gilibert I., Sawa T., Ohshima H. (2004). Nitration and nitrosation of *N*-acetyl-L-tryptophan and tryptophan in proteins by various reactive nitrogen species. *Free Radic Biol Med* 37: 671–681.

Taggart C., Cervantes-Laurean D., Kim G., McElvaney N.G., Wehr N., Moss J., Levine R.L. (2000). Oxidation of either methionine 351 or methionine 358 in alpha 1-antitrypsin causes loss of anti-neutrophil elastase activity. *J Biol Chem* 275: 27258–27265.

Takahashi M., Lu Y.B., Myint T., Fujii J., Wada Y., Taniguchi N. (1995). In vivo glycation of aldehyde reductase, a major 3-deoxyglucosone reducing enzyme: Identification of glycation sites. *Biochemistry* 34: 1433–1438.

Tao L., English A.M. (2003). Mechanism of *S*-nitrosation of recombinant human brain calbindin D28K. *Biochemistry* 42: 3326–3334.

Tarpey M., Fridovich I. (2001). Methods of detection of vascular reactive species. Nitric oxide, superoxide, hydrogen peroxide, and peroxynitrite. *Circ Res* 89: 224–236.

Taylor S.W., Fahy E., Murray J., Capaldi R.A., Ghosh S.S. (2003). Oxidative post-translational modification of tryptophan residues in cardiac mitochondrial proteins. *J Biol Chem* 278: 19587–19590.

Tell G., Scaloni A., Pellizzari L., Formisano S., Pucillo C., Damante G. (1998). Redox potential controls the structure and DNA binding activity of the paired domain. *J Biol Chem* 273: 25062–25072.

Thornalley P.J., Battah S., Ahmed N., Karachalias N., Agalou S., Babeai-Jadidi R., Danway A. (2003). Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. *Biochem J* 375: 581–592.

Tsikas D., Schwedhelm E., Stutzer F.K., Gutzki F.M., Rode I., Mehls C., Frolich J.C. (2003). Accurate quantification of basal plasma levels of 3-nitrotyrosine and 3-nitrotyrosinoalbumin by gas chromatography–tandem mass spectrometry. *J Chromatogr B* 784: 77–90.

Tsikas D., Caidahl K. (2005). Recent methodological advances in the mass spectrometric analysis of free and protein-associated 3-nitrotyrosine in human plasma. *J Chromatogr B* 814: 1–9.

Turku I.V., Li L., Aulak K.S., Stuehr D.J., Chang J., Murad F. (2003). Protein tyrosine nitration in the mithochondria from diabetic mouse heart. *J Biol Chem* 278: 33972–33977.

Upston J.M., Niu X., Brown A.J., Mashima R., Wang H., Senthilmohan R., Kettle A.J., Dean R.T., Stocker R. (2002). Disease stage-dependent accumulation of lipid and protein oxidation products in human atherosclerosis. *Am J Pathol* 160: 701–710.

van der Vliet A., Eiserich J.P., Shigenaga M.K., Cross C.E. (1999). Reactive nitrogen species and tyrosine nitration in the respiratory tract: Epiphenomena or a pathobiologic mechanism of disease? *Am J Respir Crit Care Med* 160: 1–9.

van Montfort R.L., Congreve M., Tisi D., Carr R., Jhoti H. (2003). Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B. *Nature* 423: 773–777.

Vilardo P.G., Scaloni A., Amodeo P., Barsotti C., Cecconi I,. Cappiello M., Lopez Mendez B., Rullo R., Dal Monte M., Del Corso A., Mura U. (2001). Thiol/disulfide interconversion in bovine lens aldose reductase induced by intermediates of glutathione turnover. *Biochemistry* 40: 11985–11994.

Viner R.I., Williams T.D., Schoneich C. (1999). Peroxynitrite modification of protein thiols: oxidation, nitrosylation, and *S*-glutathiolation of functionally important cysteine residue(s) in the sarcoplasmic reticulum Ca-ATPase. *Biochemistry* 38: 12408–12415.

Vogt W. (1995). Oxidation of methionyl residues in proteins: tools, targets, and reversal. *Free Radic Biol Med* 18: 93–106.

Wagner E., Luche S., Penna L., Chevallet M., van Dorsselaer A., Leize-Wagner E., Rabilloud T. (2002). A method for detection of overoxidation of cysteines: Peroxiredoxins are oxidized in vivo at the active-site cysteine during oxidative stress. *Biochem J* 366: 777–785.

Walcher W., Franze T., Weller M.G., Poschl U., Huber C.G. (2003). Liquid- and gas-phase nitration of bovine serum albumin studied by LC-MS and LC-MS-MS using monolithic columns. *J Prot Res* 2: 534–542.

Wendt S., Schlattner U., Wallimann T. (2003). Differential effects of peroxynitrite on human mitochondrial creatine kinase isoenzymes. *J Biol Chem* 278: 1125–1130.

Winterbourn C.C., Vissers M.C., Kettle A.J. (2000). Myeloperoxidase. *Curr Opin Hematol* 7: 53–58.

Wong J.H., Cai N., Balmer Y., Tanaka C.K., Vensel W.H., Hurkman W.J., Buchanan B.B. (2004). Thioredoxin targets of developing wheat seeds identified by complementary proteomic approaches. *Phytochemistry* 65: 1629–1640.

Woo H.A., Chae H.Z., Hwang S.C., Yang K.S., Kang S.W., Kim K., Rhee S.G. (2003). Reversing the inactivation of peroxiredoxins caused by cysteine sulfinic acid formation. *Science* 300: 653–656.

Wu W., Chen B.K., d'Avignon A., Hazen S.L. (1999). 3-Bromotyrosine and 3,5-dibromotyrosine are major products of protein oxidation by eosinophil peroxidase: Potential markers for eosinophil-dependent tissue injury in vivo. *Biochemistry* 38: 3538–3548.

Xiao G.G., Nel A.E., Loo J.A. (2005). Nitrotyrosine-modified proteins and oxidative stress induced by diesel exhaust particles. *Electrophoresis* 26: 280–292.

Yan L.J., Sohal R.S. (1998). Gel electrophoretic quantitation of protein carbonyls derivatized with tritiated sodium borohydride. *Anal Biochem* 265: 176–182.

Yang K.S., Kang S.W., Woo H.A., Hwang S.C., Chae H.Z., Kim K., Rhee S.G. (2002). Inactivation of human peroxiredoxin I during catalysis as the result of the oxidation of the catalytic site cysteine to cysteine-sulfinic acid. *J Biol Chem* 277: 38029–38036.

Yang Y., Loscalzo J. (2005). S-nitrosoprotein formation and localization in endothelial cells. *Proc Natl Acad Sci USA* 102: 117–122.

Yano H., Kuroda S., Buchanan B.B. (2002). Disulfide proteome in the analysis of protein function and structure. *Proteomics* 2: 1090–1096.

Ye Y.Z., Strong M., Huang Z.Q., Beckman J.S. 1996. Antibodies that recognize nitrotyrosine. *Methods Enzymol* 269: 201–209.

Yi D., Ingelse B.A., Duncan M.W., Smythe G.A. (2000). Quantification of 3-nitrotyrosine in biological tissues and fluids: Generating valid results by eliminating artifactual formation. *J Am Soc Mass Spectrom* 11: 578–586.

Young T.W., Mei F., Yang G., Thompson-Lanza J.A., Liu J., Cheng X. (2004). Activation of antioxidant pathways in Ras-mediated oncogenic transformation of human surface ovarian epithelial cells revealed by functional proteomics and mass spectrometry. *Cancer Res* 64: 4577–4584.

Yu H., Chertklow H.M., Bergman H., Schipper H.M. (2003). Aberrant profiles of native and oxidized glycoproteins in Alzheimer plasma. *Proteomics* 3: 2240–2248.

Zech B., Wilm M., van Eldik R., Brune B. (1999). Mass spectrometric analysis of nitric oxide-modified caspase-3. *J Biol Chem* 274: 20931–20936.

Zhan X., Desiderio D.M. (2004). The human pituitary nitroproteome: Detection of nitrotyrosyl-proteins with 2D Western blotting, and amino acid sequence determination with mass spectrometry. *Biochem Biophys Res Commun* 325: 1180–1186.

Zhang H., He S., Mauk A.G. (2002). Radical formation at Tyr39 and Tyr153 following reaction of yeast cytochrome c peroxidase with hydrogen peroxide. *Biochemistry* 41: 13507–13513.

Zheng L., Settle M., Brubaker G., Schmitt D., Hazen S.L., Smith J.D., Kinter M. (2005). Localization of nitration and chlorination sites on ApoAI catalyzed by myeloperoxidase in human atheroma and associated oxidative impairment in ABCA1-dependent cholesterol efflux from macrophages. *J Biol Chem* 280: 38–47.

Zheng M., Aslund F., Storz G. (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* 279: 1718–1721.

Zhu S., Basiouny K.F., Crow J.P., Matalon S. (2000). Carbon dioxide enhances nitration of surfactant protein A by activated alveolar macrophages. *Am J Physiol* 278: L1025–L1031.

4

THIOL-DISULFIDE OXIDOREDUCTION OF PROTEIN CYSTEINES: OLD METHODS REVISITED FOR PROTEOMICS

VALENTINA BONETTO AND PIETRO GHEZZI

4.1 INTRODUCTION: PROTEIN THIOLS FROM OXIDATIVE STRESS TO REDOX REGULATION

The concept of oxidative stress became very popular in the 1960s. It is based on the fact that reactive oxygen species (ROS) can interact with, and oxidize, different macromolecules. Nonprotein thiols, such as glutathione (GSH) or *N*-acetylcysteine, were considered free radical scavengers or antioxidants that could prevent ROS from damaging biological targets. Oxidation of protein cysteines has long been viewed as one of the toxic consequences of oxidative stress, and their reduction by thioltransferases is considered a "regeneration" of their activity. In fact, for most enzymes, oxidation of active site cysteines results in loss of activity.

The finding that thiol antioxidants inhibit "fine" mechanisms such as activation of transcription factors, like NF-κB, and production of cytokines including TNF, has thrown some light on the role of redox processes in fields other than pathology and toxicology. In some instances small thiols can act even without a true "oxidative stress."

The concept of redox regulation, where small changes in the redox balance in the cell, not necessarily associated with a toxic insult, can regulate cell functions

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

is becoming growingly accepted. Oxidation or reduction of protein thiols and disulfides can occur during normal metabolic processes. Addition of small thiols will shift the redox balance toward a more reduced state, implying that molecules like GSH can act as reductants, even when there are no excessive ROS to be scavenged. A widely accepted definition of the redox state of the cell is the ratio of GSH to GSSG. However, this is clearly an oversimplification, since the redox state, and particularly the thiol-disulfide ratio, varies in different compartments (cytosol, extracellular, endoplasmic reticulum), as discussed below.

4.2 DIFFERENT REDOX STATES OF PROTEIN CYSTEINES

According to classical biochemistry textbooks, protein cysteines are found either as free thiols or in disulfide bonds. However, cysteines can also undergo other forms of oxidation, most of them known for a long time. These forms of oxidation are becoming particularly important as a means of redox regulating proteins, and thus the focus of most proteomics studies. Clearly, whether the cysteines exist in a reduced or oxidized state (i.e., the ratio between their reduced and oxidized states) depends on the GSH/GSSG ratio.

4.2.1 Disulfides

Disulfide bonds are formed in the endoplasmic reticulum. These bonds are important for the stability and folding of the protein (Sevier and Kaiser, 2002), and the state of the cysteine is viewed as part of the tertiary structure of the protein. Free cysteines are normally prevalent in the cytoplasm because the intracellular environment is highly reducing, mainly due to the high GSH concentrations. A possible exception are hyperthermophilic bacteria where intracellular proteins are rich in disulfide bonds, probably are a key factor in stabilizing the thermostable proteins (Mallick et al., 2002). On the other hand, secreted proteins are as rich in disulfide bonds as the extracellular environment, under aerobic conditions, is an oxidizing one. A relatively new finding, which is not obvious to the reader of a textbook, is that under physiological conditions a cysteine normally listed as "free" may be present as a disulfide, and vice versa, a disulfide bond can be reduced. We stress the point "under physiological conditions." Obviously all disulfide bonds are reduced when a protein is treated with a chemical reductant, a common procedure before electrophoresis.

In the framework of redox regulation, some cysteines are normally present in two different oxidoreduction states. This was known for many oxidoreducing enzymes in which formation of disulfide bonds is part of the catalytic process, including the thiol-disulfide oxidoreductases thioredoxin and glutathione reductase (Arner and Holmgren, 2000; Mannervik et al., 1983; Rietsch and Beckwith, 1998; Prinz et al., 1997), or in the endoplasmic reticulum during folding (Molinari and Helenius, 1999). However, it is becoming clear that a number of other proteins can form reversible disulfide bonds, thus changing their properties and making them targets of redox regulation. These include the transcription

factor OxyR and the chaperonin Hsp33 (Choi et al., 2001; Jakob et al., 1999). As recently shown using proteomic techniques, disulfide bonds are also formed in the cytoplasm upon exposure of cells to oxidative stress (Brennan et al., 2004; Cumming et al., 2004).

4.2.2 Mixed Disulfides

Protein cysteines can form mixed disulfides with low-molecular-weight thiols. Since GSH, and to a lesser extent free cysteine, are the most abundant thiols in the cell, glutathionylated and cysteinylated proteins will be the main mixed disulfides. The fact that GSH can form mixed disulfides with proteins has been known since the late 1960s (Modig, 1968; Harding, 1969; Harrap et al., 1973). Studies by Brigelius et al. (1982, 1983) indicate that a considerable amount—up to 20% of total glutathione—is present in the form of protein-bound mixed disulfide, and this can increase up to 50% under oxidative stress (Gilbert, 1984).

Cysteinylation has attracted far less attention. The intracellular concentrations of cysteine are lower than those of GSH; a recent study on human cell lines reported 5 to 10 times lower concentrations (Hultberg and Hultberg, 2004). Cysteine may be found at much higher concentrations in tumors (Koch and Evans, 1996). Extracellular cysteine is more abundant than GSH (it was recently estimated that 99.5% of GSH in human blood was in erythrocytes and 97% of Cys was in plasma (Mills and Lang, 1996). Thus extracellular proteins may be prevalently cysteinylated. In fact, while hemoglobin in red blood cells is glutathionylated (Mawatari and Murakami, 2004), plasma proteins such as albumin (Brennan et al., 1999) and transthyretin (Terazaki et al., 1998) are mainly cysteinylated. Cysteinylation has also been reported for many proteins including secreted cytokines, such as MIF/GIF (Watarai et al., 2000), and the extracellular domain of cytokine receptors such as the high affinity interleukin-6 (IL-6) receptor and its gp130 signal transducer (Cole et al., 1999; Moritz et al., 2001). The common finding of cysteinylation is reflected by the fact that reduction of cysteinylated peptides by specific thioltransferases, particularly by a protein disulfide oxidoreductase known as gamma interferon-inducible lysosomal thiol reductase (GILT) (Arunachalam et al., 2000; Maric et al., 2001), is a key step in antigen processing and presentation (Collins et al., 1991; Chen et al., 1999; Haque et al., 2001). Other factors can determine whether a protein can be glutathionylated or cysteinylated. Cysteine has a different ability to react from GSH because of its smaller size and net neutral charge (Bump et al., 1992; Ruoso and Hedley, 2004). For the same reasons glutathionylation and cysteinylation can affect protein functions differently. Adding a GSH or cysteine molecule not only blocks a free cysteine but attaches to the protein an amino acid or a tripeptide with different steric hindrance and isoelectric point. Therefore the consequences on protein functions are not (necessarily) the same as alkylating, or mutating, a cysteine. This has clearly been shown with protein kinase C where different forms of *S*-thiolations may have different effects (Chu et al., 2001, 2004).

4.2.3 Higher Oxidation States

Cysteines can form other oxidation products, including sulfenic (Cys-SOH), sulfinic (Cys-SO_2H), and sulfonic acids (Cys-SO_3H). While the latter are considered irreversible forms of oxidation, sulfenic acids are not, and react with GSH to give a glutathionylated protein (Barrett et al., 1999).

4.3 METHODOLOGIES TO IDENTIFY AND QUANTIFY THE REDOX STATE OF PROTEIN CYSTEINES

4.3.1 Methods Based on Reagents That Label Free Cysteine

The identification and quantification of all thiol modifications rely mainly, although not exclusively, on the use of thiol-reactive reagents. These include reagents labeled with biotin, with a fluorochrome, or with chemical entities against which antibodies could be raised. A partial list of the reagents used to detect SH groups is provided in Table 4.1.

Among all these Cys labels we like to highlight the ones that are used in connection with the most advanced proteomics tools allowing accurate quantification of the redox state of a complex mixture of proteins. An interesting approach

TABLE 4.1 Selected Reagents Used to Label Protein Thiols

Reagent	References
Cy5 maleimide	Maeda et al., 2004
Cleavable ICAT reagent	Sethuraman et al., 2004
(4-Iodobutyl)triphenyl-phosphonium	Lin et al., 2002[a]
Monobromobimane	Yano et al., 2001; Berggren et al., 2001
Maleimidobutyrylbiocytin	Garant et al., 1999
Oregon green maleimide	Essex et al., 2001[b]
N-Biotinoylaminoethyl methanethiosulfonate	Ennion and Evans, 2002[b]
Sulfo-NHS-LC-Biotin	Ennion and Evans, 2002[b]
N-Byotinoyl-*N′*-(maleimidihexanoil)hydrazine	Hare and Lee, 1989[b,c]
Fluorescein-5- maleimide	Birge et al., 1991
[^{3}H]-NEM	Birge et al., 1991
[^{14}C]-NEM	Brooker and Slayman, 1983; Frillingos and Kaback, 1996
NEM-biotin	Lind et al., 2002
N-[6-(Biotinamido)hexyl]-39-(29 pyridyldithio)propionamide	Jaffrey et al., 2001
N-(Biotinoyl)-*N′*-(iodoacetyl)ethylendiamine	Kim et al., 2000[b]

[a] In whole cells specifically labels mitochondrial proteins.
[b] Cell impermeable: in whole cells has been used to label surface thiols.
[c] Has been used in conjunction with radioactive metabolic labeling to immunopurify labeled proteins with streptavidin columns; then proteins were detected on gels by autoradiography.

was described by Sethuraman et al. (2004a) to quantify oxidant-sensitive protein thiols using the isotope-coded affinity tag (ICAT) reagents. ICAT reagents have been extensively used in quantitative proteomics to evaluate the abundance of expressed proteins (Gygi et al., 1999). The ICAT reagent consists of an affinity tag (biotin), a linker (which incorporates alternatively heavy and light stable isotopes), and an iodoacetamide (IAM) moiety that reacts with free cysteines. Two protein mixtures representing two cell states are treated with the isotopically light and heavy ICAT reagents, respectively, combined and proteolyzed. The labeled peptides are isolated by avidin affinity chromatography and analyzed usually by LC-MS/MS. Peptides are quantified by measuring in the MS mode the relative signal intensities for pair of peptide ions of identical sequence tagged with the light and heavy forms of the ICAT reagent. Peptide sequence information is obtained by MS/MS in a successive scan. The ICAT approach applied to redox proteomics is based on the measurement of the decrease in ICAT labeling when reactive cysteines are exposed to oxidants under nonreducing conditions (Fig. 4.1). This approach is potentially more powerful than conventional methods, which require Cys labeling and gel separation. In fact this way the identification of the protein and the quantification of the degree of oxidation of the specific cysteine are made in the same analysis (Sethuraman et al., 2004b). Nonetheless, it is possibly applicable to gel-based approaches (Smolka et al., 2002), still the most commonly used and the best choice for resolving complex proteomes.

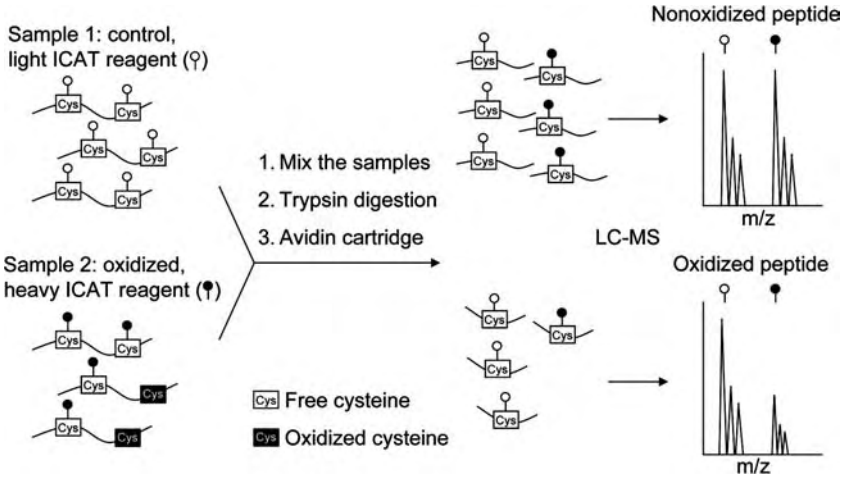


FIGURE 4.1 Schematic representation of the ICAT approach to redox proteomics. Samples 1 and 2, in a different redox state, are labeled with light and heavy ICAT reagents, respectively. The samples are mixed and subjected to trypsin digestion. The peptides are purified by an avidin affinity cartridge and analyzed by LC-MS. The oxidized cysteines are not susceptible to labeling by ICAT reagent, so there is a lower intensity of the peak corresponding to the heavy-labeled peptide, susceptible to oxidation, coming from sample 2. The sequence of the peptide and the identity of the protein can be derived from MS/MS analysis. Figure adapted from Sethuraman et al. (2004a).

Cys-reactive fluorescent dyes, instead, have been specifically designed for gel-based proteomics. In general, fluorescent detection of proteins separated in 2DE is gaining popularity for its higher sensitivity and higher dynamic range compared to the conventional colorimetric stains. The first generation of cyanine (Cy) dyes (minimal dyes), which label ε-amino groups of lysine residues, are typically used in 2D difference gel electrophoresis (DIGE). However, the lysine content of proteins is relatively high, therefore in a DIGE experiment the dye to protein ratio has to be kept low to avoid multiple dye additions per protein molecule. The cysteine-labeling maleimide Cy3 and Cy5 dyes, a further development of this technique, label cysteine (which is less prevalent than lysine) to saturation (saturation dyes), in reducing and denaturing conditions, to obtain enhanced sensitivity of protein detection (Shaw et al., 2003). The sensitivity and dynamic range of these dyes make them highly suitable for scarce sample DIGE analysis, as demonstrated in the comparative study of the proteome of two cell stem populations (Evans et al., 2004). For redox proteomics applications, Cy maleimide labeling is performed under native conditions in absence of reducing reagents to achieve selective protein detection (Maeda et al., 2004). Maleimide groups react with cysteine thiols with a certain specificity if the reaction is carried out at a pH near neutrality. Maeda et al. (2004) used Cy5 maleimide to reveal the protein targets of thioredoxin in barley seeds. In this work they showed that Cy5 maleimide was able to detect a much larger number of proteins than another fluorescent dye, monobromobimane. However, the two labeling detect different proteins because of their different thiol affinity, which suggests a possible complementary use.

Of course, this is an observation applicable to all the Cys-reactive reagents listed in Table 4.1. The efficiency of the Cys labeling is definitely connected to the specific capability of the thiol reagent to reach free Cys. Moreover bulky reagents such as Cy dyes and ICAT tags, but not only them, have shown to decrease protein solubility and affect electrophoretic mobility. Therefore, for comprehensive studies, combinations of techniques are recommended. It will be interesting to monitor future developments in Cys labeling.

4.3.2 Methods Based on Different Electrophoretic Mobility: Diagonal Electrophoresis

Brown and Hartley (1966) were the first to talk of a diagonal method, by paper electrophoresis, to determine which Cys residues are disulfide bonded in a protein. The protein was digested with trypsin and chymotrypsin and the peptides were separated in the first dimension under conditions in which disulfides are retained. The paper was then exposed to performic acid, which is able to cleave the disulfide bond and oxidize Cys residue to cysteic acid. The electrophoresis in the second dimension revealed the peptides originally linked, as they lied off from the diagonal. Diagonal electrophoresis on a matrix of SDS-PAGE was introduced by Sommer and Traut (1974) to analyze ribosomal proteins. In their work intact proteins were analyzed, and a reducing step was introduced before the second dimension to reveal the disulfide linkages.

Recent papers have applied on a proteomic scale evolved versions of these methods, reporting the "disulfide proteome" of different biological systems and identifying the disulfide proteins and the nature of their disulfide bonds. Briefly, protein extracts are separated by SDS-PAGE in the first dimension under nonreducing condition. After electrophoresis the entire lane is excised, subjected to reducing treatment and applied horizontally on another gel. Proteins without disulfide bonds are recovered on the diagonal line, while proteins with inter- or intramolecular disulfides migrate above and below the diagonal line, respectively (Fig. 4.2). The disulfide proteomes of control and treated samples are then compared and the differences quantified.

This method is, however, not convenient when the question is not especially focused on the nature of the disulfide bond. In fact the proteins that lie on the diagonal line (which can also be redox-regulated but not via disulfide bonds) are not well resolved and therefore not easily identified by conventional peptide mass fingerprinting analysis. For this type of study, a combination of Cys labeling with a thiol-reactive reagent and IEF/SDS-PAGE (2-DE gel) is a better approach, as described in Section 4.3.1.

By diagonal electrophoresis, Buchanan and co-workers could identify protein disulfides from *Arachis hypogea* seeds, which are targeted by a special reductant, thioredoxin *h* (Yano et al., 2001, 2002). This elegant strategy may have broad applications not only in other plant systems but also in microorganisms

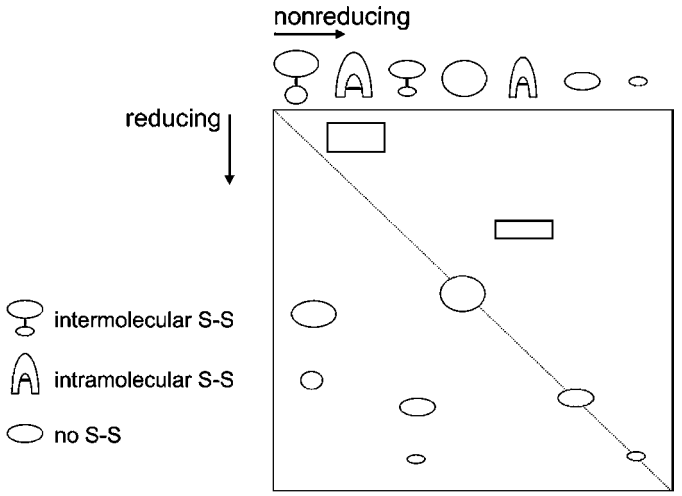


FIGURE 4.2 Principle of diagonal electrophoresis. The proteins with intermolecular, intramolecular, or without disulfide bonds are separated in SDS-PAGE under nonreducing conditions in the first dimension (horizontal direction) and under reducing condition in the second dimension (vertical direction). Protein recovered in the diagonal line are the proteins without disulfide bonds, while proteins recovered above or below the diagonal line are the ones with intramolecular or intermolecular disulfides. Figure adapted from Yano et al. (2001).

and animals. Cumming et al. (2004) identified disulfide-bonded proteins in a mammalian neuronal cell line exposed to various oxidative insults. They show that disulfide bond formation within families of cytoplasmic proteins is specifically dependent on the nature and concentration of the oxidant. Similar results, showing a widespread intermolecular protein disulfide formation in adult cardiac myocytes under a series of stimuli which alter the redox status of the cell, were obtained by the Eaton's group (Brennan et al., 2004).

4.4 METHODS TO DETECT SPECIFIC MODIFICATIONS

4.4.1 Methods Based on a Series of Alkylation, Reduction, and Labeling Steps

As described above, the methods to study the redox state of protein cysteines are based on labeled thiol reagents. So, when a protein is oxidized, it will lose its label. However, with these methods one cannot distinguish different forms of oxidation. Also the sensitivity of methods where detection is based on loss of a label (e.g., biotin) is not elevated.

The various methods to identify proteins that are in an oxidized state in a sample, where most of them are reduced, rely on sequential steps of alkylation and reduction. These methods are exemplified by the so-called biotin-switch method described to detect *S*-nitrosylated proteins (Jaffrey et al., 2001) and nowadays available as a commercial kit (Nitro-Glo™ kit from Perkin Elmer). In these methods the sample, which contains both oxidized and reduced cysteines, is first alkylated, for instance, with NEM or IAM, to block free SH. Then the disulfides (or nitrosothiols) are reduced and the new SH is labeled (e.g., with biotin) to allow detection on a gel. In this approach the use of more or less selective reducing agents will allow detection of a specific form of oxidation. For instance, *S*-nitrosothiols are rather selectively reduced by ascorbate (Jaffrey et al., 2001), arsenite will reduce sulfenic acids (Saurin et al., 2004), while stronger reductants such as DTT will reduce both nitrosothiols and disulfides. An outline of the method based on sequential alkylation and reduction, compared with the classical approach of labeling a sample (with and without DTT treatment) with a thiol reagent, is given in Figure 4.3.

Other methods can be used to identify the redox state of a known protein. For instance, one can alkylate the free SH with an alkylating reagent bound to a large molecule (e.g., polyethylene glycol) to distinguish the two forms of a protein, reduced and oxidized, in a single lane on a gel (Pepinsky et al., 2000; Wu et al., 2000). However, these methods are not suitable for proteomics studies.

4.4.2 Methods Based on Incorporation of Labeled Glutathione to Identify Glutathionylated Proteins

Several methods have been applied successfully to the identification of glutathionylated proteins in living cells. In the method of Rokutan et al. (1991), cells

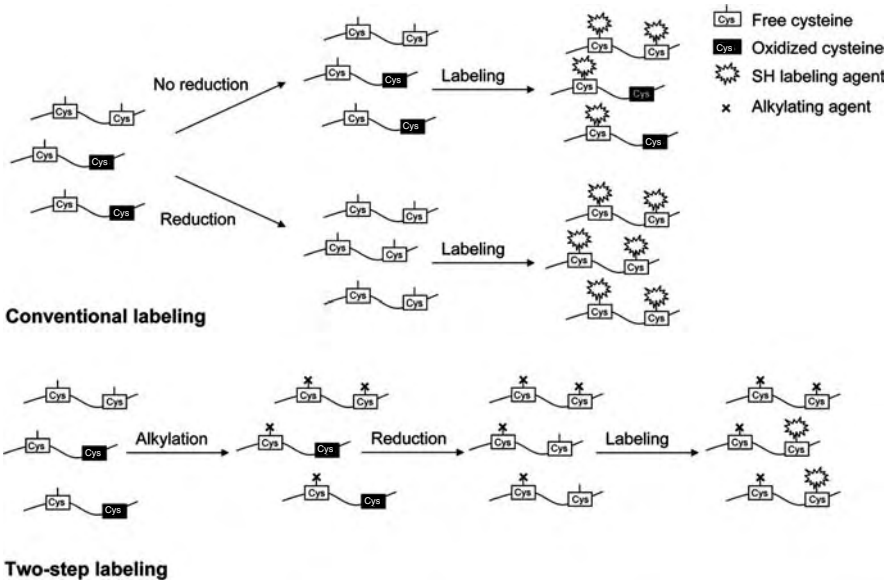


FIGURE 4.3 Schematic representation of two approaches to identify oxidized proteins. By the "conventional" methods the sample is either reduced or not; then it is labeled with a thiol reagent (biotinylated, radioactive, or fluorescent). The existence of oxidized cysteines is detected on a gel as decreased labeling of the reduced cysteines. By the alkylation-reduction methods, the free SH in the sample are blocked with an alkylating agent (e.g., IAM, NEM). As this will not react with oxidized cysteines, a subsequent reduction will render those available to a labeled SH reagent. This second method also requires a series of internal controls (e.g., no alkylation, or no reduction). In both cases selective reducing agents can be used to specifically detect some oxidation forms (e.g., *S*-nitrosothiols vs. mixed disulfides).

are metabolically labeled with ^{35}S-cysteine in the presence of high concentrations of cycloheximide, allowing the labeling of the intracellular GSH pool without incorporating radioactivity into proteins by protein synthesis. Then cells are exposed to oxidative stress and a number of proteins can be identified following 2-DE under nonreducing condition and autoradiography. We could show that protein-associated radioactivity represents *S*-thiolation, as it is removed by DTT, and is mostly glutathionylation, as shown by experiments with inhibitors of GSH synthesis (Ghezzi et al., 2002). Using this approach, we could identify several proteins previously not known to be glutathionylated in T lymphocytes, primary rat hepatocytes, and human HepG2 hepatoma cells, under basal and oxidative stress conditions (Fratelli et al., 2002, 2003; Casagrande et al., 2002).

In a similar way, biotin-labeled cysteine, instead of radioactively labeled, was used (Eaton et al., 2002). Biotin-cysteine rapidly crosses the plasma membrane and the biotin moiety allows a range of procedures that exploit the affinity of biotin for avidin derivatives. By this technique a number of *S*-thiolated proteins

were identified in cardiac tissue following ischemia and reperfusion (Eaton et al., 2002). With a similar strategy Sullivan et al. (2000) used a membrane-permeant analogue of glutathione, biotinylated glutathione ethyl ester, to identify proteins that might be regulated by glutathionylation in conjunction with TNF-α stimulation.

Methods based on labeled GSH have several drawbacks. Only proteins that are present in high quantities may be identified. For instance, some regulatory proteins, such as the p50 NF-κB subunit, shown to undergo glutathionylation in a cell-free system (Pineda-Molina et al., 2001), were never found with these approaches. Furthermore a protein that is extensively glutathionylated under basal (nonstressed) conditions may not incorporate further labeled GSH because it is already fully oxidized. Last but not least, some technical details have to be considered: (1) disulfides are unstable and can be easily reduced, leading to a loss of signal, therefore rapid analysis times are mandatory, together with maintaining the sample at an acidic pH whenever possible; (2) disulfides can undergo thiol–disulfide exchange with other protein thiols, and hence it is necessary to block free SH (e.g., with NEM) to "freeze" the thiol/disulfide state of proteins; (3) gel electrophoresis must be run under nonreducing conditions, which is not optimal, and special protocol have to be set up (Fratelli et al., 2003); (4) the identification of the site(s) of glutathionylation by MALDI mass spectrometry required tryptic digestion under nonreducing condition, and therefore the digestion in an aqueous-organic solvent to allow efficient generation of tryptic peptides is recommended (Casagrande et al., 2002; Ghezzi et al., 2005b).

4.4.3 Immunological Methods

The chemistry of thiol disulfides, and the inherent instability of disulfides and *S*-nitrosothiols, makes it particularly difficult to raise antibodies against specific modifications, the way is done for phosphoproteins or for other types of oxidative modifications, for example tyrosine nitration. Nevertheless, antibodies have been developed against some kinds of oxidative modifications:

Anti-glutathione Anti-glutathione antibodies have been described and are commercially available (e.g., Advanced Immunochemicals, Chemicon, Virogen). Chemicon antibody was shown to detect glutathionylated hemoglobin as well as (putative) glutathionylated forms of spectrins, actin, and GAPDH (Mawatari and Murakami, 2004). Virogen antibodies were used in published papers to identify glutathionylated actin (Pastore et al., 2003). In most cases these antibodies have unlikely been produced using a glutathionylated protein since the immunogen as mixed disulfide would have been reduced in vivo, as discussed above. If antibodies are raised against GSH coupled by standard methods to a carrier protein (e.g., hemocyanin), GSH may not be bound to a cysteine residue in the carrier, so that the resulting molecule will be different from a glutathionylated one. Nevertheless, this apparently recognizes at least some glutathionylated protein. Recently Townsend et al. (2004) used monoclonal antibodies directed against

glutathionylated cysteine residues and thus identified about 50 glutathionylated proteins in response to nitrative stress, indicating the usefulness of this approach.

Anti-S-nitrosocysteine Another antibody which was recently described was generated against *S*-nitrosothiols (Gow et al., 2002; Lorch et al., 2000). They were raised against cysteine coupled to bovine serum albumin (BSA) and then *S*-nitrosylated. To date these anti-nitrosocysteine antibodies have been used for immunohistochemical studies, and were demonstrated to recognize, in Western blots, *S*-nitrosylated BSA, but not native BSA (Gow et al., 2002), and also skeletal muscle ryanodine receptor (Sun et al., 2001). Similar anti-*S*-nitrosocysteine antibodies (commercially available) were shown to recognize, in immunoprecipitation experiments, inhibitory kappa-B kinase (IKK) beta (Reynaert et al., 2004). Anti-*S*-nitrosocysteine antibodies were also used to detect cysteine *S*-nitrosation of catalase in Western blots (Kocis et al., 2002).

Anti-sulfonylated Cysteine Antibodies to oxidized peroxiredoxins (Prx) were generated against a peptide containing a cysteine (the *N*-terminal conserved cysteine) sulfonylated with formic acid and used to identify oxidized Prx in Western blot (Woo et al., 2003). Interestingly these antibodies detected both sulfonylated and sulfinylated, but not nonoxidized, Prx (Woo et al., 2003). A similar method, using antibodies generated against sulfonylated peptides, has been applied to study the oxidation state of cysteine in protein tyrosine phosphatases (Persson et al., 2004, 2005).

It should be mentioned that to our knowledge, these antibodies recognize only the specific peptide, and thus cannot be used in proteomics studies to identify unknown proteins undergoing cysteine oxidation.

4.5 METHODS FOR ENRICHING REDOX-REGULATED PROTEINS

Identification of oxidized proteins by redox proteomics has led to the identification of several proteins undergoing glutathionylation, disulfide formation, and sulfinic acid oxidation. One criticism often made to most of these studies is that they identified only proteins present in large amounts in the cell, often missing "fancy" proteins such as transcription factors and other regulatory proteins. In fact we have noted that many of the proteins identified were often found not only in studies seeking the cysteine oxidation forms mentioned above but also other forms of oxidation, namely carbonylation, which targets different amino acids including lysine, arginine, proline, threonine (Stadtman, 1993), and tyrosine nitration (Ghezzi et al., 2005a). It is legitimate to suspect that some proteins are more easily identified because of their higher expression or the ease with which they can be extracted and separated electrophoretically.

4.5.1 Enrichment of Proteins with Specific Forms of Cysteine Oxidation

One way to circumvent these problems is to enrich the proteins of interest before electrophoresis. This can be done in a number of ways: (1) When a biotin-switch method is used and the modified proteins end up, after reduction, with a

biotin tag, affinity separation by streptavidin or antibiotin can be used (Jaffrey et al., 2001; Kuncewicz et al., 2003; Martinez-Ruiz and Lamas, 2004). (2) The same approach can be used when glutathionylation or cysteinylation are studied by looking at incorporation of biotin-labeled glutathione (Sullivan et al., 2000) or cysteine (Eaton et al., 2002, 2003). (3) The ability of proteins to undergo glutathionylation can be studied using GSH or GSNO (Klatt et al., 2000) affinity columns. (4) Glutathionylated proteins can bind as well to glutaredoxin bound to a resin via a monothiol mechanism (Lind et al., 2002).

All these methods have been successfully used to identify glutathionylated proteins. Although it is too early to tell which method will become the gold standard, it appears that the biotin-based methods have been used in more than one study. These techniques combined with other approaches could further improve the enriching step. For instance, many proteins undergoing oxidoreduction are protein disulfide oxidoreductases with a CXXC active site and/or contain another type of vicinal dithiol. Since vicinal dithiols bind to arsenicals, arsenical-based affinity chromatography has been used to purify vicinal dithiols-containing protein, that represent up to 5% of the total soluble proteins in leukemia cells (Gitler et al., 1997; Kalef et al., 1993).

4.5.2 Membrane Proteins

The redox state of protein cysteines in the cell is compartmentalized, as mentioned above. While cysteines of intracellular proteins are mostly reduced (with the exception of the endoplasmic reticulum), those outside the cells are mostly present as disulfides. In the plasma membrane the cysteines on the exofacial side are exposed to an oxidizing environment. They are mostly oxidized, but a number of them are maintained in a reduced state by systems involving intracellular glutathione (Laragione et al., 2003) and protein disulfide isomerase (Jiang et al., 1999). Specifically labeling exofacial thiols to study their redox state calls for non–cell permeable thiol reagents. While some reagents may not be suitable for proteomics studies, the one used for this purpose was biotinylated iodoacetamide (*N*-(biotinoyl)-*N′*-(iodoacetyl)ethylendiamine, BIAM). This reagent labels membrane protein SH (Dominici et al., 1999). We have used it in proteomics studies to investigate those membrane proteins whose exofacial thiols were reduced by *N*-acetylcysteine and identified VLA4 among them (Laragione et al., 2003). It should be mentioned that very few protein spots could be identified in this way and this limitation could be overcome in the future by combining better methods to extract membrane proteins and to enrich the proteins of interest by affinity reagents.

4.6 STRUCTURAL AND PHYSICOCHEMICAL DETERMINANTS FOR THE SUSCEPTIBILITY OF CYSTEINES TOWARD OXIDATION

The oxidation state of cysteine residues has a fundamental role in determining the three-dimensional (3D) structure of a protein. In particular, disulfide bridges

have a strong impact in the thermodynamic stability of a protein and drive protein folding, as demonstrated in the pioneering work of Anfinsen (1973). In the absence of an experimentally determined structure, the correct prediction of the disulfide bridge's location can strongly enhance the performance of ab initio modeling studies in predicting the 3D structure. Even if in silico prediction is not completely reliable starting from the primary structure, several bioinformatics methods have been developed, as reviewed in Fiser and Simon (2002). These methods are based on statistics (Fiser et al., 1992), neural networks (Fariselli and Casadio, 1999; Martelli et al., 2002) or multiple sequence alignments (Fariselli et al., 1999; Fiser and Simon, 2000). A recent method, available on the Web at http://neural.dsi.unifi.it/cysteines, combines neural networks and evolutionary information (Vullo and Frasconi, 2004).

In the case of specific Cys modifications, such as glutathionylation, little is known on the structural determinants favoring the modification. Factors contributing the specific susceptibility of certain Cys residues toward glutathionylation have been proposed, including solvent exposure of the Cys residue, which is a major determinant in the glutathionylation of Trx (Casagrande et al., 2002), and a cationic environment that renders the thiol group highly reactive and particularly susceptible to oxidation (Klatt et al., 1999; Mallis et al., 2000; Pineda-Molina et al., 2001). We identified by MALDI mass spectrometry the Cys targets of glutathionylation (Cys52 and Cys62) in Cyclophilin A (CypA), and we defined the reasons for the susceptibility of these residues to the modification by *in silico* calculations (Ghezzi et al., 2005b). A clear correlation with their measured reactivity cannot be made by looking at the solvent exposure of cysteine residues. The reactivity of cysteines must depend also on specific interactions with other residues on the protein. It is known that the cysteine side chains are stronger nucleophiles when present as the thiolate anion. It is then plausible to expect that local interactions in proteins might modulate the ease of thiolate formation and therefore cysteine reactivity. The formation of the anionic thiolate is favored and disfavored, respectively, by nearby basic and acidic amino acid residues through electrostatic interactions (Friedman, 1973). To estimate the susceptibility of the various CypA Cys residues to glutathionylation, we investigated the solvent accessibility and the chemical reactivity of the four Cys residues in CypA. Molecular dynamic simulation showed that Cys52 and Cys161 exposed a larger surface of their side chains in 1,000 ps of observation than Cys62 and Cys115. In fact Cys62 exposes at most 0.5 $Å^2$ of its side chain surface between 500 and 700 ps of simulation, while the Cys115 side chain was never on the surface during the entire dynamics. To further define the reactivity of Cys residues in CypA, the electrostatic work needed for the deprotonation of each Cys thiol was computed. For each conformer, such electrostatic work was calculated as the difference between the electrostatic energy of a given conformer with only one Cys as thiolate and of the same conformer with all the Cys in their neutral form. The same procedure was repeated for all four Cys residues. The energy involved in the conversion of a thiol to the corresponding thiolate fluctuates and is, on average, significantly different for each Cys residue. The changes in electrostatic

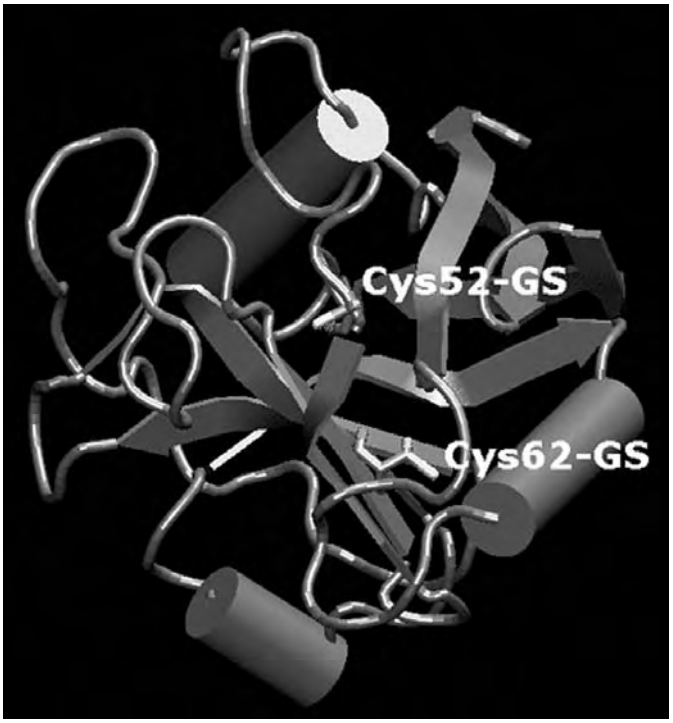


FIGURE 4.4 3D structure of Cyclophilin A. The glutathionylated Cys (Cys-SG) 52 and 62 are indicated. As confirmed by MD simulation, Cys 62 exposed at most 0.5 $Å^2$ of its side chain surface, but it undergoes glutathionylation, possibly because of the favorable electrostatic environment.

energy follow the order Cys52 < Cys62 < Cys115 < Cys161, meaning that Cys52 and Cys62 form the thiolate with greater ease than Cys115 and Cys161, and can therefore be expected to be more reactive. From our electrostatic energy computations it is clear that even when a Cys residue has a small surface exposure, as in the case of Cys62, electrostatic factors can lead to susceptibility to glutathionylation (Fig. 4.4).

4.7 PERSPECTIVE

The challenge of redox proteomics to analyze protein thiol-disulfides is complicated by the relative instability of thiols and disulfides. The use of more sophisticated technologies will allow the targets of oxidative damage in disease to be identified. But the greater challenge is to identify the proteins undergoing subtle changes occurring in redox regulation, that is, those proteins that undergo oxidoreduction under physiological conditions.

LIST OF ABBREVIATIONS

BIAM, *N*-(biotinoyl)-*N′*-(iodoacetyl)ethylendiamine
BSA, bovine serum albumin
Cy, cyanine
CypA, cyclophilin A
DIGE, difference gel electrophoresis
DTT, dithiothreitol
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
GILT, inducible lysosomal thiol reductase
GSH, glutathione
GSSG, glutathione disulfide (oxidized glutathione)
IAM, iodoacetamide
ICAT, isotope-coded affinity tag
IL-6, interleukin-6
LC, liquid chromatography
MALDI, matrix-assisted laser desorption/ionization
MS, mass spectrometry
MS/MS, tandem mass spectrometry
NEM, *N*-ethylmaleimide
Prx, peroxiredoxin
ROS, reactive oxygen species
TNF, tumor necrosis factor
Trx, thioredoxin

REFERENCES

Anfinsen C.B. (1973). Principles that govern the folding of protein chains. *Science* 181: 223–230.

Arner E.S., Holmgren A. (2000). Physiological functions of thioredoxin and thioredoxin reductase. *Eur J Biochem* 267: 6102–6109.

Arunachalam B., Phan U.T., Geuze H.J., Cresswell P. (2000). Enzymatic reduction of disulfide bonds in lysosomes: Characterization of a gamma-interferon-inducible lysosomal thiol reductase (GILT). *Proc Natl Acad Sci USA* 97: 745–750.

Barrett W.C., DeGnore J.P., Keng Y.F., Zhang Z.Y., Yim M.B., Chock P.B. (1999). Roles of superoxide radical anion in signal transduction mediated by reversible regulation of protein-tyrosine phosphatase 1B. *J Biol Chem* 274: 34543–34546.

Berggren K.N., Chernokalskaya E., Lopez M.F., Beechem J.M., Patton W.F. (2001). Comparison of three different fluorescent visualization strategies for detecting Escherichia coli ATP synthase subunits after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Proteomics* 1: 54–65.

Birge R.B., Bartolone J.B., Cohen S.D., Khairallah E.A., Smolin L.A. (1991). A comparison of proteins *S*-thiolated by glutathione to those arylated by acetaminophen. *Biochem Pharmacol* 42 Suppl: S197–S207.

Brennan J.P., Wait R., Begum S., Bell J.R., Dunn M.J., Eaton P. (2004). Detection and mapping of widespread intermolecular protein disulfide formation during cardiac oxidative stress using proteomics with diagonal electrophoresis. *J Biol Chem* 279: 41352–41360.

Brennan S.O., Fellowes A.P., George P.M. (1999). Albumin banks peninsula: A new termination variant characterised by electrospray mass spectrometry. *Biochim Biophys Acta* 1433: 321–326.

Brigelius R., Lenzen R., Sies H. (1982). Increase in hepatic mixed disulphide and glutathione disulphide levels elicited by paraquat. *Biochem Pharmacol* 31: 1637–1641.

Brigelius R., Muckel C., Akerboom T.P., Sies H. (1983). Identification and quantitation of glutathione in hepatic protein mixed disulfides and its relationship to glutathione disulfide. *Biochem Pharmacol* 32: 2529–2534.

Brooker R.J., Slayman C.W. (1983). [14C]N-ethylmaleimide labeling of the plasma membrane [H^+]-ATPase of *Neurospora crassa*. *J Biol Chem* 258: 222–226.

Brown J.R., Hartley B.S. (1966). Location of disulphide bridges by diagonal paper electrophoresis: The disulphide bridges of bovine chymotrypsinogen A. *Biochem J* 101: 214–228.

Bump E.A., Cerce B.A., al-Sarraf R., Pierce S.M., Koch C.J. (1992). Radioprotection of DNA in isolated nuclei by naturally occurring thiols at intermediate oxygen tension. *Radiat Res* 132: 94–104.

Casagrande S., Bonetto V., Fratelli M., Gianazza E., Eberini I., Massignan T., Salmona M., Chang G., Holmgren A., Ghezzi P. (2002). Glutathionylation of human thioredoxin: A possible crosstalk between the glutathione and thioredoxin systems. *Proc Natl Acad Sci USA* 99: 9745–9749.

Chen W., Yewdell J.W., Levine R.L., Bennink J.R. (1999). Modification of cysteine residues in vitro and in vivo affects the immunogenicity and antigenicity of major histocompatibility complex class I-restricted viral determinants. *J Exp Med* 189: 1757–1764.

Choi H., Kim S., Mukhopadhyay P., Cho S., Woo J., Storz G., Ryu S. (2001). Structural basis of the redox switch in the OxyR transcription factor. *Cell* 105: 103–113.

Chu F., Chen L.H., O'Brian C.A. (2004). Cellular protein kinase C isozyme regulation by exogenously delivered physiological disulfides—Implications of oxidative protein kinase C regulation to cancer prevention. *Carcinogenesis* 25: 585–596.

Chu F., Ward N.E., O'Brian C.A. (2001). Potent inactivation of representative members of each PKC isozyme subfamily and PKD via S-thiolation by the tumor-promotion/progression antagonist glutathione but not by its precursor cysteine. *Carcinogenesis* 22: 1221–1229.

Cole A.R., Hall N.E., Treutlein H.R., Eddes J.S., Reid G.E., Moritz R.L., Simpson R.J. (1999). Disulfide bond structure and *N*-glycosylation sites of the extracellular domain of the human interleukin-6 receptor. *J Biol Chem* 274: 7207–7215.

Collins D.S., Unanue E.R., Harding C.V. (1991). Reduction of disulfide bonds within lysosomes is a key step in antigen processing. *J Immunol* 147: 4054–4059.

Cumming R.C., Andon N.L., Haynes P.A., Park M., Fischer W.H., Schubert D. (2004). Protein disulfide bond formation in the cytoplasm during oxidative stress. *J Biol Chem* 279: 21749–21758.

Dominici S., Valentini M., Maellaro E., Del Bello B., Paolicchi A., Lorenzini E., Tongiani R., Comporti M., Pompella A. (1999). Redox modulation of cell surface protein thiols in U937 lymphoma cells: The role of gamma-glutamyl transpeptidase-dependent H_2O_2 production and *S*-thiolation. *Free Radic Biol Med* 27: 623–635.

Eaton P., Byers H.L., Leeds N., Ward M.A., Shattock M.J. (2002). Detection, quantitation, purification, and identification of cardiac proteins *S*-thiolated during ischemia and reperfusion. *J Biol Chem* 277: 9806–9811.

Eaton P., Jones M.E., McGregor E., Dunn M.J., Leeds N., Byers H.L., Leung K.Y., Ward M.A., Pratt J.R., Shattock M.J. (2003). Reversible cysteine-targeted oxidation of proteins during renal oxidative stress. *J Am Soc Nephrol* 14: S290–S296.

Ennion S.J., Evans R.J. (2002). Conserved cysteine residues in the extracellular loop of the human P2X(1) receptor form disulfide bonds and are involved in receptor trafficking to the cell surface. *Mol Pharmacol* 61: 303–311.

Essex D.W., Li M., Miller A., Feinman R.D. (2001). Protein disulfide isomerase and sulfhydryl-dependent pathways in platelet activation. *Biochemistry* 40: 6070–6075.

Evans C.A., Tonge R., Blinco D., Pierce A., Shaw J., Lu Y., Hamzah H.G., Gray A., Downes C.P., Gaskell S.J., Spooncer E., Whetton A.D. (2004). Comparative proteomics of primitive hematopoietic cell populations reveals differences in expression of proteins regulating motility. *Blood* 103: 3751–3759.

Fariselli P., Casadio R. (1999). A neural network based predictor of residue contacts in proteins. *Protein Eng* 12: 15–21.

Fariselli P., Riccobelli P., Casadio R. (1999). Role of evolutionary information in predicting the disulfide-bonding state of cysteine in proteins. *Proteins* 36: 340–346.

Fiser A., Cserzo M., Tudos E., Simon I. (1992). Different sequence environments of cysteines and half cystines in proteins: Application to predict disulfide forming residues. *FEBS Lett* 302: 117–120.

Fiser A., Simon I. (2000). Predicting the oxidation state of cysteines by multiple sequence alignment. *Bioinformatics* 16: 251–256.

Fiser A., Simon I. (2002). Predicting redox state of cysteines in proteins. *Methods Enzymol* 353: 10–21.

Fratelli M., Demol H., Puype M., Casagrande S., Eberini I., Salmona M., Bonetto V., Mengozzi M., Duffieux F., Miclet E., Bachi A., Vandekerckhove J., Gianazza E., Ghezzi P. (2002). Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. *Proc Natl Acad Sci USA* 99: 3505–3510.

Fratelli M., Demol H., Puype M., Casagrande S., Villa P., Eberini I., Vandekerckhove J., Gianazza E., Ghezzi P. (2003). Identification of proteins undergoing glutathionylation in oxidatively stressed hepatocytes and hepatoma cells. *Proteomics* 3: 1154–1161.

Friedman M. (1973). *The Chemistry and Biochemistry of the Sulfhydryl Group in Amino Acids, Peptides, and Proteins*, Pergamon Press, Oxford.

Frillingos S., Kaback H.R. (1996). Cysteine-scanning mutagenesis of helix VI and the flanking hydrophilic domains on the lactose permease of *Escherichia coli*. *Biochemistry* 35: 5333–5338.

Garant M.J., Kole S., Maksimova E.M., Bernier M. (1999). Reversible change in thiol redox status of the insulin receptor alpha-subunit in intact cells. *Biochemistry* 38: 5896–5904.

Ghezzi P., Bonetto V., Fratelli M. (2005a). Thiol-disulfide balance: from the concept of oxidative stress to that of redox regulation. *Antioxid Redox Signal* 7: 964–972.

Ghezzi P., Casagrande S., Massignan T., Basso M., Bellacchio E., Mollica L., Biasini E., Tonelli R., Eberini I., Gianazza E., Dai W.W., Fratelli M., Salmona M., Sherry B., Bonetto V. (2005b). Redox regulation of cyclophilin A by glutathionylation, *Proteomics* 6: 817–825.

Ghezzi P., Romines B., Fratelli M., Eberini I., Gianazza E., Casagrande S., Laragione T., Mengozzi M., Herzenberg L.A. (2002). Protein glutathionylation: Coupling and uncoupling of glutathione to protein thiol groups in lymphocytes under oxidative stress and HIV infection. *Mol Immunol* 38: 773–780.

Gilbert H.F. (1984). Redox control of enzyme activities by thiol/disulfide exchange. *Methods Enzymol* 107: 330–351.

Gitler C., Zarmi B., Kalef E. (1997). General method to identify and enrich vicinal thiol proteins present in intact cells in the oxidized, disulfide state. *Anal Biochem* 252: 48–55.

Gow A.J., Chen Q., Hess D.T., Day B.J., Ischiropoulos H., Stamler J.S. (2002). Basal and stimulated protein *S*-nitrosylation in multiple cell types and tissues. *J Biol Chem* 277: 9637–9640.

Gygi S.P., Rist B., Gerber S.A., Turecek F., Gelb M.H., Aebersold R. (1999). Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* 17: 994–999.

Haque M.A., Hawes J.W., Blum J.S. (2001). Cysteinylation of MHC class II ligands: Peptide endocytosis and reduction within APC influences T cell recognition. *J Immunol* 166: 4543–4551.

Harding J.J. (1969). Glutathione-protein mixed disulphides in human lens. *Biochem J* 114: 88P–89P.

Hare J.F., Lee E. (1989). Metabolic behavior of cell surface biotinylated proteins. *Biochemistry* 28: 574–580.

Harrap K.R., Jackson R.C., Riches P.G., Smith C.A., Hill B.T. (1973). The occurrence of protein-bound mixed disulfides in rat tissues. *Biochim Biophys Acta* 310: 104–110.

Hultberg B., Hultberg M. (2004). High glutathione turnover in human cell lines revealed by acivicin inhibition of gamma-glutamyltranspeptidase and the effects of thiol-reactive metals during acivicin inhibition. *Clin Chim Acta* 349: 45–52.

Jaffrey S.R., Erdjument-Bromage H., Ferris C.D., Tempst P., Snyder S.H. (2001). Protein *S*-nitrosylation: A physiological signal for neuronal nitric oxide. *Nat Cell Biol* 3: 193–197.

Jakob U., Muse W., Eser M., Bardwell J.C. (1999). Chaperone activity with a redox switch. *Cell* 96: 341–352.

Jiang X.M., Fitzgerald M., Grant C.M., Hogg P.J. (1999). Redox control of exofacial protein thiols/disulfides by protein disulfide isomerase. *J Biol Chem* 274: 2416–2423.

Kalef E., Walfish P.G., Gitler C. (1993). Arsenical-based affinity chromatography of vicinal dithiol-containing proteins: Purification of L1210 leukemia cytoplasmic proteins and the recombinant rat c-erb A beta 1 T3 receptor. *Anal Biochem* 212: 325–334.

Kim J.R., Yoon H.W., Kwon K.S., Lee S.R., Rhee S.G. (2000). Identification of proteins containing cysteine residues that are sensitive to oxidation by hydrogen peroxide at neutral pH. *Anal Biochem* 283: 214–221.

Klatt P., Molina E.P., De Lacoba M.G., Padilla C.A., Martinez-Galesteo E., Barcena J.A., Lamas S. (1999). Redox regulation of c-Jun DNA binding by reversible *S*-glutathiolation. *FASEB J* 13: 1481–1490.

Klatt P., Pineda Molina E., Perez-Sala D., Lamas S. (2000). Novel application of *S*-nitrosoglutathione-Sepharose to identify proteins that are potential targets for *S*-nitrosoglutathione-induced mixed-disulphide formation. *Biochem J* 349: 567–578.

Koch C.J., Evans S.M. (1996). Cysteine concentrations in rodent tumors: unexpectedly high values may cause therapy resistance. *Int J Cancer* 67: 661–667.

Kocis J.M., Kuo W.N., Liu Y., Guruvadoo L.K., Langat J.L. (2002). Regulation of catalase: Inhibition by peroxynitrite and reactivation by reduced glutathione and glutathione *S*-transferase. *Front Biosci* 7: 175–180.

Kuncewicz T., Sheta E.A., Goldknopf I.L., Kone B.C. (2003). Proteomic analysis of *S*-nitrosylated proteins in mesangial cells. *Mol Cell Proteomics* 2: 156–163.

Laragione T., Bonetto V., Casoni F., Massignan T., Bianchi G., Gianazza E., Ghezzi P. (2003). Redox regulation of surface protein thiols: Identification of integrin alpha-4 as a molecular target by using redox proteomics. *Proc Natl Acad Sci USA* 100: 14737–14741.

Lin T.K., Hughes G., Muratovska A., Blaikie F.H., Brookes P.S., Darley-Usmar V., Smith R.A., Murphy M.P. (2002). Specific modification of mitochondrial protein thiols in response to oxidative stress: A proteomics approach. *J Biol Chem* 277: 17048–17056.

Lind C., Gerdes R., Hamnell Y., Schuppe-Koistinen I., von Lowenhielm H.B., Holmgren A., Cotgreave I.A. (2002). Identification of *S*-glutathionylated cellular proteins during oxidative stress and constitutive metabolism by affinity purification and proteomic analysis. *Arch Biochem Biophys* 406: 229–240.

Lorch S.A., Foust R., 3rd, Gow A., Arkovitz M., Salzman A.L., Szabo C., Vayert B., Geffard M., Ischiropoulos H. (2000). Immunohistochemical localization of protein 3-nitrotyrosine and *S*-nitrosocysteine in a murine model of inhaled nitric oxide therapy. *Pediatr Res* 47: 798–805.

Maeda K., Finnie C., Svensson B. (2004). Cy5 maleimide labelling for sensitive detection of free thiols in native protein extracts: Identification of seed proteins targeted by barley thioredoxin h isoforms. *Biochem J* 378: 497–507.

Mallick P., Boutz D.R., Eisenberg D., Yeates T.O. (2002). Genomic evidence that the intracellular proteins of archaeal microbes contain disulfide bonds. *Proc Natl Acad Sci USA* 99: 9679–9684.

Mallis R.J., Poland B.W., Chatterjee T.K., Fisher R.A., Darmawan S., Honzatko R.B., Thomas J.A. (2000). Crystal structure of *S*-glutathiolated carbonic anhydrase III. *FEBS Lett* 482: 237–241.

Mannervik B., Axelsson K., Sundewall A.C., Holmgren A. (1983). Relative contributions of thioltransferase- and thioredoxin-dependent systems in reduction of low-molecular-mass and protein disulphides. *Biochem J* 213: 519–523.

Maric M., Arunachalam B., Phan U.T., Dong C., Garrett W.S., Cannon K.S., Alfonso C., Karlsson L., Flavell R.A., Cresswell P. (2001). Defective antigen processing in GILT-free mice. *Science* 294: 1361–1365.

Martelli P.L., Fariselli P., Malaguti L., Casadio R. (2002). Prediction of the disulfide-bonding state of cysteines in proteins at 88% accuracy. *Protein Sci* 11: 2735–2739.

Martinez-Ruiz A., Lamas S. (2004). Detection and proteomic identification of *S*-nitrosylated proteins in endothelial cells. *Arch Biochem Biophys* 423: 192–199.

Mawatari S., Murakami K. (2004). Different types of glutathionylation of hemoglobin can exist in intact erythrocytes. *Arch Biochem Biophys* 421: 108–114.

Mills B.J., Lang C.A. (1996). Differential distribution of free and bound glutathione and cyst(e)ine in human blood. *Biochem Pharmacol* 52: 401–406.

Modig H. (1968). Cellular mixed disulphides between thiols and proteins, and their possible implication for radiation protection. *Biochem Pharmacol* 17: 177–186.

Molinari M., Helenius A. (1999). Glycoproteins form mixed disulphides with oxidoreductases during folding in living cells. *Nature* 402: 90–93.

Moritz R.L., Hall N.E., Connolly L.M., Simpson R.J. (2001). Determination of the disulfide structure and *N*-glycosylation sites of the extracellular domain of the human signal transducer gp130. *J Biol Chem* 276: 8244–8253.

Pastore A., Tozzi G., Gaeta L.M., Bertini E., Serafini V., Di Cesare S., Bonetto V., Casoni F., Carrozzo R., Federici G., Piemonte F. (2003). Actin glutathionylation increases in fibroblasts of patients with Friedreich's ataxia: a potential role in the pathogenesis of the disease. *J Biol Chem* 278: 42588–42595.

Pepinsky R.B., Rayhorn P., Day E.S., Dergay A., Williams K.P., Galdes A., Taylor F.R., Boriack-Sjodin P.A., Garber E.A. (2000). Mapping sonic hedgehog-receptor interactions by steric interference. *J Biol Chem* 275: 10995–11001.

Persson C., Kappert K., Engstrom U., Ostman A., Sjoblom T. (2005). An antibody-based method for monitoring in vivo oxidation of protein tyrosine phosphatases. *Methods* 35: 37–43.

Persson C., Sjoblom T., Groen A., Kappert K., Engstrom U., Hellman U., Heldin C.H., den Hertog J., Ostman A. (2004). Preferential oxidation of the second phosphatase domain of receptor-like PTP-alpha revealed by an antibody against oxidized protein tyrosine phosphatases. *Proc Natl Acad Sci USA* 101: 1886–1891.

Pineda-Molina E., Klatt P., Vazquez J., Marina A., Garcia de Lacoba M., Perez-Sala D., Lamas S. (2001). Glutathionylation of the p50 subunit of NF-kappaB: A mechanism for redox-induced inhibition of DNA binding. *Biochemistry* 40: 14134–14142.

Prinz W.A., Aslund F., Holmgren A., Beckwith J. (1997). The role of the thioredoxin and glutaredoxin pathways in reducing protein disulfide bonds in the *Escherichia coli* cytoplasm. *J Biol Chem* 272: 15661–15667.

Reynaert N.L., Ckless K., Korn S.H., Vos N., Guala A.S., Wouters E.F., van der Vliet A., Janssen-Heininger Y.M. (2004). Nitric oxide represses inhibitory kappaB kinase through *S*-nitrosylation. *Proc Natl Acad Sci USA* 101: 8945–8950.

Rietsch A., Beckwith J. (1998). The genetics of disulfide bond metabolism. *Ann Rev Genet* 32: 163–184.

Rokutan K., Thomas J.A., Johnston R.B. Jr. (1991). Phagocytosis and stimulation of the respiratory burst by phorbol diester initiate *S*-thiolation of specific proteins in macrophages. *J Immunol* 147: 260–264.

Ruoso P., Hedley D.W. (2004). Inhibition of gamma-glutamyl transpeptidase activity decreases intracellular cysteine levels in cervical carcinoma. *Cancer Chemother Pharmacol* 54: 49–56.

Saurin A.T., Neubert H., Brennan J.P., Eaton P. (2004). Widespread sulfenic acid formation in tissues in response to hydrogen peroxide. *Proc Natl Acad Sci USA* 101: 17982–17987.

Sethuraman M., McComb M.E., Heibeck T., Costello C.E., Cohen R.A. (2004a). Isotope-coded affinity tag approach to identify and quantify oxidant-sensitive protein thiols. *Mol Cell Proteomics* 3: 273–278.

Sethuraman M., McComb M.E., Huang H., Huang S., Heibeck T., Costello C.E., Cohen R.A. (2004b). Isotope-coded affinity tag (ICAT) approach to redox proteomics: Identification and quantitation of oxidant-sensitive cysteine thiols in complex protein mixtures. *J Proteome Res* 3: 1228–1233.

Sevier C.S., Kaiser C.A. (2002). Formation and transfer of disulphide bonds in living cells. *Nat Rev Mol Cell Biol* 3: 836–847.

Shaw J., Rowlinson R., Nickson J., Stone T., Sweet A., Williams K., Tonge R. (2003). Evaluation of saturation labelling two-dimensional difference gel electrophoresis fluorescent dyes. *Proteomics* 3: 1181–1195.

Smolka M., Zhou H., Aebersold R. (2002). Quantitative protein profiling using two-dimensional gel electrophoresis, isotope-coded affinity tag labeling, and mass spectrometry. *Mol Cell Proteomics* 1: 19–29.

Sommer A., Traut R.R. (1974). Diagonal polyacrylamide-dodecyl sulfate gel electrophoresis for the identification of ribosomal proteins crosslinked with methyl-4-mercaptobutyrimidate. *Proc Natl Acad Sci USA* 71: 3946–3950.

Stadtman E.R. (1993). Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Ann Rev Biochem* 62: 797–821.

Sullivan D.M., Wehr N.B., Fergusson M.M., Levine R.L., Finkel T. (2000). Identification of oxidant-sensitive proteins: TNF-alpha induces protein glutathiolation. *Biochemistry* 39: 11121–11128.

Sun J., Xin C., Eu J.P., Stamler J.S., Meissner G. (2001). Cysteine-3635 is responsible for skeletal muscle ryanodine receptor modulation by NO. *Proc Natl Acad Sci USA* 98: 11158–11162.

Terazaki H., Ando Y., Suhr O., Ohlsson P.I., Obayashi K., Yamashita T., Yoshimatsu S., Suga M., Uchino M., Ando M. (1998). Post-translational modification of transthyretin in plasma. *Biochem Biophys Res Commun* 249: 26–30.

Townsend D., Fazliyev F., findlay V., Saavedra J., Keefer L., Tew K. *Proceedings of the AACR*, Abstract 5316, 2004, pp.

Vullo A., Frasconi P. (2004). Disulfide connectivity prediction using recursive neural networks and evolutionary information. *Bioinformatics* 20: 653–659.

Watarai H., Nozawa R., Tokunaga A., Yuyama N., Tomas M., Hinohara A., Ishizaka K., Ishii Y. (2000). Posttranslational modification of the glycosylation inhibiting factor (GIF) gene product generates bioactive GIF. *Proc Natl Acad Sci USA* 97: 13251–13256.

Woo H.A., Kang S.W., Kim H.K., Yang K.S., Chae H.Z., Rhee S.G. (2003). Reversible oxidation of the active site cysteine of peroxiredoxins to cysteine sulfinic acid: Immunoblot detection with antibodies specific for the hyperoxidized cysteine-containing sequence. *J Biol Chem* 278: 47361–47364.

Wu H.H., Thomas J.A., Momand J. (2000). p53 protein oxidation in cultured cells in response to pyrrolidine dithiocarbamate: A novel method for relating the amount of p53 oxidation in vivo to the regulation of p53-responsive genes. *Biochem J* 351: 87–93.

Yano H., Kuroda S., Buchanan B.B. (2002). Disulfide proteome in the analysis of protein function and structure. *Proteomics* 2: 1090–1096.

Yano H., Wong J.H., Lee Y.M., Cho M.J., Buchanan B.B. (2001). A strategy for the identification of proteins targeted by thioredoxin. *Proc Natl Acad Sci USA* 98: 4794–4799.

5

CARBONYLATED PROTEINS AND THEIR IMPLICATION IN PHYSIOLOGY AND PATHOLOGY

RODNEY L. LEVINE AND EARL. R. STADTMAN

5.1 INTRODUCTION

Accumulating experimental evidence supports the proposal that many of the changes that occur during aging and during the evolution of certain diseases are a consequence of oxidative stress and resulting cellular damage (Harman, 1956). The list of diseases and processes in which oxidative damage is implicated reads like a textbook of pathology and includes atherosclerosis, cancer, neurodegenerative diseases such as Alzheimer and Parkinson, and the aging process (Table 5.1). At present the implicating evidence is heavily correlative, with a large number of published studies documenting the close correlation of markers of oxidative stress and the progression of the process under study. Thus the cells of older organisms, be they worms or humans, carry an increased burden of oxidatively damaged macromolecules including nucleic acids, lipids, and proteins. In this chapter we focus on one class of oxidative modification of proteins, those that cause the introduction of a carbonyl* group into the protein.

A major goal of biological researchers of the last 50 years is the understanding of the mechanisms of regulation of cellular metabolism. By the mid-1970s it was clear that two major mechanisms were the control of the rate of synthesis of specific proteins (gene regulation) and the control of the activity of already

*In the context of oxidative modification of proteins, carbonyl refers to moieties with the reactivity of aldehydes and ketones—in other words, a subset of the general class of carbonyl groups. Thus the carbonyl group of the peptide bond is excluded because it does not have the chemical reactivity of an aldehyde or ketone.

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield
Published 2006 by John Wiley & Sons, Inc.

TABLE 5.1 Selected Studies of Carbonylated Proteins

Condition or Exposure	Selected References
Aceruloplasminemia	Kaneko et al., 2002
Acrolein exposure	Burcham and Fontaine, 2001
Actin oxidation	Dalle-Donne et al., 2001, 2002
Acute respiratory distress syndrome	Quinlan et al., 1994; Lenz et al., 1999; Winterbourn et al., 2000
Adenosine treatment	Parihar and Pandit, 2003; Sakata et al., 2000
Aging	Stadtman and Levine, 2000; Sohal et al., 1993; Musci et al., 1993; Agarwal and Sohal, 1994, 1996; Yan et al., 1997a; de la Cruz et al., 1996; Yan and Sohal, 1998; Montine et al., 2002; Jana et al., 2002; Richert et al., 2002; Mutlu Turkoglu et al., 2003; Soreghan et al., 2003; Navarro and Boveris, 2004; Poon et al., 2004; Judge et al., 2005
Alcohol exposure	Wieland and Lauterburg, 1995; Grattagliano et al., 1996; Castegna et al., 2002; Abraham et al., 2002
Algae extract exposure	Nizard et al., 2004
Allethrin exposure	Gupta et al., 1999
Alzheimer's disease	Hensley et al., 1995; Smith et al., 1998; Aksenov et al., 2001; Korolainen et al., 2002; Butterfield, 2004
Aminoguanidine treatment	Miyata et al., 1998; Gogasyavuz et al., 2002; Stadler et al., 2005
Aminolevulinic acid exposure	Demasi et al., 1996; Tomas-Zapico et al., 2002
Amyloidosis	Ando et al., 1997; Miyata et al., 2000
Amyotrophic lateral sclerosis	Bowling et al., 1993; Ferrante et al., 1997; Andrus et al., 1998; Hensley et al., 2002
Angiotensin II treatment	Haugen et al., 2000
Apolipoprotein E knockout	Choi et al., 2004
Apomorphine treatment	Moreira et al., 2003; Khaliulin et al., 2004
Arabidopsis thaliana life cycle	Johansson et al., 2004
Arsenic exposure	Ramanathan et al., 2003
Arteriosclerotic lesions	Miyata et al., 1998
Ascorbate exposure or treatment	Levine, 1983b; Barja et al., 1994; Cadenas et al., 1994; Carty et al., 2000; Suh et al., 2003; Ramanathan et al., 2003
Asthma	Foreman et al., 1999; Nadeem et al., 2003
Ataxia telangiectasia	Barlow et al., 1999
Atrial fibrillation	Mihm et al., 2001
Bcl-2 expression level	Hochman et al., 2000; Lee et al., 2001a
Benzo(a)pyrene exposure	Kim and Lee, 1997; Selvendiran et al., 2004
Berry phenolics	Viljanen et al., 2004
Beta-thalassemia major	Livrea et al., 1996
Bilirubin as antioxidant	Neuzil and Stocker, 1993; Dennery et al., 1995; Minetti et al., 1998

TABLE 5.1 (*continued*)

Condition or Exposure	Selected References
Bombesin administration	Gulluoglu et al., 1999
Brain regional differences	Floor and Wetzel, 1998
Bromobenzene exposure	Benedetti et al., 1986
Burn injury	Haycock et al., 1997; Fagan et al., 1999; Ritter et al., 2003
Caenorhabditis elegans life cycle	Ishii et al., 1990; Adachi et al., 1998; Yasuda et al., 1999; Nakamura et al., 1999
Caloric restriction	Youngman et al., 1992; Lass et al., 1998; Zainal et al., 2000; Forster et al., 2000; Nagai et al., 2000; Pamplona et al., 2002; Judge et al., 2004; Ramsey et al., 2004
Carbon tetrachloride exposure	Sundari et al., 1997
Carcinogenesis	Uchida et al., 1995; Chen et al., 2000; Sultana and Saleem, 2004
Carnitine as antioxidant	Packer et al., 1991
Carnosine treatment	Nagasawa et al., 2001; Hipkiss et al., 2002
Cataract formation	Garland et al., 1988; Varma et al., 1994; Boscia et al., 2000; Sastre et al., 2005
Cellular senescence	Chen et al., 1995; Favetta et al., 2004
Cephalosporin treatment	Jung et al., 1997
Cerebral malaria	Sanni et al., 1999
Chronic fatigue syndrome	Smirnova and Pall, 2003
Chronic arterial insufficiency	Rutkowska et al., 2005
Chronic, intermittent hypoxia	Veasey et al., 2004
Cigarette smoke and smoking	Reznick et al., 1992; Wurzel et al., 1995; Eiserich et al., 1995; Pignatelli et al., 2001
Cisplatin treatment	Senturker et al., 2002
Clofibrate treatment	Qu et al., 1999; Nicholls-Grzemski et al., 2000
Coal dust exposure	Pinho et al., 2004
Cocaine exposure	Devi and Chan, 1999
Coenzyme Q levels or treatment	Forsmark-Andrée et al., 1995; Kwong et al., 2002
Cold or immobilization stress	Sahin and Gumuslu, 2004b; Sahin and Gumuslu, 2004a
Cold stress	Kingston-Smith and Foyer, 2000; Selman et al., 2002
Confocal microscopy	Harris et al., 1994; Pompella et al., 1996; Aguilaniu et al., 2003
Copper exposure or deficiency	Yan et al., 1997b; Cockell and Belonje, 2002; Argirova and Ortwerth, 2003; Shanmuganathan et al., 2004
Coronary artery bypass surgery or cardiopulmonary bypass	Pantke et al., 1999; Matata and Galinanes, 2000; Matata et al., 2000
Crotyl alcohol exposure	Fontaine et al., 2002
Cystic fibrosis	Brown and Kelly, 1994; Dominguez et al., 1998; Kettle et al., 2004

(*continued overleaf*)

TABLE 5.1 (***continued***)

Condition or Exposure	Selected References
Dehydroepiandrosterone treatment	Pelissier et al., 2004
Dementia with Lewy bodies	Lyras et al., 1998
Diabetes	Grattagliano et al., 1998; Suzuki et al., 1999; Baynes and Thorpe, 1999; Miyata et al., 2003; Oh-Ishi et al., 2003; De et al., 2003
Dideoxycytidine treatment	Skuta et al., 1999
Diesel exhaust exposure	Blomberg et al., 1998; Madden et al., 2003
Dietary antioxidant assessment	Young et al., 2002
Dimethylthiourea treatment	Pahlmark et al., 1993
Dopa treatment	Lyras et al., 2002
Dopamine exposure	Hermida-Ameijeiras et al., 2004
Doxorubicin exposure	Tesoriere et al., 1994; DeAtley et al., 1998
Drug allergy	Mates et al., 2000
Eales's disease	Rajesh et al., 2004
Endotoxin exposure	Cadenas et al., 1998; Barreiro et al., 2005
Equine joint diseases	Dimock et al., 2000
Escherichia coli life cycle	Dukan and Nystrom, 1999; Dukan et al., 2000; Ballesteros et al., 2001
Estrogen treatment	Winter and Liehr, 1991, 1996
Etoposide treatment	Senturker et al., 2002; England et al., 2004
Exercise	Witt et al., 1992; Sohal et al., 1993; Saxton et al., 1994; Radak et al., 1997; Goto et al., 1999; Bejma et al., 2000; Chevion et al., 2003
Extracellular matrix oxidation	Mattana et al., 1995, 1998
Familial candidiasis	Gangemi et al., 2003
Fibrinogen oxidation	Shacter et al., 1995; Belisario et al., 1997
Fibromyalgia	Eisinger et al., 1996
Fish oil supplementation	Sen et al., 1997; Sato et al., 1998; Wander and Du, 2000
Food quality	Iqbal et al., 2004; Rowe et al., 2004
Fruit and vegetable intake	Dragsted et al., 2004
Fullerene exposure	Kamat et al., 1998, 2000
γ-Glutamylcysteine ethyl ester treatment	Drake et al., 2003
Gentamicin induced nephrotoxicity	Pedraza-Chaverri et al., 2004
Gestational diabetes	Coughlan et al., 2004
Ginkgo biloba treatment	Stackman et al., 2003
Ginseng treatment	Fu and Ji, 2003
Glutathione manipulation	Palamanda and Kehrer, 1992; Gupta et al., 2000a
Glutathione reductase expression	Mockett et al., 1999

TABLE 5.1 (*continued*)

Condition or Exposure	Selected References
Glutathione peroxidase-1 knockout	Ho et al., 1997; Lei, 2001
Gracile axonal degeneration	Castegna et al., 2004
Green tea administration	Song et al., 2002
Growth hormone deficiency	Ozbey et al., 2003
Helicobacter and gastritis	Li et al., 2001; Park et al., 2004
Hemin or porphyrin exposure	Silvester et al., 1998; Regan et al., 2004
Heparin exposure	Finotti and Pagetta, 1997; Finotti et al., 2001
Hepatitis C infection	De Maria et al., 1996; Koken et al., 2002
Hexachlorocyclohexane exposure	Videla et al., 2004
Histone carbonylation	Wondrak et al., 2000
Hormone replacement treatment	Telci et al., 2002
Huntington's disease	Alam et al., 2000
Hydatidiform mole	Harma et al., 2004
Hydralazine treatment	Burcham et al., 2000
Hydrogen peroxide exposure	Ciolino and Levine, 1997; Tamarit et al., 1998; Robinson et al., 1999; Costa et al., 2002; Senturker et al., 2002; Mostertz and Hecker, 2003; Canton et al., 2004; England and Cotter, 2004
Hydroxynonenal exposure	Palamanda and Kehrer, 1992; Hyun et al., 2002b; Horakova et al., 2002
Hyperbaric oxygen	Lenz et al., 1992; Akin et al., 2002; Chavko et al., 2003; Kot et al., 2003
Hyperoxia	Starke-Reed and Oliver, 1989; Winter and Liehr, 1991; Sohal et al., 1993; Singhal et al., 2002
Hypertension	Tanito et al., 2004
Hypochlorous acid exposure	Schraufstatter et al., 1990; Vissers and Winterbourn, 1991; Reddy et al., 1994; Yan et al., 1996; Yang et al., 1997; Dalle-Donne et al., 2001; Zavodnik et al., 2004
Hyponatremia correction	Mickel et al., 1990
Immunoglobulin oxidation	Margiloff et al., 1998
Inflammatory bowel disease (colitis)	Lih-Brody et al., 1996; Odetti et al., 1998; Blackburn et al., 1999; Akin et al., 2002
Iron oxide exposure	Stroh et al., 2004
Iron nitrilotriacetate exposure	Ma et al., 1998; Zainal et al., 1999; Iqbal et al., 1999
Iron administration	Lucesoli and Fraga, 1995; Sen et al., 1997; Dal-Pizzol et al., 2001; Michelis et al., 2003; Gropper et al., 2003; Anraku et al., 2004; Rehema et al., 2004

(*continued overleaf*)

TABLE 5.1 (*continued*)

Condition or Exposure	Selected References
Irradiation of meat	Rababah et al., 2004
Ischemia-reperfusion, cerebral hemorrhage, or stroke	Oliver et al., 1990; Park and Kehrer, 1991; Folbergrova et al., 1993; Grune et al., 1993; Liu et al., 1993; Ayene et al., 1992; Chang et al., 1998; He et al., 1999; Takagi et al., 1999; Robinson et al., 1999; Hall et al., 2000; Mantle et al., 2001; Singhal et al., 2002; Canton et al., 2004
Kainate exposure	Shin et al., 2004; Kwon et al., 2004
Keratin oxidation	Thiele et al., 1999
Kwashiorkor	Cho et al., 2000
Lactosylation of casein	Scaloni et al., 2002
Lead exposure	Scortegagna et al., 1998
Leptin treatment	Erkasap et al., 2003
Lichen sclerosus	Sander et al., 2004
Lindane exposure	Valencia et al., 2004
Lipid hydroperoxide exposure	Refsgaard et al., 2000
Lipoic acid treatment	Marangon et al., 1999; Perricone et al., 1999; Kayali et al., 2004; Poon et al., 2005; Samuel et al., 2005
Lung surfactant inactivation	Andersson et al., 1999
Lysosome oxidation	Yin et al., 1995
Magnesium deficiency	Stafford et al., 1993; Astier et al., 1996; Eisinger et al., 1996; Rimbach and Pallauf, 1999
Malonate exposure	Maragos et al., 2004
Malondialdehyde exposure	Burcham and Kuhan, 1996
Maternal diabetes	Cederberg et al., 2001
Melatonin treatment	Kim et al., 2000; Okatani et al., 2002; Tomas-Zapico et al., 2002; Mayo et al., 2003; Sener et al., 2004
Menadione exposure	Tamarit et al., 1998
Meningitis	Schaper et al., 2002
Metal-catalyzed oxidation	Fucci et al., 1983; Levine, 1983b; Lee and Shacter, 1995
Methamphetamine exposure	Gluck et al., 2001
Methionine sulfoxide reductase expression	Moskovitz et al., 2001; Moskovitz and Stadtman, 2003
Methylene blue exposure	Schneider, Jr. et al., 1998
Morphine exposure	Zhang et al., 2004a
Muscle stimulation	Nagasawa et al., 2000
Muscular dystrophy	Murphy and Kehrer, 1989; Haycock et al., 1996, 1998
Myocarditis	Yuan et al., 2004
N-tert-butyl-α-phenylnitrone (PBN)	Carney et al., 1991; Dubey et al., 1995; Howard et al., 1996; Butterfield et al., 1997

TABLE 5.1 (*continued*)

Condition or Exposure	Selected References
N-acetylcysteine treatment	Banaclocha et al., 1997; Martinez et al., 2000; Pocernich et al., 2000
Near-infrared irradiation	Kujawa et al., 2004
Neurofilament oxidation	Troncoso et al., 1995
Neutrophil activation	Oliver, 1987; Karsek-Staples and Webster, 1993
Nitric oxide or peroxynitrite exposure	Ischiropoulos et al., 1995; Tien et al., 1999; Bizzozero et al., 2004
Nitropropane exposure	Mirzaei and Regnier, 2005
Nitropropionic acid	La Fontaine et al., 2000
Nitrosobenzene exposure	Khan et al., 2000
Nitroxide treatment	Howard et al., 1996; Damiani et al., 2002; Senturker et al., 2002; Toth et al., 2003
Olive oil ingestion	Vissers et al., 2001
Oxygen tension	Saarinen and Murhammer, 2003; Favetta et al., 2004
Ozone exposure	Cross et al., 1992; Kelly and Birch, 1993; Berlett et al., 1996; Mudway and Kelly, 1998; Cotovio et al., 2001
Pancreatitis	Reinheckel et al., 1998; bu-Zidan et al., 2000; Gilgenast et al., 2001; Winterbourn et al., 2003
Paraquat or diquat exposure	Wolfgang et al., 1991; Winter and Liehr, 1991; Rikans and Cai, 1993; Gupta et al., 1994, 2000b; Blakeman et al., 1995; Rikans et al., 1997; Tamarit et al., 1998; Blakeman et al., 1998; Kingston-Smith and Foyer, 2000
Parkinson's disease and parkin expression or mutation	Yoritaka et al., 1996; Alam et al., 1997; Floor and Wetzel, 1998; Hyun et al., 2002a
Perinatal white matter injury	Inder et al., 2002
Peroxiredoxin expression	Wang et al., 2004a, 2004b; Lee and Park, 2004; Cha et al., 2004
Phloxine B photooxidation	Mutoh et al., 2005
Phototherapy	Kaplan et al., 2005
Pinealectomy	Reiter et al., 1999
Pneumoperitoneum and pneumoretroperitoneum	Polat et al., 2003; Samli et al., 2004
Porphyrin photooxidation	Silvester et al., 1998
Pre-eclampsia and pregnancy-induced hypertension	Zusterzeel et al., 2000, 2002; Vanderlelie et al., 2005
Premature rupture of membranes	Stuart et al., 2005
Progeric syndromes	Smith et al., 1991
Progressive supranuclear palsy	Park et al., 2001

(*continued overleaf*)

TABLE 5.1 (***continued***)

Condition or Exposure	Selected References
Proteasome inhibition	Lee et al., 2001b; Drake et al., 2002
Psoriasis	Dimon-Gadal et al., 2000
Purpurin-18 photodynamic treatment	Magi et al., 2004
Pyridoxamine treatment	Nagaraj et al., 2002
Pyrrolidine dithiocarbamate treatment	Zhu et al., 2002; Bruck et al., 2004; Shin et al., 2004
Pyruvate as antioxidant	Varma and Devamanoharan, 1995
Quercetin treatment	Kahraman et al., 2003
Respiratory distress syndrome and bronchopulmonary dysplasia	Gladstone, Jr. and Levine, 1994; Varsila et al., 1995; Buss et al., 2000; Schock et al., 2001
Rheumatoid and osteoarthritis	Chapman et al., 1989; Mantle et al., 1999; Renke et al., 2000
Rutin supplementation	Funabiki et al., 1999
Saccharomyces cerevisiae life cycle	Jakubowski et al., 2000; Aguilaniu et al., 2001, 2003; Reverter-Branch et al., 2004
Salen manganese treatment	Bayne and Sohal, 2002; Browne et al., 2004
Scutellaria baicalensis extract (flavones) treatment	Choi et al., 2002
Selenium status	Falciglia et al., 2003; Moskovitz and Stadtman, 2003
Sepsis	Fagan et al., 1996; Kantrow et al., 1997; Winterbourn et al., 2000; Abu-Zidan et al., 2002
Spinal cord injury	Jin et al., 2004; Luo and Shi, 2004
Statin treatment	Passi et al., 2003
Streptococcus agalactiae life cycle	Nair et al., 2003
Succinate dehydrogenase deficiency	Ishii et al., 1998
Sunscreen component exposure	Damiani et al., 2000
Superoxide dismutase expression or activity	Bonnes-Taourel et al., 1993; Maria et al., 1995; Cardozo-Pelaez et al., 1998; Williams et al., 1998; Ibrahim et al., 2000; O'Brien et al., 2004
Tagatose as antioxidant	Paterna et al., 1998
Tardive dyskinesia	Tsai et al., 1998
Tat exposure	Aksenov et al., 2003
Taurine treatment	Eppler and Dawson, Jr., 2001
Temporomandibular joint disease	Zardeneta et al., 2000
Teratogenesis	Winn and Wells, 1997; Wells et al., 1997
Thioredoxin levels	Takagi et al., 1999; Okuyama et al., 2003; Hattori et al., 2004

TABLE 5.1 (*continued*)

Condition or Exposure	Selected References
Thyroid status	Rahaman et al., 2001; Das and Chainy, 2004
Total parenteral nutrition	Belli et al., 2003
Tracheal obstruction	Supinski et al., 1999
Transcriptional or translational errors	Dukan et al., 2000
Trichloroethylene exposure	DuTeaux et al., 2004
Trisomy 16 (mouse)	Hanbauer et al., 1998
Trypanosoma cruzi infection	Wen and Garg, 2004
Urea exposure	Zhang et al., 2004b
Uremia	Miyata and Kurokawa, 1999; Himmelfarb et al., 2000
UV irradiation	Hu and Tappel, 1992; Podda et al., 1998; Reverter-Branch et al., 2004; Damiani et al., 2000, 2002; Sander et al., 2002; Debacq-Chainiaux et al., 2005
Varicocele	Chen et al., 2001
Viral infection	Wang et al., 2001
Vitamin A treatment	Tesoriere et al., 1994
Vitamin E treatment or status	Garibaldi et al., 1994; Marangon et al., 1999; Sen et al., 1997; Shin and Yamada, 2002; Sumien et al., 2003; Shin, 2003
X or γ irradiation	Agarwal and Sohal, 1993; Sukharev et al., 1997; Shin and Yamada, 2002; Shin, 2003
Xanthine oxidase exposure	Karsek-Staples and Webster, 1993; Varma and Devamanoharan, 1995
Zidovudine treatment	Szabados et al., 1999
Zinc deficiency	Oteiza et al., 1995; Tate, Jr. et al., 1999; Gomez et al., 2003

synthesized proteins (feedback regulation and covalent modification). Work on the elucidation of these mechanisms left little time to investigate an obvious third mechanism: regulation of the rate of degradation of proteins, although it was well known that the basal rates varied among proteins and that they changed in response to changes in the cellular environment (Schimke and Doyle, 1970).

Bacterial glutamine synthetase occupies a central position in metabolism and thus is exquisitely regulated by a complex system that includes control of gene expression, reversible covalent modification, and feedback inhibition (Stadtman, 2001). It seemed reasonable that the degradation of glutamine synthetase would also be regulated. Preliminary investigation in *Escherichia coli* and *Klebsiella aerogenes* established that it was one of the proteins rapidly degraded when the cells were stressed by nitrogen starvation, and the process was likely under metabolic control because it was inhibited by uncouplers of oxidative phosphorylation (dinitrophenol) and accelerated by inhibitors of protein synthesis

(chloramphenicol) (Fulks, 1977). Dissection of the time course of the degradative process revealed that glutamine synthetase lost catalytic activity before it was degraded, suggesting a two-step process (Levine et al., 1981). Cell-free extracts of the nitrogen-starved cells rapidly inactivated both endogenous glutamine synthetase and exogenously added, purified glutamine synthetase. Thus it appeared that glutamine synthetase was undergoing a modification that "marked" it for subsequent proteolytic degradation. The marking reaction had three requirements: (1) oxygen, (2) iron, and (3) reducing equivalents, supplied by NADH or NADPH. These are the three necessary and sufficient elements of classical mixed-function oxidation reactions, typified by cytochrome P-450. This recognition led to the demonstration that a purified cytochrome P-450 system as well as an ascorbate model system catalyzed the oxidative inactivation of glutamine synthetase (Levine et al., 1981) and that this was sufficient to mark the protein for degradation by a purified bacterial protease or the mammalian proteosome (Roseman and Levine, 1987; Rivett and Levine, 1990). This in turn led to a large number of studies from many laboratories on metal-catalyzed oxidation of proteins and its relationship to protein turnover (Stadtman, 1990).

What was the modification? Was it a conformational change, perhaps induced by the binding of small molecules that were generated by a mixed-function oxidation stimulated by nutrient starvation? Or was it a covalent modification, perhaps mediated by enzymes that were specifically activated by the stress of starvation? Investigators in the seemingly unrelated field of the biology of aging were wrestling with the same questions. By the late 1970s they had established that many enzymes purified from older animals had a lower specific activity than did the same enzyme purified from younger animals (Rothstein, 1982), and they applied a variety of biochemical and biophysical techniques in the search for conformational or covalent modifications. Of course, even when a conformational change is detected, one does not know whether this is a consequence of an induced change in shape or a consequence of a covalent modification.

Unfortunately, even today the various available techniques may fail to detect conformational differences between proteins with dramatically different functional characteristics. For example, purified glutamine synthetase subjected to metal-catalyzed oxidation completely loses its catalytic activity. Yet neither circular dichroism nor sedimentation velocity measurements detected a difference between the native and the oxidized proteins (Rivett and Levine, 1990). Similarly the failure to detect a covalent modification with available techniques does not rule out the existence of a modification, although the availability of modern mass spectrometers has certainly increased the detection of modifications.

Returning to the situation 25 years ago, no covalent modifications had been detected in the modified enzymes purified from older animals, nor in the glutamine synthetase inactivated by exposure to model systems that mimicked the extracts from the Gram-negative bacteria. By default this lack of evidence for a covalent modification led to the favoring of an induced conformational change as the mechanistic explanation for loss of catalytic activity. However, continuing studies revealed a subtle but definite difference in the ultraviolet spectra of the

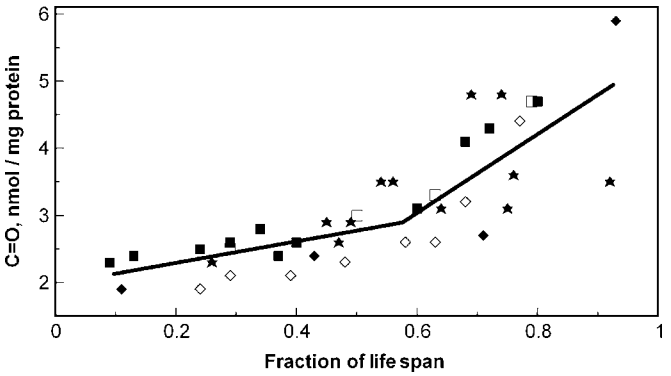


FIGURE 5.1 Carbonyl content of protein from different tissues determined in various laboratories. Observes the dramatic increase in oxidized protein during the last third of the life span. The data points were taken from published reports: ■, human dermal fibroblasts in tissue culture (Oliver et al., 1987); ★, human lens (Garland, 1990); □, *C. elegans* (Adachi et al., 1998); ♦, rat liver (Starke-Reed and Oliver, 1989); and ◇, house fly (Sohal et al., 1993).

native and inactivated glutamine synthetases (Fig. 5 in Levine et al., 1981). The difference spectra persisted when the proteins were fully denatured, providing strong evidence for the existence of a covalent modification.

Subsequent investigations led to the recognition that the initial step in the degradation of glutamine synthetase was an oxidative modification that introduced carbonyl groups into the protein, which reacted with classical carbonyl reagents such as 2,4-dinitrophenylhydrazine (Levine, 1983a). Since the parallel between the modified glutamine synthetase and modified enzymes from older animals had already been recognized, it was logical to determine the carbonyl content of tissues and cells during aging. The initial investigations have been replicated in many laboratories, and they have led to the generalization that the carbonyl content of tissues from a variety of species increases dramatically in the last third of life span (Fig. 5.1).

5.2 TYPES OF OXIDATIVE MODIFICATIONS AND CHOICE OF MARKER

The chemistry of the reactions that give rise to carbonyl groups is discussed in detail in chapter 1. To recapitulate, reactive species can react directly with the protein or they can react with molecules such as sugars and lipids, generating products that then react with the protein. Within the protein, either the peptide bond or the side chain may be targeted. Many of the reactions are mediated by free radicals, usually in a site-specific fashion (Stadtman and Levine, 2000). The reactions are frequently influenced by redox cycling metal cations, especially iron or copper. The protein may be cleaved to yield lower molecular weight products,

or it may be cross-linked to give higher molecular weight products. Classification of the oxidative modifications of proteins is usually based on these characteristics, but there is no generally accepted scheme for classification. At present we find it helpful to separate the reactions into those that oxidize and cleave the peptide bond and those that modify side chains (Stadtman and Berlett, 1999). The latter includes modification by the oxidation products of reducing sugars and lipid and generates products including pentosidine and the Michael addition products of alkenals such as 4-hydroxy-2-nonenal (Stadtman and Berlett, 1999).

The oxidative modifications can also be grouped into those that are quite specific, both in the residue oxidized and the product generated, and those that can alter multiple residues and may give rise to several products. Examples of a specific oxidation are the conversion of phenylalanine residues to *o*-tyrosine and of tyrosine to dityrosine (Leeuwenburgh et al., 1997). As mentioned, carbonyl group introduction into side chains is an example of a global modification. These can arise from direct oxidation of most residues or from secondary reaction with the primary oxidation products such as 4-hydroxy-2-nonenal. The choice of a specific or global assay may depend on the purpose of the study being undertaken, and in many cases either may be a useful marker for oxidative stress or damage. The specific modifications reported to date affect a tiny fraction of the "at-risk" residues or proteins, while the global modifications often affect a substantial fraction of the proteins in the sample. For example, dityrosine is clearly increased in atherosclerotic regions of human aorta, with the highest level reported in early fatty streaks (Leeuwenburgh et al., 1997). However, the actual content of dityrosine was 1 residue for each 3300 tyrosine residues. In contrast, the carbonyl content increases drastically in the last third of life span (Fig. 5.1), reaching a level such that on average one out of every three protein molecules carries the modification (Stadtman and Levine, 2000). Since oxidative modifications that give rise to carbonyl groups generally cause loss of catalytic or structural function in the affected proteins, it is likely that the level of oxidatively modified proteins observed during aging will have serious deleterious effects on cellular and organ function. This has been demonstrated experimentally for several enzymes. For example, γ-cystathionase of the lens undergoes oxidative modification with carbonylation leading to complete loss of enzymatic activity in over half of the lenses from 25-month-old rats (Sastre et al., 2005).

5.3 METHODOLOGICAL CONSIDERATIONS

Methods for determination of carbonyl content have been discussed in a number of reviews of the methodology (Dalle-Donne et al., 2005; Levine et al., 2000). The most common method is based on the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine to form a 2,4-dinitrophenylhydrazone, which can then be detected and quantitated spectrophotometrically or immunochemically. Assays have been developed that employ precipitation, solvent extraction, gel filtration, or electrophoresis for removal of excess reagent. Detection by ultraviolet

spectrophotometry can be done in standard spectrophotometers, in-line HPLC spectrophotometers, or 96-well plate readers. Since excellent antibodies directed against the dinitrophenyl group are commercially available, Western blot, dot blot (Robinson et al., 1999; Davies et al., 2001), immunocytochemical (Smith et al., 1998), and ELISA techniques are accessible (Buss et al., 1997). The Western blot technique developed by Shacter and colleagues (Shacter et al., 1994) and independently by Keller and colleagues (Keller et al., 1993) has emerged as the most popular incarnation of the assay, after either one- or two-dimensional blotting (Talent et al., 1998; Levine et al., 2000; Conrad et al., 2001; Reinheckel et al., 2000). This popularity is probably because antibodies are readily available commercially as well as a Western blotting kit (OxyBlot Protein Oxidation Kit, Chemicon, Temecula, CA).

Results from the various assays have been widely published in a variety of journals, from basic to clinical. It is awkward to specifically retrieve these citations, since protein carbonylation is not yet an official search term for Medline. However, a recent search using variations on the term "protein carbonyl" returned over 5000 citations, and at least 1000 report protein carbonyl determinations under a variety of conditions. A kit for ELISA analysis is also marketed, although there is not yet a sufficient literature to assess its usefulness and validity (Zentech, Dunedin, New Zealand).

As with any assay performed on a variety of extracts from biological sources, certain pitfalls have been recognized and techniques for avoiding them have been pointed out (Levine et al., 2000; Reznick and Packer, 1994). These will not be detailed here, but the importance of considering them deserves emphasis. Failure to recognize artifacts can naturally lead to erroneous conclusions, a point emphasized by Sohal and colleagues (Dubey et al., 1995).

While the 2,4-dinitrophenylhydrazine reagent has been most widely used to date, other reagents may be preferable because the resulting 2,4-dinitrophenylhydrazone is not stable to alkaline conditions (Jones et al., 1956) nor, in our experience, is it stable to storage. The hydrazone cannot simply be reduced to improve its stability because the relatively strong reducing agents that are required for reduction always cleave the hydrazone to yield an amino acid (Meister, 1965). Stability during multidimensional separations has not been systematically evaluated, although it has been employed very successfully in proteomic studies of carbonylation using 2D electrophoresis (Nakamura and Goto, 1996; Robinson et al., 1999; Conrad et al., 2001; Barreiro et al., 2005). Also, and not surprisingly, the nitro groups render the derivative rather susceptible to decomposition in the mass spectrometer, hampering identification of the specific residue that has been modified in the protein.

Other carbonyl-reactive compounds have been used with good results in proteomic studies, especially biotin hydrazide (Harris et al., 1994; Hensley et al., 2002; Oh-Ishi et al., 2003; Soreghan et al., 2003; Yoo and Regnier, 2004). Reduction of the Schiff base that forms with the carbonyl group yields a very stable amino group. The biotin label also facilitates affinity purification of labeled proteins (Mirzaei and Regnier, 2005).

As with any proteomic analysis, studies of carbonylated proteins are biased toward those proteins present in greater amount. The determination of the average extent of carbonylation is much more informative than the total carbonyl content, since the latter depends on the amount of protein present. As a result, abundant proteins with a low "specific carbonyl content" will appear prominent on immunoblots, while proteins present at low levels but with a high specific carbonyl content may be missed. For example, albumin is the most abundant protein in plasma and is always prominent in carbonyl Western blots, but its specific carbonyl content is usually low, ≤ 0.05 mol carbonyl/mol protein. On the other hand, carbonic anhydrase III is present at relatively high amounts in liver and also has a high specific carbonyl content, around 0.4 mol carbonyl/mol protein (Cabiscol and Levine, 1995). Determination of the carbonyl content of less abundant proteins requires that they be purified, most conveniently by immunoprecipitation. Machado and colleagues utilized this approach to demonstrate that oxidative inactivation of tyrosine hydroxylase occurs in the brain during aging (de la Cruz et al., 1996). Viña and colleagues used a combination of techniques to demonstrate oxidative inactivation of γ-cystathionase in the lens during aging (Sastre et al., 2005).

Carbonylated proteins are typically dysfunctional or nonfunctional, so the issue has physiological and pathological significance (Fucci et al., 1983; Shacter et al., 1994; de la Cruz et al., 1996; Dalle-Donne et al., 2001). Thus identification of carbonylated proteins should be followed by functional assessment of the protein, be it an enzyme or structural protein. These functional studies may identify metabolic or structural defects caused by oxidative modification (Tamarit et al., 1998; Cabiscol et al., 2000; Barreiro et al., 2005).

5.4 SELECTED STUDIES

The tremendous increase in papers reporting studies of protein carbonylation makes it impossible to compile a truly comprehensive bibliography. Thus Table 5.1 includes only selected references, and we apologize to the authors whose publications are not listed. The topics listed in Table 5.1 demonstrate that protein carbonylation assays have been applied widely. Areas with particularly large numbers of published papers include inflammation, neurodegenerative diseases, toxicology, ischemia-reperfusion, cataract formation, diabetes, caloric restriction, and aging.

5.5 CARBONYLATION DURING AGING

In this section we discuss carbonylation during aging as an example of a biologically important process in which oxidative modification of proteins is established to be correlated with functional changes. Figure 5.1 shows the typical exponential increase in tissue carbonyls in the last third of life span reported in most,

although not all, studies of carbonyl during aging. The mechanism causing this accumulation is of obvious interest but has not been established. One or more of several possibilities could cause an increase in the steady state level of oxidatively modified proteins. These include (1) an increase in the rate of oxidizing species, (2) a decrease in scavenging of those species, (3) an increased susceptibility of the protein to oxidation, and (4) a decrease in the rate of removal of oxidized species. Evidence for all of these has been published from many laboratories and compilations of reviews are available (Davies, 2000; Dukan et al., 2000; Schöneich, 2001). In budding yeast the carbonyl content of the mother cell increases with the number of replications. A beautiful series of studies by Aguilaniu and colleagues established that the carbonylated proteins were selectively retained by the mother cell, generating daughters with low carbonyl content (Aguilaniu et al., 2003). If a mechanism for selective retention of carbonylated proteins also operates in higher organisms, it would be of obvious importance in understanding the age-related increase in cellular carbonyl content.

Studies by Sohal and his colleagues established that mitochondria from older flies and rodents have an increased rate of generation of reactive oxygen species compared to younger animals (Sohal and Weindruch, 1996), providing support for the first mechanism. Nyström and co-workers provide a counterpoint that supports an increased susceptibility of proteins to oxidation (Dukan et al., 2000; Ballesteros et al., 2001). Their studies in *E. coli* establish that transcriptional or translational errors produce proteins that are more susceptible to oxidative modification. Oxidative modification of proteins involved in gene regulation, transcription, and translation could well increase the error rate in proteins, amplifying the entire process. While nucleic acids and proteins are often thought of as chemically unrelated macromolecules, they are clearly intertwined in cellular regulation. Thus alterations in DNA or RNA may affect the level of oxidized proteins, and vice versa. Disorders of DNA repair such as ataxia-telangiectasia and Werner's syndrome are associated with oxidative stress and oxidative modification of proteins (Barlow et al., 1999; Oliver et al., 1987). Indeed Werner's syndrome, clinically notable for premature aging, was recognized as carrying an increased burden of oxidatively damaged macromolecules well before the defective protein was identified and shown to be a DNA helicase and exonuclease (Oliver et al., 1987; Gray et al., 1997; Shen et al., 1998). *E. coli* held in stationary phase experience oxidative stress, which carbonylates proteins, increases DNA damage, and decreases viability (Dukan and Nystrom, 1999). Dukan and Nyström provide evidence supporting their conclusion that poor survival is a consequence of dysfunction caused by protein oxidation and is not due to DNA damage.

There is as yet no experimentally obtained evidence to establish the mechanism of the marked rate of increase in carbonylated proteins in the last third of lifespan, a pattern that would be expected when the rate of production of modified proteins exceeds the capacity of the degradative system in removing them. And it would occur regardless of whether the imbalance is caused by increased production or by inhibition or inactivation of the degrading machinery. This is a well-established

phenomenon in enzymology and pharmacology and can be observed even with a single enzyme that follows Michaelis-Menten kinetics. In this case increasing the input of a substrate will lead to an increased rate of product formation, thus keeping the substrate concentration relatively low. However, when the enzyme reaches its maximum velocity, any additional input of substrate will cause the substrate concentration to increase by the amount of additional input. The final result is a pattern similar to that in Figure 5.1.

As illustrated by many of the studies listed in Table 5.1, a large number of investigations on protein carbonyl are based on a hypothesis that emerged from the initial work summarized in the background section: carbonyl-bearing proteins are oxidatively damaged, either as a by-product of normal metabolism or as a consequence of disease processes. However, the initial work with bacteria also generated the hypothesis that normal, regulated cellular processes utilize the oxidative modification of specific proteins as a mechanism for triggering their degradation. For example, iron regulatory protein 2 (IRP2) is selectively but very rapidly degraded in iron-sufficient cells. It is stable and therefore functional only in iron-depleted cells (Guo et al., 1995; Iwai et al., 1995). A series of experiments in collaboration with Tracey Rouault and her colleagues established that the following occur, again both in vitro and in vivo (Iwai et al., 1998):

1. IRP2 binds iron and undergoes metal-catalyzed oxidative modification in the presence of oxygen, with introduction of carbonyl groups.
2. The oxidatively modified IRP2 is ubiquitinylated.
3. The modified IRP2 is then degraded by the proteasome.

This oxidative process yields an elegantly simple mechanism for coordinated regulation of cellular iron metabolism. When iron is deficient, IRP2 is stable and active. When iron becomes sufficient, it—or perhaps heme—binds to the protein and catalyzes an oxidative modification that suffices to trigger degradation of the entire 107 kD protein by the proteasome. Thus, oxidative modification of proteins need not arise only as an undesirable by-product of an oxygen-based metabolism; it can also function as a mechanism for cellular regulation.

REFERENCES

Abraham P., Wilfred G., Ramakrishna B. (2002). Oxidative damage to the hepatocellular proteins after chronic ethanol intake in the rat. *Clin Chim Acta* 325: 117–125.

Abu-Zidan F.M., Plank L.D., Windsor J.A. (2002). Proteolysis in severe sepsis is related to oxidation of plasma protein. *Eur J Surg* 168: 119–123.

Adachi H., Fujiwara Y., Ishii N. (1998). Effects of oxygen on protein carbonyl and aging in *Caenorhabditis elegans* mutants with long (*age-1*) and short (*mev-1*) life spans. *J Gerontol Biol Sciences* 53A: B240–B244.

Agarwal S., Sohal R.S. (1994). DNA oxidative damage and life expectancy in houseflies. *Proc Natl Acad Sci USA* 91: 12332–12335.

Agarwal S., Sohal R.S. (1993). Relationship between aging and susceptibility to protein oxidative damage. *Biochem Biophys Res Commun* 194: 1203–1206.

Agarwal S., Sohal R.S. (1996). Relationship between susceptibility to protein oxidation, aging, and maximum life span potential of different species. *Exp Gerontol* 31: 365–372.

Aguilaniu H., Gustafsson L., Rigoulet M., Nystrom T. (2001). Protein oxidation in G0 cells of *Saccharomyces cerevisiae* depends on the state rather than rate of respiration and is enhanced in pos9 but not yap1 mutants. *J Biol Chem* 276: 35396–35404.

Aguilaniu H., Gustafsson L., Rigoulet M., Nystrom T. (2003). Asymmetric inheritance of oxidatively damaged proteins during cytokinesis. *Science* 299: 1751–1753.

Akin M.L., Gulluoglu B.M., Uluutku H., Erenoglu C., Elbuken E., Yildirim S., Celenk T. (2002). Hyperbaric oxygen improves healing in experimental rat colitis. *Undersea Hyperb Med* 29: 279–285.

Aksenov M.Y., Aksenova M.V., Butterfield D.A., Geddes J.W., Markesbery W.R. (2001). Protein oxidation in the brain in Alzheimer's disease. *Neuroscience* 103: 373–383.

Aksenov M.Y., Hasselrot U., Wu G., Nath A., Anderson C., Mactutus C.F., Booze R.M. (2003). Temporal relationships between HIV-1 Tat-induced neuronal degeneration, OX-42 immunoreactivity, reactive astrocytosis, and protein oxidation in the rat striatum. *Brain Res* 987: 1–9.

Alam Z.I., Daniel S.E., Lees A.J., Marsden D.C., Jenner P., Halliwell B. (1997). A generalised increase in protein carbonyls in the brain in Parkinson's but not incidental Lewy body disease. *J Neurochem* 69: 1326–1329.

Alam Z.I., Halliwell B., Jenner P. (2000). No evidence for increased oxidative damage to lipids, proteins, or DNA in Huntington's disease. *J Neurochem* 75: 840–846.

Andersson S., Kheiter A., Merritt T.A. (1999). Oxidative inactivation of surfactants. *Lung* 177: 179–189.

Ando Y., Nyhlin N., Suhr O., Holmgren G., Uchida K., el S.M., Yamashita T., Terasaki H., Nakamura M., Uchino M., Ando M. (1997). Oxidative stress is found in amyloid deposits in systemic amyloidosis. *Biochem Biophys Res Commun* 232: 497–502.

Andrus P.K., Fleck T.J., Gurney M.E., Hall E.D. (1998). Protein oxidative damage in a transgenic mouse model of familial amyotrophic lateral sclerosis. *J Neurochem* 71: 2041–2048.

Anraku M., Kitamura K., Shinohara A., Adachi M., Suenga A., Maruyama T., Miyanaka K., Miyoshi T., Shiraishi N., Nonoguchi H., Otagiri M., Tomita K. (2004). Intravenous iron administration induces oxidation of serum albumin in hemodialysis patients. *Kidney Int* 66: 841–848.

Argirova M.D., Ortwerth B.J. (2003). Activation of protein-bound copper ions during early glycation: study on two proteins. *Arch Biochem Biophys* 420: 176–184.

Astier C., Rock E., Lab C., Gueux E., Mazur A., Rayssiguier Y. (1996). Functional alterations in sarcoplasmic reticulum membranes of magnesium-deficient rat skeletal muscle as consequences of free radical-mediated process. *Free Radic Biol Med* 20: 667–674.

Ayene I.S., Dodia C., Fisher A.B. (1992). Role of oxygen in oxidation of lipid and protein during ischemia/reperfusion in isolated perfused rat lung. *Arch Biochem Biophys* 296: 183–189.

Ballesteros M., Fredriksson A., Henriksson J., Nystrom T. (2001). Bacterial senescence: protein oxidation in non-proliferating cells is dictated by the accuracy of the ribosomes. *EMBO J* 20: 5280–5289.

Banaclocha M.M., Hernandez A.I., Martinez N., Ferrandiz M.L. (1997). *N*-acetylcysteine protects against age-related increase in oxidized proteins in mouse synaptic mitochondria. *Brain Res* 762: 256–258.

Barja G., Lopez-Torres M., Perez-Campo R., Rojas C., Cadenas S., Prat J., Pamplona R. (1994). Dietary vitamin C decreases endogenous protein oxidative damage, malondialdehyde, and lipid peroxidation and maintains fatty acid unsaturation in the guinea pig liver. *Free Radic Biol Med* 17: 105–115.

Barlow C., Dennery P.A., Shigenaga M.K., Smith M.A., Morrow J.D., Roberts L.J., Wynshaw-Boris A., Levine R.L. (1999). Loss of the ataxia-telangiectasia gene product causes oxidative damage in target organs. *Proc Natl Acad Sci USA* 96: 9915–9919.

Barreiro E., Gea J., Di F.M., Kriazhev L., James S., Hussain S.N. (2005). Protein carbonyl formation in the diaphragm. *Am J Respir Cell Mol Biol* 32: 9–17.

Bayne A.C., Sohal R.S. (2002). Effects of superoxide dismutase/catalase mimetics on life span and oxidative stress resistance in the housefly, Musca domestica. *Free Radic Biol Med* 32: 1229–1234.

Baynes J.W., Thorpe S.R. (1999). Role of oxidative stress in diabetic complications: a new perspective on an old paradigm. *Diabetes* 48: 1–9.

Bejma J., Ramires P., Ji L.L. (2000). Free radical generation and oxidative stress with ageing and exercise: differential effects in the myocardium and liver. *Acta Physiol Scand* 169: 343–351.

Belisario M.A., Di D.C., Pelagalli A., Della M.R., Staiano N. (1997). Metal-ion catalyzed oxidation affects fibrinogen activity on platelet aggregation and adhesion. *Biochimie* 79: 449–455.

Belli D.C., Albrecht R., La Scala G.C., Desjeux J.F., Pelissier M.A. (2003). Homocysteine prevents total parenteral nutrition (TPN)-induced cholestasis without changes in hepatic oxidative stress in the rat. *J Pediatr Gastroenterol Nutr* 36: 200–205.

Benedetti A., Pompella A., Fulceri R., Romani A., Comporti M. (1986). 4-Hydroxynonenal and other aldehydes produced in the liver in vivo after bromobenzene intoxication. *Toxicol Pathol* 14: 457–461.

Berlett B.S., Levine R.L., Stadtman E.R. (1996). Comparison of the effects of ozone on the modification of amino acid residues in glutamine synthetase and bovine serum albumin. *J Biol Chem* 271: 4177–4182.

Bizzozero O.A., DeJesus G., Howard T.A. (2004). Exposure of rat optic nerves to nitric oxide causes protein *S*-nitrosation and myelin decompaction. *Neurochem Res* 29: 1675–1685.

Blackburn A.C., Doe W.F., Buffinton G.D. (1999). Protein carbonyl formation on mucosal proteins in vitro and in dextran sulfate-induced colitis. *Free Radic Biol Med* 27: 262–270.

Blakeman D.P., Ryan T.P., Jolly R.A., Petry T.W. (1998). Protein oxidation: examination of potential lipid-independent mechanisms for protein carbonyl formation. *J Biochem Mol Toxicol* 12: 185–190.

Blakeman D.P., Ryan T.P., Jolly R.A., Petry T.W. (1995). Diquat-dependent protein carbonyl formation. Identification of lipid-dependent and lipid-independent pathways. *Biochem Pharmacol* 50: 929–935.

Blomberg A., Sainsbury C., Rudell B., Frew A.J., Holgate S.T., Sandstrom T., Kelly F.J. (1998). Nasal cavity lining fluid ascorbic acid concentration increases in healthy human volunteers following short term exposure to diesel exhaust. *Free Radic Res* 28: 59–67.

Bonnes-Taourel D., Guerin M.C., Torreilles J., Ceballos-Picot I., de Paulet A.C. (1993). 4-Hydroxynonenal content lower in brains of 25 month old transgenic mice carrying the human CuZn superoxide dismutase gene than in brains of their non-transgenic littermates. *J Lipid Mediat* 8: 111–120.

Boscia F., Grattagliano I., Vendemiale G., Micelli-Ferrari T., Altomare E. (2000). Protein oxidation and lens opacity in humans. *Invest Ophthalmol Vis Sci* 41: 2461–2465.

Bowling A.C., Schulz J.B., Brown R.H., Jr., Beal M.F. (1993). Superoxide dismutase activity, oxidative damage, and mitochondrial energy metabolism in familial and sporadic amyotrophic lateral sclerosis. *J Neurochem* 61: 2322–2325.

Brown R.K., Kelly F.J. (1994). Evidence for increased oxidative damage in patients with cystic fibrosis. *Pediatr Res* 36: 487–493.

Browne S.E., Roberts L.J., Dennery P.A., Doctrow S.R., Beal M.F., Barlow C., Levine R.L. (2004). Treatment with a catalytic antioxidant corrects the neurobehavioral defect in ataxiatelangiectasia mice. *Free Radic Biol Med* 36: 938–942.

Bruck R., Schey R., Aeed H., Hochman A., Genina O., Pines M. (2004). A protective effect of pyrrolidine dithiocarbamate in a rat model of liver cirrhosis. *Liver Int* 24: 169–176.

bu-Zidan F.M., Bonham M.J., Windsor J.A. (2000). Severity of acute pancreatitis: a multivariate analysis of oxidative stress markers and modified Glasgow criteria. *Br J Surg* 87: 1019–1023.

Burcham P.C., Fontaine F. (2001). Extensive protein carbonylation precedes acrolein-mediated cell death in mouse hepatocytes. *J Biochem Mol Toxicol* 15: 309–316.

Burcham P.C., Kerr P.G., Fontaine F. (2000). The antihypertensive hydralazine is an efficient scavenger of acrolein. *Redox Rep* 5: 47–49.

Burcham P.C., Kuhan Y.T. (1996). Introduction of carbonyl groups into proteins by the lipid peroxidation product, malondialdehyde. *Biochem Biophys Res Commun* 220: 996–1001.

Buss H., Chan T.P., Sluis K.B., Domigan N.M., Winterbourn C.C. (1997). Protein carbonyl measurement by a sensitive ELISA method. *Free Radic Biol Med* 23: 361–366.

Buss I.H., Darlow B.A., Winterbourn C.C. (2000). Elevated protein carbonyls and lipid peroxidation products correlating with myeloperoxidase in tracheal aspirates from premature infants. *Pediatr Res* 47: 640–645.

Butterfield D.A. (2004). Proteomics: A new approach to investigate oxidative stress in Alzheimer's disease brain. *Brain Res* 1000: 1–7.

Butterfield D.A., Howard B.J., Yatin S., Allen K.L., Carney J.M. (1997). Free radical oxidation of brain proteins in accelerated senescence and its modulation by *N*-tert-butylalpha-phenylnitrone. *Proc Natl Acad Sci USA* 94: 674–678.

Cabiscol E., Levine R.L. (1995). Carbonic anhydrase III. Oxidative modification in vivo and loss of phosphatase activity during aging. *J Biol Chem* 270: 14742–14747.

Cabiscol E., Piulats E., Echave P., Herrero E., Ros J. (2000). Oxidative stress promotes specific protein damage in *Saccharomyces cerevisiae*. *J Biol Chem* 275: 27393–27398.

Cadenas S., Rojas C., Barja G. (1998). Endotoxin increases oxidative injury to proteins in guinea pig liver: Protection by dietary vitamin C. *Pharmacol Toxicol* 82: 11–18.

Cadenas S., Rojas C., Perez-Campo R., Lopez-Torres M., Barja G. (1994). Effect of dietary vitamin C and catalase inhibition of antioxidants and molecular markers of oxidative damage in guinea pigs. *Free Radic Res* 21: 109–118.

Canton M., Neverova I., Menabo R., Van Eyk J., Di Lisa F. (2004). Evidence of myofibrillar protein oxidation induced by postischemic reperfusion in isolated rat hearts. *Am J Physiol Heart Circ Physiol* 286: H870–H877.

Cardozo-Pelaez F., Song S., Parthasarathy A., Epstein C.J., Sanchez-Ramos J. (1998). Attenuation of age-dependent oxidative damage to DNA and protein in brainstem of Tg Cu/Zn SOD mice. *Neurobiol Aging* 19: 311–316.

Carney J.M., Starke-Reed P.E., Oliver C.N., Landrum R.W., Cheng M.S., Wu J.F., Floyd R.A. (1991). Reversal of age-related increase in brain protein oxidation, decrease in enzyme activity, and loss in temporal and spatial memory by chronic administration of the spin-trapping compound *N*-tert-butyl-α-phenylnitrone. *Proc Natl Acad Sci USA* 88: 3633–3636.

Carty J.L., Bevan R., Waller H., Mistry N., Cooke M., Lunec J., Griffiths H.R. (2000). The effects of vitamin C supplementation on protein oxidation in healthy volunteers. *Biochem Biophys Res Commun* 273: 729–735.

Castegna A., Thongboonkerd V., Klein J., Lynn B.C., Wang Y.L., Osaka H., Wada K., Butterfield D.A. (2004). Proteomic analysis of brain proteins in the gracile axonal dystrophy (gad) mouse, a syndrome that emanates from dysfunctional ubiquitin carboxyl-terminal hydrolase L-1, reveals oxidation of key proteins. *J Neurochem* 88: 1540–1546.

Castegna A., Aksenov M., Aksenova M., Thongboonkerd V., Klein J.B., Pierce W.M., Booze R., Markesbery W.R., Butterfield D.A. (2002). Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part I: Creatine kinase BB, glutamine synthase, and ubiquitin carboxy-terminal hydrolase L-1. *Free Rad Biol Med* 33: 562–571.

Cederberg J., Siman C.M., Eriksson U.J. (2001). Combined treatment with vitamin E and vitamin C decreases oxidative stress and improves fetal outcome in experimental diabetic pregnancy. *Pediatr Res* 49: 755–762.

Cha M.K., Kim W.C., Lim C.J., Kim K., Kim I.H. (2004). *Escherichia coli* periplasmic thiol peroxidase acts as lipid hydroperoxide peroxidase and the principal antioxidative function during anaerobic growth. *J Biol Chem* 279: 8769–8778.

Chang C.Y., Lai Y.C., Cheng T.J., Lau M.T., Hu M.L. (1998). Plasma levels of antioxidant vitamins, selenium, total sulfhydryl groups and oxidative products in ischemic-stroke patients as compared to matched controls in Taiwan. *Free Radic Res* 28: 15–24.

Chapman M.L., Rubin B.R., Gracy R.W. (1989). Increased carbonyl content of proteins in synovial fluid from patients with rhematoid arthritis. *J Rheumatol* 16: 15–18.

Chavko M., Auker C.R., McCarron R.M. (2003). Relationship between protein nitration and oxidation and development of hyperoxic seizures. *Nitric Oxide* 9: 18–23.

Chen Q., Fischer A., Reagan J.D., Yan L.J., Ames B.N. (1995). Oxidative DNA damage and senescence of human diploid fibroblast cells. *Proc Natl Acad Sci USA* 92: 4337–4341.

Chen S.S., Chang L.S., Wei Y.H. (2001). Oxidative damage to proteins and decrease of antioxidant capacity in patients with varicocele. *Free Radic Biol Med* 30: 1328–1334.

Chen X., Ding Y.W., Yang G., Bondoc F., Lee M.J., Yang C.S. (2000). Oxidative damage in an esophageal adenocarcinoma model with rats. *Carcinogenesis* 21: 257–263.

Chevion S., Moran D.S., Heled Y., Shani Y., Regev G., Abbou B., Berenshtein E., Stadtman E.R., Epstein Y. (2003). Plasma antioxidant status and cell injury after severe physical exercise. *Proc Natl Acad Sci USA* 100(9): 5119–5123.

Cho M.K., Kim Y.G., Lee M.G., Kim S.G. (2000). The effect of cysteine on the altered expression of class alpha and mu glutathione *S*-transferase genes in the rat liver during protein-caloric malnutrition. *Biochim Biophys Acta* 1502: 235–246.

Choi J., Conrad C.C., Malakowsky C.A., Talent J.M., Yuan C.S., Gracy R.W. (2002). Flavones from *Scutellaria baicalensis Georgi* attenuate apoptosis and protein oxidation in neuronal cell lines. *Biochim Biophys Acta* 1571: 201–210.

Choi J., Forster M.J., McDonald S.R., Weintraub S.T., Carroll C.A., Gracy R.W. (2004). Proteomic identification of specific oxidized proteins in ApoE-knockout mice: relevance to Alzheimer's disease. *Free Radic Biol Med* 36: 1155–1162.

Ciolino H.P., Levine R.L. (1997). Modification of proteins in endothelial cell death during oxidative stress. *Free Radic Biol Med* 22: 1277–1282.

Cockell K.A., Belonje B. (2002). The carbonyl content of specific plasma proteins is decreased by dietary copper deficiency in rats. *J Nutr* 132: 2514–2518.

Conrad C.C., Choi J., Malakowsky C.A., Talent J.M., Dai R., Marshall P., Gracy R.W. (2001). Identification of protein carbonyls after two-dimensional electrophoresis. *Proteomics* 1: 829–834.

Costa V.M., Amorim M.A., Quintanilha A., Moradas-Ferreira P. (2002). Hydrogen peroxide-induced carbonylation of key metabolic enzymes in *Saccharomyces cerevisiae*: The involvement of the oxidative stress response regulators Yap1 and Skn7. *Free Radic Biol Med* 33: 1507–1515.

Cotovio J., Onno L., Justine P., Lamure S., Catroux P. (2001). Generation of oxidative stress in human cutaneous models following in vitro ozone exposure. *Toxicol in Vitro* 15: 357–362.

Coughlan M.T., Vervaart P.P., Permezel M., Georgiou H.M., Rice G.E. (2004). Altered placental oxidative stress status in gestational diabetes mellitus. *Placenta* 25: 78–84.

Cross C.E., Reznick A.Z., Packer L., Davis P.A., Suzuki Y.J., Halliwell B. (1992). Oxidative damage to human plasma proteins by ozone. *Free Radic Res Commun* 15: 347–352.

Dal-Pizzol F., Klamt F., Frota M.L. Jr., Andrades M.E., Caregnato F.F., Vianna M.M., Schroder N., Quevedo J., Izquierdo I., Archer T., Moreira J.C. (2001). Neonatal iron exposure induces oxidative stress in adult Wistar rat. *Brain Res Dev Brain Res* 130: 109–114.

Dalle-Donne I., Rossi R., Giustarini D., Gagliano N., Di Simplicio P., Colombo R., Milzani A. (2002). Methionine oxidation as a major cause of the functional impairment of oxidized actin. *Free Radic Biol Med* 32: 927–937.

Dalle-Donne I., Rossi R., Giustarini D., Gagliano N., Lusini L., Milzani A., Di Simplicio P., Colombo R. (2001). Actin carbonylation: from a simple marker of protein oxidation to relevant signs of severe functional impairment. *Free Radic Biol Med* 31: 1075–1083.

Dalle-Donne I., Scaloni A., Giustarini D., Cavarra E., Tell G., Lungarella G., Colombo R., Rossi R., Milzani A. (2005). Proteins as biomarkers of oxidative/nitrosative stress in diseases: The contribution of redox proteomics. *Mass Spectrom Rev* 24: 55–99.

Damiani E., Carloni P., Biondi C., Greci L. (2000). Increased oxidative modification of albumin when illuminated in vitro in the presence of a common sunscreen ingredient: protection by nitroxide radicals. *Free Radic Biol Med* 28: 193–201.

Damiani E., Castagna R., Greci L. (2002). The effects of derivatives of the nitroxide tempol on UVA-mediated in vitro lipid and protein oxidation. *Free Radic Biol Med* 33: 128–136.

Das K., Chainy G.B. (2004). Thyroid hormone influences antioxidant defense system in adult rat brain. *Neurochem Res* 29: 1755–1766.

Davies K.J. (2000). An overview of oxidative stress. *IUBMB Life* 50: 241–244.

Davies S., Elliott M.H., Floor E., Truscott T.G., Zareba M., Sarna T., Shamsi F.A., Boulton M.E. (2001). Photocytotoxicity of lipofuscin in human retinal pigment epithelial cells. *Free Radic Biol Med* 31: 256–265.

de la Cruz C.P., Revilla E., Venero J.L., Ayala A., Cano J., Machado A. (1996). Oxidative inactivation of tyrosine hydroxylase in substantia nigra of aged rat. *Free Rad Biol Med* 20: 53–61.

De Maria N., Colantoni A., Fagiuoli S., Liu G.J., Rogers B.K., Farinati F., Van Thiel D.H., Floyd R.A. (1996). Association between reactive oxygen species and disease activity in chronic hepatitis C. *Free Radic Biol Med* 21: 291–295.

De C.R., Rocca B., Vitacolonna E., Falco A., Marchesani P., Ciabattoni G., Landolfi R., Patrono C., Davi G. (2003). Lipid and protein oxidation contribute to a prothrombotic state in patients with type 2 diabetes mellitus. *J Thromb Haemost* 1: 250–256.

DeAtley S.M., Aksenov M.Y., Aksenova M.V., Carney J.M., Butterfield D.A. (1998). Adriamycin induces protein oxidation in erythrocyte membranes. *Pharmacol Toxicol* 83: 62–68.

Debacq-Chainiaux F., Borlon C., Pascal T., Royer V., Eliaers F., Ninane N., Carrard G., Friguet B., de Longueville F., Boffe S., Remacle J., Toussaint O. (2005). Repeated exposure of human skin fibroblasts to UVB at subcytotoxic level triggers premature senescence through the TGF-ß1 signaling pathway. *J Cell Sci* 118: 743–758.

Demasi M., Penatti C.A., DeLucia R., Bechara E.J. (1996). The prooxidant effect of 5-aminolevulinic acid in the brain tissue of rats: Implications in neuropsychiatric manifestations in porphyrias. *Free Radic Biol Med* 20: 291–299.

Dennery P.A., McDonagh A.F., Spitz D.R., Rodgers P.A., Stevenson D.K. (1995). Hyperbilirubinemia results in reduced oxidative injury in neonatal Gunn rats exposed to hyperoxia. *Free Radic Biol Med* 19: 395–404.

Devi B.G., Chan A.W. (1999). Effect of cocaine on cardiac biochemical functions. *J Cardiovasc Pharmacol* 33: 1–6.

Dimock A.N., Siciliano P.D., McIlwraith C.W. (2000). Evidence supporting an increased presence of reactive oxygen species in the diseased equine joint. *Equine Vet J* 32: 439–443.

Dimon-Gadal S., Gerbaud P., Therond P., Guibourdenche J., Anderson W.B., Evain-Brion D., Raynaud F. (2000). Increased oxidative damage to fibroblasts in skin with and without lesions in psoriasis. *J Invest Dermatol* 114: 984–989.

Dominguez C., Gartner S., Linan S., Cobos N., Moreno A. (1998). Enhanced oxidative damage in cystic fibrosis patients. *BioFactors* 8: 149–153.

Dragsted L.O., Pedersen A., Hermetter A., Basu S., Hansen M., Haren G.R., Kall M., Breinholt V., Castenmiller J.J., Stagsted J., Jakobsen J., Skibsted L., Rasmussen S.E., Loft S., Sandstrom B. (2004). The 6-a-day study: Effects of fruit and vegetables on markers of oxidative stress and antioxidative defense in healthy nonsmokers. *Am J Clin Nutr* 79: 1060–1072.

Drake J., Sultana R., Aksenova M., Calabrese V., Butterfield D.A. (2003). Elevation of mitochondrial glutathione by gamma-glutamylcysteine ethyl ester protects mitochondria against peroxynitrite-induced oxidative stress. *J Neurosci Res* 74: 917–927.

Drake S.K., Bourdon E., Wehr N., Levine R., Backlund P., Yergey A., Rouault T.A. (2002). Numerous proteins in Mammalian cells are prone to iron-dependent oxidation and proteasomal degradation. *Dev Neurosci* 24: 114–124.

Dubey A., Forster M.J., Sohal R.S. (1995). Effect of the spin-trapping compound *N*-tert-butyl-alpha-phenylnitrone on protein oxidation and life span. *Arch Biochem Biophys* 324: 249–254.

Dukan S., Farewell A., Ballesteros M., Taddei F., Radman M., Nystrom T. (2000). Protein oxidation in response to increased transcriptional or translational errors. *Proc Natl Acad Sci USA* 97: 5746–5749.

Dukan S., Nystrom T. (1999). Oxidative stress defense and deterioration of growth-arrested *Escherichia coli* cells. *J Biol Chem* 274: 26027–26032.

DuTeaux S.B., Berger T., Hess R.A., Sartini B.L., Miller M.G. (2004). Male reproductive toxicity of trichloroethylene: sperm protein oxidation and decreased fertilizing ability. *Biol Reprod* 70: 1518–1526.

Eiserich J.P., van der Vliet A., Handelman G.J., Halliwell B., Cross C.E. (1995). Dietary antioxidants and cigarette smoke-induced biomolecular damage: A complex interaction. *Am J Clin Nutr* 62: 1490S–1500S.

Eisinger J., Zakarian H., Pouly E., Plantamura A., Ayavou T. (1996). Protein peroxidation, magnesium deficiency and fibromyalgia. *Magnes Res* 9: 313–316.

England K., Cotter T. (2004). Identification of carbonylated proteins by MALDI-TOF mass spectroscopy reveals susceptibility of ER. *Biochem Biophys Res Commun* 320: 123–130.

England K., O'Driscoll C., Cotter T.G. (2004). Carbonylation of glycolytic proteins is a key response to drug-induced oxidative stress and apoptosis. *Cell Death Differ* 11: 252–260.

Eppler B., Dawson R. Jr. (2001). Dietary taurine manipulations in aged male Fischer 344 rat tissue: Taurine concentration, taurine biosynthesis, and oxidative markers. *Biochem Pharmacol* 62: 29–39.

Erkasap N., Uzuner K., Serteser M., Koken T., Aydin Y. (2003). Gastroprotective effect of leptin on gastric mucosal injury induced by ischemia-reperfusion is related to gastric histamine content in rats. *Peptides* 24: 1181–1187.

Fagan J.M., Ganguly M., Stockman H., Ferland L.H., Toner M. (1999). Posttranslational modifications of cardiac and skeletal muscle proteins by reactive oxygen species after burn injury in the rat. *Ann Surg* 229: 106–114.

Fagan J.M., Ganguly M., Tiao G., Fischer J.E., Hasselgren P.O. (1996). Sepsis increases oxidatively damaged proteins in skeletal muscle. *Arch Surg* 131: 1326–1331.

Falciglia H.S., Johnson J.R., Sullivan J., Hall C.F., Miller J.D., Riechmann G.C., Falciglia G.A. (2003). Role of antioxidant nutrients and lipid peroxidation in premature

infants with respiratory distress syndrome and bronchopulmonary dysplasia. *Am J Perinatol* 20: 97–107.

Favetta L.A., Robert C., King W.A., Betts D.H. (2004). Expression profiles of p53 and p66shc during oxidative stress-induced senescence in fetal bovine fibroblasts. *Exp Cell Res* 299: 36–48.

Ferrante R.J., Browne S.E., Shinobu L.A., Bowling A.C., Baik M.J., MacGarvey U., Kowall N.W., Brown R.H., Beal M.F. (1997). Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. *J Neurochem* 69: 2064–2074.

Finotti P., Pagetta A. (1997). Heparin-induced structural modifications and oxidative cleavage of human serum albumin in the absence and presence of glucose—Implications for transcapillary leakage of albumin in hyperglycaemia. *Eur J Biochem* 247: 1000–1008.

Finotti P., Pagetta A., Ashton T. (2001). The oxidative mechanism of heparin interferes with radical production by glucose and reduces the degree of glycooxidative modifications on human serum albumin. *Eur J Biochem* 268: 2193–2200.

Floor E., Wetzel M.G. (1998). Increased protein oxidation in human substantia nigra pars compacta in comparison with basal ganglia and prefrontal cortex measured with an improved dinitrophenylhydrazine assay. *J Neurochem* 70: 268–275.

Folbergrova J., Kiyota Y., Pahlmark K., Memezawa H., Smith M.L., Siesjo B.K. (1993). Does ischemia with reperfusion lead to oxidative damage to proteins in the brain? *J Cereb Blood Flow Metab* 13: 145–152.

Fontaine F.R., Dunlop R.A., Petersen D.R., Burcham P.C. (2002). Oxidative bioactivation of crotyl alcohol to the toxic endogenous aldehyde crotonaldehyde: Association of protein carbonylation with toxicity in mouse hepatocytes. *Chem Res Toxicol* 15: 1051–1058.

Foreman R.C., Mercer P.F., Kroegel C., Warner J.A. (1999). Role of the eosinophil in protein oxidation in asthma: Possible effects on proteinase/antiproteinase balance. *Int Arch Allergy Immunol* 118: 183–186.

Forsmark-Andrée P., Dallner G., Ernster L. (1995). Endogenous ubiquinol prevents protein modification accompanying lipid peroxidation in beef heart submitochondrial particles. *Free Rad Biol Med* 19: 749–757.

Forster M.J., Sohal B.H., Sohal R.S. (2000). Reversible effects of long-term caloric restriction on protein oxidative damage. *J Gerontol A Biol Sci Med Sci* 55: B522–B529.

Fu Y., Ji L.L. (2003). Chronic ginseng consumption attenuates age-associated oxidative stress in rats. *J Nutr* 133: 3603–3609.

Fucci L., Oliver C.N., Coon M.J., Stadtman E.R. (1983). Inactivation of key metabolic enzymes by mixed-function oxidation reactions: Possible implication in protein turnover and ageing. *Proc Natl Acad Sci USA* 80: 1521–1525.

Fulks R.M. (1977). Regulation of glutamine synthetase degradation in *Klebsiella aerogenes*. *Fed Proc* 36: 919.

Funabiki R., Takeshita K., Miura Y., Shibasato M., Nagasawa T. (1999). Dietary supplement of G-rutin reduces oxidative damage in the rodent model. *J Agric Food Chem* 47: 1078–1082.

Gangemi S., Saija A., Tomaino A., Cimino F., Merendino R.A., Minciullo P.L., Briuglia S., Merlino M.V., Dallapiccola B., Ferlazzo B., Salpietro D.C. (2003). Protein

carbonyl group content in patients affected by familiar chronic nail candidiasis. *Mediators Inflamm* 12: 247–249.

Garibaldi S., Aragno I., Odetti P., Marinari U.M. (1994). Relationships between protein carbonyls, retinol and tocopherols level in human plasma. *Biochem Mol Biol Int* 34: 729–736.

Garland D. (1990). Role of site-specific, metal-catalyzed oxidation in lens-aging and cataract: a hypothesis. *Exp Eye Res* 50: 677–682.

Garland D., Russell P., Zigler J.S. (1988). Oxidative modification of lens proteins. In: Simic M.G., Taylor K.S., Ward J.F., von Sontag V. (eds.), *Oxygen Radicals in Biology and Medicine*, Plenum Press, New York, pp. 347–353.

Gilgenast O., Brandt-Nedelev B., Wiswedel I., Lippert H., Halangk W., Reinheckel T. (2001). Differential oxidative injury in extrapancreatic tissues during experimental pancreatitis: Modification of lung proteins by 4-hydroxynonenal. *Dig Dis Sci* 46: 932–937.

Gladstone I.M. Jr., Levine R.L. (1994). Oxidation of proteins in neonatal lungs. *Pediatrics* 93: 764–768.

Gluck M.R., Moy L.Y., Jayatilleke E., Hogan K.A., Manzino L., Sonsalla P.K. (2001). Parallel increases in lipid and protein oxidative markers in several mouse brain regions after methamphetamine treatment. *J Neurochem* 79: 152–160.

Gogasyavuz D., Kucukkaya B., Ersoz H.O., Yalcin A.S., Emerk K., Akalin S. (2002). Effects of aminoguanidine on lipid and protein oxidation in diabetic rat kidneys. *Int J Exp Diabetes Res* 3: 145–151.

Gomez N.N., Fernandez M.R., Zirulnik F., Gil E., Scardapane L., Ojeda M.S., Gimenez M.S. (2003). Chronic zinc deficiency induces an antioxidant adaptive response in rat lung. *Exp Lung Res* 29: 485–502.

Goto S., Nakamura A., Radak Z., Nakamoto H., Takahashi R., Yasuda K., Sakurai Y., Ishii N. (1999). Carbonylated proteins in aging and exercise: Immunoblot approaches. *Mech Ageing Dev* 107: 245–253.

Grattagliano I., Vendemiale G., Boscia F., Micelli-Ferrari T., Cardia L., Altomare E. (1998). Oxidative retinal products and ocular damages in diabetic patients. *Free Radic Biol Med* 25: 369–372.

Grattagliano I., Vendemiale G., Sabba C., Buonamico P., Altomare E. (1996). Oxidation of circulating proteins in alcoholics: Role of acetaldehyde and xanthine oxidase. *J Hepatol* 25: 28–36.

Gray M.D., Shen J.C., Kamath-Loeb A.S., Blank A., Sopher B.L., Martin G.M., Oshima J., Loeb L.A. (1997). The Werner syndrome protein is a DNA helicase. *Nat Genet* 17: 100–103.

Gropper S.S., Kerr S., Barksdale J.M. (2003). Non-anemic iron deficiency, oral iron supplementation, and oxidative damage in college-aged females. *J Nutr Biochem* 14: 409–415.

Grune T., Siems W.G., Schneider W. (1993). Accumulation of aldehydic lipid peroxidation products during postanoxic reoxygenation of isolated rat hepatocytes. *Free Radic Biol Med* 15: 125–132.

Gulluoglu B.M., Kurtel H., Gulluoglu M.G., Aktan A.O., Yegen B.C., Dizdaroglu F., Yalin R., Yegen B.C. (1999). Bombesin ameliorates colonic damage in experimental colitis. *Dig Dis Sci* 44: 1531–1538.

Guo B., Phillips J.D., Yu Y., Leibold E.A. (1995). Iron regulates the intracellular degradation of iron regulatory protein 2 by the proteasome. *J Biol Chem* 270: 21645–21651.

Gupta A., Gupta A., Datta M., Shukla G.S. (2000a). Cerebral antioxidant status and free radical generation following glutathione depletion and subsequent recovery. *Mol Cell Biochem* 209: 55–61.

Gupta A., Nigam D., Gupta A., Shukla G.S., Agarwal A.K. (1999). Effect of pyrethroid-based liquid mosquito repellent inhalation on the blood-brain barrier function and oxidative damage in selected organs of developing rats. *J Appl Toxicol* 19: 67–72.

Gupta S., Husser R.C., Geske R.S., Welty S.E., Smith C.V. (2000b). Sex differences in diquat-induced hepatic necrosis and DNA fragmentation in Fischer 344 rats. *Toxicol Sci* 54: 203–211.

Gupta S., Rogers L.K., Smith C.V. (1994). Biliary excretion of lysosomal enzymes, iron, and oxidized protein in Fischer-344 and Sprague-Dawley rats and the effects of diquat and acetaminophen. *Toxicol Appl Pharmacol* 125: 42–50.

Hall N.C., Packard B.A., Hall C.L., De Court, Wagner K.R. (2000). Protein oxidation and enzyme susceptibility in white and gray matter with in vitro oxidative stress: Relevance to brain injury from intracerebral hemorrhage. *Cell Mol Biol (Noisy-le-Grand)* 46: 673–683.

Hanbauer I., Galdzicki Z., Rapoport S.I., Scortegagna M. (1998). Evidence of increased oxidative stress in hippocampal primary cultures of trisomy 16 mouse studies on metallothionein-I/II. *Restor Neurol Neurosci* 12: 87–93.

Harma M., Harma M., Kocyigit A. (2004). Comparison of protein carbonyl and total plasma thiol concentrations in patients with complete hydatidiform mole with those in healthy pregnant women. *Acta Obstet Gynecol Scand* 83: 857–860.

Harman D. (1956). Aging: A theory based on free radical and radiation chemistry. *J Gerontol* 11: 298–300.

Harris M.E., Carney J.M., Hua D.H., Leedle R.A. (1994). Detection of oxidation products in individual neurons by fluorescence microscopy. *Exp Neurol* 129: 95–102.

Hattori I., Takagi Y., Nakamura H., Nozaki K., Bai J., Kondo N., Sugino T., Nishimura M., Hashimoto N., Yodoi J. (2004). Intravenous administration of thioredoxin decreases brain damage following transient focal cerebral ischemia in mice. *Antioxid Redox Signal* 6: 81–87.

Haugen E.N., Croatt A.J., Nath K.A. (2000). Angiotensin II induces renal oxidant stress in vivo and heme oxygenase-1 in vivo and in vitro. *Kidney Int* 58: 144–152.

Haycock J.W., Mac N.S., Mantle D. (1998). Differential protein oxidation in Duchenne and Becker muscular dystrophy. *Neuroreport* 9: 2201–2207.

Haycock J.W., MacNeil S., Jones P., Harris J.B., Mantle D. (1996). Oxidative damage to muscle protein in Duchenne muscular dystrophy. *Neuroreport* 8: 357–361.

Haycock J.W., Ralston D.R., Morris B., Freedlander E., MacNeil S. (1997). Oxidative damage to protein and alterations to antioxidant levels in human cutaneous thermal injury. *Burns* 23: 533–540.

He K., Nukada H., McMorran P.D., Murphy M.P. (1999). Protein carbonyl formation and tyrosine nitration as markers of oxidative damage during ischaemia-reperfusion injury to rat sciatic nerve. *Neuroscience* 94: 909–916.

Hensley K., Floyd R.A., Gordon B., Mou S., Pye Q.N., Stewart C., West M., Williamson K. (2002). Temporal patterns of cytokine and apoptosis-related gene expression in spinal cords of the G93A-SOD1 mouse model of amyotrophic lateral sclerosis. *J Neurochem* 82: 365–374.

Hensley K., Hall N., Subramaniam R., Cole P., Harris M., Aksenov M., Aksenova M., Gabbita S.P., Wu J.F., Carney J.M. (1995). Brain regional correspondence between Alzheimer's disease histopathology and biomarkers of protein oxidation. *J Neurochem* 65: 2146–2156.

Hermida-Ameijeiras A., Mendez-Alvarez E., Sanchez-Iglesias S., Sanmartin-Suarez C., Soto-Otero R. (2004). Autoxidation and MAO-mediated metabolism of dopamine as a potential cause of oxidative stress: Role of ferrous and ferric ions. *Neurochem Int* 45: 103–116.

Himmelfarb J., McMonagle E., McMenamin E. (2000). Plasma protein thiol oxidation and carbonyl formation in chronic renal failure. *Kidney Int* 58: 2571–2578.

Hipkiss A.R., Brownson C., Bertani M.F., Ruiz E., Ferro A. (2002). Reaction of carnosine with aged proteins: another protective process? *Ann NY Acad Sci* 959: 285–924.

Ho Y.S., Magnenat J.L., Bronson R.T., Cao J., Gargano M., Sugawara M., Funk C.D. (1997). Mice deficient in cellular glutathione peroxidase develop normally and show no increased sensitivity to hyperoxia. *J Biol Chem* 272: 16644–16651.

Hochman A., Liang H., Offen D., Melamed E., Sternin H. (2000). Developmental changes in antioxidant enzymes and oxidative damage in kidneys, liver and brain of bcl-2 knockout mice. *Cell Mol Biol (Noisy-le-Grand)* 46: 41–52.

Horakova E., Ondrejickova O., Vajdova M., Korytar P., Durackova Z., Schaur R.J. (2002). Mechanisms of iron-induced oxidative modifications of creatine kinase in rat brain in vitro. Possible involvement of HNE. *Gen Physiol Biophys* 21: 327–336.

Howard B.J., Yatin S., Hensley K., Allen K.L., Kelly J.P., Carney J., Butterfield D.A. (1996). Prevention of hyperoxia-induced alterations in synaptosomal membrane-associated proteins by N-tert-butyl-alpha-phenylnitrone and 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (Tempol). *J Neurochem* 67: 2045–2050.

Hu M.L., Tappel A.L. (1992). Potentiation of oxidative damage to proteins by ultraviolet-A and protection by antioxidants. *Photochem Photobiol* 56: 357–363.

Hyun D.H., Lee M., Hattori N., Kubo S., Mizuno Y., Halliwell B., Jenner P. (2002a). Effect of wild-type or mutant Parkin on oxidative damage, nitric oxide, antioxidant defenses, and the proteasome. *J Biol Chem* 277: 28572–28577.

Hyun D.H., Lee M.H., Halliwell B., Jenner P. (2002b). Proteasomal dysfunction induced by 4-hydroxy-2,3-trans-nonenal, an end-product of lipid peroxidation: A mechanism contributing to neurodegeneration? *J Neurochem* 83: 360–370.

Ibrahim W., Lee U.S., Yen H.C., St Clair D.K., Chow C.K. (2000). Antioxidant and oxidative status in tissues of manganese superoxide dismutase transgenic mice. *Free Radic Biol Med* 28: 397–402.

Inder T., Mocatta T., Darlow B., Spencer C., Volpe J.J., Winterbourn C. (2002). Elevated free radical products in the cerebrospinal fluid of VLBW infants with cerebral white matter injury. *Pediatr Res* 52: 213–218.

Iqbal M., Pumford N.R., Tang Z.X., Lassiter K., Wing T., Cooper M., Bottje W. (2004). Low feed efficient broilers within a single genetic line exhibit higher oxidative stress and protein expression in breast muscle with lower mitochondrial complex activity. *Poult Sci* 83: 474–484.

Iqbal M., Sharma S.D., Rahman A., Trikha P., Athar M. (1999). Evidence that ferric nitrilotriacetate mediates oxidative stress by down-regulating DT-diaphorase activity: Implications for carcinogenesis. *Cancer Lett* 141: 151–157.

Ischiropoulos H., Al-Mehdi A.B., Fisher A.B. (1995). Reactive species in ischemic rat lung injury: Contribution of peroxynitrite. *Am J Physiol* 269: L158–L164.

Ishii N., Fujii M., Hartman P.S., Tsuda M., Yasuda K., Senoo-Matsuda N., Yanase S., Ayusawa D., Suzuki K. (1998). A mutation in succinate dehydrogenase cytochrome b causes oxidative stress and ageing in nematodes. *Nature* 394: 694–697.

Ishii N., Takahashi K., Tomita S., Keino T., Honda S., Yoshino K., Suzuki K. (1990). A methyl viologen-sensitive mutant of the nematode *Caenorhabditis elegans*. *Mutat Res* 237: 165–171.

Iwai K., Drake S.K., Wehr N.B., Weissman A.M., LaVaute T., Minato N., Klausner R.D., Levine R.L., Rouault T.A. (1998). Iron-dependent oxidation, ubiquitination, and degradation of iron regulatory protein 2: implications for degradation of oxidized proteins. *Proc Natl Acad Sci USA* 95: 4924–4928.

Iwai K., Klausner R.D., Rouault T.A. (1995). Requirements for iron-regulated degradation of the RNA binding protein, iron regulatory protein 2. *EMBO J* 14: 5350–5357.

Jakubowski W., Bilinski T., Bartosz G. (2000). Oxidative stress during aging of stationary cultures of the yeast *Saccharomyces cerevisiae*. *Free Radic Biol Med* 28: 659–664.

Jana C.K., Das N., Sohal R.S. (2002). Specificity of age-related carbonylation of plasma proteins in the mouse and rat. *Arch Biochem Biophys* 397: 433–439.

Jin Y., McEwen M.L., Nottingham S.A., Maragos W.F., Dragicevic N.B., Sullivan P.G., Springer J.E. (2004). The mitochondrial uncoupling agent 2,4-dinitrophenol improves mitochondrial function, attenuates oxidative damage, and increases white matter sparing in the contused spinal cord. *J Neurotrauma* 21: 1396–1404.

Johansson E., Olsson O., Nystrom T. (2004). Progression and specificity of protein oxidation in the life cycle of *Arabidopsis thaliana*. *J Biol Chem* 279: 22204–22208.

Jones L.A., Holmes J.C., Seligman R.B. (1956). Spectrophotometric studies of some 2,4-dinitrophenyhydrazones. *Anal Chem* 28: 191–198.

Judge S., Jang Y.M., Smith A., Hagen T., Leeuwenburgh C. (2005). Age-associated increases in oxidative stress and antioxidant enzyme activities in cardiac interfibrillar mitochondria: Implications for the mitochondrial theory of aging. *FASEB J* 19(3): 419–421.

Judge S., Judge A., Grune T., Leeuwenburgh C. (2004). Short-term CR decreases cardiac mitochondrial oxidant production but increases carbonyl content. *Am J Physiol Regul Integr Comp Physiol* 286: R254–R259.

Jung Y., Chay K., Song D., Yang S., Lee M., Ahn B. (1997). Protein carbonyl formation in blood plasma by cephalosporins. *Arch Biochem Biophys* 345: 311–317.

Kahraman A., Erkasap N., Serteser M., Koken T. (2003). Protective effect of quercetin on renal ischemia/reperfusion injury in rats. *J Nephrol* 16: 219–224.

Kamat J.P., Devasagayam T.P., Priyadarsini K.I., Mohan H. (2000). Reactive oxygen species mediated membrane damage induced by fullerene derivatives and its possible biological implications. *Toxicology* 155: 55–61.

Kamat J.P., Devasagayam T.P., Priyadarsini K.I., Mohan H., Mittal J.P. (1998). Oxidative damage induced by the fullerene C60 on photosensitization in rat liver microsomes. *Chem Biol Interact* 114: 145–159.

Kaneko K., Nakamura A., Yoshida K., Kametani F., Higuchi K., Ikeda S. (2002). Glial fibrillary acidic protein is greatly modified by oxidative stress in aceruloplasminemia brain. *Free Radic Res* 36: 303–306.

Kantrow S.P., Taylor D.E., Carraway M.S., Piantadosi C.A. (1997). Oxidative metabolism in rat hepatocytes and mitochondria during sepsis. *Arch Biochem Biophys* 345: 278–288.

Kaplan M., Gold V., Hammerman C., Hochman A., Goldschmidt D., Vreman H.J., Stevenson D.K. (2005). Phototherapy and photo-oxidation in premature neonates. *Biol Neonate* 87: 44–50.

Karsek-Staples J.A., Webster R.O. (1993). Ceruloplasmin inhibits carbonyl formation in endogenous proteins. *Free Rad Biol Med* 14: 115–125.

Kayali R., Cakatay U., Akcay T., Altug T. (2006). Effect of alpha-lipoic acid supplementation on markers of protein oxidation in post-mitotic tissues of ageing rat. *Cell Biochem Funct*, 26: 79–85.

Keller R.J., Halmes N.C., Hinson J.A., Pumford N.R. (1993). Immunochemical detection of oxidized proteins. *Chem Res Toxicol* 6: 430–433.

Kelly F.J., Birch S. (1993). Ozone exposure inhibits cardiac protein synthesis in the mouse. *Free Radic Biol Med* 14: 443–446.

Kettle A.J., Chan T., Osberg I., Senthilmohan R., Chapman A.L., Mocatta T.J., Wagener J.S. (2004). Myeloperoxidase and protein oxidation in the airways of young children with cystic fibrosis. *Am J Respir Crit Care Med* 170: 1317–1323.

Khaliulin I., Schneider A., Houminer E., Borman J.B., Schwalb H. (2004). Apomorphine prevents myocardial ischemia/reperfusion-induced oxidative stress in the rat heart. *Free Radic Biol Med* 37: 969–976.

Khan M.F., Wu X., Ansari G.A. (2000). Contribution of nitrosobenzene to splenic toxicity of aniline. *J Toxicol Environ Health A* 60: 263–273.

Kim K.B., Lee B.M. (1997). Oxidative stress to DNA, protein, and antioxidant enzymes (superoxide dismutase and catalase) in rats treated with benzo(a)pyrene. *Cancer Lett* 113: 205–212.

Kim S.J., Reiter R.J., Qi W., Tan D.D., Cabrera J. (2000). Melatonin prevents oxidative damage to protein and lipid induced by ascorbate-Fe^{3+}-EDTA: Comparison with glutathione and alpha-tocopherol. *Neuroendocrinol Lett* 21: 269–276.

Kingston-Smith A.H., Foyer C.H. (2000). Bundle sheath proteins are more sensitive to oxidative damage than those of the mesophyll in maize leaves exposed to paraquat or low temperatures. *J Exp Bot* 51: 123–130.

Koken T., Serteser M., Kahraman A., Gokce C. (2002). Oxidative stress markers in hepatitis C infected hemodialysis patients. *J Nephrol* 15: 302–307.

Korolainen M.A., Goldsteins G., Alafuzoff I., Koistinaho J., Pirttila T. (2002). Proteomic analysis of protein oxidation in Alzheimer's disease brain. *Electrophoresis* 23: 3428–3433.

Kot J., Sicko Z., Wozniak M. (2003). Oxidative stress during oxygen tolerance test. *Int Marit Health* 54: 117–126.

Kujawa J., Zavodnik I.B., Lapshina A., Labieniec M., Bryszewska M. (2004). Cell survival, DNA, and protein damage in B14 cells under low-intensity near-infrared (810 nm) laser irradiation. *Photomed Laser Surg* 22: 504–508.

Kwon Y.S., Park D.H., Shin E.J., Kwon M.S., Ko K.H., Kim W.K., Jhoo J.H., Jhoo W.K., Wie M.B., Jung B.D., Kim H.C. (2004). Antioxidant propolis attenuates kainate-induced neurotoxicity via adenosine A1 receptor modulation in the rat. *Neurosci Lett* 355: 231–235.

Kwong L.K., Kamzalov S., Rebrin I., Bayne A.C., Jana C.K., Morris P., Forster M.J., Sohal R.S. (2002). Effects of coenzyme Q_{10} administration on its tissue concentrations, mitochondrial oxidant generation, and oxidative stress in the rat. *Free Radic Biol Med* 33: 627–638.

La Fontaine M.A., Geddes J.W., Banks A., Butterfield D.A. (2000). 3-nitropropionic acid induced in vivo protein oxidation in striatal and cortical synaptosomes: Insights into Huntington's disease. *Brain Res* 858: 356–362.

Lass A., Sohal B.H., Weindruch R., Forster M.J., Sohal R.S. (1998). Caloric restriction prevents age-associated accrual of oxidative damage to mouse skeletal muscle mitochondria. *Free Radic Biol Med* 25: 1089–1097.

Lee J.H., Park J.W. (2004). Role of thioredoxin peroxidase in aging of stationary cultures of *Saccharomyces cerevisiae*. *Free Radic Res* 38: 225–231.

Lee M., Hyun D.H., Marshall K.A., Ellerby L.M., Bredesen D.E., Jenner P., Halliwell B. (2001a). Effect of overexpression of BCL-2 on cellular oxidative damage, nitric oxide production, antioxidant defenses, and the proteasome. *Free Radic Biol Med* 31: 1550–1559.

Lee M.H., Hyun D.H., Jenner P., Halliwell B. (2001b). Effect of proteasome inhibition on cellular oxidative damage, antioxidant defences and nitric oxide production. *J Neurochem* 78: 32–41.

Lee Y.J., Shacter E. (1995). Role of carbohydrates in oxidative modification of fibrinogen and other plasma proteins. *Arch Biochem Biophys* 321: 175–181.

Leeuwenburgh C., Rasmussen J.E., Hsu F.F., Mueller D.M., Pennathur S., Heinecke J.W. (1997). Mass spectrometric quantification of markers for protein oxidation by tyrosyl radical, copper, and hydroxyl radical in low density lipoprotein isolated from human atherosclerotic plaques. *J Biol Chem* 272: 3520–3526.

Lei X.G. (2001). Glutathione peroxidase-1 gene knockout on body antioxidant defense in mice. *BioFactors* 14: 93–199.

Lenz A.G., Jorens P.G., Meyer B., De B.W., Van O.F., Bossaert L., Maier K.L. (1999). Oxidatively modified proteins in bronchoalveolar lavage fluid of patients with ARDS and patients at-risk for ARDS. *Eur Respir J* 13: 169–174.

Lenz A.-G., Krombach F., Maier K.L. (1992). Oxidative stress in vivo and in vitro: Modulation by quartz dust and hyperbaric atmosphere. *Free Rad Biol Med* 12: 1–10.

Levine R.L. (1983b). Oxidative modification of glutamine synthetase. II. Characterization of the ascorbate model system. *J Biol Chem* 258: 11828–11833.

Levine R.L. (1983a). Oxidative modification of glutamine synthetase. I. Inactivation is due to loss of one histidine residue. *J Biol Chem* 258: 11823–11827.

Levine R.L., Oliver C.N., Fulks R.M., Stadtman E.R. (1981). Turnover of bacterial glutamine synthetase: Oxidative inactivation precedes proteolysis. *Proc Natl Acad Sci USA* 78: 2120–2124.

Levine R.L., Wehr N., Williams J.A., Stadtman E.R., Shacter E. (2000a). Determination of carbonyl groups in oxidized proteins. *Methods Mol Biol* 99: 15–24.

Li C.Q., Pignatelli B., Ohshima H. (2001). Increased oxidative and nitrative stress in human stomach associated with cagA+ *Helicobacter pylori* infection and inflammation. *Dig Dis Sci* 46: 836–844.

Lih-Brody L., Powell S.R., Collier K.P., Reddy G.M., Cerchia R., Kahn E., Weissman G.S., Katz S., Floyd R.A., McKinley M.J., Fisher S.E., Mullin G.E. (1996). Increased

oxidative stress and decreased antioxidant defenses in mucosa of inflammatory bowel disease. *Dig Dis Sci* 41: 2078–2086.

Liu Y, Rosenthal RE, Starke-Reed PE, Fiskum G. (1993). Inhibition of post cardiac arrest brain protein oxidation by acetyl-L-carnitine. *Free Radic Biol Med* 15: 667–670.

Livrea M.A., Tesoriere L., Pintaudi A.M., Calabrese A., Maggio A., Freisleben H.J., D'Arpa D., D'Anna R., Bongiorno A. (1996). Oxidative stress and antioxidant status in betathalassemia major: iron overload and depletion of lipid-soluble antioxidants. *Blood* 88: 3608–3614.

Lucesoli F., Fraga C.G. (1995). Oxidative damage to lipids and DNA concurrent with decrease of antioxidants in rat testes after acute iron intoxication. *Arch Biochem Biophys* 316: 567–571.

Luo J., Shi R. (2004). Diffusive oxidative stress following acute spinal cord injury in guinea pigs and its inhibition by polyethylene glycol. *Neurosci Lett* 359: 167–170.

Lyras L., Perry R.H., Perry E.K., Ince P.G., Jenner A., Jenner P., Halliwell B. (1998). Oxidative damage to proteins, lipids, and DNA in cortical brain regions from patients with dementia with Lewy bodies. *J Neurochem* 71: 302–312.

Lyras L., Zeng B.Y., McKenzie G., Pearce R.K., Halliwell B., Jenner P. (2002). Chronic high dose L-DOPA alone or in combination with the COMT inhibitor entacapone does not increase oxidative damage or impair the function of the nigro-striatal pathway in normal cynomologus monkeys. *J Neural Transm* 109: 53–67.

Ma Y., Kawabata T., Hamazaki S., Ogino T., Okada S. (1998). Sex differences in oxidative damage in ddY mouse kidney treated with a renal carcinogen, iron nitrilotriacetate. *Carcinogenesis* 19: 1983–1988.

Madden M.C., Dailey L.A., Stonehuerner J.G., Harris B.D. (2003). Responses of cultured human airway epithelial cells treated with diesel exhaust extracts will vary with the engine load. *J Toxicol Environ Health A* 66: 2281–2297.

Magi B., Ettorre A., Liberatori S., Bini L., Andreassi M., Frosali S., Neri P., Pallini V., Di Stefano A. (2004). Selectivity of protein carbonylation in the apoptotic response to oxidative stress associated with photodynamic therapy: A cell biochemical and proteomic investigation. *Cell Death Differ* 11: 842–852.

Mantle D., Falkous G., Walker D. (1999). Quantification of protease activities in synovial fluid from rheumatoid and osteoarthritis cases: Comparison with antioxidant and free radical damage markers. *Clin Chim Acta* 284: 45–58.

Mantle D., Siddique S., Eddeb F., Mendelow A.D. (2001). Comparison of protein carbonyl and antioxidant levels in brain tissue from intracerebral haemorrhage and control cases. *Clin Chim Acta* 312: 185–190.

Maragos W.F., Young K.L., Altman C.S., Pocernich C.B., Drake J., Butterfield D.A., Seif I., Holschneider D.P., Chen K., Shih J.C. (2004). Striatal damage and oxidative stress induced by the mitochondrial toxin malonate are reduced in clorgyline-treated rats and MAO-A deficient mice. *Neurochem Res* 29: 741–746.

Marangon K., Devaraj S., Tirosh O., Packer L., Jialal I. (1999). Comparison of the effect of alpha-lipoic acid and alpha-tocopherol supplementation on measures of oxidative stress. *Free Radic Biol Med* 27: 1114–1121.

Margiloff L., Chaplia L., Chow A., Singhal P.C., Mattana J. (1998). Metal-catalyzed oxidation of immunoglobulin G impairs Fc receptor-mediated binding to macrophages. *Free Radic Biol Med* 25: 780–785.

Maria C.S., Revilla E., Ayala A., de la Cruz C.P., Machado A. (1995). Changes in the histidine residues of Cu/Zn superoxide dismutase during aging. *FEBS Lett* 374: 85–88.

Martinez M., Hernandez A.I., Martinez N. (2000). *N*-Acetylcysteine delays age-associated memory impairment in mice: role in synaptic mitochondria. *Brain Res* 855: 100–106.

Matata B.M., Galinanes M. (2000). Cardiopulmonary bypass exacerbates oxidative stress but does not increase proinflammatory cytokine release in patients with diabetes compared with patients without diabetes: regulatory effects of exogenous nitric oxide. *J Thorac Cardiovasc Surg* 120: 1–11.

Matata B.M., Sosnowski A.W., Galinanes M. (2000). Off-pump bypass graft operation significantly reduces oxidative stress and inflammation. *Ann Thorac Surg* 69: 785–791.

Mates J.M., Perez-Gomez C., Olalla L., Segura J.M., Blanca M. (2000). Allergy to drugs: antioxidant enzymic activities, lipid peroxidation and protein oxidative damage in human blood. *Cell Biochem Funct* 18: 77–84.

Mattana J., Margiloff L., Chaplia L., Chow A., Singhal P.C. (1998). Metal-catalyzed oxidation of extracellular matrix increases macrophage nitric oxide generation. *Kidney Int* 54: 1581–1592.

Mattana J., Margiloff L., Singhal P.C. (1995). Oxidation of mesangial matrix using a mixed function oxidase system augments adhesion of macrophages: Possible role of macrophage scavenger receptors. *Biochem Biophys Res Commun* 212: 63–69.

Mayo J.C., Tan D.X., Sainz R.M., Natarajan M., Lopez-Burillo S., Reiter R.J. (2003). Protection against oxidative protein damage induced by metal-catalyzed reaction or alkylperoxyl radicals: Comparative effects of melatonin and other antioxidants. *Biochim Biophys Acta* 1620: 139–150.

Meister A. (1965). *Biochemistry of the Amino Acids*, 2nd ed., Academic Press, New York.

Michelis R., Gery R., Sela S., Shurtz-Swirski R., Grinberg N., Snitkovski T., Shasha S.M., Kristal B. (2003). Carbonyl stress induced by intravenous iron during haemodialysis. *Nephrol Dial Transplant* 18: 924–930.

Mickel H.S., Starke-Reed P.E., Oliver C.N. (1990). Protein oxidation and myelinolysis occur in brain following rapid correction of hyponatremia. *Biochem Biophys Res Commun* 172: 92–97.

Mihm M.J., Yu F., Carnes C.A., Reiser P.J., McCarthy P.M., Van Wagoner D.R., Bauer J.A. (2001). Impaired myofibrillar energetics and oxidative injury during human atrial fibrillation. *Circulation* 104: 174–180.

Minetti M., Mallozzi C., Di Stasi A.M., Pietraforte D. (1998). Bilirubin is an effective antioxidant of peroxynitrite-mediated protein oxidation in human blood plasma. *Arch Biochem Biophys* 352: 165–174.

Mirzaei H., Regnier F.E. (2005). Affinity chromatography of oxidized proteins. *Anal Chem* 77(8): 2386–2392.

Miyata T., Inagi R., Asahi K., Yamada Y., Horie K., Sakai H., Uchida K., Kurokawa K. (1998). Generation of protein carbonyls by glycoxidation and lipoxidation reactions with autoxidation products of ascorbic acid and polyunsaturated fatty acids. *FEBS Lett* 437: 24–28.

Miyata T., Ishikawa N., van Ypersele de Strihou C. (2003). Carbonyl stress and diabetic complications. *Clin Chem Lab Med* 41: 1150–1158.

Miyata T., Kurokawa K. (1999). Carbonyl stress: increased carbonyl modification of proteins by autoxidation products of carbohydrates and lipids in uremia. *Int J Artif Organs* 22: 195–198.

Miyata T., Ueda Y., Saito A., Kurokawa K. (2000). "Carbonyl stress" and dialysis-related amyloidosis. *Nephrol Dial Transplant* 15 Suppl 1: 25–28.

Mockett R.J., Sohal R.S., Orr W.C. (1999). Overexpression of glutathione reductase extends survival in transgenic *Drosophila melanogaster* under hyperoxia but not normoxia. *FASEB J* 13: 1733–1742.

Montine K.S., Montine T.J., Morrow J.D., Frei B., Milatovic D., Eckenstein F., Quinn J.F. (2002). Mouse cerebral prostaglandins, but not oxidative damage, change with age and are responsive to indomethacin treatment. *Brain Res* 930: 75–82.

Moreira J.C., Dal-Pizzol F., Bonatto F., da Silva E.G., Flores D.G., Picada J.N., Roesler R., Henriques J.A. (2003). Oxidative damage in brains of mice treated with apomorphine and its oxidized derivative. *Brain Res* 992: 246–251.

Moseley R., Hilton J.R., Waddington R.J., Harding K.G., Stephens P., Thomas D.W. (2004). Comparison of oxidative stress biomarker profiles between acute and chronic wound environments. *Wound Repair Regen* 12: 419–429.

Moskovitz J., Bar-Noy S., Williams W.M., Requena J., Berlett B.S., Stadtman E.R. (2001). Methionine sulfoxide reductase (MsrA) is a regulator of antioxidant defense and lifespan in mammals. *Proc Natl Acad Sci USA* 98: 12920–12925.

Moskovitz J., Stadtman E.R. (2003). Selenium-deficient diet enhances protein oxidation and affects methionine sulfoxide reductase (MsrB) protein level in certain mouse tissues. *Proc Natl Acad Sci USA* 100: 7486–7490.

Mostertz J., Hecker M. (2003). Patterns of protein carbonylation following oxidative stress in wild-type and sigB *Bacillus subtilis* cells. *Mol Genet Genomics* 269: 640–648.

Mudway I.S., Kelly F.J. (1998). Modeling the interactions of ozone with pulmonary epithelial lining fluid antioxidants. *Toxicol Appl Pharmacol* 148: 91–100.

Murphy M.E., Kehrer J.P. (1989). Oxidation state of tissue thiol groups and content of protein carbonyl groups in chickens with inherited muscular dystrophy. *Biochem J* 260: 359–364.

Musci G., di Patti M.C.B., Fagiolo U., Calabrese L. (1993). Age-related changes in human ceruloplasmin. Evidence for oxidative modifications. *J Biol Chem* 268: 13388–13395.

Mutlu-Turkoglu U., Ilhan E., Oztezcan S., Kuru A., ykac-Toker G., Uysal M. (2003). Age-related increases in plasma malondialdehyde and protein carbonyl levels and lymphocyte DNA damage in elderly subjects. *Clin Biochem* 36: 397–400.

Mutoh N., Kawabata M., Nakagawa C.W., Kitajima S. (2005). Pro-oxidant action of phloxine B on fission yeast *Schizosaccharomyces pombe*. *Yeast* 22: 91–97.

Nadeem A., Chhabra S.K., Masood A., Raj H.G. (2003). Increased oxidative stress and altered levels of antioxidants in asthma. *J Allergy Clin Immunol* 111: 72–78.

Nagai M., Takahashi R., Goto S. (2000). Dietary restriction initiated late in life can reduce mitochondrial protein carbonyls in rat livers: Western blot studies. *Biogerontology* 1: 321–328.

Nagaraj R.H., Sarkar P., Mally A., Biemel K.M., Lederer M.O., Padayatti P.S. (2002). Effect of pyridoxamine on chemical modification of proteins by carbonyls in diabetic rats: Characterization of a major product from the reaction of pyridoxamine and methylglyoxal. *Arch Biochem Biophys* 402: 110–119.

Nagasawa T., Hayashi H., Fujimaki N., Nishizawa N., Kitts D.D. (2000). Induction of oxidatively modified proteins in skeletal muscle by electrical stimulation and its suppression by dietary supplementation of (-)-epigallocatechin gallate. *Biosci Biotechnol Biochem* 64: 1004–1010.

Nagasawa T., Yonekura T., Nishizawa N., Kitts D.D. (2001). In vitro and in vivo inhibition of muscle lipid and protein oxidation by carnosine. *Mol Cell Biochem* 225: 29–34.

Nair S., Poyart C., Beretti J.L., Veiga-Fernandes H., Berche P., Trieu-Cuot P. (2003). Role of the *Streptococcus agalactiae* ClpP serine protease in heat-induced stress defence and growth arrest. *Microbiology* 149: 407–417.

Nakamura A., Goto S. (1996). Analysis of protein carbonyls with 2,4-dinitrophenyl hydrazine and its antibodies by immunoblot in two-dimensional gel electrophoresis. *J Biochem (Tokyo)* 119: 768–774.

Nakamura A., Yasuda K., Adachi H., Sakurai Y., Ishii N., Goto S. (1999). Vitellogenin-6 is a major carbonylated protein in aged nematode, *Caenorhabditis elegans*. *Biochem Biophys Res Commun* 264: 580–583.

Navarro A., Boveris A. (2004). Rat brain and liver mitochondria develop oxidative stress and lose enzymatic activities on aging. *Am J Physiol Regul Integr Comp Physiol* 287: R1244–R1249.

Neuzil J., Stocker R. (1993). Bilirubin attenuates radical-mediated damage to serum albumin. *FEBS Lett* 331: 281–284.

Nicholls-Grzemski F.A., Belling G.B., Priestly B.G., Calder I.C., Burcham P.C. (2000). Clofibrate pretreatment in mice confers resistance against hepatic lipid peroxidation. *J Biochem Mol Toxicol* 14: 335–345.

Nizard C., Poggioli S., Heusele C., Bulteau A.L., Moreau M., Saunois A., Schnebert S., Mahe C., Friguet B. (2004). Algae extract protection effect on oxidized protein level in human stratum corneum. *Ann N Y Acad Sci* 1019: 219–222.

O'Brien K.M., Dirmeier R., Engle M., Poyton R.O. (2004). Mitochondrial protein oxidation in yeast mutants lacking manganese-(MnSOD) or copper- and zinc-containing superoxide dismutase (CuZnSOD): evidence that MnSOD and CuZnSOD have both unique and overlapping functions in protecting mitochondrial proteins from oxidative damage. *J Biol Chem* 279: 51817–51827.

Odetti P., Valentini S., Aragno I., Garibaldi S., Pronzato M.A., Rolandi E., Barreca T. (1998). Oxidative stress in subjects affected by celiac disease. *Free Radic Res* 29: 17–24.

Oh-Ishi M., Ueno T., Maeda T. (2003). Proteomic method detects oxidatively induced protein carbonyls in muscles of a diabetes model Otsuka Long-Evans Tokushima Fatty (OLETF) rat. *Free Radic Biol Med* 34: 11–22.

Okatani Y., Wakatsuki A., Reiter R.J., Miyahara Y. (2002). Melatonin reduces oxidative damage of neural lipids and proteins in senescence-accelerated mouse. *Neurobiol Aging* 23: 639–644.

Okuyama H., Nakamura H., Shimahara Y., Araya S., Kawada N., Yamaoka Y., Yodoi J. (2003). Overexpression of thioredoxin prevents acute hepatitis caused by thioacetamide or lipopolysaccharide in mice. *Hepatology* 37: 1015–1025.

Oliver C.N. (1987). Inactivation of enzymes and oxidative modification of proteins by stimulated neutrophils. *Arch Biochem Biophys* 253: 62–72.

Oliver C.N., Ahn B.W., Moerman E.J., Goldstein S., Stadtman E.R. (1987). Age-related changes in oxidized proteins. *J Biol Chem* 262: 5488–5491.

Oliver C.N., Starke-Reed P.E., Stadtman E.R., Liu G.J., Carney J.M., Floyd R.A. (1990). Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/reperfusion-induced injury to gerbil brain. *Proc Natl Acad Sci USA* 87: 5144–5147.

Oteiza P.I., Olin K.L., Fraga C.G., Keen C.L. (1995). Zinc deficiency causes oxidative damage to proteins, lipids and DNA in rat testes. *J Nutr* 125: 823–829.

Ozbey N., Telci A., Cakatay U., Yurci A., Molvalilar S. (2003). Determination of oxidative protein and lipid damage in adult hypopituitary patients with GH deficiency. *J Endocrinol Invest* 26: 1001–1007.

Packer L., Valenza M., Serbinova E., Starke-Reed P., Frost K., Kagan V. (1991). Free radical scavenging is involved in the protective effect of L-propionyl-carnitine against ischemia-reperfusion injury of the heart. *Arch Biochem Biophys* 288: 533–537.

Pahlmark K., Folbergrova J., Smith M.L., Siesjo B.K. (1993). Effects of dimethylthiourea on selective neuronal vulnerability in forebrain ischemia in rats. *Stroke* 24: 731–736.

Palamanda J.R., Kehrer J.P. (1992). Inhibition of protein carbonyl formation and lipid peroxidation by glutathione in rat liver microsomes. *Arch Biochem Biophys* 293: 103–109.

Pamplona R., Portero-Otin M., Requena J., Gredilla R., Barja G. (2002). Oxidative, glycoxidative and lipoxidative damage to rat heart mitochondrial proteins is lower after 4 months of caloric restriction than in age-matched controls. *Mech Ageing Dev* 123: 1437–1446.

Pantke U., Volk T., Schmutzler M., Kox W.J., Sitte N., Grune T. (1999). Oxidized proteins as a marker of oxidative stress during coronary heart surgery. *Free Radic Biol Med* 27: 1080–1086.

Parihar M.S., Pandit M.K. (2003). Free radical induced increase in protein carbonyl is attenuated by low dose of adenosine in hippocampus and mid brain: implication in neurodegenerative disorders. *Gen Physiol Biophys* 22: 29–39.

Park A.M., Li Q., Nagata K., Tamura T., Shimono K., Sato E.F., Inoue M. (2004). Oxygen tension regulates reactive oxygen generation and mutation of *Helicobacter pylori*. *Free Radic Biol Med* 36: 1126–1133.

Park L.C., Albers D.S., Xu H., Lindsay J.G., Beal M.F., Gibson G.E. (2001). Mitochondrial impairment in the cerebellum of the patients with progressive supranuclear palsy. *J Neurosci Res* 66: 1028–1034.

Park Y., Kehrer J.P. (1991). Oxidative changes in hypoxic-reoxygenated rabbit heart: A consequence of hypoxia rather than reoxygenation. *Free Radic Res Commun* 14: 179–185.

Passi S., Stancato A., Aleo E., Dmitrieva A., Littarru G.P. (2003). Statins lower plasma and lymphocyte ubiquinol/ubiquinone without affecting other antioxidants and PUFA. *BioFactors* 18: 113–124.

Paterna J.C., Boess F., Staubli A., Boelsterli U.A. (1998). Antioxidant and cytoprotective properties of D-tagatose in cultured murine hepatocytes. *Toxicol Appl Pharmacol* 148: 117–125.

Pedraza-Chaverri J., Barrera D., Maldonado P.D., Chirino Y.I., ias-Ruvalcaba N.A., Medina-Campos O.N., Castro L., Salcedo M.I., Hernandez-Pando R. (2004). Sallylmercaptocysteine scavenges hydroxyl radical and singlet oxygen in vitro and attenuates

gentamicin-induced oxidative and nitrosative stress and renal damage in vivo. *BMC Clin Pharmacol* 4: 5.

Pelissier M.A., Trap C., Malewiak M.I., Morfin R. (2004). Antioxidant effects of dehydroepiandrosterone and 7alpha-hydroxy-dehydroepiandrosterone in the rat colon, intestine and liver. *Steroids* 69: 137–144.

Perricone N., Nagy K., Horvath F., Dajko G., Uray I., Nagy I. (1999). Alpha lipoic acid (ALA) protects proteins against the hydroxyl free radical-induced alterations: Rationale for its geriatric topical application. *Arch Gerontol Geriatr* 29: 45–56.

Pignatelli B., Li C.Q., Boffetta P., Chen Q., Ahrens W., Nyberg F., Mukeria A., Bruske-Hohlfeld I., Fortes C., Constantinescu V., Ischiropoulos H., Ohshima H. (2001). Nitrated and oxidized plasma proteins in smokers and lung cancer patients. *Cancer Res* 61: 778–784.

Pinho R.A., Bonatto F., Andrades M., Frota M.L., Jr., Ritter C., Klamt F., Dal-Pizzol F., Uldrich-Kulczynski J.M., Moreira J.C. (2004). Lung oxidative response after acute coal dust exposure. *Environ Res* 96: 290–297.

Pocernich C.B., La F.M., Butterfield D.A. (2000). In-vivo glutathione elevation protects against hydroxyl free radical-induced protein oxidation in rat brain. *Neurochem Int* 36: 185–191.

Podda M., Traber M.G., Weber C., Yan L.J., Packer L. (1998). UV-irradiation depletes antioxidants and causes oxidative damage in a model of human skin. *Free Radic Biol Med* 24: 55–65.

Polat C., Yilmaz S., Serteser M., Koken T., Kahraman A., Dilek O.N. (2003). The effect of different intraabdominal pressures on lipid peroxidation and protein oxidation status during laparoscopic cholecystectomy. *Surg Endosc* 17: 1719–1722.

Pompella A., Cambiaggi C., Dominici S., Paolicchi A., Tongiani R., Comporti M. (1996). Single-cell investigation by laser scanning confocal microscopy of cytochemical alterations resulting from extracellular oxidant challenge. *Histochem Cell Biol* 105: 173–178.

Poon H.F., Castegna A., Farr S.A., Thongboonkerd V., Lynn B.C., Banks W.A., Morley J.E., Klein J.B., Butterfield D.A. (2004). Quantitative proteomics analysis of specific protein expression and oxidative modification in aged senescence-accelerated-prone 8 mice brain. *Neuroscience* 126: 915–926.

Poon H.F., Farr S.A., Thongboonkerd V., Lynn B.C., Banks W.A., Morley J.E., Klein J.B., Butterfield D.A. (2005). Proteomic analysis of specific brain proteins in aged SAMP8 mice treated with alpha-lipoic acid: Implications for aging and age-related neurodegenerative disorders. *Neurochem Int* 46: 159–168.

Qu B., Li Q.T., Wong K.P., Ong C.N., Halliwell B. (1999). Mitochondrial damage by the "prooxidant" peroxisomal proliferator clofibrate. *Free Radic Biol Med* 27: 1095–1102.

Quinlan G.J., Evans T.W., Gutteridge J.M. (1994). Oxidative damage to plasma proteins in adult respiratory distress syndrome. *Free Radic Res* 20: 289–298.

Rababah T., Hettiarachchy N., Horax R., Eswaranandam S., Mauromoustakos A., Dickson J., Niebuhr S. (2004). Effect of electron beam irradiation and storage at 5 degrees C on thiobarbituric acid reactive substances and carbonyl contents in chicken breast meat infused with antioxidants and selected plant extracts. *J Agric Food Chem* 52: 8236–8241.

Radak Z., Asano K., Lee K.C., Ohno H., Nakamura A., Nakamoto H., Goto S. (1997). High altitude training increases reactive carbonyl derivatives but not lipid peroxidation in skeletal muscle of rats. *Free Radic Biol Med* 22: 1109–1114.

Rahaman S.O., Ghosh S., Mohanakumar K.P., Das S., Sarkar P.K. (2001). Hypothyroidism in the developing rat brain is associated with marked oxidative stress and aberrant intraneuronal accumulation of neurofilaments. *Neurosci Res* 40: 273–279.

Rajesh M., Sulochana K.N., Coral K., Punitham R., Biswas J., Babu K., Ramakrishnan S. (2004). Determination of carbonyl group content in plasma proteins as a useful marker to assess impairment in antioxidant defense in patients with Eales' disease. *Indian J Ophthalmol* 52: 139–144.

Ramanathan K., Shila S., Kumaran S., Panneerselvam C. (2003). Protective role of ascorbic acid and alpha-tocopherol on arsenic-induced microsomal dysfunctions. *Hum Exp Toxicol* 22: 129–136.

Ramsey J.J., Hagopian K., Kenny T.M., Koomson E.K., Bevilacqua L., Weindruch R., Harper M.E. (2004). Proton leak and hydrogen peroxide production in liver mitochondria from energy-restricted rats. *Am J Physiol Endocrinol Metab* 286: E31–E40.

Reddy V.Y., Desrochers P.E., Pizzo S.V., Gonias S.L., Sahakian J.A., Levine R.L., Weiss S.J. (1994). Oxidative dissociation of human a2macroglobulin tetramers into dysfunctional dimers. *J Biol Chem* 269: 4683–4691.

Refsgaard H.H., Tsai L., Stadtman E.R. (2000). Modifications of proteins by polyunsaturated fatty acid peroxidation products. *Proc Natl Acad Sci USA* 97: 611–616.

Regan R.F., Chen J., venisti-Zarom L. (2004). Heme oxygenase-2 gene deletion attenuates oxidative stress in neurons exposed to extracellular hemin. *BMC Neurosci* 5: 34.

Rehema A., Zilmer K., Klaar U., Karro H., Kullisaar T., Zilmer M. (2004). Ferrous iron administration during pregnancy and adaptational oxidative stress (Pilot study). *Medicina (Kaunas)* 40: 547–552.

Reinheckel T., Korn S., Mohring S., Augustin W., Halangk W., Schild L. (2000). Adaptation of protein carbonyl detection to the requirements of proteome analysis demonstrated for hypoxia/reoxygenation in isolated rat liver mitochondria. *Arch Biochem Biophys* 376: 59–65.

Reinheckel T., Nedelev B., Prause J., Augustin W., Schulz H.U., Lippert H., Halangk W. (1998). Occurrence of oxidatively modified proteins: an early event in experimental acute pancreatitis. *Free Radic Biol Med* 24: 393–400.

Reiter R.J., Tan D., Kim S.J., Manchester L.C., Qi W., Garcia J.J., Cabrera J.C., El-Sokkary G., Rouvier-Garay V. (1999). Augmentation of indices of oxidative damage in life-long melatonin-deficient rats. *Mech Ageing Dev* 110: 157–173.

Renke J., Popadiuk S., Korzon M., Bugajczyk B., Wozniak M. (2000). Protein carbonyl groups' content as a useful clinical marker of antioxidant barrier impairment in plasma of children with juvenile chronic arthritis. *Free Radic Biol Med* 29: 101–104.

Reverter-Branch G., Cabiscol E., Tamarit J., Ros J. (2004). Oxidative damage to specific proteins in replicative and chronological-aged *Saccharomyces cerevisiae*: Common targets and prevention by caloric restriction. *J Biol Chem* 279: 31983–31989.

Reznick A.Z., Cross C.E., Hu M.-L., Suzuki Y.J., Khwaja S., Safadi A., Motchnik P.A., Packer L., Halliwell B. (1992). Modification of plasma proteins by cigarette smoke as measured by protein carbonyl formation. *Biochem J* 286: 607–611.

Reznick A.Z., Packer L. (1994). Oxidative damage to proteins: Spectrophotometric method for carbonyl assay. *Methods Enzymol* 233: 357–363.

Richert S., Wehr N.B., Stadtman E.R., Levine R.L. (2002). Assessment of skin carbonyl content as a noninvasive measure of biological age. *Arch Biochem Biophys* 397: 430–432.

Rikans L.E., Ardinska V., Hornbrook K.R. (1997). Age-associated increase in ferritin content of male rat liver: implication for diquat-mediated oxidative injury. *Arch Biochem Biophys* 344: 85–93.

Rikans L.E., Cai Y. (1993). Diquat-induced oxidative damage in BCNU-pretreated hepatocytes of mature and old rats. *Toxicol Appl Pharmacol* 118: 263–270.

Rimbach G., Pallauf J. (1999). Effect of dietary phytate on magnesium bioavailability and liver oxidant status in growing rats. *Food Chem Toxicol* 37: 37–45.

Ritter C., Andrades M., Guerreiro M., Zavaschi L., Gelain D.P., Souza L.F., Ribeiro C.A., Clausell N., Menna-Barreto S., Moreira J.C., Dal-Pizzol F. (2003). Plasma oxidative parameters and mortality in patients with severe burn injury. *Intensive Care Med* 29: 1380–1383.

Rivett A.J., Levine R.L. (1990). Metal-catalyzed oxidation of *Escherichia coli* glutamine synthetase: Correlation of structural and functional changes. *Arch Biochem Biophys* 278: 26–34.

Robinson C.E., Keshavarzian A., Pasco D.S., Frommel T.O., Winship D.H., Holmes E.W. (1999). Determination of protein carbonyl groups by immunoblotting. *Anal Biochem* 266: 48–57.

Roseman J.E., Levine R.L. (1987). Purification of a protease from *Escherichia coli* with specificity for oxidized glutamine synthetase. *J Biol Chem* 262: 2101–2110.

Rothstein M. (1982). *Biochemical Approaches to Aging*, Academic Press, New York.

Rowe L.J., Maddock K.R., Lonergan S.M., Huff-Lonergan E. (2004). Influence of early postmortem protein oxidation on beef quality. *J Anim Sci* 82: 785–793.

Rutkowska M., Strzyzewski K., Iskra M., Piorunska-Stolzmann M., Majewski W. (2005). Increased protein carbonyl groups in the serum of men with chronic arterial occlusion and the effect of postoperative treatment. *Med Sci Monit* 11: CR79–CR83.

Saarinen M.A., Murhammer D.W. (2003). The response of virally infected insect cells to dissolved oxygen concentration: Recombinant protein production and oxidative damage. *Biotechnol Bioeng* 81: 106–114.

Sahin E., Gumuslu S. (2004a). Alterations in brain antioxidant status, protein oxidation and lipid peroxidation in response to different stress models. *Behav Brain Res* 155: 241–248.

Sahin E., Gumuslu S. (2004b). Cold-stress-induced modulation of antioxidant defence: Role of stressed conditions in tissue injury followed by protein oxidation and lipid peroxidation. *Int J Biometeorol* 48: 165–171.

Sakata C., Tanaka H., Takemura S., Hirohashi K., Minamiyama Y., Nakamura A., Inoue M., Kinoshita H. (2000). Post-ischemic intraportal adenosine administration protects against reperfusion injury of canine liver. *J Hepatobiliary Pancreat Surg* 7: 78–85.

Samli M.M., Guler C., Demirbas M., Koken T. (2004). The effect of carbon dioxide pneumoretroperitoneum on free oxygen radicals in rabbit retroperitoneoscopy model. *Surg Laparosc Endosc Percutan Tech* 14: 153–156.

Samuel S., Kathirvel R., Jayavelu T., Chinnakkannu P. (2005). Protein oxidative damage in arsenic induced rat brain: Influence of DL-alpha-lipoic acid. *Toxicol Lett* 155: 27–34.

Sander C.S., Ali I., Dean D., Thiele J.J., Wojnarowska F. (2004). Oxidative stress is implicated in the pathogenesis of lichen sclerosus. *Br J Dermatol* 151: 627–635.

Sander C.S., Chang H., Salzmann S., Muller C.S., Ekanayake-Mudiyanselage S., Elsner P., Thiele J.J. (2002). Photoaging is associated with protein oxidation in human skin in vivo. *J Invest Dermatol* 118: 618–625.

Sanni L.A., Fu S., Dean R.T., Bloomfield G., Stocker R., Chaudhri G., Dinauer M.C., Hunt N.H. (1999). Are reactive oxygen species involved in the pathogenesis of murine cerebral malaria? *J Infect Dis* 179: 217–222.

Sastre J., Martin J.A., Gomez-Cabrera M.C., Pereda J., Borras C., Pallardo F.V., Vina J. (2005). Age-associated oxidative damage leads to absence of gamma-cystathionase in over 50 % of rat lenses: Relevance in cataractogenesis. *Free Radic Biol Med* 38: 575–582.

Sato A., Huang M.Z., Watanabe S., Okuyama H., Nakamoto H., Radak Z., Goto S. (1998). Protein carbonyl content roughly reflects the unsaturation of lipids in muscle but not in other tissues of stroke-prone spontaneously hypertensive strain (SHRSP) rats fed different fats and oils. *Biol Pharm Bull* 21: 1271–1276.

Saxton J.M., Donnelly A.E., Roper H.P. (1994). Indices of free-radical-mediated damage following maximum voluntary eccentric and concentric muscular work. *Eur J Appl Physiol Occup Physiol* 68: 189–193.

Scaloni A., Perillo V., Franco P., Fedele E., Froio R., Ferrara L., Bergamo P. (2002). Characterization of heat-induced lactosylation products in caseins by immunoenzymatic and mass spectrometric methodologies. *Biochim Biophys Acta* 1598: 30–39.

Schaper M., Gergely S., Lykkesfeldt J., Zbaren J., Leib S.L., Tauber M.G., Christen S. (2002). Cerebral vasculature is the major target of oxidative protein alterations in bacterial meningitis. *J Neuropathol Exp Neurol* 61: 605–613.

Schimke R.T., Doyle D. (1970). Control of enzyme levels in animal tissues. *Ann Rev Biochem* 39: 929–976.

Schneider J.E. Jr., Tabatabaie T., Maidt L., Smith R.H., Nguyen X., Pye Q., Floyd R.A. (1998). Potential mechanisms of photodynamic inactivation of virus by methylene blue. I. RNA-protein crosslinks and other oxidative lesions in Q beta bacteriophage. *Photochem Photobiol* 67: 350–357.

Schock B.C., Sweet D.G., Ennis M., Warner J.A., Young I.S., Halliday H.L. (2001). Oxidative stress and increased type-IV collagenase levels in bronchoalveolar lavage fluid from newborn babies. *Pediatr Res* 50: 29–33.

Schöneich C. (2001). Editorial. *Exp Gerontol* 36: 1423–1424.

Schraufstatter I.U., Browne K., Harris A., Hyslop P.A., Jackson J.H., Quehenberger O., Cochrane C.G. (1990). Mechanisms of hypochlorite injury of target cells. *J Clin Invest* 85: 554–562.

Scortegagna M., Chikhale E., Hanbauer I. (1998). Lead exposures increases oxidative stress in serum deprived e14 mesencephalic cultures: Role of metallothionein and glutathione. *Restor Neurol Neurosci* 12: 95–101.

Selman C., Grune T., Stolzing A., Jakstadt M., McLaren J.S., Speakman J.R. (2002). The consequences of acute cold exposure on protein oxidation and proteasome activity in short-tailed field voles, microtus agrestis. *Free Radic Biol Med* 33: 259–265.

Selvendiran K., Banu S.M., Sakthisekaran D. (2004). Protective effect of piperine on benzo(a)pyrene-induced lung carcinogenesis in Swiss albino mice. *Clin Chim Acta* 350: 73–78.

Sen C.K., Atalay M., Agren J., Laaksonen D.E., Roy S., Hanninen O. (1997). Fish oil and vitamin E supplementation in oxidative stress at rest and after physical exercise. *J Appl Physiol* 83: 189–195.

Sener G., Paskaloglu K., Toklu H., Kapucu C., yanoglu-Dulger G., Kacmaz A., Sakarcan A. (2004). Melatonin ameliorates chronic renal failure-induced oxidative organ damage in rats. *J Pineal Res* 36: 232–241.

Senturker S., Tschirret-Guth R., Morrow J., Levine R., Shacter E. (2002). Induction of apoptosis by chemotherapeutic drugs without generation of reactive oxygen species. *Arch Biochem Biophys* 397: 262–272.

Shacter E., Williams J.A., Levine R.L. (1995). Oxidative modification of fibrinogen inhibits thrombin-catalyzed clot formation. *Free Radic Biol Med* 18: 815–821.

Shacter E., Williams J.A., Lim M., Levine R.L. (1994). Differential susceptibility of plasma proteins to oxidative modification. Examination by Western blot immunoassay. *Free Rad Biol Med* 17: 429–437.

Shanmuganathan A., Avery S.V., Willetts S.A., Houghton J.E. (2004). Copper-induced oxidative stress in *Saccharomyces cerevisiae* targets enzymes of the glycolytic pathway. *FEBS Lett* 556: 253–259.

Shen J.C., Gray M.D., Oshima J., Kamath-Loeb A.S., Fry M., Loeb L.A. (1998). Werner syndrome protein. I. DNA helicase and dna exonuclease reside on the same polypeptide. *J Biol Chem* 273: 34139–34144.

Shin E.J., Jhoo J.H., Kim W.K., Jhoo W.K., Lee C., Jung B.D., Kim H.C. (2004). Protection against kainate neurotoxicity by pyrrolidine dithiocarbamate. *Clin Exp Pharmacol Physiol* 31: 320–326.

Shin S.J. (2003). Vitamin E modulates radiation-induced oxidative damage in mice fed a high-lipid diet. *J Biochem Mol Biol* 36: 190–195.

Shin S.J., Yamada K. (2002). Adequate intakes of vitamin E and protein prevent increases of oxidative damage to DNA, lipids, and protein induced by total body irradiation in mice. *Nutr Cancer* 44: 169–174.

Silvester J.A., Timmins G.S., Davies M.J. (1998). Protein hydroperoxides and carbonyl groups generated by porphyrin-induced photo-oxidation of bovine serum albumin. *Arch Biochem Biophys* 350: 249–258.

Singhal A.B., Wang X., Sumii T., Mori T., Lo E.H. (2002). Effects of normobaric hyperoxia in a rat model of focal cerebral ischemia-reperfusion. *J Cereb Blood Flow Metab* 22: 861–868.

Skuta G., Fischer G.M., Janaky T., Kele Z., Szabo P., Tozser J., Sumegi B. (1999). Molecular mechanism of the short-term cardiotoxicity caused by 2′,3′-dideoxycytidine (ddC): modulation of reactive oxygen species levels and ADP-ribosylation reactions. *Biochem Pharmacol* 58: 1915–1925.

Smirnova I.V., Pall M.L. (2003). Elevated levels of protein carbonyls in sera of chronic fatigue syndrome patients. *Mol Cell Biochem* 248: 93–95.

Smith C.D., Carney J.M., Starke-Reed P.E., Oliver C.N., Stadtman E.R., Floyd R.A., Markesbery W.R. (1991). Excess brain protein oxidation and enzyme dysfunction in normal aging and Alzheimer disease. *Proc Natl Acad Sci USA* 88: 10540–10543.

Smith M.A., Sayre L.M., Anderson V.E., Harris P.L., Beal M.F., Kowall N., Perry G., Richey H.P., Beckman J.S., Rudnicka-Nawrot M., Richey P.L., Praprotnik D., Mulvihill P., Miller C.A., Cras P., Siedlak S.L. (1998). Cytochemical demonstration of

oxidative damage in Alzheimer disease by immunochemical enhancement of the carbonyl reaction with 2,4-dinitrophenylhydrazine. *J Histochem Cytochem* 46: 731–735.

Sohal R.S., Agarwal S., Dubey A., Orr W.C. (1993). Protein oxidative damage is associated with life expectancy of houseflies. *Proc Natl Acad Sci USA* 90: 7255–7259.

Sohal R.S., Weindruch R. (1996). Oxidative stress, caloric restriction, and aging. *Science* 273: 59–63.

Song D.U., Jung Y.D., Chay K.O., Chung M.A., Lee K.H., Yang S.Y., Shin B.A., Ahn B.W. (2002). Effect of drinking green tea on age-associated accumulation of Maillard-type fluorescence and carbonyl groups in rat aortic and skin collagen. *Arch Biochem Biophys* 397: 424–429.

Soreghan B.A., Yang F., Thomas S.N., Hsu J., Yang A.J. (2003). High-throughput proteomic-based identification of oxidatively induced protein carbonylation in mouse brain. *Pharm Res* 20: 1713–1720.

Stackman R.W., Eckenstein F., Frei B., Kulhanek D., Nowlin J., Quinn J.F. (2003). Prevention of age-related spatial memory deficits in a transgenic mouse model of Alzheimer's disease by chronic *Ginkgo biloba* treatment. *Exp Neurol* 184: 510–520.

Stadler K., Jenei V., Somogyi A., Jakus J. (2005). Beneficial effects of aminoguanidine on the cardiovascular system of diabetic rats. *Diabetes Metab Res Rev* 21(2): 189–196.

Stadtman E.R. (2001). The story of glutamine synthetase regulation. *J Biol Chem* 276: 44357–44364.

Stadtman E.R. (1990). Metal ion-catalyzed oxidation of proteins: Biochemical mechanism and biological consequences. *Free Rad Biol Med* 9: 315–325.

Stadtman ER, Berlett BS. Reactive oxygen-mediated protein oxidation in aging and disease. In: Colton C., Gilbert D.J. (eds.), *Reactive Oxygen Species in Biological Systems*, Kluwer Academic/Plenum, New York, pp. 657–675.

Stadtman E.R., Levine R.L. (2000). Protein oxidation. *Ann NY Acad Sci* 899: 191–208.

Stafford RE, Mak TM, Kramer JH, Weglicki WB. (1993). Protein oxidation in magnesium deficient rat brains and kidneys. *Biochem Biophys Res Commun* 196: 596–600.

Starke-Reed P.E., Oliver C.N. (1989). Protein oxidation and proteolysis during aging and oxidative stress. *Arch Biochem Biophys* 275: 559–567.

Stroh A., Zimmer C., Gutzeit C., Jakstadt M., Marschinke F., Jung T., Pilgrimm H., Grune T. (2004). Iron oxide particles for molecular magnetic resonance imaging cause transient oxidative stress in rat macrophages. *Free Radic Biol Med* 36: 976–984.

Stuart E.L., Evans G.S., Lin Y.S., Powers H.J. (2005). Reduced collagen and ascorbic acid concentrations and increased proteolytic susceptibility with prelabor fetal membrane rupture in women. *Biol Reprod* 72: 230–235.

Suh J., Zhu B.Z., Frei B. (2003). Ascorbate does not act as a pro-oxidant towards lipids and proteins in human plasma exposed to redox-active transition metal ions and hydrogen peroxide. *Free Radic Biol Med* 34: 1306–1314.

Sukharev S.A., Pleshakova O.V., Moshnikova A.B., Sadovnikov V.B., Gaziev A.I. (1997). Age- and radiation-dependent changes in carbonyl content, susceptibility to proteolysis, and antigenicity of soluble rat liver proteins. *Comp Biochem Physiol B Biochem Mol Biol* 116: 333–338.

Sultana S., Saleem M. (2004). Salix caprea inhibits skin carcinogenesis in murine skin: inhibition of oxidative stress, ornithine decarboxylase activity and DNA synthesis. *J Ethnopharmacol* 91: 267–276.

Sumien N., Forster M.J., Sohal R.S. (2003). Supplementation with vitamin E fails to attenuate oxidative damage in aged mice. *Exp Gerontol* 38: 699–704.

Sundari P.N., Wilfred G., Ramakrishna B. (1997). Does oxidative protein damage play a role in the pathogenesis of carbon tetrachloride-induced liver injury in the rat? *Biochim Biophys Acta* 1362: 169–176.

Supinski G., Nethery D., Stofan D., Hirschfield W., DiMarco A. (1999). Diaphragmatic lipid peroxidation in chronically loaded rats. *J Appl Physiol* 86: 651–658.

Suzuki D., Miyata T., Saotome N., Horie K., Inagi R., Yasuda Y., Uchida K., Izuhara Y., Yagame M., Sakai H., Kurokawa K. (1999). Immunohistochemical evidence for an increased oxidative stress and carbonyl modification of proteins in diabetic glomerular lesions. *J Am Soc Nephrol* 10: 822–832.

Szabados E., Fischer G.M., Toth K., Csete B., Nemeti B., Trombitas K., Habon T., Andrei D., Sumegi B. (1999). Role of reactive oxygen species and poly-ADP-ribose polymerase in the development of AZT-induced cardiomyopathy in rat. *Free Radic Biol Med* 26: 309–317.

Takagi Y., Mitsui A., Nishiyama A., Nozaki K., Sono H., Gon Y., Hashimoto N., Yodoi J. (1999). Overexpression of thioredoxin in transgenic mice attenuates focal ischemic brain damage. *Proc Natl Acad Sci USA* 96: 4131–4136.

Talent J.M., Kong Y., Gracy R.W. (1998). A double stain for total and oxidized proteins from two-dimensional fingerprints. *Anal Biochem* 263: 31–38.

Tamarit J., Cabiscol E., Ros J. (1998). Identification of the major oxidatively damaged proteins in *Escherichia coli* cells exposed to oxidative stress. *J Biol Chem* 273: 3027–3032.

Tanito M., Nakamura H., Kwon Y.W., Teratani A., Masutani H., Shioji K., Kishimoto C., Ohira A., Horie R., Yodoi J. (2004). Enhanced oxidative stress and impaired thioredoxin expression in spontaneously hypertensive rats. *Antioxid Redox Signal* 6: 89–97.

Tate D.J., Jr., Miceli M.V., Newsome D.A. (1999). Zinc protects against oxidative damage in cultured human retinal pigment epithelial cells. *Free Radic Biol Med* 26: 704–713.

Telci A., Cakatay U., Akhan S.E., Bilgin M.E., Turfanda A., Sivas A. (2002). Postmenopausal hormone replacement therapy use decreases oxidative protein damage. *Gynecol Obstet Invest* 54: 88–93.

Tesoriere L., Ciaccio M., Valenza M., Bongiorno A., Maresi E., Albiero R., Livrea M.A. (1994). Effect of vitamin A administration on resistance of rat heart against doxorubicin-induced cardiotoxicity and lethality. *J Pharmacol Exp Ther* 269: 430–436.

Thiele J.J., Hsieh S.N., Briviba K., Sies H. (1999). Protein oxidation in human stratum corneum: Susceptibility of keratins to oxidation in vitro and presence of a keratin oxidation gradient in vivo. *J Invest Dermatol* 113: 335–339.

Tien M., Berlett B.S., Levine R.L., Chock P.B., Stadtman E.R. (1999). Peroxynitrite-mediated modification of proteins at physiological carbon dioxide concentration: pH Dependence of carbonyl formation, tyrosine nitration, and methionine oxidation. *Proc Natl Acad Sci USA* 96: 7809–7814.

Tomas-Zapico C., Martinez-Fraga J., Rodriguez-Colunga M.J., Tolivia D., Hardeland R., Coto-Montes A. (2002). Melatonin protects against delta-aminolevulinic acid-induced oxidative damage in male Syrian hamster Harderian glands. *Int J Biochem Cell Biol* 34: 544–553.

Toth A., Halmosi R., Kovacs K., Deres P., Kalai T., Hideg K., Toth K., Sumegi B. (2003). Akt activation induced by an antioxidant compound during ischemia-reperfusion. *Free Radic Biol Med* 35: 1051–1063.

Troncoso JC, Costello AC, Kim JH, Johnson GVW. (1995). Metal-catalyzed oxidation of bovine neurofilaments in vitro. *Free Radic Biol Med* 18: 891–899.

Tsai G., Goff D.C., Chang R.W., Flood J., Baer L., Coyle J.T. (1998). Markers of glutamatergic neurotransmission and oxidative stress associated with tardive dyskinesia. *Am J Psychiatry* 155: 1207–1213.

Uchida K., Fukuda A., Kawakishi S., Hiai H., Toyokuni S. (1995). A renal carcinogen ferric nitriloacetate mediates a temporary accumulation of aldehyde-modified proteins within cytosolic compartment of rat kidney. *Arch Biochem Biophys* 317: 405–411.

Valencia C., Cornejo P., Romanque P., Tapia G., Varela P., Videla L.A., Fernandez V. (2004). Effects of acute lindane intoxication and thyroid hormone administration in relation to nuclear factor-kappaB activation, tumor necrosis factor-alpha expression, and Kupffer cell function in the rat. *Toxicol Lett* 148: 21–28.

Vanderlelie J., Venardos K., Clifton V.L., Gude N.M., Clarke F.M., Perkins A.V. (2005). Increased biological oxidation and reduced anti-oxidant enzyme activity in pre-eclamptic placentae. *Placenta* 26: 53–58.

Varma S.D., Devamanoharan P.S. (1995). Oxidative denaturation of lens protein: Prevention by pyruvate. *Ophthalmic Res* 27: 18–22.

Varma S.D., Devamanoharan P.S., Mansour S., Teter B. (1994). Studies on Emory mouse cataracts: Oxidative factors. *Ophthalmic Res* 26: 141–148.

Varsila E., Pesonen E., Andersson S. (1995). Early protein oxidation in the neonatal lung is related to development of chronic lung disease. *Acta Paediatr* 84: 1296–1299.

Veasey S.C., Davis C.W., Fenik P., Zhan G., Hsu Y.J., Pratico D., Gow A. (2004). Long term intermittent hypoxia in mice: protracted hypersomnolence with oxidative injury to sleepwake brain regions. *Sleep* 27: 194–201.

Videla L.A., Tapia G., Varela P., Cornejo P., Guerrero J., Israel Y., Fernandez V. (2004). Effects of acute gamma-hexachlorocyclohexane intoxication in relation to the redox regulation of nuclear factor-kappaB, cytokine gene expression, and liver injury in the rat. *Antioxid Redox Signal* 6: 471–480.

Viljanen K., Kylli P., Kivikari R., Heinonen M. (2004). Inhibition of protein and lipid oxidation in liposomes by berry phenolics. *J Agric Food Chem* 52: 7419–7424.

Vissers M.C., Winterbourn C.C. (1991). Oxidative damage to fibronectin. I. The effects of the neutrophil myeloperoxidase system and HOCl. *Arch Biochem Biophys* 285: 53–59.

Vissers M.N., Zock P.L., Wiseman S.A., Meyboom S., Katan M.B. (2001). Effect of phenolrich extra virgin olive oil on markers of oxidation in healthy volunteers. *Eur J Clin Nutr* 55: 334–341.

Wander R.C., Du S.H. (2000). Oxidation of plasma proteins is not increased after supplementation with eicosapentaenoic and docosahexaenoic acids. *Am J Clin Nutr* 72: 731–737.

Wang Y., Feinstein S.I., Manevich Y., Ho Y.S., Fisher A.B. (2004a). Lung injury and mortality with hyperoxia are increased in peroxiredoxin 6 gene-targeted mice. *Free Radic Biol Med* 37: 1736–1743.

Wang Y., Manevich Y., Feinstein S.I., Fisher A.B. (2004b). Adenovirus-mediated transfer of the 1-cys peroxiredoxin gene to mouse lung protects against hyperoxic injury. *Am J Physiol Lung Cell Mol Physiol* 286: L1188–L1193.

Wang Y., Oberley L.W., Murhammer D.W. (2001). Evidence of oxidative stress following the viral infection of two lepidopteran insect cell lines. *Free Radic Biol Med* 31: 1448–1455.

Wells P.G., Kim P.M., Laposa R.R., Nicol C.J., Parman T., Winn L.M. (1997). Oxidative damage in chemical teratogenesis. *Mutat Res* 396: 65–78.

Wen J.J., Garg N. (2004). Oxidative modification of mitochondrial respiratory complexes in response to the stress of *Trypanosoma cruzi* infection. *Free Radic Biol Med* 37: 2072–2081.

Wieland P., Lauterburg B.H. (1995). Oxidation of mitochondrial proteins and DNA following administration of ethanol. *Biochem Biophys Res Commun* 213: 815–819.

Williams M.D., Van Remmen H., Conrad C.C., Huang T.T., Epstein C.J., Richardson A. (1998). Increased oxidative damage is correlated to altered mitochondrial function in heterozygous manganese superoxide dismutase knockout mice. *J Biol Chem* 273: 28510–28515.

Winn L.M., Wells P.G. (1997). Evidence for embryonic prostaglandin H synthase-catalyzed bioactivation and reactive oxygen species-mediated oxidation of cellular macromolecules in phenytoin and benzo[a]pyrene teratogenesis. *Free Radic Biol Med* 22: 607–621.

Winter M.L., Liehr J.G. (1996). Possible mechanism of induction of benign prostatic hyperplasia by estradiol and dihydrotestosterone in dogs. *Toxicol Appl Pharmacol* 136: 211–219.

Winter M.L., Liehr J.G. (1991). Free radical-induced carbonyl content in protein of estrogen-treated hamsters assayed by sodium boro[^{3}H]ydride reduction. *J Biol Chem* 266: 14446–14450.

Winterbourn C.C., Bonham M.J., Buss H., bu-Zidan F.M., Windsor J.A. (2003). Elevated protein carbonyls as plasma markers of oxidative stress in acute pancreatitis. *Pancreatology* 3: 375–382.

Winterbourn C.C., Buss I.H., Chan T.P., Plank L.D., Clark M.A., Windsor J.A. (2000). Protein carbonyl measurements show evidence of early oxidative stress in critically ill patients. *Crit Care Med* 28: 143–149.

Witt E.H., Reznick A.Z., Viguie C.A., Starke-Reed P.E., Packer L. (1992). Exercise, oxidative damage, and effects of antioxidant manipulation. *J Nutr* 122: 766–773.

Wolfgang G.H., Jolly R.A., Petry T.W. (1991). Diquat-induced oxidative damage in hepatic microsomes: effects of antioxidants. *Free Radic Biol Med* 10: 403–411.

Wondrak G.T., Cervantes-Laurean D., Jacobson E.L., Jacobson M.K. (2000). Histone carbonylation in vivo and in vitro. *Biochem J* 351(3): 769–777.

Wurzel H., Yeh C.C., Gairola C., Chow C.K. (1995). Oxidative damage and antioxidant status in the lungs and bronchoalveolar lavage fluid of rats exposed chronically to sigarette smoke. *J Biochem Toxicol* 10: 11–17.

Yan L.J., Levine R.L., Sohal R.S. (1997a). Oxidative damage during aging targets mitochondrial aconitase. *Proc Natl Acad Sci USA* 94: 11168–11172.

Yan L.J., Lodge J.K., Traber M.G., Packer L. (1997b). Apolipoprotein B carbonyl formation is enhanced by lipid peroxidation during copper-mediated oxidation of human low-density lipoproteins. *Arch Biochem Biophys* 339: 165–171.

Yan L.J., Sohal R.S. (1998). Mitochondrial adenine nucleotide translocase is modified oxidatively during aging. *Proc Natl Acad Sci USA* 95: 12896–12901.

Yan L.J., Traber M.G., Kobuchi H., Matsugo S., Tritschler H.J., Packer L. (1996). Efficacy of hypochlorous acid scavengers in the prevention of protein carbonyl formation. *Arch Biochem Biophys* 327: 330–334.

Yang C.Y., Gu Z.W., Yang H.X., Yang M., Gotto A.M., Jr., Smith C.V. (1997). Oxidative modifications of apoB-100 by exposure of low density lipoproteins to HOCl in vitro. *Free Radic Biol Med* 23: 82–89.

Yasuda K., Adachi H., Fujiwara Y., Ishii N. (1999). Protein carbonyl accumulation in aging dauer formation-defective (daf) mutants of *Caenorhabditis elegans*. *J Gerontol A Biol Sci Med Sci* 54: B47–B51.

Yin D., Yuan X., Brunk U.T. (1995). Test-tube simulated lipofuscinogenesis. Effect of oxidative stress on autophagocytotic degradation. *Mech Ageing Dev* 81: 37–50.

Yoo B.S., Regnier F.E. (2004). Proteomic analysis of carbonylated proteins in two-dimensional gel electrophoresis using avidin-fluorescein affinity staining. *Electrophoresis* 25: 1334–1341.

Yoritaka A., Hattori N., Uchida K., Tanaka M., Stadtman E.R., Mizuno Y. (1996). Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. *Proc Natl Acad Sci USA* 93: 2696–2701.

Young J.F., Steffensen C.L., Nielsen J.H., Jensen S.K., Stagsted J. (2002). Chicken model for studying dietary antioxidants reveals that apple (Cox's Orange)/broccoli (*Brassica oleracea L.* var. *italica*) stabilizes erythrocytes and reduces oxidation of insoluble muscle proteins and lipids in cooked liver. *J Agric Food Chem* 50: 5058–5062.

Youngman L.D., Park J.Y., Ames B.N. (1992). Protein oxidation associated with aging is reduced by dietary restriction of protein or calories. *Proc Natl Acad Sci USA* 89: 9112–9116.

Yuan Z., Shioji K., Nimata M., Kishimoto C. (2004). Cardioprotective effects of carvedilol on acute autoimmune myocarditis. *Mol Cell Biochem* 259: 223–227.

Zainal T.A., Oberley T.D., Allison D.B., Szweda L.I., Weindruch R. (2000). Caloric restriction of rhesus monkeys lowers oxidative damage in skeletal muscle. *FASEB J* 14: 1825–1836.

Zainal T.A., Weindruch R., Szweda L.I., Oberley T.D. (1999). Localization of 4-hydroxy-2- nonenal-modified proteins in kidney following iron overload. *Free Radic Biol Med* 26: 1181–1193.

Zardeneta G., Milam S.B., Schmitz J.P. (2000). Iron-dependent generation of free radicals: Plausible mechanisms in the progressive deterioration of the temporomandibular joint. *J Oral Maxillofac Surg* 58: 302–308.

Zavodnik I.B., Lapshina E.A., Zavodnik L.B., Labieniec M., Bryszewska M., Reiter R.J. (2004). Hypochlorous acid-induced oxidative stress in Chinese hamster B14 cells: Viability, DNA and protein damage and the protective action of melatonin. *Mutat Res* 559: 39–48.

Zhang Y.T., Zheng Q.S., Pan J., Zheng R.L. (2004a). Oxidative damage of biomolecules in mouse liver induced by morphine and protected by antioxidants. *Basic Clin Pharmacol Toxicol* 95: 53–58.

Zhang Z., Dmitrieva N.I., Park J.H., Levine R.L., Burg M.B. (2004b). High urea and NaCl carbonylate proteins in renal cells in culture and in vivo, and high urea causes 8-oxoguanine lesions in their DNA. *Proc Natl Acad Sci USA* 101: 9491–9496.

Zhu B.Z., Carr A.C., Frei B. (2002). Pyrrolidine dithiocarbamate is a potent antioxidant against hypochlorous acid-induced protein damage. *FEBS Lett* 532: 80–84.

Zusterzeel P.L., Mulder T.P., Peters W.H., Wiseman S.A., Steegers E.A. (2000). Plasma protein carbonyls in nonpregnant, healthy pregnant and preeclamptic women. *Free Radic Res* 33: 471–476.

Zusterzeel P.L., Steegers-Theunissen R.P., Harren F.J., Stekkinger E., Kateman H., Timmerman B.H., Berkelmans R., Nieuwenhuizen A., Peters W.H., Raijmakers M.T., Steegers E.A. (2002). Ethene and other biomarkers of oxidative stress in hypertensive disorders of pregnancy. *Hypertens Pregnancy* 21: 39–49.

6

S-NITROSATION OF CYSTEINE THIOLS AS A REDOX SIGNAL

YANHONG ZHANG AND NEIL HOGG

6.1 INTRODUCTION

The post-translational modification of cysteinyl residues in proteins has long been thought of as a potential mechanism by which oxidative signals can be transduced into cellular events. Cysteine is an underrepresented amino acid in mammalian proteins (2.26% in humans as compared to 3.23% expected) and rarer in prokaryotes (~1% occurrence) (Miseta et al., 2000). It plays a structural role in the formation of disulfide bonds, particularly in extracellular proteins, and is often found as a metal ligand in, for example, iron sulfur clusters and zinc thiolate structures. Indeed the metal binding sequence of $C-(X)_2-C$ appears to be an early evolutionary development (Miseta et al., 2000). The strong nucleophilicity of the thiolate anion gives cysteine unique reactivity among the amino acids, which allows a subset of important catalytic reactions highlighted by the cysteine proteases. In addition, however, cysteine residues, not necessarily in the active site, appear to play an important regulatory role through their propensity to be oxidatively modified. This is the domain of the redox signaling paradigm in which alterations in cellular redox status can lead to the modulation of protein expression and other cellular events. A number of different cysteine modifications have been recognized. Perhaps the most well studied is the dynamic formation of inter- or intrapeptide disulfides, or disulfides with low molecular weight thiols (this latter modification is referred to as protein *S*-thiolation). In addition, however, thiols can react with electrophilic compounds such as aldehydes formed from nonenzymatic lipid oxidation, and other lipid oxidation products (Levonen et al., 2004),

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

and this is being increasingly recognized as a novel mechanism of transducing lipid and fatty acid oxidation to changes in cell phenotype. In this chapter we will deal with a third mode of cysteine modification that is mediated by nitric oxide (NO)—the formation of *S*-nitrosothiols. These compounds, thioesters of nitrite with the generic structure of R–S–N=O, have been observed at low levels under basal conditions in several tissues. The formation of *S*-nitrosothiols has been suggested to be an important post-translational cysteine modification that is involved in NO-mediated signal transduction. In vitro experiments have readily established that the nitrosation of enzyme cysteinyl residues can affect activity if the cysteine residue in question is involved in the catalytic, binding or regulatory functions of the protein; in addition *S*-nitrosation has been invoked in the regulation of several in vivo pathways. There is consequently a tremendous potential for *S*-nitrosation as a transducer of NO-dependent cellular events, so much so that parallels to phosphorylation have been made (Stamler et al., 2001; Gow et al., 2004).

It must be stated at the outset that the study of *S*-nitrosation, as with many other oxidative protein modifications (e.g., tyrosine nitration), is a difficult endeavor. Such investigations are prone to artifacts and currently used methodologies have given levels of *S*-nitrosothiols that are often at odds by orders of magnitude. It is tempting to use the difficulties involved in the accurate assessment of *S*-nitrosation as a reason to lower the burden of proof required to definitively establish this modification as an element in a signal transduction pathway. However, this temptation should be strongly resisted.

As yet there is no reliable method for the immunological detection of protein *S*-nitrosothiols, and radiolabeling is not a viable option. While the methodological advancement in the analytical detection of *S*-nitrosothiols has allowed increasingly accurate assessments of their physiological and cellular levels, there is a requirement for significant methodological development in order to assess robustly the role of this modification in signaling processes. In this chapter we will examine the formation and transport of *S*-nitrosothiols in cells and discuss the role of *S*-nitrosation in the context of signal transduction pathways.

6.2 MECHANISMS OF FORMATION OF *S*-NITROSOTHIOLS

S-Nitrosothiols have been detected in vivo at low concentrations (Gaston et al., 1993; Jia et al., 1996; Kluge et al., 1997; Feelisch et al., 2002). Increases in *S*-nitrosothiol content have been observed during endotoxemia (Jourd'heuil et al., 2000) and during infusion of exogenous nitrovasodilators (Janero et al., 2004). However, NO itself is not a nitrosating agent and will not nitrosate thiols to form *S*-nitrosothiols. This statement should be italicized, bolded, and underlined, as to-date, despite the wealth of accumulated knowledge on this subject, many publications still contain schemes that show NO reacting with thiols to form *S*-nitrosothiols, and the underlying biological chemistry of this modification (one of the most crucial issue in the field of *S*-nitrosation as a post-translational modification) is often glossed over. NO reacts directly with thiols by a slow oxidative

mechanism to form disulfides (Pryor et al., 1982; Hogg et al., 1996; Folkes et al., 2004). How NO becomes a nitrosating agent in biological systems is currently unknown, but several possibilities exist. The first involves the one electron oxidation of NO to form the nitrosonium cation (NO^+). While this species is thought to have little reactivity in aqueous solution, hydrolyzing immediately to nitrite, if it is formed in the active site of an enzyme in the vicinity of a thiol, it has the possibility of being trapped by a thiolate before release. Another possibility is that NO is oxidized to nitrogen dioxide (NO_2), which rapidly combines with NO to form dinitrogen trioxide (N_2O_3), which in turn can directly nitrosate thiols. A third proposed mechanism involves oxidation of the thiol to a thiyl radical followed by the radical–radical recombination with NO. Finally, a fourth possibility involves the direct reduction of an electron acceptor by the thiol/NO adduct. Note that it is only in this latter mechanism that the direct reaction between NO and thiol is invoked. While specific enzyme-catalyzed nitrosation reactions are possible, none have yet been identified in cells.

While reactions in simple chemical systems are informative, care should always be taken when extrapolating these findings to biological systems. However, studies of the reaction between NO and glutathione (GSH), in the presence of oxygen, illustrate that even such supposedly simple models contain rich and complex chemistry. In such systems, nitrosation of the GSH, to form *S*-nitrosoglutathione (GSNO), can occur by four possible routes (Fig. 6.1). Three of these (pathways 1, 2, and 3) are limited by the third-order reaction between NO and oxygen (Wink et al., 1996) to form nitrogen dioxide (Goldstein et al., 1996a,b). Nitrogen dioxide may then react with NO to form N_2O_3, which can directly nitrosate thiols (pathway 1) (Kharitonov et al., 1995). Alternatively, the reaction between GSH and nitrogen dioxide can generate thiyl radical and nitrite, and a radical–radical reaction between NO and the thiyl radical can give rise to the nitrosated thiol (pathway 2) (Goldstein et al., 1996b; Jourd'heuil et al., 2003). The downstream production of superoxide, upon reaction with NO, can give rise to peroxynitrite (Beckman et al., 1990). This latter species has been shown to nitrosate thiols with less than 1% efficiency (pathway 3) (Moro et al., 1994). The mechanism by which peroxynitrite directly nitrosates thiols is uncertain, although direct addition, followed by hydrogen peroxide elimination has been suggested (van der Vliet et al., 1998). However, it has also been reported that peroxynitrite will oxidize thiols to thiyl radicals that may react with NO to form *S*-nitrosothiols. Espey and co-workers (Espey et al., 2002) have shown that the nitrosating propensity of NO, as measured by increases in diaminonaphthaline triazole fluorescence, can be enhanced at low rates but inhibited at high rates of superoxide formation, suggesting that the presence of an additional superoxide source could enhance nitrosation via peroxynitrite formation. However, other observations have suggested superoxide may reduce *S*-nitrosothiols to form peroxynitrite (Trujillo et al., 1998), indicating that the role of superoxide in *S*-nitrosothiol formation is likely complex.

Considering its third-order rate law, the NO/oxygen reaction should be very slow at physiological concentrations of NO ($\leq$0.1 μM) and oxygen (20–50 μM).

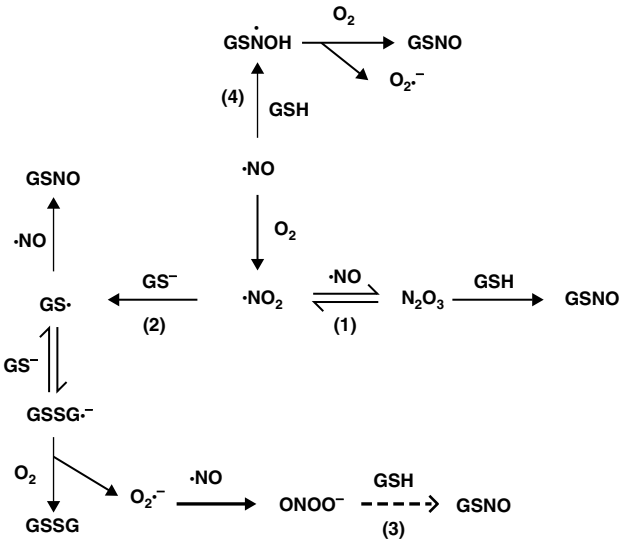


FIGURE 6.1 Possible pathways for the formation of GSNO from GSH, NO, and oxygen. The formation of GSNO from GSH, NO, and oxygen can occur via multiple pathways. Pathways 1, 2, and 3 depend on the formation of nitrogen dioxide from the oxygen-dependent oxidation of NO. In pathway 1, dinitrogen trioxide directly nitrosates GSH to form GSNO. In pathway 2, nitrogen dioxide oxidizes GSH to form thiyl radical, which combines with NO to form GSNO (referred to as oxidative nitrosylation). Pathway 3 occurs via superoxide formation from downstream reactions of the thiyl radical. Superoxide can react with NO to form peroxynitrite, which can either directly nitrosate thiols or generate additional thiyl radicals. Pathway 4 invokes the direct reduction of oxygen, or another electron acceptor, by a putative NO/GSH adduct. While speculative, this is the only pathway that is not constrained by the NO/oxygen reaction.

It has been shown that the reaction of NO with oxygen is accelerated in hydrophobic environments due to the high solubility of both reactants in the hydrophobic phase. Therefore, the NO/oxygen reaction may be facilitated in the hydrophobic interior of membranes (Liu et al., 1998) and proteins (Rafikova et al., 2002). This does not automatically imply that such hydrophobic environments will accelerate the rate of *S*-nitrosothiol formation, as the rate limit of this process in biological systems is likely the NO formation rate. However, it is possible that such processes could divert NO from other fates and so increase the yield of *S*-nitrosation. A report that N_2O_3 has a half-life of over seven minutes within serum albumin is difficult to square with this molecule's known physical and chemical properties (Rafikova et al., 2002). There is evidence, however, that oxygen-dependent NO consumption can occur more rapidly in hepatocytes with a first-order dependence on NO (Thomas et al., 2002), suggesting that, at least in some cases, NO consumption mechanisms are not limited by the third-order NO/oxygen reaction.

A fourth mechanism for thiol nitrosation in the simple chemical system, proposed by Gow and co-workers (Gow et al., 1997) (pathway 4 in Fig. 6.1),

involved the direct reaction between NO and the thiol to generate a putative free radical intermediate (RSNOH), which then reduces oxygen to form superoxide and *S*-nitrosothiol. Under anaerobic condition RSNOH radical was shown to react with an electron acceptor, such as oxidized nicotine nucleotides (NAD^+), to form *S*-nitrosothiol. However, the rate of *S*-nitrosothiol formation observed in their system had a second-order dependency on NO concentration, inconsistent with their proposed mechanism that would predict a first-order dependency on NO concentration. The basic premise of this study was that the majority of kinetic studies of nitrosation were performed at high NO concentrations, but at low concentrations the mechanisms could be distinctly different. While the mechanism proposed in this study remains speculative, the idea that a more physiological route of *S*-nitrosation is masked by the NO/oxygen reaction when using nonphysiological level of NO is highly pertinent.

Biological nitrosation may well be catalyzed by enzymatic processes, and much interest has been placed in attempting to uncover such catalysts with little success. Metalloproteins that are amenable to one-electron redox chemistry are ideal sites for catalyzing nitrosation, and plasma ceruloplasmin has been invoked as a potential locus of *S*-nitrosothiol formation (Inoue et al., 1999). The fact that there is little to no *S*-nitrosothiol present in plasma (<20 nM) (Marley et al., 2001) suggests that this route of *S*-nitrosothiol formation is not very active, perhaps due to the avid NO scavenging activity of hemoglobin (Wang et al., 2004). In the cytosol no definitive mechanism for *S*-nitrosothiol formation has been uncovered. It has been suggested that hemoglobin can self-nitrosate the β-93 cysteinyl residue by a process of NO migration from heme to thiol (McMahon et al., 2002), although this mechanism has been disputed (Xu et al., 2003). Reductive nitrosylation of ferric hemoglobin by NO has been shown to generate *S*-nitrosothiols under anaerobic conditions (Luchsinger et al., 2003), but it is hard to conceive how this reaction would compete with diffusion limited NO scavenging by ferrous hemoglobin. The bedbug (*Cimex lectularius*) nitrophorins exhibit reversible *S*-nitrosation of the heme thiolate ligand, in parallel with reductive nitrosylation of the heme upon reaction with NO (Weichsel et al., 2005). Although both "free" iron and copper ions have the potential to be important mediators of *S*-nitrosothiols formation, their role in the intracellular environment remains speculative.

It has long been speculated that peroxidases have the capacity to promote nitrosation through the ability of NO to reduce both compound I and compound II, presumable forming NO^+ (Glover et al., 1999). However, it has not been possible to "capture" this intermediate as an *S*-nitrosothiol because of its rapid rate of hydrolysis to nitrite. Peroxidases have also been shown to oxidize inorganic nitrite to nitrogen dioxide (van der Vliet et al., 1997), which, in the presence of NO, should lead to nitrosation either through N_2O_3 or thiyl radical-mediated mechanisms. The *N*-nitrosation of a quinoline derivative has recently shown to be enhanced by myeloperoxidase/H_2O_2 (Lakshmi et al., 2005), but to date there are no reports of *S*-nitrosation by these mechanisms.

One question that has not been particularly well addressed in the literature is how much total *S*-nitrosothiols can be formed upon exposure of cells to NO. Published reports vary dramatically. In endotoxin/cytokine activated macrophages, levels have been reported to be between 20 and 200 pmol/mg protein (Eu et al., 2000; Feelisch et al., 2002; Zhang et al., 2004a). Basal endothelial cell levels have been reported to be 15 pmol/mg protein, rising to over 40 pmol/mg upon stimulation with calcium ionophore (Gow et al., 2002). Other reports in endothelial cells have reported untenable high levels of cellular *S*-nitrosothiols at levels of 20 nmol/mg protein (Hoffman et al., 2001; Hoffmann et al., 2003) (assuming an average protein molecular weight of 70 kDa, this amounts to over 1 *S*-nitrosothiol/protein).

The known chemistry of NO-mediated thiol nitrosation discussed above indicates that the rate-limiting step of nitrosation (the reaction of NO with oxygen) is slow under biological conditions and is not competitive with the diffusion of NO. However, evidence exists (albeit indirect) that the location of nitric oxide synthase (NOS) can alter nitrosation-mediated signaling pathways (Barouch et al., 2002). This would allow the control of nitrosation based upon selective trafficking of NOS. As illustrated and described in Figure 6.2, nitrosation via intercrine or autocrine mechanisms occurs through the diffusion of NO to the extracellular environment or to neighboring cells before it is converted to a nitrosating agent, likely through reaction with oxygen. In contrast, intracrine mechanisms require that NO be "captured" by a thiol before it leaves the cell (i.e., this reaction is competitive with diffusion), and so decreasing the distance between the target thiol and the source of NO would increase the efficiency of nitrosation. This could be achieved if both the target protein and NOS bind to the same "adaptor" protein or by localization of NOS to a specific organelle or cell membrane. While this idea is intriguing, it cannot be reconciled with the known chemistry and diffusibility of NO and therefore would require a novel, rapid, and concerted mechanism of biological nitrosation. A simple test for intracrine mechanisms, in cell culture, is that it should not be affected by the presence of an extracellular NO scavenger such as oxyhemoglobin, as this mechanism requires that the NO that forms the *S*-nitrosothiol does not diffuse out of the cell.

We examined intracellular *S*-nitrosothiol formation by either exposing cells to exogenous NO, in the form of the NO donor spermine NONOate (Zhang et al., 2004b), or activating iNOS expression in RAW 264.7 cells using endotoxin (Zhang et al., 2004a). Exposure of cells to 500 μM spermine NONOate for 1 hour resulted in an intracellular *S*-nitrosothiol level of 30 to 50 pmol/mg protein. Endogenous NO, generated at a rate of about 5 to 6 μM per hour, resulted in the formation of even lower levels of intracellular *S*-nitrosothiols (approximately 20 pmol/mg protein after 15 hours). Examination of the mechanism of *S*-nitrosothiols formation indicated that the *S*-nitrosation reaction is not confined within the cell that generates the NO. On the contrary, we showed, using oxyhemoglobin, that the NO that is responsible for *S*-nitrosothiol formation must have spent some time in the extracellular environment (Zhang et al., 2004a). We also demonstrated that the extracellular conversion of NO to nitrogen dioxide (and

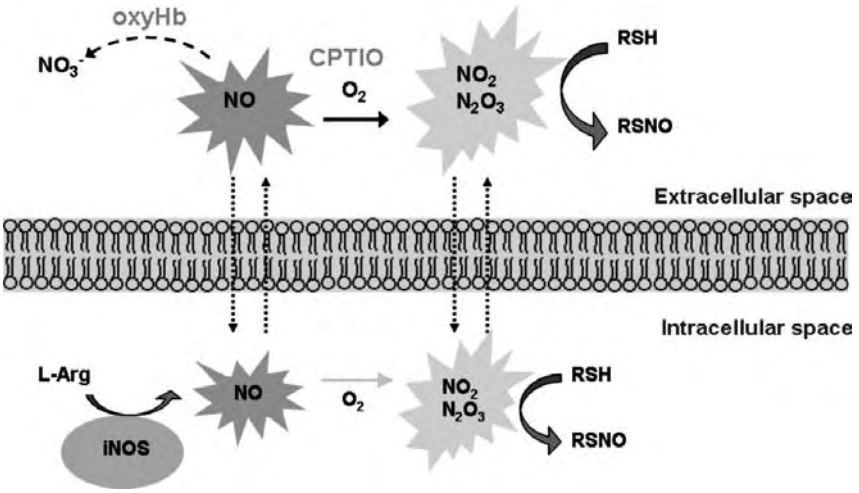


FIGURE 6.2 *S*-Nitrosation in the context of the cell: Intercrine versus intracrine mechanisms. This figure illustrates the so-called intercrine and intracrine mechanisms of nitrosation. For intercrine/autocrine routes of nitrosation (*black arrows*), an NO molecule is generated by NOS and diffuses out of the cell. At some point in time the NO molecule reacts with oxygen to form nitrogen dioxide and dinitrogen trioxide, which (see Fig. 6.1) may both lead to *S*-nitrosothiol formation. These nitrosating intermediates may themselves diffuse across cell membranes to nitrosate thiols in either the intracellular or extracellular space. The result of this mode of nitrosation is that the nitrosothiol may be formed many cell diameters away from the site of NO synthesis. For intracrine modes of nitrosation (*light gray arrow*), the NO molecule forms an *S*-nitrosothiol within the vicinity of its site of synthesis, allowing for enhancement of nitrosation by co-localizing NO synthase and the target on, for example, an adapter protein. For this mechanism the nitrosation reaction would have to be competitive with diffusion (i.e., a rate constant of $10^9 - 10^{10}$ M^{-1} s^{-1}), far faster than the rate-limiting step of any currently known mechanisms of thiol nitrosation. A differentiating test for these modes of nitrosation is the inclusion of an extracellular NO scavenger (e.g., oxyhemoglobin or 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide, CPTIO) that will interfere with the intercrine pathway while leaving intracrine pathway untouched.

consequently N_2O_3) only slightly inhibits intracellular nitrosation. From these data we can conclude that both NO and its major nitrosating oxidation products (NO_2/N_2O_3) are freely diffusible across cell membranes and can nitrosate target thiols distant from their point of generation and that intracrine nitrosation (i.e., the directed local nitrosation of a thiolic target within the same cell as the source of NO) is not a significant mechanism of nitrosation observed in this system. While the precise mechanism of nitrosation in cells is not understood, our data strongly suggest that the nitrosation reaction is not competitive with diffusion and requires a steady state level of NO that is created by both intracellular formation and diffusion from neighboring cells and the extracellular space.

Once *S*-nitrosothiols have been formed in the cell, the process of transnitrosation, in which a thiolate anion nucleophilically attacks the nitrogen atom of an *S*-nitrosothiol, can result

$$\mathrm{RSNO} + \mathrm{R'SH} \rightleftharpoons \mathrm{RSH} + \mathrm{R'SNO} \quad (1)$$

in the transfer of the nitroso group to the thiol (reaction 1). This reaction follows second-order reversible kinetics (Hogg, 1999) and can be a route to form novel *S*-nitrosothiols. A consensus motif (with acidic or basic amino acids adjacent to the cysteine residue) has been proposed to facilitate transnitrosation between low-molecular-weight *S*-nitrosothiols and protein thiols via acid–base catalysis (Stamler et al., 1997). As the thiol pK_a is crucial for transnitrosation and the pK_a of a thiol may be altered by amino acids that are close in space, it is likely that the thiol environment rather than a consensus sequence is an important factor for protein *S*-nitrosothiol formation (Ascenzi et al., 2000).

6.3 CELLULAR TRANSDUCTION OF THE *S*-NITROSO GROUP

In order to fully appreciate the role of *S*-nitrosothiols in NO-mediated signaling pathways, it is essential to understand the mechanisms by which *S*-nitrosothiol or their bound *S*-nitroso groups are transferred between the extracellular and intracellular spaces. Exogenously added *S*-nitrosothiols are used as NO donor molecules to study NO-mediated nitrosation events. It is often assumed that these compounds spontaneously decay to form NO, which diffuses across the cell membrane to elicit a biological response. However, it is far from clear whether the cellular responses are due to NO or *S*-nitrosothiols. A thorough understanding of the mechanisms of *S*-nitrosothiol transport will allow differentiation of the direct NO-mediated effects from *S*-nitrosothiol-mediated events.

Several mechanisms have been proposed for the transport of *S*-nitrosothiols across cell membranes. Protein disulfide isomerase (PDI) is an enzyme that assists in protein folding in endoplasmic reticulum by catalyzing protein disulfide bond formation, reduction or isomerization. Recently PDI has been found to localize on the plasma membrane of mammalian cells (Turano et al., 2002) and is termed as cell surface-PDI (cs-PDI). It was reported that inhibition of cs-PDI expression, using an antisense oligodeoxynucleotide, decreased cs-PDI activity as well as cyclic guanosine monophosphate (cGMP) generation upon *S*-nitrosothiol exposure (Zai et al., 1999). Ramachandran et al. (2001) proposed that cs-PDI catalyzes NO release from extracellular *S*-nitrosothiols; NO then accumulates in the cell membrane and reacts with oxygen to produce N_2O_3 (Zai et al., 1999), which nitrosates intracellular thiols. Recently a report suggested that metabolism of *S*-nitrosothiols by platelets is due to the denitrosation activity of PDI (Root et al., 2004). γ-Glutamyl transpeptidase (γ-GT) is a membrane-bound enzyme that catalyzes the hydrolysis of the γ-glutamyl residue of GSH, glutathione disulfide, and glutathione conjugates, and also the transfer of the

γ-glutamyl moiety to amino acids and peptides (Curthoys et al., 1979). γ-GT has been shown to decompose GSNO to glutamate and *S*-nitrosocysteinylglycine without the release of NO (Hogg et al., 1997). It has been suggested that the sequential actions of γ-GT and dipeptide permease transport *S*-nitroso group into cells in a *Salmonella typhimurium* model (De Groote et al., 1995). Anion exchanger (AE1, also referred to as band 3) at the red blood cell (RBC) membrane has been suggested to be involved in the transfer of the *S*-nitroso group across cell membranes as well (Pawloski et al., 2001).

There are several studies that have invoked stereoselective effects of *S*-nitrosothiols, suggesting that the bioactivity of *S*-nitrosothiols was mediated by interaction with a chiral target on a cell surface. In particular, both L-*S*-nitrosocysteine (CysNO) and L-*S*-nitrosopenicillamine (SNAP) were reported to affect hemodynamic parameters to a greater extent than their D-isomers, although the rate of degradation of both isomers was identical (Davisson et al., 1996, 1997). These studies suggest that the bioactivity of *S*-nitrosothiols is not due to spontaneous NO release but requires association with a cell surface receptor.

Studies in cell culture have also revealed stereoselective effects. For example, it was shown that the combination of NO and L-cysteine resulted in noradrenaline release from rat hippocampus, by a mechanism that was inhibitable by L-leucine and not D-leucine and also by the amino acid transporter system L (L-AT) inhibitor, 2-aminobicyclo[2.2.1]-heptane-2-carboxylate (BCH) (Satoh et al., 1996, 1997). These studies suggested that the L-AT system was important for the uptake of L-CysNO. Mallis and Thomas observed the intracellular formation of over 15 nmol/mg GSNO after exposure of NIH 3T3 cells to 1 mM CysNO for 3 hours (Mallis et al., 2000). Their data indicate that under these conditions over half of the cellular GSH is *S*-nitrosated. Interestingly GSNO and SNAP were much less effective unless cysteine was present. As the nitroso group can be transferred from *S*-nitrosothiol to cysteine to form CysNO, these data point to a selective uptake of CysNO by cells. In addition, it has been recognized that CysNO can cross the RBC membrane and result in the "loading" of RBC with *S*-nitrosothiols.

We have recently examined the uptake of *S*-nitrosothiols in RAW 264.7 cells (Zhang et al., 2004b) and in bovine aortic endothelial cells (BAEC, manuscript in preparation) by directly monitoring intracellular *S*-nitrosothiols levels by tri-iodide-based chemiluminescence measurements. In agreement with Mallis and Thomas (Mallis et al., 2000), we observed that treatment with L-CysNO resulted in a far higher increase in intracellular *S*-nitrosothiols than treatment with GSNO or SNAP. However, the majority (>95%) of the *S*-nitrosothiol was associated with the high molecular weight component of the cell (>3 kDa). This difference may be related to the fact that RAW 264.7 cells and BAEC contain significant levels of the GSNO metabolizing enzyme GSH-dependent formaldehyde dehydrogenase (Jensen et al., 1998; Liu et al., 2001). The Km of this enzyme for GSNO was determined to be 28 μM (Jensen et al., 1998), suggesting that this enzyme may not be so efficient at metabolizing the low levels of GSNO generated from basal NO formation, but would likely be

important in suppressing increases in cellular *S*-nitrosothiols when cells are placed under nitrosative stress, as exposure of cells to L-CysNO can result in the formation of mM concentrations of intracellular *S*-nitrosothiol. Again in agreement with Mallis and Thomas (Mallis et al., 2000), we observed that uptake of GSNO and SNAP by cells was stimulated by the presence of cysteine. However, we also showed that *S*-nitrosothiol uptake from GSNO, but not from SNAP, was promoted by cystine. Uptake of *S*-nitrosothiols was stereoselective with a much greater uptake of L-CysNO and a much greater stimulation of uptake by the L isomers of cysteine and cystine. In addition intracellular *S*-nitrosothiols formation was inhibited by L- (but not D-) leucine and the L-AT inhibitor BCH. From these data we concluded that the L-AT is the major route by which L-CysNO enters cells. The nitroso group of GSNO per se is not taken up into cells directly but requires transnitrosation to CysNO. Cystine-dependent uptake of *S*-nitrosothiols from GSNO was inhibitable by L-glutamate (Zhang et al., 2004b), suggesting that cystine requires uptake through the x_c^- transporter, intracellular reduction, and export as cysteine. Although much less efficient, cystine also resulted in an increase of intracellular *S*-nitrosothiols as a result of exposure of cells to *S*-nitrosated bovine serum albumin (BSA-SNO), indicating that this route of transport may also apply to protein *S*-nitrosothiols. Recent work (Li et al., 2005) using knockout and small interfering RNA techniques has positively identified both L-AT1 and L-AT2 as major transporters of L- CysNO.

It has been suggested that, in order for the nitroso group of an *S*-nitrosothiol to enter a cell, it needs to be converted to NO, which diffuses through the cell membrane and nitrosates intracellular thiols (Ramachandran et al., 2001; Zai et al., 1999). We tested this notion using the avid NO scavenger oxymyoglobin (Zhang et al., 2004b). This scavenger had no effect on the formation of intracellular *S*-nitrosothiols after exposure to GSNO/cystine, indicating that NO is not an intermediate in this transport process.

One of the major conclusions of these studies is that the effect of exposing cells to *S*-nitrosothiols depends not only on the nature of the *S*-nitrosothiol but on the composition of the medium to which the cells are exposed. Despite the fact that *S*-nitrosothiols are equated with NO in most studies, the effects of exogenous *S*-nitrosothiols on intracellular *S*-nitrosation may have little to do with the cell biology of NO. This is of immense importance to a full understanding of the *S*-nitrosothiol signaling paradigm as it suggests that a large number of studies that invoke intracellular nitrosation as an important mechanism of NO-mediated signal transduction may be observing NO-independent effects of *S*-nitrosothiols.

6.4 *S*-NITROSOTHIOLS AND REDOX PROTEOMICS

Unfortunately, the analysis of the *S*-nitrosated proteome under physiologically interesting conditions is currently beyond the available techniques. The most common technique used for proteomic analysis of *S*-nitrosation, the so-called biotin switch assay developed by Jaffrey et al. (2001), works well in cases where

cells are loaded with *S*-nitrosothiols by incubation with CysNO. Under these conditions many proteins can be observed to be nitrosated, and we have positively identified elongation factor 2, heat-shock protein 90β, and 65 kDa macrophage protein related to L-plastin (Zhang et al., 2005) in RAW 264.7 cells, and many more await identification. While this method has proved useful in *S*-nitrosothiols analysis, it has several limitations. One problem with this assay is that it relies on ascorbate to reduce *S*-nitrosothiols to thiols without significant reduction of disulfides; however, the rates of reaction of *S*-nitrosothiols with ascorbate are very slow (Holmes et al., 2000; Kashiba-Iwatsuki et al., 1996; Zhang et al., 2005). Using the conditions originally employed by Jaffrey et al. (2001) (1 mM ascorbate for 1 hour), we found that only about 15% of GSNO will be reduced and less than 1% of BSA-SNO. Consequently this method may only allow detection of the tip of the iceberg, and perhaps more important, the "tip" that is observed will be those proteins more susceptible to ascorbate-mediated reduction and not a true representation of the *S*-nitrosated proteome. In addition, the absolute sensitivity of this method is not high enough to detect the low pmol/mg levels of *S*-nitrosothiols observed after NO exposure, and several studies that have used this technique to examine the proteome of *S*-nitrosated proteins have enhanced the level of nitrosation by either incubating lysates with GSNO (Foster et al., 2004) or using acidified nitrite (Rhee et al., 2005). The challenge to the field is to identify the nitrosated proteins within the 20 to 200 pmol/mg protein of *S*-nitrosothiols that are observed from NO exposure to cells, rather than 10 to 20 nmol/mg protein of *S*-nitrosothiols that are generated upon exposure of cells to *S*-nitrosothiols. This will require agents that can specifically, and more rapidly, label the *S*-nitrosothiols group that can be detected with high sensitivity.

6.5 *S*-NITROSOTHIOLS AS AN INTRACELLULAR SIGNAL

S-Nitrosothiols were implicated in the mechanism of activation of guanylyl cyclase by organic nitroso compounds and have been shown to elicit vasorelaxing effects through cyclic guanosine monophosphate (cGMP) formation (Ignarro et al., 1980). However, as well as the classic heme targets of NO, it became clear that *S*-nitrosothiols can also modify thiol groups. The reversible modification of protein thiols by *S*-nitrosothiols was examined as early as 1984 in a study of the bacteriostatic action of thiol modifying agents (Chung et al., 2004). Park (1988) later demonstrated that the inhibition of alcohol dehydrogenase by GSNO involves specific modification of a protein thiol and postulated, by analogy to reaction observed with low molecular weight cysteine targets, that both *S*-nitrosation and *S*-thiolation of the protein occur. It is the ability of nitrosothiols to reversibly transfer their nitroso functional group to protein thiols (a reaction referred to as transnitrosation) that has generated significant interest in their potential role as reversible modifiers of enzymatic activity. There are numerous reports of protein inhibition via this process, and transnitrosation has been shown to occur in vivo. The fact that *S*-nitrosothiols are formed in vivo as metabolites

of NO has allowed the development of the idea that these compounds are more than just pharmacological thiol modifying agents, they represent intermediates in endogenous NO-dependent signaling mechanisms.

The idea that *S*-nitrosothiols can act as intracellular signals is based on a series of observations. The first and perhaps most common observation is that *S*-nitrosation of a protein thiol can alter the activity of enzymes in which a thiol group plays an important role. For example, *S*-nitrosation of the caspase family of cysteine proteases at the active site cysteine will render them catalytically inactive (Rossig et al., 2001). In most of these studies nitrosation is achieved by incubating purified protein with a low molecular weight *S*-nitrosothiol (e.g., GSNO or SNAP), which will nitrosate the protein thiol via transnitrosation. It is this reaction that allows for the potential formation and repair of protein *S*-nitrosothiols and gives support to the idea that nitrosation and denitrosation of protein thiols is a dynamic process. There are also several demonstrations of NO-dependent cellular changes that are altered or inhibited by mutating a particular protein thiol, indicating that the thiol group is involved in some aspects of NO-mediated signaling. These observations give strength to the idea that post-translational *S*-nitrosation of thiols may play a role in NO-mediated signaling processes. However, there are currently a limited set of tools available for the detection of specific protein *S*-nitrosation and because of this, the burden of proof is often lessened in contrast to, for example, phosphorylation studies.

It has been stated that over 100 proteins have been shown to be nitrosated (Stamler et al., 2001). Such a large number would appear to indicate that nitrosation is general multipurpose signaling mechanism of NO. However, one needs to use careful filters when assessing whether such a modification has been observed in a physiologically relevant situation. The data discussed in this review indicate that conditions that have been previously adjudged to be physiologically informative (i.e., the use of *S*-nitrosothiols in cell culture as NO donors) may rather be examining the NO-independent pharmacology of *S*-nitrosothiols. It is clear that no pathway has yet met the criteria recently laid down by Lancaster and Gaston (Lancaster, Jr. et al., 2004) that were suggested to be required before *S*-nitrosation is definitively identified as a signal.

The evidence and uncertainties surrounding the *S*-nitrosothiol formation in intracellular signaling can be best illustrated by examining a specific pathway: the Ras oncogene pathway. The first observations that NO is involved in the activation of guanine nucleotide exchange in p21 Ras came from experiments that exposed Jurkat cells to NO gas (Lander et al., 1995). It was observed that GDP/GTP exchange was stimulated in cells at a concentration of 10 nM NO, but purified Ras required a concentration of 75 μM NO for maximal activation. The fact that a bolus concentration of 75 μM NO causes extensive protein *S*-nitrosation is in good agreement with the known thiol nitrosating activity of NO in the presence of oxygen, especially when added as a nonphysiologically high bolus concentration of 2 mM (before dilution) (Zhang et al., 2002). The fact that thiol nitrosation by chemical means activated this enzyme led to the

speculation that thiol/NO interactions may represent a mechanism of Ras control. The difference in sensitivity between cells and purified protein was explained by the presence of so-far unknown cellular oxidizing mechanisms that enhance nitrosation pathways. Clearly, these early studies indicated mechanistic differences between the activation of Ras by NO in cells, and protein thiol nitrosation via the NO/oxygen reaction. Interestingly this study (Lander et al., 1995) also showed that the activation of p21Ras by NO was reversed by the addition of hemoglobin 10 minutes after the addition of NO. This was used as evidence confirming a protein *S*-nitrosothiol as an intermediate but in fact supports the opposite view point as, unless a transnitrosation reaction between Ras and hemoglobin cysteine residues is invoked (a process that would be extremely slow), an *S*-nitrosated protein should be immune to decomposition by oxygenated hemoglobin. Measurement of *S*-nitrosothiol formation by Saville assay in the purified protein gave a value of 1 mol/mol protein despite the fact that p21Ras has five cysteine residues. It should be noted that even the site that is nitrosated requires 100-fold excess of NO, and so nitrosation from NO is not a very efficient process. It was noted that an antibody that activates Ras inhibited nitrosation, suggesting that the nitrosatable thiols could be physically blocked to inhibit nitrosation. Remarkably NO caused a huge conformational change in Ras protein as observed by circular dichroism (Lander et al., 1995). While this original paper set the scene for nitrosation of Ras as a potentially important control mechanism, it did not show that Ras nitrosation was important for NO-mediated alterations in Ras activity in vivo. Unfortunately, studies of downstream events were conducted using sodium nitroprusside (SNP), an agent that has been shown to cause a severe oxidative stress to cells in culture (Hogg et al., 1995), and is therefore not an appropriate agent to use as an NO donating agent. In a later study this group identified, by mass spectrometry, the formation of an *S*-nitrosothiol at Cys118 after incubation of the protein with NO (Teng et al., 1999). A mutant containing a serine instead of a cysteine at this position could not be modified by NO. While NO could enhance GTPase activity in wild type protein, it had no effect on the mutant. These data were used to conclude that NO has a direct effect on Cys118. This study also showed that overexpression of a mutant in cells led to inhibition of NO-mediated downstream signaling events. This work closely relates the activation of Ras by NO to Cys118 but does not establish thiol nitrosation as the mechanism of action.

A more recent study has refuted many of the above findings and concluded, based on NMR data, that nitrosation of Ras in fact causes no major changes in protein structure or nucleotide exchange but that the "chemical process of nitrosylation (i.e., nitrosation) is responsible for the observed effects" (Williams et al., 2003). In other words, the enhancement of guanine nucleotide exchange was only observed in the presence of a low molecular weight *S*-nitrosothiol. These data are significantly in conflict with the previous studies that observed enhancement with NO gas, whose mechanism of thiol nitrosation is completely different from that of low molecular weight *S*-nitrosothiols (NO autoxidation vs. transnitrosation). This study effectively uncouples thiol nitrosation from the effects of NO on guanine nucleotide exchange and indicates that this protein will behave differently

when it is exposed to NO gas than when it is modified by transnitrosation. In addition, the fact that enhanced guanine nucleotide exchange requires the presence of high concentrations of low molecular weight *S*-nitrosothiols (which cannot be detected inside cells even under conditions of high level NO exposure) dilutes the in vivo relevance of these studies. While the link between Ras and *S*-nitrosation is intriguing, the evidence that the *S*-nitrosation of Ras is responsible for its in vivo activation by NO is circumstantial, and many other explanations are possible for the observed effects, such as cysteine *S*-thiolation and upstream effects of NO that affect Ras signaling.

This detailed discussion indicates that even the "poster child" of *S*-nitrosothiol-mediated signaling pathways has so far not been shown categorically controlled via thiol *S*-nitrosation in response to NO formation. However, the evidence strongly suggests that NO exposure can activate Ras by a mechanism that involves a protein thiol.

The inhibition of caspase-3 by *S*-nitrosation is similar in many ways to the Ras story in that there is a great deal of evidence indicating that thiol nitrosation can inhibit enzyme activity in vitro, but little evidence that this takes place in vivo. This first study again linked the inhibition of caspase activity after exposure to "NO donors" with that ability of purified proteins to become *S*-nitrosated, again using agents that should not be regarded as NO donors (SNP and SNAP) and methods of *S*-nitrosothiol detection (direct spectrophotometric changes and the Saville reaction) that are too insensitive to detect intracellular levels of *S*-nitrosothiols. In 1997 a high profile study entitled "*S*-nitrosylation inhibits apoptosis" used the same approach of associating cellular effects of SNAP with direct effects of SNAP on purified proteins. However, in this case direct detection of nitrosated caspase was attempted in vivo and was associated with a functional response. Mannick et al. (1999) examined the nitrosation and denitrosation of caspase-3 in response to FAS-ligand and proposed that this pro-apoptotic stimulus at least partially initiated apoptosis through the activation (by "denitrosation") of caspase-3. Although this issue remains controversial, this is one of the few examples where an attempt was made to directly assess *S*-nitrosation of an intracellular protein in response to a signal.

The more recent development of the "biotin switch" assay for the on-gel or on-blot detection protein *S*-nitrosation has resulted in a rash of recent studies reporting *S*-nitrosation of intracellular proteins. However, a note of caution should be sounded, as this method is indirect and, as mentioned above, contains its own subset of methodological concerns. Without corroborative analytical determination of the presence of *S*-nitrosation, this method should not be used as a gold-standard for specific protein *S*-nitrosation.

6.6 CONCLUSIONS

In this chapter we have attempted to discuss many of the issues involved in the study of protein *S*-nitrosation as an intracellular signaling paradigm. These issues

include the appropriate choice of NO donating agent, the NO-independent effects of exogenous *S*-nitrosothiols, the poor nitrosating efficiency of endogenous NO, and the difficulties in determining whether a specific intracellular protein is *S*-nitrosated. It is beyond doubt that many processes that have implicated *S*-nitrosation as a mechanism of NO-mediated signal transduction have instead evolved the NO-independent effects of exogenous *S*-nitrosothiols. We now have a much more robust, though still incomplete, appreciation of how *S*-nitrosothiols are formed, how they are transported, and how they are metabolized. In addition, methodological advancements will hopefully provide definitive answers to the most pressing questions concerning the specificity and regulation of NO-mediated *S*-nitrosation.

ACKNOWLEDGMENTS

We would like to thank Agnes Keszler, Barbora Piknova, Katarzyna Broniowska, and Nicholas Kettenhofen for discussions and contributions to this work. We would also acknowledge the National Institute of General Medicine (Grant GM55792) and the American Heart Association (Grant 0310032Z) for financial support.

LIST OF ABBREVIATIONS

γ- GT, γ-glutamyl transpeptidase
BAEC, bovine aortic endothelial cells
BCH, 2-aminobicyclo[2.2.1]-heptane-2-carboxylate
BSA-SNO, *S*-nitrosated bovine serum albumin
cGMP, cyclic guanosine monophosphate
CPTIO, 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide
Cs-PDI, cell surface protein disulfide isomerase
CysNO, *S*-nitrosocysteine
GSH, glutathione
GSNO, *S*-nitrosoglutathione
L-AT, amino acid transporter system L
NO, nitric oxide
NOS, nitric oxide synthase
PDI, protein disulfide isomerase
RBC, red blood cell
SNAP, *S*-nitrosopenicillamine
SNP, sodium nitroprusside

REFERENCES

Ascenzi P., Colasanti M., Persichini T., Muolo M., Polticelli F., Venturini G., Bordo D., Bolognesi M. (2000). Re-evaluation of amino acid sequence and structural consensus rules for cysteine-nitric oxide reactivity. *Biol Chem* 381: 623–627.

Barouch L.A., Harrison R.W., Skaf M.W., Rosas G.O., Cappola T.P., Kobeissi Z.A., Hobai I.A., Lemmon C.A., Burnett A.L., O'Rourke B., Rodriguez E.R., Huang P.L., Lima J.A., Berkowitz D.E., Hare J.M. (2002). Nitric oxide regulates the heart by spatial confinement of nitric oxide synthase isoforms. *Nature* 416: 337–339.

Beckman J.S., Beckman T.W., Chen J., Marshall P.A., Freeman B.A. (1990). Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 87: 1620–1624.

Chung K.K.K., Thomas B., Li X., Pletnikova O., Troncoso J.C., Marsh L., Dawson V.L., Dawson T.M. (2004). *S*-Nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. *Science* 304: 1328–1331.

Curthoys N.P., Hughey R.P. (1979). Characterization and physiological function of rat renal gamma-glutamyltranspeptidase. *Enzyme* 24: 383–403.

Davisson R.L., Travis M.D., Bates J.N., Johnson A.K., Lewis S.J. (1997). Stereoselective actions of *S*-nitrosocysteine in central nervous system of conscious rats. *Am J Physiol* 272: H2361–H2368.

Davisson R.L., Travis M.D., Bates J.N., Lewis S.J. (1996). Hemodynamic effects of L- and D-*S*-nitrosocysteine in the rat: Stereoselective *S*-nitrosothiol recognition sites. *Circ Res* 79: 256–262.

De Groote M.A., Granger D., Xu Y., Campbell G., Prince R., Fang F.C. (1995). Genetic and redox determinants of nitric oxide cytotoxicity in a *Salmonella typhimurium* model. *Proc Natl Acad Sci USA* 92: 6399–6403.

Espey M.G., Thomas D.D., Miranda K.M., Wink D.A. (2002). Focusing of nitric oxide mediated nitrosation and oxidative nitrosylation as a consequence of reaction with superoxide. *Proc Natl Acad Sci USA* 99: 11127–11132.

Eu J.P., Liu L., Zeng M., Stamler J.S. (2000). An apoptotic model for nitrosative stress. *Biochemistry* 39: 1040–1047.

Feelisch M., Rassaf T., Mnaimneh S., Singh N., Bryan N.S., Jourd'heuil D., Kelm M. (2002). Concomitant *S*-, *N*-, and heme-nitros(yl)ation in biological tissues and fluids: Implications for the fate of NO in vivo. *FASEB J* 16: 1775–1785.

Folkes L.K., Wardman P. (2004). Kinetics of the reaction between nitric oxide and glutathione: Implications for thiol depletion in cells. *Free Radic Biol Med* 37: 549–556.

Foster M.W., Stamler J.S. (2004). New insights into protein *S*-nitrosylation: Mitochondria as a model system. *J Biol Chem* 279: 25891–25897.

Gaston B., Reilly J., Drazen J.M., Fackler J., Ramdev P., Arnelle D., Mullins M.E., Sugarbaker D.J., Chee C., Singel D.J. (1993). Endogenous nitrogen oxides and bronchodilator *S*-nitrosothiols in human airways. *Proc Natl Acad Sci USA* 90: 10957–10961.

Glover R.E., Koshkin V., Dunford H.B., Mason R.P. (1999). The reaction rates of NO with horseradish peroxidase compounds I and II. *Nitric Oxide* 3: 439–444.

Goldstein S., Czapski G. (1996a). Kinetics of nitric oxide autoxidation in aqueous solution in the absence and presence of various reductants: The nature of the oxidizing intermediates. *J Am Chem Soc* 118: 3419–3425.

Goldstein S., Czapski G. (1996b). Mechanism of the nitrosation of thiols and amines by oxygenated NO solutions: The nature of the nitrosating intermediates. *J Am Chem Soc* 118: 3419–3425.

Gow A.J., Buerk D.G., Ischiropoulos H. (1997). A novel reaction mechanism for the formation of *S*-nitrosothiol in vivo. *J Biol Chem* 272: 2841–2845.

Gow A.J., Chen Q., Hess D.T., Day B.J., Ischiropoulos H., Stamler J.S. (2002). Basal and stimulated protein *S*-nitrosylation in multiple cell types and tissues. *J Biol Chem* 277: 9637–9640.

Gow A.J., Farkouh C.R., Munson D.A., Posencheg M.A., Ischiropoulos H. (2004). Biological significance of nitric oxide-mediated protein modifications. *Am J Physiol Lung Cell Mol Physiol* 287: L262–L268.

Hoffman J., Haendeler J., Zeiher A.M., Dimmeler S. (2001). TNF-α and oxLDL reduce protein *S*-nitrosylation in endothelial cells. *J Biol Chem* 276: 41383–41387.

Hoffmann J., Dimmeler S., Haendeler J. (2003). Shear stress increases the amount of *S*-nitrosylated molecules in endothelial cells: Important role for signal transduction. *FEBS Lett* 551: 153–158.

Hogg N. (1999). The kinetics of *S*-transnitrosation—A reversible second-order reaction. *Anal Biochem* 272: 257–262.

Hogg N., Singh R.J., Kalyanaraman B. (1996). The role of glutathione in the transport and catabolism of nitric oxide. *FEBS Lett* 382: 223–228.

Hogg N., Singh R.J., Konorev E., Joseph J., Kalyanaraman B. (1997). *S*-Nitrosoglutathione as a substrate for gamma-glutamyl transpeptidase. *Biochem J* 323: 477–481.

Hogg N., Struck A., Goss S.P., Santanam N., Joseph J., Parthasarathy S., Kalyanaraman B. (1995). Inhibition of macrophage-dependent low density lipoprotein oxidation by nitric-oxide donors. *J Lipid Res* 36: 1756–1762.

Holmes A.J., Williams D.L.H. (2000). Reaction of ascorbic acid with *S*-nitrosothiols: Clear evidence for two distinct reaction pathways. *J Chem Soc Perkin Trans* 2: 1639–1644.

Ignarro L.J., Edwards J.C., Gruetter D.Y., Barry B.K., Gruetter C.A. (1980). Possible involvement of *S*-nitrosothiols in the activation of guanylate cyclase by nitroso compounds. *FEBS Lett* 110: 275–278.

Inoue K., Akaike T., Miyamoto Y., Okamoto T., Sawa T., Otagiri M., Suzuki S., Yoshimura T., Maeda H. (1999). Nitrosothiol formation catalyzed by ceruloplasmin: Implication for cytoprotective mechanism in vivo. *J Biol Chem* 274: 27069–27075.

Jaffrey S.R., Erdjument-Bromage H., Ferris C.D., Tempst P., Snyder S.H. (2001). Protein *S*-nitrosylation: A physiological signal for neuronal nitric oxide. *Nat Cell Biol* 3: 193–197.

Janero D.R., Bryan N.S., Saijo F., Dhawan V., Schwalb D.J., Warren M.C., Feelisch M. (2004). Differential nitros(yl)ation of blood and tissue constituents during glyceryl trinitrate biotransformation in vivo. *Proc Natl Acad Sci USA* 101: 16958–16963.

Jensen D.E., Belka G.K., Du Bois G.C. (1998). S-Nitrosoglutathione is a substrate for rat alcohol dehydrogenase class III isoenzyme. *Biochem J* 331: 659–668.

Jia L., Bonaventura C., Bonaventura J., Stamler J.S. (1996). *S*-Nitrosohaemoglobin: A dynamic activity of blood involved in vascular control. *Nature* 380: 221–226.

Jourd'heuil D., Jourd'heuil F.L., Feelisch M. (2003). Oxidation and nitrosation of thiols at low micromolar exposure to nitric oxide: Evidence for a free radical mechanism. *J Biol Chem* 278: 15720–15726.

Jourd'heuil D., Gray L., Grisham M.B. (2000). *S*-Nitrosothiol formation in blood of lipopolysaccharide-treated rats. *Biochem Biophys Res Commun* 273: 22–26.

Kashiba-Iwatsuki M., Yamaguchi M., Inoue M. (1996). Role of ascorbic acid in the metabolism of *S*-nitroso-glutathione. *FEBS Lett* 389: 149–152.

Kharitonov V.G., Sundquist A.R., Sharma V.S. (1995). Kinetics of nitrosation of thiols by nitric oxide in the presence of oxygen. *J Biol Chem* 270: 28158–28164.

Kluge I., Gutteck-Amsler U., Zollinger M., Do K.Q. (1997). *S*-Nitrosoglutathione in rat cerebellum: Identification and quantification by liquid chromatography-mass spectrometry. *J Neurochem* 69: 2599–2607.

Lakshmi V.M., Nauseef W.M., Zenser T.V. (2005). Myeloperoxidase potentiates nitric oxide-mediated nitrosation. *J Biol Chem* 280: 1746–1753.

Lancaster J.R., Jr., Gaston B. (2004). NO and nitrosothiols: spatial confinement and free diffusion. *Am J Physiol Lung Cell Mol Physiol* 287: L465–L466.

Lander H.M., Ogiste J.M., Pearce S.F., Levi R., Novogrodski A. (1995). Nitric oxide-stimulated guanine nucleotide exchange on $p21^{ras}$. *J Biol Chem* 270: 7017–7020.

Levonen A.L., Landar A., Ramachandran A., Ceaser E.K., Dickinson D.A., Zanoni G., Morrow J.D., Darley-Usmar V.M. (2004). Cellular mechanisms of redox cell signalling: Role of cysteine modification in controlling antioxidant defences in response to electrophilic lipid oxidation products. *Biochem J* 378: 373–382.

Li S., Whorton A.R. (2005). Identification of stereoselective transporters for *S*-Nitroso-L-cysteine: Role of LAT1 and LAT2 in biological activity of *S*-nitrosothiols. *J Biol Chem* 280: 20102–20110.

Liu L., Hausladen A., Zeng M., Que L., Heitman J., Stamler J.S. (2001). A metabolic enzyme for *S*-nitrosothiol conserved from bacteria to humans. *Nature* 410: 490–494.

Liu X., Miller M.J.S., Joshi M.S., Thomas D.D., Lancaster J.R. Jr. (1998). Accelerated reaction of nitric oxide with O_2 within the hydrophobic interior of biological membranes. *Proc Natl Acad Sci USA* 95: 2175–2179.

Luchsinger B.P., Rich E.N., Gow A.J., Williams E.M., Stamler J.S., Singel D.J. (2003). Routes to *S*-nitroso-hemoglobin formation with heme redox and preferential reactivity in the beta subunits. *Proc Natl Acad Sci USA* 100: 461–466.

Mallis R.J., Thomas J.A. (2000). Effect of *S*-nitrosothiols on cellular glutathione and reactive protein sulfhydryls. *Arch Biochem Biophys* 383: 60–69.

Mannick J.B., Hausladen A., Liu L., Hess D.T., Zeng M., Miao Q.X., Kane L.S., Gow A.J., Stamler J.S. (1999). Fas-induced caspase denitrosylation. *Science* 284: 651–654.

Marley R., Patel R.P., Orie N., Ceaser E., Darley-Usmar V., Moore K. (2001). Formation of nanomolar concentrations of *S*-nitroso-albumin in human plasma by nitric oxide. *Free Radic Biol Med* 31: 688–696.

McMahon T.J., Moon R.E., Luschinger B.P., Carraway M.S., Stone A.E., Stolp B.W., Gow A.J., Pawloski J.R., Watke P., Singel D.J., Piantadosi C.A., Stamler J.S. (2002). Nitric oxide in the human respiratory cycle. *Nat Med* 8: 711–717.

Miseta A., Csutora P. (2000). Relationship between the occurrence of cysteine in proteins and the complexity of organisms. *Mol Biol Evol* 17: 1232–1239.

Moro M.A., Darley-Usmar V.M., Goodwin D.A., Read N.G., Zamora-Pino R., Feelisch M., Radomski M.W., Moncada S. (1994). Paradoxical fate and biological action of peroxynitrite on human platelets. *Proc Natl Acad Sci USA* 91: 6702–6706.

Park J.W. (1988). Reaction of *S*-nitrosoglutathione with sulfhydryl groups in protein. *Biochem Biophys Res Commun* 152: 916–920.

Pawloski J.R., Hess D.T., Stamler J.S. (2001). Export by red blood cells of nitric oxide bioactivity. *Nature* 409: 622–626.

Pryor W.A., Church D.F., Govindan C.K., Crank G. (1982). Oxidation of thiols by nitric oxide and nitrogen dioxide: Synthetic utility and toxicological implications. *J Org Chem* 47: 159–161.

Rafikova O., Rafikov R., Nudler E. (2002). Catalysis of *S*-nitrosothiols formation by serum albumin: The mechanism and implication in vascular control. *Proc Natl Acad Sci USA* 99: 5913–5918.

Ramachandran N., Root P., Jiang X.-M., Hogg P.J., Mutus B. (2001). Mechanism of transfer of NO from extracellular *S*-nitrosothiols into the cytosol by cell-surface protein disulfide isomerase. *Proc Natl Acad Sci USA* 98: 9539–9544.

Rhee K.Y., Erdjument-Bromage H., Tempst P., Nathan C.F. (2005). *S*-Nitroso proteome of *Mycobacterium tuberculosis*: Enzymes of intermediary metabolism and antioxidant defense. *Proc Natl Acad Sci USA* 102: 467–472.

Root P., Sliskovic I., Mutus B. (2004). Platelet cell surface disulfide isomerase mediated *S*-Nitrosoglutathione consumption. *Biochem J* 382: 575–580.

Rossig L., Fichtlscherer B., Breitschopf K., Haendeler J., Zeiher A.M., Mulsch A., Dimmeler S. (2001). Nitric oxide inhibits caspase-3 by *S*-nitrosation in vivo. *J Biol Chem* 274: 6823–6826.

Satoh S., Kimura T., Toda M., Maekawa M., Ono S., Narita H., Miyazaki H., Murayama T., Nomura Y. (1997). Involvement of L-type-like amino acid transporters in *S*-nitrosocystiene-stimulated noradrenaline release in the rat hippocampus. *J Neurochem* 69: 2197–2205.

Satoh S., Kimura T., Toda M., Miyazaki H., Ono S., Narita H., Murayama T., Nomura Y. (1996). NO donors stimulate noradrenaline release from rat hippocampus in a calmodulin-dependent manner in the presence of L-cysteine. *J Cell Physiol* 169: 87–96.

Stamler J.S., Lamas S., Fang F.C. (2001). Nitrosylation: The prototypic redox-based signaling mechanism. *Cell* 106: 675–683.

Stamler J.S., Toone E.J., Lipton S.A., Sucher N.J. (1997). (S)NO signals: Translocation, regulation, and a consensus motif. *Neuron* 18: 691–696.

Teng K.K., Esposito D.K., Schwartz G.D., Lander H.M., Hempstead B.L. (1999). Activation of c-Ha-Ras by nitric oxide modulates survival responsiveness in neuronal PC12 cells. *J Biol Chem* 274: 37315–37320.

Thomas D.D., Liu X., Kantrow S.P., Lancaster J.R., Jr. (2002). The biological lifetime of nitric oxide: Implications for the perivascular dynamics of nitric oxide and oxygen. *Proc Natl Acad Sci USA* 98: 355–360.

Trujillo M., Alvarez M.N., Peluffo G., Freeman B.A., Radi R. (1998). Xanthine oxidase-mediated decomposition of *S*-nitrosothiols. *J Biol Chem* 273: 7828–7834.

Turano C., Coppari S., Altieri F., Ferraro A. (2002). Proteins of the PDI family: Unpredicted non-ER locations and functions. *J Cell Physiol* 193: 154–163.

van der Vliet A., Chr't Hoen P.A., Wong P.S.Y., Bast A., Cross C.E. (1998). Formation of *S*-nitrosothiols via direct nucleophilic nitrosation of thiols by peroxynitrite with elimination of hydrogen peroxide. *J Biol Chem* 273: 30255–30262.

van der Vliet A., Eiserich J.P., Halliwell B., Cross C.E. (1997). Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite: A potential additional mechanism of nitric oxide-dependent toxicity. *J Biol Chem* 272: 7617–7625.

Wang X., Tanus-Santos J.E., Reiter C.D., Dejam A., Shiva S., Smith R.D., Hogg N., Gladwin M.T. (2004). Biological activity of nitric oxide in the plasmatic compartment. *Proc Natl Acad Sci USA* 101: 11477–11482.

Weichsel A., Maes E.M., Andersen J.F., Valenzuela J.G., Shokhireva T.K., Walker F.A., Montfort W.R. (2005). Heme-assisted *S*-nitrosation of a proximal thiolate in a nitric oxide transport protein. *Proc Natl Acad Sci USA* 102: 594–599.

Williams J.G., Pappu K., Campbell S.L. (2003). Structural and biochemical studies of p21Ras *S*-nitrosylation and nitric oxide-mediated guanine nucleotide exchange. *Proc Natl Acad Sci USA* 100: 6376–6381.

Wink D.A., Beckman J.S., Ford P.C. (1996). Kinetics of nitric oxide reaction in liquid and gas phase. In: Feelisch M., Stamler J.S. (eds.), *Methods in Nitric Oxide Research*, Wiley, New York, pp. 29–37.

Xu X., Cho M., Spencer N.Y., Patel N., Huang Z., Shields H., King S.B., Gladwin M.T., Hogg N., Kim-Shapiro D.B. (2003). Measurements of nitric oxide on the heme iron and β-93 thiol of heman hemoglobin during cycles of oxygenation and deoxygenation. *Proc Natl Acad Sci USA* 100: 11303–11308.

Zai A., Rudd M.A., Scribner A.W., Loscalzo J. (1999). Cell-surface protein disulfide isomerase catalyzes transnitrosation and regulates intracellular transfer of nitric oxide. *J Clin Invest* 103: 393–399.

Zhang Y., Hogg N. (2002). Mixing artifacts from bolus addition of nitric oxide to oxymyoglobin: Implications for *S*-nitrosohtiol formation. *Free Radic Biol Med* 32: 1212–1219.

Zhang Y., Hogg N. (2004a). The formation and stability of *S*-nitrosothiols in RAW 264.7 cells. *Am J Physiol Lung Cell Mol Physiol* 287: L467–L474.

Zhang Y., Hogg N. (2004b). The mechanism of transmembrane *S*-nitrosothiol transport. *Proc Natl Acad Sci USA* 101: 7891–7896.

Zhang Y., Keszler A., Broniowska K.A., Hogg N. (2005). Characterization and application of the biotin-switch assay for the identification of *S*-nitrosated proteins. *Free Radic Biol Med* 38: 874–881.

7

DETECTION OF GLYCATED AND GLYCO-OXIDATED PROTEINS

ANNUNZIATA LAPOLLA, ELISA BASSO, AND PIETRO TRALDI

7.1 INTRODUCTION

The reaction between sugars and amino groups was first studied by Maillard in 1912 (Maillard, 1912). In his experiments Maillard added glycine to a glucose water solution and observed that the liquid turned yellow and, successively, brown, and that carbon dioxide formed. This was the beginning of a wide number of investigations on the reactivity of different amino acids with different saccharides, leading to the design of a reactivity scale. Thus, among amino acids, alanine was found to be the most reactive and, among sugars, xylose and arabinose were observed to react very rapidly, in contrast to lactose and maltose, which exhibit slow reaction kinetics.

Maillard immediately recognized the relevance that this reaction could have in the natural world, mainly in biology but also in different areas of sciences as phytochemistry, geology, and medicine. He was the first to suggest that it had importance in diabetes, and his findings still remain a milestone in the study involved in the complications of diabetes (Trivelli et al., 1971).

In looking at the Maillard reaction pattern taking place between a reducing sugar and an amino group present in a protein chain (see Fig. 7.1), we can distinguish three different stages (Hodge, 1955; Watkins et al., 1985): *early* (condensation of the sugar on the protein followed by the formation of the so-called Amadori product), *intermediate* (leading to the formation of reactive dicarbonyl compounds acting as propagators), and *late* consisting of the formation of brown

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

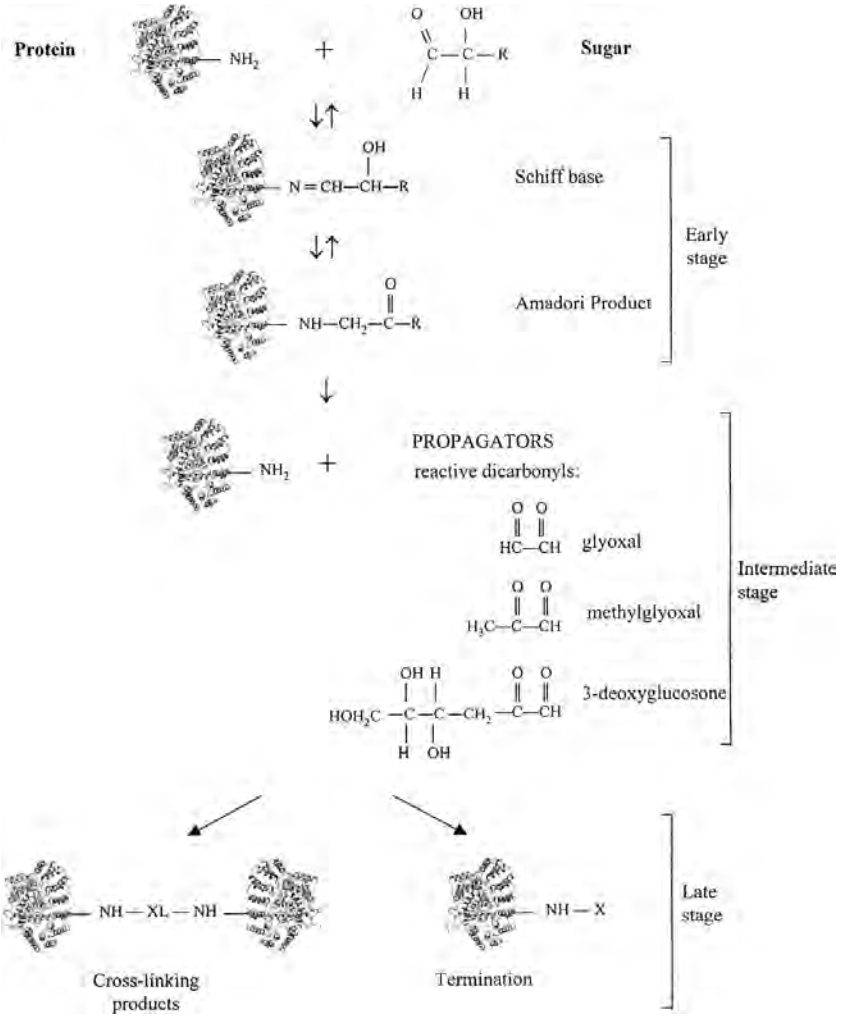


FIGURE 7.1 Maillard reaction pattern.

products, among which protein cross-linking products play an important role from the pathophysiological point of view. It is well known that a different reactivity of the protein amino groups with respect to their position (and consequently to their availability to react) in the tertiary protein structure is to be expected. In particular, the reaction of glucose with primary amino groups of proteins (i.e., terminal ones) and the ε-amino group of lysines belonging to the protein chains is useful in explaining some aspects of diabetes. It should further be noted that in the intermediate stage of the reaction concurrent oxidations may take place and, among browning products, the presence of glyco-oxidated species linked to the protein can be expected (Wolff et al., 1991).

We were interested in learning more about the products arising from the reaction between ε-amino group of lysine and glucose, so we studied the reaction products of α-protected lysine and glucose (Lapolla et al., 1991). At the time of our study the most powerful techniques available, namely LC-UV, FAB, and LC-MS, were employed. We proceeded as follows: Protected lysine and D-glucose were dissolved in sodium phosphate buffer, and the solution was incubated for 28 days at 37°C and then lyophilized. The LC-UV chromatogram (320 nm) of the reaction mixture (Fig. 7.2) shows the three main peaks obtained at retention times of 6, 11, and 12 minutes, respectively. Direct analysis by FAB/MS of the whole reaction mixture (Fig. 7.3*a*) gave a spectrum clearly different from that of pure protected lysine. Ions at *m*/*z* 285 and 263 were detectable, and on the basis of collisional experiments, structures **1** and **2**, respectively, were proposed for them (Fig. 7.3*b*). A separative step was believed to be essential for their structural identification and LC-MS measurements were made. The total ion chromatogram obtained by this approach under the same LC conditions employed to yield the chromatogram of Figure 7.2 is shown in Figure 7.4*a*. Practically no chromatographic resolution was obtained, indicating the presence of many unresolved molecular species. The discrepancy with the chromatogram of Figure 7.2 may be explained by the fact that under these conditions only components exhibiting absorption at the UV wavelength employed can be detected. Careful examination of the MS spectra at varying retention times (e.g., see Fig 7.4*b*) allowed the molecular species present in the complex mixture to be efficiently described. The possible reaction pathways leading to these species are shown in Scheme 7.1. These structures were proposed on the basis of studies of Maillard reaction occurring in physiological conditions.

As in most chemical reactions the yield depended largely on the concentrations of the reactants. Consequently, it is reasonably to suppose that the glyco-oxidation processes take place in vivo in higher yield at high glucose concentrations, as found in patients with diabetes (Wolff et al., 1991).

Preliminary studies (based mainly on spectroscopic UV absorption and fluorometric data, due to the spectroscopic characteristics of browning products) showed the occurrence of glycation processes in in vitro conditions when proteins were incubated with glucose (or other reducing sugars) in high concentrations—several orders of magnitude higher than physiological ones. Our study led to the identification of the most reactive sites of physiological proteins and suggested that measurement of the circulating protein glycation levels could be used to monitor the "glycemic stress" experienced by diabetic patients during the half-life of the protein. It was from these considerations that the hemoglobin glycation level (HbA_{1c}) was proposed for diabetic monitoring: it still represents an important analytical datum for physicians in describing a patient's pathological state. Mass spectrometry is an excellent tool to use to describe the hemoglobin glycation process. Zhang et al. (2001) and Roberts et al. (2001) studied this process by means of LC/electrospray mass spectrometry, and the reference IFCC method for HbA_{1c} is based on a mass spectrometric method (Jeppsson et al., 2002).

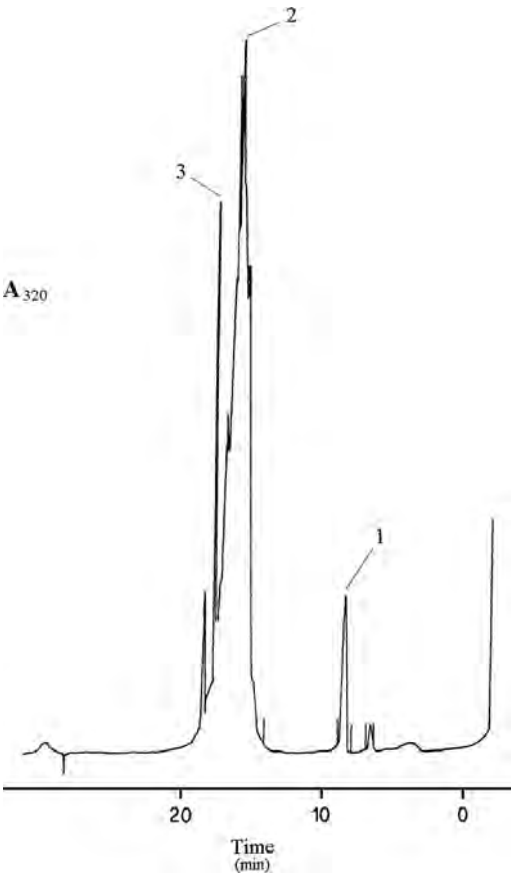


FIGURE 7.2 HPLC chromatogram obtained by detection at 320 nm of the α-protected lysine and glucose reaction environment.

In clinical practice, measurements of nonenzymatic glycation products have a double mean. In addition, evaluation of these products, depending on the half-life of the glycated protein that is measured, can give an estimate of the extent of exposure to glucose and therefore the subject metabolic control. In addition, evaluation of these compounds involves verifying the relationship between glycation and modifications observed in tissue, allowing clarification of the pathogenesis of chronic complications and evaluation of the efficacy of various kinds of therapy. In this context, measurements of some early-stage glycation products, such as HbA_{1c} and glycated serum proteins, are routinely used to assess the metabolic control in diabetic patients.

Glycated serum proteins are products of the nonenzymatic reaction between the free amino groups of serum proteins and glucose. These early glycation products formed during the half-lives of serum proteins are indicators of metabolic control

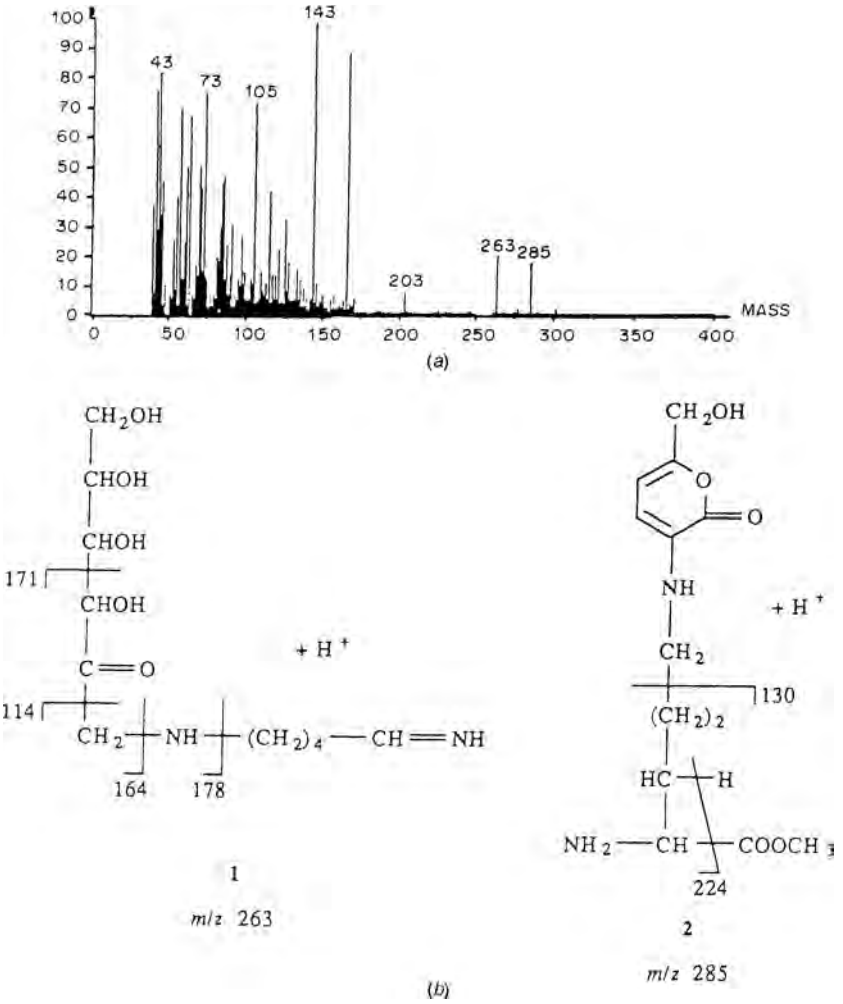


FIGURE 7.3 FAB mass spectra of (*a*) whole reaction mixture and (*b*) possible structures of the ions at *m/z* 263 and 285.

in diabetic patients (Furth, 1997). The most common method of evaluating the serum proteins is to determine the presence of fructosamine by means of a colorimetric assay based on the ability of ketoamine to reduce the nitroblue tetrazolium dye and produce a compound adsorbing at 525 nm (Johnson et al., 1983). However, this method has low specificity and some limitations. Also the reducing agents (ascorbic acid, uric acid, and glutathione) and the products adsorbing at 525 nm (triglycerides, bilirubin) can interfere with the dosage when present in serum at high concentration. For these reasons use of glycated serum is limited to certain situations: when evaluation of HbA_{1c} level cannot be used as in cases

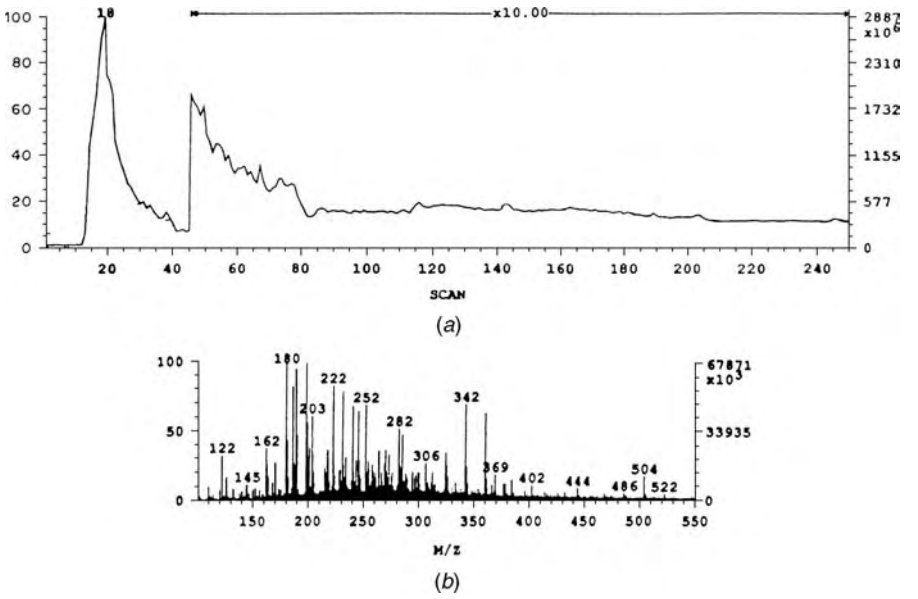
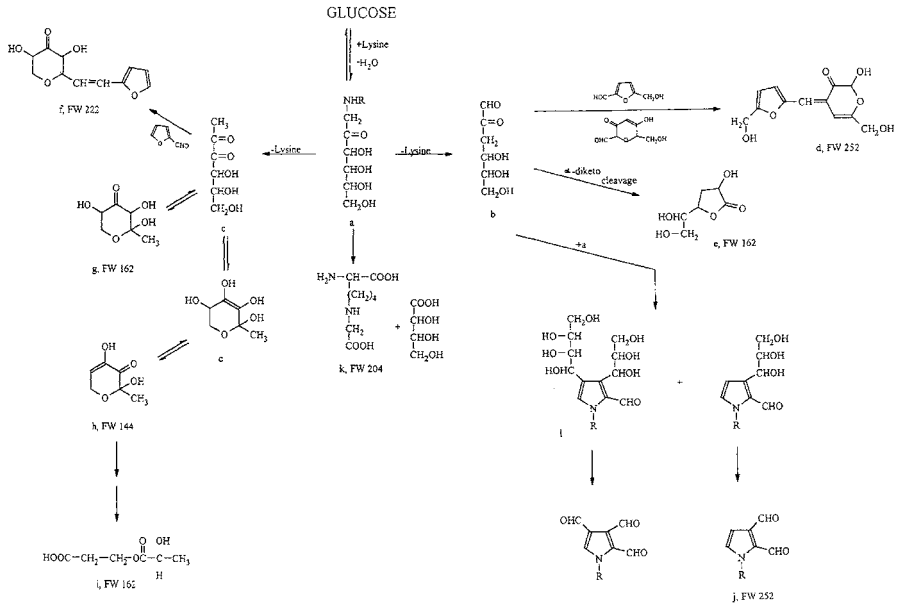


FIGURE 7.4 Reconstructed ion chromatogram for HPLC-MS analysis of α-protected lysine-glucose reaction mixture under plasma-spray conditions: (*a*) mass spectra obtained at different elution times by HPLC-MS analysis and (*b*) mass spectrum of scan No. 18.



SCHEME 7.1 Possible pathways leading to structures compatible with the molecular weight found by HPLC/MS analysis, where $R = -(CH_2)_4$ –CH(NH)–COOH.

of hemoglobinopathy, hemolytic anemia, impairment of red blood cell survival, and so forth, or in diabetic pregnancy, when major changes of therapy have to be made (American Diabetes Association, 2004; Lapolla et al., 1998).

Among the intermediate glycated products, 3-deoxyglucosone, glyoxal, and methylglyoxal can be evaluated in serum. Higher levels of 3-deoxyglucosone have been detected in the plasma of diabetic patients with complications with respect to those without them (Kusunoki et al., 2003; Beisswenger et al., 2001), thus signifying the importance of this compound. Unfortunately, 3-deoxyglucosone is difficult to measure owing to its high reactivity (Lal et al., 1997).

Levels of glyoxal and methylglyoxal higher of those found in healthy subjects have been reported in diabetic and nephropathic patients (Miyata et al., 1999). We recently showed that metabolic control could be achieved with optimized insulin therapy in type 2 diabetic patients with poor metabolic control who could not normalize glyoxal and methylglyoxal after six months, unlike HbA_{1c} and fructosamine (Lapolla et al., 2003a). Our data strongly suggest that a stable period of metabolic control can normalize the intermediate nonenzymatic glycation products. The most specific methods proposed to measure glyoxal and methylglyoxal are LC and GC/MS (Lapolla et al., 2003b; McLellan et al., 1992).

Because of the change in color of AGEs, spectroscopic methods were initially utilized to evaluate AGEs levels in the substrates of interest (Brownlee et al., 1988). However, these methods had severe limitations because they were only capable of indicating the general level of glycation but not identify the glycated proteins structurally.

Recently RIA (Radio Immuno Assay) and ELISA (Enzyme-Linked Immuno Sorbent Assay) methods have been developed that measure the polyclonal antibodies arising in AGEs, which are obtained in vitro by reacting ribonuclease with glucose (Mitsuhashi et al., 1997). However, detection of high AGEs levels in the plasma of patients with diabetes and end-stage renal disease (Furth, 1997; Schiel et al., 2003; Schinzel et al., 2001) has its limitations because the reactivity of AGEs with other substances cannot be excluded (Mitsuhashi et al., 1997). In particular, the problems with ELISA of AGEs other than incomplete epitope recognition are (1) qualitative and not quantitative detection, since the multiple epitopes are known to have different affinities for the antibodies, and (2) more AGE epitope in the blocking protein than in the samples assayed, thus decreasing nonspecific binding and giving rise to large background response levels.

Among AGEs, *N*-carboxymethyl-lysine (CML) (Ahmed et al., 1986) and pentosidine (Sell and Monnier, 1989) have been characterized: they are considered glyco-oxidation products because pro-oxidizing conditions are needed for their formation (Furth, 1997). CML is a nonfluorescent compound, deriving from the oxidative degradation of Amadori products. High levels of CML have been found in the collagen of diabetic patients, as is related to the presence of retinopathy and nephropathy (Schleicher et al., 1997; Boehm et al., 2004), and high levels are also found in patients with end-stage renal disease (Weiss et al., 2000). In addition it has been shown that lipid peroxidation may be a source of CML

in diabetes and atherosclerosis (Fu et al., 1996). CML may be measured by GC/MS (Ahmed et al., 1986) and by an ELISA technique, which uses monoclonal antibodies (Boehm et al., 2004).

Pentosidine is a fluorescent cross-link product deriving from the reaction of glucose with lysine, arginine, and ribose, forming an imidazopyridine ring. In plasma 95% of pentosidine is linked to proteins and 5% is in free form. Because of its low concentration, free pentosidine can only be detected in plasma in end-stage renal disease (Miyata et al., 1996). Increased levels of pentosidine have been found in the collagen, plasma, and red cells of patients with diabetes and end-stage renal disease (Weiss et al., 2000; Sell et al., 1992; Odetti et al., 1992; Monnier et al., 1992). In a recent paper we evaluated the plasma levels of pentosidine in type 2 diabetic patients with poor metabolic control, at baseline, and after 10 months of improved metabolic control. The results showed that after that period patients had near normal levels of the fasting plasma glucose and HbA_{1c}, and reduced, but not normalized, pentosidine levels. Clearly, this calls for a longer period over which to improve metabolic control in order to reduce both this parameter and lift the burden of chronic diabetic complications (Lapolla et al., 2005a). An LC method is available to measure pentosidine accurately (Sell and Monnier, 1989).

Pyrraline, also called pyrrole-lysine, is an AGE nonoxidatively formed from 3-deoxyglucosone. Although pyrraline has been detected in plasma proteins and tissue collagen (Hayase et al., 1989), the quantities involved are very small. Pyrraline also undergoes degradation, as a result it cannot be considered a useful parameter (Miyata and Monnier, 1992); LC and ELISA techniques are available to measure it (Hayase et al., 1989; Miyata and Monnier, 1992).

With the new mass spectrometric methods available for the study of the proteome, we became interested in their application to protein glycation and glyco-oxidation, and we report the results to date in the next section. Most of our results were obtained by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. As a simple overview of this method, it is sufficient to say that the MALDI-MS consists in an interaction of a laser beam with a solid state sample composed of a suitable matrix (usually an aromatic acid) in which the analyte is present at low level (the analyte/matrix molar ratio is generally of the order of 1/1,000). A small volume of the solid state sample is vaporized by ion/molecule reactions taking place in the matrix. In the high-density vapor cloud of proteins, close to the crystal surface, is the formation of very abundant protonated molecules. The laser is pulse-operated (100 ns), and consequently the mass analyzer usually employed is the time-of-flight (TOF) type. This instrumental setup generally covers a mass range up to 500,000 Da, a mass resolution in the order of 10^3 to 10^4, and sensitivity usually at the femtomol level.

7.2 MALDI-MS IN THE STUDY OF IN VITRO GLYCATED PROTEINS

In the early 1990s MALDI mass spectrometry was just beginning to be commercialized. Only one instrument, of rather low performance, was then available

but we thought of testing its effectiveness in our protein glycation investigations. Before we started on the glycation processes occurring in vivo, we carried out some preliminary studies on in vitro glycated proteins. Our aim was to evaluate the capabilities and the limits of the MALDI technique in this specific way.

Our first experiments in applying MALDI-MS were thus devoted to the study of in vitro glycation of bovine serum albumin (BSA) (Lapolla et al., 1993). The protein (1.5 g of BSA) was incubated in pseudophysiological conditions (15 ml sodium phosphate buffer, 0.05 M, pH 7.5) at various glucose concentrations (0.02 M, 0.2 M, 2 M, 5 M). Although the instrumental performances were low, interesting results were obtained. By drawing samples at varying incubation times (from 0 to 28 days) a clear-cut increase in the mass value of BSA protonated molecules was observed (see for example Fig. 7.5). The low resolution of the instrument did not allow clear definition of the various components of the peaks. However, from only the mean mass value, corresponding to the maximum of the peak, it was evident that the molecular weight values of BSA increase with time and that these data could be related to different number of glucose molecules condensing on the protein. This number was easily calculated, since the condensation of each glucose molecule leads to a mass increase of 162 Da. The difference in mass between glycated and unglycated BSA peaks divided by 162 gives the *minimum* number of glucose molecule condensing on the protein. Thus, for example, the difference in mass between the peak E (m/z 68,549) and that of unglycated BSA (peak A, m/z 66,429), calculated at 2,120 Da, indicates that after 28 days of incubation with 0.2 M glucose, 13 glucose molecules have condensed on the protein. We would like to emphasize that, this number must be considered the minimum number because it includes the condensation of intact glucose molecules. If further dehydration-oxidation reactions take place, the mass value is lower than 162 Da, and consequently a higher number of these species will lead to the same increase in mass. It is important to emphasize that, since the time of this early study, our view of the protein glycation process has somewhat changed. As shown by Thornalley et al. (2003), our early study was a simplification of the series of processes that occur in vitro. In particular, glucose addition can also take place on arginine residues. The enlarged peak shape is not only due to instrumental problems (low resolution and high kinetic energy spread) but also to the co-presence of species with a different number of glucose molecules and their dehydration-oxidation products.

However, if the molecular weight of the incubated proteins is plotted against the incubation time, a trend similar to that shown in Figure 7.6 is obtained, revealing a saturation level that corresponds to the occurrence of glycation on all the reactive sites of the protein. To some extent these results are in contrast to those obtained by Odetti et al. (1990) based on spectroscopic measurements of reaction mixtures. In that case a linear relationship was found between absorbance and incubation time. The difference could be explained by considering the Maillard reaction pathway. In the Odetti et al. case both the glycated protein and propagators were measured, whereas in our case only the protein was studied.

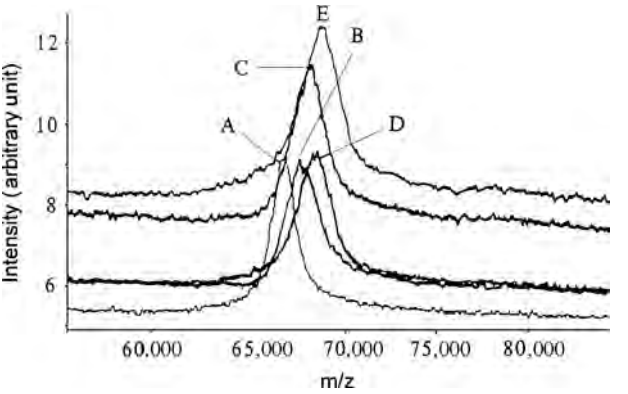


FIGURE 7.5 MALDI spectra of BSA incubated with glucose at 0.2 M concentration (pH 7.5; 37°C) recorded at different incubation times: (*A*), incubation time, 0 days; molecular mass, 66,429 Da. (*B*), incubation time, 7 days; molecular mass, 67,585 Da. (*C*), incubation time, 14 days; molecular mass, 67,897 Da. (*D*), incubation time, 21 days; molecular mass, 68,260 Da. (*E*), incubation time, 28 days; molecular mass, 68,549 Da. (Reprinted from Lapolla et al., 1993, by permission of Elsevier Science Publishers.)

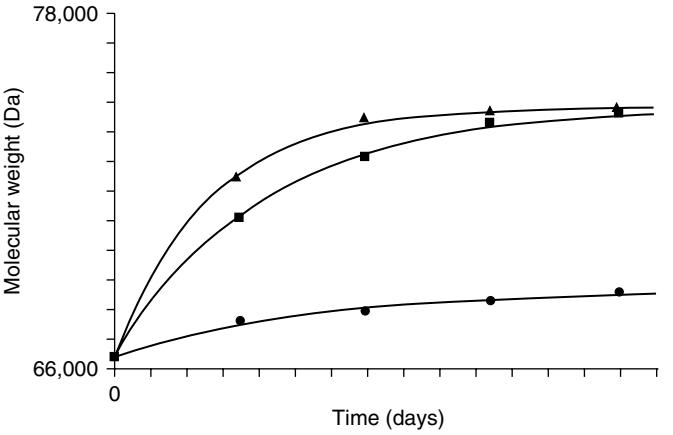
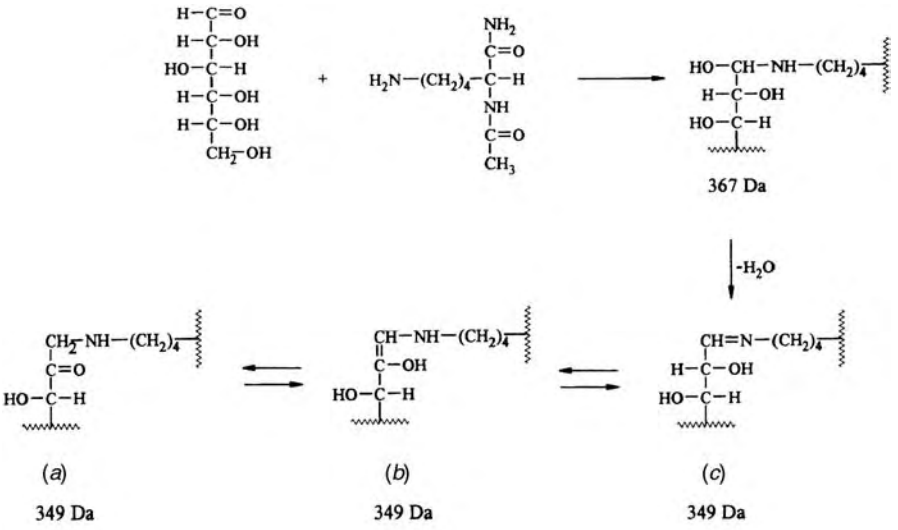


FIGURE 7.6 Plots of molecular mass (Da) against incubation time (days) related to BSA incubated with glucose at different concentration: • glucose concentration 0.2 M; □ glucose concentration 2 M; △ glucose concentration 5 M. (Reprinted from Lapolla et al., 1993, by permission of Elsevier Science Publishers.)

We made some further studies devoted to the in vitro glycation of various proteins with several sugars (Lapolla et al., 1994, 1996a). Since the results turned out to be similar to those mentioned above, we are confident about the validity of MALDI-MS for studying protein glycation.

Nevertheless, our approach did not furnish any evidence of the cross-linking of products. In order to examine this formation, we investigated the reaction between *N*-α-acetyl-L-lysinamide and glucose. To identify the products, we used a powerful technique based on the coupling of capillary zone electrophoresis with electrospray mass spectrometry (CZE-ESI-MS) (Lapolla et al., 1995a). The reaction of glucose (0.1 M) with *N*-α-acetyl-L-lysinamide was performed under pseudophysiological conditions (pH 7.4, 37°C, phosphate buffer). The reaction mixture yielded the CZE total ion current electropherogram shown in Figure 7.7*a*, with four large varying peaks. The ESI spectra are shown in Figure 7.7, panels *b* through *f*. The spectra are quite complex, because of the overlap of various molecular species exhibiting the same migration time. However, if the Maillard reaction mechanisms are taken into account, most of the compounds indicated by these ESI spectra can be explained. The first stage of the reaction of the lysine derivative with glucose is likely to consist of an addition, leading to an adduct with molecular weight 367 (see Scheme 7.2). Interestingly the peak corresponding to this protonated adduct is present in the mass spectra of the component with t_M 6.61 at m/z 368 together with the species cationized with Na^+ (m/z 390).

The next reaction in the Maillard pattern was the elimination of the water molecule, leading to structure **a** (Scheme 7.2), which rearranges, through the intermediate structure **b**, to the ketoamine **c**. These isomeric structures had a molecular weight of 349 Da, and the related $[M + H]^+$ species (m/z 350) are well represented in the shoulder of the major peak, at t_M of 8.08 min (Fig. 7.7*d*) and also appear at t_M of 8.43 min (Fig. 7.7*e*). Other species due to dehydration processes were also detected. Interestingly, the detection of high molecular weight species can be explained by the reaction of a glycated lysine with a



SCHEME 7.2 Reactions between a lysine derivative and glucose.

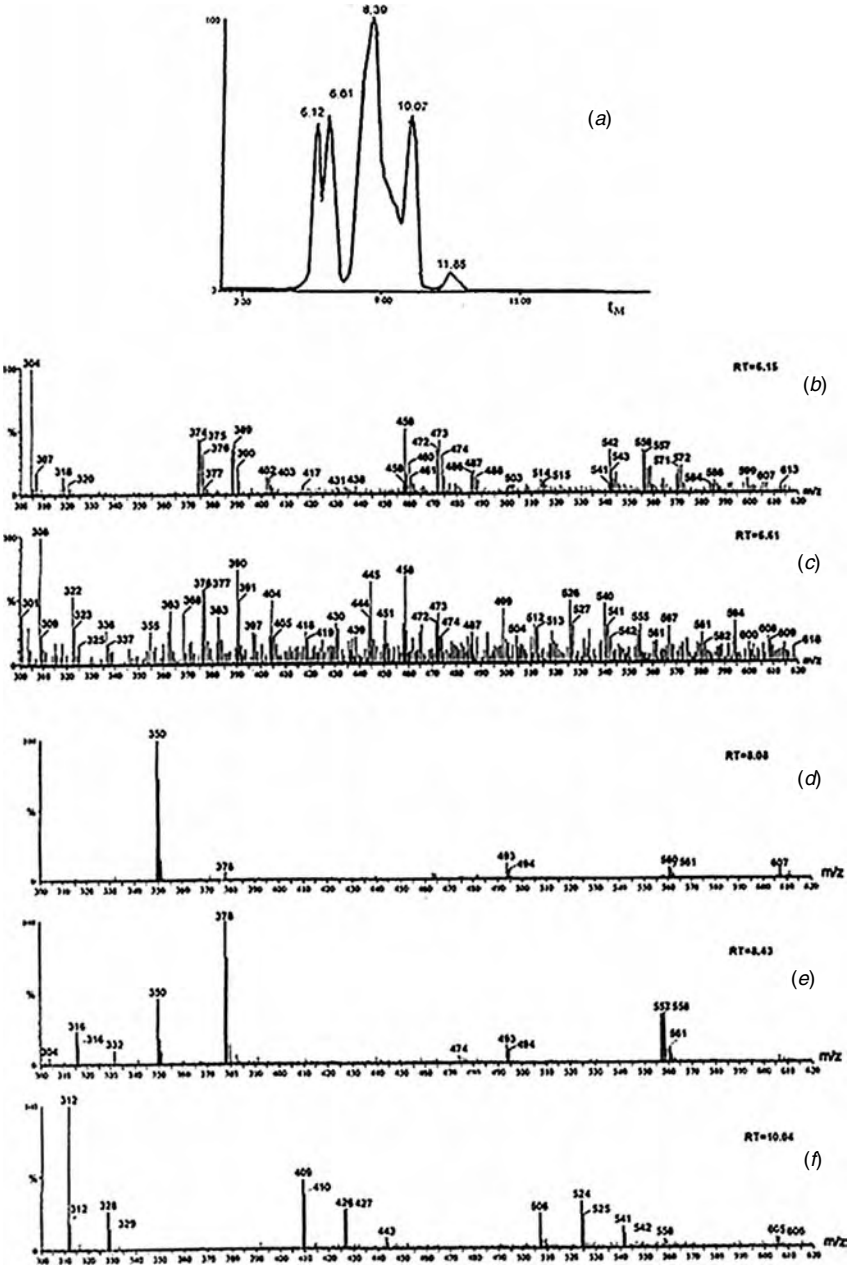


FIGURE 7.7 CZE total ion current (TIC) electropherogram obtained for the reaction mixture arising from 30 days of incubation under pseudophysiological conditions (pH 7.4; 37°C; phosphate buffer) of 0.1 M *N*-α-acetyl-L-lysinamide with 1 M glucose (*a*). ES mass spectra under the TIC peaks with migration times (t_M) of (*b*) 6.15 min and (*c*) 6.61 min. ES mass spectra under the TIC peaks with migration times (t_M) of (*d*) 8.08 min, (*e*) 8.43 min, and (*f*) 10.04 min.

lysine molecule, thus paralleling the cross-linking reaction observed in the case of a glycated protein with a nonglycated protein.

7.3 MALDI-MS IN THE EVALUATION OF GLYCATION LEVELS OF HSA AND IgG IN DIABETIC PATIENTS

Because of our positive preliminary results from the in vitro experiments, we were confident of the validity of the MALDI/MS technique applied to glyco-oxidation processes. So we undertook a systematic study of plasma proteins with healthy subjects, well-controlled, and poorly controlled diabetic patients (Lapolla et al., 1995b) (clinical and metabolic data are listed in Table 7.1). The MALDI/MS spectrum of the plasma protein fraction from a healthy subject is shown in Figure 7.8*a*. It may be considered as a valid map of the various proteins in plasma. The species detected around m/z 150,000 arise from IgG, whereas the most abundant species at m/z 66,680 is due to HSA, which shows the doubly protonated species at m/z 33,375. The minor peak at m/z 79,679 is probably due to prothrombin. Owing to its high abundance, we based our study on HSA and first proved its identity by comparison with a standard HSA sample. This produced only two peaks, the higher peak is at m/z 66,680 corresponding to $[M + H]^+$ species and the lower one at m/z 33,375, due to $[M + 2H]^+$ species, thus confirming our hypothesis.

Analysis of the plasma protein samples from the poorly controlled diabetic patients showed completely different mass values (Fig. 7.8*b*). The peak due to HSA was shifted at m/z 68,033, corresponding to a mass increase (Δm) of 1,353 Da with respect to that shown in Figure 7.8*a*. This increase may be explained by the occurrence of glyco-oxidation on plasma proteins, that is, by the number of glucose molecules that react with the ε-amino groups of lysines present in the protein chain. The condensed molecules can even undergo further

TABLE 7.1 Clinical and Metabolic Data

Subjects	*n*	Male/ Female	Age (years)	Disease Duration (years)	Fasting Plasma Glucose Level (mmol/l)	HbA_{1c} (%)	Furosine (μg/mg Protein)
Poorly controlled diabetic patients	20	10/10	64 ± 7.6^a	9.3 ± 8.7	$20.2 \pm 4.3^{b,c}$	$10.6 \pm 1.9^{b,c}$	$0.47 \pm 0.08^{b,c}$
Well-controlled diabetic patients	10	5/5	60 ± 11	12.3 ± 8	7.96 ± 1.1^d	7.25 ± 0.63^d	0.33 ± 0.03^d
Healthy subjects	10	4/6	56 ± 9.6	—	5.46 ± 0.4	5.57 ± 0.43	0.23 ± 0.02

Note: Data are mean ± SD.
[a] $p < 0.05$
[b] $p < 0.001$ compared to healthy subjects
[c] $p < 0.001$ compared to well-controlled diabetic patients
[d] $p < 0.001$ compared to healthy subjects

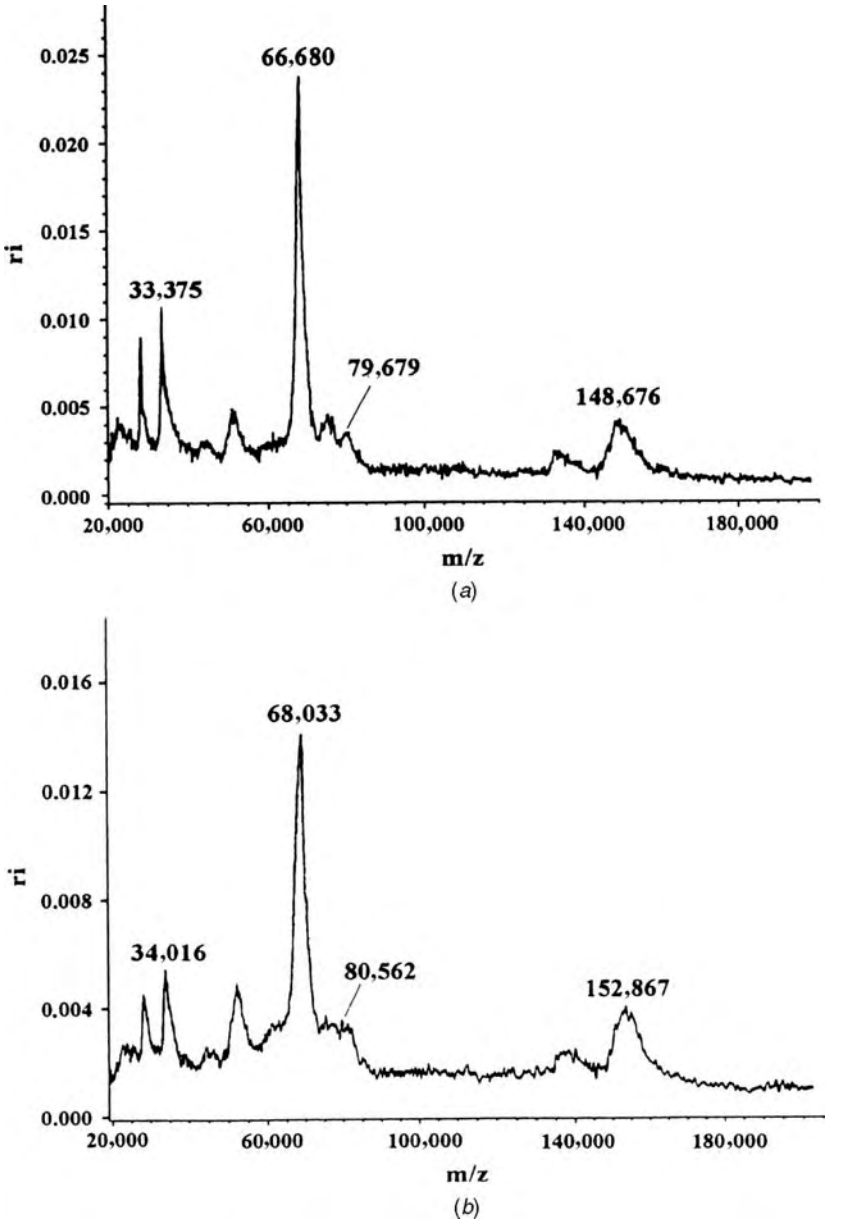


FIGURE 7.8 MALDI/MS spectra of plasma protein fractions observed from (*a*) healthy subjects A and (*b*) poorly controlled diabetic patient 4. The mass values reported on the spectra are those directly determined by the instrument, with a mass accuracy of 0.01% to 0.1%. (Reprinted from Lapolla et al., 1995, by permission of Springer-Verlag.)

oxidation according to Amadori findings. Figure 7.9 shows the great difference in Δm values (defined as the mass difference between the glycated HSA and genuine HSA) obtained for healthy subjects and diabetics with well controlled and badly controlled metabolism. Figure 7.10 shows the histograms for the plasma glucose, furosine, and HbA_{1c} levels of the 10 healthy subjects, the 10 patients with well-controlled diabetes, and the 20 poorly controlled diabetic patients. The values measured for the first two groups of subjects are quite close, with the values of healthy subjects in most case being nearly zero. So the difference in Δm (Fig. 7.9) is more meaningful in patients with poorly controlled diabetes, where Δm values vary from 439 to 2,403 Da. These values correspond to the condensation of 3 to 15 glucose units on HSA. Again, it must be noted that the figures do not take into account any further oxidative modifications of glucose, which would give a species of lower molecular weight. These results fit the earlier findings of Ghiggeri et al. (1985), confirming that in HSA, 16 active sites are prone to react with glucose molecules.

However, the use of mass measurement of proteins to assess the extent of glycation has been criticized by others. The arguments are that (1) the mass increment cannot be interpreted as only fructose-lysine (FL) residues, (2) large increments of mass of albumin in vivo have not be found by others (Thornalley et al., 2000), and (3) the kinetics of glycation in vivo and the half-life of albumin are not consistent with high modification of albumin by glucose in vivo (Baynes

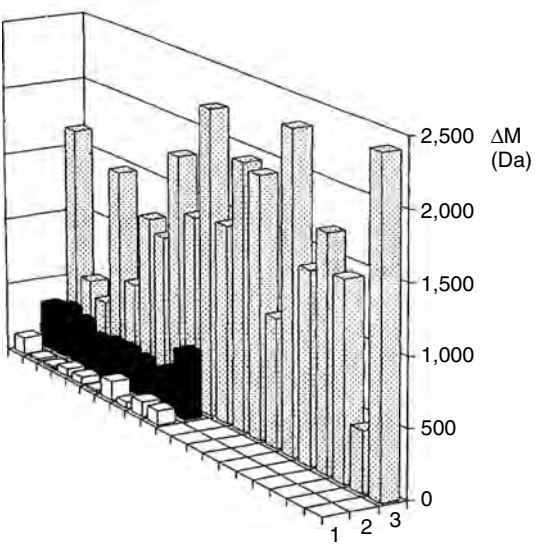


FIGURE 7.9 The Δm values, defined as the difference in molecular weight of plasma HSA of the subjects under study with respect to that of standard HSA, obtained for healthy subjects (1), 10 patients with well-controlled diabetes (2), and 20 with poorly controlled diabetes (3). (Reprinted from Traldi et al., 1997, by permission of John Wiley & Sons.)

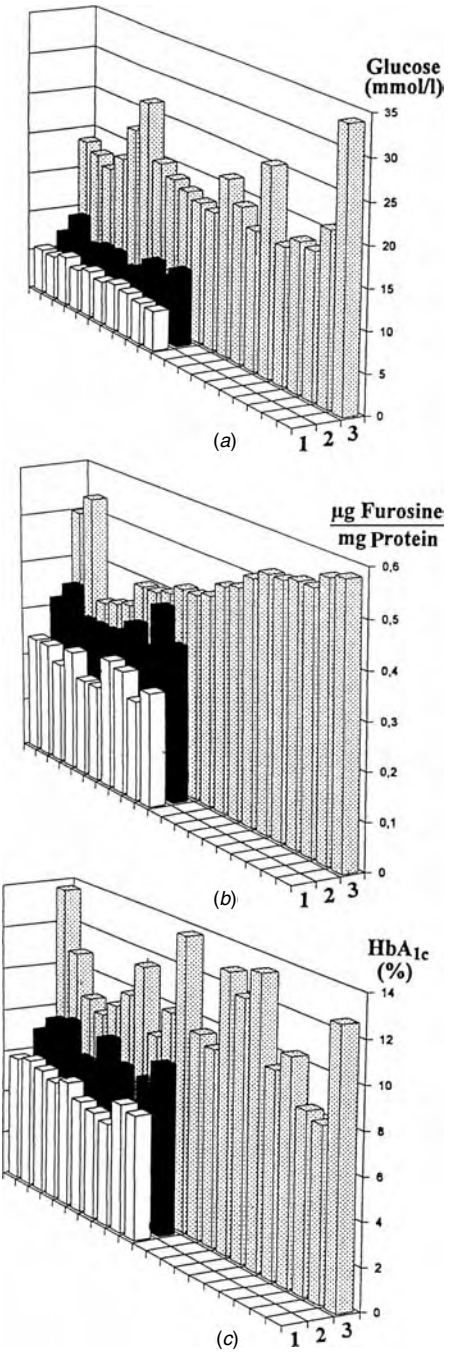


FIGURE 7.10 (*a*) plasma glucose, (*b*) furosine levels, (*c*) HbA_{1c} determined for 10 healthy (1), 10 patients with well-controlled diabetes (2), and 20 patients with poorly controlled diabetes (3). (Reprinted from Lapolla et al., 1995, by permission of Springer-Verlag.)

et al., 1984), and biochemical measurements of FL residues do not indicate more than one FL residue per albumin molecule in vivo—even in diabetic patients (Yamamoto et al., 1989).

We emphasize that our research was not performed on a single sample but on plasma samples coming from two different populations: one of healthy subjects, and the other of diabetic patients with different levels of metabolic control but never at the point of hyperlipemia. Furthermore, we need to stress that the investigations based on digestion of in vivo glycated proteins can led to misleading results; as it will be shown later, the enzyme action on a glycated protein is very different from that observed for unglycated proteins. The MALDI-MS technique has also proved to be highly effective in the study of glyco-oxidation occurring in in vivo conditions. However, the drawback is that although information on the number of glucose units condensing on the protein is easy to obtain, the information on their oxidation/dehydration level is not.

The spectra of Figure 7.11 show the large mass increase in IgG experienced by the poorly controlled diabetic patient in a study similar to that of HSA performed for this protein (Lapolla et al., 2000). Figure 7.12 shows the mass increase observed in the 10 healthy subjects, the 10 well-controlled, and the 10 poorly controlled diabetic patients. Note the high variability in the number of glucose molecules condensed on the protein within each category. This may confirm a recent hypothesis on the existence of different phenotypes with respect to protein glycation (Hempe et al., 2002).

Moreover, the IgG glycation reflects the different functionality of this immunoprotein (Kennedy et al., 1994). In order to examine this point, in a further experimental study we compared the papain digestion products of in vitro glycated IgG with those obtained by papain digestion of the whole plasma of diabetic patients (Lapolla et al., 2000). Our results showed that the Fab fragment is the most prone to glycation, indicating that the possible failure of the action of the protein may be due to its antigen-binding properties. In order to confirm this finding, we passed to molecular modeling of the whole protein and the calculation of solvent accessible surface (SAS) of the lysine belonging to the protein chain (representing the reactive sites for the glycation processes). The latter results fit the experimental ones, that is, that the Fab fragment is the most prone to glycation.

7.4 HbA_{1c} AND THE REAL GLOBIN GLYCATION AND GLYCO-OXIDATION

The metabolic control of diabetic patients is usually evaluated by measuring fasting plasma glucose (i.e., the concentration of glucose in plasma at the moment of blood sample drawing) and by HbA_{1c}, defined as the percentage of glycated β globin coming from nonenzymatic glycation of hemoglobin. Since the mean half-life of hemoglobin is estimated to be about 120 days, HbA_{1c} measurements allow evaluation of the "glycation stress" undergone by the subject in the previous four to six weeks (Schwartz, 1995).

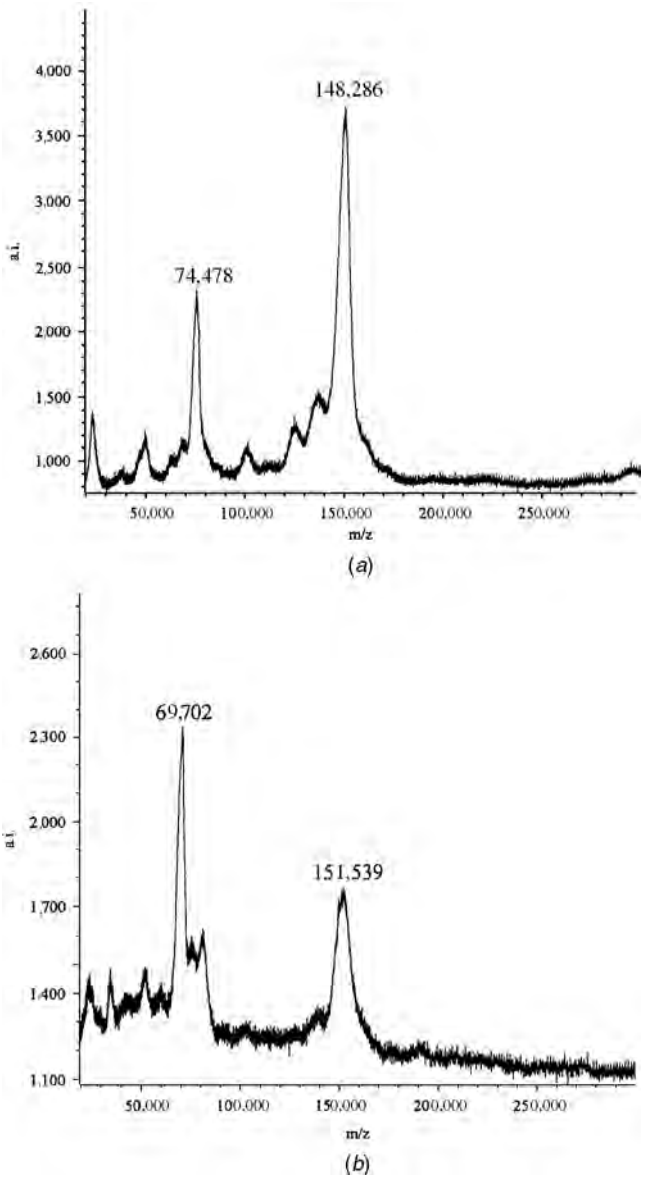


FIGURE 7.11 MALDI mass spectra of (*a*) standard IgG and (*b*) IgG contained in a protein plasma fraction of a poorly controlled diabetic subjects.

It was therefore surprising to find that MALDI mass spectrometry showed both globins (α and β) to be glycated to a similar extent (Lapolla et al., 1996b). The spectrum in Figure 7.13 clearly indicates the presence of unglycated α and β globins (at m/z 15,127 and m/z 15,868), together with ion at m/z 15,289

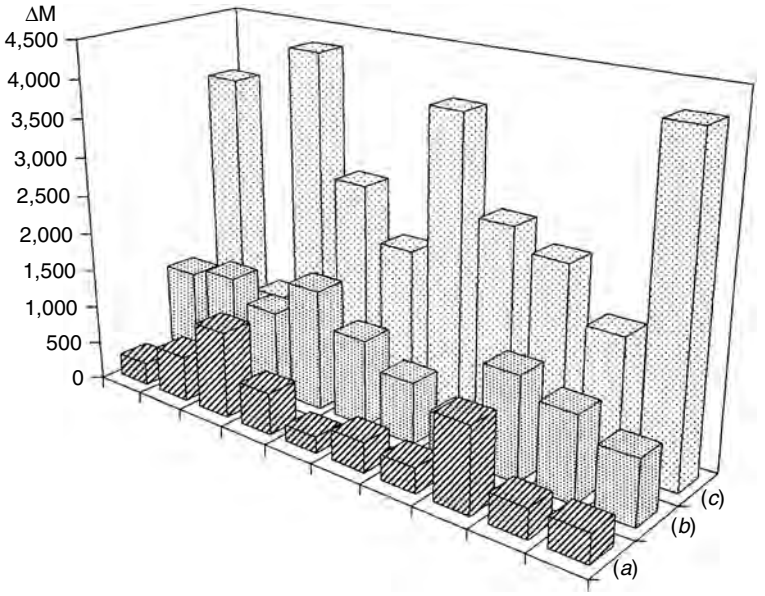


FIGURE 7.12 Histograms of Δm values of IgG obtained for 10 healthy subjects (*a*), 10 well-controlled (*b*), and 10 poorly controlled diabetic patients (*c*). Δm values are simply calculated as differences between IgG molecular weight obtained for the various subjects and that corresponding to standard IgG (14,8286 Da). (Reprinted from Lapolla et al., 2000, by permission of Elsevier.)

and 16,030 corresponding to the condensation of glucose molecule on α and β globin, respectively. Careful examination of the spectra also revealed other species, for example, those due to diglycated β globin, and proteins containing dehydration/oxidation products of glucose. These preliminary results show that the current view of HbA_{1c} cannot be correct because the best linear relationship is found between the percentage of the entire set of glycated and glyco-oxidated α and β globins and HbA_{1c}. A plot showing no significance was obtained between the percentage of glycated β globin (obtained by MS) and HbA_{1c}. With regard to the methods usually employed for HbA_{1c} measurements this is not surprising. The LC method of HbA_{1c} evaluation strips the glycated and unglycated species of both α and β globins, and consequently does not give sufficiently high specificity. When electrospray ionization was employed to evaluate the globin glycation levels (Zhang et al., 2001; Roberts et al., 2001), the peaks related to the glycated and unglycated globins are obtained on the basis of the observed multiple charge clusters. Both investigations therefore agree with the occurrence of glycation of both α and β globins. The results were further confirmed by more extensive studies based on both MALDI/MS and electrospray measurements (Lapolla et al., 1999). Investigations by different sample treatments and by analysis of the glycated β globin fraction obtained by preparative chromatography show the

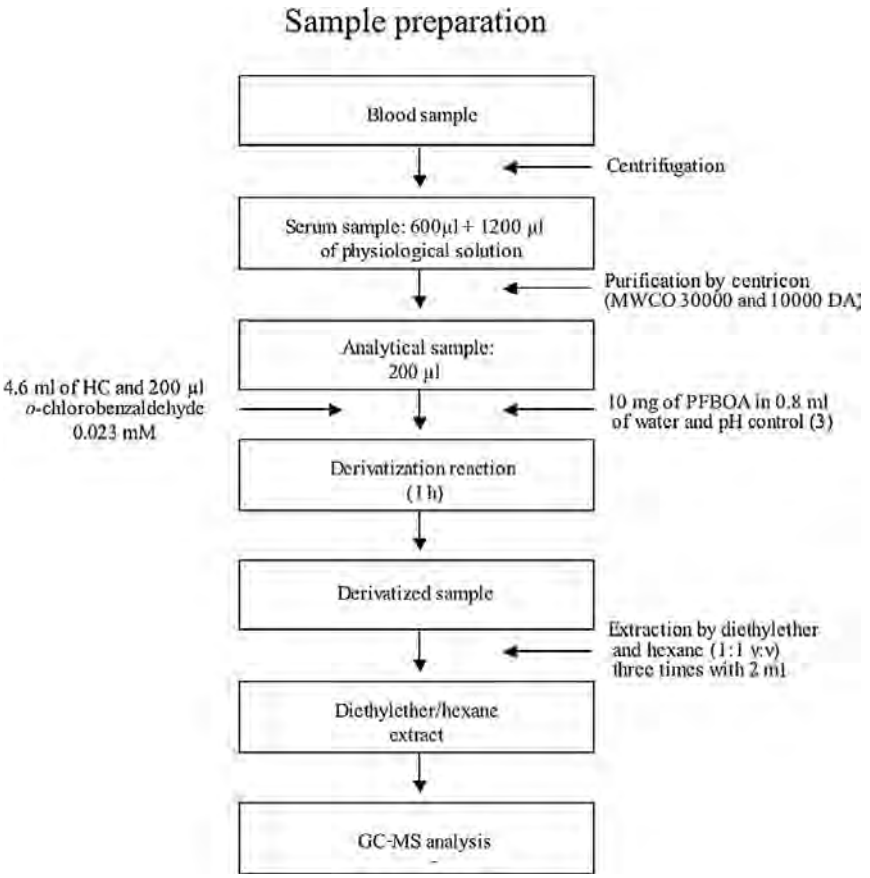


FIGURE 7.13 MALDI mass spectrum of erythrocyte globins from a healthy subject. (Reprinted from Lapolla et al., 1996b, by permission of John Wiley & Sons.)

presence of both glycated and glyco-oxidated globins. Recently the International Federation of Clinical Chemistry (IFCC) organized a working group on HbA_{1c}, with the aim of achieving an international system of standardization, developing and validating a reference method able to measure HbA_{1c} with high specificity, in order to produce a series of control materials, certified, of human origin, to serve as secondary materials of reference, and to establish new reference and decision limits. This working group has constructed a reference standard for HbA_{1c}, which has been quantified by mass spectrometry; from this basic reference, secondary standards have been constructed for use as calibrators for routine HbA_{1c} measurements (Jeppsson et al., 2002).

In a recent investigation the MALDI-MS method was applied to study the glycation and glyco-oxidation products of a population of diabetic patients, some of whom had chronic complications (Lapolla et al., 2004a). Interestingly, the linear

relationship of the percentage of the all set of glycated and glyco-oxidated α and β globins versus HbA_{1c} of patients without complications showed a slope differing from that of patients with chronic complications. The difference indicates that accurate measurements of glycated and glyco-oxidated species are in some way related to the pathological condition of the patients or, alternatively, to different phenotypes inside the diabetic population. A GC-MS method for the identification and quantitation of *N*-(carboxymethyl)valine adducts in hemoglobin has been proposed by Cai and Hurst (1999), and more recently, for the detection of AGEs in hemoglobin, a LC-MS/MS approach has been investigated by Thornalley et al. (2003). In this extensive study underivatized analytes were detected and quantified in physiological fluids and in enzymatic hydrolyzates of cellular and extracellular proteins.

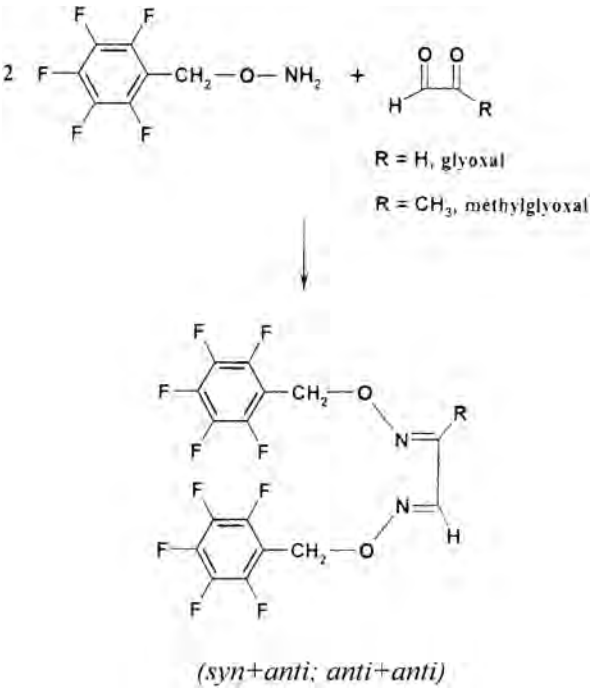
7.5 DETERMINATION OF DICARBONYL COMPOUND LEVELS IN DIABETIC AND NEPHROPATHIC PATIENTS

In the intermediate Maillard reaction pattern (see Fig. 7.1) a series of reactive dicarbonyl compounds are released from the protein and, after a series of rearrangements, give rise to advanced glycation end-products (AGEs). As described in the introduction, AGEs are responsible for tissue damage by means of a series of mechanisms, the most important of which are cross-linking formation, intracellular accumulation, and interactions with specific cellular receptors. In the case of diabetic patients the high level of plasma glucose promotes increased formation and accumulation of AGEs (Thornalley, 1996), leading to chronic diabetic complications such as macroangiopathy, nephropathy, and retinopathy. The same problems arise when AGEs accumulation is due to impaired excretion, as in nephropathies (Makita et al., 1991, 1994; Miyata et al., 1999; Schinzel et al., 2001).

Many studies have demonstrated that the three main dicarbonyl compounds—deoxyglucosones, glyoxal, and methylglyoxal—are usually excreted by the kidney. However, in the case of renal failure, they accumulate in plasma, leading to worsening of macroangiopathy (Makita et al., 1994; Miyata et al., 1999). Consequently, the development of analytical methods for evaluating the levels of these compounds has been and still is of great interest. In this framework various methods have been proposed based on liquid chromatography (LC) (McLellan et al., 1992; Thornalley et al., 1999) after suitable derivatization and LC/ESI tandem mass spectrometry (Odani et al., 1998, 1999). Nevertheless, the development of simple methods based on low-cost instrumentation with sufficiently high enough specificity is definitively of interest. We took up the challenge of developing a GC/MS method for glyoxal and methylglyoxal quantification after derivatization with *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride (PFBOA) (Lapolla et al., 2003a). As shown in Scheme 7.3, this reactant leads to the formation of two different oximes, *syn* and *anti*, from each carbonyl group. Glyoxal and methylglyoxal, both containing two carbonyl groups, are quantified

by the sum of chromatographic peaks of their *syn* + *anti* and *anti* + *anti* *O*-PFB-dioximes (the formation of the *syn* + *syn* dioxime is not observed, probably for reason of steric hindrance). Ortho-chlorobenzaldehyde was chosen as internal standard and quantified on the basis of the sum of signals of *syn* and *anti* *O*-PFB-monoximes. Nine 600 μl plasma samples, three from healthy subjects, three from poorly controlled diabetic patients, and three from patients affected by chronic renal failure, were analyzed (Lapolla et al., 2003a). The sample treatment is shown in Figure 7.14. By GC-MS, we detected higher abundances of glyoxal and methylglyoxal in the sample from the nephropathic patient. Quantitative results for the nine samples are listed in Table 7.2. Each datum is the mean value of four different measurements performed on different days; the related standard deviations are also shown. In the case of glyoxal, a mean value of 17.3 μg/l was found in healthy subjects—clearly lower than those obtained for poorly controlled diabetic patients (26.4 μg/l) and those affected by chronic renal failure (27.2 μg/l). An analogous trend was observed for methylglyoxal, namely 8.7 μg/l for healthy subjects, 16.8 μg/l for poorly controlled diabetic patients, and 16.6 μg/l for nephropathic patients.

The same method was used for another study (Lapolla et al., 2003b). Ten diabetic patients were evaluated before and after improvement of glycemic control.



SCHEME 7.3 Formation of *syn*- and *anti*-oximes by derivatization of glyoxal and methylglyoxal with PFBOA.

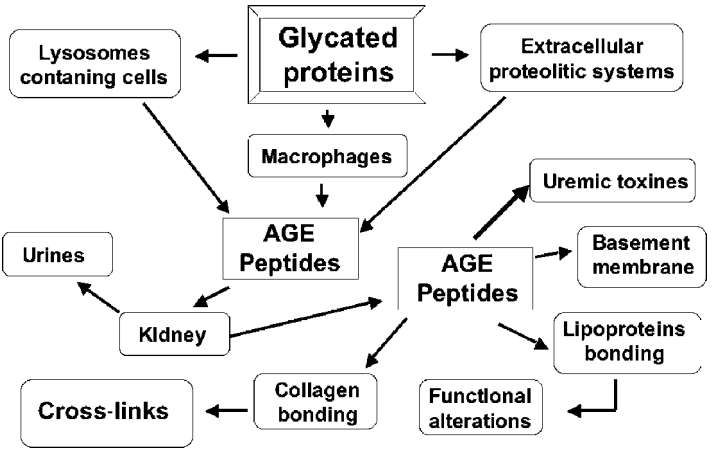


FIGURE 7.14 Flowchart of plasma sample preparation procedure.

TABLE 7.2 Quantitative Evaluation of Glyoxal and Methylglyoxal in Plasma Samples from Healthy, Diabetic, and Nephropathic Subjects

Sample (μg/L)	Glyoxal	SD	Methylglyoxal	SD
Healthy subject 1	20.2	1.8	10.3	1.9
Healthy subject 2	8.3	0.3	2.7	0.1
Healthy subject 3	23.5	2.1	13.2	1.1
Mean value	17.3		8.7	
Diabetic subject 1	23.4	0.5	14.9	1.4
Diabetic subject 2	27.7	3.6	19.7	0.9
Diabetic subject 3	28.1	0.03	15.7	2.8
Mean value	26.4		16.8	
Nephropathic subject 1	27.5	3.6	14.9	2.0
Nephropathic subject 2	22.4	0.5	15.8	3.0
Nephropathic subject 3	31.6	2.1	18.6	0.2
Mean value	27.2		16.4	

Fasting plasma glucose, HbA_{1c}, AGEs, pentosidine, glyoxal, and methylglyoxal levels were all measured. The percentage decreases in levels of fasting plasma glucose, HbA_{1c}, and AGEs were greater than those of pentosidine, glyoxal, and methylglyoxal. These results may be explained by the different positions of these compounds in the Maillard reaction pathways: the two sets of metabolic parameters give a different picture of the patients' metabolic control. Measurement of glyoxal and methylglyoxal is particularly important when evaluating the possible effect of oxidative stress. Other metabolic pathways may contribute to glyoxal production, and the observed minor decrease in these compounds may, in theory, be ascribed to such effect.

In conclusion, our data showed that good glycemic control in diabetic patients is not sufficient to normalize glyoxal and methylglyoxal levels. In view of the high toxicity of these compounds, their slight decrease even after therapy must be noted, and new therapeutic approaches, such as the possible use of antioxidants, may be required, parallel with optimization of glycemic control.

It must also be stressed that in conditions of intracellular hyperglycemia, as occurs in diabetes, the accumulation of triosephosphates in pericytes and endothelial cells has been shown (McLellan et al., 1992). This leads to increased formation of methylglyoxal and, consequently, of AGEs (Thornalley, 1999). Intracellular accumulation of AGEs is an important pathogenetic mechanism contributing to the development of chronic diabetic complications, so the assessment of intracellular formation of methylglyoxal is important in monitoring intracellular damage. In red blood cells incubated at a high glucose concentration in short-term culture, an increase in the flux of metabolites through the pentose-phosphate pathway, and a consequent increase in methylglyoxal production have recently been demonstrated (Thornalley et al., 2001). It would be particularly interesting to carry out a similar study of glyoxal and methylglyoxal levels in red blood cells, since the result could yield information on the time required for normalization of their concentration in improving metabolic control, and this would enable evaluation of potential intracellular damage. Other metabolic pathways may contribute to glyoxal and methylglyoxal production (McLellan et al., 1992), and their lower decreases in the levels of these compounds observed in the present study could be, in principle, ascribed to them. However, in our opinion, the similar behavior of pentosidine indicates that the pathways, contrary to the Maillard reaction, contribute very little to glyoxal and methylglyoxal formation.

7.6 AGE/PEPTIDES: AN IN VITRO STUDY AND IN VIVO PRELIMINARY RESULTS

As AGE modified proteins are chemically altered, biological mechanisms act in their recognition and turnover (Makita et al., 1991). The fate of a glycated protein is shown in Figure 7.15. In particular, macrophages, via the AGE receptor system, appear to be the main agents responsible for AGE-modified tissue removal. The resulting small peptides so generated are transported to the kidney for clearance (Gugliucci and Bendayan, 1996; Horiuchi et al., 1996; Dean, 1975; Skolnik et al., 1991). It should be emphasized that such AGE peptides, in being a highly reactive species, can interact with both circulating and tissue proteins, leading to modifications more severe than those due to simple glycation.

In the case of renal failure the excretion of AGE peptides is impaired, and their accumulation in plasma has been demonstrated in diabetic and nondiabetic patients with end-stage renal disease (Ritz et al., 1994; Korbert et al., 1993). Their reactivity against plasma lipoproteins and collagen explains the progression of chronic complications in diabetic patients with renal failure as well as the activation of vascular pathologies in end-stage renal disease (Bucala et al., 1994; Gugliucci and Menini, 1998).

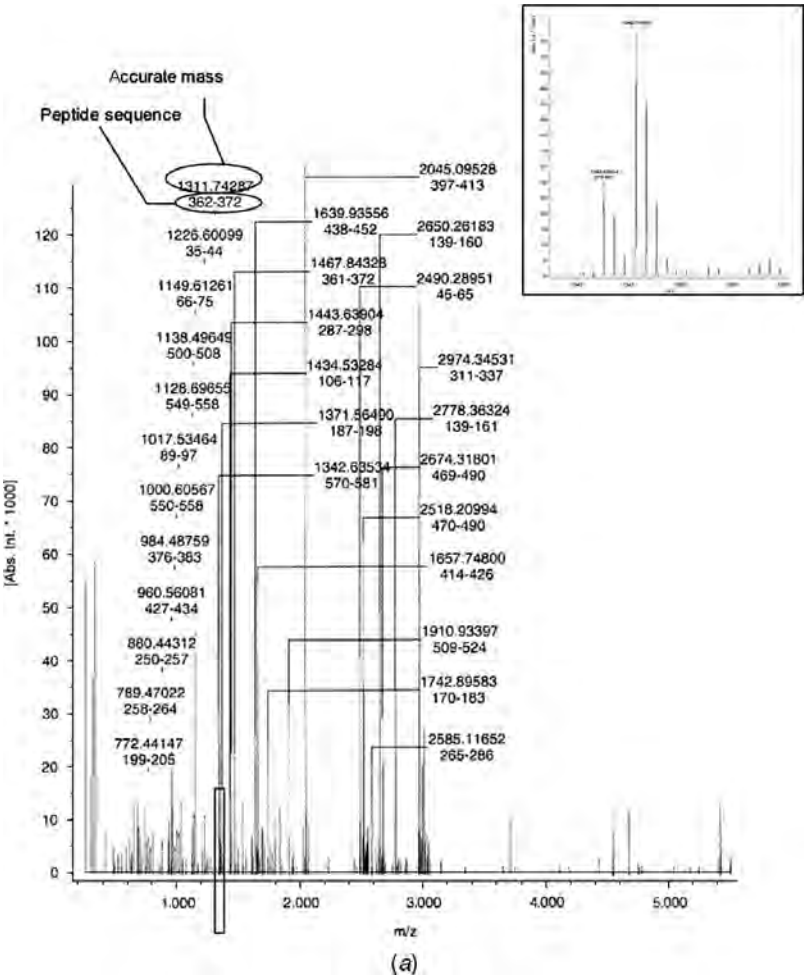


FIGURE 7.15 Fate of a glycated protein.

AGE peptides were first studied by spectroscopic methods and LC separation. The term AGE peptides is currently applied to the AGE product present in plasma, separated by ultrafiltration with membranes at molecular mass cut-off of 10,000 Da (Makita et al., 1992, 1994; Gugliucci and Bendayan, 1996; Papanastasiou et al., 1994).

In view of the great complexity of low molecular weight protein fraction of plasma, a direct approach for determining AGE peptides by high-specificity methods such as LC/MS and MALDI/MS seems to be unproductive. For these reasons a preliminary step, based on in vitro glycation and enzymatic digestion of physiologically important proteins, was believed to be necessary. Since HSA is the most abundant protein in plasma, it was thought necessary to perform its

in vitro glycation followed by digestion of glycated HSA and unglycated HSA by different enzymes and compare the resulting maps by mass spectrometric methods, in order to achieve the structural identification of the AGE peptides.

A first study, based on digestion with proteinase K of both glycated and unglycated HSA, according to the approach proposed by Gugliucci and Bendayan (1996), followed by LC-MS analysis, showed that digestion mixtures are very complex (Lapolla et al., 2002). Although clear-cut differences were found between the digestion products of glycated and unglycated HSA, no structural identification was possible, due to the low specificity of the mass spectrometric approach. More specific approaches were needed, and for accurate mass measurements and MS^n experiments, this framework seemed to be highly attractive. The former approach yielded the elemental composition of a selected species and the latter supplied important information of the amino acid sequence. In addition, we considered the use of more specific enzymes in order to obtain a digestion mixture simpler than that obtained by proteinase K, so trypsin and endoproteinase Lys-C were employed.

Fourier transform mass spectrometry (FT/MS) provided information on the digestion mixture obtained by the action of trypsin on in vitro glycated HSA and untreated protein (Marotta et al., 2003). In view of the high specificity of the FT-MS approach, the spectra detected throughout the chromatographic run were added and, by means of a high entropy deconvolution program, identified most of the molecular species. The spectra resulting for unglycated and glycated HSA are shown in Figures 7.16*a* and *b*, respectively. These data show the significant decrease in the number of components for glycated HSA with respect to those originating by enzymatic digestion of unglycated protein. The power of FT-MS is clearly proved by the resolution in Figure 7.16*b*, which gives an accurate mass determination of most of the ionic species detected. Since the HSA structure is known, the Bio Tools 2.1 program and the MASCOT database source engine could be used to identify the peptides generated by the enzymatic digestion.

This approach yielded a large number of data, and the comparison between the observed masses for the species generated from glycated and unglycated HSA revealed some interesting points. First of all, some species are common to both proteins and probably arise from cleavage of parts of protein chain, which have been not subjected to glycation. Others are typical of glycated HSA only and, in theory, may represent glycated peptides, namely AGE peptides as was the aim of our study.

From the quantity of data we can generalize that by this approach, and looking only at the most abundant ionic species, 91 peptides were detected in the digestion mixture of unglycated HSA with molecular masses ranging from 300 to 5,700 Da. In the case of glycated HSA only 35 abundant peptides were detected, most of them with molecular masses in the range of 1,340 to 3,330 Da. As expected, the automatic program could not be used to identify most of the species coming from glycated HSA, so the modifications due to glycation had to be hypothesized on the basis of the Maillard reaction pattern. The data so obtained do fit previous results (Lapolla et al., 2000) achieved by digestion with proteinase K, that is,

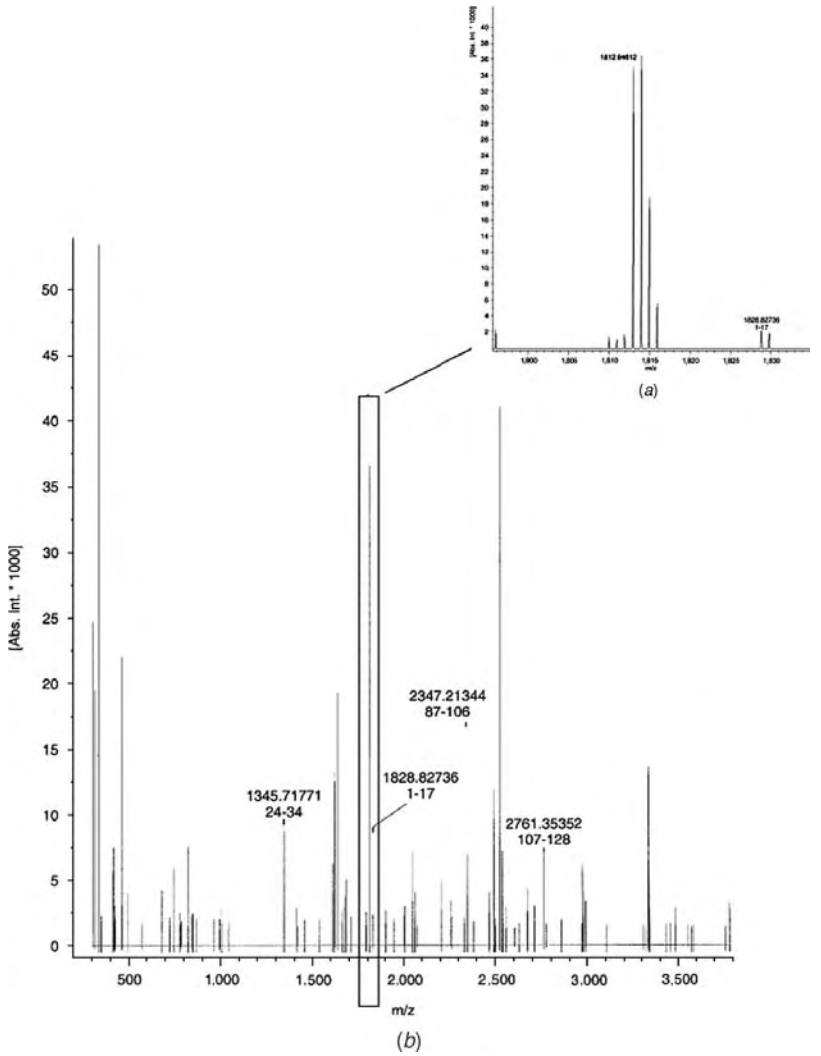


FIGURE 7.16 ESI mass spectra obtained from the entire set of ion species detected during TIC chromatogram runs of trypsin digestion products of (*a*) control HSA and (*b*) in vitro glycated HSA. (Reprinted from Marotta et al., 2003, by permission of John Wiley & Sons.)

that glycated proteins are less prone to enzymatic digestion. These results are particularly important when considered in vivo: they indicate that the lifetime of a glycated protein is different from that of its unglycated analogue and that, when digested, the peptides are different from those coming from the genuine protein. From the pathophysiological point of view, this aspect may explain the toxicity of AGE peptides; in other words, owing to their different structure, AGE

peptides are different from those originating from physiological proteins. AGEs are not recognized by receptors' sites, and consequently their excretion through the kidney is impaired and they accumulate, as hypothesized.

Another important point was the presence of a large number of species originating from glycation-induced cross-linking processes, probably through intramolecular reactions. This fact could greatly influence the behavior of the glycated protein, leading to a tertiary structure different from that of the unglycated protein, which could explain its reactivity.

We have attempted to identify the specific glycated peptides involved by applying MALDI-MS and LC-ESI-MS^2 to nonenzymatically glycated HSA by enzymatic digestion with trypsin and endoproteinase Lys-C (Lapolla et al., 2004b). This study was a logical evolution of the previous one. In theory, it could lead to a structural identification of AGE peptides whose presence is to be verified in plasma samples from healthy, diabetics, and end-stage renal failure subjects. We were aware of the very different protein degradation mechanism operating in in vitro experiments and in physiological conditions, but from the glycated peptide maps obtained by the various approaches, we hoped to obtain results that can be transferred to in vivo studies. Briefly, our general strategy was as follows: unglycated and in vitro glycated HSA were digested by trypsin or Lys-C. Digestions were performed without any derivatization of the sulfhydryl groups, to approach as close as possible to conditions at systemic level. MALDI-MS measurements were performed on the untreated glycated protein in order to obtain the total number of glucose molecules condensed on the protein. The digestion mixtures of both unglycated and glycated HSA were analyzed both by MALDI-MS (to yield their fingerprints) and by LC by means of various detection systems: UV (214 and 280 nm), ESI-MS, and ESI-MS^2. The experimental data were rationalized by comparison with database and theoretical data from molecular modeling.

The first step—that is, MALDI-MS of in vitro glycated HSA—showed that the mean number of glucose molecules condensed on protein was 14; however, from the shape of the molecular peak, the upper number ranged from 1 to 29. When digested by trypsin, unglycated and glycated HSA gave the MALDI-MS spectra of Figure 7.17, showing that clear-cut differences exist between the two digestion mixtures. At first the most conspicuous result was the complete disappearance of ionic species at m/z 1,899.0 (which, in the case of the unglycated HSA sample, was the most abundant species) and 2,056.1 in the glycated HSA digest. Some other peptides remained in both mixtures, such as those at m/z 1,467.8 and 1,623.8. However, the spectra of Figure 7.17*b* show a few new species, generally of low abundance, in the glycated HSA digest. This is the case of ions at m/z 1,179.6, 1,405.7, 1,700.9, 2,165.1, and 2,413.0.

The same tryptic digests were analyzed by LC. In the unglycated HSA digest, UV detection at 214 nm revealed its great complexity: some of the peaks detected here disappeared in the sample from the glycated HSA, and in the latter case new species were detectable. Similar results were obtained with UV detection at 280 nm, further confirming the fact that glycated HSA is less prone to enzymatic digestion. Passing to LC-ESI-MS experiments, we found that the total ion

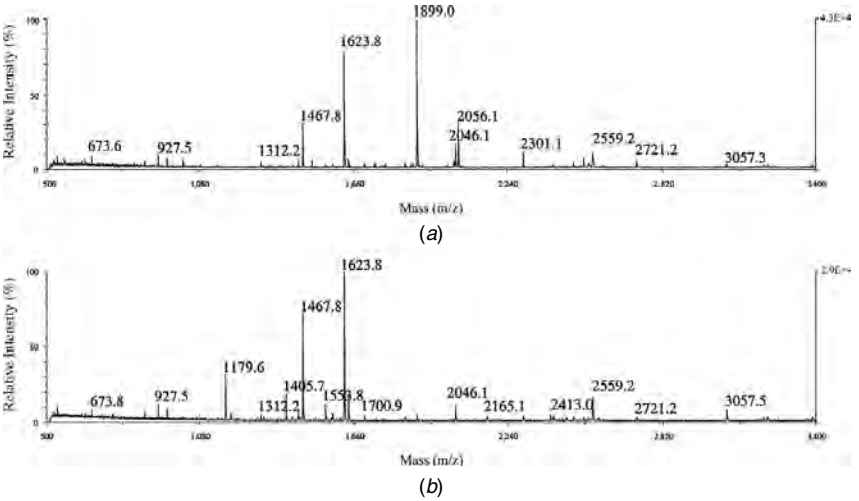


FIGURE 7.17 MALDI/MS spectra of tryptic digest of (*a*) unglycated HSA and (*b*) glycated HSA.

chromatograms (Fig. 7.18) confirm the UV data. These chromatograms were the starting point of an extensive investigation, based on collisional experiments, by which we identified the various peptides produced by enzymatic digestion.

The ESI and MALDI-MS data for the unglycated HSA digest are shown in Table 7.3. The sequences in column 4 were obtained by comparing the mass values of the various peptides with those calculated by the protein digestion tool MS-Digest (Protein Prospector version 4.0.5). It must be emphasized that the comparison of LC-ESI-MS and MALDI-MS data (Table 7.3) indicates that the former approach is more effective, since only 27 out of the 56 peptides detected by LC-ESI-MS also appear in the MALDI-MS spectra. However, under MALDI-MS, other peptides, undetectable in ESI/MS conditions, were identified, as reported in the note to Table 7.3. As to the validity of these results, it should be noted that the identified peptides comprise 78% of the whole protein.

Data pertaining to the tryptic digest of glycated HSA show most of the peptides detected in the unglycated HSA digest, although in lower abundance. However, as we expected, new peptides were revealed—some glycated and others, as discussed below, originating from a different enzymatic action. For easier discussion of the data, only peptides characteristic of the glycated HSA are listed in Table 7.4. These possibly glycated peptides show mass values ranging from 738.3 to 3,940.0 Da. In theory, they originate from the HSA where the glucose molecule(s) condensed, with and without further dehydration. For example, the peptide detected at a retention time of 27.0 minutes shows a mass value of 1,198.7 corresponding to the sequence 137–144, in which the dehydrated glucose molecule condensed on ^{137}K. Interestingly, the unglycated peptide with

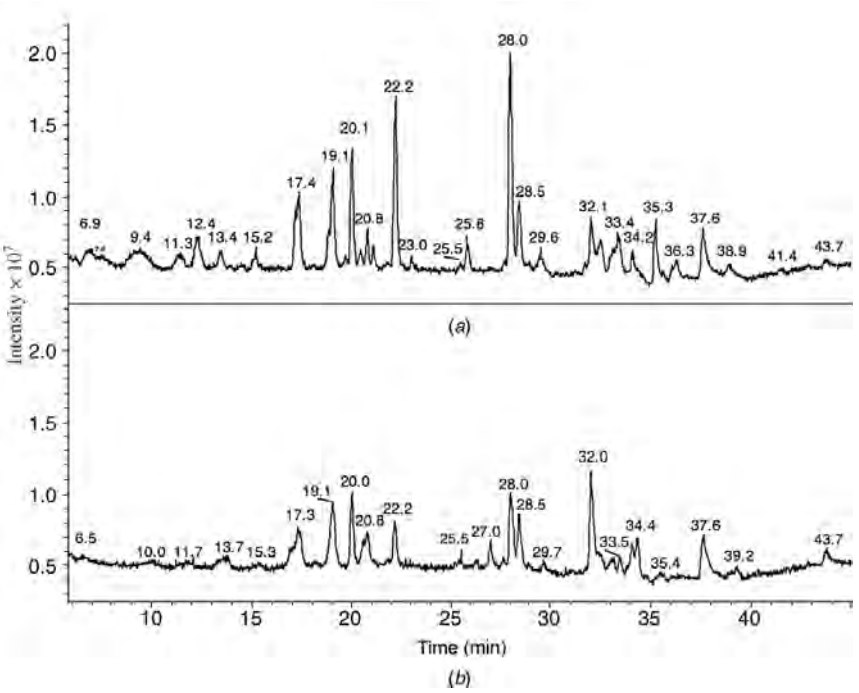


FIGURE 7.18 Total ion current (TIC) chromatograms of tryptic digest of (*a*) unglycated HSA and (*b*) glycated HSA.

the same sequence is present in the digestion mixture of the unglycated HSA at m/z 1,055.5 ($rt = 18.7$ min).

However, some profound changes did occur during the enzyme action. First of all, some enzymatic cleavages never observed in the unglycated HSA become operative, proving that glycation greatly modifies enzymatic action. For example, the peak eluting at 17 minutes is due to the glycated peptide with sequence 275–286; the same sequence, obviously not glycated, is completely absent in the case of the unglycated HSA. A further example is shown by the data related to the sequence of Scheme 7.4. The sequence 181–200 of unglycated HSA undergoes different cleavages when glycated. Thus the upper part of Scheme 7.4 shows that the unglycated sequence is enzymatically cleaved at ^{181}K, ^{195}K, and ^{197}R, whereas in the case of glycation (at either ^{190}K or ^{195}K), cleavages occur at ^{186}R and ^{197}R.

The sequence data, first obtained by comparison with the databases, were subjected to MS/MS experiments on doubly charged ions. With regard to the peptides from unglycated HSA, spectra similar to that shown in Figure 7.19*a* for the ion at m/z 826.0 (corresponding to the sequence 226–240) were obtained, revealing ions of the y and b series.

TABLE 7.3 Protonated Ions of Trypsin Digestion Products Common to Unglycated and Glycated HSA, Detected in Both ESI and MALDI Conditions

rt (min)	$[M + H]^+$ ESI	Charge State	Sequence	$[M + H]^+$ MALDI
6.9	1518.7[a]	1+, 2+	182–195	
7.1	1074.5[a]	1+, 2+	182–190	
8.9	880.4	1+, 2+	226–233	
9.6	875.3	1+, 2+	219–225	875.3
11.5	1022.4	1+, 2+	476–484	1022.6
11.7	1296.4[a]	2+	349–359	
12.2	789.4	1+, 2+	234–240	
13.4	1318.5	1+, 2+	82–93	
13.5	673.3	1+, 2+	213–218	673.7
15.3	1189.6	1+, 2+	277–286	
17.2	1226.7	1+, 2+	11–20	
17.4	1430.8	1+, 2+	275–286	
18.5	1252.4	2+	223–233	
18.7	1055.5	1+, 2+	137–144	
18.9	927.5	1+, 2+	138–144	927.7
19.1	1149.9	1+, 2+	42–51	1149.7
19.4	1737.6[a]	2+	219–233	
19.7	1016.9[a]	1+	65–73	
20.0	1640.1	1+, 2+	414–428	
20.5	1019.4	1+, 2+	210–218	1019.7
20.8	960.4	1+, 2+	403–410	960.8
21.1	1128.7	1+, 2+	525–534	
21.2	3244.9	3+	546–574	
21.9	2381.2	2+, 3+	198–218	
22.2	1001.6	1+, 2+	526–534	
	1013.9	1+, 2+, +Na	575–585	
23.0	2413.8	2+, 3+	241–262	2414.0
24.6	1854.1	2+	485–500	1853.3
25.5	3184.6	2+, 3+	234–262	
25.9	1600.6	1+, 2+	390–402	
	983.7	1+	352–359	
27.7	3239.4[a]	2+, 3+	52–81	3239.0
27.9	2488.8	2+, 3+	501–521	2488.0
28.0	2045.2	2+, 3+	373–389	2045.4
	1342.8	1+, 2+	546–557	
28.5	1468.0	1+, 2+	337–348	1467.6
29.0	1311.7	1+, 2+	338–348	1311.7
29.5	2722.4	2+, 3+	115–137	2720.9
29.8	2935.0	2+, 3+	137–159	
31.8	2543.2	2+, 3+	390–410	2543.0
32.1	3543.2	2+, 3+	445–475	3542.0
32.2	2918.4	2+, 3+	287–313	
32.4	1651.7	1+, 2+	226–240	

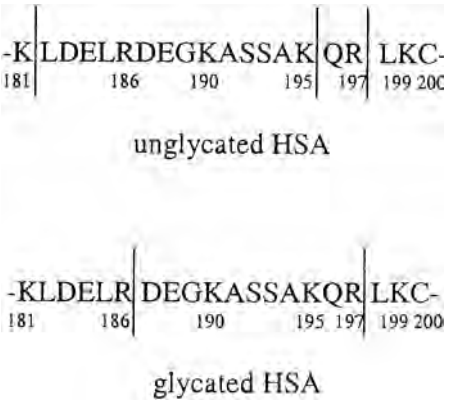
(*continued overleaf*)

TABLE 7.3 (*continued*)

rt (min)	$[M + H]^+$ ESI	Charge State	Sequence	$[M + H]^+$ MALDI
32.5	3362.6	3+	287–317	
32.6	3215.4	2+, 3+	445–472	3213.0
33.2	2559.2	2+, 3+	445–466	2558.0
33.5	3058.4	2+, 3+	446–472	3057.0
	2055.6	2+, 3+	145–160	2055.5
33.9	1639.7	1+, 2+	324–336 1Met-ox	1639.5
34.1	1898.9[a]	1+, 2+	145–159	1898.4
34.2	2403.0	2+	446–466	
34.4	746.4	1+	21–27	
35.3	1898.9	1+, 2+	146–160	1898.4
36.1	1742.8	1+, 2+	146–159	1742.5
36.3	2301.0	2+, 3+	318–336	2300.2
37.6	1623.9	1+, 2+	324–336	1623.4

Note: Further ionic species were detected in MALDI conditions only: 1,083.8 (sequence 138–145), 1,502.6 (sequence 210–222), 1,699.5 (sequence 429–444), 3,385.6 (sequence 403–432), 1,779.8[a] (sequence 324–337), 1,875.0[a] (sequence 65–81).

[a]Peptides present only in the digestion products of unglycated HSA.



SCHEME 7.4 Differences in the enzyme action occurring in unglycated HSA (*up*) and glycated HSA (*down*).

In the case of peptides containing a glucose moiety, completely different behavior was observed, as shown in the MS/MS spectrum of the doubly charged ion at m/z 907.3, corresponding to the sequence 226–240 with a glucose molecule condensed on ^{233}K (see Fig. 7.19*b*). In this case only a highly favored water loss is observed, and ions due to the **y** and **b** series are practically undetectable. However, what at first may be viewed as a negative aspect is really a highly efficient diagnostic tool in identifying glucose-containing peptides among the digestion products of the glycated HSA.

TABLE 7.4 Protonated Ions of Trypsin Digestion Products Presented Only in Glycated HSA Detected in ESI Spectra and Compared with MALDI-MS Data

rt (min)	$[M+H]^+$ ESI	Charge State	$[M+H]^+$ MALDI	Sequence (+ Mass Increase)	Modified Amino Acid
11.5	1458.7	1+, 2+		349–359 (+ 162)	^{351}K
13.5	1512.4	2+			
17.0	1592.7	1+, 2+		275–286 (+ 162)	^{276}K
19.0	1179.5	1+, 2+	1179.5		
20.6	1290.7	1+, 2+		525–534 (+ 162)	^{525}K
21.6	2542.6	2+, 3+		198–218 (+ 162)	^{199}K or ^{205}K or ^{209}R or ^{212}K
24.3	3940.0	3+		440–472 (+ 162)	^{444}K or ^{445}R or ^{466}K
25.2	1578.6	1+, 2+	1579	187–199 (+ 162)	^{190}K or ^{195}K or ^{197}R
25.5	1056.5	1+, 2+			
26.3	894.4	1+, 2+			
27.0	1198.7	1+, 2+	1198.6	137–144 (+ 144)	^{137}K
27.6	2207.6	2+, 3+		373–389 (+ 162)	^{378}K
28.2	738.3	1+, 2+			
29.7	1405.8	1+, 2+	1405.5		
31.6	3523.9	3+		287–317 (+ 162)	^{313}K
32.1	1812.9	1+, 2+		226–240 (+ 162)	^{233}K
32.4	2003.0	2+		542–557 (+ 162)	^{545}K
33.1	2303.8	2+		539–557 (+ 162)	^{541}K or ^{545}K
	1396.5	1+, 2+		223–233 (+ 144)	^{225}K
35.5	2462.8	2+, 3+	2462.2	318–336 (+ 162)	^{323}K
	2185.8	2+, 3+		223–240 (+ 162)	^{225}K
39.3	2164.2	2+	2164.1		

Similar treatment of unglycated and glycated HSA was performed by Lys-C, and the related data clearly show that not all the K-sites are glycated. In fact, the glycated peptides identified by both MALDI-MS and LC-ESI-MS comprise only 54% of the whole protein. This result may be due to two phenomena: (1) a different enzymatic action on the glycated protein, which leads to preferential production of some glycated peptides, and (2) the occurrence of favored glycation processes on specific lysine residues belonging to the protein chain.

The experimental data indicate that ^{233}K, ^{276}K, ^{378}K, ^{545}K, and ^{525}K are privileged glycation sites. To further support to these results, we used molecular modeling to identify the most exposed lysine residues, which are more readily available to possible glycation processes. The theoretical values so calculated are in good agreement with the MS values. However, some discrepancies appeared, such as those related to glycated residues ^{545}K, ^{525}K, and ^{413}K, which were detected even though their SAS values are particularly low. This may be due to at least a partial tertiary structural modification of the protein, induced by glycation and/or by acid catalysis (Garlick and Mazer, 1983).

From these studies we drew up some general rules that could be applied to AGE peptide identification in plasma samples: (1) glycated proteins are less prone

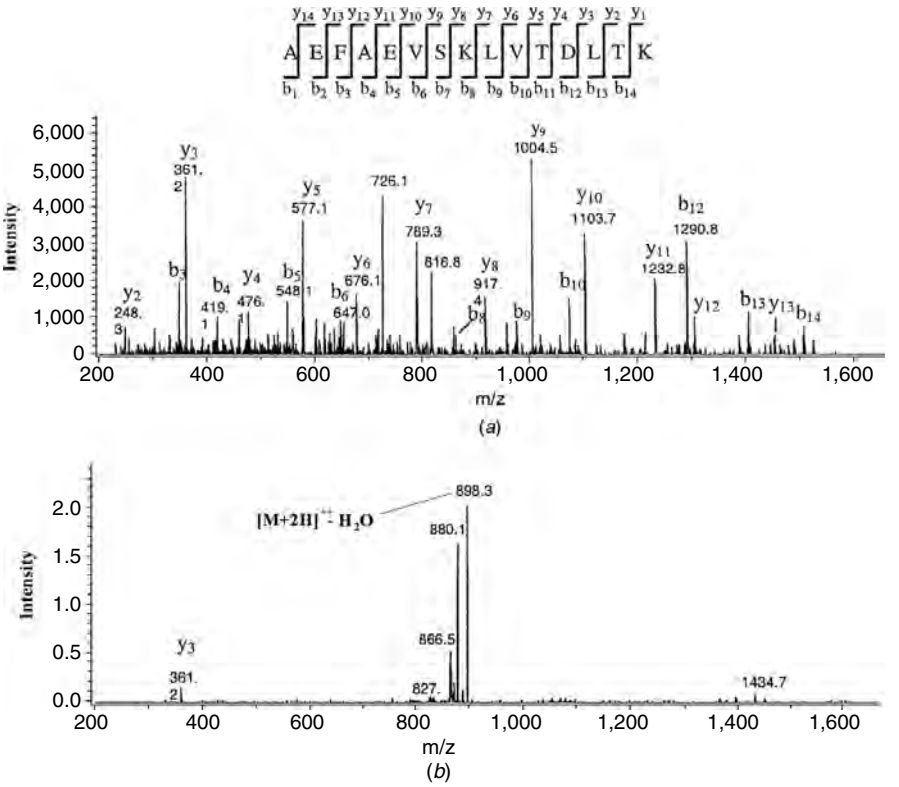


FIGURE 7.19 MS/MS spectrum of doubly charged ions corresponding to sequence 226–240 (*a*) unglycated (*m/z* 826.0) and (*b*) containing a glucose molecule condensed on ^{233}K (*m/z* 907.3).

to enzymatic digestion than the unglycated one, (2) enzymes cleave glycated and unglycated HSA in different positions, and (3) MS/MS experiments performed on doubly charged species of glycated peptides show that water loss is highly favored, while the typical y and b ions are detected in low abundance.

After these preliminary investigations we undertook a study based on ESI and MALDI mass spectrometry of serum samples from healthy, diabetic, and nephropathic subjects (Lapolla et al., 2005b). Ten type 2 diabetic patients with poor metabolic control (mean age 62 ± 6 years, mean disease duration 10 ± 5 years), 10 nondiabetic patients with end-stage renal disease (mean age 61 ± 9 years, mean disease duration 8 ± 5 years), and 10 healthy subjects (mean age 60 ± 5 years) were evaluated. Blood samples were centrifuged at 4,000 g and 1 ml aliquots of serum were diluted twice with physiological solution. The resulting samples were treated first with a centricon 30,000 Da cutoff and further purified by a centricon at 10,000 Da cutoff. The final filtrates were analyzed by LC-ESI-MS and MALDI. As expected, the LC-ESI-MS chromatograms of the

30 serum samples were all complex, due to high number of peptides with molecular weight ranging from 800 to 2,000 Da. However, among the chromatograms of samples from subjects belonging to the same group, some analogies could be made, but definite differences were observed for the subjects in other groups. This is clearly evidenced by the three-dimensional plots of m/z values, in terms of retention time and relative ion abundance, shown in Figure 7.20. In samples from healthy subjects a wide distribution of ionic species is observed in the retention time range of 23 to 26 minutes. Samples from diabetic and nephropathic patients yield different plots, like those shown in Figure 7.20*b* and *c*, respectively. In these cases the most abundant species were detected in the range of 25 to 26.5 minutes.

These data were used to study the presence, among low molecular weight serum peptides, of the species found by enzymatic digestion of in vitro glycated HSA. This study was simply carried out by reconstructing the ion chromatograms related of the m/z values of these possible AGE peptides. Unfortunately, this approach did not give any significant results: none of the peptides identified in the previous studies yielded significant ion chromatograms. Most gave rise to chromatographic peaks with a signal-to-noise ratio lower than 2, and consequently without any analytical meaning. Only for one ionic species, that at m/z 1,396, was an abundant signal obtained, whose intensity was on the order of 3×10^5 DAC in healthy subjects, but it showed an increment in both diabetic and nephropathic patients ($6-9 \times 10^5$ DAC). One species with this m/z value was observed among the digestion products of the glycated HSA, and assigned to the amino acid sequence of HSA 223–233 (i.e., FPKAEFAEVSK) with ^{225}K glycated. However, further experiments showed that this ion, detected at m/z 1,369, did not have the same structure as the isobaric species identified among the digestion products of in vitro glycated HSA. It not only had a different retention time but its MS/MS spectrum (see Fig. 7.21) revealed a completely different sequence. Favored primary water loss, leading to the fragment at m/z 1,378, indicated that it was a glycated peptide, but its sequence did not plot with the HSA structure. In other words, this species may be assumed to be an AGE peptide, but it does not originate by in vivo digestion of glycated HSA.

In parallel with the LC-MS study, we took further measurements by MALDI-MS. As in the previous study on enzymatic digestion of in vitro glycated HSA, in which we used LC-ESI-MS and MALDI-MS, we were seeking, to some extent, to produce complementary results. In these experimental conditions, similar results were obtained. Again, clear-cut similarities between samples coming from the same group of subjects were observed, whereas some differences among the three groups of subjects were detected.

An example of the MALDI-MS spectra of low molecular weight serum fractions from one healthy subject, one diabetic patient, and one nephropathic patient is shown in Figure 7.22. Note that some ionic species are common to all three spectra (e.g., those at m/z 1,520, 1,524, 1,617), although they exhibit different abundances. However, only in the patients were abundant ionic species detected at m/z 1,488. Analogously, the ion at m/z 1,561 appeared only in the diabetic

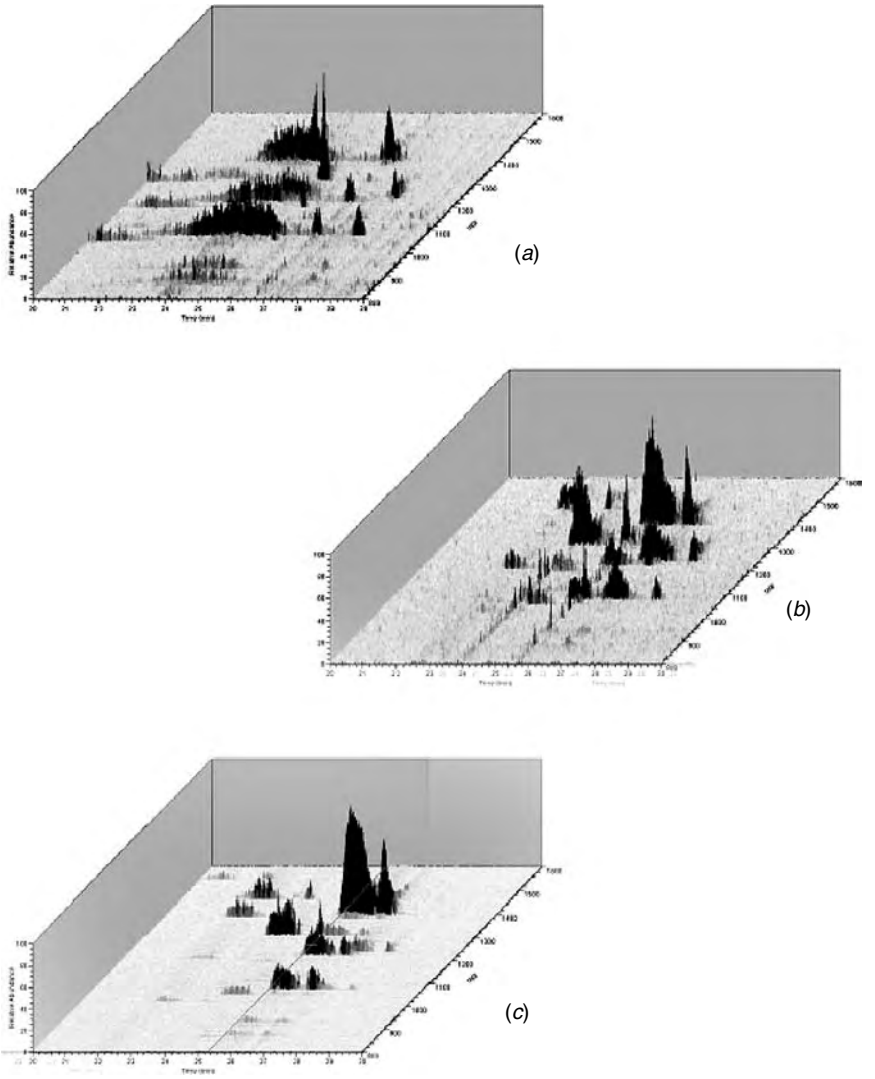


FIGURE 7.20 Three-dimensional plots (m/z values, retention times, and ion abundances) of the serum low molecular weight peptide fractions from (*a*) a healthy subject, (*b*) a poorly controlled diabetic patient, and (*c*) a nephropathic patient.

patient spectra. The abundance of these ionic species was measured for all 30 subjects and the results, listed in Table 7.5, confirm the trends revealed by the spectra in Figure 7.22. Unfortunately, the same ions were in very low abundance in the LC-ESI-MS runs: they gave peaks with a signal-to-noise ratio of 2 to 3, so attempts to characterize them by MS/MS failed.

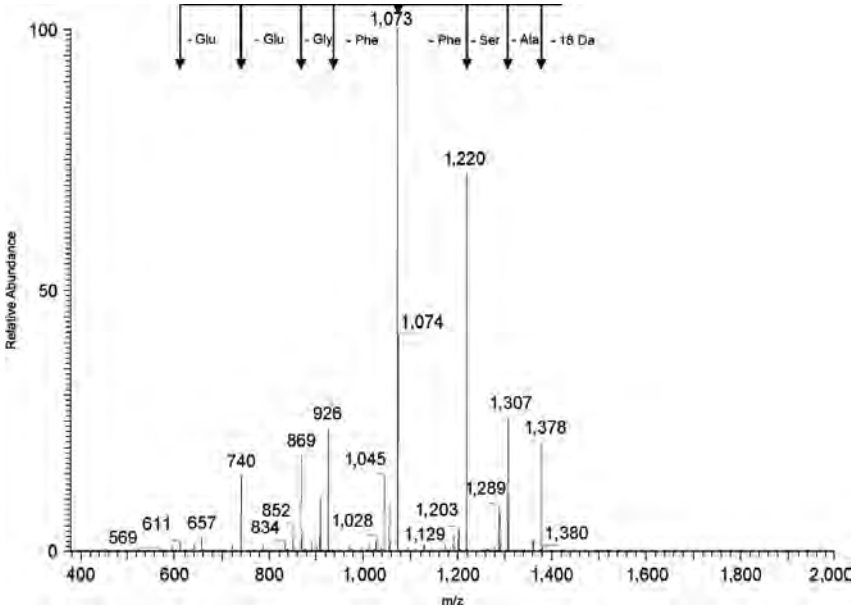


FIGURE 7.21 MS/MS spectrum of the ions at m/z 1396 detected by ESI in the serum low molecular weight fractions of the subjects under investigation.

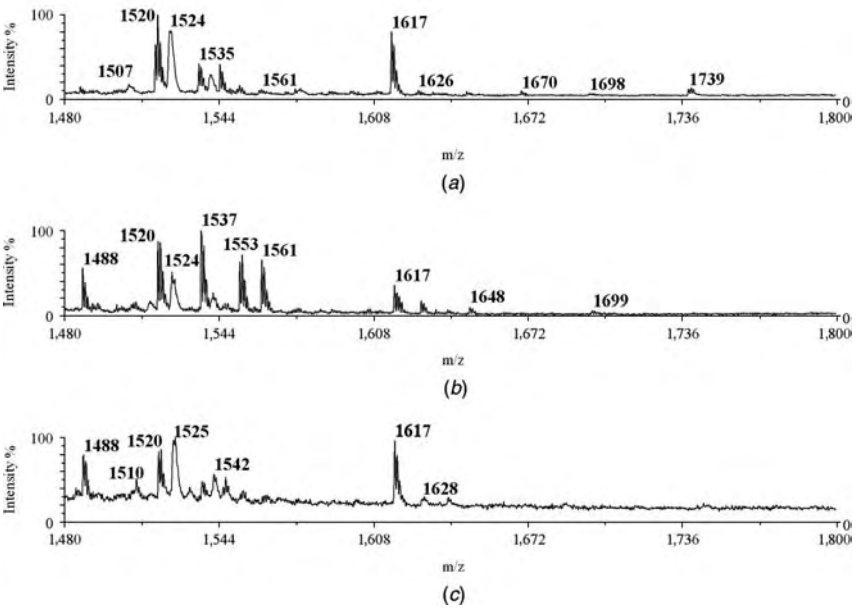


FIGURE 7.22 MALDI/MS spectra of the serum low molecular weight peptide fractions from (*a*) a healthy subject, (*b*) a poorly controlled diabetic patient, and (*c*) a nephropathic patient.

TABLE 7.5 Relative Abundances of Ions at *m/z* 1488 and 1561 in MALDI-MS Spectra of Serum Samples of Subjects

	m/z 1488			*m/z* 1561		
	Healthy Subjects	Nephropathic Patient	Diabetic Patient	Healthy Subjects	Nephropathic Patient	Diabetic Patient
1	+	+++	++			+++
2		+++	++			++
3	+	+	+++	+		+++
4	+	++	++		+	++
5		+++	++			++
6		+++	+++			+++
7	+	++	++		+	+++
8	+	+++	++			+++
9		+++	+++	+	+	++
10	+	+++	+++			++

Note: + = 5–10%, ++ = 11–50%, +++ => 50%.

In conclusion, this study allowed us to exclude the presence, in the low molecular weight peptide serum fraction, of abundant glycated species originating by the in vivo digestion process of glycated HSA analogous to the process occurring in vitro. The development of an easily reproducible cleanup procedure was essential to our obtaining a clearly defined set of serum peptides. Nevertheless, further sample treatments (e.g., 1D and 2D gel electrophoresis, 2D LC methods) will be necessary because of recent developments in quantitative LC-MS/MS measurements that report the putative AGE peptides detected by immunoassay in plasma to be instead AGE free adducts (Thornalley et al., 2003). Peptides enriched with AGEs were found only in portal venous plasma and not in peripheral venous plasma (Ahmed et al., 2004). It should be noted therefore that mass spectrometric techniques (LC-MS/MS) may be used to quantify AGE residues in proteins and peptides and AGE free adducts (glycated amino acids). It is the latter than accumulate in uremia (Thornalley et al., 2003). There are peptides from incomplete proteolysis in vivo (Ivanov et al., 2000; Greive et al., 2001), but recent studies indicate that they are not enriched with AGEs. This controversial point is surely to be a matter of discussion in the near future.

7.7 EXPECTED FUTURE TRENDS

The data obtained until now by mass spectrometry in the study of protein glycation are highly interesting. Physicians have, as a result, new powerful and reliable methods to describe the pathological state of diabetic and nephropathic patients.

In the near future a more extensive use of this analytical approach can be expected, in particular, together with effective pre-separation techniques and/or a

sample preparation procedure for MALDI-MS analysis that privileges the concentration of glycated and glyco-oxidated species. Because of the effectiveness of the glyco-oxidated species as markers of oxidative stress, their structural characterization will be fundamental to the development of powerful instruments devoted to their quantification. However, as important will be the continuing collaboration among physicians, biochemists, and mass spectrometrists. Their combined contributions will be the basis for fruitful investigations in this medically relevant field.

LIST OF ABBREVIATIONS

AGEs, advanced glycation end-products
BSA, bovine serum albumin
CML, *N*-carboxymethyl-lysine
CZE-ESI-MS, capillary zone electrophoresis with electrospray mass spectrometry
ELISA, Enzyme-Linked Immuno Sorbent Assay
FL, fructose-lysine
FT-MS, Fourier transform–mass spectrometry
GC-MS, gas-chromatography–mass spectrometry
HbA_{1c}, hemoglobin glycation level
HSA, human serum albumin
IFCC, International Federation of Clinical Chemistry
LC-MS, liquid chromatography/mass spectrometry
MALDI-MS, matrix-assisted laser desorption–ionization mass spectrometry
PFBOA, *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride
RIA, Radio Immuno Assay
SAS, solvent accessible surface
TIC, total ion current
TOF, time of flight

REFERENCES

Ahmed M.U., Thorpe S.R., Baynes J.W. (1986). Identification of *N* epsilon-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. *J Biol Chem* 261: 4889–4894.

Ahmed N., Thornalley P.J., Luthen R., Haussinger D., Sebekova K., Schinzel R., Voelker W., Heidland A. (2004). Processing of protein glycation, oxidation and nitrosation adducts in the liver and the effect of cirrhosis. *J Hepatol* 41: 913–919.

American Diabetes Association: Clinical Recommendations. (2004). *Diabetes Care* 27: S91–S93.

Baynes J.W., Thorpe S.R., Murtiashaw M.H. (1984). Nonenzymatic glycosylation of lysine residues in albumin. *Methods Enzymol* 106: 88–98.

Beisswenger P.J., Howell S.K., O'Dell R.M., Wood M.E., Touchette A.D., Szwergold B.S. (2001). Alfa-dicarbonyls increase in the postprandial period and reflect the degree of hyperglycemia. *Diabetes Care* 24: 726–732.

Boehm B.O., Schilling S., Rosinger S., Lang G.E., Lang G.K., Kientsch-Engel R., Stahl P. (2004). Elevated serum levels of *N*(epsilon)-carboxymethyl-lysine, an advanced glycation end product, are associated with proliferative diabetic retinopathy and macular oedema. *Diabetologia* 47: 1376–1379.

Brownlee M., Cerami A., Vlassara H. (1988). Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N Engl J Med* 318: 1315–1321.

Bucala R., Makita Z., Vega G., Grundy S., Koshinsky T., Cerami A., Vlassara H. (1994). Modification of low density lipoprotein by advanced glycation end products contributes to the dyslipidemia of diabetes and renal insufficiency. *Procl Natl Acad Sci USA* 91: 9441–9445.

Cai J., Hurst H.E. (1999). Identification and quantitation of *N*-(carboxymethyl)valine adducts in hemoglobin by gas chromatography/mass spectrometry. *J Mass Spectrom* 34: 537–543.

Dean R.T. (1975). Lysosomal enzymes as agents of turnover of soluble cytoplasmic protein. *Eur J Biochem* 58: 9–14.

Fu M.X., Requena J.R., Jenkins A.J., Lyons T.J., Baynes J.W., Thorpe S.R. (1996). The advanced glycation end product, Nepsilon-(carboxymethyl)lysine, is a product of both lipid peroxidation and glycoxidation reaction. *J Biol Chem* 271: 9982–9986.

Furth A.J. (1997). Glycated proteins in diabetes. *Br J Biomed Sci* 54: 192–200.

Garlick R.L., Mazer J.S. (1983). The principal site of nonenzymatic glycosylation of human serum albumin in vivo. *J Biol Chem* 258: 6142–6146.

Ghiggeri G.M., Candiano G., Delfino G., Queirolo C., Vecchio G., Gianazza E., Righetti P.G. (1985). Reaction of human serum albumin with aldoses. *Carbohydr Res* 145: 113–122.

Greive K.A., Balazs N.D.H., Comper W.D. (2001). Protein fragments in urine have been considerably underestimated by various protein assays. *Clin Chem* 47: 1717–1719.

Gugliucci A., Bendayan M. (1996). Renal fate of circulating advanced glycated end products (AGE): Evidence for reabsorption and catabolism of AGE-peptides by renal proximal tubular cells. *Diabetologia* 39: 149–160.

Gugliucci A., Menini T. (1998). Circulating advanced glycation peptides in streptozotocin-induced diabetic rats: Evidence for preferential modification of IgG light chains. *Life Sci* 62: 2141–2150.

Hayase F., Nagaraj R.H., Miyata S., Njoroge F.G., Monnier V.M. (1989). Aging of proteins: Immunological detection of a glucose-derived pyrrole formed during Maillard reaction in vivo. *J Biol Chem* 264: 3758–3764.

Hempe J.M., Gomez R., McCarter R.J., Chalew S.A. (2002). High and low haemoglobin glycation phenotypes in type 1 diabetes: A challenge for interpretation of glycemic control. *J Diab Compl* 16: 313–320.

Hodge S.E. (1955). The Amadori rearrangement. *Adv Carbohydr Chem* 10: 169–205.

Horiuchi S., Higashi T., Ikeda K., Saishoji T., Jinnouchi Y., Sano H., Shibayama R., Sakamoto T., Araki N. (1996). Advanced glycation end products and their recognition by macrophage and macrophage-derived cells. *Diabetes* 45: S73–S76.

Ivanov V.T., Yatskin O.N., Kalinina O.A., Philippova M.M., Karelin A.A., Blishchenko E.Y. (2000). Tissue-specific peptide pools: Generation and function. *Pure Appl Chem* 72: 355–363.

Jeppsson J.O., Kobold U., Barr J., Finke A., Hoelzel W., Hoshino T., Miedema K., Mosca A., Mauri P., Thienpont L., Umemoto M., Weykamp C. (2002). Approved IFCC reference method for the measurement of HbA_{1c} in human blood. *Clin Chem* 40: 78–89.

Johnson R.N., Metcalf P.A., Baker J.R. (1983). Fructosamine: A new approach to the estimation of serum glycosylprotein. An index of diabetic control. *Clin Chim Acta* 127: 87–95.

Kennedy D.M., Skillen A.W., Self G.H. (1994). Glycation of monoclonal antibodies impairs their ability to bind antigen. *Clin Exp Immunol* 98: 245–251.

Korbert S.M., Makita Z., Firanek C.A., Vlassara H. (1993). Advanced glycosylation end products in continuous ambulatory peritoneal dialysis patients. *Am J Kidney Dis* 22: 588–591.

Kusunoki H., Miyata S., Ohara T., Liu B.F., Uriuhara A., Kojima H., Suzuki K., Miyazaki H., Yamashita Y., Inaba K., Kasuga M. (2003). Relation between serum 3-deoxyglucosone and development of diabetic microangiopathy. *Diabetes Care* 26: 1889–1894.

Lal S., Kappler F., Walker M., Orchard T.J., Beisswenger P.J., Szwergold B.S., Brown T.R. (1997). Quantitation of 3-deoxyglucosone levels in human plasma. *Arch Biochem Biophys* 342: 254–260.

Lapolla A., Gerhardinger C., Crepaldi G., Fedele D., Palumbo M., Dalzoppo D., Porter C.J., Ghezzo E., Seraglia R., Traldi P. (1991). Mass spectrometric approaches in structural identification of the reaction products arising from the interaction between glucose and lysine. *Talanta* 38: 405–412.

Lapolla A., Gerhardinger C., Baldo L., Fedele D., Keane A., Seraglia R., Catinella S., Traldi P. (1993). A study on in vitro glycation processes by matrix-assisted laser desorption ionization mass spectrometry. *Biochim Biophys Acta* 1225: 33–38.

Lapolla A,, Baldo L., Aronica R., Gerhardinger C., Fedele D., Elli G., Seraglia R., Catinella S., Traldi P. (1994). Matrix-assisted laser desorption/ionization mass spectrometric studies on protein glycation. 2. The reaction of ribonuclease with hexoses. *Biol Mass Spectrom* 23: 241–248.

Lapolla A., Fedele D., Aronica R., Curcuruto O., Hamdan M., Catinella S., Seraglia R., Traldi P. (1995a). Investigation of the reaction between *N*-α-acetyl-L-lysinamide and glucose by electrospray mass spectrometry. *J Mass Spectrom Rapid Commun Mass Spectrom*: S69–S76.

Lapolla A., Fedele D., Seraglia R., Catinella S., Baldo L., Aronica R., Traldi P. (1995b). A new effective method for the evaluation of glycated intact plasma protein in diabetic subjects. *Diabetologia* 38: 1076–1081.

Lapolla A., Fedele D., Aronica R., Baldo L., D'Alpaos M., Seraglia R., Traldi P. (1996a). The in vitro glycation of lysozyme and the influence of buffer concentration investigated by mass spectrometry. *Rapid Commun Mass Spectrom* 10: 1512–1518.

Lapolla A., Fedele D., Aronica R., Garbeglio M., D'Alpaos M., Seraglia R., Traldi P. (1996b). The in vivo glyco-oxidation of α- and β-globins investigated by matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun Mass Spectrom* 10: 1133–1135.

Lapolla A., Poli T., Barison A., Fedele D. (1998). Fructosamine assay: an index of medium term metabolic control parameters in diabetic disease. *Diabetes Res Clin Pract* 4: 231–235.

Lapolla A., Fedele D., Plebani M., Garbeglio M., Seraglia R., D'Alpaos M., Aricò C.N., Traldi P. (1999). Direct evaluation of glycated and glyco-oxidized globins by matrix-assisted laser desorption/ionization mass spectrometry. *Radio Commun Mass Spectrom* 13: 8–14.

Lapolla A., Fedele D., Garbeglio M., Martano L., Tonani R., Seraglia R., Favretto D., Fedrigo M.A., Traldi P. (2000). Matrix-assisted laser desorption/ionization mass spectrometry, enzymatic digestion, and molecular modeling in the study of non-enzymatic glycation of IgG. *J Am Soc Mass Spectrom* 11: 153–159.

Lapolla A., Fedele D., Senesi A., Aricò N.C., Reitano R., Favretto D., Seraglia R., Astner H., Traldi P. (2002). Advanced glycation end-products/peptides: A preliminary investigation by LC and LC/MS. *Il Farmaco* 57: 845–852.

Lapolla A., Flamini R., Tonus T., Fedele D., Senesi A., Reitano R., Marotta E., Pace G., Seraglia R., Traldi P. (2003a). An effective derivatization method for quantitative determination of glyoxal and methyl-glyoxal in plasma samples by gas chromatography/mass spectrometry. *Rapid Commun Mass Spectrom* 17: 876–878.

Lapolla A., Flamini R., Dalla Vedova A., Senesi A., Reitano R., Fedele D., Basso E., Seraglia R., Traldi P. (2003b). Glyoxal and methyl-glyoxal in diabetic patients: Quantitative determination by a new GC/MS method. *Clin Chem Lab Med* 41: 1166–1173.

Lapolla A., Tubaro M., Reitano R., Aricò N.C., Ragazzi E., Seraglia R., Vogliardi S., Traldi P., Fedele D. (2004a). The complexity of non-enzymatic glycation product sets of human globins. *Diabetologia* 47: 1712–1715.

Lapolla A., Fedele D., Reitano R., Aricò N.C., Seraglia R., Traldi P., Marotta E., Tonani R. (2004b). Enzymatic digestion and mass spectrometry in the study of advanced glycation end products/peptides. *J Am Soc Mass Spectrom* 15: 496–509.

Lapolla A., Reitano R., Baccarin L., Sartore G., Plebani M., Fedele D. (2005a). Pentosidine plasma levels and relation with metabolic control in diabetic patients. *Horm Metab Res* 37: 252–256.

Lapolla A., Fedele D., Reitano R., Bonfante L., Gulzzo M, Seraglia R., Tubaro M., Traldi P. (2005b). Mass spectrometric study of in vivo production of advanced glycation end products/peptides. *J Mass Spectrom* 40: 969–972.

Maillard L.C. (1912). Action des acides amines sur le sucres: Formation des melanoidines per voie methodique. *CR Acad Sci* 154: 66–68.

Makita Z., Radoff S., Raifield E.J., Yang Z., Skolnik E., Delaney V., Friedman E.A., Cerami A., Vlassara H. (1991). Advanced glycosylation end products in patients with diabetic nephropathy. *N Engl J Med* 325: 836–842.

Makita Z., Vlassara H., Cerami A., Bucala R. (1992). Immunochemical detection of advanced glycosylation end products in vivo. *J Biol Chem* 267: 5133–5138.

Makita Z., Bucala R., Rayfield E.S., Freidmann E.A., Kaufman A.M., Korbert S.M., Barth R.H., Winston J.A., Fuh H., Manogue K.R., Cerami A., Vlassara H. (1994). Reactive glycosylation endproducts in diabetic uraemia and treatment of renal failure. *Lancet* 343: 1519–1522.

Marotta E., Lapolla A., Fedele D., Senesi A., Reitano R., Witt M., Seraglia R., Traldi P. (2003). Accurate mass measurements by Fourier transform mass spectrometry in the study of advanced glycation end products/peptides. *J Mass Spectrom* 38: 196–205.

McLellan A.C., Phillips S.A., Thornalley P.J. (1992). The assay of methylglyoxal in biological system by derivatization with 1,2-diamino-4,5-dimethoxybenzene. *Anal Biochem* 206: 17–23.

Mitsuhashi T., Vlassara H., Founds H.W., Li Y.M. (1997). Standardizing the immunological measurement of advanced glycation endproducts using normal human serum. *J Immunol Methods* 207: 79–88.

Miyata S., Monnier V.M. (1992). Immunohistochemical detection of advanced glycosylation end products in diabetic tissues using monoclonal antibody to pyrraline. *J Clin Invest* 89: 1102–1112.

Miyata T., Ueda Y., Ida Y. (1996). Accumulation of albumin linked and free form pentosidine in the circulation of uremic patients with end-stage renal failure: Renal implication in the pathophysiology of pentosidine. *J Am Soc Nephrol* 7: 1198–1206.

Miyata T., van Ypersele de Strihou C., Thorpe S., Baynes J.W. (1999). Alteration in nonenzymatic biochemistry in uremia: Origin and significance of "carbonyl stress" in long term uremic complications. *Kidney Int* 55: 389–399.

Monnier V.M., Sell D.R., Miyata S., Nagaraj R.H., Odetti P., Lapolla A. (1992). Advanced Maillard reaction products as a marker for tissue damage in diabetes and uremia: Relevance to diabetic nephropathy. *Acta Diabetol* 29: 130–135.

Odani H., Shinzato T., Usami J., Matsumoto Y., Brinkmann Frye E., Baynes J.W., Maeda K. (1998). Imidazolium crosslinks derived from reaction of lysine with glyoxal and methylglyoxal are increased in serum proteins of uremic patients: Evidence for increased oxidative stress in uremia. *FEBS Lett* 427: 381–385.

Odani H., Shinzato T., Matsumoto Y., Usami J., Maeda K. (1999). Increase in three alpha,beta-dicarbonyl compound levels in human uremic plasma: Specific in vivo determination of intermediates in advanced Maillard reaction. *Biochem Biophys Res Commun* 256: 89–93.

Odetti P.R., Borgoglio A., De Pascale A., Rolandi R., Adezati R. (1990). Prevention of diabetes-increased aging effect on rat collagen-linked fluorescence by amino guanidine and rutin. *Diabetes* 39: 796–801.

Papanastasiou P., Grass L., Rodela H., Patrikarea A., Oreopoulos D., Diamandis E.P. (1994). Immunological quantification of advanced glycosylation end-products in the serum of patients on haemodialysis or CAPD. *Kidney Int* 46: 216–222.

Ritz E., Deppisch R., Nawroth P. (1994). Toxicity of uraemia: Does it come of AGE? *Nephrol Dial Transplant* 9: 1–2.

Roberts N.B., Amara A.B., Morris M., Green B.N. (2001). Long-term evaluation of electrospray ionization mass spectrometric analysis of glycated hemoglobin. *Clin Chem* 47: 316–321.

Schiel R., Franke S., Appel T., Voigt U., Ross I.S., Kientsch-Engel R., Stein G., Muller U.A. (2003). Improvement in quality of diabetes control and concentrations of AGE-products in patients with type 1 and insulin-treated type 2 diabetes mellitus studied over a period of 10 years (JEVIN). *J Diabetes Complications* 17: 90–97.

Schinzel R., Munch G., Heidland A., Sebekova K. (2001). Advanced glycation end products in end-stage renal disease and their removal. *Nephron* 87: 295–303.

Schleicher E.D., Wagner E., Nerlich A.G. (1997). Increased accumulation of the glycoxidation product *N*(epsilon)-(carboxymethyl)lysine in human tissues in diabetes and aging. *J Clin Invest* 99: 457–468.

Schwartz J.C. (1995). The role of glycohemoglobin and other proteins in diabetes management. *Diabetes Rev* 3: 269–287.

Sell D.R., Monnier V.M. (1989). Structure elucidation of a senescence cross-link from extracellular matrix. Implication of pentoses in the aging process. *J Biol Chem* 264: 21597–21602.

Sell D.R., Lapolla A., Odetti P., Fogarthy J., Monnier V.M. (1992). Pentosidine formation in skin correlates with severity of complications in individuals with long-standing IDDM. *Diabetes* 41: 1286–1292.

Skolnik E.Y., Yang Z., Makita Z., Radoff S., Kirkstein M., Vlassara H. (1991). Human and rat mesangial cell receptors for glucose-modified proteins: Potential role in kidney tissue remodeling and diabetic nephropathy. *J Exp Med* 174: 931–939.

Thornalley P.J. (1996). Advanced glycation and the development of diabetic complications: Unifying the involvement of glucose, methyl-glyoxal and oxidative stress. *Endocrinol Metab* 3: 149–166.

Thornalley P.J., Langborg A., Minhas H.S. (1999). Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. *Biochem J* 344: 109–116.

Thornalley P.J. (1999). The clinical significance of glycation. *Clin Lab* 45: 263–273.

Thornalley P.J., Argirova M., Ahmed N., Mann V.M., Argirov O.K., Dawnay A. (2000). Mass spectrometric monitoring of albumin in uremia. *Kidney Int* 58: 2228–2234.

Thornalley P.J., Jahan I., Ng R. (2001). Suppression of the accumulation of triosephosphate and increased formation of methylglyoxal in human red blood cells during hyperglycemia by thiamine in vitro. *J Biochem* 129: 543–549.

Thornalley P.J., Battah S., Ahmed N., Karachalias N., Agalou S., Babaei-Jadidi R., Dawnay A. (2003). Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. *Biochem J* 375: 581–592.

Trivelli L.A., Ranney H.M., Lai H.T. (1971). Haemoglobin components in patients with diabetes mellitus. *N Engl J Med* 284: 353–357.

Watkins N.G., Thorpe S.R., Baynes J.W. (1985). Glycation of amino acid groups in proteins. *J Biol Chem* 260: 10629–10636.

Weiss M.F., Erhard P., Kader-Attia F.A., Wu Y.C., Deoreo P.B., Araki A., Glomb M.A., Monnier V.M. (2000). Mechanisms for the determination of glycoxidation products in end-stage renal disease. *Kidney Int* 57: 2571–2585.

Wolff S.P., Jiang Z.Y., Hunt J.V. (1991). Protein glycation and oxidative stress in diabetes mellitus and ageing. *Free Radic Biol Med* 10: 339–352.

Yamamoto Y., Tahara Y., Cha T., Noma Y., Fukuda M., Yamato E., Yoneda H., Hashimoto F., Ohboshi C., Hirota M., Iida M., Shin S., Shima K. (1989). Radioimmunoassay of glycated serum protein using monoclonal antibody to glucitollysine and Coomassie-brilliant blue-coated polystyrene beads. *Diabetes Res* 11: 45–49.

Zhang X., Medzihradszhy K.F., Cunningham J., Lee P.D.K., Rognerud C.L., Ou C.N., Harmatz P., Witkowska H.E. (2001). Characterization of glycated hemoglobin in diabetic patients: Usefulness of electrospray mass spectrometry in monitoring the extent and distribution of glycation. *J Chromatogr B* 759: 1–15.

8

MUDPIT (MULTIDIMENSIONAL PROTEIN IDENTIFICATION TECHNOLOGY) FOR IDENTIFICATION OF POST-TRANSLATIONAL PROTEIN MODIFICATIONS IN COMPLEX BIOLOGICAL MIXTURES

STEFANI N. THOMAS, BING-WEN LU, TATIANA NIKOLSKAYA, YURI NIKOLSKY, AND AUSTIN J. YANG

8.1 INTRODUCTION

A substantial degree of complexity is conferred upon the proteome by the post-translational modification of proteins. One of the hallmarks of the proteome is its ability to dynamically regulate protein expression in response to internal and external stress. The stress can be physiological, pathological, developmental, pharmacological, or aging related, and often results in the formation of various protein post-translational modifications. Proteomic analyses of expressed cellular components that are post-translationally modified will significantly help clarify the dynamics of oxidative stress. Currently, more than one hundred types of post-translational modifications are known, including glycosylation, ubiquitination, phosphorylation, oxidation, and acetylation (O'Donovan et al., 2001). Of particular interest is the oxidative damage of proteins in human diseases—a modification that leads to aberrant biological functions and to cell death.

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

The generation of free radicals and the oxidative injury they impart are characteristic of the processes of aging and various age-related neurodegenerative disorders. While oxidation converts proteins to forms that are more susceptible to proteases, thereby marking them for degradation by proteolysis, oxidative modifications can also promote the formation of cross-linked protein aggregates that are resistant to removal by proteases (Aksenov et al., 2001). The free radical theory of aging contends that the accumulation of oxidative damage to macromolecules, cells, and tissues underlies the basis of age-related functional deficits (Yu, 1996). Evidence supporting the involvement of oxidative stress in aging is widespread (Stadtman, 2001; Shacter, 2000). For instance, protein carbonyl concentration, a measure of protein oxidation, increases progressively with age in the mouse brain (Dubey et al., 1996). Hence, the analysis of protein carbonyl concentrations in humans could clarify the proteome dynamics in human diseases. In this chapter we provide a broad overview of the use of *mu*lti*d*imensional *p*rotein *i*dentification *t*echnology (MudPIT) to identify post-translational modifications in protein complexes using protein carbonylation as a primary example. In addition, we introduce the bioinformatics tools used for proteomic data validation and visualization.

8.2 PROTEOMIC ANALYSIS OF OXIDATIVELY MODIFIED PROTEINS

Reactive oxygen species (ROS) play important physiological roles and have the ability to cause extensive cellular damage. In order to investigate the role of oxidative stress and ROS in the pathogenesis and progression of disease, the applicable analytical procedures must be optimized. Proteomic studies can provide substantial insight into the cellular and molecular mechanisms of various disease processes including the elucidation of post-translational modification of proteins. Proteomic approaches often include the use of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) to separate and visualize proteins followed by mass spectrometry (MS) for protein identification. The strength of experiments involving this 2D-PAGE/MS approach is the ability to examine differences in the post-translational states of high abundance proteins; however, a more sensitive and rapid alternative can be employed, namely the coupling of liquid chromatography with electrospray ionization tandem mass spectrometry (LC-MS/MS). LC-MS/MS is frequently adopted to analyze complex protein mixtures, which are often underrepresented via 2D-PAGE/MS approaches, namely low-abundance proteins that play important roles in signal transduction.

8.2.1 Affinity Purification of Carbonylated Proteins

As with any biological marker of disease states, a valid biological marker of oxidative stress should be: (1) specific for the reactive species involved; (2) a chemically and biologically stable product; (3) determined by an assay that is specific, sensitive, and reproducible; (4) a major product of oxidative modification that may be

directly implicated in the onset or progression of disease, and (5) representative of the balance between the generation of oxidative damage and clearance (Dalle-Donne et al., 2005). The protein carbonyl content is widely used as a marker to determine the level of protein oxidation that is caused either by direct oxidation of amino acid side chains (e.g., proline and arginine to γ-glutamylsemialdehyde, lysine to aminoadipic semialdehyde, and threonine to aminoketobutyrate) or by indirect reactions with oxidative by-products [lipid peroxidation derivatives such as 4-hydroxynonenal (HNE), malondialdehyde (MDA), and advanced glycation end-products (AGEs)] (Wei and Lee, 2002; Shacter, 2000).

The serious consequence of oxidative impairment of protein is protein dysfunction. For example, it has recently been reported that HNE causes a direct inactivation of lysosomal cathepsin B activity in vitro (Crabb et al., 2002). Mass spectrometric analysis of HNE-conjugated cathepsin B showed that the loss of cathepsin B enzymatic activity is directly attributed to HNE's adduction to His150 and Cys290 within the active site of the protein (Crabb et al., 2002). In another study Keller and co-workers observed the detrimental effects of lipid peroxidation on neuronal plasticity (Keller et al., 1997). Isolated synaptosomes treated with 4-hydroxynonenal disrupt mitochondrial function and impair glutamate transport. Although the specific molecular mechanisms involved in these alterations are not known, biochemical analyses suggest that there is a correlation between the adduction of HNE to a glutamate transporter and the loss of glutamate transporter function (Keller et al., 1997).

Traditionally protein carbonylation has been identified through the derivatization of carbonyl groups by 2,4-dinitrophenylhydrazine (DNPH). The stable dinitrophenyl hydrazone product that forms can be detected by various immunoassays (enzyme-linked immunosorbent assay, immunohistochemistry, or Western blot) or by spectrophotometric assay (Levine et al., 1990; Smith et al., 1998; Nakamura and Goto, 1996). Spectrophotometric DNPH assays can be coupled to protein fractionation by high-performance liquid chromatography (HPLC) to give greater specificity and sensitivity than measuring total carbonyls in a protein mixture (Levine et al., 1994). Providing even more sensitivity are Western blot assays, which can detect as little as 1 pmol carbonyl in a protein sample and require as little as 50 ng protein oxidized to the extent of 0.5 mol carbonyl/mol protein (Levine et al., 1994).

The aforementioned methods are sufficient for detection of the protein carbonyl content within a complex biological mixture. However, two of the goals of proteomic investigations of protein post-translational modification are (1) the identification of proteins that are susceptible to modification and (2) the determination of the exact amino acid residues where the modifications occur. Therefore, it is advantageous to employ a protein carbonylation detection procedure that is compatible with mass spectrometry. To this end, our group has recently developed a method to affinity purify carbonylated proteins based on a biocytin hydrazide and streptavidin methodology coupled with LC-MS/MS (Fig. 8.1) (Soreghan et al., 2003). By this method we were able to identify hundreds of carbonylated proteins in a single experiment.

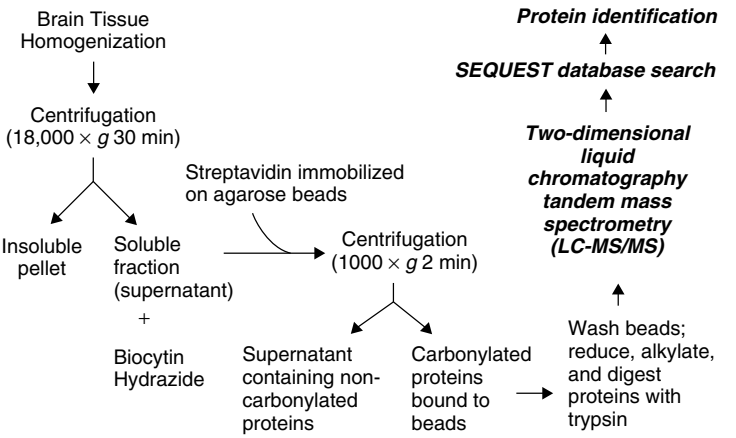


FIGURE 8.1 Schematic representation of MudPIT identification of carbonylated proteins from mouse brain homogenate.

In our biocytin hydrazide-streptavidin affinity purification scheme, soluble proteins from tissue homogenate of an aged mouse brain were reacted with biocytin hydrazide in order to induce the formation of hydrazone conjugates with the carbonyl groups of oxidatively modified proteins (Fig. 8.2). These biocytin-labeled conjugates were then affinity purified by streptavidin immobilized on agarose beads. The carbonylated proteins bound to the agarose beads were then reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin. The tryptic peptides generated from this approach were identified using MudPIT, as described in the following section. (This method can also be applied to the analysis of insoluble carbonylated proteins in the tissue homogenate following treatment with 70% formic acid.)

A general concern regarding the affinity purification approach is that nonspecific binding of noncarbonylated proteins to the streptavidin-agarose beads might occur. A recent mass spectrometric and isotope-coded affinity tag (ICAT) based approach addresses quantitatively the issue of specificity during protein affinity purification procedures (Ranish et al., 2003). To differentiate specific complex components from nonspecific co-purifying proteins, Ranish and co-workers prepared a control purification in which the complex of interest was not enriched and the samples were labeled with either a "light" or "heavy" stable isotope tag. Following tryptic digestion and LC-MS/MS analysis, true components of the complex of interest were distinguished by their increased abundance in the purified sample in comparison to the nonenriched control sample. It was demonstrated that this approach distinguishes between specific complex components and co-purifying proteins, even when the specific components are more than 20 times less abundant than the co-purifying proteins. In sum, the application of this quantitative mass spectrometry technique permitted not only identification of the specific protein complex components in partially purified samples but also detection of quantitative differences in protein abundance within the specific complexes.

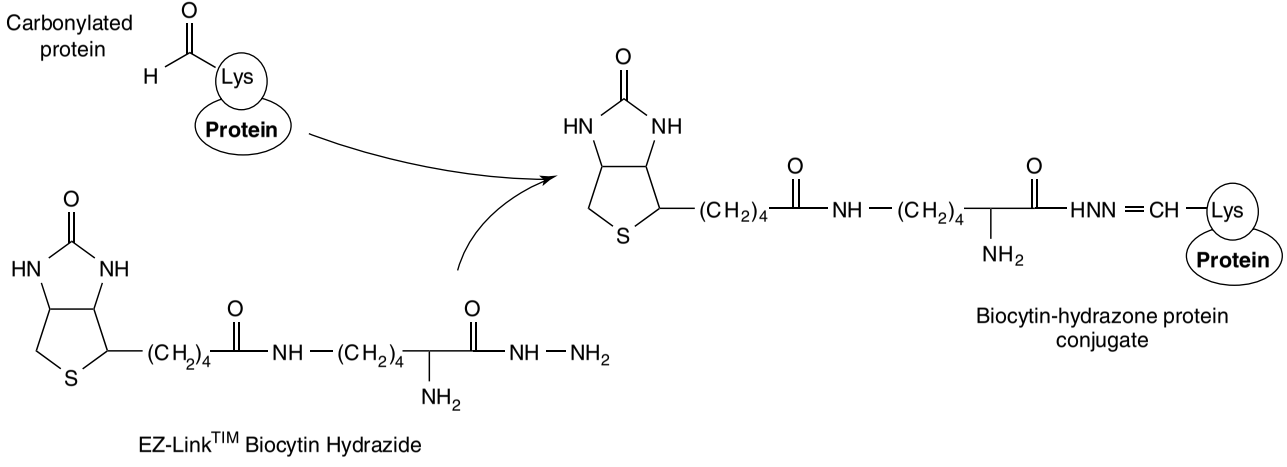


FIGURE 8.2 Isolation of carbonylated proteins by a biocytin hydrazide and streptavidin affinity methodology.

8.2.2 MudPIT Incorporating Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS)

The analysis of post-translationally modified proteins requires resolution of the proteins in a sample, followed by protein identification. Any purification steps that enrich for proteins with a post-translational modification of interest will aid in subsequent proteomic analyses. In the case of selectively analyzing oxidatively modified proteins, particularly those that are subject to carbonylation, one approach that can be employed is the biocytin hydrazide/streptavidin affinity approach recently described by our group (Soreghan et al., 2003).

The most widely used method of protein resolution and identification is 2D-PAGE followed by mass spectrometry (Washburn and Yates, 2000). In this technique the proteins are separated in the first dimension by isoelectric point (pI) and in the second dimension by molecular weight. Typically, individual protein spots on the gel are excised, digested, and are analyzed via mass spectrometry. However, if numerous protein spots must be selected, the analysis can become time-consuming and laborious. Hence, an alternative two-dimensional protein separation scheme was established employing non–gel-based chromatography systems to resolve and identify thousands of proteins from a biological sample (Washburn et al., 2001; McCormack et al., 1997; Yates et al., 1995; Giddings, 1984). The result of these efforts is a technique termed MudPIT.

A standard MudPIT experiment uses two-dimensional liquid chromatography consisting of a strong cation exchange column (SCX) to separate peptides based on their charge, in tandem with a reversed-phase column to resolve peptides based on their hydrophobicity. To achieve peptide separation in the first dimension, a salt gradient is created with solvents such as 500 mM sodium chloride (Nagele et al., 2003, 2004) or 500 mM ammonium acetate (Washburn et al., 2001). Hydrophobic separation in the second dimension is accomplished by an acetonitrile gradient. Peptide fragments are eluted directly into the ion source of a tandem mass spectrometer, which isolates and induces fragmentation of these peptides resulting in a mass spectrum of the fragment ions for each peptide that is acquired. A computer algorithm is then used to match the experimental tandem mass spectra with theoretical spectra generated from a sequence database to identify peptide sequences within the protein mixture.

The direct identification of proteins by MudPIT bypasses the potential limitations of gel electrophoresis, including protein insolubility, limited fractionation ranges, and limited recovery of material. When applied to the large-scale analysis of the proteome of the *Saccharomyces cerevisiae* strain BJ5460 (Washburn et al., 2001), this MudPIT strategy resulted in the assignment of 5,540 peptides to MS spectra, leading to the detection and identification of 1,484 proteins. Of these identified proteins, 131 had three or more predicted transmembrane domains, indicating the robustness of this approach in identifying membrane proteins, which are often difficult to detect by standard proteomic analyses involving 2D-PAGE. Additional studies have applied MudPIT to the characterization of complex biological mixtures such as rat liver (Jiang et al., 2005), developing mouse brain phosphoproteome (Ballif et al., 2004), sumoylated proteins in human

embryonic kidney cells (Manza et al., 2004), human erythroid leukemia cell line K562 (Resing et al., 2004), human heart mitochondria (Gaucher et al., 2004), and human epidermoid carcinoma cell line A431 (Chelius et al., 2003). MudPIT has been demonstrated to achieve a dynamic range of 10,000 to 1 between the most abundant and least abundant proteins/peptides in a complex mixture (Wolters et al., 2001). The sensitivity and dynamic range of this proteomic approach make it amenable for use in the discernment of protein post-translational modifications in complex biological samples.

In order to optimize the comprehensive proteomic analysis of a given sample while attempting to identify various post-translational modifications, it is often advantageous to use multiple proteases—as opposed to a single protease such as trypsin—to increase the protein sequence coverage by identified peptides (MacCoss et al., 2002). For example, a nonspecific protease approach coupled with MudPIT analysis of rat brain homogenate led to the identification of 79 post-translational modifications on 51 proteins (Wu et al., 2003). Another complementary step can be taken in MudPIT analyses to optimize protein identification coverage. This approach, known as gas phase fractionation, involves the iterative mass spectrometric interrogation of a sample over multiple unique m/z ranges (Yi et al., 2002).

8.2.3 MudPIT Analysis of Carbonylated Proteins in Mouse Brain

In our group's work toward the goal of employing MudPIT to understand the effects of post-translational modifications of proteins during the course of aging and neurodegeneration, we have reported a sensitive detection method for the occurrence of protein carbonylation in aged mice (Soreghan et al., 2003). This method entails MudPIT analysis coupled with a hydrazide biotin-streptavidin methodology. The use of this functional proteomic approach resulted in the identification of more than 100 carbonylated proteins. Furthermore, several of the identified classes of proteins, such as transmembrane, large molecular weight, and low abundance proteins, are those that are traditionally challenging to detect by 2D-PAGE alone.

In the presently described study (Soreghan et al., 2003), protein carbonylation in the brains of aged (18-month-old) and young (3-week-old) C57B mice was analyzed by protein carbonyl labeling using a hydrazide biotin-streptavidin methodology. The carbonylated proteins were derivatized with biocytin hydrazide, affinity-purified with streptavidin immobilized on agarose beads, and were subjected to trypsin digestion. The resultant peptide fragments were then separated on a reversed-phase (RP) column for resolution based on hydrophobicity. The peptide fragments were then eluted directly into the ion source of a tandem mass spectrometer, which isolates and induces fragmentation of the peptides, resulting in a mass spectrum of the fragment ions for each peptide that is recorded. Each raw tandem mass spectrum was then processed as follows: (1) spectra derived from singly or multiply charged parent ions were identified, and (2) the selected charged ions were then subjected to a final protein identification analysis

through the SEQUEST algorithm using the following cross-correlation score parameters: >1.5 for $+1$ ions, >2.0 for $+2$ ions, and >2.5 for $+3$ ions.

Among the classes of proteins identified from the aged mice brain samples were cytoskeletal proteins, mitochondrial proteins known to be involved in both redox regulation and ATP generation, and cell-signaling regulatory proteins including several proteins and receptors that are tightly associated with insulin and the insulin-like growth factor signal transduction pathway, as well as phosphatases involved in controlling the phosphorylation state of numerous key signaling proteins. The degree to which any of the identified proteins are rendered dysfunctional by a carbonyl modification and the extent to which the modified proteins might act in a causal role in the aging process or in neurodegenerative diseases is the subject of more elaborate investigations currently being undertaken by our group.

8.2.4 Peptide Sequencing and Protein Assignment via Database Searching of MS/MS Spectra

Since the sequence information for a single peptide is contained in one tandem mass spectrum, it is theoretically possible to use a mass ruler to determine, based on peak mass differences, the exact amino acid sequence of the peptide. Database-searching algorithms have become an indispensable tool for the interpretation of tandem mass spectra (Peng and Gygi, 2001) given that de novo sequencing approaches are often not feasible due to factors such as (1) ion series being rarely complete, (2) more than one ion sequence being usually present, (3) fragment ions being present in varying abundances, frequently with losses of water or ammonia, and (4) some amino acids having similar or identical molecular masses.

SEQUEST and Mascot are the two main algorithms that are used to analyze MS/MS data. In the SEQUEST algorithm, peptides with molecular masses matching that of the parent peptide ion sequence from the tandem mass spectrometer are extracted from a protein database (Yates et al., 1995). Each peptide is then given a preliminary score based on the number of predicted fragment ions from the database peptide that match the acquired fragment ions in the tandem mass spectrometer. The top 500 matching peptides are then subjected to a more extensive ion-matching algorithm in order to generate cross-correlation scores. Ultimately a list of peptides with their corresponding correlation scores is produced, and protein identifications are evaluated based on these scores.

One important caveat is that any mass spectrometry based protein identification is wholly dependent on the sequences that have been deposited into the database that is used for searching. For example, for any peptide sequence the SEQUEST algorithm will identify the most likely match for a protein in the database. As a result, if a protein does not exist in the database or if the sequence is incorrect, the best-matched protein assigned by SEQUEST will be incorrect. The implementation of statistical analysis following database searching will serve to reduce occurrences of false protein assignments.

8.2.5 Bioinformatic Determination of Amino Acid Sites of Post-translational Oxidative Modifications

Traditionally identification of post-translational modifications by tandem mass spectrometric analysis requires users to manually inspect thousands of MS/MS spectra, which can be tedious and time-consuming. Alternatively, when using algorithms such as Mascot and SEQUEST to analyze MS/MS data, users can specify various amino acid modifications in the database search criteria. The specific mass shifts corresponding to the indicated post-translational modifications will then be taken into account when assigning peptides to the experimental MS/MS spectra.

Another computer algorithm that can be used to aid in the analysis of protein post-translational modifications is the *s*coring *al*gorithm for *s*pectral *a*nalysis, or SALSA (Hansen et al., 2001; Liebler et al., 2002). SALSA is a pattern recognition algorithm used to determine site-specific post-translational modifications. With the use of SALSA, users can automatically search for post-translational modifications based on the spacing of the m/z values associated with the ion series of an amino acid sequence. Our group has recently used SALSA as a novel approach for the detection of sites of amino acid side chain modification within copper-oxidized ß-amyloid (Aß) 1–40 (Schiewe et al., 2004). The SALSA algorithm independently scores spectra for product ions, neutral losses, charged losses, and ion pairs. Together these characteristics indicate the presence of specific amino acid modifications in peptide ions subjected to MS/MS. SALSA evaluates each MS/MS scan in a data file for these specific criteria in a user-specified hierarchy. The ranking of MS/MS spectra according to their SALSA scores allows the user to quickly identify those MS/MS scans corresponding to the modified peptide(s) of interest.

8.3 STATISTICAL VALIDATION AND INTERPRETATION OF MudPIT DATA

The generation of very large numbers (hundreds of thousands) of MS/MS spectra from such high-throughput proteomic approaches such as MudPIT creates a formidable challenge when correct peptide assignments must be discerned from false identifications among database search results (Keller et al., 2002; Nesvizhskii et al., 2003). A robust solution to this problem has not yet been achieved, although recent reports have begun to improve the confidence of sequence assignments using a probabilistic model for peptide identification (Sadygov et al., 2004), linear discriminant analysis (Keller et al., 2002), or machine learning algorithms (Anderson et al., 2003) to evaluate the scores generated by SEQUEST. To validate peptide assignments, peptide properties other than their fragmentation pattern, such as exact mass measurements, have also been adopted (Smith et al., 2002).

An additional approach to solving the problem of deciphering correct peptide assignments from false identifications is a novel programmatic approach, which

significantly improves sensitivity and confidence in peptide assignments and protein representation and is readily available to the general research community (Resing et al., 2004). Key features of this approach include (1) integrating results of two database search programs to increase detection sensitivity of correctly identified peptides, (2) implementing new filtering criteria based on peptide chemical properties to increase discrimination between correct versus incorrect sequence assignments, (3) focusing the search strategy to improve accuracy in sequence assignments, and (4) developing a peptide-centric nomenclature for protein profiling to accurately report ambiguities in protein identification due to sequence redundancy in the database. The following section will describe the statistical models, PeptideProphet and ProteinProphet, which have been developed to assess the validity of peptide and protein identifications based on database searches.

8.3.1 Statistical Models to Assess the Quality of Peptide and Protein Assignments

PeptideProphet and ProteinProphet are automated and robust statistical programs that allow for the standardization of data analysis from large-scale proteomic experiments such as those incorporating MudPIT for the identification of protein post-translational modifications (Nesvizhskii et al., 2003). The fact that PeptideProphet and ProteinProphet are probability-based programs permits comparison of data, independent of the mass spectrometric instrument, database, and search engine. The use of such powerful statistical tools ensures an objective measurement of the quality of proteomic data.

Figure 8.3 is a graphical representation of the PeptideProphet and ProteinProphet statistical validation of SEQUEST-generated peptide and protein identifications from a study conducted in our lab incorporating MudPIT to analyze carbonylated proteins in aged mouse brain. PeptideProphet is based on use of an expectation maximization algorithm to derive a mixture model of correct and incorrect peptide assignments from the data (Fig. 8.3*a*). It uses the observed information about each assigned peptide in the data set, learns to distinguish correct from incorrect peptide assignments, and, finally, computes a probability for each assignment being correct. The program computes discriminant scores (F scores) for correct and incorrect peptide assignments using a set of scores generated from SEQUEST search results; this set includes Xcorr (cross-correlation score between theoretical and experimental MS/MS spectra), ΔC_n (the relative difference between the first and second highest Xcorr score for all peptides of a given protein queried from the database), Sp Rank (a measure of how well the assigned peptide scored using a preliminary correlation metric), d_M (the absolute difference between the masses of the precursor peptide and the assigned peptide), and NTT (number of tryptic termini = 0, 1, or 2). Probability scores (p_{comp}) of peptide assignments that are assigned to each acquired spectrum are then calculated based on the distribution of discriminant (F) scores.

The list of peptide sequences and their respective p_{comp} scores obtained from PeptideProphet is then used to determine a minimal list of proteins (derived from database entries to which the peptide sequences were compared) that can be

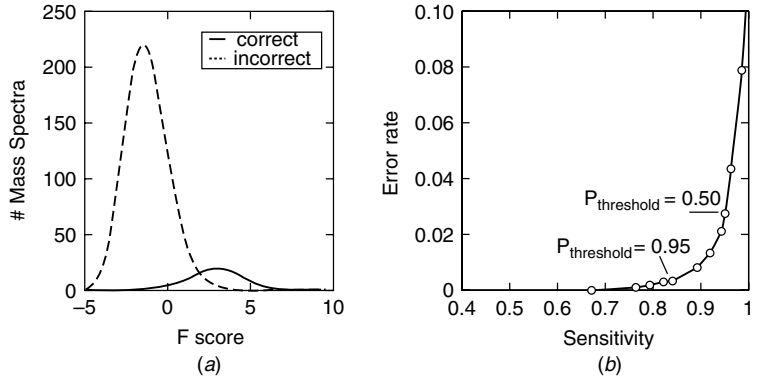


FIGURE 8.3 PeptideProphet and ProteinProphet statistical validation of SEQUEST-generated peptide and protein identifications. (*a*) Correct and incorrect peptide discriminating scores (F scores) determined by PeptideProphet plotted against a portion of the number of mass spectra generated from a typical LC-MS/MS run of mouse brain tissue homogenate. (*b*) ProteinProphet error rates (percentage of total identifications that are false) and sensitivity rates (percentage of current correct identifications out of total correct identifications) of identified proteins.

correlated to the observed data and to compute a probability (P_{comp}) that each protein is indeed present in the original peptide mixture. This is accomplished by employing the ProteinProphet program, which uses an expectation maximization algorithm similar to that used by PeptideProphet. ProteinProphet uses the following scoring system to determine the probability that a protein is present in a specific sample: (1) NTT: number of tryptic termini; (2) NSP: number of sibling peptides (different peptide sequences matching the same protein identification); (3) TOT: number of MS/MS spectra matching the same peptide; and (4) peptide probability score (p_{comp}). After these analyses the probability of each protein is ranked from 0 (incorrect) to 1 (correct). Proteins that are represented by numerous peptides, high percentage sequence coverage, or extremely strong single ion elution profiles are then retained. The distribution of error rates versus sensitivity from our group's MudPIT analysis of carbonylated proteins in aged mouse brain suggests a false positive protein identification rate of 3% and 95% sensitivity when the probability threshold is smaller than 0.5 (Fig. 8.3*b*).

As indicated in the outset of this section discussing the statistical validation and interpretation of data generated from MudPIT analyses, several computational tools have been developed that permit the rapid, consistent, and transparent analysis of large-scale proteomic datasets. However, in the immediate future, it is not feasible to expect all research groups to agree to use the same statistical tools or approaches to analyze their data. As an alternative, it would be reasonable for members of the general research community to statistically validate their proteomic data using tools that have detailed statistical models, that have been extensively tested using reference data sets, and that are made available to the

TABLE 8.1 Available Proteomic Tools for Database Searching, Statistical Validation of Data, Analysis of Post-translational Modifications, and Data Visualization

Program	Reference	Web Site
	Database search tools	
Sequest	Eng et al., 1994	http://www.thermo.com
Mascot	Perkins et al., 1999	http://www.matrixscience.com
	Statistical validation of peptide and protein identifications	
PeptideProphet	Keller et al., 2002	http://www.proteomecenter.org/software.php
ProteinProphet	Nesvizhskii et al., 2003	http://www.proteomecenter.org/software.php
	Bioinformatic analysis of post-translational modifications	
SALSA	Hansen et al., 2001; Liebler et al., 2002	http://www.thermo.com
	Data visualization	
GoSurfer	Zhong et al., 1994	http://www.gosurfer.org
MetaCore	Nikolsky et al., 2005	http://genego.com

public (Nesvizhskii and Aebersold, 2004). A list of the programs that our group uses to evaluate our proteomic data is included as Table 8.1.

8.3.2 Issues to Consider in the Statistical Validation of Proteomic Data Sets

To analyze tandem mass spectrometry data generated by our group, we employ the automated and statistically robust probability-based programs, PeptideProphet and ProteinProphet. We are currently incorporating the use of these mathematical models to filter mass spectrometric data generated from the analysis of protein carbonylation in a mouse model of Alzheimer's disease in an effort to identify and characterize protein targets of oxidative stress. Among the factors to be considered when employing statistical tools to validate proteomic data are (1) the number of constituent peptides of a given identified protein, (2) repetitive peptide sequences from proteins belonging to the same gene family, (3) protein identifications based on single peptide hits, and (4) borderline identifications. The aforementioned factors will be discussed within the context of Peptide- and ProteinProphet.

Identified proteins with several constituent peptides are often referred to as "multi-hit" proteins and typically have a high probability. Table 8.2 displays the PeptideProphet identification of isoform 8 of α-tubulin. The validity of this protein assignment is high due to its high probability (1.00), sequence coverage (22%), and multiple constituent peptides (8). In addition, the number of tryptic termini (NTT) is two for seven out of the eight identified peptides, and the number of sibling peptides (NSP) and total number of MS/MS spectra matching the same peptide (TOT) exceed one.

TABLE 8.2 Protein with Multiple Constituent Peptides as Identified by ProteinProphet

gi|8394493|ref|NP_059075.1| ***1.00***
coverage: 22.0%
>gi|8394493|ref|NP_059075.1| (NM_017379) tubulin, alpha 8; tubulin alpha 8 [Mus musculus]

	Peptide Sequence	Probability	Ntt	Nsp	Tot
*wt-1.00	1_FDGALNVDLTEFQTNLVPYPR	1.00/1.00	2	6	2
*wt-1.00	1_LISQIVSSITASLR	1.00/1.00	2	6	2
*wt-1.00	1_VGINYQPPTVVPGGDLAK	1.00/1.00	2	6	4
*wt-1.00	1_QLFHPEQLITGK	1.00/1.00	2	6	7
*wt-1.00	1_TIQFVDWC*PTGFK	1.00/1.00	2	6	3
*wt-1.00	1_FDLMYAK	1.00/1.00	2	6	7
*wt-1.00	1_NLDIERPTYTNLNR	1.00/1.00	2	6	8
*wt-1.00	1_PPTVVPGGDLAK	0.99/0.95	1	6	2

Note: *C indicates modified cysteine residue (carboxyamidomethylation).

A main reason why protein inference based on peptide assignments is a challenging task, even when statistical models are employed to validate those assignments, is the issue pertaining to peptide degeneracy—instances where peptide sequences are present in more than a single entry in the protein sequence database. Often it is not possible to distinguish the origins of a single peptide if the peptide sequence is located within a highly conserved domain of a particular gene family. An example of such a case is given in Table 8.3. The peptide with the amino acid sequence L(I)QI(L)WDTAGQER is a constituent peptide of two different proteins, GTP-binding protein Rab 3D, and Rab33B, therefore complicating the assignment of a unique protein to these peptide sequences. A similar situation occurs with another peptide, LLLIGNSSVGK, which is common to both GTP-binding protein Rab 3D and Rab 3A. In both cases ProteinProphet did not make a unique protein assignment but rather identified the name of a protein group. The identification of a specific valid protein will require the presence of a gene-specific peptide in order to be positively identified.

Last, there is the issue pertaining to protein identifications which are based on single peptide "hits." It is sometimes the case that such proteins are present in low abundance in biological samples. Hence, when employing proteomic techniques such as MudPIT, it is advantageous to undergo measures that will enrich the starting material for a particular protein(s) of interest. Although there are instances where MS/MS data are generated for only one peptide of a given protein, it is still possible for valid protein assignments to be made. The statistical model used in ProteinProphet penalizes, but does not exclude, peptides corresponding to single-hit proteins and rewards those corresponding to multi-hit proteins (Nesvizhskii et al., 2003). Such is the case for the first four proteins listed in Table 8.4. The

TABLE 8.3 ProteinProphet Identification of Multiple Proteins from a Single Gene Family (RAS Oncogene Family)

[PROTEIN GROUP 1: gi_mus_musculus_ras_protein_rab33b_oncogene_dbj_source_family_member_ mouse_gtp_related_rab3a_ref_rab_binding] ***1.00***				
−1 gi|346947|pir||A45384 ***1.00*** **>gi|346947|pir||A45384 GTP-binding protein rab3D - mouse**				
Peptide Sequence	Probability	Ntt	Nsp	Tot
wt-0.99 1_LLLIGNSSVGK	1.00/1.00	2	3	4
—1_ILIIGNSSVGK				
wt-0.99 1_LQIWDTAGQER	1.00/1.00	2	3	4
−2 gi|8394133|ref|NP_058554.1| ***0.00*** **>gi|8394133|ref|NP_058554.1| (NM_016858) RAB33B, member of RAS oncogene family [Mus musculus]**				
wt-0.01 1_LQIWDTAGQER	0.99/1.00	2	0	4
–1_IQLWDTAGQER				
−3 gi|6679593|ref|NP_033027.1| ***0.00*** **>gi|6679593|ref|NP_033027.1| (NM_009001) RAB3A, member RAS oncogene family [Mus musculus]**				
wt-0.01 1_LLLIGNSSVGK	0.99/1.00	2	0	4
–1_ILIIGNSSVGK				

peptides identified from these proteins are all tryptic peptides (NTT = 2) and the probabilities of the proteins are significantly higher than a filter cutoff value of 0.5. The last two proteins listed in Table 8.4 are examples of borderline data. As is the case with the first four proteins listed in Table 8.4, these are single-hit proteins, but their probabilities are lower than the filter cutoff of 0.5 and are thus not acceptable as valid protein identifications.

When undertaking proteomic studies involving MudPIT, the statistical filtering of large-scale data sets becomes a critical factor. The implementation of such statistical tools as Peptide- and ProteinProphet, which yield computed probabilities that can be used to estimate false-positive error rates resulting from data

TABLE 8.4 ProteinProphet Protein Identifications Based on Single Peptides

gi|65923|pir||DEMSLM ***1.00*** **>gi|65923|pir||DEMSLM L-lactate dehydrogenase (EC 1.1.1.27) chain M - mouse**				
Peptide sequence	Probability	Ntt	Nsp	Tot
*wt-1.00 1_SADTLWGIQK	1.00/1.00	2	0	5
gi|21263432|sp|Q91VR2|ATPG_MOUSE ***1.00*** **>gi|21263432|sp|Q91VR2|ATPG_MOUSE ATP synthase gamma chain, mitochondrial precursor**				
*wt-1.00 1_THSDQFLVSFK	1.00/1.00	2	0	1
gi|400622|sp|P31648|S6A1_MOUSE ***0.98*** **>gi|400622|sp|P31648|S6A1_MOUSE Sodium- and chloride-dependent GABA transporter 1**				
*wt-1.00 1_VADGQISTEVSEAPVASDKPK	0.98/1.00	2	0	4
gi|6678195|ref|NP_033331.1| ***0.98*** **>gi|6678195|ref|NP_033331.1| (NM_009305) synaptophysin; Syp I [Mus musculus]**				
*wt-1.00 1_LHQVYFDAPSC*VK	0.98/1.00	2	0	2
gi|6755686|ref|NP_035630.1| ***0.45*** **>gi|6755686|ref|NP_035630.1| (NM_011500) striatin, calmodulin binding protein; striatin [Mus musculus]**				
*wt-1.00 1_AAGDGAAAAGAAR	0.45/0.75	1	0	1
gi|484964|pir||PN0510 ***0.44*** **>gi|484964|pir||PN0510 integrin beta-3 chain - mouse (fragment)**				
*wt-1.00 1_MC*SGHGQC*NCGDCVCDSDWTGYYC*NC*R	0.44/0.74	1	0	1

filtering, can serve as a reliable means of publishing large-scale data sets of protein identifications.

8.3.3 Functional Categorization and Pathway Analysis of Proteomic Data Sets

Difficulties stemming from information overflow and the management of large data sets generated from proteomic analyses involving MudPIT often arise. To overcome this challenge, our group has recently adopted Systems Reconstruction

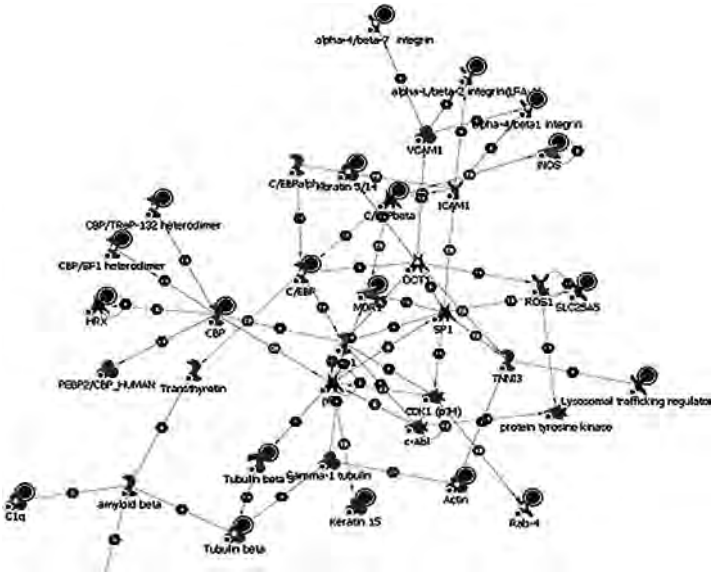


FIGURE 8.4 Functional network analysis and re-constructed protein–protein interaction pathways of oxidatively modified (carbonylated) proteins in brain homogenate of a mouse model of Alzheimer's disease. Concentric circles adjacent to icons in the network indicate carbonylated proteins that were identified from a biocytin-hydrazide/streptavidin affinity purification procedure followed by MudPIT analysis.

technology to assist us with our proteomic data visualization and integration. MetaCore™ is a program developed and based on Systems Reconstruction technology, which contains over 11,500 human pathways (both metabolic and signal transduction), over 12,000 biochemical reactions, and more than 250 manually curated maps for major functional pathways of cellular processes (Nikolsky et al., 2005). MetaCore™ represents an integrated database on human and mammalian signaling, regulatory, and metabolic pathways, which are interconnected via associations between genes, proteins, metabolites, pathways, and human diseases. These maps and pathway networks represent the backbone for the integration of several types of experimental data, such as mRNA expression, protein expression (e.g., from 2D-PAGE or mass spectrometry), protein–protein interaction assays (yeast two-hybrid systems, co-immunoprecipitation), metabolic profiles, and enzymatic activity. Thus, MetaCore™ is a tool that can be effectively used to visualize proteomic datasets generated from MudPIT experiments designed to identify post-translational protein modifications in complex biological mixtures. An example of a functional network analysis using MetaCore™ is shown in Figure 8.4. In order to visualize datasets generated from our group's proteomic analysis of affinity-purified carbonylated proteins in brain homogenate of a mouse model of Alzheimer's disease, protein-protein interaction pathways of identified carbonylated proteins were re-constructed as presented in Figure 8.4.

8.4 CONCLUDING REMARKS

MudPIT is a methodology that can be effectively applied for the comprehensive high-throughput analyses of proteomes. The high sensitivity of this method makes it amenable for proteomic studies designed to analyze post-translational protein modifications. It should be noted that the affinity purification method described earlier for the detection of carbonylated proteins represents a method that can be applied to only a fraction of the total amount of oxidatively modified proteins. However, there are numerous alternative methods of detection of protein oxidation in biological samples (Shacter, 2000) that can be incorporated with MudPIT analysis. The shotgun proteomics approach to the identification of oxidatively modified proteins as detailed here, in addition to the use of various bioinformatics and computational tools, will aid in the systematic elucidation of the various proteome dynamics underlying human diseases associated with oxidative stress.

ACKNOWLEDGMENTS

This work was supported by grants awarded by the National Institutes of Health to A.Y. (MH59786, AG025323-01). S.T. is supported by a pre-doctoral training fellowship awarded by the Society for Neuroscience. We would also like to thank Diane Cripps for critical discussion pertaining to the manuscript.

LIST OF ABBREVIATIONS

AGE, advanced glycation end-product
DNPH, 2,4-dinitrophenylhydrazine
2D-PAGE, two-dimensional polyacrylamide gel electrophoresis
HNE, 4-hydroxynonenal
HPLC, high-performance liquid chromatography
ICAT, isotope-coded affinity tag
LC-MS/MS, liquid chromatography tandem mass spectrometry
MDA, malondialdehyde
MS, mass spectrometry
MudPIT, multidimensional protein identification technology
NSP, number of sibling peptides
NTT, number of tryptic termini
ROS, reactive oxygen species
RP, reversed-phase
SALSA, scoring algorithm for spectral analysis
SCX, strong cation exchange
TOT, total number of MS/MS spectra matching the same peptide
Xcorr, cross-correlation score

REFERENCES

Aksenov M.Y., Aksenova M.V., Butterfield D.A., Geddes J.W., Markesbery W.R. (2001). Protein oxidation in the brain in Alzheimer's disease. *Neuroscience* 103: 373–383.

Anderson D.C., Li W., Payan D.G., Noble W.S. (2003). A new algorithm for the evaluation of shotgun peptide sequencing in proteomics: Support vector machine classification of peptide MS/MS spectra and SEQUEST scores. *J Proteome Res* 2: 137–146.

Ballif B.A., Villen J., Beausoleil S.A., Schwartz D., Gygi S.P. (2004). Phosphoproteomic analysis of the developing mouse brain. *Mol Cell Proteomics* 3: 1093–1101.

Chelius D., Zhang T., Wang G., Shen R.F. (2003). Global protein identification and quantification technology using two-dimensional liquid chromatography nanospray mass spectrometry. *Anal Chem* 75: 6658–6665.

Crabb J.W., O'Neil J., Miyagi M., West K., Hoff H.F. (2002). Hydroxynonenal inactivates cathepsin B by forming Michael adducts with active site residues. *Protein Sci* 11: 831–840.

Dalle-Donne I., Scaloni A., Giustarini D., Cavarra E., Tell G., Lungarella G., Colombo R., Rossi R., Milzani A. (2005). Proteins as biomarkers of oxidative/nitrosative stress in diseases: the contribution of redox proteomics. *Mass Spectrom Rev* 24: 55–99.

Dubey A., Forster M.J., Lal H., Sohal R.S. (1996). Effect of age and caloric intake on protein oxidation in different brain regions and on behavioral functions of the mouse. *Arch Biochem Biophys* 333: 189–197.

Eng J.K., McCormack A.L., Yates JR. (1994). An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J Am Soc Mass Spectrom* 5: 976–989.

Gaucher S.P., Taylor S.W., Fahy E., Zhang B., Warnock D.E., Ghosh S.S., Gibson B.W. (2004). Expanded coverage of the human heart mitochondrial proteome using multidimensional liquid chromatography coupled with tandem mass spectrometry. *J Proteome Res* 3: 495–505.

Giddings J.C. (1984). Two-dimensional separations: Concept and promise. *Anal Chem* 56: 1258A–1260A, 1262A, 1264A passim.

Hansen B.T., Jones J.A., Mason D.E., Liebler D.C. (2001). SALSA: A pattern recognition algorithm to detect electrophile-adducted peptides by automated evaluation of CID spectra in LC-MS-MS analyses. *Anal Chem* 73: 1676–1683.

Jiang X.S., Dai J., Sheng Q.H., Zhang L., Xia Q.C., Wu J.R., Zeng R. (2005). A comparative proteomic strategy for subcellular proteome research: ICAT approach coupled with bioinformatics prediction to ascertain rat liver mitochondrial proteins and indication of mitochondrial localization for catalase. *Mol Cell Proteomics* 4: 12–34.

Keller A., Nesvizhskii A.I., Kolker E., Aebersold R. (2002). Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal Chem* 74: 5383–5392.

Keller J.N., Mark R.J., Bruce A.J., Blanc E., Rothstein J.D., Uchida K., Waeg G., Mattson M.P. (1997). 4-Hydroxynonenal, an aldehydic product of membrane lipid peroxidation, impairs glutamate transport and mitochondrial function in synaptosomes. *Neuroscience* 80: 685–696.

Levine R.L., Garland D., Oliver C.N., Amici A., Climent I., Lenz A.G., Ahn B.W., Shaltiel S., Stadtman E.R. (1990). Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* 186: 464–478.

Levine R.L., Williams J.A., Stadtman E.R., Shacter E. (1994). Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol* 233: 346–357.

Liebler D.C., Hansen B.T., Davey S.W., Tiscareno L., Mason D.E. (2002). Peptide sequence motif analysis of tandem MS data with the SALSA algorithm. *Anal Chem* 74: 203–210.

MacCoss M.J., McDonald W.H., Saraf A., Sadygov R., Clark J.M., Tasto J.J., Gould K.L., Wolters D., Washburn M., Weiss A., Clark J.I., Yates J.R. III. (2002). Shotgun identification of protein modifications from protein complexes and lens tissue. *Proc Natl Acad Sci USA* 99: 7900–7905.

Manza L.L., Codreanu S.G., Stamer S.L., Smith D.L., Wells K.S., Roberts R.L., Liebler D.C. (2004). Global shifts in protein sumoylation in response to electrophile and oxidative stress. *Chem Res Toxicol* 17: 1706–1715.

McCormack A.L., Schieltz D.M., Goode B., Yang S., Barnes G., Drubin D., Yates J.R. 3rd. (1997). Direct analysis and identification of proteins in mixtures by LC/MS/MS and database searching at the low-femtomole level. *Anal Chem* 69: 767–776.

Nagele E., Vollmer M., Horth P. (2004). Improved 2D nano-LC/MS for proteomics applications: a comparative analysis using yeast proteome. *J Biomol Tech* 15: 134–143.

Nagele E., Vollmer M., Horth P. (2003). Two-dimensional nano-liquid chromatography–mass spectrometry system for applications in proteomics. *J Chromatogr A* 1009: 197–205.

Nakamura A., Goto S. (1996). Analysis of protein carbonyls with 2,4-dinitrophenyl hydrazine and its antibodies by immunoblot in two-dimensional gel electrophoresis. *J Biochem (Tokyo)* 119: 768–774.

Nesvizhskii A.I., Aebersold R. (2004). Analysis, statistical validation and dissemination of large-scale proteomics datasets generated by tandem MS. *Drug Discov Today* 9: 173–181.

Nesvizhskii A.I., Keller A., Kolker E., Aebersold R. (2003). A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem* 75: 4646–4658.

Nikolsky Y., Ekins S., Nikolskaya T., Bugrim A. (2005). A novel method for generation of signature networks as biomarkers from complex high throughput data. *Toxicol Lett* 158: 20–29.

O'Donovan C., Apweiler R., Bairoch A. (2001). The human proteomics initiative (HPI). *Trends Biotechnol* 19: 178–181.

Peng J., Gygi S.P. (2001). Proteomics: The move to mixtures. *J Mass Spectrom* 36: 1083–1091.

Perkins D.N., Pappin D.J., Creasy D.M., Cottrell J.S. (1999). Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 20: 3551–3567.

Ranish J.A., Yi E.C., Leslie D.M., Purvine S.O., Goodlett D.R., Eng J., Aebersold R. (2003). The study of macromolecular complexes by quantitative proteomics. *Nat Genet* 33: 349–355.

Resing K.A., Meyer-Arendt K., Mendoza A.M., Aveline-Wolf L.D., Jonscher K.R., Pierce K.G., Old W.M., Cheung H.T., Russell S., Wattawa J.L., Goehle G.R., Knight R.D., Ahn N.G. (2004). Improving reproducibility and sensitivity in identifying human proteins by shotgun proteomics. *Anal Chem* 76: 3556–3568.

Sadygov R.G., Liu H., Yates J.R. (2004). Statistical models for protein validation using tandem mass spectral data and protein amino acid sequence databases. *Anal Chem* 76: 1664–1671.

Schiewe A.J., Margol L., Soreghan B.A., Thomas S.N., Yang A.J. (2004). Rapid characterization of amyloid-beta side-chain oxidation by tandem mass spectrometry and the scoring algorithm for spectral analysis. *Pharm Res* 21: 1094–1102.

Shacter E. (2000). Quantification and significance of protein oxidation in biological samples. *Drug Metab Rev* 32: 307–326.

Smith M.A., Sayre L.M., Anderson V.E., Harris P.L., Beal M.F., Kowall N., Perry G. (1998). Cytochemical demonstration of oxidative damage in Alzheimer disease by immunochemical enhancement of the carbonyl reaction with 2,4-dinitrophenylhydrazine. *J Histochem Cytochem* 46: 731–735.

Smith R.D., Anderson G.A., Lipton M.S., Pasa-Tolic L., Shen Y., Conrads T.P., Veenstra T.D., Udseth H.R. (2002). An accurate mass tag strategy for quantitative and high-throughput proteome measurements. *Proteomics* 2: 513–523.

Soreghan B.A., Yang F., Thomas S.N., Hsu J., Yang A.J. (2003). High-throughput proteomic-based identification of oxidatively induced protein carbonylation in mouse brain. *Pharm Res* 20: 1713–1720.

Stadtman E.R. (2001). Protein oxidation in aging and age-related diseases. *Ann NY Acad Sci* 928: 22–38.

Washburn M.P., Wolters D., Yates J.R. III. (2001). Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat Biotechnol* 19: 242–247.

Washburn M.P., Yates J.R. III. (2000). Analysis of the microbial proteome. *Curr Opin Microbiol* 3: 292–297.

Wei Y.H., Lee H.C. (2002). Oxidative stress, mitochondrial DNA mutation, and impairment of antioxidant enzymes in aging. *Exp Biol Med (Maywood)* 227: 671–682.

Wolters D.A., Washburn M.P., Yates J.R. 3rd. (2001). An automated multidimensional protein identification technology for shotgun proteomics. *Anal Chem* 73: 5683–5690.

Wu C.C., MacCoss M.J., Howell K.E., Yates J.R. III. (2003). A method for the comprehensive proteomic analysis of membrane proteins. *Nat Biotechnol* 21: 532–538.

Yates J.R. 3rd, Eng J.K., McCormack A.L., Schieltz D. (1995). Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. *Anal Chem* 67: 1426–1436.

Yi E.C., Marelli M., Lee H., Purvine S.O., Aebersold R., Aitchison J.D., Goodlett D.R. (2002). Approaching complete peroxisome characterization by gas-phase fractionation. *Electrophoresis* 23: 3205–3216.

Yu B.P. (1996). Aging and oxidative stress: modulation by dietary restriction. *Free Radic Biol Med* 21: 651–668.

Zhong S., Storch K.F., Lipan O., Kao M.C., Weitz C.J., Wong W.H. (2004). GoSurfer: A graphical interactive tool for comparative analysis of large gene sets in gene ontologytrade mark space. *Appl Bioinformatics* 3: 261–264.

9

USE OF A PROTEOMIC TECHNIQUE TO IDENTIFY OXIDANT-SENSITIVE THIOL PROTEINS IN CULTURED CELLS

MARK B. HAMPTON, JAMES W. BATY, AND CHRISTINE C. WINTERBOURN

9.1 INTRODUCTION

The oxidation and reduction of cysteine residues in proteins is recognized as an important post-translational signaling mechanism. The key attributes of a good signal transduction pathway are all present: selective targeting of specific cysteine residues, a modification known to alter the structure and/or activity of influential thiol proteins, and a comprehensive network of cellular reductants to quickly convert an oxidized cysteine back to its reduced form. However, in comparison to more recognized post-translational modifications such as phosphorylation, glycosylation, and ubiquitination, cysteine oxidation (including *S*-nitrosylation) appears to have been slower in capturing the attention of the wider scientific community.

Part of the tempered enthusiasm may result from the traditional concept of protein oxidation as a damaging reaction rather than a controlled signaling mechanism. Technology, or lack thereof, is also likely to have played a central role in the slow development of this field. Except for peroxiredoxins (Woo et al., 2003), there is a notable absence of antibodies to proteins with an oxidized cysteine. Compare this to the number of commercially available antibodies directed against

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

proteins with phosphorylated serine, threonine, or tyrosine. It could be argued that the key thiol proteins to warrant attention have not been convincingly identified, but inadequate methodology itself has contributed to this problem. There are no specific inhibitors of cysteine oxidation and reduction, in contrast to selective kinase and phosphatase inhibitors, to test whether a particular modification is important in cell signaling. Also, while there is no shortage of putative targets, global comparisons of thiol protein modification in cells during signaling events are only just beginning. In this chapter we discuss a proteomic technique for measuring oxidized thiols and its use for monitoring changes in oxidatively stressed cells.

9.2 FLUORESCENCE LABELING AND PROTEOMIC ANALYSIS OF OXIDIZED THIOL PROTEINS

Methods for detecting changes in the redox state of cysteine predominantly utilize the ability of iodoacetamide or maleimide derivatives to alkylate reduced thiols. The alkylating agents can be tagged with fluorescent (Wu et al., 1998), biotinylated (Bayer et al., 1987; Kim et al., 2000), or radioactive moieties (Gitler et al., 1997; Denu and Tanner, 1998; Lee et al., 1998). Alkylating agents do not modify oxidized thiols, and protein oxidation can be quantified as a decrease in probe incorporation. The simplest procedure is to label reduced proteins. However, the majority of intracellular thiols are in a reduced form, making it difficult to detect changes in a few proteins within a sea of labeled spots. Also, if a protein has several cysteine residues that are labeled by the probe, and only one cysteine is a target for oxidation, then it will be very difficult to observe decreased labeling.

An alternative is to label reversibly oxidized thiol proteins. Procedures have been developed in which intact cells are disrupted in the presence of *N*-ethylmaleimide (NEM) to rapidly and irreversibly block reduced thiols (Bayer et al., 1987; Gitler et al., 1997). The oxidized thiols are then reduced with dithiothreitol (DTT) and the thiol-reactive probe is added. We adopted this approach, using 5-iodoacetamidofluorescein (IAF) as the probe (Fig. 9.1).

The next part of the process is to visualize the labeled proteins and identify those that change in cells following treatment with oxidants. Labeled proteins can be selectively removed from the extract by using anti-fluorescein antibodies, or streptavidin-agarose in the case of biotinylated probes (Saurin et al., 2004; Sullivan et al., 2000). The isolated proteins are then separated by gel electrophoresis and protein stained. Bands of interest can be excised for identification, and because unlabeled proteins have been removed, 1D separation may be adequate. However, a disadvantage of this method is that unlabeled proteins may be isolated as a result of protein–protein interactions in the extracts. Indeed, Saurin et al. recently discovered two proteins that presented as being labeled with biotinylated maleimide but did not have any cysteine residues (Saurin et al., 2004). An alternative is to dispense with an initial separation and use the labeled probe to visualize modified proteins within the entire extract, using two-dimensional (2D) electrophoresis to maximize protein separation.

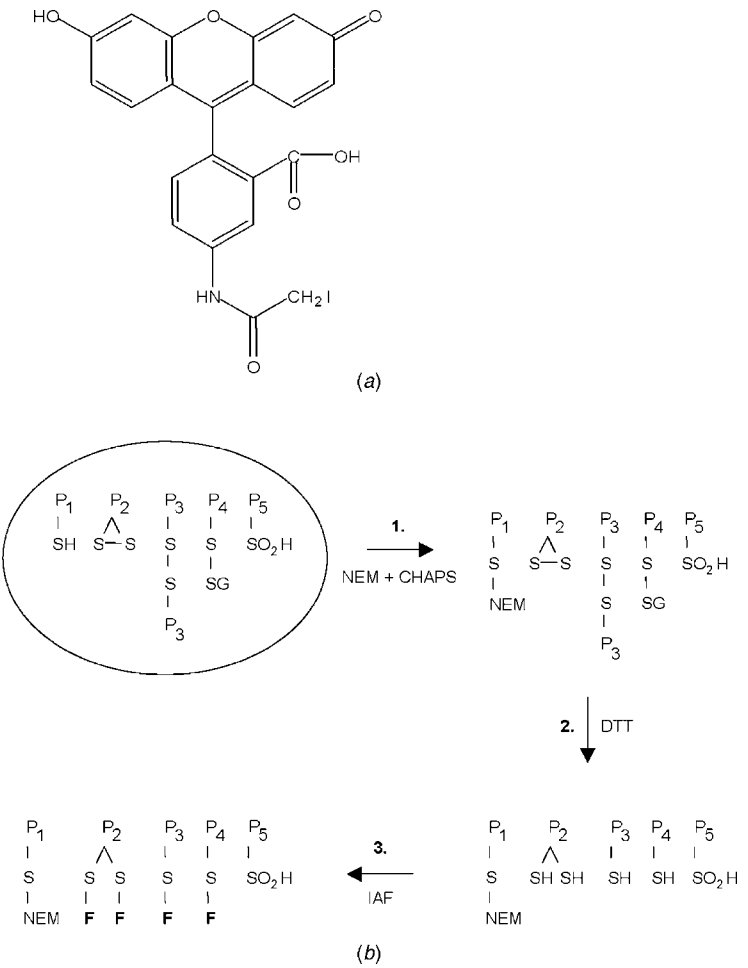


FIGURE 9.1 Labeling of oxidized thiol proteins. (*a*) Structure of 5-iodoacetamidofluorescein (IAF). (*b*) Schematic outline of protocol to label oxidized thiol proteins. P = protein, G = glutathione, and F = fluorescein.

We have developed a proteomic method in which oxidized thiols are labeled with IAF and separated by 2D electrophoresis (Baty et al., 2002). Gels are scanned using a FX Molecular Imager and images analyzed using PDQuest software from Bio-Rad Laboratories (Hercules, CA, USA). IAF-labeled spots are excised for protein identification by MALDI-TOF mass spectrometry of tryptic digests in combination with searching of online databases. Fluorescent spot cutters are ideal for this purpose, as labeled proteins are invisible to the naked eye. Alternatively, gels can be overlaid on a copy of the scanned image printed at the original scale. After spots are excised, the gel is rescanned to ensure that the spot of interest has been removed. A full protocol is presented in the appendix.

9.3 DETECTION OF THIOL PROTEIN OXIDATION IN JURKAT CELLS

To illustrate the capacity of the method to identify changes in cells under oxidative stress, we describe the results of treating Jurkat T lymphoma cells with hydrogen peroxide or with dinitrochlorobenzene (DNCB), which disrupts thiol homeostasis by inactivating thioredoxin reductase and lowering glutathione levels. When unstressed cells are analyzed, approximately 400 proteins show consistent labeling with IAF, indicating the presence of oxidized cysteine residues (Fig. 9.2*a*).

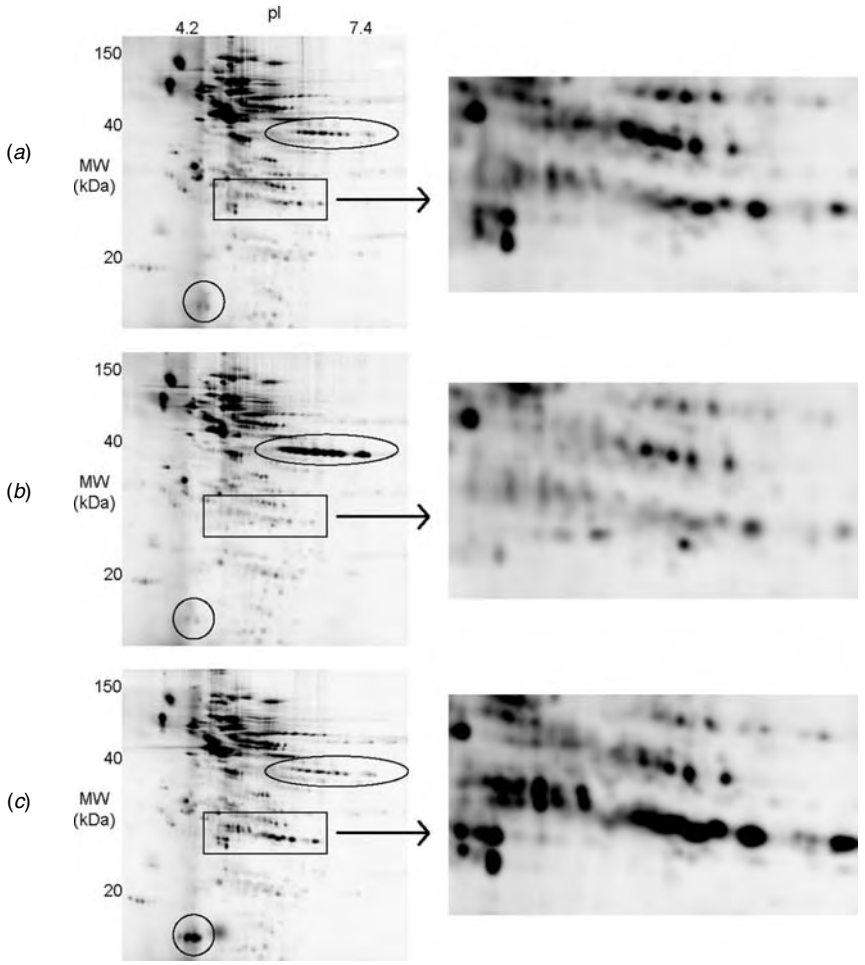


FIGURE 9.2 2D electrophoresis of IAF-labeled thiol proteins from Jurkat cells. (*a*) Untreated cells. (*b*) Treated with 200 μM hydrogen peroxide for 10 min. (*c*) Treated with 30 μM DNCB for 10 min. Enlarged images of the boxed regions are shown adjacent to each gel.

These constitute only a small fraction of the total cysteine residues as IAF-labeled reduced proteins are greatly overexposed when scanned at an identical sensitivity. IAF is an extremely sensitive stain; indeed, a comparison with silver staining of the same gel shows not only a different pattern of labeling but also a number of spots that are detectable only with IAF (Fig. 9.3). Fluorescent stains have a broad linear range for quantification, and a 40,000-fold difference in fluorescence intensity between the darkest and faintest spots can be detected (Fig. 9.3). The main limitation in detecting changes in the redox state of these low abundance proteins is not labeling, rather that they have to be sufficiently separated from the major thiol proteins on the gel.

On treatment of the cells with 200 μM hydrogen peroxide (for a short time to avoid any effects of altered protein expression), a striking feature is that most

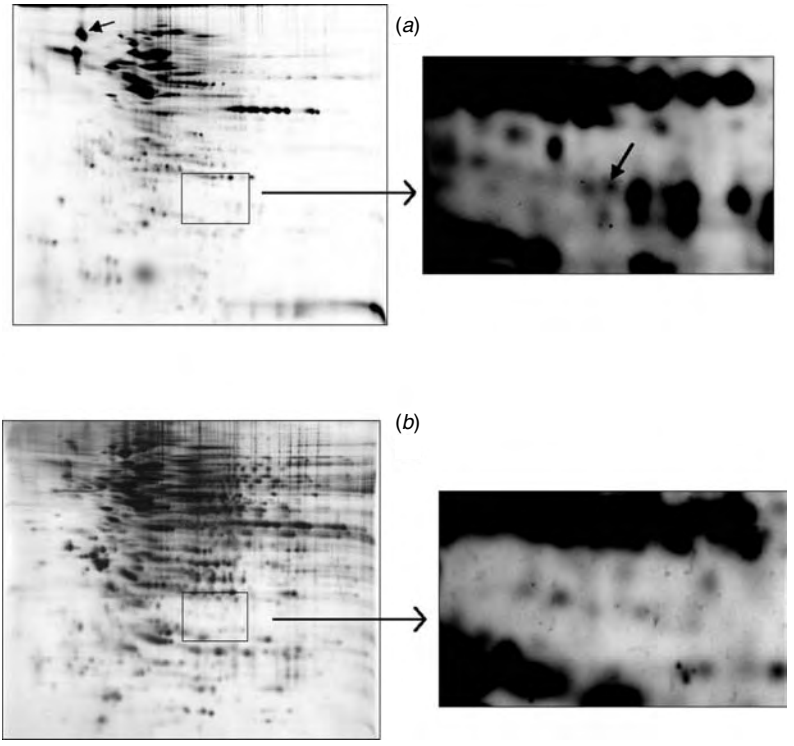


FIGURE 9.3 Comparison of IAF labeling with protein staining. Oxidized thiol proteins from hydrogen peroxide-treated Jurkat cells were labeled with IAF and separated by 2D electrophoresis with 15% SDS-PAGE in the second dimension: (*a*) fluorescence scan; (*b*) silver nitrate staining of the same gel. Enlarged and darkened images of the boxed regions are shown adjacent to each gel. The arrows in panel *a* indicate heavily and faintly labeled spots that show a 40,000 difference in intensity. Panel *b* indicates that many proteins do not have oxidized cysteine residues; however, the zoomed images show that some IAF-labeled spots do not appear on the silver stain.

of the thiol proteins remain unmodified. We have observed a consistent twofold increase or decrease in IAF labeling of 52 spots. The most obvious changes are highlighted in Figure 9.2*b*. In particular, a series of spots of approximately 37 kDa, which have been identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), show a conspicuous increase in intensity, and a cluster of spots in the 25 kDa area, identified as peroxiredoxins, show decreased IAF labeling (Fig. 9.2*b*). All the isoforms of GAPDH are extremely sensitive, with reversible oxidation also observed at 20 μM hydrogen peroxide. The other changes affect proteins with a variety of functions, few of which have been considered to be particularly oxidant sensitive (Baty et al., 2005).

A considerably different pattern of labeling is observed with DNCB. Under these conditions we have detected changes in 42 spots, with only 11 of these also changing with hydrogen peroxide (Baty et al., 2005). No oxidation of GAPDH is seen, and rather than decreasing, the peroxiredoxins substantially increase in intensity, along with thioredoxin spots at 15 kDa (Fig. 9.2*c*).

The increased labeling of these proteins in the presence of DNCB demonstrates that without thioredoxin reductase activity, the cells cannot maintain thioredoxin in its reduced form, which itself is necessary to keep the peroxiredoxins reduced. The accumulation of oxidized thioredoxin within 10 minutes indicates that this redox cycle is active even in unstressed Jurkat cells. The system is robust while the thioredoxin reductase is functional, however, because oxidized thioredoxin does not accumulate in the presence of 200 μM hydrogen peroxide.

9.4 DETECTION OF REVERSIBLE AND IRREVERSIBLE THIOL OXIDATION

Increases in spot intensity represent mild oxidation of a thiol to a disulfide or sulfenic acid that is reducible with DTT. This accounts for many of the changes observed in stressed cells. In theory, the technique should detect only reversibly oxidized proteins and not the more highly oxidized sulfinic ($–SO_2H$) and sulfonic acid ($–SO_3H$) derivatives of cysteine that are not reduced by DTT. However, for the significant number of proteins that show a decrease in IAF labeling following treatment with hydrogen peroxide, the most likely explanation is irreversible oxidation to these forms. The overoxidation phenomenon has been well characterized for peroxiredoxins (Wagner et al., 2002). Irreversible oxidation would not be detectable if a protein went from being completely reduced to irreversibly oxidized since neither form is labeled with IAF in our protocol (Fig. 9.1). However, if some of the protein is already reversibly oxidized without peroxide treatment, then redox cycling and overoxidation would result in decreased labeling (Fig. 9.4).

An alternative explanation for decreased thiol labeling of a spot is that the protein shifts its position on the gel. Oxidation has the capability of modifying the pI of a protein. A reversible oxidation will not do this in our system because any cysteine not blocked with NEM is reduced and reacted with IAF, and neither

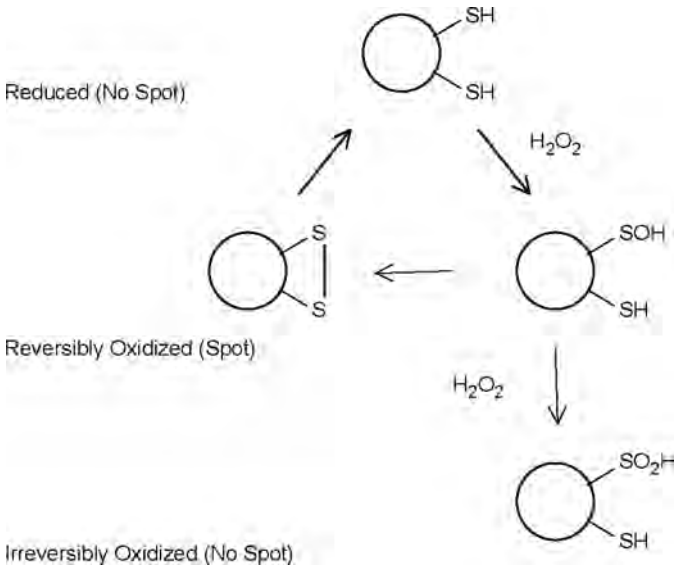


FIGURE 9.4 Schematic diagram showing inter-conversion of reduced, reversibly oxidized, and irreversibly oxidized thiol proteins.

agent alters pI or molecular weight. Overoxidation has been shown to lower the pI of the peroxiredoxins, thereby altering their position on a gel (Wagner et al., 2002; Wood et al., 2003), so the loss of irreversibly oxidized proteins is potentially of combination of protein movement and absence of IAF labeling. Secondary modifications such as phosphorylation or cleavage could also cause a protein to disappear from a 2D gel, and this needs to be considered before it is assumed that irreversible oxidation is occurring. Irreversible oxidation of the peroxiredoxins was confirmed in our system by Western blotting with an antibody to the overoxidized forms (Baty et al., 2005).

9.5 OXIDIZED THIOL COMPARED WITH REDUCED THIOL MEASUREMENTS

An advantage of measuring oxidized thiols as against reduced cell thiols is the improved sensitivity of detecting small increases in oxidation. The initial blocking of reduced thiol proteins minimizes artifacts because of oxidation during sample preparation, which can complicate labeling of reduced thiols. Also NEM inhibits the cysteine-dependent glutathione and thioredoxin pathways that mediate thiol reduction. Targeted thiols are thereby maintained in the oxidized form during extract preparation and labeling.

IAF labeling reveals many cellular thiol proteins with a proportion of their cysteine present in an oxidized form. The majority of these will be structural proteins containing disulfide bonds, and those that undergo redox cycling and exist in

a partially oxidized form. Nonspecific labeling, resulting from either incomplete blocking with NEM or IAF binding to proteins in a cysteine-independent manner, may also be observed. At physiological pH the major protein residues that react with iodoacetamides are cysteines. Reaction with methionine and histidine is possible, albeit at much slower rates. Nonspecific labeling can be detected by adding IAF to NEM-treated extracts without addition of DTT. If this background is of concern, then it may be necessary to decrease the concentration of IAF.

The alternative of labeling reduced thiols has the potential to detect both reversible and irreversible oxidation. However, a limitation is the large number of cysteine residues that are insensitive to oxidation. A change in a sensitive protein may be obscured by heavily stained neighboring spots, or more critically, if a protein with many cysteines has only one that is sensitive, its loss may go undetected. This problem can be partially circumvented by controlling the pH of labeling. The thiolate anion is the species that reacts with iodoacetamide derivatives, and at physiological pH most cysteines are protonated. The proximity of positively charged lysine, arginine, and histidine residues lowers the pK_a, thereby promoting reactivity. Undertaking labeling at neutral or slightly acidic pH provides selectivity for the subset of reactive cysteine residues. However, while cysteines with a lower pK_a are also favored as oxidant targets, additional factors, such as reactive sensor proteins facilitating the oxidation of associated proteins (Delaunay et al., 2002), mean that pK_a is not be the sole predictor of whether a cysteine residue will be sensitive to oxidation in cells.

9.6 MORE SELECTIVE THIOL LABELING PROTOCOLS

Analysis of both the oxidized and reduced thiol proteomes is likely to provide more information on cellular oxidative changes than either method alone. Complementary labeling or separation procedures that focus on specific cysteine modifications are also valuable for probing the mechanism of oxidation. Whereas reduction with DTT enables detection of all forms of reversible cysteine oxidation, arsenite selectively reduces sulfenic acid (Parker and Allison, 1969) and ascorbate reduces *S*-nitrosothiols (Jaffrey et al., 2001; Kashiba-Iwatsuki et al., 1997). Immobilized phenylarsine oxide has been used to select for vicinal thiol proteins prior to labeling (Gitler et al., 1997). Glutathionylation of proteins can be detected by growing cells with labeled cysteine or glutathione ester (Fratelli et al., 2002). A biotinylated form of glutathione has also been used to label glutathionylated proteins (Sullivan et al., 2000), but it is not immediately obvious that the biotin tag has no effect on the redox properties of glutathione or its interaction with the enzymes that mediate disulphide exchange. As an alternative, a modified form of glutaredoxin has been used to reduce and label glutathionylated proteins (Lind et al., 2002). Diagonal 2D electrophoresis enables detection of intermolecular disulphide bonds (Brennan et al., 2004). The first dimension is a nonreducing gel. Proteins with intermolecular disulfides run at a higher molecular weight than they do under reducing conditions in the second dimension and therefore lie above the diagonal.

9.7 IDENTIFICATION OF OXIDANT-SENSITIVE PROTEINS

IAF labeling is a very sensitive tool for detecting oxidant-sensitive proteins. However, identifying these proteins is more challenging. High copy number proteins provide little problem, but for others where clear changes are evident, a major limitation is insufficient protein. With sensitivity greater than for silver staining, recoveries from the gel are often too low for MALDI-TOF analysis. The other potential problem is overlapping proteins that are not labeled with IAF. Both of these problems should be overcome by a combination of cellular fractionation and anti-IAF immunoaffinity to purify and concentrate labeled proteins.

It is important to validate changes identified in a proteomic screen and confirm that a putative target is actually being oxidized, in the same way that real time PCR is used to validate results obtained from microarray analysis. Artifactual changes in spot intensity are possible, particularly for minor proteins, and peptide mass fingerprints obtained by MALDI-TOF may not be definitive. Techniques for monitoring changes to specific thiol proteins in cell extracts utilize the same principles of labeling as described above, but include the use of antibodies to the protein of interest. For example, separation of proteins labeled with biotinylated iodoacetamide, using streptavidin-agarose, into pellet and supernatant fractions followed by Western blotting will enable determination of the ratio of protein in the reduced and oxidized fractions. Immunoprecipitation of the protein of interest has also been undertaken before measurement of probe incorporation (Lee et al., 1998), but this approach is subject to interference by co-precipitating proteins. Probes that alter the electrophoretic properties of proteins have also been used in combination with Western blotting. Maleimide conjugated to polyethyleneglycol increases the molecular weight of a protein (Makmura et al., 2001), and iodoacetic acid decreases the pI of proteins as detected on an isoelectric focusing gel (Thomas et al., 1995). With both of these approaches it is theoretically possible to detect the number of cysteines labeled in any protein by quantal shifts in protein mobility. We have had experience with each of these techniques, which have their own advantages and disadvantages. A common limitation is the ability of the antibody to recognize the labeled protein, and better techniques for selective monitoring of thiol proteins would be valuable.

9.8 CONCLUSIONS AND FUTURE DIRECTIONS

Proteomic analysis of reversibly oxidized thiol proteins has provided insight into the cellular effects of hydrogen peroxide. It has shown that oxidation is quite restricted and in many respects not closely linked to thioredoxin-dependent antioxidant pathways. More work is needed to identify key changes associated with redox signaling. It will be interesting to compare the changes seen with other oxidative stresses and to monitor cells treated with growth factors and cytokines or at different stages of the apoptosis process, where redox changes are implicated.

As well as identifying proteins that are oxidant sensitive, proteomic analysis of thiol oxidation provides a useful benchmark for studies that examine only selected proteins. A number of investigations have used immunological techniques to examine whether a particular cell protein such as a phosphatase is oxidized. In order to appreciate whether such proteins are particularly sensitive, these changes should be related to a global oxidation profile through a combination of the two approaches.

Once a protein has been identified as a redox-signaling target, molecular techniques can be employed. Site-directed mutagenesis can identify the susceptible cysteine in a protein and provide information on how neighboring residues influence susceptibility. It will also be of interest to determine if oxidation occurs directly or is facilitated by a more reactive redox sensor. Expression of the mutant protein will enable assessment of its role in the response of cells to either oxidative stress or signaling molecules proposed to use thiol oxidation as part of their transduction pathways. In the current literature few proteins stand out as warranting such attention. As the new proteomic technologies become more widely adopted, we can look forward to the identification of key targets, and a rapid progression in the field of redox signaling.

APPENDIX: PROTOCOL FOR MEASURING OXIDIZED THIOL PROTEINS IN CELLS BY 2D ELECTROPHORESIS

A. IAF Labeling of Oxidized Thiol Proteins

1. Harvest cells by centrifugation at 1,000 g for 1 min. Wash in phosphate-buffered saline (PBS) and resuspend at 5×10^7 cells/ml in extract buffer (40 mM HEPES, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, complete™ protease inhibitors, pH 7.4) containing 100 mM fresh NEM for 15 min at room temperature.
2. Add CHAPS (1% final), vortex and incubate for 15 min at room temperature.
3. Centrifuge for 5 min at 16,000 g, and pipette 75 μl onto a spin column equilibrated with extract buffer containing 1% CHAPS. Use spin column according to manufacturer instructions.
4. Add DTT (1 mM final) to the sample collected from the column and incubate for 10 min at room temperature. The Bio-Rad *DC* protein assay can be used at this step to measure protein concentration.
5. Add fresh 5-iodoacetamidofluorescein (IAF, stock 10 mM in DMSO) from Molecular Probes (Eugene, OR, USA) to a final concentration of 200 μM, and incubate for 10 min at room temperature in the dark.
6. Put samples through a second spin column equilibrated with sample/rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 0.2% biolytes, bromophenol blue crystals).

B. 2D Electrophoresis of IAF-Labeled Proteins

Electrophoresis is performed according to standard protocols, as outlined briefly below.

1. *IPG strip rehydration and sample absorption.* Pipette the sample/rehydration solution into one side of tray channel that is sitting with a long edge slightly raised, and avoiding the ends of each channel. This ensures that all of the sample gets absorbed into the IPG strip. Using forceps remove an IPG strip from packet, peel plastic backing of IPG strip and place strip gel side down into tray channel on top of the sample. Do this slowly and try to prevent bubbles forming. Apply mineral oil into channel so that strip is submerged to prevent dehydration by evaporation. It is important to place tray on a balanced surface so that there is even absorption along the strips. If the sample is labeled with IAF, wrap tray in tin foil. Passive rehydration takes at least 6 hours for 7 cm strips and overnight for 17 cm strips.
2. *Isoelectric focusing (IEF).* Using forceps, place electrode wicks (4 per strip) onto a paper towel, and pipette 1 drop of milliQ water onto each wick. Blot off excess water. Insert wicks on top of the electrode wires in the focusing try (2 at each end). Take the IPG strip from the rehydration tray and let the excess oil and unabsorbed sample flow off onto a clean paper towel. Place rehydrated strip gel side down in focusing channel with the end labeled positive at the positive end of the tray. Ensure good contact with electrodes by depressing the strip lightly. Overlay the strip with mineral oil and place the lid on the focusing try so that lid pressure tabs press on the IPG strip directly over the electrodes. Strips are focused according to manufacturer instructions. We use 17 cm pH 3–10 Bio-Rad ReadyStrips™ that are focused on a Bio-Rad Protean IEF Cell. The program is 300 V for 1 h, 1,000 V for 1 h, 3,000 V for 1 h, and 6,000 V for 12.5 h. For IAF-labeled samples, cover the entire IEF cell with a box. At the end of the IEF the strips can either be prepared straight away for SDS-PAGE second dimension, or they can be placed in a rehydration tray gel side up, wrapped in tin foil and stored at -80°C.
3. *Equilibration of IPG strips for second dimension SDS-PAGE.* If strips have been frozen, let them thaw for a few minutes before equilibration. Place the strips in rehydration tray channel gel side up. Then pipette equilibration buffer (6 M urea, 0.375 M Tris-HCl pH 8.8, 20% glycerol, 2% SDS and bromophenol blue crystals) on top of the IPG strips (2–3 ml/strip). Incubate on shaker for 10 min, then transfer strips to clean channels and repeat. The final step is to place the IEF strips on top of a pre-poured SDS-PAGE gel, avoiding bubbles forming between strip and gel. Pipette 1 to 2 ml of melted 0.5% agarose over strip to hold it in place and allow to set before running the second dimension.
4. *Scanning of gels.* At the completion of electrophoresis, scan gels. We use a Bio-Rad Molecular Imager® FX (Bio-Rad Laboratories, Hercules, CA,

USA) with an excitation wavelength of 488 nm and an emission/detection wavelength of 530 nm.

LIST OF ABBREVIATIONS

DNCB, dinitrochlorobenzene
DTT, dithiothreitol
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
IAF, 5-iodoacetamidofluorescein
MALDI-TOF, matrix-assisted laser desorption–ionization time of flight
NEM, *N*-ethylmaleimide

REFERENCES

Baty J.W., Hampton M.B., Winterbourn C.C. (2002). Detection of oxidant sensitive thiol proteins by fluorescence labeling and two-dimensional electrophoresis. *Proteomics* 2: 1261–1266.

Baty J.W., Hampton M.B., Winterbourn C.C. (2005). Proteomic detection of hydrogen peroxide-sensitive thiol proteins in Jurkat cells. *Biochem J* 389(Pt 3): 785–795.

Bayer E.A., Safars M., Wilchek M. (1987). Selective labeling of sulfhydryls and disulfides on blot transfers using avidin-biotin technology: Studies on purified proteins and erythrocyte membranes. *Anal Biochem* 161: 262–271.

Brennan J.P., Wait R., Begum S., Bell J.R., Dunn M.J., Eaton P. (2004). Detection and mapping of widespread intermolecular protein disulfide formation during cardiac oxidative stress using proteomics with diagonal electrophoresis. *J Biol Chem* 279: 41352–41360.

Delaunay A., Pflieger D., Barrault M.B., Vinh J., Toledano M.B. (2002). A thiol peroxidase is an H_2O_2 receptor and redox-transducer in gene activation. *Cell* 111: 471–481.

Denu J.M., Tanner K.G. (1998). Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: Evidence for a sulfenic acid intermediate and implications for redox regulation. *Biochemistry* 37: 5633–5642.

Fratelli M., Demol H., Puype M., Casagrande S., Eberini I., Salmona M., Bonetto V., Mengozzi M., Duffieux F., Miclet E., Bachi A., Vandekerckhove J., Gianazza E., Ghezzi P. (2002). Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. *Proc Natl Acad Sci USA* 99: 3505–3510.

Gitler C., Zarmi B., Kalef E. (1997). General method to identify and enrich vicinal thiol proteins in intact cells in the oxidized, disulfide state. *Anal Biochem* 252: 48–55.

Jaffrey S.R., Erdjument-Bromage H., Ferris C.D., Tempst P., Snyder S.H. (2001). Protein *S*-nitrosylation: A physiological signal for neuronal nitric oxide. *Nat Cell Biol* 3: 193–197.

Kashiba-Iwatsuki M., Kitoh K., Kasahara E., Yu H., Nisikawa M., Matsuo M., Inoue M. (1997). Ascorbic acid and reducing agents regulate the fates and functions of *S*-nitrosothiols. *J Biochem (Tokyo)* 122: 1208–1214.

Kim J.R., Yoon H.W., Kwon K.S., Lee S.R., Rhee S.G. (2000). Identification of proteins containing cysteine residues that are sensitive to oxidation by hydrogen peroxide at neutral pH. *Anal Biochem* 283: 214–221.

Lee S., Kwon K., Kim S., Rhee S.G. (1998). Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. *J Biol Chem* 273: 15366–15372.

Lind C., Gerdes R., Hamnell Y., Schuppe-Koistinen I., von Lowenhielm H.B., Holmgren A., Cotgreave I.A. (2002). Identification of *S*-glutathionylated cellular proteins during oxidative stress and constitutive metabolism by affinity purification and proteomic analysis. *Arch Biochem Biophys* 406: 229–240.

Makmura L., Hamann M., Areopagita A., Furuta S., Munoz A., Momand J. (2001). Development of a sensitive assay to detect reversibly oxidized protein cysteine sulfhydryl groups. *Antioxid Redox Signal* 3: 1105–1118.

Parker D.J., Allison W.S. (1969). The mechanism of inactivation of glyceraldehyde 3-phosphate dehydrogenase by tetrathionate, *o*-iodosobenzoate, and iodine monochloride. *J Biol Chem* 244: 180–189.

Saurin A.T., Neubert H., Brennan J.P., Eaton P. (2004). Widespread sulfenic acid formation in tissues in response to hydrogen peroxide. *Proc Natl Acad Sci USA* 101: 17982–17987.

Sullivan D.M., Wehr N.B., Fergusson M.M., Levine R.L., Finkel T. (2000). Identification of oxidant-sensitive proteins: TNF-alpha induces protein glutathiolation. *Biochemistry* 39: 11121–11128.

Thomas J.A., Zhao W., Hendrich S., Haddock P. (1995). Analysis of cells and tissues for *S*-thiolation of specific proteins. *Methods Enzymol* 251: 423–429.

Wagner E., Luche S., Penna L., Chevallet M., Van Dorsselaer A., Leize-Wagner E., Rabilloud T. (2002). A method for detection of overoxidation of cysteines: Peroxiredoxins are oxidized in vivo at the active-site cysteine during oxidative stress. *Biochem J* 366: 777–785.

Woo H.A., Kang S.W., Kim H.K., Yang K.S., Chae H.Z., Rhee S.G. (2003). Reversible oxidation of the active site cysteine of peroxiredoxins to cysteine sulfinic acid: Immunoblot detection with antibodies specific for the hyperoxidized cysteine-containing sequence. *J Biol Chem* 278: 47361–47364.

Wood Z.A., Schroder E., Robin Harris J., Poole L.B. (2003). Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem Sci* 28: 32–40.

Wu Y., Kwon K., Rhee S.G. (1998). Probing cellular protein targets of H_2O_2 with fluorescein-conjugated iodoacetamide and antibodies to fluorescein. *FEBS Lett* 440: 111–115.

10

ICAT (ISOTOPE-CODED AFFINITY TAG) APPROACH TO REDOX PROTEOMICS: IDENTIFICATION AND QUANTIFICATION OF OXIDANT-SENSITIVE PROTEIN THIOLS

MAHADEVAN SETHURAMAN, MARK E. MCCOMB, HUA HUANG, SEQUIN HUANG, TYLER HEIBECK, CATHERINE E. COSTELLO, AND RICHARD A. COHEN

10.1 INTRODUCTION

Generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), as well as their elimination by antioxidant defense mechanisms, is normally tightly regulated. Oxidative or nitrosative stress during aging and disease results from excessive generation of ROS/RNS and/or impaired antioxidant defenses. Regulation of cellular homeostasis through post-translational modification of proteins is one of the major responses to oxidative and nitrosative stress (Stadtman and Berlett, 1998). Proteins containing Cys thiol groups are particularly susceptible to oxidation by free radicals, electrophilic molecules, and nitric oxide donors (Ghezzi and Bonetto, 2003; Cotgreave and Gerdes, 1998). One or more reduced thiol groups are essential for the function of many proteins. Because modifications of reactive Cys thiols can alter the function of proteins whenever thiols are involved in catalysis or modulation of activity, they represent

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

not only a major mechanism of normal cell signaling via *S*-nitrosation (Stamler, 1994) or *S*-glutathiolation (Klatt and Lamas, 2000; Mallis et al., 2002; Adachi et al., 2004a,b) but also a mechanism by which disease and aging interfere with protein function by irreversible thiol oxidation (Kim et al., 2000; Adachi et al., 2004a). Thus it is essential to identify the proteins containing oxidant-sensitive Cys residues, their relative sensitivity to oxidation, and their participation in physiological and pathological cell function. The existing methods for identification of oxidant-sensitive Cys in redox proteomics are based on the reaction of analogues of iodoacetamide (IAM) with free Cys thiols, and the fact that thiol oxidation prevents binding (Kim et al., 2000; Wu et al., 1998; Lee et al., 1998). As a step in the development of a proteomic approach to identify post-translational modification of Cys residues in proteins involved in redox signaling or those affected by disease, this review describes a method to identify and quantify oxidant-sensitive protein thiols by mass spectrometric peptide fingerprinting using a thiol-specific, acid-cleavable isotope-coded affinity tag (ICAT™) reagent (Applied Biosystems, USA).

10.2 OXIDANT-SENSITIVE CYS

Among the amino acids in proteins, Cys thiols are one of those most susceptible to oxidation (Kim et al., 2000). The pK_a value of most protein Cys thiols is approximately 8.5. However, certain Cys residues have lower pK_a values, depending on their local charge environment, and exist as thiolate anions at physiological pH. Because the thiolate anion ($Cys\text{-}S^-$) is more readily oxidized than is the reduced Cys thiol group (Cys-SH) (Winterbourn and Metodiewa, 1999), Cys residues with low pK_a at physiological pH may confer to some proteins the potential for regulation by reactive species such as hydrogen peroxide, nitric oxide and peroxynitrite. Thus, for assessment of the effects of oxidant stress, it is important to identify these proteins and the sites of the sensitive Cys.

10.3 CHALLENGES IN REDOX PROTEOMICS

High-throughput techniques for the analysis of the redox status of individual proteins in complex mixtures are lacking. Identification of oxidant-sensitive Cys in proteins, and analysis of the oxidative post-translational modifications like *S*-glutathiolation, *S*-nitrosation, and irreversible modification of Cys have been a major challenge in redox proteomics. Recently Lind and colleagues developed a technique to identify *S*-glutathiolated proteins during oxidative stress and constitutive metabolism (Lind et al., 2002). The method is based on the specific reduction of mixed disulfides by glutaredoxin, their reaction with the Cys-specific alkylating agent *N*-ethylmaleimide-biotin, affinity purification of biotin-tagged proteins, and identification by mass spectrometry. They identified 43 novel cellular protein substrates for *S*-glutathiolation after treating ECV304 cells with the thiol oxidant, diamide. Many of the proteins detected possess critical Cys

thiols that are likely to be involved in redox regulation of the respective proteins in intact cells. Another approach has been described recently combining ^{35}S labeling of intracellular glutathione pools followed by proteome analysis using nonreducing, two-dimensional electrophoresis and MS finger fingerprinting to identify *S*-glutathiolated proteins in human T cell blasts exposed to oxidative stress induced by diamide or hydrogen peroxide (Fratelli et al., 2002). However, while these methods identify the proteins with oxidatively modified Cys residues, these methods do not identify the Cys residue involved. Identification of the particular Cys residue involved is very important to understand the biological impact of different oxidative modifications on the function of proteins. Moreover the existing methods lack the ability to provide quantitative information about the thiol modifications.

10.4 IODOACETAMIDE-BASED REDOX PROTEOMICS

Analogues of iodoacetamide (IAM), a thiol-specific alkylating agent, react with Cys thiolate anions more readily than with Cys thiols. The existing methods for the identification of oxidant-sensitive Cys are based on the reaction of IAM with free Cys thiols, and the fact that thiol oxidation prevents binding (Kim et al., 2000; Wu et al., 1998; Lee et al., 1998). Shown in the scheme of Figure 10.1 is a protein with one reactive and two nonreactive Cys; the reactive Cys exists as a thiolate anion at physiological pH because it is low pK_a

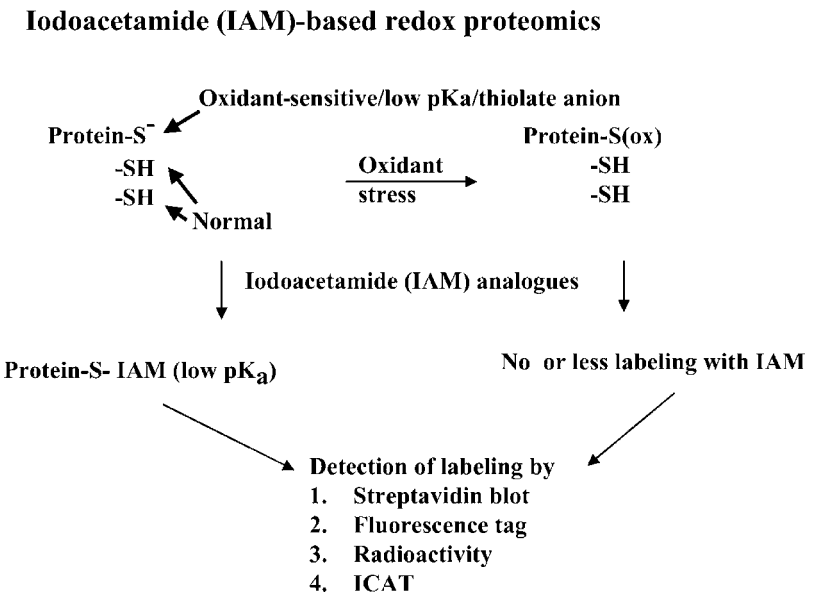


FIGURE 10.1 Diagram of iodoacetamide (IAM) based methods in redox proteomics to identify and quantitate Cys oxidation.

thiol. Because the thiolate anion is prone to oxidation compared to a free thiol group, this reactive Cys is targeted under oxidant stress conditions and modified reversibly (*S*-nitrosation or *S*-glutathiolation) or irreversibly (sulfenyl, sulfinyl, or sulfonyl oxidation). When this protein is subjected to normal conditions and then labeled with iodoacetamide (IAM) analogues, all the thiols would be labeled by IAM. After exposure to oxidant stress that oxidizes the thiolate anion, however, only two thiols would be available for labeling. Thus the extent of thiol oxidation can be monitored by labeling with iodoacetamide analogues, and depending on the iodoacetamide analogue used, various detection methods can be used for monitoring the labeling.

Biotinylated iodoacetamide (BIAM) has been widely used to analyze Cys oxidation by differential labeling of Cys in normal and oxidant stress states followed by streptavidin blotting (Kim et al., 2000). The extent of BIAM labeling can be readily measured by SDS-PAGE and blot analysis of the biotinylated proteins using HRP-conjugated streptavidin and chemiluminescent detection. A recent example showed less BIAM-labeling of creatine kinase following treatment with hydrogen peroxide (1 mM) at pH 7.1 (Fig. 10.2). This indicates that Cys of creatine kinase are oxidized upon treatment with hydrogen peroxide. Wu et al. recently described a procedure to detect proteins with reactive Cys residues in

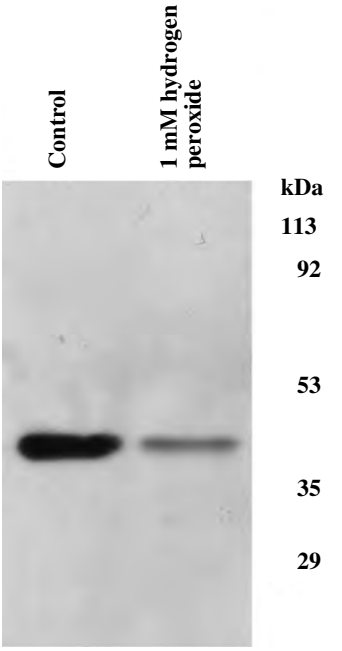


FIGURE 10.2 Effect of hydrogen peroxide (1 mM) on biotinylated iodoacetamide (BIAM) labeling of creatine kinase as assayed by HRP-conjugated streptavidin blot analysis. (Reprinted from Sethuraman et al., 2004a, by the permission of The American Society for Biochemistry and Molecular Biology.)

human A431 cells by using fluorescein-conjugated iodoacetamide (Wu et al., 1998). Two-dimensional SDS-PAGE with fluorescence detection was used to detect labeled proteins, and tyrosine phosphatase 1B was identified by mass spectrometry as one protein whose labeling was changed by intracellular H_2O_2. Radiolabeled iodoacetamide has also been used to quantitate labeling of reactive Cys under different conditions (Lee et al., 1998). As a step toward the identification of reactive protein thiols, we recently developed an approach using an iodoacetamide analogue incorporated into an acid-cleavable isotope-coded affinity tag (cICAT™) reagent (Applied Biosystems, USA) to identify and quantify oxidant-sensitive protein thiols. Proof of principle was achieved using purified creatine kinase (Sethuraman et al., 2004a). A similar approach by stable isotope differential alkylation in combination with mass spectrometric analysis has been used recently to determine the overall level of reversible Cys oxidation (Schilling et al., 2004).

10.5 ICAT APPROACH TO REDOX PROTEOMICS

Isotope-coded affinity tag (ICAT)™ reagents that are iodoacetamide analogues have been used extensively in quantitative proteomics to evaluate the abundance of expressed proteins (Gygi et al., 1999; Hansen et al., 2003) based on tagging free Cys in proteins that are labeled after isolation under strong reducing conditions. We determined the feasibility of using the acid cleavable IAM-based ICAT reagent (Applied Biosystems, Foster City, CA; catalog 4337335) to quantitate the extent of thiol oxidation under nonreducing conditions. When Cys thiols are labeled under these conditions, oxidized or modified Cys will prevent binding of the ICAT reagent. The decrease in labeling of specific oxidant-sensitive Cys can then be quantified from the ratio of ICAT labeling, and the Cys residue can be identified from the LC/MS analysis of the proteolyzed peptides. After exposing equivalent samples of creatine kinase to hydrogen peroxide or control conditions, the side chains of cysteinyl residues in proteins were derivatized with either the light (^{12}C) or heavy (^{13}C) isotope form of the acid-cleavable ICAT reagent (Fig. 10.3). The two samples were mixed and digested with trypsin to generate peptide fragments. The peptides were desalted by subjecting to purification through a strong cation exchange (SCX) column followed by affinity isolation of tagged peptides using an avidin cartridge. The isolated peptides were separated and analyzed by capillary LCMS/MS. The quantitation and sequence identification were carried out with automated multistage MS. The approach was successfully validated for creatine kinase by showing that H_2O_2 (1 mM, 10 min) significantly decreased labeling of a specific oxidant-sensitive Cys.

10.6 VALIDATION OF THE ICAT APPROACH USING THE RECOMBINANT PROTEIN CREATINE KINASE

Creatine kinase, which catalyzes the reversible transfer of the γ-phosphate group of ATP to the guanidine group of creatine, has four Cys residues, among which

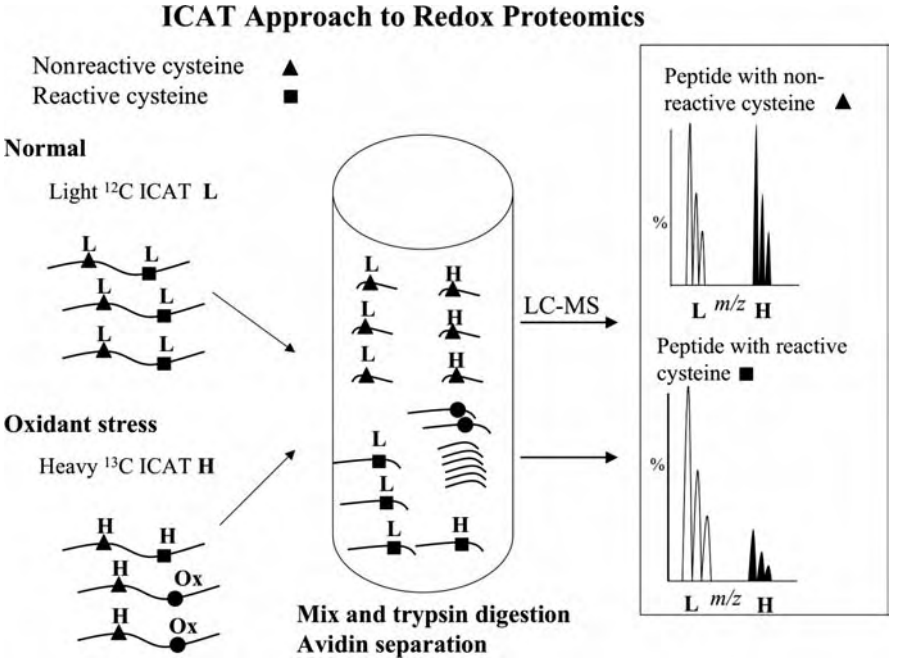


FIGURE 10.3 ICAT approach to redox proteomics identification and quantitation of oxidant-sensitive thiols. Proteins with nonreactive Cys thiols ▲ and reactive Cys thiols ■ are exposed to oxidant stress and normal conditions before labeling with ICAT reagent. Some of the reactive Cys thiols are oxidized, depending on the level of the oxidant stress, and oxidized thiols are designated as ●. Following this, labeling of the free thiols, ▲ and ■, under normal and oxidant stress conditions, is performed with light **L** and heavy **H** ICAT reagent. ICAT-labeled samples are mixed and digested with trypsin, followed by purification by HPLC cation exchange and avidin affinity cartridges (Sethuraman et al., 2004a). Affinity-captured peptides are analyzed by LC-MS and MS/MS. As shown for the reactive Cys, the oxidized Cys thiols are not susceptible to labeling by ICAT reagent, and hence the signal intensity is decreased compared with that of the light-labeled peptide. For nonreactive Cys residues, the peptides in samples prepared under normal and oxidant stress conditions exhibit equivalent signal intensity in MS. From the relative peak intensities of the MS of light and heavy ICAT-labeled peptides, the ratio of oxidized thiols in the samples can be estimated. Identity of the peptide sequences can be derived from MS/MS analysis of these peptides. (Reprinted with modifications from Sethuraman et al., 2004b, by the permission of The American Chemical Society.)

only one, Cys^{283} is known to have a redox-sensitive thiol that plays an important role in regulating catalytic activity of the enzyme (Furter et al., 1993). In this study only this oxidant-sensitive Cys was shown to be oxidized by differential ICAT labeling.

The total ion chromatogram (TIC) for the LC-MS/MS analysis of ICAT-labeled tryptic peptides of creatine kinase is shown in Figure 10.4*a*. The region of the

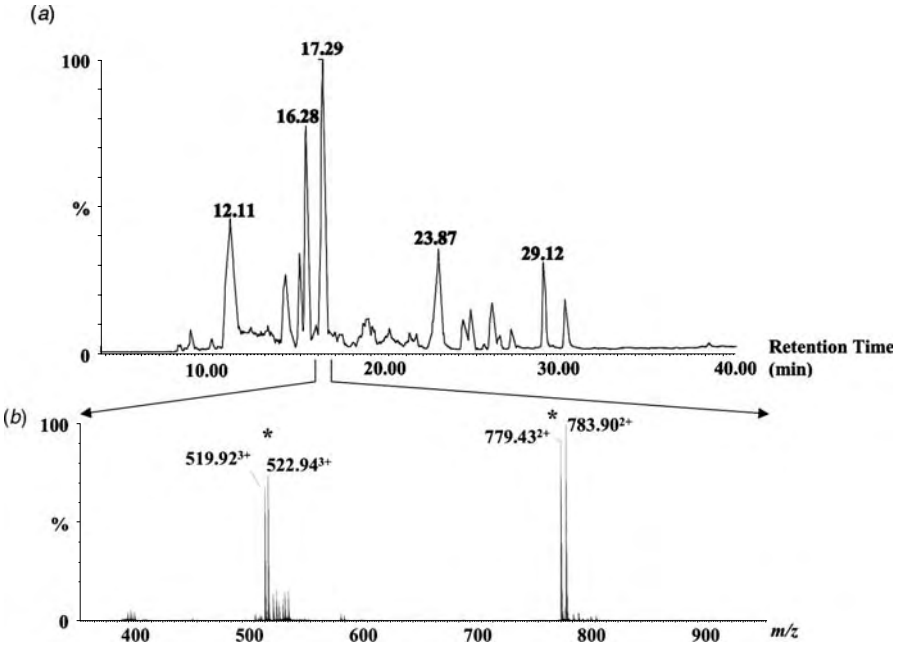


FIGURE 10.4 LC-MS of ICAT-labeled peptides. (*a*) Total ion chromatogram for the LC-ESIMS/MS of the ICAT-labeled tryptic peptides from creatine kinase. The numbers at the top of the peaks refer to the retention time of the molecular ion selected for fragmentation. (*b*) Molecular ion regions in the mass spectra of ions eluted between 16.60 and 17.40 min; the peptides identified as ICAT-labeled peptides are indicated by asterisks. (Reprinted from Sethuraman et al., 2004a, by the permission of American Society for Biochemistry and Molecular Biology.)

summed mass spectrum of peptides eluting between 16.6 and 17.4 min, which contains the signals from the peptide with the redox-sensitive Cys^{283} (*m*/*z* 519.92 and *m*/*z* 522.94, $z = 3$ and *m*/*z* 779.43 and *m*/*z* 783.90, $z = 2$), is shown in Figure 10.4*b*. The ICAT-labeled peptides are indicated by asterisks, and they have the expected *m*/*z* differences of 3 and 4.5, respectively, for the triply and doubly charged ions, as would be expected for the 9 Da mass difference introduced by the 9-^{13}C atoms in the ICAT tag (Hansen et al., 2003). Tandem MS analysis was performed on this peptide, eluting at 16.6 to 17.4 min, which contains the reactive Cys^{283}, and a second peptide that eluted from 11.5 to 12.6 min, which contains the nonreactive Cys^{146}. The molecular ion regions in the mass spectra of these two Cys-containing peptides are shown in Figure 10.5. For the peptide containing Cys^{283}, labeling with ICAT was dramatically reduced after hydrogen peroxide treatment (1 mM, 10 min, Fig. 10.5*a*). As estimated from the peak areas of the single ion chromatogram reconstructed from MS of the peptide, 92% of Cys^{283} was oxidized by hydrogen peroxide. Experiments with varying concentrations of hydrogen peroxide showed that 1 and 10 μM hydrogen

peroxide had no measurable effect on ICAT labeling of Cys^{283}, whereas 100 μM hydrogen peroxide caused a 38% reduction in labeling (Fig. 10.5*a*). For the peptide containing Cys^{146}, the labeling with ICAT was not affected, even by 1 mM hydrogen peroxide, as shown by the similar magnitude of the MS peaks of the light and heavy ICAT-labeled peptides (Fig. 10.5*b*).

The collision-induced dissociation (CID) mass spectrum (Fig. 10.6*a*) recorded from the doubly charged precursor ion with $[M + 2H]^{2+}$ m/z 779.40 was used to confirm the sequence of the peptide containing the redox-sensitive Cys^{283}. The b and y ions in MS^2 showed that this peptide spans residues Val^{280}-Arg^{292} of creatine kinase. As expected, the acid-cleaved ICAT modification adds 227 Da to the mass of the Cys^{283}. This creatine kinase peptide is derived by a chymotrypsin-like digestion at the *C*-terminal tyrosine of the tryptic peptide that spans residues Ala^{267}-Arg^{292}. The tandem mass spectrum shown in Figure 10.6*b* corresponds to the tryptic peptide of creatine kinase that spans residues Gly^{139}-Arg^{148} and contains Cys^{146}.

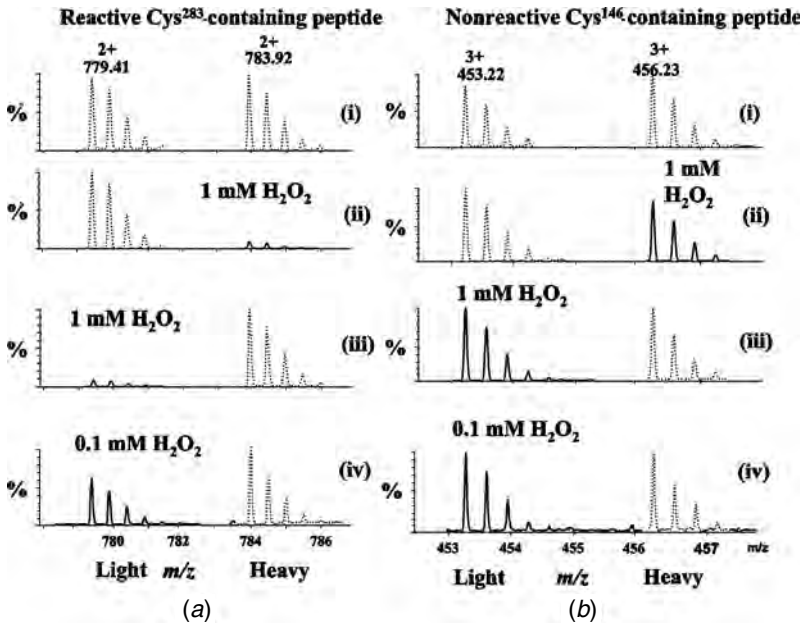


FIGURE 10.5 Effect of hydrogen peroxide on ICAT labeling. (*a*) MS of the ICAT-labeled peptide (peptide 1) containing reactive Cys^{283}. (*b*) The MS of ICAT-labeled peptide 2 containing the nonreactive Cys^{146}. (· · · · · ·) Samples not pretreated with hydrogen peroxide before labeling; (_____) samples pretreated with hydrogen peroxide (0.1 and 1 mM) before labeling. (*i*) No hydrogen peroxide treatment; (*ii*) hydrogen peroxide (1 mM) treated sample labeled with heavy ICAT; (*iii*) hydrogen peroxide (1 mM) treated sample labeled with light ICAT; (*iv*) hydrogen peroxide (0.1 mM) treated sample labeled with light ICAT. (Reprinted from Sethuraman et al., 2004a, by the permission of The American Society for Biochemistry and Molecular Biology.)

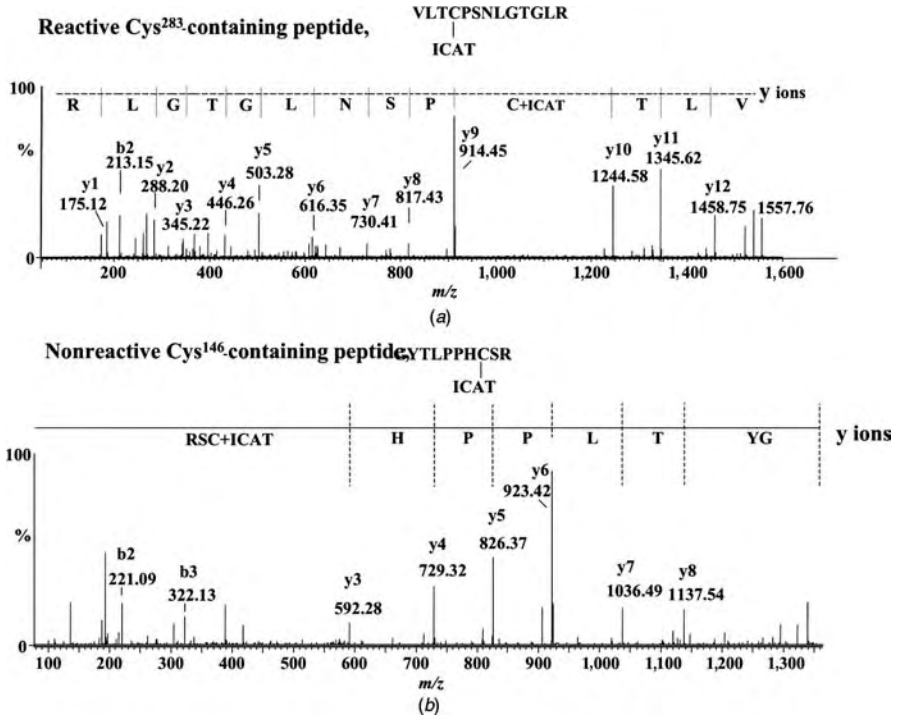


FIGURE 10.6 (*a*) Deconvolved CID MS/MS spectrum of ICAT-labeled peptide $[M + 2H]^{2+} m/z$ 779.41 identified as the creatine kinase peptide spanning residues Val^{280}-Arg^{292} containing Cys^{283} with the reactive thiol. (*b*) Deconvolved CID MS/MS spectrum of ICAT-labeled peptide $[M + 3H]^{3+} m/z$ 453.22 identified as the creatine kinase peptide spanning residues Gly^{139}-Arg^{148} containing Cys^{146} with the nonreactive thiol. (Reprinted from Sethuraman et al., 2004a, by the permission of The American Society for Biochemistry and Molecular Biology.)

10.7 ICAT APPROACH TO THE COMPLEX PROTEIN MIXTURES

This ICAT method has also been successfully applied to complex protein mixtures (Sethuraman et al., 2004b). A membrane particulate fraction obtained from rabbit heart by sucrose gradient ultracentrifugation was dialyzed in Tris buffer (pH 7.1) and treated with hydrogen peroxide (10 mM). Control samples and those treated with hydrogen peroxide were subjected to light or heavy ICAT labeling, respectively, and mixed. The labeled proteins were digested with trypsin, and the tryptic peptides were subjected to strong cation exchange (SCX) chromatography by using a KCl (0–350 mM) step gradient (Alpert, 1990). Four chromatographic fractions were subjected to avidin affinity purification to capture the ICAT-labeled peptides followed by acid cleavage of the biotin linker, and the peptides were subjected to LC-MS/MS.

The TIC for the LC-MS/MS analysis of ICAT-labeled tryptic peptides from one cation exchange fraction is shown in Figure 10.7*a*. Selected regions of the mass spectra of the ions eluting between 16.3 and 16.8 (i) and between 31.5 and 32.0 min (ii) are shown in Figure 10.7*b* and 7*d*, respectively. The region of the mass spectrum around *m/z* 614 (see the insert in Fig. 10.7b) shows the signal from a pair of ICAT-labeled peptides from the same sample, corresponding to a doubly charged, light and heavy ICAT-labeled pair of peptides. From the LC-MS/MS of the precursor ion (Fig. 10.7*c*), the peptide was identified as being derived from sarcomeric creatine kinase, which is an isoform of the cytosolic enzyme. This peptide, spanning residues Gly^{134}-Arg^{143}, contains Cys^{141}, and it was labeled equally by light and heavy ICAT, despite exposure to hydrogen peroxide prior to labeling with heavy ICAT. In the insert in Figure 10.7*d*, the doubly charged peptide pair exhibits a 42% decrease in labeling by the heavy ICAT as calculated from the area of the relevant reconstructed single ion chromatogram. From the LC-MS/MS spectrum of the precursor ion (Fig. 10.7*e*), the peptide was also identified as originating from sarcomeric creatine kinase,

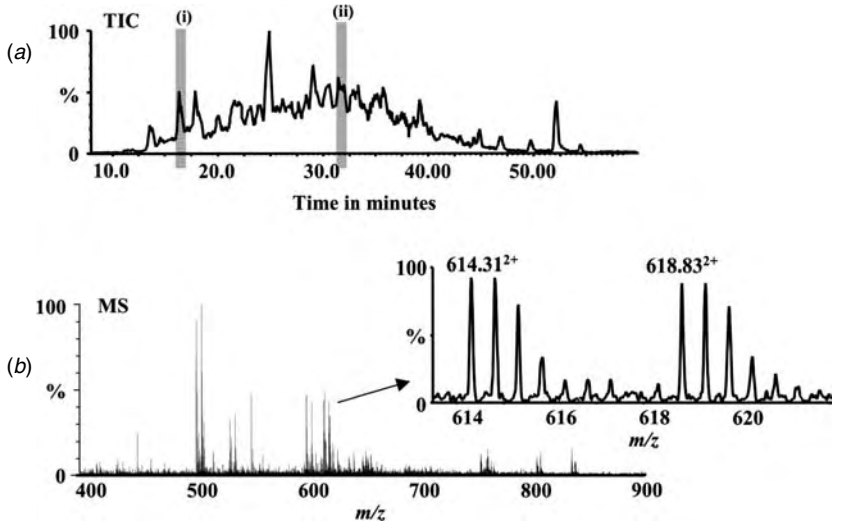


FIGURE 10.7 LC-MS/MS of ICAT-labeled tryptic peptides. (*a*) Total ion chromatogram (TIC) for LC-ESI-MS/MS of ICAT-labeled tryptic peptides from HPLC cation exchange fraction 2 of the membrane protein mixture. (*b*) The *m/z* 400–900 region of the mass spectrum of the peptides eluting between 16.3 and 16.8 min. *Inset*: Expanded view of the mass spectrum around *m/z* 614. (*c*) LC-MS/MS spectrum of $[M + 2H]^{2+}$ *m/z* 614.31 corresponding to the peptide (Gly^{134}-Arg^{143}) of sarcomeric creatine kinase containing the nonreactive Cys^{141}. (*d*) The *m/z* 400–1,250 region of the mass spectrum of the peptides eluting between 31.5 and 32.0 min. *Inset*: Expanded view of the mass spectrum of the peptides around *m/z* 953. (*e*) LC-MS/MS of $[M + 2H]^{2+}$ *m/z* 953.08 corresponding to the peptide (Leu^{272}-Arg^{287}) of sarcomeric creatine kinase containing the reactive Cys^{278}. (Reprinted from Sethuraman et al., 2004b, by the permission of The American Chemical Society.)

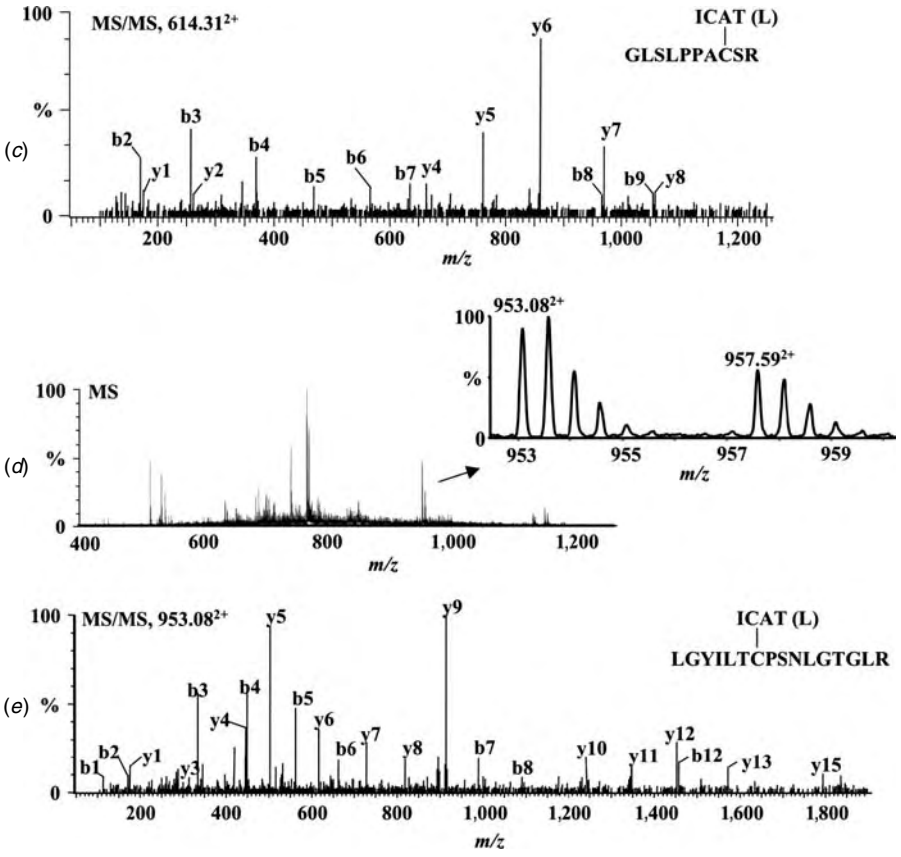


FIGURE 10.7 (*continued*)

spanning residues Leu^{272}-Arg^{287}, and containing the ICAT-labeled, Cys^{278}. This Cys residue is homologous to the reactive Cys^{283} in the cytosolic form of creatine kinase that was oxidized by hydrogen peroxide, as we showed above in the recombinant protein. Earlier studies have indicated that oxidation of the reactive Cys residue inactivates the enzyme (Kim et al., 2000; Wendt et al., 2003). In the mass spectra from a control protein sample that was not treated with hydrogen peroxide, there was no change in the relative abundance of the signal from the heavy ICAT-labeled peptide containing this Cys residue (Fig. 10.8).

Table 10.1 lists the oxidant-sensitive and -insensitive Cys containing peptides and their respective proteins identified from the membrane protein mixture, as well as the apparent extent of oxidation of the Cys residues. Among the additional proteins identified was glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key enzyme in glycolysis (Fig. 10.9*a*). Cys^{149} in GAPDH has been identified as an oxidant-sensitive thiol that is *S*-nitrosated by nitric oxide and regulates enzyme activity (Souza and Radi, 1998). We also identified proteins

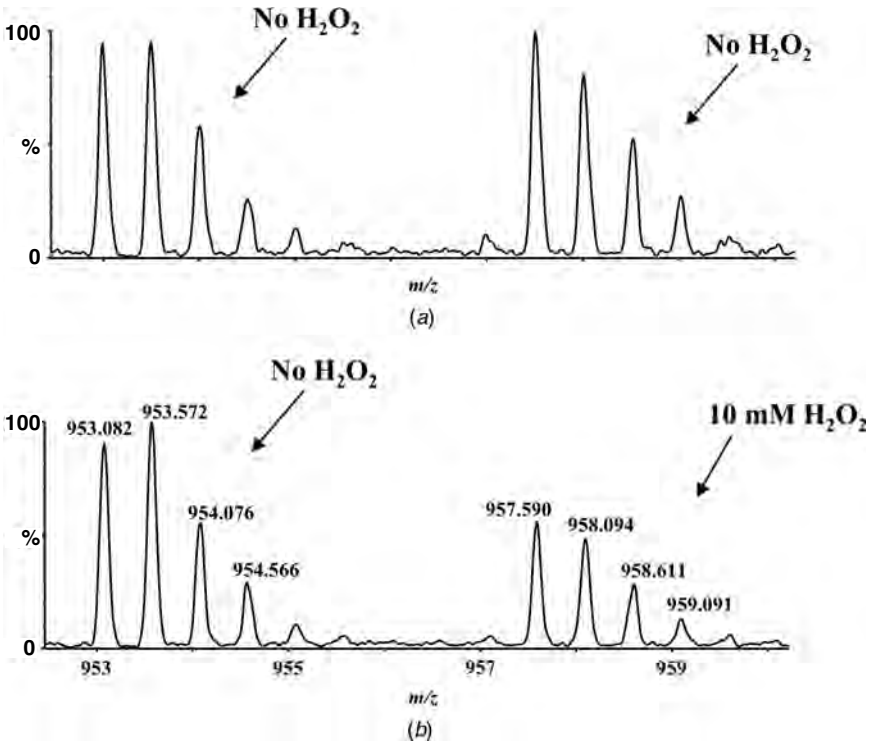


FIGURE 10.8 Effect of hydrogen peroxide on ICAT labeling. MS of the ICAT-labeled peptide pair (Leu^{272}-Arg^{287}) of sarcomeric creatine kinase containing reactive Cys^{278} from rabbit heart particulate fraction in which the heavy ICAT-labeled sample was treated with (*a*) 0 mM or (*b*) 10 mM hydrogen peroxide. (Reprinted from Sethuraman et al., 2004b, by the permission of The American Chemical Society.)

with novel oxidant-sensitive Cys residues. Among them is Cys^{838} in the β-subunit of phosphorylase-B kinase (Fig. 10.9*b*). This protein has been identified as a target of *S*-glutathiolation, although the Cys residues involved have not been identified (Fratelli et al., 2002). Peptides from voltage-dependent anion selective channels (VDAC) 1, 2, and 3 (Fig. 10.9*c*) were identified and found to contain Cys that were oxidized by between 45% and 55%. VDAC proteins play an essential role in cellular metabolism and are part of the mitochondrial permeability transition pore complex important in apoptosis (Liberatori et al., 2004). Cys oxidation has not previously been reported to regulate the activity of VDAC proteins, although oxidants are known to regulate apoptosis at multiple steps. Interestingly, likely because the heart was not flushed of blood prior to homogenization, we found hemoglobin peptides and identified Cys^{93} of the hemoglobin β chain (Fig. 10.9*d*), which was oxidized 49% by hydrogen peroxide. This Cys residue forms bioactive nitrosothiols when nitric oxide is transferred from the heme iron (Jia et al., 1996; Xu et al., 2003).

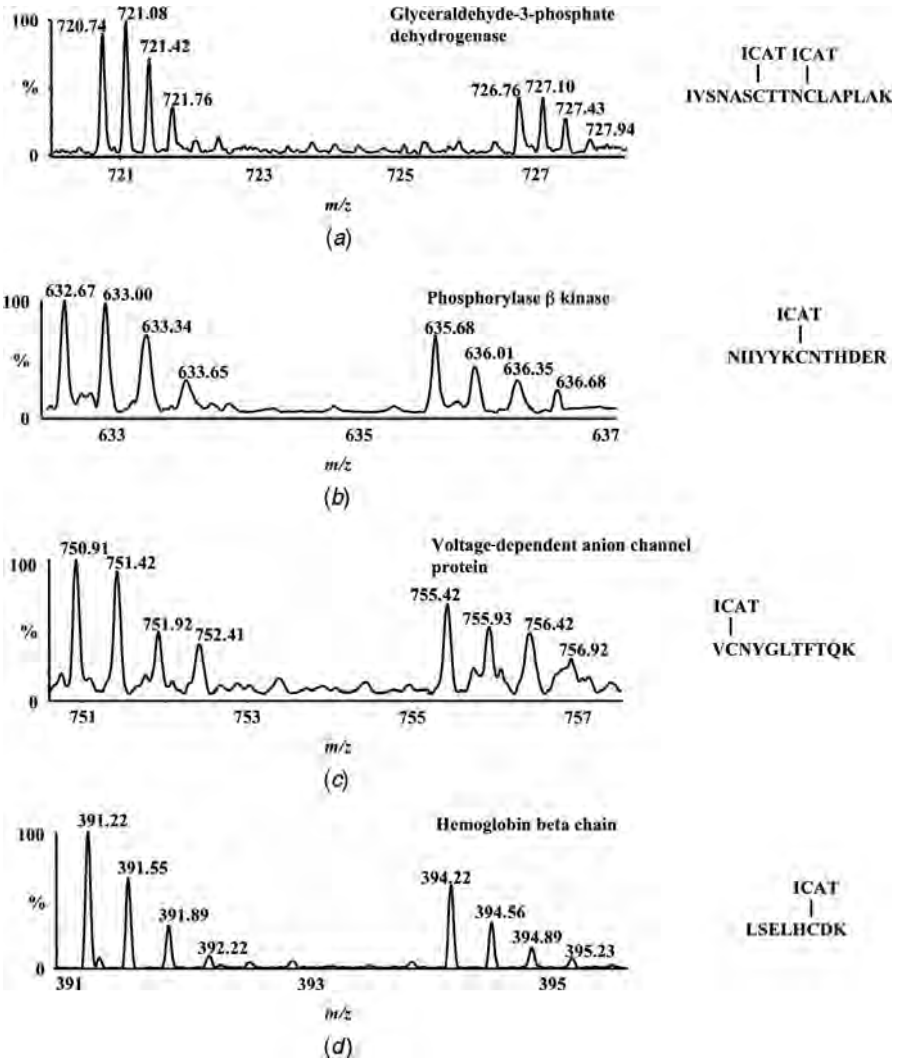


FIGURE 10.9 Identification and quantitation of oxidant-sensitive Cys thiols in complex protein mixtures. Mass spectra of heavy (labeled after oxidation of the protein mixture with 10 mM hydrogen peroxide) and light (no hydrogen peroxide treatment) ICAT-labeled peptide pairs containing oxidant-sensitive Cys of glyceraldehyde-3-phosphate dehydrogenase (*a*), phosphorylase ß kinase (*b*), voltage-dependent anion channel protein 3 (*c*), and hemoglobin ß chain (*d*). The extent of oxidation of Cys in various proteins in the rabbit heart particulate fraction was quantified from the change in intensity of the MS peak of the heavy ICAT-labeled peptide from the hydrogen peroxide-treated sample and is listed in Table 10.1. (Reprinted from Sethuraman et al., 2004b, by the permission of The American Chemical Society.)

TABLE 10.1 Identification and Quantification of Cys Oxidation in Rabbit Heart Particulate Membranes

Name	Description	Start	End	Sequence	%Cys Oxidized[a]
KCRS_RABIT	Creatine kinase sarcomeric mitochondrial precursor	134	143	GLSLPPACSR	9.8
		272	287	LGYILTCPSNLGTGLR	42.3
G3P_RABIT	Glyceraldehyde phosphate dehydrogenase	232	245	VPTPNVSVVDLTCR	26.9
		143	159	IVSNASCTTNCLAPLAK	72.2
ALFA_RABIT	Fructose biphosphate aldolase	69	86	VNPCIGGVILFHETLYQK	12.3
		201	207	CQYVTEK	24.3
		331	341	ALANSLACQGK	24.1
ATA2_RABIT	Sarcoplasmic endoplasmic reticulum Ca2+ ATPase	437	451	VGEATETALTCLVEK	32.5
		372	397	VDGDTCSLNEFTITGSTYAPIGEVHK	15.1
		468	476	ANACNSVIK	28.6
PIGR_RABIT	Polymeric immunoglobulin receptor precursor	353	374	SPPVLKGFPGGSVTIRCPYNPK	24.0
		587	595	ARCPVPRRR	99.6
		595	612	RQWYPLSRKLRTSCPEPR	13.7
KPBB_RABIT	Phosphorylase B kinase beta regulatory chain	276	287	QTLCSLLPRESR	< 5.0
		832	844	NIIYYKCNTHDER	62.3
K6PF_RABIT	Phosphofructose kinase muscle type	108	128	GITNLCVIGGDGSLTGADTFR	< 5.0
		630	648	CNENYTTDFIFNLYSEEGK	48.0
POR1_RABIT	Voltage-dependent anion-selective channel protein 1	224	235	YQIDPDACFSAK	45.6
		120	138	EHINLGCDVDFDIAGPSIR	30.1
POR2_RABIT	Voltage-dependent anion-selective channel protein 2	46	64	SCSGVEFSTSGSSNTDTGK	54.4

POR3_RABIT	Voltage-dependent anion-selective channel protein 3	64	74	VCNYGLTFTQK	48.6
LCAT_RABIT	Phosphatidylcholine-sterol acyltransferase [Precursor]	64	77	LDKPSVVNWMCYRK	43.9
RETB_RABIT	Plasma retinol binding protein	172	181	QRQEELCLSR	28.1
FA10_RABIT	Coagulation factor	334	344	RNVAPACLPQK	39.0
HBB_RABIT	Hemoglobin beta 1 chain	88	95	LSELHCDK	49.6
PRTS_RABIT	Vitamin K dependent protein S precurson fragment	442	452	QKKHCLVTVEK	89.6
MDHM_RAT[b]	Malate dehydrogenase	92	104	GCDVVVIPAGVPR	26.0
DHSA_HUMAN[b]	Succinate dehydrogenase mitochondrial	233	246	GVIALCIEDGSIHR	26.7
NUFM_RAT[b]	NADH-ubiquinone oxidoreductase	7	22	TTGLVGLAVCDTPHER	31.5

[a]The precision of the peak area measurement was estimated by measuring the MS peak areas for 15 pairs of peptides in an experiment in which light and heavy ICAT labeling was performed in identical samples without treating either with hydrogen peroxide. A 95% confidence interval (2 times the SD) was calculated for the change in peak area for each peptide pair. This value equaled ±13%. In twice-repeated experiments, 85% of peptides were detected in both experiments.
[b]Proteins found in subsequent search of Swiss-Prot database.

Source: Reprinted from Sethuraman et al. (2004b) by permission of The American Chemical Society.

10.8 PERSPECTIVES

Because the redox proteomic technique acquires information on ICAT-labeled peptides regardless of the extent of oxidation, the prevalence of oxidant-sensitive Cys among the total number of Cys residues detected was considered (Fig. 10.10). Only 10% of the thiols in Cys-containing peptides were oxidized by more than 50%, and only 5% were oxidized by more than 70%. Thus, of the total Cys detected, those sensitive to the high concentrations of H_2O_2 to which they were exposed are relatively rare, suggesting specificity and functional relevance of oxidant-sensitive Cys.

This ICAT approach is superior in some respects to previously described methods for redox proteomics in which fluorescein-conjugated iodoacetamide (Wu et al., 1998) or [^{3}H]iodoacetic acid (Lee et al., 1998) were used, because (1) gel separation of proteins is not required and (2) simultaneous identification of the peptide/protein and quantitation of the degree of Cys oxidation of the involved Cys residue is accomplished. Proteomic methods using gel separation and mass spectrometric protein identification (Lind et al., 2002; Fratelli et al., 2002; Martinez-Ruiz and Lamas, 2004) have been used to identify potential *S*-glutathiolated or *S*-nitrosated proteins but generally have not enabled identification of the Cys residue involved.

By knowing which proteins and, within these, which Cys residues are susceptible to oxidation, this ICAT-based proteomic approach will provide the opportunity to understand the relationship between oxidant sensitivity of particular Cys residues and their participation in physiological, reversible oxidative post-translational modifications, such as *S*-nitrosation and *S*-glutathiolation, as well as their irreversible oxidation by disease. Because there is variable reactivity of Cys

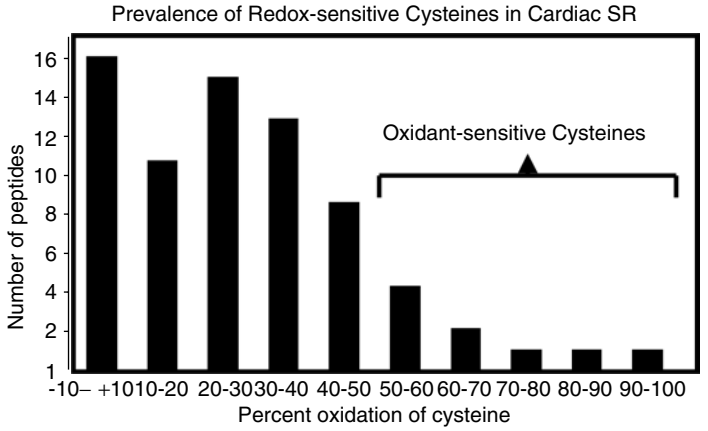


FIGURE 10.10 Prevalence of redox-sensitive Cys in cardiac sarcoplasmic reticulum membrane fraction.

to oxidants, it is not unexpected that specific Cys residues will be involved in oxidant signaling. Proteomic studies will enable identification of other proteins and the Cys within them that are susceptible to oxidation in a particular normal sample, as well as degree to which the Cys residues are oxidized by oxidant stresses and disease. Additionally we intend to use this approach to further understand the relationship between redox sensitivity of Cys and physiologically significant post-translational modifications such as *S*-glutathiolation and *S*-nitrosation. Examples of this type of functional regulation of proteins include specific reactive Cys residues in the small GTPase, Ras (Adachi et al., 2004b), and the sarcoplasmic reticulum calcium ATPase (Adachi et al., 2004a) that are *S*-glutathiolated and regulate functional activity of the proteins. Moreover the most reactive Cys residues in proteins may be those most susceptible to irreversible oxidization in disease states, and thereby prevent their physiological regulation, as was recently demonstrated for the sarcoplasmic reticulum calcium ATPase in atherosclerotic arteries (Adachi et al., 2004a). Finally, this method addresses a potential vulnerability in the usual use of the ICAT approach. That is, if Cys thiols are irreversibly oxidized in an experimental sample and the peptides contain these residues are selected for quantitation, then ICAT analysis may falsely indicate a decrease in protein abundance. Knowledge of which proteins in a normal sample have Cys that are susceptible to oxidant modifications should help investigators to avoid this misinterpretation when control and diseased samples are analyzed.

LIST OF ABBREVIATIONS

BIAM, biotinylated IAM
CID, collision induced dissociation
Cys, cysteine
ESI, electrospray ionization
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
HPLC, high-performance LC
HRP, horseradish peroxidase
IAM, iodoacetamide
ICAT, isotope-coded affinity tag
LC, liquid chromatography
MS, mass spectrometry
MS/MS, tandem MS
RNS, reactive nitrogen species
ROS, reactive oxygen species
SCX, strong cation exchange
SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis
TIC, total ion chromatogram
VDAC, voltage-dependent anion-selective channels

ACKNOWLEDGMENTS

This project has been funded in whole or in part with funds from the National Institutes of Health, the National Heart, Lung, and Blood Institute sponsored Boston University Cardiovascular Proteomics Center (Contract HHSN 268200248178C) and the National Center for Research Resources (Grant P41 RR10888). MS was supported by NIH Postdoctoral Research Fellowship T32 HL07224. TH was supported by NIH predoctoral Research Fellowship T32 HL007969.

REFERENCES

Adachi T., Weisbrod R.M., Pimentel D.R., Ying J., Sharov V.S., Schoneich C., Cohen R.A. (2004a). *S*-Glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. *Nat Medicine* 10: 1200–1207.

Adachi T., Pimentel D.R., Heibeck T., Hou X., Lee Y.J., Jiang B., Ido Y., Cohen R.A. (2004b). *S*-Glutathiolation of Ras mediates redox-sensitive signaling by angiotensin II in vascular smooth muscle cells. *J Biol Chem* 279: 29857–29862.

Alpert A.J. (1990). Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. *J Chromatogr* 499: 177–196.

Cotgreave I.A., Gerdes G.R. (1998). Recent trends in glutathione biochemistry—Glutathione–protein interactions: A molecular link between oxidative stress and cell proliferation? *Biochem Biophys Res Commun* 242: 1–9.

Fratelli M., Demol H., Puype M., Casagrande S., Eberini I., Salmona M., Bonetto V., Mengozzi M., Duffieux F., Miclet E., Bachi A., Vandekerckhove J., Gianazza E., Ghezzi P. (2002). Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. *Proc Natl Acad Sci USA* 99: 3505–3510.

Furter R., Furter-Graves E.M., Wallimann T. (1993). Creatine kinase: The reactive Cys is required for synergism but is nonessential for catalysis. *Biochemistry* 32: 7022–7029.

Ghezzi P., Bonetto V. (2003). Redox proteomics: Identification of oxidatively modified proteins. *Proteomics* 3: 1145–1153.

Gygi P.S., Rist G., Gerber S.A., Turecek F., Gelb M.H., Aebersold R. (1999). Quantitative analysis of complex protein mixtures using isotope coded affinity tags. *Nature Biotech* 17: 994–997.

Hansen K.C., Schmitt-Ulms G., Chalkley R.J., Hirsch J., Baldwin M.A., Burlingame A.L. (2003). Mass spectrometric analysis of protein mixtures at low levels using cleavable ^{13}C-isotope-coded affinity tag and multidimensional chromatography. *Mol Cell Proteomics* 2: 299–314.

Jia L., Bonaventura C., Bonaventura J., Stamler J.S. (1996). *S*-Nitroso-haemoglobin: A dynamic activity of blood involved in vascular control. *Nature* 6571: 221–226.

Kim J.R., Yoon H.W., Kwon K.S., Lee S.R., Rhee S.G. (2000). Identification of proteins containing Cys residues that are sensitive to oxidation by hydrogen peroxide at neutral pH. *Anal Biochem* 283: 214–221.

Klatt P., Lamas S. (2000). Regulation of protein function by *S*-glutathiolation in response to oxidative and nitrosative stress. *Eur J Biochem* 267: 4928–4944.

Lee S.R., Kwon K.S., Kim S.R., Rhee S.G. (1998). Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. *J Biol Chem* 273: 15366–15372.

Liberatori S., Canas B., Tani C., Bini L., Buonocore G., Zimmermann J.G., Mishra O.P., Papadopoulos M.D., Bracci R., Pallini V. (2004). Proteomic approach to the identification of voltage-dependent anion channel protein isoforms in guinea pig brain synaptosomes. *Proteomics* 4: 1335–1340.

Lind C., Gerdes R., Hamnell Y., Koistinen I.S., von Lowenhielm H.B., Holmgren A., Cotgreave I.A. (2002). Identification of *S*-glutathionylated cellular proteins during oxidative stress and constitutive metabolism by affinity purification and proteomic analysis *Arch Biochem Biophys* 406: 229–240.

Mallis R.J., Hamann M.J., Zhao W., Zhang T., Hendrich S., Thomas J.A. (2002). Irreversible thiol oxidation in carbonic anhydrase III: Protection by *S*-glutathiolation and detection in aging rats. *Biol Chem* 383: 649–662.

Martinez-Ruiz A., Lamas S. (2004). Detection and proteomic identification of *S*-nitrosylated proteins in endothelial cells. *Arch Biochem Biophys* 423: 192–199.

Schilling B., Yoo C.B., Collins C.J., Gibson B.W. (2004). Determining Cys oxidation status using differential alkylation. *Int J Mass Spectrometry* 236: 117–127.

Sethuraman M., McComb M.E., Heibeck T., Costello C.E., Cohen R.A. (2004a). Isotope-coded affinity tag approach to identify and quantify oxidant-sensitive protein thiols. *Mol Cell Proteomics* 3: 273–278.

Sethuraman M., McComb M.E., Huang H., Huang S., Heibeck T., Costello C.E., Cohen R.A. (2004b). Isotope-coded affinity tag approach to redox proteomics: Identification and quantitation of oxidant sensitive Cys thiols in complex protein mixtures. *J Proteome Res* 3: 1228–1233.

Souza J.M., Radi R. (1998). Glyceraldehyde-3-phosphate dehydrogenase inactivation by peroxynitrite. *Arch Biochem Biophys* 360: 187–194.

Stadtman E.R., Berlett G.S. (1998). Reactive oxygen-mediated protein oxidation in aging and disease. *Drug Metab Rev* 30: 225–243.

Stamler J.S. (1994). Redox signaling: Nitrosylation and related target interactions of nitric oxide. *Cell* 78: 931–936.

Wendt S., Schlattner U., Wallimann T. (2003). Differential effects of peroxynitrite on human mitochondrial creatine kinase isoenzymes: Inactivation, octamer destabilization, and identification of involved residues. *J Biol Chem* 278: 1125–1130.

Winterbourn C.C., Metodiewa D. (1999). Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide. *Free Radic Biol Med* 27: 322–328.

Wu Y., Kwon K.S., Rhee S.G. (1998). Probing cellular protein targets of hydrogen peroxide with fluorescein-conjugated iodoacetamide and antibodies to fluorescein. *FEBS Lett* 440: 111–115.

Xu X., Cho M., Spencer N.Y., Patel N., Huang Z., Shields H., King S.B., Gladwin M.T., Hogg N., Kim-Shapiro D.B. (2003). Measurements of nitric oxide on the heme iron and -93 thiol of human hemoglobin during cycles of oxygenation and deoxygenation. *Proc Natl Acad Sci USA* 100: 11303–11308.

11

QUANTITATIVE DETERMINATION OF FREE AND PROTEIN-ASSOCIATED 3-NITROTYROSINE AND *S*-NITROSOTHIOLS IN THE CIRCULATION BY MASS SPECTROMETRY AND OTHER METHODOLOGIES: A CRITICAL REVIEW AND DISCUSSION FROM THE ANALYTICAL AND REVIEW POINT OF VIEW

DIMITRIOS TSIKAS

11.1 INTRODUCTION

11.1.1 The L-Arginine/Nitric Oxide Pathway as a Producer of Reactive Nitrogen Species

The unique oxidation of one N atom of the guanidine group of L-arginine by constitutive and inducible nitric oxide synthases (NOS; EC 1.14.13.39), which practically occurs in all types of cell (Marletta, 1993; Förstermann et al., 1994; Andrew and Mayer, 1999), produces the small, free-diffusable, radical, bioactive gas nitric oxide ($^{\bullet}$NO) (Hibbs et al., 1987; Ignarro et al., 1987; Iyengar et al., 1987; Palmer et al., 1988a,b; Schmidt et al., 1988) (Fig. 11.1). A key step in the

This work is dedicated to my father Stefanos Tsikas (†) (1932–2005).

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

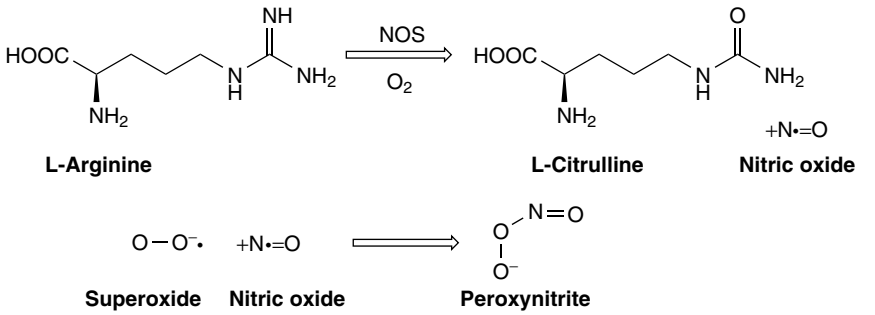


FIGURE 11.1 The L-arginine/nitric oxide pathway as a producer of reactive nitrogen species. (*Upper panel*) Formation of nitric oxide ($^{\bullet}NO$) and L-citrulline from L-arginine by the catalytical action of nitric oxide synthase (NOS). (*Lower panel*) formation of peroxynitrite ($ONOO^-$) from the reaction of $^{\bullet}NO$ with superoxide ($O_2^{-\cdot}$).

metabolic fate of $^{\bullet}NO$ is its reaction with superoxide ($O_2^{-\cdot}$). The superoxide radical anion is ubiquitous in mammalian cells and is also constitutively produced by all known NOS isozymes (Pou et al., 1992; Xia and Zweier, 1997; Vásquez-Vivar et al., 1998). $^{\bullet}NO$ reacts very rapidly with superoxide in aqueous solution to form peroxynitrite ($ONOO^-$) (Fig. 11.1). The rate constant for this reaction ($\sim 7 \times 10^9$ M^{-1} s^{-1}; Huie and Padmaja, 1993) is much greater than that of the reaction of $^{\bullet}NO$ with heme compounds ($k < 10^8$ M^{-1} s^{-1}) and comparable to the rate constant at which superoxide is dismutated by superoxide dismutase (SOD) enzymes ($\sim 2 \times 10^9$ M^{-1} s^{-1}; Halliwell and Gutteridge, 1999). This is of particular interest because SOD may compete with the reaction of superoxide with $^{\bullet}NO$, and superoxide dismutation may result in enhanced bioavailability of $^{\bullet}NO$ produced from L-arginine by NOS (Hobbs et al., 1994).

The conjugate acid of peroxynitrite, namely peroxynitrous acid (ONOOH; $pK_a \approx 6.8$), is a strong oxidant and potent nitrating agent, and as such harmful to cells (Radi et al., 2001). Reaction of peroxynitrite with biomolecules may lead to impaired function, toxicity, and alterations in signaling pathways (see other chapters of this book). Recent investigations on peroxynitrite decomposition using relatively pure preparations of peroxynitrite and improved analytical methods indicate that the final decomposition products of peroxynitrite in aqueous phase within a large pH range comprise nitrate, nitrite and dioxygen (Pfeiffer et al., 1997; Kissner and Koppenol, 2002). At physiological blood-pH peroxynitrite was found to decompose to nitrite and dioxygen at a molar ratio of 2:1 (Pfeiffer et al., 1997).

Peroxynitrite and other reactive nitrogen species (RNS) including nitrylchloride (Halliwell, 1997) preferentially react with the aromatic ring of tyrosine and the sulfhydryl group of cysteine—both of the soluble amino acids and the residues in proteins—of cysteinyl-thiols and other thiols to form 3-nitrotyrosine and *S*-nitrosothiols (RSNO) (Fig. 11.2) and/or *S*-nitrothiols ($RSNO_2$). In particular, nitration of tyrosine (Ischiropoulos, 1998; Radi et al.,

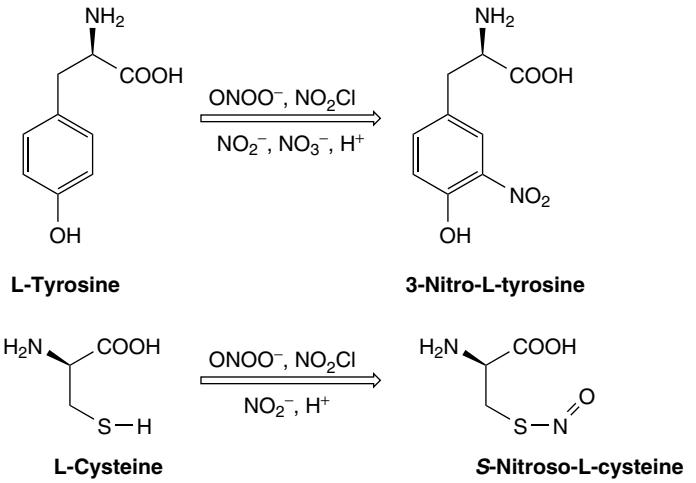


FIGURE 11.2 Nitration and nitrosylation of L-tyrosine and L-cysteine by reactive nitrogen species. (*Upper panel*): Reaction of L-tyrosine with peroxynitrite ($ONOO^-$) or nitrylchloride to form 3-nitro-L-tyrosine. (*Lower panel*): Reaction of L-cysteine with $ONOO^-$ to form *S*-nitroso-L-cysteine. Under acidic conditions L-tyrosine and L-cysteine are also nitrated and nitros(yl)ated by nitrite and nitrate.

2001) and *S*-nitros(yl)ation of cysteine moieties in proteins may influence their functions; for example, they may inhibit or enhance enzyme activity if these amino acids are involved in the catalytic process (see other chapters of this book).

It is worth mentioning that the endogenous *S*-nitrosothiols *S*-nitrosohemoglobin and *S*-nitrosoalbumin exert potent biological activity (Giustarini et al., 2003, 2004; Schechter and Gladwin, 2003; Frehm et al., 2004; Stamler, 2004), unlike 3-nitrotyrosine. In addition to the potential physiological roles of *S*-nitrosothiols, this class of compounds has also attracted attention because of its therapeutic potential as $^{\bullet}NO$ donating drugs (Al-Sa'doni and Ferro, 2000; Richardson and Benjamin, 2002). Interestingly the synthetic *S*-nitroso-*N*-acetylpenicillamine (SNAP) has been shown in vivo in the rabbit not to induce tolerance, unlike the organic nitrate glycerol trinitrate (Shaffer et al., 1992). It may reasonably be assumed that all *S*-nitrosothiols will not induce tolerance.

11.1.2 *S*-Nitrosothiols

In 1992 it was reported for the first time that $^{\bullet}NO$ circulates in plasma of healthy humans primarily as *S*-nitrosoalbumin (at a concentration of about 7 μM), and was suggested that *S*-nitrosoalbumin may be a physiological reservoir of $^{\bullet}NO$ by which $^{\bullet}NO$-related biological actions such as vasodilation are regulated in humans (Stamler et al., 1992a). This highly interesting finding has initiated much scientific work in this area. However, to date there is no solid confirmation of

$$\text{R1-S–NO} + \text{R2-S–H} \underset{}{\overset{K_{eq}}{\rightleftharpoons}} \text{R1-S–H} + \text{R2-S–NO}$$

$$K_{eq} = \frac{[\text{R1SH}] \times [\text{R2SNO}]}{[\text{R1SNO}] \times [\text{R2SH}]}$$

FIGURE 11.3 Equilibrium *S*-transnitrosylation reaction between thiols (R1SH, R2SH) and the conjugate *S*-nitrosothiols (R1SNO, R2SNO). K_{eq} is the equilibrium constant of the reaction. [R1SH], [R2SH], [R1SNO], [R2SNO] are equilibrium concentrations of the respective reactants.

the originally reported values and hypothesis (see below). The concentration of *S*-nitrosohemoglobin and *Fe*-nitrosohemoglobin in blood has been reported to be below 1000 nM (Jia et al., 1996; Cannon et al., 2001).

One of the most characteristic chemical reactions of *S*-nitrosothiols is the *S*-transnitrosylation reaction, namely the transfer of the nitrosyl group ($^{+}$NO) to other thiols (Arnelle and Stamler, 1995) (Fig. 11.3). In vitro, *S*-transnitrosylation reactions have been demonstrated by gas chromatography–mass spectrometry (GC–MS) (Tsikas et al., 1999a) and by ^{15}N nuclear magnetic resonance spectroscopy using ^{15}N-labeled *S*-nitrosothiols (Wang et al., 1999). Interestingly the transfer of $^{+}$NO from intravenously infused exogenous *S*-nitrosoalbumin to intravenously administered L-cysteine has also been shown to occur in vivo in the rabbit (Scharfstein et al., 1994); however, the amounts used in that study were very high. *S*-Transnitrosylation of endogenous albumin in the rat from intravenously infused ^{15}N-labeled *S*-nitrosoglutathione at the relatively low infusion rate of 80 nmol/min was demonstrated by means of the GC–MS methodology (Tsikas et al., 2001a). In that study the half-life of ^{15}N-labeled *S*-nitrosoalbumin in vivo in the rat was estimated to be of the order of 10 minutes. In vitro in human blood, the half-life of ^{15}N-labeled *S*-nitrosoalbumin (added at 25 μM) was determined to be 5.5 hours (Tsikas et al., 1999b). Interestingly the low-molecular-mass (LMM) *S*-nitrosocysteine, which has been considered to be the endothelium-derived relaxing factor (EDRF) (Myers et al., 1990), has a half-life of 42 seconds, thus even shorter than the half-life of $^{\bullet}$NO of 100 seconds under similar experimental conditions, namely in aqueous phosphate buffer of pH 7.0 at 25°C (Tsikas, 2005a).

Because *S*-transnitrosylation are equilibrium reactions and precede rapidly (Meyer et al., 1994; Arnelle and Stamler, 1995; Tsikas et al., 1999a), the formation of the first *S*-nitrosothiol is the limiting step of the *S*-nitrosothiol cascade. Formation of a single *S*-nitrosothiol would necessarily lead to formation of *S*-nitrosothiols of all endogenous thiols via *S*-transnitrosylation reactions. Endogenous LMM *S*-nitrosothiols most likely include *S*-nitrosocysteine, *S*-nitrosocysteinylglycine, and *S*-nitrosoglutathione, with *S*-nitrosothiol concentrations being dependent on the thiol concentrations in accordance with the respective equilibrium constants (K_{eq}), which are close to unity for most putative endogenous *S*-nitrosothiols (Tsikas et al., 1999a).

Several mechanisms that may lead to *S*-nitrosothiols in vivo have been suggested. However, the origin of endogenous *S*-nitrosothiols is still uncertain. In the presence of reduced glutathione (GSH) at mM concentrations in NOS incubates, formation of *S*-nitrosoglutathione at μM concentrations, namely similar to those of L-arginine-derived L-citrulline and nitrite, has been reported (Schmidt et al., 1996; Mayer et al., 1998). In NOS incubates containing GSH and L-[*guanidino*-$^{15}N_2$]-arginine as the substrate, formation of *S*-nitrosoglutathione only in the lower nM range could be demonstrated by GC–MS (Tsikas et al., 2000a). Nevertheless, in consideration of the nM concentrations of circulating *S*-nitrosohemoglobin and *S*-nitrosoalbumin, NOS-dependent *S*-nitrosoglutathione production rate could be enough to produce nM concentrations of *S*-nitrosohemoglobin and *S*-nitrosoalbumin in the blood (see Section 11.3.2).

One mechanism by which *S*-nitrosoglutathione may be formed in GSH-containing NOS incubates could be the reaction of GSH with $ONOO^-$. It should be noted that only a very small part of $ONOO^-$ (0–300 μM) of the order of 2% may *S*-nitrosylate GSH (0–5 mM) to *S*-nitrosoglutathione (Tsikas et al., 2001b) (Fig. 11.4). Theoretically both *S*-nitrosothiols (RSNO) and *S*-nitrothiols ($RSNO_2$) could be formed by the reaction of thiols with $ONOO^-$. RSNO and $RSNO_2$ have almost identical chromatographic and spectrophotometric properties (Balazy et al., 1998; Tsikas et al., 2001b). By means of the liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) methodology it has been shown that *S*-nitroglutathione ($GSNO_2$) but not *S*-nitrosoglutathione (GSNO) is formed from the reaction of GSH with $ONOO^-$ (Balazy et al., 1998). By contrast,

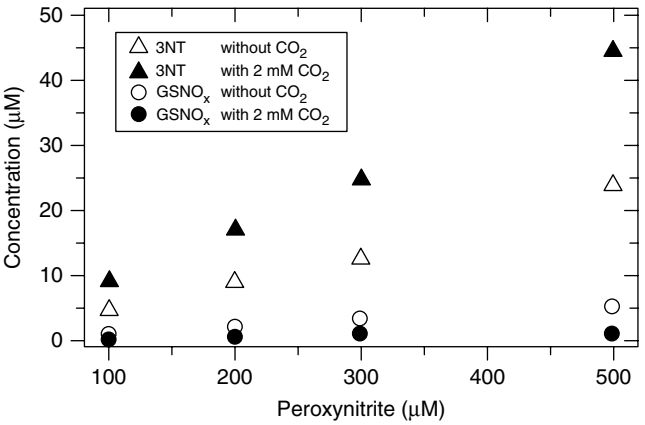


FIGURE 11.4 Peroxynitrite-dependent formation of *S*-nitro(so)glutathione ($GSNO_x$) from glutathione (1 mM) and of 3-nitro-L-tyrosine (3NT) from L-tyrosine (1 mM) in aqueous 100 mM phosphate buffer at pH 7.4 in the absence or presence of carbon dioxide (2 mM) supplied as sodium bicarbonate (20 mM). Separate incubations of peroxynitrite with glutathione or L-tyrosine were performed. *S*-Nitrosoglutathione and 3-nitro-L-tyrosine were determined by HPLC. Note that carbon dioxide decreases $GSNO_x$ formation but increases 3NT formation.

based on the observation that *S*-nitrosoglutathione and H_2O_2 were formed at similar levels after reaction of GSH with $ONOO^-$, it has been suggested that $ONOO^-$ *S*-nitrosylates GSH to form *S*-nitrosoglutathione (van der Vliet et al., 1998). Eventually it should be noted that a molecular orbital study suggested that $RSNO_2$ may rearrange to form intermediates such as RSONO and RS(O)NO that may release $^{\bullet}NO$ (Cameron et al., 1995). Nevertheless, the products formed and the underlying mechanisms of the reaction of thiols with peroxynitrite are still insufficiently investigated and incompletely understood.

An interesting alternative mechanism that may lead to RSNO could involve formation of *S*-nitrosohemoglobin (SNOHb) from the intramolecular transfer of the ^{+}NO group of *Fe*-nitrosohemoglobin (HbFeNO) to the sulfhydryl group of β-Cys93 of hemoglobin (Gow and Stamler, 1998), with HbFeNO being produced from the reaction of $^{\bullet}NO$ (Gow and Stamler, 1998) or nitrite (Gladwin et al., 2004) with hemoglobin. Frequently dinitrogen trioxide (N_2O_3) is discussed as a potent *S*-nitrosylating species (Jourd'heuil et al., 2000; Zhang and Hogg, 2005). However, unlike in gas phase, formation of N_2O_3 from autoxidation of $^{\bullet}NO$ in aqueous phase is rather unlikely (Pogrebnaya et al., 1975; Ford et al., 1993). Eventually serum albumin and other proteins have been suggested to catalyze formation of LMM and high-molecular-mass (HMM) *S*-nitrosothiols by oxidizing in the protein hydrophobic core $^{\bullet}NO$ to ^{+}NO, which is then transferred to the sulfhydryl group of LMM thiols and other functionalities (Nedospasov et al., 2000; Rafikova et al., 2002). However, further studies involving application of mass spectrometry (MS)-based analytical approaches such as liquid chromatography–mass spectrometry (LC–MS) and LC–MS/MS are required to delineate this important challenging issue.

11.1.3 3-Nitrotyrosine

The reaction of peroxynitrite, nitrylchloride, nitrate, and nitrite with L-tyrosine, phenol, and other phenolic compounds has been subject of numerous investigations from several points of view. 3-Nitrotyrosine has been suggested for the first time as a new marker for endogenous nitrosation and nitration of protein-associated tyrosine on the basis of results which were obtained using the exogenous nitrating agent tetranitromethane but not peroxynitrite in vivo in the rat (Ohshima et al., 1990). In that study intraperitoneal injection of tetranitromethane (0.5 to 2.5 μmol per rat) led to formation of 3-nitrotyrosine in plasma proteins and hemoglobin. Furthermore 3-nitrotyrosine orally given to rats was found to be metabolized to 3-nitro-4-hydroxyphenylacetic acid (NHPA) and 3-nitro-4-hydroxyphenyllactic acid (NHPL), which were excreted in the urine (Ohshima et al., 1990). About 44% and 5% of orally administered 3-nitrotyrosine were found to be excreted, respectively, revealing NHPA as the major urinary metabolite of 3-nitrotyrosine in the rat (Ohshima et al., 1990). It was reported that NHPA is also excreted in the urine of six smokers and five nonsmokers at mean excretion rates of 2.7 μg/day (17.8 nmol/day) and 2.9 μg/day (19.1 nmol/day), respectively (Ohshima et al., 1990). Considering the

mean urine volume reported in that study, a mean urinary NHPA concentration of 15 nM in humans is calculated. This order of magnitude for the urinary excretion rate of NHPA was confirmed by gas chromatography–mass spectrometry/mass spectrometry (GC–MS/MS) in young healthy volunteers, who excreted about 2.3 nmol of NHPA per mmol of creatinine (Keimer et al., 2003).

Formation of 3-nitrotyrosine was also demonstrated from the reaction of soluble L-tyrosine as well as L-tyrosine moieties in bovine serum albumin (BSA) with nitrite at acidic pH, with maximum nitratrion yield occuring at pH of about 2.5 for L-tyrosine (Ohshima et al., 1990). Unlike low-yield nitration of L-tyrosine to 3-nitrotyrosine, *S*-nitrosylation of thiols is also pH-dependent but almost quantitative at pH values of and below 3.4, namely the pK_a value of nitrous acid (Tsikas and Frölich, 2002a; Tsikas, 2003). It should be mentioned that the most popular method of synthesis of LMM and HMM *S*-nitrosothiols is based on the reaction of thiols with nitrite in acidic solution (Saville, 1958; Stamler et al., 1992b). The importance of acid-catalyzed, nitrite-, and nitrate-dependent formation of 3-nitrotyrosine and *S*-nitrosothiols for the quantitative determination of the endogenous compounds is discussed below in detail (see Section 11.2.2).

Special attention has been and is still attributed to the reaction of peroxynitrite with L-tyrosine to form 3-nitrotyrosine as well as with phenol and other phenolic compounds to form the respective nitro derivatives. The most important characteristic of the peroxynitrite-mediated nitration of soluble and protein-associated L-tyrosine is that it may occur in aqueous solution at neutral and alkaline pH values, while nitration by nitrite and nitrate strictly requires acidic conditions. The exact mechanism of the reaction of L-tyrosine with peroxynitrite to form 3-nitrotyrosine in aqueous buffered solutions is still unknown. In particular it is incompletely understood which species actually nitrates L-tyrosine to 3-nitro-L-tyrosine, namely peroxynitrite itself ($ONOO^-$), $^{\bullet}NO_2$ or $^{+}NO_2$ (Ducrocq et al., 1999; Halliwell et al., 1999; Radi et al., 2001). Like *S*-nitrosylation of thiols, only a small portion of $ONOO^-$ may nitrate L-tyrosine to 3-nitro-L-tyrosine (Fig. 11.4).

One of the most characteristic reactions of peroxynitrite is that with ubiquitous carbon dioxide (CO_2). This important reaction may be of particular importance as the physiologically abundantly present CO_2 may influence peroxynitrite-mediated reactions including nitration of tyrosine and aromatics (Ducrocq et al., 1999; Gow et al., 1996; Lemercier et al., 1997; Uppu et al., 1996), *S*-nitrosylation, and oxidation of thiols (Denicola et al., 1996; Jourd'heuil et al., 1999; Lymar and Hurst, 1998; Zhang et al., 1997) (Fig. 11.4). One-electron oxidations by peroxynitrite are attributed to reactive intermediates such as the $^{\bullet}CO_3^-$ and $^{\bullet}NO_2$ radicals (Lymar and Hurst, 1998; Goldstein and Czapski, 1999), which are assumed to be formed by homolytic decomposition of the intermediate reaction product of CO_2 and $ONOO^-$, namely nitrosoperoxycarbonate ($ONOOCO_2^-$). It should be pointed out that in some circumstances peroxynitrite-mediated 3-nitrotyrosine formation may lack, in particular, when peroxynitrite is generated at physiological pH, for example, by using the xanthine oxidase/hypoxanthine system and $^{\bullet}NO$ donors such as spermine NONOate (Pfeiffer and Mayer, 1998). For the sake of completeness it should also be mentioned that 3-nitrotyrosine formation may be

independent of peroxynitrite, but may depend on RNS produced by peroxidases such as myeloperoxidase (Brennan et al., 2002). Eventually peroxynitrite was also found to react with other endogenous and exogenous compounds including uric acid (Skinner et al., 1998), ascorbic acid (Squadrito et al., 1995), and ebselen (Masumoto et al., 1996), in addition to aromatics, thiols, and carbon dioxide.

11.1.4 Quantity of Circulating *S*-Nitrosothiols and 3-Nitrotyrosine as Indicator of Health and Disease

Generally, it is assumed that enhanced nitration of soluble and protein-associated tyrosine leads to cellular dysfunction and disease. In this chapter the term "protein-associated 3-nitrotyrosine" is preferred because the frequently used term "protein-bound 3-nitrotyrosine" is misleading. Circulating free and protein-associated 3-nitrotyrosine are widely used as biomarkers of oxidative stress in humans. Thus the levels of free and protein-associated 3-nitrotyrosine in the circulation are used to quantify the state of oxidative stress in vivo in humans (Duncan, 2003).

Because of the •NO-related biological actions of *S*-nitrosothiols, generally these compounds are not considered to be biomarkers of oxidative stress, but rather a reservoir for •NO (Stamler et al., 1992a; Stamler, 2004). Circulating levels of *S*-nitrosothiols may be elevated both due to elevated •NO formation and due to impaired release of •NO from these compounds. On the other hand, circulating levels of *S*-nitrosothiols may be decreased both due to impaired •NO formation and due to enhanced release of •NO from these compounds. Therefore the significance of the quantity of circulating *S*-nitrosothiols in humans is at present unclear and controversial. It has been suggested that the quantity of *S*-nitrosothiols in the blood is of no relevance (Stamler, 2004), but other authors disagree on this opinion (Tsikas, 2004).

Reference values are indispensable in evaluating the impact not only of clinical studies, but also that of analytical methods. Establishment of standard values for free 3-nitrotyrosine, protein-associated 3-nitrotyrosine, and *S*-nitrosoproteins in human plasma and erythrocytes is an indispensable prerequisitive for defining pathological conditions, for deciding on the necessity for taking pharmacological or nutritional measures or change of habit, for monitoring therapy success on the basis of a biochemical laboratory parameter, and last but not least for evaluating the validity of the analytical methods used (Tsikas, 2004). Recent advances in the methods of analysis make it possible to suggest reference intervals for 3-nitrotyrosine (Tsikas and Caidahl, 2005). Circulating *S*-nitrosothiols have evaded the definition of reference intervals to date. Therefore the most urgent and important future prospect in the area of *S*-nitrosothiol research is the establishment of reference intervals for circulating *S*-nitrosothiols, namely *S*-nitrosoalbumin and *S*-nitrosohemoglobin—the most abundant and most stable *S*-nitrosothiols in the blood.

Today numerous analytical methods based on different analytical approaches are available for the quantitative determination of free and protein-associated

3-nitrotyrosine and *S*-nitrosothiols. In particular, with respect to 3-nitrotyrosine, the most reliable analytical methods are based on mass spectrometry (MS) because of the inherent accuracy of the MS and especially of the tandem MS (i.e., MS/MS) methodology. This chapter discusses current analytical methodologies for the quantitative determination of endogenously produced 3-nitrotyrosine and *S*-nitrosothiols in human circulation. Special emphasis is given to quantitative MS-based analytical methods, such as GC–MS/MS and LC–MS/MS, which are generally accepted as reference methods and the gold standard as well as for accuracy control, in particular, in clinical research (Lawson et al., 1985). On the basis of reported MS-based and non–MS-based analytical approaches and circulating levels of 3-nitrotyrosine and *S*-nitrosothiols measured in health and disease, the reliability of analytical methods, the suitability of 3-nitrotyrosine as a biomarker of •NO-associated oxidative stress, and the function of *S*-nitrosothiols as a reservoir for •NO in humans are critically discussed. It should be already pointed out here that this field of research is rich in analytical mines and pitfalls such as abundant artifactual formation of 3-nitrotyrosine (Tsikas and Caidahl, 2005) and *S*-nitrosothiols (Tsikas, 2003). Furthermore the importance of analytical chemistry, in particular, in clinical research, has been neglected thus far. These issues are critically discussed and potential solutions are recommended.

11.2 METHODS OF ANALYSIS

11.2.1 General Considerations

Need for Chemical Conversion and Derivatization The *S*-nitroso group of endogenous *S*-nitrosothiols, in particular, of *S*-nitrosocysteinylthiols, possesses weak absorptivity ($\varepsilon \approx 800\ M^{-1}\ cm^{-1}$) at the characteristic broad UV absorption maximum around 340 nm (Balazy et al., 1998). Therefore LMM *S*-nitrosothiols including *S*-nitrosocysteine, *S*-nitroso-*N*-acetylcysteine, and *S*-nitrosoglutathione cannot be analyzed by UV absorbance detection at 340 nm, for example, by high-performance liquid chromatography (HPLC), at concentrations below approximately 0.3 μM (Gaston, 1999; Tsikas et al., 1999a). On the other hand, the absorptivity of the *S*-nitroso group of the HMM *S*-nitrosocysteinyl thiols *S*-nitrosoalbumin and *S*-nitrosohemoglobin is of the same order of magnitude as that of the LMM *S*-nitrosocysteinylthiols *S*-nitrosocysteine, *S*-nitroso-*N*-acetylcysteine, and *S*-nitrosoglutathione (Meyer et al., 1994), and it is furthermore much lower than the absorptivity of other moieties in the proteins. In addition the precursor nonnitrosylated molecules, namely albumin and hemoglobin, are present in high molar excess over *S*-nitrosoalbumin and *S*-nitrosohemoglobin and cannot be selectively eliminated. Consequently *S*-nitrosoalbumin and *S*-nitrosohemoglobin and most likely other HMM *S*-nitrosocysteinyl thiols cannot be quantified at all by UV absorbance detection around 340 nm in biological matrices (Tsikas et al., 1999b).

The *S*-nitroso group of putative endogenous LMM *S*-nitrosothiols such as *S*-nitrosocysteine, *S*-nitroso-*N*-acetylcysteine, and *S*-nitrosoglutathione is

thermally highly unstable. Authentic as well as chemically modified LMM *S*-nitrosothiols are, in general, not accessible to quantitative analysis by gas chromatography (GC)-based approaches such as GC–MS without loss of the *S*-nitroso group due to thermal decomposition. *S*-Nitroso-*N*-acetylcysteine could be measured in human plasma and urine by GC–MS after HPLC separation and derivatization despite thermal decomposition of its *S*-nitroso group (Tsikas et al., 1996). However, endogenous *S*-nitroso-*N*-acetylcysteine cannot be detected in human plasma and urine at concentrations above 1 nM, suggesting that renal *N*-acetylation of *S*-nitrosocysteine does not represent a major metabolic pathway in humans (i.e., the mercapturic acid pathway), unlike the non-nitrosylated amino acid L-cysteine (Tsikas et al., 1996).

In principle, native as well as derivatized LMM and HMM *S*-nitrosothiols can be analyzed by LC–MS and LC–MS/MS (Mirza et al., 1995; Tsikas et al., 1996, 2000b; Ferranti et al., 1997; Kluge et al., 1997; Balazy et al., 1998). However, the utility of these MS-based techniques for the quantitative determination of endogenous LMM and HMM *S*-nitrosothiols in the circulation or in the urine of humans has not been demonstrated thus far.

The inability to quantify in blood and urine endogenous *S*-nitrosothiols that still carry their *S*-nitroso groups makes it necessary to measure *S*-nitrosothiols indirectly, namely after conversion of the *S*-nitroso group into species such as $^{\bullet}$NO or nitrite that are accessible for analysis by various methodologies, including chemiluminescence, HPLC, and GC–MS (for a review, see: Butler and Rhodes, 1997; Giustarini et al., 2003; Yang et al., 2003; Stamler, 2004). However, this proceeding is associated with analytical problems and requires, for instance, elimination of blank nitrite or accurate determination of its concentration and subtraction from the total value (i.e., blank nitrite + *S*-nitrosothiol-derived nitrite). Because nitrite levels from endogenous and exogenous sources are usually above *S*-nitrosothiol levels, such analytical methods may become inaccurate mainly due to interfering nitrite.

An alternative analytical approach is based on the measurement of thiols formed by ascorbate-catalyzed reduction of the $^{+}$NO group of HMM *S*-nitrosothiols that releases the reduced forms of the thiols which are subsequently biotinylated (Jaffrey and Snyder, 2001; Jaffrey et al., 2001). However, this method has not been developed and validated for plasma *S*-nitrosothiols, and specificity, selectivity, and potential interferences such as mixed disulfides (Ckless et al., 2004) are currently missing. Nevertheless, this method has been applied to proteomic analysis of *S*-nitrosylated proteins in various cells (Kuncewicz et al., 2003; Zhang et al., 2005) and even to quantify *S*-nitrosoproteins in human plasma (Gandley et al., 2005). A similar method has been described for the measurement of *S*-nitrosoglutathione in human plasma after its conversion to GSH by 2-mercaptoethanol (Tsikas et al., 1999c). This method is based on the measurement of GSH by HPLC with fluorescence detection after precolumn derivatization with *o*-phthaldehyde. However, no endogenous *S*-nitrosoglutathione could be detected in plasma of healthy humans above the limit of quantitation of 100 nM for *S*-nitrosoglutathione (Tsikas et al., 1999c).

Unlike *S*-nitrosothiols, 3-nitrotyrosine is thermally and chemically much more stable and possesses an approximately six times higher molar absorptivity at characteristic UV (276 nM) and visible (428 nm, $\varepsilon \approx 4{,}600\ M^{-1}\ cm^{-1}$; Schwedhelm et al., 1998) wavelengths in acidic and alkaline aqueous solutions. The physicochemical properties of 3-nitrotyrosine allow a more easier analytical handling with biological matrices containing this compound and a more reliable quantitative analysis of authentic and derivatized 3-nitrotyrosine by means of a variety of methodologies such as HPLC with UV absorbance, electrochemical, and fluorescence detection (Kamisaki et al., 1996; Herce-Pagliai et al., 1998; Liu et al., 1998; Hensley et al., 2000; Duncan, 2003). However, HPLC with various detection techniques does not provide sufficient sensitivity and specificity for accurate quantitative determination of free 3-nitrotyrosine in human plasma (Tsikas and Caidahl, 2005), which are only offered by MS-based methodologies. Quantitative determination of 3-nitrotyrosine in human plasma by MS-based methods requires one derivatization step in LC–MS/MS and at least two derivatization steps in GC–MS/MS (see Section 11.2.3).

Methods of Quantitation—The Internal Standard The MS methodology is the single analytical technology in which quantitation is carried out by using stable-isotope-labeled analogues of the endogenous compounds that serve as internal standards. Thus, for the quantification of endogenous *S*-nitrosothiols and 3-nitrotyrosine, the respective stable-isotope-labeled analogue, such as *S*-[^{15}N]nitrosoalbumin (Tsikas et al., 1999b) or 3-nitro-L-[$^{2}H_3$]tyrosine (Schwedhelm et al., 1998), is added to the respective biological matrix, such as plasma, at a relevant final concentration. Endogenous compound and internal standard undergo all chemical and physical changes during the whole analytical process, namely derivatization, extraction, and chromatographic separation (Hill and Whelan, 1984; Schoeller, 1986; Duncan, 2003). However, ions derived from derivatives of endogenous substances and their respective stable-isotope-labeled analogues are separated in the mass spectrometer due to their distinctly different mass-to-charge ratios (m/z) (Hill and Whelan, 1984; see also Section 11.2.3). MS analysis reveals GC peaks of which the ratio of the areas or heights corresponds to the ratio of the concentrations in the biological sample. Thus the concentration of an endogenous substance in the biological matrix is simply determined by multiplying the peak area ratio or peak height ratio measured by the known concentration of the internal standard externally added (Hill and Whelan, 1984; Schoeller, 1986). Generally, use of stable-isotope-labeled analogues as internal standards in MS-based methods generates linear standard curves within a large concentration range of several orders of magnitude (Schoeller, 1986). The reliability of this mode of quantitation has been demonstrated practically for all members of the L-arginine/$^{\bullet}$NO pathway including *S*-nitrosothiols and 3-nitrotyrosine (Tsikas, 2005b).

In all the other methodologies, calculation of concentrations of endogenous substances in biological matrices is performed by using calibration curves that are generated in matrices distinctly different from the biological matrices, usually

in aqueous solution. Moreover in many of these methodologies the calibrator is completely different from the endogenous target substance. Thus usually aqueous nitrite is used as a calibrator for *S*-nitrosohemoglobin in a chemiluminescence-based method, which produces chemiluminescence signals that regularly have completely different peak shapes (i.e., different analytical characteristics) from those caused by the endogenous substances in a blood sample (Feelisch et al., 2002; Bryan et al., 2004). This proceeding does not consider at all the well-known matrix-associated effects, including unknown recovery of the final species to be detected, such as nitrite for *S*-nitrosohemoglobin, in contrast to the use of stable-isotope-labeled analogues of the endogenous compounds. Strictly speaking, measurement of absolute concentrations of endogenous substances in biological samples such as blood and urine is not possible by non–MS-based analytical methods. This circumstance is an important reason for the great discrepancies between different analytical methods with respect to basal levels of endogenous *S*-nitrosothiols and 3-nitrotyrosine, especially in comparison to MS-based approaches (see Section 11.2.3).

Another important issue that should attract special attention concerns the quantification of protein-associated *S*-nitrosothiols and protein-associated 3-nitrotyrosine both by physicochemical and immunological methods. Unlike LMM *S*-nitrosothiols and 3-nitrotyrosine, synthesis and structural characterization of unlabeled and stable-isotope-labeled reference substances of HMM *S*-nitrosothiols and protein-associated 3-nitrotyrosine for the purpose of quantification is much more difficult and frequently not satisfactory from the analytical point of view. Several strategies have been suggested to overcome these shortcomings.

Specific detection of a certain HMM *S*-nitrosothiol, such as *S*-nitrosoalbumin in plasma and *S*-nitrosohemoglobin in red blood cells, or a certain HMM 3-nitrotyrosine, such as 3-nitrotyrosinoalbumin in plasma, is a practicable way (Tsikas et al., 1999d, 2003a). Human serum albumin (HSA) contains a single *S*-nitrosable sulfhydryl group located at cysteine-34. Thus the concentration of *S*-nitroso groups in *S*-nitrosoalbumin is identical with the concentration of the entire *S*-nitrosoalbumin molecule. The major advantages of measuring *S*-nitrosoalbumin and 3-nitrotyrosinoalbumin, instead of the whole spectrum of HMM *S*-nitrosothiols and 3-nitrotyrosinoproteins in plasma, are the use of standardized protocols for synthesis, analysis, and detection, a better comparability of analytical and clinical results, and the establishment of reference values for *S*-nitrosoalbumin and 3-nitrotyrosinoalbumin.

The HSA molecule contains 18 tyrosine residues (Peters, 1985). Until now we do not know whether there is any preference for RNS in vivo as well as in vitro when synthesizing 3-nitrotyrosinated HSA by means of peroxynitrite or other nitrating agents. Also we do not know whether an albumin molecule is multiply nitrated on tyrosine residues in vivo. Certainly this also applies to other plasma proteins. HSA is by 76% identical with bovine serum albumin (BSA), which contains 21 tyrosine residues (Peters, 1985). Mass spectrometry investigations on tyrosine nitration in BSA by the exogenous tetranitromethane indicated some

site-specific nitration, thus with four out of 21 tyrosine residues being nitrated to much higher degree than a fifth tyrosine residue located at position 163 (Petersson et al., 2001; see also Sarver et al., 2001). However, it should be pointed out that the model nitrating agent tetranitromethane requires much more space than peroxynitrite or nitrylchloride and can therefore be considerably more specific in nitrating tyrosine residues in BSA and HSA, in particular, those being readily accessible on the protein surface. So, unlike HMM *S*-nitrosothiols, determination of the concentration of 3-nitrotyrosinated plasma protein molecules is practically not possible. Thus the measurement of 3-nitrotyrosinoproteins is reduced to the measurement of 3-nitrotyrosine produced by chemical or enzymic hydrolysis of the proteins to the single amino acids. The problem mentioned above has been satisfactorily solved by measuring protein-associated tyrosine in addition to 3-nitrotyrosine and by reporting the molar ratio of 3-nitrotyrosine to tyrosine both derived from proteins (Tsikas and Caidahl, 2005).

Antibody-based methods for 3-nitrotyrosinoproteins, such as immunohistochemical staining methods, ELISA assays, and solid phase immunoradiochemical methods, are very frequently used in vivo in humans although not being satisfactorily validated (Duncan, 2003). In these immunological methods quantitation is based on the use of structurally not characterized "nitrated BSA" or "nitro-BSA," and the nature of the antibodies produced is not well known. Consequently considerable variations may reasonably be expected in the preparation both of the calibrator and the antibodies, not only between different researcher groups but also within the same laboratory including the manufacturers. In addition, because of considerable structural differences between HSA and BSA as well between their nitrated forms, it is uncertain what and which quantity of 3-nitrotyrosinoproteins in human blood is detected by such immunological assays. It is worth mentioning that the 3-nitrotyrosine-to-tyrosine molar ratio cannot be determined by these methods. Therefore quantitative determination of 3-nitrotyrosinoproteins in human plasma by immunological methods is not without ambiguity (Duncan, 2003), and levels are usually reported as "nitro-BSA equivalents" (Khan et al., 1998; Oldreive et al., 2001). Thus methods such as the competitive ELISA can at best be described as "semi-quantitative" (Khan et al., 1998). Consequently a reliable comparison on the basis of reported plasma levels is not possible, not only between immunological and physicochemical methods but also between immunological assays themselves and even between ELISA methods (see Section 11.3).

11.2.2 Methodological Problems and Pitfalls

Circulating *S*-nitrosothiols have been detected in plasma of healthy humans at concentrations ranging between 1 nM and 10 μM, thus covering four orders of magnitude (see Section 11.3). Also reported 3-nitrotyrosine basal levels in human plasma range between 1 nM and at least 60 nM (see Section 11.3). The issue concerning the wide variability of reported basal plasma levels for *S*-nitrosothiols (Rossi et al., 2001; Tsikas and Frölich, 2002b, 2003; Tsikas, 2003,

2004; Giustarini et al., 2004; Giustarini and Rossi, 2004; Stamler, 2004; Tsikas et al., 2004) and 3-nitrotyrosine (Tsikas et al., 2002a; Duncan, 2003; Tsikas and Caidahl, 2005) is subject of intensive discussion. The extremely great variability in the levels of *S*-nitrosothiols and 3-nitrotyrosine in human plasma at the basal state is an impressive indicator of existing methodological problems in this field of research.

Earlier in this section there were addressed some analytical issues that may lead to diverging values, in particular, in approaches based on different principles such as mass spectrometry and immunology. However, we must search for other reasons when we want to satisfactorily explain the great divergencies among analytical methods that are based on the same methodology such as the mass spectrometry, in particular, with respect to 3-nitrotyrosine measurement (Tsikas and Caidahl, 2005). Several major methodological shortcomings have been identified. An important kind of these problems implies sample handling issues including procedures to draw blood, generate and storage plasma or serum, to extract and derivatize samples, and artifactual formation both of 3-nitrotyrosine and *S*-nitrosothiols. A second but no less important source for divergencies in 3-nitrotyrosine plasma levels in GC–MS and GC–MS/MS methods is lack of specificity (see Section 11.2.3). The pressing and unavoidable need for chemical conversion and derivatization may involve a certain risk for abundant artifactual formation of *S*-nitrosothiols and 3-nitrotyrosine, in particular, under acidic conditions (see below). Therefore many precautions have to be taken to eliminate/minimize this risk. These precautionary measures may involve additional chromatographic procedures such as solid phase extraction (SPE) and HPLC, so the whole method may become complicated, time-consuming, and not suitable for high-throughput analysis. On the other hand, use of HPLC not only eliminates interferences but also increases the method's sensitivity by increasing the signal-to-noise ratio (Tsikas and Caidahl, 2005).

Artifactual Formation of S-Nitrosothiols and 3-Nitrotyrosine Nitrite and nitrate are not only physiologically present in blood, they are also ubiquitous as contamination in every laboratory material including distilled water, chemicals such as buffer salts, monovettes, and ultrafiltration devices (Smith et al., 2002; Tashimo et al., 2003; Tsikas, 2005b). Cysteine and tyrosine, both as soluble amino acids and as residues in plasmatic and erythrocytic proteins, physiologically occur at μM concentrations, GSH even at mM concentrations in red blood cells. Consequently acidification of biological samples or other samples generated in the course of analytical procedures, such as plasma ultrafiltrate and plasma extracts that contain thiols or tyrosine, would inevitably generate artifactually *S*-nitrosothiols and 3-nitrotyrosine. Even when the extent of artifactual formation is low with respect to nitrite and nitrate, the concentration of artifactually formed *S*-nitrosothiols and 3-nitrotyrosine may be of the same order and even exceed by far the basal concentration of the endogenous compounds.

In most analytical methods, use of acidic conditions is absolutely required, either during sample derivatization, including acid-catalyzed hydrolysis of proteins, or during HPLC chromatography. Theoretically artifactual formation of

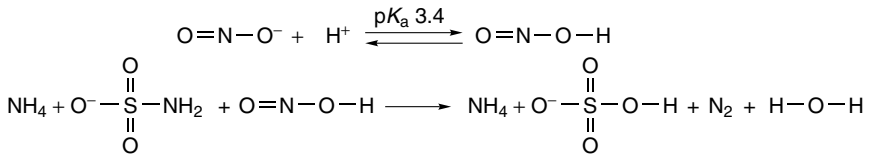


FIGURE 11.5 The nitrite/nitrous acid system and reaction of ammonium sulfamate [$(NH_4)OS(O)_2\ NH_2$] with nitrite under acidic conditions to form molecular nitrogen.

S-nitrosothiols and 3-nitrotyrosine can be avoided by removing nitrite and nitrate. However, because of the ubiquity of these anions and the incompletness of the removal procedures available so far, measures based on nitrite and nitrate removal by chemical means cannot entirely avoid artifactual formation of *S*-nitrosothiols and 3-nitrotyrosine (Kluge et al., 1997; Tsikas, 2003). Moreover the majority of these procedures, the most frequently used being based on ammonium sulfamate or sulfanilamide, are in principle diazotization reactions and absolutely require acidic conditions (Tsikas, 2003) (Fig. 11.5). Therefore artifactual formation—in particular, of *S*-nitrosothiols—may compete with the diazotization reaction and remove nitrite prior to its reaction with thiols. To prevent artifactual formation of *S*-nitrosothiols, thiol-specific agents such as *N*-ethylmaleimide (NEM) are used at mM concentrations, at high molar excess over thiol concentration (Feelisch et al., 2002; Yang et al., 2003). Nevertheless, these measures have shown not to completely avoid artifactual formation of *S*-nitrosothiols (Kluge et al., 1997; Tsikas, 2003).

Artifactual acid-catalyzed formation of 3-nitrotyrosine during sample treatment has been thoroughly investigated and repeatedly demonstrated by several groups (Shigenaga et al., 1997; Schwedhelm et al., 1999; Frost et al., 2000; Yi et al., 2000; Delatour et al., 2002a; Gaut et al., 2002; Söderling et al., 2003), and it is a well-recognized serious analytical problem (Duncan, 2003; Tsikas and Caidahl, 2005). Basically this problem can be effectively overcome by separating tyrosine from nitrite and nitrate by HPLC (Schwedhelm et al., 1999), by reducing 3-nitrotyrosine to 3-aminotyrosine by dithionite (Söderling et al., 2003), and also by using exogenous aromatic compounds such as phenol at mM concentration (Shigenaga, 1999). HPLC separation and dithionite reduction are presently superior to the use of phenol (Tsikas and Frölich, 2004; Tsikas and Caidahl, 2005).

Quantification of 3-nitrotyrosine as a residue in proteins may be associated with artifactual formation of 3-nitrotyrosine from acid-catalyzed hydrolysis, which is usually performed in 6 M HCl at 110°C for up to 24 hours (Ohshima et al., 1990), and with contribution of proteolytic enzymes to 3-nitrotyrosine by self-digestion. Several solutions have been suggested to circumvent the problem arising from chemical proteolysis. They include hydrolysis in alkaline solution (Frost et al., 2000), HCl hydrolysis in the gas phase (Yi et al., 2000), and addition of phenol (e.g., 1%, w/v) to act as a scavenger for nitration (Shigenaga, 1999). However, use of phenol has been shown to reduce but not to eliminate

the problem (Shigenaga, 1999). The major problem associated with the use of proteolytic enzymes may be the great variability in the contribution by self-digested proteins (Shigenaga et al., 1997; Söderling et al., 2003; Tsikas et al., 2003a). Therefore accurate quantification of protein-associated 3-nitrotyrosine in human plasma requires accurate quantitation and consideration of the extent of contribution of digesting enzymes by self-digestion.

Interference of Nitrite and Nitrate in the Measurement of S-Nitrosothiols

In methods of analysis of *S*-nitrosothiols, which are actually based on the measurement of nitrite such as that using the diaminonaphthalene (DAN) reagent (Damiani and Burini, 1986), leaving out of ammonium sulfamate or using this reagent at neutral pH would certainly not remove nitrite (Tsikas, 2003). In such cases we may reasonably assume that the method would not specifically measure *S*-nitrosothiols but nitrite and *S*-nitrosothiols *in toto*, with nitrite most likely being by far the major fraction. Thus determination of *S*-nitrosothiol concentration would require separate accurate determination of nitrite, the concentration of which in plasma may be much higher than that of *S*-nitrosothiols, for example, 0.5 to 3 μM versus approximately 0.2 μM (Tsikas, 2005b). Application of such an assay (i.e., use of ammonium sulfamate at almost neutral pH value) to quantify *S*-nitrosothiols in human serum and cell culture media revealed *S*-nitrosothiol levels that were almost identical with those of nitrite (Marzinzig et al., 1997), strongly suggesting that this method is not specific for *S*-nitrosothiols but actually measures nitrite plus *S*-nitrosothiols. Nevertheless, this method has been applied by other groups (Massy et al., 2003; Wlodek et al., 2003), apparently without any modification of the original method (Marzinzig et al., 1997), to measure *S*-nitrosothiols in plasma of patients suffering from chronic renal diseases. Because these groups did not measure and did not consider nitrite concentration, there is reasonable doubt about the value of these measurements (Tsikas, 2003; Tsikas and Frölich, 2003; Giustarini and Rossi, 2004; Tsikas et al., 2004). Furthermore both groups (i.e., Massy et al., 2003; Wlodek et al., 2003) have used the same method (i.e., Marzinzig et al., 1997) to measure *S*-nitrosothiols in plasma, but the *S*-nitrosothiol basal levels measured in their control groups differed almost by a factor of 20, namely 8.8 μM (Wlodek et al., 2003) and 0.45 μM (Massy et al., 2003). Regardless of these analytical problems and identified pitfalls, the same method has been used in a prospective study (Massy et al., 2004) that has revealed increased plasma *S*-nitrosothiol concentration to predict cardiovascular outcomes in end-stage renal disease patients (for discussion, see Giustarini and Rossi, 2004; see also Section 11.3.2).

In methods of analysis of *S*-nitrosothiols that are based on the measurement of nitrite, the order of the concentration of endogenous plasma nitrite, or blank nitrite in plasma extracts may be a decisive analytical factor and must therefore be quantified as accurately as possible. Nonconsideration of nitrite that comes not from *S*-nitrosothiols will be falsely attributed to *S*-nitrosothiols, resulting in overestimated *S*-nitrosothiol levels. When nitrite and *S*-nitrosothiol concentrations are of the same order of magnitude, this situation is presumably the case for

circulating *S*-nitrosothiols (see Section 11.3.2), and the comparably high blank nitrite level will increase the limit of quantitation of the method and consequently decrease the sensitivity of the method. Accurate quantification of small changes in *S*-nitrosothiol levels, as they may occur in the course of disease or pharmacological treatment, will then be not possible.

Artifactual formation of *S*-nitrosothiols via *S*-nitrothiol formation from nitrate and thiols requires very strong acidic conditions such as the use of concentrated sulfuric acid, which is not common practice. Presumably this kind of artifactual formation of *S*-nitrosothiols and *S*-nitrothiols can be neglected. However, photolysis of the chemically inert nitrate, in particular, in the presence of thiols, has been reported to produce $^{\bullet}$NO and *S*-nitrosothiols (Dejam et al., 2003). In quantitative methods of *S*-nitrosothiol analysis, which are based on photolysis, in particular, in the widely used photolysis-chemiluminescence method (Stamler et al., 1992a,b; Tyurin et al., 2001; see also Giustarini et al., 2003), thiol-catalyzed photolytic conversion of nitrate and to $^{\bullet}$NO and *S*-nitrosothiols may lead to falsely overstimated *S*-nitrosothiol levels (Dejam et al., 2003).

Blood Sampling and Sample Storage Accurate quantification of *S*-nitrosothiols and 3-nitrotyrosine requires a protocol that involves proper conditions not only to prevent their artifactual formation but also to ensure maximum stability until analysis. This issue is of fundamental importance for *S*-nitrosothiols, which are chemically considerably less stable than 3-nitrotyrosine and may moreover undergo rapid oxidation and decomposition in blood ex vivo. To date there is no confirmation of the originally reported finding that LMM *S*-nitrosothiols physiologically occur in human plasma at basal concentration of 7000 nM (Stamler et al., 1992a). On the basis of their *S*-transnitrosylation equilibrium constants and the concentration of LMM and HMM thiols in human plasma and under consideration of an *S*-nitrosoalbumin basal concentration of the order of 200 nM (Tsikas et al., 1999d, 2002b), it is estimated that LMM *S*-nitrosothiols such as *S*-nitrosocysteine and *S*-nitrosoglutathione may not be present in human plasma at basal concentrations above 2 to 5 nM (Tsikas et al., 1999a). It is assumed that LMM *S*-nitrosothiol levels in human plasma at the basal state are below 25 nM (Gladwin et al., 2000). It is worth mentioning that *S*-nitrosocysteine and *S*-nitrosoglutathione, externally added to human plasma or blood at these concentrations, cannot be recovered from these matrices (Tsikas et al., 1999e, 2001a; Gladwin et al., 2000), unlike when added to plasma ultrafiltrate (Tsikas et al., 1999c). HMM *S*-nitrosothiols such as *S*-nitrosoalbumin are considerably more stable in blood (Tsikas et al., 1999b,d) and plasma in vitro (Butler and Rhodes, 1997) and in vivo (Tsikas et al., 2001a). To the best knowledge of the author, respective studies on *S*-nitrosohemoglobin are missing (see Feelisch et al., 2002; Yang et al., 2003).

Despite considerable stability of *S*-nitrosoalbumin and *S*-nitrosohemoglobin, blood sampling, generation of plasma, and erythrocyte fractions are performed as quickly as possible at low temperature (e.g., at 5°C), and when necessary after taking additional measures to stabilize the *S*-nitrosothiols (Yang et al., 2003). Detection of *S*-nitrosothiols should also follow as quickly as possible (Tsikas and

Frölich, 2004). In practice, however, samples are frozen, preferably at −80°C, until being measured. It has been reported that *S*-nitrosoalbumin concentrations in plasma are 0.25 to 1.0 μM in samples that have been stored for two to three weeks, but appear higher if measurements are made immediately after samples have been taken (Butler and Rhodes, 1997). Although there is supporting finding for a decrease in *S*-nitrosoalbumin concentration upon storage at 4°C over several weeks even in the presence of the chelator EDTA (Tsikas et al., 1999b), this issue has not been investigated thoroughly thus far.

Unexpectedly, freezing of solutions of nitrite and thiols or tyrosine in sodium but not potassium phosphate buffer of neutral pH has been reported to produce artifactually *S*-nitrosothiols and 3-nitrotyrosine, respectively (Daiber et al., 2004). Although freezing of plasma samples and plasma extracts did not lead to such artifactual formation (Tsikas and Frölich, 2004), this finding is an insistent warning to •NO investigators that this field of research is rich in unexpected analytical mines and pitfalls. It should be pointed out that when analyzing *S*-nitrosothiols, freezing and storage of samples from in vitro studies and extracts from plasma or serum is not recommended, since even relatively stable *S*-nitrosothiols such as *S*-nitrosoalbumin can decompose rather than be formed artifactually (Tsikas and Frölich, 2004). Preferably *S*-nitrosothiol-containing biological samples should be analyzed immediately after their generation.

11.2.3 Mass Spectrometry Based Methodologies

Mass Spectrometry-Based Analysis of S-Nitrosothiols *S*-Nitroso-*N*-acetyl-L-cysteine and *S*-nitrosoalbumin are the sole endogenous *S*-nitrosothiols that have been determined quantitatively in human plasma by GC–MS based methodologies (Tsikas et al., 1996, 1999d, 2002b). Because *S*-nitroso-*N*-acetyl-L-cysteine could not be found in human plasma and urine at significant concentrations, namely above 1 nM (Tsikas et al., 1996), here the discussion is focused on the GC–MS methods for the quantification of *S*-nitrosoalbumin in human plasma. The principles of these GC–MS methods are shown schematically in Figure 11.6 (Tsikas et al., 1999d, 2002b). Both methods are based on the use of ^{15}N-labeled *S*-nitrosoalbumin as the internal standard on an affinity chromatography extraction of endogenous and ^{15}N-labeled *S*-nitrosoalbumin from plasma, on the conversion of the *S*-nitroso groups into nitrite and ^{15}N-labeled nitrite, respectively, and the final GC–MS detection of unlabeled and ^{15}N-labeled nitrite as pentafluorobenzyl derivatives by GC–MS (Tsikas et al., 1994). Quantification of nitrite by this GC–MS method has been demonstrated to be free of interferences by GC–MS/MS (Tsikas et al., 1999f).

Two *S*-nitroso-group-specific approaches based on different mechanisms have been applied in these methods to convert *S*-nitroso groups into nitrite. The most frequently used approach is the well-known method of Saville that uses $HgCl_2$ (Saville, 1958). The second method applies a reagent consisting of L-cysteine and Cu^{2+} ions. $HgCl_2$ in aqueous solution is used at a high molar excess over thiol and *S*-nitrosothiol concentration, such as at a final concentration of 1 mM (Tsikas et al., 1999d). It is assumed that Hg^{2+} ions interact with

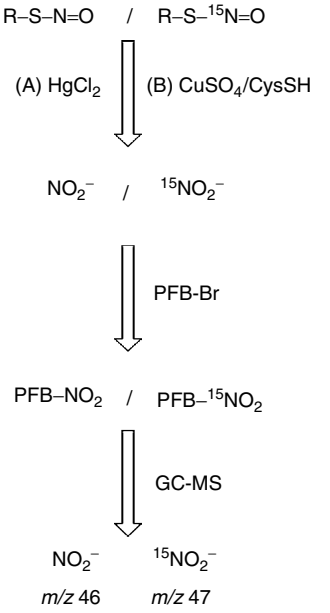


FIGURE 11.6 Schematic of the principles of the GC–MS methods for the quantitative analysis of *S*-nitrosoalbumin (R–S–N=O) in human plasma by GC–MS, using ^{15}N-labeled *S*-nitrosoalbumin (R–S–^{15}N=O) as internal standard, and two approaches to convert the *S*-nitroso group to nitrite, using (*A*) $HgCl_2$ (Tsikas et al., 1999d) and (*B*) $CuSO_4$ and cysteine (Tsikas et al., 2002b). Nitrite (NO_2^-) produced from endogenous *S*-nitrosoalbumin and ^{15}N-labeled nitrite ($^{15}NO_2^-$) produced from the internal standard ^{15}N-labeled *S*-nitrosoalbumin are converted with pentafluorobenzyl bromide (PFB-Br) to the pentafluorobenzyl derivatives that are analyzed by GC–MS. Quantification is carried out by selected-ion monitoring of the ions with mass-to-charge ratios (*m*/*z*) of 46 (NO_2^-) for *S*-nitrosoalbumin and *m*/*z* 47 ($^{15}NO_2^-$) for ^{15}N-labeled *S*-nitrosoalbumin.

the S-atom of the *S*-nitroso group, whereby the intermediatedly formed nitrosonium cation ($^+$NO) is attacked by one molecule of water to finally produce the nitrite anion (Saville, 1958). The use of L-cysteine and Cu^{2+} most likely produces the unstable *S*-nitrosocysteine via a specific *S*-transnitrosylation reaction (Tsikas et al., 2002b). *S*-Nitrosocysteine is highly sensitive toward Cu^{1+} ions—intermediatedly produced via reduction of Cu^{2+} by L-cysteine—that reduce the *S*-nitroso group to $^\bullet$NO, which further autoxidizes to nitrite (Pogrebnaya et al., 1975; Ford et al., 1993). Unlike the $HgCl_2$ reagent, the method using the L-cysteine/Cu^{2+} reagent requires only autocatalytically amounts of Cu^{2+}, namely at a final added concentration of 500 nM, while L-cysteine is used typically at a final concentration of 50 μM. It is worth mentioning that both approaches revealed very similar plasma levels for *S*-nitrosoalbumin in healthy humans, namely 181 nM using $HgCl_2$ (Tsikas et al., 1999d) and 205 nM using L-cysteine/Cu^{2+} (Tsikas et al., 2002b).

The GC–MS methods reported for *S*-nitrosoalbumin (Tsikas et al., 1999d, 2002b) and LMM *S*-nitrosothiols (Tsikas et al., 1996, 1999e) are the only methods that use stable-isotope-labeled analogues as internal standards, such as *S*-[^{15}N]nitrosoalbumin for *S*-nitrosoalbumin. Synthesis of pure and well-characterized unlabeled *S*-nitrosoalbumin and *S*-[^{15}N]nitrosoalbumin preparations is of particular importance. Hydrochloric acid-catalyzed *S*-nitrosylation of the cysteine-34 moiety of albumin is widely used, but it is unspecific because other amino acids of the protein are also abundantly nitrosylated/nitrated and may moreover denature the protein (Tsikas et al., 1999b). In dependence on the analytical method used, these impurities may lead to $^{\bullet}$NO or nitrite formation unrelated to the *S*-nitroso group. It should also be mentioned that preparation of *S*-nitrosoalbumin and *S*-[^{15}N]nitrosoalbumin from commercially available HSA is associated with serious problems arising from the need of reductive treatment such as with 2-mercaptoethanol or dithiothreitol (Tsikas et al., 1999b). The most useful method of synthesis of *S*-nitrosoalbumin is the specific *S*-nitrosylation of albumin in freshly isolated native plasma by means of butylnitrite, which proceeds at plasma pH. Unlabeled and ^{15}N-labeled butylnitrite can be easily synthesized in the laboratory starting from commercially available *n*-butanol and unlabeled or ^{15}N-labeled nitrite (Noyes, 1943). It is recommended to isolate and purify the *S*-nitrosoalbumin by affinity chromatography (Tsikas et al., 1999b). This procedure usually provides *S*-[^{15}N]nitrosoalbumin preparations (in 50 mM potassium buffer, pH 7, containing 1.5 M KCl; buffer B) at a final yield of about 50%, an SDS-PAGE electrophoretic purity of 90%, and a mean concentration of *S*-[^{15}N]nitrosoalbumin of 188 μM in the stock solution. The concentration of *S*-[^{15}N]nitrosoalbumin in its stock solution is accurately determined by GC–MS using unlabeled nitrite as the internal standard after treatment with $HgCl_2$ or L-cysteine/Cu^{2+} (Tsikas et al., 1999b).

The analytical procedure of the quantification of *S*-nitrosoalbumin in human plasma (0.4 ml aliquot) starts with the addition of the internal standard *S*-[^{15}N]nitrosoalbumin at a final plasma concentration of 1 μM (Fig. 11.6). The plasma sample is diluted with a 1.6 ml aliquot of buffer A (50 mM potassium buffer, pH 7) and extracted on a 1 ml HiTrapBlue Sepharose cartridge, which is subsequently washed with a 2 ml aliquot of buffer A. Retained endogenous albumin and *S*-nitrosoalbumin and externally added *S*-[^{15}N]nitrosoalbumin are eluted from the cartridge with a 2 ml aliquot of buffer B. Proteins are concentrated by ultrafiltering the eluate to a remaining protein fraction of 0.4 ml, which corresponds to the initial plasma volume subjected to affinity extraction. Because of a recovery rate of about 50%, the concentration of endogenous *S*-nitrosoalbumin and *S*-[^{15}N]nitrosoalbumin in this fraction is each half of that in the original plasma but most important in the same molar ratio. To two 100 μl aliquots of the concentrated eluate $HgCl_2$ or L-cysteine/Cu^{2+} are added and incubated for 1 hour at room temperature. In further two 100 μl aliquots the concentration of blank nitrite is determined after addition of [^{15}N]nitrite (at 1 μM) as the internal standard in the absence of $HgCl_2$ or L-cysteine/Cu^{2+}. After derivatization with pentafluorobenzyl bromide (PFB-Br) and extraction with

toluene, GC–MS analysis is performed by selected ion monitoring of *m/z* 46 for *S*-nitrosoalbumin and nitrite, and of *m/z* 47 for *S*-[^{15}N]nitrosoalbumin and [^{15}N]nitrite. The concentration of endogenous *S*-nitrosoalbumin in the plasma sample is determined by difference, namely by subtracting the blank nitrite concentration (without $HgCl_2$ or L-cysteine/Cu^{2+}) from the total concentration (with $HgCl_2$ or L-cysteine/Cu^{2+}). Typically blank nitrite concentration in buffer B amounts to 290 nM (Tsikas et al., 1999d). Typical GC–MS chromatograms from the analysis of *S*-nitrosoalbumin in human plasma are shown in Figure 11.7.

Theoretically interferences in these GC–MS methods may include LMM *S*-nitrosothiols bound to albumin, albumin itself due to its very high molar excess over *S*-nitrosoalbumin in the eluate, and *S*-nitrosohemoglobin and hemoglobin, which behave very closely during the affinity extraction step (Tsikas

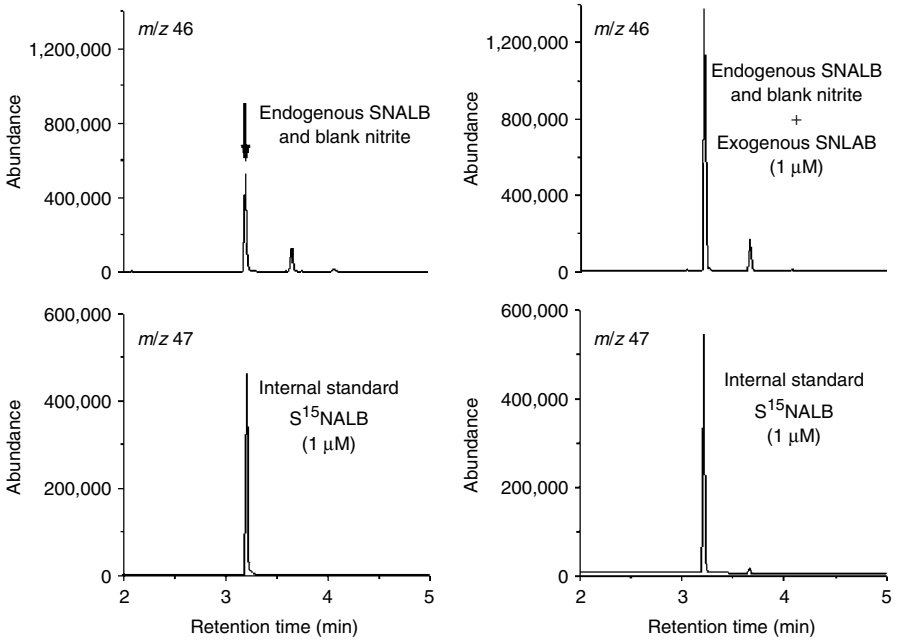


FIGURE 11.7 Partial GC–MS chromatograms from the quantification of *S*-nitrosoalbumin (SNALB) in plasma samples from a healthy volunteer without (*left panel*) and after addition of synthetic SNALB at a final concentration of 1 μM (*right panel*). ^{15}N-Labeled *S*-nitrosoalbumin (S^{15}NALB) was added to plasma at a final concentration of 1 μM. SNALB and S^{15}NALB were extracted from plasma (0.4 ml) by affinity column chromatography, concentrated by ultrafiltration, treated with $HgCl_2$, derivatized with pentafluorobenzyl bromide (PFB-Br), and quantitated by selected-ion monitoring of *m/z* 46 for SNALB (*upper traces*) and *m/z* 47 for S^{15}NALB (*lower traces*) as described (Tsikas et al., 1999d). The peaks eluting at 3.24 minutes correspond to SNALB and S^{15}NALB. Note that the SNALB peak contains also blank nitrite (approximately 300 nM). The concentration of endogenous SNALB in this plasma sample is calculated to be 200 nM.

et al., 1999d). Interferences by *S*-nitrosohemoglobin and hemoglobin are simply eliminated by avoiding hemolysis. LMM *S*-nitrosothiols such as *S*-nitrosocysteine and *S*-nitrosoglutathione were found not to interfere when present in plasma at concentrations below 24 μM, which is most likely at least three orders of magnitude higher than physiological levels. Albumin at concentrations of up to 300 μM in the eluate was found not to interfere with the analysis of *S*-nitrosoalbumin (Tsikas et al., 1999d).

Preanalytical factors such as anticoagulants, which may influence the stability of *S*-nitrosoalbumin, were found not to affect quantification of *S*-nitrosoalbumin in human plasma. Thus in plasma from blood taken from a healthy volunteer by using monovettes that contained EDTA, citrate or heparin, endogenous *S*-nitrosoalbumin was measured at mean concentrations of 120, 120, and 110 nM, respectively (Tsikas et al., 1999d). *S*-Nitrosoalbumin has been shown to be relatively stable in vivo in the rat (Tsikas et al., 2001a) and in vitro in human blood (Tsikas et al., 1999b). The concentration of *S*-nitrosoalbumin in the blood in vivo may change in the blood in vitro after taking of the blood as a consequence of the disturbance of the physiological homeostasis (Stamler, 2004). Mass spectrometry is the sole methodology that allows to investigate this issue on a quantitative basis. For this purpose the internal standard *S*-[^{15}N]nitrosoalbumin is added to EDTA monovettes into which blood is collected by dropping. From the same volunteer, blood is also regularly drawn by using EDTA monovettes; then plasma is generated, and *S*-[^{15}N]nitrosoalbumin is added. By this procedure and quantification by GC–MS no differences in the plasma concentration of *S*-nitrosoalbumin were found in both cases, indicating that the time point of addition of the internal standard *S*-[^{15}N]nitrosoalbumin to the blood or plasma is not crucial (Tsikas et al., 1999d). Furthermore this finding suggests that when plasma is generated immediately after blood taking under mild conditions (e.g., centrifugation at 4°C, 1000 g, 10 min), the concentration of *S*-nitrosoalbumin in the native, untreated plasma will not change significantly.

The GC–MS method for the quantitative determination of *S*-nitrosoalbumin in human plasma has been thoroughly validated (Tsikas et al., 1999d) in accordance with generally accepted methods and criteria (Shah et al., 1992), in addition to studies on interferences discussed above. Validation parameters include precision and accuracy, and limits of detection and quantitation. This issue is discussed in more details below (see Section 11.4.1). In the relevant concentration range of up to 10 μM, endogenous and externally added *S*-nitrosoalbumin was determined with intra-assay and inter-assay precision (relative standard deviation, RSD, in %) less than 10%, and with an accuracy (recovery, in %) ranging between 98% and 111% (Tsikas et al., 1999d). Under the method's conditions the lowest concentration of *S*-[^{15}N]nitrosoalbumin that could be detected as pentafluorobenzyl derivative was 0.2 nM, which corresponds to an injected amount of 67 amol of *S*-[^{15}N]nitroso groups. The lowest concentration of externally added *S*-nitrosoalbumin to a plasma sample (with a basal *S*-nitrosoalbumin concentration of 100 nM), which could be detected with acceptable imprecision (RSD $<$20%) and inaccuracy ($<$20%) (Shah et al., 1992), was 100 nM. Because

S-nitrosoalbumin is measured as nitrite by the method, blank nitrite is the most important analytical parameter that affects the limit of quantitation and the specificity of this method. In the absence of any nitrite the GC–MS method would allow accurate quantification of SNALB in human plasma at concentrations below 1 nM (Tsikas et al., 1999d).

Measurement by this GC–MS method of *S*-nitrosoalbumin in plasma of healthy humans at the basal state revealed concentrations of the order of 150 to 200 nM with high precision (RSD, 7.5% and 10.5%) and accuracy (recovery, 93% and 104%) from accompanying quality control samples at the level of 2 and 5 μM, respectively (Tsikas et al., 1999d, 2002b). The physiological occurrence of *S*-nitrosoalbumin in human plasma at a concentration of the order of 200 nM is strongly supported by platelet aggregation measurements as well as by analysis of *S*-nitroso-*N*-acetyl-L-cysteine formed from *S*-nitrosoalbumin by *S*-transnitrosylation using *N*-acetyl-L-cysteine (Tsikas et al., 1999d). Thus washed human platelets were incubated with extracts from unspiked plasma and plasma spiked with 2 μM of *S*-nitrosoalbumin. Significant inhibition of collagen-induced platelet aggregation and detection of *S*-nitroso-*N*-acetyl-L-cysteine at concentrations above the limit of detection of the HPLC method of 170 nM for *S*-nitroso-*N*-acetyl-L-cysteine were observed only with plasma samples spiked with 2 μM of *S*-nitrosoalbumin (Tsikas et al., 1999d). The values of *S*-nitrosoalbumin in plasma of healthy humans measured by this GC–MS method are approximately 35 times smaller than those reported for the first time by photolysis-based chemiluminescence (Stamler et al., 1992a).

Mass Spectrometry-Based Analysis of 3-Nitrotyrosine Unlike *S*-nitrosothiols, the MS methodology has been applied by several groups to quantify free 3-nitrotyrosine and protein-associated 3-nitrotyrosine in human plasma and serum (Table 11.1). In these methods protein-associated 3-nitrotyrosine is analyzed as 3-nitrotyrosine after chemical or enzymic digestion. The analytical methods listed in Table 11.1 differ in their basic principles, including GC or liquid chromatography (LC), ionization technique (e.g., chemical ionization or electrospray ionization), and mass spectrometry (MS) or mass spectrometry-mass spectrometry (i.e., tandem mass spectrometry, MS/MS). These methods are characterized by different sensitivity, selectivity, and specificity as well as by varying accessibility to interferences, such as artifactual formation of free 3-nitrotyrosine and protein-associated 3-nitrotyrosine and co-elution of unknown substances with similar mass spectrometric properties (Duncan, 2003; Tsikas and Caidahl, 2005). Application of these methodologies to healthy normal humans revealed basal levels for circulating free 3-nitrotyrosine ranging between 0.4 and 64 nM, namely by two orders of magnitude (Table 11.1). Great discrepancies were also found for protein-associated 3-nitrotyrosine; thus the molar ratio of protein-associated 3-nitrotyrosine/protein-associated tyrosine ranged from $1.6{:}1 \times 10^6$ to $35{:}1 \times 10^6$ (Table 11.1). The wide range of reported basal plasma levels of free 3-nitrotyrosine and protein-associated 3-nitrotyrosine in humans

TABLE 11.1 Reported Plasma Free 3-Nitrotyrosine and/or Protein-Associated 3-Nitrotyrosine Mean Levels in Healthy Humans at the Basal State or in the Rat by Mass Spectrometry Based Methodologies

Species	NO_2Tyr (in nM)	NO_2TyrProt ($\times 1{:}10^6$ or pmol/mg)	Method	LOD/LOQ	Purification Step	Reference
Human ($n = 8$)	2.8	N.M.	GC–MS/MS	4 amol/0.125 nM	HPLC	Schwedhelm et al., 1999
Human ($n = 8$)	64	$35 \times 1{:}10^6$	GC–MS	N.R.	SPE	Frost et al., 2000
Human	<4.4	N.M.	LC–MS/MS	1 fmol/4.4 nM	SPE	Yi et al., 2000
Human ($n = 3$)	11	N.M.	GC–MS	70 amol	SPE	Gaut et al., 2002
Human	N.D.	N.R.	LC–MS/MS	3.2 fmol	SPE	Gaut et al., 2002
Rat ($n = 6$)	N.M.	$4–18 \times 1{:}10^6$	LC–MS/MS	N.R.	SPE	Delatour et al., 2002a
Rat ($n = 4$)	<1.4	N.M.	LC–MS/MS	73 fmol/1.4 nM	SPE	Delatour et al., 2002b
Human ($n = 12$)	0.74	0.6 pmol/mg	GC–MS/MS	30 amol/0.3 nM	Dithionite	Söderling et al., 2003
Human ($n = 18$)	0.73	$1.55 \times 1{:}10^6$	GC–MS/MS	N.R.	HPLC	Tsikas et al., 2003a

Note: LOD, limit of detection; LOQ, limit of quantification; N.M., not measured; N.R., not reported; SPE, solid phase extraction; N.D., not detectable.

expressly demonstrates the existence of severe analytical problems in MS-based methods for 3-nitrotyrosine. For the sake of completeness it should be mentioned that non–MS-based methods such as HPLC with fluorescence, UV absorbance or electrochemical detection, as well as immunological methods such as ELISA also provided greatly varying concentrations for free 3-nitrotyrosine and protein-associated 3-nitrotyrosine in human plasma at the basal state, with values frequently being much higher than those provided by MS-based methods (Duncan, 2003; Tsikas and Caidahl, 2005).

Derivatization in GC–MS and GC–MS/MS aims at making possible analysis of polar and nonvolatile compounds such as amino acids by increasing their volatility and thermal stability. Usual derivatization procedures in the GC–MS and GC–MS/MS analysis of 3-nitrotyrosine involve esterification of the carboxylic group, acylation of the α-amino group, and etherification of the 4-hydroxy group (Tsikas and Caidahl, 2005) (Fig. 11.8). In some methods the 3-nitro group of 3-nitrotyrosine is reduced to the amino group, which must also be acylated (Crowley et al., 1998; Söderling et al., 2003). In LC–MS and LC–MS/MS derivatization is not common. Thus 3-nitrotyrosine has been analyzed in human and rat plasma without any derivatization (Yi et al., 2000; Brennan et al., 2002; Delatour et al., 2002a,b; Gaut et al., 2002; Wang et al., 2003). However, preparation of the butyl ester of 3-nitrotyrosine has been demonstrated to significantly improve sensitivity, such as to lower the limit of quantitation by a factor of approximately 3, namely from 4.4 (Yi et al., 2000) to 1.4 nM (Delatour et al., 2002b). Apparently derivatization in LC–MS/MS aims at improving sensitivity in LC–MS/MS, at least when electrospray ionization is used.

Different procedures have been described for the esterification of the carboxylic group, including use of pentafluorobenzyl bromide (Jiang and Balazy, 1998), 3 M HCl in methanol (Schwedhelm et al., 1999; Söderling et al., 2003), 1-propanol (Schwedhelm et al., 1999) or 1-butanol (Delatour et al., 2002a,b), and silylating agents (Frost et al., 2000; Gaut et al., 2002). The α-amino group and the 3-amino group from reduced 3-nitrotyrosine (Crowley et al., 1998; Söderling et al., 2003) are acylated using perfluorated acylating agents such as pentafluoropropionic (Schwedhelm et al., 1999) or heptafluorobutyric anhydride (Frost et al., 2000; Söderling et al., 2003). The 4-hydroxy group of 3-nitrotyrosine has been etherified in order to improve the chromatographic and mass spectrometric properties of 3-nitrotyrosine (Schwedhelm et al., 1999; Söderling et al., 2003).

Hydrochloric acid-catalyzed esterification of 3-nitrotyrosine leads to artifactual formation of 3-nitrotyrosine in the presence of tyrosine. Therefore, artifactual formation of 3-nitrotyrosine may occur both in GC–MS(–MS) and LC–MS(–MS) methods. Three strategies have been developed and used to avoid artifactual formation of 3-nitrotyrosine from endogenous tyrosine and endogenous as well as exogenous nitrite and nitrate during the HCl-catalyzed preparation of the methyl, propionyl or butyl esters. One strategy uses HPLC to isolate 3-nitrotyrosine from plasma ultrafiltrate, thereby separating 3-nitrotyrosine from plasma tyrosine, nitrite, and nitrate (Schwedhelm et al., 1999; Tsikas et al., 2003a). The

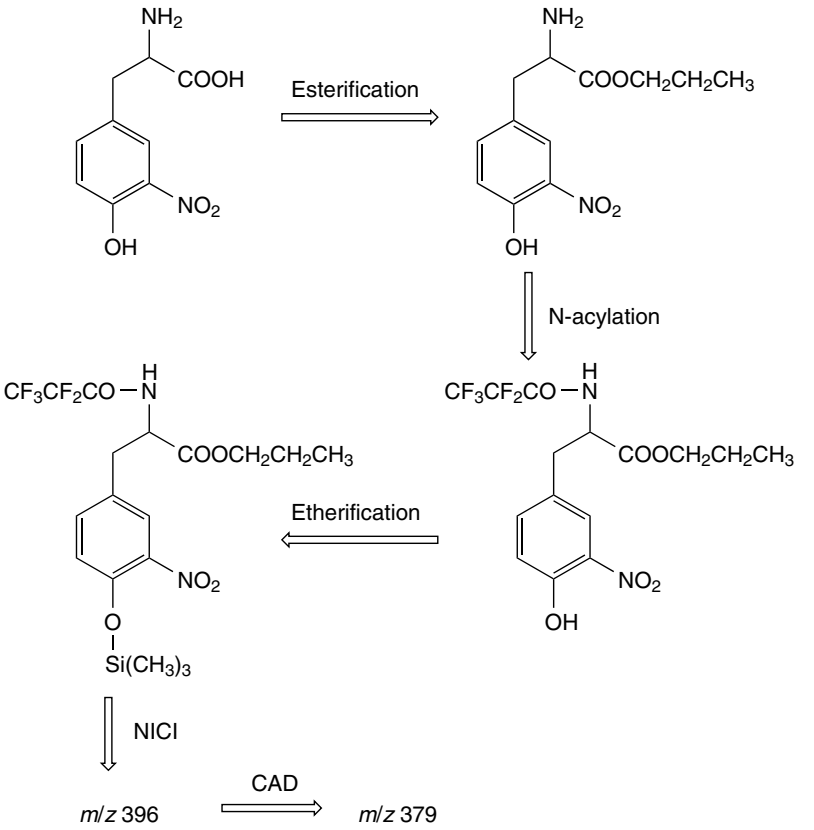


FIGURE 11.8 Derivatization reactions for 3-nitrotyrosine for the quantitative determination in human plasma by GC–MS/MS in the negative-ion chemical ionization (NICI) mode (Schwedhelm et al., 1999). The carboxyl group of 3-nitrotyrosine is esterified to the propyl ester by *n*-propanol/HCl. Subsequently the α-amino group is acylated by pentafluoropropionic anhydride in ethyl acetate. Finally, the aromatic hydroxyl group is converted to its trimethylsilyl ether. The thermally stable derivative of 3-nitrotyrosine is separated gas chromatographically from other compounds, is ionized in the NICI mode, the anion with *m*/*z* 396 is selected by the first quadrupole of the GC–MS/MS instrument, is subjected to collision-activated dissociation (CAD) in the second quadrupole, and the specific product ion with *m*/*z* 379 is selected by the third quadrupole and finally detected. The corresponding ions for the internal standard 3-nitro-L-[$^2H_3$]-tyrosine are *m*/*z* 399 and *m*/*z* 382. Note that the derivatives of unlabeled and 3-nitro-L-[$^2H_3$]-tyrosine are not separated gas chromatographically, but they are discriminated mass spectrometrically (see Fig. 11.9).

second strategy selectively extracts 3-nitrotyrosine from plasma by solid phase extraction after precipitation of plasma proteins by organic solvents (Delatour et al., 2000b). An alternative and effective approach to circumvent acid-catalyzed formation of 3-nitrotyrosine is based on the reduction of the 3-nitro group to

the 3-amino group in combination with sophisticated derivatization/extraction procedures (Söderling et al., 2003). Interestingly, GC–MS/MS and LC–MS/MS methods having applied these procedures revealed very similar 3-nitrotyrosine plasma levels at the basal state in humans (Schwedhelm et al., 1999; Söderling et al., 2003; Tsikas et al., 2003a) and in rats (Delatour et al., 2002b), being of the order of 1 nM. This level for free 3-nitrotyrosine in plasma of healthy humans is supported by a LC–MS/MS method with improved sensitivity (Wang et al., 2003; Svatikova et al., 2004); however, the method applied in these studies has not been fully reported so far.

Co-derivatization of 3-nitrotyrosine and tyrosine extracted from plasma has also been reported by two groups using similar derivatization procedures (Frost et al., 2000; Gaut et al., 2002). Interestingly, among the MS-based methods, these two GC–MS methods have been reported to provide the highest levels in human plasma at the basal state for free 3-nitrotyrosine (Table 11.1), namely 64 nM (Frost et al., 2000) and 11 nM (Gaut et al., 2002), and protein-associated 3-nitrotyrosine, namely $35 \times 1{:}10^6$ (Frost et al., 2000). Investigations by these groups on artifactual formation of 3-nitrotyrosine during the derivatization procedures finally applied have not been reported. Although strong acidic conditions did not prevail in these studies (Frost et al., 2000; Gaut et al., 2002), artifactual formation of 3-nitrotyrosine from tyrosine co-extracted from plasma and blank nitrite and nitrate cannot be excluded. An alternative explanation for the very high plasma levels of 3-nitrotyrosine in those studies could be co-elution of unknown compounds, which produce ions with the same m/z value as the 3-nitrotyrosine derivatives (Frost et al., 2000; Gaut et al., 2002). Such an interference is very likely and has been identified by GC–MS/MS. Thus, despite HPLC separation of 3-nitrotyrosine from tyrosine, nitrite, and nitrate, analysis of the samples by GC–MS revealed several times higher 3-nitrotyrosine plasma levels than quantification by GC–MS/MS (Tsikas and Caidahl, 2005). It should also be emphasized that 3-nitrotyrosine cannot be quantified in plasma by LC–MS, underlining the superiority of the MS-MS technology coupled to LC or GC.

On the basis of the data provided by GC–MS/MS (Schwedhelm et al., 1999; Söderling et al., 2003; Tsikas et al., 2003a) and LC–MS/MS (Yi et al., 2000; Delatour et al., 2002a,b; Wang et al., 2003; Svatikova et al., 2004), the use of a range of 0.5 to 3 nM for free 3-nitrotyrosine and the order of magnitude of 0.6 pmol/mg plasma protein or a molar ratio of 0.4 to $1.6 \times 1{:}10^6$ for protein-associated 3-nitrotyrosine in plasma of healthy humans as reference values appear reasonably justified (Tsikas and Caidahl, 2005). At present, circulating free 3-nitrotyrosine seems to be best quantitable in human plasma at the basal state by the GC–MS/MS technology, because of higher sensitivity and specificity as compared with the LC–MS/MS and the GC–MS methodologies, respectively. Thus the lowest reported limits of quantitation of 0.125 nM (Schwedhelm et al., 1999) and 0.3 nM (Söderling et al., 2003) have been provided by the GC–MS/MS technology. A typical chromatogram from the GC–MS/MS quantification of free 3-nitrotyrosine in human plasma at the basal state is shown in Figure 11.9.

11.3 *S*-NITROSOTHIOLS AND 3-NITROTYROSINE IN HEALTH AND DISEASE

Health and disease can also be defined on the basis of the quantity in biological fluids of a certain biochemical parameter that is specific for a particular biological function, such as serum creatinine for kidney filter function. Therefore quantitative analysis is of particular significance for finding out biomarkers specific for certain diseases and for defining physiological and pathological ranges and states. Circulating 3-nitrotyrosine is frequently used as a biomarker of harmful reactive nitrogen species, with elevated levels indicating enhanced harm to health. Circulating *S*-nitrosothiols are double-edged sword, with the interpretation of their role being rather wishful thinking and ambiguous. Our understanding of the significance of *S*-nitrosothiols and 3-nitrotyrosine in health and disease has

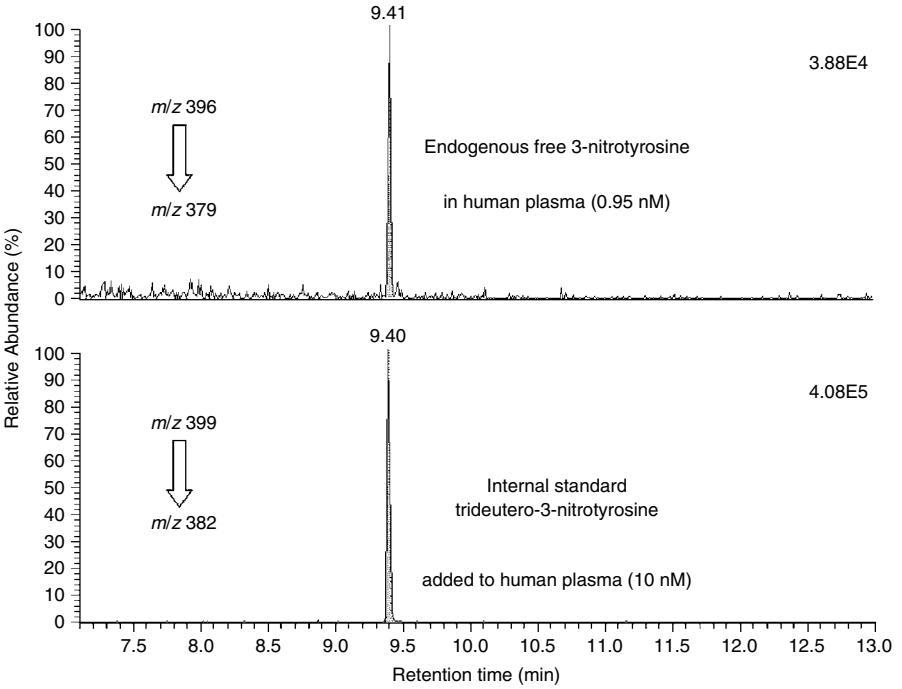


FIGURE 11.9 Partial GC–MS/MS chromatograms from the quantification of free 3-nitrotyrosine in plasma from a healthy volunteer. Plasma (2 ml) was spiked with the internal standard 3-nitro-L-[$^2H_3$]-tyrosine (10 nM); endogenous and the trideutero-3-nitrotyrosine were isolated from ultrafiltrate (200 μl) by HPLC and SPE, derivatized and quantitated by selected-reaction monitoring of the ions *m*/*z* 379 generated from *m*/*z* 396 (upper trace) for endogenous 3-nitrotyrosine and the ions *m*/*z* 382 generated from *m*/*z* 399 (lower trace) for the internal standard as described (Schwedhelm et al., 1999) (see Fig. 11.8). The concentration of endogenous free 3-nitrotyrosine in this plasma sample is determined to be 0.95 nM.

been confounded by problems associated with the analytical approaches that have been employed to quantify the free and protein-associated forms of these compounds in human circulation. Duncan recently stated that: "Our understanding of the biological significance of reactive nitrogen species will only advance when we ensure that validated analytical data form the foundations of our understanding." (Duncan, 2003). Duncan also predicted with regard to 3-nitrotyrosine that: "Some of the high levels and dramatic changes observed in earlier work may not prove real." (Duncan, 2003). Indeed, recent advances in the analytical chemistry of 3-nitrotyrosine, especially in tandem mass spectrometry, let this prediction come true (Tsikas and Caidahl, 2005), expressly emphasizing the importance of carefully validated quantitative analytical methods in this area of research. *S*-Nitrosothiols are still a problem child and a challenging analytical task. In consideration of the great discrepancies regarding basal levels of *S*-nitrosothiols, Stamler expressed the opinion that the quantity of these compounds is not of relevance (Stamler, 2004). However, this would mean a capitulation to analytical difficulties. Moreover nonconsideration of circulating intervals for circulating *S*-nitrosothiols would give free rein to consider any used analytical method to be adequate and any measured value to be valid (Tsikas, 2004). Thus it is somewhat odd to rate findings of studies in which basal levels of *S*-nitrosoalbumin have been reported to be in the low nM range (Cannon et al., 2001; Ng et al., 2004) and in the low μM range (Tyurin et al., 2001; Massy et al., 2004), in particular, when analytical shortcomings are obvious. In consideration of the importance of accurate quantitative analysis in vivo in humans, below previous studies on circulating *S*-nitrosothiols (Table 11.2) and 3-nitrotyrosine (Table 11.3) in health and disease are critically discussed in particular from the analytical standpoint.

11.3.1 *S*-Nitrosothiols

The first reported plasma levels of *S*-nitrosothiols in five healthy humans were measured by photolysis-chemiluminescence to be 7.2 ± 5.7 μM for total *S*-nitrosothiols of which 6.9 ± 5.5 μM being attributed to *S*-nitrosoproteins (Stamler et al., 1992a). The method's linearity for various *S*-nitrosothiols including *S*-nitroso-bovine serum albumin, which was prepared from bovine serum albumin with acidified nitrite (Stamler et al., 1992b), sensitivity as well as intra-assay variability were reported, but data on the method's accuracy were missing (Stamler et al., 1992a). The photolysis-chemiluminescence method originally used to quantitate *S*-nitrosothiols in human plasma (Stamler et al., 1992a) was reported several years later in detail by the same group (Alpert et al., 1997). Unfortunately, in that study no *S*-nitrosothiol levels was determined in human plasma. Nevertheless, detailed description of the analytical method made clear that potential analytical problems may occur due to the variability in the efficiency of the photolysis to release $^{\bullet}$NO from certain *S*-nitrosothiols and due to interferences by other species including nitrite, *N*-nitroso, and *Fe*-nitroso compounds (Alpert et al., 1997).

TABLE 11.2 Reported Venous Plasma or Serum Mean Levels of *S*-Nitrosoalbumin and Other *S*-Nitrosoproteins in Health and Disease Measured by Various Methodologies in Clinical Studies

Health/Disease	RSNO (in nM)	Study	Method	Detected Species	Remark
Health ($n = 5$)	7190	Stamler et al., 1992a	Photolysis-CL	Nitric oxide	
Health ($n = 64$?)	450	Marzinzig et al., 1997	$HgCl_2$/DAN-fluorimetry	Nitrite	NEM; pH 7.4
Sepsis ($n =$?)	1300	Marzinzig et al., 1997	$HgCl_2$-fluorimetry	Nitrite	NEM; pH 7.4
Health ($n = 23$)	181	Tsikas et al., 1999d	$HgCl_2$/GC–MS	Nitrite	Tsikas et al., 1994
Liver disease ($n = 40$)	161	Tsikas et al., 1999d	$HgCl_2$/GC–MS	Nitrite	Tsikas et al., 1994
Renal disease ($n = 6$)	147	Tsikas et al., 1999d	$HgCl_2$/GC–MS	Nitrite	Tsikas et al., 1994
Health ($n = 10$)	63	Gladwin et al., 2000	Iodide/iodine-CL	Nitric oxide	$^{\bullet}$NO analyzer
Health ($n = 5$)	62	Jourd'heuil et al., 2000	$HgCl_2$/Griess-HPLC	Nitrite	LOD, 20 nM
Normal pregnancy ($n = 21$)	5100	Tyurin et al., 2001	Photolysis/DAF-2-fluorimetry	Nitric oxide	Kojima et al., 1998
Preeclampsia ($n = 21$)	6300	Tyurin et al., 2001	Photolysis/DAF-2-fluorimetry	Nitric oxide	Kojima et al., 1998
Health ($n = 11$)	250	Moriel et al., 2001	$HgCl_2$/KI-CL	Nitric oxide	$^{\bullet}$NO analyzer
Hypercholestrolemia ($n = 18$)	540	Moriel et al., 2001	$HgCl_2$/KI-CL	Nitric oxide	$^{\bullet}$NO analyzer
Health ($n = 10$)	205	Tsikas et al., 2002b	Cu^{2+}/Cysteine	GC–MS	Tsikas et al., 1994
Health ($n = 12$)	450	Massy et al., 2003	$HgCl_2$-fluorimetry	Nitrite	Marzinzig et al., 1997
Renal disease ($n = 22$)	2250	Massy et al., 2003	$HgCl_2$-fluorimetry	Nitrite	Marzinzig et al., 1997
Health ($n =$?)	8800	Wlodek et al., 2003	$HgCl_2$-fluorimetry	Nitrite	Marzinzig et al., 1997
Renal disease ($n =$?)	11,250	Wlodek et al., 2003	$HgCl_2$-fluorimetry	Nitrite	Marzinzig et al., 1997

Note: CL, chemiluminescence; DAN, 1,2-diaminonaphthalene; NEM, *N*-ethylmaleimide; LOD, limit of detection.

TABLE 11.3 Reported Circulating Free 3-Nitrotyrosine and Protein-Associated 3-Nitrotyrosine NO_2TyrProt as 3-Nitrotyrosine/Tyrosine Molar Ratio Levels in Health and Disease Measured by Various Methodologies in Methodological and Clinical Studies

Health/Disease/Treatment	NO_2Tyr (in nM)	NO_2TyrProt ($\times 1{:}10^6$, in pmol/mg or in nM)	Method	Reference	Remark
Health ($n = 9$)	31	N.M.	HPLC-fluorescence	Kamisaki et al., 1996	LOQ, 6 nM
Health ($n = 2$)	N.M.	5.1 pmol/mg	HPLC/GC-TEA	Petruzzelli et al., 1997	LOD, 88 pmol
Smokers ($n = 11$)	N.M.	571 pmol/mg	HPLC/GC-TEA	Petruzzelli et al., 1997	LOD, 88 pmol
Health ($n = 4$)	N.D.	N.D.	ELISA	ter Steege et al., 1998	LOQ, 0.2 nM
Celiac disease ($n = 12$)	N.D.	1270 nM	ELISA	ter Steege et al., 1998	LOQ, 0.2 nM
Health ($n = 4$)	N.D.	120 nM	ELISA	Khan et al., 1998	IC_{50}, 5–100 nM
Systemic sclerosis ($n = 20$)	N.D.	293 nM	ELISA	Khan et al., 1998	IC_{50}, 5–100 nM
Health ($n = 8$)	2.8	N.M.	GC–MS/MS	Schwedhelm et al., 1999	LOQ, 0.125 nM
Health ($n = 9$)	14	N.M.	HPLC-UV	Strand et al., 2000	Mobile phase pH 2.0
Septic shock ($n = 16$)	102	N.M.	HPLC-UV	Strand et al., 2000	Mobile phase pH 2.0
Health ($n = 25$)	N.M.	N.D.	ELISA	Ceriello et al., 2001	LOQ, 10 nM
Diabetes type II ($n = 40$)	N.M.	251 nM	ELISA	Ceriello et al., 2001	LOQ, 10 nM
Health ($n = 12$)	0.74	0.6 pmol/mg	GC–MS/MS	Söderling et al., 2003	
Health ($n = 18$)	0.73	$1.55 \times 1{:}10^6$	GC–MS/MS	Tsikas et al., 2003a	
Health ($n = 10$)	0.75	$1.40 \times 1{:}10^6$	GC–MS/MS	Tsikas and Caidahl, 2005	
Renal disease ($n = 12$)	2.24	$1.78 \times 1{:}10^6$	GC–MS/MS	Tsikas and Caidahl, 2005	

Note: N.M., not measured; LOQ, limit of quantitation; N.D., not detectable.

Potential interferences by nitrate and LMM thiols in photolysis-chemiluminescence were not investigated in those studies (Stamler et al., 1992a; Alpert et al., 1997), but lately by another group (Dejam et al., 2003). L-Cysteine was used at the very high, nonrelevant concentration of 1 mM—in plasma LMM thiol concentration is below 20 μM (Mansoor et al., 1992)—and, most important, the effect of the most abundant thiol in plasma (i.e., albumin) was not investigated (Dejam et al., 2003). Nevertheless, plasma nitrate and thiols should be considered as potential interferences in all those methods of analysis of *S*-nitrosothiols that are based on direct photolysis of plasma without any analytical steps to remove thiols and nitrate.

It is noteworthy that quantification of *S*-nitrosothiols in healthy pregnancy plasma by batch-fluorimetry after photolysis yielded *S*-nitrosoalbumin levels of 5.1 ± 0.7 μM and total *S*-nitrosoprotein levels of 9.4 ± 1.5 μM (Tyurin et al., 2001), which are among the highest levels reported thus far (Giustarini et al., 2004). It should be pointed out that the method used in that study (i.e., Tyurin et al., 2001) had been reported by other group (Kojima et al., 1998) for in vitro qualitative analysis of $^{\bullet}NO$ only, but not for quantitative determination of *S*-nitrosothiols in plasma or serum. The same group (i.e., Gandley et al., 2005) applied the so-called biotin switch assay (Jaffrey and Snyder, 2001) to determine *S*-nitrosoalbumin levels in plasma and found only approximately 2.9 μM in pooled plasma in healthy pregnancy (Gandley et al., 2005). The reliability of the biotin switch method for the accurate quantification of *S*-nitrosoalbumin in human plasma, the selectivity of ascorbate-catalyzed reduction of the *S*-nitroso group, and the comparison of this assay with other quantitative methods remain, however, to be demonstrated. The biotin switch assay has been recently characterized and applied to identify *S*-nitrosoproteins (Zhang et al., 2005). The results of this study indicate that the sensitivity and specificity of the biotin switch assay are closely related to the concentrations of ascorbate and *S*-nitrosothiol as well as the structures of the *S*-nitrosothiol species (Zhang et al., 2005). Thus *S*-nitrosoalbumin has been found to be stable against reduction by ascorbate, indicating that likely many factors, including neighboring amino acids, thiol pK_a, and steric issues, can dramatically affect ascorbate-mediated *S*-nitrosothiol decomposition (Zhang et al., 2005). The biotin switch method seems to be suitable for qualitative detection of *S*-nitrosylated proteins such as in brain lysates and in selected neuronal proteins (Jaffrey and Snyder, 2001; Jaffrey et al., 2001) or in cell culture in vitro by incubating cells with very high irrelevant concentrations of *S*-nitrosocysteine (Zhang et al., 2005) rather than for quantitative measurements in human plasma.

Although the conversion of the *S*-nitroso group to nitrite by means of $HgCl_2$ (Saville, 1958) or cysteine/Cu^{2+} (Tsikas et al., 1999d, 2002b) are specific, application of methods based on these principles to quantitative analysis requires elimination of nitrite nonrelated to *S*-nitrosothiols or consideration (i.e., accurate measurement) of blank nitrite concentration in the respective matrix. By evidence (Tsikas, 2003; Ng et al., 2004), elimination of nitrite by ammonium sulfamate requires acidic conditions. Therefore in all those studies

where ammonium sulfamate was used under plasma-pH conditions and plasma nitrite concentrations were not determined or simply neglected (Marzinzig et al., 1997; Massy et al., 2003, 2004; Wlodek et al., 2003), the values reported for plasma *S*-nitrosothiols and the conclusions made are questionable.

In sepsis, serum *S*-nitrosothiols were reported to be elevated as compared to healthy humans, namely 1.3 μM versus 0.45 μM, but also nitrite + nitrate levels were found to be elevated, namely 64 μM versus 34 μM, respectively (Marzinzig et al., 1997). Also in urine from septic patients *S*-nitrosothiols were reported to be elevated as compared to healthy humans, namely 8.9 μM versus < 0.15 μM, but also nitrite + nitrate were found to be elevated, namely 2050 μM versus 504 μM, respectively (Marzinzig et al., 1997). As the respective nitrite levels had not been determined in that study, and because nitrite levels in serum, plasma, and urine even of healthy humans amounted to 0.5 to 3 μM (Tsikas, 2005a,b), we may reasonably assume that the greatest portion of the reported *S*-nitrosothiol levels in sepsis is attributed to nitrite (Tsikas, 2003).

Other studies (Massy et al., 2003, 2004; Wlodek et al., 2003) in which the same method of *S*-nitrosothiol analysis was used (i.e., Marzinzig et al., 1997) can be discussed from the same standpoint. Thus *S*-nitrosothiol plasma mean levels were measured to be 8.8 μM in healthy humans (Wlodek et al., 2003)—it should be noted that the same method revealed only 0.45 μM for plasma *S*-nitrosothiols in healthy humans (Marzinzig et al., 1997)—and 11.0 μM in patients with chronic renal failure, with corresponding nitrite + nitrate plasma levels being 120 μM and 135 μM, and plasma nitrite levels not being reported (Wlodek et al., 2003). This and other analytical shortcomings in that study (Wlodek et al., 2003) question the finding of elevated *S*-nitrosothiol plasma levels in chronic renal failure (Tsikas et al., 1999d, 2004; Giustarini and Rossi, 2004). Application of the same method (i.e., Marzinzig et al., 1997) by the other group (Massy et al., 2003) to patients on chronic hemodialysis revealed elevated levels for plasma *S*-nitrosothiols (i.e., 2.25 ± 1.17 μM) as compared to healthy humans (i.e., 0.45 ± 0.45 μM). Again, plasma nitrite had not been measured in that study and its contribution to *S*-nitrosothiols, in particular, in patients suffering from chronic renal failure, is unknown. It should be emphasized that in chronic renal failure, nitrate (Tsikas et al., 1999d), free 3-nitrotyrosine (Fig. 11.10) (Tsikas and Caidahl, 2005), and other LMM compounds such as asymmetric dimethylarginine (i.e., ADMA; Zoccali et al., 2001) were found to accumulate in the blood, unlike *S*-nitrosoalbumin, that is, 147 ± 55 nM in chronic renal failure ($n = 6$) versus 181 ± 150 nM in healthy humans ($n = 23$; Tsikas et al., 1999d) and protein-associated 3-nitrotyrosine (Tsikas and Caidahl, 2005), as measured by fully validated and reliable MS-based methods. Interestingly elevated circulating and urinary levels of nitrate (Arkenau et al., 2002; Tsikas et al., 1999d) and ADMA (Tsikas et al., 2003b) were also found in chronic liver disease, with nitrate levels positively correlating with disease stage and parameters of hyperdynamic circulation (Arkenau et al., 2002). By contrast, plasma *S*-nitrosoalbumin levels in hepatic disease (161 ± 274 nM, $n = 40$) did not differ from those in health (181 ± 150 nM, $n = 23$) and did not correlate

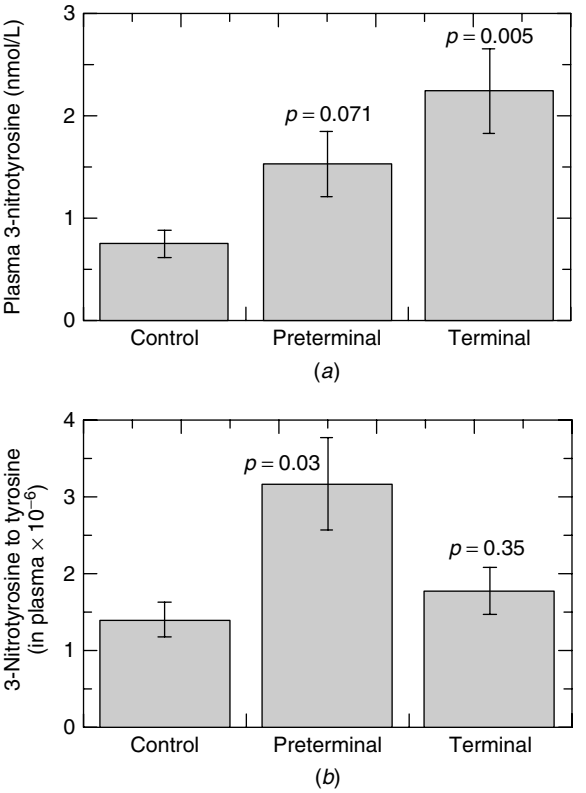


FIGURE 11.10 Plasma levels of (*a*) free 3-nitrotyrosine and (*b*) protein-associated 3-nitrotyrosine (expressed as the molar ratio of 3-nitrotyrosine to tyrosine) in health (control; mean age 51.2 years, $n = 10$) and chronic renal failure at two disease stages (preterminal, terminal). The preterminal (mean age 59.1 years, $n = 15$) and terminal (mean age 52.8 years, $n = 12$) groups comprised of patients with serum creatinine levels above 530 μM but not on dialysis and serum creatinine levels above 700 μM, respectively. Values are shown as mean ± SEM. Free and protein-associated 3-nitrotyrosine were determined by GC–MS/MS as described (Tsikas et al., 2003a).

with plasma levels of nitrate, albumin, or stage of hepatic disease (Tsikas et al., 1999d).

Nitrite, nitrate, and *S*-nitrosothiols are frequently measured by iodide-chemiluminescence on commercially available •NO analyzers (e.g., NOA^{TMS280}; Sievers Instruments Inc.). Quantification of total *S*-nitrosothiols in plasma by such analyzers is based on a calibration curve prepared using aqueous nitrite solution and by subtracting the signal obtained without pretreatment from that obtained after $HgCl_2$ treatment (Moriel et al., 2001). Application of such an assay to a human study revealed that *S*-nitrosothiol plasma levels are elevated in hypercholesterolemia as compared to health (540 nM versus

250 nM; Moriel et al., 2001). Other groups applied commercially available •NO analyzers but different experimental protocols to measure *S*-nitrosothiols, nitrite and nitrate (Yang et al., 2003). It is worth mentioning that *S*-nitrosothiol levels in plasma of healthy humans measured by •NO analyzers of the same type may depend on the procedures used to distinguish between nitrite and *S*-nitrosothiols; for example, they may amount to 250 nM (Moriel et al., 2001) or 63 nM (Gladwin et al., 2000).

S-Nitrosothiols in plasma (and serum) have been repeatedly measured in health and disease by many groups. By contrast, erythrocytic *S*-nitrosohemoglobin (SNOHb) has been measured by few groups and almost exclusively in health (Giustarini et al., 2004). This fact reflects analytical difficulties regarding this specific *S*-nitrosothiol rather than underestimation of the SNOHb's significance in human organism. Photolysis-chemiluminescence was applied to measure SNOHb and *Fe*-nitrosohemoglobin (HbFeNO) in rat blood (Jia et al., 1996). At the basal state, endogenous levels were measured as 311 nM for SNOHb and 536 nM for HbFeNO in arterial blood, and 32 nM for SNOHb and 894 nM for HbFeNO in venous blood (Jia et al., 1996), suggesting considerable concentration gradients between venous and arterial blood. A batch-fluorimetry method based on the use of $HgCl_2$ and DAN reagents revealed that the levels of SNOHb in red blood cells from venous blood of healthy humans are below 100 nM, namely below the detection limit of the method, while *S*-nitrosoalbumin plasma levels were determined to be 62 nM by a similar method (Jourd'heuil et al., 2000). By using ozone-based chemiluminescence and two different methods to release •NO from LMM and HMM *S*-nitrosothiols; that is, use of Cu^{1+}/L-cysteine or I_3^-, LMM *S*-nitrosothiols in serum was found to be below 25 nM, whereas HMM *S*-nitrosothiols in serum were determined to be 45 nM from arterial blood and 63 nM from venous blood (Gladwin et al., 2000). In the same study using the same analytical method, SNOHb was determined to be 161 nM in whole arterial blood and 142 nM in whole venous blood, suggesting no significant concentration gradient (Gladwin et al., 2000). It was reported that the technique used in that study had been validated using laboratory-synthesized SNOHb, the specificity of β-Cys-93 modification being confirmed by LC–MS with electrospray ionization after enzymatic digestion. Unfortunately, the respective data and other potentially interesting details had not been reported (Gladwin et al., 2000). Thus not only different concentrations have been reported for erythrocytic SNOHb, but there are also qualitative discrepancies with regard to arteriovenous differences in SNOHb levels. Arteriovenous SNOHb gradient is of particular interest because it forms the basis for understanding the role of hemoglobin in the regulation of •NO vasoactivity (Frehm et al., 2004; Giustarini et al., 2004).

In summary, in the literature there is no further example for the development and application of so wide a spectrum of analytical approaches and methods in recent years for *S*-nitrosothiols that has yielded highly divergent values, within a range of almost four orders of magnitude, and has led to numerous deceptive

conclusions. The value of the methodology for the accurate quantitative measurement of plasmatic and erythrocytic *S*-nitrosothiols has been underestimated, although there is pressing and convincing evidence for its pivotal significance to understanding of the *S*-nitrosothiol roles in the vasculature (Giustarini et al., 2004). The significance of circulating *S*-nitrosothiols in health and disease is not as nearly as well understood.

11.3.2 3-Nitrotyrosine

β-Blockers such as nebivolol, carvedilol, and metoprolol have been associated with additional antioxidative effects in vitro. A fully validated GC–MS/MS method (Schwedhelm et al., 1999) was applied to study effects of these β-blockers at standard antihypertensive doses in healthy humans with normal clinical history (Troost et al., 2000; Fahlbusch et al., 2004). These β-blockers were found to have no statistically significant effects on free 3-nitrotyrosine (NO_2Tyr) and protein-associated 3-nitrotyrosine (NO_2TyrProt) as compared with the placebo group (Troost et al., 2000; Fahlbusch et al., 2004). Nevertheless, carvedilol and metoprolol showed a trend toward reduction of NO_2Tyr and NO_2TyrProt below basal levels (Fahlbusch et al., 2004). This was the first clinical study to show that NO_2Tyr and NO_2TyrProt levels can be lowered below normal levels.

Increased vascular superoxide production is currently discussed as a mechanism leading to nitrate tolerance in nonintermittent therapy. Application of a GC–MS/MS method (Schwedhelm et al., 1999; Tsikas et al., 2003a) in the frame of a clinical study showed that administration of therapeutically relevant doses of the organic nitrates pentaerythrityl tetranitrate (PETN) or isosorbide dinitrate (ISDN) to healthy humans did not result in increase of circulating NO_2Tyr and NO_2TyrProt and urinary NHPA as compared to the data before drug administration (Keimer et al., 2003). These findings contradict previous report on statistically significant increases (by a factor of 3) in free 3-nitrotyrosine levels in urine upon administration of glycerol trinitrate (Schwemmer et al., 2000). In that study 3-nitrotyrosine in urine had been determined by using a nonvalidated GC method with flame ionization detection that revealed basal excretion rates for 3-nitrotyrosine of 248 nmol/day (Schwemmer et al., 2000). This excretion rate is approximately 54 times higher than that measured by a fully validated GC–MS/MS method, namely 4.6 nmol/day (Tsikas et al., 2005), and 4 times higher than those measured by others using a commercially available ELISA method (Ferdinandy et al., 2000; Szilvássy et al., 2001; Radák et al., 2003). In the same study (i.e., Schwemmer et al., 2000) an ELISA method was also applied to measure 3-nitrotyrosine in urine and plasma. It was reported that: “In principle, plasma and urine n-tyr content, as determined by the ELISA method, closely correlated with values obtained by gas chromatography. ... Thus, we used the ELISA as confirmatory method (data not shown) to further support the n-tyr values obtained by gas chromatography” (Schwemmer et al., 2000). In that study, n-tyr means 3-nitrotyrosine and the ELISA method was used

according to a method reported by another group (Khan et al., 1998). From the analytical standpoint, especially on the basis of quantitative data, the author's findings (Schwemmer et al., 2000) do not allow one to definitively conclude that organic nitrates enhance oxidative stress.

The highest plasma levels of NO_2Tyr and NO_2TyrProt measured from the application of GC–MS/MS methods (Schwedhelm et al., 1999; Tsikas et al., 2003a) in human studies were observed in patients suffering from end-stage renal disease (Tsikas and Caidahl, 2005) (Fig. 11.10). In comparison with the control group comprising of healthy volunteers (0.75 nM), the patients had higher NO_2Tyr levels, namely 1.5 nM in the preterminal group and 2.3 nM in the terminal group. Quantification of NO_2TyrProt by a GC–MS/MS method resulted in approximate values of 20 nM for 3-nitrotyrosinoalbumin in plasma of healthy volunteers and in slightly elevated levels in patients suffering from end-stage renal disease (Fig. 11.10). Unchanged NO_2TyrProt plasma levels have been reported by other group for chronic hemodialysis patients in comparison to healthy volunteers as measured by a commercially available ELISA method (Massy et al., 2003), with reported levels being approximately 4,000 nM, or 200 times higher than those measured by GC–MS/MS (Tsikas and Caidahl, 2005). Although renal disease may be associated with elevated oxidative stress, it may be assumed that the elevated plasma levels, in particular, of NO_2Tyr (Fig. 11.10), are largely due to accumulation rather than due to highly elevated oxidative stress. It is worth mentioning that even the well-dialyzable nitrate anion may accumulate in plasma of patients suffering from chronic renal diseases (56.5 μM) as compared to healthy volunteers (35.6 μM), unlike *S*-nitrosoalbumin (Tsikas et al., 1999d) and NO_2TyrProt (Fig. 11.10).

Application of the GC–MS/MS methodology to clinical studies clearly suggests that the changes in NO_2Tyr plasma levels resulting from therapeutical intervention, disease, or age should be considered rather moderate (Tsikas and Caidahl, 2005). This is also supported by an improved LC–MS/MS method, although not fully described so far (Wang et al., 2003; Svatikova et al., 2004). Obstructive sleep apnea (OSA) has been linked to cardiovascular disease, endothelial dysfunction, and oxidative stress, generated by repetitive nocturnal hypoxemia and reperfusion. OSA patients and healthy controls were found to have almost identical plasma levels of NO_2Tyr, namely 0.66 nM in the OSA group and 0.62 nM in the control group (Svatikova et al., 2004).

NO_2TyrProt was determined by a LC–MS/MS method in patients with established coronary artery disease (CAD) and in patients with no clinically evident CAD (Shishehbor et al., 2003a). NO_2TyrProt ratios were $9 \times 1{:}10^6$ in the patients' plasma with CAD and $5 \times 1{:}10^6$ in the patients' plasma without CAD. In an interventional study the same group has found by LC–MS/MS that treatment with atorvastatin of hypercholesterolemic subjects with no known CAD led to a significant reduction (by 25%) in NO_2TyrProt levels from $15 \times 1{:}10^6$ at baseline to $11 \times 1{:}10^6$ after 12 weeks (Shishehbor et al., 2003b). Unfortunately, these studies did not report on NO_2TyrProt levels in healthy volunteers nor on NO_2Tyr

levels in the patients investigated. Furthermore the range of the NO_2TyrProt values reported by this group (Shishehbor et al., 2003a,b) is unusually wide and the reported levels are considerably greater than those measured in the patients suffering from end-stage renal disease (Tsikas and Caidahl, 2005). Last, it should be pointed out that the LC–MS/MS method used in those clinical studies (Shishehbor et al., 2003a,b) had not been originally developed for plasma samples and has not been reported in detail in a chemical analysis oriented journal (Brennan et al., 2002).

Immunodot and Western blot have been used to measure nitrated and oxidized plasma proteins in smokers and lung cancer patients (Pignatelli et al., 2001). It has been reported that cigarette smoking increases oxidative stress and that during lung cancer development, formation of reactive nitrogen species results in nitration and oxidation of plasma proteins (Pignatelli et al., 2001). However, in plasma of healthy nonsmokers NO_2TyrProt has been detected at 170 pmol/mg protein (Pignatelli et al., 2001). This value is at least 280 times higher than the NO_2TyrProt basal levels provided by fully validated GC–MS/MS methods (Söderling et al., 2003; Tsikas et al., 2003a). Therefore the results of this and other studies reporting very high levels for NO_2TyrProt from the use of analytically insufficiently characterized methods should be treated with caution. The sequential HPLC/GC thermal analysis technique has also been used to measure NO_2TyrProt in plasma of healthy cigarette smokers and nonsmokers (Petruzzelli et al., 1997). This method was incorporated into molecular epidemiologic studies for lung chronic inflammatory and neoplastic disorders in which exposure to oxidants posed an important risk factor (Petruzzelli et al., 1997). It should be, however, mentioned that the HPLC/GC thermal analysis technique lacks in sensitivity and selectivity. Moreover it is of limited value in quantitative analysis because NO_2TyrProt was measured only in 11% of the nonsmokers group and in 55% of cigarette smokers (Petruzzelli et al., 1997).

The essential objections to the use of antibody-based methods such as ELISA for quantitative measurement of 3-nitrotyrosine were addressed in Section 11.2.1 and elsewhere (Duncan, 2003). Although some ELISA methods for 3-nitrotyrosine are homemade, some of their analytical characteristics have been reported, and assays have been used to quantitate 3-nitrotyrosine in human plasma in health and disease (ter Steege et al., 1998; Ceriello et al., 2001; Oldreive et al., 2001). Surprisingly plasma 3-nitrotyrosine in healthy humans could not be found by the two ELISA methods, despite low limits of detection of 0.2 nM (ter Steege et al., 1998) and 10 nM (Ceriello et al., 2001) having been reported satisfactorily, whereas a third ELISA method allowed determination of basal plasma levels of protein-associated 3-nitrotyrosine (Oldreive et al., 2001). Nevertheless, application of these ELISA methods to measure protein-associated 3-nitrotyrosine in disease (ter Steege et al., 1998; Ceriello et al., 2001) revealed highly elevated levels of 1270 nM in patients suffering from celiac disease (ter Steege et al., 1998) and 251 nM in patients with type II diabetes mellitus (Ceriello et al., 2001). It should be noted that such high levels for protein-associated 3-nitrotyrosine can be observed by ELISA (Khan et al., 1998)

and GC–MS/MS (Tsikas et al., 2003a) only from in vitro nitration of BSA by peroxynitrite at the extraordinarily high concentrations of 100 to 500 μM. The apparently drastic increases in protein-associated 3-nitrotyrosine measured by ELISA methods (ter Steege et al., 1998; Ceriello et al., 2001) give rise to doubt the reliability of such ELISA assays in quantitative measurement of 3-nitrotyrosine. Several ELISA methods for 3-nitrotyrosine are commercially available, and some of these methods (e.g., HyCultBiotechnology; ⟨www.hbt.nl⟩) are cited in the literature (ter Steege et al., 1998), indicating that the marketed assays are based on published assays. Some dubious ELISA methods have frequently been used in quantitative measurements of protein-associated 3-nitrotyrosine in human plasma within clinical studies that follow the protocols provided by the manufacturers, and there is no explicit mention that the protocols of the assays are valid and proper for quantitative analysis in plasma or serum.

HPLC, both with UV absorbance and fluorescence detection, has proved to be too insensitive for the quantification of NO_2Tyr in human plasma at the basal state (Duncan, 2003; Tsikas and Caidahl, 2005). Nevertheless, it has been reported that NO_2Tyr plasma levels are highly elevated in human septic shock (i.e., 59 nM, with peak plasma concentration of 160 nM) as compared to those of healthy, medication-free, nonsmoking volunteers (i.e., 14 nM) as measured by HPLC analysis with UV absorbance detection at 355 nm of only a 20 μl aliquot of plasma ultrafiltrate (Strand et al., 2000), whereas the limit of detection of such HPLC methods is clearly above 20 nM from injection of 200 μl aliquots (Du et al., 2004; Liu et al., 1998; Tsikas and Frölich, 2004). In the study mentioned above (i.e., Strand et al., 2000) no data were reported on the validity of the HPLC method, and neither was the acid-catalyzed artifactual formation of 3-nitrotyrosine from endogenous tyrosine and nitrite and nitrate investigated during HPLC analysis under the acidic conditions of the mobile phase, which had a pH value of 2.0. It is worth mentioning that in septic shock, nitrite and nitrate levels in blood are highly elevated (Ellis et al., 1998; Strand et al., 2000). In human septic shock *S*-nitrosothiols were found by means of a commercially available assay kit not to be elevated as compared to healthy controls, namely 2.9 versus 2.2 μM (Strand et al., 2000).

In summary, MS-based analytical methods, in particular, GC–MS/MS, are much more complex and time-consuming, and they have relatively low throughput as compared with antibody-based approaches. This may limit the type of studies that can be performed by MS-based approaches. "However, it is far preferable to have limited good quality data, than it is to be flooded with complex and confounding results" (Duncan, 2003). At present good quality data are limited on 3-nitrotyrosine, and the usefulness and reliability of circulating 3-nitrotyrosine as a biomarker of RNS-mediated oxidative stress in vivo in humans remain to be established. Application of the GC–MS/MS and LC–MS/MS methodologies to clinical studies clearly shows that the basal levels of NO_2Tyr and NO_2TyrProt are relatively low and that the changes resulting from disease and therapeutical intervention should be considered very moderate (Tsikas and Caidahl, 2005).

11.4 CONSIDERATIONS FROM THE ANALYTICAL AND REVIEW POINTS OF VIEW

Solid knowledge about 3-nitrotyrosine and *S*-nitrosothiols is gained only through the development, validation, and application of reliable analytical methods, including results from studies, being extensively reported in chemical analysis oriented and peer-reviewed journals. Worldwide, several groups have applied different MS-based methods to the quantification of 3-nitrotyrosine in human blood circulation. Unfortunately, only one group used MS methodology to quantify *S*-nitrosoalbumin in human plasma. By providing accurate levels for 3-nitrotyrosine in the plasma of humans and animals at the basal state, and by identifying analytical shortcomings and pitfalls, MS technology, notably tandem mass spectrometry (i.e., MS/MS) technique, has greatly contributed to form a solid foundation for our understanding of 3-nitrotyrosine's physiology and pathophysiology. By way of tandem mass spectrometry methodology we have reliable reference intervals for concentrations of 3-nitrotyrosine in the healthy human (Tsikas and Caidahl, 2005). However, unlike 3-nitrotyrosine, circulating *S*-nitrosothiols have evaded the definition of reference ranges to date. The basal levels provided by the GC–MS technique for plasma *S*-nitrosoalbumin, which are of the order of 150 to 210 nM (Tsikas et al., 1999d, 2002b) are used as reference values, but they require confirmation by other MS-based methods. Therefore the most critical challenge is to establish reference intervals for circulating *S*-nitrosoproteins, namely *S*-nitrosoalbumin and *S*-nitrosohemoglobin. The physiological and pathological roles of these biologically potent $^{\bullet}$NO derivatives are at present uncertain and questionable.

Although our discussion is confined to circulating 3-nitrotyrosine and *S*-nitrosothiols in in vivo studies, which, in principle, can be translated to in vitro studies, the quantitative determination of 3-nitrotyrosine and *S*-nitrosothiols in vitro is limited by severe analytical problems and pitfalls (Tsikas and Frölich, 2004). This is mostly because less attention has been given, in general, to the quantitative aspects of the analytical methodologies used in the in vitro studies even though the purpose behind these analytical methods is to obtain quantitative information (Rafikova et al., 2002; Haendeler et al., 2004).

11.4.1 Aspects of Method Validation and Quality Control

Method validation is universally recognized as a necessary part of a comprehensive system of quality assurance. In analytical chemistry the application of this principle to 3-nitrotyrosine and *S*-nitrosothiols has importance in that only validated analytical methods should be used for the quantitative determination of these biomarkers in biological fluids, especially in human blood. The guiding principles for validation of analytical methods, previously provided for the quantitative determination of drugs in animals and humans (Shah et al., 1992), can be adopted with minor modifications for physiological substances, including 3-nitrotyrosine and *S*-nitrosothiols. Minimum recommendations on procedures

that should be employed to ensure adequate validation of single-laboratory analytical methods have been recently reported (Thompson et al., 2002). Requirements for study of method performance characteristics include applicability, selectivity, calibration and linearity, trueness, precision, recovery, range, detection limit, limit of determination or limit of quantification, sensitivity, ruggedness, fitness for purpose, matrix variation, and measurement uncertainty (Thompson et al., 2002). Unfortunately, there are various interpretations of the relevant concepts of trueness, accuracy or inaccuracy, uncertainty, and precision or imprecision (Dybkaer, 1998), and some of these validation parameters are incorrectly used in the literature.

Quantitative analysis is often a challenge, but the problems posed by 3-nitrotyrosine and *S*-nitrosothiols are particularly significant. It is therefore essential that method validation—that is, calibration, linearity, accuracy, and precision—be performed within the relevant concentration range for the respective compound in a certain matrix. It is further important that method validation be carried out using well-characterized synthetic standards for 3-nitrotyrosine and the respective *S*-nitrosothiol, such as unlabeled or labeled *S*-nitrosoalbumin but not an *S*-nitrosothiol different from the target molecule such as *S*-nitrosoglutathione or even nitrite for *S*-nitrosohemoglobin.

Both chemical and immunological methods of quantitative analysis of 3-nitrotyrosine and *S*-nitrosothiols are indirect. Nitrite and nitrate are permanent escorts of *S*-nitrosothiols and 3-nitrotyrosine, either as contamination or as endogenous sources. The occurrence and the extent of these and other potentially interfering compounds, including tyrosine, should be determined and reported in analytical and clinical studies. Also procedures used to remove such species, for example, nitrite by the use of ammonium sulfamate, and the final adjusted pH value, should be fully reported.

Analytical methods for quantitative analysis of 3-nitrotyrosine and *S*-nitrosothiols should be published and fully elaborated in peer-reviewed chemical analysis-oriented journals prior to use in experimental and clinical studies. The work should describe specific analytical problems and provide, if valid, the kind and extent of interferences.

In the case of comparisons of different analytical methods and/or for the purpose of validation of one method by another method, frequently only the coefficient of correlation from the linear regression analysis is reported. This practice is usually inappropriate because use of the correlation alone is misleading (Bland and Altmam, 1986; see also Porter, 1999). In addition to the coefficient of correlation, the whole regression equation from linear regression between the data obtained from the methods should be reported. However, the most appropriate statistical method available so far for assessing agreement between two methods of quantitative analysis, especially of clinical measurement, is based on graphical techniques (Bland and Altmam, 1986), and this should be preferably used.

Methods of analysis of 3-nitrotyrosine and *S*-nitrosothiols for use especially in human studies should have the same value as those used in clinical chemistry. One important approach to the study of those errors that are the responsibility of

the research group, and the procedures used to recognize and minimize them, is the incorporation of a quality control (QC) system in clinical studies similar to those proposed for clinical chemistry (Büttner et al., 1979). The QC system should involve concomitant analysis of QC samples in addition to study samples and should report analytical data on the method's accuracy and precision obtained from the analysis of the QC samples. In the field of •NO clinical research, QC is not yet an integral and routine part of clinical studies, but it is sporadically used by few groups for some •NO metabolites including nitrite and nitrate (Keimer et al., 2003), *S*-nitrosothiols (Tsikas et al., 1999d), and 3-nitrotyrosine (Fahlbusch et al., 2004).

11.4.2 Considerations from the Review Point of View

Increasingly "ready-to-use" chemical and immunological methodologies for the analysis of 3-nitrotyrosine, *S*-nitrosothiols, and species produced thereof, such as nitrite or •NO, are being provided commercially. Apart from other analytical difficulties, it should be pointed out that commercial availability does not automatically guarantee the accuracy of the results obtained by these methodologies. Consequently analytical quantitative methods adopted from other groups or simply used "as recommended by the manufacturer" have to be treated the same way and with as much carefulness as original methods. So performance characteristics of the assay in the own laboratory should be reported.

In clinical journals, which are primarily focused on the results rather than on the analytical methods applied, there is a general belief that adopted and commercially available assays are fundamentally reliable for accurate quantitative measurement. Particularly in those journals, the analytical methods used should be adequately described and cited with reference to the main original work published previously, preferably in analysis-oriented journals. Despite space constraints it is essential that the journal's policy demand and also offer the opportunity for authors to describe the methodologies used in full detail and to provide essential information in a "Supplementary Materials" section, if needed. The possibility of reporting satisfactorily analytical methods in experimental and clinical journals is nicely documented in the literature for *S*-nitrosothiols, nitrite, and nitrate in the frame of an animal study (Ng et al., 2004). Statements in the methodological part of the manuscript such as "measured as described elsewhere" or "as recommended by the manufacturer" should not be allowed. These methodological issues should be stated explicitly in the instructions for authors of the respective journal. Submitted work not fulfilling these criteria should not be considered for publication. Open and clear description of analytical methods applied enables the reader to evaluate the reliability of the methods, the presence of potential pitfalls, and the efficacy of measures undertaken to overcome them. One important instrument to monitor the overall reliability of the analytical approaches used in clinical studies is the use of a QC system. The same as in clinical chemistry (Büttner et al., 1979) and in human studies on bioavailability, bioequivalence, and pharmacokinetic studies of drugs (Shah et al., 1992), establishment of QC

systems in clinical studies is highly recommended, and should be demanded by clinically oriented journals.

Finally, in nonanalytically oriented journals the submitted work should be refereed not only from the biological and/or pharmacological point of view—the latter being presently common practice—but also from the analytical point of view, preferably by experienced analysts familiar with the analysis of 3-nitrotyrosine, *S*-nitrosothiols, nitrite, and nitrate.

11.5 CONCLUDING REMARKS AND FUTURE PROSPECTS

In recent years free and protein-associated 3-nitrotyrosine and *S*-nitrosothiols from endogenous sources, notably *S*-nitrosoalbumin and *S*-nitrosohemoglobin, have been thoroughly investigated in vitro and in vivo by different investigators from several points of view and by means of a variety of analytical methodologies. However, at present there are limited data of good quality on circulating 3-nitrotyrosine and *S*-nitrosothiols. The number of confounding analytical methods and biological results is huge. Many of the proposed roles of circulating 3-nitrotyrosine and *S*-nitrosothiols in health and disease are questionable. Reported 3-nitrotyrosine and *S*-nitrosothiols levels in plasma and red blood cells of healthy humans range from not detectable (i.e., <1 nM) to μM concentrations. Mass spectrometry based methodologies have helped define the reference interval values for 3-nitrotyrosine, but circulating *S*-nitrosothiols have evaded such definition. Quantitative analyses of circulating 3-nitrotyrosine and *S*-nitrosothiols are no doubt a great analytical challenge, but real progress in this area is only possible if validated, accurate, clear, and entirely reported methods are developed, reported, and used. Importantly the methods of quantitative analysis of 3-nitrotyrosine and *S*-nitrosothiols should openly address potential analytical problems and the methods' limitations. Clinically oriented journals have further a responsibility to this area of research to be more attentive to analytical chemistry both from the methodological and the review points of view with regard to authors' own methodologies as well as to adopted and, most important, to commercially available assays. It is urgent that the future prospect studies in methodological and clinical include the development, establishment, and routine use of QC systems for 3-nitrotyrosine and *S*-nitrosothiols and other members of the L-arginine/$^{\bullet}$NO family.

LIST OF ABBREVIATIONS

BSA, bovine serum albumin
CAD, collision-activated dissociation or coronary artery disease
DAN, diaminonaphthalene
EDRF, endothelium-derived relaxing factor
GC, gas chromatography
GC–MS, gas chromatography–mass spectrometry

GC–MS/MS, gas chromatography–mass spectrometry/mass spectrometry
GSH, reduced glutathione
GSNO, *S*-nitrosoglutathione
$GSNO_2$, *S*-nitroglutathione
HbFeNO, *Fe*-nitrosohemoglobin
HMM, high-molecular-mass
HPLC, high-performance liquid chromatography
HSA, human serum albumin
LC, liquid chromatography
LC–MS, liquid chromatography–mass spectrometry
LC–MS/MS, liquid chromatography–mass spectrometry/mass spectrometry
LMM, low-molecular-mass
MS, mass spectrometry
m/*z*, mass-to-charge ratio
NEM, *N*-ethylmaleimide
NHPA, 3-nitro-4-hydroxyphenylacetic acid
NHPL, 3-nitro-4-hydroxyphenyllactic acid
NO, nitric oxide
NOS, nitric oxide synthase
NO_2Tyr, free 3-nitro-L-tyrosine
NO_2TyrProt, protein-associated 3-nitro-L-tyrosine
OSA, obstructive sleep apnea
PFB-Br, pentafluorobenzyl bromide
QC, quality control
RNS, reactive nitrogen species
RSNO, *S*-nitrosothiol
$RSNO_2$, *S*-nitrothiol
SNALB, *S*-nitrosoalbumin
SNOHb, *S*-nitrosohemoglobin
SOD, superoxide dismutase
SPE, solid phase extraction
UV, ultraviolet

REFERENCES

Alpert C., Ramdev N., George D., Loscalzo J. (1997). Detection of *S*-nitrosothiols and other nitric oxide derivatives by photolysis-chemiluminescence spectrometry. *Anal Biochem* 245: 1–7.

Al-Sa'doni H., Ferro A. (2000). S-Nitrosothiols: a class of nitric oxide-donor drugs. *Clin Sci* 98: 507–520.

Andrew P.J., Mayer B. (1999). Enzymatic function of nitric oxide synthases. *Cardiovasc Res* 43: 521–531.

Arkenau H.T., Stichtenoth D.O., Frölich J.C., Manns M.P., Böker K.H.W. (2002). Elevated nitric oxide levels in patients with chronic liver diseases and cirrhosis correlate with disease stage and parameters of hyperdynamic circulation. *Z Gastroenterol* 40: 907–912.

Arnelle D.R., Stamler J.S. (1995). NO^+, $NO^\bullet$, and NO^- donation by *S*-nitrosothiols: Implications for regulation of physiological functions by *S*-nitrosylation and acceleration of disulfide formation. *Arch Biochem Biophys* 318: 279–285.

Balazy M.J., Kaminski P.M., Mao K., Tan J., Wolin M.S. (1998). *S*-Nitroglutathione, a product of the reaction between peroxynitrite and glutathione that generates nitric oxide. *J Biol Chem* 273: 32009–32015.

Bland J.M., Altman D.G. (1986). Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1: 307–310.

Brennan M.L., Wu W., Fu X., Shen Z., Song W., Frost H., Vadseth C., Narine L., Lenkiewicz E., Borchers M.T., Lusis A.J., Lee J.J., Lee N.A., Abu-Soud H.M., Ischiropoulos H., Hazen S.L. (2002). A tale of two controversies: defining both the role peroxidases in nitrotyrosine formation in vivo using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. *J Biol Chem* 277: 17415–17427.

Bryan N.S., Rassaf T., Rodriguez J., Feelisch M. (2004). Bound NO in human blood red cells: Fact or artifact? *Nitric Oxide* 10: 221–228.

Butler A.R., Rhodes P. (1997). Chemistry, analysis, and biological roles of *S*-nitrosothiols. *Anal Biochem* 249: 1–9.

Büttner J., Borth R., Boutwell J.H., Broughton P.M.G., Bowyer R.C. (1979). International Federation of Clinical Chemistry. Committee on Standards. Expert Panel on Nomenclature and Principles of Quality Control in Clinical Chemistry. Approved recommendation (1978) on quality control in clinical chemistry. Part 1. General principles and terminology. *Clin Chim Acta* 98: 129F–143F.

Cameron D.R., Borrajo A.M.P., Bennett B.M., Thatcher G.R.J. (1995). Organic nitrates, thionitrates, peroxynitrites, and nitric oxide: A molecular orbital study of the $RXNO_2 \Leftrightarrow RXONO$ (X = O, S) rearrangement, a reaction of potential biological significance. *Can J Chem* 73: 1627–1638.

Cannon R.O. III, Schechter A.N., Panza J.A., Ognibene F.P., Pease-Fye M.E., Waclawiw M.A., Shelhamer J.M., Gladwin M.T. (2001). Effects of inhaled nitric oxide on regional blood flow are consistent with intravascular nitric oxide delivery. *J Clin Invest* 108: 279–287.

Ceriello A., Mercuri F., Quagliaro L., Assaloni R., Motz E., Tonutti L., Taboga C. (2001). Detection of nitrotyrosine in the diabetic plasma: Evidence of oxidative stress. *Diabetologia* 44: 834–838.

Ckless K., Reynaert N.L., Taatjes D.J., Lounsbury K.M., van der Vliet A., Janssen-Heininger Y. (2004). In situ detection and visualization of *S*-nitrosylated proteins following chemical derivatization: Identification of RAN GTPase as a target for *S*-nitrosylation. *Nitric Oxide* 11: 216–227.

Crowley J.R., Yarasheski K., Leeuwenburgh C., Turk J., Heinecke J.W. (1998). Isotope dilution mass spectrometric quantification of 3-nitrotyrosine in proteins and tissues is facilitated by reduction to 3-aminotyrosine. *Anal Biochem* 259: 127–135.

Daiber A., Bachschmid M., Kavakli C., Frein D., Wendt M., Ullrich V., Munzel T. (2004). A new pitfall in detecting biological end products of nitric oxide—Nitration, nitros(yl)ation and nitrite/nitrate artefact during freezing. *Nitric Oxide* 9: 44–52.

Damiani P., Burini G. (1986). Fluorometric determination of nitrite. *Talanta* 33: 649–652.

Dejam A., Kleinbongard P., Rassaf T., Hamada S., Gharini P., Rodriguez J., Feelisch M., Kelm M. (2003). Thiols enhance NO formation from nitrate photolysis. *Free Radic Biol Med* 35: 1551–1559.

Delatour T., Richoz J., Vuichoud J., Stadler R.H. (2002a). Artifactual nitration controlled measurement of protein-bound 3-nitro-L-tyrosine in biological fluids and tissues by isotope dilution liquid chromatography electrospray ionization tandem mass spectrometry. *Chem Res Toxicol* 15: 1209–1217.

Delatour T., Guy P.A., Stadler R.H., Turesky R.J. (2002b). 3-Nitrotyrosine butyl ester: A novel derivative to assess tyrosine nitration in rat plasma by liquid chromatography-tandem mass spectrometry detection. *Anal Biochem* 302: 10–18.

Denicola A., Freemann B.A., Trujillo M., Radi R. (1996). Peroxynitrite reaction with carbon dioxide/bicarbonate: Kinetics and influence on peroxynitrite-mediated oxidations. *Arch Biochem Biophys* 333: 49–58.

Du M., Wu W., Ercal N., Ma Y. (2004). Simultaneous determination of 3-nitro tyrosine, *o*-, *m*-. and *p*-tyrosine in urine samples by liquid chromatography-ultraviolet absorbance detection with pre-column cloud point extraction. *J Chromatogr B* 803: 321–329.

Ducrocq C., Blanchard B., Pignatelli B., Ohshima H. (1999). Peroxynitrite: An endogenous oxidizing and nitrating agent. *Cell Mol Life Sci* 55: 1068–1077.

Duncan M.W. (2003). A review of approaches to the analysis of 3-nitrotyrosine. *Amino Acids* 25: 351–361.

Dybkaer R. (1998). Terminological problems around "accuracy" and "uncertainty." *Scand J Clin Lab Invest* 58: 35–46.

Ellis G., Adatia I., Yazdanpanah M., Makela S.K. (1998). Nitrite and nitrate analyses: A clinical biochemistry perspective. *Clin Biochem* 31: 195–220.

Fahlbusch S.A., Tsikas D., Mehl C., Gutzki F.M., Böger R.H., Frölich J.C., Stichtenoth D.O. (2004). Effects of carvedilol on oxidative stress in human endothelial cells and healthy volunteers. *Eur J Clin Pharmacol* 60: 83–88.

Feelisch M., Rassaf T., Mnaimneh S., Singh N., Bryan N.S., Jourd'heuil D., Kelm M. (2002). Concomitant *S*-, *N*- and heme-nitros(yl)ation in biological tissues and fluids: implications for the fate of NO in vivo. *FASEB J* 16: 1775–1785.

Ferdinandy P., Danial H., Ambrus I., Rothery R.A., Schulz R. (2000). Peroxynitrite is a major contributor to cytokine-induced myocardial contractile failure. *Circ Res* 87: 241–247.

Ferranti P., Malorni A., Mamone G., Sannolo N., Marino G. (1997). Characterisation of *S*-nitrosohaemoglobin by mass spectrometry. *FEBS Lett* 400: 19–24.

Ford P.C., Wink D.A., Standbury D.M. (1993). Autoxidation kinetics of aqueous nitric oxide. *FEBS Lett* 326: 1–3.

Förstermann U., Closs E.I., Pollock J.S., Nakane M., Schwarz P., Gath I., Kleinert H. (1994). Nitric oxide synthase isozymes: Characterization, purification, molecular cloning, and functions. *Hypertension* 23: 1121–1131.

Frehm E.J., Bonaventura J., Gow A.J. (2004). *S*-Nitrosohaemoglobin: an allosteric mediator of NO group function in mammalian vasculature. *Free Radic Biol Med* 37: 442–453.

Frost M.T., Halliwell B., Moore K.P. (2000). Analysis of free and protein-bound nitrotyrosine in human plasma by a gas chromatography/mass spectrometry method that avoids nitration artifacts. *Biochem J* 345: 453–458.

Gandley R.E., Tyurin V.A., Huang W., Arroyo A., Daftary A., Harger G., Jiang J., Pitt B., Taylor R.N., Hubel C.A., Kagan V.E. (2005). *S*-Nitrosoalbumin-mediated relaxation is enhanced by ascorbate and copper: Effects in pregnancy and preeclampsia plasma. *Hypertension* 45: 1–7.

Gaston B. (1999). Nitric oxide and thiol groups. *Biochim Biophys Acta* 1411: 323–333.

Gaut J.P., Byun J., Tran H.D., Heinecke J.W. (2002). Artifact-free quantification of free 3-chlorotyrosine, 3-bromotyrosine, and 3-nitrotyrosine in human plasma by electron capture-negative chemical ionization gas chromatography mass spectrometry and liquid chromatography-electrospray ionization tandem mass spectrometry. *Anal Biochem* 300: 252–259.

Giustarini D., Milzani A., Colombo R., Dalle-Donne I., Rossi R. (2003). Nitric oxide and *S*-nitrosothiols in human blood. *Clin Chim Acta* 330: 85–98.

Giustarini D., Milzani A., Colombo R., Dalle-Donne I., Rossi R. (2004). Nitric oxide, *S*-nitrosothiols and haemoglobin: Is methodology the key? *TRENDS Pharmacol Sci* 25: 311–316.

Giustarini D., Rossi R. (2004). Plasma *S*-nitrosothiols and chronic renal failure. *Am J Physiol Renal Physiol* 287: F1294–F1295.

Gladwin M.T., Shelhamer J.H., Schechter A.N., Pease-Fye M.E., Waclawiw M.A., Panza J.A., Ognibene F.P., Cannon R.O. III (2000). Role of circulating nitrite and *S*-nitrosohaemoglobin in the regulation of regional blood flow in humans. *Proc Natl Acad Sci USA* 97: 11482–11487.

Gladwin M.T., Crawford J.H., Patel R.P. (2004). The biochemistry of nitric oxide, nitrite, and haemoglobin: Role in blood flow regulation. *Free Radic Biol Med* 36: 707–714.

Goldstein S., Czapski G. (1999). Viscosity effects on the reaction of peroxynitrite with CO_2: evidence for radical formation in a solvent cage. *J Am Chem Soc* 121: 2444–2447.

Gow A., Duran D., Thom S.R., Ischiropoulos H. (1996). Carbon dioxide enhancement of peroxynitrite-mediated protein tyrosine nitration. *Arch Biochem Biophys* 333: 42–48.

Gow A.J., Stamler J.S. (1998). Reactions between nitric oxide and haemoglobin under physiological conditions. *Nature* 391: 169–173.

Haendeler J., Hoffmann J., Zeiher A.M., Dimmeler S. (2004). Antioxidant effects of statins via *S*-nitrosylation and activation of thioredoxin in endothelial cells: A novel vasculoprotective function of statins. *Circulation* 110: 856–861.

Halliwell B. (1997). What nitrates tyrosine? Is nitrotyrosine specific as a biomarker of peroxynitrite formation in vivo? *FEBS Lett* 411: 155–160.

Halliwell B., Gutteridge J.M.C. (1999). *Free Radicals in Biology and Medicine*, 3rd ed., Oxford University Press, Oxford.

Halliwell B., Zhao K., Whiteman M. (1999). Nitric oxide and peroxynitrite. The ugly, the uglier and the not so good. *Free Rad Res* 31: 651–669.

Hensley K., Williamson K.S., Floyd R.A. (2000). Measurement of 3-nitrotyrosine and 5-nitro-γ-tocopherol by high-performance liquid chromatography with electrochemical detection. *Free Radic Biol Med* 28: 520–528.

Herce-Pagliai C., Kotecha S., Shuker D.E.G. (1998). Analytical methods for 3-nitrotyrosine as a marker of exposure to reactive nitrogen species: a review. *Nitric Oxide* 2: 324–336.

Hibbs J.B. Jr, Taintor R.R., Vavrin Z. (1987). Macrophage cytotoxicity: Role for L-arginine deiminase and imino nitrogen oxidation to nitrite. *Science* 235: 473–476.

Hill R.E., Whelan D.T. (1984). Mass spectrometry and clinical chemistry. *Clin Chim Acta* 139: 231–294.

Hobbs A.J., Fukuto J.M., Ignarro L.J. (1994). Formation of free nitric oxide from L-arginine by nitric oxide synthase: Direct enhancement of generation by superoxide dismutase. *Proc Natl Acad Sci USA* 91: 10992–10996.

Huie R.E., Padmaja S. (1993). The reaction of NO with superoxide. *Free Radic Res Commun* 18: 195–199.

Ignarro L.J., Buga G.M., Wood K.S., Byrns R.E., Chaudhuri G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci USA* 84: 9265–9269.

Ischiropoulos H. (1998). Biological tyrosine nitration: A pathophysiological function of nitric oxide and reactive oxygen species. *Arch Biochem Biophys* 356: 1–11.

Iyengar R., Stuehr D.J., Marletta M.A. (1987). Macrophage synthesis of nitrite, nitrate, and *N*-nitrosamines: Precursors and role of the respiratory burst. *Proc Natl Acad Sci USA* 84: 6369–6373.

Jaffrey S.R., Snyder S.H. (2001). The biotin switch method for the detection of *S*-nitrosylated proteins. *Sci STKE*: L1.

Jaffrey S.R., Erdjument-Bromage H., Ferris C.D., Tempst P., Snyder S.H. (2001). Proteins *S*-nitrosylation: A physiological signal for neuronal nitric oxide. *Nat Cell Biol* 3: 193–197.

Jia L., Bonaventura C., Bonaventura J., Stamler J.S. (1996). *S*-Nitrosohaemoglobin: A dynamic activity of blood involved in vascular control. *Nature* 380: 221–226.

Jiang H., Balazy M. (1998). Detection of 3-nitrotyrosine in human platelets exposed to peroxynitrite by a new gas chromatography/mass spectrometry assay. *Nitric Oxide* 2: 350–359.

Jourd'heuil D., Miranda K.M., Kim S.M., Espey M.G., Vodovotz Y., Laroux S., Mai C.T., Miles A.M., Grisham M.B., Wink D.A. (1999). The oxidative and nitrosative chemistry of the nitric oxide/superoxide reaction in the presence of bicarbonate. *Arch Biochem Biophys* 365: 92–100.

Jourd'heuil D., Hallén K., Feelisch M., Grisham M.B. (2000). Dynamic state of *S*-nitrosothiols in human plasma and whole blood. *Free Radic Biol Med* 28: 409–417.

Kamisaki Y., Wada K., Nakamoto K., Kishimoto Y., Kitano M., Itoh T. (1996). Sensitive determination of nitrotyrosine in human plasma by isocratic high-performance liquid chromatography. *J Chromatogr B* 685: 343–347.

Keimer R., Stutzer F.K., Tsikas D., Troost R., Gutzki F.M., Frölich J.C. (2003). Lack of oxidative stress during sustained therapy with isosorbide dinitrate and pentaerythrityl tetranitrate in healthy humans: a randomized, double-blind crossover study. *J Cardiovasc Pharmacol* 41: 284–292.

Khan J., Brennan D.M., Bradley N., Gao B., Bruckdorfer R., Jacobs M. (1998). 3-Nitrotyrosine in the proteins of human plasma determined by an ELISA method. *Biochem J* 330: 795–801.

Kissner R., Koppenol W. (2002). Product distribution of peroxynitrite decay as a function of pH, temperature, and concentration. *J Am Chem Soc* 124: 234–239.

Kluge I., Gutteck-Amsler U., Zollinger M., Do K.Q. (1997). *S*-Nitrosoglutathione in rat cerebellum: Identification and quantification by liquid chromatography–mass spectrometry. *J Neurochem* 69: 2599–2607.

Kojima H., Nakatsubo N., Kikuchi K., Kawahara S., Kirino Y., Nagoshi H., Hirata Y., Nagano T. (1998). Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Anal Chem* 70: 2446–2453.

Kuncewicz T., Sheta E.A., Goldknopf I.L., Kone B.C. (2003). Proteomic analysis of *S*-nitrosylated proteins in messangial cells. *Mol Cell Proteomics* 2: 156–163.

Lawson A.M., Gaskell S.J., Hjelm M. (1985). Methodological aspects on quantitative mass spectrometry used for accuracy control in clinical chemistry. *J Clin Chem Clin Biochem* 23: 433–441.

Lemercier J.N., Padmaja S., Cueto R., Squadrito G.L., Uppu R.M., Pryor W.A. (1997). Carbon dioxide modulation of hydroxylation and nitration of phenol by peroxynitrite. *Arch Biochem Biophys* 345: 160–170.

Liu H., Huang T., Kissinger C.B., Kissinger P.T. (1998). Comparison of detection for liquid chromatographic determination of 3-nitro-L-tyrosine. *J Chromatogr B* 713: 289–295.

Lymar S.V., Hurst J.K. (1998). CO_2-Catalyzed one-electron oxidations by peroxynitrite: properties of the reactive intermediated. *Inorg Chem* 37: 294–301.

Mansoor M.A., Svardal A.M., Ueland P.M. (1992). Determination of the in vivo redox status of cysteine, cysteinylglycine, homocystine, and glutathione in human plasma. *Anal Biochem* 200: 218–229.

Marletta M.A. (1993). Nitric oxide synthase structure and mechanism. *J Biol Chem* 268: 12231–12234.

Marzinzig M., Nussler A.K., Stadler J., Marzinzig E., Barthlen W., Nussler N.C., Berger H.G., Morris S.M., Brückner U.B. (1997). Improved methods to measure end products of nitric oxide in biological fluids: Nitrite, nitrate, and *S*-nitrosothiols. *Nitric Oxide* 1: 177–189.

Massy Z.A., Borederie D., Nguyen T., Drüeke T.B., Ekindjian O.G., Lacour B. (2003). Increased plasma *S*-nitrosothiol levels in chronic haemodialysis patients. *Nephrol Dial Transplant* 18: 153–157.

Massy Z.A., Fumeron C., Borederie D., Tuppin P., Nguyen-Khoa T., Benoit M.O., Jacquot C., Buisson C., Drüeke T.B., Ekindjian O.G., Lacour B., Iliou M.C. (2004). Increased plasma *S*-nitrosothiol concentrations predict cardiovascular outcomes among patients with end-stage renal disease: A prospective study. *J Am Soc Nephrol* 15: 470–476.

Masumoto H., Kissner R., Koppenol W.H., Sies H. (1996). Kinetic study of the reaction of ebselen with peroxynitrite. *FEBS Lett* 398: 179–182.

Mayer B., Pfeiffer S., Schrammel A., Koesling D., Schmidt K., Brunner F. (1998). A new pathway of nitric oxide/cyclic GMP signaling involving *S*-nitrosoglutathione. *J Biol Chem* 273: 3264–3270.

Meyer D.J., Kramer H., Özer N., Coles B., Ketterer B. (1994). Kinetics and equilibria of *S*-nitrosothiol-thiol exchange between glutathione, cysteine, penicillamines and serum albumin. *FEBS Lett* 345: 177–180.

Mirza U.A., Chait B.T., Lander H.M. (1995). Monitoring reactions of nitric oxide with peptides and proteins by electrospray ionization-mass spectrometry. *J Biol Chem* 270: 17185–17188.

Moriel P., Pereira I.R.O., Bertolami M.C., Abdalla S.P. (2001). Is ceruloplasmin an important catalyst for *S*-nitrosothiol generation in hypercholesterolemia? *Free Radic Biol Med* 30: 318–326.

Myers P.R., Minor R.L. Jr, Guerra R. Jr, Bates J.N., Harrison D.G. (1990). Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble *S*-nitrosocysteine than nitric oxide. *Nature* 345: 161–163.

Nedospasov A., Rafikov R., Beda N., Nudler E. (2000). An autocatalytic mechanism of protein nitrosylation. *Proc Natl Acad Sci USA* 97: 13543–13548.

Ng E.S.M., Jourd'heuil D., McCord J.M., Hernandez D., Yasui M., Knight D., Kubes P. (2004). Enhanced *S*-nitroso-albumin formation from inhaled NO during ischemia/reperfusion. *Circ Res* 94: 559–565.

Noyes W.A. (1943). *n*-Butyl nitrite. *Org Syn Coll* 2: 108–109.

Ohshima H., Friesen M., Brouet I., Bartsch H. (1990). Nitrotyrosine as a new marker for endogenous nitrosation and nitration of proteins. *Food Chem Toxic* 28: 647–652.

Oldreive C., Bradley N., Bruckdorfer R., Rice-Evans C. (2001). Lack of influence of dietary nitrate/nitrite on plasma nitrotyrosine levels measured using a competitive inhibition of binding ELISA assay. *Free Rad Res* 35: 377–386.

Palmer R.M.J., Ashton D.S., Moncada S. (1988a). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 333: 664–666.

Palmer R.M.J., Rees D.D., Ashton D.S., Moncada S. (1988b). L-Arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem Biophys Res Commun* 153: 1251–1256.

Peters T. Jr (1985). Serum albumin. *Adv Prot Chem* 37: 161–245.

Petersson A.S., Steen H., Kalume D.E., Caidahl K., Roepstorff P. (2001). Investigation of tyrosine nitration in proteins by mass spectrometry. *J Mass Spectrom* 36: 616–625.

Petruzzelli S., Puntoni R., Mimotti P., Pulera N., Baliva F., Fornai E., Giuntini C. (1997). Plasma 3-nitrotyrosine in cigarette smokers. *Am J Respir Crit Care Med* 156: 1902–1907.

Pfeiffer S., Gorren A.C.F., Schmidt K., Werner E.R., Hansert B., Bole D.S., Mayer B. (1997). Metabolic fate of peroxynitrite in aqueous solution: Reaction with nitric oxide and pH-dependent decomposition to nitrite and oxygen in a 2:1 stoichiometry. *J Biol Chem* 272: 3465–3470.

Pfeiffer S., Mayer B. (1998). Lack of tyrosine nitration by peroxynitrite generated at physiological pH. *J Biol Chem* 273: 27280–27285.

Pignatelli B., Li C.Q., Boffetta P., Chen Q., Ahrens W., Nyberg F., Mukeria A., Bruske-Hohlfeld I., Fortes C., Constantinescu V., Ischiropoulos H., Ohshima H. (2001). Nitrated and oxidized plasma proteins in smokers and lung cancer patients. *Cancer Res* 61: 778–784.

Pogrebnaya V.L., Usov A.P., Baranov A.V., Nesterenko A.I., Bez'yazychnyi P.I. (1975). Oxidation of nitric oxide by oxygen in the liquid phase. *J Appl Chem USSR* 48: 1004–1007.

Porter A.M.W. (1999). Misuse of correlation and regression in three medical journals. *J Royal Soc Med* 92: 123–128.

Pou S., Pou W.S., Bredt D.S., Snyder S.H., Rosen G.M. (1992). Generation of superoxide by purified brain nitric oxide synthase. *J Biol Chem* 267: 24173–24176.

Radák Z., Ogonovszky H., Dubecz J., Sasvari M., Pucsok J., Berkes I., Csont T., Ferdinandy P. (2003). Super-marathon race increases serum and urinary nitrotyrosine and carbonyl levels. *Eur J Clin Pharmacol* 33: 726–730.

Radi R., Peluffo G., Alvarez M.N., Naviliat M., Cayota A. (2001). Unravelling peroxynitrite formation in biological systems. *Free Radic Biol Med* 30: 463–488.

Rafikova O., Rafikov R., Nudler E. (2002). Catalysis of *S*-nitrosothiols formation by serum albumin: The mechanism and implication in vascular control. *Proc Natl Acad Sci USA* 99: 5913–5918.

Richardson G., Benjamin N. (2002). Potential therapeutic uses for *S*-nitrosothiols. *Clin Sci* 102: 99–105.

Rossi R., Giustarini D., Milzani A., Colombo R., Dalle-Donne I., Di Simplicio P. (2001). Physiological levels of *S*-nitrosothiols in human plasma. *Circ Res* 89: e47.

Sarver A., Scheffler N.K., Shetlar M.D., Gibson B.W. (2001). Analysis of peptides and proteins containing nitrotyrosine by matrix-assisted laser desorption/ionization mass spectrometry. *J Am Soc Mass Spectrom* 12: 439–448.

Saville B. (1958). A scheme for the colorimetric determination of microgram amounts of thiols. *Analyst* 83: 670–672.

Scharfstein J.S., Keaney J.F. Jr, Slivka A., Welch G.N., Vita J.A., Stamler J.S. (1994). In vivo transfer of nitric oxide between a plasma protein-bound reservoir and low molecular weight thiols. *J Clin Invest* 94: 1432–1439.

Schechter A.N., Gladwin M.T. (2003). Haemoglobin and the paracrine functions of nitric oxide. *N Engl J Med* 348: 1483–1485.

Schmidt H.H.H.W., Nau H., Wittfoht W., Gerlach J., Prescher K.E., Klein M.M., Niroomand F., Böhme E. (1988). Arginine is a physiological precursor of endothelium-derived nitric oxide. *Eur J Pharmacol* 154: 213–216.

Schmidt H.H.H.W., Hofmann H., Schindler U., Shutenko Z.S., Cunningham D.D., Feelisch M. (1996). No NO from NO synthase. *Proc Natl Acad Sci USA* 93: 14492–14497.

Schoeller D.A. (1986). Mass spectrometry: calculations. *J Clin Pharmacol* 26: 396–399.

Schwedhelm E., Sandmann J., Tsikas D. (1998). Synthesis of 3-nitro-L-[$^2H_3$]tyrosine for use as an internal standard for quantification of 3-nitro-L-tyrosine by gas chromatography–mass spectrometry. *J Labelled Cpds Radiopharm* 41: 773–780.

Schwedhelm E., Tsikas D., Gutzki F.M., Frölich J.C. (1999). Gas chromatographic–tandem mass spectrometric quantification of free 3-nitrotyrosine in human plasma at the basal state. *Anal Biochem* 276: 195–203.

Schwemmer M., Fink B., Köckerbauer R., Bassenge E. (2000). How urine analysis reflects oxidative stress—Nitrotyrosine as a potential marker. *Clin Chim Acta* 297: 207–216.

Shaffer J.E., Han B.J., Chern W.H., Lee F.W. (1992). Lack of tolerance to a 24-hour infusion of *S*-nitroso-*N*-acetylpenicillamine (SNAP) in conscious rabbits. *J Pharmacol Exp Ther* 260: 286–293.

Shah V.P., Midha K.K., Dighe S., McGilveray I.J., Skelly J.P., Yacobi A., Layloff T., Viswanathan C.T., Cook C.E., McDowall R.D., Pittmann K.A., Spector S. (1992). Analytical methods validation: Bioavailability, bioequivalence, and pharmacokinetic studies. *J Pharmaceut Sci* 81: 309–312.

Shigenaga M.K., Lee H.H., Blount B.C., Christen S., Shigeno E.T., Yip H., Ames B.N. (1997). Inflammation and NO_x-induced nitration: assay for 3-nitrotyrosine by HPLC with electrochemical detection. *Proc Natl Acad Sci USA* 94: 3211–3216.

Shigenaga M.K. (1999). Quantitation of protein-bound 3-nitrotyrosine by high-performance liquid chromatography with electrochemical detection. *Methods Enzymol* 301: 27–40.

Shishehbor M.H., Aviles R.J., Brennan M.L., Fu X., Goormastic M., Pearce G.L., Gokce N., Keaney J.F. Jr, Penn M.S., Sprecher D.L., Vita J.A., Hazen S.L. (2003a). Association of nitrotyrosine levels with cardiovascular disease and modulation by statin therapy. *JAMA* 289: 1675–1680.

Shishehbor M.H., Brennan M.L., Aviles R.J., Fu X., Penn M.S., Sprecher D.L., Hazen S.L. (2003b). Statins promote potent systemic antioxidant effects through specific inflammatory pathways. *Circulation* 108: 426–431.

Skinner K.A., Whites C.R., Patel R., Tan S., Barnes S., Kirk M., Darley-Usmar V., Parks D.A. (1998). Nitrosation of uric acid by peroxynitrite: Formation of a vasoactive nitric oxide donor. *J Biol Chem* 273: 24491–24497.

Smith C.C.T., Stanyer L., Betteridge D.J. (2002). Evaluation of methods for the extraction of nitrite and nitrate in biological fluids employing high-performance anion-exchange liquid chromatography for their determination. *J Chromatogr B* 779: 201–209.

Söderling A.S., Ryberg H., Gabrielson A., Lärstad M., Torén K., Niari S., Caidahl K. (2003). A derivatization assay using gaschromatography/negative chemical ionization tandem mass spectrometry to quantify 3-nitrotyrosine in human plasma. *J Mass Spectrom* 38: 1187–1196.

Squadrito G.L., Jin X., Pryor W.A. (1995). Stopped-flow kinetic study of the reaction of ascorbic acid with peroxynitrite. *Arch Biochem Biophys* 322: 53–59.

Stamler J.S., Jaraki O., Osborne J., Simon D.I., Keaney J., Vita J., Singel D., Valeri C.R., Loscalzo J. (1992a). Nitric oxide circulates in mammalian plasma primarily as an *S*-nitroso adduct of serum albumin. *Proc Natl Acad Sci USA* 89: 7674–7677.

Stamler J.S., Simon D.I., Osborne J., Mullins M.E., Jaraki O., Michel T., Singel D.J., Loscalzo J. (1992b). *S*-Nitrosylation of proteins with nitric oxide: Synthesis and characterization of biological active compounds. *Proc Natl Acad Sci USA* 89: 444–448.

Stamler J. (2004). *S*-Nitrosothiols in the blood: Roles, amounts, and methods of analysis. *Circ Res* 94: 414–417.

Strand Ø.A., Leone A., Gierccksky K.E., Kirkebøen K.A. (2000). Nitric oxide indices in human septic shock. *Crit Care Med* 28: 2779–2785.

Svatikova A., Wolk R., Wang H.H., Otto M.E., Bybee K.A., Singh R.J., Somers V.K. (2004). Circulating free nitrotyrosine in obstructive sleep apnea. *Am J Physiol Regul Comp Physiol* 287: R284–R287.

Szilvássi Z., Csont T., Páli T., Droy-Lefaix M.T., Ferdinandy P. (2001). Nitric oxide, peroxynitrite and cGPM in atherosclerosis-induced hypertension in rabbits: Beneficial effects of cicletanine. *J Vasc Res* 38: 39–46.

Tashimo O., Ishibashi T., Yoshida J., Tsuchida H., Nishio M. (2003). Interference with Saville's method in the determination of low-molecular weight *S*-nitrosothiols by ultrafiltration. *Nitric Oxide* 9: 148–152.

ter Steege J.C.A., Koster-Kamphuis L., van Straaten E.A., Forget P.P., Buurman W.A. (1998). Nitrotyrosine in plasma of celiac disease patients as detected by a new sandwich ELISA. *Free Radic Biol Med* 25: 953–963.

Thompson M., Ellison S.L.R., Wood R. (2002). Harmonized guidelines for single-laboratory validation of methods of analysis. *Pure Appl Chem* 74: 835–855.

Troost R., Schwedhelm E., Rojczyk S., Tsikas D., Frölich J.C. (2000). Nebivolol decreases systemic oxidative stress in healthy volunteers. *Br J Clin Pharmacol* 50: 377–379.

Tsikas D., Böger R.H., Bode-Böger S.M., Gutzki F.M., Frölich J.C. (1994). Quantification of nitrite and nitrate in human urine and plasma as pentafluorobenzyl derivatives by gas chromatography–mass spectrometry using their ^{15}N-labelled analogs. *J Chromatogr B* 661: 185–191.

Tsikas D., Rossa S., Stichtenoth D.O., Raida M., Gutzki F.M., Frölich J.C. (1996). Is *S*-nitroso-*N*-acetyl-L-cysteine a circulating or an excretory metabolite of nitric oxide (NO) in man? Assessment by gas chromatography-mass spectrometry. *Biochem Biophys Res Commun* 220: 939–944.

Tsikas D., Sandmann J., Rossa S., Gutzki F.M., Frölich J.C. (1999a). Investigations of *S*-transnitrosylation reactions between low- and high-molecular-weight *S*-nitroso compounds and their thiols by high-performance liquid chromatography and gas chromatography-mass spectrometry. *Anal Biochem* 270: 231–241.

Tsikas D., Sandmann J., Rossa S., Gutzki F.M., Frölich J.C. (1999b). Measurement of *S*-nitrosoalbumin by gas chromatography-mass spectrometry. I. Preparation, purification, isolation, characterization and metabolism of *S*-[^{15}N]nitrosoalbumin in human blood in vitro. *J Chromatogr B* 726: 1–12.

Tsikas D., Sandmann J., Holzberg D., Pantazis P., Raida M., Frölich J.C. (1999c). Determination of *S*-nitrosoglutathione in human and rat plasma by high-performance liquid chromatography with fluorescence and ultraviolet absorbance detection after precolumn derivatization with *o*-phthaldehyde. *Anal Biochem* 273: 32–40.

Tsikas D., Sandmann J., Gutzki F.M., Stichtenoth D.O., Frölich J.C. (1999d). Measurement of *S*-nitrosoalbumin by gas chromatography-mass spectrometry. II. Quantitative determination of *S*-nitrosoalbumin in human plasma using *S*-[^{15}N]nitrosoalbumin as internal standard. *J Chromatogr B* 726: 13–24.

Tsikas D., Sandmann J., Rossa S., Gutzki F.M., Frölich J.C. (1999e). Gas chromatographic–mass spectrometric detection of *S*-nitroso-cysteine and *S*-nitroso-glutathione. *Anal Biochem* 272: 117–122.

Tsikas D., Gutzki F.M., Sandmann J., Schwedhelm E., Frölich J.C. (1999f). Quantification of plasma and urinary nitrate after its reduction to nitrite and to the pentafluorobenzyl derivative by gas chromatography–tandem mass spectrometry. *J Chromatogr B* 731: 285–301.

Tsikas D., Sandmann J., Denker K., Frölich J.C. (2000a). Is *S*-nitrosoglutathione formed in nitric oxide synthase incubates? *FEBS Lett* 483: 83–85.

Tsikas D., Raida M., Sandmann J., Rossa S., Forssmann W.G., Frölich J.C. (2000b). Electrospray ionization mass spectrometry of low-molecular-mass *S*-nitroso compounds and their thiols. *J Chromatogr B* 742: 99–108.

Tsikas D., Sandmann J., Lueßen P., Savva A., Rossa S., Stichtenoth D.O., Frölich J.C. (2001a). *S*-Transnitrosylation of albumin in human plasma and blood in vitro and in vivo in the rat. *Biochim Biophys Acta* 1546: 422–434.

Tsikas D., Denker K., Frölich J.C. (2001b). Artifactual-free analysis of *S*-nitrosoglutathione and *S*-nitroglutathione by neutral-pH, anion-pairing, high-performance liquid

chromatography: Study on peroxynitrite-mediated *S*-nitration of glutathione to *S*-nitroglutathione under physiological conditions. *J Chromatogr A* 915: 107–116.

Tsikas D., Frölich J.C. (2002a). Is circulating nitrite a directly acting vasodilator? *Clin Sci* 103: 107–110.

Tsikas D., Frölich J.C. (2002b). *S*-Nitrosoalbumin plasma levels in healthy and disease: facts or artifacts? Value of analytical chemistry in nitric oxide clinical research. *Circ Res* 90: e39.

Tsikas D., Schwedhelm E., Frölich J.C. (2002a). Methodological considerations on the detection of 3-nitrotyrosine in the cardiovascular system. *Circ Res* 90: e70.

Tsikas D., Sandmann J., Frölich J.C. (2002b). Measurement of *S*-nitrosoalbumin by gas chromatography-mass spectrometry. III. Quantitative determination in human plasma after specific conversion of the *S*-nitroso group to nitrite by cysteine and Cu^{2+} via intermediate formation of *S*-nitrosocysteine and nitric oxide. *J Chromatogr B* 772: 335–346.

Tsikas D. (2003). Measurement of physiological *S*-nitrosothiols: a problem child and a challenge. *Nitric Oxide* 9: 53–55.

Tsikas D., Schwedhelm E., Stutzer F.K., Gutzki F.M., Rode I., Mehls C., Frölich J.C. (2003a). Accurate quantification of basal plasma levels of 3-nitrotyrosine and 3-nitrotyrosinoalbumin by gas chromatography-tandem mass spectrometry. *J Chromatogr B* 784: 77–90.

Tsikas D., Rode I., Becker T., Nashan B., Klempnauer J., Frölich J.C. (2003b). Elevated plasma and urine levels of ADMA and 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in end-stage liver disease. *Hepatology* 38: 1063–1064.

Tsikas D., Frölich J.C. (2003). Are plasma *S*-nitrosothiol levels elevated in chronic renal failure? *Nephrol Dial Transplant* 918: 2199–2201.

Tsikas D. (2004). *S*-Nitrosoalbumin and other *S*-nitrosothiols in the blood. Is their quantity of no relevance? *Circ Res* 94: e106.

Tsikas D., Frölich J.C., Kielstein J.T. (2004). Nitric oxide synthesis in chronic renal failure: Are plasma *S*-nitrosothiol levels elevated? *Clin Chim Acta* 339: 195–197.

Tsikas D., Frölich J.C. (2004). Trouble with the analysis of nitrite, nitrate, *S*-nitrosothiols and 3-nitrotyrosine: freezing-induced artifacts? *Nitric Oxide* 11: 209–213.

Tsikas D. (2005a). Methods of quantitative analysis of the nitric oxide metabolites nitrite and nitrate in human biological fluids. *Free Radic Res* 39: 797–815.

Tsikas D. (2005b). Analysis of the L-arginine/nitric oxide pathway: The unique role of mass spectrometry. *Curr Pharmac Anal* 1: 15–30.

Tsikas D., Caidahl K. (2005). Recent methodological advances in the mass spectrometric analysis of free and protein-associated 3-nitrotyrosine in human plasma. *J Chromatogr B* 814: 1–9.

Tsikas D., Mitschke A., Suchy M.T., Gutzki F.M., Stichtenoth D.O. (2005). Determination of 3-nitrotyrosine in human urine at the basal state by gas chromatography-tandem mass spectrometry and evaluation of the excretion after oral intake. *J Chromatogr B*, 827: 146–156.

Tyurin V.A., Liu S.X., Tyurina Y.Y., Sussman N.B., Hubel C.A., Roberts J.M., Taylor R.N., Kagan V.E. (2001). Elevated levels of *S*-nitrosoalbumin in preeclampsia plasma. *Circ Res* 88: 1210–1215.

Uppu R.M., Squadrito G.L., Pryor W.A. (1996). Acceleration of peroxynitrite oxidations by carbon dioxide. *Arch Biochem Biophys* 327: 335–343.

Vásquez-Vivar J., Kalyanaraman B., Martásek P., Hogg N., Masters B.S.S., Karoui H., Tordo H., Pritchard K.A. (1998). Superoxide generation by endothelial nitric oxide synthase: The influence of cofactors. *Proc Natl Acad Sci USA* 95: 9229–9225.

van der Vliet A., 't Hoen P.A.C., Wong P.S.Y., Bast A., Cross C.E. (1998). Formation of *S*-nitrosothiols via direct nucleophilic nitrosation of thiols by peroxynitrite with elimination of hydrogen peroxide. *J Biol Chem* 273: 30255–30262.

Wang K., Hou Y., Zwang W., Ksebati M.B., Xian M., Cheng J.P., Wang P.G. (1999). ^{15}N NMR and electronic properties of *S*-nitrosothiols. *Bioinorg Medic Chem Lett* 9: 2897–2902.

Wang H.H., Lagerstedt S.A., Heinecke J.W., Singh R.J. (2003). LC–MS/MS quantification of circulating free nitrotyrosine. Evidence of elevated free nitrotyrosine in hypertensive patients. In: *Proceedings of the 1st ASMS Conference on Mass Spectrometry Allied Topics TPC 050*.

Wlodek P.J., Kucharczyk J., Sokolovska M.M., Milkowski A., Markiewicz O.B., Smolenski O.B., Wlodek L.B. (2003). Alteration in plasma levels of nonproteins sulfhydryl compounds and *S*-nitrosothiols in chronic renal failure. *Clin Chim Acta* 327: 87–94.

Xia Y., Zweier J.L. (1997). Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. *Proc Natl Acad Sci USA* 94: 6954–6958.

Yang B.K., Vivas E.X., Reiter C.D., Gladwin M.T. (2003). Methodologies for the sensitive and specific measurement of *S*-nitrosothiols, iron-nitrosyls, and nitrite in biological samples. *Free Rad Res* 37: 1–10.

Yi D., Ingelse B.A., Duncan M.W., Smythe G.A. (2000). Quantification of 3-nitrotyrosine in biological tissues and fluids: Generating valid results by eliminating artifactual formation. *J Am Soc Mass Spectrom* 11: 578–586.

Zhang H., Squadrito G.L., Uppu R.M., Lemercier J.N., Cueto R., Pryor W.A. (1997). Inhibition of peroxynitrite-mediated oxidation of glutathione by carbon dioxide. *Arch Biochem Biophys* 339: 183–189.

Zhang Y., Hogg N. (2005). *S*-Nitrosothiols: Cellular formation and transport. *Free Radic Biol Med* 38: 831–838.

Zhang Y., Keszler A., Broniowska K.A., Hogg N. (2005). Characterization and application of the biotin-switch assay for the identification of *S*-nitrosated proteins. *Free Radic Biol Med* 38: 874–881.

Zoccali C., Bode-Böger S.M., Mallamaci F., Benedetto F.A., Tripepi G., Malatino L.S., Catalioti A., Bellanuova I., Fermo I., Frölich J.C., Böger R.H. (2001). Plasma concentration of asymmetrical dimethylarginine and mortality in patients with end-stage renal disease: A prospective study. *Lancet* 358: 2113–2117.

PART II

CELLULAR ASPECTS OF PROTEIN OXIDATION

12

THE COVALENT ADVANTAGE: A NEW PARADIGM FOR CELL SIGNALING MEDIATED BY THIOL REACTIVE LIPID OXIDATION PRODUCTS

DALE A. DICKINSON, VICTOR M. DARLEY-USMAR, AND AIMEE LANDAR

12.1 INTRODUCTION

We are constantly exposed to pathological toxins that are derived from metabolic defects, environmental pollutants, and the diet. Cells can distinguish specific molecular components that derive from these stress stimuli. At low concentrations the cell can respond through the activation of signal transduction pathways, leading to the synthesis of cytoprotective proteins or intracellular antioxidants, while exposure to high concentrations of these same stimuli can lead to cell death mediated by apoptosis. The precise molecular mechanisms through which the initial environmental stress generates molecules capable of interacting with proteins ("receptors") is only now emerging. It is now possible to identify the mechanisms through which proteins change function on exposure to oxidants through the application of proteomics techniques capable of identifying the post-translational modifications of low-abundance proteins.

The central concept that underlies the field of "redox cell signaling" is that changes in the reductive or oxidative capacity of the cell lead to post-translational

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

modification of proteins by ROS/RNS (Fukagawa et al., 2000; Levonen et al., 2001a; Cooper et al., 2002; Droge, 2002; Kim et al., 2002; Landar and Darley-Usmar, 2003; Matsumoto et al., 2003). The mechanisms of ROS/RNS-mediated post-translational modification are distinct from phosphorylation, which primarily involves Ser, Thr, and Tyr residues, and these mechanisms instead involve modification at other specific amino acids, typically Cys, Lys, and His. It is important to note that cross-talk is also mediated by modulation of the activity of kinases or phosphatases (Rhee et al., 2000; Xu et al., 2002). Redox signaling pathways play a regulatory role in cell differentiation and apoptosis (Ischiropoulos, 1998; Patel et al., 2000) and adaptation to stress. Loss of control of these signaling pathways are thought to contribute to the pathogenesis of several diseases, including atherosclerosis and cancer (Dempke et al., 2001; Hanna et al., 2002).

The interaction of lipids with oxidants, either through enzymatic mechanisms such as the cyclooxygenases or nonenzymatic processes, can generate lipid oxidation products with a high degree of structural and chemical variability, some of which are capable of irreversible protein modification (Subbanagounder et al., 2000; Berliner et al., 2001; Marathe et al., 2002; Uchida, 2003; Ceaser et al., 2004). The amplification of these signals also appears to involve the further generation of the primary oxidants through activation of specific systems in the cell, including NADPH oxidases, nitric oxide synthases, and mitochondria (Griendling et al., 2000; Ramachandran et al., 2002). At low levels both complex mixtures of oxidized lipids and specific electrophilic cyclopentenone lipids can protect cells against inflammation and apoptosis (Darley-Usmar et al., 1991; Gotoh et al., 1993; Maggi et al., 2000; Rossi et al., 2000; Levonen et al., 2001b; Shen and Sevanian, 2001; Moellering et al., 2002; Bea et al., 2003; Itoh et al., 2004). It is now clear from our own work and that of others that at low concentrations an electrophile responsive proteome orchestrates these cytoprotective effects. This review describes the mechanisms of formation of electrophilic lipid oxidation products and their impact on cell function. A particular emphasis is placed on the hypothesis that the formation of a covalent interaction with the protein targets with electrophilic lipids enhances their biological effects, the covalent advantage.

12.2 CYCLOOXYGENASE AND THE CONVERSION OF NONREACTIVE LIPIDS TO THIOL SWITCHING MOLECULES

The conversion of unsaturated fatty acids to reactive lipid oxidation products can occur either enzymatically or through nonspecific lipid peroxidation, both of which can lead to reactive electrophilic lipids. A large class of these endogenously generated compounds are the prostaglandins (PGs), a family of cyclic C_{20} fatty acids with potent and diverse biological properties, which depend on the type of PG and the type of cell. Prostaglandins are synthesized from arachidonic acid (AA) and other nonreactive polyunsaturated fatty acids (eicosaenoic acids) derived from the phospholipid pool of the cell membrane. The regulated,

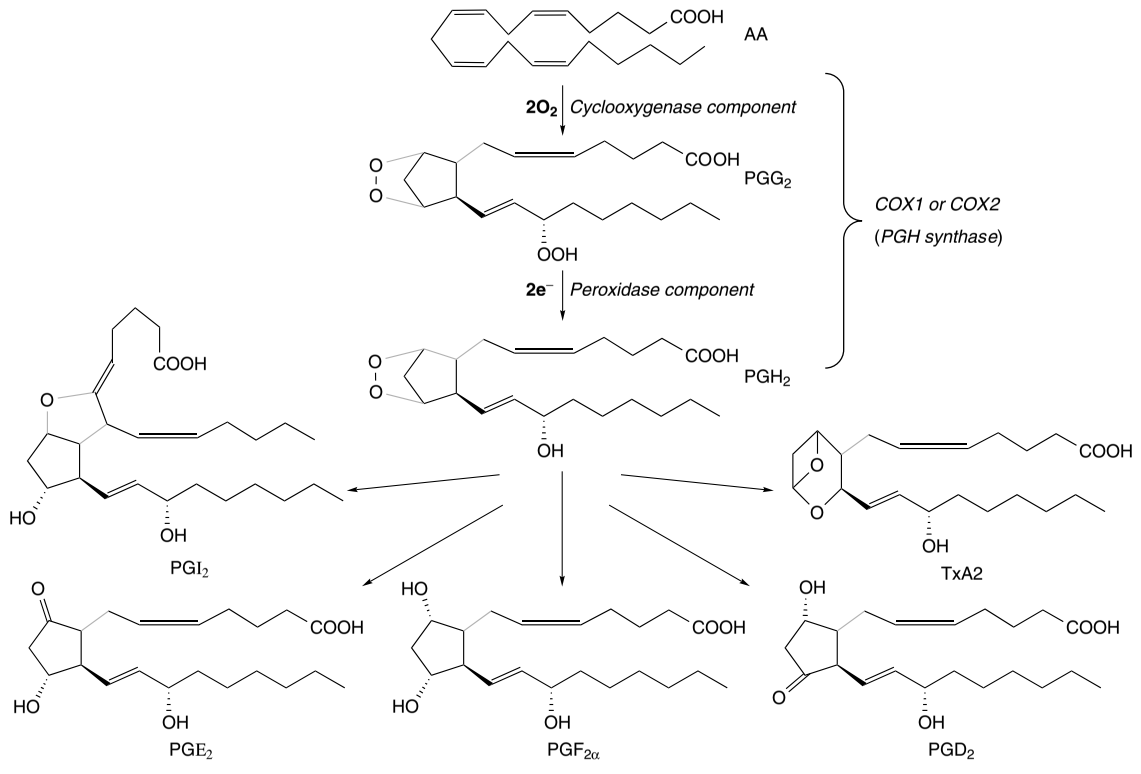


FIGURE 12.1 Enzymatic formation of the prostaglandins. Arachidonic acid from the plasma membrane is converted to PGG_2 via the cyclooxygenase component of COX. PGG_2 is rapidly converted by the same enzyme, through the peroxidase component, to form PGH_2. PGH_2 is then converted to one of several prostaglandins through specific enzymatic processes. These include PGI_2 (prostacyclin), PGE_2, $PGF_{2\alpha}$, PGD_2, and TxA_2 (thromboxane). Which PGs are produced, and in which various relative amounts, is cell-type dependent and is determined by the expression of the specific enzymes that convert PGH_2.

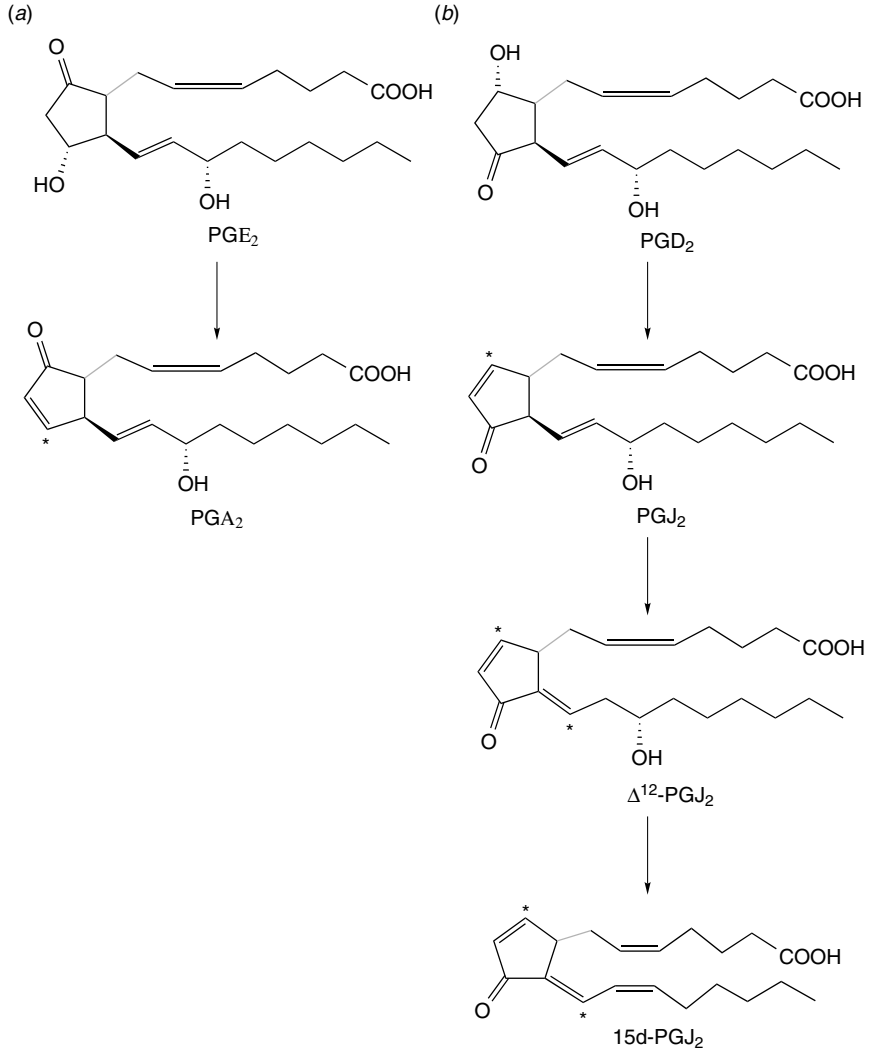


FIGURE 12.2 Generation of electrophilic cyclopentenone prostaglandins from nonelectrophilic parent compounds. The cyclopentenone ring of PGE_2 and PGD_2 undergoes dehydration to form PGA_2 and PGJ_2, respectively, each of which has a single electrophilic carbon (indicated with an asterisk). PGJ_2 can then be converted to Δ12-PGJ_2 via a double-bond isomerization, resulting in two electrophilic carbon centers. This short-lived intermediate rapidly undergoes a second dehydration step to form 15d-PGJ_2.

enzymatic production of prostaglandins is mediated by the enzyme cyclooxygenase. There are two forms of cyclooxygenase (COX), a constitutive form (COX-1) and an inducible form (COX-2). Expression of COX-2 is induced by pro-inflammatory cytokines, and COX-2 is a major target for nonsteroidal

anti-inflammatory drugs such as aspirin and indomethacin (Rouzer and Marnett, 2003). The cyclopentenone prostaglandins (cy-PGs) can also be produced from the nonspecific peroxidation of arachidonic acid (Chen et al., 1999; Straus and Glass, 2001; Cox et al., 2002; Hubatsch et al., 2002; Shibata et al., 2002; Subbanagounder et al., 2002).

The pathways for the enzymatic biosynthesis of prostaglandins are illustrated in Figure 12.1. The first step in the pathway involves the intracellular release of AA from plasma membrane phospholipids via the action of phospholipase A_2. Arachidonic acid is then converted sequentially to PGG_2 and PGH_2 by the cyclooxygenase and peroxidase activities, respectively, of cyclooxygenase (also called prostaglandin endoperoxide H synthase). PGH_2 is an unstable intermediate, which is converted enzymatically to a series of biologically active prostanoids, each of which has its own specific receptor(s). These include PGE_2, PGD_2, and $PGF_{2\alpha}$. Alternatively, PGH_2 may be converted to thromboxane (TxA2) or prostacyclin (PGI_2). The cy-PGs PGA_2, PGA_1, and PGJ_2 are formed by dehydration within the cyclopentane ring of PGE_2, PGE_1, and PGD_2, respectively (Fig. 12.2). Migration of the 13,14-double bond of PGJ_2 results in the formation of Δ^{12}-PGJ_2. This product can then undergo further dehydration by loss of the 15-hydroxyl group, to form 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2). The unique characteristic of the cy-PGs is the presence of an α, β-unsaturated carbonyl group in the cyclopentane ring, yielding an electrophilic carbon atom. This allows this portion of the molecule to form Michael adducts with cellular nucleophiles and bind covalently to specific proteins.

12.3 LIPID PEROXIDATION AND THE NONENZYMATIC FORMATION OF LIPID ADDUCTS CAPABLE OF MODIFYING PROTEINS

As with the enzymatic pathway, the nonenzymatic oxidation of polyunsaturated fatty acids (PUFA) results in products that fundamentally change the biochemistry of an unsaturated fatty acid from a molecule unreactive with nucleophilic centers in proteins to those that are highly reactive. First, a PUFA such as arachidonic acid is converted to a lipid peroxide, which subsequently forms reactive electrophiles capable of adduct formation with proteins. In contrast to the oxidized lipids that are formed enzymatically by the lipoxygenase and cyclooxygenase enzymes as discussed in the previous section, uncontrolled lipid peroxidation leads to the loss of stereospecificity while forming a mixture of diverse products (Yin and Porter, 2005). The electrophilic centers derive from the further reaction of lipid peroxides through enzymatic or nonenzymatic mechanisms dependent on transition metals such as copper and iron from heme proteins (Spiteller and Spiteller, 1998). The biological significance of nonenzymatic lipid peroxidation was first discovered from the nonenzymatic decomposition of arachidonic acid to form the family of compounds now known as the isoprostanes (Moore et al., 1995). This is a group of prostaglandin-like compounds that are formed through ROS/RNS-catalyzed

peroxidation of esterified arachidonic acid within the phospholipids of cellular membranes. This is followed by phospholipase-catalyzed release of the isoprostane moiety. The in vivo formation of isoprostanes increases as a function of oxidative stress (Moore et al., 1998; Witztum and Berliner, 1998; Pratico et al., 2001, 2004; Morrow, 2005). The biological properties of specific isoprostanes have been assessed, and it is now clear that in addition to being markers of oxidative stress and antagonists of the action of prostaglandins, they may exert unique biological effects (Roberts et al., 1999). Notable examples include the disease of rhabdomyolysis in which myoglobin is released into the plasma, oxidizes arachidonic acid in the presence of lipid peroxides, and leads to the vasoconstriction in the kidney (Banerjee et al., 1992; Moore et al., 1998). It is interesting to speculate that similar mechanisms may underlie the vascular pathologies associated with hemolysis.

Structurally analogous to the enzymatically generated prostaglandins, there have been described isoprostanes containing F-prostane, D/E-prostane, and A_2/J_2 cyclopentenone ring structures (Fig. 12.3). Analogous to the cyclopentenone prostaglandins, the cyclopentenone isoprostanes form via dehydration of the D/E-isoprostanes within the cyclopentane ring. Each of these can form 4 regioisomers, which in turn can each form 4 stereoisomers. One particular cyclopentenone

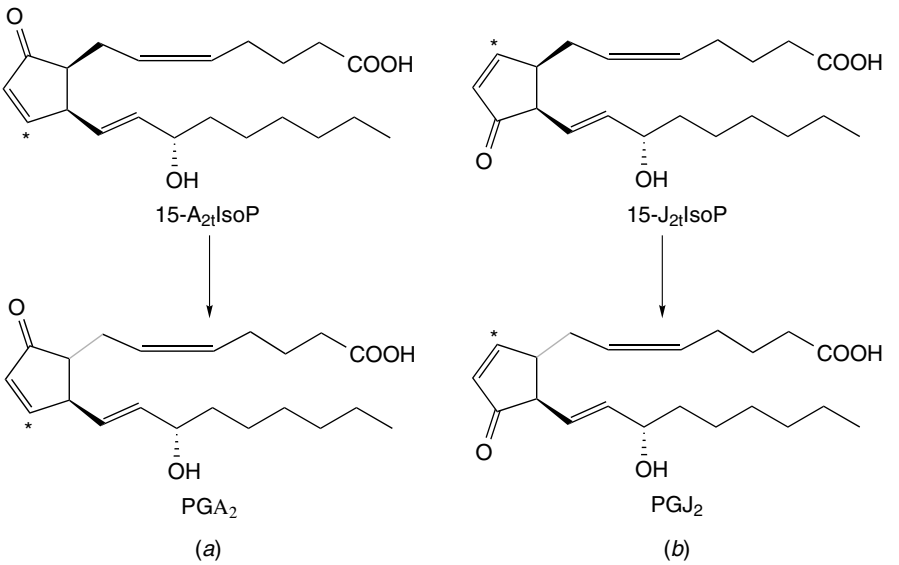


FIGURE 12.3 Similarities between isoprostanes and prostaglandins. The isoprostanes are prostaglandin-like compounds, which are formed through free radical mediated peroxidation of AA within the plasma membrane. Two common isoprostanes detectable in vivo, 15-A_{2t}-IsoP (also called 8-iso-PGA_2) and 15-J_{2t}-IsoP are drawn above. Note the only structural difference between PGA_2 and 15-A_{2t}-IsoP is the chirality about the asymmetric carbon C-8. This is seen again in 15-J_{2t}-IsoP when compared to PGJ_2.

isoprostane, 15-A_{2t}-isoprostane, has been shown to increase in the livers of animals exposed to oxidative stress (Chen et al., 1999).

12.4 THE THIOL SWITCH AND REDOX CELL SIGNALING

One of the primary targets for electrophiles are the nucleophilic amino acids. Among these cysteine is particularly important because it serves numerous functions in proteins. These include regulation of enzyme active sites and conformational changes leading to modulation of cell signaling proteins (Cooper et al., 2002; Ghezzi and Bonetto, 2003; Giles et al., 2003a, 2003b). Cysteine residues have the potential to control "redox" cell signaling pathways, since thiol chemistry offers the possibility of modification by structurally diverse species, including those derived from oxidized lipids, peroxides, or nitric oxide (Cleeter et al., 1994; Cooper et al., 2002; Ghezzi and Bonetto, 2003). Redox signaling contributes to the control of cell development, differentiation, growth, death, and adaptation, and it has been implicated in diverse physiological and pathological processes (Fukagawa et al., 2000; Forman and Torres, 2001; Stamler et al., 2001; Droge, 2002; Xu et al., 2002; Landar and Darley-Usmar, 2003). To date perhaps the best understood thiol switching mechanism is the *S*-nitrosylation pathway, which has been implicated in a broad range of signal transduction pathways including inhibition of apoptosis (Marshall et al., 2000; Stamler et al., 2001). It is important to note that the electrophilic cyclopentenone structures are limited in their reactivity with cellular nucleophiles, probably exclusively to those involving cysteine residues with a low pK_a. This concept is supported by our own proteomics studies, and those of others, with cyclopentenone electrophiles, which react with a very small proteome (composed of probably only 20–30 proteins) within the cell (Levonen et al., 2004; Sanchez-Gomez et al., 2004). We have termed this group of proteins the electrophile responsive proteome to emphasize its limited and apparently specific nature (Ceaser et al., 2004). We hypothesize that it is through the modification of thiols on proteins by electrophilic lipids that a major class of oxidized lipid signaling pathways appears to be mediated.

Indeed increasing attention is being paid to the nonreceptor mediated actions of electrophilic lipids including cyclooxygenase products. We propose that the electrophilic lipid oxidation products from COX-2 are capable of contributing to the beneficial cytoprotective effects of this enzyme. Some of the possible isomers of the J and A series cyclopentenone electrophilic lipids are known to be formed in biological systems and have been shown to exert biological effects through mechanisms independent of the PPAR system (Chen et al., 1999; Amici et al., 2001; Levonen et al., 2001b, 2003; Clay et al., 2002). It has been proposed that the cy-PGs may participate in the resolution of inflammation through inhibition of NF-κB activity (Amici et al., 2001).

As mentioned previously many of the cytoprotective properties of these compounds are mediated through the electrophile response element (EpRE), which regulates genes important for protection against nitrosative or oxidative

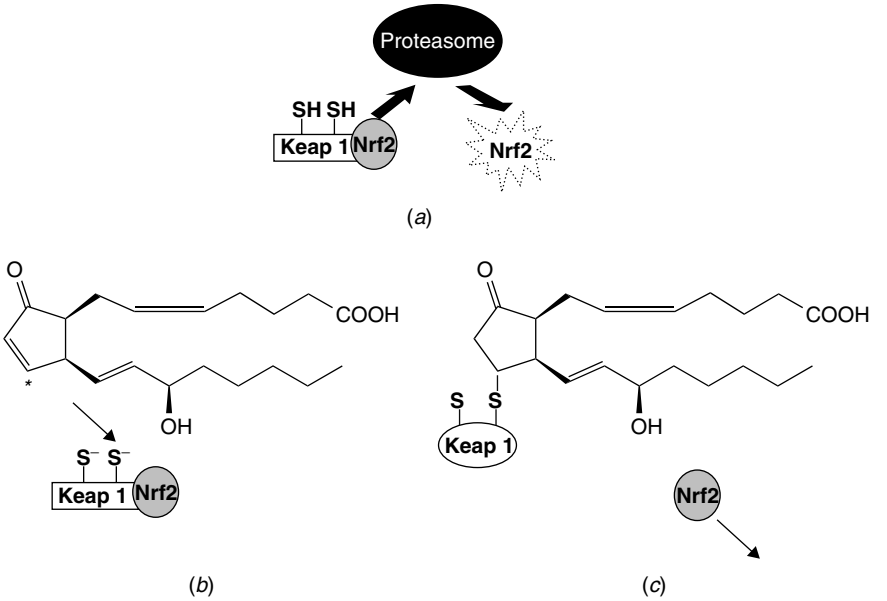


FIGURE 12.4 Activation of EpRE by cyclopentenones. In the cytosol during resting conditions, Nrf2 is bound to Keap1 homodimers (*a*) and directed to the proteasome for degradation. (*b*) The sulfhydryl groups on Keap1 are able to conjugate with electrophilic carbon centers, such as those found on the electrophile 15d-PGJ_2. The covalent modification of Keap1 by 15d-PGJ_2 allows for release of Nrf2. (*c*) This free Nrf2 is now able to translocate to the nucleus where it can bind to EpRE elements and, along with other bZIP proteins such as JunD, mediate EpRE-driven transcription.

stress including the enzymes that synthesize GSH (Nguyen et al., 2003b). The mechanism by which EpRE is activated upon exposure of the cell to oxidants or electrophiles is through the dissociation of the transcription factor Nrf2 from its cytoplasmic binding protein Keap1 and binding to the EpRE sequence (Fig. 12.4) (Levonen et al., 2003; Nguyen et al., 2003b; Dickinson et al., 2004). Keap1 directs Nrf2 to the proteasome for degradation, and this is a major regulatory mechanism for controlling the levels of Nrf2 available to migrate to the nucleus (Itoh et al., 2003; McMahon et al., 2003). This results in the expression of cytoprotective enzymes such as glutamyl cysteine ligase (GCL) and heme oxygenase (Dinkova-Kostova et al., 2002; Levonen et al., 2003; Itoh et al., 2004). Our published studies demonstrate the direct modification of Keap1 by electrophilic lipid oxidation products, including those such as the J and A series isoprostanes (Dickinson et al., 2003, 2004; Ceaser et al., 2004; Levonen et al., 2004). The dominating structural motif responsible for the biological actions of these compounds is the presence of the electrophilic carbon, since closely related compounds that do not possess this group are unable to activate the EpRE (Levonen et al., 2004). Furthermore, only those compounds that are

electrophilic, or can form an electrophile through subsequent dehydrations and rearrangements, are capable of inducing the induction of GSH synthesis, which we have shown is meditated via the EpRE (Levonen et al., 2004). It is also clear that nonenzymatic prostaglandin analogues such as 15-A_{2t}-isoprostane can form covalent adducts with the Nrf2 regulatory protein Keap1, allowing for regulation of cytoprotective gene expression (Levonen et al., 2004).

As expected cross-talk with other signal transduction pathways is mediated by several kinase pathways, including the p38 and ERK1/2 MAP-kinases, PI3-kinase, and PKC (Wild and Mulcahy, 2000; Zipper and Mulcahy, 2000; Go et al., 2004). Furthermore, the Nrf2 gene itself has two EpRE elements in its promoter, allowing a positive feedback regulation of Nrf2 to further enhance protection against oxidative or nitrosative stress (Bloom et al., 2002; Nguyen et al., 2002). Thus, it is possible that electrophilic cyclopentenone lipid products, which have been detected in atherosclerotic lesions (Shibata et al., 2002), may be contributing to cytoprotection against cardiovascular disease.

12.5 BIOLOGICAL RESPONSES TO ENDOGENOUS ELECTROPHILIC LIPID PRODUCTION

Can the uncontrolled introduction of environmental or nonspecific lipid peroxidation reactions disrupt electrophilic signaling pathways? The answer to this question is still not clear, but some of the earliest evidence for a role of lipid oxidation reactions in vivo was the detection of electrophilic lipids adducted to proteins in atherosclerotic lesions (Palinski et al., 1989; Yla-Herttuala et al., 1989). In human atherosclerotic lesions the immunoreactivity against COX-2, prostaglandin D synthase, and 15d-PGJ_2 are increased (Shibata et al., 2002; Cipollone et al., 2004). A potentially anti-atherogenic role of COX-2 has been proposed, and the enzyme appears to be expressed in endothelial cells overlying vascular lesions (Baker et al., 1999; Schonbeck et al., 1999; Belton et al., 2000). It is not known whether electrophilic lipids promote or inhibit the progression of the disease, since we currently do not understand the basic mechanisms through which they act on the endothelium. It is tempting to speculate that some of the deleterious effects of cyclooxygenase inhibitors may be due in part to the loss of the ability to generate endogenous lipid electrophiles capable of inducing intracellular antioxidant defenses.

12.6 A NEW PARADIGM OF OXIDIZED LIPID SIGNALING—THE COVALENT ADVANTAGE

Can the formation of a covalent bond between a lipid oxidation product and a protein in a cellular setting have a biological advantage? Critical to answering this question is understanding the fact that electrophilic lipid oxidation products are found at low concentrations in the free form in biological systems. For example, in a recent report the levels of free 15d-PGJ_2 were measured in vivo

and the concentrations found to be lower than that required for the activation of PPARγ (Bell-Parikh et al., 2003). However, this result should not be extrapolated to exclude the possibility that non-PPARγ mechanisms may be activated by electrophilic cyclopentenones. Excluding such a possibility on the basis of concentration alone clearly does not encompass the potential of these compounds to have biological effects through mechanisms involving the direct post-translational modification of proteins. The quantitative estimation of electrophilic lipid oxidation products is complicated by the reactivity of the α,β-unsaturated ketone group, which, as mentioned above, renders them susceptible to conjugation. Electrophilic lipid oxidation products, including 4-HNE, 15-A_{2t}-isoprostane, and the A and J series prostaglandins, are conjugated to GSH by glutathione-*S*-transferases and, as we have demonstrated, proteins (Cox et al., 2002; Hubatsch et al., 2002; Levonen et al., 2004). For these reasons the measured concentrations of free compounds do not represent the levels that cells are exposed to locally or the flux of electrophiles generated by nonenzymatic or enzymatic mechanisms of lipid peroxidation. In addition it is known that COX-2 can be induced in endothelial cells (Okahara et al., 1998; Cao et al., 2001; Gryglewski et al., 2001; Rikitake et al., 2001), and it is likely that the local formation of cyclopentenone prostaglandins will lead to modification of nearby protein thiols.

To address the potential relevance of covalent and noncovalent mechanisms to control redox signaling, a comparison of both mechanisms is instructive. Reversible binding of a ligand to a receptor has, at first glance, a clear advantage over a covalent modification in terms of cell signaling in that the reversibility of the system is responsive to changes in the concentration of the agonist. A good example of this model is the activation of soluble guanylate cyclase (sGC) by NO. Once NO is removed, synthesis of cGMP ceases, and its removal by phosphodiesterases allows a dynamic response in vessel tone. Furthermore the receptor, in this case sGC, may be reused multiple times.

To illustrate the potential of a covalent linkage to activate a signal transduction pathway at biologically relevant concentrations of the electrophilic lipids, we can simulate the receptor occupancy of the agonist with and without covalent bond formation with the receptor. In this model we have set the receptor concentration at 1 nM and the K_d for binding of the electrophilic lipid agonist at 10 nM. The results of the kinetics of the simulation of this reaction are shown in Figure 12.5 for both reversible and irreversible binding of the agonist. As predicted for the simple binding kinetics, where the agonist (10 nM) is allowed to freely diffuse, the receptor shows 50% occupancy under these conditions and this is achieved over a time period of a few seconds (Fig. 12.5*a*). In contrast, when covalent binding is allowed, activation approaches 100% over a similar time period with the same concentration of agonist. In the second scenario the agonist concentration is decreased to 1 nM, and as shown in Figure 12.5*b*, only when the formation of a covalent bond with the receptor is allowed, does full activation of the receptor occur. The activation of the receptor occurs over a slower time scale, and this offers an additional level of control over the activation of the signaling pathway. For example, if the turnover of the receptor is faster

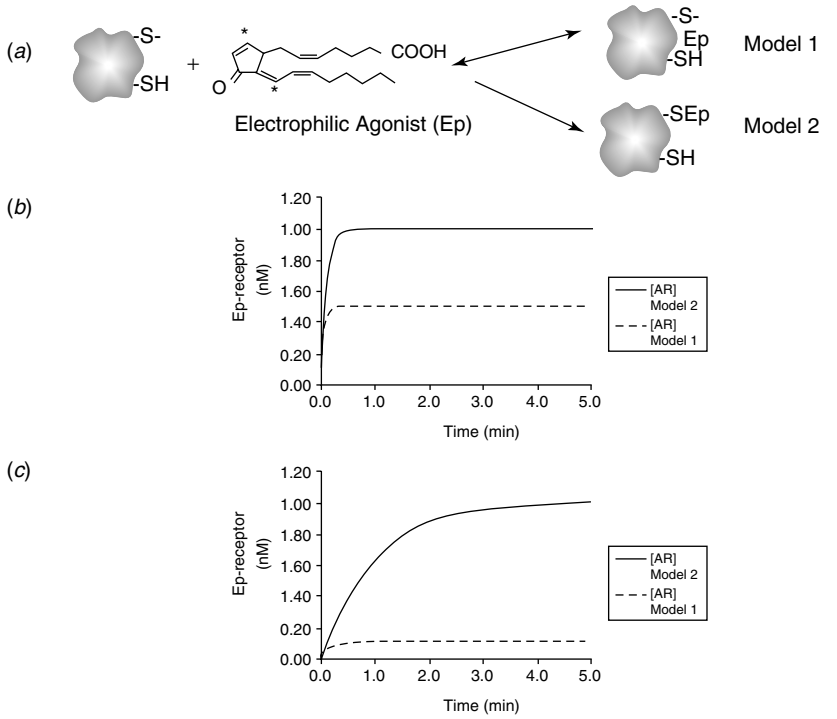


FIGURE 12.5 Simulation of the reversible and irreversible model for the binding of an electrophile to a receptor. (*a*) The scheme shows the two models simulated to demonstrate the covalent advantage in activation of a cell signaling pathway (Ep = an electrophilic lipid agonist). (*b*) The computer simulation shows the occupancy of a receptor (R = 1 nM) exposed to an agonist (A = 10 nM) with an affinity constant of 10 nM using a simple reversible binding model (model 1) compared with the occupancy of the receptor with formation of an irreversible covalent interaction between receptor and agonist (model 2). The concentration of the agonist has been fixed such that binding does not deplete the available concentration. The receptor shows 50% occupancy achieved over a time period of a few seconds for model 1 and full occupancy with model 2. (*c*) The computer simulation shows the same models under the same conditions with the exception that the concentration of agonist is set at 1 nM.

than the time taken to accumulate the agonist–receptor covalent interaction, then activation of the signaling pathway will not occur. The covalent binding models the exposure of the receptor to a low but constant flux of electrophile such as would occur with activation of cyclooxygenase. These data demonstrate the covalent advantage in which a low flux of reactive electrophile can lead to full activation of a receptor even though the concentration is well below the binding constant for the lipid. Clearly, the biological setting is complicated by other factors, for example, competition with detoxification pathways for the electrophile by enzymes such as the glutathione *S*-transferases (GSTs). An interesting point

arises in this regard: if the K_m for the electrophile for these enzymes is in the μM range, as reported for representative compounds, then if the concentration of the electrophile is 10 nM, little or no competition for a reactive signaling molecule will occur (Sheehan et al., 2001). We propose that only under extreme conditions, as may occur in some pathologies associated with inflammation, will significant metabolism through this route occur. In the covalent advantage paradigm specificity is possible because of the high reactivity of thiols in proteins that have a low pK_a (Sanchez-Gomez et al., 2004). As discussed above, a number of proteins have been identified that can form stable, covalent adducts with electrophilic lipids that have the potential to control cell signaling (Shibata et al., 2003a, 2003b; Zhuang et al., 2003b; Cheron et al., 2004; Levonen et al., 2004; Zhang et al., 2004; Milne et al., 2005). With respect to reversibility it is now clear from studies reported in the literature that proteasomal-dependent turnover of proteins is also an important regulatory step in controlling transcriptional regulation (Mullally et al., 2001; Itoh et al., 2003; Nguyen et al., 2003a; Shibata et al., 2003b; Levonen et al., 2004). We propose that at low concentrations and low levels of modification, the reactivity with a subproteome within the cell leads to cytoprotection, while at high concentrations and high levels of modification, it will lead to apoptosis (Dickinson et al., 2003; Ceaser et al., 2004; Levonen et al., 2004).

12.7 IMPLICATIONS FOR THE PATHOPHYSIOLOGY OF DISEASE

Under conditions of increased free fatty acids and inflammation it is possible that the activation of cytoprotective pathways by electrophilic lipids is deleterious. For example, several lines of evidence suggest that there is a connection between COX-2, obesity, and cancer (Leris and Mokbel, 2001; Na and Surh, 2003; Raju and Bird, 2003). Products of COX-2 have been shown to promote cellular proliferation and angiogenesis. An important role for COX-2 has been proposed in cancer etiology through a number of mechanisms (Dempke et al., 2001; Davies et al., 2003). It is already known that some of the products of COX-2 and its related metabolites can act through activation of PPARγ such as 15d-PGJ_2 (Willson et al., 1996; Na and Surh, 2003). Interestingly these cy-PGs are electrophilic and, as mentioned above, have the capacity to initiate cell signaling through the post-translational modification of proteins, which then mediate effects via PPARγ-independent mechanisms. There are a number of reports in the cancer field that focus on the pro-apoptotic effects of 15d-PGJ_2 (Clay et al., 2002; Emi and Maeyama, 2004). However, we have shown that in endothelial cells 15d-PGJ_2 exerts a biphasic effect in a number of different cell lines, with low concentrations being cytoprotective and higher concentrations being pro-apoptotic (Levonen et al., 2001b). While the current studies in the cancer field promote the interesting possibility that 15d-PGJ_2 may be used as a pharmacological anticancer agent, we propose that low levels of 15d-PGJ_2 produced endogenously under certain conditions such as obesity may promote cell survival and proliferation. For example, we have shown that at low concentrations

15d-PGJ_2 can protect cells from oxidative stress through the induction of GSH synthesis (Levonen et al., 2001b, 2003; Moellering et al., 2002; Ceaser et al., 2003). As mentioned above, electrophilic lipids such as 15d-PGJ_2 can also evoke other cytoprotective responses such as the induction of heme oxygenase-1 (HO-1), through the transcriptional regulation of the EpRE (Koppal et al., 2000; Lee et al., 2003; Zhuang et al., 2003a, 2003b; Itoh et al., 2004). Increased levels of these enzymes lead to increased resistance to apoptosis and decreased cytotoxicity from the redox cycling therapeutic agents, such as adriamycin (Fang et al., 2004; Gamcsik et al., 2004). However, it remains unknown whether increases in COX-2-derived electrophilic lipids from elevated free fatty acids present in obesity predispose mammary cells to develop cancer.

In cardiovascular disease, in contrast to cancer, induction of cytoprotective genes may be beneficial. It is then interesting to speculate that the introduction of other molecules, which are also reactive with protein nucleophiles, may disrupt these signaling pathways, leading to exacerbation of an underlying pathological condition such as atherosclerosis. This may be what is contributing in part to the increased risk to cardiovascular disease associated with exposure to tobacco smoke. Epidemiological evidence strongly suggests that cigarette smoking increases the incidence of cardiovascular disease (CVD) in both men and women (Doll and Peto, 1976; Doll et al., 1980; Willett et al., 1987). Furthermore, passive smoking (environmental tobacco smoke) is associated with approximately a 30% increase in risk of CVD, compared with an 80% increase in active smokers (Glantz and Parmley, 1991; Law et al., 1997). Atherosclerosis is a particularly important component of CVD, contributing significantly to the overall mortality and morbidity of this disease. Our current understanding of the pathobiology of atherosclerosis suggests that these alterations of endothelial function play a pivotal role in the development and progression of the disease and its associated clinical complications (Schulz et al., 2004). Lifestyle factors, such as obesity discussed above, are clearly significant contributory factors to the progression of vascular dysfunction, but the molecular mechanisms underlying these effects remain largely obscure.

As we have shown, electrophilic lipids at low concentrations have a protective role. One possibility is that reactive electrophiles such as acrolein present in cigarette smoke cause cell dysfunction through perturbation of the electrophile responsive proteome. Acrolein has been shown to induce apoptosis in several cell types, a process associated with the depletion of cellular GSH and the generation of intracellular oxidants (Nardini et al., 2002). Acrolein is also known to be able to form adducts with cellular proteins (Burcham and Fontaine, 2001). Other nonenzymatic products from arachidonic acid that are highly reactive with nucleophiles can also be formed. These include rearrangements of PGH_2 to form the levuglandins E_2 and D_2 (Fig. 12.6*a*). These products are classified as γ-ketoaldehydes, and they are known to react rapidly with the ε-amino group of lysine. The data suggest the hypothesis that environmentally induced electrophilic toxins may cause dysfunction in cytoprotective redox signaling pathways through post-translational modification of proteins.

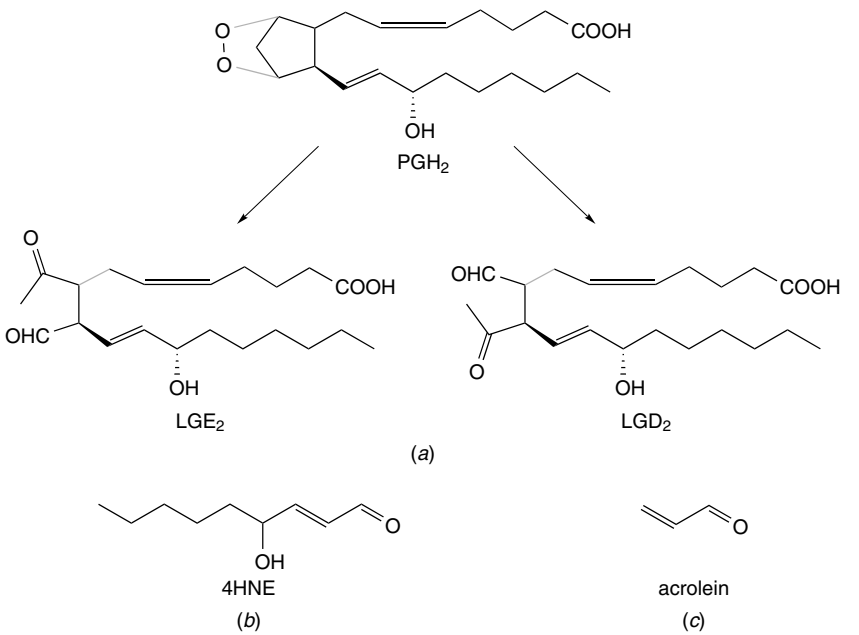


FIGURE 12.6 Nonenzymatic generation of reactive lipid electrophiles. (*a*) The cyclooxygenase product PGH_2, in addition to its enzymatic conversion to the various prostaglandins (as in Fig. 12.1), can also undergo nonenzymatic rearrangements to form the levuglandins LGE_2 and LGD_2. These compounds are γ-ketoaldehydes that can react rapidly with lysine residues in proteins to form covalent adducts. (*b*) The endogenously produced electrophile 4-HNE is often considered to be an end-product of lipid peroxidation. (*c*) Acrolein is the highly reactive electrophile found in cigarette smoke.

12.8 SUMMARY

It is now becoming clear that activation of the electrophile responsive proteome plays a central role in the coordination of the cellular response to oxidative and nitrosative stress. This subproteome is characterized by a family of proteins with reactive nucleophiles that can accumulate a covalent modification over time and so modify cellular responses. Interestingly this paradigm gives a different perspective to the role of GSH and related enzymes with respect to redox signaling pathways (Fig. 12.7). We propose that one of their primary functions, with respect to cell signaling, is to "insulate" the thiol switching proteins in these pathways from unregulated activation and instead to maintain local control within specific signaling domains. In this model the covalent advantage is to allow redox signaling pathways to sense the low flux of electrophiles generated by controlled oxidation of lipids within the cell. This raises a possible mechanism for how intracellular sources of reactive oxygen and nitrogen species in the cell can participate in redox cell signaling. In this respect we propose that

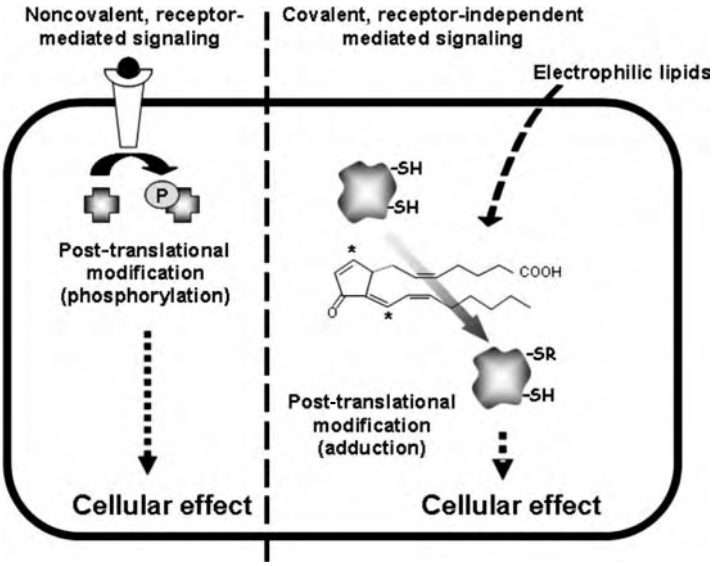


FIGURE 12.7 The covalent advantage in redox cell signaling. (*a*) The more classical signaling mediated by prostaglandins involves the reversible, noncovalent binding of an agonist, such as the nonelectrophilic prostanoid PGE_2, to a receptor. This causes a change in the receptor, which leads to the activation of intracellular signaling cascades through a series of post-translational modifications, typically phosphorylation, ultimately resulting in a cellular effect. (*b*) The signaling mediated by electrophilic lipids involves the covalent binding of a reactive agonist, such as the electrophilic prostanoid 15d-PGJ_2, to a protein, for example, Keap1, resulting in a change in the function of that adducted protein, eventually resulting in a cellular effect.

the mitochondrion is just such a potential site at which hydrogen peroxide signaling can be transduced through the formation of secondary lipid electrophiles to coordinate the bioenergetics of the cell with the commands for cell growth, differentiation, or apoptosis.

ACKNOWLEDGMENTS

The authors are grateful for support from the National Institutes of Health (HL58031, ES10167, AA13395, HL70610) to VDU, and from the American Heart Association, Southeast Affiliate, to DAD.

LIST OF ABBREVIATIONS

15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2
AA, arachidonic acid

COX, cyclooxygenase
CVD, cardiovascular disease
cy-PGs, cyclopentenone prostaglandins
EpRE, electrophile response element
GCL, glutamate-cysteine ligase
GSH, glutathione
GST, glutathione-*S*-transferase
4-HNE, 4-hydroxy-2-nonenal
HO-1, heme oxygenase-1
Keap-1, kelch like erythroid cell derived protein with CNC homology (ECH)–associated protein-1
NOS, nitric oxide synthase
Nrf2, nuclear factor-erythroid 2 related factor
PGs, prostaglandins
PKC, protein kinase C
PPAR, peroxisome proliferator activated receptor
PUFA, polyunsaturated fatty acid
RNS, reactive nitrogen species
ROS, reactive oxygen species
sGC, soluble guanylate cyclase

REFERENCES

Amici C., Belardo G., Rossi A., Santoro M.G. (2001). Activation of I kappa b kinase by herpes simplex virus type 1: A novel target for anti-herpetic therapy. *J Biol Chem* 276: 28759–28766.

Baker C.S., Hall R.J., Evans T.J., Pomerance A., Maclouf J., Creminon C., Yacoub M.H., Polak J.M. (1999). Cyclooxygenase-2 is widely expressed in atherosclerotic lesions affecting native and transplanted human coronary arteries and colocalizes with inducible nitric oxide synthase and nitrotyrosine particularly in macrophages. *Arterioscler Thromb Vasc Biol* 19: 646–655.

Banerjee M., Kang K.H., Morrow J.D., Roberts L.J., Newman J.H. (1992). Effects of a novel prostaglandin, 8-epi-PGF2 alpha, in rabbit lung in situ. *Am J Physiol* 263: H660–663.

Bea F., Hudson F.N., Chait A., Kavanagh T.J., Rosenfeld M.E. (2003). Induction of glutathione synthesis in macrophages by oxidized low-density lipoproteins is mediated by consensus antioxidant response elements. *Circ Res* 92: 386–393.

Bell-Parikh L.C., Ide T., Lawson J.A., McNamara P., Reilly M., FitzGerald G.A. (2003). Biosynthesis of 15-deoxy-delta12,14-PGJ2 and the ligation of PPARgamma. *J Clin Invest* 112: 945–955.

Belton O., Byrne D., Kearney D., Leahy A., Fitzgerald D.J. (2000). Cyclooxygenase-1 and -2-dependent prostacyclin formation in patients with atherosclerosis. *Circulation* 102: 840–845.

Berliner J.A., Subbanagounder G., Leitinger N., Watson A.D., Vora D. (2001). Evidence for a role of phospholipid oxidation products in atherogenesis. *Trends Cardiovasc Med* 11: 142–147.

Bloom D., Dhakshinamoorthy S., Jaiswal A.K. (2002). Site-directed mutagenesis of cysteine to serine in the DNA binding region of Nrf2 decreases its capacity to upregulate antioxidant response element-mediated expression and antioxidant induction of NAD(P)H:quinone oxidoreductase1 gene. *Oncogene* 21: 2191–2200.

Burcham P.C., Fontaine F. (2001). Extensive protein carbonylation precedes acrolein-mediated cell death in mouse hepatocytes. *J Biochem Mol Toxicol* 15: 309–316.

Cao C., Matsumura K., Shirakawa N., Maeda M., Jikihara I., Kobayashi S., Watanabe Y. (2001). Pyrogenic cytokines injected into the rat cerebral ventricle induce cyclooxygenase-2 in brain endothelial cells and also upregulate their receptors. *Eur J Neurosci* 13: 1781–1790.

Ceaser E.K., Moellering D.R., Shiva S., Ramachandran A., Landar A., Venkartraman A., Crawford J., Patel R., Dickinson D.A., Ulasova E., Ji S., Darley-Usmar V.M. (2004). Mechanisms of signal transduction mediated by oxidized lipids: The role of the electrophile-responsive proteome. *Biochem Soc Trans* 32: 151–155.

Ceaser E.K., Ramachandran A., Levonen A.L., Darley-Usmar V.M. (2003). Oxidized low-density lipoprotein and 15-deoxy-delta 12,14-PGJ2 increase mitochondrial complex I activity in endothelial cells. *Am J Physiol Heart Circ Physiol* 285: H2298–2308.

Chen Y., Morrow J.D., Roberts L.J., 2nd (1999). Formation of reactive cyclopentenone compounds in vivo as products of the isoprostane pathway. *J Biol Chem* 274: 10863–10868.

Cheron A., Peltier J., Perez J., Bellocq A., Fouqueray B., Baud L. (2004). 15-deoxy-Delta12,14-prostaglandin J2 inhibits glucocorticoid binding and signaling in macrophages through a peroxisome proliferator-activated receptor gamma-independent process. *J Immunol* 172: 7677–7683.

Cipollone F., Rocca B., Patrono C. (2004). Cyclooxygenase-2 expression and inhibition in atherothrombosis. *Arterioscler Thromb Vasc Biol* 24: 246–255.

Clay C.E., Monjazeb A., Thorburn J., Chilton F.H., High K.P. (2002). 15-Deoxy-delta12,14-prostaglandin J2-induced apoptosis does not require PPARgamma in breast cancer cells. *J Lipid Res* 43: 1818–1828.

Cleeter M.W., Cooper J.M., Darley-Usmar V.M., Moncada S., Schapira A.H. (1994). Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide: Implications for neurodegenerative diseases. *FEBS Lett* 345: 50–54.

Cooper C.E., Patel R.P., Brookes P.S., Darley-Usmar V.M. (2002). Nanotransducers in cellular redox signaling: modification of thiols by reactive oxygen and nitrogen species. *Trends Biochem Sci* 27: 489–492.

Cox B., Murphey L.J., Zackert W.E., Chinery R., Graves-Deal R., Boutaud O., Oates J.A., Coffey R.J., Morrow J.D. (2002). Human colorectal cancer cells efficiently conjugate the cyclopentenone prostaglandin, prostaglandin J(2), to glutathione. *Biochim Biophys Acta* 1584: 37–45.

Darley-Usmar V.M., Severn A., O'Leary V.J., Rogers M. (1991). Treatment of macrophages with oxidized low-density lipoprotein increases their intracellular glutathione content. *Biochem J* 278: 429–434.

Davies G., Salter J., Hills M., Martin L.A., Sacks N., Dowsett M. (2003). Correlation between cyclooxygenase-2 expression and angiogenesis in human breast cancer. *Clin Cancer Res* 9: 2651–2656.

Dempke W., Rie C., Grothey A., Schmoll H.J. (2001). Cyclooxygenase-2: A novel target for cancer chemotherapy? *J Cancer Res Clin Oncol* 127: 411–417.

Dickinson D.A., Levonen A.L., Moellering D.R., Arnold E.K., Zhang H., Darley-Usmar V.M., Forman H.J. (2004). Human glutamate cysteine ligase gene regulation through the electrophile response element. *Free Radic Biol Med* 37: 1152–1159.

Dickinson D.A., Moellering D.R., Iles K.E., Patel R.P., Levonen A.L., Wigley A., Darley-Usmar V.M., Forman H.J. (2003). Cytoprotection against oxidative stress and the regulation of glutathione synthesis. *Biol Chem* 384: 527–537.

Dinkova-Kostova A.T., Holtzclaw W.D., Cole R.N., Itoh K., Wakabayashi N., Katoh Y., Yamamoto M., Talalay P. (2002). Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc Natl Acad Sci USA* 99: 11908–11913.

Doll R., Gray R., Hafner B., Peto R. (1980). Mortality in relation to smoking: 22 years' observations on female British doctors. *Br Med J* 280: 967–971.

Doll R., Peto R. (1976). Mortality in relation to smoking: 20 Years' observations on male British doctors. *Br Med J* 2: 1525–1536.

Droge W. (2002). Free radicals in the physiological control of cell function. *Physiol Rev* 82: 47–95.

Emi M., Maeyama K. (2004). The biphasic effects of cyclopentenone prostaglandins, prostaglandin J(2) and 15-deoxy-Delta(12,14)-prostaglandin J(2) on proliferation and apoptosis in rat basophilic leukemia (RBL-2H3) cells. *Biochem Pharmacol* 67: 1259–1267.

Fang J., Sawa T., Akaike T., Greish K., Maeda H. (2004). Enhancement of chemotherapeutic response of tumor cells by a heme oxygenase inhibitor, pegylated zinc protoporphyrin. *Int J Cancer* 109: 1–8.

Forman H.J., Torres M. (2001). Redox signaling in macrophages. *Mol Aspects Med* 22: 189–216.

Fukagawa N.K., Timblin C.R., Buder-Hoffman S., Mossman B.T. (2000). Strategies for evaluation of signaling pathways and transcription factors altered in aging. *Antioxid Redox Signal* 2: 379–389.

Gamcsik M.P., Bierbryer R.G., Millis K.K. (2004). Noninvasive monitoring of glutathione turnover in perfused MCF-7 cells. *Free Radic Biol Med* 37: 961–968.

Ghezzi P., Bonetto V. (2003). Redox proteomics: Identification of oxidatively modified proteins. *Proteomics* 3: 1145–1153.

Giles N.M., Giles G.I., Jacob C. (2003a). Multiple roles of cysteine in biocatalysis. *Biochem Biophys Res Commun* 300: 1–4.

Giles N.M., Watts A.B., Giles G.I., Fry F.H., Littlechild J.A., Jacob C. (2003b). Metal and redox modulation of cysteine protein function. *Chem Biol* 10: 677–693.

Glantz S.A., Parmley W.W. (1991). Passive smoking and heart disease: Epidemiology, physiology, and biochemistry. *Circulation* 83: 1–12.

Go Y.M., Gipp J.J., Mulcahy R.T., Jones D.P. (2004). H_2O_2-dependent activation of GCLC-ARE4 reporter occurs by mitogen-activated protein kinase pathways without oxidation of cellular glutathione or thioredoxin-1. *J Biol Chem* 279: 5837–5845.

Gotoh N., Graham A., Nikl E., Darley-Usmar V.M. (1993). Inhibition of glutathione synthesis increases the toxicity of oxidized low-density lipoprotein to human monocytes and macrophages. *Biochem J* 296: 151–154.

Griendling K.K., Sorescu D., Lassegue B., Ushio-Fukai M. (2000). Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. *Arterioscler Thromb Vasc Biol* 20: 2175–2183.

Gryglewski R.J., Chlopicki S., Uracz W., Marcinkiewicz E. (2001). Significance of endothelial prostacyclin and nitric oxide in peripheral and pulmonary circulation. *Med Sci Monit* 7: 1–16.

Hanna I.R., Taniyama Y., Szocs K., Rocic P., Griendling K.K. (2002). NAD(P)H oxidase-derived reactive oxygen species as mediators of angiotensin II signaling. *Antioxid Redox Signal* 4: 899–914.

Hubatsch I., Mannervik B., Gao L., Roberts L.J., Chen Y., Morrow J.D. (2002). The cyclopentenone product of lipid peroxidation, 15-A(2t)-isoprostane (8-isoprostaglandin A(2)), is efficiently conjugated with glutathione by human and rat glutathione transferase A4–4. *Chem Res Toxicol* 15: 1114–1118.

Ischiropoulos H. (1998). Living and dying with reactive species. Focus on "peroxynitrite induces apoptosis of HL-60 cells by activation of a caspase-3 family protease." *Am J Physiol* 274: C853–C854.

Itoh K., Mochizuki M., Ishii Y., Ishii T., Shibata T., Kawamoto Y., Kelly V., Sekizawa K., Uchida K., Yamamoto M. (2004). Transcription factor Nrf2 regulates inflammation by mediating the effect of 15-deoxy-delta(12,14)-prostaglandin J(2). *Mol Cell Biol* 24: 36–45.

Itoh K., Wakabayashi N., Katoh Y., Ishii T., O'Connor T., Yamamoto M. (2003). Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes Cells* 8: 379–391.

Kim S.O., Merchant K., Nudelman R., Beyer W.F., Jr., Keng T., DeAngelo J., Hausladen A., Stamler J.S. (2002). OxyR: A molecular code for redox-related signaling. *Cell* 109: 383–396.

Koppal T., Petrova T.V., Van Eldik L.J. (2000). Cyclopentenone prostaglandin 15-deoxy-Delta(12,14)-prostaglandin J(2) acts as a general inhibitor of inflammatory responses in activated BV-2 microglial cells." *Brain Res* 867: 115–121.

Landar A., Darley-Usmar V.M. (2003). Nitric oxide and cell signaling; modulation of redox tone and protein modification. *Amino Acids* 25: 313–321.

Law M.R., Morris J.K., Wald N.J. (1997). Environmental tobacco smoke exposure and ischaemic heart disease: an evaluation of the evidence. *Br Med J* 315: 973–980.

Lee T.S., Tsai H.L., Chau L.Y. (2003). Induction of heme oxygenase-1 expression in murine macrophages is essential for the anti-inflammatory effect of low dose 15-deoxy-delta 12,14-prostaglandin J2. *J Biol Chem* 278: 19325–19330.

Leris C., Mokbel K. (2001). The prevention of breast cancer: An overview. *Curr Med Res Opin* 16: 252–257.

Levonen A.L., Dickinson D.A., Moellering D.R., Mulcahy R.T., Forman H.J., Darley-Usmar V.M. (2001b). Biphasic effects of 15-deoxy-delta(12,14)-prostaglandin J(2) on glutathione induction and apoptosis in human endothelial cells. *Arterioscler Thromb Vasc Biol* 21: 1846–1851.

Levonen A.L., Landar A., Ramachandran A., Ceaser E.K., Dickinson D.A., Zanoni G., Morrow J.D., Darley-Usmar V.M. (2004). Cellular mechanisms of redox cell signalling:

Role of cysteine modification in controlling antioxidant defences in response to electrophilic lipid oxidation products. *Biochem J* 378: 373–382.

Levonen A.L., Patel R.P., Brookes P., Go Y.M., Jo H., Parthasarathy S., Anderson P.G., Darley-Usmar V.M. (2001a). Mechanisms of cell signaling by nitric oxide and peroxynitrite: from mitochondria to MAP kinases. *Antioxid Redox Signal* 3: 215–229.

Maggi L.B., Jr., Sadeghi H., Weigand C., Scarim A.L., Heitmeier M.R., Corbett J.A. (2000). Anti-inflammatory actions of 15-deoxy-delta 12,14-prostaglandin J2 and troglitazone: Evidence for heat shock-dependent and -independent inhibition of cytokine-induced inducible nitric oxide synthase expression. *Diabetes* 49: 346–355.

Marathe G.K., Zimmerman G.A., Prescott S.M., McIntyre T.M. (2002). Activation of vascular cells by PAF-like lipids in oxidized LDL. *Vascul Pharmacol* 38: 193–200.

Marshall H.E., Merchant K., Stamler J.S. (2000). Nitrosation and oxidation in the regulation of gene expression. *Faseb J* 14: 1889–1900.

Matsumoto A., Comatas K.E., Liu L., Stamler J.S. (2003). Screening for nitric oxide-dependent protein-protein interactions. *Science* 301: 657–661.

McMahon M., Itoh K., Yamamoto M., Hayes J.D. (2003). Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. *J Biol Chem* 278: 21592–21600.

Milne G.L., Musiek E.S., Morrow J.D. (2005). The cyclopentenone (A2/J2) isoprostanes—Unique, highly reactive products of arachidonate peroxidation. *Antioxid Redox Signal* 7: 210–220.

Moellering D.R., Levonen A.L., Go Y.M., Patel R.P., Dickinson D.A., Forman H.J., Darley-Usmar V.M. (2002). Induction of glutathione synthesis by oxidized low-density lipoprotein and 1-palmitoyl-2-arachidonyl phosphatidylcholine: Protection against quinone-mediated oxidative stress. *Biochem J* 362: 51–59.

Moore K.P., Darley-Usmar V., Morrow J., Roberts L.J. II (1995). Formation of F2-isoprostanes during oxidation of human low-density lipoprotein and plasma by peroxynitrite." *Circ Res* 77: 335–341.

Moore K.P., Holt S.G., Patel R.P., Svistunenko D.A., Zackert W., Goodier D., Reeder B.J., Clozel M., Anand R., Cooper C.E., Morrow J.D., Wilson M.T., Darley-Usmar V., Roberts L.J., 2nd. (1998). A causative role for redox cycling of myoglobin and its inhibition by alkalinization in the pathogenesis and treatment of rhabdomyolysis-induced renal failure. *J Biol Chem* 273: 31731–31737.

Morrow J.D. (2005). Quantification of isoprostanes as indices of oxidant stress and the risk of atherosclerosis in humans. *Arterioscler Thromb Vasc Biol* 25: 279–286.

Mullally J.E., Moos P.J., Edes K., Fitzpatrick F.A. (2001). Cyclopentenone prostaglandins of the J series inhibit the ubiquitin isopeptidase activity of the proteasome pathway. *J Biol Chem* 276: 30366–30373.

Na H.K., Surh Y.J. (2003). Peroxisome proliferator-activated receptor gamma (PPAR-gamma) ligands as bifunctional regulators of cell proliferation. *Biochem Pharmacol* 66: 1381–1391.

Nardini M., Finkelstein E.L., Reddy S., Valacchi G., Traber M., Cross C.E., van der Vliet A. (2002). Acrolein induced cytotoxicity in cultured human bronchial epithelial cells. Modulation by alpha-tocopherol and ascorbic acid. *Toxicology* 170: 173–185.

Nguyen T., Sherratt P.J., Huang H.C., Yang C.S., Pickett C.B. (2003a). Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the

antioxidant response element. Degradation of Nrf2 by the 26 S proteasome. *J Biol Chem* 278: 4536–4541.

Nguyen T., Sherratt P.J., Pickett C.B. (2003b). Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. *Ann Rev Pharmacol Toxicol*. 43: 233–260.

Okahara K., Sun B., Kambayashi J. (1998). Upregulation of prostacyclin synthesis-related gene expression by shear stress in vascular endothelial cells. *Arterioscler Thromb Vasc Biol* 18: 1922–1926.

Palinski W., Rosenfeld M.E., Yla-Herttuala S., Gurtner G.C., Socher S.S., Butler S.W., Parthasarathy S., Carew T.E., Steinberg D., Witztum J.L. (1989). Low density lipoprotein undergoes oxidative modification in vivo. *Proc Natl Acad Sci USA* 86: 1372–1376.

Patel R.P., Moellering D., Murphy-Ullrich J., Jo H., Beckman J.S., Darley-Usmar V.M. (2000). Cell signaling by reactive nitrogen and oxygen species in atherosclerosis. *Free Radic Biol Med* 28: 1780–1794.

Pratico D., Lawson J.A., Rokach J., FitzGerald G.A. (2001). The isoprostanes in biology and medicine. *Trends Endocrinol Metab* 12: 243–247.

Pratico D., Rokach J., Lawson J., FitzGerald G.A. (2004). F2-isoprostanes as indices of lipid peroxidation in inflammatory diseases. *Chem Phys Lipids* 128: 165–171.

Raju J., Bird R.P. (2003). Energy restriction reduces the number of advanced aberrant crypt foci and attenuates the expression of colonic transforming growth factor beta and cyclooxygenase isoforms in Zucker obese (fa/fa) rats. *Cancer Res* 63: 6595–6601.

Ramachandran A., Levonen A.L., Brookes P.S., Ceaser E., Shiva S., Barone M.C., Darley-Usmar V. (2002). Mitochondria, nitric oxide, and cardiovascular dysfunction. *Free Radic Biol Med* 33: 1465–1474.

Rhee S.G., Bae Y.S., Lee S.R., Kwon J. (2000). Hydrogen peroxide: a key messenger that modulates protein phosphorylation through cysteine oxidation. *Sci STKE* 2000: PE1.

Rikitake Y., Hirata K., Kawashima S., Takeuchi S., Shimokawa Y., Kojima Y., Inoue N., Yokoyama M. (2001). Signaling mechanism underlying COX-2 induction by lysophosphatidylcholine. *Biochem Biophys Res Commun* 281: 1291–1297.

Roberts L.J., 2nd, Brame C.J., Chen Y., Morrow J.D. (1999). Novel eicosanoids: Isoprostanes and related compounds. *Methods Mol Biol* 120: 257–285.

Rossi A., Kapahi P., Natoli G., Takahashi T., Chen Y., Karin M., Santoro M.G. (2000). Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IkappaB kinase. *Nature* 403: 103–108.

Rouzer C.A., Marnett L.J. (2003). Mechanism of free radical oxygenation of polyunsaturated fatty acids by cyclooxygenases. *Chem Rev* 103: 2239–2304.

Sanchez-Gomez F.J., Cernuda-Morollon E., Stamatakis K., Perez-Sala D. (2004). Protein thiol modification by 15-deoxy-Delta12,14-prostaglandin J2 addition in mesangial cells: Role in the inhibition of pro-inflammatory genes. *Mol Pharmacol* 66: 1349–1358.

Schonbeck U., Sukhova G.K., Graber P., Coulter S., Libby P. (1999). Augmented expression of cyclooxygenase-2 in human atherosclerotic lesions. *Am J Pathol* 155: 1281–1291.

Schulz E., Anter E., Keaney J.F., Jr. (2004). Oxidative stress, antioxidants, and endothelial function. *Curr Med Chem* 11: 1093–1104.

Sheehan D., Meade G., Foley V.M., Dowd C.A. (2001). Structure, function and evolution of glutathione transferases: Implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochem J* 360: 1–16.

Shen L., Sevanian A. (2001). OxLDL induces macrophage gamma-GCS-HS protein expression: A role for oxLDL-associated lipid hydroperoxide in GSH synthesis. *J Lipid Res* 42: 813–823.

Shibata T., Kondo M., Osawa T., Shibata N., Kobayashi M., Uchida K. (2002). 15-deoxy-delta 12,14-prostaglandin J2: A prostaglandin D2 metabolite generated during inflammatory processes. *J Biol Chem* 277: 10459–10466.

Shibata T., Yamada T., Ishii T., Kumazawa S., Nakamura H., Masutani H., Yodoi J., Uchida K. (2003a). Thioredoxin as a molecular target of cyclopentenone prostaglandins. *J Biol Chem* 278: 26046–26054.

Shibata T., Yamada T., Kondo M., Tanahashi N., Tanaka K., Nakamura H., Masutani H., Yodoi J., Uchida K. (2003b). An endogenous electrophile that modulates the regulatory mechanism of protein turnover: Inhibitory effects of 15-deoxy-delta(12,14)-prostaglandin J2 on proteasome. *Biochemistry* 42: 13960–13968.

Spiteller P., Spiteller G. (1998). Strong dependence of the lipid peroxidation product spectrum whether Fe^{2+}/O_2 or Fe^{3+}/O_2 is used as oxidant. *Biochim Biophys Acta* 1392: 23–40.

Stamler J.S., Lamas S., Fang F.C. (2001). Nitrosylation. the prototypic redox-based signaling mechanism. *Cell* 106: 675–683.

Straus D.S., Glass C.K. (2001). Cyclopentenone prostaglandins: New insights on biological activities and cellular targets. *Med Res Rev* 21: 185–210.

Subbanagounder G., Watson A.D., Berliner J.A. (2000). Bioactive products of phospholipid oxidation: Isolation, identification, measurement and activities. *Free Radic Biol Med* 28: 1751–1761.

Subbanagounder G., Wong J.W., Lee H., Faull K.F., Miller E., Witztum J.L., Berliner J.A. (2002). Epoxyisoprostane and epoxycyclopentenone phospholipids regulate monocyte chemotactic protein-1 and interleukin-8 synthesis: Formation of these oxidized phospholipids in response to interleukin-1beta. *J Biol Chem* 277: 7271–7281.

Uchida K. (2003). 4-Hydroxy-2-nonenal: A product and mediator of oxidative stress. *Prog Lipid Res* 42: 318–343.

Wild A.C., Mulcahy R.T. (2000). Regulation of gamma-glutamylcysteine synthetase subunit gene expression: Insights into transcriptional control of antioxidant defenses. *Free Radic Res* 32: 281–301.

Willett W.C., Green A., Stampfer M.J., Speizer F.E., Colditz G.A., Rosner B., Monson R.R., Stason W., Hennekens C.H. (1987). Relative and absolute excess risks of coronary heart disease among women who smoke cigarettes. *New Engl J Med* 317: 1303–1309.

Willson T.M., Lehmann J.M., Kliewer S.A. (1996). Discovery of ligands for the nuclear peroxisome proliferator-activated receptors. *Ann N Y Acad Sci* 804: 276–283.

Witztum J.L., Berliner J.A. (1998). Oxidized phospholipids and isoprostanes in atherosclerosis. *Curr Opin Lipidol* 9: 441–448.

Xu D., Rovira I.I., Finkel T. (2002). Oxidants painting the cysteine chapel: Redox regulation of PTPs. *Dev Cell* 2: 251–252.

Yin H., Porter N.A. (2005). New insights regarding the autoxidation of polyunsaturated fatty acids. *Antioxid Redox Signal* 7: 170–184.

Yla-Herttuala S., Palinski W., Rosenfeld M.E., Parthasarathy S., Carew T.E., Butler S., Witztum J.L., Steinberg D. (1989). Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J Clin Invest* 84: 1086–1095.

Zhang X., Lu L., Dixon C., Wilmer W., Song H., Chen X., Rovin B.H. (2004). Stress protein activation by the cyclopentenone prostaglandin 15-deoxy-delta12,14-prostaglandin J2 in human mesangial cells. *Kidney Int* 65: 798–810.

Zhuang H., Kim Y.S., Namiranian K., Dore S. (2003a). Prostaglandins of J series control heme oxygenase expression: Potential significance in modulating neuroinflammation. *Ann NY Acad Sci* 993: 208–216; discussion 287–288.

Zhuang H., Pin S., Li X., Dore S. (2003b). Regulation of heme oxygenase expression by cyclopentenone prostaglandins. *Exp Biol Med (Maywood)* 228: 499–505.

Zipper L.M., Mulcahy R.T. (2000). Inhibition of ERK and p38 MAP kinases inhibits binding of Nrf2 and induction of GCS genes. *Biochem Biophys Res Commun* 278: 484–492.

13

EARLY MOLECULAR EVENTS DURING RESPONSE TO OXIDATIVE STRESS IN HUMAN CELLS BY DIFFERENTIAL PROTEOMICS

GIANLUCA TELL

13.1 INTRODUCTION

13.1.1 General Overview on Cellular Redox State

Reactive oxygen species (e.g., H_2O_2, $OH^{\bullet}$, $O^{\bullet -}{}_2$, collectively known as ROS) cause extensive cellular damage but can also play important physiological functions. Cells are provided with efficient molecular strategies to strictly control the intracellular ROS level and to maintain the balance between oxidant and antioxidant molecules together with antenna molecules, so called because they are involved in the control of signal transduction pathways downstream ROS. Oxidative stress, resulting from an imbalance between the generation of ROS and the antioxidant defense capacity of the cell (Sies, 1985), affects major cellular components, including lipids, proteins, and DNA. This phenomenon is closely associated to a number of human disorders, in particular, many degenerative diseases such as cardiovascular diseases, diabetes, cancer, and neurodegenerative disorders (Bray, 1999; Dalle-Donne et al., 2005), and with almost all liver pathologies (Loguercio and Federico, 2003; Poli and Parola, 1997; Cesaratto et al., 2004). All these conditions appear to be mostly related to chronic oxidative stress. However, the acute exposure to high levels of ROS seems also to be responsible for the development of different damage such as during ischemia/reperfusion

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

(I/R) in liver (Berrevoet et al., 2003; McCord, 1985). In addition to ROS, reactive nitrogen species (collectively known as RNS) derived from NO, which is produced in higher organisms by the oxidation of one of the terminal guanidonitrogen atoms of L-arginine (Palmer et al., 1988), such as the NO radical ($NO^{\bullet}$), the nitrosonium cation (NO^{+}), the nitroxyl anion (NO^{-}), or peroxynitrite ($ONOO^{-}$), play important roles in controlling cellular redox state (Stamler et al., 1992; Barrett et al., 2005; Marshall et al., 2004; Reynaert et al., 2004; Jaffrey et al., 2001; Hare and Stamler, 2005; Kroncke et al., 2002; Kroncke, 2001; Forman et al., 2004). Interestingly, it seems that some of the physiological effects of RNS may be mediated through the intermediate formation of *S*-nitroso-cysteine or *S*-nitroso-glutathione (Gow and Stamler, 1998). However, being the objects of other chapters of the present book, the role of RNS will not be discussed in detail.

Besides producing cell damage, ROS serve as molecular second messengers within the cell. They are generated during triggering of membrane-bound receptors by cytokines, hormones, growth factors, and other soluble mediators such as extracellular ATP leading to particular cellular responses (Lander, 1997).

Therefore ROS represent a double purpose. They can act either positively or negatively on cell functioning. But how can a rise in ROS levels trigger such different responses? This might be explained by the fact that different biological outcomes reflect subtle differences in the intensity and duration of the oxidative stress produced on a cell and/or on the cellular context affected by the oxidative stress condition. It is then not surprising that the role of ROS, either as apoptotic molecule or as stimulator of cell proliferation, depends on the cell type, on the intensity of the stress produced, and on the location in the cell where the ROS are generated.

13.1.2 Major Types of Oxygen Free Radicals and Their Derivatives

Eukaryotic cells are continually subjected to ROS exposure, which derives from external environment (e.g., by the action of ionizing radiation on atmospheric molecular oxygen). But, particularly, ROS is the endogenous by-product of the oxidative phosphorylation events that occur during respiration of all aerobic organisms. Another major cause of ROS production is represented by phagocytic NADPH oxidases during inflammatory responses and by nonphagocytic NADPH oxidases, as recently pointed out for different cell systems (Griendling et al., 2000; Takeya and Sumimoto, 2003).

ROS comprise the **superoxide anion**, which has a short half-life and is an intermediate during reduction of molecular oxygen to **hydrogen peroxide**. Hydrogen peroxide is the most abundant ROS with the longest half-life; it can easily pass through cellular membranes. Hydrogen peroxide is commonly used in laboratories to generate a prooxidant cellular status. Moreover H_2O_2 can give rise to the highly reactive **hydroxyl radical**, which is the most reactive ROS for the biological macromolecules, acting also as a mutagen produced upon ionizing radiation exposure. Because of its very short half-life, it can exert its effects locally in the site of its own production (Fig. 13.1).

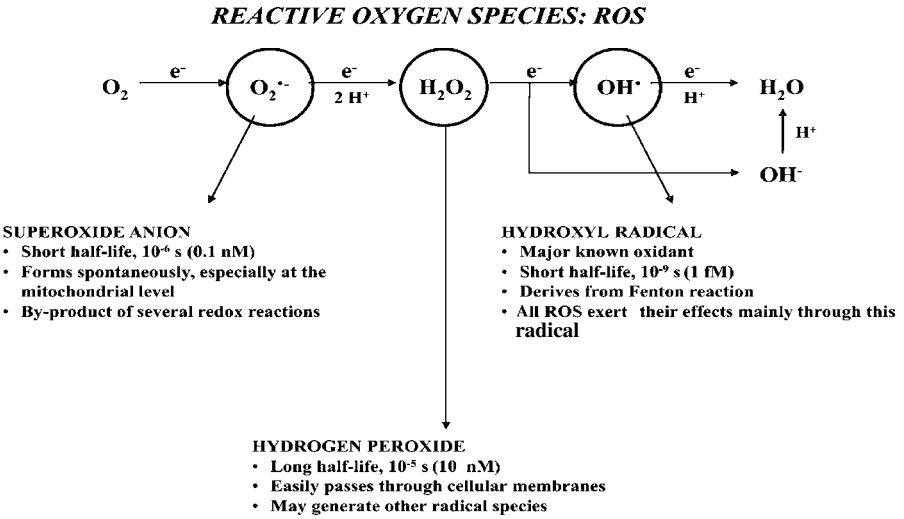


FIGURE 13.1 General features of reactive oxygen species (ROS).

Mitochondria are the major source of cellular ROS in nonphagocytic cells during the respiratory process, but ROS can be also produced by membrane-associated enzymes such as NADPH oxidases. Low levels of potentially toxic oxygen metabolites are physiologically generated in cells and tissues during oxidative phosphorylation (Mikkelsen and Wardman, 2003). The resulting moderate levels of ROS play an essential role in the modulation of several physiological functions of the cell, including gene expression, signal transduction, and defense against invading pathogens. Under normal conditions it is estimated that up to 1% of the mitochondrial electron flow primarily leads to the formation of superoxide anion that is generated by the univalent reduction of molecular oxygen. This process is mediated by enzymes such as NADP(H) oxidases and xanthine oxidase (Fig. 13.2, reactions a and b) or nonenzymatically by redox-reactive compounds, such as the semi-ubiquinone compound of the mitochondrial electron transport chain (Fig. 13.2, reaction c). Interference with electron transport can dramatically increase superoxide production. Superoxide dismutases (SODs) catalyze dismutation of two superoxide anions into hydrogen peroxide and molecular oxygen (Fridovich, 1978) (Fig. 13.2, reaction d). H_2O_2 can react with reduced transition metals, via the Fenton reaction, to produce the highly reactive hydroxyl radical (Fig. 13.2, reaction e), a far more damaging molecule to the cell. Alternatively, H_2O_2 may be converted into water by the enzymes catalase (CAT) and glutathione peroxidase (GPX) (Fig. 13.2, reactions f and g). During the glutathione peroxidase reaction, glutathione is oxidized to glutathione disulfide, which can be then reconverted to glutathione by glutathione reductase in an NADPH-consuming process (Fig. 13.2, reaction h).

The role played by NADPH oxidases in ROS production as second messengers is becoming clearer and clearer. The function of NADPH oxidase in ROS

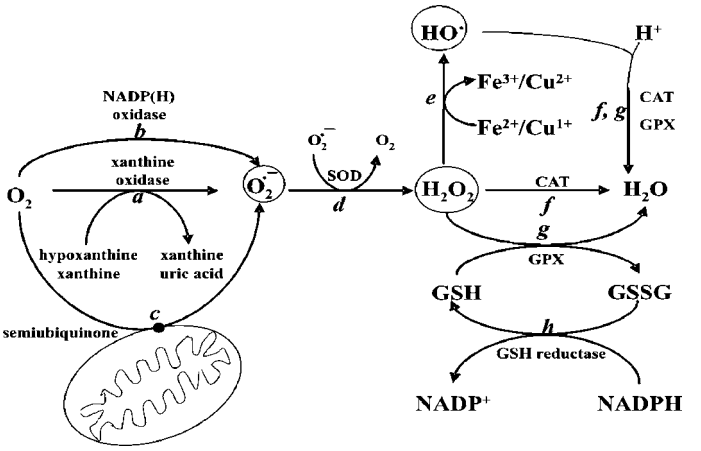


FIGURE 13.2 Main intracellular pathways for the formation of ROS. SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase.

production has been classically associated with host defense against pathogens in phagocytes (H_2O_2 is produced by activated macrophages during an inflammatory response at a very high rate, leading to a concentration, in the proximity of the cell, of about 10–100 μM). Interestingly new evidence suggests that the bactericidal role of ROS is not directly due to damaging agents against the pathogen but rather to signaling molecules that induce protease release from neutrophils to kill the microorganisms (Reeves et al., 2002). In this regard the oxidants act primarily as a signal than as ultimate effector molecules. The role of NADPH oxidase in the regulation of intracellular signaling cascades has been clearly elucidated in a number of different cell systems, ranging from thyroid cells, cardiac myocytes, endothelial cells, fibroblasts, to smooth muscle cells (for a review, see Droge, 2002). The rate of ROS production by the nonphagocytic NADPH oxidase is about only 30% of that of neutrophils. However, a lot of extracellular stimuli (hormones, cytokines, soluble mediators such as ATP, mechanical stresses, local metabolic changes) require this signaling mechanism to exert their biological effects (Table 13.1). A leading role for this class of enzymes in controlling biological functions has been recently proposed, after observation that a NADPH oxidase homologue (i.e., Nox1), expressed in colonic epithelial and vascular smooth muscle cells, is able to transform NIH3T3 cells and produce highly aggressive tumors (Suh et al., 1999); NADPH oxidase is also strongly upregulated in prostate cancer (Lim et al., 2005). Interestingly its transforming abilities are due to the sustained increase in hydrogen peroxide production, which is in turn required to maintain the transformed state (Arnold et al., 2001).

Frequently different reactive oxygen species coexist in the reactive cellular environment resulting in a difficult identification of the real effector molecules responsible for a given biological effect. Hence, in many of the studies where ROS are measured, the techniques do not discriminate between different ROS

TABLE 13.1 Examples of Intracellular ROS Production during Cellular Response to Receptor Triggering or Environmental Stimulation

Cell System	Stimulus	Reference
Vascular smooth muscle cells	Thrombin, PDGF, TNF-α angiotensin	Bae et al., 1997; Griendling et al., 2000
Fibroblasts	IL-1, TNF-α, PAF, angiotensin	Jones et al., 1994
Endothelial cells	Mechanical stress	Yeh et al., 1999
Cardiac myocytes	Reoxygenation	Suzuki et al., 1999
Prostate cancer cells	Extracellular ATP	Sauer et al., 2001
Neuronal cells	EGF, NGF	Bae et al., 1997; Suzukawa et al., 2000

and RNS. Therefore, at present, it is difficult to understand which is the cause and which is the effect in redox signaling.

Oxidative stress may cause reversible and/or irreversible modifications on sensitive proteins. Reversible modifications, usually occurring at cysteine residues, may have a dual role: (1) protection from irreversible damage and (2) modulation of protein function (redox regulation). Irreversible modifications induced by ROS, such as di-tyrosine formation, protein–protein cross-linking or lysine, and arginine carbonylation, are generally associated with permanent loss of function and may lead to either the unfolding and degradation of the damaged proteins or to their progressive accumulation into cytoplasmic inclusions, as observed in age-related neurodegenerative disorders (Dalle-Donne et al., 2005).

13.1.3 A Model of Redox Signaling Is Represented by Cellular Oxidative Stress Response

Redox signaling implies the use of redox chemistry in controlling cellular responses through signal transduction mechanisms. Redox signaling is a highly conserved biological mechanism during phylogenesis of living organisms. Apparently it is a molecular strategy widely used by different organisms (ranging from prokaryotes to higher eukaryotes and plants) and probably as a consequence of the evolutive pressure represented by the abundance of oxygen in the earth atmosphere. Cellular membranes protect the intracellular reducing environment from the highly oxidizing extracellular environment. Therefore it is not by chance that ROS can commonly act as intracellular second messengers: it is an efficient way to perturb the biological system and to induce a meaningful response by the cell. ROS are present in cells and tissues at low but measurable concentrations, which are determined by the balance between the rate of their production versus their clearance by different antioxidant enzymes (SOD, GPX, and CAT) and compounds (glutathione, ascorbate, α-tocopherol, β-carotene) (Halliwell and Gutteridge, 1989). The role of ROS as second messengers is strengthened by the fact that the triggering of a large majority of receptors (comprising cytokine receptors,

purinergic receptors, and tyrosine kinases receptors) lead to intracellular ROS rise by nonphagocytic NADPH oxidases (Droge, 2002).

A complex functional modulation of several proteins occurs upon cellular oxidative stress (Nakamura et al., 1993; Staal et al., 1994; Sun et al., 2000). In particular, this is accomplished through modification of proteins, since several amino acids such as tryptophan, tyrosine, histidine, and particularly cysteine are direct targets of ROS (Davies, 1987). ROS-mediated protein modification can affect both protein structure and function and protein stability. It is largely known that oxidized proteins are highly sensitive to proteolytic attack by proteasomes (Grune et al., 1997). By controlling the functional activity of cellular effectors (i.e., proteins), it is clear that ROS can also affect signal transduction mechanisms and, eventually, influence cellular gene expression. It is becoming increasingly evident that mammalian cells can modulate patterns of protein expression in response to hydroperoxide stress, by activating redox-sensitive transcription factors such as Egr-1 and by stimulating cellular kinases such as the mitogen-activated protein kinase (MAPK) family (Droge, 2002). This oxidative-dependent cellular signaling pathway alteration may frequently lead, with a still unknown mechanism, to cell apoptosis. However, sensor polypeptides specific for H_2O_2 and other ROS are still to be identified and little is known about early effects on biological macromolecules regulating ROS-induced cellular effects. It is clear that the comprehension of stimulus-specific mechanisms of oxidant-dependent cell apoptosis would be extremely important to develop effective therapies for a number of human pathologies.

13.2 CELLULAR RESPONSE TO OXIDATIVE STRESS: FROM MEMBRANE RECEPTORS TO GENE EXPRESSION CONTROL

In striking contrast to the conventional idea that reactive oxygen is mostly a trigger for oxidative damage of biological structures, it is now commonly accepted that low physiologically relevant concentration of ROS can regulate a variety of key molecular mechanisms that may be linked to important processes such as inflammation, cell proliferation, mechanical stress, cell communication, aging, and apoptosis (Droge, 2002). Redox-based regulation of gene expression represents a fundamental regulatory mechanism in cell biology for all aerobic organisms. Therefore a lot of efforts are currently devoted to (1) the identification of the molecular targets of ROS and (2) the dissection of the molecular mechanisms responsible for the ROS-induced biological responses. To these aims, Redox proteomics techniques have made a considerable contribution over the last few years.

There is strong evidence that intracellular ROS production is elicited by different cell types upon triggering of several membrane receptors (Fig. 13.3) including T cell receptor (Williams and Kwon, 2004) and purinergic receptors (Sauer et al., 2001) and that the oxidative burst is required for the subsequent signal transduction pathway. A paradigmatical example of this is represented by the EGF- and

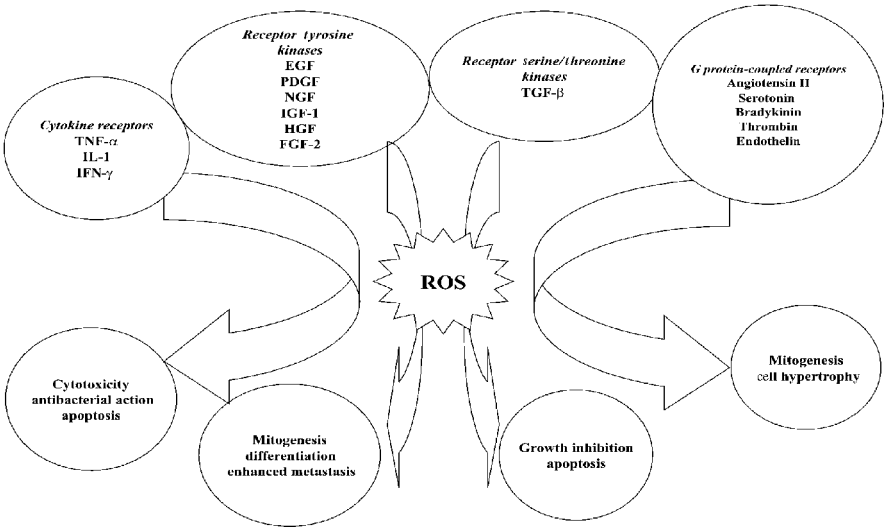


FIGURE 13.3 Receptor-triggering mediated generation of ROS in nonphagocytic cells as deduced by the cited literature. The term "kinase" refers to any of the numerous intracellular kinases that are activated upon oxidative stress, as reported in the text.

NGF-induced tyrosine phosphorylation of various cellular proteins, including the autophosphorylation of the growth factor receptor itself, that are inhibited by elimination of hydrogen peroxide by catalase treatment (Bae et al., 1997; Suzukawa et al., 2000). This ROS production is, in turn, able to mediate a positive feedback effect on signal transduction from these receptors by synergistically enhancing the intracellular signaling effects (Droge, 2002). However, the molecular mechanisms mediating these effects are not yet completely understood. Since, as is evident, exposure to a range of physiologically significant concentrations of ROS able to induce a cellular oxidative stress condition without killing cells can exert stimulating responses such as transformation, adaptation, and repair, the target proteins involved in this kind of biological response have good therapeutic potential. So ROS activity may be viewed in two ways: as they cause extensive cell damage leading to death, they generate an oxidative stress, and as at low levels they act as second messenger molecules, they are involved in redox signaling. Clearly, no sharp distinction, in biological terms, between these two conditions can be drawn.

13.2.1 The Problem of the *Primum movens* (Redox Sensors) in Monitoring the Intracellular Redox State and Its Conversion into a Biological Response: The Role of "Antenna" Molecules

The main features of second messengers are (1) the rapid generation upon receptor triggering, (2) the necessity to be short-lived, and (3) the specificity of action on downstream effectors. Indeed, while the first two are undoubtedly

characteristics of ROS, their specificity of action is highly questionable, at least at present. In fact, because of their intrinsic nature and stabilities, all of ROS react with almost all nearby molecules depending on their diffusion abilities. At the moment the comprehension of the molecular basis for its specificity of action is under debate, and it may hold the key to understanding the role that ROS play in controlling so different biological effects. So there is much to be gained by the identification of all the major target molecules of ROS and, particularly, of the induced post-translational modifications occurring on these targets.

In contrast to the rough specificity of action of ROS, NO represents the prototypical redox signaling molecule fulfilling all the requirements for a second messenger, including specificity in chemical reactivity (Jaffrey et al., 2001; Kroncke, 2001; Kroncke et al., 2002; Marshall et al., 2004; Hare et al., 2005; Boon et al., 2005) (see Chapters 2 and 6 in this volume for details).

13.2.2 Role of Kinases, Phosphatases, and Intracellular Redox Sensors in Redox Signaling

Kinases and Phosphatases: Mitogen-Activated Protein Kinases (MAPK), Protein Tyrosine Phosphatase (PTP), Protein Kinase C (PKC), Protein Kinase A (PKA), Src, and Ras Consolidated evidence from different cell systems has led to the idea that mitogen-activated protein kinase (MAPK) pathways control cellular responses to change in intracellular redox. This has been proved for the extracellular regulated kinase 1/2 (Erk-1 and Erk-2) as well as for the c-Jun *N*-terminal kinase (JNK) and p38 MAPK pathways (Fig. 13.4). The cascades are regulated by phosphorylation/dephosphorylation on serine and threonine residues upon receptor tyrosine kinases, G protein-coupled receptor, and growth factor receptor triggering. In particular, both the c-Jun *N*-terminal kinase (JNK) and p38 are strongly activated by ROS or by a slight change in the intracellular redox state (Abe et al., 1996; Guyton et al., 1996; Allen and Tresini, 2000; Hehner et al., 2000). Hydrogen peroxide exogenously applied to human lens cells and hepatocytes and endogenously produced upon angiotensin II and PDGF stimulation has been shown to induce activation of Erk-1 and Erk-2 (Abe et al., 1996; Sundaresan et al., 1995; Czaja et al., 2003; Paron et al., 2004; Cesaratto et al., 2005).

The intracellular oxidant scavengers also can directly activate signaling pathways, as is the case of JNK. It has been demonstrated that in the mouse fibroblast cell line 3T3-4A, the glutathione S-transferase P_i (GSTp) is physically associated with JNK and catalytically inhibits it, therefore limiting the degree of phosphorylation of Jun under nonstressed conditions in normal growing cells. Mild oxidative stress conditions are able to induce the dissociation of GSTp-JNK complex, leading to JNK activation (Adler et al., 1999). The tripeptide glutathione (L-γ-glutamyl-L-cysteinylglycine) is the most abundant nonprotein thiol in eukaryotic cells, and it serves as a "marker" molecule in the condensation reaction catalyzed by GST between GSH itself and xenobiotic hydrophobic chemical substrates, such as the cytochrome P450 products. The result is increased water solubility

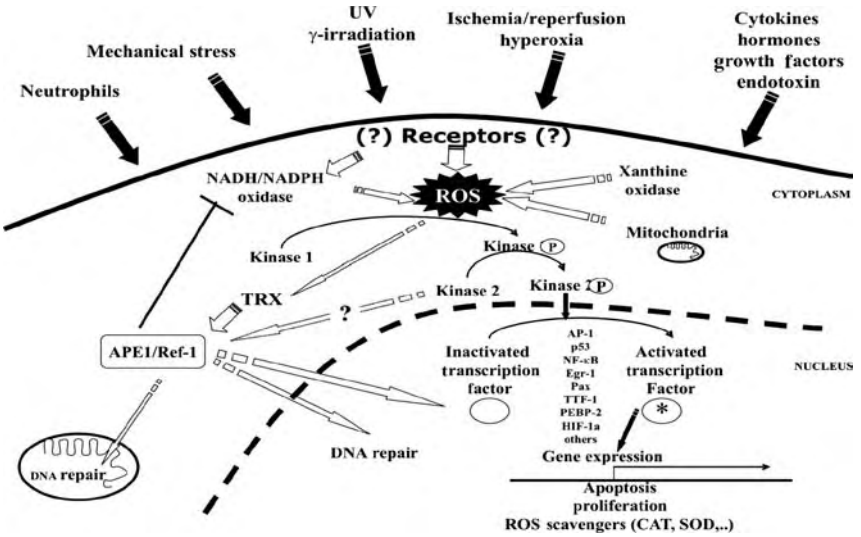


FIGURE 13.4 ROS involvement in the activation of cellular responses.

of the glutathionylated products, which allows for efficient excretion (Wu et al., 2004). Moreover GSH has a major role as an antioxidant. It maintains the reduced state of cellular protein thiol groups and is even used by peroxidases to catalyze the reduction of hydroperoxides to water. This way it confers protection against protein thiol oxidation. During oxidative stress conditions the intracellular concentrations of oxidized glutathione (GSSG) and protein-glutathione mixed disulfides rapidly increase. It is being increasingly observed that glutathionylation represents a means to control protein kinase functions, as most recently demonstrated by Cross and Templeton (2004). The mitogen-activated protein kinase kinase (MEKK1) becomes actively glutathionylated at Cys^{1238} upon oxidative stress induced by menadione. This modification interferes with the optimal binding and coordination of ATP in the glycine-rich loop of subdomain I of the kinase domain, which inhibits any kinase catalytic activity. Similarly other protein kinases, such as protein kinase C (PKC) and the cAMP-dependent protein kinase A (PKA), have been reported to be regulated by cysteine glutathionylation within their catalytic domains in response to oxidation (Ward et al., 2000; Humphries et al., 2002). Modification of cysteine residues in protein kinase activation loops might block kinase activation by preventing the activation-loop phosphorylation or by changing the protein–substrate interactions.

The PKCα isoform of the well-known serine/threonine kinase protein kinase C, which is typically activated by the lipid second messenger diacylglycerol through binding to a conserved cysteine-rich region within the amino terminal C1 domain (Ono et al., 1989), may also be activated by hydrogen peroxide in the absence of its natural agonist by a mechanism involving tyrosine phosphorylation in the catalytic domain (Konishi et al., 1997).

The cellular phosphorylation state is maintained under strict control by the coordinated action of kinases and protein tyrosine phosphatases (PTP). The PTP superfamily, composed of almost no different enzymes that catalyze the dephosphorylation of tyrosine residues in proteins, shares a highly conserved CX_5R motif in its active site and an identical catalytic mechanism of action (Tonks and Neel, 2001). The conserved Cys residue in the catalytic site exists as a thiolate anion at neutral pH, due to the acidic pK_a of this Cys residue (pK_a 4.7–5.4). This thiolate anion contributes to the formation of a thiol-phosphate intermediate in the catalytic mechanism of PTPs. Because of its chemical characteristics this Cys residue is also a target of redox regulation during oxidative stress. In fact it has been demonstrated that H_2O_2 is able to oxidize the Cys residues to cysteine-sulfenic acid (Cys-SOH), leading to PTP inactivation (Cho et al., 2004). This PTP inactivation in turn may change the intracellular phosphorylative equilibrium toward a higher phosphorylation rate. The biological relevance of these observations has been recently reinforced by finding that growth factor stimulation of eukaryotic cells by EGF and PDGF results in a selective reduction in the extent of chemical modification of active site cysteine (Lee et al., 1998; Meng et al., 2002). Moreover recent studies suggest that some PTPs, such as PTEN, Cdc25, and LMW-PTP, can even form a reversible disulfide bond between the Cys at the active site and a neighboring cysteine of another PTP molecule and/or non-PTPs. In either case the formation of a transient disulfide confers a certain efficiency to the redox regulation of PTPs as well as protection of Cys-SOH from further oxidation.

Interestingly the nonreceptor tyrosine kinase Src and the membrane-bound G-proteins of the *ras* gene family have been confirmed to be a target of ROS action. Since about 30% of all cancers involve disregulation of these two classes of proteins, many efforts are currently devoted to the study of whether ROS is active in the tumorigenic process (Leonard et al., 2004).

Cross-talk between oxidative events and nitrosative signaling has been recently investigated by Barrett et al. (Barrett et al., 2005). These authors showed that inhibition of PTPs by mild oxidative stresses, generated by ionizing radiation or low doses of hydrogen peroxide, is dependent on active *S*-nitrosylation of PTPs by means of a Ca^{2+}-dependent nitric-oxide synthase (NOS). That is, an oxidative event is converted by the cell into a nitrosative one because of the better redox signaling properties of NO.

Intracellular "Redox Sensors": Peroxiredoxins, Thioredoxin, and APE1/Ref-1

Recently Wood and co-workers (2003) proposed that the cellular switch that determines whether hydrogen peroxide acts as a signaling molecule or as a deleterious oxidant is based on the ubiquitous peroxidases, 2-Cys peroxiredoxins (2-Cys Prxs). They are a peculiar type of peroxidase because the protein itself is the reducing substrate (Wood et al., 2003). These enzymes contain an activated cysteine at the active site that, under oxidative conditions, is oxidized to cysteine sulfenic acid or to a disulfide, or the cysteine may be further subjected to overoxidation to a sulfinic or sulfonic acid, which inactivates the enzyme. Nevertheless,

the firewall created by Prxs against hydrogen peroxide can be re-established through a mechanism of retro-reduction involving the thioredoxin-thioredoxin reductase system (Chae et al., 1994) and sestrins (Budanov et al., 2004). The mechanism of cyclic oxidation-reduction catalyzed by auxiliary enzymes has been clearly demonstrated for the 2-Cys containing Prxs (Prx I, II, III, and IV), while the mechanism of functioning and retro-reduction for the 1-Cys PrxVI is still under debate. However, different independent studies suggest that the functioning mechanism for the 1-Cys and the 2-Cys containing Prxs are very different (Cesaratto et al., 2005; Chevallet et al., 2003). When the intracellular levels of hydrogen peroxide production are strictly controlled, it is not surprising that the enzymes take part in intracellular signaling. In fact it has been demonstrated that Prxs are required for Myc-mediated transformation and apoptosis (Wonsey et al., 2002), regulate NF-κB activation (Kang et al., 1998), and play a role in tumorigenic processes (Noh et al., 2001).

The thioredoxin system was one of the early molecular mechanisms responsible for the reduction of exposed disulphide bridges in oxidized proteins by means of its NADPH-dependent disulfide oxidoreductase mechanism. This small 12-kDa protein, also known as the adult T cell leukemia-derived factor, may therefore control the oxidation state of protein thiols affecting the function of important nodes of the intracellular redox network (Nakamura et al., 1997). The presence of a catalytic site, WCGPCK, is a common feature of all the Trxs, both in prokaryotes and eukaryotes from yeast, animals, and plants. The proposed mechanism of functioning involves a reversible oxidation of the two Cys residues at the catalytic site forming a disulfide bridge, which is further retroreduced by action of a selenoenzyme thioredoxin reductase in the presence of NADPH. Interestingly the antioxidant properties of Trxs, which are also present in the extracellular fluids such as plasma, include removal of hydrogen peroxide and free radicals (Spector et al., 1988; Schallreuter and Wood, 1986), therefore constituting a general protection tool for cells against oxidative stress.

The human apurinic/apyrimidinic endonuclease 1/redox effector factor-1 (hAPE1/Ref-1) is a perfect paradigm of the functional complexity of a biological macromolecule. First, it plays a crucial role, by both redox-dependent and redox-independent mechanisms, as a transcriptional co-activator for different transcription factors, both ubiquitous (i.e., AP-1, Egr-1, NF-κB, p53, HIF) and tissue specific (i.e., PEBP-2, Pax-5 and -8, TTF-1), in controlling different cellular processes such as apoptosis, proliferation, and differentiation in Trx-dependent and Trx-independent mechanisms. Second, it acts as an apurinic/apyrimidinic endonuclease, during the second step of the DNA base excision repair (BER) pathway, which is responsible for the repair of DNA alkylation and oxidative DNA damages. Third, it controls the intracellular ROS production by negatively regulating the activity of the Ras-related GTPase, Rac1. Despite these known functions of APE1/Ref-1, information is still scanty about the molecular mechanisms responsible for the coordinated control of its several activities. Most evidence shows that the expression and subcellular localization of APE1/Ref-1 are finely tuned. The protein APE1/Ref-1 is ubiquitous, but its expression

patterns differ according to the different cell types. The subcellular localization of APE1/Ref-1 is mainly nuclear, but additionally cytoplasmic localization has been reported, the latter being associated with mitochondria and/or presence within the endoplasmic reticulum. It is not by chance that both expression and/or subcellular localization are altered in metabolic and proliferative disorders such as in tumors and aging. Moreover the reason for the different post-translational modifications in modulating APE1/Ref-1 functional activity is starting to be revealed (for a general review, see Tell et al., 2005).

Cytoskeleton Reorganization Several in vitro studies show that oxidant-stressed mammalian cells undergo profound changes in the structure of the actin cytoskeleton associated with profound morphological changes (e.g., see Fig. 13.5). However, the molecular mechanisms involved in this regulation are far from being understood. One line of evidence suggests that actin polymerization (F-actin) is controlled by activation of the HSP25/27 modulator upon phosphorylation along the MAPK pathway in response to oxidative stress or as an alteration of the redox status of actin or of some actin regulatory proteins (Dalle-Donne et al., 2001). In many cell types, such as fibroblasts and hepatocytes, ROS can induce a total disruption of the F-actin cytoskeleton organization (Huot et al., 1997), and in others, such as endothelial cells, hydrogen peroxide can induce the rapid formation of focal adhesion complexes and a major reorganization of the actin cytoskeleton, leading to the formation of a dense transcytoplasmatic stress fiber network (Huot et al., 1998). The noxious effects of oxidative stress on the cytoskeleton's organization are associated with morphological cellular changes due to the formation of surface blebs on plasma membrane (Dalle-Donne et al.,

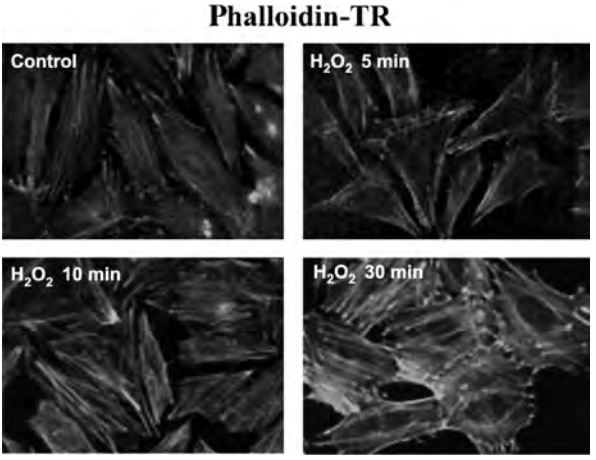


FIGURE 13.5 Formation of transcytoplasmatic actin stress fibers in the human osteoblast-like HOBIT cell line after treatment with sublethal doses of H_2O_2 over short time periods as evidenced by phalloidin-rhodamin fluorescence staining.

2001). The molecular basis of these morphological changes seems to reside in the interference of oxidative stress with intracellular Ca^{2+} homeostasis. The rise in intracellular Ca^{2+} upon ROS exposure of cells might be responsible for the dissociation of actin microfilaments from α-actinin, which serves in the stabilization of F-actin with actin-binding proteins within the cytoskeleton. The additional effect of intracellular Ca^{2+} mobilization may be due to its activator role on intracellular proteases, such as calpain, leading to elimination of the plasma membrane anchorage to the cytoskeleton by cleavage of actin-binding proteins themselves.

The direct effect of the intracellular rise in ROS on actin molecules has been well documented, and actin residues as target of oxidative stress have been mapped in vitro. Fast and reversible formation of mixed disulfides between actin sulfhydryls and the sulfhydryl of GSH has been described in cultured gastric mucosal cells (Rokutan et al., 1994). This post-translational modification, occurring under nonlethal oxidative stress serves as a protection mechanism against inactivation by the intra- and/or intermolecular disulfide bond formation of actin caused by oxidative bursts and prevents excessive actin polymerization, thereby preserving microfilament dynamics under oxidative stress condition. Further post-translational modification occurring in the actin molecule during oxidative stress is represented by carbonylation and cysteine oxidation (Dalle-Donne et al., 2001). In vitro studies have led to mapping of the cysteine residues that are easily subjected to oxidation. Cys^{374} has been demonstrated to be the most reactive molecule and the oxidation process, which may also involve the 16 methionine residues of the actin itself, induces conformational changes on the molecule, as assayed by proteolytic susceptibility studies (Milzani et al., 2000).

In using a differential proteomics approach to the study of the human lens cell's early response to oxidative stress, we found altered expression of several cytoskeletal proteins. Among these, α-tubulin was upregulated after oxidative stress, pointing out a role for this protein in stress-protection in the lens epithelium. This finding, together with the observation that also β-actin is upregulated in response to the oxidative stimulus in other cell systems (Clarkson et al., 2002), strengthens the hypothesis that cytoskeletal proteins are indirectly involved in cellular response to oxidative stress (Liu and Sundqvist, 1995). These findings parallel recent data obtained by a cDNA microarray approach in which β-tubulin was found significantly upregulated during treatment of SR-01-04 epithelial lens cells with 50 μM H_2O_2 (Goswami et al., 2003). Furthermore in our cell system we found a certain degree of vimentin degradation after oxidative stimulus, possibly related to triggering of apoptotic pathways following H_2O_2 exposure. In fact vimentin has been identified as a caspase-9 substrate. Caspase-9 is a well-known protease activated by cellular damage during oxidative stress (Nakanishi et al., 2001). Altogether our evidence for involvement of cytoskeletal proteins (e.g., vimentin and tubulin) in dysregulation after oxidative insult must be viewed in light of recent papers on lens opacization during cataractogenesis (Clark et al., 1999).

13.3 GENE EXPRESSION CONTROL DURING CELL RESPONSE TO OXIDATIVE STRESS: REDOX-REGULATED TRANSCRIPTION FACTORS

The complex functional modulation of several proteins occurring upon change in intracellular redox status includes the modulation of the cellular gene array by specific transcriptional regulators. Up to now, only in bacteria the transcription factor OxyR has been shown to be a specific transductional sensor to H_2O_2 (Zheng et al., 1998). Also mammalian cells modulate patterns of gene expression in response to hydroperoxide stress, by activating transcription factors such as Egr-1 (Gashler and Sukhatme, 1995), HIF-1α (Ema et al., 1999), NF-κB, and AP-1 (Marshall et al., 2000). Presently it is not entirely known whether the redox sensitivity of transcription factors is designed to directly sense changes in the intracellular redox state or to recognize redox-related signals of upstream effectors. To this second aim, a master "redox sensor" for the cell is represented by APE1/Ref-1, whose redox activity over different transcription factors acts as a mediator. However, the molecular mechanisms responsible for the fine-tuning of biological effects played by ROS in modulating the gene expression profile is still to be understood in detail. This redox regulation mainly occurs by controlling the redox status of specific Cys residues located within the DNA-binding domain of redox-regulated transcription factors or within their regulatory regions, such as the transactivation domain (Tell et al., 2002). Up to now, several TFs containing specific Cys residues have been shown to be the target of redox regulation by APE1/Ref-1 nuclear redox activity, such as AP-1 (Xanthoudakis and Curran, 1992), NF-κB (Nishi et al., 2002), Myb (Xanthoudakis et al., 1992), PEBP2 (Akamatsu et al., 1997), HLF (Ema et al., 1999), NF-Y (Nakshatri et al., 1996), Egr-1 (Huang and Adamson, 1993), HIF-1α (Huang et al., 1996), the ATF/CREB family (Xanthoudakis et al., 1992), and p53 (Gaiddon et al., 1999), and PAX proteins (Tell et al., 2005). In order to properly bind to specific DNA target sequences, these TFs require that critical Cys residues be in the reduced state. Therefore, by maintaining these Cys in the reduced state, APE1/Ref-1 provides a redox-dependent mechanism for regulation of target gene expression. APE1/Ref-1 contains two cysteine residues located within the redox active domain (Cys^{65} and Cys^{93}), and previous in vitro studies showed that Cys^{65} should be the redox-active site of the protein (Walker et al., 1993). In agreement with the molecular model describing redox regulation exerted by APE1/Ref-1, Cys^{65} should interact with the sensitive cysteine residues within the DNA binding domains of TFs. However, Jayaraman et al. suggest that the stimulatory role played by APE1/Ref-1 on p53 activity may also occur in a redox-independent way (Jayaraman et al., 1997). This has been recently corroborated by the work of Ordway and colleagues (Ordway et al., 2003), in which these authors provide the first in vivo evidence that the Cys^{65} residue of APE1/Ref-1 is, unexpectedly, not essential for redox regulation of AP-1 DNA-binding. However, these authors did not completely exclude a possible presence of compensatory phenomena. In any case this evidence challenges previous hypotheses about the

molecular mechanisms by which APE1/Ref-1 exerts its redox-dependent activities on specific transcription factors.

Another mode of redox-regulation of transcription factor activity is provided by the redox sensitivity of protein degradation, as is the case of HIF-1α during cellular normoxic conditions. During hypoxic conditions the ubiquitin pathway responsible for the maintenance of the proper turnover of the protein is inhibited, causing an upregulation of HIF-1α expression.

Furthermore very recent studies point to chromatin remodeling as a way by which oxidative stress controls gene expression. The acetylation state of histones controls the compaction of the DNA within the nucleus of eukaryotic cells. In particular, the balance between histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities determines the DNA compaction degree. Acetylation by HAT of specific Lys residues on the *N*-terminal tail of core histones results in uncoiling of the DNA and increased accessibility to transcription factors binding. In contrast, histone deacetylation by HDAC inhibits gene expression by promoting DNA winding, therefore limiting access to transcription factors. Also transcriptional co-activators, such as CBP/p300, possess HAT activity and actively contribute to transcriptional activation. Recent evidence suggests that oxidative stress may activate gene expression by interfering with the balance between HAT and HDAC activities (Kilty and Rahman, 2004). In fact ROS can enhance complex formation between CBP/p300 and NF-κB or can inhibit HDAC activities, leading to enhancement of inflammatory responses by turning on inflammatory gene expression (Rahman et al., 2004; Moodie et al., 2002).

13.4 THE POWER OF DIFFERENTIAL PROTEOMICS IN DETECTING EARLY MOLECULAR MARKERS OF OXIDATIVE STRESS: EXAMPLES FROM HUMAN CELL LINES

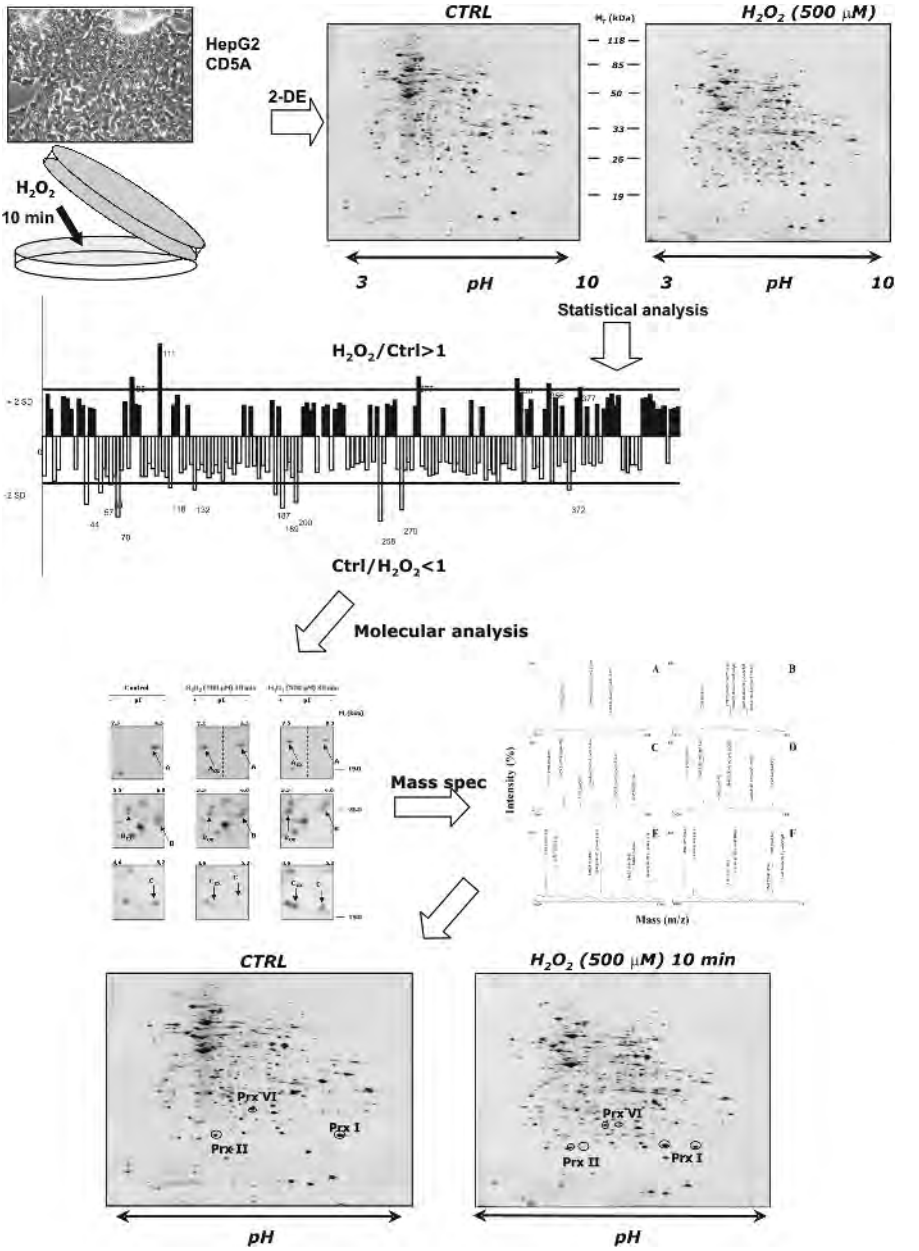
In order to understand the molecular bases of cellular responses to oxidative stress, it is important to identify those genes whose expression or function are directly modified by oxidative stress. Modification of gene expression can be tested by two different approaches. The first consists in studying expression of single candidate genes such as those already known to be involved in cellular response to oxidative stress (*AP-1, NF-κB*, *MAPK, PKC*, etc.). However, it is clear that effects due to oxidative stress are mediated by modification of a complex array of genes. Thus the study of single candidate genes may not be adequate to solve the complexity of cell response to oxidative stress. The second approach consists in technologies, such as differential display, DNA microarrays, or proteomics, which are able to simultaneously measure modifications of a very large number of gene products. At present, knowledge of eukaryotic cell response to oxidative stress in terms of polypeptide products is still scanty because most studies have been carried out only at the RNA level.

In our recent work the molecular modifications upon oxidative stress have been studied using a differential proteomics approach in two models of eukaryotic cells, which are physiologically highly exposed to ROS burst, such as human lens and hepatocyte cell lines (Paron et al., 2004; Cesaratto et al., 2005). This methodology, based on the use of two-dimensional gel electrophoresis (2DE) comparative analysis (by separating proteins according to their physicochemical features, i.e., their pI in the first dimension and their apparent molecular mass in the second one) and MALDI-TOF mass spectrometry identification of differentially expressed proteins, other than yielding a quantitative evaluation on the expression levels of cellular proteins in response to a particular stimulus provided us with important qualitative information on post-translational modifications occurring to each protein (see Fig. 13.6). These processes are particularly important when early cellular events upon a stimulus are considered. During these processes, signaling cascades are triggered independently from protein neosynthesis. To identify direct molecular targets of oxidative stress, and not secondary epiphenomena due, for instance, to apoptotic processes, it is important to develop a cellular model in which the cellular response to the stimulus, expressed in terms of molecular markers of oxidative stress, cell viability, and cellular redox state, is well defined. Moreover it is important to expose the cells for the minimal time to the oxidative injury at conditions of maximal cell viability. In order to generate an oxidative stress condition, we generally use hydrogen peroxide as it easily passes biological membranes. H_2O_2 effects are mainly distinguishable in acute and chronic effects. Experimentally the former are mimicked by high doses (100 μM–mM ranges) for short time treatment (minutes to hours), while the latter are obtained by treatment of cells with low doses of H_2O_2 (nM–10 μM ranges) for longer times (hours to days). Short-time effects of H_2O_2 on cells are associated with protein post-translational modifications, such as phosphorylation, glutathionylation, and Cys-oxidation. In order to study early modifications, human lens and hepatocyte cells were treated with 500 μM H_2O_2. This dose in fact induced block of cellular proliferation without affecting cell viability and causing only a mild decrease in the GSH/GSSG ratio, a common marker of cellular oxidative stress. In both cases we have been able to identify, in total cell extracts, a series of protein modifications occurring at the quantitative and qualitative levels. Quantitative differences, as demonstrated by changes in their expression profiles, occurred on cytoskeletal proteins (tubulin 1α and vimentin) and on enzymes (phosphoglycerate kinase 1, ATP synthase β, enolase α, nucleophosmin, and heat shock cognate 54 kDa protein); qualitative differences, showed by changes in proteins pI as a result of overoxidation, occurred on Prx I, Prx II, PrxVI, RKIP, and glyceraldehyde 3-phosphate dehydrogenase. Moreover mass mapping experiments demonstrated the specific modification of peroxiredoxin active site thiol into sulfinic and/or sulfonic acid, thus explaining the increase in negative charge measured for these proteins. The oxidation kinetics of all peroxiredoxins was extremely rapid (within seconds of H_2O_2 exposure) and sensitive, occurring at H_2O_2 doses unable to affect the common markers of cellular oxidative stress, including ERKs phosphorylation, and recovery experiments demonstrated a quite

different behavior between the retroreduction process of 1-Cys and 2-Cys containing Prxs, thus suggesting a functional difference between different classes of Prxs. The in vivo relevance of our study, in the hepatic cell model, was further reinforced by the finding that overoxidation of PrxI indeed occurs during I/R upon liver transplantation and is dependent on the time of warm ischemia (Cesaratto et al., 2005). Therefore this approach could be of relevance in developing new pharmacological therapies to prevent oxidant-based pathologies.

Fratelli et al. recently applied a proteomics approach, which takes advantages of in vivo labeling with ^{35}S-GSH, to find mixed disulfides between glutathione and the cysteine residues of some reactive proteins (glutathionylation) in T lymphocytes and in hepatocytes exposed to oxidative stress (Fratelli et al., 2002, 2003). Interestingly some of the identified proteins (e.g., vimentin, PrxI, nucleophosmin, enolase, and phosphoglycerate kinase) were common to our cell systems. Therefore our work extends these investigations with additional complementary and encouraging results.

Relatively few methods actually exist for examining disulfide bond formation within cells. One of the most powerful methods, termed diagonal SDS-PAGE, was successfully used by Cumming et al. very recently (Cumming et al., 2004). In this analysis, oxidized proteins are separated in the first dimension under nonreducing conditions and in the orthogonal dimension under reducing conditions. Protein species not aligned along the diagonal are affected by redox regulation via formation of disulfide bridges. By this approach, Cumming et al. identified a series of protein disulfide bonds even in basal nonstressed conditions and formation of new disulfides upon oxidant treatment. They found out a large number of disulfide-bonded proteins, including those involved in glycolysis, cytoskeletal structure, signal transduction, molecular chaperoning, cell growth, and translation. It is to be noted that also in this case some of the identified proteins (PrxI, enolase, etc.) corresponded to those found by us and by Fratelli et al. (2002, 2003). However, despite the high reproducibility the main conceptual problem is represented by the fact that by analyzing only the proteome profile from whole cell extracts, these methods can only focus on major expressed protein species. In order to study poorly expressed ROS-sensing proteins (100–1,000 copies/cell) that may be involved in initiating signal transduction pathways or in controlling gene expression downstream, such as kinases and transcription factors, it is necessary to specifically enrich for proteins of the proper subcellular compartment (i.e., the nucleus), to affinity purify them prior to proteomic analysis, or to develop new strategies to monitor the post-translational modifications on proteins induced by oxidative stress conditions. An elegant approach, called the biotin switch method, has been recently reported for the isolation of *S*-nitrosated proteins (Jaffrey et al., 2001). According to this method free sulfhydryl groups are initially blocked with methyl methanethiosulfonate and, successively, protein-*S*-nitrosocysteine groups are reduced to free thiols by treatment with ascorbate. Then newly released protein thiols are further derivatized with a cleavable biotin-containing reagent and affinity purified by using streptavidin agarose beads. The precipitated material


HepG2
CD5A
2-DE
H_2O_2
10 min
CTRL
H_2O_2 (500 μM)
M_r (kDa)
118
85
50
33
28
19
3
pH
10
Statistical analysis
H_2O_2/Ctrl>1
+2 SD
-2 SD
Ctrl/H_2O_2<1
Molecular analysis
Mass spec
Intensity (%)
Mass (m/z)
CTRL
H_2O_2 (500 μM) 10 min
Prx VI
Prx II
Prx I
pH

is then released for 1D or 2D gel analysis. This methodology allowed the *S*-nitrosylation occurring on PTP to be monitored (Barrett et al., 2005) and on the regulatory phosphatase IKKβ controlling the activation of NF-κB transcription factor (Reynaert et al., 2004), confirming that enrichment procedures are needed to fully comprehend the molecular mechanisms involved in ROS signaling. Nowadays global screening and selective detection of ROS/RNS-modified species separated on different 2D gels are possible by means of different specific staining procedures (Dalle-Donne et al., 2005). In particular, protein carbonyls can be detected and quantified on 2DE maps by measuring the incorporation of tritium following reduction with ^{3}H-borohydride (Yan and Sohal, 1998) or following derivatization with 2,4-dinitrophenylhydrazine (DNPH) (Levine et al., 1990). Very recently detection of carbonylated protein species has been facilitated by introduction of anti-DNP antibodies to allow carbonyl detection by Western blotting analysis (Dalle-Donne et al., 2005). Additionally sensitive and specific immunodetection of sulfinylated peroxiredoxins has been reported by Woo et al. (2003) allowing the oxidized state of these important redox signaling proteins to be monitored. The availability of antibodies specific for sulfinylated peptides should provide insight into protein function similar to that achieved with antibodies to peptides containing phosphoserine or phosphothreonine.

A major limitation in the study of those proteins involved in the cellular response to an acute dose of extracellular H_2O_2 is the fact that due to short lifetime and diffusibility of exogenously applied ROS, it is unlikely that responsive proteins are the same group of proteins that are oxidized by endogenous generation of ROS. Therefore, to have a more complete view, it is advisable to explore proteomic changes occurring upon generation of intracellular oxidative stress. This can be done by using GSH depleting agents, such as buthionine sulfoximine (BSO) and diamide that easily penetrate cellular membranes (Cumming et al., 2004), by using mild generation of H_2O_2 by glucose oxidase, or by using treatment with *tert*-butyl hydroperoxide (*t*-BOOH), a precursor in the formation

FIGURE 13.6 Early markers of oxidative stress by differential proteomics approach. Schematic view of the differential proteomics approach used to identify early molecular targets of oxidative stress as recently used in human hepatocytes (HepG2) and human lens (CD5A) cell lines (Paron et al., 2004; Cesaratto et al., 2005). Cells were treated over short time periods with acute sublethal doses of H_2O_2 and the total cell extracts separated by 2DE analysis. Statistical analysis allows the identifications of upregulated and downregulated protein spots following oxidative burst (the histograms show the mean logarithmic value of the ratio of treated to control cells in accord with the normalized intensities of the matched spots). Differently expressed protein spots are excised from the gel and identified by MALDI-MS. Post-translational modifications are further characterized by extensive peptide mapping experiments using trypsin or endoprotease AspN as in situ hydrolyzing agents in the presence of alkylating reagents. This approach allows identification of the nature of the acidification (visible as a shift in the *pI* of the three circled proteins occurring upon treatment with H_2O_2 of the cells), due to oxidation of the thiol group of Cys residues in the catalytic site to sulfinic and sulfonic acid, occurring at PrxI, PrxII, and PrxVI.

of malondialdehyde that is a membrane-permeant oxidant extensively used as a model of oxidative stress in different systems (Rabilloud et al., 2002).

All these studies are clearly initial investigations and they demonstrate the promising nature of differential proteomics approaches in the detection of early molecular markers of oxidative stress. In this sense these methodologies well satisfy the necessity of optimal target identification for the so-called redox gene therapy as has been already faced to overcome problems associated with oxidative damage-based pathologies. In fact classical anti-oxidant genes such as *SOD, CAT* or *GPX*, often failed to respond in a meaningful manner to oxidative stress. Therefore, rather than delivering classical oxidant scavengers, the overexpression of early oxidative-stress–response genes (e.g., *PRX*) could be most efficient in protecting cells from acute doses of ROS.

13.5 CONCLUSIONS

Since oxidative stress is a common pathogenetic event occurring in several human disorders, ranging from acute and chronic diseases to cancers, the identification of a pattern of molecular alterations present at early stages of oxidative damage would be of great help in monitoring the progression of the disease and in its early recognition. To address this important target, further innovative approaches deriving from postgenomic discoveries are needed to better understand the complex molecular mechanisms of oxidative stress cell responses and to burst the antioxidant ability of eukaryotic cells.

Information regarding the nature of ROS, as well as the localization and the effects of oxidative stress, may be obtained from the analysis of discrete biomarkers isolated from tissues and biological fluids. Biomarkers are cellular indicators of the physiological state and changes during a disease at a specific time. However, the presence of oxidatively damaged molecules could simply reflect secondary epiphenomena rather than having a causal role. Although a clear definition of the cause–effect role cannot be given at present, a growing body of evidence indicates that high levels of ROS induce distinct pathological consequences that greatly amplify and propagate organ injury, leading to irreversible cell and tissue degeneration.

To understand the molecular bases of oxidative stress-induced human diseases, it is important to identify those genes whose expression/function is directly modified by oxidative stress. Since it has been shown that the biological response to oxidative stress is mediated by the modification of a complex array of genes, the study of single candidate genes may not be adequate to solve the complexity of oxidative stress-based human pathologies.

The term proteomics describes the possibility to apply global experimental procedures to evaluate gene expression in terms of single protein species characteristic of each cell type (Zhu et al., 2003). Proteomics approaches compete with other experimental approaches devoted to investigate global expression profiles such as DNA microarrays, but in dealing with the real effectors of biological

functions, it should be able to provide much more valuable information. The possibility of understanding the early molecular effects of oxidative stress by the proteomic approach makes it a powerful tool to use in generating new hypotheses for future studies.

By using a proteomic approach, we recently studied the early molecular targets of oxidative stress in human epithelial lens and hepatocyte cells (Paron et al., 2004; Cesaratto et al., 2005). These are some of the many ways in which a new development of proteomics, called redox proteomics, may be useful in the study of molecular modifications induced by oxidative stress. However, the road we just started is long and still partially hazy; nevertheless, it is clear that a scientific adventure has just begun. Proteomics studies hold promise of detection of early molecular markers of oxidative stress. No doubt, much more efforts must be devoted to the study of protein modifications upon oxidative stress at each single subcellular compartment. In particular, the involvement of membrane-bound receptors in controlling cellular response toward an oxidative burst deserves much more attention for the possible therapeutical implications as pharmacological targets. The ROS-sensing proteins that initiate signal transduction pathways are at amounts too low to be detected by presently used differential proteomics strategies unless methods are used to affinity purify them prior to proteomic analysis. In addition to the biotin switch method, used to affinity purify *S*-nitrosylated proteins, and to the specific immunodetection assays developed to study oxidized or carbonylated proteins, techniques need to be developed to identify oxidatively modified proteins.

Proteomics techniques well satisfy the identification of optimal targets for redox gene therapy, such as has been already demonstrated to overcome problems associated with oxidative damage during oxidative-based liver pathologies (Wheeler et al., 2001). The future will tell us if, rather than delivering through transgene vectors, classical oxidant scavengers, such as SOD or CAT, the overexpression of early genes responsive to oxidative stress could be efficient in protecting cells from acute doses of ROS and provide an optimal tool for a large number of human pathologies.

ACKNOWLEDGMENTS

This work was supported by grants from MIUR (FIST 2003 and FIRB RBNE0155LB-03) to G.T. The author thanks Drs. Franco Quadrifoglio and Elisa Tell for reading the manuscript and for improvements to the English style.

LIST OF ABBREVIATIONS

APE1/Ref-1, apurinic apyrimidinic endonuclease 1/redox effector factor-1
AP-1, activator protein-1

CAT, catalase
EGF, epidermal growth factor
Egr-1, early growth response protein-1
ERK, extracellular signal regulated kinase
GSH, reduced glutathione
GSSG, oxidized glutathione
GSTp, glutathione *S*-transferase P_i
GPX, glutathione peroxidase
HAT, hystone acetyltransferase
HDAC, hystone deacetylase
HIF-1, hypoxia inducible factor-1
I/R, Ischemia/Reperfusion
JNK, Jun *N*-terminal kinase
MALDI-TOF, matrix-assisted laser desorption ionization–time-of-flight spectroscopy
MAPK, mitogen-activated protein kinase
MEKK1, mitogen-activated protein kinase kinase 1
NF-κB, nuclear factor-κ B
NGF, nerve growth factor
Pax, paired box containing protein
PDGF, platelet derived growth factor
PEBP-2, polyoma enhancer binding protein-2
PKC, protein kinase C
PKA, protein kinase A
Prx, peroxiredoxin
PTP, protein tyrosine phosphatase
ROS, reactive oxygen species
SOD, superoxide dismutases
TFs, transcription factors
Trx, thioredoxin
TTF-1, thyroid transcription factor-1

REFERENCES

Abe J, Kusuhara M., Ulevitch R.J., Berk B.C., Lee J.D. (1996). Big mitogen-activated protein kinase 1 (BMK1) is a redox-sensitive kinase. *J Biol Chem* 271: 16586–16590.

Adler V., Yin Z., Fuchs S.Y., Benezra M., Rosario L., Tew K.D., Pincus M.R., Sardana M., Henderson C.J., Wolf C.R., Davis R.J., Ronai Z. (1999). Regulation of JNK signaling by GSTp. *EMBO J* 18: 1321–1334.

Akamatsu Y., Ohno T., Hirota K., Kagoshima H., Yodoi J., Shigesada K. (1997). Redox regulation of the DNA binding activity in transcription factor PEBP2: The roles of two conserved cysteine residues. *J Biol Chem* 272: 14497–14500.

Allen R.G., Tresini M. (2000). Oxidative stress and gene regulation. *Free Radic Biol Med* 28: 463–499.

Arnold R.S., Shi J., Murad E., Whalen A.M., Sun C.Q., Polavarapu R., Parthasarathy S., Petros J.A., Lambeth J.D. (2001). Hydrogen peroxide mediates the cell growth and transformation caused by the mitogenic oxidase Nox1. *Proc Natl Acad Sci USA* 98: 5550–5555.

Bae Y.S., Kang S.W., Seo M.S., Baines I.C., Tekle E., Chock P.B., Rhee S.G. (1997). Epidermal growth factor (EGF)-induced generation of hydrogen peroxide: Role in EGF receptor-mediated tyrosine phosphorylation. *J Biol Chem* 272: 217–221.

Barrett D.M., Black S.M., Todor H., Schmidt-Ullrich R.K., Dawson K.S., Mikkelsen R.B. (2005). Inhibition of protein-tyrosine phosphatases by mild oxidative stresses is dependent on *S*-nitrosylation. *J Biol Chem* 280: 14453–14461.

Berrevoet F., Schafer T., Vollmar B., Menger M.D. (2003). Ischemic preconditioning: Enough evidence to support clinical application in liver surgery and transplantation? *Acta Chir Belg* 103: 485–489.

Boon E.M., Huang S.H., Marletta M.S. (2005). A molecular basis for NO selectivity in soluble guanylate cyclase. *Nat Chem Biol* 1: 53–59.

Bray T.M. (1999). Antioxidants and oxidative stress in health and disease: introduction. *Proc Soc Exp Biol Med* 222: 195.

Budanov A.V., Sablina A.A., Feinstein E., Koonin E.V., Chumakov P.M. (2004). Regeneration of peroxiredoxins by p53-regulated sestrins, homologs of bacterial AhpD. *Science* 304: 596–600.

Cesaratto L., Vascotto C., Calligaris S., Tell G. (2004). The importance of redox state in liver damage. *Ann. Hepatol* 3: 86–92.

Cesaratto L., Vascotto C., D'Ambrosio C., Scaloni A., Baccarani U., Paron I., Damante G., Calligaris S., Quadrifoglio F., Tiribelli C., Tell G. (2005). Overoxidation of Peroxiredoxins as an immediate and sensitive marker of oxidative stress in HepG2 cells and its application to the redox effects induced by ischemia/reperfusion in human liver. *Free Rad Res* 39: 255–268.

Chae H.Z., Chung S.J., Rhee S.G. (1994). Thioredoxin-dependent peroxide reductase from yeast. *J Biol Chem* 269: 27670–27678.

Chevallet M., Wagner E., Luche S., van Dorsselaer A., Leize-Wagner E., Rabilloud T. (2003). Regeneration of Peroxiredoxins during recovery after oxidative stress. *J Biol Chem* 278: 37146–37153.

Cho S.H., Lee C.H., Ahn Y., Kim H., Kim H., Ahn C.Y., Yang K.S., Lee S.R. (2004). Redox regulation of PTEN and protein tyrosine phosphatases in H_2O_2 mediated cell signaling. *FEBS Lett* 560: 7–13.

Clark J.I., Matsushima H., David L.L., Clark J.M. (1999). Lens cytoskeleton and transparency: A model. *Eye* 13: 417–424.

Clarkson M.R., Murphy M., Gupta S., Lambe T., Mackenzie H.S., Godson C., Martin F., Brady H.R. (2002). High glucose-altered gene expression in mesangial cells: Actin-regulatory protein gene expression is triggered by oxidative stress and cytoskeletal disassembly. *J Biol Chem* 277: 9707–9712.

Cross J.V., Templeton D.J. (2004). Oxidative stress inhibits MEKK1 by site-specific glutathionylation in the ATP-binding domain. *Biochem J* 381: 675–683.

Cumming R.C., Andon N.L., Haynes P.A., Park M., Fischer W.H., Schubert D. (2004). Protein disulfide bond formation in the cytoplasm during oxidative stress. *J Biol Chem* 279: 21748–21758.

Czaja M.J., Liu H., Wang Y. (2003). Oxidant-induced hepatocyte injury from menadione is regulated by ERK and AP-1 signaling. *Hepatology* 37: 1405–1413.

Dalle-Donne I., Rossi R., Milzani A., Di Simplicio P., Colombo R. (2001). The actin cytoskeleton response to oxidants: From small heat shock protein phosphorylation to changes in the redox state of actin itself. *Free Radic Biol Med* 31: 1624–1632.

Dalle-Donne I., Scaloni A., Giustarini D., Cavarra E., Tell G., Lungarella G., Colombo R., Rossi R., Milzani A. (2005). Proteins as biomarkers of oxidative/nitrosative stress in diseases: The contribution of redox Proteomics. *Mass Spectrom Rev* 24: 55–99.

Davies K.J. (1987). Protein damage and degradation by oxygen radicals. I. General aspects. *J Biol Chem* 262: 9895–9901.

Droge W. (2002). Free radicals in the physiological control of cell function. *Physiol Rev* 82: 47–95.

Ema M., Hirota K., Mimura J., Abe H., Yodoi J., Sogawa K., Poellinger L., Fujii-Kuriyama Y. (1999). Molecular mechanisms of transcription activation by HLF and HIF1 alpha in response to hypoxia: Their stabilization and redox signal-induced interaction with CBP/p300. *EMBO J* 18: 1905–1914.

Forman H.J., Fukuto J.M., Torres M. (2004). Redox signaling: Thiol chemistry defines which reactive oxygen and nitrogen species can act as second messengers. *Am J Physiol Cell Physiol* 287: C246–C256.

Fratelli M., Demol H., Puype M., Casagrande S., Eberini I., Salmona M., Bonetto V., Mengozzi M., Duffieux F., Miclet E., Bachi A., Vandekerckhove J., Gianazza E. Ghezzi P. (2002). Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. *Proc Natl Acad Sci USA* 99: 3505–3510.

Fratelli M., Demol H., Puype M., Casagrande S., Villa P., Eberini I., Vandekerckhove J., Gianazza E., Ghezzi P. (2003). Identification of proteins undergoing glutathionylation in oxidatively stressed hepatocytes and hepatoma cells. *Proteomics* 3: 1154–1161.

Fridovich I. (1978). The biology of oxygen radicals. *Science* 201: 875–880.

Gaiddon C., Moorthy N.C., Prives C. (1999). Ref-1 regulates the transactivation and pro-apoptotic functions of p53 in vivo. *EMBO J* 18: 5609–5621.

Gashler A., Sukhatme V.P. (1995). Early growth response protein 1 (Egr-1): Prototype of a zinc-finger family of transcription factors. *Prog Nucleic Acid Re Mol Biol* 50: 191–224.

Goswami S., Sheets N.L., Zavadil J., Chauhan B.K., Bottinger E.P., Reddy V.K., Kantorow M., Cvekl A. (2003). Spectrum and range of oxidative stress responses of human lens epithelial cells to H_2O_2 insult. *Invest Ophthalmol Vis Sci* 44: 2084–2093.

Gow A.J., Stamler J.S. (1998). Reactions between nitric oxide and haemoglobin under physiological conditions. *Nature* 391: 169–173.

Griendling K.K., Sorescu D., Ushio-Fukai M. (2000). NAD(P)H oxidase: Role in cardiovascular biology and disease. *Circ Res* 86: 494–501.

Grune T., Reinheckel T., Davies K.J. (1997). Degradation of oxidized proteins in mammalian cells. *FASEB J* 11: 526–534.

Guyton K.Z., Liu Y., Gorospe M., Xu Q., Holbrook N.J. (1996). Activation of mitogen-activated protein kinase by H_2O_2: Role in cell survival following oxidant injury. *J Biol Chem* 271: 4138–4142.

Halliwell B., Gutteridge J.M.C. (1989). *Free Radicals in Biology and Medicine.* 2nd ed, Clarendon, Oxford.

Hare J.M., Stamler J.S. (2005). NO/redox disequilibrium in the failing heart and cardiovascular system. *J Clin Invest* 115: 509–517.

Hehner S.P., Breitkreutz R., Shubinsky G., Unsoeld H., Schulze-Osthoff K., Schmitz M.L., Droge W. (2000). Enhancement of T cell receptor signaling by a mild oxidative shift in the intracellular thiol pool. *J Immunol* 165: 4319–4328.

Huang R.P., Adamson E.D. (1993). Characterization of the DNA-binding properties of the early growth response-1 (Egr-1) transcription factor: Evidence for modulation by a redox mechanism. *DNA Cell Biol* 12: 265–273.

Huang L.E., Arany Z., Livingston D.M., Bunn H.F. (1996). Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. *J Biol Chem* 271: 32253–32259.

Humphries K.M., Juliano C., Taylor S.S. (2002). Regulation of cAMP-dependent protein kinase activity by glutathionylation. *J Biol Chem* 277: 43505–43511.

Huot J., Houle F., Marceau F., Landry J. (1997). Oxidative stress-induced actin reorganization mediated by the p38 mitogen-activated protein kinase/heat shock protein 27 pathway in vascular endothelial cells. *Circ Res* 80: 383–392.

Huot J., Houle F., Rousseau S., Deschesnes R.G.., Shah G.M., Landry J. (1998). SAPK2/p38-dependent F-actin reorganization regulates early membrane blebbing during stress-induced apoptosis. *J Cell Biol* 143: 1361–1373.

Jaffrey S.R., Erdjument-Bromage H., Ferris C.D., Tempst P., Snyder S.H. (2001). Protein *S*-nitrosylation: A physiological signal for neuronal nitric oxide. *Nat Cell Biol* 3: 193–197.

Jayaraman L., Murthy K.G., Zhu C., Curran T., Xanthoudakis S., Prives C. (1997). Identification of redox/repair protein Ref-1 as a potent activator of p53. *Genes Dev* 11: 558–570.

Jones S.A., Wood J.D., Coffey M.J., Jones O.T. (1994). The functional expression of p47-phox and p67-phox may contribute to the generation of superoxide by an NADPH oxidase-like system in human fibroblasts. *FEBS Lett* 355: 178–182.

Kang S.W., Chae H.Z., Seo M.S., Kim K., Baines I.C., Rhee S.G. (1998). Mammalian peroxiredoxin isoforms can reduce hydrogen peroxide generated in response to growth factors and tumor necrosis factor-alpha. *J Biol Chem* 273: 6297–6302.

Kilty I., Rahman I. (2004). Oxidative stress and cigarette smoke alter chromatin remodeling but differentially regulate NF-kappaB activation and proinflammatory cytokine release in alveolar epithelial cells. *FASEB J* 18: 1897–1899.

Konishi H., Tanaka M., Takemura Y., Matsuzaki H., Ono Y., Kikkawa U., Nishizuka Y. (1997). Activation of protein kinase C by tyrosine phosphorylation in response to H_2O_2. *Proc Natl Acad Sci USA* 94: 11233–11237.

Kroncke K.D. (2001). Cysteine-Zn^{2+} complexes: Unique molecular switches for inducible nitric oxide synthase-derived NO. *FASEB J* 15: 2503–2507.

Kroncke K.D., Klotz L.O., Suschek C.V., Sies H. (2002). Comparing nitrosative versus oxidative stress toward zinc finger-dependent transcription: Unique role for NO. *J Biol Chem* 277: 13294–13301.

Lander H.M. (1997). An essential role for free radicals and derived species in signal transduction. *FASEB J* 11: 118–124.

Lee S.R., Kwon K.S., Kim S.R., Rhee S.G. (1998). Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. *J Biol Chem* 273: 15366–15372.

Leonard S.S., Harris G.K., Shi X. (2004). Metal-induced oxidative stress and signal transduction. *Free Radic Biol Med* 37: 1921–1942.

Levine R.L., Garland D., Oliver C.N., Amici A., Climenti I., Lenz A., Ahn B., Shaltiel S., Stadtman E.R. (1990). Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* 186: 464–478.

Lim S.D., Sun C., Lambeth J.D., Marshall F., Amin M., Chung L., Petros J.A., Arnold R.S. (2005). Increased Nox1 and hydrogen peroxide in prostate cancer. *Prostate* 62: 200–207.

Liu S.M., Sundqvist T. (1995). Effects of hydrogen peroxide and phorbol myristate acetate on endothelial transport and F-actin distribution. *Exp Cell Res* 217: 1–7.

Loguercio C., Federico A. (2003). Oxidative stress in viral and alcoholic hepatitis. *Free Radic Biol Med* 34: 1–10.

Marshall H.E., Merchant K., Stamler J.S. (2000). Nitrosation and oxidation in the regulation of gene expression. *FASEB J* 14: 1889–1900.

Marshall H.E., Hess D.T., Stamler J.S. (2004). *S*-Nitrosylation: Physiological regulation of NF-kappaB. *Proc Natl Acad Sci USA* 101: 8841–8842.

McCord J.M. (1985). Oxygen-derived free radicals in postischemic tissue injury. *N Engl J Med* 312: 159–163.

Meng T.C., Fukada T., Tonks N.K. (2002). Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo. *Mol Cell* 9: 387–399.

Mikkelsen R.B., Wardman P. (2003). Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms. *Oncogene* 22: 5734–5754.

Milzani A., Rossi R., Di Simplicio P., Giustarini D., Colombo R., Dalle-Donne I. (2000). The oxidation produced by hydrogen peroxide on Ca-ATP-G-actin. *Protein Sci* 9: 1774–1782.

Moodie F.M., Marwick J.A., Anderson C.S., Szulakowski P., Biswas S.K., Bauter M.R., Morgan K.T., Ni H., Brown H.R., Yoon L., Qualls C.W. Jr, Crosby L.M., Reynolds R., Gaskill B., Anderson S.P., Kepler T.B., Brainard T., Liv N., Easton M., Merrill C., Creech D., Sprenger D., Conner G., Johnson P.R., Fox T., Sartor M., Richard E., Kuruvilla S., Casey W., Benavides G. (2002). Application of cDNA microarray technology to in vitro toxicology and the selection of genes for a real-time RT-PCR-based screen for oxidative stress in Hep-G2 cells. *Toxicol Pathos* 30: 435–451.

Nakamura K., Hori T., Sato N., Sugie K., Kawakami T., Yodoi J. (1993). Redox regulation of a src family protein tyrosine kinase p56lck in T cells. *Oncogene* 8: 3133–3139.

Nakamura H., Nakamura K., Yodoi J. (1997). Redox regulation of cellular activation. *Ann Rev Immunol* 15: 351–69.

Nakanishi K., Maruyama M., Shibata T., Morishima N. (2001). Identification of a caspase-9 substrate and detection of its cleavage in programmed cell death during mouse development. *J Biol Chem* 276: 41237–41244.

Nakshatri H., Bhat-Nakshatri P., Currie R.A. (1996). Subunit association and DNA binding activity of the heterotrimeric transcription factor NF-Y is regulated by cellular redox. *J Biol Chem* 271: 28784–28791.

Nishi T., Shimizu N., Hiramoto M., Sato I., Yamaguchi Y., Hasegawa M., Aizawa S., Tanaka H., Kataoka K., Watanabe H., Handa H. (2002). Spatial redox regulation of a critical cysteine residue of NF-kappa B in vivo. *J Biol Chem* 277: 44548–44556.

Noh D.Y., Ahn S.J., Lee R.A., Kim S.W., Park I.A., Chae H.Z. (2001). Overexpression of peroxiredoxin in human breast cancer. *Anticancer Res* 21: 2085–2090.

Ono Y., Fujii T., Igarashi K., Kuno T., Tanaka C., Kikkawa U., Nishizuka Y. (1989). Phorbol ester binding to protein kinase C requires a cysteine-rich zinc-finger-like sequence. *Proc Natl Acad Sci USA* 86: 4868–4871.

Ordway J.M., Eberhart D., Curran T. (2003). Cysteine 64 of Ref-1 is not essential for redox regulation of AP-1 DNA binding. *Mol Cell Biol* 23: 4257–4266.

Palmer R.M.J., Rees D.D., Ashton D.S., Moncada S. (1988). L-Arginine is the physiological precursor for the formation of nitric oxide in endothelium dependent relaxation. *Biochem Biophys Res Commun* 153: 1251–1256.

Paron I., D'Elia A., D'Ambrosio C., Scaloni A., D'Aurizio F., Prescott A., Damante G., Tell G. (2004). A Proteomics approach to identify early molecular targets of oxidative stress in human epithelial lens cells. *Biochem J* 378: 929–937.

Poli G., Parola M. (1997). Oxidative damage and fibrogenesis. *Free Radic Biol Med* 22: 287–305.

Rabilloud T., Heller M., Gasnier F., Luche S., Rey C., Aebersold R., Benahmed M., Louisot P., Lunardi J. (2002). Proteomics analysis of cellular response to oxidative stress: Evidence for in vivo overoxidation of peroxiredoxins at their active site. *J Biol Chem* 277: 19396–19401.

Rahman I., Marwick J., Kirkham P. (2004). Redox modulation of chromatin remodeling: impact on histone acetylation and deacetylation, NF-kappaB and pro-inflammatory gene expression. *Biochem Pharmacol* 68: 1255–1267.

Reeves E.P., Lu H., Jacobs H.L., Messina C.G., Bolsover S., Gabella G., Potma E.O., Warley A., Roes J., Segal A.W. (2002). Killing activity of neutrophils is mediated through activation of proteases by K^+ flux. *Nature* 416: 291–297.

Reynaert N.L., Ckless K., Korn S.H., Vos N., Guala A.S., Wouters E.F.M., van der Vliet A., Janssen-Heininger Y.M.W. (2004). Nitric oxide represses inhibitory κB kinase through *S*-nitrosylation. *Proc Natl Acad Sci USA* 101: 8945–8950.

Rokutan K., Johnston R.B. Jr, Kawai K. (1994). Oxidative stress induces *S*-thiolation of specific proteins in cultured gastric mucosal cells. *Am J Physiol* 266: G247–G254.

Sauer H., Klimm B., Hescheler J., Wartenberg M. (2001). Activation of p90RSK and growth stimulation of multicellular tumor spheroids are dependent on reactive oxygen species generated after purinergic receptor stimulation by ATP. *FASEB J* 15: 2539–2541.

Schallreuter K.U., Wood J.M. (1986). The role of thioredoxin reductase in the reduction of free radicals at the surface of the epidermis. *Biochem Biophys Res Commun* 136: 630–637.

Sies H. (1985). Oxidative stress: Introductory remarks. In: Sies H. (ed.), *Oxidative Stress*, Academic Press, London, pp. 1–9.

Spector A., Yan G.Z., Huang R.R., McDermott M.J., Gascoyne P.R., Pigiet V. (1988). The effect of H_2O_2 upon thioredoxin-enriched lens epithelial cells. *J Biol Chem* 263: 4984–4990.

Staal F.J., Anderson M.T., Staal G.E., Herzenberg L.A., Gitler C., Herzenberg L.A. (1994). Redox regulation of signal transduction: tyrosine phosphorylation and calcium influx. *Proc Natl Acad Sci USA* 91: 3619–3622.

Stamler J.S., Single D., Loscalzo J. (1992). Biochemistry of nitric oxide and its redox-activated forms. *Science* 258: 1898–1902.

Suh Y.A., Arnold R.S., Lassegue B., Shi J., Xu X., Sorescu D., Chung A.B., Griendling K.K., Lambeth J.D. (1999). Cell transformation by the superoxide-generating oxidase Mox1. *Nature* 401: 79–82.

Sun X., Wu F., Datta R., Kharbanda S., Kufe D. (2000). Interaction between protein kinase C delta and the c-Abl tyrosine kinase in the cellular response to oxidative stress. *J Biol Chem* 275: 7470–7473.

Sundaresan M., Yu Z.X., Ferrans V.J., Irani K., Finkel T. (1995). Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. *Science* 270: 296–299.

Suzukawa K., Miura K., Mitsushita J., Resau J., Hirose K., Crystal R., Kamata T. (2000). Nerve growth factor-induced neuronal differentiation requires generation of Rac1-regulated reactive oxygen species. *J Biol Chem* 275: 13175–13178.

Suzuki Y.J., Ford G.D. (1999). Redox regulation of signal transduction in cardiac and smooth muscle. *J Mol Cell Cardiol* 31: 345–353.

Takeya R., Sumimoto H. (2003). Molecular mechanism of activation of superoxide-producing NADPH oxidases. *Mol Cells* 26: 271–277.

Tell G., Damante G., Caldwell D., Kelley M.R. (2005). The Intracellular Localization of APE1/Ref-1: More than a Passive Phenomenon? *Antioxid Redox Signal* 7: 367–384.

Tell G., Pines A., Paron I., D'Elia A., Bisca A., Kelley M.R., Manzini G., Damante G. (2002). Redox effector factor-1 regulates the activity of thyroid transcription factor 1 by controlling the redox state of the N transcriptional activation domain. *J Biol Chem* 277: 14564–14574.

Tonks N.K., Neel B.G. (2001). Combinatorial control of the specificity of protein tyrosine phosphatases. *Curr Opin Cell Biol* 13: 182–195.

Walker L.J., Robson C.N., Black E., Gillespie D., Hickson I.D. (1993). Identification of residues in the human DNA repair enzyme HAP1 (Ref-1) that are essential for redox regulation of Jun DNA binding. *Mol Cell Biol* 13: 5370–5376.

Ward N.E., Stewart J.R., Ioannides C.G., O'Brian C.A. (2000). Oxidant-induced *S*-glutathiolation inactivates protein kinase C-alpha (PKC-alpha): A potential mechanism of PKC isozyme regulation. *Biochemistry* 39: 10319–10329.

Wheeler M.D., Kono H., Yin M., Rusyn I., Froh M., Condor H.D., Mason R.P., Samulski R.J., Thurman R.G. (2001). Delivery of the Cu/Zn-superoxide dismutase gene with adenovirus reduces early alcohol-induced liver injury in rats. *Gastroenterology* 120: 1241–1250.

Williams M.S., Kwon J. (2004). T cell receptor stimulation, reactive oxygen species, and cell signaling. *Free Radic Biol Med* 37: 1144–1151.

Wonsey D.R., Zeller K.I., Dang C.V. (2002). The c-Myc target gene PRDX3 is required for mitochondrial homeostasis and neoplastic transformation. *Proc Natl Acad Sci USA* 99: 6649–6654.

Woo H.A., Kang S.W., Kim H.K., Yang K.S., Chae H.Z., Rhee S.G. (2003). Reversible oxidation of the active site cysteine of peroxiredoxins to cysteine sulfinic acid: Immunoblot detection with antibodies specific for the hyperoxidized cysteine-containing sequence. *J Biol Chem* 278: 47361–47364.

Wood Z.A., Poole L.B., Karplus P.A. (2003). Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* 300: 650–653.

Wu G., Fang Y.Z., Yang S., Lupton J.R., Turner N.D. (2004). Glutathione metabolism and its implications for health. *J Nutr* 134: 489–492.

Xanthoudakis S., Curran T. (1992). Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity. *EMBO J* 11: 653–665.

Xanthoudakis S., Miao G., Wang F., Pan Y.C., Curran T. (1992). Redox activation of Fos-Jun DNA binding activity is mediated by a DNA repair enzyme. *EMBO J* 11: 3323–3335.

Yan L.J., Sohal R.S. (1998). Gel electrophoretic quantitation of protein carbonyls derivatized with tritiated sodium borohydride. *Anal Biochem* 265: 176–182.

Yeh L.H., Park Y.J., Hansalia R.J., Ahmed I.S., Deshpande S.S., Goldschmidt-Clermont P.J., Irani K., Alevriadou B.R. (1999). Shear-induced tyrosine phosphorylation in endothelial cells requires Rac1-dependent production of ROS. *Am J Physiol* 276: C838–C847.

Zheng M., Aslund F., Storz, G. (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* 279: 1718–1721.

Zhu H., Bilgin M., Snyder M. (2003). Proteomics. *Ann Rev Biochem* 72: 783–812.

14

OXIDATIVE DAMAGE TO PROTEINS: STRUCTURAL MODIFICATIONS AND CONSEQUENCES IN CELL FUNCTION

ELISA CABISCOL AND JOAQUIM ROS

14.1 INTRODUCTION

Oxidative damage to macromolecules is an inescapable price for the evolution of aerobic metabolism. The co-evolution of protective agents, enzymes, or low molecular weight antioxidants can at best reduce the magnitude of damage. Repairing such damage has been almost limited to DNA, probably because of chemical feasibility and strong selective pressure. For other kinds of damaged macromolecules, such as proteins, clearing by turnover seems to be the main option. This strategy may be satisfactory for the perpetuation of unicellular organisms, although it is not always available or sufficient for multicellular organisms. For instance, accumulation of oxidatively damaged proteins is often associated with senescence and various disease states.

Nevertheless, although oxidative damage to a protein can result in a loss of function, it cannot represent a deleterious effect for the whole cell. For instance, the glycolytic pathway is shut down under oxidative stress, by inactivating key enzymes, to favor channeling glucose to the pentose phosphate pathway, thus providing NADPH for antioxidant response.

Proteins described in this chapter have been grouped in several sections: glycolysis, pyruvate metabolism, tricarboxylic acid cycle, electron transport chain and oxidative phosphorylation, antioxidant defenses, molecular chaperones, and cytoskeleton. They have been selected on the bases of their significance for cell

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

physiology and metabolism. This means that many laboratories focus their investigations on such proteins, and consequently modifications of such proteins are well documented. All these data have been compiled in Table 14.1. However, we are aware that important works on specific enzymes have not been included in this chapter. One example is the pioneering studies on glutamine synthetase performed by Levine and Stadtman at NIH (Levine et al., 1981; Levine, 1983a,b). Other examples are the studies on albumin and hemoglobin oxidation.

For many years the elucidation of structural modifications on proteins as a consequence of oxidative stress has been limited to in vitro studies and not easily solved. The main reason was, and still is, the lack of tools available. The increased use of mass spectrometry to study proteins is changing the tendency. It helps elucidate structural modifications and, in combination with two-dimensional gel electrophoresis and liquid chromatography, improves the in vivo identification of damaged proteins.

14.2 GLYCOLYSIS

Glucose is the main fuel of most organisms, so it occupies a central position in metabolism. In higher plants and animals, glucose has three major fates: it may be stored (as a polysaccharide or as sucrose), oxidized to pentoses via the pentose phosphate pathway, or oxidized as a three-carbon compound (pyruvate) via glycolysis. During the sequential reactions of glycolysis, some of the free energy released from glucose is conserved in the form of ATP and NADH.

Because of the major role of glycolysis in energy metabolism, its enzymes have been extensively studied as a target under both exogenous and endogenously produced oxidative stress situations. In consequence, the role of protein oxidation in several pathologies and also in aging has been evaluated.

Over the last few years the number of articles that have appeared describing oxidative modifications in glycolytic enzymes has been increasing exponentially, and it is impossible to mention all of them in this review. Only examples of oxidatively damaged enzymes identified by several authors and under several situations have been included.

The breakdown of the six-carbon glucose into two molecules of the three-carbon pyruvate occurs in ten steps (Fig. 14.1). In four of them, the catalytic enzyme has been extensively identified as a target under oxidative stress situations: fructose 1,6-bisphosphate aldolase, triose phosphate isomerase (TPI), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and enolase. The oxidative modifications mainly described to occur in such enzymes are *S*-thiolation, carbonylation, and tyrosine nitration, although other modifications have also been described.

14.2.1 Fructose 1,6-Bisphosphate Aldolase

Fructose 1,6-bisphosphatase aldolase, often called simply aldolase, catalyzes a reversible aldol condensation. Fructose 1,6-bisphosphate is cleaved to yield two

TABLE 14.1 Oxidatively Damaged Proteins

Name	Observed Modification	Origin	Condition/Treatment	References	Comments
			Glycolysis		
Fructose 1,6-bisphosphate aldolase	*S*-thiolation	Yeast	Hydrogen peroxide	Shenton and Grant, 2003	Not inactivated
		Mammalian	Hydrogen peroxide and diamide	Fratelli et al., 2002	Human T lymphocytes
			Diamide	Lind et al., 2002	ECV304 endothelial cells
	Carbonylation	Yeast	Copper	Shanmuganathan et al., 2004	
			Aging	Reverter-Branchat et al., 2004	Chronological and replicative aging
		Mammalian	*E. coli* lipopolysaccharide	Barreiro et al., 2005	Inactivated. Rat diaphragm
			VP16 (etoposide)	England et al., 2004	HL60 cells
	Modification by RNS	Mammalian	*E. coli* lipopolysaccharide	Aulak et al., 2001	Lung and liver
			Damaging light	Miyagi et al., 2002	Rat retina
			Ischemia-reperfusion	Koeck et al., 2004	Isolated mitochondria from rat liver. Inactivated. Nitration of Tyr363 and Tyr342
TPI Triose phosphate isomerase	*S*-thiolation	Yeast	Hydrogen peroxide	Shenton and Grant, 2003	Not inactivated
		Mammalian	Ischemia-reperfusion	Eaton et al., 2002	Rat heart
			Diamide	Fratelli et al., 2002	Human T lymphocytes

(*continued overleaf*)

TABLE 14.1 (*continued*)

Name	Observed Modification	Origin	Condition/Treatment	References	Comments
	Carbonylation	Yeast	Hydrogen peroxide	Costa et al., 2002	Not inactivated
		Mammalian	VP16 (etoposide)	England et al., 2004	HL60 cells
	Modification by RNS	Mammalian	Alzheimer's disease	Castegna et al., 2003	Human brain
GAPDH Glyceraldehyde 3-phosphate dehydrogenase	Inactivated	Yeast	Hydrogen peroxide	Grant et al., 1999	Tdh2 inactivated
	S-thiolation	Yeast	Hydrogen peroxide	Shenton and Grant, 2003	Tdh3 Inactivated. Glutathiolated candidate: Cys149
				Grant et al., 1999	Tdh3 but not Tdh2 was *S*-thiolated in vivo. Both inactivated
		Mammalian	Hydrogen peroxide	Schuppe-Koistinen et al., 1994	Human endothelial cells
			Phagocytosis-associated respiratory burst	Ravichandran et al., 1994	Human blood monocytes
			Ischemia-reperfusion	Eaton et al., 2002	Rat heart
		In vitro	Hydrogen peroxide	Shenton et al., 2002	Cell free extracts from yeast. Tdh2 and Tdh3 thiolated
	Carbonylation	Yeast	Hydrogen peroxide	Costa et al., 2002	Tdh2 and Tdh3 carbonylated and inactivated
			Copper	Shanmuganathan et al., 2004	Tdh2 and Tdh3 carbonylated
			Hydrogen peroxide and menadione	Cabiscol et al., 2000	Inactivated

		Δ*sod1* and Δ*sod2* mutants	O'Brien et al., 2004	Tdh2 and Tdh3 carbonylated
		Aging	Reverter-Branchat et al., 2004	Replicative and chronological aging. Inactivated in chronological aging
	Mammalian	*E. coli* lipopolysaccharide	Barreiro et al., 2005	Rat diaphragm
	In vitro	Hydroxynonenal	Uchida and Stadtman, 1993	Purified from rabbit muscle. Inactivated. Loss of SH (Cys), His and Lys.
			Ishii et al., 2003	Purified from rabbit muscle. Five peptides contained HNE adducts
Modification by RNS	Yeast	Peroxynitrite	Buchczyk et al., 2000	Nitration and inactivation
	Mammalian	Peroxynitrite	Buchczyk et al., 2001	Nitration and inactivation. Cultured rat astrocytes
		Aging	Kanski et al., 2005	Nitrated. Rat heart
		E. coli lipopolysaccharide	Szweda et al., 1993	Nitrated. Rat liver
ADP-ribosylation	Mammalian	Hydrogen peroxide	Colussi et al., 2000	Inactivated. U937 cells. Ribosylation protect these cells from apoptosis
		NAD plus nitroprusside	Kots et al., 1992	Human erythrocytes
	In vitro	NAD plus nitric oxide	Zhang and Snyder, 1992	Rat brain. Cytosolic preparations.
		Nitric oxide and nitric oxide-generating agents	Dimmeler et al., 1992	Purified from human platelet

(*continued overleaf*)

TABLE 14.1 (*continued*)

Name	Observed Modification	Origin	Condition/Treatment	References	Comments
	NADH modification	In vitro	NADH and low molecular weight thiols	Rivera-Nieves et al., 1999	Purified from rabbit muscle. Covalent attachment of NADH at Cys149, the active-site thiol
Enolase	*S*-thiolation	Yeast	Hydrogen peroxide	Shenton and Grant, 2003	Eno2 inactivated
		Mammalian	Hydrogen peroxide and diamide	Fratelli et al., 2002	Human T lymphocytes. Glutathionylated and inhibited
	Carbonylation	Bacteria	Hydrogen peroxide	Tamarit et al., 1998	*E. coli*. Inactivated
		Yeast	Copper	Shanmuganathan et al., 2004	
			Menadione	Cabiscol et al., 2000	Eno2 inactivated
			Δ*sod1* mutants	O'Brien et al., 2004	Eno2 carbonylated
			Aging	Reverter-Branchat et al., 2004	Eno2 and Eno1 carbonylated. Lower levels in Eno1
		Mammalian	VP16 (etoposide)	England et al., 2004	α-enolase carbonylated. HL60 cells.
			Alzheimer's disease	Shin et al., 2004	α-enolase carbonylated in transgenic brain mice (AD model)
				Castegna et al., 2002	Human AD brain
	Modification by RNS	Mammalian	Aging	Kanski et al., 2004	Rat heart
			Alzheimer's disease	Castegna et al., 2003	α-enolase and γ-enolase nitrated in human AD brain
				Shin et al., 2004	α-enolase nitrated (3-nitrotyrosine). Transgenic mice (AD model)

PDHC Pyruvate dehydrogenase complex	Inactivation	Mammalian	Ischemia-reperfusion	Fukuchi et al., 1998	Gerbil cortex
				Schoder et al., 1998	Dog heart
				Zaidan and Sims, 1997	Dorsolateral striatum
			Alzheimer's disease	Sheu et al., 1985	Human AD brain
			Ischemia-reperfusion	Martin et al., 2005	Purified porcine heart mitochondria. Possible sulfhydryl oxidation
		In vitro	Xanthine oxidase/hypoxanthine	Tabatabaie et al., 1996	Purified from porcine heart. Possible sulfhydryl oxidation.
	Carbonylation	Bacteria	Hydrogen peroxide and menadione	Cabiscol et al., 2000	*E. coli.* E2 subunit carbonylated. Highly inactivated. Lipoic acid affected.
		Yeast	Hydrogen peroxide and menadione	Cabiscol et al., 2000	E2 subunit carbonylated. Highly inactivated. Lipoic acid affected
			Δ*sod1* mutant cells	O'Brien et al., 2004	E1 subunit carbonylated
		In vitro	Hydroxynonenal	Humphries and Szweda, 1998	Purified from porcine heart. HNE modified lipoic acid in both purified enzyme preparations and rat heart mitochondria. Inactivated
	Cys adducts	In vitro	Acrolein	Pocernich and Butterfield, 2003	Purified enzyme. Inactivated. Acrolein bind to lipoic acid.

(*continued overleaf*)

TABLE 14.1 (*continued*)

Name	Observed Modification	Origin	Condition/Treatment	References	Comments
PDC Pyruvate decarboxylase	Carbonylation	Yeast	Hydrogen peroxide and menadione	Cabiscol et al., 2000	Inactivation
			Hydrogen peroxide	Yoo and Regnier, 2004	Major target
			Copper	Shanmuganathan et al., 2004	
			Aging	Reverter-Branchat et al., 2004	Carbonylated in both replicative and chronological aging.
ADH Alcohol dehydrogenase	*S*-thiolation	Yeast	Hydrogen peroxide	Shenton and Grant, 2003	ADHI inactivated
	Carbonylation	Yeast	Copper	Shanmuganathan et al., 2004	
			Aging	Reverter-Branchat et al., 2004	ADHI carbonylated in replicative and chronological aging. Inactivated during stationary phase. ADH II not carbonylated
ADHE Alcohol dehydrogenase E	Inactivation	Bacteria	Shift from anaerobic to aerobic conditions	Membrillo-Hernández et al., 2000	*E. coli.* Inactivated by metal-catalyzed oxidation
	Carbonylation	Bacteria	Shift from anaerobic to aerobic conditions	Echave et al., 2002	*E. coli.* Highly carbonylated and inactivated
			Hydrogen peroxide	Tamarit et al., 1998	*E. coli.* Inactivated by metal-catalyzed oxidation
				Echave et al., 2003	*E. coli.* Inactivated by metal-catalyzed oxidation

Tricarboxylic acid cycle

Aconitase	Inactivation	Bacteria	Hyperoxia	Flint et al., 1993	*E. coli.* Release of one iron atom from the cluster
			Peroxynitrite	Keyer and Imlay, 1997	Fully inactivated in *E. coli.* Toxicity of iron released from aconitase
		Mammalian	Hyperoxia	Gardner et al., 1994	Human epithelial-like A549 cells and rat lung
			Superoxide generators	Gardner et al., 1995	Mammalian cells. Inactivation and iron-dependent reactivation
			Paraquat	Patel et al., 1996	Rat cortical cultures. Selective, reversible, and SOD-sensitive inactivation
			Methylphenyltetra-hydropyridine (MPTP)	Liang and Patel, 2004	Mice. PD model. Inactivation. Source of mitochondrial iron with a pathogenic role in PD
			Huntington's disease	Tabrizi et al., 1999	Human HD brain
			Supranuclear palsy	Albers et al., 2001	Human HD brain
		In vitro	Hydrogen peroxide, ferricyanide and persulfate	Verniquet et al., 1991	Purified aconitase from potato. Inactivation gives a paramagnetic $[3Fe–4S]^{1+}$ cluster
			Xanthine/xanthine oxidase	Vasquez-Vivar et al., 2000	Purified from bovine heart. Inactivation by superoxide increases hydroxyl radicals (·OH) through the Fenton reaction in mitochondria

(*continued overleaf*)

TABLE 14.1 (*continued*)

Name	Observed Modification	Origin	Condition/Treatment	References	Comments
			Aerobic conditions	Gardner and Fridovich, 1991	Cell extracts from *E. coli* mutants (Δ*sod*). Inactivated by superoxide. Oxidative attack on the prosthetic Fe–S cluster
	Carbonylation	Yeast	Hydrogen peroxide	Cabiscol et al., 2000	Inactivated. Carbonylated peptide included amino acids around Fe–S cluster
		Drosophila	Aging	Yan et al., 1997	Major carbonylated protein. Inactivation results in a reductive stress (NADH accumulation)
	Modification by RNS	In vitro	Nitric oxide	Gardner et al., 1997	Purified from *E. coli* and bovine heart. *S*-nitrosation of the [4Fe–4S] center
			Nitric oxide, peroxynitrite, and superoxide	Castro et al., 1994	Purified from pig heart. Peroxynitration. Inactivation reversed by incubation with thiols and ferrous iron.
		Mammalian	Aging	Kanski et al., 2005	Rat cardiac tissue. Nitrotyrosine-containing protein
α-KGDHC Alpha-ketoglutarate dehydrogenase complex	Inactivation	Mammalian	Ischemia-reperfusion	Sadek et al., 2002	Isolated mitochondria from rat heart

			Alzheimer's disease	Gibson et al., 1999	Human PD brain
			Parkinson's disease	Mizuno et al., 1994	Human PD brain
			Azide and *E. coli* lipopolysaccharide	Park et al., 1999	Microglia. Inactivation
	Carbonylation	Bacteria	Hydrogen peroxide and menadione	Cabiscol et al., 2000	*E. coli.* E2 subunit carbonylated. Highly inactivated. Lipoic acid affected
		Yeast	Hydrogen peroxide and menadione	Cabiscol et al., 2000	E2 subunit carbonylated. Highly inactivated. Lipoic acid affected
		In vitro	Hydroxynonenal	Humphries and Szweda, 1998	Purified from porcine heart. HNE modified lipoic acid in both purified enzyme preparations and rat heart mitochondria. Inactivated
	Modification by RNS	In vitro	Peroxynitrite	Park et al., 1999	Isolated enzyme. Inactivated. Nitrated Tyr residues from all three subunits
	Cys adducts	In vitro	Acrolein	Pocernich and Butterfield, 2003	Purified enzyme. Inactivated. Acrolein binds lipoic acid
			Electron transport chain and oxidative phosphorylation		
Complex I	Inactivation	Mammalian	Parkinson's disease	Mizuno et al., 1995	Human PD brain
				Parker et al., 1989	Platelet mitochondria purified from PD patients. Inactivation
				Schapira et al., 1990	Human PD brain. Inactivated in substantia nigra

(*continued overleaf*)

TABLE 14.1 (*continued*)

Name	Observed Modification	Origin	Condition/Treatment	References	Comments
			Glutathione depletion	Jha et al., 2000	Dopaminergic PC12 cells in which levels of GSH can be down-regulated. Selective and reversible inhibition as a result of thiol oxidation
	Carbonylation	Mammalian	*Trypanosoma cruzi* infection	Wen and Garg, 2004	Murine heart. Several subunits identified as carbonylated proteins
	Modification by RNS	Mammalian	Methylphenyltetra-hydropyridine	Ferrante et al., 1999	Mice. PD model
				Pennathur et al., 1999	Mice. PD model
			Peroxynitrite exposure	Murray et al., 2003	Enriched mitochondrial preparation from bovine heart. 3-Nitrotyrosine formation
	Trp modification	Mammalian	None	Taylor et al., 2003	Human heart mitochondria. *N*-formylkynurenine present in nine subunits
Complex II	Inactivation	Mammalian	Hydrogen peroxide	Long et al., 2004	Rat cardiomyocytes. Inactivated
			Peroxynitrite	Murray et al., 2003	Enriched mitochondrial preparation from bovine heart.
			Δ*sod2* null mice	Hinerfeld et al., 2004	Cortex. Loss of activity was mainly due to a reduction in the protein level

			3-Nitropropionic acid	Saulle et al., 2004	Mice. HD model. Striatal spiny neurons and cholinergic interneurons
			Huntington's disease	Seo et al., 2004	Human brain. Caudate-putamen from HD patients
				Tabrizi et al., 1999	Inactivation confined to striatum within the HD human brain. Not affected in fibroblast.
Complex III	Inactivation	Mammalian	Ischemia-reperfusion	Rouslin, 1983	Dog adult heart
				Veitch et al., 1992	Isolated rat heart
			Ischemia	Lesnefsky et al., 2001	Rabbit heart. Highly inhibited
		In vitro	Hematoporphyrin	Miki et al., 1991	Purified from beef heart mitochondria. Photosensitizer: generates singlet oxygen. Two His residues destroyed, presumably ligands for the Fe–S cluster of Rieske's protein
	Carbonylation	Yeast	Δ*sod1* and Δ*sod2* mutants	O'Brien et al., 2004	
		Mammalian	*E. coli* lipopolysaccharide	Barreiro et al., 2005	Rats treated with LPS. Diaphragm protein carbonylation measured.
			Trypanosoma cruzi infection	Wen and Garg, 2004	Murine heart. Several subunits carbonylated
	Trp modification	Mammalian	None	Taylor et al., 2003	Human heart mitochondria. *N*-formylkynurenine in two oxidized peptides

(*continued overleaf*)

TABLE 14.1 (*continued*)

Name	Observed Modification	Origin	Condition/Treatment	References	Comments
Complex IV	Inactivation	Mammalian	Alzheimer's disease	Cardoso et al., 2004	Platelet mitochondria isolated from AD patients
				Curti et al., 1997	Fibroblasts from AD patients
				Mutisya et al., 1994	Frontal, temporal, parietal, and occipital cortex from AD patients
				Parker et al., 1990	Platelet mitochondria isolated from AD patients
			Ischemia	Davey et al., 1997	Mitochondria isolated from the hippocampal area
				de la Torre et al., 1997	Brain rat. Inactivated in hippocampus and parietal cortex
				Lesnefsky et al., 2004	Isolated rabbit heart
	S-thiolation	Mammalian	Diamide	Fratelli et al., 2002	Glutathiolation. Human T lymphocytes
Complex V	*S*-thiolation	Mammalian	Ischemia-reperfusion	Eaton et al., 2003	Isolated rat kidneys. α-chain thiolated
	Carbonylation	Bacteria	Hydrogen peroxide, paraquat and menadione	Tamarit et al., 1998	*E. coli*. β-subunit carbonylated
		Yeast	Δ*sod2* mutants	O'Brien et al., 2004	
		Mammalian	*Trypanosoma cruzi* infection	Wen and Garg, 2004	Murine hearts. γ-chain
	Modification by RNS	Mammalian	Peroxynitrite	Morgan et al., 2002b	Mitochondria from bovine heart

			Alzheimer's disease	Shin et al., 2004	Transgenic mice (AD model). Tyrosine nitration (3-NT) in ATP5b
			Aging	Kanski et al., 2005	Rat heart. Protein nitration of F1-ATP synthase
	Trp modification	Mammalian	None	Taylor et al., 2003	Human heart mitochondria. *N*-formylkynurenine in several oxidized peptides, specially subunit δ
				Bienvenut et al., 2002	Bovine heart. *N*-formylkynurenine in subunit δ
			Antioxidant enzymes		
SOD Superoxide dismutase	Inactivation	Mammalian	Xanthine/xanthine oxidase	Salo et al., 1990	Red blood cells. Inactivation followed by degradation
		In vitro	Hydrogen peroxide	Hodgson and Fridovich, 1975	Purified from bovine erythrocytes. Modification of a histidine residue at the active center
	Modification by RNS	Mammalian	*E. coli* lipopolysaccharide	Aulak et al., 2001	Proteins from rat liver. Detected as anti-nitrotyrosine positive
		In vitro	Peroxynitrite	MacMillan-Crow et al., 1998	Purified enzyme. Human MnSOD expressed in *E. coli*. Nitration of Tyr34
	Glycation	In vitro	Glucose	Ookawara et al., 1992	Purified from human and horse erythrocytes. Release of Cu from the enzyme after glycation contribute to fragmentation by Fenton reaction

(*continued overleaf*)

TABLE 14.1 (*continued*)

Name	Observed Modification	Origin	Condition/Treatment	References	Comments
Catalase	Inactivation	In vitro	Superoxide Hydrogen peroxide	Kono and Fridovich, 1982 Lardinois et al., 1996	From bovine liver. Enzymes from bovine liver and *Aspergillus niger.*
	Carbonylation	In vitro	Singlet oxygen	Kim et al., 2001	From bovine liver. Photoactivation by visible light and methylene blue or rose bengal. Inactivation, aggregation and fragmentation of purified enzyme also occurs
		Yeast	Aging	Reverter-Branchat et al., 2004	Concomitant loss of activity. Not observed under calorie restriction
	Modification by RNS	Mammalian	*E. coli* lipopolysaccharide	Aulak et al., 2001	Proteins from rat liver. Detected as anti-nitrotyrosine positive
GPX Glutathione peroxidase	Carbonylation	Mammalian	Hydroxynonenal	Kinter and Roberts, 1996	Cultured fibroblasts. Depletion of GSH pools. Inactivation by modification of selenocysteine is predicted
	Glycation	In vitro	3-deoxyglucosone	Niwa and Tsukushi, 2001	Purified enzyme from human erythrocytes. Inactivation by formation of AGEs.
	Modification by RNS	Mammalian	*S*-nitro-*N*-acetyl-DL-penicillamine	Asahi et al., 1995	A nitric oxide generating compound. Inactivation. Lymphoma cell line.

		In vitro	*S*-nitro-*N*-acetyl-DL-penicillamine	Asahi et al., 1997	A nitric oxide generating compound. Bovine cellular. Inactivation by formation of selenenyl sulfide.
			Peroxynitrite	Padmaja et al., 1998	Bovine erythrocytes. Inactivation by formation of selenenic acid at the selenocysteine in the active center. Other species are not excluded
			Molecular chaperones		
DnaK	Carbonylation	Bacteria	Hydrogen peroxide, paraquat	Tamarit et al., 1998	*E. coli.* Highly carbonylated with hydrogen peroxide or paraquat but only slightly carbonylated with menadione
			Hydrogen peroxide	Dukan and Nyström, 1998	*E. coli*
HSP60	Carbonylation	Yeast	Hydrogen peroxide, menadione	Cabiscol et al., 2000	
			Hydrogen peroxide	Costa et al., 2002	
		Mammalian	Purpurin-18	Magi et al., 2004	Photodynamic therapy. Cultured fibroblasts
	Modification by RNS	In vitro	Peroxynitrite	Khor et al., 2004	Purified from *E. coli*. Inactivated dose-dependent. Formation of Tyr203 nitration. Negligible effects by nitric oxide.
	Cys modification	In vitro	Hypochlorous acid	Khor et al., 2004	Purified from *E. coli*. Cys converted to sulfonic acid. Conversion of oligomeric into monomeric form.

(*continued overleaf*)

TABLE 14.1 (*continued*)

Name	Observed Modification	Origin	Condition/Treatment	References	Comments
	Met modification	In vitro	Hypochlorous acid	Khor et al., 2004	Purified from *E. coli*. It contributes to inactivation by conversion to methionine sulfoxide. Partially reactivated by Met sulfoxide reductase.
ER chaperones	Carbonylation	Mammalian	Aging	Rabek et al., 2003	Mice liver. Main targets are BiP, calreticulin and protein disulfide isomerase
Alpha crystallin	Argpyrimidine	In vitro	Methylglyoxal	Derham and Harding, 2002	Purified from rabbit lenses. Decreased chaperone activity
				Nagaraj et al., 2003	Purified from human and bovine lenses. Enhanced chaperone activity.
			Cytoskeleton		
Actin	S-thiolation	Mammalian	Diamide	Lind et al., 2002	Cultured endothelial-like cells. Method based on specific reduction of mixed disulfides by glutaredoxin and reaction with *N*-ethylmaleimide-biotin
			Friedreich's ataxia (FRDA)	Pastore et al., 2003	Fibroblasts of patients with FRDA. Impaired microfilament organization.
			Diamide, hydrogen peroxide	Fratelli et al., 2002	Human T lymphocytes. Vimentin, profilin, tropomyosin, cofilin and myosin were also identified

	In vitro	Reduced glutathione	Dalle-Donne et al., 2003	Purified from rabbit skeletal muscle. Glutathionylation of Cys374 decreases the capacity to polymerize. This capacity is restored after dethiolation
Carbonylation	Mammalian	Ischemia-reperfusion	Powell et al., 2001	Observation made in post-ischemic rat hearts. Decreased contractile function
		Alzheimer's disease (AD)	Aksenov et al., 2001	Brain sections of AD patients. Increased protein oxidation in hippocampus and parahippocampal gyrus, superior and middle temporal gyri. Not in cerebellum
	In vitro	Hypochlorous acid	Dalle-Donne et al., 2001a	Purified from rabbit skeletal muscle. Aggregation of carbonylated actin
Modification by RNS	Mammalian	*E. coli* lipopolysaccharide	Aulak et al., 2001	Proteins from rat liver. Detected as anti-nitrotyrosine positive
Cys modification	Yeast		Haarer and Amberg, 2004	*S. cerevisiae* actin. Formation of disulfide bridges.
	In vitro	Sickle cell anemia	Shartava et al., 1995.	From red blood cells from patients with SCA. Formation of disulfide bridges.

(*continued overleaf*)

TABLE 14.1 (*continued*)

Name	Observed Modification	Origin	Condition/Treatment	References	Comments
				Bencsath et al., 1996	From red blood cells from patients with SCA. Formation of disulfide bridges
	Met Modification	In vitro	Chloramine T	Dalle-Donne et al., 2002	Purified from rabbit skeletal muscle. Formation of Methionine sulfoxide preferentially at Met44, Met47, and Met355 residues.
			Hydrogen peroxide	Milzani et al., 2000	Protein purified from rabbit skeletal muscle. Conversion of six Met residues to Methionine sulfoxide
Tubulin	Carbonylation	Mammalian	Alzheimer's disease	Aksenov et al., 2001	Sample from superior and middle temporal gyri. Low levels of carbonyl content compared to actin.
	Modification by RNS	In vitro	Peroxynitrite	Landino et al., 2002	From porcine brains. Detection of nitrated tyrosine residues and formation of inter- and intrasubunit disulfide bridges

different triose phosphates, glyceraldehyde 3-phosphate, an aldose, and dihydroxyacetone phosphate, a ketose.

Aldolases can be classified into two groups that have different catalytic mechanisms. Class I aldolases are found in vertebrates and form a Schiff-base intermediate between the C-2 carbonyl group of the substrate and the ε-amino group of a lysine residue. Class II aldolases, primary occurring in bacteria and yeast, do not form covalent enzyme-substrate intermediates but require bivalent transition-metal ions such as zinc (Marsh and Lebherz, 1992). Whereas class II contains a single isoform, class I includes three tissue-specific isoenzymes in vertebrate tissues: aldolase A predominates in skeletal muscle and red blood cells, aldolase B in liver, kidney, and small intestine, and aldolase C in neuronal tissues and smooth muscle (Horecker et al., 1972). Their sequence homology is not too high (only 68% from A to B, and 81% from A to C) and correlates with differences in their preference for substrate. Aldolase B is believed to preferentially perform the gluconeogenic reaction, Fru-1,6-P2 synthesis.

Cysteine SH groups are among the most easily oxidized residues in proteins, and their oxidation is described in more detail in Chapter 4. Like other glycolytic enzymes, aldolase A has been described to be *S*-thiolated after several oxidative stress situations. In *S. cerevisiae*, aldolase was identified as a target of *S*-thiolation after H_2O_2 treatment, but this modification had no effect on its activity (Shenton and Grant, 2003). It may indicate that the Cys targets of thiolation do not affect the active site of the enzyme. Inhibition of aldolase would prevent ribose 5-phosphate generated from the oxidative phase of the pentose phosphate pathway from being recycled back to glucose 6-phosphate. A common form of *S*-thiolation, glutathiolation (formation of mixed disulfides between glutathione and cysteines in proteins), was described in human peripheral blood mononuclear cells after diamide and H_2O_2 treatment (Fratelli et al., 2002) and also in ECV304 endothelial-like cells during constitutive metabolism, but the effect of such modification on enzyme activity was not evaluated.

Carbonylation has been repeatedly described in aldolase. In yeast, it was identified after Cu treatment (Shanmuganathan et al., 2004), and also during both chronological and replicative aging (Reverter-Branchat et al., 2004). Production of reactive oxygen species (ROS) is a common feature of treatment of cells with cytotoxic drugs. In this context, VP16, or etoposide, has been described to induce cell death mediated by ROS production. This drug promoted carbonylation of aldolase C in HL60 cells (England et al., 2004).

Protein tyrosine nitration increases in vivo as a result of oxidative stress. Diseases associated with protein nitration include cardiovascular, neurodegenerative, and inflammatory diseases, as well as diabetes. Protein nitration also occurs under certain conditions like ischemia-reperfusion and aging. All three isoforms of mammalian aldolase have been recently identified as being nitrated. Tyrosine nitration was described in lung epithelial cells and liver (Aulak et al., 2001) as well as in retina (Miyagi et al., 2002) under different inflammatory conditions. To reveal the consequences of tyrosine nitration, Koeck and collaborators (Koeck et al., 2004a) studied in vitro the impact of peroxynitrite on the

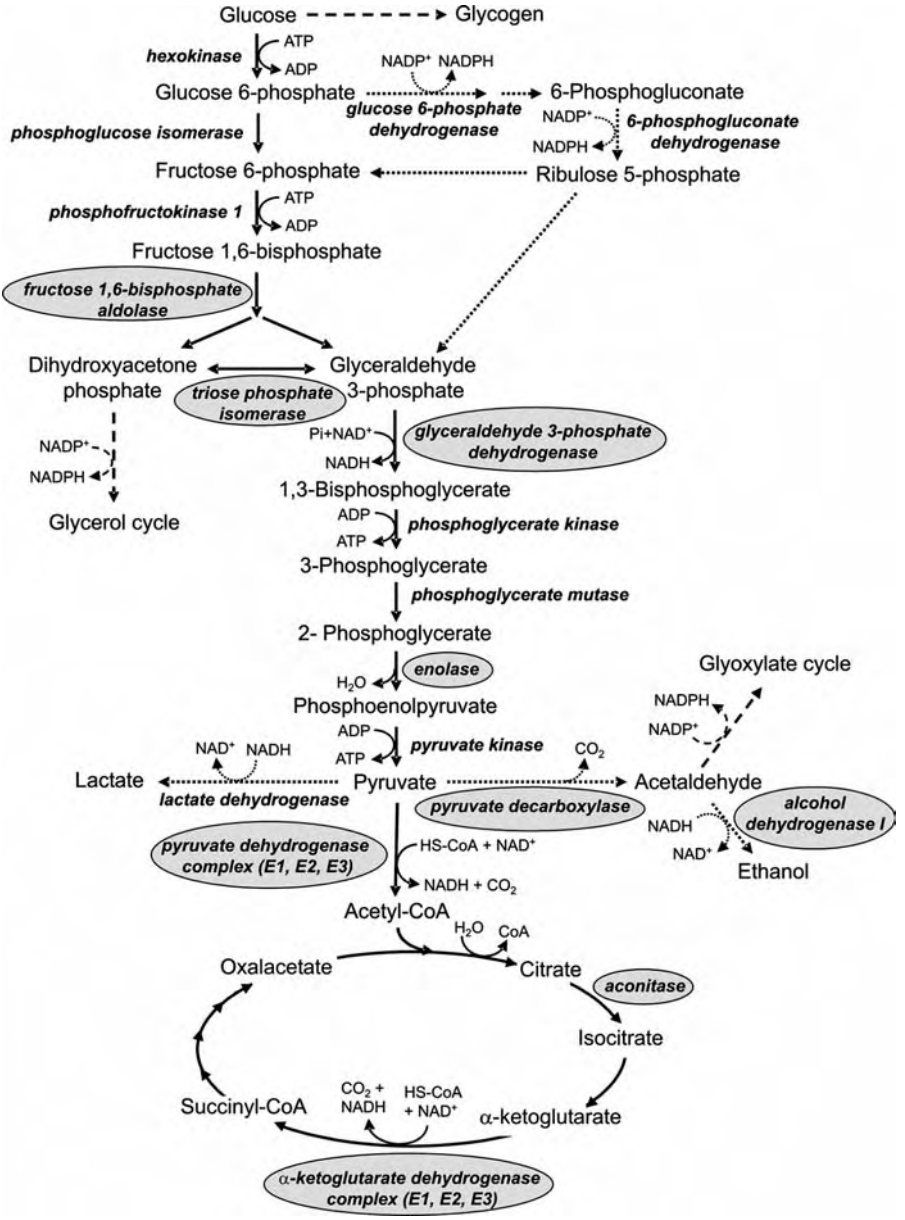


FIGURE 14.1 Scheme of glycolysis, fates of pyruvate, and TCA cycle with alternative routes like NADPH-producing steps of the pentose phosphate pathway, glycerol pathway, and glyoxylate pathway. The main oxidatively damaged proteins are circled.

glycolytic functions of aldolase A. They showed a peroxynitrite concentration-dependent decrease in aldolase activity with a concomitant increase in nitrotyrosine immunoreactivity (both V_{max} and K_m for fructose 1,6-bisphosphate were affected). Tyrosine 363, critical for its catalytic activity, was shown by tandem mass spectrometric analysis to be the most susceptible to nitration with a modification of Tyr342 occurring only after nitration of Tyr363. These tyrosine nitrations likely cause the decline in enzyme activity.

The physiological effects of aldolase A nitration are very complex, and depend on the nitrated residues in vivo and therefore on the local environment like the distance to sources of peroxynitrite and the presence of antioxidants. These conditions may be different from the conditions used for the in vitro nitration by peroxynitrite. Several mutations are associated with aldolase activities that are 4% to 11% of the basal levels. However, the content of ATP (93% of controls) is not significantly altered (Kreuder et al., 1996). Thus, to cause any significant metabolic impact in the case where aldolase A is the only modified protein in a tissue, a nitration at least at Tyr363 and Tyr342 in most of its population would have to occur. This is very improbable regardless of the nitration mechanisms and conditions in vivo. The situation likely changes when enzymes downstream of aldolase become nitrated simultaneously.

14.2.2 Triose Phosphate Isomerase

Only one of the two triose phosphates formed by aldolase—glyceraldehyde 3-phosphate—can be directly degraded in the subsequent steps of glycolysis. The other product, dihydroxyacetone phosphate, is rapidly and reversibly converted to glyceraldehyde 3-phosphate by the dimeric triose phosphate isomerase (TPI).

Yeast (like mammals) contains a single TPI (Tpi1p) that has been described to be both *S*-thiolated (Shenton and Grant, 2003) and carbonylated (Costa et al., 2002) after treatment of yeast cells with H_2O_2. Curiously neither *S*-thiolation nor carbonylation results in enzyme inactivation at H_2O_2 concentrations that inactivate GAPDH as much as 80% to 90%. Our group identified TPI as an abundant protein highly resistant to carbonylation under yeast aging (Reverter-Branchat et al., 2004). In yeast, protection of Tpi1p may allow the conversion of glyceraldehyde-3-phosphate, which accumulates as a result of GAPDH inactivation, into dihydroxyacetone phosphate. The increase in dihydroxyacetone phosphate levels favors the production of NADPH by the glycerol cycle, which is also induced by H_2O_2 (Lee et al., 1999).

Mammalian TPI has also been described to be carbonylated during etopside-induced apoptosis of HL60 cells (England et al., 2004). An oxidative stress results upon VP16 treatment, but although glucose utilization decreases in these cells, there was no experimental evidence indicating inactivation of TPI. A number of other glycolytic enzymes were also carbonylated, which can be the cause of dramatic glycolysis impairment. By contrast, the pentose phosphate pathway activity was not decreased.

S-Thiolation of TPI (like GAPDH) has been described in the pathophysiological oxidant stress associated with ischemia and reperfusion (Eaton et al., 2002)

and also in human T lymphocytes after diamide (a strong thiol-selective oxidant) treatment, but not upon H_2O_2 treatment (Fratelli et al., 2002).

Nitration of tyrosine residues associated with several neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), and Alzheimer's disease (AD) represents a pathological event. Brain tissue from AD patients showed increased levels of nitrated proteins and reduced glucose metabolism. Several glycolytic enzymes are nitrated and TPI is one of them (Castegna et al., 2003), but no enzyme activity assays have been performed.

Although several oxidative modifications of TPI have been clearly described, to our knowledge, their effects on mammalian TPI activity are just a matter of speculation. We believe that TPI, although more or less sensitive to amino acid modifications, is highly resistant to inactivation. This assumption is based on the experimental results published in yeast, where enzyme activity was not affected by *S*-thiolation (Shenton and Grant, 2003) or carbonylation (Costa et al., 2002). Nevertheless, it is interesting to note that in hereditary hemochromatosis, a disease characterized by a systemic deficiency of TPI, patients suffer from wide range cardiac pathologies (Guertl et al., 2000).

14.2.3 Glyceraldehyde 3-Phosphate Dehydrogenase

This enzyme catalyzes the reversible oxidation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate. Glyceraldehyde 3-phosphate is covalently bound to the enzyme during the reaction. The aldehyde group of glyceraldehyde 3-phosphate reacts with the –SH group of an essential cysteine residue in the active site. GAPDH, active as a homotetramer, is one of the most abundant soluble proteins and probably the most extensively studied enzyme modified as a result of different oxidative stress insults.

In *S. cerevisiae*, GAPDH is encoded by three unlinked genes designated *TDH1, TDH2*, and *TDH3*. None of the *TDH* genes is individually essential for cell viability, but a functional copy of either *TDH2* or *TDH3* is required because a *tdh2 tdh3* double mutant is nonviable. These results implied that Tdh1 may perform a function distinct from that of Tdh2 or Tdh3, since Tdh1 alone is unable to support growth. Tdh1 constitutes roughly 3% of the total GAPDH protein in exponential cells, but its expression is induced when cells enter stationary phase, in heat-shocked cells or by a NADH-reductive stress. Grant and colleagues (1999) described enzyme inactivation of both Tdh2 and Tdh3 isoenzymes following culture exposure to H_2O_2. Nevertheless, despite a high degree of sequence homology (98%), only Tdh3 activity was restored after a 2 hour recovery period, indicating that inactivation of Tdh3 was reversible. The authors found that protein modification is tightly regulated. A model was suggested where both enzymes are required for survival during conditions of oxidative stress, playing complementary roles depending on their ability to undergo *S*-thiolation. *S*-thiolation, and hence protection of Tdh3 against irreversible oxidation, was required for survival following a challenge with high levels of oxidants, which would irreversibly oxidize Tdh2. However, the nonthiolated Tdh2 is also necessary during a prolonged exposure

to low levels of oxidants. Under these conditions the continued *S*-thiolation of Tdh3 will negatively affect cell growth, but some Tdh2 will avoid oxidation to provide the necessary GAPDH activity for growth.

The model agrees with the fact that V_{max} of Tdh3 is approximately two- to threefold lower than that of Tdh2 (McAlister and Holland, 1985). Additional experimental observation showed that when H_2O_2 is added to cell-free extracts, both Thd2 and Tdh3 are *S*-thiolated (Shenton et al., 2002). This indicates that cellular factors are likely to be responsible for the difference in GAPDH *S*-thiolation observed in vivo rather than structural differences between isoenzymes.

Like most of the thiolated proteins, Tdh3 was detected to form a reaction with glutathione (GSH), with a 90% decrease in activity (Shenton and Grant, 2003). Although it was not determined, the highly reactive Cys149 present in the active site is the most probable candidate for the reaction. Interestingly the dipeptide γ-Glu-Cys may be able to replace GSH in the *S*-thiolation reaction (Grant et al., 1999). In mammalian samples, GAPDH has also been identified as a target of *S*-thiolation in human endothelial cells (Schuppe-Koistinen et al., 1994), human blood monocytes (Ravichandran et al., 1994), and during reperfusion of ischemic rat heart (Eaton et al., 2002).

GADPH has been described as a carbonylated protein under several oxidative stress conditions and organisms. In yeast, both Tdh2 and Thd3 appeared as carbonylated proteins after Cu treatment (Shanmuganathan et al., 2004), menadione or H_2O_2 treatment (Cabiscol et al., 2000; Costa et al., 2002), paraquat treatment (O'Brien et al., 2004), and also in replicative and chronologically aged cells (Reverter-Branchat et al., 2004). When measured, carbonylation correlates with loss of activity, in a range that varies from 50% to 90% inactivation. In rat diaphragm, GADPH was carbonylated in septic animals injected with *E. coli* lipopolysaccharide, known to induce an inflammatory and oxidative stress situation (Barreiro et al., 2005).

It was reported more than 10 years ago that GADPH reacts with compounds such as 4-hydroxynonenal (HNE), introducing a secondary carbonyl into the protein (Uchida and Stadtman, 1993). HNE is a well-known product of membrane lipid peroxidation, with cytotoxic action due to its facile reactivity with proteins (Uchida and Stadtman, 1992). When purified rabbit muscle enzyme was treated with HNE, the loss of enzyme activity was associated with the loss of free sulfhydryl groups. Histidine and lysine residues were also modified. The HNE-dependent loss of amino acid residues was accompanied by the generation of protein-linked carbonyl derivatives—a Michael addition-type reaction—in which the carbonyl function of HNE would be preserved (Uchida and Stadtman, 1993). Recently five HNE adducts at His164, Cys244, Cys281, His327, and Lys331 were identified by mass spectrometry and revealed that both His164 and Cys281 (outside the active center) were rapidly modified (Ishii et al., 2003). The authors speculated that HNE first reacts with Cys281, rendering His164 also susceptible to electrophilic attack by HNE. Cys149, present at the catalytic center, was not observed as modified by HNE, suggesting that the HNE inactivation of

GAPDH is not due to the modification of the catalytic center but to the selective modification of amino acids primarily located at the surface of the molecule.

Nitration of GAPDH has been described both in yeast and cultured rat astrocytes after treatment with peroxynitrite, where the activity was strongly inhibited (Buchczyk et al., 2000). In addition enzyme nitration has been found in rat heart as a consequence of biological aging (Kanski et al., 2005), but the modified amino acid was not determined.

Nitric oxide radicals can lead to GAPDH inactivation also by favoring the modification of the active cysteine residue by ADP-ribosylation (Dimmeler et al., 1992; Kots et al., 1992; Zhang and Snyder, 1992). ADP-ribosylation is a reversible modification that can be catalyzed by enzymes, like poly(ADP-ribosyl)polymerase and a number of mono (ADP-ribosyl) transferases, by bacterial toxins, and also stimulated by NO, which add the ADP-ribose moiety of NAD^+ onto acceptor proteins. It has been demonstrated that ADP-ribosylation of GAPDH after H_2O_2 treatment on U937 cells (human promonocytes derived from histiocytic lymphoma) protects these cells from apoptosis (Colussi et al., 2000). Although modification of Cys149 was proposed, to our knowledge, proper identification has not been performed.

Modification of GAPDH by NADH, in contrast to NAD^+, proceeds in the presence of low molecular weight thiols, is independent of NO, and is mediated by superoxide (Rivera-Nieves et al., 1999). The authors demonstrated that in the presence of superoxide, NADH establishes a linkage to Cys149 that appears to involve the nicotinamide moiety and is chemically distinct from the ADP-ribosyl linkages.

In summary, it seems probable that the reactive Cys149 thiol moiety may be involved in multiple types of reaction as a result of oxidative stress, resulting in a decrease in enzyme activity. Reversible cysteine modifications, including *S*-thiolation, can be regarded as a protective mechanism by which reactive protein thiols are protected from irreversible or terminal oxidation by the formation of a mixed disulfide with a low molecular weight thiol. Several authors pointed out the protective effect of arresting the glycolytic flow under such situations. However, GAPDH displays a number of diverse activities unrelated to its glycolytic function (see Sirover, 1999, for a review). These include its role in membrane fusion, microtubule bundling, phosphotransferase activity, nuclear RNA export, DNA replication, and DNA repair. These new activities may be related to the subcellular localization and oligomeric structure of GAPDH in vivo. Furthermore other investigations suggest that GAPDH is involved in apoptosis, age-related neurodegenerative disease, prostate cancer, and viral pathogenesis (reviewed in Sirover, 1999). How oxidative modification affects these activities and cell physiology will have to be evaluated in the future.

14.2.4 Enolase

Enolase promotes reversible removal of a molecule of water from 2-phosphoglycerate to yield phosphoenolpyruvate, a compound with high phosphoryl group

transfer potential. Yeast has two enolases acting as a homodimer, *ENO1* (also named *ENOA* and *HSP48*) and *ENO2* (also named *ENOB*). Enolase I was found to be repressed and enolase II simultaneously induced by glucose. Enolase catalyzes the first common step of glycolysis and gluconeogenesis. Gluconeogenic enolase I shows substrate inhibition for 2-phosphoglycerate (glycolytic substrate) and glycolytic enolase II is substrate-inhibited by phosphoenolpyruvate (gluconeogenic substrate). Mammalian enolase is composed of 3 isoenzyme subunits, alpha (also named EnoA and Eno1), beta (EnoB and Eno3), and gamma (EnoG and Eno2), which can form homodimers or heterodimers, the latter being cell-type and development specific. The α/α homodimer is expressed in embryo and in most adult tissues. The α/β heterodimer and the β/β homodimer are found in striated muscle, and the α/γ heterodimer and the γ/γ homodimer in neurons (Ueta et al., 2004).

Enolase is the fourth glycolytic enzyme that has been extensively described to be modified by oxidative stress. *S*-thiolation of enolase II in yeast after H_2O_2 treatment decreased enolase activity by at least 70% (Shenton and Grant, 2003). In human blood mononuclear cells, enolase A was glutathionylated—and inhibited—after cell exposure to diamide or H_2O_2 (Fratelli et al., 2002). Enolase contains six potential target sites, but identification of thiolated sites has not yet been performed.

More information exists on carbonylation of enolase. In *E. coli*, enolase is one of the main carbonylated proteins after H_2O_2 treatment (0.92 nmol carbonyl/mg protein) (Tamarit et al., 1998). In *S. cerevisiae*, Eno2p was carbonylated after several stress situations, like menadione treatment, where a 50% inactivation was observed (Cabiscol et al., 2000), Cu^{2+} treatment (Shanmuganathan et al., 2004), and also in cells lacking CuZn SOD (*sod1* mutants) (O'Brien et al., 2004). During the study of yeast aging, enolase 2 appeared as a highly carbonylated protein both in replicative and chronologically aged cells (Reverter-Branchat et al., 2004). Under chronological aging an interesting observation was the different oxidative modification of enolase isoenzymes. Although enolase 2 involved in glycolytic flux appeared to be one of the first oxidized proteins, enolase 1 expressed in the postdiauxic shift and involved in gluconeogenesis showed a marked resistance to oxidation. Whether this means an evolutionary adaptation to stressing conditions in yeast cells as they age is still unknown.

Peroxide production was detected after VP16 treatment in HL-60 cells, resulting in carbonylation of several proteins, including α-enolase (England et al., 2004). Along with other glycolytic enzymes also carbonylated, the authors demonstrated a dramatic decrease in glucose utilization. By contrast, the pentose phosphate pathway activity was not decreased.

In AD, like in several neurological diseases, oxidative stress has been implicated as an important event in the progression of the pathology, and protein carbonyl levels are increased in several regions of the brain (Markesbery, 1997; Butterfield and Kanski, 2001). Alpha enolase was described to be carbonylated both in AD human brain (Castegna et al., 2002) and in transgenic (Tg2576) mice, used as a model for studying AD (Shin et al., 2004). It is interesting that

α/γ and γ/γ isoforms are the predominant dimers in the brain, representing the neuron-specific enolases. The γ-type enolase subunit is mainly located in neurons, while α-type subunits are in glial cells (Deloulme et al., 1997). The reduced glucose metabolism observed in AD brain suggests a possible relationship between glycolytic impairment and AD hypometabolism.

In addition to α-enolase carbonylation, Shin and colleagues (2004) also identified the same enzyme as modified by nitration in transgenic AD model mice. Nitration of α-enolase was also confirmed in AD human brain (Castegna et al., 2003). Moreover α-enolase was identified as a 3-nitrotyrosine containing protein in heart of aged rats (Kanski et al., 2005).

Enolase was formerly described as a heat shock protein in yeast because it shares some degree of homology with DnaK (an *E. coli* Hsp70p). DnaK has been found associated to RNase E in a multicomponent ribonucleolytic complex (RNA degradosome) essential for RNA processing and degradation (Py et al., 1996). RNase E, a major endonuclease, is associated with polynucleotide phosphorylase (PNPase), RhIB helicase, and enolase. The role of enolase within the degradosome has been totally mysterious. Recently Morita and collaborators (2004) in an elegant work concluded that enolase plays a role in the regulation of ptsG mRNA (encoding the major glucose transporter $IICB^{glc}$) stability in response to metabolic stress. Increased degradation of 16S ribosomal RNA has been described to occur in mitochondria in response to H_2O_2 exposure (Borras et al., 2003). In this situation, proteins involved in the RNA degradosome, such as enolase, would be oxidized preferentially as a consequence of the reactive oxygen species generated by metal ions associated with RNA.

EnoA is a multifunctional enzyme that, besides its role in glycolysis, plays a part in various processes such as growth control, hypoxia tolerance, and allergic responses. It may also function in the intravascular and pericellular fibrinolytic system due to its ability to act as a receptor and activator of plasminogen on the cell surface of several cell types such as leukocytes and neurons (Pancholi, 2001). How oxidative modification of enolase may affect all this functions remains unanswered.

It seems clear that, under an oxidative stress situation, inactivation—both reversible and irreversible—of selected glycolytic enzymes plays a key role in glucose metabolization, blocking glycolysis. This would be an active cell reaction programmed for self-defense, allowing glucose to be derived through the pentose phosphate pathway, known to be resistant to oxidative stress. The NADPH synthesized will be used for antioxidant defense systems, finally increasing cell viability.

14.3 PYRUVATE METABOLISM

Pyruvate represents an important junction in carbohydrate metabolism (Fig. 14.1), and some of the enzymes responsible for its metabolization have been described to be sensitive to oxidative stress. Under aerobic conditions pyruvate can be

oxidized to form acetyl-CoA by the pyruvate dehydrogenase complex (PDHC). Acetyl-CoA enters the citric acid cycle and is oxidized to CO_2 and H_2O. NADH produced in glycolysis is reoxidized to NAD^+ by passage of its electrons to O_2 in mitochondrial respiration. However, under hypoxic conditions such as very active muscle, in submerged plant parts, or in lactic acid bacteria, the NADH generated cannot be reoxidized by O_2. NAD^+ is regenerated from NADH by reduction of pyruvate to lactate, a reaction catalyzed by lactate dehydrogenase (LDH). Some tissues and cell types (e.g., erythrocytes, lacking mitochondria) produce lactate even under aerobic conditions. Although PDHC is highly susceptible to oxidation, as will be described in this section, lactate dehydrogenase is highly resistant to inactivation under oxidative stress, as reported by several labs (Bogaert et al., 1994; Tabatabaie et al., 1996; Zhang and Snyder, 1992; Morgan et al., 2002a).

In yeast and other microorganisms, when glucose is in excess, this sugar is fermented to ethanol and CO_2 in a two-step process. Enzymes from the citric acid cycle and respiratory chain are repressed. In the first step, pyruvate is decarboxylated in an irreversible reaction catalyzed by pyruvate decarboxylase (PDC). In the second step, acetaldehyde is reduced to ethanol through the action of alcohol dehydrogenase (ADH), and NADH is oxidized. Ethanol and CO_2 are thus the end products of alcohol fermentation. PDC is absent in vertebrates and other microorganisms that carry out lactic acid fermentation. ADH is present in many organisms that metabolize ethanol, including humans. Both PDC and ADH have been described to be modified under several oxidative stress situations (Cabiscol et al., 2000; Reverter-Branchat et al., 2004; Shanmuganathan et al., 2004; Shenton and Grant, 2003).

Lactate and ethanol are common products of microbial fermentations, but they are by no means the only possible ones. *E. coli*, the most extensively studied prokaryote organism, carries out what is called a mixed-acid fermentation in the absence of oxygen. The fermentation products include ethanol, formate, acetate, glycerol, D-lactate, succinate, CO_2, and H_2. One of the major products is ethanol, which is synthesized from acetyl-CoA by two consecutive Fe^{2+} and NADH-dependent reductions, catalyzed by alcohol dehydrogenase E (AdhE). AdhE is very sensitive to oxidative stress (Tamarit et al., 1998; Membrillo-Hernández et al., 2000; Echave et al., 2003) and will be described in this section.

14.3.1 Pyruvate Dehydrogenase Complex

PDHC is a cluster of three enzymes located in the mitochondrial matrix of eukaryotic cells and in the cytosol of prokaryotes. The complex is greater than 7 million Daltons, but the number of copies of each enzyme, and therefore the size of the complex, varies among organisms. PDHC has remarkably similarities in protein structure, cofactor requirements, and reaction mechanism to two other important enzyme complexes: α-ketoglutarate dehydrogenase (α-KGDHC) and the branched-chain α-ketoacid dehydrogenase of the oxidative pathways of several amino acids.

PDHC catalyzes the oxidative decarboxylation of pyruvate to form acetyl-CoA, NADH, and CO_2. This reaction constitutes the bridge between anaerobic

and aerobic energy metabolism. The PDHC is a multisubunit complex composed of three major subunits: E1, E2, and E3. The E1 (pyruvate dehydrogenase) subunit requires thiamine pyrophosphate as a cofactor and is a tetramer that contains two α and two β subunits. The E2 (dihydrolipoyl transacetylase) subunit requires lipoic acid and coenzyme A (CoA). The E3 (dihydrolipoyl dehydrogenase) subunit has FAD covalently bound and requires NAD^+ as a coenzyme. Activity of this complex is regulated by a multitude of factors, including phosphorylation (PDHC is inactivated by PDH kinase, whereas PDH phosphatase activates the enzyme complex), pyruvate, Ca^{2+} and Mg^{2+} concentrations, and ratios of ATP/ADP, acetyl-CoA/CoA, and NADH/NAD^+.

It was reported more than two decades ago that cerebral glucose metabolism and oxygen utilization are impaired markedly during postischemic reperfusion (Pulsinelly et al., 1982). Thereafter decreased PDHC activity after ischemia was extensively described and proposed as a possible explanation for reduced cerebral glucose utilization in these conditions (Zaidan and Sims, 1997; Fukuchi et al., 1998; Schoder et al., 1998). The decrease in enzyme activity is reperfusion dependent, as no change in enzyme activity is detected after ischemia alone, so a site-specific protein oxidation is the more likely explanation (Martin et al., 2005).

PDHC is also affected in other neurological disorders associated with oxidative stress, like AD (Sheu et al., 1985). Brain lipid peroxidation and decreased brain glucose utilization are characteristic of AD. It has been published that acrolein, a by-product of lipid peroxidation that accumulates within the brain during AD, decreases PDHC and α-KGDHC activities (Pocernich and Butterfield, 2003). By HPLC-MS techniques the authors found that acrolein binds lipoic acid, a component of E2 subunit of both complexes, which may explain the loss of activity. Their inactivation may be partially responsible for the dysfunction of mitochondria and loss of energy found in AD brain and potentially contributing to the neurodegeneration.

The reactivity of sulfhydryl groups of lipoic acid bound to PDHC and α-KGDHC was well established in studies in vitro (Humphries and Szweda, 1998). This reactivity makes them a target for oxidative stress, as it was described both in *S. cerevisiae* and *E. coli* (Cabiscol et al., 2000). After H_2O_2 and menadione treatment, E2 subunits of PDHC and α-KGDHC were inactivated and identified as heavily carbonylated proteins. Increased carbonylation paralleled with the modification of their cofactor. One possible explanation for the decline in immunodetection of lipoic acid and the increased detection of carbonyl groups is the formation of a Michael adduct with HNE. We demonstrated, by mass spectrometry, that lipoic acid was modified after oxidative stress, but the exact nature of modification still remains unknown (unpublished results).

In addition to secondary modification of cofactors, like binding to lipid peroxidation products, the protein subunits may also be direct targets of oxidative stress. Purified porcine heart PDHC was highly sensitive to inactivation when exposed to a hydroxyl radical generating system or a superoxide radical generating system (Martin et al., 2005; Tabatabaie et al., 1996). Sulfhydryl protective reagents,

like dithiothreitol (DTT) and GSH, prevented enzyme inactivation, which implicates sulfhydryl oxidation. However, incubation of inactivated PDHC with DTT could not reactivate the enzyme, which points to the irreversibility of the inactivation process. The activity of lactate dehydrogenase, an enzyme resistant to inactivation by ischemia reperfusion, was not affected by this system, and consequently levels of lactate are used as an index of stress damage.

Indirect evidence suggests that loss of PDHC enzyme activity after cardiac arrest and resuscitation contributes to the prolonged elevation of brain lactate levels. Administration of lipoic acid is neuroprotective, but its mechanism of action is ascribed to either direct antioxidant activity or regulation of gene transcription (Clark et al., 2001). Also post-ischemic infusion of acetyl-L-carnitine is neuroprotective (it reduces brain lactate/pyruvate ratio and improves neurological outcome), possibly by providing alternative oxidative fuel in the presence of reduced PDHC activity (Martin et al., 2005). Other authors demonstrated neuroprotection by administration of ketone bodies, such as β-hydroxybutyrate in animal models of ischemia, PD, and AD (Kashiwaya et al., 2000; Ottani et al., 2003).

Although interventions that compensate for a loss of PDHC activity may prove clinically effective, an alternative approach is to inhibit those mechanisms responsible for damage to the enzyme complex. If this is true, antioxidants should prove useful. However, although preclinical studies with antioxidants have demonstrated neuroprotection, results from the few clinical trials testing antioxidants are disappointing (van der Worp et al., 2002). An alternative approach to minimizing oxidative stress during post-ischemic reperfusion is to limit the delivery of oxygen to the brain. The few reported comparisons strongly suggest that hyperoxic resuscitation is detrimental (Zwemer et al., 1994). Since other enzymes besides PDHC are affected by oxidative stress, it seems more promising to try to reduce radical concentration, either by decreasing their generation or by eliminating them after formation.

14.3.2 Pyruvate Decarboxylase

PDC is a thiamine pyrophosphate-dependent enzyme implicated in the nonoxidative conversion of pyruvate to acetaldehyde and carbon dioxide during alcoholic fermentation. Most of the produced acetaldehyde is subsequently reduced to ethanol, but some is required for cytosolic acetyl-CoA production for biosynthetic pathways. The enzyme is also one out of five 2-oxo acid decarboxylases (PDC1, PDC5, PDC6, ARO10, and THI3) able to decarboxylate more complex 2-oxo acids (alpha-ketoacids) than pyruvate, which seem mainly involved in amino acid catabolism.

PDC works as a cytoplasmic homotetramer and binds one Mg^{2+} ion and one thiamine pyrophosphate per subunit, being allosterically activated by substrate. Protein expression is strongly induced by high concentrations of fermentable carbon sources and under anaerobic growth conditions and is repressed by ethanol. In yeast, there are three PDC, isoenzyme 1 (*PDC1*) is by far the most abundant,

isoenzyme 2 (*PDC5*) is present at very low amount under fermentative conditions, and isoenzyme 3 (*PDC6*) is a minor isoform.

In yeast, PDC was one of the main carbonylated protein after H_2O_2 or menadione treatment, which concomitantly resulted in enzyme inactivation (60% and 80% decrease after H_2O_2 and menadione treatment, respectively) (Cabiscol et al., 2000). Additionally a new method to detect carbonylated proteins in 2D gel electrophoresis using avidin-fluorescein affinity staining also identified PDC1 as a major target following oxidative stress with H_2O_2 (Yoo and Regnier, 2004). Similar results were obtained after Cu^{2+} treatment using the more traditional DNPH derivatization (Shanmuganathan et al., 2004). In yeast aging, PDC also appeared carbonylated both in replicative and chronologically aged cells (Reverter-Branchat et al., 2004). To our knowledge, the exact nature of modification is unknown. The role of PDC oxidation decreasing glucose consumption via glycolysis will be discussed next, since the second step of alcoholic fermentation is also a target for oxidation.

14.3.3 Alcohol Dehydrogenase

The oxidoreductases catalyzing the interconversion of alcohol, aldehydes, and ketones can be divided into three major categories: (1) NAD(P)-dependent alcohol dehydrogenases (ADHs), (2) NAD(P)-independent ADHs, and (3) FAD-dependent alcohol oxidases. The first category can, in turn, be divided into three groups that have a possible common ancestor based on the coenzyme-binding site: group I medium-chain zinc-dependent dehydrogenases, group II short-chain zinc-independent dehydrogenases, and group III "iron-activated" dehydrogenases.

Yeast alcohol dehydrogenase is a member of a large family of zinc-containing ADHs divided in four divergent groups: vertebrates, plants, eukaryotic microorganisms, and prokaryotic bacteria. *S. cerevisiae*, a member of the third group, has three isoenzymes: ADH I, ADH II, and ADH III (see Leskovak et al., 2002, for a review). ADH I is the constitutive form and the predominant isoenzyme under glucose fermentation. ADH II is another cytoplasmic form, which is repressed by glucose, and its transcription increases dramatically when cells are shifted from glucose to ethanol growth conditions. Thus the physiologic role of ADH II is the conversion of ethanol to acetaldehyde (the opposite way of ADH I). ADH III is found in the mitochondria. Another ADH has been described, ADH IV, included by homology in the iron-containing ADH family. The function of ADH IV remains unknown because in yeast *ADH4* is not expressed under laboratory conditions, except upon insertion of a Ty element at the *ADH4* locus or amplification of *ADH4*.

After oxidative stress (H_2O_2 treatment), ADH I was inhibited by approximately 70% in a reversible way. Enzyme activity was recovered when cells were transferred into fresh media and activity was restored to approximately 60% of the pre-stress activity levels (Shenton and Grant, 2003). The same article demonstrates that ADH I was *S*-thiolated under this situation, but analysis of the location of the sulfhydryl group attacked was not performed.

Carbonylation of ADH I was observed after Cu treatment (Shanmuganathan et al., 2004) and also in yeast aging, where enzyme activity declined progressively up to 75% activity after 60 days culture (stationary phase) (Reverter-Branchat et al., 2004). It is interesting that even though during diauxic phase ADH II is strongly induced, it never appeared as a carbonylated protein.

As mentioned earlier, inactivation of glycolytic enzymes leads to the impairment of glucose utilization, which is proposed to be a protection system under oxidative stress. Following this idea, reduced glycolysis has proven to increase longevity in several organisms (Guarente and Kenyon, 2000) and correlates with the enhancement of the gluconeogenic and energy storage pathways observed during replicative aging in yeast (Lin et al., 2001).

14.3.4 Alcohol Dehydrogenase E

ADHE is a multifunctional protein that catalyzes the sequential reduction of acetyl-CoA to acetaldehyde and then to ethanol under fermentative conditions in *E. coli*. The NH_2-terminal region of the ADHE protein is highly homologous to aldehyde:NAD^+ oxidoreductases, whereas the COOH-terminal region is homologous to a family of Fe^{2+}-dependent ethanol:NAD^+ oxidoreductases. This fusion protein also functions as a pyruvate formate lyase deactivase because it converts the active radical form of pyruvate-formate lyase into the nonradical form (Kessler et al., 1991). ADHE belongs to the group III Fe-activated dehydrogenases and shares a high degree of structural homology with other microbial ADHs (Reid and Fewson, 1994).

ADHE is abundantly synthesized (3×10^4 copies per cell) during anaerobic growth. When shifted to aerobic conditions, transcription of the *adhE* gene is reduced and maintained within 10% of the range found under anaerobiosis. In fact, under aerobic conditions, ADHE has no assigned function, accounts for about 1% of total protein, and is inactivated by metal-catalyzed oxidation (Membrillo-Hernandez et al., 2000; Echave et al., 2002). This is the main reason why *E. coli* cannot grow on ethanol as the sole carbon and energy source. In this disabling process the amino acid chains of ADHE are thought to be covalently attacked by the highly reactive hydroxyl radicals locally generated by the Fe^{2+} bound to the active site of the alcohol:NAD^+ oxidoreductase domain. Not surprisingly ADHE has been identified as one of the major carbonylated proteins when *E. coli* cells were submitted to H_2O_2 stress (Tamarit et al., 1998; Echave et al., 2003).

The case of the *adhE* gene product and its role in general fermentation is analogous to that of the *fucO* gene product, propanediol oxidoreductase (POR). POR catalyzes the reduction of L-lactaldehyde to L-1,2-propanediol in the fermentation of L-fucose. Like ADHE, POR belongs to the family of iron-activated dehydrogenases, and is inactivated by metal-catalyzed oxidation under aerobic conditions and H_2O_2 treatment, resulting in carbonyl formation (Cabiscol et al., 1994). POR is not a fusion protein and presents homology only with the ethanol dehydrogenase domain of ADHE, where the iron-binding site is located.

Based on this homology and studies performed with POR and another iron-activated dehydrogenase, ADH II from *Zymomonas mobilis*, our group demonstrated that the amino acid attacked by hydroxyl radical made in this cage system is most probably His277 (position 735 in ADHE), (Cabiscol et al., 1994; Lu et al., 1998; Tamarit et al., 1997). The crystal structure of POR has been recently solved and demonstrated that the iron ion is tetrahedrally coordinated by three histidine (His200, His263, and His277) and an aspartate (Asp196) residues (Montella et al., 2005). It seems possible that oxidation of His277 results in a carbonyl group either by a primary modification (formation of 2-oxo-histidine) (Uchida, 2003) or a secondary modification (lipid or carbohydrate modification) (Berlett and Stadtman, 1997).

ADH E	C^{720}**H**SMAHKLGSQFHIP**H**GLANA
POR	V^{262}**H**GMAHPLGAFYNTP**H**GVANA
ADH II	V^{262}**H**AMAHQLGGYYNLP**H**GVCNA
Bold: iron-binding	Underlined: oxidized His residue

Nevertheless, its function in the presence of oxygen remained ignored for a long time. Our group demonstrated that *E. coli* cells deleted in the *adhE* gene cannot grow aerobically in minimal media, are extremely sensitive to oxidative stress, and show division defects (Echave et al., 2003). Additionally the purified enzyme is highly reactive with hydrogen peroxide (with a K_i of 5 μM), causing inactivation due to a metal-catalyzed oxidation system. All these results lead us to propose that the enzyme has a protective role against oxidative stress acting as a H_2O_2 scavenger in *E. coli* cells grown under aerobic conditions.

14.4 TRICARBOXYLIC ACID CYCLE

Cellular respiration occurs in three major stages. First, organic fuel molecules—glucose, fatty acids, and some amino acids—are oxidized to yield two-carbon fragments in the form of the acetyl group of acetyl-CoA. In the second stage, the acetyl groups are fed into the tricarboxylic acid (TCA) cycle, which enzymatically oxidizes them to CO_2. The energy released by oxidation is conserved in the reduced electron carriers NADH and $FADH_2$. In the third stage of respiration, these reduced coenzymes are oxidized. The electrons are transferred to O_2 via the respiratory chain. In the course of electron transfer, the large amount of energy released is conserved in the form of ATP by the oxidative phosphorylation.

This section will discuss the role of two enzymes that have been extensively described as sensitive to oxidative stress: aconitase and α-KGDHC. Enzymes highly resistant to oxidation are present in the TCA cycle, like malate dehydrogenase (MDH) and the eukaryotic fumarase. MDH has been used as a control enzyme in several studies because it is highly resistant to inactivation (Cabiscol et al., 2002; Rodriguez-Manzaneque et al., 2002; Zhang and Snyder, 1992). Although it has been described to be nitrated both during aging (Kanski et al., 2005)

and after ischemia reperfusion (Koeck et al., 2004b), there are no data on its effects on activity. *E. coli* contains three fumarases (FumA, FumB, and FumC). FumA and B belong to the class I fumarase family, work as a homodimer, and contain a 4Fe-4S cluster. FumC belongs to the class II fumarase/aspartate family, acts as a homotetramer, and does not present the Fe-S cluster. In eukaryotic cells, fumarase is present inside mitochondria and in the cytosol, but these two isoforms are produced by alternative initiation of one gene *FumH*. FumH belongs to the class II fumarase/aspartate family, and consequently it does not use a Fe-S cluster to catalyze the dehydration reaction. This fact makes FumH resistant to oxidation, as it was described in the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) model of PD, a situation where aconitase became inactivated but fumarase activity was preserved (Liang and Patel, 2004).

14.4.1 Aconitase

More formally, aconitate hydratase catalyzes the reversible transformation of citrate to isocitrate, through the intermediate formation of the tricarboxylic acid cis-aconitate, which normally does not dissociate from the active site. Aconitase, which acts as a monomer, contains an iron-sulfur center [4Fe-4S], acting both in the binding of the substrate at the active site and in the catalytic addition or removal of H_2O. In this cluster only three of the iron atoms are ligated directly to cysteines of the protein backbone. The fourth iron is only ligated to inorganic sulfur of the cluster and has a free coordination site that participates in the binding of substrates to the active site interacting with carboxyl and hydroxyl groups of substrates.

E. coli contains two major aconitases, ACO1 (AcnA) and ACO2 (AcnB). They are distantly related monomeric Fe–S proteins that contain different arrangements of four structural domains. AcnA has been designated as an aerobic stationary phase enzyme that is specifically induced by iron and oxidative stress, whereas AcnB functions as the major citric acid cycle enzyme during exponential growth. The biochemical and kinetic properties of the purified enzymes showed that AcnA is more stable than AcnB, has a higher affinity for citrate, and operates optimally over a wider pH range. This is in agreement with its role as a maintenance or survival enzyme during nutritional or oxidative stress (Jordan et al., 1999). In contrast, the better performance at high substrate concentrations and greater instability of AcnB indicate that it is specifically adapted to function as the main catabolic enzyme. AcnB inactivation in response to oxidative or pH stress would rapidly modulate energy metabolism, either directly or indirectly by regulating post-transcriptional gene expression. Electron paramagnetic resonance and magnetic circular dichroism spectroscopy showed that the iron-sulfur clusters of the bacterial aconitases (and their binding sites) strongly resemble those of the mammalian enzymes (Jordan et al., 1999).

In eukaryotes, two aconitase isoenzymes exist, one localized to the mitochondrial matrix (acting in the TCA cycle) and the other (iron responsive protein-1) in the cytoplasm. Since the role of iron responsive protein-1 is not related to energy production, we will focus on the mitochondrial enzyme.

There are hundreds of articles studying aconitase susceptibility to oxidative stress. The particular sensitivity of mitochondrial aconitase to oxidative damage is related to the iron-sulfur cluster $[4Fe–4S]^{2+}$ in its active site. Studies in vitro established that the enzyme is particularly sensitive to reaction with superoxide (Castro et al., 1994; Gardner and Fridovich, 1991; Gardner et al., 1994, 1995). This sensitivity is attributed to the presence of the unligated iron atom. Upon oxidation the cluster assumes an unstable valence promoting the loss of the labile Fe^{2+} atom, which causes release of one iron atom from the cluster (Flint et al., 1993). Several oxidants, including H_2O_2, ferricyanide, and persulfate, can inactivate the enzyme and give a paramagnetic $[3Fe–4S]^{1+}$ cluster (Verniquet et al., 1991).

It has been described that under normal redox conditions the release of aconitase iron by superoxide anion and the resultant inactivation can be reversed (Patel et al., 1996). However, inactivation of aconitase is expected to have pleiotropic effects. On one hand, inhibition of the important energy-yielding and synthetic reactions of the TCA cycle and, on the other hand, inactivation of aconitase can cause the accumulation of citrate or other metabolic intermediates. Several enzymes have been shown to be allosterically regulated by citrate. Furthermore, given the relatively high citrate concentration present, further elevation of citrate concentration could conceivably alter the osmotic balance and metal-ion homeostasis of the cell. Keyer and Imlay demonstrated the resultant toxicity of iron released from aconitase in *E. coli* (Keyer and Imlay, 1997).

Importantly, purified mitochondrial aconitase has been shown to be a source of hydroxyl radical formation, presumably via Fenton chemistry initiated by the co-released Fe^{2+} and H_2O_2 (Vasquez-Vivar et al., 2000). Hydroxyl radical may cause carbonylation and irreversible inactivation of aconitase through metal-dependent, site-specific oxidations, according to the mechanism proposed by Stadtman (1990). Carbonylation and inactivation of aconitase was described in yeast cells treated with H_2O_2 (Cabiscol et al., 2000). Major carbonyl content was detected in a peptide encompassing Val334 to Asp527, which interestingly includes amino acid sequences around the iron-sulfur cluster, and so agrees with the site specificity of metal-catalyzed oxidation.

In *Drosophila melanogaster* aconitase was estimated to account for approximately 15% of the total mitochondrial matrix proteins. Yan and collaborators (1997) showed that mitochondrial aconitase was inactivated and accumulated as the major carbonylated protein during aging of the fly. Experimental inactivation of aconitase by either hyperoxia or fluoroacetate shortened life span, indicating a causal relationship between aconitase activity and life span. The fact that citrate accumulates during aging (Zahavi and Tahori, 1965) implies that aconitase inactivation has functional importance. Thus its oxidative inactivation may block normal electron flow to oxygen, leading to an accumulation of reduced metabolites such as NADH. This “reductive stress” can cause increased production of ROS, initiating a cascade that dramatically increases the cellular burden of oxidative damage (Yan et al., 1997).

Aging is the most important risk factor for developing PD. Mitochondrial oxidative stress, bioenergetic decline, and iron overload are important hallmarks of this disease. As we will describe in the next section, inhibition of the mitochondrial electron transport chain at complex I is thought to be a critical biochemical lesion that accounts for both ATP depletion and increased steady state mitochondrial superoxide radical formation in human and animal models of PD. Liang and Patel (2004) tested whether neuronal damage in the MPTP model of PD results, in part, from superoxide radical toxicity via inactivation of iron-sulfur proteins. The authors showed that administration of MPTP in mice resulted in aconitase inactivation, iron accumulation, and dopamine depletion. All these phenomena were significantly attenuated in transgenic mice overexpressing mitochondrial *Sod2* and exacerbated in partial deficient *Sod2* mice. Inactivation of aconitase has also been demonstrated in the brains of patients with Hungtinton's disease (Tabrizi et al., 1999) and progressive supranuclear palsy (Albers et al., 2001). In this context it seems that aconitase inactivation will worsen the already ongoing energy crisis.

The dehydration/rehydration reaction catalyzed by aconitase has been modeled using complexes of cobalt, and it is not necessarily dependent on iron-sulfur clusters. It is evidenced by the comparable reaction catalyzed by eukaryotic fumarase, which is not a [4Fe-4S] enzyme and is resistant to inactivation in the MPTP model of PD (Liang and Patel, 2004). According to Gardner and Fridovich (1991), the advantage of utilizing an oxidant-sensitive enzyme for the interconversion of citrate and isocitrate may arise because aconitase may act as a "circuit breaker," which, by its sensitivity to O_2/O_2^-, shuts off much of the flow of electrons from substrates under conditions of incipient oxidative stress.

In this context, in *E. coli*, TCA cycle impairment would lead to growth inhibition, and a decreased growth rate has been shown to decrease the lethality of radiation (UV and ionizing) (Gillies and Alper, 1959) or the lethality of the O_2^- generator paraquat (Minakami and Fridovich, 1990) to *E. coli*. A similar conclusion came from the work with *S. cerevisiae* (Cabiscol et al., 2000), where it was evidenced the importance of arresting mitochondrial metabolism under oxidative stress conditions to prevent deleterious production of ROS. Under such stress the exogenous addition of succinate (which causes a stimulation of electron transfer to coenzyme Q) increased the production of ROS, which in turn reduced cell viability. Inactivation of aconitase as well as other TCA cycle enzymes involved in energy metabolism should have a beneficial effect by stopping growth, and thus minimize cell damage. This is consistent with the fact that oxidative stress induces growth arrest and DNA damage genes (GADD) to stop growth and preserve cell integrity (Crawford and Davies, 1994; Zerbini et al., 2004).

Aconitases are also important cellular targets of nitric oxide (NO) toxicity, and NO-derived species, rather than NO per se, have been proposed to mediate their inactivation. In vitro experiments (Castro et al., 1994) showed that $ONOO^-$ reacts with isolated pig heart mitochondrial aconitase at 1.4×10^5 M^{-1} s^{-1}, resulting in a significant loss of enzymatic activity. Aconitase activity was totally recovered after postincubation with thiols and ferrous iron. Superoxide inactivated

mitochondrial aconitase at $(3.5 \pm 2) \times 10^6$ M^{-1} s^{-1}, a reaction rate three orders of magnitude slower than that found by incubation with NO. Castro and collaborators (Castro et al., 1994) suggested that the mechanism by which NO and superoxide inactivates aconitase may rely on the formation of $ONOO^-$. From studies with *E. coli* and bovine heart aconitases, Gardner and colleagues proposed an *S*-nitrosation of the aconitase [4Fe–4S] center catalyzed by the solvent-exposed electron withdrawing iron atom (Gardner et al., 1997). For example, aconitase was identified as a nitrotyrosine-containing protein in rat cardiac tissue during aging (Kanski et al., 2005).

14.4.2 Alpha-Ketoglutarate Dehydrogenase Complex

Alpha-KGDHC, also named 2-oxoglutarate dehydrogenase, converts α-ketoglutarate to succinyl-CoA and CO_2; NAD^+ serves as electron acceptor and CoA as the carrier of the succinyl group. The energy of α-ketoglutarate oxidation is conserved in the formation of the thioester bond of succinyl-CoA. As described above, this reaction is identical to the pyruvate dehydrogenase reaction, and both complexes shared similar structure and function (Roche and Patel, 1989). The E1 components of the two complexes are structurally similar, but their amino acid sequences differ and they have different binding specificities. The E2 components are very similar, both having covalently bound lipoyl moieties, and the subunits of E3 for the two complexes are virtually identical. These structural similarities, specially the presence of highly reactive lipoamide moiety, make α-KGDHC as susceptible to oxidation as PDHC. In fact, as described before, several oxidative stress situations affect both complexes in a similar manner, like acrolein treatment (Pocernich and Butterfield, 2003) or HNE treatment (Humpries and Szweda, 1998). Acrolein (2-propen-1-al) and HNE, as lipid peroxidation by-products, decreased PDHC and α-KGDHC activities significantly in a dose-dependent manner because they bind to lipoic acid. A similar result was found in *S. cerevisiae* and *E. coli* after H_2O_2 or menadione treatment, where lipoic acid was also modified (Cabiscol et al., 2000). See the PDHC paragraph for further details and consequences of its inactivation.

Reperfusion of ischemic myocardial tissue results in an increase in mitochondrial free radical production and in a decline in respiratory activity. TCA cycle enzymes α-KGDHC and aconitase were susceptible to reperfusion-induced inactivation with no decline in the activities of other TCA cycle enzymes (Sadek et al., 2002). Oxidative stress and diminished metabolism occur in several neurodegenerative disorders. Brains from AD patients exhibit several indicators of oxidative stress and have reduced activities of the α-KGDHC (Gibson et al., 1999). Additionally α-KGDHC activity has been described to be reduced in brains from PD patients (Mizuno et al., 1994).

The results suggested that α-KGDHC participates in a deleterious cascade of events related to oxidative stress that are critical in selective neuronal loss in neurodegenerative diseases.

Nitric oxide derived from microglial inflammatory responses partially inactivates α-KGDHC, and this effect is potentiated by inhibition of complex IV

(cytochrome oxidase) (Park et al., 1999). In vivo inactivation of α-KGDHC by $ONOO^-$, which was formed by azide-mediated ROS and lipopolysaccharide-induced NO, was observed, confirming that the complex is sensitive to both species (Park et al., 1999). The reactivity of NO toward α-KGDHC is suggested to reside in its ability to interact with sulfhydryl groups essential for catalytic activity, whereas $ONOO^-$ nitrates tyrosine residues of all three subunits irreversibly (Park et al., 1999).

14.5 ELECTRON TRANSPORT CHAIN AND OXIDATIVE PHOSPHORYLATION

Oxidative phosphorylation, coupled to the electron transport chain, is the final step of cellular respiration in aerobic organisms. The energy of oxidation of coenzymes NADH and $FADH_2$, by donating a pair of electrons to a specific set of membrane-bound carriers (the electron transport chain), drives the synthesis of ATP. This process is called oxidative phosphorylation and is catalyzed by a membrane protein complex (ATP synthase). In eukaryotes this process occurs in mitochondria, where the great majority of O_2 consumption by respiring cells occurs. Five complexes are involved: complexes I to IV are implicated in the electron transport chain and complex V in the oxidative phosphorylation.

The leakage of electrons from the electron transport chains located on the inner membrane of mitochondria is the source of O_2^-, which then produces H_2O_2 by dismutation. It is believed that mitochondria are the major intracellular source of ROS, but there are substantial controversies regarding the exact amounts of ROS production at the different sites of the respiratory chain. Although it is generally believed that during normal cell respiration 1% to 4% of the oxygen reduced by mitochondria is converted to superoxide, some authors indicate that under normal conditions this percentage can be reduced down to 0.1% (Fridovich, 2004). In any case, even with the lower estimations, a superoxide concentration of 5 μM per second would accumulate inside the cell.

Nitric oxide is present in mitochondria in around 1 μM (Brown, 1995) and is generated by the mitochondrial nitric oxide synthase. Besides the role of NO as a physiological mediator (which is not the focus of this chapter), it can be converted into a pathological molecule. Peroxynitrite ($ONOO^-$), formed by the reaction of O_2^- with NO, is implicated as one of the major oxidants responsible for mitochondrial damage. The short-lived but highly reactive $ONOO^-$ damages mitochondria by modifying DNA, lipids, and proteins. Protein modification can occur by nitration of tyrosines to generate 3-nitrotyrosine (3NT), by *S*-nitrosylation of cysteines, and by oxidation of tryptophan residues to produce, for example, *N*-formylkynurenine.

Because of its high ATP demand, heart and brain consumes O_2 rapidly, which can in turn lead to increased O_2^- formation. The brain contains multiple antioxidant defenses, but there is substantial evidence that superoxide and H_2O_2 contribute to the pathogenesis of several neurodegenerative diseases, especially

those associated with aging. In this context, all complexes have been described to be affected in some way by the ROS generation inside mitochondria.

14.5.1 Complex I

Complex I, also called NADH:ubiquinone oxidoreductase and NADH dehydrogenase, is an enormous enzyme complex composed of many (ea. 40–45) different polypeptide chains, including an FMN-containing flavoprotein and at least six iron-sulfur centers. Complex I catalyzes two simultaneously and obligately coupled processes: (1) the exergonic transfer to ubiquinone of a hydride ion from NADH and a proton from the matrix, and (2) the endergonic transfer of four protons from the matrix to the intermembrane space. Complex I is therefore a proton pump driven by the energy of electron transfer.

$$NADH + H^{+}Q \rightarrow NAD^{+} + QH_2$$

There is growing evidence that mitochondrial damage underlies the pathology of several neurological disorders. Particularly, inhibition of complex I activity has been a reported feature of PD (Mizuno et al., 1995; Parker et al., 1989; Schapira et al., 1990). Several model systems have been developed. In rodents, Parkinsonian symptoms were observed as a result of treatment with specific inhibitors of complex I, such as rotenone (Betarbet et al., 2000) and MPTP (Heikkila et al., 1984).

The formation of 3NT was observed in the MPTP-induced PD animal model (Ferrante et al., 1999; Pennathur et al., 1999). Murray and colleagues (2003) undertook a highly detailed analysis of peroxynitrite-induced modification of proteins from an enriched mitochondrial preparation from bovine heart. Immunological and mass spectrometric approaches coupled with two-dimensional PAGE were used to show that peroxynitrite modification resulting in a 3NT signature is predominantly associated with 49 kDa subunit (NDUFS2), TYKY (NDUFS8), B17.2 (17.2 kDa differentiation associated protein), B15 (NDUFB4), and B14 (NDUFA6). Subunits B15 (NDUFB4) and B14 (NDUFA6) contained the highest degree of nitration. The most reactive site in subunit B14 was Tyr122, while the most reactive region in B15 contained three closely spaced tyrosines, Tyr46, Tyr50, and Tyr51.

In the same study the authors observed the double oxidation of a tryptophan residue in subunit B17.2, resulting in the formation of *N*-formylkynurenine, as well as oxidation of methionine in the tryptic peptide WVITYYEMNGK. Formation of *N*-formylkynurenine was also described by the same group to be overrepresented in complex I of human heart mitochondria (Taylor et al., 2003). Nine subunits of complex I had *N*-formylkynurenine-containing tryptic peptides.

PD patients and MPTP treated animals have dramatically decreased levels of glutathione (Perry et al., 1982; Sriram et al., 1998). This tripeptide plays multiple roles in the nervous system both as an antioxidant and a redox modulator. The decreases in mitochondrial activities in GSH-depleted cells appear to be caused

by a selective inhibition of complex I activity as a result of thiol oxidation (Jha et al., 2000). Selective inhibition of complex I (complexes II, III, and IV were not affected) activity following GSH depletion can be reversed by treatment with DTT. This reversibility suggests that, likely, a mixed disulfide or intramolecular disulfide is formed from vicinal thiols, rather than the formation of a sulfinic (SO_2H) or sulfonic (SO_3H) acid.

It has been described that in synaptic mitochondria, complex I exerts a major control over oxidative phosphorylation. At a 25% inhibition, energy metabolism is disturbed resulting in decreased ATP synthesis; complex III and IV inhibition in the range of 70% to 80% is required to exert similar effects (Davey et al., 1998). Such a molecular modification may contribute to the altered functioning of the complex and plays a key role in the onset of PD.

Other pathological situations presented deficiencies in the activities of the mitochondrial respiratory complexes and reduced mitochondrial ATP generation capacity, like in chagasic hearts infected by *Trypanosoma cruzi*. In infected murine hearts, Wen and Garg separated the individual components of the respiratory complexes, and carbonyl adducts were detected by Western blotting (Wen and Garg, 2004). Substantial carbonylation of complexes I, III, and V was detected. In complex I, NDUFS1, NDUFS2, and NDUFV1, which form the catalytic core, and UQCRC1, UQCRC2, and UQCRQ, the subunits of the core subcomplex, were identified as carbonylated proteins. The extent of oxidative modifications of the subunits correlated with the catalytic defects of the respiratory complexes in the infected myocardium.

14.5.2 Complex II

Mammalian complex II, also named succinate-ubiquinone oxidoreductase and succinate dehydrogenase, is the smallest complex in the respiratory chain. It functions both as a respiratory chain component and an essential enzyme of the TCA cycle. It contains two types of prosthetic groups and at least four different proteins. One protein has a covalently bound FAD and a 4Fe–4S cluster; a second (and in some species a third) iron-sulfur protein is present. Electrons pass from succinate to FAD, then through the Fe–S centers to ubiquinone.

$$\text{Succinate} + \text{Q} \rightarrow \text{Fumarate} + \text{QH}_2$$

Many articles described complex II inactivation under several oxidative stress situations (Long et al., 2004; Murray et al., 2003). Inactivation of complex II seems to be especially important in the pathogenesis of Huntington's disease (Saulle et al., 2004; Seo et al., 2004; Tabrizi et al., 1999), and a decrease in the activity of complex II alone is sufficient to cause neurological disorders in humans (Ackrell, 2002). Despite these reports the susceptibility of complex II to oxidative stress is still a matter of controversy. Several groups reported that complex II seems to be more resistant to oxidative stress than other complexes of the electron transport chain (Riobo et al., 2001; Taylor et al., 2003; Wen and

Garg, 2004). Whether this is due to its distance to radical-generating sites or due to its structure making it more resistant is still unclear. On the other side, a recent study on mice that lack mitochondrial superoxide dismutase (*SOD2* null mice) described inactivation of complex II compared to control mice (Hinerfeld et al., 2004). However, as the authors pointed out, this loss of activity was mainly due to a reduction in the levels of complex II subunits.

As described previously, nine subunits of complex I from human heart mitochondria had *N*-formylkynurenine-containing tryptic peptides after peroxynitrite treatment (Taylor et al., 2003). Interestingly *N*-formylkynurenine was not detected in any subunit of complex II. A report on the crystal structure of the bacterial analogue of mitochondrial complex II revealed that the redox centers are arranged in a manner that prevents generation of ROS at the flavin adenine dinucleotide (Yankovskaya et al., 2003). The absence of oxidized tryptophan in complex II therefore supports the hypothesis that the degree of tryptophan oxidation reflects the exposure of proteins to ROS-generating sites.

14.5.3 Complex III

Complex III, also known as cytochrome bc1 complex and ubiquinone:ferricytochrome c oxidoreductase, couples the transfer of electrons from ubiquinol (QH_2) to cytochrome c with the transport of protons from the matrix to the intermembrane space.

$$QH_2 + 2\text{cyt } c_1(\text{oxidized}) + 2H^+ \rightarrow Q + 2\text{cyt } c_1(\text{reduced}) + 4H^+$$

Mammalian complex III contains three catalytic subunits: cytochrome *b*, which contains two noncovalently attached heme *b* groups; cytochrome *c*1, which contains a *c*-type heme group bound through covalent thiol-ether linkages; and the iron-sulfur protein, which bears a 2Fe–2S cluster coordinated by two cysteines and two histidines of the apoprotein.

Myocardial ischemia decreased complex III activity in the adult heart following 60 minutes of in vivo coronary occlusion in the dog (Rouslin, 1983) and following global ischemia in the isolated rat heart (Veitch et al., 1992). Aged heart sustains greater injury during ischemia and reperfusion compared to adult hearts (Lesnefsky et al., 2001). These authors found that ischemia damaged the iron-sulfur protein, which contains a 2Fe–2S redox-active iron-sulfur cluster. Ischemic damage to the iron-sulfur protein resulted in loss of the EPR signal of the cluster without loss of the iron-sulfur protein peptide, suggesting that ischemia disrupted the cluster without degradation of the subunit. Conserved cysteine and histidine residues are ligands for the Fe atoms in the cluster. In vitro oxidative damage to the histidine residues leads to loss of complex III activity (Miki et al., 1991). In addition to the ligands of the Fe atoms, the integrity of the cluster requires that the native conformation of iron-sulfur protein be preserved. An intramolecular disulfide bond between two highly conserved cysteine residues remote from the cluster itself is required to preserve the integrity of the cluster (Denke et al., 1998). These sites provide likely targets for ischemic damage,

including possibly oxidative processes, to selectively disrupt the 2Fe–2S cluster without a loss of the peptide.

Additionally, carbonylation of complex III has been observed in the diaphragm of septic rats (Barreiro et al., 2005), yeast mutants lacking MnSOD or CuZn-SOD (O'Brien et al., 2004), and murine hearts infected by *Trypanosoma cruzi* (Wen and Garg, 2004). Wen and Garg identified carbonyl adducts located at CYC1 and core protein 1 (UQCRC1), and most likely core protein 2 (UQCRC2) (Wen and Garg, 2004). It is possible that oxidative modifications of CYC1 and core proteins contribute to a loss in complex III activity in the cardiac mitochondria of infected murine hearts, albeit through different mechanisms. CYC1, along with Reiske [2Fe–2S] protein and cytochrome b, forms the inter-membrane-associated, central catalytic domain of complex III that is involved in energy conservation and electron transfer from ubiquinol to cytochrome c.

Formation of *N*-formylkynurenine from tryptophan residues has been described in complex III of human heart mitochondria. However, only two oxidized peptides were observed, compared with the highly modified complex I and V (Taylor et al., 2003).

14.5.4 Complex IV

Complex IV, also known as cytochrome c oxidase and ferrocytochrome c:oxygen oxidoreductase, carries electrons from cytochrome c to molecular oxygen, reducing it to H_2O. Complex IV is a large enzyme (13 subunits in eukaryotes) of the inner mitochondrial membrane. Subunit II contains two Cu ions complexed with the –SH groups of two cysteine residues in a binuclear center (called Cu_A) that has some similarities to the 2Fe–2S centers of iron-sulfur proteins. Subunit I contains two heme groups, designated a and a_3, and another copper ion (Cu_B). Heme a_3 and Cu_B form a second binuclear center. To convert O_2 to $2H_2O$, the enzyme consumes four substrate H^+ from the matrix for every four electrons passing through this complex.

$$4\text{cyt c}_{(\text{reduced})} + 8H^+ + O_2 \rightarrow 4\text{cyt c}_{(\text{oxidized})} + 4H^+ + 2H_2O$$

Bacteria contain a much simpler form, with only three or four subunits, but is still capable of catalyzing both electron transfer and proton pumping. This four-electron reduction of O_2 involves redox centers that carry only one electron at a time. This transfer must avoid the release of incompletely reduced intermediates, such as superoxide, that can damage cellular components.

It is well documented (see the following references only as examples) that selective reduction of mitochondrial cytochrome oxidase activity occurs in AD (Cardoso et al., 2004; Curti et al., 1997; Mutisya et al., 1994; Parker et al., 1990) and ischemia (Davey et al., 1997; de la Torre et al., 1997; Lesnefsky et al., 2004). However, to our knowledge, the reasons for the defect are unknown.

Glutathiolation of cytochrome oxidase has been reported in T cell blasts under oxidative stress (diamide or H_2O_2 treatment) (Fratelli et al., 2002), and the binding of nitric oxide to cytochrome oxidase, acting as a regulatory molecule of the mitochondrial respiratory chain, is well documented (see Chapter 6).

Generally, inhibition of the electron transport chain during ischemia lower damage to mitochondria. The limitation of electron flow during ischemia preserves cardiolipin content, cytochrome c content, and the rate of oxidation through cytochrome oxidase (Lesnefsky et al., 2004). The mitochondrial electron transport chain contributes to ischemic mitochondrial damage, which in turn augments myocyte injury during subsequent reperfusion.

14.5.5 Complex V

Complex V, more formally, ATP synthase, catalyzes the synthesis of ATP from ADP and inorganic phosphate accompanied by the flow of protons from the intermembrane space to the matrix side. According to the chemiosmotic model, the proton motive force (the electrochemical energy inherent in the difference in proton concentration and separation of charge across the mitochondrial inner membrane) drives the synthesis of ATP as protons flow passively back into the matrix through ATP synthase. To emphasize this crucial role of the proton-motive force, the equation for ATP synthesis is sometimes written as

$$\mathrm{ADP} + \mathrm{Pi} + n\mathrm{H}^+_\mathrm{P} \rightarrow \mathrm{ATP} + \mathrm{H_2O} + n\mathrm{H}^+_\mathrm{N}$$

Mitochondrial ATP synthase is an F-type ATPase similar in structure and mechanism to the ATP synthases of chloroplast and eubacteria. Complex V has two distinct components: F_1, a peripheral membrane protein, and F_0, which is integral to the membrane. F_1 has 10 subunits of four different types, with the composition $\alpha_3\beta_3\gamma\delta_3$. Each of the 3 β subunits has one catalytic site for ATP synthesis. The F_0 complex making up the proton pore is composed of 3 subunits, a, b, and c, in the proportion ab_2c_{10-12}. According to Boyer (1997), ATP synthase is a "splendid molecular machine," and to understand how it works, crystal structure determination is crucial (Abrahams et al., 1994; Stock et al., 1999).

H_2O_2, paraquat, and menadione stress performed under aerobic conditions resulted in carbonylation of the catalytic β-subunit of F_0F_1-ATPase in *E. coli* (Tamarit et al., 1998). The same result was found in *S. cerevisiae* under H_2O_2 stress (Cabiscol and Ros, date not known). Similarly, in yeast *sod2* mutants (lacking mitochondrial MnSOD), the α-chain of ATP synthase become carbonylated (O'Brien et al., 2004). A γ-chain that is essential for ATP synthesis by complex V also appeared carbonylated in chagasic hearts infected by *Trypanosoma cruzi* (Wen and Garg, 2004). However, apparently some contradictory data exist. In SOD2 deficient mice (sod2-/-) complex V appeared to be insensitive to the endogenously generated oxidative stress (complexes I, II, III, and IV were sensitive) (Hinerfeld et al., 2004).

Peroxynitrite reacts with mitochondrial membranes from bovine heart to significantly inhibit the activities of complexes I, II, and V (50–80%) (Morgan

et al., 2002b). Tyrosine nitration was determined in ATP5b from a murine model of AD (Tg2576 mice) by proteomic techniques (2D gels and immunochemical detection of 3NT) (Shin et al., 2004). The same approach allowed identifying, among others, F_1 ATP as a cardiac protein from whole rat hearts that suffer protein nitration as a consequence of aging (Kanski et al., 2005). Another consequence of peroxynitrite attack to amino acid residues is the formation of *N*-formylkynurenine, adding 32 atomic mass units to tryptophan residues. According to Taylor and collaborators (Taylor et al., 2003), complex V is one of the most *N*-formylkynurenine-containing components of the oxidative phosphorylation machinery. Three subunits with oxidized tryptophan residues were observed in this complex, the most oxidized being subunit δ. Interestingly a homologous peptide from subunit d was also reported to contain *N*-formylkynurenine in bovine heart (Bienvenut et al., 2002), suggesting that there may be a specificity for tryptophan oxidation in certain sequences across species. It is interesting that the three oxidized subunits come from the membrane-bound F_0 component of the complex and not from the subunits of the F_1 component, which have been described as highly carbonylated subunits.

Biotin-cysteine was used to study protein *S*-thiolation in isolated rat kidneys subjected to ischemia and reperfusion. After 40 minutes of ischemia, total protein *S*-thiolation increased significantly. With the use of gel filtration chromatography followed by affinity purification with streptavidin-agarose, *S*-thiolated proteins were purified and separated by SDS-PAGE. With a combination of MALDI-TOF and LC-MS/MS analysis of protein bands digested with trypsin, a number of *S*-thiolation substrates were identified, including the ATP synthase alpha chain (Eaton et al., 2003). Whether complex V may be functionally regulated by *S*-thiolation and its consequences in cell metabolism will have to be addressed.

14.6 ANTIOXIDANT DEFENSES

Aerobic metabolism produces partial reduction products of oxygen such as superoxide, which then is converted to hydrogen peroxide. Estimations of generation of superoxide radicals made in vitro indicated that there were 1% to 2% of the oxygen molecules consumed. Nevertheless, as indicated earlier in this chapter, it seems clear that under normal conditions leakage of electrons from respiratory chain represents 0.1% of the total electron flow (Fridovich, 2004). Considering the damaging effects of superoxide anion, one of the main consequences is the oxidation of iron-sulfur clusters of several dehydratases. The release of iron from these enzymes contributes to increase the labile iron pool, which in turn potentiates the formation of ROS.

These and other ROS can also be generated by pro-oxidant agents such as radiation or redox cycling agents. Living organisms have evolved systems to prevent the damage that ROS can produce. In bacteria, the protective responses against reactive oxygen species involve the expression, among others, of SODs, catalase, alkyl-hydroperoxide reductase, glutathione reductase, glutaredoxin I, and

the product of *oxyS* gene (Storz and Imlay, 1999). In *S. cerevisiae*, treatment with hydrogen peroxide activates Yap1p transcription factor, which governs the expression of antioxidant enzymes such as SOD, glutathione reductase, glucose-6-P-dehydrogenase (Lee et al., 1999; Toledano et al., 2003). Proteomic approaches indicated that treatment of yeast cells with hydrogen peroxide, in addition to the already mention proteins, thioredoxim reductase, cytochrome c-peroxidase, several proteasome subunits, and heat shock proteins were also induced. In mammalian cells response to oxidative stress has similarities to response in *S. cerevisiae*. Thioredoxin reductase, peroxyredoxins, and heme oxygenase are, among others, enzymes induced in lymphocytes by treatment with hydrogen peroxide or UV irradiation (Dröge, 2002).

14.6.1 Superoxide Dismutase

Disproportionation of superoxide can be carried out by Fe-, Mn-, and copper-zinc superoxide dismutases. Iron, manganese, and copper participate in the reaction by alternate cycles of oxidation/reduction. When present, zinc atom stabilizes the enzyme and does not participate in the catalytic cycle. There are several types of SOD: one of them, SOD1, has been localized in the cytosol, but it has also been identified in peroxisomes, lysosomes, and nucleus. In humans, SOD1 has been related to the development of amyotrophic lateral sclerosis (Fridovich, 1995; Wiedau-Pazos et al., 1996; Yim et al., 1996). In yeast, SOD1 and its co-chaperone CCS has been localized in the intermembrane space of mitochondria (Sturtz et al., 2001). Mn-SOD (SOD2) is present in mitochondria. Bacteria, algae, and higher plants, but not animal tissues, contain Fe-SOD localized in the cell matrix (Fridovich, 1995; Halliwell and Gutteridge, 1999). SOD3 has been found associated with extracellular elements. This is why it is also referred as EC-SOD. It is clear that if these enzymes become themselves damaged, loosing their function, the impact of oxidative stress on a given cell or tissue will be exacerbated. In this context yeast mutants lacking SOD1 or SOD2 showed increased oxidative damage to mitochondrial proteins, compared to wild-type cells (O'Brien et al., 2004). Also SOD2 null mice, which display neurodegenerative disorders, show that mitochondrial proteins such as α-KGDHC and succinate dehydrogenase are especially vulnerable to oxidative stress. As the authors pointed out, the results obtained gave insights on how mitochondrial oxidative stress links to neurodegenerative disorders (Hinerfeld et al., 2004).

Experiments of in vitro inactivation of SOD by hydrogen peroxide were first reported about 30 years ago (Hodgson and Fridovich, 1975). The data suggested that H_2O_2 reduces Cu^{2+} to Cu^{+}, which then reacts with another H_2O_2 molecule to generate Cu^{2+}–OH^{-}. This species will inactivate the enzyme by modification of a histidine residue in the active center. In vivo inactivation of SOD was demonstrated in red blood cells via a continuous flux of superoxide and hydrogen peroxide generated by xanthine plus xanthine oxidase. This system produced an inactivation even higher than that observed after treatment with 15 mM H_2O_2. Given that a great portion of such hydrogen peroxide can be removed

by catalase or glutathione peroxidase present in red blood cells, the authors suggested that inactivation occurred by the presence of H_2O_2 in the catalytic center as a consequence of the dismutation reaction (Salo et al., 1990). Once inactivated, the protein was susceptible to proteolytic degradation. This sequence of events—oxidative modification prior to degradation—was proposed earlier, using glutamine synthetase as a model (Levine et al., 1981).

Experiments carried out to analyze the effect of peroxynitrite on Mn-SOD showed that Tyr34 present in the catalytic center was the tyrosine residue most prone to nitration, and only three out of the nine tyrosine residues were converted to the nitrated forms. These modifications led to complete inactivation of the enzyme (MacMillan-Crow et al., 1998). These results gave an explanation to previous observations about the oxidative modifications underlying the chronic rejections of renal allografts (MacMillan-Crow et al., 1996).

Glycation of SOD has also been observed (Ookawara et al., 1992). Incubation of SOD with glucose to promote glycation of the enzyme resulted in a release of Cu. This metal could contribute to promote the formation of hydroxyl radical by Fenton reaction. As a result, the enzyme became inactivated, and in addition, fragmentation occurred between Pro62 and His63 residues, yielding peptides of 15 and 5 kDa.

A proteomic approach to identify proteins nitrated in vivo as a consequence of *E. coli* lipopolysaccharide treatment showed that MnSOD, as well as catalase, were identified as antinitrotyrosine immunopositive proteins (Aulak et al., 2001).

14.6.2 Catalase

Catalases are enzymes that contain a ferric heme group and catalyze a disproportionation reaction of H_2O_2 to H_2O and O_2 (Nicholls and Schonbaum, 1963). In this reaction the enzyme cycles between ferric state and compound I, which is an intermediate state comprising an oxyferryl center and a porphyrin radical structure (Chuang and Van Wart, 1992). Additional catalytically inactive, oxidized forms of catalase can be formed. One of them, compound II, contains iron (IV) without the porphyrin radical (Chuang et al., 1989). Compound III seems to be an oxy-ferrous compound that can be formed, among others, by superoxide anion or by addition of hydrogen peroxide to compound II (Lardinois, 1995). Manganese-containing catalases have been described in several strains of *Lactobacillus* and *Streptococcus* (Beyer and Fridovich, 1985).

Escherichia coli has two catalases HPI and HPII encoded by *katG* and *katE* genes, respectively (Schellhorn, 1995). The *E. coli* cells scavenge hydrogen peroxide at low concentrations by means of alkyl hydroperoxide reductase (Ahp). This enzyme has a low K_m for hydrogen peroxide compared to that of catalase, which will be important at high concentrations of the peroxide (Seaver and Imlay, 2001). Yeast cells also have two catalases: A and T, located, respectively, in the peroxisome and the cytosol; they are encoded by *CTA1* and *CTT1*, respectively (Jamieson, 1998). In animal tissues catalase activity is present in all major organs, mainly in liver, and in erythrocytes (Halliwell and Gutteridge, 1999).

In vitro experiments using purified enzymes showed inhibition of catalase by superoxide radical generated by a continuous flux of O_2^- using xanthine-xanthine oxidase system (Kono and Fridovich, 1982). The authors found that there are two ways of inhibition of catalase by O_2^-: (1) a rapid inactivation, due to the formation of compound III, which was prevented by adding Mn-superoxide dismutase to the assay, and (2) a slow inactivation, due to the formation of compound I, which was prevented by ethanol. Irreversible inactivation of catalase can also occur in the presence of hydrogen peroxide (De Luca et al., 1995; Lardinois et al., 1996). When exposed to a source of singlet oxygen using methylene blue, rose bengal, and visible light, the catalase as well as SOD are irreversibly inactivated. Besides loosing activity, these enzymes aggregate and fragment, and protein carbonyl groups increased two- to threefold compared to basal levels of untreated enzymes (Kim et al., 2001).

Oxidative modification of catalase—protein carbonyl formation—has also been described in chronological aged yeast grown with 2% glucose, while under caloric restriction conditions the carbonylation of catalase was not observed (Reverter-Branchat et al., 2004). This fact could account for the increased viability of yeasts observed under caloric restriction culture conditions.

A different mechanism of catalase inactivation, which involves glycation mediated by ribose and fructose, has also been described (Yan and Harding, 1997). As mentioned above, catalase was identified as a tyrosine-nitrated protein during inflammatory challenge (Aulak et al., 2001).

14.6.3 Glutathione Peroxidase

Glutathione peroxidases (Gpx) comprise a family of selenium-containing enzymes that catalyze reduction of peroxides at the expense of glutathione. Classical Gpx are multimeric enzymes, while those that have specificity for phospholipid hydroperoxides (PHGpx) are monomeric. In mammalian systems at least five forms of Gpx have been described (Gpx1–5). Gpx1 is the most abundant and the first to be identified, Gpx2 is expressed in the gastrointestinal tract, Gpx3 is the plasma form, Gpx4 is a membrane-associated enzyme, having a phospholipid hydroperoxide Gpx activity, and Gpx5 is the epididymis-specific secretory Gpx (Brigelius-Flohé, 1999; Brigelius-Flohé et al., 2002). All of them possess Se-Cys at their active site, except Gpx5, which is a Cys-containing Gpx. Yeasts produce three Gpx, which are of the PHGpx type (Avery and Avery, 2001; Avery et al., 2004; Inoue et al., 1999). Among them, Gpx3 is especially relevant because it acts as a sensor for hydrogen peroxide and is responsible for the oxidation of the Yap1 transcription factor, which triggers the stress response to hydrogen peroxide (Delaunay et al., 2002).

Structural modifications of Gpxs leading to enzyme inactivation have been shown using NO donors. According to the work of Asahi and co-workers, inactivation by peroxynitrite involves modification of selenocysteine residue giving a selenenyl sulfide (Asahi et al., 1995, 1997). It has been also suggested that such inactivation could not be directly executed by $ONOO^-$ but by $ONOOCO_2^-$. This

assumption is based on the higher reactivity (10^3 times faster) of peroxynitrite with CO_2 than with Gpx (Padmaja et al., 1998). The physiological consequence is an increase in intracellular peroxides that are responsible for cellular damage. Such accumulation has also been demonstrated in cultures of cells from thoracic aorta treated with NO donors (Koh et al., 2001).

Modification by 3-deoxyglucosone has also been reported to inactivate Gpx (Niwa and Tsukushi, 2001). 3-Deoxyglucosone is a highly reactive carbonyl compound that comes from dehydration and rearrangements of Amadori products. Also 3-deoxyglucosone is an intermediate compound in the formation of pyrraline, an advanced glycation end product (AGE). This and other AGEs, such as pentosidine and imidazolone, have been implicated in development of diabetic complications (Portero-Otin et al., 2002). Inactivation of Gpx by HNE has been described in cultured fibroblasts (Kinter and Roberts, 1996). HNE is a toxic aldehyde derived from polyunsaturated fatty acid oxidation. It is highly reactive with thiol groups, either those present in amino acid residues of proteins or in small molecules such as glutathione (Esterbauer et al., 1991). A consequence of such reactivity is the depletion of glutathione pools, and thus the weakening of the protection systems against oxidative stress.

14.7 MOLECULAR CHAPERONES

Heat shock proteins (Hsps)—often called stress proteins—are a family of proteins highly conserved among species that carry out essential functions such as protein translocation, folding, and assembly under normal cellular conditions (where Hsps account for 5–10% of total proteins). Under stress conditions, which include heat shock, glucose deprivation, exposure to free radicals, infection by pathogens, and tissue injuries, they are overexpressed. A common aspect of these stress-inducing conditions is that they give rise to proteins with nonnative conformations, which are targets for molecular chaperones. Under stress situations where the ATP levels decrease, "holding" chaperones bind to damaged proteins to prevent their aggregation. After the stress, when ATP levels are restored, "folding" chaperones are able to refold protein substrates; if damage is too severe, modified proteins are subjected to degradation (Dougan et al., 2002; Hartl and Hayer-Hartl, 2002). There are excellent reviews on how these chaperones work and cooperate (Lund, 2001; Mayer et al., 2000b).

The molecular chaperones present both in prokaryotic and eukaryotic cells can be grouped in several systems: Hsp100/Clp, Hsp90, Hsp70, Hsp60, small Hsp (sHsp). The Hsp100/Clp proteins are involved in increased tolerance to high temperatures, proteolysis of specific cellular substrates, and regulation of transcription (Schirmer et al., 1996). They can be divided into two groups: group I proteins that contain two nucleotide-binding domains in their structures, and group II proteins that only have a single domain. Sequence analysis of these groups of Hsps revealed that they have high similarity to proteins of the AAA family (ATPases associated with several cellular activities). These findings led

to propose a definition of an AAA+ superfamily, which also included Lon and FtsH proteases (Lund, 2001).

Members of the group I are ClpA, ClpX, or ClpB in *E. coli* and Hsp104 in *S. cerevisiae*. ClpB and Hsp104 are both involved in resolubilizing protein aggregates in cooperation with the Hsp70 system (Glover and Lindquist, 1998; Mogk et al., 1999; Zolkiewski, 1999). Members of the Hsp90 system are involved in cell proliferation, differentiation, and apoptosis; in the last few years they have been the focus of many laboratories due to their clinical importance as targets of anticancer drugs (Chiosis et al., 2004; Piper, 2001). From a structural point of view, they form a homodimer and have an *N*-terminal ATPase activity domain, a *C*-terminal dimerization domain and a linker domain (Young et al., 2001). Under stress situations Hsp90 acts as a "holdase," allowing refolding of proteins by other chaperones as the stress has ceased (Freeman and Morimoto, 1996). To our knowledge, there are very few reports on oxidative modification of these two molecular chaperone systems (Nardai et al., 2000; Reverter-Branchat et al., 2004).

14.7.1 Hsp70

The Hsp70 system is a highly conserved group of chaperones. In bacteria the *dnaK* operon codes for DnaK and DnaJ proteins. In eukaryotic cells members of this system are, among others, Hsc70, BiP or Hsp72. They have an *N*-terminal domain with ATPase activity and a *C*-terminal domain that binds to proteins (Rudiger et al., 1997). They cooperate with Hsp40 proteins (DnaJ in bacteria) (Hartl and Hayer-Hartl, 2002). DnaK and DnaJ bind and protect unfolded regions of proteins, and their function is dependent on GrpE. The DnaK system is also involved in protection and recovery of thermolabile proteins. It has been described to interact with ClpB to disaggregate proteins after heat shock (Mogk et al., 1999). Small heat shock proteins (sHsps) also cooperate in this process (Mogk et al., 2003). The sHsps have molecular masses ranging between 15 and 40 kDa (members of this group are IbpA/IbpB in *E. coli* and Hsp25 in eukaryotic cells). They have the ability to bind to a great number of nonnative polypeptides; such interaction seems to be a prerequisite for the refolding by Hsp70 or Hsp60 systems (Veinger et al., 1998).

DnaK was described as one of the major targets when *E. coli* cells were stressed with hydrogen peroxide or with the superoxide generating compound, paraquat (Tamarit et al., 1998). In stationary phase *E. coli* cultures oxidative damage to DnaK was also observed (Dukan and Nyström, 1998). Increased damage to this and other proteins was observed in *E. coli* mutants of *rpoS* and *oxyR* regulons, indicating that these regulons are involved in defense against oxidation of specific proteins during stationary phase. Since DnaK participates in σ^{32} degradation, damage to this chaperone will increase the stability of σ^{32}, allowing the induction of heat-shock genes (Nyström, 1994). Also overexpression of DnaK protects proteins, such as AdhE, from oxidative damage (Echave et al., 2002).

In yeast, Hsp70 proteins are divided in four groups: SSA, SSB, SSC, and SSD (Mager and Moradas-Ferreira, 1993). Among them, carbonylation of Ssa1

and Ssa2 have been detected during aging (Reverter-Branchat et al., 2004), and by treatment with hydrogen peroxide (Yoo and Regnier, 2004). Yoo and Regnier report the use of biotin-hydrazide to derivatize protein carbonyls and avidin-fluorescein as detection system. Biotin derivatization also facilitates purification of oxidized proteins by affinity chromatography using avidin columns (Mirzaei and Regnier, 2005).

14.7.2 Hsp60

The Hsp60 group, also known as chaperonins, is divided into two groups. Group I is present in bacteria (GroEL), mitochondria, and chloroplasts (Fenton and Horwich, 1997). GroEL subunits (57 kDa) form two stacked heptameric rings and cooperate with the co-chaperone GroES (10 kDa). The group II (TRiC) localizes at eukaryotic cytosol, they have eight subunits per ring, and they do not need a co-chaperone (Dunn et al., 2001).

In *E. coli,* GroEL assists the folding of newly synthesized polypeptides. Under stress situations, where it can function as a "holdase," its synthesis is induced and it can bind to partially unfolded polypeptides to prevent their irreversible denaturation (Llorca et al., 1998).

A recent publication showed that GroEL is modified and inactivated by incubation of purified GroEL either with peroxynitrite ($ONOO^-$) or hypochlorous acid (HOCl), but it is almost insensitive to hydrogen peroxide (Khor et al., 2004). Only high concentrations of hydrogen peroxide (20–60 mM) caused exposure of hydrophobic surfaces or dissociation into monomers after 16 hours in 10 mM peroxide, as well as partial loss of secondary structure (Melkani et al., 2004). An accurate analysis of oxidative modifications of GroEL showed that treatment with 1 mM hypochlorous acid modifies several Cys residues to cysteine sulfonic acid, and Met residues to methionine sulfoxide (Khor et al., 2004). It is not clear how the modification of Cys residues can prevent the assembly of this chaperonin; nevertheless, oxidation of Met residues contributed the most to GroEL inactivation. These findings are highly interesting since, in coronary heart disease, increased levels of myeloperoxidase activity have been reported. Production of HOCl by this enzyme would modify the structure of GroEL and thus contributing to disease progression (Zhang et al., 2001).

In *S. cerevisiae* the mitochondrial chaperone Hsp60, which is the eukaryotic homologue of GroEL, becomes severely carbonylated under oxidative stress promoted by hydrogen peroxide or menadione (Cabiscol et al., 2000). Photodynamic therapy, which is used for treatment of some types of cancers, produces, among other effects, carbonylation of specific proteins including Hsp60 in cultured eukaryotic cells (Magi et al., 2004). Hsp60 is an essential mitochondrial chaperone involved in folding of proteins imported into mitochondrial matrix, and Hsp60 deletion mutants are not viable (Cheng et al., 1990). Using mutants of Hsp60 with a doxycycline-regulated $tetO_7$ promoter, it was shown that protein carbonylation in the yeast cell increased under low Hsp60 expression levels and Hsp60 itself was heavily damaged. As a consequence of the lowered levels of

the chaperone, the free-iron pool increased from lack of correct protein folding, which is especially relevant when proteins with iron-sulfur clusters are affected. This would be the main reason for exacerbated increase in protein carbonylation observed (Cabiscol et al., 2002).

Members of the Hsp60 and Hsp70 families have been found modified by glutathione in T lymphocytes stressed with hydrogen peroxide or diamide (Fratelli et al., 2002), as well as cyclophilin, a chaperone involved in thermal resistance (Sykes et al., 1993). Carbonylation of this chaperone has also been reported under hydrogen peroxide stress in yeast (Costa et al., 2002).

The question of why these molecular chaperones are susceptible to oxidation is not clear. It is known that they interact with proteins modified as a consequence of oxidative treatment. Since protein hydroperoxides can be present in damaged proteins (Dean et al., 1993), protein hydroperoxides can in turn generate further radicals that can attack and damage other proteins, such as chaperones. In fact, incubation of peptides and protein peroxides with several enzymes results in enzyme inactivation, which is exacerbated by addition of iron ions to the assay (Morgan et al., 2002a).

14.7.3 Chaperones of Endoplasmic Reticulum

Chaperones present in the endoplasmic reticulum (ER) are involved in the so-called quality control pathway. Among them, BiP and Grp94 assist protein folding and assembly. BiP is an ER-chaperone of the Hsp70 family, which has a weak ATPase activity and surveys the folding status of the proteins in the ER. When BiP binds to misfolded proteins, production of ER chaperones increases and a degradation program that involves proteasome is activated (Rutkowski and Kaufman, 2004). Protein disulfide isomerase acts as an enzyme catalyzing the formation of disulfide bridges and as a chaperone that cooperates with BiP (Mayer et al., 2000a; Wang and Tsou, 1993). Calreticulin, calnexin, and the associated co-chaperone ERp57 are essential components of a maturation system that assists correct folding of glycoproteins in ER (Ellgaard et al., 1999; Lynch and Michalak, 2003). Other proteins with chaperone function are involved in more specific tasks. For example, receptor-associated protein acts on LDL receptor (Bu and Schwartz, 1998). Some of the chaperones in the ER have been also described to suffer oxidative modifications. Although mitochondria are the major source of reactive oxygen species, the endoplasmic reticulum produces oxidants that can potentially damage proteins (Tu et al., 2000). Stress in ER could be a consequence of nutrient deprivation, calcium fluxes, and accumulation of misfolded proteins (Kaufman, 1999; Schroder and Kaufman, 2005). Isolation of an enriched fraction of the endoplasmic reticulum from old mice liver showed carbonylation of specific proteins when compared to livers of young animals (Rabek et al., 2003). Mass spectrometry analysis showed that the major targets were BiP (Grp78), calreticulin, and protein disulfide isomerase. Oxidative modification of such proteins implies that protein folding, glycosylation, and disulfide-bond formation are severely compromised in aged mouse liver. In addition other reports

described that the sarco/endoplasmic reticulum calcium ATPase pump can be inhibited by oxidation of sulfhydryl groups and nitration of specific tyrosine residues (van der Vlies et al., 2003; Viner et al., 1999a, 1999b). These processes will affect not only the ER function but also the cellular metabolism. Only proteins that reach the native conformation will be transported through the ER membrane; lack of the above-mentioned protein functions will lead to accumulation of misfolded proteins, contributing to perturbation of cell function (Berlett and Stadtman, 1997).

14.7.4 Alpha-Crystallin

Alpha-crystallin acts as a molecular chaperone in the lens, preventing aggregation of lens proteins that occurs during aging (Horwitz, 1992). Studies of alpha-crystallin eye lens provide evidences about methylglyoxal modification of this protein. Methylglyoxal is derived either from spontaneous loose of phosphate from glyceraldehyde-3-P or from dihydroxyacetone phosphate. It is also a product derived from lipid peroxidation or threonine metabolism. Main targets are arginine and lysine residues (Glomb and Monnier, 1995). Modification of arginine leads to formation of argpyrimidine, an adduct that has been reported to naturally occur in human serum proteins and cornea (Shamsi et al., 1998). Using specific monoclonal antibodies, argpyrimidine derivatives have been localized in some arterial walls of rat brains submitted to ischemia-reperfusion as well as in the intima and media of small artery walls on kidneys from diabetic patients (Oya et al., 1999). Modification of lysine residues by methylglyoxal produces *N*-carboxyethyl-lysine (Ahmed et al., 1986). Carboxymethyl-lysine is formed by reaction of glyoxal with lysine residues (Wells-Knecht et al., 1995; Ahmed et al., 1997).

The effect of methylglyoxal modifications in alpha-crystallin are somehow controversial; while a report shows that the chaperone activity is enhanced by formation of argpyrimidine derivatives of three arginine residues (Nagaraj et al., 2003), a previous article found decreased chaperone activity after modification (Derham and Harding, 2002). Enhanced activity by in vivo formation of argpyrimidine has also been reported for a small heat-shock protein, Hsp27, which has an anti-apoptotic function (Sakamoto et al., 2002).

14.8 CYTOSKELETON

The cytoskeleton consists of proteins that cooperate in the structure and dynamics of a cell. These functions are cell shape, cell movement, cell replication, cell signaling, cell differentiation, and apoptosis. The cytoskeleton of eukaryotic cells has three major components:

1. Actin that forms microfilaments. These structures play a pivotal role in maintaining cell morphology and integrity of plasma membrane (Stossel,

1989). Filamin and fodrin, as well as myosin, are proteins associated with actin filaments.

2. Microtubules composed of alpha, beta tubulin, and microtubule-associated proteins, such as the well-known kinesin and dynein, or a key component of neuronal cytoskeleton, the tau protein (Kosik, 1990).
3. Intermediate filament proteins classified in five different subtypes. One of them, lamin, is a component of the supporting nucleus structure (Shelton et al., 1982); vimentin is present in mesenchymal cells, and synemin is present in muscle cells (Granger and Lazarides, 1980; Gabbiani et al., 1981).

One of the most studied cytoskeleton proteins is actin. In vitro assays with purified actin showed that hypochlorous acid, which is biologically produced by macrophages during the respiratory burst, affects the Cys374 residue and some Met residues. This leads to loss of actin dynamics and filament disruption. Actin exposed to chloramine T showed modification of Met residues 44, 47, and 355 to the sulfoxide derivatives. In addition oxidation of Met residues 176, 190, and 269 causes inhibition of actin polymerization and destabilize actin filaments, which depolymerize (Dalle-Donne et al., 2001a, 2002).

One of the most documented modifications of actin filaments is the formation of glutathionyl derivatives. A proteomic analysis of glutathionylated proteins during oxidative stress by diamide revealed that actin is one of the specific proteins targeted (Lind et al., 2002). These results have also been reported in oxidatively stressed human T lymphocytes (Fratelli et al., 2002). In this work, in addition to actin, other cytoskeletal proteins such as vimentin, myosin, tropomyosin, cofilin, and profilin were also detected as glutathionylation targets.

It is interesting that besides these in vitro approaches, oxidative modifications of cytoskeleton proteins have been reported to occur in several neurodegenerative pathologies. Actin modification has been described in brains of patients with AD (Aksenov, 2001) and in mouse models of ALS (Collard et al., 1995). Also nitration of actin has been observed in an inflammatory response after injection of *E. coli* lipopolysaccharide (Aulak et al., 2001). Carbonyl formation in actin was observed in post-ischemic rat hearts, which resulted in a significant loss of contractile function (Powell et al., 2001). Direct observation of oxidized actin has been reported in erythrocyte cytoskeleton of patients with sickle cell anemia (Bencsath et al., 1996). In fact red blood cells loose plasticity due to formation of an intramolecular Cys285–Cys374 disulfide bridge, which is responsible for an anomalous stabilization of membrane cytoskeleton (Shartava et al., 1995, 1997). For several years it has been thought that glutaredoxin and thioredoxin systems would be responsible for reduction of this disulfide bridge. Nevertheless, studies carried out on yeast cells demonstrated that the disulfide bridge between Cys285–Cys374 on yeast actin is reduced by Oye2p (Old yellow enzyme), a NADPH reductase, which is highly induced under oxidative stress (Haarer and Amberg, 2004).

Fibroblasts of patients with Friedreich's ataxia also show glutathionylated actin (Pastore et al., 2003). In this pathology, frataxin, a protein involved in

iron metabolism is present at very low levels. As a consequence, these patients accumulate iron in mitochondria. It is conceivable that in these cells and tissues, iron can promote oxidative stress, which could explain neurodegenerative process observed in these patients. Oxidative modification of actin will affect dynamics of cytoskeleton since glutathionylated actin has reduced capacity to polymerize (Dalle-Donne et al., 2003), and it has been suggested that Cys374 is the most likely glutathionylation site in vivo (Wang et al., 2001). Although the answer to how these modifications can perturb actin dynamics is still uncertain, a model of oxidative stress mediated modifications was proposed (Dalle-Donne et al., 2001b). There has been some controversy about the mechanism of glutathionylation of actin in vivo (Dalle-Donne et al., 2003; Wang et al., 2001). Clear evidence points out that glutathionylation of Cys residues does not proceed by a thiol disulfide exchange mechanism with GSSG, but via the formation of an activated form of specific thiol groups of the protein, which then react with the reduced glutathione (Dalle-Donne et al., 2003). This is mainly based on the highly abundance of GSH under physiological conditions compared to oxidized glutathione. Nevertheless, cells constantly exposed to stress situations that could be generated endogenously (e.g., by high levels of iron) can unbalance the ratio GSH/GSSG. Oxidized glutathione could then react with free protein thiols.

Although the primary effects on actin oxidation are modifications at Cys residues, oxidation of six Met residues to methionine sulfoxide has also been reported (Milzani et al., 2000). At this point it is important to comment an interesting aspect concerning the significance of oxidation of Met residues based on elegant experiments carried out with glutamine synthetase as a model (Levine et al., 1996, 1999). The authors proposed that accessible Met residues could act as endogenous antioxidants of proteins. They found that 8 out of the 16 Met residues can be oxidized to Met-sulfoxide with mild effect on enzyme activity. Given that Met-sulfoxide can be repaired by methionine sulfoxide reductases, cycles of oxidation/reduction can take place, and thus the net reaction would be the neutralization of reactive species. As the authors mentioned, this mechanism could be the "last chance antioxidant defense system for proteins" (Levine et al., 1996).

Another important component of cytoskeleton is tubulin, a heterodimer of alpha and beta subunits, which are components of the microtubules, in association to MAPs—microtubule-associated proteins (Anderson, 1979). Tubulin can be oxidatively modified in vitro by peroxynitrite, inducing the formation of inter- and intrasubunit disulfide bridges (Landino et al., 2002), which are responsible for the inhibition of microtubule polymerization.

As cited above, an important and exciting question rises from the fact that oxidation of such abundant proteins could be envisaged as radical sinks (Landino et al., 2004a,b). The authors suggest that this buffering capacity would protect other targets sensitive to reactive oxygen species. The system could be very effective provided that disulfide bridges or Met-sulfoxide have specific repair systems: glutaredoxin and thioredoxin systems or methionine sulfoxide reductase.

14.9 CONCLUSIONS

From this review there are several points that deserve a comment. The first one concerns the selectivity of protein targets. Why only few proteins are affected by oxidative stress? Why in some cases several types of modifications occurred simultaneously on the same protein? Why a specific amino acid in a particular position in a protein can be modified in several ways? There is not an easy way to answer these questions. It could be merely a consequence of protein structure. One might think that the presence of highly reactive groups that are necessary for catalytic activity (in the case of enzymes) would be prone to modification. Additionally protein-bound transition metals are sources of free radicals that attack surrounding amino acids. Alternatively, the presence of these groups could have been evolutively favored to inactivate proteins mainly with two purposes: either to preserve their integrity (e.g., reversible *S*-thiolation) or to avoid an undesirable function (e.g., impairment of glycolytic flux to favor pentose phosphate pathway). In either case one might think that preserving cell viability is the main goal.

Proximity to the site of reactive species generation seems to be especially relevant to protein oxidation. One of the most representative examples is generation of ROS at the respiratory chain complexes. Some of the complex I subunits are specifically damaged by peroxynitrite produced as a consequence of superoxide generation from that complex.

It is clear from the most recent publications that proteomics has been increasingly used to analyze protein modifications occurring in vivo. This approach has a clear applicability to diseases where oxidative stress is involved in their pathophysiology. The challenge in the near future will be to analyze how these protein modifications affect the whole cell metabolism, in other words, the metabolome. This will give clues to understand the real impact to the cell.

LIST OF ABBREVIATIONS

3NT, 3-nitrotyrosine
AD, Alzheimer's disease
ADH, alcohol dehydrogenase
ADHE, alcohol dehydrogenase E
AGE, advanced glycation end-products
ALS, amyotrophic lateral sclerosis
DTT, dithiothreitol
ER, endoplasmic reticulum
FBP aldolase, fructose 1,6-bisphosphate aldolase
GAPDH, glyceraldehyde 3-phosphate dehydrogenase
Gpx, glutathione peroxidase
GSH, reduced glutathione
GSSG, oxidized glutathione

HD, Huntington's disease
HNE, 4-hydroxy-2-nonenal
Hsp, heat-shock protein
α-KGDHC, alpha-ketoglutarate dehydrogenase complex
LC, liquid chromatography
LDH, lactate dehydrogenase
MALDI-TOF, matrix-assisted laser desorption ionization–time of flight
MDH, malate dehydrogenase
MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
PD, Parkinson's disease
PDC, pyruvate decarboxylase
PDHC, pyruvate dehydrogenase complex
PHGpx, phospholipid hydroperoxide Gpx
POR, propanediol oxidoreductase
RNS, reactive nitrogen species
ROS, reactive oxygen species
SOD, superoxide dismutase
TCA, tricarboxylic acid
TPI, triose phosphate isomerase

REFERENCES

Abrahams J.P., Leslie A.G.W., Lutter R., Walker J.E. (1994). The structure of F1-ATPase from bovine heart mitochondria determined at 2.8 Å resolution. *Nature* 370: 621–628.

Ackrell B.A. (2002). Cytopathies involving mitochondrial complex II. *Mol Aspects Med* 23: 369–384.

Ahmed M.U., Thorpe S.R., Baynes J.W. (1986). Identification of *N*-epsilon-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. *J Biol Chem* 261: 4889–4894.

Ahmed M.U., Brinkmann Frye E., Degenhardt T.P., Thorpe S.R., Baynes J.W. (1997). *N*-epsilon-(carboxyethyl)lysine, a product of the chemical modification of proteins by methylglyoxal, increases with age in human lens proteins. *Biochem J* 324: 565–570.

Aksenov M.Y., Aksenova M.V., Butterfield D.A., Geddes J.W., Markesbery W.R. (2001). Protein oxidation in the brain in Alzheimer's disease. *Neuroscience* 103: 373–383.

Albers D.S., Swerdlow R.H., Manfredi G., Gajewski C., Yang L., Parker W.D. Jr, Beal M.F. (2001). Further evidence for mitochondrial dysfunction in progressive supranuclear palsy. *Exp Neurol* 168: 196–198.

Anderson P.J. (1979). The structure and amount of tubulin in cells and tissues. *J Biol Chem* 254: 2168–2171.

Asahi M., Fujii J., Suzuki K., Seo H.G., Kuzuya T., Hori M., Tada M., Fujii S., Taniguchi N. (1995). Inactivation of glutathione peroxidase by nitric oxide. Implication for cytotoxicity. *J Biol Chem* 270: 21035–21039.

Asahi M., Fujii J., Takao T., Kuzuya T., Hori M., Shimonishi Y., Taniguchi N. (1997). The oxidation of selenocysteine is involved in the inactivation of glutathione peroxidase by nitric oxide donor. *J Biol Chem* 272: 19152–19157.

Aulak K.S., Miyagi M., Yan L., West K.A., Massillon D., Crabb J.W., Stuehr D.J. (2001). Proteomic method identifies proteins nitrated in vivo during inflammatory challenge. *Proc Natl Acad Sci USA* 98: 12056–12061.

Avery A.M., Avery S.V. (2001). *Saccharomyces cerevisiae* expresses three phospholipid hydroperoxide glutathione peroxidases. *J Biol Chem* 276: 33730–33735.

Avery A.M., Willetts S.A., Avery S.V. (2004). Genetic dissection of the phospholipid hydroperoxidase activity of yeast gpx3 reveals its functional importance. *J Biol Chem* 279: 46652–46658.

Barreiro E., Gea J., Di Falco M., Kriazhev L., James S., Hussain S.N. (2005). Protein carbonyl formation in the diaphragm. *Am J Respir Cell Mol Biol* 32: 9–17.

Bencsath F.A., Shartava A., Monteiro C.A., Goodman S.R. (1996). Identification of the disulfide-linked peptide in irreversibly sickled cell beta-actin. *Biochemistry* 35: 4403–4408.

Berlett B.S., Stadtman E.R. (1997). Protein oxidation in aging, disease and oxidative stress. *J Biol Chem* 272: 20313–20316.

Betarbet R., Sherer T.B., MacKenzie G., Garcia-Osuna M., Panov A.V., Greenamyre J.T. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat Neurosci* 3: 1301–1306.

Beyer W.F. Jr, Fridovich I. (1985). Pseudocatalase from *Lactobacillus plantarum*: Evidence for a homopentameric structure containing two atoms of manganese per subunit. *Biochemistry* 24: 6460–6467.

Bienvenut W.V., Deon C., Pasquarello C., Campbell J.M., Sanchez J.C., Vestal M.L., Hochstrasser D.F. (2002). Matrix-assisted laser desorption/ionization–tandem mass spectrometry with high resolution and sensitivity for identification and characterization of proteins. *Proteomics* 2: 868–876.

Bogaert Y.E., Rosenthal R.E., Fiskum G. (1994). Postischemic inhibition of cerebral cortex pyruvate dehydrogenase. *Free Radic Biol Med* 16: 811–820.

Borras C., Sastre J., Garcia-Sala D., Lloret A., Pallardo F.V., Vina J. (2003). Mitochondria from females exhibit higher antioxidant gene expression and lower oxidative damage than males. *Free Radic Biol Med* 34: 546–552.

Boyer P.D. (1997). The ATP synthase—A splendid molecular machine. *Ann Rev Biochem* 66: 717–749.

Brigelius-Flohé R. (1999). Tissue-specific functions of individual glutathione peroxidases. *Free Radic Biol Med* 27: 951–965.

Brigelius-Flohe R., Wingler K., Muller C. (2002). Estimation of individual types of glutathione peroxidases. *Methods Enzymol* 347: 101–112.

Brown G.C. (1995). Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase. *FEBS Lett* 369: 136–139.

Bu G., Schwartz A.L. (1998). RAP, a novel type of ER chaperone. *Trends Cell Biol* 8: 272–276.

Buchczyk D.P., Briviba K., Hartl F.U., Sies H. (2000). Responses to peroxynitrite in yeast: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a sensitive intracellular target for nitration and enhancement of chaperone expression and ubiquitination. *Biol Chem* 381: 121–126.

Butterfield D.A., Kanski J. (2001). Brain protein oxidation in age-related neurodegenerative disorders that are associated with aggregated proteins. *Mech Ageing Dev* 122: 945–962.

Cabiscol E., Aguilar J., Ros J. (1994). Metal-catalyzed oxidation of Fe^{2+} dehydrogenases: Consensus target sequence between propanediol oxidoreductase of *Escherichia coli* and alcohol dehydrogenase II of *Zymomonas mobilis*. *J Biol Chem* 269: 6592–6597.

Cabiscol E., Badia J., Baldoma L., Hidalgo E., Aguilar J., Ros J. (1992). Inactivation of propanediol oxidoreductase of *Escherichia coli* by metal-catalyzed oxidation. *Biochim Biophys Acta* 1118: 155–160.

Cabiscol E., Belli G., Tamarit J., Echave P., Herrero E., Ros J. (2002). Mitochondrial Hsp60, resistance to oxidative stress, and the labile iron pool are closely connected in *Saccharomyces cerevisiae*. *J Biol Chem* 277: 44531–44538.

Cabiscol E., Piulats E., Echave P., Herrero E., Ros J. (2000). Oxidative stress promotes specific protein damage in *Saccharomyces cerevisiae*. *J Biol Chem* 275: 27393–27398.

Cardoso S.M., Proenca M.T., Santos S., Santana I., Oliveira C.R. (2004). Cytochrome c oxidase is decreased in Alzheimer's disease platelets. *Neurobiol Aging* 25: 105–110.

Castegna A., Aksenov M., Thongboonkerd V., Klein J.B., Pierce W.M., Booze R., Markesbery W.R., Butterfield D.A. (2002). Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part II: Dehydropyrimidinase-related protein, α-enolase and heat shock cognate 71. *J Neurochem* 82: 1524–1532.

Castegna A., Thongboonkerd V., Klein J.B., Lynn B., Markesbery W.R., Butterfield D.A. (2003). Proteomic identification of nitrated proteins in Alzheimer's disease brain. *J Neurochem* 85: 1394–1401.

Castro L., Rodríguez M., Radi R. (1994). Aconitase is readily inactivated by peroxynitrite, but not by its precursor, nitric oxide. *J Biol Chem* 269: 29409–29415.

Cheng M.Y., Hartl F.U., Horwich A.L. (1990). The mitochondrial chaperonin hsp60 is required for its own assembly. *Nature* 348: 455–458.

Chiosis G., Vilenchik M., Kim J., Solit D. (2004). Hsp90: The vulnerable chaperone. *Drug Discov Today* 9: 881–888.

Chuang W.J., Heldt J., Van Wart H.E. (1989). Resonance Raman spectra of bovine liver catalase compound II: Similarity of the heme environment to horseradish peroxidase compound II. *J Biol Chem* 264: 14209–14215.

Chuang W.J., Van Wart H.E. (1992). Resonance Raman spectra of horseradish peroxidase and bovine liver catalase compound I species: Evidence for predominant 2A2u pi-cation radical ground state configurations. *J Biol Chem* 267: 13293–13301.

Clark W.M., Rinker L.G., Lessov N.S., Lowery S.L., Cipolla M.J. (2001). Efficacy of antioxidant therapies in transient focal ischemia in mice. *Stroke* 32: 1000–1004.

Collard J.F., Cote F., Julien J.P. (1995). Defective axonal transport in a transgenic mouse model of amyotrophic lateral sclerosis. *Nature* 375: 61–64.

Colussi C., Albertini M.C., Coppola S., Rovidati S., Galli F., Ghibelli L. (2000). H2O2-induced block of glycolysis as an active ADP-ribosylation reaction protecting cells from apoptosis. *FASEB J* 14: 2266–2276.

Costa V.M., Amorim M.A., Quintanilha A., Moradas-Ferreira P. (2002). Hydrogen peroxide-induced carbonylation of key metabolic enzymes in *Saccharomyces cerevisiae*: The involvement of the oxidative stress response regulators Yap1 and Skn7. *Free Radic Biol Med* 33: 1507–1515.

Crawford D.R., Davies K.J.A. (1994). Adaptive response and oxidative stress. *Environ Health Perspect* 102: 25–28.

Curti D., Rognoni F., Gasparini L., Cattaneo A., Paolillo M., Racchi M., Zani L., Bianchetti A., Trabucchi M., Bergamaschi S., Govoni S. (1997). Oxidative metabolism in cultured fibroblasts derived from sporadic Alzheimer's disease (AD) patients. *Neurosci Lett* 236: 13–16.

Dalle-Donne I., Giustarini D., Rossi R., Colombo R., Milzani A. (2003). A. Reversible *S*-glutathionylation of Cys374 regulates actin filament formation by inducing structural changes in the actin molecule. *Free Radic Biol Med* 34: 23–32.

Dalle-Donne I., Rossi R., Giustarini D., Gagliano N., Di Simplicio P., Colombo R., Milzani A. (2002). Methionine oxidation as a major cause of the functional impairment of oxidized actin. *Free Radic Biol Med* 32: 927–937.

Dalle-Donne I., Rossi R., Giustarini D., Gagliano N., Lusini L., Milzani A., Di Simplicio P., Colombo R. (2001a). Actin carbonylation: From a simple marker of protein oxidation to relevant signs of severe functional impairment. *Free Radic Biol Med* 31: 1075–1083.

Dalle-Donne I., Rossi R., Milzani A., Di Simplicio P., Colombo R. (2001b). The actin cytoskeleton response to oxidants: from small heat shock protein phosphorylation to changes in the redox state of actin itself. *Free Radic Biol Med* 31: 1624–1632.

Davey G.P., Canevari L., Clark J.B. (1997). Threshold effects in synaptosomal and non-synaptic mitochondria from hippocampal CA1 and paramedian neocortex brain regions. *J Neurochem* 69: 2564–2570.

Davey G.P., Peuchen S., Clark J.B. (1998). Energy thresholds in brain mitochondria: Potential involvement in neurodegeneration. *J Biol Chem* 273: 12753–12757.

de la Torre J.C., Cada A., Nelson N., Davis G., Sutherland R.J., Gonzalez-Lima F. (1997). Reduced cytochrome oxidase and memory dysfunction after chronic brain ischemia in aged rats. *Neurosci Lett* 223: 165–168.

Dean R.T., Gieseg S., Davies M.J. (1993). Reactive species and their accumulation on radical-damaged proteins. *Trends Biochem Sci* 18: 437–441.

Delaunay A., Pflieger D., Barrault M.B., Vinh J., Toledano M.B. (2002). A thiol peroxidase is an H_2O_2 receptor and redox-transducer in gene activation. *Cell* 111: 471–481.

Deloulme J.C., Helies A., Ledig M., Lucas M., Sensenbrenner M. (1997). A comparative study of the distribution of alpha- and gamma-enolase subunits in cultured rat neural cells and fibroblasts. *Int J Dev Neurosci* 15: 183–194.

DeLuca D.C., Dennis R., Smith W.G. (1995). Inactivation of an animal and a fungal catalase by hydrogen peroxide. *Arch Biochem Biophys* 320: 129–134.

Denke E., Merbitz-Zahradnik T., Hatzfeld O.M., Snyder C.H., Link T.A., Trumpower B.L. (1998). Alteration of the midpoint potential and catalytic activity of the rieske-iron sulfur protein by changes of amino acids forming hydrogen bonds to the iron-sulfur clusters. *J Biol Chem* 273: 9085–9093.

Derham B.K., Harding J.J. (2002). Effects of modifications of alpha-crystallin on its chaperone and other properties. *Biochem J* 364: 711–717.

Dimmeler S., Lottspeich F., Brune B. (1992). Nitric oxide causes ADP-ribosylation and inhibition of glyceraldehyde-3-phosphate dehydrogenase. *J Biol Chem* 267: 16771–16774.

Dougan D.A., Mogk A., Bukau B. (2002). Protein folding and degradation in bacteria: To degrade or not to degrade? That is the question. *Cell Mol Life Sci* 59: 1607–1616.

Dröge W. (2002). Free radicals in the physiological control of cell function. *Physiol Rev* 82: 47–95.

Dukan S., Nyström T. (1998). Bacterial senescence: Stasis results in increased and differential oxidation of cytoplasmic proteins leading to developmental induction of the heat shock regulon. *Genes Dev* 12: 3431–3441.

Dunn A.Y., Melville M.W., Frydman J. (2001). Cellular substrates of the eukaryotic chaperonin TRiC/CCT. *J Struct Biol* 135: 176–184.

Eaton P., Byers H.L., Leeds N., Ward M.A., Shattock M.J. (2002). Detection, quantitation, purification, and identification of cardiac proteins *S*-thiolated during ischemia and reperfusion. *J Biol Chem* 277: 9806–9811.

Eaton P., Jones M.E., McGregor E., Dunn M.J., Leeds N., Byers H.L., Leung K.Y., Ward M.A., Pratt J.R., Shattock M.J. (2003). Reversible cysteine-targeted oxidation of proteins during renal oxidative stress. *J Am Soc Nephrol* 14: S290–S296.

Echave P., Esparza-Ceron M.A., Cabiscol E., Tamarit J., Ros J., Membrillo-Hernandez J., Lin E.C. (2002). DnaK dependence of mutant ethanol oxidoreductases evolved for aerobic function and protective role of the chaperone against protein oxidative damage in *Escherichia coli*. *Proc Natl Acad Sci USA* 99: 4626–4631.

Echave P., Tamarit J., Cabiscol E., Ros J. (2003). Novel antioxidant role of alcohol dehydrogenase E from *Escherichia coli*. *J Biol Chem* 278: 30193–30198.

Ellgaard L., Molinari M., Helenius A. (1999). Setting the standards: Quality control in the secretory pathway. *Science* 286: 1882–1888.

England K., O'Driscoll C., Cotter T.G. (2004). Carbonylation of glycolytic proteins is a key response to drug-induced oxidative stress and apoptosis. *Cell Death Differ* 11: 252–260.

Esterbauer H., Schaur R.J., Zollner H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* 11: 81–128.

Fenton W.A., Horwich A.L. (1997). GroEL-mediated protein folding. *Protein Sci* 6: 743–760.

Ferrante R.J., Hantraye P., Brouillet E., Beal M.F. (1999). Increased nitrotyrosine immunoreactivity in substantia nigra neurons in MPTP treated baboons is blocked by inhibition of neuronal nitric oxide synthase. *Brain Res* 823: 177–182.

Flint D.H., Smyk R.E., Tuminello J.F., Draczynska L.B., Brown O.R. (1993). The inactivation of dihydroxy-acid dehydratase in *Escherichia coli* treated with hyperbaric oxygen occurs because of the destruction of its Fe–S cluster, but the enzyme remains in the cell in a form that can be reactivated. *J Biol Chem* 268: 25547–25552.

Fratelli M., Demol H., Puype M., Casagrande S., Eberini I., Salmona M., Bonetto V., Mengozzi M., Duffieux F., Miclet E., Bachi A., Vandekerckhove J., Gianazza E., Ghezzi P. (2002). Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. *Proc Natl Acad Sci USA* 99: 3505–3510.

Freeman B.C., Morimoto R.I. (1996). The human cytosolic molecular chaperones hsp90, hsp70 (hsc70) and hdj-1 have distinct roles in recognition of a non-native protein and protein refolding. *EMBO J* 15: 2969–2979.

Fridovich I. (1995). Superoxide radical and superoxide dismutases. *Ann Rev Biochem* 64: 97–112.

Fridovich I. (2004). Mitochondria: Are they the seat of senescence? *Aging Cell* 3: 13–16.

Fukuchi T., Katayama Y., Kamiya T., McKee A., Kashiwagi F., Terashi A. (1998). The effect of duration of cerebral ischemia on brain pyruvate dehydrogenase activity, energy metabolites, and blood flow during reperfusion in gerbil brain. *Brain Res* 792: 59–65.

Gabbiani G., Schmid E., Winter S., Chaponnier C., de Ckhastonay C., Vandekerckhove J., Weber K., Franke W.W. (1981). Vascular smooth muscle cells differ from other smooth muscle cells: Predominance of vimentin filaments and a specific alpha-type actin. *Proc Natl Acad Sci USA* 78: 298–302.

Gardner P.R., Costantino G., Szabo C., Salzman A.L. (1997). Nitric oxide sensitivity of the aconitases. *J Biol Chem* 272: 25071–25076.

Gardner P.R., Fridovich I. (1991). Superoxide sensitivity of the *Escherichia coli* aconitase. *J Biol Chem* 266: 19328–19333.

Gardner P.R., Nguyen D.D., White C.W. (1994). Aconitase is a sensitive and critical target of oxygen poisoning in cultured mammalian cells and in rat lungs. *Proc Natl Acad Sci USA* 91: 12248–12252.

Gardner P.R., Raineri I., Epstein L.B., White C.W. (1995). Superoxide radical and iron modulate aconitase activity in mammalian cells. *J Biol Chem* 270: 13399–13405.

Gibson G.E., Park L.C.H., Zhang H., Sorbi S., Calingasan N.Y. (1999). Oxidative stress and a key metabolic enzyme in Alzheimer brains, cultures cells, and an animal model of chronic oxidative deficits. *Ann NY Acad Sci* 893: 79–94.

Gillies N.E., Alper T. (1959). Reduction in the lethal effects of radiations on *Escherichia coli* beta by treatment with chloramphenicol. *Nature* 183: 237–238.

Glomb M.A., Monnier V.M. (1995). Mechanism of protein modification by glyoxal and glycolaldehyde, reactive intermediates of the Maillard reaction. *J Biol Chem* 270: 10017–10026.

Glover J.R., Lindquist S. (1998). Hsp104, Hsp70, and Hsp40: A novel chaperone system that rescues previously aggregated proteins. *Cell* 94: 73–82.

Granger B.L., Lazarides E. (1980). Synemin: A new high molecular weight protein associated with desmin and vimentin filaments in muscle. *Cell* 22: 727–738.

Grant C.M., Quinn K.A., Dawes I.W. (1999). Differential protein *S*-thiolation of glyceraldehyde-3-phosphate dehydrogenase isoenzymes influences sensitivity to oxidative stress. *Mol Cell Biol* 19: 2650–2656.

Guarente L., Kenyon C. (2000). Genetic pathways that regulate ageing in model organisms. *Nature* 408: 255–262.

Guertl B., Noehammer C., Hoefler G. (2000). Metabolic cardiomyopathies. *Int J Exp Pathol* 81: 349–372.

Haarer B.K., Amberg D.C. (2004). Old yellow enzyme protects the actin cytoskeleton from oxidative stress. *Mol Biol Cell* 15: 4522–4531.

Halliwell B., Gutteridge J.M.C. (1999). *Free Radicals in Biology and Medicine*, 3rd ed., Oxford University Press, Oxford.

Hartl F.U., Hayer-Hartl M. (2002). Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* 295: 1852–1858.

Heikkila R.E., Manzino L., Cabbat F.S., Duvoisin R.C. (1984). Protection against the dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine by monoamine oxidase inhibitors. *Nature* 311: 467–469.

Hinerfeld D., Traini M.D., Weinberger R.P., Cochran B., Doctrow S.R., Harry J., Melov S. (2004). Endogenous mitochondrial oxidative stress: Neurodegeneration, proteomic analysis, specific respiratory chain defects, and efficacious antioxidant therapy in superoxide dismutase 2 null mice. *J Neurochem* 88: 657–667.

Hodgson E.K., Fridovich I. (1975). The interaction of bovine erythrocyte superoxide dismutase with hydrogen peroxide: Inactivation of the enzyme. *Biochemistry* 14: 5294–5299.

Horecker B.L., Tsolas O.J., Lai C.Y. (1972). Aldolases. In: *The Enzymes, Vii*, 3rd ed., Academic Press, New York, pp. 213–258.

Horwitz J. (1992). Alpha-crystallin can function as a molecular chaperone. *Proc Natl Acad Sci USA* 89: 10449–10453.

Humpries K.M., Szweda L.I. (1998). Selective inactivation of alpha-ketoglutarate dehydrogenase and pyruvate dehydrogenase: Reaction of lipoic acid with 4-hydroxy-2-nonenal. *Biochemistry* 37: 15835–15841.

Inoue Y., Matsuda T., Sugiyama K., Izawa S., Kimura A. (1999). Genetic analysis of glutathione peroxidase in oxidative stress response of *Saccharomyces cerevisiae*. *J Biol Chem* 274: 27002–27009.

Ishii T., Tatsuda E., Kumazawa S., Nakayama T., Uchida K. (2003). Molecular basis of enzyme inactivation by an endogenous electrophile 4-hydroxy-2-nonenal: Identification of modification sites in glyceraldehyde-3-phosphate dehydrogenase. *Biochemistry* 42: 3474–3480.

Jamieson D.J. (1998). Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. *Yeast* 14: 1511–1527.

Jha N., Jurma O., Lalli G., Liu Y., Pettus E.H., Greenamyre J.T., Liu R.M., Forman H.J., Andersen J.K. (2000). Glutathione depletion in PC12 results in selective inhibition of mitochondrial complex I activity: Implications for Parkinson's disease. *J Biol Chem* 275: 26096–26101.

Jordan P.A., Tang Y., Bradbury A.J., Thomson A.J., Guest J.R. (1999). Biochemical and spectroscopic characterization of *Escherichia coli* aconitases (AcnA and AcnB). *Biochem J* 344: 739–746.

Kanski J., Behring A., Pelling J., Schoneich C. (2005). Proteomic identification of 3-nitrotyrosine-containing rat cardiac proteins: Effects of biological aging. *Am J Physiol Heart Physiol* 288: 371–381.

Kashiwaya Y., Takeshima T., Mori N., Nakashima K., Clarke K., Veech R.L. (2000). D-beta-hydroxybutyrate protects neurons in models of Alzheimer's and Parkinson's disease. *Proc Natl Acad Sci USA* 97: 5440–5444.

Kaufman R.J. (1999). Stress signaling from the lumen of the endoplasmic reticulum: Coordination of gene transcriptional and translational controls. *Genes Dev* 13: 1211–1233.

Kessler D., Leibrecht I., Knappe J. (1991). Pyruvate-formate-lyase-deactivase and acetyl-CoA reductase activities of *Escherichia coli* reside on a polymeric protein particle encoded by adhE. *FEBS Lett* 281: 59–63.

Keuder J., Borkhardt A., Repp R., Pekrun A., Göttsche B., Gottschalk U., Reichmann H., Schachmayer W., Schlegel K., Lampert F. (1996). Brief report: Inherited metabolic myopathy and hemolysis due to a mutation in aldolase A. *N Engl J Med* 334: 1100–1104.

Keyer K., Imlay J.A. (1997). Inactivation of dehydratase [4Fe–4S] clusters and disruption of iron homeostasis upon cell exposure to peroxynitrite. *J Biol Chem* 272: 27652–27659.

Khor H.K., Fischer M.T., Schöneich C. (2004). Potential role of methionine sulfoxide in the inactivation of the chaperone GroEL by hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$). *J Biol Chem* 279: 19486–19493.

Kim S.Y., Kwon O.J., Park J.W. (2001). Inactivation of catalase and superoxide dismutase by singlet oxygen derived from photoactivated dye. *Biochimie* 83: 437–444.

Kinter M., Roberts R.J. (1996). Glutathione consumption and glutathione peroxidase inactivation in fibroblast cell lines by 4-hydroxy-2-nonenal. *Free Radic Biol Med* 21: 457–462.

Koeck T., Levinson B., Hazen S.L., Crabb J.W., Stuehr D.J., Aulak K.S. (2004a). Tyrosine nitration impairs mammalian aldolase A activity. *Mol Cell Proteomics* 3: 548–557.

Koeck T., Fu X., Hazen S.L., Crabb J.W., Stuehr D.J., Aulak K.S. (2004b). Rapid and selective oxygen-regulated protein tyrosine denitration and nitration in mitochondria. *J Biol Chem* 279: 27257–27262.

Koh Y.H., Suzuki K., Che W., Park Y.S., Miyamoto Y., Higashiyama S., Taniguchi N. (2001). Inactivation of glutathione peroxidase by NO leads to the accumulation of H_2O_2 and the induction of HB-EGF via c-Jun NH2-terminal kinase in rat aortic smooth muscle cells. *FASEB J* 15: 1472–1474.

Kono Y., Fridovich I. (1982). Superoxide radical inhibits catalase. *J Biol Chem* 257: 5751–5754.

Kosik K.S. (1990). Tau protein and neurodegeneration. *Mol Neurobiol* 4: 171–179.

Kots A.T., Skurat A.V., Sergienko E.A., Bulgarina T.V., Severin E.S. (1992). Nitroprusside stimulates the cysteine-specific mono(ADP-ribosylation) of glyceraldehyde-3-phosphate dehydrogenase from human erythrocytes. *FEBS Lett* 300: 9–12.

Landino L.M., Hasan R., McGaw A., Cooley S., Smith A.W., Masselam K., Kim G. (2002). Peroxynitrite oxidation of tubulin sulfhydryls inhibits microtubule polymerization. *Arch Biochem Biophys* 398: 213–220.

Landino L.M., Iwig J.S., Kennett K.L., Moynihan K.L. (2004b). Repair of peroxynitrite damage to tubulin by the thioredoxin reductase system. *Free Radic Biol Med* 36: 497–506.

Landino L.M., Moynihan K.L., Todd J.V., Kennett K.L. (2004a). Modulation of the redox state of tubulin by the glutathione/glutaredoxin reductase system. *Biochem Biophys Res Commun* 314: 555–560.

Lardinois O.M. (1995). Reactions of bovine liver catalase with superoxide radicals and hydrogen peroxide. *Free Radic Res* 22: 251–274.

Lardinois O.M., Mestdagh M.M., Rouxhet P.G. (1996). Reversible inhibition and irreversible inactivation of catalase in presence of hydrogen peroxide. *Biochim Biophys Acta* 1295: 222–238.

Lee J., Gordon C., Lagniel G., Spector D., Garin J., Labarre J., Toledano M.B. (1999). Yap1 and Skn7 control two specialized oxidative stress regulons in yeast. *J Biol Chem* 274: 16040–16046.

Leskovak V., Trivic S., Pericin D. (2002). The three zinc-containing alcohol dehydrogenases from baker's yeast, *Saccharomyces cerevisiae*. *FEMS Yeast Res* 2: 481–494.

Lesnefsky E.J., Chen Q., Moghaddas S., Hassan M.O., Tandler B., Hoppel C.L. (2004). Blockade of electron transport during ischemia protects cardiac mitochondria. *J Biol Chem* 279: 47961–47967.

Lesnefsky E.J., Gudz T.I., Migita C.T., Ikeda-Saito M., Hassan M.O., Turkaly M.J., Hoppel C.L. (2001). Ischemic injury to mitochondrial electron transport in the aging heart: Damage to the iron-sulfur protein subunit of electron transport complex III. *Arch Biochem Biophys* 385: 117–128.

Levine R.L. (1983a). Oxidative modification of glutamine synthetase. I. Inactivation is due to loss of one histidine residue. *J Biol Chem* 258: 11823–11827.

Levine R.L. (1983b). Oxidative modification of glutamine synthetase. II. Characterization of the ascorbate model system. *J Biol Chem* 258: 11828–11833.

Levine R.L., Berlett B.S., Moskovitz J., Mosoni L., Stadtman E.R. (1999). Methionine residues may protect proteins from critical oxidative damage. *Mech Ageing Develop* 107: 323–332.

Levine R.L., Mosoni L., Berlett B.S., Stadtman E.R. (1996). Methionine residues as endogenous antioxidants in proteins. *Proc Natl Acad Sci USA* 93: 15036–15040.

Levine R.L., Oliver C.N., Fulks R.M., Stadtman E.R. (1981). Turnover of bacterial glutamine synthetase: oxidative inactivation precedes proteolysis. *Proc Natl Acad Sci USA* 78: 2120–2124.

Liang L.P., Patel M. (2004). Iron-sulfur enzyme mediated mitochondrial superoxide toxicity in experimental Parkinson's disease. *J Neurochem* 90: 1076–1084.

Lin S.S., Manchester J.K., Gordon J.I. (2001). Enhanced gluconeogenesis and increased energy storage as hallmarks of aging in *Saccharomyces cerevisiae*. *J Biol Chem* 276: 36000–36007.

Lind C., Gerdes R., Hamnell Y., Schuppe-Koistinen I., von Lowenhielm H.B., Holmgren A., Cotgreave I.A. (2002). Identification of *S*-glutathionylated cellular proteins during oxidative stress and constitutive metabolism by affinity purification and proteomic analysis. *Arch Biochem Biophys* 406: 229–240.

Llorca O., Galan A., Carrascosa J.L., Muga A., Valpuesta J.M. (1998). GroEL under heat-shock: Switching from a folding to a storing function. *J Biol Chem* 273: 32587–32594.

Long X., Goldenthal MJ, Wu GM, Marin-Garcia J (2004). Mitochondrial Ca^{2+} flux and respiratory enzyme activity decline are early events in cardiomyocyte response to H_2O_2. *J Mol Cell Cardiol* 37: 63–70.

Lu Z, Cabiscol E., Obradors N., Tamarit J., Ros J., Aguilar J., Lin E.C. (1998). Evolution of an *Escherichia coli* protein with increased resistance to oxidative stress. *J Biol Chem* 273: 8308–8316.

Lund P. (2001). *Molecular Chaperones in the cell*, Oxford University Press, New York.

Lynch J., Michalak M. (2003). Calreticulin is an upstream regulator of calcineurin. *Biochem Biophys Res Commun* 311: 1173–1179.

MacMillan-Crow L.A., Crow J.P., Kerby J.D., Beckman J.S., Thompson J.A. (1996). Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. *Proc Natl Acad Sci USA* 93: 11853–11858.

MacMillan-Crow L.A., Crow J.P., Thompson J.A. (1998). Peroxynitrite-mediated inactivation of manganese superoxide dismutase involves nitration and oxidation of critical tyrosine residues. *Biochemistry* 37: 1613–1622.

Mager W.H., Ferreira P.M. (1993). Stress response of yeast. *Biochem J* 290: 1–13.

Magi B., Ettorre A., Liberatori S., Bini L., Andreassi M., Frosali S., Neri P., Pallini V., Di Stefano A. (2004). Selectivity of protein carbonylation in the apoptotic response to oxidative stress associated with photodynamic therapy: A cell biochemical and proteomic investigation. *Cell Death Differ* 11: 842–852.

Markesbery W.R. (1997). Oxidative stress hypothesis in Alzheimer's disease. *Free Rad Biol Med* 23: 134–147.

Marsh J.J., Lebherz H.G. (1992). Fructose-bisphosphate aldolases: An evolutionary history. *Trends Biochem Sci* 17: 110–113.

Martin E., Rosenthal R.E., Fiskum G. (2005). Pyruvate dehydrogenase complex: Metabolic link to ischemic brain injury and target of oxidative stress. *J Neurosci Res* 79: 240–247.

Mayer M., Kies U., Kammermeier R., Buchner J. (2000a). BiP and PDI cooperate in the oxidative folding of antibodies in vitro. *J Biol Chem* 275: 29421–29425.

Mayer M.P., Rudiger S., Bukau B. (2000b). Molecular basis for interactions of the DnaK chaperone with substrates. *Biol Chem* 381: 877–885.

McAlister L., Holland M.J. (1985). Isolation and characterization of yeast strains carrying mutations in the glyceraldehyde-3-phosphate dehydrogenase genes. *J Biol Chem* 260: 15013–15018.

Melkani G.H., McNamara C., Zardeneta G., Mendoza J.A. (2004). Hydrogen peroxide induces the dissociation of GroEL into monomers that can facilitate the reactivation of oxidatively inactivated rhodanese. *Int J Biochem Cell Biol* 36: 505–518.

Membrillo-Hernandez J., Echave P., Cabiscol E., Tamarit J., Ros J., Lin E.C.C. (2000). Evolution of the *adhE* gene product of *Escherichia coli* from a functional reductase to a dehydrogenase: Genetic and biochemical studies of the mutant proteins. *J Biol Chem* 275: 33869–33875.

Miki T., Yu L., Yu C.A. (1991). Hematoporphyrin-promoted photoinactivation of mitochondrial ubiquinol-cytochrome c reductase: Selective destruction of the histidine ligands of the iron-sulfur cluster and protective effect of ubiquinone. *Biochemistry* 30: 230–238.

Milzani A., Rossi R., Di Simplicio P., Giustarini D., Colombo R., DalleDonne I. (2000). The oxidation produced by hydrogen peroxide on Ca-ATP-G-actin. *Protein Sci* 9: 1774–1782.

Minakami H., Fridovich I. (1990). Relationship between growth of *Escherichia coli* and susceptibility to the lethal effect of paraquat. *FASEB J* 4: 3239–3244.

Mirzaei H., Regnier F. (2005). Affinity chromatographic selection of carbonylated proteins followed by identification of oxidation sites using tandem mass spectrometry. *Anal Chem* 77: 2386–2392.

Miyagi M., Sakaguchi H., Darrow R.W., Yan L., West K.A., Aulak K.S., Stuehr D.J., Hollyfield J.G., Organisciak D.T., Crabb W. (2002). Evidence that light modulates protein nitration in rat retina. *Mol Cell Proteomics* 1: 293–303.

Mizuno Y., Ikebe S., Hattori N, Nakagawa-Hattori Y., Mochizuki H., Tanaka M., Ozawa T. (1995). Role of mitochondria in the etiology and pathogenesis of Parkinson's disease. *Biochim Biophys Acta* 1271: 265–274.

Mizuno Y., Matsuda S., Yoshino H., Mori H., Hattori N., Ikebe S.I. (1994). An immunohistochemical study on a-ketoglutarate dehydrogenase complex in Parkinson's disease. *Ann Neurol* 35: 204–210.

Mogk A., Deuerling E., Vorderwulbecke S., Vierling E., Bukau B. (2003). Small heat shock proteins, ClpB and the DnaK system form a functional triade in reversing protein aggregation. *Mol Microbiol* 50: 585–595.

Mogk A., Tomoyasu T., Goloubinoff P., Rudiger S., Roder D., Langen H., Bukau B. (1999). Identification of thermolabile *Escherichia coli* proteins: Prevention and reversion of aggregation by DnaK and ClpB. *EMBO J* 18: 6934–6949.

Montella C., Bellsolell L., Perez-Luque R., Badia J., Baldoma L., Coll M., Aguilar J. (2005). Crystal structure of an iron-dependent group III dehydrogenase that interconverts L-lactaldehyde and L-1,2-propanediol in *Escherichia coli*. *J Bacteriol* 187: 4957–4966.

Morgan P.E., Dean R.T., Davies M.J. (2002a). Inhibition of glyceraldehyde-3-phosphate dehydrogenase by peptide and protein peroxides generated by singlet oxygen attack. *Eur J Biochem* 269: 1916–1925.

Morgan P.E., Dean R.T., Davies M.J. (2002b). Inactivation of cellular enzymes by carbonyls and protein-bound glycation/glycoxidation products. *Arch Biochem Biophys* 403: 259–269.

Morita T., Kawamoto H., Mizota T., Inada T., Aiba H. (2004). Enolase in the RNA degradosome plays a crucial role in the rapid decay of glucose transporter mRNA in the response to phosphosugar stress in *Escherichia coli*. *Mol Microbiol* 54: 1063–1075.

Murray J., Taylor S.W., Zhang B., Ghosh S.S., Capaldi R.A. (2003). Oxidative damage to mitochondrial complex I due to peroxynitrite: Identification of reactive tyrosines by mass spectrometry. *J Biol Chem* 278: 37223–37230.

Mutisya E.M., Bowling A.C., Beal M.F. (1994). Cortical cytochrome oxidase activity is reduced in Alzheimer's disease. *J Neurochem* 63: 2179–2184.

Nagaraj R.H., Oya-Ito T., Padayatti P.S., Kumar R., Mehta S., West K., Levison B., Sun J., Crabb J.W., Padival A.K. (2003). Enhancement of chaperone function of alpha-crystallin by methylglyoxal modification. *Biochemistry* 42: 10746–10755.

Nardai G., Sass B., Eber J., Orosz G., Csermely P. (2000). Reactive cysteines of the 90-kDa heat shock protein, Hsp90. *Arch Biochem Biophys* 384: 59–67.

Nicholls P., Schonbaum G.R. (1963). Catalases. In: Boyer P.D., Lardy H., Myrback K. (eds)., *The Enzymes*, Vol. 8, Academic Press, Orlando, pp. 147–225.

Niwa T., Tsukushi S. (2001). 3-deoxyglucosone and AGEs in uremic complications: Inactivation of glutathione peroxidase by 3-deoxyglucosone. *Kidney Int* 78: S37–S41.

Nyström T. (1994). The glucose starvation stimulon of Escherichia coli: Induced and repressed synthesis of enzymes of central metabolic pathways and the role of acetyl-phosphate in gene expression and starvation survival. *Mol Microbiol* 12: 833–843.

O'Brien K.M., Dirmeier R., Engle M., Poyton R.O. (2004). Mitochondrial protein oxidation in yeast mutants lacking manganese-(MnSOD) or copper- and zinc-containing superoxide dismutase (CuZnSOD): Evidence that MnSOD and CuZnSOD have both unique and overlapping functions in protecting mitochondrial proteins from oxidative damage. *J Biol Chem* 279: 51817–51827.

Ookawara T., Kawamura N., Kitagawa Y., Taniguchi N. (1992). Site-specific and random fragmentation of Cu,Zn-superoxide dismutase by glycation reaction. Implication of reactive oxygen species. *J Biol Chem* 267: 18505–18510.

Ottani A., Saltini S., Bartiromo M., Zaffe D., Renzo B.A., Ferrari A., Bertolini A., Genedani S. (2003). Effect of γ-hydroxybutyrate in two rat models of focal cerebral damage. *Brain Res* 986: 181–190.

Oya T., Hattori N., Mizuno Y., Miyata S., Maeda S., Osawa T., Uchida K. (1999). Methyl glyoxal modification of protein. *J Biol Chem* 274: 18492–18502.

Padmaja S., Squadrito G.L., Pryor W.A. (1998). Inactivation of glutathione peroxidase by peroxynitrite. *Arch Biochem Biophys* 349: 1–6.

Pancholi V. (2001). Multifunctional alpha-enolase: Its role in diseases. *Cell Mol Life Sci* 58: 902–920.

Park L.C., Zhang H., Sheu K.F., Calingasan N.Y., Kristal B.S., Lindsay J.G., Gibson G.E. (1999). Metabolic impairment induces oxidative stress, compromises inflammatory responses, and inactivates a key mitochondrial enzyme in microglia. *J Neurochem* 72: 1948–1958.

Parker W.D. Jr, Boyson S.J., Parks J.K. (1989). Abnormalities of the electron transport chain in idiopathic Parkinson's disease. *Ann Neurol* 26: 719–723.

Parker W.D. Jr, Filley C.M., Parks J.K. (1990). Cytochrome oxidase deficiency in Alzheimer's disease. *Neurology* 40: 1302–1303.

Pastore A., Tozzi G., Gaeta L.M., Bertini E., Serafini V., Di Cesare S., Bonetto V., Casoni F., Carrozzo R., Federici G., Piemonte F. (2003). Actin glutathionylation increases in fibroblasts of patients with Friedreich's ataxia: A potential role in the pathogenesis of the disease. *J Biol Chem* 278: 42588–42595.

Patel M., Day P.J., Crapo J.D., Fridovich I., McNamara J.O. (1996). Requirement for superoxide in excitotoxic cell death. *Neuron* 16: 345–355.

Pennathur S., Jackson-Lewis V., Przedborski S., Heinecke J.W. (1999). Mass spectrometric quantification of 3-nitrotyrosine, *ortho*-tyrosine, and *o*,*o*′-dityrosine in brain tissue of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice, a model of oxidative stress in Parkinson's disease. *J Biol Chem* 274: 34621–34628.

Perry T.L., Godin D.V., Hansen S. (1982). Parkinson's disease: A disorder due to nigral glutathione deficiency? *Neurosci Lett* 33: 305–310.

Piper P.W. (2001). The Hsp90 chaperone as a promising drug target. *Curr Opin Investig Drugs* 2: 1606–1610.

Pocernich C.B., Butterfield D.A. (2003). Acrolein inhibits NADH-linked mitochondrial enzyme activity: Implications for Alzheimer's disease. *Neurotox Res* 5: 515–520.

Portero-Otin M., Pamplona R., Bellmunt M.J., Ruiz M.C., Prat J., Salvayre R., Negre-Salvayre A. (2002). Advanced glycation end product precursors impair epidermal growth factor receptor signaling. *Diabetes* 51: 1535–1542.

Powell S.R., Gurzenda E.M., Wahezi S.E. (2001). Actin is oxidized during myocardial ischemia. *Free Radic Biol Med* 30: 1171–1176.

Pulsinelly W.A., Levy D.E., Duffy T.E. (1982). Regional cerebral blood flow and glucose metabolism following transient forebrain ischemia. *Ann Neurol* 11: 499–502.

Py B., Higgins C.F., Krisch H.M., Carpousis A.J. (1996). A DEAD-box RNA helicase in the Escherichia coli RNA degradosome. *Nature* 381: 169–172.

Rabek J.P., Boylston W.H. 3rd, Papaconstantinou J. (2003). Carbonylation of ER chaperone proteins in aged mouse liver. *Biochem Biophys Res Commun* 305: 566–572.

Ravichandran V., Seres T., Moriguchi T., Thomas J.A., Johnston R.B. Jr (1994). *S*-Thiolation of glyceraldehyde-3-phosphate dehydrogenase induced by the phagocytosis-associated respiratory burst in blood monocytes. *J Biol Chem* 269: 25010–25015.

Reid M.F., Fewson C.A. (1994). Molecular characterization of microbial alcohol dehydrogenases. *Crit Rev Microbiol* 20: 13–56.

Reverter-Branchat G., Cabiscol E., Tamarit J., Ros J. (2004). Oxidative damage to specific proteins in replicative and chronological-aged *Saccharomyces cerevisiae*: Common targets and prevention by calorie restriction. *J Biol Chem* 279: 31983–31989.

Riobo N.A., Clementi E., Melani M., Boveris A., Cadenas E., Moncada S., Poderoso J.J. (2001). Nitric oxide inhibits mitochondrial NADH:ubiquinone reductase activity through peroxynitrite formation. *Biochem J* 359: 139–145.

Rivera-Nieves J., Thompson W.C., Levine R.L., Moss J. (1999). Thiols mediate superoxide-dependent NADH modification of glyceraldehyde-3-phosphate dehydrogenase. *J Biol Chem* 274: 19525–19531.

Roche T.E., Patel M.S. (editors) (1989). Alpha-keto acid dehydrogenase complexes: Organization, regulation, and biomedical ramifications. *Ann NY Acad Sci* 573: 1–474.

Rodriguez-Manzaneque M.T., Tamarit J., Belli G., Ros J., Herrero E. (2002). Grx5 is a mitochondrial glutaredoxin required for the activity of iron/sulfur enzymes. *Mol Biol Cell* 13: 1109–1121.

Rouslin R. (1983). Mitochondrial complexes I, II, III, IV, and V in myocardial ischemia and autolysis. *Am J Physiol* 244: H743–H748.

Rudiger S., Germeroth L., Schneider-Mergener J., Bukau B. (1997). Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries. *EMBO J* 16: 1501–1507.

Rutkowski D.T., Kaufman R.J. (2004). A trip to the ER: coping with stress. *Trends Cell Biol* 14: 20–28.

Sadek H.A., Humphries K.M., Szweda P.A., Szweda L.I. (2002). Selective inactivation of redox-sensitive mitochondrial enzymes during cardiac reperfusion. *Arch Biochem Biophys* 406: 222–228.

Sakamoto H., Mashima T., Yamamoto K., Tsuruo T. (2002). Modulation of heat-shock protein 27 (Hsp27) anti-apoptotic activity by methylglyoxal modification. *J Biol Chem* 277: 45770–45775.

Salo D.C., Pacifici R.E., Lin S.W., Giulivi C., Davies K.J. (1990). Superoxide dismutase undergoes proteolysis and fragmentation following oxidative modification and inactivation. *J Biol Chem* 265: 11919–11927.

Saulle E., Gubellini P., Picconi B., Centonze D., Tropepi D., Pisani A., Morari M., Marti M., Rossi L., Papa M., Bernardi G., Calabresi P. (2004). Neuronal vulnerability following inhibition of mitochondrial complex II: A possible ionic mechanism for Huntington's disease. *Mol Cell Neurosci* 25: 9–20.

Schapira A.H., Cooper J.M., Dexter D., Clark J.B., Jenner P., Marsden C.D. (1990). Mitochondrial complex I deficiency in Parkinson's disease. *J Neurochem* 54: 823–827.

Schellhorn H.E. (1995). Regulation of hydroperoxidase (catalase) expression in *Escherichia coli*. *FEMS Microbiol Lett* 131: 113–119.

Schirmer E.C., Glover J.R., Singer M.A., Lindquist S. (1996). HSP100/Clp proteins: A common mechanism explains diverse functions. *Trends Biochem Sci* 21: 289–296.

Schoder H., Knight R.J., Kofoed K.F., Schelbert H.R., Buxton D.B. (1998). Regulation of pyruvate dehydrogenase activity and glucose metabolism in post-ischemic myocardium. *Biochim Biophys Acta* 1406: 62–72.

Schroder M., Kaufman R.J. (2005). ER stress and the unfolded protein response. *Mutat Res* 569: 29–63.

Schuppe-Koistinen I., Moldeus P., Bergman T., Cotgreave I.A. (1994). *S*-Thiolation of human endothelial cell glyceraldehyde-3-phosphate dehydrogenase after hydrogen peroxide treatment. *Eur J Biochem* 221: 1033–1037.

Seaver L.C., Imlay J.A. (2001). Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*. *J Bacteriol* 183: 7173–7181.

Seo H., Sonntag K.C., Isacson O. (2004). Generalized brain and skin proteasome inhibition in Huntington's disease. *Ann Neurol* 56: 319–328.

Shamsi F.A., Partal A., Sady C., Glomb M.A., Nagaraj R.H. (1998). Immunological evidence for methylglyoxal-derived modifications in vivo: Determination of antigenic epitopes. *J Biol Chem* 273: 6928–6936.

Shanmuganathan A., Avery S.V., Willetts S.A., Houghton J.E. (2004). Copper-induced oxidative stress in *Saccharomyces cerevisiae* targets enzymes of the glycolytic pathways. *FEBS Let* 556: 253–259.

Shartava A., Korn W., Shah A.K., Goodman S.R. (1997). Irreversibly sickled cell beta-actin: Defective filament formation. *Am J Hematol* 55: 97–103.

Shartava A., Monteiro C.A., Bencsath F.A., Schneider K., Chait B.T., Gussio R., Casoria-Scott L.A., Shah A.K., Heuerman C.A., Goodman S.R. (1995). A posttranslational modification of beta-actin contributes to the slow dissociation of the spectrin-protein 4.1-actin complex of irreversibly sickled cells. *J Cell Biol* 128: 805–818.

Shelton K.R., Guthrie V.H., Cochran D.L. (1982). Oligomeric structure of the major nuclear envelope protein lamin B. *J Biol Chem* 257: 4328–4332.

Shenton D., Grant C.M. (2003). Protein *S*-thiolation targets glycolysis and protein synthesis in response to oxidative stress in yeast *S. cerevisiae*. *Biochem J* 374: 513–519.

Shenton D., Perrone G., Quinn K.A., Dawes I.W., Grant C.M. (2002). Regulation of protein *S*-thiolation by glutaredoxin 5 in the yeast *Saccharomyces cerevisiae*. *J Biol Chem* 277: 16853–16859.

Sheu K.F., Kim Y.T., Blass J.P., Weksler M.E. (1985). An immunochemical study of the pyruvate dehydrogenase deficit in Alzheimer's disease brain. *Ann Neurol* 17: 444–449.

Shin S.J., Lee S.E., Boo J.H., Kim M., Yoon Y.D., Kim S.I., Mook-Jung I. (2004). Profiling proteins related to amyloid deposited brain of Tg2576 mice. *Proteomics* 4: 3359–3368.

Sirover M.A. (1999). New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. *Biochim Biophys Acta* 1432: 159–184.

Sriram K., Shankar S.K., Boyd M.R., Ravindranath V. (1998). Thiol oxidation and loss of mitochondrial complex I precede excitatory amino acid-mediated neurodegeneration. *J Neurosci* 18: 10287–10296.

Stadtman E.R. (1990). Metal ion-catalyzed oxidation of proteins: Biochemical mechanism and biological consequences. *Free Radical Biol Med* 9: 315–325.

Stock D., Leslie A.G.W., Walker J.E. (1999). Molecular architecture of the rotary motor in ATP synthase. *Science* 286: 1700–1705.

Storz G., Imlay J.A. (1999). Oxidative stress. *Curr Opin Microbiol* 2: 188–194.

Stossel T.P. (1989). From signal to pseudopod: How cells control cytoplasmic actin assembly. *J Biol Chem* 264: 18261–18264.

Sturtz L.A., Diekert K., Jensen L.T., Lill R., Culotta V.C. (2001). A fraction of yeast Cu,Zn-superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria: A physiological role for SOD1 in guarding against mitochondrial oxidative damage. *J Biol Chem* 276: 38084–38089.

Sykes K., Gething M.J., Sambrook J. (1993). Proline isomerases function during heat shock. *Proc Natl Acad Sci USA* 90: 5853–5857.

Tabatabaie T., Potts J.D., Floyd R.A. (1996). Reactive oxygen species-mediated inactivation of pyruvate dehydrogenase. *Arch Biochem Biophys* 336: 290–296.

Tabrizi S.J., Cleeter M.W., Xuereb J., Taanman J.W., Cooper J.M., Schapira A.H. (1999). Biochemical abnormalities and excitotoxicity in Huntington's disease brain. *Ann Neurol* 45: 25–32.

Tamarit J., Cabiscol E., Aguilar J., Ros J. (1997). Differential inactivation of alcohol dehydrogenase isoenzymes in *Zymomonas mobilis* by oxygen. *J Bacteriol* 179: 1102–1104.

Tamarit J., Cabiscol E., Ros J. (1998). Identification of the major oxidatively damaged proteins in *Escherichia coli* cells exposed to oxidative stress. *J Biol Chem* 273: 3027–3032.

Taylor S.W., Fahy E., Murray J., Capaldi R.A., Ghosh S.S. (2003). Oxidative post-translational modification of tryptophan residues in cardiac mitochondrial proteins. *J Biol Chem* 278: 19587–19590.

Toledano M.B., Delaunay A., Biteau B., Spector D., Azevedo D. (2003). Oxidative stress responses in yeast. In: Hohmann S, Mager P.W.H (eds.), *Topics in Current Genetics*. Vol. 1, Springer-Verlag, Berlin, pp. 241–303.

Tu B.P., Ho-Schleyer S.C., Travers K.J., Weissman J.S. (2000). Biochemical basis of oxidative protein folding in the endoplasmic reticulum. *Science* 290: 1571–1574.

Uchida K. (2003). Histidine and lysine as targets of oxidative modification. *Amino Acids* 25: 249–257.

Uchida K., Stadtman E.R. (1992). Modification of histidine residues in proteins by reaction with 4-hydroxynonenal. *Proc Natl Acad Sci USA* 89: 4544–4548.

Uchida K., Stadtman E.R. (1993). Covalent attachment of 4-hydroxynonenal to glyceraldehyde-3-phosphate dehydrogenase: A possible involvement of intra- and intermolecular cross-linking reaction. *J Biol Chem* 268: 6388–6393.

Ueta H., Nagasawa H., Oyabu-Manabe Y., Toida K., Ishimura K., Hori H. (2004). Localization of enolase in synaptic plasma membrane as an alphagamma heterodimer in rat brain. *Neurosci Res* 48: 379–386.

van der Vlies D., Woudenberg J., Post J.A. (2003). Protein oxidation in aging: Endoplasmic reticulum as a target. *Amino Acids* 25: 397–407.

van der Worp H.B., Kappelle L.J., Algra A., Bar P.R., Orgogozo J.M., Ringelstein E.B., Bath P.M., van Gijn J. (2002). The effect of tirilazad mesylate on infarct volume of patients with acute ischemic stroke. *Neurology* 58: 133–135.

Vasquez-Vivar J., Kalyanaraman B., Kennedy M.C. (2000). Mitochondrial aconitase is a source of hydroxyl radical. An electron spin resonance investigation. *J Biol Chem* 275: 14064–14069.

Veinger L., Diamant S., Buchner J., Goloubinoff P. (1998). The small heat-shock protein IbpB from *Escherichia coli* stabilizes stress-denatured proteins for subsequent refolding by a multichaperone network. *J Biol Chem* 273: 11032–11037.

Veitch K., Hombreckx A., Caucheteux D., Pouleur H., Hue L. (1992). Global ischaemia induces a biphasic response of the mitochondrial respiratory chain: Anoxic pre-perfusion protects against ischaemic damage. *Biochem J* 281: 709–715.

Verniquet F., Gaillard J., Neuburger M., Douce R. (1991). Rapid inactivation of plant aconitase by hydrogen peroxide. *Biochem J* 276: 643–648.

Viner R.I., Ferrington D.A., Williams T.D., Bigelow D.J., Schoneich C. (1999a). Protein modification during biological aging: Selective tyrosine nitration of the SERCA2a isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase in skeletal muscle. *Biochem J* 340: 657–669.

Viner R.I., Williams T.D., Schoneich C. (1999b). Peroxynitrite modification of protein thiols: Oxidation, nitrosylation, and S-glutathiolation of functionally important cysteine residue(s) in the sarcoplasmic reticulum Ca-ATPase. *Biochemistry* 38: 12408–12415.

Wang C.C., Tsou C.L. (1993). Protein disulfide isomerase is both an enzyme and a chaperone. *FASEB J* 7: 1515–1517.

Wang J., Boja E.S., Tan W., Tekle E., Fales H.M., English S., Mieyal J.J., Chock P.B. (2001). Reversible glutathionylation regulates actin polymerization in A431 cells. *J Biol Chem* 276: 47763–47766.

Wells-Knecht K.J., Zyzak D.V., Litchfield J.E., Thorpe S.R., Baynes J.W. (1995). Mechanism of autoxidative glycosylation: Identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose. *Biochemistry* 34: 3702–3709.

Wen J.J., Garg N. (2004). Oxidative modification of mitochondrial respiratory complexes in response to the stress of *Trypanosoma cruzi* infection. *Free Radic Biol Med* 37: 2072–2081.

Wiedau-Pazos M., Goto J.J., Rabizadeh S., Gralla E.B., Roe J.A., Lee M.K., Valentine J.S., Bredesen D.E. (1996). Altered reactivity of superoxide dismutase in familial amyotrophic lateral sclerosis. *Science* 271: 515–518.

Yan H., Harding J.J. (1997). Glycation-induced inactivation and loss of antigenicity of catalase and superoxide dismutase. *Biochem J* 328: 599–605.

Yan L.J., Levine R.L., Sohal R.S. (1997). Oxidative damage during aging targets mitochondrial aconitase. *Proc Natl Acad Sci USA* 94: 11168–11172.

Yankovskaya V., Horsefield R., Tornroth S., Luna-Chavez C., Miyoshi H., Leger C., Byrne B., Cecchini G., Iwata S. (2003). Architecture of succinate dehydrogenase and reactive oxygen species generation. *Science* 299: 700–704.

Yim M.B., Kang J.H., Yim H.S., Kwak H.S., Chock P.B., Stadtman E.R. (1996). A gain-of-function of an amyotrophic lateral sclerosis-associated Cu,Zn-superoxide dismutase mutant: An enhancement of free radical formation due to a decrease in Km for hydrogen peroxide. *Proc Natl Acad Sci USA* 93: 5709–5714.

Yoo B.S., Regnier F.E. (2004). Proteomic analysis of carbonylated proteins in two-dimensional gel electrophoresis using avidin-fluorescein affinity staining. *Electrophoresis* 25: 1334–1341.

Young J.C., Moarefi I., Hartl F.U. (2001). Hsp90: A specialized but essential protein-folding tool. *J Cell Biol* 154: 267–273.

Zahavi M., Tahori A.S. (1965). Citric acid accumulation with age in houseflies and other diptera. *J Insect Physiol* 11: 811–816.

Zaidan E., Sims N.R. (1997). Reduced activity of the pyruvate dehydrogenase complex but not cytochrome c oxidase is associated with neuronal loss in the striatum following short-term forebrain ischemia. *Brain Res* 772: 23–28.

Zerbini L.F., Wang Y., Czibere A., Correa R.G., Cho J.Y., Ijiri K., Wei W., Joseph M., Gu X., Grall F., Goldring M.B., Zhou J.R., Libermann T.A. (2004). NF-kappaB-mediated repression of growth arrest- and DNA-damage-inducible proteins 45alpha and gamma is essential for cancer cell survival. *Proc Natl Acad Sci USA* 101: 13618–13623.

Zhang J., Snyder S.H. (1992). Nitric oxide stimulates auto-ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase. *Proc Natl Acad Sci USA* 89: 9382–9385.

Zhang R., Brennan M.L., Fu X., Aviles R.J, Pearce G.L., Penn M.S., Topol E.J., Sprecher D.L., Hazen S.L. (2001). Association between myeloperoxidase levels and risk of coronary artery disease. *JAMA* 286: 2136–2142.

Zolkiewski M. (1999). ClpB cooperates with DnaK, DnaJ, and GrpE in suppressing protein aggregation. A novel multi-chaperone system from *Escherichia coli*. *J Biol Chem* 274: 28083–28086.

Zwemer C.F., Whitesall S.E., D'Alecy L.G. (1994). Cardiopulmonary-cerebral resuscitation with 100% oxygen exacerbates neurological dysfunction following nine minutes of normothermic cardiac arrest in dogs. *Resuscitation* 27: 159–170.

15

OXIDATIVE DAMAGE AND CELLULAR SENESCENCE: LESSONS FROM BACTERIA AND YEAST

THOMAS NYSTRÖM

15.1 MICROBIAL SENESCENCE

Exponentially growing bacterial populations are not age structured, and there is no adult form of cells. A bacterial "mother" cell ceases being a mother at the time of cytokinesis and becomes two "daughters" of the same age. Thus, an exponentially growing cell's generation time can be described but the concept of a life span for such dividing individual microbes is ambiguous.

On the other hand, the terms life span, longevity, and cellular age can be useful when microbial cells enter a growth-arrested state (stationary phase) due to, for example, carbon depletion. Progressive physiological alterations can be followed as a function of the chronological age of such individual stationary phase cells, and the life span of these cells is finite. For example, the average life span, measured as the time of sustained reproductive ability, of stationary phase *E. coli* and *S. cerevisae* cells is around four and seven days, respectively, when starved for exogenous carbon (e.g., Ericsson et al., 2000; Ballesteros et al., 2001; Fabrizio and Longo, 2003). Such a loss in reproductive ability during stasis has been argued to be the nearest microbes come to a "natural" death of the kind familiar among higher organisms (Postgate, 1976). Indeed, loss of reproductive ability of stationary phase yeast cells is often referred to as chronological aging. Even so, it should be stressed that it is a form of accidental death conceptually distinct from mandatory aging in higher organisms. In line with this notion scientists

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

dealing with prokaryotic stationary phase death have been more cautious and used the term conditional senescence to make a distinction between this phenomenon and mandatory aging (Nyström 1995, 1999, 2002a, b, 2003, 2004). All the same, many molecular alterations occurring during conditional senescence of stationary phase bacteria and yeast are similar to those of aging organisms (see below; Nyström, 1999, 2002a, b, 2003).

Apart from experiencing conditional senescence during starvation, the yeast *S. cerevisiae* belongs to the exclusive club of organisms displaying replicative senescence. Most laboratory strains of *S. cerevisiae* can complete, on average, 20 to 30 divisions followed by death. During these progressive divisions, the cells undergo age-related changes, including an increased generation time, increase in size, decline in mating ability, and accumulation of extrachromosomal rDNA circles (ERCs). Thus, with each division the mother cell becomes phenotypically older and more deteriorated but generates offspring exhibiting a full replicative potential. In other words, the acquired aging phenotype of the mother cell is somehow expunged in the progeny during the process of asymmetrical division (Jazwinski, 2004). This argues for the existence of a senescence factor that accumulates in the mother cells but is prevented from transmission to the offspring (Egilmez and Jazwinski, 1989). ERCs (Sinclair and Guarente, 1997) and dysfunctional mitochondria (Jazwinski, 2004) have been suggested to be such senescence factors, but conclusive evidence for one primary senescence factor in yeast is still lacking. Asymmetrical cytokinesis is not restricted to eukaryotic microbes, the bacteria *Caulobacter* is a prime example of an organism displaying asymmetrical cell morphologies and functions (stalk cells and swarmer cells) during vegetative reproduction and the stalk cells have, like yeast mother cells, been shown to show signs of aging as a function of repeated cell division (Ackermann et al., 2003).

This chapter reviews data that links microbial senescence (conditional and replicative) to oxidative damage of proteins, especially protein carbonylation. Where appropriate, protein carbonylation in the microbial model systems is related to data obtained for animal aging models and plants.

15.2 PROTEIN CARBONYLATION—AN IRREVERSIBLE OXIDATIVE DAMAGE TO PROTEINS

Proteins can become modified by a large number of reactions involving reactive oxygen species (ROS). Among these reactions, carbonylation has attracted a great deal of attention due to its presumably irreversible and unrepairable nature. Carbonyl derivatives are formed by a direct metal catalyzed oxidative (MCO) attack on the amino acid side chains of proline, arginine, lysine, and threonine. In addition, carbonyl derivatives on lysine, cysteine, and histidine can be formed by secondary reactions with reactive carbonyl compounds on carbohydrates (glycoxidation products), lipids, and advanced glycation/lipoxidation end-products (Dalle-Donne et al., 2003; Levine, 2002). A large number of studies have shown that

protein carbonylation increases with the age of cells, organelles, and tissues of varied species. Such an increase in protein carbonyls can be quite substantial, eventually reaching a level of one out of every third protein molecule carrying this modification (Stadtman and Levine, 2000). In view of the fact that carbonylation is reducing, or totally abrogating, the targeted proteins catalytic functions and triggers the formation of high molecular, potentially cytotoxic, aggregates, this modification is likely to perturb physiological activities in the aging organism. Indeed, carbonylation has in some cases been linked to age-dependent deterioration of specific enzymes, such as the aconitase and the nucleotide translocator ANT (e.g., Yan et al., 1997; Yan and Sohal, 1998). Diseases associated with increased carbonylation include Parkinson's disease, Alzheimer's disease, cancer, cataractogenesis, diabetes, and sepsis (e.g., Dalle-Donne et al., 2003; Levine, 2002). In some model organisms, the levels of carbonylated-damaged proteins have been shown to be associated with the physiological age or life expectancy rather than chronological age. For example, carbonyl levels are higher in crawlers (low life expectancy) than fliers in a cohort of houseflies of the same chronological age (Sohal et al., 1993). Moreover, specific environmental insults, such as irradiation (Jayko and Garrison 1958), hypochlorous acid (Headlam and Davies, 2004), and ozone exposure (Berlett et al., 1996) introduce carbonyl groups in proteins.

Aging-, starvation-, and stress-induced carbonylation does not affect the proteome uniformly. Examples of carbonylated proteins include the Hsp70 and Hsp60 chaperones, the histone-like protein, H-NS, elongation factors, EF-Tu and EF-G, glutamine synthetase, glutamate synthase, aconitase, malate dehydrogenase, and pyruvate kinase (Dukan and Nyström, 1998, 1999; Tamarit et al., 1998). Some of these proteins have been demonstrated to be specifically carbonylated in bacteria, oxidation-stressed yeast cells (Cabiscol et al., 2000), aging flies (Sohal, 2002; Yan et al., 1997), plants (Johansson et al., 2004; Kristensen et al., 2004), and Alzheimer's diseased brain (Castegna et al., 2002). Thus, there is a similar pattern of carbonylation in distantly related organisms. The molecular basis for the conserved sensitivity of some proteins to carbonylation is not understood, but it is likely that MCO is an intrinsic problem for proteins containing transition metals. However, only a few of the carbonylated proteins are known to bind metals.

It appears as the classical enzymes involved in ROS detoxification: namely superoxide dismutases, catalases, and peroxidases, are key members of the cellular defense also against protein carbonylation, but, in contrast to its essential role in attenuating illegitimate disulfide bond formation, the glutathione reductase system appears less important, at least in *E. coli* (Dukan and Nyström, 1998, 1999). Another important factor in the generation of protein carbonyls during aging and oxidative stress is the intracellular availability of free iron (Stadtman, 1992; Stadtman and Levine, 2000). For example, carbonylation levels are markedly higher in yeast mutants lacking the iron storage protein YFH1p, a homologue to the human frataxin (Desmyter et al., 2004). Interestingly, the human ferritin L gene can partly compensate for the lack of functional YFH1p; that is, it counteracts elevated carbonylation, and also prolongs the replicative life span of the

yeast cells (Desmyter et al., 2004). In yeast, increased carbonylation has also been linked to an increased tendency of the aging mitochondria to produce ROS rather than a diminished activity (or abundance) of antioxidant defense systems (Aguilaniu et al., 2001; see also Jazwinski, 2004).

Differential carbonylation during aging also relates to the activity of the cellular protease systems. It has been shown that the function of the proteasome decrease during aging in several human tissues as well as in senescent primary cultures, which may result in the accumulation of damaged proteins (Friguet et al., 2000; Shringarpure and Davies, 2002; Sitte et al., 2000). It has been suggested that this decreased proteolysis is a consequence of the accumulation of protease-resistant aggregates, "aggresomes," that bind to the proteasome (Grune et al., 2004). In other words, the aggresomes are argued to clog up the proteasomes, and as a consequence damaged (e.g., carbonylated) and potentially protease-susceptible substrates accumulate with time (Grune et al., 2004).

Recent data indicates that carbonylation of proteins may occur also in the absence of an increased ROS production, diminished ROS defense, elevated iron availability, or reduced protease activity. This pathway of carbonylation is rather linked to an increased production of substrates available for oxidative attack (see next section).

15.3 BACTERIAL SENESCENCE AND PROTEIN CARBONYLATION

In a reproductively arrested population of *E. coli* cells the levels of oxidative defense proteins increase, and the population become increasingly resistant to external oxidative stresses (Matin, 1991; Hengge-Aronis, 2002). Yet the levels of oxidation damaged proteins in such an *E. coli* population increase (Dukan and Nyström, 1998, 1999). In addition, it has been demonstrated that no strict correlation exists between respiratory activity and protein oxidation (or life span) in growth-arrested *E. coli* cells (Ballesteros et al., 2001). Similar results have been obtained with growth-arrested G_0 cells of the yeast *S. cerevisiae* (Aguilaniu et al., 2001). Thus, the rate of respiration in a nongrowing aerobic system does not, per se, determine the degree of oxidative damage to the proteins of the system.

Instead, the use of proteomics demonstrated that the sudden increase in protein oxidation during the early stages of senescence in *E. coli* is strongly associated with the production of aberrant protein isoforms, seen as protein stuttering on two-dimensional gels (Ballesteros et al., 2001). (The phenomenon called protein stuttering has been shown to be the result of erroneous incorporation of amino acids into proteins and can be detected on autoradiograms of two-dimensional gels as satellite spots with similar molecular weights to the authentic protein but separated from it in the isoelectric focusing dimension; O'Farrell, 1978.) Moreover, the level of protein carbonylation has been found to increase upon treatment of cells with antibiotics, such as streptomycin, causing mistranslation (Dukan et al., 2000). Other means of producing aberrant proteins have generated a similar increased oxidation of proteins. The conditions tested include (1) addition of

puromycin, which causes premature translation termination, (2) overproduction of a mutated 16S rRNA, which, when incorporated into ribosomes, render them prone to mistranslate, and (3) introduction of a mutation in *mutT,* causing a decreased transcriptional fidelity and increased mutation rates. During these treatments, the rate of superoxide production and the activities of the superoxide dismutases and catalases were unchanged, and the expression of oxidative stress defense genes did not increase (Dukan et al., 2000). In other words, protein oxidation of aberrant proteins does not appear to be sensed by the oxidative defense regulons and does not require increased generation of reactive oxygen species.

Frameshifting (Wenthzel et al., 1998; Barak et al., 1996), missense errors (O'Farrell, 1978), and stop codon read-through (Ballesteros et al., 2001) increase during senescence in *E. coli* cells. This fact, together with results showing that aberrant proteins are more susceptible to oxidation, raises the possibility that carbonylation in nonproliferating cells may be caused by an increased mistranslation. This notion was tested directly by assaying protein oxidation in a mutant strain (*rpsL141)* harboring intrinsically hyperaccurate ribosomes. Notably, this mutant retains its translational fidelity during stasis, and it was demonstrated that protein carbonylation is drastically attenuated in the early stages of stasis in the cells carrying the *rpsL141* allele (Ballesteros et al., 2001). Thus, the elevated oxidation of proteins in nonproliferating cells may be due to an increased availability of substrates (aberrant proteins) available for oxidative attack, and these substrates surge during stasis due to a reduced fidelity of the translational apparatus. It is not at present clear why aberrant proteins are more susceptible to carbonylation. Possibly a slight misfolding of the corrupted polypeptide exposes oxidation sensitive targets that are normally hidden during the coupled translation-folding process.

The rapid carbonylation of mistranslated or otherwise aberrant proteins points to an important physiological role of carbonylation in protein quality control. Since carbonylated proteins are more susceptible to proteolytic degradation than their nonoxidized counterparts (Grune et al., 2003, 2004; Bota and Davies, 2002; Dukan et al., 2000), the rapid carbonylation of an erroneous protein may ensure that such a polypeptide is directed to the proteolysis apparatus. This could effectively reduce incorporation of mistranslated proteins into mature machines (e.g., ribosomes and RNA and DNA polymerases) involved in information transfer. In line with this notion, carbonylated proteins generated as a result of increased mistranslation were found to be less stable than the average bulk protein (Dukan et al., 2000) and the degree of carbonylated proteins in mature ribosomes is small in healthy cells of *E. coli* (Desnues et al., 2003). However, carbonylation may be a mixed blessing, since heavily oxidized forms of proteins tend to form high molecular weight aggregates that escape degradation (Bota and Davies, 2002). It has been suggested that the decline in proteasomal activity during aging (e.g., Agarwal and Sohal, 1994; Bota et al., 2002) may in fact be closely connected to a gradual accumulation of proteolysis-resistant aggregates of oxidized proteins that bind and inhibit proteasomal function (Grune et al., 2004).

15.4 REPLICATIVE SENESCENCE AND SEGREGATION OF CARBONYLATED PROTEINS

Propagation of a cell's genetic endowment involves two distinct processes: mitosis and cytokinesis. The latter of these processes may proceed in a symmetrical or asymmetrical fashion. In *E. coli* this process is predominantly symmetrical, whereas the yeast *Saccharomyces cerevisiae* displays asymmetrical cytokinesis as daughter and mother cells exhibit distinct morphologies. In addition, some genetic material is unequally distributed during yeast cytokinesis. Specifically, the daughter cell does not inherit extrachromosomal rDNA circles. These ERCs accumulate in mother cells during growth and have been suggested to cause replicative senescence (Sinclair and Guarente, 1997). Cells lacking the silent information regulator (or sirtuin), Sir2p, an NAD-dependent histone deacetylase, contain more ERC than their wild-type counterparts and have a reduced replicative potential. In addition, elevated levels of Sir2p extend life span. Homologues of Sir2p are present in a wide range of organisms (from bacteria to mammals) and Sir2p has now been shown to be an aging determinant in organisms ranging from yeast, worms, and flies (Guarente, 2000; Sinclair, 2002; Tissenbaum and Guarente, 2001; Rogina and Helfand, 2004).

Apart from the accumulation of ERCs, age-related changes in dividing yeast include an increased generation time, increase in size, decline in mating ability, and reduced mitochondrial functions (Jazwinski, 2004). In addition, by a technique that combines dinitrophenyl hydrazine derivatization of protein carbonyls with immunocytochemistry to visualize oxidatively damaged proteins in situ, it was demonstrated that oxidized proteins accumulate with replicative age (Aguilaniu et al., 2003). The in situ technique, together with age fractionation experiments, demonstrated that carbonylated proteins are not inherited by daughter cells during mitotic cell division. The ability of mother cells to retain carbonylation-damaged proteins is diminished with the replicative age of the mother. Moreover, a screen for mutants with an abrogated ability to retain carbonylated proteins in the mother cell identified the sirtuin, Sir2p, as an essential component in segregating oxidative damage during mitosis (Aguilaniu et al., 2003).

The asymmetrical distribution of carbonylated proteins in mother and daughter cells is not an effect of differential proteolysis in the old and young cells (Aguilaniu et al., 2003). Instead, data point toward a true segregation mechanisms involving the cytoskeleton. Specifically, drugs that debilitate actin filament assembly inhibit damage segregation during cytokinesis (i.e., give a phenocopy of *sir2*), and actin staining indicates that *sir2* mutants are somewhat defective in actin filament organization during cytokinesis (Aguilaniu et al., 2003, supplementary material). Thus, it appears as the cytoskeleton has a dual function in ensuring that daughters inherit a full complement of genomic material and active mitochondria while preventing inheritance of old and damaged macromolecules. Future analysis may clarify whether damage segregation is restricted to asymmetrically dividing microbes or if this phenomenon is a common

feature also in eukaryotic development and cellular rejuvenation, such as during stem cell renewal or generation of the germ cell line.

15.5 YEAST SENESCENCE, PROTEIN OXIDATION, AND ONCOGENESIS

Many genes demonstrated to regulate yeast life span have proved to have mammalian homologues, some of which are implicated in tumorigenesis. For example, the human homologue of the *SIR2* gene mentioned above deacetylates and negatively controls the tumor suppressor p53, thereby promoting cell survival (Luo et al., 2001; Vaziri et al., 2001). On the other hand, an overactive form of p53 was recently shown to accelerate aging in mice (Tyner et al., 2002). This implies that there may be links between oncogenesis and senescence and *RAS* is another example of a mammalian oncogene that is present in yeast and acts as a gerontogene.

In mammalian cells, transformation of fibroblasts with the oncogenic allele of *RAS* (v-Ha-*RAS*) triggers rapid senescence in a p53 dependent way (Ferbeyre et al., 2002). Likewise a high PKA activity, caused by either the $RAS2^{val19}$ allele or *bcy1* loss-of-function mutations shortens the replicative life span of yeast (Sun et al., 1994), but the underlying molecular causation is not known. It has been shown that conditional senescing yeast cells undergo a respiratory state transition (reduced membrane ATPase activity) that generates an increased membrane potential, elevated ROS production, and increased oxidative damage to proteins (Aguilaniu et al., 2001). This behavior of the mitochondria was recently linked to the activity of the Ras pathway (Hlavata et al., 2003). Specifically, characterization of the oncogenic $RAS2^{val19}$ mutant with respect to its replicative life span, free radical biology, and oxidative metabolism revealed novel and hitherto unknown phenotypes of yeast cells expressing oncogenic *RAS*. These phenotypes include increased occurrence of petite mutants, increased membrane potential and endogenous production of ROS, elevated levels of carbonylated proteins, including mitochondrial proteins, and a nonphosphorylating activity of the mitochondrial electron transport chain. Furthermore, mitochondrial phosphorylating activity in the $RAS2^{val19}$ mutant was not linked to a constitutively high cAMP/PKA activity but could be restored by ectopic production of UCP1, a mammalian uncoupling protein. In addition, ectopic expression of UCP1 reduced protein carbonylation (Hlavata et al., 2003). However, the effect of the oncogenic *RAS* allele on the replicative life span is primarily asserted via the PKA-dependent pathway, since a reduction cAMP, but not UCP1 overproduction, suppressed premature aging of the $RAS2^{val19}$ mutant.

15.6 PERSPECTIVE

Animals display a seemingly unavoidable increase in carbonylated proteins, as they grow older (Levine, 2002). Moreover, the carbonyl content increases at

an accelerated rate in the last third of an animal's life span (Levine, 2002), namely after termination of the reproductive period. Thus, it is possible that carbonylation levels are important determinants in setting the pace of aging, but if the carbonylation load of young individuals is sufficiently low not to affect the fitness of the offspring, the increased load of damage of the soma in late life is impervious to natural selection. Thus, providing the soma with more proficient defense systems against protein carbonylation may make no evolutionary sense. However, vegetatively reproducing microbes, in which there is no distinction between soma and germ line, are faced with a different problem.

Reproduction of most prokaryotes is accomplished by binary fission. Cytokinesis proceeds in a symmetrical fashion with a nonconservative dispersion of cytoplasmic material and damaged constituents are distributed equally, more or less, to both cells produced. Thus, the cells produced are of the same age, there is no adult form, and the population is not age structured. The generation of damage and its removal is balanced during growth, and titration of damaged constituents is an important factor in keeping oxidative damage at bay. In fact, it appears as the rapid process of growth and binary fission in *E. coli* makes some primary oxidative defense systems superfluous; cells that lack all cytoplasmic superoxide dismutase activity grow remarkably well aerobically in rich media with a generation time only slightly reduced compared to wild-type cells (Carlioz and Touati, 1986). However, this mode of damage titration is only effective during rapid growth and cell proliferation. Growth arrest imparts a special problem with respect to oxidation management and *E. coli sod* mutants survive growth arrest extremely poorly (Dukan and Nyström, 1999; Benov and Fridovich, 1995). Interestingly, *E. coli* cells subjected to growth arrest breaks up into two populations exhibiting markedly different loads of carbonylated proteins (Desnues et al., 2003). The cells displaying low carbonyl levels remain reproductively competent, whereas cells with a high carbonyl load become genetically dead (nonculturable). Whether this starvation-induced heterogeneity in carbonylation and fitness is programmed or purely stochastic remains to be elucidated.

The yeast *S. cerevisiae* is, in contrast to *E. coli*, dividing in an asymmetrical fashion. This sets the scene for another possibility of ensuring fitness during vegetative reproduction. The yeast has evolved a Sir2p-dependent system that retains oxidatively damaged proteins in the mother cell compartment during mitotic cytokinesis (Aguilaniu et al., 2003). Thus the progeny, exhibiting a full reproductive potential in contrast to the mother cell, starts out with a markedly reduced load of damage compared to the ancestor cell. It would be interesting to know why natural selection has provided some yeasts with this type of cytokinesis, whereas other yeasts, such as *Schizosaccharomyces pombe*, have remained faithful to a symmetrical mode of fission.

If a high carbonyl load is a genuine hazard to fitness, organisms producing reproductive organs at the closing stages of their life should have evolved means of keeping protein damage low throughout their life span or, alternatively, be equipped with systems that can clear out damage prior to reproduction. Interestingly, the plant *Arabidopsis thaliana* has evolved defense systems to do just

that (Johansson et al., 2003). Carbonylation first increases with the age of the plant, similar to animals but drops abruptly prior to the vegetative to reproductive transition (Johansson et al., 2003). This demonstrates that increased protein carbonylation is not universally and inevitably related to the age of a biological tissue. In addition, it highlights that, in both animals and plants, production of offspring occurs at a time at which the overall oxidative damage in the organism is low. In animals this coincides with the early to middle stages of the organism's life cycle, whereas in *A. thaliana* it signifies the end point of its developmental progression.

The microbial and plant model systems thus provide examples of novel defense systems against oxidative deterioration, which includes both clear-out and segregation of oxidized proteins that may ensure the fitness of the offspring. Identifying the players and the exact molecular mechanisms involved may shed some light also on the molecular reasons for the escalating oxidative damage in aging animals. In addition, it may give hints on measures that may tinker with the deterioration of the aging proteome.

ACKNOWLEDGMENT

Past and present colleagues of the "Nyström lab" are greatly acknowledged for contributing to the work described in this review. I thank The Swedish Research Council, VR, for long-term support of the cellular senescence project and the Göran Gustafsson Foundation for an award in Molecular Biology.

LIST OF ABBREVIATIONS

ERCs, extrachromosomal rDNA circles
MCO, metal catalyzed oxidative
ROS, reactive oxygen species

REFERENCES

Ackermann M., Stearns S.C., Jenal U. (2003). Senescence in a bacterium with asymmetric division. *Science* 300: 1920.

Agarwal S., Sohal R.S. (1994). Aging and proteolysis of oxidized proteins. *Arch Biochem Biophys* 309: 24–28.

Aguilaniu H., Gustafsson L., Rigoulet M., Nyström T. (2001). Protein oxidation in G_0 cells of *Saccharomyces cerevisiae* depends on the state rather than rate of respiration and is enhanced in *pos9* but not *yap1* mutants. *J Biol Chem* 276: 35396–35404.

Aguilaniu H., Gustafsson L., Rigoulet M., Nyström T. (2003). Asymmetric inheritance of oxidatively damaged proteins during cytokinesis in *Saccharomyces cerevisiae*: A Sir2p dependent mechanism. *Science* 299: 1751–1753.

Ballesteros M., Fredriksson Å., Henriksson J., Nyström T. (2001). Bacterial senescence: Protein oxidation in non-proliferating cells is dictated by the accuracy of the ribosomes. *EMBO J* 18: 5280–5289.

Barak Z., Gallant J., Lindsley D., Kwieciszewki B., Heidel D. (1996). Enhanced ribosome frameshifting in stationary phase cells. *J Mol Biol* 263: 140–148.

Benov L., Fridovich I. (1995). A superoxide dismutase mimic protects *sodA sodB Escherichia coli* against aerobic heating and stationary-phase death. *Arch Biochem Biophys* 322: 291–294.

Berlett B.S., Levine R.L., Stadtman E.R. (1996). Comparison of the effects of ozone on the modification of amino acid residues in glutamine synthetase and bovine serum albumin. *J Biol Chem* 271: 4177–4182.

Bota D.A., Davies K.J. (2002). Lon protease preferentially degrades oxidized mitochondrial aconitase by an ATP-stimulated mechanism. *Nat Cell Biol* 4: 674–680.

Bota D.A., Van Remmen H., Davies K.J. (2002). Modulation of Lon protease activity and aconitase turnover during aging and oxidative stress. *FEBS Lett* 532: 103–106.

Cabiscol E., Piulats E., Echave P., Herrero E., Ros J. (2000). Oxidative stress promotes specific protein damage in *Saccharomyces cerevisiae*. *J Biol Chem* 275: 27393–27398.

Carlioz A., Touati D. (1986). Isolation of superoxide dismutase mutants in *Escherichia coli*: Is superoxide dismutase necessary for aerobic life? *EMBO J* 5: 623–630.

Castegna A., Aksenov M., Aksenova M., Thongboonkerd V., Klein J.B., Pierce W.M., Booze R., Markesbery W.R., Butterfield D.A. (2002). Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part I: Creatine kinase BB, glutamine synthase, and ubiquitin carboxy-terminal hydrolase L-1. *Free Radic Biol Med* 33: 562–571.

Dalle-Donne I., Giustarini D., Colombo R., Rossi R., Milzani A. (2003). Protein carbonylation in human diseases. *Trends Mol Med* 9: 169–176.

Desmyter L., Dewaele S., Verstraelen J., Nyström T., Contreras R., Chen C. (2004). Expression of the human ferritin light chain in a budding yeast frataxin mutant affects aging and cell death. *Exp Gerontol* 39: 707–715.

Desnues B., Gregori G., Dukan S., Aguilaniu H., Nyström T. (2003). Differential oxidative damage and expression of stress regulons in culturable and nonculturable cells of *Escherichia coli*. *EMBO Rep* 4: 400–405.

Dukan S., Farewell A., Ballesteros M., Taddei F., Radman M., Nyström T. (2000). Protein oxidation in response to increased transcriptional or translational errors. *Proc Natl Acad Sci USA* 97: 5746–5749.

Dukan S., Nyström T. (1998). Bacterial senescence: Stasis results in increased and differential oxidation of cytoplasmic proteins leading to developmental induction of the heat shock regulon. *Genes Dev* 12: 3431–3441.

Dukan S., Nyström T. (1999). Oxidative stress defense and deterioration of growth-arrested *Escherichia coli* cells. *J Biol Chem* 274: 26027–26032.

Egilmez N.K., Jazwinski S.H. (1989). Evidence for the involvement of a cytoplasmic factor in the aging of the yeast *Saccharomyces cerevisiae*. *J Bacteriol* 171: 37–42.

Ericsson M., Hanstorp D., Hagberg P., Enger J., Nyström T. (2000). Sorting out bacterial viability with optical tweezers. *J Bacteriol* 182: 5551–5555.

Fabrizio P., Longo V.D. (2003). The chronological life span of *Saccharomyces cerevisiae*. *Aging Cell* 2: 73–81.

Ferbeyre G., de Stanchina E., Lin A.W., Querido E., McCurrach M.E., Hannon G.J., Lowe S.W. (2002). Oncogenic ras and p53 cooperate to induce cellular senescence. *Mol Cell Biol* 22: 3497–3508.

Friguet B., Bulteau A.L., Chondrogianni N., Conconi M., Petropoulos I. (2000). Protein degradation by the proteasome and its implications in aging. *Ann NY Acad Sci* 908: 143–154.

Grune T., Jung T., Merker K., Davies K.J.A. (2004). Decreased proteolysis caused by protein aggregates, inclusion bodies, plaques, lipofuscin, ceroid, and "aggresomes" during oxidative stress, aging, and disease. *Int J Biochem Cell Biol* 36: 2519–2530.

Grune T., Merker K., Sandig G., Davies K.J. (2003). Selective degradation of oxidatively modified protein substrates by the proteasome. *Biochem Biophys Res Commun* 305: 709–718.

Guarente L. (2000). Sir2 links chromatin silencing, metabolism, and aging. *Genes Dev* 14: 1021–1026.

Headlam H.A., Davies M.J. (2004). Markers of protein oxidation: Different oxidants give rise to variable yields of bound and released carbonyl products. *Free Radic Biol Med* 36: 1175–1184.

Hengge-Aronis R. (2002). Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase. *Microbiol Mol Biol Rev* 66: 373–395.

Hlavata L., Aguilaniu H., Pichova A., Nyström T. (2003). The oncogenic RAS2(val19) mutation locks respiration, independently of PKA, in a mode prone to generate ROS. *EMBO J* 22: 3337–3345.

Jayko M.E., Garrison W.M. (1958). Formation of >C=O bonds in the radiation-induced oxidation of protein in aqueous systems. *Nature* 181: 413–414.

Jazwinski S.M. (2004). Yeast replicative life span—The mitochondrial connection. *FEMS Yeast Res* 5: 119–125.

Johansson E.C., Olsson O., Nyström T. (2004). Progression and specificity of protein oxidation in the life cycle of *Arabidopsis thaliana*. *J Biol Chem* 279: 22204–22208.

Kristensen B.K., Askerlund P., Bykova N.V., Egsgaard H., Moller I.M. (2004). Identification of oxidised proteins in the matrix of rice leaf mitochondria by immunoprecipitation and two-dimensional liquid chromatography–tandem mass spectrometry. *Phytochemistry* 65: 1839–1851.

Levine R.L. (2002). Carbonyl modified proteins in cellular regulation, aging, and disease. *Free Radic Biol Med* 32: 790–796.

Luo J., Nikolaev A.Y., Imai S., Chen D., Su F., Shiloh A., Guarente L., Gu W. (2001). Negative control of p53 by Sir2alpha promotes cell survival under stress. *Cell* 107: 137–148.

Matin A. (1991). The molecular basis of carbon-starvation-induced general resistance in *Escherichia coli*. *Mol Microbiol* 5: 3–10.

Nyström T. (1995). The trials and tribulations of growth arrest. *Trends Microbiol* 3: 131–136.

Nyström T. (1999). Starvation, cessation of growth and bacterial aging. *Curr Opin Microbiol* 2: 214–219.

Nyström T. (2002a). Aging in bacteria. *Curr Opin Microbiol* 5: 596–601.

Nyström T. (2002b). Translational fidelity, protein oxidation, and senescence: Lessons from bacteria. *Aging Res Rev* 1: 693–703.

Nyström T. (2003). Viable but non-culturable bacteria: programmed survival forms or cells at death's door? *BioEssays* 25: 204–211.

Nyström T. (2004). Stationary phase physiology. *Ann Rev Microbiol* 58: 161–181.

O'Farrell P.H. (1978). The suppression of defective translation by ppGpp and its role in the stringent response. *Cell* 14: 545–557.

Postgate J.R. (1976). Death of macrobes and microbes. *Symp Soc Gen Microbiol* 26: 1–18.

Rogina B., Helfand S.L. (2004). Sir2 mediates longevity in the fly through a pathway related to calorie restriction. *Proc Natl Acad Sci USA* 101: 15998–16003.

Shringarpure R., Davies K.J. (2002). Protein turnover by the proteasome in aging and disease. *Free Rad Biol Med* 32: 1084–1089.

Sinclair D.A. (2002). Paradigms and pitfalls of yeast longevity research. *Mech Ageing Dev* 123: 857–867.

Sinclair D.A., Guarente L. (1997). Extrachromosomal rDNA circles—A cause of aging in yeast. *Cell* 91: 1033–1042.

Sitte N., Merker K., von Zglinicki T., Grune T., Davies K.J.A. (2000). Protein oxidation and degradation during cellular senescence of human BJ fibroblasts. Part I: Effects of proliferative senescence. *FASEB J* 14: 2495–2502.

Sohal R.S. (2002). Role of oxidative stress and protein oxidation in the aging process. *Free Radic Biol Med* 33: 37–44.

Sohal R.S., Agarwal S., Dubey A., Orr W.C. (1993). Protein oxidative damage is associated with life expectancy of houseflies. *Proc Natl Acad Sci USA* 90: 7255–7259.

Stadtman E.R. (1992). Protein oxidation and aging. *Science* 257: 1220–1224.

Stadtman E.R., Levine R.L. (2000). Protein oxidation. *Ann NY Acad Sci* 899: 191–208.

Sun J., Kale S.P., Childress A.M., Pinswasdi C., Jazwinski S.M. (1994). Divergent roles of RAS1 and RAS2 in yeast longevity. *J Biol Chem* 269: 18638–18645.

Tamarit J., Cabiscol E., Ros J. (1998). Identification of the major oxidatively damaged proteins in *Escherichia coli* cells exposed to oxidative stress. *J Biol Chem* 273: 3027–3032.

Tissenbaum H.A., Guarente L. (2001). Increased dosage of a *sir-2* gene extends lifespan in *Caenorhabditis elegans*. *Nature* 410: 227–230.

Tyner S.D., Venkatachalam S., Choi J., Jones S., Ghebranious N., Igelmann H., Lu X., Soron G., Cooper B., Brayton C., Hee Park S., Thompson T., Karsenty G., Bradley A., Donehower L.A. (2002). p53 Mutant mice that display early ageing-associated phenotypes. *Nature* 415: 45–53.

Yan L.J., Sohal R.S. (1998). Mitochondrial adenine nucleotide translocase is modified oxidatively during aging. *Proc Natl Acad Sci USA* 95: 12896–12901.

Yan L.J., Levine R.L., Sohal R.S. (1997). Oxidative damage during aging targets mitochondrial aconitase. *Proc Natl Acad Sci USA* 94: 11168–11172.

Vaziri H., Dessain S.K., Ng Eaton E., Imai S.I., Frye R.A., Pandita T.K., Guarente L., Weinberg R.A. (2001). hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* 107: 149–159.

Wenthzel A.M., Stancek M., Isaksson L.A. (1998). Growth phase dependent stop codon readthrough and shift of translation reading frame in *Escherichia coli*. *FEBS Lett* 421: 237–242.

PART III

REDOX PROTEOMIC ANALYSIS IN HUMAN DISEASES

16

PROTEINS AS SENSITIVE BIOMARKERS OF HUMAN CONDITIONS ASSOCIATED WITH OXIDATIVE STRESS

Isabella Dalle-Donne, Ranieri Rossi, Fabrizio Ceciliani, Daniela Giustarini, Roberto Colombo, and Aldo Milzani

16.1 INTRODUCTION

Reactive oxygen and nitrogen species (ROS/RNS) are both physiologically necessary and potentially destructive (Mikkelsen and Wardman, 2003). It is now known these species are not only simply a by-product of normal oxidative metabolism or a tool through which phagocytes accomplish antimicrobial action. Moderate levels of ROS play specific roles in the modulation of several cellular events, including signal transduction, proliferative response, and gene expression (Halliwell and Gutteridge, 1999; Mikkelsen and Wardman, 2003). High ROS/RNS levels can cause damage to key cellular components such as lipids, proteins, and nucleic acids, possibly leading to subsequent cell death by necrosis or apoptosis. Oxidation of any of these substrates, if unchecked, can theoretically contribute to disease development (Fig. 16.1). Indeed, an increasing amount of evidence suggests that the production of ROS/RNS, and subsequently oxidative/nitrosative stress and damage, is linked to either the primary or the secondary pathophysiological mechanisms of multiple disorders, including cancer, diabetes, atherosclerosis, coronary heart disease, cataracts, the degenerative processes involved in age-related diseases, and even the rate of aging (Halliwell and Gutteridge, 1999;

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

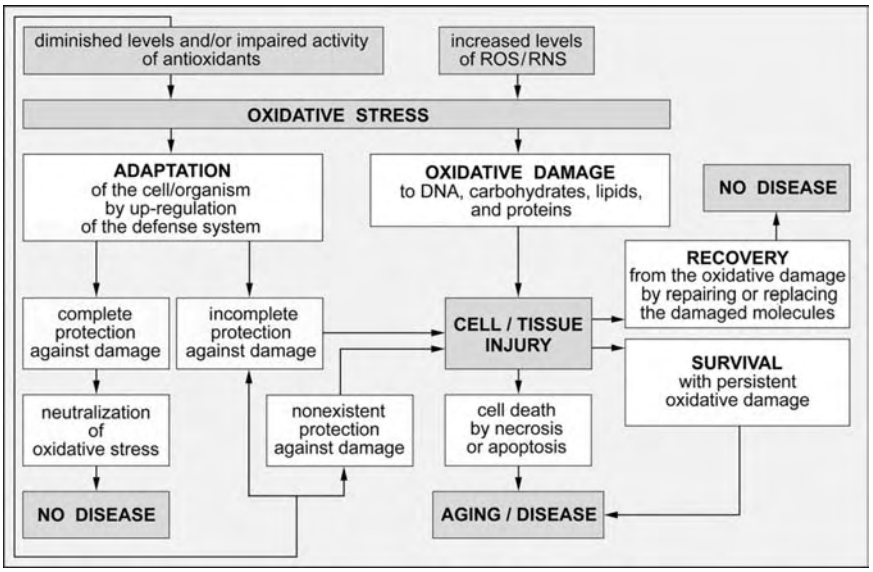


FIGURE 16.1 Reactive species, oxidative damage, and cellular responses to oxidative stress. Oxidants are generated as a result of normal intracellular metabolism in mitochondria and peroxisomes, as well as from a variety of cytosolic enzyme systems. In addition a number of external agents can trigger ROS/RNS production. A sophisticated enzymatic and nonenzymatic antioxidant defense system counteracts and regulates overall ROS/RNS levels to maintain physiological homeostasis. Overproduction of ROS/RNS and/or diminished levels and/or impaired activity of antioxidants result in the deleterious condition known as oxidative stress. Increased oxidative stress can be detrimental and lead to cell death or to an acceleration in aging and age-related diseases. The impairment caused by increased ROS/RNS is thought to result from random damage to proteins, lipids, and DNA. In addition to these effects, a rise in ROS/RNS levels may also constitute a stress signal that activates specific redox-sensitive signaling pathways. Once activated, these diverse signaling pathways may have either damaging or potentially protective functions.

Halliwell, 2000, 2001; Beal, 2002; Stocker and Keaney, 2003; Dalle-Donne et al., 2003a, 2005; Klaunig and Kamendulis, 2004).

Under normal physiological conditions, cells and tissues counterbalance the production of ROS/RNS with an abundance of endogenous antioxidant systems (Table 16.1) acting in several ways including removal of O_2, scavenging ROS/RNS or their precursors, inhibiting ROS formation, and binding metal ions needed for catalysis of ROS generation. An antioxidant is any substance (exogenous—natural or synthetic—or endogenous compound), which, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell and Gutteridge, 1999). In other words, an antioxidant is defined as a substance being effective against oxidative damage (preventing or delaying it) when present in much smaller quantity than the substance that it protects.

TABLE 16.1 Antioxidant Systems to Scavenge or Otherwise Eliminate ROS/RNS

Endogenous antioxidant systems
Albumin
Antioxidant enzymes (e.g., SOD, catalase, GSH peroxidase, GSH reductase, thioredoxin, thioredoxin reductase, peroxiredoxins, methionine sulfoxide reductase, heme oxygenase)
Bcl-2
Bilirubin
Ceruloplasmin
Coenzyme Q_{10} (ubiquinone)
GSH and other low-molecular-weight thiols
Protein thiols
Lipoic acid
Transferrin, ferritin, lactoferrin
Metallothioneines
Uric acid
Exogenous antioxidant systems
Anthocyanosides
Carotenoids
Flavonoids
Polyphenols
Vitamin A
Vitamin C (ascorbic acid)
Vitamin E (α-tocopherol)

The natural antioxidant defense system consists of enzymes and a variety of other nonenzymatic, low-molecular-weight molecules, including ascorbate, pyruvate, flavonoids, polyphenols, and carotenoids; most of them are derived from dietary sources. The latter easily penetrate into cells and accumulate in specific locations near targets of ROS/RNS attack. They are dissipated during their reaction with various ROS and can be replenished through the diet. Oxidative stress can be defined as an imbalance between oxidants and antioxidants in favor of the former, potentially leading to damage (Sies, 1991). Endogenous important and widespread antioxidant systems include the tripeptide glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) and enzymes such as superoxide dismutases (SOD), catalase, thioredoxins, glutaredoxins (or thioltransferases), and glutathione peroxidases (Table 16.1). SOD are localized to the cytosol and mitochondria, and they function to reduce a superoxide anion to hydrogen peroxide and water, whereas catalase, located in peroxisomes, is responsible for the removal of high levels of hydrogen peroxide.

16.2 OXIDATIVE STRESS IN HUMAN DISEASES AND ANIMAL MODELS

When the generation of reactive species exceeds the rate at which endogenous antioxidant defenses can scavenge oxidants, or when reduction in the endogenous

antioxidant defense system, due to environmental or genetic factors, occurs (e.g., mutations affecting antioxidant enzymes or depletions of antioxidants), proteins, lipids, DNA, and other macromolecules become targets for oxidative modifications (Fig. 16.1). Accumulation of altered forms of nucleic acids, lipids, and proteins can result in loss or decrease of cellular and tissue functions or gain of harmful functions, and, ultimately, death. Therefore, surviving neurons in human neurological disorders without evidence of biological oxidation could be the cells with the most effective antioxidant capacity. For example, neurons surviving in Huntington's disease brains have strongly induced expression of the potent antioxidant enzyme mitochondrial Mn-SOD (Browne et al., 1999). However, oxidative damage of biological targets does not necessarily translate to a pathogenic phenotype, since a multitude of repair processes can be activated to sustain physiological function. In keeping with this view, succumbing neurons may be the least proficient in repair capacity. Likewise in Alzheimer's disease brains decreased repair activity of methionine sulfoxide reductase has been demonstrated: this enzymatic activity is essential for repair of oxidized Met residues and may regulate the lifespan of mammals (Gabbita et al., 1999; Moskovitz et al., 2001). Increased production of ROS/RNS is usually thought to be more relevant than diminished antioxidant defense, and an unbalanced production of ROS/RNS has been postulated to play a role in the pathogenesis of a number of human disorders.

Neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS), are defined by the progressive loss of specific neuronal cell populations and are associated with protein aggregates. A common feature of these diseases is extensive evidence of oxidative stress, which might be responsible for the dysfunction or death of neuronal cells that contributes to disease pathogenesis (Butterfield et al., 2001; Butterfield, 2002; Butterfield and Lauderback, 2002; Barnham et al., 2004). Oxidation and nitration of proteins, DNA, and lipids are markers of neurodegeneration in postmortem tissues. However, it is impossible to determine with certainty by postmortem analysis whether oxidative stress has a primary role in neurodegeneration or is a secondary end-stage epiphenomenon (Ischiropoulos and Beckman, 2003).

Like the other neurodegenerative diseases, ALS, also known as Lou Gehring's disease, a fatal disorder causing gradual degeneration of lower motor neurons in the spinal cord and of upper motor neurons in the cerebral cortex, is characterized by the deposition of a misfolded protein in neural tissue, in this instance Cu/Zn-SOD. More than 100 mutations in the Cu/Zn-SOD gene have been associated with the familial forms of the disease. About 15% to 20% of patients with the familial ALS, which is clinically indistinguishable from the more common sporadic ALS, carry mutations in the gene encoding for the Cu/Zn-SOD (Valentine, 2002; Barnham et al., 2004). Through transgenic mouse studies it has been shown that these mutations, rather than causing a loss of function, lead to a toxic gain of function by Cu/Zn-SOD that results in neuronal degeneration (Valentine, 2002; Barnham et al., 2004). The nature of this gain of function is widely debated, and there are two main theories that might not be mutually exclusive: one suggests

that the toxicity is due to misfolded aggregated forms of SOD, whereas the other proposes that SOD becomes a pro-oxidant protein generating ROS (Barnham et al., 2004). A variety of markers of oxidative damage, including protein-bound 3-nitrotyrosine, are indeed increased in ALS spinal cords (Cleveland and Rothstein, 2001). Notably there has been presented the first case of a novel Cu/Zn-SOD mutation in a patient with genetically proved sporadic ALS (Alexander et al., 2002).

PD is associated with a profound and selective loss of dopaminergic neurons in the nigrostriatal pathway of the brain, as well as a more widespread (but inadequately characterized) neuronal loss in other brain regions. There seem to be multiple, divergent causes of PD, yet the pathogenesis of this disease appears to be converging on common mechanisms: mitochondrial impairment, oxidative stress, and protein mishandling, all of which are tightly linked (Greenamyre and Hastings, 2004). A common by-product of many types of mitochondrial impairment is increased production of ROS, and this may be the source of the oxidative damage found in PD brains. The importance of the genetics underlying susceptibility to PD is increasingly recognized. Despite the overall rarity of the familial forms of PD (<10% of cases), the identification of single genes linked to the disease has yielded crucial insights into possible mechanisms of PD pathogenesis. Disease-causing mutations implicate aberrant protein handling and oxidative stress as key events in PD pathogenesis. Postmortem studies have consistently implicated oxidative damage (Jenner, 2003) in PD pathogenesis, but the source of this damage is not yet clear. Leading candidates for production of ROS include dopamine metabolism and dysfunction of mitochondria. Epidemiological studies suggest that environmental chemicals, such as pesticides, might be crucial factors in PD pathogenesis. Together these studies suggest that environmental chemicals, disrupted mitochondrial complex I activity, and oxidative stress may all participate in the killing of dopaminergic neurons in PD (Greenamyre and Hastings, 2004).

The "free radical theory" of aging is built on a comparable argument: an age-dependent increase in the oxidative modifications of biomolecules caused by an age-dependent increase in the steady state levels of ROS. In recent years much support has been accumulated for the involvement of oxidative stress in aging (Finkel and Holbrook, 2000; Hekimi and Guarente, 2003). It is now well established that biological aging correlates with the accumulation of oxidized biomolecules such as oxidized proteins (Berlett and Stadtman, 1997), lipids, DNA bases, advanced glycation end-products (AGEs), and lipofuscin (Schoneich, 2005) in most tissues.

In many human diseases, oxidative stress is a consequence and not a cause of the primary disease process. For example, pre-eclampsia, a pregnancy-specific syndrome, is characterized by a low perfusion of the placenta secondary to abnormal placentation. Ischemia of the fetus-placental unit triggers signals to increase its own blood supply, which paradoxically causes vasoconstriction in vital organs of the mother (Page et al., 2000). Activated neutrophils and endothelial cells increase the production of ROS, which, in the hypoxic placenta, release

cytokines and generate additional oxidative stress. Indeed, on the basis of the hypothesis that oxidative stress plays an important role in the pathogenesis of pre-eclampsia, clinical trials that have tested antioxidant administration to women in early pregnancy have shown the benefits of such treatment: decreased oxidative stress, endothelial activation, and lower prevalence of pre-eclampsia (Roberts and Cooper, 2001).

PD may serve as an excellent example to discuss the significance of oxidative processes as a central but not an initiating event for the development of clinical disease. The existing working hypothesis places oxidative processes at the narrowest point of a funnel through which environmental, genetic, and endogenous risk factors flow to adversely impact cellular function and viability (Ischiropoulos and Beckman, 2003).

The production of ROS/RNS, and the resultant oxidative/nitrosative stress, is an ubiquitous phenomenon in all eukaryotic organisms that require atmospheric oxygen to survive. ROS/RNS are often produced by very simple pathways, and the specific enzymes that evolved to detoxify them are usually conserved among the various species. Therefore several animal models can be very useful to study in vivo the consequences of oxidative stress, both endogenous and exogenous. Invertebrate models, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, are mainly used to study "gerontogenes" (i.e., genes affecting the aging rate) and the genetic base of oxidative stress associated with aging. Moreover it is possible to create, via genetic manipulation, animal models (essentially mice) that can spontaneously generate endogenous ROS/RNS.

Mice are favorite subjects of investigators doing research on human aging and oxidative stress-related diseases. Their relatively short life span makes them easier to study than other longer lived mammals. The very powerful technique of gene knock out has been utilized in mice to generate several models of ROS/RNS toxicity. Most interesting are those that reveal the molecular basis of some mitochondrial diseases. For instance, the mitochondrial form of SOD (Mn-SOD) is one of these main defenses against ROS. Transgenic mouse (SOD2-/-) was generated by inactivating the SOD2 (EC 1.15.1.1) gene (Li et al., 1995). The SOD2-/- phenotype includes tissue-specific diseases caused by the inhibition of the respiratory chain enzymes NADH-dehydrogenase (complex I) and succinate dehydrogenase (complex II) but also inactivation of aconitase. Overall, inhibition of these enzymes induces an increase in mitochondrial ROS, and accumulation of oxidative mitochondrial DNA damage, that can result in biochemical modifications similar to those of mitochondrial miopathy or Friedreich's ataxia (Melov et al., 1999).

The dog is widely used experimentally for investigations on free radicals mediated dysfunction in some diseases, such as diabetes mellitus and cardiovascular diseases. The pathogenesis of diabetes in dogs closely resembles that of humans, and the involvement of oxygen-derived free radicals in the endothelial dysfunction of coronary arterioles has been demonstrated in diabetic dogs (Ammar et al., 2000). In particular, the incidence rate of type 1 diabetes is rising in both dogs and humans, a trend that has been explained with the increase in

adverse environmental conditions acting on a background of complex genetic factors (Rand et al., 2004). The canine model has been utilized also for studies on neurodegeneration, which displays some common morphological characteristics, most of which are observed in the brain of several mammalian species, including dogs. Oxidative stress is an important contributor of the pathogenesis of AD, and its role in the spontaneous canine model, called "canine counterpart of senile dementia of the Alzheimer type" (ccSDAT), has been investigated, in particular, for what concerns the diffusion of the toxic products of lipid peroxidation from the site of primary formation (brain) to other organs, or blood. End-products of lipid peroxidation have been found increased also in erythrocytes of demented dogs, and their detection in blood has been proposed as a tool for diagnostic purposes (Skoumalova et al., 2003).

Accumulating evidence in human diseases and animal models thereof points to many interrelated mechanisms during pathogenesis that increase the production of ROS/RNS and/or decrease the antioxidant protection against oxidative insult, although the exact contributions of such mechanisms are not entirely clear. The presence of oxidative damage, defined as any molecular damage caused by direct attack of ROS/RNS during oxidative stress (Halliwell and Whiteman, 2004), could simply reflect secondary epiphenomena rather than have a causal role, as in PD. The oxidative damage should meet defined requirements to be implicated as a significant mechanism of cell/tissue injury in human disease (Halliwell and Whiteman, 2004). Basically, to establish the role of oxidative damage in disease, it is essential to measure it reliably and accurately.

16.3 BIOMARKERS OF OXIDATIVE STRESS STATUS (BOSS)

The primary cellular target of oxidative stress can vary depending on the cell type, the absolute level and duration of oxidant production, the species of ROS/RNS generated (radical vs. nonradical oxidant), its site of generation (intra- vs. extracellular), and the proximity of the oxidant to a specific cellular substrate (Halliwell and Gutteridge, 1999). The extent of damage to particular targets depends on a number of factors, including the concentration of target, the rate constant for reaction of oxidant with the target, the location of the target when compared to the site of oxidant formation, the occurrence of secondary damaging events (chain reactions and damage transfer processes), the occurrence of oxidant-scavenging reactions, and repair reactions. The first two of these determine the rate at which any particular reaction is likely to occur; the others modulate the extent of reaction that occurs.

Methods for the analysis of ROS/RNS are available: for instance, those for measuring $O_2^{\bullet -}$, $ONOO^-$, and H_2O_2 (Tarpey and Fridovich, 2001). However, ROS/RNS are generally too reactive and/or have a too brief half-life (even much shorter than seconds) to measure them directly in cells/tissues or body fluids. Further low intracellular concentrations, and the efficient and redundant systems that have evolved to scavenge ROS/RNS, require that any detection technique must be sensitive and specific enough to compete with antioxidant defenses against the

species in question (Warner et al., 2004). Additionally, the methods applied must have intracellular access to monitor the intracellular milieu. This undoubtedly has contributed to confusion surrounding the roles of these species in disease. Anyway, the concomitant presence of ROS/RNS and other biomolecules may yield specific products, generating a sort of specific chemical footprint of their occurrence. Since molecular products from oxidative stress are generally more stable than reactive species (i.e., products of DNA damage, products of lipid peroxidation, and oxidatively modified proteins are more stable than the reactive species that effected their modification), most commonly ROS/RNS have been tracked by measuring stable metabolites (e.g., nitrate/nitrite) and/or levels of their oxidation target products (Pryor, 2001; Griffiths et al., 2002; Halliwell and Whiteman, 2004).

Information regarding the nature of the ROS/RNS, as well as the localization and the effects of oxidative stress, may be gleaned from the analysis of discrete biomarkers of oxidative stress status (BOSS) (Pryor, 2001) isolated from tissues and biological fluids, such as plasma and urine. BOSS is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention. Therefore BOSS have been used to evaluate the efficacy of dietary supplements or synthetic antioxidants on oxidative damage (and so far thought to be useful and reliable cues of the effects of these compounds on oxidative stress related diseases arising subsequently) but with mixed results, which may reflect expectations we have regarding the information that BOSS can yield. Biomarkers may yield information on three progressive levels of disease outcome: (1) as measurable endpoints of damage to biomolecules such as lipids, DNA bases, proteins; (2) as functional markers of, for example, blood flow, platelet aggregation, or cognitive function; and (3) as endpoints related to specific disease. While the clinical symptoms of a disease are endpoints in themselves, they are not suitable in many cases for early detection, and therefore prevention of diseases associated with oxidative stress. A series of biomarkers would be preferred, each validated in sequence. To this end, the association between a biomarker and a disease should be defined. For the clinician there is hope that biomarkers will help diagnose symptomatic and presymptomatic disease or provide surrogate endpoints to demonstrate clinical efficacy of new treatments. No biomarker is likely to fulfill all these functions, so we need to know how each has been validated in order to understand their uses and limitations and be aware of potential pitfalls.

The most intuitive goal for a biomarker is to help diagnose disease. The usefulness of the ideal biomarker of oxidative damage lies in its ability to provide early indication of disease and/or its progression (Fig. 16.2). Characteristics of a valid BOSS are summarized in Table 16.2. Validation of biomarkers requires two different steps. One is the analytical validation, including development of procedures, analysis of reference materials, and quality control. The second is the validation of the fact that changes in their level do reflect the later development of disease. No currently used biomarker has yet fulfilled the key requirement of

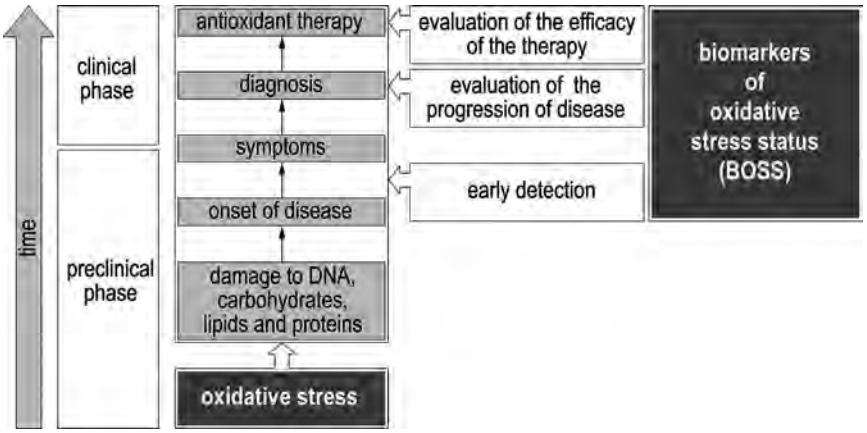


FIGURE 16.2 Use of BOSS in the detection of disease initiation and/or progression as well as in assessing effective therapies.

the ideal biomarker: that it is predictive of the later development of disease. No biomarker meets all the "technical" requirements (Table 16.2), but some are better than others. Case-control studies on stored samples should be used to test the efficiency of the biomarkers. Care must be taken to define and establish references or baseline profiles from normal tissues, cells, or body fluids. The use of a panel of biomarkers would enhance the positive predictive value of a test and minimize the proportion of false-positive and false-negative results. A key methodologic issue concerns the potential for artifacts in estimates of baseline levels of BOSS. That is, oxidation of samples can occur during normal sample handling, processing, and analysis such that measured levels are not at all reflective of the levels encountered in vivo. This issue has plagued investigations of many different biomarkers of oxidation. For instance, recent reports (Frost et al., 2000; Yi et al., 2000; Tsikas and Caidahl, 2005) have documented that 3-nitrotyrosine can be generated ex vivo during sample processing, illustrating the limitations of some quantitative methods that do not use isotopically labeled internal standards (e.g., immunochemical, spectroscopic, and electrochemical detection) that can distinguish in vivo from ex vivo generation. These findings highlight the importance of developing robust analytical methods and validating them by in vivo settings. Indeed, some advances in the mass spectrometric quantification of tyrosine modification have been described (Frost et al., 2000; Yi et al., 2000; Aulak et al., 2001; Gaut et al., 2002; Tsikas and Caidahl, 2005).

Many BOSS in biological samples are available (Table 16.3), including GSH, GSSG, various measures of lipid oxidation, oxidized DNA bases, and oxidized proteins (Davies et al., 1999; Winterbourn and Buss, 1999; Levine et al., 2000; Pryor, 2001; Tarpey and Fridovich, 2001; Griffiths et al., 2002; Dalle-Donne et al., 2003b; Halliwell and Whiteman, 2004; Montuschi et al., 2004). The following briefly describes some of the more commonly used biomarkers of oxidative damage and includes selected examples of human studies.

TABLE 16.2 Principal Characteristics of a Valid BOSS

A valid BOSS should be:

- Accessible in a target tissue or a valid surrogate one able to quantitatively reflect the oxidative modification of the target tissue
- Cheap, noninvasive, and quick to measure by untrained staff (especially a clinical biomarker)
- A chemically and biologically stable product, not susceptible to artifactual induction, oxidation or loss during sample handling, processing, analysis, and storage
- Determined by an assay that is specific, sensitive, and reproducible
- Easy to detect and measurable across population
- Free of confounding factors from dietary intake
- Highly reactive
- A major product of oxidative/nitrosative damage that may be implicated directly in the onset and/or progression of disease
- Present at a high enough concentration for the biomarker to be a significant product
- Representative of the balance between oxidative/nitrosative damage generation and clearance
- Specific for the reactive species in question
- Its levels should not vary widely in the same subjects under the same conditions at different times
- The coefficient of variation between different assays of the same samples should be small in comparison with the differences be among subjects

16.3.1 Glutathione

There is no doubt that the most used BOSS is glutathione (GSH). Because blood glutathione concentrations may reflect glutathione status in other less accessible tissues, measurement of both reduced (GSH) and oxidized glutathione (glutathione disulfide, GSSG) in blood has been considered essential as an index of whole-body GSH status and a useful indicator of oxidative stress in humans. Several methods have been optimized in order to identify and quantify glutathione forms in human samples. They include spectrophotometric, fluorometric, and bioluminometric assays, often applied to HPLC analysis, as well as the more recently developed gas chromatography–mass spectrometry (GC–MS) and high-performance liquid chromatography–electrospray ionization–mass spectrometry (HPLC–ESI–MS) techniques (Pastore et al., 2003b). Further, a wide variety of methods have been introduced for the determination of GSH and GSSG in human blood, whose measurement, especially that of GSSG, could result overestimated if samples are not properly processed (Rossi et al., 2002; Giustarini et al., 2003a, 2004b).

It has been well established that a decrease in GSH concentration is associated with aging and the pathogenesis of many diseases, including rheumatoid arthritis

TABLE 16.3 Biomarkers for the Determination of Oxidative/Nitrosative Damage in Biological Samples

Antioxidant enzyme activity
Nonenzymatic antioxidant proteins
Low-molecular-weight antioxidants
DNA
Aldehyde/other base adducts
Nitrated, deaminated, oxidized bases
Carbohydrates
Glycoxidation products
Lipids
2-Propenal (acrolein)
4-Hydroxynonenal
Isoleukotrienes
Isoprostanes
Malondialdehyde
Oxysterols
Proteins
3-Bromotyrosine
3,5-Dibromotyrosine
3-Chlorotyrosine
3,5-Dichlorotyrosine
3-Nitrotyrosine
Methionine sulfoxide
o,*o*′-Dityrosine
o-Tyrosine
Protein carbonyls
S-Glutathionylated proteins
S-Nitrosated proteins

(RA), ALS, AIDS, AD, alcoholic liver disease, cataract genesis, respiratory distress syndrome, cardiovascular disease, and Werner syndrome (Pastore et al., 2003b). Further, there is a drastic depletion in cytoplasmic levels of GSH within the substantia nigra of PD patients (Bharath et al., 2002). Depletion of total GSH (GSH + 2GSSG + protein-bound fraction) and a decreased GSH/GSSG ratio are BOSS in ischemic brain (Warner et al., 2004), and decreased levels of GSH are consistently observed in both types of diabetes mellitus (Pastore et al., 2003b). Low GSH and a high GSSG/GSH ratio have been found in blood of patients with various diseases, including breast and lung cancer, coronary heart surgery, and pre-eclampsia (Pantke et al., 1999; Pastore et al., 2003b). The GSH system is also altered in lung inflammatory conditions. For instance, GSH levels are elevated in the epithelial lining fluid of chronic smokers, whereas

decrease rapidly in patients with mild asthma during an asthma exacerbation. Similarly GSH levels in the epithelial lining fluid are decreased in idiopathic pulmonary fibrosis, asbestosis, acute respiratory distress syndrome, and in HIV-positive patients (Comhair and Erzurum, 2002). Marked total GSH decreases occurred in old patients with chronic diseases including cancer and genitourinary, gastrointestinal, cardiovascular, and musculoskeletal diseases, the deficit being due to lower GSH concentrations and not to higher GSSG. These findings suggest that the decrease in GSH is a risk factor for chronic diseases that may be used to monitor the severity and progress of the diseases. Conversely, high levels of total GSH in the blood occur in old persons who are in excellent physical and mental health. Furthermore a high blood GSH level was correlated with long life span in the mouse, rat, and in healthy elderly human beings (Lang et al., 2000, 2002). GSH can be reversibly bound to protein thiol groups, by a mechanism called *S*-glutathionylation, leading to the formation of *S*-glutathionylated proteins, which have been investigated as possible BOSS in some human diseases (see Section 16.4.3).

16.3.2 Oxidative DNA Damage

ROS/RNS can induce the formation of several base adducts in DNA, which are implicated in mutagenesis, carcinogenesis, and neurologic disorders (Cooke et al., 2003). An abundant marker of DNA damage by ROS attack is 8-hydroxy-2′-deoxyguanosine (8-OHdG). Although DNA can be oxidized to produce many different oxidized products, oxidation of the C-8 of guanine is one of the more common oxidative events. 8-OHdG can be measured in human DNA samples (e.g., lymphocyte and placental DNA) and in urine. This BOSS can be quantitated by HPLC with electrochemical detection, GC–MS, liquid chromatography–mass spectrometry (LC–MS), and enzyme-linked immunosorbent assay (ELISA) (Halliwell and Whiteman, 2004). Urine is readily obtained, and thus many epidemiologic studies use urinary 8-OHdG to assess rates of "whole-body" oxidative DNA damage. Although convenient, this BOSS has some limitations: 8-OHdG in urine may be influenced by metabolic rate (oxygen consumption), as it is a function not only of oxidation of DNA but also of excision repair. For this reason the use of urinary 8-OHdG as a sole biomarker should be undertaken with caution, particularly in intervention studies; agents that increase repair would increase excretion of 8-OHdG in urine, which could be misinterpreted as an increase in oxidative DNA damage (Halliwell, 1999). The major problems arise from artifactual oxidative damage to DNA and consequent 8-OHdG formation during isolation of DNA, its preparation for analysis, and the analysis itself (Halliwell and Whiteman, 2004).

Increased oxidative damage to DNA was reported in neurodegenerative conditions such as AD, PD, and ALS (Zhang et al., 1999; Lovell et al., 1999; Bogdanov et al., 2000; Jenner, 2003), and in Friedreich's ataxia (Schulz et al., 2000). Elevated levels of oxidized DNA have been noted in many tumors, strongly implicating such damage in the etiology of cancer (Cooke et al., 2003). Particularly, it is widely believed that oxidative DNA damage over the long

human life span is a significant contributor to the age-related development of the major cancers, such as those of the colon, prostate, rectum, and breast (Halliwell, 2002). Therefore diets or therapeutic agents that decrease oxidative DNA damage would delay or prevent the onset of cancer. The observation that diets rich in fruits and vegetables can decrease both oxidative DNA damage and cancer incidence is consistent with this. By contrast, agents increasing oxidative DNA damage, such as cigarette smoke, several other carcinogens, and chronic inflammation, usually increase risk of cancer development. Numerous studies have attempted to establish a relationship between levels of oxidative DNA damage and cancer. Although such studies have supported the hypothesis that oxidative DNA damage may be an important risk factor for carcinogenesis, it has been argued that the mere presence of 8-OHdG in DNA is unlikely to be necessary or sufficient to cause tumor formation. On the other hand, elevated oxidative DNA damage may not always be associated with increased cancer development. There are many pathological conditions (e.g., AD, PD, ALS, multiple sclerosis, Friedreich's ataxia, cystic fibrosis, type I and II diabetes mellitus, RA) in which levels of oxidative DNA damage are elevated, as evidenced by elevated levels of 8-OHdG in blood cells and/or increased urinary excretion of 8-OHdG, with no increased incidence of carcinogenesis (Halliwell, 2002; Cooke et al., 2003). In conclusion, no one to date has proved that oxidative DNA damage is a valid biomarker of subsequent cancer development.

16.3.3 Lipid Peroxidation Products

ROS-mediated damage to cellular biomembranes results in lipid peroxidation, a process that generates a variety of relatively stable decomposition end-products, including aldehydes, such as malondialdehyde (MDA), 4-hydroxynonenal (HNE), and acrolein (Uchida, 2003; Carini et al., 2004), and isoprostanes (Cracowski et al., 2002; Montuschi et al., 2004), which can then be measured in plasma as an indirect index of oxidative stress. There is increasing evidence that α,β-unsaturated aldehydes (HNE and acrolein), generated endogenously during the lipid peroxidation of polyunsaturated fatty acids, are involved in the onset and progression of many pathologies such as cardiovascular (atherosclerosis, long-term complications of diabetes), and neurodegenerative diseases (Halliwell, 2000; Poli and Schaur, 2000; Uchida, 2000; Carini et al., 2004). The biological activities of MDA, HNE, and acrolein include cross-linking with DNA and proteins. Modification of amino acids by α,β-unsaturated aldehydes, which may cause considerably more cytotoxicity in vivo than MDA (Esterbauer et al., 1991; Uchida, 2003; Halliwell and Whiteman, 2004), commonly occurs on the nucleophilic residues Cys, His, and Lys. Lipid hydroperoxides and aldehydes can also be absorbed from the diet and then excreted in urine. It follows that measurements of urinary MDA and HNE can be confounded by diet and should not be used as an index of whole-body lipid peroxidation unless diet is controlled.

Malondialdehyde Malondialdehyde (MDA) is a physiological ketoaldehyde produced by peroxidative decomposition of unsaturated lipids as a by-product

of arachidonic metabolism. Excessive production of MDA, as a result of tissue injury, can combine with free amino groups of proteins—MDA mainly reacts with Lys residues by Michael addition—resulting into MDA-modified protein adducts. Modifications of proteins by MDA could conceivably alter their biological properties. Moreover MDA-modified proteins are immunogenic, and autoantibodies against MDA-modified Lys residues have been demonstrated in the serum of both rabbits and humans (Stocker and Keaney, 2003). Some studies have reported that the titer of these autoantibodies is associated with the burden, and may predict progression, of atherosclerosis and myocardial infarction. Higher titers of autoantibodies have also been associated with coronary artery disease (Stocker and Keaney, 2003). The clinical relevance of the reaction between MDA and proteins is highlighted in atherosclerosis. This is a major cause of coronary heart disease and strokes. MDA-LDL, in addition to oxidized LDL, mediates several pro-inflammatory and pro-atherogenic processes, all of which ultimately lead to foam cell generation (Berliner and Heinecke, 1996). MDA plasma concentration is increased in diabetes mellitus, and it is found in the atherosclerotic plaques promoted by diabetes (Slatter et al., 2000). Adducts of apolipoprotein B-100 Lys residues with MDA and HNE have been characterized extensively in human atherosclerotic lesions (Heinecke, 2002; Stocker and Keaney, 2003). Elevated MDA levels were found in pre-eclampsia (Yoneyama et al., 2002) as well as in both plasma and breath condensate in asthmatics (Wood et al., 2003). MDA is increased in PD brains, whereas increased TBARS (the most prevalent substrate of which is MDA) have been observed in the plasma of ALS patients as well as in AD brains (Barnham et al., 2004).

The most widely used technique to evaluate lipid peroxidation is the thiobarbituric acid-reactive substances (TBARS) assay that includes MDA. TBA reacts with MDA to produce a stable chromogen that can be quantified by either spectrophotometry or HPLC. Although this technique is easy to use (mainly the spectrophotometric assay), the use of the TBARS test with human fluids is problematic for several reasons. First, aldehydes other than MDA may react with TBA to produce a compound that absorbs in the same range as MDA. Second, the decomposition of lipid peroxides during the test itself may mask the actual MDA content in the fluid before testing. Third, the presence or absence of metal ions or other undefined radicals affects the rate of this decomposition, making reliability a problem. Finally, most TBA-reactive material in human body fluids is not related to lipid peroxidation and may produce false-positive results (Halliwell and Gutteridge, 1999; Halliwell and Whiteman, 2004). Significant improvement to the TBA test can be made by using HPLC to isolate the MDA-TBA chromogen before analysis (Halliwell and Gutteridge, 1999; Halliwell and Whiteman, 2004). Therefore, although the TBARS assay is accepted as an index of oxidative stress, this method quantitates MDA-like material and does not specifically measure MDA or lipid peroxidation.

Hydroxynonenal and Acrolein 4-Hydroxy-*trans*-2-nonenal (HNE) is the major and toxic aldehyde generated by free radical attack on ω-6 polyunsaturated fatty

acids (arachidonic and linoleic acid) (Uchida, 2003). HNE exhibits a wide range of biological activities, including inhibition of protein and DNA synthesis, inactivation of enzymes, stimulation of phospholipase C, reduction of gap-junction communication, stimulation of neutrophil chemotaxis, modulation of platelet aggregation, and modulation of the expression of various genes (Esterbauer et al., 1991; Poli and Schaur, 2000; Uchida, 2003; Carini et al., 2004). In addition, HNE may be an important mediator of oxidative stress-induced apoptosis (Carini et al., 2004). The α,β-unsaturated aldehydes, HNE and acrolein, highly react with proteins, forming stable covalent adducts to His, Lys, and Cys residues through Michael addition, known as advanced lipoxidation end-products (ALEs) (Uchida and Stadtman, 1992; Butterfield, 2002; Butterfield et al., 2002). Carbonyl groups are thereby introduced into proteins. Both HNE and acrolein can diffuse from their point of production to cause oxidative modification of distant proteins, so acting as a "toxic second messenger" (Butterfield, 2002; Butterfield et al., 2002).

Numerous studies have demonstrated increased lipid peroxidation, as assessed by increased levels of HNE, acrolein, isoprostanes, and neuroprostanes, in AD brain relative to age-matched controls, whereas lipid peroxidation is not a significant feature of usual aging (Sayre et al., 2001; Butterfield, 2002; Butterfield et al., 2002; Montine et al., 2002b; Uchida, 2003; Barnham et al., 2004). In particular, the glial glutamate transporter GLT-1 (EAAT2) has increased binding of HNE in AD brain (Butterfield, 2002; Butterfield et al., 2002). Neurofilament proteins are major targets of HNE modification (Wataya et al., 2002). Notably it has recently been shown that the phosphorylation-dependent adduction/carbonylation of tau by HNE promotes and contributes to the generation of the major conformational changes associated with neurofibrillary tangles (Liu et al., 2005). The concentration of both free and protein-bound HNE is also elevated in PD brain tissue, in the cerebrospinal fluid (CSF) of ALS patients, as well as in human atherosclerotic lesions (Butterfield, 2002; Butterfield et al., 2002; Jenner, 2003; Stocker and Keaney, 2003; Barnham et al., 2004). Further, increased levels of acrolein and HNE protein adducts are found in cardiovascular disease (Uchida, 2000). Acrolein reacts with Lys residues of apolipoprotein A-I (apoA-I), the major protein of high-density lipoprotein (HDL), which plays a critical role in mobilizing cholesterol from artery wall macrophages. Acrolein adducts colocalized with apoA-I in human atherosclerotic lesions. Moreover, the ability of acrolein-modified apoA-I to remove cholesterol from cultured cells is impaired, suggesting that carbonylation might interfere with apoA-I's normal function of promoting cholesterol removal from artery wall cells, thus playing a critical role in atherogenesis (Shao et al., 2005). Increased concentrations of HNE-protein adducts have been reported in the lungs of smokers with and without chronic obstructive pulmonary disease (COPD). Notably HNE concentrations in the pulmonary epithelium, airway endothelium, and, particularly, in neutrophils of COPD patients were found to be inversely associated with lung function (Rahman et al., 2002). COPD patients also showed higher diaphragm levels of both protein carbonyls and HNE-protein adducts. Furthermore, a negative correlation was found between carbonyl groups and airway obstruction (i.e., reactive carbonyl

levels correlated with the disease severity) and between HNE-protein adducts and respiratory muscle strength (i.e., HNE-protein adduct formation correlated with respiratory muscle function) (Barreiro et al., 2005).

In the last decade fundamental improvements in the analysis of protein-HNE adducts by MS, and in particular, by matrix-assisted laser desorption/ionization (MALDI) and ESI tandem MS, have been made (Carini et al., 2004).

Isoprostanes F_2-Isoprostanes are a family of 64 prostaglandin-like compounds generated in vivo by nonenzymatic free radical catalyzed peroxidation of arachidonic acid, and then cleaved and released into the circulation by phospholipases(s) before excretion in the urine as free isoprostanes. Several of these compounds possess potent biological activity, as evidenced mainly through their pulmonary and renal vasoconstrictive effects, and have short half-lives. Reports have shown that F_2-isoprostanes are authentic biomarkers of lipid peroxidation and can be used as potential in vivo indicators of oxidant stress in various clinical conditions, as well as in evaluations of antioxidants or drugs for their free radical scavenging properties (Janssen, 2001; Roberts and Morrow, 2000, 2002; Basu, 2004; Halliwell and Whiteman, 2004; Montuschi et al., 2004).

Tissue that does not contain isoprostanes is yet to be reported. They have also been found in measurable quantities in most of the biological fluid analyzed, including plasma, urine, synovial fluid, bronchoalveolar fluid, bile, lymph, microdialysis fluid from various organs, and amniotic, pericardial, and seminal fluid, although plasma and urine samples are the ones that are commonly analyzed. However, the basal level in plasma and urine varies widely among species, and also among individuals. This observed variation in isoprostane concentration is assumed to be due to the variation in the rate of formation and/or metabolism (Basu, 2004).

Measurement of F_2-isoprostanes is at present regarded as one of the major reliable approaches for the assessment of oxidant stress status or free radical mediated lipid peroxidation in vivo (Roberts and Morrow, 2002; Basu, 2004; Halliwell and Whiteman, 2004; Montuschi et al., 2004). F_2-Isoprostanes are chemically stable but are rapidly metabolized. With the currently available assay methods—GC–MS, LC–MS, LC–MS/MS, enzyme immunoassays (EIA), radioimmunoassays (RIA)—they are found in detectable amounts in many tissues and body fluids, even in the normal basal state, which allows researchers to assess any fluctuation in their levels following any degree of oxidant stress or lipid peroxidation in vivo. For quantitation of lipid peroxidation, measurements of F_2-isoprostanes have a clear advantage over the currently available methods such as assay of MDA, TBARS, lipid hydroperoxides, or conjugated diene, which are hampered by various methodological limitations (Basu, 2004).

Isoprostanes are very well suited as BOSS. (1) They can be measured accurately down to picomolar levels with analytic techniques such as HPLC, GC–MS, LC–MS, or RIA. The former three techniques are supremely able to discriminate among the different types of isoprostanes but require extensive preparation of the material (e.g., phospholipid extraction, alkaline hydrolysis) and expensive instrumentation. RIA are somewhat easier to perform and are widely commercially

available; however, many of these are not able (or have not yet been shown to be able) to distinguish between the prostanoids and the isoprostanes, let alone among the different types of isoprostanes. (2) They are stable in isolated samples of body fluids, and can be detected in breath condensates, providing an exceedingly noninvasive route for their measurement. (3) Their measured values do not exhibit diurnal variations nor are affected by lipid content in the diet, but do vary markedly in clinical and experimental conditions characterized by oxidative stress, and these values closely parallel disease severity. (4) They are specific products of peroxidation. (5) They are present in detectable amounts in all normal tissues and biological fluids, thus allowing definition of a normal range.

Most of the assays of isoprostanes to date have focused on assessment in body fluids of 8-*iso*-$PGF_{2\alpha}$, which is a major product of the total lipid peroxidation process in vivo. Several other F_2-isoprostanes of the $ipF_{2\alpha}$-IV series are also found in high concentrations in the urine and are considered to be reliable parameters of oxidant stress.

Dozens of diseases and experimental conditions with diverse etiologies have been shown to be associated with marked increases in urinary, plasma, and tissue levels of F_2-isoprostanes (Janssen, 2001; Basu, 2004; Montuschi et al., 2004). However, they should be considered not just mere markers, but also "mediators" of disease, as they evoke important biological responses on virtually every cell type found within the lung. In fact the isoprostanes may mediate many of the features of the disease states for which they are used as indicators (Janssen, 2001). 8-*iso*-$PGF_{2\alpha}$ is generated in substantial amounts in otherwise "normal" individuals exposed to cigarette smoke, allergen, ozone, or hyperoxia, and during ventilated ischemia. It is also markedly elevated, serving as a BOSS, in the BAL fluid, plasma, urine, or exhaled breath condensate (a noninvasive method for direct measurement of oxidant stress in the lungs) in several pulmonary diseases such as asthma, COPD, interstitial lung disease, cystic fibrosis, pulmonary hypertension, acute chest syndrome, sickle cell disease, acute lung injury including acute respiratory distress syndrome (ARDS), and severe respiratory failure in infants as well as in chronic healthy smokers (Montuschi et al., 2000a,b, 2004; Wood et al., 2003; Basu, 2004). Systemic and synovial fluid levels of 8-*iso*-$PGF_{2\alpha}$ were higher in patients with RA, psoriatic arthritis, reactive arthritis, and osteoarthritis than in healthy controls. Similarly, a number of cardiovascular conditions feature marked elevations in isoprostane levels, including during and after cardiopulmonary bypass, renal, cerebral, and myocardial ischemia-reperfusion injury, unstable angina, heart failure, coronary heart disease, acute ischemic stroke, hypercholesterolemia, and atherosclerosis. Recently urinary 8-*iso*-$PGF_{2\alpha}$, measured by GC–MS/MS, was found to be a novel, sensitive, and independent risk marker in patients with coronary heart disease, in addition to known risk factors of this pathology, namely diabetes mellitus, hypercholesterolemia, hypertension, obesity, and smoking (Schwedhelm et al., 2004). Elevated levels of 8-*iso*-$PGF_{2\alpha}$ have also been found in plasma or urinary samples from type 2 diabetic patients (Basu, 2004).

Levels of F_2- and F_4-isoprostanes (arising from oxidation of docosahexaenoic acid, an abundant unsaturated fatty acid in the CNS, and often called neuroprostanes) are elevated in the CSF from AD patients (Montine et al., 1998, 2002a; Pratico et al., 2000; Halliwell, 2001; Butterfield, 2002; Butterfield et al., 2002; Barnham et al., 2004), and may even be elevated prior to the development of AD (Pratico et al., 2001, 2002), consistent with the view that peroxidation may be an important step in progressive neuronal injury leading to clinically manifested disease (Halliwell, 2001; Pratico et al., 2001, 2002; Butterfield, 2002; Butterfield et al., 2002). It has been claimed that urinary and plasma levels of isoprostanes are also elevated in AD patients to an extent correlated with the degree of cognitive impairment (Pratico et al., 2000, 2002), although this was not confirmed in other studies (Montine et al., 2002a; Bohnstedt et al., 2003). The reason for this discrepancy is possibly because the samples are not taken at the same stage of the disease or various drugs that the patients use might have affected the outcome. A majority of AD studies have shown that isoprostanes are localized in the tissues or in the CSF. Finally, 8-*iso*-$PGF_{2\alpha}$ has been found to be a useful marker of oxidative damage and lipid peroxidation in disease states as diverse as multiple sclerosis, systemic lupus erythematosus, several hepatic pathologies (e.g., acute and chronic alcoholic liver disease), and inflammatory diseases (Janssen, 2001; Montuschi et al., 2004; Pratico et al., 2004).

16.4 PROTEINS AS BIOMARKERS OF OXIDATIVE STRESS STATUS

Proteins are major targets for ROS/RNS when these are formed in vivo either in intra- or extracellular environments, as these are the major component of most biological systems. It has been estimated that proteins can scavenge 50% to 75% of reactive radicals such as $^{\bullet}OH$ (Davies et al., 1999).

Exposure of proteins to ROS/RNS may alter every level of protein structure from primary to quaternary (if multimeric proteins), resulting in major physical changes in protein structure. Oxidative damage to proteins is induced either directly by ROS/RNS or indirectly by reaction of secondary by-products of oxidative stress and can occur via different mechanisms, resulting in peptide backbone cleavage, cross-linking, and/or modification of the side chain of virtually every amino acid (Berlett and Stadtman, 1997; Dean et al., 1997; Stadtman and Berlett, 1997; Davies et al., 1999; Davies, 2004; and Chapter 1 in this volume). Most protein damage is nonrepairable, and oxidative changes of protein structure can have a wide range of downstream functional consequences (Fig. 16.3), such as inhibition of enzymatic and binding activities, increased susceptibility to aggregation and proteolysis, increased or decreased uptake by cells, and altered immunogenicity (Stadtman, 1990; Dean et al., 1997; Grune et al., 2003; Ischiropoulos, 2003; Kelly and Mudway, 2003; Requena et al., 2003; Stadtman and Levine, 2003; Stadtman et al., 2003; Dalle-Donne et al., 2005). However, not all proteins are equally sensitive to oxidative damage, and oxidation susceptibility depends on the structure of the protein (e.g., sequence motifs, residues exposed on the molecular surface, bound metal atoms). Oxidative damage to proteins may be important

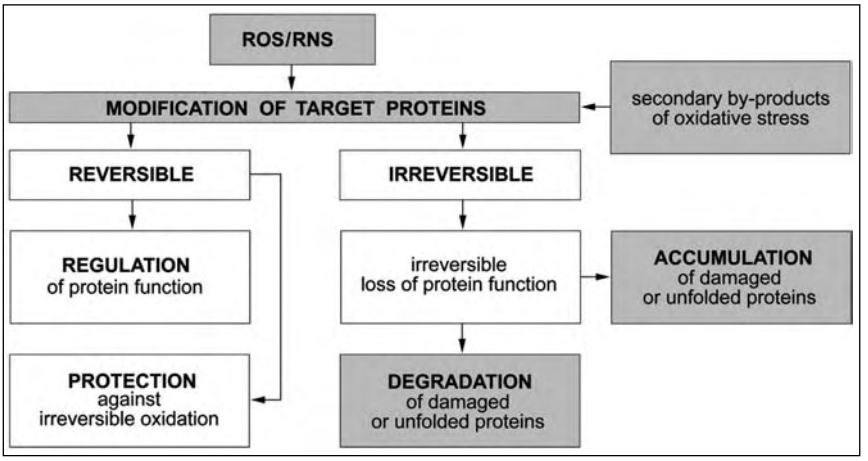


FIGURE 16.3 Consequences of reactive species on protein function and fate. ROS/RNS may cause oxidative modifications on sensitive target proteins. Reversible modifications, usually at Cys and Met residues, may have a dual role of modulation of protein function and protection from irreversible modification. Irreversible modifications are usually associated with permanent loss of protein function and may lead either to the degradation of the damaged proteins by the proteasome system and other proteases or to their progressive accumulation.

in vivo not only in its own right (affecting the function of receptors, enzymes, transport proteins, etc.) but also because it can contribute to secondary damage to other biomolecules, for example, inactivation of DNA repair enzymes and loss of fidelity of damaged DNA polymerases in replicating DNA (Halliwell and Gutteridge, 1999). The major fate of oxidized proteins is catabolism by proteosomal and lysosomal pathways, but some materials appear to be poorly degraded and accumulate within cells (Fig. 16.3) (Dean et al., 1997; Grune et al., 2003; and Chapter 17 in this volume). The accumulation of such damaged material may contribute to a range of human pathologies.

16.4.1 Protein Carbonyls

For many ROS/RNS the modifications that they carry out on proteins have been characterized, and several of their molecular targets are known. A number of reactions determining the cleavage of the protein backbone have been elucidated, resulting in the generation of protein carbonyls (Berlett and Stadtman, 1997; Dean et al., 1997; Stadtman and Levine, 2003; Davies, 2004; and Chapter 1 in this volume). Nevertheless, although peptide backbone cleavage can be easily detected with isolated proteins, its use as a marker of protein oxidation in vivo is very limited because of the occurrence of other proteins in complex systems and the potential role of proteases in polypeptide hydrolysis. Thus backbone fragmentation is rarely used to quantify protein oxidation in complex systems. Differently, some side chain oxidation products can be employed

as sensitive, specific markers of oxidative damage, and the product profile can, in some cases, provide valuable information on the species involved. Therefore, the use of stable products of amino acid side chain oxidation as potential markers for assessing oxidative damage in vivo is amply diffused and applicated in the study of human disease (Beal, 2002; Heinecke, 2002; Turko and Murad, 2002; Brennan and Hazen, 2003; Dalle-Donne et al., 2003a,b, 2005; Stocker and Keaney, 2003; and most chapters in Part III of this volume), but the field has emerged in its own right over the last decade, propelled by redox proteomics and MS techniques (Aulak et al., 2001; Baty et al., 2002; Fratelli et al., 2002, 2003; Heinecke, 2002; Ahmed and Thornalley, 2003; Butterfield and Castegna, 2003; Ghezzi and Bonetto, 2003; Requena et al., 2003; Thornalley et al., 2003; Butterfield, 2004; Dalle-Donne et al., 2005; and most chapters in Part I of this volume).

Protein carbonyls may be generated by the oxidation of several amino acid side chains (Lys, Arg, Pro, Thr), by the formation of Michael adducts between Lys, His, and Cys residues and α,β-unsaturated aldehydes, forming ALEs, and also by glycation/glycoxidation of Lys amino groups, forming AGEs (Berlett and Stadtman, 1997; Dean et al., 1997; Stadtman and Berlett, 1997; Dalle-Donne et al., 2003a,b; Stadtman and Levine, 2003; Davies, 2004; and Chapter 5 in this volume). The formation of carbonyl compounds is actually employed as the most general and widely used marker of severe protein oxidation both in vitro and in vivo, with a number of assays developed for the quantitation of these species (Winterbourn and Buss, 1999; Levine et al., 2000; Dalle-Donne et al., 2003b). As a marker of oxidative damage to proteins, carbonyls have been shown to accumulate during aging, ischemia-reperfusion injury, chronic inflammation, cystic fibrosis, and a great number of age-related diseases, such as diabetes and its related complications and atherosclerosis, in a variety of organisms (Berlett and Stadtman, 1997; Dean et al., 1997; Stadtman and Berlett, 1997; Miyata et al., 1999; Thorpe and Baynes, 1999; Heinecke, 2002; Levine, 2002; Sohal, 2002; Thornalley, 2002; Dalle-Donne et al., 2003a, 2005; Stocker and Keaney, 2003; Kettle et al., 2004).

Specific carbonylated proteins have been detected in both the brain and plasma of AD subjects (Dalle-Donne et al., 2003a, 2005; Butterfield and Castegna, 2003; Butterfield, 2004; and Chapter 18 in this volume). The identification of specific proteins that are more affected by carbonylation, and consequently more prone to inactivation, in AD brain has been amply investigated by Butterfield and colleagues, who first used redox proteomics to address this issue (see Chapter 18 in this volume). The observation of carbonylated proteins in plasma—a body fluid easily obtainable without invasive procedures and, more interesting and unlike brain samples, before the death of the subject—suggests that these oxidized species are useful as (possibly presymptomatic) diagnostic biomarkers for AD.

The carbonyl group content in plasma proteins (mainly albumin and γ-globulines) of children with different forms of juvenile chronic arthritis was significantly higher than in healthy fellows, and more important, the carbonyls increased parallelwise with the activity of the disease. Correlation between the carbonyl level and the activity or the type of juvenile chronic arthritis indicates that plasma

protein carbonyl groups are a good marker of inflammatory process activity and may allow use of carbonyls as a clinical marker of antioxidant barrier impairment in this group of patients, of use in monitoring of possible pharmacologic treatment (Renke et al., 2000).

The plasma content of protein carbonyls, as well as free F_2-isoprostanes, and protein-reduced thiols differ significantly between chronic kidney disease patients and healthy subjects; furthermore such BOSS are significantly higher in patients with diabetes and hypercholesterolemia (Oberg et al., 2004).

Winterbourn and co-workers have determined that severe sepsis and major trauma patients had elevated protein carbonyl concentrations in both plasma and BAL fluid, which correlates well with ALE measurements and indexes of neutrophilia and neutrophil activation (Winterbourn et al., 2000), and that patients with acute pancreatitis had significantly increased concentration of protein carbonyls in plasma, which were related to disease severity, thus confirming that this protein modification is a useful plasma marker of oxidative injury (Winterbourn et al., 2003).

16.4.2 Oxidative Tyrosine Modifications

Elevated levels of stable halogenated Tyr residues have been detected in proteins isolated from atherosclerotic plaques as well as plasma and airways secretions of patients with asthma, ARDS, and cystic fibrosis, and are widely used as markers for damage mediated by hypohalous acids (HOCl, HOBr) in these diseases (van der Vliet et al., 2000; Stocker and Keaney, 2003; Kettle et al., 2004; Pattison and Davies, 2004; Dalle-Donne et al., 2005). The major products are 3-chlorotyrosine and 3-bromotyrosine (Cl-Tyr and Br-Tyr, respectively), but dihalogenated compounds (3,5-dichlorotyrosine and 3,5-dibromotyrosine) are formed with high excesses of HOCl and HOBr. Dramatic selective enrichment in 3-nitrotyrosine (NO_2-Tyr), a post-translational modification specific for protein oxidation by $^{\bullet}NO$-derived oxidants, and Cl-Tyr content within apolipoprotein A-I (apoA-I), the major protein constituent within HDL, recovered from human plasma and atherosclerotic lesions has been demonstrated using proteomic and MS techniques. Analysis of serum also demonstrates that the NO_2-Tyr and Cl-Tyr contents of apoA-I are markedly higher in individuals with established coronary artery disease (Bergt et al., 2004; Pennathur et al., 2004; Zheng et al., 2004). These observations suggest that elevated levels of Cl-Tyr and NO_2-Tyr in circulating HDL might represent specific markers for clinically significant atherosclerosis. Remarkably HDL from human aortic atherosclerotic intima had an 8-fold higher level of Cl-Tyr than plasma HDL. Moreover, the level of Cl-Tyr was 13-fold higher in HDL isolated from plasma of subjects with established coronary artery disease than in HDL from plasma of healthy subjects (Bergt et al., 2004). Elevated levels of Cl-Tyr have also been detected in LDL isolated from human atherosclerotic lesions (Hazen and Heinecke, 1997; Leeuwenburgh et al., 1997; Heinecke, 2002). The mean level of NO_2-Tyr in HDL isolated from human aortic atherosclerotic intima was 6-fold higher than that in circulating HDL. Moreover, levels of plasma HDL from patients with established coronary

artery disease contained twice as much NO_2-Tyr as HDL from plasma of healthy subjects (Pennathur et al., 2004). It is noteworthy that the level of NO_2-Tyr in lesion HDL was very similar to that previously reported for lesion LDL, which contains much a higher level of NO_2-Tyr than does circulating LDL (Heinecke, 2002), indicating that both lipoproteins are nitrated to a similar extent in the human artery wall. The clinical relevance of protein Tyr nitration has been recently emphasized by the observation of a strong association between protein NO_2-Tyr levels and coronary artery disease risk. Circulating levels of protein NO_2-Tyr may serve as an independent biomarker to assess atherosclerosis risk, burden, and incident cardiac events, as well as to monitor the vasculoprotective action of drugs such as statins (hydroxymethylglutaryl coenzyme A reductase inhibitors) (Shishehbor et al., 2003a,b).

Tyrosine nitration is one of the earliest markers found in AD brains, in the plaques of multiple sclerosis brains, and in degenerating upper and lower motor neurons in ALS patients (Ischiropoulos and Beckman, 2003; Dalle-Donne et al., 2005). Nitrated α-synuclein selectively accumulates in Lewy bodies and protein inclusions in a wide range of pathologies (AD, PD, synucleinopathies, tauopathies) (Dalle-Donne et al., 2005). NO_2-Tyr is formed in diverse inflammatory diseases, including ARDS, severe asthma, inflammatory bowel disease, chronic renal failure, RA, as well as in type 1 and 2 diabetes, and cystic fibrosis (Dalle-Donne et al., 2005). On the other hand, basal protein nitration has been detected under physiological conditions in most tissues, including plasma and the human pituitary, and some of these nitrated proteins have been identified. In the normal human pituitary, two-dimensional Western blotting and LC–MS/MS analysis have recently been used to detect and characterize four nitrated proteins, including actin, that participate in neurotransmission, cellular immunity, and cellular structure and motility (Zhan and Desiderio, 2004). These data are consistent with the emerging perspective that low levels of Tyr nitration may be a physiological regulator of a signaling pathway (Greenacre and Ischiropoulos, 2001; Turko and Murad, 2002; Zhan and Desiderio, 2004). Although protein Tyr nitration is a low-yield process in vivo, and under inflammatory conditions one to five NO_2-Tyr residues per 10,000 Tyr residues (100–500 μmol/mol) are detected, relatively limited number of proteins are preferential targets of nitration, and within these proteins only one or a few specific Tyr can be nitrated (Greenacre and Ischiropoulos, 2001).

A biomarker worth further development for human use might be *o*,*o*′-dityrosine (di-Tyr), which is apparently not metabolized and is easily detectable also in urine. Elevated levels of di-Tyr have been reported in atherosclerosis, where its accumulation positively correlates with disease severity, AD, cystic fibrosis, end-stage renal disease, and acute inflammation with or without sepsis (Dalle-Donne et al., 2005). Dityrosine has also been proposed as a marker of organismal oxidative stress, like atherosclerosis, acute inflammation, and systemic bacterial infections. In this regard di-Tyr concentrations were found 100-fold higher in LDL isolated from atherosclerotic lesions than in normal ones, and humans suffering from systemic bacterial infections had twice the concentration of di-Tyr in urine

than those of healthy individuals (Heinecke, 2002). Moreover, di-Tyr has the advantage of being metabolically stable because, once the 3′-3′ carbon–carbon bond is formed, it is only released after enzymatic hydrolysis of the oxidatively modified protein (Giulivi et al., 2003).

16.4.3 *S*-Nitrosation and *S*-Glutathionylation

Both *S*-nitrosated and *S*-glutathionylated proteins have been investigated as possible markers of oxidative/nitrosative stress in correlation with pathophysiological conditions such as Friedreich's ataxia, renal cell carcinoma, and diabetes (Klatt and Lamas, 2000; Pastore et al., 2003a; Giustarini et al., 2003b, 2004a; Dalle-Donne et al., 2005). A significant increase in glutathionyl-hemoglobin and glutathionyl-actin has been found in the blood and fibroblasts, respectively, of patients with Friedreich's ataxia (Pastore et al., 2003a; Giustarini et al., 2004a). Glutathionyl-hemoglobin is also increased in patients suffering from type 1 and type 2 diabetes, hyperlipidemia, and uremia associated with hemodialysis or peritoneal dialysis (Giustarini et al., 2003b, 2004a). Elevated protein *S*-nitrosation has been reported in RA, multiple sclerosis, bronchopulmonary dysplasia, and after lung transplantation; further, increased levels of *S*-nitroso-albumin have been measured in pre-eclampsia, hemodialysis, and hypercholesterolemia (Dalle-Donne et al., 2005). Parkin is a ubiquitin E3 ligase involved in the ubiquitination of proteins that are important in the survival of dopamine neurons in PD. In addition parkin may play a more general role in the ubiquitin proteasomal pathway by participating in the removal and/or detoxification of abnormally folded or damaged proteins. Mutations in parkin that lead to a loss of parkin's ubiquitin E3 ligase activity are the most common cause of hereditary PD (autosomal recessive juvenile Parkinsonism). It has recently been shown that parkin is *S*-nitrosated in vitro, as well as in vivo in a mouse model of PD and in postmortem brains of patients with PD and diffuse Lewy body disease. This *S*-nitrosation markedly inhibits parkin's ubiquitin E3 ligase activity and its protective function. In this manner *S*-nitrosation of wild-type parkin would affect the ubiquitin-proteasome system degradative pathway and contribute to both protein aggregation and the degenerative process, and therefore the Parkinsonian phenotype, in these disorders (Chung et al., 2004; Yao et al., 2004).

16.4.4 Methionine Oxidation

Surface-exposed Met residues of proteins are readily oxidized by almost all forms of ROS to methionine sulfoxide (MetO). But, in contrast to the oxidation of other amino acid residues (except the formation of cystine and mixed disulfides, which can be reduced back to the corresponding thiols by a battery of reductases and isomerases, and possibly Tyr nitration by enzymatic "denitration"; Aulak et al., 2004), the oxidation of Met residues is readily reversed by the action of methionine sulfoxide reductases that catalyze the thioredoxin-dependent reduction of MetO back to Met. Based on this consideration, it was proposed that the oxidation-reduction of Met residues of proteins may serve an important

antioxidant function; that is, Met residues may, in some cases, serve as internal scavengers and protect critical amino acyl residues from oxidative attack (Levine et al., 1996; Stadtman et al., 2003; Requena et al., 2004). Given its reversibility, MetO has not been used much as a biomarker of oxidative damage in human studies. Anyway, the oxidation of protein Met residues is associated with the development of some diseases, including respiratory distress syndrome, emphysema, bronchiolitis obliterans syndrome, cataract formation, and reperfusion injury (Stadtman et al., 2003; Davies, 2004; Dalle-Donne et al., 2005). In addition, a decrease in methionine sulfoxide reductase activities in various brain regions, paralleled by increased levels of MetO in these regions, is associated with the development of AD (Gabbita et al., 1999).

16.4.5 Pros and Cons of Using Proteins as Biomarkers of Oxidative Damage

Compared to measuring products of lipid peroxidation and oxidative DNA damage, proteins offer some advantages as biomarkers of oxidative stress/damage (Table 16.4). There are a number of sensitive assays available for detection of oxidatively modified proteins (e.g., Davies et al., 1999; Winterbourn and Buss, 1999; Levine et al., 2000; Pryor, 2001; Jaffrey et al., 2001; Baty et al., 2002; Griffiths et al., 2002; Dalle-Donne et al., 2003b, 2005; Fratelli et al., 2003; Butterfield et al., 2004; Carini et al., 2004; Halliwell and Whiteman, 2004). Thus, from a purely technical perspective, oxidized proteins serve as suitable BOSS.

TABLE 16.4 Principal Advantages and Disadvantages in the Use of Proteins as Biomarkers of Oxidative/Nitrosative Damage

Advantages
• Products of protein oxidative/nitrosative modifications are relatively stable
• Proteins can retain the fingerprint of the initial oxidative insult that mediates damage
• Proteins have unique biological functions, so there are unique functional consequences resulting from their oxidative/nitrosative modifications
• Sensitive assays are available for detection of oxidized proteins
• Some oxidized proteins have long half-lives
• The nature of the protein modification can give significant information as to the type of ROS/RNS involved in the oxidation process
Disadvantages
• Twenty different amino acids can be attacked by ROS/RNS in multiple ways
• There is no single universal marker for protein oxidative/nitrosative modification
• It may be necessary to set up several different sensitive assays in order to find the most appropriate assay for the type of ROS/RNS involved

Many amino acid oxidation products are superior to their lipid oxidation product counterpart in terms of stability during sample storage. For example, protein carbonyls form early and remain circulating for longer periods in the blood compared with other parameters of oxidative stress, such as GSSG or MDA; their elevation in serum is stable for at least four hours (Pantke et al., 1999). The chemical stability of protein carbonyls makes them suitable targets for laboratory measurement and is also useful for their storage: their stability on storage has been demonstrated for 10 years at $-80^{\circ}C$ (Stadtman and Levine, 2003). Species like F_2-isoprostanes are readily generated during sample storage, processing, and analysis. Further, unlike MS methods for quantifying amino acid oxidation products, assays for lipid oxidation products have not yet been reported that routinely monitor for artifactual formation during sample storage and analysis, such as through incorporation of isotopically labeled parent lipids. The costs of such an assay would be prohibitive for widespread use. Thus, a significant advantage of protein oxidation products as markers of disease risk is that they are more readily usable in banked specimens. Archival specimens from preexisting clinical studies, which will undoubtedly play a critical role in validating the clinical utility of any oxidation marker, cannot be employed for examining lipid oxidation products unless extensive precautionary measures were taken to prevent artificial oxidation. Stable species like Cl-Tyr and NO_2-Tyr, which are not formed during prolonged storage in a freezer, are thus ideally suited from a stability standpoint for serving as reliable BOSS in vivo. These species suffer, however, from the rather complex analytical methods currently required for their accurate quantification using MS. While commercial ELISA kits are currently available for NO_2-Tyr quantification, such assays are far from accurate, with values typically over an order of magnitude off from those determined from more rigorous quantification methods like stable isotope dilution tandem MS (Brennan and Hazen, 2003).

The product profile can in some cases provide valuable information on the species involved, incorporating elements of the original oxidant. Then the use of some protein oxidation products as BOSS has the potential not only to determine the extent of oxidative injury but also to identify the source of the oxidant. Such information is important for predicting the consequences of oxidation as well as for providing a basis for designing appropriate interventions to alleviate injury. For example, for HOCl, oxidative reactions are fastest but tend to be nonspecific, so oxidation products are not useful as specific markers for HOCl (with the possible exception of glutathione sulfonamide). Chlorination reactions tend to be much slower than oxidations, and smaller amounts of chlorinated products are therefore formed. However, by incorporating chlorine into the target molecule, they are more specific. The most favored chlorination reaction of HOCl is with amine groups. However, the chloramines produced should be short-lived in biological fluids and unsuitable biomarkers. Chlorination of Tyr residues is slower than for amines, but the end-products are stable and more suitable and valuable as biomarkers of HOCl formation. Thus, the analysis of protein levels of Cl-Tyr and 3,5-dichlorotyrosine by GC and MS is currently the best method available for probing the involvement of oxidation by myeloperoxidase and its products in

the pathology of particular diseases (Hazen et al., 1999; Winterbourn and Kettle, 2000; Kettle et al., 2004). Tyrosyl radical (the oxidizing intermediate generated by myeloperoxidase and other peroxidases) forms di-Tyr as the major product, whereas nitration of protein Tyr residues is one of the molecular footprints left by $^{\bullet}$NO-derived oxidants (RNS), such as peroxynitrite anion ($ONOO^-$) and nitrogen dioxide ($^{\bullet}NO_2$), during oxidative damage to tissues. This contrasts with lipid peroxidation, where propagation reactions involving the initial lipid oxidation products result in loss of the information that tells us about the initial oxidative insult. Diversely, carbonyls can be induced by almost all types of ROS, and hence they do not shed significant light on the source of the oxidative stress. In addition carbonyls are relatively difficult to induce compared to MetO and cysteinyl derivatives, and they may thus be reflective of more severe cases of oxidative stress (Dalle-Donne et al., 2001, 2002). Indeed detection of elevated levels of protein carbonyls is generally a sign not only of oxidative stress but also of a disease-associated dysfunction (Berlett and Stadtman, 1997; Stadtman and Berlett, 1997; Levine, 2002; Dalle-Donne et al., 2003c).

Proteins show some disadvantages as BOSS. Analyzing protein oxidative damage products is an order of magnitude more complex than dealing with DNA: rather than four bases and one sugar there are 20 different amino acids, each of which can be potentially attacked by ROS/RNS in multiple ways, generating a huge variety of products. Therefore there is no a single universal marker for protein oxidative damage. However, carbonyl formation at different side chains is a major mechanism and seems to be a common phenomenon during oxidation, and quantification of protein carbonyls allows the determination of the extent of oxidative modification. So protein carbonyl content is actually the most general indicator and by far the most commonly used marker of protein oxidation (Berlett and Stadtman, 1997; Beal, 2002; Levine, 2002; Levine et al., 2002; Dalle-Donne et al., 2003a,b). Because so many different protein oxidation products can be formed, it may be necessary to set up several different sensitive assays in order to find the most appropriate one for the type of ROS/RNS involved.

It should be highlighted that ROS/RNS are not only a cause of structural and functional damage; they are also physiologically important mediators in biological signaling processes, being widely used as second messengers to propagate pro-inflammatory or growth stimulatory signals (Thannickal and Fanburg, 2000; Mikkelsen and Wardman, 2003). Abnormally high levels of ROS/RNS may therefore lead to dysregulation of redox-sensitive signaling pathways. The redox-sensitive targets in these pathways are often signaling proteins with redox-sensitive Cys residues that are oxidized to sulfenic acid moieties and mixed disulfides, thereby altering the signaling function of the protein (Klatt and Lamas, 2000; Giustarini et al., 2004a; Martinez-Ruiz and Lamas, 2004).

16.5 CONCLUSIONS

Whether ROS/RNS activity has a causal or propagating role in human diseases associated with oxidative stress remains generally unresolved (Ischiropoulos

and Beckman, 2003; Stocker and Keaney, 2003). Nevertheless, the finding of increased oxidative stress in pathological conditions such as AD, chronic and acute inflammatory diseases, and diabetes has suggested the use of BOSS for the development of new diagnostic, therapeutic, and preventive strategies for preventing or delaying the development of complications such as atherosclerosis and coronary artery disease. Consequently, the availability of biomarkers that can provide an accurate assessment of the degree of oxidative damage will become important in clinical trials aimed at investigating the effectiveness of antioxidant therapy for preventing or reducing the risks of complications. While there is ample experimental evidence demonstrating the protective effects of antioxidants in in vitro models, for example, of neurodegeneration, and in some animal models, the clinical evidence that antioxidants act as protective drugs is still relatively scarce and/or controversial (Gilgun-Sherki et al., 2002, 2003). Nevertheless, approaches directed at inhibiting the oxidative modifications caused by ROS/RNS open new avenues for the treatment of inflammatory, vascular, and neurodegenerative diseases (Sano et al., 1997; Desnuelle et al, 2001; Christen, 2002; Gilgun-Sherki et al., 2002, 2003; Stocker and Keaney, 2003; Barnham et al., 2004).

A key issue is that the effectiveness of a given biological antioxidant may depend on the ROS/RNS involved. Carotenoids, for example, are highly efficient antioxidants when the oxidizing species is singlet oxygen, but their effectiveness for other ROS is more questionable. Unquestionably, the relative importance of antioxidants as protective agents depends on which ROS/RNS is generated, how it is generated, where it is generated, what target of damage is measured, and the severity of the damage (Halliwell and Gutteridge, 1999). This is particularly relevant and extremely important in the setting of disease research, in that the key ROS are rarely known (mainly because the possible sources for the overproduction of reactive species are widespread), although there are a few limited situations wherein the etiology is known (e.g., erythropoietic protoporphyria is known to be a singlet oxygen-mediated condition). Indeed, since methods currently available for the direct measurement of ROS/RNS are of limited applicability to humans (Halliwell and Whiteman, 2004), most clinical studies focus on the measurement of oxidative damage. This is to some extent logical, since it is the damage caused by reactive species that is important rather than the total amount of such species generated. So far the understanding is that some protein biomarkers of oxidative damage can be useful indicators of the type of ROS involved (see above). For instance, the detection of elevated levels of Cl-Tyr in the bronchoalveolar fluid from children with cystic fibrosis led Kettle and colleagues to conclude that HOCl is produced early in cystic fibrosis and that it is a candidate for precipitating the fatal decline in lung function associated with this disease (Kettle et al., 2004).

In the last few years there has been a dramatic transformation in our ability to conduct qualitative and quantitative analysis of oxidative protein modifications. The development of redox proteomics represents an exciting new way to examine pathological processes at the molecular level, and already improvements have

been made in the understanding of many conditions. The application of redox proteomics provides major opportunities to elucidate disease mechanisms and to identify new diagnostic markers and therapeutic targets. Proteome-wide screening for modified proteins promises to be an exciting area of research in the immediate future. High-throughput methods have recently been developed for examining global protein *S*-nitrosation, *S*-glutathionylation, Tyr nitration, and advanced glycation (Aulak et al., 2001; Jaffrey et al., 2001; Baty et al., 2002; Fratelli et al., 2002, 2003; Ahmed and Thornalley, 2003; Zhan and Desiderio, 2004; Tsikas and Caidahl, 2005). Biomarkers derived from redox proteomics (Ghezzi and Bonetto, 2003; Butterfield, 2004; Dalle-Donne et al., 2005) have the potential to dramatically reduce both the time and the risk involved in target discovery and validation, better understand disease mechanisms (novel therapeutic modalities), diagnose and track diseases and drug actions, and, finally, profile patients for personalized medicine. However, the importance of detecting and quantifying BOSS in vivo, as well as of detection of more than one BOSS, should be emphasized because a single biomarker may give misleading results. It needs to be examined whether the level of a specific biomarker reflects the severity of the oxidative stress that has been exerted on the subject as well as whether this BOSS, alone or in combination with others, can serve as a true indicator for the health status of the subject and allows the monitoring of the success or failure of a treatment.

A voluminous body of research on the potential role of antioxidant therapy in preventing or retarding human diseases associated with oxidative stress has accumulated over the past several decades. Despite this effort there is much that remains unknown. The identification and functional characterization of oxidized proteins and the use of oxidized proteins such as BOSS have the potential to help fill the gaps in current knowledge. Thus, it is reasonable to believe that the next few years will bring more precise definition of the events that lead from protein oxidative modification to altered cellular function. This is what is needed if a strong experimental basis is to be developed of new diagnostic, therapeutic, and preventive strategies for human diseases associated with oxidative stress. The challenge that lies ahead is formidable and requires a multidisciplinary approach to further our understanding of the complex relationships among oxidative damage, antioxidants, and human disease.

LIST OF ABBREVIATIONS

AD, Alzheimer's disease
AGEs, advanced glycation end-products
ALEs, advanced lipoxidation end-products
ALS, amyotrophic lateral sclerosis
ARDS, adult (acute) respiratory distress syndrome
BAL, bronchoalveolar lavage
Br-Tyr, 3-bromotyrosine
Cl-Tyr, 3-chlorotyrosine

COPD, chronic obstructive pulmonary disease
CSF, cerebrospinal fluid
di-Tyr, *o*,*o*′-dityrosine
ELISA, enzyme-linked immunosorbent assay
ESI, electrospray ionization
GC, gas chromatography
GSH, reduced glutathione
GSSG, glutathione disulfide
HDL, high-density lipoprotein
HNE, 4-hydroxy-*trans*-2-nonenal
HPLC, high-performance liquid chromatography
LC, liquid chromatography
LDL, low-density lipoprotein
MALDI, matrix-assisted laser desorption/ionization
MDA, malondialdehyde
MetO, methionine sulfoxide
MS, mass spectrometry
NO_2-Tyr, 3-nitrotyrosine
8-OHdG, 8-hydroxy-2′-deoxyguanosine
PD, Parkinson's disease
RA, rheumatoid arthritis
RNS, reactive nitrogen species
ROS, reactive oxygen species
SOD, superoxide dismutase
TBARS, thiobarbituric acid-reactive substances

REFERENCES

Ahmed N., Thornalley P.J. (2003). Quantitative screening of protein biomarkers of early glycation, advanced glycation, oxidation and nitrosation in cellular and extracellular proteins by tandem mass spectrometry multiple reaction monitoring. *Biochem Soc Trans* 31: 1417–1422.

Alexander M.D., Traynor B.J., Miller N., Corr B., Frost E., McQuaid S., Brett F.M., Green A., Hardiman O. (2002). "True" sporadic ALS associated with a novel SOD-1 mutation. *Ann Neurol* 2: 680–683.

Ammar R.F., Gutterman D.D., Brooks L.A., Dellsperger K.C. (2000). Free radicals mediate endothelial dysfunction of coronary arterioles in diabetes. *Cardiovasc Res* 47: 595–601.

Aulak K.S., Miyagi M., Yan L., West K.A., Massillon D., Crabb J.W., Stuehr D.J. (2001). Proteomic method identifies proteins nitrated in vivo during inflammatory challenge. *Proc Natl Acad Sci USA* 98: 12056–12061.

Aulak K.S., Koeck T., Crabb J.W., Stuehr D.J. (2004). Dynamics of protein nitration in cells and mitochondria. *Am J Physiol Heart Circ Physiol* 286: H30–H38.

Barnham K.J., Masters C.L., Bush A.I. (2004). Neurodegenerative diseases and oxidative stress. *Nat Rev Drug Discov* 3: 205–214.

Barreiro E., de la Puente B., Minguella J., Corominas J.M., Serrano S., Hussain S.N., Gea J. (2005). Oxidative stress and respiratory muscle dysfunction in severe chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 171: 1116–1124.

Basu S. (2004). Isoprostanes: Novel bioactive products of lipid peroxidation. *Free Radic Res* 38: 105–122.

Baty J.W., Hampton M.B., Winterbourn C.C. (2002). Detection of oxidant sensitive thiol proteins by fluorescence labeling and two-dimensional electrophoresis. *Proteomics* 2: 1261–1266.

Beal M.F. (2002). Oxidatively modified proteins in aging and disease. *Free Radic Biol Med* 32: 797–803.

Bergt C., Pennathur S., Fu X., Byun J., O'Brien K., McDonald T.O., Singh P., Anantharamaiah G.M., Chait A., Brunzell J., Geary R.L., Oram J.F., Heinecke J.W. (2004). The myeloperoxidase product hypochlorous acid oxidizes HDL in the human artery wall and impairs ABCA1-dependent cholesterol transport. *Proc Natl Acad Sci USA* 101: 13032–13037.

Berlett B.S., Stadtman E.R. (1997). Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem* 272: 20313–20316.

Berliner J., Heinecke J.W. (1996). The role of oxidized lipoproteins in atherogenesis. *Free Radic Biol Med* 20: 707–727.

Bharath S., Hsu M., Kaur D., Rajagopalan S., Andersen J.K. (2002). Glutathione, iron and Parkinson's disease. *Biochem Pharmacol* 64: 1037–1048.

Bogdanov M., Brown R.H., Matson W., Smart R., Hayden D., O'Donnell H., Flint Beal M., Cudkowicz M. (2000). Increased oxidative damage to DNA in ALS patients. *Free Radic Biol Med* 29: 652–658.

Bohnstedt K.C., Karlberg B., Wahlund L.O., Jonhagen M.E., Basun H., Schmidt S. (2003). Determination of isoprostanes in urine samples from Alzheimer patients using porous graphitic carbon liquid chromatography–tandem mass spectrometry. *J Chromatogr B* 796: 11–19.

Brennan M.L., Hazen S.L. (2003). Amino acid and protein oxidation in cardiovascular disease. *Amino Acids* 25: 365–374.

Browne S.E., Ferrante R.J., Beal M.F. (1999). Oxidative stress in Huntington's disease. *Brain Pathol* 9: 147–163.

Butterfield D.A., Lauderback C.M. (2002). Lipid peroxidation and protein oxidation in Alzheimer's disease brain: Potential causes and consequences involving amyloid beta-peptide associated free radical oxidative stress. *Free Radic Biol Med* 32: 1050–1060.

Butterfield D.A., Castegna A., Lauderback C.M., Drake J. (2002). Evidence that amyloid beta-peptide-induced lipid peroxidation and its sequelae in Alzheimer's disease brain contribute to neuronal death. *Neurobiol Aging* 23: 655–664.

Butterfield D.A., Drake J., Pocernich C., Castegna A. (2001). Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid beta-peptide. *Trends Mol Med* 7: 548–554.

Butterfield D.A., Castegna A. (2003). Proteomics for the identification of specifically oxidized proteins in brain: Technology and application to the study of neurodegenerative disorders. *Amino Acids* 25: 419–425.

Butterfield D.A. (2004). Proteomics: A new approach to investigate oxidative stress in Alzheimer's disease brain. *Brain Res* 1000: 1–7.

Butterfield D.A. (2002). Amyloid beta-peptide (1–42)-induced oxidative stress and neurotoxicity: Implications for neurodegeneration in Alzheimer's disease brain. *Free Radic Res* 36: 1307–1313.

Carini M., Aldini G., Facino R.M. (2004). Mass spectrometry for detection of 4-hydroxy-trans-2-nonenal (HNE) adducts with peptides and proteins. *Mass Spectrom Rev* 23: 281–305.

Christen Y. (2000). Oxidative stress and Alzheimer disease. *Am J Clin Nutr* 71: 621S–629S.

Chung K.K., Thomas B., Li X., Pletnikova O., Troncoso J.C., Marsh L., Dawson V.L., Dawson T.M. (2004). *S*-Nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. *Science* 304: 1328–1331.

Cleveland D.W., Rothstein J.D. (2001). From Charcot to Lou Gehrig: Deciphering selective motor neuron death in ALS. *Nat Rev Neurosci* 2: 806–819.

Comhair S.A., Erzurum S.C. (2002). Antioxidant responses to oxidant-mediated lung diseases. *Am J Physiol Lung Cell Mol Physiol* 283: L246–L255.

Cooke M.S, Evans M.D., Dizdaroglu M., Lunec J. (2003). Oxidative DNA damage: Mechanisms, mutation, and disease. *FASEB J* 17: 1195–1214.

Cracowski J.L., Durand T., Bessard G. (2002). Isoprostanes as a biomarker of lipid peroxidation in humans: Physiology, pharmacology and clinical implications. *Trends Pharmacol Sci* 23: 360–366.

Dalle-Donne I., Giustarini D., Colombo R., Rossi R., Milzani A. (2003a). Protein carbonylation in human diseases. *Trends Mol Med* 9: 169–176.

Dalle-Donne I., Rossi R., Giustarini D., Gagliano N., Di Simplicio P., Colombo R., Milzani A. (2002). Methionine oxidation as a major cause of the functional impairment of oxidized actin. *Free Radic Biol Med* 32: 927–937.

Dalle-Donne I., Rossi R., Giustarini D., Gagliano N., Lusini L., Milzani A., Di Simplicio P., Colombo R. (2001). Actin carbonylation: from a simple marker of protein oxidation to relevant signs of severe functional impairment. *Free Radic Biol Med* 31: 1075–1083.

Dalle-Donne I., Rossi R., Giustarini D., Milzani A., Colombo R. (2003b). Protein carbonyl groups as a biomarkers of oxidative stress. *Clin Chim Acta* 329: 23–38.

Dalle-Donne I., Scaloni A., Giustarini D., Cavarra E., Tell G., Lungarella G., Colombo R., Rossi R., Milzani A. (2005). Proteins as biomarkers of oxidative stress in diseases: The contribution of redox proteomics. *Mass Spectrom Rev* 24: 55–99.

Davies M.J. (2005). The oxidative environment and protein damage. *Biochem Biophys Acta*, 1703: 93–109.

Davies M.J., Fu S., Wang H., Dean R.T. (1999). Stable markers of oxidant damage to proteins and their application in study of human disease. *Free Radic Biol Med* 27: 1151–1161.

Dean R.T., Fu S., Stocker R., Davies M.J. (1997). Biochemistry and pathology of radical-mediated protein oxidation. *Biochem J* 324: 1–18.

Desnuelle C., Dib M., Garrel C., Favier A. (2201). A double-blind, placebo-controlled randomized clinical trial of alpha-tocopherol (vitamin E) in the treatment of amyotrophic lateral sclerosis: ALS Riluzole-Tocopherol Study Group. *Amyotroph Lateral Scler Other Motor Neuronal Disord* 2: 9–18.

Esterbauer H., Schaur R.J., Zollner H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* 11: 81–128.

Finkel T., Holbrook N.J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* 408: 239–247.

Fratelli M., Demol H., Puype M., Casagrande S., Eberini I., Salmona M., Bonetto V., Mengozzi M., Duffieux F., Miclet E., Bachi A., Vandekerckhove J., Gianazza E., Ghezzi P. (2002). Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. *Proc Natl Acad Sci USA* 99: 3505–3510.

Fratelli M., Demol H., Puype M., Casagrande S., Villa P., Eberini I., Vandekerckhove J., Gianazza E., Ghezzi P. (2003). Identification of proteins undergoing glutathionylation in oxidatively stressed hepatocytes and hepatoma cells. *Proteomics* 3: 1154–1161.

Frost M.T., Halliwell B., Moore K.P. (2000). Analysis of free and protein-bound nitrotyrosine in human plasma by a gas chromatography/mass spectrometry method that avoids nitration artifacts. *Biochem J* 345: 453–458.

Gabbita S.P., Aksenov M.Y., Lovell M.A., Markesbery W.R. (1999). Decrease in peptide methionine sulfoxide reductase in Alzheimer's disease brain. *J Neurochem* 73: 1660–1666.

Gaut J.P., Byun J., Tran H.D., Heinecke J.W. (2002). Artifact-free quantification of free 3-chlorotyrosine, 3-bromotyrosine, and 3-nitrotyrosine in human plasma by electron capture-negative chemical ionisation gas chromatography mass spectrometry and liquid chromatography–electrospray ionization tandem mass spectrometry. *Anal Biochem* 300: 252–259.

Ghezzi P., Bonetto V. (2003). Redox proteomics: Identification of oxidatively modified proteins. *Proteomics* 3: 1145–1153.

Gilgun-Sherki Y., Melamed E., Offen D. (2003). Antioxidant treatment in Alzheimer's disease: Current state. *J Mol Neurosci* 21: 1–11.

Gilgun-Sherki Y., Rosenbaum Z., Melamed E., Offen D. (2002). Antioxidant therapy in acute central nervous system injury: Current state. *Pharmacol Rev* 54: 271–284.

Giulivi C., Traaseth N.J., Davies K.J. (2003). Tyrosine oxidation products: analysis and biological relevance. *Amino Acids* 25: 227–232.

Giustarini D., Dalle-Donne I., Colombo R., Milzani A., Rossi R. (2003a). An improved HPLC measurement for GSH and GSSG in human blood. *Free Radic Biol Med* 35: 1365–1372.

Giustarini D., Dalle-Donne I., Colombo R., Petralia S., Giampaoletti S., Milzani A., Rossi R. (2003b). Protein glutathionylation in erythrocytes. *Clin Chem* 49: 327–330.

Giustarini D., Dalle-Donne I., Colombo R., Milzani A., Rossi R. (2004b). Interference of plasmatic glutathione and hemolysis on glutathione disulfide levels in human blood. *Free Radic Res* 38: 1101–1106.

Giustarini D., Rossi R., Milzani A., Colombo R., Dalle-Donne I. (2004a). *S*-Glutathionylation: From redox regulation of protein functions to human diseases. *J Cell Mol Med* 8: 201–212.

Greenacre S.A., Ischiropoulos H. (2001). Tyrosine nitration: Localisation, quantification, consequences for protein function and signal transduction. *Free Radic Res* 34: 541–581.

Greenamyre J.T., Hastings T.G. (2004). Parkinson's—Divergent causes, convergent mechanisms. *Science* 304: 1120–1122.

Griffiths H.R., Moller L., Bartosz G., Bast A., Bertoni-Freddari C., Collins A., Cooke M., Coolen S., Haenen G., Hoberg A.M., Loft S., Lunec J., Olinski R., Parry J., Pompella A., Poulsen H., Verhagen H., Astley S.B. (2002). Biomarkers. *Mol Aspects Med* 23: 101–208.

Grune T., Merker K., Sandig G., Davies K.J. (2003). Selective degradation of oxidatively modified protein substrates by the proteasome. *Biochem Biophys Res Commun* 305: 709–718.

Halliwell B., Gutteridge J.M.C. (1999). *Free Radicals in Biology and Medicine*, Oxford University Press, New York.

Halliwell B., Whiteman M. (2004). Measuring reactive species and oxidative damage in vivo and in cell culture: How should you do it and what do the results mean? *Br J Pharmacol* 142: 231–255.

Halliwell B. (2000). Lipid peroxidation, antioxidants and cardiovascular disease: How should we move forward? *Cardiovasc Res* 47: 410–418.

Halliwell B. (2001). Role of free radicals in the neurodegenerative diseases: Therapeutic implications for antioxidant treatment. *Drugs Aging* 18: 685–716.

Halliwell B. (2002). Effect of diet on cancer development: is oxidative DNA damage a biomarker? *Free Radic Biol Med* 32: 968–974.

Halliwell B. (1999). Establishing the significance and optimal intake of dietary antioxidants: the biomarker concept. *Nutr Rev* 57: 104–113.

Hazen S.L., Heinecke J.W. (1997). 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. *J Clin Invest* 99: 2075–2081.

Hazen S.L., Hsu F.F., Gaut J.P., Crowley J.R., Heinecke J.W. (1999). Modification of proteins and lipids by myeloperoxidase. *Methods Enzymol* 300: 88–105.

Heinecke J.W. (2002). Oxidized amino acids: culprits in human atherosclerosis and indicators of oxidative stress. *Free Radic Biol Med* 32: 1090–1101.

Hekimi S., Guarente L. (2003). Genetics and the specificity of the aging process. *Science* 299: 1351–1354.

Ischiropoulos H. (2003). Biological selectivity and functional aspects of protein tyrosine nitration. *Biochem Biophys Res Commun* 305: 776–783.

Ischiropoulos H., Beckman J.S. (2003). Oxidative stress and nitration in neurodegeneration: Cause, effect, or association? *J Clin Invest* 111: 163–169.

Jaffrey S.R., Erdjument-Bromage H., Ferris C.D., Tempst P., Snyder S.H. (2001). Protein S-nitrosylation: A physiological signal for neuronal nitric oxide. *Nat Cell Biol* 3: 193–197.

Janssen L.J. (2001). Isoprostanes: an overview and putative roles in pulmonary pathophysiology. *Am J Physiol Lung Cell Mol Physiol* 280: L1067–L1082.

Jenner P. (2003). Oxidative stress in Parkinson's disease. *Ann Neurol* 53: S26–S36.

Kelly F.J., Mudway I.S. (2003). Protein oxidation at the air–lung interface. *Amino Acids* 25: 375–396.

Kettle A.J., Chan T., Osberg I., Senthilmohan R., Chapman A.L., Mocatta T.J., Wagener J.S. (2004). Myeloperoxidase and protein oxidation in the airways of young children with cystic fibrosis. *Am J Respir Crit Care Med* 170: 1317–1323.

Klatt P., Lamas S. (2000). Regulation of protein function by *S*-glutathiolation in response to oxidative and nitrosative stress. *Eur J Biochem* 267: 4928–4944.

Klaunig J.E., Kamendulis L.M. (2004). The role of oxidative stress in carcinogenesis. *Annu Rev Pharmacol Toxicol* 44: 239–267.

Lang C.A., Mills B.J., Mastropaolo W., Liu M.C. (2000). Blood glutathione decreases in chronic diseases. *J Lab Clin Med* 135: 402–405.

Lang C.A., Mills B.J., Lang H.L., Liu M.C., Usui W.M., Richie J. Jr., Mastropaolo W., Murrell S.A. (2002). High blood glutathione levels accompany excellent physical and mental health in women ages 60 to 103 years. *J Lab Clin Med* 140: 413–417.

Leewenburgh C., Rasmussen J.E., Hsu F.F., Mueller D.M., Pennathur S., Heinecke J.W. (1997). Mass spectrometric quantification of markers for protein oxidation by tyrosyl radical, copper, and hydroxyl radical in low density lipoprotein isolated from human atherosclerotic plaques. *J Biol Chem* 272: 3520–3526.

Levine R.L., Mosoni L., Berlett B.S., Stadtman E.R. (1996). Methionine residues as endogenous antioxidants in proteins *Proc Natl Acad Sci USA* 93: 15036–15040.

Levine R.L., Wehr N., Williams J.A., Stadtman E.R., Shacter E. (2000). Determination of carbonyl groups in oxidized proteins. *Methods Mol Biol* 99: 15–24.

Levine R.L. (2002). Carbonyl modified proteins in cellular regulation, aging, and disease. *Free Radic Biol Med* 32: 790–796.

Li Y., Huang T.T., Carlson E.J., Melov S., Ursell P.C., Olson J.L., Noble L.J., Yoshimura M.G., Berger C., Chan P.H., Wallace D.C., Epstein C.J. (1995). Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet* 11: 376–381.

Liu Q., Smith M.A., Avila J., DeBernardis J., Kansal M., Takeda A., Zhu X., Nunomura A., Honda K., Moreira P.I., Oliveira C.R., Santos M.S., Shimohama S., Aliev G., de la Torre J., Ghanbari H.A., Siedlak S.L., Harris P.L., Sayre L.M., Perry G. (2005). Alzheimer-specific epitopes of tau represent lipid peroxidation-induced conformations. *Free Radic Biol Med* 38: 746–754.

Lovell M.A., Gabbita S.P., Markesbery W.R. (1999). Increased DNA oxidation and decreased levels of repair products in Alzheimer's disease ventricular CSF. *J Neurochem* 72: 771–776.

Martinez-Ruiz A., Lamas S. (2004). *S*-Nitrosylation: A potential new paradigm in signal transduction. *Cardiovasc Res* 62: 43–52.

Melov S., Coskun P., Patel M., Tuinstra R., Cottrell B., Jun A.S., Zastawny T.H., Dizdaroglu M., Goodman S.I., Huang T.T., Miziorko H., Epstein C.J., Wallace D.C. (1999). Mitochondrial diseases in superoxide dismutase 2 mutant mice. *Proc Natl Acad Sci USA* 96: 846–851.

Mikkelsen R.B., Wardman P. (2003). Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms. *Oncogene* 22: 5734–5754.

Miyata T., van-Ypersele-de-Strihou C., Kurokawa K., Baynes J.W. (1999). Alterations in nonenzymatic biochemistry in uremia: origin and significance of "carbonyl stress" in long-term uremic complications. *Kidney Int* 55: 389–399.

Montine T.J., Markesbery W.R., Morrow J.D., Roberts L.J. 2nd (1998). Cerebrospinal fluid F2-isoprostane levels are increased in Alzheimer's disease. *Ann Neurol* 44: 410–413.

Montine T.J., Quinn J.F., Milatovic D., Silbert L.C., Dang T., Sanchez S., Terry E., Roberts L.J. 2nd, Kaye J.A., Morrow J.D. (2002a). Peripheral F2-isoprostanes and F4-neuroprostanes are not increased in Alzheimer's disease. *Ann Neurol* 52: 175–179.

Montine T.J., Neely M.D., Quinn J.F., Beal M.F., Markesbery W.R., Roberts L.J., Morrow J.D. (2002b). Lipid peroxidation in aging brain and Alzheimer's disease. *Free Radic Biol Med* 33: 620–626.

Montuschi P., Kharitonov S.A., Ciabattoni G., Corradi M., van Rensen L., Geddes D.M., Hodson M.E., Barnes P.J. (2000a). Exhaled 8-isoprostane as a new non-invasive biomarker of oxidative stress in cystic fibrosis. *Thorax* 55: 205–209.

Montuschi P., Barnes P.J., Roberts L.J. 2nd. (2004). Isoprostanes: Markers and mediators of oxidative stress. *FASEB J* 18: 1791–1800.

Montuschi P., Collins J.V., Ciabattoni G., Lazzeri N., Corradi M., Kharitonov S.A., Barnes P.J. (2000b). Exhaled 8-isoprostane as an in vivo biomarker of lung oxidative stress in patients with COPD and healthy smokers. *Am J Respir Crit Care Med* 162: 1175–1177.

Moskovitz J., Bar-Noy S., Williams W.M., Requena J., Berlett B.S., Stadtman E.R. (2001). Methionine sulfoxide reductase (MsrA) is a regulator of antioxidant defense and lifespan in mammals. *Proc Natl Acad Sci USA* 98: 12920–12925.

Oberg B.P., McMenamin E., Lucas F.L., McMonagle E., Morrow J., Ikizler T.A., Himmelfarb J. (2004). Increased prevalence of oxidant stress and inflammation in patients with moderate to severe chronic kidney disease. *Kidney Int* 65: 1009–1016.

Page N.M., Woods R.J., Gardiner S.M., Lomthaisong K., Gladwell R.T., Butlin D.J., Manyonda I.T., Lowry P.J. (2000). Excessive placental secretion of neurokinin B during the third trimester causes pre-eclampsia. *Nature* 405: 797–800.

Pantke U., Volk T., Schmutzler M., Kox W.J., Sitte N., Grune T. (1999). Oxidized proteins as a marker of oxidative stress during coronary heart surgery. *Free Radic Biol Med* 27: 1080–1086.

Pastore A., Tozzi G., Gaeta L.M., Bertini E., Serafini V., Di Cesare S., Bonetto V., Casoni F., Carrozzo R., Federici G., Piemonte F. (2003a). Actin glutathionylation increases in fibroblasts of patients with Friedreich's ataxia: A potential role in the pathogenesis of the disease. *J Biol Chem* 43: 42588–42595.

Pastore A., Federici G., Bertini E., Piemonte F. (2003b). Analysis of glutathione: Implication in redox and detoxification. *Clin Chim Acta* 333: 19–39.

Pattison D.I., Davies M.J. (2004). Kinetic analysis of the reactions of hypobromous acid with protein components: implications for cellular damage and use of 3-bromotyrosine as a marker of oxidative stress. *Biochemistry* 43: 4799–4809.

Pennathur S., Bergt C., Shao B., Byun J., Kassim S.Y., Singh P., Green P.S., McDonald T.O., Brunzell J., Chait A., Oram J.F., O'Brien K., Geary R.L., Heinecke J.W. (2004). Human atherosclerotic intima and blood of patients with established coronary artery disease contain high density lipoprotein damaged by reactive nitrogen species. *J Biol Chem* 279: 42977–42983.

Poli G., Schaur J. (2000). 4-Hydroxynonenal in the pathomechanisms of oxidative stress. *IUBMB Life* 50: 315–321.

Pratico D., Rokach J., Lawson J., FitzGerald G.A. (2004). F_2-Isoprostanes as indices of lipid peroxidation in inflammatory diseases. *Chem Phys Lipids* 128: 165–171.

Pratico D., Clark C.M., Lee V.M., Trojanowski J.Q., Rokach J., Fitzgerald G.A. (2000). Increased 8,12-*iso*-$iPF_{2\alpha}$-VI in Alzheimer's disease: correlation of a non-invasive index of lipid peroxidation with disease severity. *Ann Neurol* 48: 809–812.

Pratico D., Clark C.M., Liun F., Rokach J., Lee V.Y., Trojanowski J.Q. (2002). Increase of brain oxidative stress in mild cognitive impairment: A possible predictor of Alzheimer disease. *Arch Neurol* 59: 972–976.

Pratico D., Uryu K., Leight S., Trojanoswki J.Q., Lee V.M. (2001). Increased lipid peroxidation precedes amyloid plaque formation in an animal model of Alzheimer amyloidosis. *J Neurosci* 21: 4183–4187.

Pryor W.A. (2001). *Bio-assays for Oxidative Stress Status (BOSS)*, Elsevier Science, Amsterdam.

Rahman I., van Schadewijk A.A., Crowther A.J., Hiemstra P.S., Stolk J., MacNee W., De Boer W.I. (2002). 4-Hydroxy-2-nonenal, a specific lipid peroxidation product, is elevated in lungs of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 166: 490–495.

Rand J.S., Fleeman L.M., Farrow H.A., Appleton D.J., Lederer R. (2004). Canine and feline diabetes mellitus: Nature of nurture. *J Nutr* 134: 2072S–2079S.

Renke J., Popadiuk S., Korzon M., Bugajczyk B., Wozniak M. (2000). Protein carbonyl groups' content as a useful clinical marker of antioxidant barrier impairment in plasma of children with juvenile chronic arthritis. *Free Radic Biol Med* 29: 101–104.

Requena J.R., Levine R.L., Stadtman E.R. (2003). Recent advances in the analysis of oxidized proteins. *Amino Acids* 25: 221–226.

Requena J.R., Dimitrova M.N., Legname G., Teijeira S., Prusiner SB., Levine R.L. (2004). Oxidation of methionine residues in the prion protein by hydrogen peroxide. *Arch Biochem Biophys* 432: 188–195.

Roberts J.M., Cooper D.W. (2001). Pathogenesis and genetics of pre-eclampsia. *Lancet* 357: 53–56.

Roberts L.J., Morrow J.D. (2000). Measurement of F_2-isoprostanes as an index of oxidative stress in vivo. *Free Radic Biol Med* 28: 505–513.

Roberts L.J. II, Morrow J.D. (2002). Products of the isoprostane pathway: Unique bioactive compounds and markers of lipid peroxidation. *Cell Mol Life Sci* 59: 808–820.

Rossi R., Milzani A., Dalle-Donne I., Giustarini D., Lusini L., Colombo R., Di Simplicio P. (2002). Blood glutathione disulfide: In vivo factor or in vitro artifact? *Clin Chem* 48: 742–753.

Sano M., Ernesto C., Thomas R.G., Klauber M., Schafer K., Grundman M., Woodbury P., Growdon J., Cotman C.W., Pfeiffer E., Schneider L.S., Thal L.J. (1997). A controlled trial of selegiline, alpha-tocopherol, or both as treatments for Alzheimer's disease. *N Engl J Med* 336: 1216–1222.

Sayre L.M., Smith M.A., Perry G. (2001). Chemistry and biochemistry of oxidative stress in neurodegenerative disease. *Curr Med Chem* 8: 721–738.

Schoneich C. (2005). Mass spectrometry in aging research. *Mass Spectrom Rev* 24: 701–718.

Schulz J.B., Dehmer T., Schols L., Mende H., Hardt C., Vorgerd M., Burk K., Matson W., Dichgans J., Beal M.F., Bogdanov M.B. (2000). Oxidative stress in patients with Friedreich's ataxia. *Neurology* 55: 1719–1721.

Schwedhelm E., Bartling A., Lenzen H., Tsikas D., Maas R., Brummer J., Gutzki F.M., Berger J., Frolich J.C., Boger R.H. (2004). Urinary 8-iso-prostaglandin F2alpha as a risk marker in patients with coronary heart disease: A matched case-control study. *Circulation* 109: 843–848.

Shao B., O'brien K.D., McDonald T.O., Fu X., Oram J.F., Uchida K., Heinecke J.W. (2005). Acrolein modifies apolipoprotein a-I in the human artery wall. *Ann NY Acad Sci* 1043: 396–403.

Shishehbor M.H., Aviles R.J., Brennan M.L., Fu X., Goormastic M., Pearce G.L., Gokce N., Keaney J.F. Jr., Penn M.S., Sprecher D.L., Vita J.A., Hazen S.L. (2003a). Association of nitrotyrosine levels with cardiovascular disease and modulation by statin therapy. *J Am Med Assoc* 289: 1675–1680.

Shishehbor M.H., Brennan M.L., Aviles R.J., Fu X., Penn M.S., Sprecher D.L., Hazen S.L. (2003b). Statins promote potent systemic antioxidant effects through specific inflammatory pathways. *Circulation* 108: 426–431.

Sies H. (1991). *Oxidative Stress: Oxidants and Antioxidants*. New York: Academic Press.

Skoumalova A., Rofina J., Schwippelova Z., Gruys E., Wilhelm J. (2003). The role of free radicals in canine counterpart of senile dementia of the Alzheimer type. *Exp Geront* 38: 711–719.

Slatter D.A., Bolton C.H., Bailey A.J. (2000). The importance of lipid-derived malondialdehyde in diabetes mellitus. *Diabetologia* 43: 550–557.

Sohal R.S. (2002). Role of oxidative stress protein oxidation in the aging process: *Free Radic Biol Med* 33: 37–44.

Stadtman E.R. (1990). Metal ion-catalyzed oxidation of proteins: Biochemical mechanism and biological consequences. *Free Radic Biol Med* 9: 315–325.

Stadtman E.R., Berlett B.S. (1997). Reactive oxygen-mediated protein oxidation in aging and disease. *Chem Res Toxicol* 10: 485–494.

Stadtman E.R., Levine R.L. (2003). Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids* 25: 207–218.

Stadtman E.R., Moskovitz J., Levine R.L. (2003). Oxidation of methionine residues of proteins: biological consequences. *Antiox Redox Signal* 5: 577–582.

Stocker R., Keaney J.F. Jr. (2003). Role of oxidative modifications in atherosclerosis. *Physiol Rev* 84: 1381–1478.

Tarpey M., Fridovich I. (2001). Methods of detection of vascular reactive species: Nitric oxide, superoxide, hydrogen peroxide, and peroxynitrite. *Circ Res* 89: 224–236.

Thannickal V.J., Fanburg B.L. (2000). Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol* 279: L1005–1028.

Thornalley P.J. (2002). Glycation in diabetic neuropathy: Characteristics, consequences, causes, and therapeutic options. *Int Rev Neurobiol* 50: 37–57.

Thornalley P.J., Battah S., Ahmed N., Karachalias N., Agalou S., Babaei-Jadidi R., Dawnay A. (2003). Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. *Biochem J* 375: 581–592.

Thorpe SR., Baynes J.W. (1999). The role of oxidative stress diabetic complications: A new perspective on an old paradigm. *Diabetes* 48: 1–9.

Tsikas D., Caidahl K. (2005). Recent methodological advances in the mass spectrometric analysis of free and protein-associated 3-nitrotyrosine in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci* 814: 1–9.

Turko I.V., Murad F. (2002). Protein nitration in cardiovascular diseases. *Pharmacol Rev* 54: 619–634.

Uchida K., Stadtman E.R. (1992). Modification of histidine residues in proteins by reaction with 4-hydroxynonenal. *Proc Natl Acad Sci USA* 89: 4544–4548.

Uchida K. (2000). Role of reactive aldehyde in cardiovascular diseases. *Free Radic Biol Med* 28: 1685–1696.

Uchida K. (2003). 4-Hydroxy-2-nonenal: A product and mediator of oxidative stress. *Prog Lipid Res* 42: 318–343.

Valentine J.S. (2002). Do oxidatively modified proteins cause ALS? *Free Radic Biol Med* 33: 1314–1320.

van der Vliet A., Nguyen M.N., Shigenaga M.K., Eiserich J.P., Marelich G.P., Cross C.E. (2000). Myeloperoxidase and protein oxidation in cystic fibrosis. *Am J Physiol* 279: L537–L546.

Warner D.S., Sheng H., Batinić-Haberle I. (2004). Oxidants, antioxidants and the ischemic brain. *J Exp Biol* 207: 3221–3231.

Wataya T., Nunomura A., Smith M.A., Siedlak S.L., Harris P.L., Shimohama S., Szweda L.I., Kaminski M.A., Avila J., Price D.L., Cleveland D.W., Sayre L.M., Perry G. (2002). High molecular weight neurofilament proteins are physiological substrates of adduction by the lipid peroxidation product hydroxynonenal. *J Biol Chem* 277: 4644–4648.

Winterbourn C.C., Buss H. (1999). Protein carbonyl measurement by enzyme-linked immunosorbent assay. *Methods Enzymol* 300: 106–111.

Winterbourn C.C., Kettle A.J. (2000). Biomarkers of myeloperoxidase-derived hypochlorous acid. *Free Radic Biol Med* 29: 403–409.

Winterbourn C.C., Buss I.H., Chan T.P., Plank L.D., Clark M.A., Windsor J.A. (2000). Protein carbonyl measurements show evidence of early oxidative stress in critically ill patients. *Crit Care Med* 28: 143–149.

Winterbourn C.C., Bonham M.J., Buss H., Abu-Zidan F.M., Windsor J.A. (2003). Elevated protein carbonyls as plasma markers of oxidative stress in acute pancreatitis. *Pancreatology* 3: 375–382.

Wood L.G., Gibson P.G., Garg M.L. (2003). Biomarkers of lipid peroxidation, airway inflammation and asthma. *Eur Respir J* 21: 177–186.

Yao D., Gu Z., Nakamura T., Shi Z.Q., Ma Y., Gaston B., Palmer L.A., Rockenstein E.M., Zhang Z., Masliah E., Uehara T., Lipton S.A. (2004). Nitrosative stress linked to sporadic Parkinson's disease: *S*-Nitrosylation of parkin regulates its E3 ubiquitin ligase activity. *Proc Natl Acad Sci USA* 101: 10810–10814.

Yi D., Ingelse B.A., Duncan M.W., Smythe G.A. (2000). Quantification of 3-nitrotyrosine in biological tissues and fluids: Generating valid results by eliminating artifactual formation. *J Am Soc Mass Spectrom* 11: 578–586.

Yoneyama Y., Sawa R., Suzuki S., Doi D., Yoneyama K., Otsubo Y., Araki T. (2002). Relationship between plasma malondialdehyde levels and adenosine deaminase activities in preeclampsia. *Clin Chim Acta* 322: 169–173.

Zhan X., Desiderio D.M. (2004). The human pituitary nitroproteome: detection of nitrotyrosyl-proteins with two-dimensional Western blotting, and amino acid sequence determination with mass spectrometry. *Biochem Biophys Res Commun* 325(4): 1180–1186.

Zhang J., Perry G., Smith M.A., Robertson D., Olson S.J., Graham D.G., Montine T.J. (1999). Parkinson's disease is associated with oxidative damage to cytoplasmic DNA and RNA in substantia nigra neurons. *Am J Pathol* 154: 1423–1429.

Zheng L., Nukuna B., Brennan M.L., Sun M., Goormastic M., Settle M., Schmitt D., Fu X., Thomson L., Fox P.L., Ischiropoulos H., Smith J.D., Kinter M., Hazen S.L. (2004). Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease. *J Clin Invest* 114: 529–541.

17

DEGRADATION AND ACCUMULATION OF OXIDIZED PROTEINS IN AGE-RELATED DISEASES

PETER VOSS AND TILMAN GRUNE

17.1 OXIDATIVE MODIFICATIONS OF AMINO ACIDS AND PROTEIN DAMAGE

Oxidative changes in the structure of proteins may have different reasons. On the one hand, there are environmental factors including UV-irradiation, and on the other hand, there are cellular processes like the energy production in the mitochondria, which produce reactive oxygen species (ROS) including the hydroxyl or the superoxide radical. These ROS are responsible for a number of oxidative changes of amino acid residues, leading to damaged proteins with changed, reduced, or vanished catalytic activity. A detailed overview of several oxidative amino acid modifications, specific methods to detect them and their impact on cell metabolism is given in the preceding chapters of this volume and in the reviews from Stadtman and Levine (2003), Requena et al. (2003), and Naskalski and Bartosz (2000). Therefore, we will only give a brief overview of the most common and important amino acid modifications in oxidative damaged proteins.

Oxidative post-translational protein modifications can be divided into two major groups. On the one side, there are those changes that concern only single amino acid residues, and on the other side, there are those changes that lead to fragmentation of polypeptide chains or covalent cross-linking of two amino acids

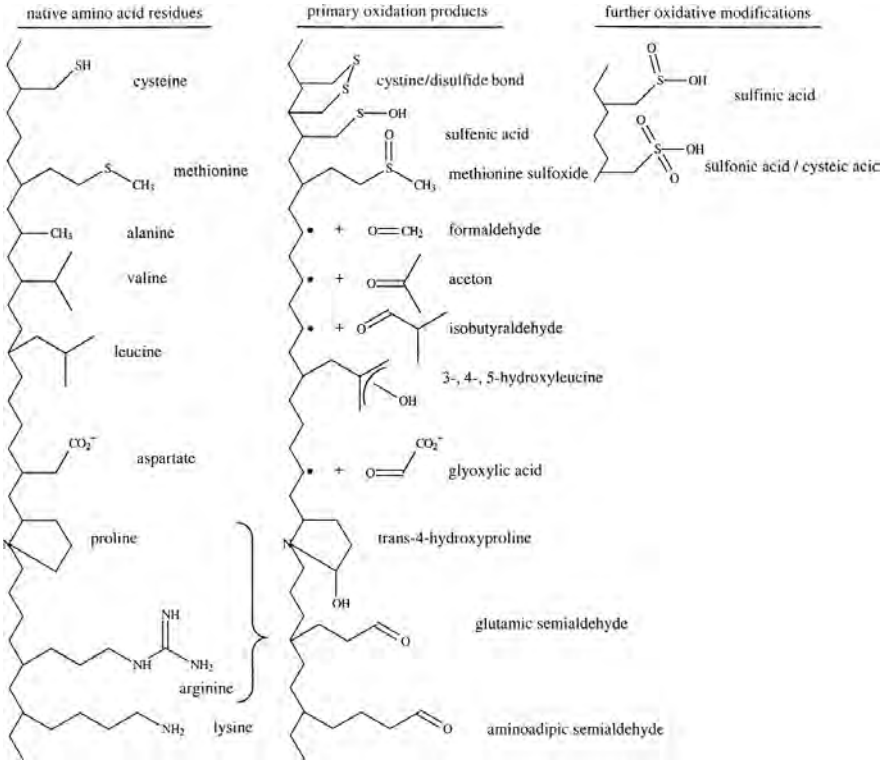
Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

either of the same or of two different proteins. Figures 17.1 and 17.2 demonstrate the most important protein modifications by oxidants.

17.1.1 Oxidative Modifications of Amino Acids in Proteins

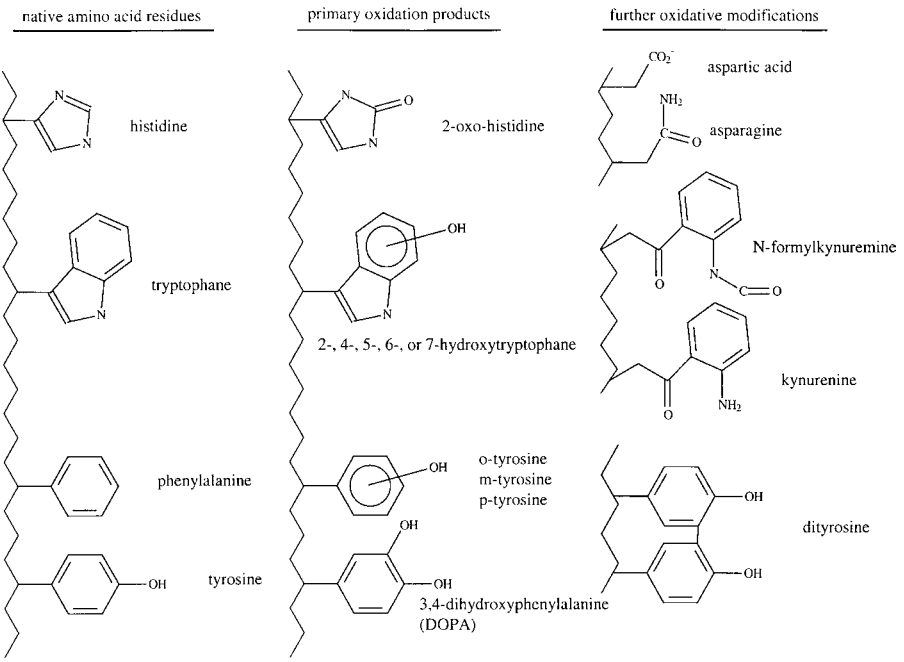
The sulfur-containing amino acids cysteine and methionine are the most susceptible to oxidative damage (Levine et al., 1996, 1999). The reaction of ROS with cysteine leads to the formation of inter- or intramolecular disulfide bonds, sulfenic acid, sulfinic acid, and sulfonic acid (Fig. 17.1*a*). These modifications are partially reversible. In vivo as well as in vitro they can at least in part be reversed by dithiothreitol (Finkel, 2000) and the glutathione system (Holmgren, 1989).

The oxidation of methionine results in methionine sulfoxide (Fig. 17.1*a*), which can either be further oxidized to methionine sulfone or reduced again



(*a*)

FIGURE 17.1(a) Oxidative modifications of amino acid residues in proteins due to ROS. 1(*a*) On the left side, the native amino acid residues of the aliphatic amino acids; in the middle column, the primary oxidation products; and on the right side, possible further modification of the amino acid residues due to oxidative stress are shown. 1(*b*) The aromatic amino acids and the histidine residue listed in the same manner as the aliphatic amino acids in panel *a*.



(*b*)

FIGURE 17.1(b) (*continued*)

to methionine by the methionine sulfoxide reductase system (Levine et al., 2000; Requena et al., 2004). The ROS reaction with histidine results in 2-oxo-histidine (Uchida and Kawakishi, 1993; Uchida, 2003), and asparagine or aspartic acid (Berlett et al., 1996). Upon oxidative stress, tryptophan forms a number of hydroxylated products, namely 2-, 4-, 5-, 6-, or 7-hydroxytryptophan or *N*-formylkynurenine and kynurenine (Armstrong and Swallow, 1969; Maskos et al., 1992).

The aliphatic side chains of amino acid residues like alanine, valine, leucine, and the acid side chain of aspartate can be removed from the protein backbone via β-scission. The remaining protein-bound carbonyl can be detected (Headlam and Davies, 2004; Requena et al., 2003), such as via ELISA, after derivatization with dinitrophenylhydrazine (DNPH). It therefore represents a useful biomarker for the oxidative damage on proteins (Dalle-Donne et al., 2003). The hydroxylation of lysine forms 3-, 4-, or 5-hydroxylysine (Garrison, 1987).

Another important target of oxidative damage are the aromatic side chains. Phenylalanine can be easily oxidized and forms para-, ortho- and meta-tyrosine (Heinecke et al., 2000; van der Vliet et al., 1994), which themselves can react further to 3,4-dihydroxyphenylalanine (Gieseg et al., 1993). If other oxidants than ROS (e.g., reactive species derived from nitrogen monoxide or hypochloric acid, HOCl) are involved in the protein damage, this will result in a variety of

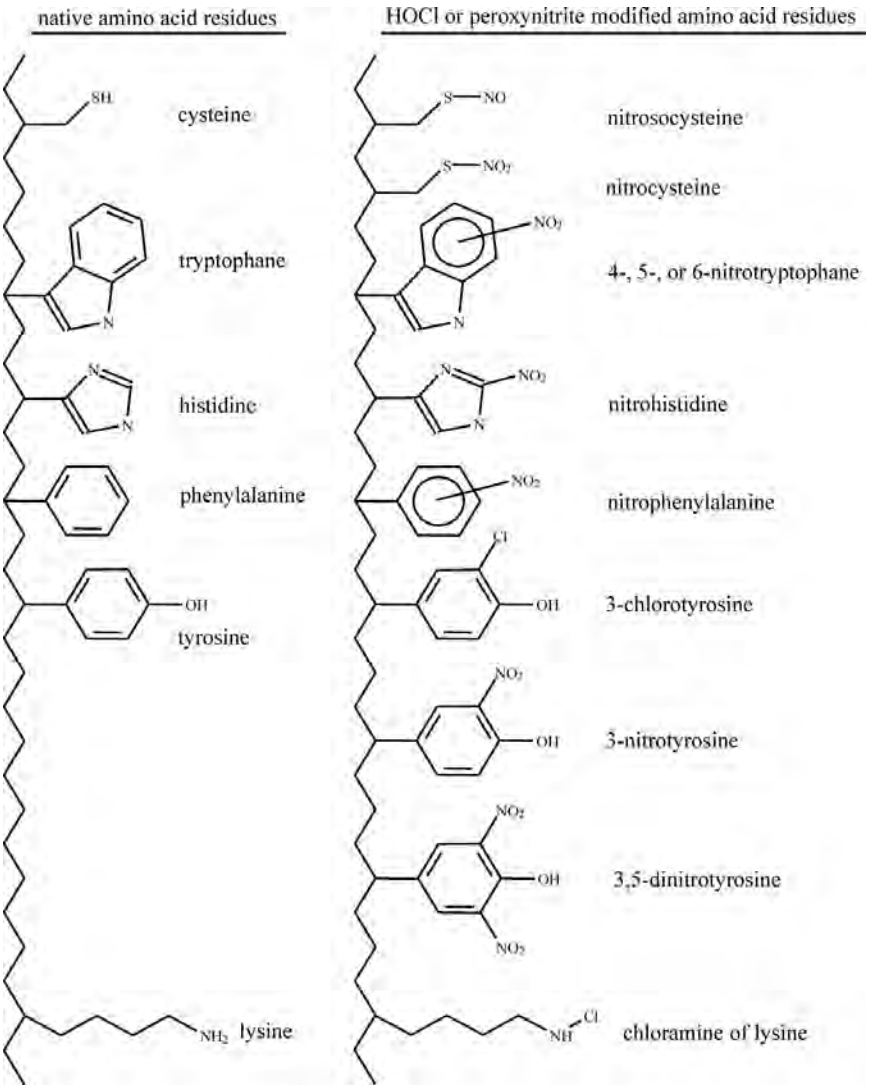


FIGURE 17.2 Oxidative modifications of amino acid residues in proteins due to RNS and HOCl. On the left side, the native amino acid residues; on the right, the corresponding oxidatively modified side chains, which occur after the reaction with HOCl or peroxynitrite are demonstrated.

further amino acid derivatives like 3-nitrotyrosine (Yi et al., 1997), 4-, 5-, and 6-nitrotryptophan (Herold, 2004), 3-chlorotyrosine (Kettle, 1996), or chloramines of lysine (Hawkins and Davies, 1999; Hawkins et al., 2003). Figure 17.2 shows the structural changes of the amino acid side chains caused by RNS and HOCl.

Besides the very common modification 4,5-hydroxyproline (Creeth et al., 1983; Poston, 1988), proline is also able to react in a similar manner as the two basic amino acids arginine and lysine. Oxidation of protein lysyl residues leads to the formation of aminoadipic semialdehyde, while prolyl and arginyl residues both build glutamic semialdehyde (Amici et al., 1989; Requena et al., 2001; Uchida, 2003).

17.1.2 Cross-Linking of Proteins due to Oxidation

All the above-mentioned modifications lead to partial unfolded proteins that show an increased hydrophobicity on the surface due to exposure of aliphatic and aromatic amino acids, which are normally hidden inside the inner catalytic parts of native proteins (Pacifici et al., 1993; Giulivi et al., 1994; Lasch et al., 2001). In order to minimize the contact surface between these hydrophobic residues and the hydrophile solvent in the partially unfolded protein, hydrophobic side chains will stick together and exclude the solvent water as far as possible.

Besides these hydrophobic interactions damaged proteins might undergo cross-linking reactions by different mechanisms (Stadtman and Levine, 2003):

- Interaction of two carbon-centered radicals after hydrogen abstraction (Garrison, 1987).
- Formation of dityrosine from two tyrosine radicals (Giulivi et al., 2003; Malencik and Anderson, 2003).
- Dimerization via disulfide bonds due to oxidation of cysteine (Garrison, 1987; Brodie and Reed, 1990; Takahashi and Goto, 1990; Zhou and Gafni, 1991).
- Interaction of protein carbonyls with the amino group of a lysine residue.
- Reaction of aldehyde groups of malondialdehyde with lysine residues.
- Reaction of glycation/glyoxidation-derived protein carbonyls with lysine or arginine residues (Grandhee and Monnier, 1991; Wells-Knecht et al., 1995; Verzyl et al., 2000).
- Interaction of aldehydes formed via Michael addition with lipid peroxidation products like 4-hydroxy-2-nonenal and lysine residues (Uchida and Stadtman, 1993; Friguet et al., 1994).

All these reactions can either be intramolecular or intermolecular. If the oxidatively modified proteins are not removed from the cellular environment, they tend to aggregate and cross-link and form nondegradable protein aggregates, which might cause disease.

Besides these reactions, which are induced by oxidative changes on the amino acid residues, ROS are able to damage the peptide backbone. The cleavage of peptide backbones due to radical-induced hydrogen abstraction can either lead to fragmentation of the protein or to aggregation due to polymerization (Easton, 1997).

17.2 DEGRADATION AND ACCUMULATION OF OXIDATIVELY MODIFIED PROTEINS

Oxidative amino acid modifications can lead to catalytic or functional inactive protein material that has to be removed from the cellular environment in order to maintain the normal metabolism. This is done either by the damaged proteins being degraded into smaller peptides, which are cleaved into single amino acids, or by the "waste material" being accumulated in separate compartments within the cell or the extracellular environment. Clearly, the advantage of the degradation is a complete removal of the waste protein material, and this provides the cell with single amino acids that can be reused for new protein synthesis. Figure 17.3 gives a schematic overview of the components involved in protein synthesis, degradation, and accumulation and their impact on each other.

17.2.1 Degradation of Oxidatively Modified Proteins

The diversity of proteins that have to be degraded makes it necessary to have different metabolic pathways responsible for the degradation of foreign, damaged, misfolded, or unused proteins. The three most important pathways for protein degradation are the lysosomal pathway, the proteasomal system, and the cytosolic calpains (Chondrogianni et al., 2002; Dunlop et al., 2002; Mehlhase and Grune, 2002; Merker and Grune, 2004). Beside these three main pathways several proteases exist with a high specificity concerning their substrates. While the lysosomal pathway is mainly used for the removal of extracellular and autophagocytosed material, the proteasomal system is responsible for the degradation of abnormal and misfolded proteins, oxidatively modified proteins, processing of antigens, removal of signal transduction and transcription factors, cell cycle progression, and apoptosis. All these different purposes are fulfilled by proteasomes found in the cytosol, attached to the endoplasmic reticulum (ER), and in the nucleus. Because of their higher substrate specificity many other proteases are only able to degrade a limited number of proteins and are therefore used for a selective regulation of metabolic processes.

The Lysosomal Pathway The lysosomal pathway is mainly responsible for the degradation of exogenous uptaken or autophagocytosed proteins. Foreign protein material is taken up by the cell and transported into the lysosomes, and the proteins are degraded by cathepsins, a protease family consisting of at least 16 members. Cathepsin A and G are serine proteases, D and E aspartate proteases and B, C, F, H, K, L, O, S, T, V, W, and X are cysteine proteases (Johnson, 2000; Turk et al., 2000; Turk et al., 2001). Besides the impact on the cell maintenance the cathepsins are also able to trigger apoptosis (Leist and Jaattela, 2001a,b; Bidere et al., 2003; Cirman et al., 2004). Cellular proteins, which are also degraded by the cathepsins, have to be transported into the lysosomes (Dean, 1975a,b; Cuervo et al., 1995).

Concerning the degradation of oxidatively modified proteins in the lysosomal compartment of cells, there are many conflicting results observed over the

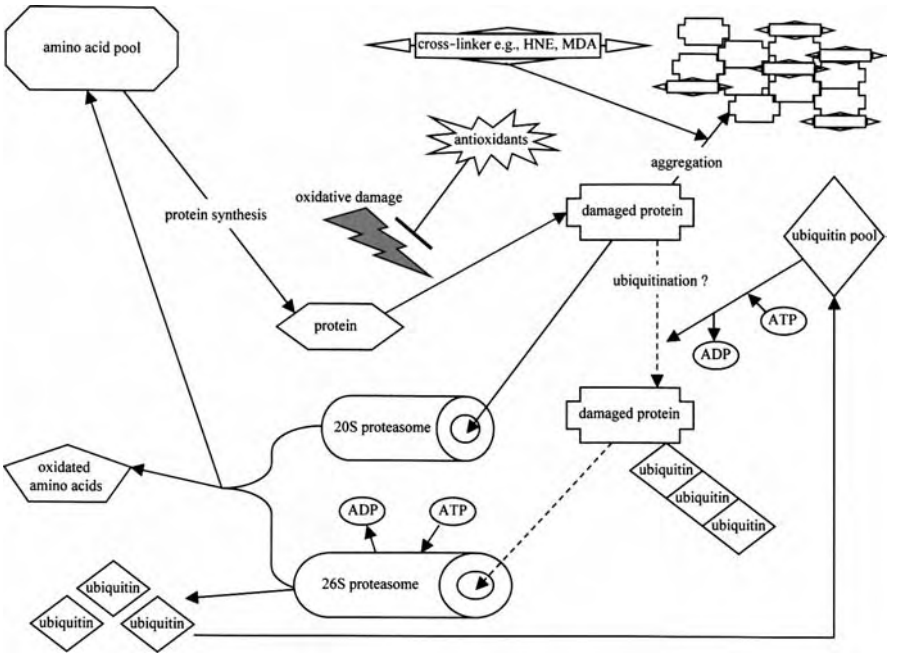


FIGURE 17.3 Degradation and accumulation of oxidatively damaged proteins. Proteins are synthesized out of the normal amino acid pool (*upper left*). Oxidative stress, which will be prevented by antioxidants, might damage these proteins. These oxidatively modified proteins either accumulate and build up large aggregates or are degraded by the proteasomal system. As it is not clear whether oxidized proteins are degraded only by the 20S proteasome or also by the 26S proteasome after ubiquitination, both pathways are indicated. Cross-linkers like HNE or MDA further enhance the process of aggregation.

last decade. While Jessup et al. (1992), Grant et al. (1992), and Dhaliwal and Steinbrecher (2000) found only poor degradation of apoB derived from oxidized LDL, 4-hydroxynonenal-derivatized apoB, and bovine serum albumin (BSA), our group could demonstrate that the uptake and degradation of extracellular proteins by macrophages and microglial cells depends on their oxidation status (Stolzing et al., 2002).

It is generally accepted that aging has an impact on the lysosomal pathway. The activity of the cathepsins declines in senescent fibroblasts (Rivett, 1985; Sitte et al., 2000a). It could also be shown that the degradation of long-lived proteins was impaired in senescent fibroblasts (Dice, 1993; Okada and Dice, 1984) as well as in the liver of old mice (Lavie et al., 1982). Undegradable protein aggregates like lipofuscin particles accumulate within the lysosomes with increasing age (Grune et al., 1997; Terman and Brunk, 1998); it therefore is not surprising that the number and size of lysosomes increase during the aging process (Sitte et al., 2001).

Although the lysosomal protein degradation is mainly a nonselective process, there are some hints that a monoubiquitination triggers receptors and other membrane proteins to the endosomal compartment and into the lysosomes (Hicke, 1999, 2001; Marques et al., 2004; Shih et al., 2000; de Wit et al., 2000, 2001). The involvement of ubiquitin in this process can be referred to as an interaction with the ubiquitin-proteasome-system, the second most important proteolytic system for the removal of oxidized intracellular proteins (Hofmann and Falquet, 2001).

The Proteasomal Pathway The proteasomal system is responsible for the degradation of abnormal, misfolded, and oxidized proteins. The proteasomes are further involved in an array of cellular processes like antigen processing, signal transduction, transcription, cell cycle progression, and apoptosis (Carrard et al., 2002; Coux et al., 1996; Davies, 2001). In order to fulfill all the above-mentioned reactions the proteasomes are found in the cytosol, attached to the endoplasmic reticulum (ER), and in the nucleus (Peters et al., 1994; Rivett, 1998; Enenkel et al., 1998). Considering the number of processes in which the proteasomal system is involved, the low specificity and therefore a broad substrate spectrum like that of the lysosomal system are not surprising.

Prevalence Multicatalytic proteases with a similar structure to the eukaryotic 20S-proteasome have been found in all forms of life, including bacteria, archaea, and eukaryotes. Bacteria and archaea do not need the proteasomal system during normal growth and express it only under stress conditions. But for eukaryotic cells the proteasomal system is essential for surviving (Baumeister et al., 1998; Coux et al., 1996; Peters et al., 1998). A very detailed overview of the development of the proteasomal-like proteases, their different structures, the molecular mechanism of the catalytic activity, the regulation of the proteolytic processes, and their impact on other cellular mechanisms is given by Zwickl and Baumeister (2002).

Structure The bacterial proteasome-like protease consists of two stacked rings of HsIV-subunits that can associate to rings of ATPases of the Clp/HSP100 subfamily. Archaea and actinomycetes form a barrel consisting of four rings, each of seven subunits, like the 20S-proteasome of eukaryotes, that can interact with ATPase rings of the AAA type (Volker and Lupas, 2002).

Eukaryotes have developed the most complicated proteasomal system. The simplest form thereof is the 20S proteasome that is often called the "core proteasome" as it plays also a central role in other proteasomal constructs. Figure 17.4 shows the structures of the 20S core proteasome, the 11S and the 19S regulator, and the different combinations of them: the 26S proteasome, the hybrid-form, and the proteasome bound to two 11S regulators. The 20S core proteasome has a cylindrical shape that consists of four stacked rings, each of seven subunits. The two outer rings contain seven different α-subunits, while the two inner rings are constructs out of seven different β-subunits. Three out of the seven β-units within one ring are catalytically active, and therefore the eukaryotic proteasome

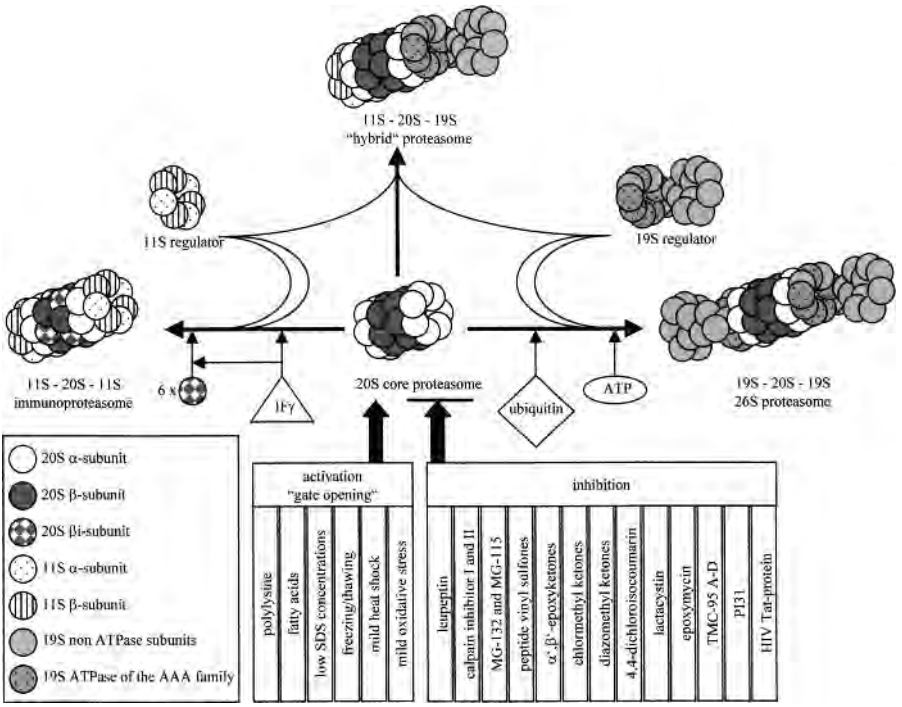


FIGURE 17.4 Proteases and regulation of the proteasomal system. The proteasomal system consist mainly of the 20S core proteasome, and the 11S and the 19S regulator. These components can build up immuno-, "hybride-", and the 26S-proteasome. While IFγ stimulates the expression of the inducible β-subunits and the assembly of the immunoproteasome, ubiquitin and ATP enhance the concentration of the 26S proteasome. The lower part of the figure shows conditions that either stimulate or inhibit the 20S core proteasome.

comprises six catalytic active sites, all located in the inner chamber between the two β-rings. The β1, β2, and β5 of each β-ring are those subunits that possess hydrolytic activities.

To regulate the activity, eukaryotes have developed two regulatory particles, the 11S and the 19S regulator. These are responsible for the substrate specificity, the substrate recognition, as well as the preparation of the substrate for the degradation and also for the product spectrum. The 11S as well as the 19S regulator can be attached either on one or on both sides of the 20S proteasome (Rechsteiner, 1998; Voges et al., 1999). Also a hybrid form with the 11S regulator on the one side and the 19S regulator on the other side has been identified.

The 11S regulator consists of two different subunits, α and β which can be incorporated in different amounts in the heptameric ring (Song et al., 1996; Zhang et al., 1999). The 19S regulator consists of two major structures (Glickman et al., 1998). The base is a ring containing six ATPases of the AAA family and three

other subunits. The lid is somewhat covering the opening of the base. The base is responsible for the stacking of the 19S regulator on the α-ring of the 20S proteasome, while the lid is responsible for the recognition and binding of the substrates, deubiquitination, unfolding, and translocation of the protein into the proteasome (Strickland et al., 2000).

The 26S proteasome contains a 20S core that is capped at one or both ends by the 19S regulator. Functionally the 26S proteasome seems to be the most important protease for the degradation of polyubiquitinated proteins.

Function Generally the proteasome operates in the following manner: the damaged protein is recognized by the protease, enters the proteasome through the narrow opening (α-annulus) in the middle of the outer α-ring, is processed in the catalytic chamber between the two β-rings, and the small polypeptides leaves the proteasome through the opening of the second α-ring (Groll et al., 1997; Löwe et al., 1995; Wenzel and Baumeister, 1995).

Whereas the 20S core proteasome is able to degrade proteins by an ATP and ubiquitin independent manner, the 26S proteasome is largely responsible for degradation of polyubiquitinated protein substrates by a mechanism in which the ATPase activity of the 19S regulator is used to facilitate unfolding of the ubiquitinated proteins and their translocation into the opening of the proteasome (Braun et al., 1999). Few exceptions of the above-mentioned conditions have been observed (Benaroudj et al., 2001; Sheaff et al., 2000; Tarcsa et al., 2000).

The first step in the removal of unused proteins is the recognition by the proteasome. In the case of the 26S proteasome this is done by the polyubiquitin chain, which binds to the polyubiquitin-binding subunits of the lid of the 19S regulator. There the polyubiquitin chain is removed and hydrolyzed into free ubiquitin, which can be reused. Because the pathway by the 20S proteasome is ubiquitin independent, there must be another mode of recognition to decide which proteins have to be degraded and which ones not. Here it is proposed that oxidatively damaged or misfolded proteins expose more hydrophobic amino acids on their surface than native folded ones. These hydrophobic areas can be recognized by the 20S proteasome and the damaged protein is hydrolyzed (Pacifici et al., 1993; Giulivi et al., 1994; Lasch et al., 2001).

To enter the small α-annulus of the core proteasome, which is normally blocked by the *N*-termini of the α-subunits, the protein has to be completely unfolded (Groll et al., 1997, 2000a). In the 26S proteasome the base of the 19S regulator, which includes ATPases, assists to unfold the protein and translocate it into the core proteasome in an energy consuming way. The 11S regulator activates the 20S proteasome, due to the fact that it facilitates the entry of a substrate into the proteasome by binding on the α-ring of the core proteasome. This leads to a conformational change that removes the *N*-terminal chains of the α-subunits, normally blocking the α-annulus. The opening is enlarged so that the protein can enter and/or leave the processing chamber easier (Whitby et al., 2000).

Within the catalytic chamber of the proteasome, between the two β-rings are located the six active centers that exhibit different catalytic activities. While the

β1-subunit cleaves the protein preferentially after acidic amino acid residues in a peptidylglutamyl-like manner, the β2-subunit, like trypsin, cuts after basic amino acids, and the β5-subunit shows a chymotrypsin-like activity, since it cleaves after hydrophobic residues. Beside these main activities mammalian subunit β2 exhibits also a "small neutral amino acid preferring" activity (SNAAP) and the β1 and β5 subunits have an additional "branched-chain amino acid preferring" activity (BrAAP) (Orlowski et al., 1993).

The core proteasome degrades the protein only partially to single amino acids. The main products are short polypeptides from 4 to 25 residues, with an average length of 7 to 9 residues (Voges et al., 1999). These peptides can be either further hydrolyzed by other cytoplasmic peptidases to single amino acid (Tamura et al., 1996; Walz et al., 1997; Tomkinson, 1999) or used as antigens as presented by the MHC-1 complex (Tanaka and Kasahara, 1998). The single amino acids that are not modified or damaged can be recycled to synthesize new proteins, while the modified have to be removed.

Regulation As the proteasomal system consists of several proteasomal forms and regulatory particles, which themselves are composed of different subunits with distinct functions, it seems to be likely that the regulation of the proteasomal activity is very complex. There is additionally a multitude of factors that might have an influence on the degradation of the different proteins.

The proteasomal activators, like the 11S and the 19S regulator, influence the substrate specificity, the activity of the catalytic centers, and the product spectra, if one or two of them bind to the 20S core proteasome (Glickman and Maytal, 2002; Hill et al., 2002). Besides the already discussed effects of the 11S and the 19S regulator on the proteolytic activities of the 20S core proteasome, the 11S regulator plays an important role in the early immune defense (Niedermann, 2002).

The cytokine interferon-γ (IFN-γ) is able to induce the synthesis of the 11S regulator (Rechsteiner et al., 2000; Strohwasser et al., 2000), on the one hand, and three alternative β-subunits are upregulated, on the other hand. The β-subunits β1i, β2i, and β5i (i for inducible) are only expressed under cytokine induction. These exhibit similar catalytic activities as their native counterparts and replace them in the 20S core proteasome, which is then called immunoproteasome as it is now a part of the immune defense of the cell (Tanaka and Kasahara, 1998; York et al., 1999; Rechsteiner et al., 2000). (Remark: The term "immunoproteasome" is not used uniquely in literature. Here we use it to describe the proteasomal forms containing the three inducible subunits).

Due to the subunit exchange the catalytic activities are altered. The chymotrypsin-, the trypsin-like as well as the peptidylglutamyl-activity, change severely (Driscoll et al., 1993; Gayzynska et al., 1993; Aki et al., 1994). The immunoproteasomes cleave substrates therefore in a different manner so that more polypeptides with hydrophobic or basic and less peptides with acidic *C*-terminus are produced. The average polypeptide length changes upon 11S regulator binding to the 20S proteasome. The MHC-I protein prefers hydrophobic and basic residues

at the *C*-terminus of the peptides for the presented antigens. As the immunoproteasome produces exactly these peptides, preferably the antigen presentation by the MHC-I is raised. Under noninterferon γ-stimulating conditions, the proteasome produces most peptides of seven to nine amino acids (Voges et al., 1999). These are too short to serve as molecules for the presentation by the MHC-1 because the MHC-1 binds only polypeptides that are at least nine amino acids long (Engelhard, 1994; Rammensee et al., 1993; Stern and Wiley, 1994). In this connection the 11S regulator plays an important role. It opens the α-annulus of the 20S core proteasome to make it easier for proteins to get into the catalytic chamber and to facilitate more rapid release of cleavage products. A shorter time in the degradation machinery will result in larger polypeptides, and that will lead to more polypeptides that are presentable by the MHC-1.

As the 26S proteasome degrades mainly polyubiquitinated proteins a defect in the ubiquitination pathway would result in a decreased activity of this pathway. There are at least three different enzyme categories necessary to mark a protein for degradation with a polyubiquitin chain (Pickart, 2000). While there exists only one E1 enzyme (ubiquitin activating enzyme), several classes of E2 enzymes (ubiquitin conjugating enzymes), and a large variety of E3 enzymes (ubiquitin ligases) are known. Since the E2/E3 enzymes are somewhat overlapping in their activity, a single defective E3 might have only a limited impact on the proteasomal protein degradation, while a mutation in E1 would stop the whole ubiquitination system, and this results in a lower 26S activity. Ulrich (2002a,b) gives a very detailed overview of the recognition and ubiquitination of substrates for the proteasome.

Several nonproteinogenic factors are able to influence the proteasomal subunits, including the phosphorylation of proteasomal subunits (Bose et al., 2004) as well as *N*-acetylation of subunits (Claverol et al., 2002). Additionally, some environmental factors are able to activate the core proteasome similar to the 11S regulator. Incubation with polylysine, fatty acids, low concentrations of detergents like SDS (Saitoh et al., 1989), repeated freeze–thaw cycles (Bajorek and Glickman, 2004), as well as a mild heat shock (Conconi et al., 1998; Beedholm et al., 2004) increase the proteasomal activity by structural changes that open the α-annulus of the 20S core proteasome.

Besides the above-mentioned mechanisms by which the proteasomal activity is increased, there are also some specific inhibitors known that downregulate the proteasomal activity. First of all, there are the derivatives of peptide-aldehydes like leupeptin, calpain inhibitor I and II, MG-132, and MG-115 (Harding et al., 1995; Lee and Goldberg, 1996; Rock et al., 1994). These small molecules bind reversibly to a threonine within the active center of the proteasome. Mainly the chymotrypsin-like activity is inhibited, but also the two other catalytic activities are down regulated at higher concentrations (Groll et al., 1997). As these inhibitors are not very specific and inhibit also other serine- and cysteine-proteases, several modifications have been performed in order to reach a higher specificity toward the proteasome. For example, the boron ester derivative of

the MG-132 aldehyde is such a specific inhibitor for the proteasome (Gardner et al., 2000).

Peptide vinyl sulfones, α′,β′-epoxyketones, chlormethyl ketones, diazomethyl ketones, and 4,4-dichloroisocoumarin are proteasomal inhibitors that form covalent adducts (Harper and Powers, 1985; Savory et al., 1993; Palmer et al., 1995; Spaltenstein et al., 1996). Also bifunctional molecules have been designed to block two activities in the proteasomal catalytic chamber (Loidl et al., 1999, 2000).

Lactacystin, epoxymicin, and TMC-95 A-D are natural proteasomal inhibitors. Their high specificity toward the inhibition of the proteasome is a common feature. Lactacystin and epoxymicin bind covalently to the protease, while TMC-95 A-D binds in a noncovalent fashion (Fenteany et al., 1995; Kohno et al., 2000; Groll et al., 2000b, 2001).

The inhibition of the proteasome is not due solely to the binding of small molecules within the catalytic chamber; whole proteins or protein complexes like PI31 or the HIV Tat-protein also inhibit the proteasome (Li et al., 1991; Chu-Ping et al., 1992; Li and Etlinger, 1992; Seeger et al., 1997). The Tat-protein binds only to the 20S core proteasome, inhibits the binding of the 11S regulator, and thus downregulates the immune response while it activates the assembled 26S proteasome at the same time. These results make proteasomal inhibitors candidates for anti-inflammatory agents (Palombella et al., 1994) or anticancer agents (Glotzer et al., 1991).

Role of Proteasome in Removal of Oxidized Proteins The proteasomal system plays an important role in the removal of oxidized proteins from the cellular environment of cells. Since Salo et al. found that the degradation of oxidatively damaged superoxide dismutase by cell free extracts of bovine erythrocytes is enhanced (Salo et al., 1988, 1990), numerous experiments in vitro as well as in vivo have been performed in order to investigate the influence of oxidation on the proteasomal protein turnover.

The degradation of different proteins like aconitase (Grune et al., 1998), ferritin (Grune et al., 2001; Shringarpure et al., 2003), crystallins (Sommerburg et al., 1998), glucose-6-phospate dehydrogenase (G6PD) and isolated histones (Ullrich et al., 1999a–c), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Buchczyk et al., 2003), and lysozyme (Shringarpure et al., 2003) in vitro by isolated proteasomes is raised due to low oxidative stress. Stronger oxidative lesions lead to a lower protein degradation.

As ATP and ubiquitin are not necessary for the degradation of oxidatively modified proteins in vitro and the fact that the activity of the 26S proteasome declines after oxidative stress while the 20S activity remains unchanged (Reinheckel et al., 1998, 2000), it seems to be possible that the 20S core proteasome is sufficient to degrade oxidatively damaged proteins.

These findings are supported by several experiments in living cells using different cell lines. Among these are the human hematopoietic cell line K562 (Reinheckel et al., 2000; Ullrich et al., 1999b; Ullrich and Grune, 2001),

Clone 9 liver cells (Grune et al., 1995, 2002), BV-2 cells (Mehlhase et al., 2000), MCR-5 fibroblasts (Sitte et al., 1998), and RAW cells (Gieche et al., 2001; Mehlhase et al., 2005). Either the whole cells are oxidized or cell lysates are used to investigate the degradation of the oxidized proteins.

To introduce the oxidative modifications into the proteins, different systems are used. The easiest assay is the incubation of proteins or cells with reactive oxygen species like H_2O_2 (Reinheckel et al., 1998; Ullrich et al., 1999b,c; Gieche et al., 2001), singlet oxygen (Grune et al., 1996, 2001), or the xanthine/xanthine-oxidase system (Grune et al., 1995; Salo et al., 1988). Besides these methods, the influence of other oxidatively active substances like peroxynitrite (Buchczyk et al., 2003; Grune et al., 2001) and hypochlorite (Reinheckel et al., 1998; Ullrich et al., 1999a) have been used to damage proteins and test their influence on proteolysis. Additionally, products of the lipid peroxidation like 4-hydroxynonenal and malonyldialdehyde also have an influence on the proteasomal degradation of proteins (Grune et al., 1995).

Role of Proteasome During Aging Process The activity of the proteasomal system toward the degradation of proteins declines with age. This decline may reflect an age-related decrease in the number of proteasomes, accumulation of post-translational modifications of the proteasome, alterations in subunit exchange processes, or a change in interactions with cofactors. This decline can be shown with aging animals as well as in human epidermis biopsies, keratinoytes, and other cell cultures (Conconi et al., 1996; Hayashi and Goto, 1998; Lee et al., 1999; Ly et al., 2000; Petropoulos et al., 2000; Sitte et al., 2000b,c; Wagner and Margolis, 1995).

The expression of proteasomes in epidermis and keratinocyte cultures is downregulated with age (Petropoulos et al., 2000). Aging models like mouse skeletal muscle and human dermal fibroblasts support this change in the expression level of the proteasome (Lee et al., 1999; Ly et al., 2000). In contrast thereof, the amount of proteasome did not seem to decline with age in rat liver cells (Anselmi et al., 1998).

The comparison of 2D gel electrophoresis images reveals that at least two proteasomal subunits are modified during the aging process (Anselmi et al., 1998; Bulteau et al., 2000). Therefore, the age-related decline in proteasomal activity can at least partially be related to structural changes of the subunits.

Other Proteases Beside the lysosomal and the proteasomal degradation systems, with a broad substrate spectrum there are a multitude of proteases that degrade only a small number of proteins in order to regulate different cellular processes like cell cycle, cell proliferation, cell differentiation, apoptosis, necrosis, and the immune response.

Caspases The family of mammalian caspases consists of 13 members that are involved in different cellular processes (Schwerk and Schulze-Osthoff, 2003). They play an important role in T cell proliferation and regulate the cell cycle

in B cells (Lakhani and Flavell, 2003; Mahidhara et al., 2003; Woo et al., 2003). Besides this, caspases are deeply involved in the apoptotic process and regulate it on different levels. They cleave DNA protective enzymes, destabilize the cytoskeleton, degrade apoptosis inhibitors, inhibit antiapoptotic-signaling cascades, and prevent cellular necrosis. Fischer et al. (2003) give a complete overview over the substrates that are cleaved via the caspase signaling pathways.

Another function of caspases is to process precursors of inflammatory cytokines. They play therefore an important role in the immune system, and they are essential for the maturation of inflammatory cytokines, including Il-1β, Il-1α, and Il-18 (Kim et al., 2002; Martinon et al., 2002; Burns et al., 2003; Tschopp et al., 2003).

Calpains Calcium-dependent neutral proteases (calpains) are cytosolic cysteine proteases that are ubiquitously expressed in all tissues and are located close to the cytoskeleton. As well as the lysosomal cathepsins and caspases, the calpains are implicated in some basic cellular processes like cell proliferation, differentiation, and apoptosis (Wang, 2000; Perrin and Huttenlocher, 2002; Rami, 2003). They show high substrate specificity, are regulated by the cellular Ca^{2+} concentration, and can selectively be inhibited by calpastatins. Some groups found an activation of calpains under oxidative stress (Dare et al., 2000; Ishihara et al., 2000; McCracken et al., 2001), while other groups found a decreased calpain activity (Cuzzocrea et al., 2001; Guttman and Johnson, 1998; Seo et al., 1999). Therefore, it can be concluded that calpains are activated during short and moderate oxidative stress. A calcium-triggered phosphorylation cascade mediates this signal. In contrast to this, severe oxidative stress leads to an inactivation of calpains. Calpains are also suggested to be involved in aging processes (Baudry et al., 1986; Ibrahim et al., 1994; Karlsson et al., 1995).

Lon Proteases Mitochondria as the compartment of energy production and oxidative phosphorylation produce large amounts of reactive oxygen species. Therefore mitochondria are equipped with proteases that selectively degrade oxidatively damaged mitochondrial proteins. In the mitochondrial matrix the lon protease seems to be responsible for this (Bota and Davies, 2002). This mitochondrial matrix serine type protease is able to degrade oxidatively modified aconitase (Watabe and Kimura, 1985; Wang et al., 1993; Bota and Davies, 2002). In murine skeletal muscles the concentration of the lon protease declines during the aging process. It is supposed that lon proteases protect the mitochondria against accumulation of oxidized protein material during oxidative stress and aging (Lee et al., 1999).

17.2.2 Accumulation of Oxidized Proteins

Moderate oxidation of amino acid residues leads to a disruption of the structure of the proteins, which causes hydrophobic areas on the outside of the protein. These areas can be recognized by proteases like the lysosomal cathepsins or the

proteasomal system. The proteases are selectively recognizing these damaged proteins and degrade them. In contrast, severe changes of the protein structure due to oxidative damage or stronger denaturing conditions lead to damaged proteins that cannot be degraded by either the proteasomal system or the lysosomal system, or by other proteases (Davies et al., 1987; Grune et al., 1997; Stadtman, 1992). This severely damaged protein material is stored within the cell, accumulates there during the aging process, and is able to act as an inhibitor of the proteasome (Friguet et al., 1994; Friguet and Szweda, 1997; Keck et al., 2003; Sitte et al., 2000a).

The main reason why the proteasomes cannot degrade the severely oxidized proteins is the cross-linking of damaged proteins either by disulfide bonds, formation of dityrosine, covalent bonds due to C-radicals or by cross-linking reagents like 4-hydroxy-nonenal (HNE), malondialdehyde (MDA) or other lipid-peroxidation products (Esterbauer et al., 1991; Friguet et al., 1994; Friguet and Szweda, 1997). These initial protein aggregates can further be oxidized, and they grow on to insoluble aggregates of high molecular weight while the cell is aging (Grune et al., 2004).

17.2.3 The Vicious Cycle of Accumulation of Oxidatively Damaged Proteins and Decreased Proteolysis

It seems clear that the aging process is a continuous, ongoing process. Oxidative stress leads to damaged proteins, which are degraded, and undegradable proteinous material, which accumulates within the cells. The accumulation starts slowly, as only a few damaged proteins escape the proteolysis and begin to form larger aggregates. The process of cross-linking is a very complex stochastic process requiring specific intermolecular interactions, such as, between the tyrosine residues of two different proteins (Grune et al., 2001; Keller et al., 1997; Merker et al., 2001). The number and the molecular weight of these aggregates increase during the aging process, since they cannot be degraded. This effect is further amplified by the age-related decreasing antioxidant status of the cells, resulting in an increased susceptibility toward oxidative stress.

Cells react by increasing compensatory amounts and activity of the proteolytic systems, but this is unable to prevent the aggregate formation. Due to the fact that the aggregates inhibit the proteasome, the growth of the aggregates is further enhanced (Fig. 17.5). One of the consequences is a continuous decline of the activity of the proteasome within the aging process (Petropoulos et al., 2000; Shringarpure et al., 2000; Sitte et al., 2000a). Because of this declined capacity for removal of oxidized proteins, the accumulation of misfolded and damaged proteins is accelerated. The vicious cycle of decreased proteolysis and accumulation of more and more oxidatively damaged proteins is rolling on until the protein aggregates cause metabolic dysfunctions or the initiation to apoptotic or necrotic events.

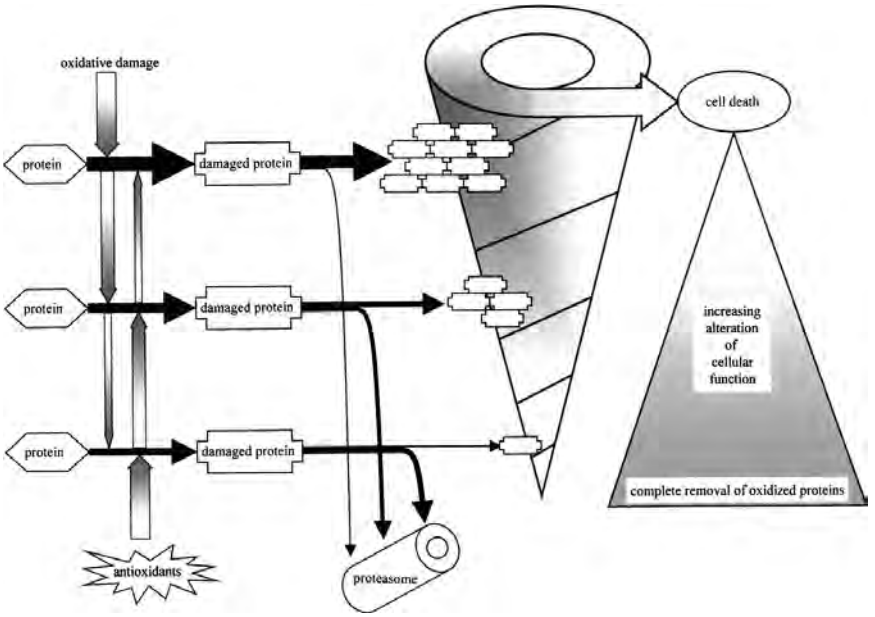


FIGURE 17.5 Vicious cycle of decreasing proteolytic activity and increasing aggregation of damaged proteins during aging and oxidative stress. During aging the content of oxidatively damaged proteins in cells is raising, whereas the antioxidative defenses as well as the activities of the proteasomal system are declining. The damaged proteins aggregate, and these aggregates cannot be degraded and they even inhibit the removal of oxidatively damaged proteins by the proteasomal system. Therefore the amount of oxidized proteins raises and the aggregation process is enhanced, accompanied by an increasing loss of function and ending eventually in cell death.

17.3 OXIDIZED PROTEINS IN AGE-RELATED DISEASES

A whole variety of age-related diseases is linked with the accumulation of oxidized or abnormal folded proteins. Some of them hit the cardiovascular system and lead to symptoms like atherosclerosis (Baker et al.; Vaisar and Heinecke; Carini et al., this volume, chapters 23, 24 and 27, respectively; Ron and Oyadomari, 2004), coronary artery disease (Baker et al.; Vaisar and Heinecke, this volume, chapters 23 and 24), hypertension (Baker et al., this volume, chapter 23), and cerebral ischemia (DeGracia and Montie, 2004; Liu et al., 2004).

Advanced glycation end-products (AGEs) resulting from the oxidation of proteins, lipids, or nucleic acids are formed at an enhanced rate during diabetes (Carini et al., this volume, chapter 27; Cellek, 2004; Smit and Lutgers, 2004), which in turn can facilitate development of atherosclerosis (Renard et al., 2004).

Abnormal protein overexpression or accumulation is also seen in some tumors. Squamous cell carcinomas of the head and neck, gastric cancer, as

well as non–small cell lung cancer are characterized by an overexpression and accumulation of p53 or beta-catenin (Hoffmann et al., 2005; Suriano et al., 2005; Wang et al., 2005).

Since postmitotic cells like neurons are more sensitive to the accumulation of protein aggregates than dividing cells (Sitte et al., 2000b,c), we will focus on the impact of accumulation of oxidized proteins in neurodegenerative diseases in this review. The neurodegenerative diseases include Alzheimer's (AD), Parkinson's (PD), Huntington's (HD), amyotrophic lateral sclerosis (ALS), and prion diseases like Creutzfeldt-Jakobs disease (CJD) (Barnham et al., 2004; Bossy-Wetzel et al., 2004; Ciechanover and Brundin, 2003; Emerit et al., 2004; Goldberg, 2003; Willner, 2004). Table 17.1 gives an overview of some neurodegenerative diseases accompanied by the accumulation of protein material in the tissue. In some of these diseases a single mutation leads to an abnormal protein structure that cannot be degraded by the proteasomal system and accumulates during lifetime. Another reason for an accumulation of oxidized proteins is a nonfunctioning or ineffective ubiquitin pathway.

The formation of aggregates is an accumulative process that might take years to develop, and oxidative stress can be one factor to enhance these severe diseases. Carney et al. (1991) demonstrated a direct correlation between age, spatial and temporal memory loss, and the accumulation of oxidized proteins in the gerbil brain.

The most prominent disease that is linked to oxidative stress and protein aggregation is Alzheimer's disease (AD). Neurofibrillary tangels (NFTs), consisting of abnormal phosphorylated and oxidized tau protein, as well as senile plaques consisting of β-amyloid proteoglycans, Apo E, and some other proteins contribute to the loss of memory, reasoning, and speech (Martin, 1999). Exposure of cells to the β-amyloid peptide lead to an increase in the H_2O_2 production and therefore to an increase in oxidative modifications (Farrer et al., 1997; Mecocci et al., 1994).

Accumulation of Lewy bodies consisting of α-synuclein, ubiquitin, and parkin is a hallmark of Parkinson's disease (PD). PD causes rest tremor, rigidity, bradykinesia, and a disturbance of gait and posture in the patients (Cole and Murphy, 2002; Rao et al., 2003; Uversky and Fink, 2002). Oxidative damage in this disease might be a consequence of a mitochondrial defect in the electron transport chain (Schapira et al., 1990) or the result of increased iron and decreased GSH levels. This situation leads to an incomplete detoxifying of H_2O_2 and (in the presence of redox active iron) to increased hydroxyl radical levels, which cause further oxidative damage (Dexter et al., 1989, 1991; Ebadi et al., 1996).

Amyotrophic lateral sclerosis (ALS) is characterized by shrinkage of motor neurons often accompanied by the accumulation of lipofuscin (Leigh et al., 1988; Rowland, 1994). In the familiar form of the disease this loss of neurons was linked to a mutation in a gene encoding superoxide dismutase (SOD), an enzyme of the antioxidative defense of the cells (Cudkowicz et al., 1997). The disease is characterized by a progressive loss of motor neuron functions leading to death via the involvement of muscles of the respiratory system (Tandan and Bradley, 1985).

TABLE 17.1 Protein Aggregation in Neurodegenerative Diseases

Name of Disease	Abbreviation	Protein Aggregated	Localization
		Tau-related diseases	
Alzheimer's disease	AD	Tau, amyloid	Extracellular/ intracellular
Parkinson's disease	PD	Synuclein, tau, parkin	Cytoplasmic
Pick's disease	PiD	Tau	Cytoplasmic
Frontotemporal dementias	FTD	Tau	Cytoplasmic
Frontotemporal dementias with parkinsonism	FTDP	Tau	Cytoplasmic
Progressive supranuclear palsy	PSP	Tau	Cytoplasmic
Corticobasal degeneration	CBD	Tau	Cytoplasmic
Alexander's disease	AxD	GFAP, tau-2	Cytoplasmic
		Polyglutamine repeat diseases	
Huntington's disease	HD	Huntingtin	Nuclear/ cytoplasmic
Spinocerebellar ataxia	SCA	Ataxin, androgen receptor (AR)	Nuclear
Spinobulbar muscular atrophy (Kennedy's syndrome)	SBMA	Androgen receptor (AR)	Nuclear
Dentato-rubral and pallido-lusyian atrophy	DRPLA	Atropin-1	Nuclear
Machado-Joseph disease	MJD	Atropin-1	Nuclear
		Prion protein diseases	
Creutzfeldt-Jakob disease	CJD	Prion protein	Extracellular/ intracellular
Fatal familial insomnia	FFI	Prion protein	Extracellular/ intracellular
Gerstmann-Sträussler-Scheinker disease	GSS	Prion protein	Extracellular/ intracellular
Kuru		Prion protein	Extracellular/ intracellular
Bovine spongiform encephalopathy	BSE	Prion protein	Extracellular/ intracellular

(*continued overleaf*)

TABLE 17.1 (*continued*)

Name of Disease	Abbreviation	Protein Aggregated	Localization
Scrapie	Sc	Prion protein	Extracellular/ intracellular
	Other diseases		
Amyotrophic lateral sclerosis	ALS	SOD-1/ synuclein	Cytoplasmic
Neuronal ceroid lipofuscinosis	NCL	Lipofuscin	Cytoplasmic
Friedreich's ataxia	FA	Frataxin	Cytoplasmic
Pantothenate kinase-associated neurodegeneration (Former: Hallervorden-Spatz)	PKAN HSV	Pantothenate kinase 2	Cytoplasmic

Patients with Huntington's disease (HD) show a progressive dementia and other psychiatric symptoms. A gene defect is responsible for an elongated Huntingtin protein with an expanded polyglutamine tract that causes aggregation of the protein (Davies and Ramsden, 2001; Rosenblatt et al., 1998; Saudou et al., 1998). Although there is no hint that oxidative damage is involved in the outbreak of HD (Alam et al., 2000), increased oxidative stress has a promoting effect, since the aggregation of the Huntingtin protein is enhanced. Up to now, eight other polyglutamine-repeat diseases are known. These include the spinocerebellar ataxia family (SCA 1, 2, 3, 6, 7, and 17), dentato-rubro and pallido-lusyian atrophy (DRPLA), and spinobulbar muscular atrophy (SBMA; Kennedy's syndrome). All these diseases are caused by mutated proteins that contain an elongated polyglutamine tail (Ross, 2002; Taylor et al., 2002; Zoghbi and Orr, 2000). This polyglutamine tends to aggregate and thus prevents the abnormal proteins from the degradation by the proteasome. The symptoms can vary from different combinations of motoric, psychiatric, cognitive, and sensoric dysfunctions, depending on the neurons affected.

Prion diseases, like Creutzfeldt-Jakob disease (CJD), fatal familial insomnia (FFI), Gerstmann-Sträussler-Scheinker disease (GSS), Kuru, bovine spongiform encephalopathy (BSE), and scrapie are connected with the proteinaceous infectious particles (prions), which were considered to be the reason for the diseases (Prusiner, 1982; McKintosh et al., 2003). Recently the involvement of the ubiquitin proteasomal system in the onset and development of the disease was demonstrated (Hooper, 2003). Patients or, in the case of BSE and scrapie, cattle or sheep, respectively, suffer from a progressive development of severe motoric disturbances and dementia. The aggregates found in the affected neurons contained mainly prion proteins with a shift in the secondary structure from an

α-helix to a β-sheet. This change seems to be sufficient to prevent the protein from the proteasomal degradation (Hooper, 2003; Ma and Lindquist, 2002; Ma et al., 2002).

17.4 SUMMARY

Oxidative stress leads to a huge number of different amino acid modifications, mostly irreversible. These modifications change the structure of proteins in a way that more hydrophobic side chains are exposed on the surface. The damaged proteins can be recognized and degraded by proteases like the lysosomal and proteasomal systems, or they accumulate and aggregate in the cell. Aggregates are able to inhibit the proteolytic systems and therefore aggregate formation is accelerated. Several severe neurodegenerative diseases like Parkinson's, Alzheimer's, and Huntington's diseases, amyotrophic lateral sclerosis, and prion diseases are also linked with the aggregation of abnormal, mutated, and oxidized proteins. This accumulation and the aggregation of oxidatively damaged proteins are cumulative and amplify during the development of the disease.

LIST OF ABBREVIATIONS

AD, Alzheimer's disease
AGE, advanced glycation end-product
ALS, amyotrophic lateral sclerosis
ATP, adenosine triphosphate
BrAAP, branched-chain amino acid preferring
BSA, bovine serum albumin
BSE, bovine spongiform encephalopathy
CJD, Creutzfeldt-Jakob disease
DNA, deoxyribonucleic acid
DNPH, dinitrophenylhydrazine
DRPLA, dentato-rubro and pallido-lusyian atrophy
ER, endoplasmic reticulum
FFI, fatal familial insomnia
G6PD, glucose-6-phosphate dehydrogenase
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
GSS, Gerstmann-Sträussler-Scheinker disease
HD, Huntington's disease
HNE, 4-hydroxy-nonenal
HOCl, hypochloric acid
IFN-γ, interferon γ

Il, interleukine
LPS, lipopolysaccharide
MDA, malondialdehyde
MHC-1, major histocompatibility complex class I
NFT, neurofibrillary tangle
PD, Parkinson's disease
RBC, red blood cell
RNS, reactive nitrogen species
ROS, reactive oxygen species
SBMA, spinobulbar muscular atrophy (Kennedy's syndrome)
SCA, spinocerebellar ataxia
SNAAP, small neutral amino acid preferring
SOD, superoxide dismutase

REFERENCES

Aki M., Shimbara N., Takashina M., Akiyama K., Kagawa S., Tamurea T., Tanahashi N., Yoshimura T., Tanaka K., Ichihara A. (1994). Interferon-gamma induces different subunit organizations and functional diversity of proteasomes. *J Biochem* 115: 257–269.

Alam Z.I., Halliwell B., Jenner P. (2000). No evidence for increased oxidative damage to lipids, proteins, or DNA in Huntington's disease. *J Neurochem* 75: 840–846.

Alvarez B., Radi R. (2003). Peroxynitrite reactivity with amino acids and proteins. *Amino Acids* 25: 295–311.

Amici A., Levine R.L., Tsai L., Stadtman E.R. (1989). Conversion of amino acid residues in proteins and amino acid homopolymers to carbonyl derivatives by metal-catalyzed oxidation reactions. *J Biol Chem* 264: 3341–3346.

Anselmi B., Conconi M., Veyrat-Durebex C., Turlin E., Bilville F., Alliot J., Friguet B. (1998). Dietary self-selection can compensate an age-related decrease of rat liver 20S proteasome activity observed with standard diet. *J Gerontol A Biol Sci Med Sci* 53: B173–B179.

Armstrong R.C., Swallow A.J. (1969). Pulse- and gamma-radiolysis of aqueous solutions of tryptophan. *Radiations Res* 41: 563–579.

Bajorek M., Glickman M.H. (2004). Keepers at the final gates: Regulatory complexes and gating of the proteasomal channel. *Cell Mol Life Sci* 61: 1–10.

Balazy M., Kaminski K.M., Mao K., Tan J., Wolin M.S. (1998). S-Nitroglutathione, a product of the reaction between peroxynitrite and glutathione that generates nitric oxide. *J Biol Chem* 273: 32009–32015.

Barnham K.J., Masters C.L., Bush A.I. (2004). Neurodegenerative diseases and oxidative stress. *Nat Rev Drug Discov* 3: 205–214.

Baudry M., DuBrin R., Beasley L., Leon M., Lynch G. (1986). Low lewels of calpain activity in Chiroptera brain: Implications for mechanisms of aging. *Neurobiol Aging* 7: 255–258.

Baumeister W., Walz J., Zühl F., Seemuller E. (1998). The proteasome: Paradigm of a self-compartmentalizing protease. *Cell* 92: 367–380.

Beedholm R., Clark B.F.C., Rattan S.I.S. (2004). Mild heat stress stimulates 20S proteasome and its 11S activator in human fibroblasts undergoing aging in vitro. *Cell Stress Chaperones* 9: 49–57.

Benaroud J.N., Tarcsa E., Cascio P., Goldberg A.L. (2001). The unfolding of substrates and ubiquitin-independent protein degradation by proteasomes. *Biochimie* 83: 311–318.

Berlett B.S., Levine R.L., Stadtman E.R. (1996). Comparison of the effects of ozone on the modification of amino acid residues in glutamine synthetase and bovine serum albumin. *J Biol Chem* 271: 4177–4182.

Bidere N., Lorenzo H.K., Carmona S., Laforge M., Harper F., Dumont C., Senik A. (2003). Cathepsin D triggers Bax activation, resulting in selective apoptosis-inducing factor (AIF) relocation in T lymphocytes entering the early commitment phase to apoptosis. *J Biol Chem* 278: 31401–31411.

Bose S., Stratford F.L., Broadfoot K.I., Mason G.G., Rivett A.J. (2004). Phosphorylation of 20S proteasome alpha subunit C8 (alpha7) stabilizes the 26S proteasome and plays a role in the regulation of proteasome complexes by gamma-interferon. *Biochem J* 378(Pt1): 177–184.

Bossy-Wetzel E., Schwarzenbacher R., Lipton S.A. (2004). Molecular pathways to neurodegeneration. *Nat Med* 10: S2–S9.

Bota D.A., Davies K.J.A. (2002). Lon protease preferentially degrades oxidized mitochondrial aconitase by an ATP-stimulated mechanism. *Nat Cell Biol* 4: 673–680.

Braun B.C., Glickman M., Kraft R., Dahlmann B., Kloetzel P.M., Finley D., Schmidt M. (1999). The base of the proteasome regulatory particle exhibits chaperone-like activity. *Nature Cell Biol* 1: 221–226.

Brodie A.E., Reed D.J. (1990). Cellular recovery of glyceraldehyde-3-phosphate dehydrogenase activity and thiol status after exposure to hydroperoxides. *Arch Biochem Biophys* 276: 212–218.

Buchczyk D.P., Grune T., Sies H., Klotz L.O. (2003). Modifications of glyceraldehyde-3-phosphate dehydrogenase induced by increasing concentrations of peroxynitrite: Early recognition by 20S proteasome. *Biol Chem* 384: 237–241.

Bulteau A., Petropoulos I., Friguet B. (2000). Age-related alterations of proteasome structure and function in aging epidermis. *Exp Gerontol* 35: 767–777.

Burns K., Martinon F., Tschopp J. (2003). New insights into the mechanism of IL-2beta maturation. *Curr Opin Immunol* 12: 26–30.

Carney J.M., Starke-Reed P.E., Oliver C.N., Landum R.W., Cheng M.S., Wu J.F., Floyd R.A. (1991). Reversal of age-related increase in brain protein oxidation, decrease in enzyme activity, and loss in temporal and spatial memory by chronic administration of the spin-trapping compound N-*tert*-butyl-alpha-phenylnitrone. *Proc Natl Acad Sci USA* 88: 3633–3636.

Carrard G., Bulteau A.L., Petropoulos I., Friguet B. (2002). Impairment of proteasome structure and function in aging. *Int J Biochem Cell Biol* 34: 1461–1474.

Cellek S. (2004). Point of NO return for nitrergic nerves in diabetes: A new insight into diabetic complications. *Curr Pharm Des* 10: 3683–3695.

Chondrogianni N., Fracoulis E.G., Gonos E.S. (2002). Protein degradation during aging: the lysosome-, the calpain- and the proteasome-dependent cellular proteolytic systems. *Biogerontology* 3: 121–123.

Chu-Ping M., Slaughter C.A., DeMartino G.N. (1992). Purification and characterization of a protein inhibitor of the 20S proteasome (macropain). *Biochim Biophys Acta* 1119: 303–311.

Ciechanover A., Brundin P. (2003). The ubiquitin proteasome system in neurodegenerative diseases: Sometimes the chicken, sometimes the egg. *Neuron* 40: 427–446.

Cirman T., Oresic K., Mazovec G.D., Turk V., Reed J.C., Myers R.M., Salvesen T.S., Turk B. (2004). Selective disruption of lysozymes in HeLa cells triggers apoptosis mediated by cleavage of Bid by multiple papain-like lysosomal cathepsins. *J Biol Chem* 279: 3578–3587.

Claverol S., Burlet-Schiltz O., Girbal-Neuhauser E., Gairin J.E., Monsarrat B. (2002). Mapping and structural dissection of human 20S proteasome using proteomic approaches. *Mol Cell Proteomics* 1: 567–578.

Cole N.B., Murphy D.D. (2002). The cell biology of alpha-synuclein: A sticky problem? *Neuromolecular Med* 1: 95–109.

Conconi M., Petropoulos I., Emod I., Turlin E., Biville F., Friguet B. (1998). Protection from oxidative inactivation of the 20S proteasome by heat-shock protein 90. *Biochem J* 333: 407–415.

Conconi M., Szweda L.I., Levine R.L., Stadtman E.R., Friguet B. (1996). Age-related decline of rat liver multicatalytic proteinase activity and protection from oxidative inactivation by heat-shock protein 90. *Mol Biol Rep* 24: 45–50.

Coux O., Tanaka K., Goldberg A.L. (1996). Structure and function of the 20S and 26S proteasomes. *Ann Rev Biochem* 65: 801–847.

Creeth J.M., Cooper B., Donald A.S.R., Clamp J.R. (1983). Studies on the limited degradation of mucous glycoproteins. *Biochem J* 211: 323–332.

Cudkowicz M.E., McKenna-Yasek D., Sapp P.E., Chin W., Geller B., Hayden D.L., Schoenfeld D.A., Hosler B.A., Horvitz H.R., Brown R.H. (1997). Epidemiology of mutations in superoxide dismutase in amyotrophic lateral sclerosis. *Ann Neurol* 41: 210–221.

Cuervo A.M., Knecht E., Terlecky S.R., Dice J.F. (1995). Activation of a selective pathway of lysosomal proteolysis in rat liver by prolonged starvation. *Am J Physiol* 269: C1200–C1208.

Cuzzocrea S., McDonald M.C., Mazzon E., Mota-Filipe H., Centorrino T., Terranova M.L., Ciccolo A., Britti D., Caputi A.P., Thiemermann C. (2001). Calpain inhibitor I reduces colon injury caused by dinitrobenzene sulphonic acid in the rat. *Gut* 48: 478–488.

Dalle-Donne I., Rossi R., Giustarini D., Milzani A., Colombo R. (2003). Protein carbonyl groups as biomarkers of oxidative stress. *Clin Chim Acta* 329: 23–28.

Dare E., Gotz M.E., Zhivotovski B., Manzo L., Cettatelli S. (2000). Antioxidants J811 and 17-ß-estradiol protect cerebellar granule cells from methylmercury-induced apoptotic cell death. *J Neurosci Res* 62: 557–565.

Davies K.J. (2001). Degradation of oxidized proteins by the 20S proteasome. *Biochimie* 83: 301–310.

Davies K.J.A., Delsignore M.E., Lin S.W. (1987). Protein damage and degradation by oxygen radicals. II. Modification of amino acids. *J Biol Chem* 262: 9902–9907.

Davies S., Ramsden D.B. (2001). Huntington's disease. *Neurobiol Dis* 3: 3–15.

De Wit R., Capello A., Boonstra J., Verkleij A.J., Post J.A. (2000). Hydrogen peroxide inhibits epidermal growth factor receptor internalization in human fibroblasts. *J Cell Physiol* 178: 17–27.

De Wit R., Makkinje M., Boonstra J., Verkleij A.J., Post J.A. (2001). Hydrogen peroxide reversibly inhibits epidermal growth factor (EGF) receptor internalization and coincident ubiquitination of the EGF receptor and Eps15. *FASEB J* 15: 305–308.

Dean R.T. (1975a). Concerning a possible mechanism for selective capture of cytoplasmic proteins by lysosomes. *Biochem Biophys Res Commun* 67: 604–609.

Dean R.T. (1975b). Direct evidence of importance of lysosomes in degradation of intracellular proteins. *Nature* 257: 414–416.

DeGracia D.J., Montie H.L. (2004). Cerebral ischemia and the unfolded protein response. *J Neurochem* 91: 1–8.

Dexter D.T., Carayon A., Javoy-Agid F., Agid Y., Wells F.R., Daniel S.E., Lees A.J., Jenner P., Marsden C.D. (1991). Alterations in the levels of iron, ferritin and other trace metals in Parkinson's disease and other neurodegenerative diseases affecting the basal ganglia. *Brain* 114: 1953–1975.

Dexter D.T., Carter C.J., Wells F.R., Javoy-Agid F., Agid Y., Lees A., Jenner P., Marsden C.D. (1989). Basal lipid peroxidation in substantia nigra is increased in Parkinson's disease. *J Neurochem* 52: 381–389.

Dhaliwal B.S., Steinbrecher U.P. (2000). Cholesterol delivered to macrophages by oxidized low density lipoprotein is sequestered in lysosomes and fails to efflux normally. *J Lipid Res* 41: 1658–1665.

Driscoll J., Brown M.G., Finley D., Monaco J.J. (1993). MHC-linked *LMP* gene products specifically alter peptidase activities of the proteasome. *Nature* 365: 262–264.

Dice J.F. (1993). Cellular and molecular mechanisms of aging. *Physiol Rev* 73: 149–159.

Dunlop R.A., Rodgers K.J., Dean R.T. (2002). Recent developments in the intracellular degradation of oxidized proteins. *Free Rad Biol Med* 33: 894–906.

Easton C.J. (1997). Free-radical reactions in the synthesis of alpha-amino acids and derivatives. *Chem Rev* 97: 53–82.

Ebadi M., Srinivasan S.K., Baxi M.D. (1996). Oxidative stress and antioxidant therapy in Parkinson's disease. *Prog Neurobiol* 48: 1–19.

Emerit J., Edeas M., Bricaire F. (2004). Neurodegenerative diseases and oxidative stress. *Biomed Pharmacother* 58: 39–46.

Enenkel C., Lehmann A., Kloetzel P.-M. (1998). Subcellular distribution of proteasomes implicates a major location of protein degradation in the nuclear envelope-ER network in yeast. *EMBO J* 17: 6144–6154.

Engelhard V.H. (1994). Structure of peptides associated with MHC class I molecules. *Curr Opin Immun* 6: 13–23.

Esterbauer H., Schur R.J., Zollner H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* 11: 81–128.

Farrer L.A., Cupples L.A., Haines J.L., Jyman B., Kukull W.A., Mayeux R., Myers R.H., Pericak-Vance M.A., Rich N., van Duijn C.M. (1997). Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease: A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium. *JAMA* 278: 1349–1356.

Fenteany G., Standaert R.F., Lane W.S., Choi S., Corey E.J., Schreiber S.L. (1995). Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science* 268: 726–731.

Finkel T. (2000). Redox-dependent signal transduction. *FEBS Lett* 476: 52–54.

Fischer U., Janicke R.U., Schulze-Osthoff K. (2003). Many cuts to ruin: A comprehensive update of caspase substrates. *Cell Death Differ* 10: 76–100.

Friguet B., Stadtman E.R., Szweda L.I. (1994). Modification of glucose-6-phosphate dehydrogenase by 4-hydroxy-2-nonenal: Formation of cross-linked protein that inhibits the multicatalytic protease. *J Biol Chem* 269: 21639–21643.

Friguet B., Szweda L.I. (1997). Inhibition of the multicatalytic proteinase (proteasome) by 4-hydroxy-2-nonenal cross-linked protein. *FEBS Lett* 405: 21–25.

Gaczynska M., Rock K.L., Goldberg A.L. (1993). γ-Interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes. *Nature* 365: 264–267.

Gardner R.C., Assinder S.J., Christie G., Mason G.G., Markwell R., Wadsworth H., McLaughlin M., King R., Chabot-Fletcher M.C., Beton J.J., Allsop D., Rivett A.J. (2000). Characterization of peptidyl boronic acid inhibitors of mammalian 20S and 26S proteasomes and their inhibition of proteasomes in cultured cells. *Biochem J* 346: 447–454.

Garrison W.M. (1987). Reaction mechanisms in radiolysis of peptides, polypeptides, and proteins. *Chem Rev* 87: 381–398.

Gieche J., Mehlhase J., Licht A., Zacke T., Sitte N., Grune T. (2001). Protein oxidation and proteolysis in RAW264.7 macrophages: Effects of PMA activation. *Biochim Biophys Acta* 1538: 321–328.

Gieseg S.P., Simpson J.A., Charton T.S., Ducan M.W., Dean R.T. (1993). Protein-bound 3,4-dihydroxyphenylalanine is a major reductant formed during hydroxyl radical damage to proteins. *Biochemistry* 32: 4780–4786.

Giulivi C, Pacifici R.E., Davies K.J.A. (1994). Exposure of hydrophobic moieties promotes the selective degradation of hydrogen peroxide-modified hemoglobin by the multicatalytic proteinase complex, proteasome. *Arch Biochem Biophys* 311: 329–341.

Giulivi C., Traaseth N.J., Davies K.J.A. (2003). Tyrosine oxidation products: analysis and biological relevance. *Amino Acids* 25: 227–232.

Glickman M.H., Maytal V. (2002). Regulating the 26S proteasome activity. In: Zwickl P., Baumeister W. (eds.), *The Proteasome-Ubiquitin Protein Degradation Pathway*, Springer-Verlag, Berlin, pp. 43–72.

Glickman M.H., Rubin D.M., Coux O., Wefes I., Pfeifer G., Cjeka Z., Baumeister W., Fried V.A., Finley D. (1998). A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. *Cell* 94: 615–623.

Glotzer M., Murray A.W., Kirschner M.W. (1991). Cyclin is degraded by the ubiquitin pathway. *Nature* 349: 132–138.

Goldberg A.L. (2003). Protein degradation and protection against misfolded or damaged proteins. *Nature* 426: 895–899.

Grandhee S., Monnier V.M. (1991). Mechanism of formation of the Maillard protein cross-link pentosidine. *J Biol Chem* 266: 11649–11653.

Grant A.J., Jessup W., Dean R.T. (1992). Accelerated endocytosis and incomplete catabolism of radical-damaged protein. *Biochim Biophys Acta* 1134: 203–209.

Groll M., Bajorek M., Koehler A., Moroder L., Rubin D., Huber R., Glickman M.H., Finley D. (2000a). A gated channel into the core particle of the proteasome. *Nat Struct Biol* 7: 1062–1067.

Groll M., Ditzel I., Löwe J., Stock D., Bochtler M., Bartunik H.D., Huber R. (1997). Structure of 20S proteasome form yeast at 2.4 Å resolution. *Nature* 386: 463–471.

Groll M., Kim K.B., Kairies N., Huber R., Crews C.M. (2000b). Crystal structure of epoxymicin: 20S Proteasome reveals a molecular basis for selectivity of α′,β′-epoxyketone proteasomal inhibitors. *J Am Chem Soc* 122: 1237–1238.

Groll M., Koguchi Y., Huber R., Kohno J. (2001). Crystal structures of the 20S proteasome TMC-95A complex: A non-covalent proteasome inhibitor. *J Mol Biol* 311: 543–548.

Grune T., Blasig I.E., Sitte N., Roloff B., Haseloff R., Davies K.J. (1998). Peroxynitrite increases the degradation of aconitase and other cellular proteins by proteasome. *J Biol Chem* 273: 10857–10862.

Grune T., Jung T., Merker K., Davies K.J.A. (2004). Decreased proteolysis caused by protein aggregates, inclusion bodies, plaques, lipofuscin, ceroid, and "aggresomes" during oxidative stress, aging, and disease. *Int J Biochem Cell Biol* 36: 2519–2530.

Grune T., Klotz L.O., Gieche J., Rudeck M., Sies H. (2001). Protein oxidation and proteolysis by the nonradical oxidants singlet oxygen or peroxynitrite. *Free Radic Biol Med* 30: 1243–1253.

Grune T., Reinheckel T., Davies K.J. (1996). Degradation of oxidized proteins in K562 human hematopoietic cells by proteasome. *J Biol Chem* 271: 15504–15509.

Grune T., Reinheckel T., Davies K.J.A. (1997). Degradation of oxidized proteins in mammalian cells. *FASEB J* 11: 526–534.

Grune T., Reinheckel T., Joshi M., Davies K.J. (1995). Proteolysis in cultured liver epithelial cells during oxidative stress: Role of the multicatalytic proteinase complex, proteasome. *J Biol Chem* 270: 2344–2351.

Grune T., Reinheckel T., Li R., North J.A., Davies K.J. (2002). Proteasome-dependent turnover of protein disulfide isomerase in oxidatively stressed cells. *Arch Biochem Biophys* 397: 407–413.

Guttman R.J., Johnson G.V. (1998). Oxidative stress inhibits calpain activity in situ. *J Biol Chem* 273: 13331–13338.

Harding C.V., France J., Song R., Farah J.M., Chatterjee S., Iqbal M., Siman R. (1995). Novel dipeptide aldehydes are proteasome inhibitors and block the MHC-I antigen-processing pathway. *J Immunol* 255: 1767–1775.

Harper J.W., Powers J.C. (1985). Reaction of serine proteases with substituted 3-alkoxy-4-chloroisocoumarins and 3-alkoxy-7-amino-4-chloroisocoumarins: new reactive mechanism-based inhibitors. *Biochemistry* 25: 7200–7213.

Hawkins C.L., Davies M.J. (1999). Hypochlorite-induced oxidation of proteins in plasma: Formation of chloramines and nitrogen-centered radicals and their role in protein fragmentation. *Biochem J* 340: 539–548.

Hawkins C.L., Pattison D.I., Davies M.J. (2003). Hypochlorite-induced oxidation of amino acids, peptides and proteins. *Amino Acids* 25: 259–274.

Hayashi T., Goto S. (1998). Age-related changes in the 20S and 26S proteasome activities in the liver of male F344 rats. *Mech Aging Dev* 102: 55–66.

Headlam H.A., Davies M.J. (2004). Markers of protein oxidation: Different oxidants give rise to variable yields of bound and released carbonyl products. *Free Rad Biol Med* 36: 1175–1184.

Heinecke J.W., Hsu F.F., Crowley J.R., Hazen S.L., Leeuwenburgh C., Mueller D.M., Rasmussen J.E., Turk J. (2000). Detecting oxidative modification of biomolecules with isotope dilution mass spectrometry: Sensitive and quantitative assays for oxidized amino acids in proteins and tissues. *Meth Enzymol* 300: 124–144.

Herold S. (2004). Nitrotyrosine, dityrosine and nitrotryptophan formation form metmyoglobin, hydrogen peroxide and nitrite. *Free Rad Biol Med* 36: 565–579.

Hicke L. (1999). Getting down with ubiquitin: turning off cell-surface receptors, transporters and channels. *Trends Cell Biol* 9: 107–112.

Hicke L. (2001). A new ticket for entry into budding vesicles-ubiquitin. *Cell* 106: 527–530.

Hill C.P., Masters E.I., Whitby F.G. (2002). The 11S regulators of 10S proteasome activity. In: Zwickl P., Baumeister W. (eds.), *The Proteasome-Ubiquitin Protein Degradation Pathway*, Springer-Verlag, Berlin, pp. 73–89.

Hoffmann T., Bier H., Donnenberg A.D., Whiteside T.L., De Leo A.B. (2005). p54 as an immunotherapeutic target in head and neck cancer. *Adv Otorhinolaryngol* 62: 151–160.

Hofmann K., Falquet L. (2001). A ubiquitin-interacting motif conserved in components of the proteasomal and lysosomal protein degradation system. *Trends Biochem Sci* 26: 347–350.

Holmgren A. (1989). Thioredoxin and glutaredoxin systems. *J Biol Chem* 264: 13963–13966.

Hopper N.M. (2003). Could inhibition of the proteasome cause mad cow disease? *Trends Biotechnol* 115: 941–948.

Ibrahim M., Upreti R.K., Kidwai A.M. (1994). Calpain from rat intestinal epithelial cells: Age-dependent dynamics during cell differentiation. *Mol Cell Biochem* 131: 49–59.

Ishihara I, Minami Y., Nishizaki T., Matsuoka T., Yamamura H. (2000). Activation of calpain precedes morphological alterations during hydrogen peroxide-induced apoptosis in neuronally differentiated mouse embryonal carcinoma P19 cell line. *Neurosci Lett* 279: 97–100.

Jessup W., Mander E.L., Dean R.T. (1992). The intracellular storage and turnover of apolipoprotein B of oxidized LDL in macrophages. *Biochim Biophys Acta* 1126: 167–177.

Johnson D.E. (2000). Non-caspase proteases in apoptosis. *Leukemia* 14: 1695–1703.

Kang Y.C., Kim P.K., Choi B.M., Chung H.T., Ha K.S., Kwon Y.G., Kim Y.M. (2004). Regulation of programmed cell death in neuronal cells by nitric oxide. *In vivo* 18: 367–376.

Karlsson J.O., Blennow K., Janson I., Bombren K., Barlsson I., Regland B., Wallin A., Gottfries C.G. (1995). Increased proteolytic activity in lymphocytes from patients with early onset Alzheimer's disease. *Neurobiol Aging* 16: 901–906.

Keck S., Nitsch R., Grune T., Ullrich O. (2003). Proteasome inhibition by paired helical filament-tau in brains of patients with Alzheimer's disease. *J Neurochem* 85: 115–122.

Keller J.N., Pang Z., Geddes J.W., Begley J.G., Germeyer A., Waeg G., Mattson M.P. (1997). Impairment of glucose and glutamate transport and induction of mitochondrial oxidative stress and dysfunction in synaptosomes by amyloid beta-peptide: Role of the lipid peroxidation product 4-hydroxynonenal. *J Neurochem* 69: 273–284.

Kettle A.J. (1996). Neutrophils convert tyrosyl residues in albumin to chlorotyrosine. *FEBS Lett* 379: 103–106.

Kim P.K., Kwon Y.G., Chung H.T., Kim YM. (2002). Regulation of caspases by nitric oxide. *Ann NY Acad Sci* 962: 42–52.

Kohno J., Koguchi Y., Niskio M., Nakao K., Kuroda M., Shimizu R., Ohnuki T., Komatsubara S. (2000). Structures of TMC-95A-D: Novel proteasome inhibitors from *Apoispore montagnei Sacc*. TC 1093. *J Org Chem* 65: 990–995.

Lakhani S., Flavell R.A. (2003). Caspases and T lymphocytes: a flip to the coin? *Immunol Rev* 193: 22–30.

Lasch P., Petras T., Ullrich O., Backmann J., Naumann D., Grune T. (2001). Hydrogen peroxide-induced structural alterations of RNAse A. *J Biol Chem* 276: 9492–9502.

Lavie L., Reznick A.Z., Gershon D. (1982). Decreased protein and puromycinyl-peptide degradation in livers of senescent mice. *Biochem J* 202: 47–51.

Lee C.K., Klopp R.G., Weindruck R., Prolla T.A. (1999). Gene expression profile of ageing and its retardation by caloric restriction. *Science* 285: 1390–1393.

Lee D.H., Goldberg A.L. (1996). Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in *Saccharomyces cerevisiae*. *J Biol Chem* 271: 27280–27284.

Leigh P.N., Anderton B.H., Dodson A., Gallo J.M., Swash M., Power D.M. (1988). Ubiquitin deposits in anterior horn cells in motor neurone disease. *Neurosci Lett* 93: 197–203.

Leist M., Jaattela M. (2001a). Triggering of apoptosis by cathepsins. *Cell Death Differ* 8: 324–326.

Leist M., Jaattela M. (2001b). Four deaths and a funeral: From caspases to alternative mechanisms. *Nat Rev Mol Cell Biol* 2: 589–598.

Levine R.L., Berlett B.S., Moskovitz J., Mosoni L., Stadtman E.R. (1999). Methionine residues may protect proteins from critical oxidative damage. *Mech Ageing Dev* 107: 323–332.

Levine R.L., Mosoni L., Berlett B.S., Stadtman E.R. (1996). Methionine residues as endogenous antioxidants in proteins. *Proc Natl Acad Sci USA* 93: 15036–15040.

Levine R.L., Moskovitz J., Stadtman E.R. (2000). Oxidation of methionine in proteins: Roles of antioxidant defense and cellular regulation. *IUBMB Life* 50: 301–307.

Li X.C., Gu M.Z., Etlinger J.D. (1991). Isolation and characterization of a novel endogenous inhibitor of the proteasome. *Biochemistry* 30: 9709–9715.

Li X.S., Etlinger J.D. (1992). Ubiquitinated proteasome inhibitor is a component of the 26S proteasome complex. *Biochemistry* 31: 11964–11967.

Liu C.L., Martone M.E., Hu B.R. (2004). Protein ubiquitination in postsynaptic densities after transient cerebral ischemia. *J Cereb Blood Flow Metab* 24: 1219–1225.

Löwe J., Stock D., Jap B., Zwickl. P., Baumeister W., Huber R. (1995). Crystal structure of the 20S proteasome from the archaeon *T. acidophilum* at 3.4 Å resolution. *Science* 268: 533–539.

Loidl G., Groll M., Musiol H.J., Huber R., Moroder L. (1999). Bivalency as a principle for proteasome inhibition. *Proc Natl Acad Sci USA* 95: 5418–5422.

Loidl G., Musiol H.J., Groll M., Huber R., Moroder L. (2000). Synthesis of bivalent inhibitors of eucaryotic proteasomes. *J Pept Sci* 66: 36–46.

Ly D.H., Lockhart D.J., Lerner R.A., Schultz P.G. (2000). Mitotic misregulation and human aging. *Science* 287: 2486–2492.

Ma J., Lindquist S. (2002). Conversion of PrP to a self-perpetuating PrPSc-like conformation in the cytosol. *Science* 198: 1785–1788.

Ma J., Wollmann R., Lindquist S. (2002). Neurotoxicity and neurodegeneration when PrP accumulates in the cytosol. *Science* 298: 1781–1785.

Mahidhara R.S., Hoffman R.A., Huang S., Wolf-Johnston A., Vodovotz Y., Simmons R.L., Billiar T.R. (2003). Nitric oxide-mediated inhibition of caspase-dependent T lymphocyte proliferation. *J Leukoc Biol* 74: 403–411.

Malencik D.A., Anderson S.R. (2003). Dityrosine as product of oxidative stress and fluorescent probe. *Amino Acids* 25: 233–247.

Martin J.B. (1999). Molecular basis of the neurodegenerative disorders. *N Engl J Med* 340: 1970–1980.

Marques C., Pereira P., Taylor A., Liang J.N., Reddy V.N., Szweda L.I., Shang F. (2004). Ubiquitin-dependent lysosomal degradation of the HNE-modified proteins in lens epithelial cells. *FASEB J* 18: 1424–1426.

Martinon F., Burns K., Tschopp J. (2002). The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 10: 426–427.

Maskos Z., Rush J.D., Koppenol W.H. (1992). The hydroxylation of phenylalanine and tyrosine: A comparison with salicylate and tryptophan. *Arch Biochem Biophys* 296: 651–654.

McCracken E., Dewar D., Hunter A.J. (2001). White matter damage following systemic injection of the mitochondrial inhibitor 3-nitropropionic acid in rat. *Brain Res* 892: 329–335.

McKintosh E., Tabrizi S.J., Collinge J. (2003). Prion diseases. *J Neurovirol* 9: 183–193.

Mecocci P., MacGarvey U., Beal M.V. (1994). Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. *Ann Neurol* 36: 747–751.

Mehlhase J., Gieche J., Ullrich O., Sitte N., Grune T. (2000). LPS-induced protein oxidation and proteolysis in BV-2 microglial cells. *IUBMB Life* 50: 1–5.

Mehlhase J., Grune T. (2002). Proteolytic response to oxidative stress in mammalian cells. *Biol Chem* 383: 559–567.

Mehlhase J., Sandig G., Pantopoulos K., Grune T. (2005). Oxidation-induced ferritin turnover in microglial cells: Role of the proteasome. *Free Radic Biol Med* 38: 276–285.

Merker K., Grune T. (2004). Age-related changes in the degradation of oxidized proteins. In: Özben T., Chevion M. (eds.), *Frontiers in Neurodegenerative Disorders and Aging: Fundamental Aspects, Clinical Perspectives and New Insights*, IOS Press, Amsterdam, pp. 1–9.

Merker K., Stolzing A., Grune T. (2001). Proteolysis, caloric restriction and aging. *Mech Ageing Dev* 122: 595–615.

Naskalski J.W., Bartosz G. (2000). Oxidative modification of protein structures. *Adv Clin Chem* 35: 161–253.

Niedermann G. (2002). Immunological functions of the proteasome. In: Zwickl P., Baumeister W. (eds.), *The Proteasome-Ubiquitin Protein Degradation Pathway*, Springer-Verlag, Berlin, pp. 91–136.

Okada A.A., Dice J.F. (1984). Altered degradation of intracellular proteins in aging human fibroblasts. *Mech Aging Dev* 26: 341–356.

Orlowski M., Cardozo C., Michaud C. (1993). Evidence for the presence of five distinct proteolytic components in the pituitary multicatalytic proteinase complex: Properties of two components cleaving bonds on the carboxyl side of branched chain and small neutral amino acids. *Biochemistry* 32: 1563–1572.

Palmer J.T., Rasnick D., Klaus J.L., Bromme D. (1995). Vinyl sulfones as mechanism-based cysteine protease inhibitors. *J Med Chem* 38: 3193–3196.

Palombella V.J., Rando O.J., Goldberg A.L., Maniatis T. (1994). The ubiquitin-proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. *Cell* 78: 773–785.

Pacifici R.E., Kono Y., Davies K.J.A. (1993). Hydrophobicity as the signal for selective degradation of hydroxyl radical-modified hemoglobin by the multicatalytic proteinase complex, proteasome. *J Biol Chem* 268: 15405–15411.

Perrin B.J., Huttenlocher A. (2002). Calpain. *Int J Biochem Cell Biol* 34: 722–725.

Peters J.-M., Franke W.W., Kleinschmidt J.A. (1994). Distinct 19S and 20S subcomplexes of the 26 S proteasome and their distribution in the nucleus and the cytoplasm. *J Biol Chem* 269: 7709–7718.

Peters J.-M., Harris J.R., Finley D. (eds.). (1998). *Ubiquitin and the Biology of the Cell*, Plenum Press, New York.

Petropoulos I., Conconi M., Wang X., Hoenel B., Bregegere F., Milner Y., Friguet B. (2000). Increase of oxidatively modified protein is associated with a decrease of proteasomal activity and content in aging epidermal cells. *J Gerontol A Biol Sci Med Sci* 55: 220–227.

Pickart C. (2000). Ubiquitin in chains. *Trends Biochem Sci* 25: 544–548.

Poston J.M. (1988). Detection of oxidized amino acid residues using P-aminobenzoic acid adducts. *Fed Proc* 46: 1979.

Prusiner S.B. (1982). Novel proteinaceous infectious particles cause scrapie. *Science* 216: 136–144.

Rami A. (2003). Ischemic neuronal death in the rat hippocampus: The calpain-calpastatin-caspase hypothesis. *Neurobiol Dis* 13: 75–88.

Rammensee H.G., Falk K., Rotzschke O. (1993). Peptides naturally presented by JHC class I molecules. *Ann Rev Immunol* 11: 213–244.

Rao G., Fisch L., Srinivasan S., D'Amico F., Okada T., Eaton C., Robbins C. (2003). Does this patient have Parkinson's disease? *JAMA* 289: 347–353.

Rechsteiner M. (1998). The 26S proteasome. In Peters J.M., Harris J.R, Finley D. (eds.). *Ubiquitin and the Biology of the Cell*, Plenum Press, New York.

Rechsteiner M., Realini C., Ustrell V. (2000). The proteasome activator 11S REG (PA28) and class I antigen presentation. *Biochem J* 345: 1–15.

Reinheckel T., Sitte., Ullrich O., Kuckelkorn U., Davies K.J., Grune T. (1998). Comparative resistance of the 20S and 26S proteasome to oxidative stress. *Biochem J* 335: 637–642.

Reinheckel T., Ullrich O., Sitte N., Grune T. (2000). Differential impairment of 20S and 26S proteasome activities in human hematopoietic K562 cells during oxidative stress. *Arch Biochem Biophys* 377: 65–68.

Renard C.B., Kramer F., Johansson F., Lamharzi N., Tannock L.R., von Herrath M.G., Chait A., Bornfeldt K.E. (2004). Diabetes and diabetes-associated lipid abnormalities

have distinct effects on initiation and progression of atherosclerotic lesions. *Clin Invest* 114: 659–668.

Requena J.R., Chao C.C., Levine R.L., Stadtman E.R. (2001). Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins. *Proc Natl Acad Sci USA* 98: 69–74.

Requena J.R., Levine R.L., Stadtman E.R. (2003). Recent advances in the analysis of oxidized proteins. *Amino Acids* 25: 221–226.

Requena J.R., Dimitrova M.N., Legname G., Teijeira S., Prusiner S.B., Levine R.L. (2004). Oxidation of methionine residues in the prion protein by hydrogen peroxide. *Arch Biochem Biophys* 432: 188–195.

Rivett A.J. (1985). Preferential degradation of the oxidatively modified form of glutamine synthetase by intracellular mammalian proteases. *J Biol Chem* 260: 300–305.

Rivett A.J. (1998). Intracellular distribution of proteasomes. *Curr Opin Immunol* 10: 110–114.

Rock K.L., Gramm C., Rothstein L., Clark K., Stein R., Dick L., Hwang D., Goldberg A.L. (1994). Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* 78: 761–771.

Ron D., Oyadomari S. (2004). Lipid phase perturbation and the unfolded protein response. *Dev Cell* 7: 287–288.

Rosenblatt A., Margolis R.L., Becher M.W., Aylward E., Franz M.L., Sherr M., Abbott M.H., Lian K.Y., Ross C.A. (1998). Does CAG repeat number predict the rate of pathological changes in Huntington's disease? *Ann Neurol* 44: 708–709.

Ross C.A. (2002). Polyglutamine pathogenesis: Emergence of unifying mechanisms for Huntingtons' disease and related disorders. *Neuron* 35: 819–822.

Rowland L.P. (1994). Amyotrophic lateral sclerosis. *Curr Opin Neurol* 8: 310–315.

Saitoh Y., Yokosawa H., Ishii S. (1989). Sodium Dodecyl sulfate-induced conformational and enzymatic changes of multicatalytic proteinase. *Biochem Biophys Res Commun* 162: 334–339.

Salo D.C., Pacifici R.E., Lin S.W., Giulivi C., Davies K.J. (1990). Superoxide dismutase undergoes proteolysis and fragmentation following oxidative modification and inactivation. *J Biol Chem* 265: 11919–11927.

Salo D.C., Lin S.W., Pacifici R.E., Davies K.J. (1988). Superoxide dismutase is preferentially degraded by a proteolytic system from red blood cells following oxidative modification by hydrogen peroxide. *Free Radic Biol Med* 5: 335–339.

Saudou F., Finkbeiner S., Devys D., Greenberg M.E. (1998). Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. *Cell* 95: 55–66.

Savory P.J., Djaballah H., Angliker H., Shaw E., Rivett A.J. (1993). Reaction of proteasomes with peptidylchloromethanes and peptidyldiazomethanes. *Biochem J* 296: 601–605.

Schapira A.H., Cooper J.M., Dexter D., Clark J.B., Jenner P., Marsden C.D. (1990). Mitochondrial complex I deficiency in Parkinsons's disease. *J Neurochem* 54: 823–827.

Schwerk C., Schulze-Osthoff K. (2003). Non-apoptotic functions of caspases in cellular proliferation and differentiation. *Biochem Pharmacol* 66: 1453–1458.

Seeger M., Ferrell K., Frank R., Dubiel W. (1997). HIV-1 tat inhibits the 20S proteasome and its 22S regulator-mediated activation. *J Biol Chem* 272: 8145–8148.

Seo M.S., Kim J.K., Lim Y., Kang S.W., Cho Y.J., Lee W.K., Kim H.J., Cho K.K., Lee K.H., Rhee S.G. (1999). Rapid degradation of Prxl and PrxII induced by silica in Rat2 cells. *Biochem Biophys Res Commun* 265: 541–544.

Sheaff R.J., Singer J.D., Swanger J., Smitherman M., Roberts J.M., Clurman B.E. (2000). Proteasomal turnover of p21Cip1 does not require p21Cip1 ubiquitination. *Molecular Cell* 5: 403–410.

Shih S.C., Sloper-Mould K.E., Hicke L. (2000). Monoubiquitin carries a novel internalization signal that is appended to activated receptors. *EMBO J* 19: 187–198.

Shringarpure R., Grune T., Mehlhase J., Davies K.J. (2003). Ubiquitin conjugation is not required for the degradation of oxidized proteins by proteasome. *J Biol Chem* 278: 311–318.

Shringarpure R., Grune T., Sitte N., Davies K.J.A. (2000). 4-Hydroxynonenal-modified amyloid-β peptide inhibits the proteasome: Possible importance for Alzheimer's disease. *Cell Mol Life Sci* 57: 1802–1809.

Sitte N., Merker K., Grune T. (1998). Proteasome-dependent degradation of oxidized proteins in MRC-5 fibroblasts. *FEBS Lett* 440: 399–402.

Sitte N., Huber M., Grune T., Ladhoff A., Doecke W.D., Zglinicki T., Davies K.J.A. (2000a). Proteasome inhibition by lipofuscin/ceroid during postmitotic ageing of fibroblasts. *FASEB J* 14: 1490–1498.

Sitte N., Merker K., von Zglinicki T., Davies K.J.A., Grune T. (2000b). Protein oxidation and degradation during cellular senescence of human BJ-fibroblasts. Part I. Effects of proliferative senescence. *FASEB J* 14: 2495–2502.

Sitte N., Merker K., von Zglinicki T., Davies K.J.A., Grune T. (2000c). Protein oxidation and degradation during cellular senescence of human BJ-fibroblasts. Part II. Aging of non-dividing cells. *FASEB J* 14: 2503–2510.

Sitte N., Merker K., Grune T., von Zglinicki T. (2001). Lipofuscin accumulation in proliferating fibroblasts in vitro: An indicator of oxidative stress. *Exp Gerontol* 36: 475–486.

Smit A., Lutgers H.L. (2004). The clinical relevance of advanced glycation endproducts (AGE) and recent developments in pharmaceutics to reduce AGE accumulation. *Curr Med Chem* 11: 2767–2784.

Sommerburg O., Ullrich O., Sitte N., von Zglinicki D., Siems W., Grune T. (1998). Dose- and wavelength-dependent oxidation of crystallins by UV light—Selective recognition and degradation by the 20S proteasome. *Free Radic Biol Med* 24: 1369–1374.

Song X., Mott J.D., von Kampen K., Pramanik B., Tanaka K., Slaughter C.A., DeMartino G.N. (1996). A model for the quaternary structure of the proteasome activator PA28. *J Biol Chem* 271: 26410–26417.

Spaltenstein A., Leban J.J., Huang J.J., Reinhardt K.R., Viveros O.H., Sigafoos J., Crouch R. (1996). Design and synthesis of novel protease inhibitors: Tripeptide α′,β′-epoxyketones as nanomolar inactivators of the proteasome. *Tet Lett* 37: 1343–1346.

Stadtman E.R. (1992). Protein oxidation and aging. *Science* 257: 1220–1224.

Stadtman E.R., Levine R.L. (2003). Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids* 25: 207–218.

Stern I.J., Wiley D.C. (1994). Antigenic peptide binding by class I and class II histocompatibility proteins. *Structure* 2: 245–251.

Stolzing A., Wengner A., Grune T. (2002). Degradation of oxidized extracellular proteins by microglia. *Arch Biochem Biophys* 400: 171–179.

Strickland E., Hakala K., Thomas P.J., DeMartino G.N. (2000). Recognition of misfolding proteins by PA700, the regulatory subcomplex of the 26 S proteasome. *J Biol Chem* 275: 5565–5572.

Strohwasser R., Salzmann U., Ruppert T., Kloetzel P.M., Holzhuetter H.G. (2000). Kinetic evidences for facilitation of peptide channeling by the proteasomal activator PA28. *Eur J Biochem* 267: 6221–6230.

Suriano G., Vrcelj N., Senz J., Ferreira P., Masoudi H., Cox K., Nabais S., Lopes C., Machado J.C., Seruca R., Carneiro F., Huntsman D.G. (2005). beta-Catenin (CTNNB1) gene amplification: A new mechanism of protein overexpression in cancer. *Genes Chromosomes Cancer* 42: 238–246.

Takahashi R., Goto S. (1990). Alteration of aminoacyl-tRNA synthetase with age: Heat-labilization of the enzyme by oxidative damage. *Arch Biochem Biophys* 227: 228–233.

Tamura T., Tamura N., Cejka Z., Hegerl R., Lottspeich F., Baumeister W. (1996). Tricorn protease—The core of a modular proteolytic system. *Science* 274: 1385–1389.

Tanaka K., Kasahara M. (1998). The MHC Class I ligand-generating system: Roles of immunoproteasomes and the interferon-gamma-inducible proteasome activator PA28. *Immunol Rev* 163: 161–176.

Tandan R., Bradley W.G. (1985). Amyotrophic lateral sclerosis. Part 1. Clinical features, pathology, and ethical issues in management. *Ann Neurol* 18: 271–280.

Tarcsa E., Szymanska G., Lecker S., O'Connor C.M., Goldberg A.L. (2000). Ca^{2+}-free calmodulin and calmodulin damaged by in vitro aging are selectively degraded by the 26S proteasome without ubiquitination. *J Biol Chem* 275: 20295–20301.

Taylor J.P., Hardy J., Fischbeck K-H. (2002). Toxic proteins in neurodegenerative diseases. *Science* 296: 1991–1995.

Terman A., Brunk U.T. (1998). Lipofuscin: Mechanisms of formation and increase with age. *APMIS* 106: 265–276.

Tomkinson B. (1999). Tripeptidyl peptidases: Enzymes that count. *Trends Biochem Sci* 24: 355–359.

Tschopp J., Martinon F., Burns K. (2003). NALPs: A novel protein family involved in inflammation. *Nat Rev Mol Cell Biol* 4: 95–104.

Turk B., Turk D., Turk V. (2000). Lysosomal cysteine proteases: More than scavengers. *Biochim Biophys Acta* 1477: 98–111.

Turk V., Turk B., Turk D. (2001). Lysosomal cysteine proteases: Facts and opportunities. *EMBO J* 20: 4629–4633.

Uchida K., Kawakishi S. (1993). 2-oxohistidine as a novel biological marker for oxidatively modified proteins. *FEBS Lett* 332: 208–210.

Uchida K., Stadtman E.R. (1993). Covalent attachment of 4-hydroxynonenal to glyceraldehyde-3-phosphate dehydrogenase. *J Biol Chem* 268: 6388–6393.

Uchida K. (2003). Histidine and lysine as targets of oxidative modification. *Amino Acids* 25: 249–257.

Ullrich O., Grune T. (2001). Proteasomal degradation of oxidatively damaged endogenous histones in K562 human leukemic cells. *Free Radic Biol Med* 31: 887–893.

Ullrich O., Reinheckel T., Sitte N., Grune T. (1999a). Degradation of hypochlorite-damaged glucose-6-phosphate dehydrogenase by the 20S proteasome. *Free Radic Biol Med* 27: 487–492.

Ullrich O., Reinheckel T., Sitte N., Hass R., Grune T., Davies K.J. (1999b). Poly-ADP ribose polymerase activates nuclear proteasome to degrade oxidatively damaged histones. *Proc Natl Acad Sci USA* 96: 6223–6228.

Ullrich O., Sitte N., Sommerburg O., Sandig V., Davies K.J., Grune T. (1999c). Influence of DNA binding on the degradation of oxidized histones by the 20S proteasome. *Arch Biochem Biophys* 362: 211–216.

Ulrich H.D. (2002a). Natural substrates of the proteasome and their recognition by the ubiquitin system. In: Zwickl P., Baumeister W. (eds.), *The Proteasome-Ubiquitin Protein Degradation Pathway*, Springer-Verlag, Berlin, pp. 137–174.

Ulrich H.D. (2002b). Natural substrates of the proteasome and their recognition by the ubiquitin system. *Curr Top Microbiol Immunol* 268: 137–174.

Uversky V.N., Fink A.L. (2002). Amino acid determinants of alspha-synuclein aggregation: Putting together pieces of the puzzle. *FEBS Lett* 522: 9–13.

van der Vliet A., Ca O.N., Halliwell B., Cross C.E., Kaur H. (1994). Aromatic hydroxylation and nitration of phenylalanine and tyrosine by peroxynitrite: Evidence for hydroxyl radical production form peroxynitrite. *FEBS Lett* 399: 89–92.

Verzyl N., de Groot J., Oldehinken E., Bank R.A., Thorpe S.R., Baynes J.W., Bayliss M.T., Bijlsma W.J., Lafeber F.P.J.G., Tekoppele J.M. (2000). Age accumulation of Maillard products in human articular cartilage collagen. *Biochem J* 350: 381–387.

Voges D., Zwickl P., Baumeister W. (1999). The 26S proteasome: A molecular machine designed for controlled proteolysis. *Annu Rev Biochem* 68: 1015–1068.

Volker C., Lupas A.N. (2002). Molecular evolution of proteasomes. In: Zwickl P., Baumeister W. (eds.), *The Proteasome-Ubiquitin Protein Degradation Pathway*, Springer-Verlag, Berlin, pp. 1–22.

Wagner B.J., Margolis J.W. (1995). Age-dependent association of isolated bovine lens multicatalytic proteinase complex (proteasome) with heat-shock protein 90, an endogenous inhibitor. *Arch Biochem Biophys* 323: 455–462.

Walz J., Tamura T., Tamura N., Grimm R., Baumeister W., Kloster A.J. (1997). Tricorn protease exists as an icosahedral supermolecule in vivo. *Mol Cell* 1: 59–65.

Wang K.K. (2000). Calpain and caspase: Can you tell the difference? *Trends Neurosci* 23: 20–26.

Wang N., Gottesman S., Willingham M.C., Gottesman M.M., Maurize M.R. (1993). A human mitochondrial ATP-dependent protease that is highly homologous to bacterial Lon protease. *Proc Natl Acad Sci USA* 90: 11247–11251.

Wang Y.C., Lin R.K., Tan Y.H., Chen J.T., Chen C.Y., Wang Y.C. (2005). Wild-type p53 overexpression and its correlation with MDM2 and p14ARF alterations: An alternative pathway to Non-small-cell lung cancer. *J Clin Oncol* 23: 154–164.

Watabe S., Kimura T. (1985). ATP-dependent protease in bovine adrenal cortex: Tissue specificity, subcellular localization and partial characterisation. *J Biol Chem* 260: 5511–5517.

Wells-Knecht K.J., Zyzak D.V., Lichtfield J.E., Thorpe S.R., Baynes J.W. (1995). Mechanism of autooxidative glycosylation: Identification of glyoxal and arabinose as intermediates in the autooxidative modification of proteins by glucose. *Biochemistry* 34: 3702–3709.

Wenzel T., Baumeister W. (1995). Conformational constraints in protein degradation by the 20S proteasome. *Nat Struct Biol* 2: 199–204.

Whitby F.G., Masters E.I., Kramer L., Knowlton J.R., Yao Y., Wang C.C., Hill C.P. (2000). Structural basis for the activation of 20S proteasomes by 11S regulators. *Nature* 408: 115–120.

Willner C. (2004). An overview of the pathophysiology of neurodegenerative disorders. *Altern Ther Health Med* 10: 26–34.

Woo M., Hakem R., Furlinger C., Hakem A., Duncan G.S., Sasaki T., Bouchard D., Lu L., Wu G.E., Paige C.J., Mak T.W. (2003). Caspase-3 regulates cell cycle in B cells: A consequence of substrate specificity. *Nat Immunol* 4: 1016–1022.

Yi D., Smythe G.A., Blount B.C., Ducan M.W. (1997). Peroxynitrite-mediated nitration of peptides: characterisation of the products by electrospray and combined gas chromatography-mass spectrometry. *Arch Biochem Biophys* 344: 253–259.

York I.A., Goldberg A.L., Mo X.Y., Rock K.L. (1999). Proteolysis and class I major histocompatibility complex antigen presentation. *Immunol Rev* 172: 49–66.

Zhang Z., Kruchinsky A., Endicott S., Realini C., Rechsteiner M., Standing K.G. (1999). Proteasome activator 11S REG or PA28. Recombinant REGα/REGβ hetero-oligomers are heptamers. *Biochemistry* 38: 5651–5658.

Zhou J.Q., Gafni A. (1991). Exposure of rat muscle phosphoglycerate kinase ton nonenzymatic MFO system generates the old form of the enzyme. *J Gerontol* 46: B217–B221.

Zoghbi H.Y., Orr H.T. (2000). Glutamine repeats and neurodegeneration. *Annu Rev Neurosci* 23: 217–247.

Zwickl P., Baumeister W. (eds.). (2002). *The Proteasome-Ubiquitin Protein Degradation Pathway*. Springer-Verlag, Berlin.

18

REDOX PROTEOMICS: A NEW APPROACH TO INVESTIGATE OXIDATIVE STRESS IN ALZHEIMER'S DISEASE

D. Allan Butterfield, Rukhsana Sultana, and H. Fai Poon

18.1 INTRODUCTION

Alzheimer's disease (AD) is an age-related neurodegenerative disease characterized by learning and memory deficits (Katzman and Saitoh, 1991). Currently AD affects about 2% of the US population (Katzman, 2000). Since the risk of AD dramatically increases beyond the age of 70, it is estimated that 22 million people worldwide will be affected by AD in the near future and the incidence of AD will increase threefold within the next 50 years (Mattson, 2004). Pathological hallmarks of AD include amyloid β (Aβ), extracellular senile plaques (SP), intracellular neurofibrillary tangles (NFT), and excessive synapse loss. In addition to pathological alterations, increased oxidative stress is also observed in AD brains. Oxidative stress in AD brains mediates protein oxidation, lipid peroxidation, and DNA oxidation, which are manifested by increased protein carbonyls, 4-hydroxyl-2-nonenal (HNE), and 8-hydroxyl-2-deoxyguanine, respectively (recently reviewed in Butterfield et al., 2001, 2002a; Butterfield and Lauderback, 2002).

Since Aβ(1−42) and oxidative stress appear to play significant roles in the pathogenesis of AD, our laboratory combined these two notions and proposed a comprehensive, Aβ(1−42)-centered model for neurodegeneration in AD brain (Butterfield et al., 2001, 2002a, b; Butterfield and Kanski, 2002; Butterfield and

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

Lauderback, 2002). In support of this model, it was demonstrated that Aβ(1–42) can induce oxidative damage to cells through its ability to produce free radicals (Butterfield et al., 1994, 2001, 2002a; Varadarajan et al., 2000; Butterfield and Kanski, 2002; Butterfield and Lauderback, 2002). Moreover Aβ(1–42) can mediate oxidative stress by the production of $O_2^{\bullet -}$ through the stimulation of NADPH oxidase (Hurst and Barrette, 1989), the production of H_2O_2 through copper or iron reduction, and nitric oxide (NO) production in macrophages in a microglial cell line (Meda et al., 1995). Also accumulation of Aβ(1–42) can induce neurotoxicity by binding to the nicotinic acetylcholine receptor (Wang et al., 2000), forming calcium and potassium channels in cell membranes (Arispe et al., 1993; Etcheberrigaray et al., 1994; Engstrom et al., 1995), decreasing glucose transport across brain endothelial cells (Blanc et al., 1997), and actuating the release of chemokines (Fiala et al., 1998) and cytokines (Akama and Van Eldik, 2000).

Protein oxidation is essential to the pathology of AD, since protein oxidation occurs in Aβ(1–42)-rich brain regions but not in the Aβ(1–42)-poor cerebellum, indicating correlation between protein oxidation and markers of AD histopathology (Hensley et al., 1995b). The backbone and side chain of proteins are targeted by free radicals for oxidative modification (Butterfield et al., 1997a; Poon et al., 2004a). Backbone protein oxidation causes protein cross-linking and/or peptide bond cleavage and decreases the availability of functional proteins available (Butterfield and Stadtman, 1997). Side chain oxidation of proteins alters their chemical structures (Subramaniam et al., 1997), thereby affecting the chemical properties and activity of proteins (Butterfield et al., 1997a; Poon et al., 2004a). Therefore both backbone and side chain protein oxidation probably impair the function of the proteins. Although increased protein oxidation in AD brains is well established, many enzymes preserve their activity in AD brains, suggesting that only particular proteins are oxidatively modified in AD (Stadtman, 1992, 2001; Agarwal and Sohal, 1994; Agrawal et al., 1996; Castegna et al., 2002a,b, 2003; Keller et al., 2004; Poon et al., 2004c). Although brain proteins are oxidized in non-AD subjects, there is an increase in the amount of proteins in the AD brain (Hensley et al., 1995b).

Although most protein oxidative modifications are irreversible (lysine and arginine carbonylation, tyrosine nitration, tryptophan nitration, dityrosine formation, protein–protein cross-linking), some oxidative modifications are reversible (glutathionylation, *S*-nitrosation). These reduced forms of oxidized proteins together with those non-oxidized proteins are considered in their reduced states, in contrast to the oxidatively modified proteins in their oxidized states. This redox state of the proteins contributes to the redox regulation of the cellular responses to oxidative stress and antioxidant status in brains (recently reviewed in Calabrese et al., 2003; Poon et al., 2004b). Initial attempts to address the redox states of these specific oxidized protein was by double immunoprecipitation, whereby oxidized proteins are first immunoprecipitated with an antibody and then, by a similar immunoprecipitation step, with the antibody used against the protein of interest

(Lauderback et al., 2001). Although this one-at-a-time method is commonly used and accepted, it is impractical and infeasible for the brain proteome.

Recent advances in proteomics allow the identification of a large number of proteins as well as their redox states, hence referred to as redox proteomics (Castegna et al., 2002a, 2002b; Ghezzi and Bonetto, 2003; Dalle-Donne et al., 2005). Our laboratory was the first to use redox proteomics to identify the oxidatively modified proteins in AD brain and models of AD in order to gain insight to the mechanisms of specific protein oxidation in AD and their roles in neurodegeneration, as discussed below. Other laboratories also used such techniques to identify oxidatively modified proteins in plasma as potential biomarkers (Choi et al., 2002; Yu et al., 2003). Therefore this review will primarily focus on the findings of the redox proteomics study in AD and its models as well as the implications of these findings.

18.2 BRAIN TISSUE AND MODELS USED IN STUDYING Aβ(1–42)-INDUCED OXIDATIVE STRESS AND NEUROTOXICITY IN AD

18.2.1 AD Brain Tissue

The sampling of AD brain tissue is described in detail elsewhere (Castegna et al., 2002a,b, 2003; Butterfield, 2004). Briefly, brain tissue samples (inferior parietal lobule and hippocampus) used for analysis were taken at autopsy from AD and control subjects in the Rapid Autopsy Program of the University of Kentucky Alzheimer's Diseases Research Center (UKADRC). No tissue was used with longer than a 4 hour postmortem interval. All AD subjects displayed progressive intellectual decline, met NINCDS ADRDA workgroup criteria for clinical diagnosis of probable AD (McKhann et al., 1984), and met accepted guidelines for the histopathological diagnosis of AD (1998). All control subjects were part of the UK ADRC normal volunteer study and whose neuropsychological test scores were within the normal range. Neuropathological evaluation of control brains revealed only age-associated gross histopathological alterations.

18.2.2 Senescence-Accelerated Mice Prone 8 (SAMP8)

The SAMP8 mouse strain undergoes a natural mutation that results in age-dependent learning and memory deficits (Flood and Morley, 1993). These SAMP8 mice produce increased amounts of Aβ and Aβ-like protein immunoreactive granular structures in their brains similar to the moieties observed in AD (Takemura et al., 1993; Morley et al., 2000). Unlike transgenic mice that have 5 to 14 times the normal amount of Aβ increased in their brains as function of age, the Aβ level of SAMP8 mice increases only 100% from 4 to 12 months (Kumar et al., 2000), an increase that is closer to the estimated 50% increase in Aβ seen in AD (Rosenberg, 2000). Moreover, brains of SAMP8 mice

show axonal dystrophy in dorsal column nuclei, small neurons in the gracile nucleus, and some well-defined or swollen axons (Kawamata et al., 1998). Age-related shrinkage of the cholinergic neurons of the laterodorsal tegmental nucleus are observed in aged SAMP8 mice brains (Kawamata et al., 1998). Therefore, SAMP8 mice serve as a useful model in the study of age-related cognitive impairment, such as AD.

18.2.3 Aβ(1–42) In vivo

In order to mimic the effect of excess accumulation of Aβ(1–42) in AD brains, we injected Aβ(1–42) into the nucleus basalis Magnocellaris (nbM) of three-month old male Wistar rats to compare the specific protein oxidation of these subjects to saline-injected control after a seven day postinjection peroid. nbM is a major source of cholinergic innervation to the cerebral cortex (Ezrin-Waters and Resch, 1986; Detari et al., 1999). Lesions of nbM reduce acetylcholinesterase (AchE)-positive fibers in the neocortex (Wellman and Sengelaub, 1991) and impair learning and memory functions (Baxter et al., 1995; Abe et al., 1998; Baxter, 2001). A similar correlation has been observed in patients with AD (Ezrin-Waters and Resch, 1986; Muir, 1997).

18.2.4 Aβ(1–42) In vitro

Synaptosomes Synaptic alteration is an early event in the pathogenesis of AD (Crystal et al., 1988; Hamos et al., 1989; Mattson et al., 1998). In particular, synaptic loss in the hippocampal dentate gyrus disrupts the communication between the hippocampus and the entorhinal cortex leading to the memory deficits associated with AD (Masliah et al., 1994). Moreover, the severity of damage in these regions corresponds with the extent of dementia, oxidative stress, and deposition of Aβ(1–42) (DeKosky and Scheff, 1990; Terry et al., 1991; Hensley et al., 1995b). Therefore, inducing protein oxidation in synaptosomes by adding Aβ(1–42) to isolated gerbil synaptosomes served as a good model for the mechanisms of Aβ(1–42) induced synaptic alterations found in AD brain (Boyd-Kimball et al., 2005a).

Primary Neuronal Culture Protein oxidation, indexed by protein carbonyls, is a toxic intermediate, and leads to conformational changes in proteins, and consequently loss of protein function (Subramaniam et al., 1997; Hensley et al., 1995b). Such oxidative inactivation was observed in creatine kinase and glutamine synthetase (Hensley et al., 1995b; Yatin et al., 1999b; Aksenov et al., 2000; Castegna et al., 2002a). In order to identify the targets of Aβ(1–42)-induced protein oxidation, thereby gaining insight into the Aβ(1–42) mediate toxicity, we used redox proteomic techniques to identify proteins in E18 fetal rat neurons that are significantly oxidized by incubating 10 μM Aβ(1–42) with these neuronal cultures for 24 hours (Boyd-Kimball et al., 2005d).

18.2.5 *Caenorhabditis elegans (C. elegans)*

Transgenic *C. elegans* that express human Aβ(1–42) through a body-wall muscle myosin promoter and an Aβ minigene (Link, 1995) have been used as an in vivo model to study Aβ toxicity and deposition (Yatin et al., 1999b; Link et al., 2001). The temperature-inducible Aβ expression system in the *C. elegans* is a good model to investigate the relationship between Aβ toxicity, fibril formation, and oxidative stress. *C. elegans* expressing human Aβ(1–42) showed that increased protein oxidation preceded fibrillar deposition of the peptide, suggesting that small soluble aggregates of the peptide are the toxic species of this peptide (Drake et al., 2003).

18.3 REDOX PROTEOMICS

18.3.1 Sample Preparation

The samples described above, in which protein oxidation is found, are first derivatized by dinitrophenylhydrazine (DNPH) to form DNP-adduction for carbonyl detection. If 3-nitrotyrosine levels are measured, no derivatization step is needed. The samples after derivatization usually contain a high concentration of ions, since an acidic buffer is used to optimize the reaction. The high level of ions in the buffer can cause variation of voltage and current during isoelectric focusing (IEF), thereby preventing successful isoelectric separation. This phenomenon usually manifested by horizontal smearing of the protein spot (Fig. 18.1). This horizontal smearing problem is usually avoided by using trichloroacetic acid (TCA) to precipitate the protein and using organic solvents to wash the pellet substantially. Commercial spin ion-removal columns (Sigma and Pierce) are available as well to remove ion from samples prior to IEF.

18.3.2 Two-dimensional (2D) Gel Electrophoresis

Two-dimensional gel electrophoresis can separate a mixture of proteins into single detectable protein spots in most cases. The 2D separation of proteins is usually achieved by two separation steps. In the first step, proteins are separated according to their isoelectric point by IEF on an IEF gel, or strip. The resulting IEF strips are then treated with dithiothreitol and iodacetamide to avoid cysteine–cysteine interaction, which will decrease the resolution of the second step of separation. In the second step, proteins are separated according to their molecular migration rate (similar to molecular weight in most cases). If cross-linking between the cysteine residues occurs, vertical smearing will appear in the 2D gel because the molecular migration rate is shape-dependent as well as molecular weight dependent (Fig. 18.2). The resulting 2D map allows comparison within and among groups of samples for statistical analysis (Tilleman et al., 2002). The ability to compare and match between different samples on 2D electrophoresis is especially important in redox proteomics. This is because in order to identify the oxidation

FIGURE 18.1 (*Bottom*) Horizontal smearing due to excessive ions during isoelectric focusing. (*Top*) Spots without horizontal smearing when ions are completely removed prior to IEF.

state of a protein, the 2D gels are transferred and developed by Western blotting. The 2D map of the gels and the blots can then be matched to determine the unit of oxidative modification per unit of proteins (specific carbonyl level or specific 3-NT level of a protein). The advantages of the 2D gel electrophoresis are its reproducibility and high resolution. However, some drawbacks to this technique are present. The solubilization of membrane proteins is still the main obstacle for 2D electrophoresis, since the ionic detergents used for solubilization of the membrane proteins can interfere with the focusing process. Additionally, there exist the technical limitations to the mass range and the detection capabilities of the method. However, our laboratory and many others are working to overcome these issues by using chaotropic agents, subcellular 2D gel electrophoresis, and so forth. High-throughput proteomic techniques, such as HPLC, have also been used to separate proteins without 2D electrophoresis (Soreghan et al., 2003). However,

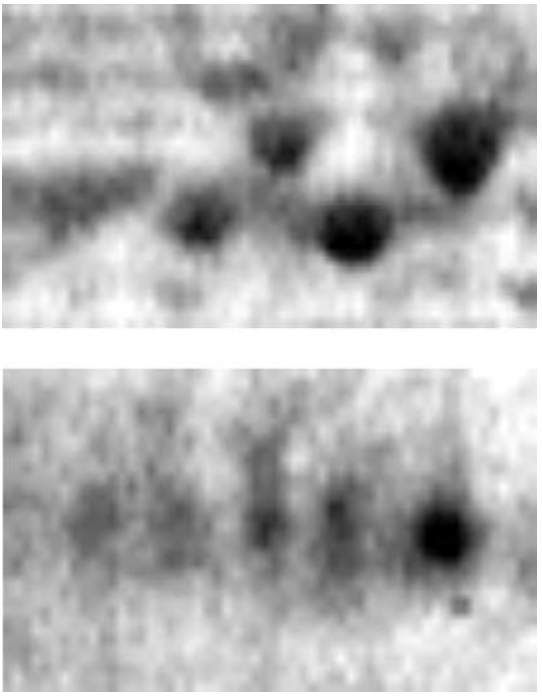


FIGURE 18.2 (*Bottom*) Vertical smearing due to excessive ions during isoelectric focusing. (*Top*) Spots without vertical smearing when the IEF strips are properly treated with dithiothreitol and iodacetamide.

the application of these techniques in redox proteomics is still at an early stage. More development in all these techniques to obtain quantitative data on the redox state of a protein may supersede the limitations of 2D electrophoresis.

18.3.3 Image Analysis and Statistical Analysis

The 2D gels traditionally were visualized by classical detection methods, including Coomassie blue and silver staining. Nevertheless, these detection methods have been problematic because of their low sensitivity (for Coomassie) or poor reproducibility and dynamic range (for silver). The recent development of fluorescent dyes, namely SYPRO™ Ruby, has overcome these problems, since the sensitive (1–2 ng) detection limits and linear dynamic range of these dyes are over three orders of magnitude (Molloy and Witzmann, 2002). The resulting 2D map images are analyzed by specially programmed software.

Image analysis allows gel-to-gel comparison and generates a large amount of data, which are accumulated from multiple 2D gels. The specialized softwares to manage these data are commercially available. Some of these software programs were evaluated in a recent survey (Righetti et al., 2004). They proved to enable

investigative matching and analysis of visualized protein spots among different gels and blots. The principles behind measuring intensity values by 2D analysis software are similar to those of densitometric measurement. After completion of spot matching, the normalized intensity of each protein spot from individual gels (or blots) is compared between groups using statistical analysis. The available software is equipped with raw-image-based alignment coupled with matching of neighboring spots, which has improved the spot-matching steps and significantly reduced analysis time. Nevertheless, hands-on processing is still necessary to ensure accuracy in spot matching between gels or blots.

18.3.4 In-gel Digestion and Mass Spectrometry

The protein of interest is excised and treated with ammonium bicarbonate and acetonitrile. After reduction/alkylation by dithiothreitol and iodacetamide, the excised spots are then digested in-gel with a protease (trypsin is commonly used) in an optimal buffer for its activity. The digested peptides are easily eluted from the gel to undergo mass spectrometry analysis. In-gel digestion not only reduces the mass of a protein into small peptides ideal for mass spectrometry, it also forms a collection of protease sequence-specific peptides that enables the identification of the protein (Fig. 18.3) (Jensen et al., 1999).

Mass spectrometry has made a great impact on proteomics by facilitating the rapid analysis of proteins, and coupled to bioinformatics approaches, this has led to the identification and characterization of thousands of proteins (Stuhler and Meyer, 2004). These advancements have resulted from the improvement of mass spectrometry by way of two ionization techniques whose developers were recent recipients of the Nobel Prize. In the past, the traditional modes of ionization fragmentation of peptides failed to provide accurate peptide mass. With the development of MALDI (matrix-assisted laser desorption/ionization) and ESI (electrospray ionization), large biological macromolecules can now be ionized without fragmentation, thus enabling the identification of proteins. In MALDI the peptide samples are mixed to an acidic matrix and dried on a plate that is subjected to laser radiation. The peptides are incorporated into the crystal lattice of the matrix during the drying process. Various compounds are used as the matrices for laser absorption. One of the most commonly used matrices for peptides is α-cyano-4-hydroxycinnamic acid because it provides high sensitivity and negligible matrix adduction during the laser beam's absorption (Beavis and Chait, 1989). When the high energy of the laser is applied to the matrix, the peptides along with the matrix particles are vaporized, and in a process that is not well understood, proton transfer occurs from the matrix to the peptide (Butterfield et al., 2003). In order to ensure sublimation of the matrix–peptides solid, the process is generally conducted in high vacuum. The positive ions of the peptides are formed in the gas phase because of the acidic nature of the matrix. In ESI, the peptide in the solution is sprayed through an outlet with a high potential difference that causes the liquid to disperse into fine droplets. The solvent of these droplets continuously evaporates until droplet fission occurs by the repulsion force of the charges on

1 MQIFVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ QRLIFAGKQL
51 EDGRTLSDYN IQKESTLHLV LRLRGG

Digested by trypsine

Start Postion		End Position	Corresponded Sequence	Molecular Weight
1	-	6	MQIFVK	764.43
7	-	11	TLTGK	518.31
12	-	27	TITLEVEPSDTIENVK	1786.92
28	-	29	AK	217.14
30	-	33	IQDK	502.28
34	-	42	EGIPPDQQR	1038.51
43	-	48	LIFAGK	647.4
49	-	54	QLEDGR	716.35
55	-	63	TLSDYNIQK	1080.55
64	-	72	ESTLHLVLR	1066.61
73	-	74	LR	287.2
75	-	76	GG	132.05

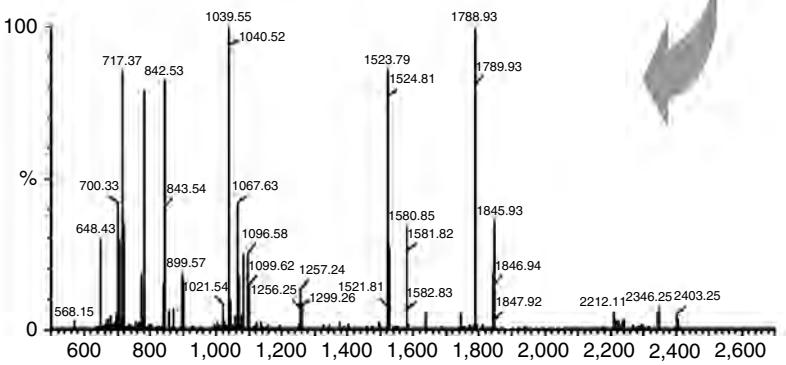


FIGURE 18.3 Digested ubiquitin into different peptides with corresponding masses. An example of the ubiquitin mass spectrum is shown at the bottom of the figure.

the small droplet surface. Eventually a single peptide ion forms for analysis by the mass spectrometer. Since both ionization techniques are very sensitive to charges, a low concentration of salt is required. The peaks of the resulting mass spectrum represent the peptide ions' mass. Other mass spectrometry methods used for redox proteomics have also been recently reviewed (Butterfield et al., 2003; Butterfield and Castegna, 2003; Butterfield, 2004).

18.3.5 Bioinformatics and Identification

Since the peaks of the resulting mass spectrum represent the peptide ions mass of the sample of interest, the peaks should be correlated to the mass of the peptides produced by the protease from an intact protein (see Fig. 18.3). Databases are available for theoretical digests of all known proteins, so matching the peptide mass data obtained from samples of interest to this theoretical digested protein database can help one successfully identify the proteins. This process, known as peptide mass fingerprinting, accounts for several factors involved in the identification of a protein, such as molecular weight, pI, and the probability of a single peptide appearing in the entire database. Many search engines can perform the matching process for each theoretical digested protein and indicate the certainty of the identification with an output of a probability score. The threshold score, which indicates if the experimental mass spectrum significantly matches the theoretical digested protein spectrum, is calculated by mathematical algorithms specific to each search engine and each experimental mass spectrum. Although false identification is conceivable, it can be avoided by taking into account the molecular weight and pI range on the 2D map in the identification. More sophisticated approaches to confirm the protein identity involve use of different proteases or different modes of mass spectrometry, such as tandem mass spectrometry or postsource decay. Moreover prior results suggest that the accuracy of protein identification by mass spectrometry is equivalent to immunochemical identification (Castegna et al., 2002a). However, immunochemical validation of protein identity is often performed in proteomics studies from our laboratory (Castegna et al., 2002a; Poon et al., 2005). The nonspecific effects of the secondary antibody used are negligible (Perluigi et al., 2005).

18.4 OXIDATIVELY MODIFIED PROTEINS IN AD AND AD MODELS BY REDOX PROTEOMICS

A summary of the oxidatively modified proteins in AD and AD models is listed in Table 18.1. These proteins will be discussed in reference to each system.

18.4.1 Oxidatively Modified Proteins in AD Brain Tissue

Creatine kinase (CK), α-enolase (ENO1), triosephosphate isomerase (TPI), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate mutase

TABLE 18.1 Oxidatively Modified Proteins in AD Brain and Different AD Models

Oxidatively modified proteins in AD brain tissue[a]
Creatine kinase, CK
α-Enolase, ENO1
Triosephosphate isomerase, TPI
α-ATPase, ATPase
Glyceraldehydes-3-phosphate dehydrogenase, GADP
Phosphoglycerate mutase 1, PGM 1
Glutamine synthase, GS
Heat-shock cognate, HSC 70
γ-Synaptosomal protein like soluble *N*-ethylmaleimidesensitive factor (NSF)
Attachment proteins, γ-SNAP
Ubiquitin hydrolase L1, UCH L1
Neuropolypeptide h3, NPH 3
β-Actin, ACT
Dihydropyrimidinase-related protein 2, DRP 2
Peptidyl-prolyl isomerase, PIN 1
Carbonic anhydrase 2, CA 2
Oxidatively modified proteins in aged senescence-accelerated mice prone 8 (SAMP8)[b]
α-Enolase, ENO1
Lactate dehydrogenase 2, LDH 2
Creatine kinase, CK
Dihydropyrimidinase-related protein 2, DRP 2
α-Spectrin, SPEC
Oxidatively modified proteins by following intracerebral injection Aβ(1–42) in vivo[c]
Phosphoglycerate mutase 1, PGM 1
14-3-3 Zeta, 14-3-3z
β-synuclein, SYN
Heat-shock protein 60, HSP60
Oxidatively modified proteins induced by Aβ(1–42) in synapotosomes in vitro[d]
β-Actin, ACT
Glial fibrillary acid protein, GFAP
ATP synthase, ATPS
Syntaxin binding protein 1, SNBP1
Glutamate dehydrogenase, GDH
Dihydropyrimidinase-related protein 2, DRP 2
Mitochondrial elongation factor—Tu, EF-Tu
Oxidatively modified proteins induced by Aβ(1–42) in primary neuronal culture in vitro[e]
14-3-3 Zeta, 14-3-3z

(*continued overleaf*)

TABLE 18.1 (*continued*)

Glyceraldehyde-3-phosphate dehydrogenase, GADP
Oxidatively modified proteins in Caenorhabditis elegans (C. elegans) that express human Aβ(1–42)[f]
Acyl-CoA dehydrogenase, ACD
Glutathione *S*-transferases, GST
Malate dehydrogenase, MDH
Arginine kinase, ARK
Guanine nucleotide-binding proteins, G protein
Adenosine kinase, AK
Lipid binding protein 6, LBP-6
Transketolase, TKL
α-Proteosome, α-PRTM
β-Proteosome, β-PRTM

[a] Increased oxidative modification of these proteins is reported in (Castegna et al., 2002a,b, 2003; Sultana et al., 2005a,b,c).
[b] Increased oxidative modification of these proteins is reported in (Poon et al., 2004c).
[c] Increased oxidative modification of these proteins is reported in (Boyd-Kimball et al., 2005c).
[d] Increased oxidative modification of these proteins is reported in (Boyd-Kimball et al., 2005a).
[e] Increased oxidative modification of these proteins is reported in (Boyd-Kimball et al., 2005d).
[f] Increased oxidative modification of these proteins is reported in (Boyd-Kimball et al., 2005b).

1 (PGM1), and α-ATPase are metabolic enzymes involved in production of ATP in brains. Oxidative inactivation of CK suggests the impairment of ATP synthesis in AD brain (Aksenova et al., 1999; Castegna et al., 2002a). CK activity is diminished in AD brain (Hensley et al., 1995b). Therefore, oxidative modification of glycolytic enzymes likely leads to their inactivation. Since glycolysis is the main source of ATP production in brain, impairment of glycolysis may lead to shortage of ATP in brain, and thus to cellular dysfunction. Moreover, such ATP shortage can induce hypothermia, causing abnormal tau phosphorylation through differential inhibition of kinase and phosphatase (Planel et al., 2004).

It is well documented that glutamine synthase (GS) activity declines in AD (Hensley et al., 1995b; Aksenov et al., 1996; Howard et al., 1996; Butterfield et al., 1997a). Since GS is particularly sensitive to inactivation by oxidant agents (Levine, 1983; Rivett and Levine, 1990; Fisher and Stadtman, 1992; Butterfield et al., 1997a), the activity decline is likely caused by the alteration of the structure of GS induced by the oxidative modification of the enzyme (Butterfield et al., 1997a, 1999; Butterfield and Stadtman, 1997; Castegna et al., 2002a). GS catalyzes the rapid amination of glutamate to form the nonneurotoxic amino acid, glutamine. This reaction maintains the optimal level of glutamate and ammonia in neurons and modulates excitotoxicity. Oxidative modification of GS suggests the glutamate–glutamine cycle in AD brains is impaired. Impairment of this important cycle may contribute to the glutamate dysregulation in AD brains (Lee et al., 2002).

Age-related oxidative stress induces heat-shock proteins (HSP) in brains. HSP are molecular chaperones that mediate folding and assembly of other proteins (Poon et al., 2004b). HSP-70 protects neurons against apoptosis by inhibiting caspase cascade activation (Mosser et al., 1997). HSC-70, the constitutive isoforms of HSP-70, is recruited by the cell as a primary defense against stress conditions. HSC-70 is involved in the degradation of misfolded proteins by binding to a particular peptide region and labeling it for proteolysis (Kouchi et al., 1999). Therefore, it was suggested that HSC-70 may be involved in the structural maintenance of proteins by coupling with the proteasome (Kouchi et al., 1999). The decline in activity of HSC-70 was suggested to be compensated by increased expression (Cuervo and Dice, 2000). This decreased activity is believed to be brought about by oxidative modification of HSC-70 (Castegna et al., 2002b). Consistent with this notion, HSC-70 was oxidatively modified in AD, indicating that oxidative inactivation of HSC-70 may cause the impaired protein degradation and aggregation observed in AD brains (Beyreuther et al., 1991).

The γ-synaptosomal protein like the soluble *N*-ethylmaleimidesensitive factor (NSF) attachment proteins (γ-SNAP) is a member of SNAPs, which play an important role in vesicular transport for neurotransmitter release, hormone secretion, and mitochondrial integrity. It is believed that oxidation of γ-SNAP contributes to learning and memory impairment in AD by altering neurotransmitter systems in brain, thereby leading to loss of synaptic circuitry (Masliah et al., 1994; Scheff and Price, 2003). The function of SNAPs is altered in AD brain (Beckers et al., 1989; Stenbeck, 1998). Consistent with this notion, we have shown the oxidative modification of γ-SNAP is significantly increased in AD brain (Sultana et al., 2005a).

Ubiquitin carboxyl terminal hydrolase L1 (UCH-L1) is an enzyme that removes ubiquitin from proteins under degradation to maintain the level of ubiquitin in the cell. UCH-L1 was found to be oxidized in AD brain (Castegna et al., 2002a). An in vitro study showed that HNE decreases the activity of recombinant UCH-L1 (Masliah et al., 1996), suggesting that oxidative modification of UCH-L1 inactivates its hydrolase activity. Therefore oxidative modification of UCH-L1 depletes the availability of free ubiquitin, consequently impairing protein degradation in cells and potentially forming protein aggregates in AD brains. Such accumulation of the damaged protein may cause synaptic deterioration and degeneration in AD brains. Indeed, loss of activity of UCH-L1 in AD brain is consistent with the observed increased protein ubiquitinylation, decreased proteasome activity, and accumulation of damaged proteins in AD brains (Butterfield, 2004). Moreover, altered UCH-L1 can itself lead to brain protein oxidation (Castegna et al., 2004b).

Neuropolypeptide h3 (NPH3) plays an important role in the structure and function of membranes by maintaining phospholipid asymmetry, a process that is important to mitochondrial and plasma membranes (Daleke and Lyles, 2000). Aβ(1–42) or HNE, which is formed by Aβ(1–42), leads to loss of synaptosomal membrane lipid bilayer asymmetry (Castegna et al., 2004a; Mohmmad Abdul et al., 2004), consistent with the notion that Aβ(1–42) contributes to

the oxidative modification of UCH-L1. Inhibition of NPH3 leads to apoptosis and consequently cell death. Moreover NPH3 upregulates the levels of choline acetyltransferase, a deficient enzyme in AD brain (Davies, 1999), suggesting the importance of NPH3 in the development of AD. Therefore oxidative modification of NPH3 possibly leads to functional abnormalities, causing cholinergic impairment, impaired mitochondria function, and apoptosis in AD.

β-Actin (ACT) and dihydropyrimidinase-related protein 2 (DRP2) are critical to neuroplasticity for memory consolidation (Lamprecht and LeDoux, 2004). The decreased protein level and increased oxidative modification of these two proteins in AD brain (Lubec et al., 1999; Castegna et al., 2002b, 2003) correspond to the oxidative modifications of proteins in AD pathology. Moreover, loss of actin could explain the loss of membrane integrity and activation of cellular events that may lead to apoptosis. DRP2 appears to maintain interneuronal communication and repair. It also interacts with collapsin to regulate dendritic elongation in brain. Oxidative inactivation of DRP2 is consistent with the shortened dendritic lengths observed in AD (Coleman and Flood, 1987). Taken together, oxidation of these proteins not only damages cellular functions but also affects neuronal plasticity, thereby impairing the memory consolidation in AD (Coleman and Flood, 1987).

Peptidyl-prolyl isomerase (PIN) is a chaperone enzyme that reversibly alters the conformation of proteins from *cis* to *trans* between a given amino acid and a proline (Schutkowski et al., 1998). Peptidyl-prolyl isomerase 1 (PIN1) recognizes phosphorylated Ser-Pro and phosphorylated Thr-Pro motifs in proteins, and thereby binds to many cell cycle regulating proteins and phosphatases that regulate phosphorylation of tau protein. PIN1 is colocalized with phosphorylated tau and also shows an inverse relationship to the expression of tau in AD brain. Studies suggest that PIN1 can reduce the production of hyperphosphorylated tau (Holzer et al., 2002; Kurt et al., 2003; Ramakrishnan et al., 2003). PIN1 was found to be oxidized in AD brain, causing structural modifications and thereby affecting the properties of its targeted proteins, such as tau. Consistent with this notion, PIN1 could restore the function of tau protein in AD (Lu et al., 1999), suggesting oxidative alteration of PIN 1 could be one of the initial events that trigger tangle formation and oxidative damage in AD brain.

Carbonic anhydrase 2 (CA 2) regulates cellular pH, CO_2, and HCO_3^- transport, and maintains H_2O and electrolyte balance (Sly and Hu, 1995) by reversible hydration of CO_2. CA 2 deficiency leads to cognitive defects, varying from disabilities to severe mental retardation, suggesting the importance of CA 2 in cognitive functions. Moreover oxidative modification of CA 2 leads to its inactivation (Poon et al., 2005b). Therefore, diminished enzyme activity observed in AD brain is likely caused by the oxidative modification of the enzyme (Meier-Ruge et al., 1984). Further, oxidized CA 2 may not be able to balance both the extracellular and intracellular pH in brain. Since pH plays a crucial role in regulating the function of enzymes, modification of CA 2 may advance the development of AD.

18.4.2 Oxidatively Modified Proteins in Aged Senescence-Accelerated Mice Prone 8 (SAMP8)

α-Enolase (ENO1) is a subunit of enolase, the other subunits being β- and γ-enolase. Two of the subunits form active enolase isoforms (αα, ββ, γγ, αβ, and αβ) that interconvert 2-phosphoglycerate to phosphoenolpyruvate. Since αγ and γγ isoforms predominate in the brain, they are called neuron-specific enolase (NSE) (Keller et al., 1994). Although the level of NSE is not significantly altered in AD brain (Kato et al., 1991), ENO1 specific carbonyl level and protein level (Schonberger et al., 2001; Castegna et al., 2002b) are increased in AD brain when compared to age-matched control, suggesting that the loss of activity by oxidative modification of α-enolase is compensated by the increased protein level. It was shown that a decline of enolase activity results in abnormal growth and reduced metabolism in brain (Tholey et al., 1982). The specific carbonyl level of ENO1 is significantly increased in SAMP8 mice, while the protein level of ENO1 is not, suggesting that the activity of α-enolase is reduced in SAMP8 brain. This result conceivably could reflect the lower ATP level in SAMP8 mice brains (Shimano, 1998).

Lactate dehydrogenase 2 (LDH 2) is a subunit of lactate dehydrogenase (LDH). LDH is a glycolytic protein that catalyzes the reversible NAD-dependent interconversion of pyruvate to lactate. LDH isoform 5 in astrocytes favors the formation of lactate (Bittar et al., 1996). Also LDH isoform 5 is more abundant in mitochondria than elsewhere in the cell (Brooks et al., 1999), indicating that lactate is the predominant monocarboxylate oxidized by mitochondria for intracellular lactate transport. Moreover production of lactate also serves as intercellular energy transfer from astrocytes to neurons because lactate is secreted by astrocytes, taken up by neurons, and converted to pyruvate, which then enters the Krebs cycle for ATP production (Deitmer, 2001). These studies together suggest that LDH plays a significant role in intra- and intercellular lactate shuttling. Lactate appears to be the main energetic compound delivered by astrocytes (Dringen et al., 1993) and is the only oxidizable energy substrate available to support neuronal recovery in the CNS (Schurr et al., 1997a, 1997b; Sahlas et al., 2002). Also many studies show that LDH activity in rat brain declines with increased age. Since aging is associated with oxidative stress (Hensley et al., 1995a; Butterfield et al., 1997b, 1999; Fu et al., 2003; Ozaki et al., 2003), the studies above suggest that the observed LDH activity loss may be due to the oxidative modification of the enzyme, and our laboratory used redox proteomics to prove that LDH-2 is significantly modified by oxidative insult in aged SAMP8 brain (Poon et al., 2004c).

CK is highly sensitive to oxidation and is found in the cytoplasm and mitochondria of the cell. As noted earlier, CK catalyzes the reversible transfer of high-energy phosphoryl between ATP and creatine phosphate (Schlegel et al., 1990; Wallimann et al., 1992; Wyss et al., 1992; Kaldis et al., 1994). In AD brain the expression of CK-BB is decreased compared to the age-matched controls (David et al., 1998). It is also well established that oxidative modification of CK decreases its activity with aging, AD, and other neurodegenerative disease

brain (Hensley et al., 1995b; Aksenova et al., 1998, 1999; Yatin et al., 1999a; Aksenov et al., 2001; Castegna et al., 2002a). Consistent with these findings, CK in aged SAMP8 brain is oxidized significantly, which affects its ability to produce ATP (Poon et al., 2004c).

DRP 2 is one of the four members of the dihydropyrimidinase-related protein family (DRP-1, -2, -3, and -4), which was originally identified in humans by their homology to dihydropyrimidinase (Hamajima et al., 1996; Wang and Strittmatter, 1996; Kato et al., 1998). Other nonhuman counterparts of the human DRPs are the chicken collapsing response mediator protein (CRMP-62) (Goshima et al., 1995), rat turned on after division (TOAD)-64 (Minturn et al., 1995), and mouse unc-33-like phosphoprotein (Ulip). The DRP family is involved in axonal outgrowth and pathfinding through transmission and modulation of extracellular signals (Goshima et al., 1995; Minturn et al., 1995; Byk et al., 1996). One of the identified extracellular signals is mediated by the protein of the collapsing-semaphorin family in collaboration with their receptor, neuropilion (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Collapsin contributes to axonal pathfinding by inducing growth cone collapse, which repels the outgrowing axon (Luo et al., 1993). It was reported that DRP-2 can induce growth cone collapse (Goshima et al., 1995; Wang and Strittmatter, 1996) by Rho-kinase phosphorylation (Arimura et al., 2000), and binding to tubulin heterodimers and bundled microtubule as carriers to promote microtubules assembly and dynamics (Gu and Ihara, 2000; Fukata et al., 2002). Many neurodegenerative diseases are associated with DRP 2. It was suggested that incorporation of DRP 2 in the neurofibrillary tangles decrease cytosolic DRP 2 and leads to abnormal neuritic and axonal growth, thereby accelerating the neuronal degeneration in AD (Yoshida et al., 1998). Decreased expression of DRP 2 protein is observed in AD (Lubec et al., 1999), and DRP 2 is oxidatively modified (Castegna et al., 2002b). The oxidative modification of DRP2 is significantly increased in aged SAMP8 mice (Poon et al., 2004c), suggesting that the DRP-2 activity loss, due to either reduced expression or oxidative modification, disrupts neural development and plasticity in the CNS, resulting in impairment of learning and memory. Oxidative modification of DRP2 may have an important role in the memory and learning deficit observed in aged SAMP8 mice.

Spectrins are a family of widely distributed filamentous proteins. α-Spectrin (SPEC), a component of the membrane-associated cytoskeleton, forms a supporting and organized scaffold for intracellular cohesion with the association of actins (Leto et al., 1988). The breakdown products of SPEC from calcium-activated proteolysis are commonly used as markers of apoptosis (Vanderklish and Bahr, 2000). It is reported that Aβ can also induce SPEC breakdown products in cultured rat cortical neurons by activating caspases (Harada and Sugimoto, 1999). Consistent with these studies, a decreased level of SPEC was observed in aged SAMP8 mouse brain, as well as an increased specific carbonyl level (Poon et al., 2004c), suggesting that the proteolytic mechanism in apoptosis involves oxidative modification and degradation of SPEC. Correspondingly loss of SPEC by oxidation or degradation could disrupt the cytoskeleton and the structure of

cells in brain, and thereby affect intercellular and intracellular communications, which could explain the learning and memory deficits observed in SAMP8 mice.

Interestingly the learning and memory deficits of aged SAMP8 mice could be reversed by a treatment of α-lipoic acid (LA) (Farr et al., 2003). LA, a coenzyme involved in production of ATP in mitochondria, is a potent antioxidant. The LDH2, DRP 2, and ENO 1 in aged SAMP8 mice treated with LA showed a lower level of oxidative modification than in aged SAMP8 mice without the LA treatment (Poon et al., 2005a). As these studies suggest, oxidative modification of these proteins may potentially reverse the memory deficits in AD. Similar to the LA treatment, the production of Aβ was decreased by an intracerebroventricular injection of a 42 mer phosphorothiolated antisense oligonucleotide (AO) directed at the Aβ region of the APP gene in order to reduce lipid peroxidation, protein oxidation (Poon et al., 2004d), and improve cognitive deficits (Kumar et al., 2000) in aged SAMP8 mice. In yet another study, the oxidative modification of aldolase, coronin 1a, and peroxiredoxin 2 proved to be significantly reduced (Poon et al., 2005a). These proteins are oxidatively modified in aged SAMP8 mice when compared to young SAMP8 mice, although their modification is not statistically significant. Aldolase is a glycolytic enzyme. Aldolase interacts with DRP-2 for redox regulation of cell growth and development in response to external oxidative stress and/or antioxidants (Bulliard et al., 1997). Peroxiredoxin 2 is an antioxidant enzyme that is exclusively expressed in neurons (Sarafian et al., 1999). The increased expression of peroxiredoxin 2 in AD, Down's syndrome, and PD (Kim et al., 2001a; Krapfenbauer et al., 2003) is likely to be in response to the increased oxidative stress.

Coronin 1a is an actin-binding protein (de Hostos et al., 1991). Coronin-like protein promotes rapid actin polymerization by reducing the lag phase of actin polymerization. It also serves as a link between oxidase and the actin cytoskeleton or/and as a docking site for translocation of the plasma membrane (Grogan et al., 1997; Reeves et al., 1999). The altered coronin level in fetal Down's syndrome (DS) brain indicates that it is involved in migration of cells and/or neuronal outgrowth (Weitzdoerfer et al., 2002). Reducing the Aβ level in aged SAMP8 mice could possibly improve the neuronal outgrowth and repair process that are indicated to be impaired by other redox proteomic in AD brain, indicating the key role of Aβ in oxidative stress of AD.

18.4.3 Oxidatively Modified Proteins by Aβ(1–42) In vivo

We used redox proteomics to identify a number of oxidatively modified proteins in rat brain following intracerebral injection of Aβ(1–42) (Boyd-Kimball et al., 2005c). Consistent with the human brain, PGM1 was found to be oxidized when Aβ(1–42) was infused into rat brain, suggesting the oxidation of PGM1 is related to Aβ(1–42).

14-3-3 Zeta protein (14-3-3z) is involved in a number of cellular functions, including signal transduction, protein trafficking, and metabolism (Dougherty and Morrison, 2004). The expression of 14-3-3z is increased in AD brain (Fountoulakis

et al., 1999) and CSF (Burkhard et al., 2001). Moreover 14-3-3z is associated with NFT in AD brain (Layfield et al., 1996) and acts as an effector for tau protein phosphorylation (Hashiguchi et al., 2000) by providing a scaffold of promotion for the tau protein polymerization (Hernández et al., 2004). Recently it was shown that 14-3-3z simultaneously binds to tau and glycogen synthase kinase 3 β (GSK3β) in a multiple protein tau phosphorylation complex (Agarwal-Mawal et al., 2003). Together, oxidative modification of 14-3-3z may alter its normal function, leading to abnormal tau phosphorylation in AD brain.

β-Synuclein (SYN) is a presynaptic protein that is involved in some way in synaptic vesicle homeostasis and neuroprotection in the CNS (Hashimoto et al., 2004). Accumulation of SYN in disease filaments associated with Lewy bodies, such as PD and AD, is well established. Association of SYN with cholinergic components, particularly in the basal forebrain (Li et al., 2002), suggests that functional SYN is necessary for normal cholinergic function. Therefore, oxidation of SYN may alter its function and cause apoptosis and the protein aggregation observed in AD brain.

Heat-shock protein 60 (HSP60) is a mitochondrial chaperone protein that is involved in folding and assembly of mitochondrial proteins. The expression of HSP60 is significantly decreased in AD (Yoo et al., 2001). Also Aβ(25–35) induces oxidation of HSP60 in fibroblasts derived from AD patients (Choi et al., 2003). Taken together, oxidation of HSP60 is likely caused by the abnormal accumulation of Aβ. The loss of function of HSP60 by oxidative modification may increase misfolded proteins and protein aggregations. These protein accumulations are particularly detrimental to mitochondria because this organelle lacks abundant antioxidant mechanisms to protect age-related oxidative stress. Therefore, oxidation of HSP60 may contribute to the mitochondrial dysfunction in AD brain.

18.4.4 Oxidatively Modified Proteins by Aβ(1–42) In vitro

Synaptosomes Actin is a core subunit of microfilaments found in both neurons and glial cells. Actin is a target of Aβ(1–42)-mediated protein oxidation. Although only β-actin (ACT) was oxidatively modified in AD brain (Aksenov et al., 2001), synaptosomes treated with Aβ(1–42) showed oxidation of both β- and γ-actin (Boyd-Kimball et al., 2005a). Actin microfilaments play a role in the neuronal cytoskeleton by maintaining the distribution of membrane proteins and segregating axonal and dendritic proteins (Beck and Nelson, 1996). Therefore oxidation of actin can lead to alteration of membrane cytoskeletal structure, decreased membrane fluidity, and retrograde/antigrade trafficking of synaptic proteins in axons. Moreover, actin is an important component in the elongation of the growth cone directed by DRP-2; thus oxidative structural alteration of actin may be involved in the loss of synapse and neuronal communication in AD (Masliah et al., 1994).

Glial fibrillary acid protein (GFAP) is an intermediate filament that contributes to the maintenance of glial cell cytoskeletal integrity and neuronal myelination. Increased expression of GFAP is associated with AD (Porchet et al., 2003;

Ross et al., 2003; Ingelsson et al., 2004). Moreover, increased expression of GFAP is involved in impaired synaptic plasticity in AD (Finch, 2002). GFAP is used as a marker for activated astrocytes (Champagne et al., 2003), a cellular repair response associated with senile plaques and neurofibrillary tangles in AD brain (Nagele et al., 2004). So increased GFAP expression is likely a cellular response to cellular insult. Consistent with this notion, activation of astrocytes is induced by Aβ(1–42) in culture (Hu et al., 1998). It is believed that oxidative stress mediates the increase in GFAP, since its increased expression is affected when oxidative stress is released by caloric restriction (Morgan et al., 1997). Since GFAP was significantly oxidized in synaptosomes treated with Aβ(1–42), it is conceivable that increased expression of GFAP is a compensatory response to its oxidative modification. Therefore oxidation of GFAP would indicate the reactivity of reactive oxygen species (ROS) generated by Aβ(1–42) in the vicinity of GFAP.

Mitochondrial dysfunction is observed in AD because of the altered expression and decreased activity of complex I (Aksenov et al., 1999; Kim et al., 2001b) and complex III (Kim et al., 2000; Verwer et al., 2000). Adding to the notion of compromised energy metabolism in AD is our finding that H^+-transporting two-sector ATPase (ATP synthase) is oxidized in synaptosomes treated with Aβ(1–42). Since ATP synthase phosphorylates ADP to produce ATP by proton transport in mitochondria, either decreased expression (Schagger and Ohm, 1995) or increased oxidation of ATP synthase in AD brain could potentially inactivate ATP synthase and contribute to a decrease in the activity of the entire electron transport chain and impaired ATP production. Taken together with the alterations in complex I and III, impaired mitochondria electron transport complex may result in significant leakage of electrons from their carrier molecules to generate ROS. This increased ROS production due to mitochondrial dysfunction suggests an alternative rationalization for the evidence of oxidative stress in AD (Kim et al., 2001b; Butterfield and Lauderback, 2002).

Syntaxin binding protein 1 (SNBP1) is a neuronal protein that shows high affinity to plasma membrane protein syntaxin, which plays a significant role in docking and release of synaptic vesicles. Association of SNBP with syntaxin enables synaptic vesicle exocytosis and neurotransmitter release (Gengyo-Ando et al., 1996; Verhage et al., 2000). SNBP1 was oxidized in synaptosomes by Aβ(1–42)-mediated oxidative stress, suggesting oxidative alteration of SNBP1 impairs the fusion of synaptic vesicles, release of neurotransmitters and, subsequently, loss of synaptic transmission and neuronal function.

Glutamate dehydrogenase (GDH) is a mitochondrial matrix enzyme that is involved in metabolic or catabolic reactions of glutamate. In the biosynthetic direction, GDH catalyzes the reversible amination of α-ketoglutarate with NADPH to yield glutamate. The conversion of glutamate to α-ketoglutarate by GDH is particularly important for eliminating excitotoxicity of glutamate. Conversion of glutamate to glutamine by glutamine synthetase (GS) is significantly impaired in AD brain (Hensley et al., 1995b) due to the oxidative modification of GS (Hensley et al., 1995b; Castegna et al., 2002a). Additionally

Aβ(1–42) induces HNE modification of the glutamate transporter EAAT2 in synaptosomes (Lauderback et al., 2001), suggesting that the EAAT2 activity decrement in AD brain is due to Aβ(1–42)-induced HNE modification (Masliah et al., 1996). Taken together, oxidative inactivation of GDH, GS, and EAAT2 results in a decreased uptake and breakdown of glutamate, resulting in accumulation of extracellular glutamate. The excessive glutamate would stimulate NMDA receptors, leading to an increase in Ca^{2+} influx and thus changes in long-term potentiation that affect learning and memory as well as activate multiple apoptosis cascades. These are all changes that may lead to neuronal death in AD.

DRP 2, as mentioned above, is involved in the formation of neuronal connections and consequently maintenance of neuronal communication. DRP 2 is a pathfinding and guidance protein for axonal outgrowth. It interacts with and modulates collapsin, a protein responsible for the elongation and guidance of dendrites. The oxidation and impaired activity of DRP 2 could result in the shortened dendritic lengths reported in AD (Flood, 1991; Hanks and Flood, 1991). Neurons with shortened neurites would be expected to impair communication with adjacent neurons, a process that could conceivably contribute to memory and cognitive loss in AD. DPR 2 is normally expressed during development. However, in AD brain, where synaptic regions of neurons are undergoing oxidative insult, it requires DRP 2 to repair synaptic regions. Oxidation of DRP 2 was observed in this model, and the finding that DRP-2 is oxidatively modified by acute exposure to Aβ(1–42) provides a connection between the in vitro protein oxidation induced by Aβ(1–42) and the in vivo protein oxidation observed in AD brain. Consequently this is supporting evidence for the role of Aβ(1–42) in the pathogenesis of AD.

Mitochondrial elongation factor-Tu (EF-Tu) binds to amino acyl-tRNA by coupling with ATP to form a complex that promotes the binding of the amino acyl-tRNA to the acceptor site of the ribosome. Thus EF-Tu is necessary for the synthesis of polypeptides encoded by the mitochondrial genome, which are the components of the electron transport chain and ATP synthetase (Cai et al., 2001; He et al., 2001). Oxidative modification of EF-Tu may impair the synthesis of the proteins that are vital to energy metabolism, and alteration of the synthesis of these proteins has been reported in AD (Chandrasekaran et al., 1997; Bonilla et al., 1999; Manczak et al., 2004). Consistent with this notion, altered glucose metabolism is also observed in AD (Messier and Gagnon, 1996; Vanhanen and Soininen, 1998; Scheltens and Korf, 2000).

Primary Neuronal Cultures Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme that converts the first oxidation-reduction reaction in the glycolytic pathway (conversion of glyceraldehyde-3-phosphate to 1,3-phosphoglycerate). Accumulation of GAPDH has been demonstrated in AD brain along with other glycolytic enzymes such as α-enolase and γ-enolase (Schonberger et al., 2001). The possible oxidative inactivation of GAPDH results in decreased ATP production, which is consistent with the altered glucose tolerance and metabolism confirmed by PET scanning studies of AD patients (Blass

et al., 1988; Vanhanen and Soininen, 1998; Messier and Gagnon, 2000; Scheltens and Korf, 2000). Such a scenario is also consistent with the oxidative modification of α-enolase and triosephosphate isomerase, along with creatine kinase in AD brain (Castegna et al., 2002b, 2003), suggesting Aβ(1–42) has a key role in oxidative stress in AD brain.

14-3-3 Zeta is a cytosolic protein found primarily in the gray matter in rats (Takahashi, 2003). As previously mentioned, 14-3-3z proteins play a role in a variety of cellular functions including metabolism, signal transduction, cell-cycle regulation, apoptosis, protein trafficking, and stress responses by binding to specific target proteins (Dougherty and Morrison, 2004). Oxidative modification of 14-3-3z in neuron treated with Aβ(1–42) indicate that the oxidation of 14-3-3z is associated with Aβ(1–42).

18.4.5 Oxidatively Modified Proteins in *Caenorhabditis elegans* *(C. elegans)* Expressing Human Aβ(1–42)

Acyl-CoA dehydrogenase (ACD) catalyzes the conversion of acyl-CoA to trans-Δ2-enoyl-CoA by coupling to the reduction of FAD to $FADH_2$. This reaction provides acetyl-CoA to the Krebs cycle for ATP formation from fatty acids. Therefore, oxidative inactivation of acyl-CoA dehydrogenase might inhibit the production of acetyl-CoA and thus reduce the production of ATP from fatty acid catabolism, conceivably related to the known energy metabolism alteration in AD brain (Prasad et al., 1998).

Glutathione S-transferases (GSTs) catalyze the reaction of reactive alkenals with glutathione, a major antioxidant that is abundant in the brain (Esterbauer et al., 1991; Xie et al., 1998; Leiers et al., 2003). Reactive alkenals, such as HNE, are products of lipid peroxidation that can be induced by Aβ(1–42) (Mark et al., 1997; Lauderback et al., 2001). Elevated HNE levels modify cysteine, lysine, and histidine residues to increase their carbonyl content in AD brain (Markesbery and Lovell, 1998). Consistent with the observation that GST activity is decreased in AD brain (Lovell et al., 1998) and HNE is bound in excess to GST (Sultana and Butterfield, 2004), oxidation of GSTs was also found in *C. elegans* expressing Aβ(1–42), suggesting the possible oxidative inactivation of GSTs would result in an increased vulnerability of neurons to reactive alkenals, and consequently to oxidative damage.

Malate dehydrogenase (MDH) catalyzes the reversible oxidation of malate to oxaloacetate coupled with the reduction of NAD^+ to NADH. MDH also facilitates the transfer of NADH across the mitochondrial membrane to complex I. Moreover, MDH participates in the malate-aspartate shuttle that passively feeds electrons from cytosolic NADH into the electron transport chain. Oxidative inactivation of MDH therefore would significantly decrease the efficiency of the citric acid cycle as well as the transport of electrons from cytosolic NADH into the mitochondrial matrix, and consequently decrease the production of ATP.

Arginine kinase (ARK) catalyzes the reversible transfer of phosphate from a phophorylated guanidino ($\sim NH–CN_2H_4^+$) substrate to ADP to provide immediate ATP requirements. This reaction supports neuronal activity from draining

ATP that is essential to other cellular functions (Zhou et al., 1998; Suzuki et al., 1999; Azzi et al., 2004). This process is achieved in mammals by creatine kinase, whose oxidative inactivation plays significant role in AD (Aksenov et al., 2000; Castegna et al., 2002a). Arginine kinase shares 41% similarity of amino acid sequence to creatine kinase, suggesting oxidative modification of arginine kinase is comparative to the modification of CK in AD brain, with similar consequences.

Guanine nucleotide-binding proteins (G proteins) are the key proteins for signal transduction from hormones, neurotransmitters, and chemokines. G proteins are activated by ligand-bound transmembrane receptors, which subsequently lead to the activation of many cellular signaling pathways, such as regulation of metabolic enzymes, ion channels, and transporters (Neves et al., 2002). Oxidative modification can hinder the phosphorylation site of the G protein sterically, thereby affecting Ca^{2+} homeostasis, second messengers, cell cycle, and neurotransmission, and ultimately lead to apoptosis.

Cytoskeletal alterations in AD are shown by the significant oxidation of ACT and the oxidative modification of β-tubulin in AD (Aksenov et al., 2001). As mentioned above, oxidation of actin can lead to many damaging cellular effects. Myosin regulatory light chain and myosin light chain 1 was also found to be oxidized in response to Aβ(1–42) in *C. elegans*. This is most likely due to the expression of Aβ(1–42) within the muscular wall of the nematode since the unc-54 muscle promoter was used as a promoter of human Aβ(1–42) expressed (Link et al., 2003). Oxidation of myosin was not detected in the *C. elegans* model expressing ypkA, which is a control for paralysis. This finding suggests that the oxidation of myosin in response to Aβ(1–42) was a direct effect of the proximity of the protein to the site of ROS production.

Adenosine kinase (AK) catalyzes the reversible phosphorylation of adenosine by ATP to form ADP and AMP to regulate intra- and extracellular levels of adenosine. Loss of function of arginine kinase and adenosine kinase would result in altered cellular phosphate storage for ATP production. High level of ATP in brain is necessary because of the large consumption of ATP by Na^+/K^+-ATPase to maintain neuronal membrane potential. Therefore decreased levels of ATP could cause impairment in membrane potential and impulse transmission, thus altering long-term potentiation, influx of Ca^{2+}, and neuronal apoptosis, all of which eventually lead to the neurodegeneration as evident in AD. Decreased levels of ATP could be especially important for the synaptic region of neurons, as it is believed to be the site of initial attack in AD neurons (Masliah et al., 1994).

Lipid binding protein 6 (LBP-6) is a fatty acid binding protein that is involved in lipid metabolism and fatty acid transport. Oxidation of LBP-6 was found in *C. elegans* induced by overexpressing Aβ(1–42), suggesting the role of Aβ(1–42) in lipid metabolism. This finding is consistent with the observation of altered cholesterol homeostasis and membrane fluidity in AD. Moreover cholesterol modulates the toxicity of Aβ(1–42) on neuronal membranes (Eckert et al., 2003). However, a recent study of APP/PS 1 double-mutant mice raised on a high-cholesterol diet did not observe additional oxidative stress by cholesterol over

that produced by Aβ(1–42) (Mohmmad Abdul et al., 2004). Conceivably mitochondrial resident fatty acid metabolism is altered by Aβ(1–42) and in AD brain. Taken together, oxidative modification of LBP-6 induced by Aβ(1–42) may play a role in the cholesterol homeostasis alteration in AD.

Transketolase (TKL) catalyzes the independent formation of NADPH and ribose-5-phosphate in the pentose phosphate pathway. NADPH is essential for a variety of cellular process such as energy metabolism and the reduction of oxidized glutathione by glutathione reductase, while ribose-5-phosphate is required for biosynthesis of nucleic acids. Moreover, the catalytic reactions of TKL can produce glyceraldehyde-3-phosphate and fructose-6-phosphate, which can produce ATP by glycolysis. Expression of TKL is significantly reduced in *C. elegans* expressing Aβ(1–42) (Link et al., 2003), and the activity of TKL is decreased in AD brain (Gibson et al., 1988). Consistent with the notion that TKL is altered in AD brain, oxidative modification of TKL could possibly inactivate the enzyme and reduce the production of NADPH, intermediates of the pentose phosphate pathway synthesis, and the synthesis of nucleic acids for DNA repair. These processes then would lead to decreased activity of protein synthesis, resulting in impaired protein turnover.

Seven α subunits and seven β subunits of proteasome (α-PTSM, β-PTSM) complex form the multi-subunit protein complex that is responsible for the proteolytic degradation of intracellular proteins. The proteasome plays an important role in the turnover of misfolded and aggregated proteins (Jayarapu and Griffin, 2004). Proteasome inactivation was reported in AD (Keller et al., 2000), and increased mitochondrial ROS production and decreased mitochondrial turnover is observed when the proteasome is inhibited (Sullivan et al., 2004). Oxidation of both proteasomal subunits suggests that aggregated Aβ(1–42) have a role in the loss of proteasome activity reported in AD. Moreover the oxidation of proteasome α subunit 4 (PTSM) in the *C. elegans* model suggests that cytoskeletal alterations also have a role in proteasome inhibition. Consistent with this notion, there is increasing evidence that cytoskeletal alterations are caused by Aβ(1–42) (Zheng et al., 2002).

18.5 CONCLUSION

The proteins that are oxidized in the AD brain and AD models are involved in five cellular functions that are reportedly impaired in AD: neuroprotection, synaptic function, mitochondria function, energy metabolism, and glutamate regulation (Fig. 18.4). Increased ROS production is related to the mitochondrial dysfunction in AD brains (Schapira, 1998; Calabrese et al., 2001). The neuroprotective entities against ROS production, such as heat-shock proteins, glutathione, and antioxidant enzymes are significantly impaired in AD brains (Calabrese et al., 2002a, 2002b). The imbalance of oxidants and antioxidants induces oxidative stress in AD, thereby leading to glutamate dysregulation (Butterfield and Pocernich, 2003) and impaired energy metabolism (Blass et al., 1988), and eventually synaptic dysfunction observed in AD (DeKosky and Scheff, 1990; Bertoni-Freddari et al.,

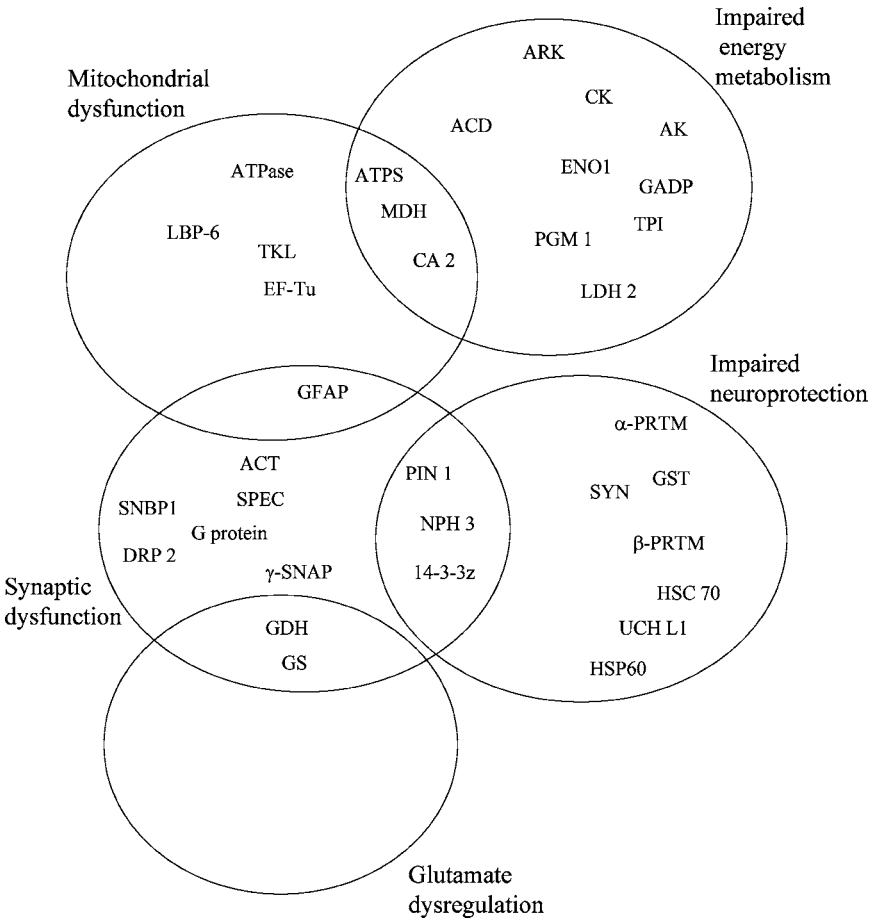


FIGURE 18.4 Venn diagram of functionality of oxidatively modified proteins in AD brain and AD models.

1992). Redox proteomics analysis not only shows that oxidatively modified proteins contribute to the impairment of neuroprotection, synaptic function, mitochondrial function, and energy metabolism in AD, but also can pinpoint the proteins that are oxidatively modified. This information provides valuable information on the mechanism of AD and it also provides potential targets for therapeutic intervention in AD.

The technology of redox proteomics has improved rapidly over recent years. Many high-throughput methods are being developed in order to improve sensitivity and detection limit of 2D gel electrophoresis (Morris and Wilson, 2004) that will enable researchers to detect low abundant proteins. When such techniques mature, a large body of information will become available to better understand the disease and to develop biomarkers for diagnosis and for indexing

therapeutic efficacy. Moreover information from proteomic experiments may lead to new hypotheses that may bring about a greater understanding of the pathogenesis and therapy of AD. Collaboration among physicians, biological chemists, software engineers will be necessary to accomplish these aims, and at the University of Kentucky, we look forward to continuing such collaborative efforts.

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Institutes of Health to D.A. Butterfield (AG-10836; AG-05119).

LIST OF ABBREVIATIONS

ACD, Acyl-CoA dehydrogenase
ACT, β-actin
AD, Alzheimer's disease
AK, adenosine kinase
AO, antisense oligonucleotide
ARK, arginine kinase
CA 2, carbonic anhydrase 2
CK, creatine kinase
CNS, central nervous system
DNPH, dinitrophenylhydrazine
DRP2, dihydropyrimidinase related proteins 2
DS, Down's syndrome
EF-Tu, elongation factor-Tu
ENO1, α-enolase
ESI, electrospray ionization
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
GDH, glutamate dehydrogenase
GFAP, glial fibrillary acid protein
GS, glutamine synthase
GSK3β, glycogen synthase kinase 3 β
GSTs, glutathione S-transferases
HNE, 4-hydroxyl-2-nonenal
HPLC, high performance liquid chromatography
HSP, heat shock proteins
IEF, isoelectric focusing

LA, α-lipoic acid
LBP-6, lipid binding protein 6
LDH, lactate dehydrogenase
MALDI, matrix assisted laser desorption/ionization
MDH, malate dehydrogenase
NFT, neurofibrillary tangles
NO, nitric oxide
NPH3, neuropolypeptide h3
NSE, neuron-specific enolase
3-NT, 3-nitrotyrosine
PGM1, phosphoglycerate mutase 1
PIN, peptidyl-prolyl isomerase
PTSM, proteasome subunit
ROS, reactive oxygen species
SAMP8, senescence-accelerated mice prone 8
γ-SNAP, γ-synaptosomal protein like soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment proteins
SNBP1, syntaxin binding protein 1
SP, senile plaques
SPEC, α-spectrin
SYN, β-synuclein
TCA, trichloroacetic acid
TKL, transketolase
TPI, triosephosphate isomerase
UCH-L1, ubiquitin carboxyl terminal hydrolase L1
UKADRC, University of Kentucky Alzheimer's Diseases Research Center

REFERENCES

Abe K., Inokawa M., Kashiwagi A., Yanagihara T. (1998). Amnesia after a discrete basal forebrain lesion. *J Neurol Neurosurg Psychiatry* 65: 126–130.

Agarwal S., Sohal R.S. (1994). Aging and proteolysis of oxidized proteins. *Arch Biochem Biophys* 309: 24–28.

Agarwal-Mawal A., Qureshi H.Y., Cafferty P.W., Yuan Z., Han D., Lin R. Paudel H.K. (2003). 14-3-3 connects glycogen synthase kinase-3 beta to tau within a brain microtubule-associated tau phosphorylation complex. *J Biol Chem* 278: 12722–12728.

Agrawal A., Shukla R., Tripathi L.M., Pandey V.C., Srimal R.C. (1996). Permeability function related to cerebral microvessel enzymes during ageing in rats. *Int J Dev Neurosci* 14: 87–91.

Akama K.T., Van Eldik L.J. (2000). Beta-amyloid stimulation of inducible nitric-oxide synthase in astrocytes is interleukin-1beta- and tumor necrosis factor-alpha (TNFalpha)-dependent, and involves a TNFalpha receptor-associated factor- and NFkappaB-inducing kinase-dependent signaling mechanism. *J Biol Chem* 275: 7918–7924.

Aksenov M., Aksenova M., Butterfield D.A., Markesbery W.R. (2000). Oxidative modification of creatine kinase BB in Alzheimer's disease brain. *J Neurochem* 74: 2520–2527.

Aksenov M.Y., Aksenova M.V., Butterfield D.A., Geddes J.W., Markesbery W.R. (2001). Protein oxidation in the brain in Alzheimer's disease. *Neuroscience* 103: 373–383.

Aksenov M.Y., Aksenova M.V., Butterfield D.A., Hensley K., Vigo-Pelfrey C., Carney J.M. (1996). Glutamine synthetase-induced enhancement of beta-amyloid peptide A beta (1–40) neurotoxicity accompanied by abrogation of fibril formation and A beta fragmentation. *J Neurochem* 66: 2050–2056.

Aksenov M.Y., Tucker H.M., Nair P., Aksenova M.V., Butterfield D.A., Estus S., Markesbery W.R. (1999). The expression of several mitochondrial and nuclear genes encoding the subunits of electron transport chain enzyme complexes, cytochrome c oxidase, and NADH dehydrogenase, in different brain regions in Alzheimer's disease. *Neurochem Res* 24: 767–774.

Aksenova M.V., Aksenov M.Y., Carney J.M., Butterfield D.A. (1998). Protein oxidation and enzyme activity decline in old brown Norway rats are reduced by dietary restriction. *Mech Ageing Dev* 100: 157–168.

Aksenova M.V., Aksenov M.Y., Payne R.M., Trojanowski J.Q., Schmidt M.L., Carney J.M., Butterfield D.A., Markesbery W.R. (1999). Oxidation of cytosolic proteins and expression of creatine kinase BB in frontal lobe in different neurodegenerative disorders. *Dement Geriatr Cogn Disord* 10: 158–165.

Arimura N., Inagaki N., Chihara K., Menager C., Nakamura N., Amano M., Iwamatsu A., Goshima Y., Kaibuchi K. (2000). Phosphorylation of collapsin response mediator protein-2 by Rho-kinase: Evidence for two separate signaling pathways for growth cone collapse. *J Biol Chem* 275: 23973–23980.

Arispe N., Rojas E., Pollard H.B. (1993). Alzheimer disease amyloid beta protein forms calcium channels in bilayer membranes: Blockade by tromethamine and aluminum. *Proc Natl Acad Sci USA* 90: 567–571.

Azzi A., Clark S.A., Ellington W.R., Chapman M.S. (2004). The role of phosphagen specificity loops in arginine kinase. *Protein Sci* 13: 575–585.

Baxter M.G. (2001). Effects of selective immunotoxic lesions on learning and memory. *Methods Mol Biol* 166: 249–265.

Baxter M.G., Bucci D.J., Gorman L.K., Wiley R.G., Gallagher M. (1995). Selective immunotoxic lesions of basal forebrain cholinergic cells: Effects on learning and memory in rats. *Behav Neurosci* 109: 714–722.

Beavis R.C., Chait B.T. (1989). Cinnamic acid derivatives as matrices for ultraviolet laser desorption mass spectrometry of proteins. *Rapid Commun Mass Spectrom* 3: 432–435.

Beck K.A., Nelson W.J. (1996). The spectrin-based membrane skeleton as a membrane protein-sorting machine. *Am J Physiol* 270: C1263–C1270.

Beckers C.J., Block M.R., Glick B.S., Rothman J.E., Balch W.E. (1989). Vesicular transport between the endoplasmic reticulum and the Golgi stack requires the NEM-sensitive fusion protein. *Nature* 339: 397–398.

Bertoni-Freddari C., Fattoretti P., Casoli T., Gambini M., Meier-Ruge W., Ulrich J. (1992). Structural dynamics of synaptic junctional areas in aging and Alzheimer's disease. *Ann NY Acad Sci* 673: 285–292.

Beyreuther K., Bush A.I., Dyrks T., Hilbich C., Konig G., Monning U., Multhaup G., Prior R., Rumble B., Schubert W., et al. (1991). Mechanisms of amyloid deposition in Alzheimer's disease. *Ann NY Acad Sci* 640: 129–139.

Bittar P.G., Charnay Y., Pellerin L., Bouras C., Magistretti P.J. (1996). Selective distribution of lactate dehydrogenase isoenzymes in neurons and astrocytes of human brain. *J Cereb Blood Flow Metab* 16: 1079–1089.

Blanc E.M., Toborek M., Mark R.J., Hennig B., Mattson M.P. (1997). Amyloid beta-peptide induces cell monolayer albumin permeability, impairs glucose transport, and induces apoptosis in vascular endothelial cells. *J Neurochem* 68: 1870–1881.

Blass J.P., Sheu R.K., Cedarbaum J.M. (1988). Energy metabolism in disorders of the nervous system. *Rev Neurol (Paris)* 144: 543–563.

Bonilla E., Tanji K., Hirano M., Vu T.H., DiMauro S., Schon E.A. (1999). Mitochondrial involvement in Alzheimer's disease. *Biochim Biophys Acta* 1410: 171–182.

Boyd-Kimball D., Castegna A., Petroze R., Poon H.F., Sultana R., Lynn B.C., Klein J.B., Butterfield D.A. (2005a). Proteomic identification of protein oxidized by Aβ(1–42) in synaptosomes: Implications for Alzheimer's disease. *Brain Res* 1044: 206–215.

Boyd-Kimball D., Poon H.F., Lynn B.C., Cai J., Pierce Jr W.M., Klein J.B., Ferguson J., Link C.D., Butterfield D.A. (2005b). Proteomic identification of proteins specifically oxidized in *Caenorhabditis elegans* expressing human Aβ(1–42): Implications for Alzheimer's disease. *Neurobiol Aging* PMID: 16099075; doi:10.1016/j.neurobiolaging.

Boyd-Kimball D., Sultana R., Poon H.F., Lynn B.C., Casamenti F., Pepeu G., Klein J.B., Butterfield D.A. (2005c). Proteomic identification of proteins specifically oxidized by intracerebral injection of amyloid β-peptide (1–42) into rat brain: Implications for Alzheimer's disease. *Neuroscience* 132: 313–324.

Boyd-Kimball D., Sultana R., Poon H.F., Mohmmad-Abdul H., Lynn B.C., Klein J.B., Butterfield D.A. (2005d). Gamma-glutamylcysteine ethyl ester protection of proteins from Abeta(1–42)-mediated oxidative stress in neuronal cell culture: A proteomics approach. *J Neurosci Res* 79: 707–713.

Brooks G.A., Dubouchaud H., Brown M., Sicurello J.P., Butz C.E. (1999). Role of mitochondrial lactate dehydrogenase and lactate oxidation in the intracellular lactate shuttle. *Proc Natl Acad Sci USA* 96: 1129–1134.

Bulliard C., Zurbriggen R., Tornare J., Faty M., Dastoor Z., Dreyer J.L. (1997). Purification of a dichlorophenol-indophenol oxidoreductase from rat and bovine synaptic membranes: Tight complex association of a glyceraldehyde-3-phosphate dehydrogenase isoform, TOAD64, enolase-gamma and aldolase C. *Biochem J* 324: 555–563.

Burkhard P.R., Sanchex J.C., Landis T., Hochstrasser D.F. (2001). CSF detection of the 14-3-3 protein in unselected patients with dementia. *Neurology* 56: 1528–1533.

Butterfield D.A. (2004). Proteomics: A new approach to investigate oxidative stress in Alzheimer's disease brain. *Brain Res* 1000: 1–7.

Butterfield D.A., Castegna A. (2003). Proteomics for the identification of specifically oxidized proteins in brain: Technology and application to the study of neurodegenerative disorders. *Amino Acids* 25: 419–425.

Butterfield D.A., Kanski J. (2002). Methionine residue 35 is critical for the oxidative stress and neurotoxic properties of Alzheimer's amyloid beta-peptide 1–42. *Peptides* 23: 1299–1309.

Butterfield D.A., Lauderback C.M. (2002). Lipid peroxidation and protein oxidation in Alzheimer's disease brain: potential causes and consequences involving amyloid beta-peptide-associated free radical oxidative stress. *Free Radic Biol Med* 32: 1050–1060.

Butterfield D.A., Pocernich C.B. (2003). The glutamatergic system and Alzheimer's disease: Therapeutic implications. *CNS Drugs* 17: 641–652.

Butterfield D.A., Stadtman E.R. (1997). Protein oxidation processes in aging brain. *Adv Cell Aging Gerontol* 2: 161–191.

Butterfield D.A., Hensley K., Harris M., Mattson M., Carney J. (1994). Beta-amyloid peptide free radical fragments initiate synaptosomal lipoperoxidation in a sequence-specific fashion: Implications to Alzheimer's disease. *Biochem Biophys Res Commun* 200: 710–715.

Butterfield D.A., Hensley K., Cole P., Subramaniam R., Aksenov M., Aksenova M., Bummer P.M., Haley B.E., Carney J.M. (1997a). Oxidatively induced structural alteration of glutamine synthetase assessed by analysis of spin label incorporation kinetics: Relevance to Alzheimer's disease. *J Neurochem* 68: 2451–2457.

Butterfield D.A., Howard B.J., Yatin S., Allen K.L., Carney J.M. (1997b). Free radical oxidation of brain proteins in accelerated senescence and its modulation by *N*-tert-butyl-alpha-phenylnitrone. *Proc Natl Acad Sci USA* 94: 674–678.

Butterfield D.A., Howard B., Yatin S., Koppal T., Drake J., Hensley K., Aksenov M., Aksenova M., Subramaniam R., Varadarajan S., Harris-White M.E., Pedigo N.W. Jr., Carney J.M. (1999). Elevated oxidative stress in models of normal brain aging and Alzheimer's disease. *Life Sci* 65: 1883–1892.

Butterfield D.A., Drake J., Pocernich C., Castegna A. (2001). Evidence of oxidative damage in Alzheimer's disease brain: Central role for amyloid beta-peptide. *Trends Mol Med* 7: 548–554.

Butterfield D.A., Castegna A., Lauderback C.M., Drake J. (2002a). Evidence that amyloid beta-peptide-induced lipid peroxidation and its sequelae in Alzheimer's disease brain contribute to neuronal death. *Neurobiol Aging* 23: 655–664.

Butterfield D.A., Griffin S., Munch G., Pasinetti G.M. (2002b). Amyloid beta-peptide and amyloid pathology are central to the oxidative stress and inflammatory cascades under which Alzheimer's disease brain exists. *J Alzheimer's Dis* 4: 193–201.

Butterfield D.A., Boyd-Kimball D., Castegna A. (2003). Proteomics in Alzheimer's disease: Insights into mechanisms into neurodegeneration. *J Neurochem* 86: 1313–1327.

Byk T., Dobransky T., Cifuentes-Diaz C., Sobel A. (1996). Identification and molecular characterization of Unc-33-like phosphoprotein (Ulip), a putative mammalian homolog of the axonal guidance-associated unc-33 gene product. *J Neurosci* 16: 688–701.

Cai H., Wang Y., McCarthy D., Wen H., Borchelt D.R., Price D.L., Wong P.C. (2001). BACE1 is the major beta-secretase for generation of Abeta peptides by neurons. *Nat Neurosci* 4: 233–234.

Calabrese V., Scapagnini G., Colombrita C., Ravagna A., Pennisi G., Giuffrida Stella A.M., Galli F., Butterfield D.A. (2003). Redox regulation of heat shock protein expression in aging and neurodegenerative disorders associated with oxidative stress: A nutritional approach. *Amino Acids* 25: 437–444.

Calabrese V., Scapagnini G., Giuffrida Stella A.M., Bates T.E., Clark J.B. (2001). Mitochondrial involvement in brain function and dysfunction: Relevance to aging, neurodegenerative disorders and longevity. *Neurochem Res* 26: 739–764.

Calabrese V., Scapagnini G., Ravagna A., Fariello R.G., Giuffrida Stella A.M., Abraham N.G. (2002a). Regional distribution of heme oxygenase, HSP70, and glutathione in brain: Relevance for endogenous oxidant/antioxidant balance and stress tolerance. *J Neurosci Res* 68: 65–75.

Calabrese V., Scapagnini G., Ravagna A., Giuffrida Stella A.M., Butterfield D.A. (2002b). Molecular chaperones and their roles in neural cell differentiation. *Dev Neurosci* 24: 1–13.

Castegna A., Aksenov M., Aksenova M., Thongboonkerd V., Klein J.B., Pierce W.M., Booze R., Markesbery W.R., Butterfield D.A. (2002a). Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part I: Creatine kinase BB, glutamine synthase, and ubiquitin carboxy-terminal hydrolase L-1. *Free Radic Biol Med* 33: 562–571.

Castegna A., Aksenov M., Thongboonkerd V., Klein J.B., Pierce W.M., Booze R., Markesbery W.R., Butterfield D.A. (2002b). Proteomic identification of oxidatively modified proteins in Alzheimer's disease brain. Part II: Dihydropyrimidinase-related protein 2, alpha-enolase and heat shock cognate 71. *J Neurochem* 82: 1524–1532.

Castegna A., Thongboonkerd V., Klein J., Lynn B.C., Wang Y.L., Osaka H., Wada K., Butterfield D.A. (2004a). Proteomic analysis of brain proteins in the gracile axonal dystrophy (gad) mouse, a syndrome that emanates from dysfunctional ubiquitin carboxyl-terminal hydrolase L-1, reveals oxidation of key proteins. *J Neurochem* 88: 1540–1546.

Castegna A., Thongboonkerd V., Klein J.B., Lynn B., Markesbery W.R., Butterfield D.A. (2003). Proteomic identification of nitrated proteins in Alzheimer's disease brain. *J Neurochem* 85: 1394–1401.

Castegna A., Thongboonkerd V., Klein J.B., Lynn B., Markesbery W.R., Butterfield D.A. (2004b). Proteomic identification of oxidatively modified proteins in gracile axonal dystrophy mice. *J Neurochem* 88: 1540–1546.

Champagne D., Pearson D., Dea D., Rochford J., Poirier J. (2003). The cholesterol-lowering drug probucol increases apolipoprotein E production in the hippocampus of aged rats: Implications for Alzheimer's disease. *Neuroscience* 121: 99–110.

Chandrasekaran K., Hatanpaa K., Rapoport S.I., Brady D.R. (1997). Decreased expression of nuclear and mitochondrial DNA-encoded genes of oxidative phosphorylation in association neocortex in Alzheimer disease. *Brain Res Mol Brain Res* 44: 99–104.

Choi J., Malakowsky C.A., Talent J.M., Conrad C.C., Carroll C.A., Weintraub S.T., Gracy R.W. (2003). Anti-apoptotic proteins are oxidized y Ab25-35 in Alzheimer's fibroblasts. *Biochim Biophys Acta* 1637: 135–141.

Choi J., Malakowsky C.A., Talent J.M., Conrad C.C., Gracy R.W. (2002). Identification of oxidized plasma proteins in Alzheimer's disease. *Biochem Biophys Res Commun* 293: 1566–1570.

Coleman P.D., Flood D.G. (1987). Neuron numbers and dendritic extent in normal aging and Alzheimer's disease. *Neurobiol Aging* 8: 521–545.

Crystal H., Dickson D., Fuld P., Masur D., Scott R., Mehler M., Masdeu J., Kawas C., Aronson M., Wolfson L. (1988). Clinico-pathologic studies in dementia: nondemented subjects with pathologically confirmed Alzheimer's disease. *Neurology* 38: 1682–1687.

Cuervo A.M., Dice J.F. (2000). Age-related decline in chaperone-mediated autophagy. *J Biol Chem* 275: 31505–31513.

Daleke D.L., Lyles J.V. (2000). Identification and purification of aminophospholipid flippases. *Biochim Biophys Acta* 1486: 108–127.

Dalle-Donne I., Scaloni A., Giustarini D., Cavarra E., Tell G., Lungarella G., Colombo R., Rossi R., Milzani A. (2005). Proteins as biomarkers of oxidative/nitrosative stress in diseases: The contribution of redox proteomics. *Mass Spectrom Rev* 24: 55–99.

David S., Shoemaker M., Haley B.E. (1998). Abnormal properties of creatine kinase in Alzheimer's disease brain: correlation of reduced enzyme activity and active site photolabeling with aberrant cytosol-membrane partitioning. *Brain Res Mol Brain Res* 54: 276–287.

Davies P. (1999). Challenging the cholinergic hypothesis in Alzheimer disease. *JAMA* 281: 1433–1434.

de Hostos E.L., Bradtke B., Lottspeich F., Guggenheim R., Gerisch G. (1991). Coronin, an actin binding protein of Dictyostelium discoideum localized to cell surface projections, has sequence similarities to G protein beta subunits. *Embo J* 10: 4097–4104.

Deitmer J.W. (2001). Strategies for metabolic exchange between glial cells and neurons. *Respir Physiol* 129: 71–81.

DeKosky S.T., Scheff S.W. (1990). Synapse loss in frontal cortex biopsies in Alzheimer's disease: Correlation with cognitive severity. *Ann Neurol* 27: 457–464.

Detari L., Rasmusson D.D., Semba K. (1999). The role of basal forebrain neurons in tonic and phasic activation of the cerebral cortex. *Prog Neurobiol* 58: 249–277.

Dougherty M.K., Morrison D.K. (2004). Unlocking the code of 14-3-3. *J Cell Sci* 117: 1875–1884.

Drake J., Link C.D., Butterfield D.A. (2003). Oxidative stress precedes fibrillar deposition of Alzheimer's disease amyloid beta-peptide (1–42) in a transgenic *Caenorhabditis elegans* model. *Neurobiol Aging* 24: 415–420.

Dringen R., Gebhardt R., Hamprecht B. (1993). Glycogen in astrocytes: Possible function as lactate supply for neighboring cells. *Brain Res* 623: 208–214.

Eckert G.P., Kirsch C., Leutz S., Wood W.G., Muller W.E. (2003). Cholesterol modulates amyloid beta-peptide's membrane interactions. *Pharmacopsychiatry* 36 Suppl 2: S136–S143.

Engstrom I., Ronquist G., Pettersson L., Waldenstrom A. (1995). Alzheimer amyloid beta-peptides exhibit ionophore-like properties in human erythrocytes. *Eur J Clin Invest* 25: 471–476.

Esterbauer H., Schaur R.J., Zollner H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* 11: 81–128.

Etcheberrigaray R., Ito E., Kim C.S., Alkon D.L. (1994). Soluble beta-amyloid induction of Alzheimer's phenotype for human fibroblast K^+ channels. *Science* 264: 276–279.

Ezrin-Waters C., Resch L. (1986). The nucleus basalis of Meynert. *Can J Neurol Sci* 13: 8–14.

Farr S.A., Poon H.F., Dogrukol-Ak D., Drake J., Banks W.A., Eyerman E., Butterfield D.A., Morley J.E. (2003). The antioxidants alpha-lipoic acid and *N*-acetylcysteine reverse memory impairment and brain oxidative stress in aged SAMP8 mice. *J Neurochem* 84: 1173–1183.

Fiala M., Zhang L., Gan X., Sherry B., Taub D., Graves M.C., Hama S., Way D., Weinand M., Witte M., Lorton D., Kuo Y.M., Roher A.E. (1998). Amyloid-beta induces chemokine secretion and monocyte migration across a human blood–brain barrier model. *Mol Med* 4: 480–489.

Finch C.E. (2002). Neurons, glia, and plasticity in normal brain aging. *Adv Gerontol* 10: 35–39.

Fisher M.T., Stadtman E.R. (1992). Oxidative modification of *Escherichia coli* glutamine synthetase: Decreases in the thermodynamic stability of protein structure and specific changes in the active site conformation. *J Biol Chem* 267: 1872–1880.

Flood D.G. (1991). Region-specific stability of dendritic extent in normal human aging and regression in Alzheimer's disease. Part II: Subiculum. *Brain Res* 540: 83–95.

Flood J.F., Morley J.E. (1993). Age-related changes in footshock avoidance acquisition and retention in senescence accelerated mouse (SAM). *Neurobiol Aging* 14: 153–157.

Fountoulakis M., Cairns N., Lubec G. (1999). Increased levels of 14-3-3 gamma and epsilon proteins in brain f patients with Alzheimer's disease and Down syndrome. *J Neural Transm* Suppl. 57: 323–335.

Fu C., Cao C.M., Xia Q., Yang J., Lu Y. (2003). [Reactive oxygen species and mitochondrial K(ATP)-sensitive channels mediated cardioprotection induced by TNF-alpha during hypoxia and reoxygenation]. *Sheng Li Xue Bao* 55: 284–289.

Fukata Y., Itoh T.J., Kimura T., Menager C., Nishimura T., Shiromizu T., Watanabe H., Inagaki N., Iwamatsu A., Hotani H., Kaibuchi K. (2002). CRMP-2 binds to tubulin heterodimers to promote microtubule assembly. *Nat Cell Biol* 4: 583–591.

Gengyo-Ando K., Kitayama H., Mukaida M., Ikawa Y. (1996). A murine neural-specific homolog corrects cholinergic defects in *Caenorhabditis elegans* unc-18 mutants. *J Neurosci* 16: 6695–6702.

Ghezzi P., Bonetto V. (2003). Redox proteomics: Identification of oxidatively modified proteins. *Proteomics* 3: 1145–1153.

Gibson G.E., Sheu K.F., Blass J.P., Baker A., Carlson K.C., Harding B., Perrino P. (1988). Reduced activities of thiamine-dependent enzymes in the brains and peripheral tissues of patients with Alzheimer's disease. *Arch Neurol* 45: 836–840.

Goshima Y., Nakamura F., Strittmatter P., Strittmatter S.M. (1995). Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33. *Nature* 376: 509–514.

Grogan A., Reeves E., Keep N., Wientjes F., Totty N.F., Burlingame A.L., Hsuan J.J., Segal A.W. (1997). Cytosolic phox proteins interact with and regulate the assembly of coronin in neutrophils. *J Cell Sci* 110: 3071–3081.

Gu Y., Ihara Y. (2000). Evidence that collapsin response mediator protein-2 is involved in the dynamics of microtubules. *J Biol Chem* 275: 17917–17920.

Hamajima N., Matsuda K., Sakata S., Tamaki N., Sasaki M., Nonaka M. (1996). A novel gene family defined by human dihydropyrimidinase and three related proteins with differential tissue distribution. *Gene* 180: 157–163.

Hamos J.E., DeGennaro L.J., Drachman D.A. (1989). Synaptic loss in Alzheimer's disease and other dementias. *Neurology* 39: 355–361.

Hanks S.D., Flood D.G. (1991). Region-specific stability of dendritic extent in normal human aging and regression in Alzheimer's disease. I. CA1 of hippocampus. *Brain Res* 540: 63–82.

Harada J., Sugimoto M. (1999). Activation of caspase-3 in beta-amyloid-induced apoptosis of cultured rat cortical neurons. *Brain Res* 842: 311–323.

Hashiguchi M., Sobue K., Paudel H.K. (2000). 14-3-3 zeta is an effector of tau protein phosphorylation. *J Biol Chem* 275: 25247–25254.

Hashimoto M., Bar-on P., Ho G., Takenouchi T., Rockenstein E., Crews L., Masliah E. (2004). beta-synuclein regulates Akt activity in neuronal cells: A possible mechanism for neuroprotection in Parkinson's disease. *J Biol Chem* 279: 23622–23629.

He H., Chen M., Scheffler N.K., Gibson B.W., Spremulli L.L., Gottlieb R.A. (2001). Phosphorylation of mitochondrial elongation factor Tu in ischemic myocardium: Basis for chloramphenicol-mediated cardioprotection. *Circ Res* 89: 461–467.

He Z., Tessier-Lavigne M. (1997). Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. *Cell* 90: 739–751.

Hensley K., Aksenova M., Carney J.M., Harris M., Butterfield D.A. (1995a). Amyloid beta-peptide spin trapping. Part I: Peptide enzyme toxicity is related to free radical spin trap reactivity. *Neuroreport* 6: 489–492.

Hensley K., Hall N., Subramaniam R., Cole P., Harris M., Aksenov M., Aksenova M., Gabbita S.P., Wu J.F., Carney J.M. (1995b). Brain regional correspondence between Alzheimer's disease histopathology and biomarkers of protein oxidation. *J Neurochem* 65: 2146–2156.

Hernández F., Cuadros R., Avila J. (2004). Zeta 14-3-3 protein favours the formation of human tau fibrillar polymers. *Neurosci Lett* 357: 143–146.

Holzer M., Gartner U., Stobe A., Hartig W., Gruschka H., Bruckner M.K., Arendt T. (2002). Inverse association of Pin1 and tau accumulation in Alzheimer's disease hippocampus. *Acta Neuropathol (Berl)* 104: 471–481.

Howard B.J., Yatin S., Hensley K., Allen K.L., Kelly J.P., Carney J., Butterfield D.A. (1996). Prevention of hyperoxia-induced alterations in synaptosomal membrane-associated proteins by *N*-tert-butyl-alpha-phenylnitrone and 4-hydroxy- 2,2,6,6-tetramethylpiperidin-1-oxyl (Tempol). *J Neurochem* 67: 2045–2050.

Hu J., Akama K.T., Krafft G.A., Chromy B.A., Van Eldik L.J. (1998). Amyloid-beta peptide activates cultured astrocytes: Morphological alterations, cytokine induction and nitric oxide release. *Brain Res* 785: 195–206.

Hurst J.K., Barrette W.C. Jr. (1989). Leukocytic oxygen activation and microbicidal oxidative toxins. *Crit Rev Biochem Mol Biol* 24: 271–328.

Ingelsson M., Fukumoto H., Newell K.L., Growdon J.H., Hedley-Whyte E.T., Frosch M.P., Albert M.S., Hyman B.T., Irizarry M.C. (2004). Early Abeta accumulation and progressive synaptic loss, gliosis, and tangle formation in AD brain. *Neurology* 62: 925–931.

Jayarapu K., Griffin T.A. (2004). Protein–protein interactions among human 20S proteasome subunits and proteassemblin. *Biochem Biophys Res Commun* 314: 523–528.

Jensen O.N., Wilm M., Shevchenko A., Mann M. (1999). Sample preparation methods for mass spectrometric peptide mapping directly from 2-DE gels. *Methods Mol Biol* 112: 513–530.

Kaldis P., Furter R., Wallimann T. (1994). The *N*-terminal heptapeptide of mitochondrial creatine kinase is important for octamerization. *Biochemistry* 33: 952–959.

Kato K., Kurobe N., Suzuki F., Morishita R., Asano T., Sato T., Inagaki T. (1991). Concentrations of several proteins characteristic of nervous tissue in cerebral cortex of patients with Alzheimer's disease. *J Mol Neurosci* 3: 95–99.

Kato Y., Hamajima N., Inagaki H., Okamura N., Koji T., Sasaki M., Nonaka M. (1998). Post-meiotic expression of the mouse dihydropyrimidinase-related protein 3 (DRP-3) gene during spermiogenesis. *Mol Reprod Dev* 51: 105–111.

Katzman R. (2000). Epidemilogy of Alzheimer's disease. *Neurobiol Aging* 21: S1.

Katzman R., Saitoh T. (1991). Advances in Alzheimer's disease. *FASEB J* 5: 278–286.

Kawamata T., Akiguchi I., Maeda K., Tanaka C., Higuchi K., Hosokawa M., Takeda T. (1998). Age-related changes in the brains of senescence-accelerated mice (SAM): Association with glial and endothelial reactions. *Microsc Res Tech* 43: 59–67.

Keller A., Berod A., Dussaillant M., Lamande N., Gros F., Lucas M. (1994). Coexpression of alpha and gamma enolase genes in neurons of adult rat brain. *J Neurosci Res* 38: 493–504.

Keller J.N., Dimayuga E., Chen Q., Thorpe J., Gee J., Ding Q. (2004). Autophagy, proteasomes, lipofuscin, and oxidative stress in the aging brain. *Int J Biochem Cell Biol* 36: 2376–2391.

Keller J.N., Hanni K.B., Markesbery W.R. (2000). Impaired proteasome function in Alzheimer's disease. *J Neurochem* 75: 436–439.

Kim S.H., Fountoulakis M., Cairns N., Lubec G. (2001a). Protein levels of human peroxiredoxin subtypes in brains of patients with Alzheimer's disease and Down syndrome. *J Neural Transm Suppl* 61: 223–235.

Kim S.H., Vlkolinsky R., Cairns N., Fountoulakis M., Lubec G. (2001b). The reduction of NADH ubiquinone oxidoreductase 24- and 75-kDa subunits in brains of patients with Down syndrome and Alzheimer's disease. *Life Sci* 68: 2741–2750.

Kim S.H., Vlkolinsky R., Cairns N., Lubec G. (2000). Decreased levels of complex III core protein 1 and complex V beta chain in brains from patients with Alzheimer's disease and Down syndrome. *Cell Mol Life Sci* 57: 1810–1816.

Kolodkin A.L., Levengood D.V., Rowe E.G., Tai Y.T., Giger R.J., Ginty D.D. (1997). Neuropilin is a semaphorin III receptor. *Cell* 90: 753–762.

Kouchi Z., Sorimachi H., Suzuki K., Ishiura S. (1999). Proteasome inhibitors induce the association of Alzheimer's amyloid precursor protein with Hsc73. *Biochem Biophys Res Commun* 254: 804–810.

Krapfenbauer K., Engidawork E., Cairns N., Fountoulakis M., Lubec G. (2003). Aberrant expression of peroxiredoxin subtypes in neurodegenerative disorders. *Brain Res* 967: 152–160.

Kumar V.B., Farr S.A., Flood J.F., Kamlesh V., Franko M., Banks W.A., Morley J.E. (2000). Site-directed antisense oligonucleotide decreases the expression of amyloid precursor protein and reverses deficits in learning and memory in aged SAMP8 mice. *Peptides* 21: 1769–1775.

Kurt M.A., Davies D.C., Kidd M., Duff K., Howlett D.R. (2003). Hyperphosphorylated tau and paired helical filament-like structures in the brains of mice carrying mutant amyloid precursor protein and mutant presenilin-1 transgenes. *Neurobiol Dis* 14: 89–97.

Lamprecht R., LeDoux J. (2004). Structural plasticity and memory. *Nat Rev Neurosci* 5: 45–54.

Lauderback C.M., Hackett J.M., Huang F.F., Keller J.N., Szweda L.I., Markesbery W.R., Butterfield D.A. (2001). The glial glutamate transporter, GLT-1, is oxidatively modified by 4-hydroxy-2-nonenal in the Alzheimer's disease brain: The role of Abeta1–42. *J Neurochem* 78: 413–416.

Layfield R., Fergusson J., Aitken A., Lowe J., Landon M., Mayer R. (1996). Neurofibrillary tangles of Alzheimer's disease brains contain 14-3-3 proteins. *Neurosci Lett* 209: 57–60.

Lee H.G., Zhu X., Ghanbari H.A., Ogawa O., Raina A.K., O'Neill M.J., Perry G., Smith M.A. (2002). Differential regulation of glutamate receptors in Alzheimer's disease. *Neurosignals* 11: 282–292.

Leiers B., Kampkotter A., Grevelding C.G., Link C.D., Johnson T.E., Henkle-Duhrsen K. (2003). A stress-responsive glutathione *S*-transferase confers resistance to oxidative stress in *Caenorhabditis elegans*. *Free Radic Biol Med* 34: 1405–1415.

Leto T.L., Fortugno-Erikson D., Barton D., Yang-Feng T.L., Francke U., Harris A.S., Morrow J.S., Marchesi V.T., Benz E.J. Jr. (1988). Comparison of nonerythroid alpha-spectrin genes reveals strict homology among diverse species. *Mol Cell Biol* 8: 1–9.

Levine R.L. (1983). Oxidative modification of glutamine synthetase. Part II: Characterization of the ascorbate model system. *J Biol Chem* 258: 11828–11833.

Li J.Y., Henning Jensen P., Dahlstrom A. (2002). Differential localization of α-, β-, and γ-synucleins in the rat CNS. *Neuroscience* 113: 463–478.

Link C.D. (1995). Expression of human beta-amyloid peptide in transgenic *Caenorhabditis elegans*. *Proc Natl Acad Sci USA* 92: 9368–9372.

Link C.D., Johnson C.J., Fonte V., Paupard M., Hall D.H., Styren S., Mathis C.A., Klunk W.E. (2001). Visualization of fibrillar amyloid deposits in living, transgenic *Caenorhabditis elegans* animals using the sensitive amyloid dye, X-34. *Neurobiol Aging* 22: 217–226.

Link C.D., Taft A., Kapulkin V., Duke K., Kim S., Fei Q., Wood D.E., Sahagan B.G. (2003). Gene expression analysis in a transgenic *Caenorhabditis elegans* Alzheimer's disease model. *Neurobiol Aging* 24: 397–413.

listed, N.a. (1998). Consensus report of the Working Group on: "Molecular and Biochemical Markers of Alzheimer's Disease." The Ronald and Nancy Reagan Research Institute of the Alzheimer's Association and the National Institute on Aging Working Group. *Neurobiol Aging* 19: 109–116.

Lovell M.A., Xie C., Markesbery W.R. (1998). Decreased glutathione transferase activity in brain and ventricular fluid in Alzheimer's disease. *Neurology* 51: 1562–1566.

Lu Q., Ceddia M.A., Price E.A., Ye S.M., Woods J.A. (1999). Chronic exercise increases macrophage-mediated tumor cytolysis in young and old mice. *Am J Physiol* 276: R482–R489.

Lubec G., Nonaka M., Krapfenbauer K., Gratzer M., Cairns N., Fountoulakis M. (1999). Expression of the dihydropyrimidinase related protein 2 (DRP-2) in Down syndrome and Alzheimer's disease brain is downregulated at the mRNA and dysregulated at the protein level. *J Neural Transm Suppl* 57: 161–177.

Luo Y., Raible D., Raper J.A. (1993). Collapsin: A protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell* 75: 217–227.

Manczak M., Park B.S., Jung Y., Reddy P.H. (2004). Differential expression of oxidative phosphorylation genes in patients with Alzheimer's disease: Implications for early mitochondrial dysfunction and oxidative damage. *Neuromolecular Med* 5: 147–162.

Mark R.J., Lovell M.A., Markesbery W.R., Uchida K., Mattson M.P. (1997). A role for 4-hydroxynonenal, an aldehydic product of lipid peroxidation, in disruption of ion homeostasis and neuronal death induced by amyloid beta-peptide. *J Neurochem* 68: 255–264.

Markesbery W.R., Lovell M.A. (1998). Four-hydroxynonenal, a product of lipid peroxidation, is increased in the brain in Alzheimer's disease. *Neurobiol Aging* 19: 33–36.

Masliah E., Alford M., DeTeresa R., Mallory M., Hansen L. (1996). Deficient glutamate transport is associated with neurodegeneration in Alzheimer's disease. *Ann Neurol* 40: 759–766.

Masliah E., Mallory M., Hansen L., DeTeresa R., Alford M., Terry R. (1994). Synaptic and neuritic alterations during the progression of Alzheimer's disease. *Neurosci Lett* 174: 67–72.

Mattson M.P. (2004). Pathways towards and away from Alzheimer's disease. *Nature* 430: 631–639.

Mattson M.P., Partin J., Begley J.G. (1998). Amyloid beta-peptide induces apoptosis-related events in synapses and dendrites. *Brain Res* 807: 167–176.

McKhann G., Drachman D., Folstein M., Katzman R., Price D., Stadlan E.M. (1984). Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* 34: 939–944.

Meda L., Cassatella M.A., Szendrei G.I., Otvos L. Jr., Baron P., Villalba M., Ferrari D., Rossi F. (1995). Activation of microglial cells by beta-amyloid protein and interferon-gamma. *Nature* 374: 647–650.

Meier-Ruge W., Iwangoff P., Reichlmeier K. (1984). Neurochemical enzyme changes in Alzheimer's and Pick's disease. *Arch Gerontol Geriatr* 3: 161–165.

Messier C., Gagnon M. (1996). Glucose regulation and cognitive functions: Relation to Alzheimer's disease and diabetes. *Behav Brain Res* 75: 1–11.

Messier C., Gagnon M. (2000). Glucose regulation and brain aging. *J Nutr Health Aging* 4: 208–213.

Minturn J.E., Fryer H.J., Geschwind D.H., Hockfield S. (1995). TOAD-64, a gene expressed early in neuronal differentiation in the rat, is related to unc-33, a *C. elegans* gene involved in axon outgrowth. *J Neurosci* 15: 6757–6766.

Mohmmad Abdul H., Wenk G.L., Gramling M., Hauss-Wegrzyniak B., Butterfield D.A. (2004). APP and PS-1 mutations induce brain oxidative stress independent of dietary cholesterol: Implications for Alzheimer's disease. *Neurosci Lett* 368: 148–150.

Molloy M.P., Witzmann F.A. (2002). Proteomics: Technologies and applications. *Brief Funct Genomic Proteomic* 1: 23–39.

Morgan T.E., Rozovsky I., Goldsmith S.K., Stone D.J., Yoshida T., Finch C.E. (1997). Increased transcription of the astrocyte gene GFAP during middle-age is attenuated by food restriction: Implications for the role of oxidative stress. *Free Radic Biol Med* 23: 524–528.

Morley J.E., Kumar V.B., Bernardo A.E., Farr S.A., Uezu K., Tumosa N., Flood J.F. (2000). Beta-amyloid precursor polypeptide in SAMP8 mice affects learning and memory. *Peptides* 21: 1761–1767.

Morris C.M., Wilson K.E. (2004). High throughput approaches in neuroscience. *Int J Dev Neurosci* 22: 515–522.

Mosser D.D., Caron A.W., Bourget L., Denis-Larose C., Massie B. (1997). Role of the human heat shock protein hsp70 in protection against stress-induced apoptosis. *Mol Cell Biol* 17: 5317–5327.

Muir J.L. (1997). Acetylcholine, aging, and Alzheimer's disease. *Pharmacol Biochem Behav* 56: 687–696.

Nagele R.G., Wegiel J., Venkataraman V., Imaki H., Wang K.C. (2004). Contribution of glial cells to the development of amyloid plaques in Alzheimer's disease. *Neurobiol Aging* 25: 663–674.

Neves S.R., Ram P.T., Iyengar R. (2002). G protein pathways. *Science* 296: 1636–1639.

Ozaki M., Haga S., Zhang H.Q., Irani K., Suzuki S. (2003). Inhibition of hypoxia/reoxygenation-induced oxidative stress in HGF-stimulated antiapoptotic signaling: role of PI3-K and Akt kinase upon rac1. *Cell Death Differ* 10: 508–515.

Perluigi M., Poon H.F., Hensley K., Pierce W.M., Klein J.B., Calabrese V., De Marco C., Butterfield D.A. (2005) Proteomic analysis of 4-hydroxy-2-nonenal-modified proteins in G93A-SOD1 transgenic mice—A model of familial amyotrophic lateral sclerosis. *Free Radic Biol Med* 38: 960–968.

Planel E., Miyasaka T., Launey T., Chui D.H., Tanemura K., Sato S., Murayama O., Ishiguro K., Tatebayashi Y., Takashima A. (2004). Alterations in glucose metabolism induce hypothermia leading to tau hyperphosphorylation through differential inhibition of kinase and phosphatase activities: Implications for Alzheimer's disease. *J Neurosci* 24: 2401–2411.

Poon H.F., Calabrese V., Scapagnini G., Butterfield D.A. (2004a). Free radicals and brain aging. *Clin Geriatr Med* 20: 329–359.

Poon H.F., Calabrese V., Scapagnini G., Butterfield D.A. (2004b). Free radicals: Key to brain aging and heme oxygenase as a cellular response to oxidative stress. *J Gerontol A Biol Sci Med Sci* 59: 478–493.

Poon H.F., Castegna A., Farr S.A., Thongboonkerd V., Lynn B.C., Banks W.A., Morley J.E., Klein J.B., Butterfield D.A. (2004c). Quantitative proteomics analysis of specific protein expression and oxidative modification in aged senescence-accelerated-prone 8 mice brain. *Neuroscience* 126: 915–926.

Poon H.F., Joshi G., Sultana R., Farr S.A., Banks W.A., Morley J.E., Calabrese V., Butterfield D.A. (2004d). Antisense directed at the A-beta region of APP decreases brain oxidative markers in aged senescence accelerated mice. *Brain Res* 1018: 86–96.

Poon H.F., Farr S.A., Banks W.A., Pierce W.M., Klein J.B., Morley J.E., Butterfield D.A. (2005). Proteomic identification of less oxidized brain proteins in aged senescence accelerated mice following administration antisense oligonucleotide directed at the Aβ region of amyloid precursor protein. *Brain Res Molec Brain Res* 138: 8–16.

Poon H.F., Farr S.A., Thongboonkerd V., Lynn B.C., Banks W.A., Morley J.E., Klein J.B., Butterfield D.A. (2005a). Proteomic analysis of specific brain proteins in aged SAMP8 mice treated with alpha-lipoic acid: Implications for aging and age-related neurodegenerative disorders. *Neurochem Int* 46: 159–168.

Poon H.F., Frasier M., Shreve N., Calabrese V., Wolozin B., Butterfield D.A. (2005b). Mitochondrial associated metabolic proteins are selectively oxidized in A30P α-synuclein transgenic mice—A model of familial Parkinson's disease. *Neurobiol Dis* 18: 492–428.

Porchet R., Probst A., Bouras C., Draberova E., Draber P., Riederer B.M. (2003). Analysis of glial acidic fibrillary protein in the human entorhinal cortex during aging and in Alzheimer's disease. *Proteomics* 3: 1476–1485.

Prasad M.R., Lovell M.A., Yatin M., Dhillon H., Markesbery W.R. (1998). Regional membrane phospholipid alterations in Alzheimer's disease. *Neurochem Res* 23: 81–88.

Ramakrishnan P., Dickson D.W., Davies P. (2003). Pin1 colocalization with phosphorylated tau in Alzheimer's disease and other tauopathies. *Neurobiol Dis.* 14: 251–264.

Reeves E.P., Dekker L.V., Forbes L.V., Wientjes F.B., Grogan A., Pappin D.J., Segal A.W. (1999). Direct interaction between p47phox and protein kinase C: Evidence for targeting of protein kinase C by p47phox in neutrophils. *Biochem J* 344: 859–866.

Righetti P.G., Castagna A., Antonucci F., Piubelli C., Cecconi D., Campostrini N., Antonioli P., Astner H., Hamdan M. (2004). Critical survey of quantitative proteomics in two-dimensional electrophoretic approaches. *J Chromatogr A* 1051: 3–17.

Rivett A.J., Levine R.L. (1990). Metal-catalyzed oxidation of *Escherichia coli* glutamine synthetase: Correlation of structural and functional changes. *Arch Biochem Biophys* 278: 26–34.

Rosenberg R.N. (2000). The molecular and genetic basis of AD: The end of the beginning: The 2000 Wartenberg lecture. *Neurology* 54: 2045–2054.

Ross G.W., O'Callaghan J.P., Sharp D.S., Petrovitch H., Miller D.B., Abbott R.D., Nelson J., Launer L.J., Foley D.J., Burchfiel C.M., Hardman J., White L.R. (2003). Quantification of regional glial fibrillary acidic protein levels in Alzheimer's disease. *Acta Neurol Scand* 107: 318–323.

Sahlas D.J., Liberman A., Schipper H.M. (2002). Role of heme oxygenase-1 in the biogenesis of corpora amylacea. *Biogerontology* 3: 223–231.

Sarafian T.A., Verity M.A., Vinters H.V., Shih C.C., Shi L., Ji X.D., Dong L., Shau H. (1999). Differential expression of peroxiredoxin subtypes in human brain cell types. *J Neurosci Res* 56: 206–212.

Schagger H., Ohm T.G. (1995). Human diseases with defects in oxidative phosphorylation. 2: F_1F_0 ATP-synthase defects in Alzheimer disease revealed by blue native polyacrylamide gel electrophoresis. *Eur J Biochem* 227: 916–921.

Schapira A.H. (1998). Mitochondrial dysfunction in neurodegenerative disorders. *Biochim Biophys Acta* 1366: 225–233.

Scheff S.W., Price D.A. (2003). Synaptic pathology in Alzheimer's disease: A review of ultrastructural studies. *Neurobiol Aging* 24: 1029–1046.

Scheltens P., Korf E.S. (2000). Contribution of neuroimaging in the diagnosis of Alzheimer's disease and other dementias. *Curr Opin Neurol* 13: 391–396.

Schlegel J., Wyss M., Eppenberger H.M., Wallimann T. (1990). Functional studies with the octameric and dimeric form of mitochondrial creatine kinase: Differential pH-dependent association of the two oligomeric forms with the inner mitochondrial membrane. *J Biol Chem* 265: 9221–9227.

Schonberger S.J., Edgar P.F., Kydd R., Faull R.L., Cooper G.J. (2001). Proteomic analysis of the brain in Alzheimer's disease: Molecular phenotype of a complex disease process. *Proteomics* 1: 1519–1528.

Schurr A., Payne R.S., Miller J.J., Rigor B.M. (1997a). Brain lactate is an obligatory aerobic energy substrate for functional recovery after hypoxia: Further in vitro validation. *J Neurochem* 69: 423–426.

Schurr A., Payne R.S., Miller J.J., Rigor B.M. (1997b). Brain lactate, not glucose, fuels the recovery of synaptic function from hypoxia upon reoxygenation: An in vitro study. *Brain Res* 744: 105–111.

Schutkowski M., Bernhardt A., Zhou X.Z., Shen M., Reimer U., Rahfeld J.U., Lu K.P., Fischer G. (1998). Role of phosphorylation in determining the backbone dynamics

of the serine/threonine-proline motif and Pin1 substrate recognition. *Biochemistry* 37: 5566–5575.

Shimano Y. (1998). [Studies on aging through analysis of the glucose metabolism related to the ATP—Production of the senescence accelerated mouse (SAM)]. *Hokkaido Igaku Zasshi* 73: 557–569.

Sly W.S., Hu P.Y. (1995). Human carbonic anhydrases and carbonic anhydrase deficiencies. *Ann Rev Biochem* 64: 375–401.

Soreghan B.A., Yang F., Thomas S.N., Hsu J., Yang A.J. (2003). High-throughput proteomic-based identification of oxidatively induced protein carbonylation in mouse brain. *Pharm Res* 20: 1713–1720.

Stadtman E.R. (1992). Protein oxidation and aging. *Science* 257: 1220–1224.

Stadtman E.R. (2001). Protein oxidation in aging and age-related diseases. *Ann NY Acad Sci* 928: 22–38.

Stenbeck G. (1998). Soluble NSF-attachment proteins. *Int J Biochem Cell Biol* 30: 573–577.

Stuhler K., Meyer H.E. (2004) MALDI: More than peptide mass fingerprints. *Curr Opin Mol Ther* 6: 239–248.

Subramaniam R., Roediger F., Jordan B., Mattson M.P., Keller J.N., Waeg G., Butterfield D.A. (1997). The lipid peroxidation product, 4-hydroxy-2-trans-nonenal, alters the conformation of cortical synaptosomal membrane proteins. *J Neurochem* 69: 1161–1169.

Sullivan P.G., Dragicevic N.B., Deng J.H., Bai Y., Dimayuga E., Ding Q., Chen Q., Bruce-Keller A.J., Keller J.N. (2004). Proteasome inhibition alters neural mitochondrial homeostasis and mitochondria turnover. *J Biol Chem* 279: 20699–20707.

Sultana R., Boyd-Kimball D., Poon H.F., Cai J., Pierce W.M., Klein J.B., Butterfield D.A. (2005a). Regional redox proteomics to identify oxidized proteins in Alzheimer's disease brain: A mechanistic approach to understand pathological and biochemical alterations in AD. Neurobiology of Aging, in press. PMID: 16271804.

Sultana R., Boyd-Kimball D., Poon H.F., Cai J., Pierce W.M., Klein J.B., Markesbery W.R., Zhou X.Z., Lu K.P., Butterfield D.A. (2005b). Oxidative modification and down-regulation of PIN-1 in Alzheimer's disease: a redox proteomics analysis. *Neurobiology of Aging*, in press: PMID: 15950321; doi:10.1016/j.neurobiolaging.

Sultana R., Butterfield D.A. (2004). Identification of oxidatively modified GST and MRP1 in Alzheimer's disease brain. *Neurochem Res* 29: 2215–2220.

Sultana R., Poon H.F., Cai J., Pierce W.M., Merchant M., Klein J.B., Markesbery W.R., Butterfield D.A. (2005c). Identification of nitrosatively modified proteins in AD brain using redox proteomics. Neurobiology of Disease, in press. PMID: 16378731.

Suzuki T., Kamidochi M., Inoue N., Kawamichi H., Yazawa Y., Furukohri T., Ellington W.R. (1999). Arginine kinase evolved twice: Evidence that echinoderm arginine kinase originated from creatine kinase. *Biochem J* 340: 671–675.

Takahashi Y. (2003). The 14-3-3 proteins: Gene, gene expression, and function. *Neurochem Res* 28: 1265–1273.

Takemura M., Nakamura S., Akiguchi I., Ueno M., Oka N., Ishikawa S., Shimada A., Kimura J., Takeda T. (1993). Beta/A4 proteinlike immunoreactive granular structures in the brain of senescence-accelerated mouse. *Am J Pathol* 142: 1887–1897.

Terry R.D., Masliah E., Salmon D.P., Butters N., DeTeresa R., Hill R., Hansen L.A., Katzman R. (1991). Physical basis of cognitive alterations in Alzheimer's disease: Synapse loss is the major correlate of cognitive impairment. *Ann Neurol* 30: 572–580.

Tholey G., Ledig M., Mandel P. (1982). Modifications in energy metabolism during the development of chick glial cells and neurons in culture. *Neurochem Res* 7: 27–36.

Tilleman E.H., de Castro S.M., Busch O.R., Bemelman W.A., van Gulik T.M., Obertop H., Gouma D.J. (2002). Diagnostic laparoscopy and laparoscopic ultrasound for staging of patients with malignant proximal bile duct obstruction. *J Gastrointest Surg* 6: 426–430; discussion 430–421.

Vanderklish P.W., Bahr B.A. (2000). The pathogenic activation of calpain: A marker and mediator of cellular toxicity and disease states. *Int J Exp Pathol* 81: 323–339.

Vanhanen M., Soininen H. (1998). Glucose intolerance, cognitive impairment and Alzheimer's disease. *Curr Opin Neurol* 11: 673–677.

Varadarajan S., Yatin S., Aksenova M., Butterfield D.A. (2000). Review: Alzheimer's amyloid beta-peptide-associated free radical oxidative stress and neurotoxicity. *J Struct Biol* 130: 184–208.

Verhage M., Maia A.S., Plomp J.J., Brussaard A.B., Heeroma J.H., Vermeer H., Toonen R.F., Hammer R.E., van den Berg T.K., Missler M., Geuze H.J., Sudhof T.C. (2000). Synaptic assembly of the brain in the absence of neurotransmitter secretion. *Science* 287: 864–869.

Verwer R.W., Jansen K.A., Sluiter A.A., Pool C.W., Kamphorst W., Swaab D.F. (2000). Decreased hippocampal metabolic activity in Alzheimer patients is not reflected in the immunoreactivity of cytochrome oxidase subunits. *Exp Neurol* 163: 440–451.

Wallimann T., Wyss M., Brdiczka D., Nicolay K., Eppenberger H.M. (1992). Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: The 'phosphocreatine circuit' for cellular energy homeostasis. *Biochem J* 281: 21–40.

Wang H.Y., Lee D.H., D'Andrea M.R., Peterson P.A., Shank R.P., Reitz A.B. (2000). Beta-amyloid (1–42) binds to alpha7 nicotinic acetylcholine receptor with high affinity. Implications for Alzheimer's disease pathology. *J Biol Chem* 275: 5626–5632.

Wang L.H., Strittmatter S.M. (1996). A family of rat CRMP genes is differentially expressed in the nervous system. *J Neurosci* 16: 6197–6207.

Weitzdoerfer R., Fountoulakis M., Lubec G. (2002). Reduction of actin-related protein complex 2/3 in fetal Down syndrome brain. *Biochem Biophys Res Commun* 293: 836–841.

Wellman C.L., Sengelaub D.R. (1991). Cortical neuroanatomical correlates of behavioral deficits produced by lesion of the basal forebrain in rats. *Behav Neural Biol* 56: 1–24.

Wyss M., Smeitink J., Wevers R.A., Wallimann T. (1992). Mitochondrial creatine kinase: A key enzyme of aerobic energy metabolism. *Biochim Biophys Acta* 1102: 119–166.

Xie C., Lovell M.A., Markesbery W.R. (1998). Glutathione transferase protects neuronal cultures against four hydroxynonenal toxicity. *Free Radic Biol Med* 25: 979–988.

Yatin S.M., Aksenov M., Butterfield D.A. (1999a). The antioxidant vitamin E modulates amyloid beta-peptide-induced creatine kinase activity inhibition and increased protein oxidation: Implications for the free radical hypothesis of Alzheimer's disease. *Neurochem Res* 24: 427–435.

Yatin S.M., Varadarajan S., Link C.D., Butterfield D.A. (1999b). In vitro and in vivo oxidative stress associated with Alzheimer's amyloid beta-peptide (1–42). *Neurobiol Aging* 20: 325–330; discussion 339–342.

Yoo B.C., Kim S.H., Cairns N., Fountoulakis M., Lubec G. (2001). Deranged expression of molecular chaperones in brains of patients with Alzheimer's disease. *Biochem Biophys Res Commun* 280: 249–258.

Yoshida H., Watanabe A., Ihara Y. (1998). Collapsin response mediator protein-2 is associated with neurofibrillary tangles in Alzheimer's disease. *J Biol Chem* 273: 9761–9768.

Yu H.L., Chertkow H.M., Bergman H., Schipper H.M. (2003). Aberrant profiles of native and oxidized glycoproteins in Alzheimer plasma. *Proteomics* 3: 2240–2248.

Zheng W.H., Bastianetto S., Mennicken F., Ma W., Kar S. (2002). Amyloid beta peptide induces tau phosphorylation and loss of cholinergic neurons in rat primary septal cultures. *Neuroscience* 115: 201–211.

Zhou G., Somasundaram T., Blanc E., Parthasarathy G., Ellington W.R., Chapman M.S. (1998). Transition state structure of arginine kinase: Implications for catalysis of bimolecular reactions. *Proc Natl Acad Sci USA* 95: 8449–8454.

19

OXIDIZED PROTEINS IN CARDIAC ISCHEMIA AND REPERFUSION

JONATHAN P. BRENNAN AND PHILIP EATON

19.1 INTRODUCTION TO CARDIAC ISCHEMIA AND REPERFUSION

Ischemic heart disease is the most common cause of death in the world accounting for over seven million deaths worldwide, and over 23% of deaths in the developed world per annum according to figures published by the World Health Organization. In this chapter we review the evidence for oxidative stress or pro-oxidizing conditions mediating injury during, as well as adaptation to, myocardial ischemia and reperfusion. The molecular targets of the oxidative stress are discussed, with a particular focus on the important role of protein oxidation.

19.1.1 Definition and Consequences of Ischemia and Reperfusion

As Hearse (1994) highlighted in his editorial addressing the definition of ischemia, it is not a straightforward issue. Having sought the opinion of 33 eminent cardiologists, he received replies "rich in diversity," that ranged from three to four hundred and four words. It was concluded that, "biochemical ischemia perhaps can be most simply defined as a condition in which coronary blood flow is inadequate to permit the maintenance of a steady state metabolism." And that, "physiological ischemia, can be defined as a condition in which coronary flow is inadequate to permit the organ to perform at a level sufficient to support the body over its full physiological range of activity." Simply, myocardial ischemia is an inadequate blood supply to the heart. Clinically ischemia is most often caused by narrowed, partial or

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

complete, blockage of one or more coronary arteries as a result of atherosclerosis, although ischemia can also occur in other settings, such as during cardiac corrective surgery or heart transplantation. This reduced blood flow results in a decreased supply of oxygen and nutrients, and an accumulation of metabolic end-products.

Reperfusion is the re-establishment of blood flow to ischemic tissue and is associated with procedures such as thrombolysis, angioplasty and coronary bypass surgery. In the setting of a "heart attack" it is essential that the myocardium is reperfused adequately and expeditiously, so to limit injury due to ischemia. In some circumstances the consequences of cardiac ischemia are not acute or extreme, and so can be remedied by elective procedures that revascularize the tissue to restore blood flow (reperfusion) and prevent ischemia. Although reperfusion is essential for survival of severely ischemic tissue, it is known that reperfusion itself may increase injury over and above that sustained during ischemia (Tennant and Wiggers, 1935). This mode of damage has attached a lot of attention because it is theoretically amenable to therapies administered simultaneously with revascularization procedures.

Ischemia and reperfusion results in cardiac injury ranging from short-term reversible injury to cell necrosis and infarction. The crucial consequence of ischemia and reperfusion is the inability of the heart to pump blood effectively, which of course has critical implications for patient survival. The extent of injury following ischemia and reperfusion is a multifactorial and complex problem, but it is particularly dependent on the duration and the "depth" of the ischemic episode. Severely ischemic tissue will ultimately become necrotic, and its survival is dependent on adequate reperfusion. Although necrosis likely accounts for the majority of tissue death during the "heart attack" scenario, apoptosis may have a role to play, particularly in the setting of long-term, low-grade ischemia. The electromechanical function of the heart is also compromised during ischemia, and this can be further exacerbated by reperfusion injury. Both ischemia and reperfusion may result in loss of the regular heart beat (arrhythmias), and there is significant evidence for a causal role of oxidative stress in this phenomenon, particularly with regard to reperfusion-induced arrhythmias. Furthermore, contractility can be severely depressed in ischemic tissue and is related to the extent of blood flow restriction, with many contributing factors explaining its occurrence, including lack of ATP, myofilament desensitization due to acidosis, or adaptive responses such as hibernation. In addition contractility can remain depressed following full reperfusion even in the absence of an infarct, a phenomenon known as myocardial stunning, with oxidative stress being a crucial component of this mechanism of dysfunction.

An in-depth review of the pathophysiology of cardiac ischemia and reperfusion is beyond the scope of this text, but many reviews are available (Hearse, 1990, 1992; Hearse and Bolli, 1992). One common and recurrent theme in cardiac ischemia-reperfusion research is the potentially damaging role of oxidative stress. However, a growing number of more contemporary studies also document the importance of oxidant species in adaptive events that can actually limit injury

during ischemia and reperfusion, particularly in the setting of cardioprotection involving ischemic preconditioning.

19.1.2 Oxidative Stress and Injury during Ischemia and Reperfusion

Oxidative stress is a change in the balance between oxidants and antioxidants toward a pro-oxidizing environment, whether this is due to increased formation of reactive oxygen species (ROS) or a reduction in the antioxidant defenses (see Fig. 19.1). Oxidative stress and ROS production in cell and cardiovascular biology were once almost exclusively considered in terms of injury, disease, and cellular dysfunction. The rationale was that the uncontrolled, aberrant, or overproduction of oxidants targeted the various cellular components, including lipids, nucleic acids, and proteins to induce an oxidative alteration. This oxidation compromised the molecular function of these biomolecules, which could ultimately irreversibly damage the cell, tissue, or organ. Oxidative stress has been implicated in most major diseases including those of the cardiovascular system.

In recent times, however, there has also been a growing recognition that oxidative stress should not solely be considered in the context of damage and dysfunction, as many contemporary studies show that oxidants are integral to normal function and control of signal transduction pathways. This, coupled with the fact that some oxidative modifications of proteins are reversible, has led to the recognition that the post-translational oxidative modification of proteins can regulate protein function in a specific manner. Therefore in this situation it may be inappropriate to use the term *oxidant stress*, although this term is used widely throughout the literature. An extensive number of studies have contributed evidence supporting a role for oxidative stress in the myocardial injury caused by ischemia-reperfusion. In general, this evidence has three components: (1) enhanced oxidative stress can be measured in the heart during ischemia and reperfusion, (2) antioxidant interventions can reduce injury caused by ischemia and reperfusion, and (3) the introduction of pro-oxidant species into the heart can cause dysfunction (arrhythmias, contractile depression) that is reminiscent of ischemia-reperfusion injury.

Enhanced Oxidative Stress during Ischemia and Reperfusion A variety of analytical approaches have shown that enhanced oxidative stress, normally interpreted as elevated ROS formation, occurs in the heart during ischemia and reperfusion. To generalize, these approaches have relied on the introduction of a reporter molecule into the tissue that reacts (with varying specificity or selectivity) with oxidants in the system to generate a signal that can be quantified. Commonly utilized methodologies in the detection of oxidized sensor molecules that report redox status include fluorescence, luminescence, and electron spin resonance (ESR) spectroscopy, as well as HPLC. One general complication with the use of these reporter molecules is that they are often used in a setting of depleted antioxidant status, such as occurs during ischemia, that reduces the tissues endogenous ability to reduce oxidant species. This antioxidant depletion may result in an increase of oxidant availability (which was not sequestered by

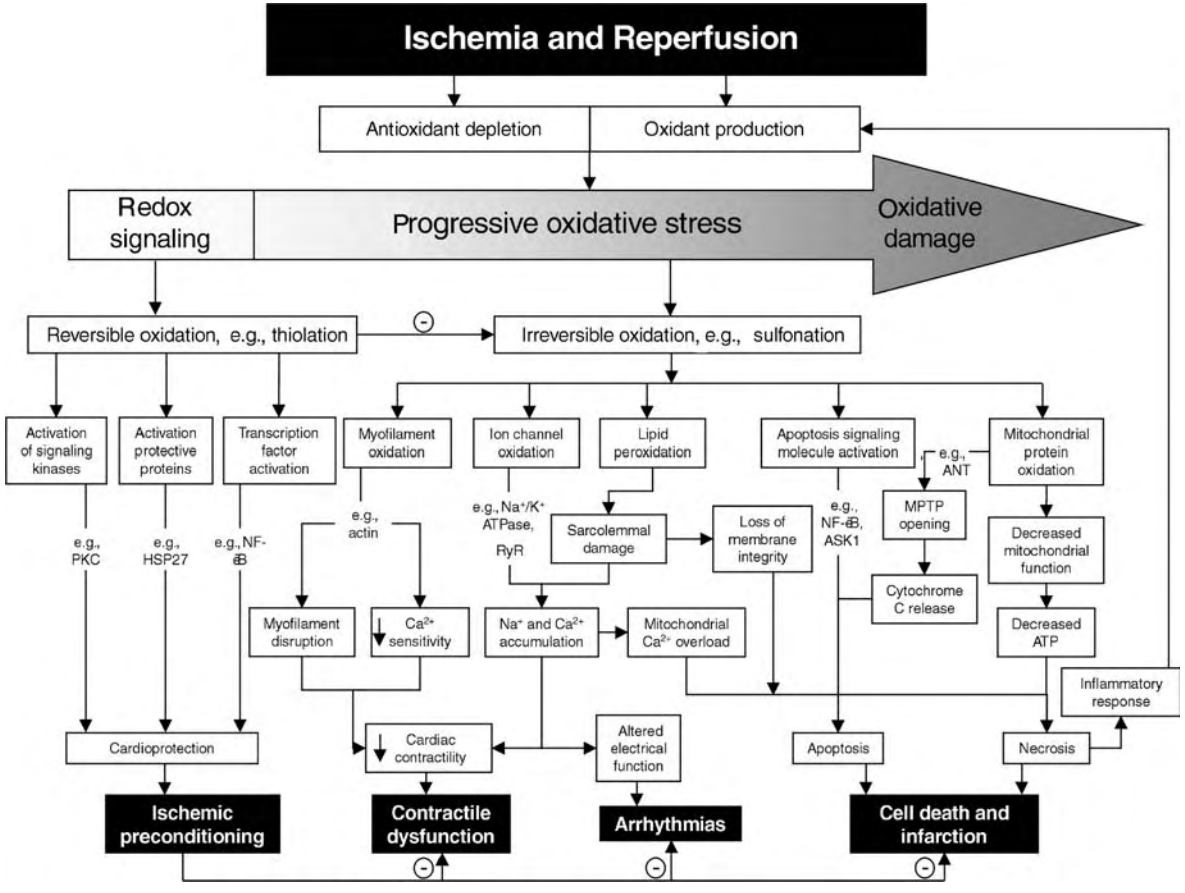


FIGURE 19.1 Schematic diagram showing how ischemia and reperfusion induces pro-oxidative changes in the heart, and how this can cause either injury or cardioprotective adaptation.

an antioxidant), allowing more oxidant to react with the reporter molecule. As highlighted above, this may potentially be interpreted as an increased oxidant concentration, whereas it more likely reflects a net increase in free or bioavailable oxidant. This determination of bioavailable oxidant may be the most relevant measure anyway. Other methods are available to measure oxidative stress that do not rely on introduction of reporter molecules, but instead directly measure the concentration of endogenous oxidant-sensitive biomolecules. There is an entire battery of such assays that measure the oxidation state of lipids, proteins, DNA, amino acids, and antioxidant molecules. From this extensive array of methods, evidence has accumulated (too much to cite in full here) that increased oxidant stress occurs both during ischemia (Arroyo et al., 1987; Maupoil et al., 1990; Grill et al., 1992; Premaratne et al., 1993; Timoshin et al., 1993, 1994; Vanden Hoek et al., 1997a) and during reperfusion (Garlick et al., 1987; Zweier et al., 1989; Bolli, 1991; Blasig et al., 1994; Vanden Hoek et al., 1997a).

The oxidant-sensitive fluorescent probe dichlorodihydrofluorescein (DCF) has been used to detect significant ROS production during simulated ischemia, which was exacerbated during simulated reperfusion in chick myocytes (Vanden Hoek et al., 1997a; Becker et al., 1999). Prior to this Garlick et al. (1987) had used *N*-tert-butyl-alpha-phenylnitrone (PBN), as a spin-trapping agent and electron spin resonance (ESR) spectroscopy, to demonstrate a burst of elevated free radical production during post-ischemic reperfusion. This radical formation peaked at four minutes and was dependent on molecular oxygen, as the increase was attenuated by anoxic reperfusion. Analysis of the ESR spectra highlighted the presence of a carbon-centered species or an alkoxyl radical. They concluded that these could be formed by secondary reactions of ROS, such as hydrogen peroxide or hydroxyl radicals, with membrane lipid components. Using a similar strategy, Zweier et al. (1989) measured ROS formation directly in isolated buffer perfused rabbit hearts during ischemia, and after reperfusion, suggesting that superoxide anion was the predominant oxidant produced.

Enhanced oxidative stress is often associated almost exclusively with reperfusion, and not ischemia. This may reflect the idea that the reduced oxygen supply limits oxygen-centered radical formation in the ischemic heart. As reported above, some studies do report elevated radical production during ischemia, but many studies have specifically focused on reperfusion, without making measurements of radical production during ischemia. This is often because their experimental methods are not amenable to the analysis of ischemia. For example, many spin trap experiments are reliant on the collection of coronary effluent, which is generally not available at this time. When methods that permit the direct measurement of ROS in ischemic hearts have been used, free radical production has been observed. Indeed there have even been reports of decreased radical production at reperfusion compared to ischemia (Maupoil et al., 1990; Timoshin et al., 1993).

The source of oxidants in cardiac ischemia and reperfusion also remains incompletely defined, and a full review of the literature is beyond the scope of this text. Briefly, significant attention has focused on mitochondria, and in particular, the electron transport chain where electrons are removed from NADH

or $FADH_2$ and transported through a chain of oxidation/reduction reactions until they are donated to molecular oxygen. Partially reduced and highly reactive metabolites of oxygen can be formed during these electron transfer reactions, particularly at complex III, as electrons can leak out and reduce O_2 to superoxide ($O_2^{-\bullet}$). Ischemia slows flow through the electron transport chain and therefore increases the time spent at the critical ubiquinol/semiquinone states at complex III, increasing likelihood of leakage of electrons and therefore the production of superoxide. Schumacker's group demonstrated that brief periods of hypoxia caused a transient increase in ROS generation in chick cardiomyocytes (Vanden Hoek et al., 1998). They attributed this increase in ROS to come predominantly from mitochondria as myxothiazol (an inhibitor of site III on the electron transport chain) attenuated the production, while NAD(P)H oxidase or nitric oxide synthase inhibitors had no effect. Other potential sources of oxidant production include neutrophils and xanthine oxidase. A number of studies suggest that neutrophils may be an important source of ROS during cardiac ischemia-reperfusion as depletion of neutrophils (Hernandez et al., 1987; Romson et al., 1983) and blocking of neutrophil–endothelial cell interaction (Hernandez et al., 1987) attenuate ischemia-reperfusion induced injury. However, ischemia-reperfusion injury has been demonstrated in Langendorff perfused hearts, a model devoid of neutrophils. The role of xanthine oxidase in ROS production is also controversial. Xanthine oxidase is a metalloflavoprotein, which is generated by a post-translational modification of xanthine dehydrogenase. It catalyzes oxidation of hypoxanthine to xanthine and xanthine to urate and requires the reduction of molecular O_2, generating $O_2^{-\bullet}$. Much evidence for xanthine oxidase as a source of ROS during ischemia-reperfusion comes from studies using its inhibitor allopurinol. For example, Brown et al. (1988) showed reperfusion of ischemic rat hearts resulted in elevated H_2O_2 levels and ventricular dysfunction, which could be partially attenuated by allopurinol. Similarly ischemic rabbit hearts pretreated with allopurinol experienced less ventricular dysfunction on reperfusion compared with untreated hearts (Terada et al., 1991). On the other hand, a variation in tissue and species distribution of xanthine oxidase calls into question the importance of this as a ubiquitous oxidant source in cardiac ischemia and reperfusion. Xanthine oxidase has been shown to be abundant in bovine and rat tissue, in particular but importantly is much lower in human tissue, and some studies have failed to identify xanthine oxidase protein or activity in human heart (Eddy et al., 1987; Linder et al., 1999; Downey et al., 1988).

As highlighted above, antioxidant depletion is also an important component of the net oxidative stress exerted on a tissue (see Fig. 19.1). A number of studies have shown loss of antioxidant molecules during cardiac ischemia and reperfusion (Guarnieri et al., 1979; Ferrari et al., 1985; Arduini et al., 1988). Haramaki et al. (1998) reported depletion of the hydrophilic antioxidants ascorbate and glutathione during reperfusion of isolated rat hearts, which did not occur during ischemia alone. The decrease in cellular glutathione during reperfusion may be explained by its export, as it accumulates in the coronary effluent (Lesnefsky et al., 1989; Rigobello and Bindoli, 1993). Tritto et al. (1998) also observed a

prominent release of oxidized glutathione disulfide (GSSG) into the coronary effluent at reperfusion. However, when hearts were treated with the antioxidant enzyme, superoxide dismutase, GSSG release was negligible and functional recovery was significantly increased, establishing a causal link between redox status and the pathogenesis of myocardial ischemia-reperfusion injury.

Protection by Antioxidants from Ischemia and Reperfusion Injury Over 20 years ago Ganote et al. (1982) added the free radical scavenger dimethyl sulfoxide to the post-ischemic rat heart, an intervention that decreased injury as measured by creatine kinase release and protection from contracture. Further evidence came from the studies of Jolly et al. (1984), who showed treatment with superoxide dismutase and catalase during reperfusion of canine hearts protected against infarction. Protection was only afforded if the antioxidants were present during initial reperfusion, and they were ineffective when administered 15 minutes into reperfusion. Similar observations were made by Vanden Hoek et al. (1997b) in isolated chick myocytes, where 2-mercaptopropionyl glycine (MPG) and 1–10-phenanthroline given throughout simulated ischemia and reperfusion significantly reduced cell death. Numerous other (too many to cite) antioxidant intervention studies have broadly made the same observations regarding antioxidant protection.

In addition, although not true chemical antioxidants, many studies have focused on the roles of disulfide reductase proteins such as the glutaredoxins and thioredoxins during cardiac ischemia-reperfusion. These proteins reduce oxidized cysteine groups on protein through an interaction with their redox-active centers (Cys-X-X-Cys) and are responsible for maintaining the intracellular environment in a reduced state. It has been shown that thioredoxin 1 expression can be induced by an oxidative stress (Prieto-Alamo et al., 2000) and has also been demonstrated to be released into the plasma in patients during reperfusion following open-heart surgery (Nakamura et al., 1998). Further to this, it has been shown that overexpression of thioredoxin 1 in heart-attenuated doxorubicin/adriamycin induced cardiotoxicity, an anticancer drug known to induce an oxidative stress that is cardiotoxic (Shioji et al., 2002). Also in an isolated perfused rat heart model thioredoxin protects against reperfusion-induced arrhythmias (Aota et al., 1996) and mouse hearts overexpressing thioredoxin 1 were resistant to ischemic injury (Turoczi et al., 2003) in a way analogous to ischemic preconditioning, which is discussed in more detail below. It has also been reported that transgenic mice overexpressing glutaredoxin 1 were more resistant to apoptotic death in the ischemic reperfused heart as well (Das, 2004). Clearly, as thioredoxin and glutaredoxin reduce protein disulfide bonds, and thioredoxin in particular has been shown to be protective during cardiac ischemia-reperfusion, this adds weight to the argument that protein oxidation plays a significant role in the injury and dysfunction associated with cardiac ischemia-reperfusion.

Oxidants Induce Cardiac Dysfunction The ability of oxidants to induce injury in many models of disease is well established. However, such studies often suffer the same general criticism, that unjustified or high amounts of oxidant are

used to initiate dysfunction, which raises issues regarding the pathophysiological relevance of the observations. Either way, oxidants introduced into the heart can cause contractile dysfunction (Miki et al., 1988; Przyklenk et al., 1990), electrical disturbances (Hearse et al., 1989), and obviously at high enough concentrations will even cause infarction.

19.1.3 Consequences of Ischemia and Reperfusion in the Heart

The evidence that oxidative stress accompanies cardiac ischemia and reperfusion is outlined above, but how this directly impacts on the function of the heart is more difficult to define. This complication arises because injury during ischemia and reperfusion has many contributing components, and it is difficult to identify the relative contribution of each factor. As highlighted below, profound biochemical and metabolic changes occur in the oxygen and nutrient deprived ischemic heart. This impacts significantly on the ionic homeostasis of the tissue, which directly compromises cardiac performance. However, oxidative stress clearly does occur during ischemia and reperfusion, and the evidence would suggest it is an important and causal mediator of dysfunction at this time (see Fig. 19.1 for a summary of the consequences of increasing oxidative stress in the heart during ischemia and reperfusion).

Metabolic Effects of Ischemia-Reperfusion With the onset of severe acute myocardial ischemia the supply of oxygen becomes insufficient to maintain oxidative phosphorylation, and anaerobic glycolysis takes over to provide high-energy phosphates (Jennings and Reimer, 1991). In an attempt to maintain the cellular level of ATP, flux through the glycolytic pathway is accelerated (Kubler and Spieckermann, 1970), leading to accumulation of glycolytic intermediates and NADH. In order to regenerate reserves of NAD^+ for continued glycolytic activity, pyruvate is reduced to lactate, which, combined with proton accumulation, leads to an acidotic environment. This acidosis may impact directly on enzyme activity, but it is also likely to decrease the reactivity of redox active protein thiol groups, as they are more reactive at alkaline pH, due to enhanced ionization of the thiol moiety. Eventually glycolysis halts, due to inhibitory acidosis and severe depletion of glycogen. In addition the inadequate synthesis of NADPH prevents glutathione reductase reducing GSSG to GSH. The loss of GSH compromises the ability of the heart to detoxify radical species; they then may oxidize cellular components, including proteins. In addition, as GSSG accumulates, it may undergo disulfide exchange reactions, resulting in accumulation of *S*-thiolated proteins (Eaton et al., 2003).

Role of Oxidative Stress in Ionic Homeostasis and Arrhythmias The depletion of ATP during ischemia leads to loss of many energy-dependent functions catalyzed by ATPases. This includes a number of ATP-dependent ion translocators, leading to a loss of ionic homeostasis. During ischemia Na^+ accumulates in cells, entering through Na^+ channels (van Emous et al., 1997), Na^+/H^+ exchange (Pike et al., 1990), $Na^+/K^+/2Cl^-$ cotransport (Rubin and Navon, 1993), with reduced

Na^+ extrusion via the Na^+/K^+ ATPase (Bersohn et al., 1982). This reduces the driving force for the Na^+/Ca^{2+} exchanger, leading to accumulation of intracellular Ca^{2+}. The consequences of intracellular Ca^{2+} accumulation are multiple, and include increased depolarization of the cell, anomalous activation of enzymes (including proteases), ischemic contracture, and mitochondrial damage, and together these are a major component of injury during ischemia and reperfusion. K^+ is also lost to the extracellular space during ischemia (Kleber, 1983; Fleet et al., 1985), which facilitates the development of arrhythmias at this time (Harris et al., 1954). In addition to effects mediated by ATP depletion, it has also been shown that ion channel and pump activity may be altered directly by the redox environment, which, as highlighted above, changes markedly during ischemia and reperfusion (Anzai et al., 2000; McStay et al., 2002; Rozanski and Xu, 2002). For example, the Na^+/K^+ ATPase is inhibited by oxidant stress during post-ischemic reperfusion (Kim and Akera, 1987; Kukreja et al., 1990) and this may be mediated by oxidation of protein thiol groups (Haddock et al., 1995). Other ion translocators with cysteines susceptible to oxidation include ryanodine receptors (Dulhunty et al., 2000), where oxidizing conditions have been shown to affect their Ca^{2+} sensitivity (Balshaw et al., 2001), and SERCA2A (Daiho et al., 2001). Furthermore the peroxidation of polyunsaturated fatty acids can alter membrane permeability, which can perturb the function of adjacent membrane proteins. It therefore becomes apparent that oxidative stress could play an integral role in the loss of ionic homeostasis during ischemia-reperfusion. Ionic disturbances disrupt the electrical activity and action potential of the myocyte. During ischemia the resting potential of the cell depolarizes due to the rapid changes in $[K]_o$, the amplitude of the action potential is reduced, and the length shortened by opening of K_{ATP} channels, while excitability and conduction velocity are also reduced (Kardesch et al., 1958). These changes can manifest themselves as arrhythmias such as ventricular premature beats (VPB), ventricular tachycardia (VT), or ventricular fibrillation (VF), all of which are characteristic of ischemia (Lubbe et al., 1978; Clark et al., 1980; Hashimoto et al., 1982; Walker et al., 1988) and have been suggested to be, to some extent, redox dependent. Nakaya et al. (1992) suggested that the oxidant-induced decrease in resting membrane potential and shortened action potential was due to decreased inward rectifier potassium current and slightly decreased calcium current and Tokube et al. (1998) have shown that K_{ATP} channels of guinea pig ventricular myocytes can be activated by a range of ROS. Moreover oxygen free radicals alone, in the absence of ischemia and reperfusion, are capable of initiating arrhythmias (Bernier et al., 1986; Hearse et al., 1989). Hearse et al. (1989) showed that Rose-Bengal derived oxidants can almost instantaneously alter the electrical function of the isolated perfused rat heart to induce arrhythmias. Furthermore the incidence of reperfusion arrhythmias can be attenuated by free radical scavengers (Bernier et al., 1986; Hearse and Bolli, 1992). Oxidants can manifest changes in ion translocator function on a millisecond time scale (Holmberg et al., 1991), which is consistent with the direct oxidation of proteinaceous components

such as thiol residues, and is unlikely to be explained by lipid peroxidation, due to the slower rate constants of such reactions.

This is not to say that ROS production is the sole cause of arrhythmias during ischemia and reperfusion, but it is evident that oxidative stress can have significant impact on ionic homeostasis, possibly by direct oxidation of ion translocators.

Oxidative Stress, Myocardial Contractility, and Stunning Reperfusion, after short periods of ischemia that do not induce necrosis, leads to a recovery of contractile response that is often tri-phasic. After a short period of initial contractile recovery, there is generally a significant decline, which slowly recovers over a period of days. This depressed contractile function with slow and complete recovery was described by Heyndrickx et al. (1975) and was later termed "stunning" by Braunwald and Kloner (1982). As with other consequences of ischemia and reperfusion, considered above, there is evidence to show oxidative stress is also an important mediator of stunning (Bolli and Marban, 1999). For example, a number of antioxidants such as SOD, catalase (Myers et al., 1985), dimethylthiourea (Bolli et al., 1987), and MPG (Myers et al., 1986) have been shown to attenuate myocardial stunning and enhance post-ischemic functional recovery. In addition a burst of free radical production at reperfusion has been demonstrated in "stunned" canine hearts (Bolli et al., 1988).

The mechanism whereby ROS depress contractile function remains unresolved, and is likely multifactorial. While the oxidative inhibition of ion channel proteins leading to Ca^{2+} accumulation is one factor that can lead to abnormal excitation–contraction coupling, another potential mechanism involves ROS damaging contractile proteins. Exposure of myofilaments to superoxide, for example, has been shown to cause significant force reduction in rat cardiac muscle (MacFarlane and Miller, 1992). Many myofilament proteins have also been shown to undergo proteolysis or be lost from ischemic tissue (Westfall and Solaro, 1992; Sato et al., 1993; Hein et al., 1994, 1995; Van Eyk et al., 1998; McDonough et al., 1999), and it has been demonstrated that oxidative modifications of proteins can render them more susceptible to proteolytic attack (Grune et al., 1997). More recently it has been shown that critical thiol groups of a number of myofilament proteins are selectively modified during ischemia or reperfusion, which may alter protein and therefore contractile function (Powell et al., 2001; Canton et al., 2004). Evidence for oxidative modification of specific cardiac proteins and the implications for dysfunction are considered in more detail below. A crucial role for the proteolytic cleavage of troponin I by calpain appears to be integral to the genesis of stunning (Gao et al., 1997; Murphy et al., 2000), but the precise details of the link between oxidative stress and protein degradation is not fully defined.

Cell Death and Ischemia and Reperfusion If the severity of ischemia endured by the heart is too great, it will lead to cell death, which is thought to primarily occur by necrosis. Cardiac cell death by apoptosis is known to occur in some circumstances but is likely relatively unimportant in the setting of severe, acute

ischemia, as occurs in the setting of a "heart attack." However, some evidence suggests that apoptosis is an important mediator of cell death following revascularization procedures where the tissue is reperfused. In this scenario, ischemia is thought to prime the tissue for apoptosis, and this is only unveiled if the tissue is re-energized by reperfusion.

A primary characteristic of necrosis is cell-swelling leading to membrane lysis, which in cardiac tissue rapidly leads to hypercontraction due to calcium flooding the cell. Although exogenously generated ROS are clearly able to damage cardiac myocytes to varying degrees, including the induction of necrosis, it remains unclear whether oxidant stress during ischemia and reperfusion is a major player in this event. Although there is evidence that antioxidant interventions can prevent necrosis (cited above), the general consensus is that perhaps this is not a major route leading to infarction. This contrasts stronger evidence for an integral role of oxidative stress during such events as arrhythmias and stunning.

Apoptosis, in contrast to necrosis, is an energy-dependent mechanism whereby cells undergo "programmed" death involving a controlled sequence of events. Traditionally this was not thought to be involved in ischemia-reperfusion induced cell death where necrotic death was thought to dominate. However, there is increasing evidence for apoptosis, particularly in cell death during reperfusion (Morita-Fujimura et al., 2001; Abbate et al., 2002; Zhao, 2004). The rationale here is that ATP is depleted during ischemia and apoptotic cell death can only proceed at reperfusion when some of the aerobic metabolic processes are recovered, as apoptosis is an energy-dependent process. In addition to the re-supply of energy, oxidative stress during reperfusion may contribute to the triggering of myocardial apoptosis. For example, apoptotic death can be reduced by antioxidant interventions (Galang et al., 2000), while exposure of myocytes to ROS generating systems triggered apoptosis via release of cytochrome C and activation of caspase 3 (von Harsdorf et al., 1999). Redox regulation of thioredoxin has been shown to alter its binding to the apoptosis signal-regulating kinase (ASK) 1. Under oxidative conditions thioredoxin is oxidized and releases ASK 1 to become activated, leading to activation of apoptotic signaling pathways (Saitoh et al., 1998). Therefore, antioxidant interventions that prevent apoptosis may function in part by attenuating thioredoxin oxidation.

Studies using isolated heart models of ischemia-reperfusion and cultured myocyte models using hypoxia or oxidative stress to induce apoptotic cell death have reported increased activation of the MAPK family members, p38 and JNK. It has also been proposed that direct activation of these kinases by ROS lead to the phosphorylation and translocation of NF-κB to the nucleus, where it binds the promoter region of TNFα to initiate cell death following oxidative stress (Meldrum, 1998). Other ROS-dependent mechanisms involving protein thiol oxidation have been implicated in the induction of apoptosis. For example, Cargnoni et al. (2002) showed NF-κB activation in isolated rat hearts during reperfusion, which was paralleled by a decrease in both GSH and protein thiols, with an accumulation of GSSG. Thiol redox status is also important in opening of the mitochondrial permeability transition pore. This complex is located

in the inner mitochondrial membrane and consists of the voltage-dependent anion channel (VDAC), the adenine nucleotide translocase (ANT), and cyclophilin D. When open, it is a nonselective channel permitting the transport of molecules less than 1.5 kDa in size, including cytochrome C, which, when released, can induce apoptosis. Opening of the pore occurs in conditions of high Ca^{2+} (Gunter and Pfeiffer, 1990), often combined with low ATP, elevated Pi, membrane depolarization, and oxidative stress (Crompton and Costi, 1988), all of which occur during early reperfusion of the ischemic heart. The mechanism leading to pore opening during reperfusion is an area of extensive research (reviewed by Halestrap and Brennerb, 2003). Recently it has been suggested that oxidation of a critical thiol on ANT may play a key role in enforcing pore opening and therefore apoptotic cell death (Costantini et al., 2000; McStay et al., 2002). In this regard ANT in cardiac tissue is susceptible to multiple forms of cysteine-targeted oxidation, including interprotein disulfide (Brennan et al., 2004) and sulfenic acid (Saurin et al., 2004) formation. In noncardiac cells GSH is released during apoptosis (van den Dobbelsteen et al., 1996), lowering the intracellular thiol reducing capacity, which exerts an oxidative stress that may be crucial to the completion of the apoptotic event.

19.2 OXIDATIVELY MODIFIED PROTEINS IN THE HEART

So far we have presented evidence for ROS-mediated injury during ischemia and reperfusion, and briefly addressed how oxidants can modify proteins, leading to alterations in function. There are an extensive array of oxidative modifications that proteins can undergo, which are dependent on the oxidant species, their concentration, and the surrounding environment, including the presence of antioxidants.

19.2.1 What Proteins Are Targeted by Oxidants and How Are They Modified?

Significant evidence supports a role for oxidative stress in the genesis of injury during ischemia and reperfusion. However, the molecular targets of this oxidative insult remain largely undefined, particularly in terms of the specific proteins involved and a molecular nature of the alterations.

Some of these oxidative modifications are irreversible and are generally associated with loss of protein function, leading to accumulation of damaged protein or their degradation. On the other hand, some oxidative reactions, particularly those at cysteinyl thiol groups, are reversible and therefore may have roles in cellular regulation or signal transduction (redox signaling) during oxidative conditions. In light of the evidence presented above, it is therefore attractive to postulate that oxidative modification of proteins play a causative role in a number of the manifestations of ischemia-reperfusion already outlined.

Protein Cysteinyl Thiols as Primary Targets Perhaps because they are the most vulnerable targets, the most studied mode of protein oxidation are those where

thiol groups on the cysteine residues are oxidized. A range of chemical species, including low molecular weight thiols, ROS, and RNS, can modify thiols. The resulting modifications include direct oxidation to sulfenic, sulfinic, sulfonic acid derivatives; inter- and intraprotein disulfide formation; *S*-thiolation by cysteine, glutathione, homocysteine, and other small thiols; *S*-nitrosylation by NO and its analogues such as nitrosoglutathione; as well as reactions with reactive oxidized lipid derivatives.

Cysteine residues directly oxidized by ROS such as hydrogen peroxide can form a sulfenic acid derivative (PSOH). This modification is known to be reversible, but it is unstable and can undergo further oxidation to form sulfinic (PSO_2H) and sulfonic (PSO_3H) acid derivatives. Until recently it was believed that these more oxidized derivatives (PSO_2H and PSO_3H) were irreversible modifications leading to loss of protein function. However, it has recently been demonstrated that the sulfinic form of some members of the 2-Cys peroxiredoxin family of enzymes can be reduced back to the free thiol by sulfiredoxin (Biteau et al., 2003; Woo et al., 2003). Some evidence suggests sulfiredoxin is specific for 2-Cys peroxiredoxins and is unable to reduce other proteins such as GAPDH-SO_2H (Woo et al., 2004), although the studies did not categorically exclude the possibility that other protein sulfinic acids are reduced by this enzyme.

Sulfenic acids are generally rapidly reduced by vicinal or accessible low molecular weight thiols, such as glutathione, to form intramolecular or mixed disulfide bonds, respectively. Protein sulfenate formation in redox signaling has recently been reviewed, and it is clear that this oxidative modification is important in the redox regulation of transcription factors, tyrosine phosphatases, members of the NAD(P)H-disulphide reductase family, cysteine-based peroxidases including peroxiredoxins, and methionine sulfoxide reductases (Claiborne et al., 1999; Choi et al., 2001; Poole et al., 2004). Recently we have reported widespread protein sulfenic acid formation within cardiac tissue when H_2O_2 is elevated (Saurin et al., 2004). However, no information is available regarding protein sulfenic acid formation during cardiac ischemia-reperfusion.

The formation of disulfide bonds between cysteine thiol groups is another oxidative modification that protein cysteines can undergo, and it has the potential to be an important regulatory mechanism. Disulfides can form in oxidative conditions between two proteins (interprotein) or within a protein (intraprotein), causing changes in protein aggregation and conformation. In line with this Cumming et al. have shown interprotein disulfide formation in the cytosolic fraction of a neuronal cell line subjected to pro-oxidative conditions induced by diamide, hydrogen peroxide, or glutamate treatment (Cumming et al., 2004). Also we have shown a significant number of proteins with diverse functions form intermolecular disulfides following oxidative stress in isolated cardiac myocytes (Brennan et al., 2004). Interprotein disulfide bonds have been shown to occur during cardiac ischemia and reperfusion, involving cross-links between actin and tropomyosin, an association that likely contributes to contractile dysfunction at this time (Powell et al., 2001; Canton et al., 2004).

The evidence for redox-modified intraprotein disulfide bonds is less clear, although it has been shown in prokaryotic cells that two intramolecular disulfide bonds are formed in the redox-sensitive chaperone Hsp33, leading to large conformational changes that increase its affinity for protein-folding intermediates and thus protect them from oxidative damage (Graumann et al., 2001; Linke and Jakob, 2003). It has been shown that cardiac troponin C is activated by the formation of an intraprotein disulfide between Cys35 and Cys84, rendering the protein Ca^{2+} independent (Putkey et al., 1993), and that catalytic activity of placental Na^{+}-H^{+} exchanger is dependent on vicinal dithiols (Kulanthaivel et al., 1990), raising the possibility that disulfide formation between them during oxidative stress may be inhibitory. Similarly thioredoxin contains critical vicinal thiols (Cys32 and Cys35). It acts by cycling between the oxidized and reduced form, which differ by the formation of an intraprotein disulfide between the two vicinal thiols. It is also directly involved in redox regulation of gene expression by promoting DNA binding of a number of transcription factors or activating MAPK pathways.

The activation of a number of transcription factors is also known to involve intraprotein disulfide bond formation. Redox-sensitive transcription factors have been studied in depth in prokaryotic cells, and a great deal is known about factors such as OxyR in relation to the mechanism of their oxidation-dependent regulation. Ground-breaking studies by Zheng et al. (1998), investigating the activation of OxyR transcription factor in *E. coli*, showed that hydrogen peroxide induced an intramolecular disulfide bond between two cysteine residues (Cys199–Cys208), resulting in a structural change in the protein that changed its DNA binding affinity. Similarly, again in bacterial cells, the Yap1 transcription factor has been shown to be activated by oxidation when hydrogen peroxide levels increase. This activation involves the glutathione peroxidase-like enzyme Gpx3 as both a sensor and transducer of the hydrogen peroxide signal to Yap1. When oxidized to sulfenic acid, Gpx3 Cys36 forms a disulfide bond with Yap1 Cys598. This intermolecular disulfide bond is then transformed into an intramolecular disulfide bond within YAP1, causing its activation (Delaunay et al., 2002).

In mammalian cells, and in particular in heart, much less is known about the specific mechanisms of transcription factor activation, although a number of established redox-controlled factors are known to be activated during cardiac ischemia and reperfusion, including NF-κB, AP-1, hypoxia-inducible factor 1α (HIF-1α), and heat-shock factor (HSF) (Guyton et al., 1996; Cargnoni et al., 2002; Chi and Karliner, 2004). Several studies have demonstrated NF-κB activation following ischemia and reperfusion in both animal and human studies (Morishita et al., 1997; Florens et al., 2001; Li et al., 2001; Valen et al., 2001; Cargnoni et al., 2002; Fan et al., 2002; Kukreja, 2002), although whether this is by direct oxidation of the transcription factor was not demonstrated. Activation can occur via two predominant pathways, activation of p38 MAPK via ROS or upstream kinases leading to the phosphorylation and translocation of NF-κB to the nucleus (Meldrum, 1998) or by direct oxidative modification

of NF-κB protein thiol groups (Cargnoni et al., 2002), although the specific oxidative modification has not been identified in studies of cardiac tissue.

Disulfides can also be formed between proteins and low-molecular-weight thiols such as glutathione, cysteine, and homocysteine in a process known as *S*-thiolation. As glutathione is the most abundant nonprotein thiol in mammalian cells, formation of protein-SSG (*S*-glutathiolation) is likely the predominant mechanism of cysteine modification. *S*-glutathiolation is also reversible by cellular reducing agents such as glutathione, as well as by enzymatic reactions, making this process a plausible and likely mechanism for the regulation of protein function. Although often considered as a protective mechanism, as formation of protein-SSG can prevent further oxidation of protein thiol groups to irreversible derivatives, it has also been shown to regulate protein function directly either by activating or inactivating a number of proteins. Collison and Thomas (1987) showed in myocardial cell cultures that creatine kinase became *S*-thiolated under conditions of oxidative stress using diamide. Supporting this, we have shown myocardial proteins become *S*-thiolated during ischemia and reperfusion in isolated rat hearts (Eaton et al., 2002a). Using *N*-biotinyl labeled cysteine perfused through isolated rat hearts during aerobic perfusion or post-ischemic reperfusion, we labeled and identified proteins that were *S*-thiolated. Western blot analysis of affinity-purified *S*-thiolated proteins with antibodies to candidate proteins demonstrated that actin, GAPDH, HSP27, protein-tyrosine phosphatase 1B, protein kinase Cα, and the G-protein Ras were *S*-thiolated during cardiac ischemia and reperfusion. MALDI-TOF mass spectrometry peptide mapping also independently identified GAPDH, actin, triosephosphate isomerase, and aconitate hydratase (aconitase) as target proteins for *S*-thiolation. The mechanism leading to protein *S*-thiolation is not clear as there are multiple routes by which this may occur in the heart during post-ischemic reperfusion, although metal-catalyzed oxidation and disulfide bond exchange are possible mechanisms. Proteins that have been identified as becoming *S*-thiolated during cardiac ischemia-reperfusion may be susceptible to oxidation in a number of different ways, not just *S*-thiolation. Some oxidations such as *S*-thiolation, sulfenation, or *S*-nitrosylation can also be an intermediate step to intraprotein disulfide bond formation (see Table 19.1 for a list of cardiac proteins that have been shown to undergo oxidative modification). The end-products of cysteine oxidation that dominate during pro-oxidative conditions will depend on many factors, including the nature and intensity of the oxidizing species and intracellular spatial distribution. This is illustrated by studies showing that cysteines within the G-protein H-Ras are differentially susceptible to various forms of oxidation, including *S*-thiolation and *S*-nitrosylation (Mallis et al., 2001).

S-Nitrosylation of protein cysteines results in the formation of nitrosothiols and is considered a major mechanism by which NO can modulate protein activity. NO production by eNOS is increased within minutes of the onset of ischemia (Depre et al., 1997; Csonka et al., 1999), but eNOS protein expression is eventually inhibited by increased tissue acidosis if ischemia is prolonged (Giraldez et al., 1997). However, a nonenzymatic NO synthesis can occur in severely ischemic

and acidotic tissue involving the direct disproportionation (acidic reduction) of nitrite to NO (Zweier et al., 1999). At reperfusion NO concentration is again increased (Wang and Zweier, 1996), which wanes with continued reperfusion to levels below baseline (Amrani et al., 1995). Raised NO during both ischemia and reperfusion may therefore result in increased protein thiol *S*-nitrosylation at this time. The process of *S*-nitrosylation and current methodologies for detecting *S*-nitrosylated proteins have recently been reviewed by Martinez-Ruiz and Lamas (2004). Briefly, the process of *S*-nitrosylation requires a reaction between a thiol group and NO, although there is some literature that indicates that this may not be a direct reaction but may require an oxidized derivative of NO (Gow et al., 1997). As with *S*-glutathiolation, some cysteine thiol groups are more susceptible than others to the reaction, depending on the chemical reactivity between the nitrosylating agent and the target. Factors that affect whether a protein becomes *S*-nitrosylated include the localization and reactivity of the target protein residue. For example, the pK_a of different cysteines and the acid–base interactions of surrounding residues influence cysteines susceptability to *S*-nitrosylation. In addition the localization of the protein itself can affect its propensity to become *S*-nitrosylated, with some being directly associated with NO synthases (Matsumoto et al., 2003). An increasing number of proteins are known to be susceptible to *S*-nitrosylation (see the supplementary data of Stamler et al., 2001). Although their potential role may be as fundamental as phosphoregulation, as with protein *S*-thiolation the full importance of this mode of reversible post-translational oxidation is yet to be clearly defined. Exactly how protein *S*-nitrosylation may be modulated during cardiac ischemia and reperfusion warrants further study.

Another class of oxidants that modifies proteins and can alter their function are reactive lipid oxidation products (RLOP). Lipid hydroperoxides (LOOH), formed when omega-6 polyunsaturated fatty acids, react with free radical species, and their breakdown products such as 4-hydroxynonenal (HNE) and malondialdehyde (MDA) have also been shown to modify cardiac proteins during cardiac ischemia. Our group has demonstrated in isolated rat hearts subject to ischemia and reperfusion that as ischemia proceeds, proteins become increasingly subjected to modification by LOOH and HNE, but these modifications are not reversed or exacerbated by reperfusion (Eaton et al., 1999; Eaton et al., 2001). The time course of these modifications likely parallels the progression of oxidative stress, as it develops during ischemia. The formation of LOOH-modified proteins may represent potentially deleterious modifications that contribute to cardiac injury. This concept is supported by studies demonstrating that exogenous application of LOOH can induce cardiac dysfunction (Iliou et al., 1993; Thollon et al., 1995). However, there is also growing evidence that modification of proteins by these lipid products, like other oxidants, have integral roles in initiating signal transduction pathways (Leonarduzzi et al., 2004). The identification of proteins modified by these reactive lipids would allow us to determine the extent to which protein–RLOP formation contributes to ischemic heart damage.

TABLE 19.1 Partial List of Proteins from Cardiac Tissue That Undergo Oxidative Modification

Protein	Reactive Thiol	Thiolated	Carbonylated	Nitrosylated	Nitrotyrosine	Disulfide (Interprotein)	Sulfenated/ Sulfonated
Unidentified	√ 21		√ 14, 23, 34, 35		√ 20, 24, 25, 26, 28, 29		
Aconitase		√ 6					√ 17
Actin		√ 6, 3	√ 15, 3, 33			√ 2, 3	√ 17
Acyl-CoA dehydro-genase						√ 2	√ 17
ANT						√ 2, 12	√ 17
ATP synthase						√ 2	√ 17
Complex 1	√ 18			√ 32		√ 2	
Creatine kinase	√ 18	√ 5			√ 13, 26	√ 2	
Cytochrome c					√ 27		
Cytochrome c oxidase							√ 17
Desmin						√ 2, 3	
G-protein Ras		√ 6					
GAPDH	√ 18	√ 6, 9				√ 2	
Heat-shock proteins		√ 6, 7				√ 2	
Ito K^+ channel		√ 16					
L-type Ca^{2+} channel	√ 22			√ 30			
Lactate dehydro-genase							√ 17

(*continued overleaf*)

TABLE 19.1 (*continued*)

Protein	Reactive Thiol	Thiolated	Carbonylated	Nitrosylated	Nitrotyrosine	Disulfide (Interprotein)	Sulfenated/ Sulfonated
Malate dehydro-genase	√ 18	√ 8				√ 2	√ 17
Myoglobin						√ 2	√ 17
Myosin heavy chain						√ 2	√ 17
Myosin light chain						√ 2	
Na^+/K^+ ATPase		√ 10					
NF-κB		√ 4					
NDPKB		√ 6				√ 2	
Peroxired-oxins						√ 2	
Phosphatidyl-cholinesterol acyltrans-ferase	√ 18						
Phosphory-lase B kinase	√ 18						
Phospho-fructose kinase	√ 18, 31						
PKA						√ 2	
PKCs		√ 6				√ 2	
Plasma retinol binding protein	√ 18						
PTP-1B		√ 6					

TABLE 19.1 (*continued*)

Protein	Reactive Thiol	Thiolated	Carbonylated	Nitrosylated	Nitrotyrosine	Disulfide (Interprotein)	Sulfenated/ Sulfonated
Ryanodine receptor		√ 11		√ 19			
SERCA	√ 18	√ 1				√ 2	√ 1
Succinate dehydro-genase	√ 18					√ 2	
Superoxide dismutase						√ 2	
Tubulin						√ 2	
Triosepho-sphate isomerase.		√ 6				√ 2	
Tropomyosin		√ 3				√ 2, 3	√ 17
Troponin						√ 2	√ 17
VDAC protein 1/2/3	√ 18						

(1) Adachi et al., 2004. (2) Brennan et al., 2004.* (3) Canton et al., 2004. (4) Cargnoni et al., 2002. (5) Collison and Thomas, 1987. (6) Eaton et al., 2002a. (7) Eaton et al., 2002b. (8) Eaton and Shattock, 2002. (9) Eaton et al., 2002c. (10) Haddock et al., 1995. (11) Hidalgo et al., 2002. (12) McStay et al., 2002. (13) Mihm and Bauer, 2002. (14) Pantke et al., 1999. (15) Powell et al., 2001. (16) Rozanski and Xu, 2002. (17) Saurin et al., 2004.* (18) Sethuraman et al., 2004.* (19) Stoyanovsky et al., 1997. (20) Zweier et al., 2001. (21) Ferrari et al., 1985. (22) Lacampagne et al., 1995. (23) Reznick et al., 1992. (24) Keira et al., 2002. (25) Hayashi et al., 2001. (26) Weinstein et al., 2000. (27) Cassina et al., 2000. (28) Liu et al., 1997. (29) Wang and Zweier, 1996. (30) Campbell et al., 1996. (31) Froede et al., 1968. (32) Jekabsone et al., 2003. (33) Schwalb et al., 2001. (34) Park et al., 1991. (35) Poston and Parenteau, 1992.

*Not all proteins identified in these studies are included here.

Other Forms of Protein Oxidation Protein carbonylation in cardiac tissue during ischemia and reperfusion has been demonstrated in a number of studies, often using the extent to which this modification occurs as an index of injury. Park et al. (1991) showed protein carbonyl content of rabbit heart tissue increased significantly after 60 minutes of hypoxia, while Poston and Parenteau (1992) showed in isolated rat hearts that protein carbonyl levels rose during ischemia, and reached four times basal levels 5 minutes into reperfusion. Increased protein carbonyl formation has also been shown in serum of patients undergoing coronary surgery and cardiopulmonary artery bypass grafting (Pantke et al., 1999). Furthermore, there is a strong negative correlation between the recovery of mechanical function and the presence of protein carbonyls (Reznick et al., 1992), and cardioprotection by ischemic preconditioning is associated with reduced protein carbonyl

formation (Schwalb et al., 2001; Khaliulin et al., 2004). Other studies have shown carbonylation of myofibrillar proteins, including actin, following ischemia and reperfusion (Powell et al., 2001; Schwalb et al., 2001; Canton et al., 2004), the extent of which was associated with depressed contractile function.

Tyrosine residues can be nitrated to form 3-nitrotyrosine, mainly following oxidation by peroxynitrite, which itself is formed by reaction of nitric oxide and superoxide radicals (Beckman, 1996). Again, this modification was largely considered to be irreversible, but evidence is emerging that suggests tyrosine nitration may be reversible (Balabanli et al., 1999; Koeck et al., 2004). There is a wealth of data supporting peroxynitrite formation in cardiac ischemia-reperfusion, but its role in mediating injury remains largely undefined, with both cytoprotective and cytotoxic effects having been demonstrated (Ishida et al., 1996; Wang and Zweier, 1996; Yasmin et al., 1997; Jensen et al., 1998; Zweier et al., 2001). Tyrosine nitration in post-ischemic hearts has been shown by Zweier et al. (2001), who measured a marked increase in peroxynitrite at 2 minutes of reperfusion, using luminol to detect it. This increase was blocked by nitric oxide synthase inhibitors and by superoxide dismutase, treatments that also enhanced functional recovery. Additional immunohistological analysis confirmed that the nitrotyrosine was formed in post-ischemic hearts, but not in aerobic controls. They therefore concluded that peroxynitrite production at reperfusion resulted in protein nitration that was causal in myocardial injury at this time. Similar results were also shown in an in vivo study, which again used immunohistochemical methods to demonstrate nitrotyrosine formation following ischemia and reperfusion (Liu et al., 1997). Peroxynitrite treatment of an isolated heart caused impairment of high-energy phosphate metabolism, resulting in cardiac dysfunction (Lee et al., 2003). Some proteins, such as creatine kinase, have been identified as a substrate for tyrosine nitration, which causes enzyme inhibition (Mihm and Bauer, 2002). However, more detailed studies investigating the full extent of protein tyrosine nitration and its functional consequences are required.

19.3 ESTABLISHED TARGETS OF POST-TRANSLATIONAL OXIDATION

There are an increasing number of specific proteins that are known to be directly regulated by oxidative post-translational modifications. Proteins regulated by oxidation include ion translocators (Shattock and Matsuura, 1993), structural proteins (Chai et al., 1994), metabolic enzymes (Miller et al., 1990; Cheung et al., 1998), DNA isomerases (Wang et al., 2001), and signaling proteins. Signal transduction proteins that are directly regulated by oxidative modification of cysteine residues include protein phosphatases (Mahadev et al., 2001), protein kinases (Ward et al., 1998; Gopalakrishna and Jaken, 2000), G-proteins (Mallis et al., 2001), and membrane receptors (Liu et al., 1999), although many of these are yet to be shown directly in cardiac tissue. In the following section we discuss in more detail the specific proteins that are known to be susceptible to oxidative

modifications in the heart (see Table 19.1 for an overview of cardiac proteins identified as being oxidatively modified during oxidative stress).

19.3.1 Metabolic Enzymes

Many metabolic enzymes are known to have reactive cysteine residues, the oxidation of which causes inactivation (Gilbert, 1990). Many studies have shown that GAPDH is a target for oxidation, and it has been shown to undergo a variety of oxidative modifications, including *S*-glutathiolation, carbonylation, *S*-nitrosylation, and interprotein disulfide formation (Ravichandran et al., 1994; Mohr et al., 1996; Eaton et al., 2002c; Brennan et al., 2004; Cahuana et al., 2004). GAPDH contains two cysteine residues within its catalytic domain but only Cys149 is essential for catalytic activity, and it is this thiol site that is believed to be particularly susceptible to oxidative modification resulting in loss of enzyme activity (Ravichandran et al., 1994; Mohr et al., 1996; Souza and Radi, 1998). Indeed, in hearts subject to ischemia and reperfusion, GAPDH activity has been shown to be reduced (Knight et al., 1996). Interestingly this inactivation has been shown to be partly reversible, as application of thiol-reducing agents following ischemia restored lost activity (Souza and Radi, 1998; Eaton et al., 2002c) while similar treatment of GAPDH from reperfused tissue failed to do this (Knight et al., 1996; Eaton et al., 2002c). This indicates that in contrast to a primarily reversible oxidative modification during ischemia, reperfusion induces an irreversible modification to the enzyme, which is consistent with the known burst of radical production at this time.

Many other metabolic enzymes have been shown to contain critical thiol groups susceptible to oxidation including phosphofructokinase (Gilbert, 1982), fructose-1,6-bisphosphate (Nakashima et al., 1970), glycogen synthase (Ernest and Kim, 1974), and 3-hydroxy-3-methylglutaryl-CoA reductase (Cappel and Gilbert, 1989), while triosephosphate isomerase (Taegtmeyer et al., 1997) and aconitase (Eaton et al., 2002a) have been shown to be oxidatively modified following cardiac ischemia and reperfusion. Inhibition of these enzymes therefore is likely to contribute to the metabolic deficit that is known to occur during reperfusion. Creatine kinase has also been demonstrated to be susceptible to a range of oxidative modifications including *S*-glutathiolation (Collison and Thomas, 1987; Miller et al., 1990), carbonylation (Aksenov et al., 2001), nitration (Mihm and Bauer, 2002), and interprotein disulfide formation (Brennan et al., 2004). Furthermore cardiac creatine kinase has been shown to be inhibited by peroxynitrite as well as diamide. It was also inhibited in skeletal muscle by GSSG treatment, which was associated with *S*-thiolation of the active site cysteine (Reddy et al., 2000). This inhibition was reversed by subsequent GSH treatment. In this sense *S*-glutathiolation may be considered a protective modification guarding against other irreversible modifications such as sulfonation. Either way, the inhibition of this metabolic enzyme during cardiac oxidative stress can clearly compromise energy metabolism.

19.3.2 Structural and Myofibrillar Proteins

One key event during cardiac ischemia is the loss of contractile function, which generally returns at reperfusion, but generally in a compromised state due to stunning and arrhythmias. Clearly, oxidation of myofibrillar proteins is one mechanism that may contribute to loss of contractility, as discussed above. The myofibrillar proteins actin and tropomyosin have been shown to be oxidized upon reperfusion, showing elements of reversible and irreversible modifications (Canton et al., 2004). This study provided evidence that less oxidation of myofilament proteins occurred during ischemia and reperfusion compared to hydrogen peroxide treatment of hearts, although mixed disulfides involving actin and tropomyosin were detected in the post-ischemic preparations. This is in line with evidence presented by our group where we identified actin, using biotin-labeled cysteine, as one of a number of proteins to become *S*-thiolated in hearts subject to ischemia-reperfusion (Eaton et al., 2002a). Evidence of carbonylation of actin was also presented by Canton et al. (2004), as Oxyblot analysis of this modification showed a 2.8-fold increase in oxidized actin following ischemia and reperfusion. This supports data previously presented that also showed an 80% increase in carbonyl modified actin following ischemia and reperfusion in isolated rat hearts (Powell et al., 2001). The exact effect of oxidation on actin is unclear, although it can inhibit its polymerization and cause depolymerization (Milzani et al., 1997; Dalle-Donne et al., 2003). Oxidative modification may also enhance cross-linking of actin, which can be detrimental to contraction by interfering with thick and thin filament interactions. In support of this hypothesis actin was one of the myofilament proteins identified as forming interprotein disulfides following oxidant stress in isolated cardiac myocytes, along with tubulin, troponin I, myosin light chain, myosin heavy chain, tropomyosin, and desmin (Brennan et al., 2004). In light of these data, oxidative modification of myofilament and structural proteins during ischemia and reperfusion provides another plausible explanation for loss of function at this time.

19.3.3 Ion Channels

A number of ion translocators are also susceptible to oxidative modification, altering channel function and therefore ion homeostasis. As discussed above, these modifications may play a key role in arrhythmias. Many ion translocators are known to be susceptible to oxidative modification, including the Na^+/K^+ ATPase (Haddock et al., 1995), K^+ channels (Rozanski and Xu, 2002), the ryanodine receptor, SERCA (Adachi et al., 2004), and the mitochondrial permeability transition pore (McStay et al., 2002). The activity of Na^+/K^+ ATPase is reduced during myocardial ischemia and reperfusion (Kim and Akera, 1987) and Haddock et al. (1995) demonstrated that this may be due to modification of protein thiol groups. Using isolated myocytes, they showed that thiol-blocking agents were able to inhibit Na^+/K^+ pump current, and this was reversed by DTT. They also showed depletion of GSH from animals, using diethyl maleate, had similar inhibitory effects on pump current. Along similar lines Rozanski et al. have demonstrated that GSSG decreased the repolarizing transient outward K^+ current

when applied to either the external or internal solutions (Rozanski and Xu, 2002). Therefore redox modulation of ion channels may also contribute to acute changes in cardiac action potential under conditions of oxidative stress, such as ischemia and reperfusion.

Altered Ca^{2+} handling is also a key feature of cardiac ischemia-reperfusion, and it is well established that Ca^{2+} handling proteins such as SERCA and the ryanodine receptor are redox sensitive. Adachi et al. (2004) have shown that peroxynitrite can directly stimulate SERCA activity via *S*-glutathiolation of Cys674. Interestingly they also found that NO-dependent relaxation as well as *S*-glutathiolation and activation of SERCA were decreased by atherosclerosis, and was explained in part by oxidation of Cys674 to sulfonic acid. The ryanodine receptor complex is responsible for Ca^{2+} release from the sarcoplasmic reticulum in a process called calcium-induced calcium release in response to a cardiac action potential. Modulation of its activity would therefore have a significant impact on excitation–contraction coupling of the heart. The ryanodine receptor complex contains 364 cysteines, the majority of which are maintained in a reduced state by the prevailing intracellular redox conditions. However, these thiols are sensitive to oxidation, and this alters the Ca^{2+} sensitivity of the ryanodine receptor (Balshaw et al., 2001).

Incubation of skeletal muscle vesicles with the oxidant thimerosal increased their susceptibility to stimulation by Ca^{2+} and decreased the inhibitory effect of Mg^{2+}, while GSSG and *S*-nitrosoglutathione stimulated Ca^{2+} release from sarcoplasmic reticulum (Hidalgo et al., 2002). In addition, the channel is known to be modulated by *S*-nitrosylation, although this has been shown to be both stimulatory and inhibitory (Stoyanovsky et al., 1997; Zahradnikova et al., 1997).

19.3.4 Molecular Chaperones

HSP27 is a molecular chaperone that exists as a high-molecular-weight aggregate that is broken down following phosphorylation by stress-activated kinase pathways such as p38 MAPK, which activates MAPKAPK2, and phosphorylates HSP27 (Stokoe et al., 1992). Rat HSP27 contains a single cysteine residue at Cys141, and it has been shown that this becomes *S*-thiolated during oxidant stress, causing disaggregation of the multimeric HSP27. We demonstrated that *S*-thiolation of HSP27 increased 3.6-fold during post-ischemic reperfusion and that hydrogen peroxide disaggregated HSP27 (Eaton et al., 2002b). It has also been shown in a cell model that there is a direct relationship between the multimeric aggregate size of HSP27 and the concentration of GSH, as well as evidence that there is interplay between the HSP27 and GSH that regulates the cellular levels of these molecules (Mehlen et al., 1997). The oligomeric state of HSP27 regulates its ability to act as a molecular chaperone and controls its ability to inhibit the polymerization of actin that, as we have already discussed, is also a known target of *S*-thiolation (Chai et al., 1994).

HSP60 is predominantly a mitochondrial protein and is upregulated by an accumulation of unfolded proteins within the mitochondria, although its expression is also known to be enhanced by oxidized low-density lipoproteins, a process

that occurs during atherosclerosis (Frostegard et al., 1996). HSP60 is known to be susceptible to *S*-glutathiolation following an oxidative stress, as demonstrated by Fratelli et al. (2002) in human T lymphocytes.

In relation to cardiac ischemia and reperfusion, overexpression of HSP60 in rat neonatal myocytes protected cells against simulated ischemia and reoxygenation-induced apoptosis, by maintaining mitochondrial integrity and capacity for ATP generation (Lin et al., 2001). This raises the possibility that HSP60 activity within mitochondria preserves metabolic enzymes and protects the energy production machinery from direct oxidation or detrimental changes in protein aggregation.

HSP70 is also susceptible to oxidative modifications such as *S*-glutathiolation (Fratelli et al., 2002; Lind et al., 2002), and it has been shown in vitro that this modification activates chaperone activity, preventing protein aggregation. It is also known to be potently induced by ischemia and may play a role in myocardial protection. Overexpression of constitutive HSP70 in H9c2 cardiac myoblasts confers protection from a range of oxidative stresses, including exposure to hydrogen peroxide, hydroxyl radicals, menadione, and hypoxia followed by reoxygenation (Chong et al., 1998), while overexpression of the inducible form of HSP70 in a transgenic mouse increased the resistance of the heart to ischemic injury (Marber et al., 1995). Even direct injection of adenovirus encoding HSP70 into the myocardium reduced infarct following ischemia and reperfusion in rabbit heart (Okubo et al., 2001). It is suggested that HSP70 may prevent changes in the tertiary structure caused by oxidative stress or promote correct refolding of denatured proteins during reperfusion. Furthermore it has been suggested that that *S*-glutathionylation of the HSPs may potentiate their respective protein chaperoning activities.

19.3.5 Signaling Molecules

Many signaling molecules can be directly regulated by the post-translational redox modifications, including kinases, phosphatases, and transcription factors. These redox-dependent sensors then transduce the signal via an extensive signaling web, which results in widespread changes in phosphoregulation. An extensive review of the redox control of signaling molecules is beyond the scope of this review, as reviews are available (Suzuki and Ford, 1999; Allen and Tresini, 2000; Thannickal and Fanburg, 2000; Finkel, 2001; Forman et al., 2002; Esposito et al., 2004), including a number focused on cardiovascular health and disease (Das, 2001; Yoshizumi et al., 2001; Sabri et al., 2003). However, as redox signaling is crucial to how the heart responds to limit injury during ischemia and reperfusion, the role of signaling molecules as oxidant sensors is discussed below in the section on ischemic preconditioning.

19.4 OXIDATIVE STRESS IN MYOCARDIAL ADAPTATION TO ISCHEMIA AND REPERFUSION

As well as a role for oxidative stress and protein oxidation in cardiac dysfunction, it is also known that redox signaling is crucial to the genesis of protection

by ischemic preconditioning. Ischemic preconditioning, discovered in 1986 by Reimer, Murry, and Jennings (Reimer et al., 1986; Murry et al., 1986), is the phenomenon where brief periods of sublethal ischemia and reperfusion significantly attenuate damage (measured in terms of infarction, loss of contractility, or incidence of arrhythmias) incurred during a subsequent prolonged duration of ischemia. This classical preconditioning protection is lost after about two hours, but eventually a "second window of protection" known as "delayed preconditioning" arises. While there are similarities in the triggers of both these forms of protection, it is generally thought that classical protection is mediated by acute changes in signal transduction, whereas the delayed form likely involves expression of new gene products, such as stress inducible heat-shock proteins (Marber et al., 1993, 1995; Yellon and Marber, 1994).

19.4.1 Free Radical Generation and Ischemic Preconditioning

ROS generation during the ischemic preconditioning stimulus is thought to be an important component of the triggering of protection. The evidence for this includes exogenous application of oxidants induces preconditioning-like protection (Tritto et al., 1997), antioxidant compounds given during the trigger phase block protection, and elevated levels of oxidants can be detected during the trigger cycles of ischemia and reperfusion (Vanden Hoek et al., 1998). Murry (Murry et al., 1988) was the first to show an effect of antioxidants when he showed superoxide dismutase partially attenuated preconditioning in his canine model. Tanaka et al. (1994) reported that both superoxide dismutase (SOD) and *N*-2-mercaptopropionyl glycine (MPG) attenuated the infarct size limiting effect of ischemic preconditioning in the rabbit. Similarly Chen et al. (1995) showed, in isolated perfused rat hearts, that *N*-acetylcysteine (NAC) given throughout the trigger stimulus blocked preconditioning protection. However, the triggers of preconditioning are more complex than outlined here. For example, Baines et al. (1997) found that MPG blocked preconditioning induced by one cycle of ischemia and reperfusion, but not when multiple cycles were used. They suggested that other factors are released that induce protection by alternate routes to those invoked by oxidants. However, another possibility is that multiple cycles overdrive the antioxidant blockade, such that an adequate oxidant load was reached to invoke protection. Regardless of the finer details of the mechanism of preconditioning, it is clear that oxidative stress is an important trigger for protection, and one key goal is to understand the redox signal transduction in this system.

Clearly, the oxidative conditions that accompany the preconditioning trigger may cause reversible oxidative protein modifications. There are at least four possible ways in which these reversible modifications of proteins could protect the ischemic myocardium: (1) They could activate signaling cascades leading to cardioprotection. This could, for example, include the inhibition of a redox-sensitive phosphatase (Barrett et al., 1999) or the activation of a kinase. (2) Oxidation of proteins could *directly* activate cardioprotective stress responses. For example, glutathiolation of HSP27 causes the breakdown of its normal aggregated state and translocation to the cytoskeleton; this in turn may affect its chaperone activity or

may be directly cardioprotective (Eaton et al., 2002b). (3) Reversible oxidations such as *S*-glutathiolation, *S*-nitrosylation or sulfenic acid formation of protein thiols during pre-treatments or preconditioning cycles may prevent further more harmful irreversible modification by ROS bursts during index ischemia and reperfusion, thus preventing or decreasing the cell injury and death associated with the oxidative burst at reperfusion. (4) Another mechanism for ROS-induced protection has been proposed by Schumacker's group (Vanden Hoek et al., 2000). They suggest that the increase in ROS during the preconditioning and index ischemia can actually attenuate harmful ROS production at reperfusion. This presents a dual role for ROS in preconditioning, at induction when low levels may activate kinase pathways and at reperfusion, when their production is attenuated.

It is perhaps fair to say that that the most studied signaling molecules in ischemic preconditioning are the protein kinase C (PKC) isozymes, particularly, delta and epsilon (Mackay and Mochly-Rosen, 2001; Saurin et al., 2002; Inagaki et al., 2003), and p38 mitogen activated protein kinase (MAPK) (Nakano et al., 2000; Ping and Murphy, 2000; Saurin et al., 2000; Fryer et al., 2001), although many other kinases have been studied. p38MAPK activation during ischemia may be via thioredoxin, as highlighted above, but there is little direct evidence of this and activation by upstream kinases is likely more important. However, inhibition of thioredoxin 1 abolished the cardioprotection afforded by ischemic preconditioning as evidenced by decreased ventricular functional recovery and increased infarct size and cardiomyocyte apoptosis (Turoczi et al., 2003). PKC activation (particularly delta and epsilon) can be directly regulated by oxidative post-translational modification, such as *S*-thiolation (Chu et al., 2001, 2003, 2004). So one possible link between the dual integral roles of PKC and oxidative stress in the mechanism of preconditioning protection is that the kinase itself is directly sensitive to oxidants generated at this time.

To generalize, preconditioning is largely induced by oxidants and inhibited by interventions that inhibit protein phosphorylation, such as the broad-spectrum kinase inhibitor staurosporine as well as the tyrosine kinase inhibitor genistein (Schulz et al., 2001). These observations are consistent with the possibility that the protective flux through kinase pathways is a result of the inhibition of tyrosine phosphatases by oxidation of a catalytically essential cysteine residue (Claiborne et al., 1999).

In studies from this laboratory (Eaton et al., 2005), preconditioning increased protein *S*-thiolation by 160%, which was blocked by the antioxidant mercaptopropionylglycine, an intervention that, as outlined above, also blocks the genesis of protection. Agonists that initiate protection (phorbol 12-myristate 13-acetate or phenylephrine) or oxidants (hydrogen peroxide or diamide) also induced efficient protein *S*-thiolation. Preconditioning agonist induced *S*-thiolation was significantly attenuated by diphenyleneiodonium (a flavoprotein inhibitor) as well as by the PKC inhibitor bisindolylmaleimide I. Studies such as these support the central role of oxidative stress in modulation of cardioprotection, and help define the molecular redox mechanisms that may be important.

The fact that delayed preconditioning is dependent on expression of new proteins leads to the possibility that this may be modulated, at least in part, by redox-regulated transcription factors that can be directly activated by oxidant stress. There is a wealth of evidence that transcriptional regulation by post-translational oxidative modification is an archetypal mechanism, as it is present in bacteria (Hausladen et al., 1996; Zheng et al., 1998; Georgiou, 2002; Kim et al., 2002) as well as in mammalian tissues (De Nigris et al., 2001; Cargnoni et al., 2002; Haddad, 2002).

19.5 CONCLUSIONS, THERAPEUTIC IMPLICATIONS, AND FUTURE DIRECTIONS

Much evidence has accrued showing oxidative stress as an integral component of injury during ischemia and reperfusion, but at the same time it has a crucial role in the genesis of cardioprotection that limits damage. This is not really a paradoxical situation and reflects the differential effects of pro-oxidative events in the heart depending on the exact set of circumstances. Clearly, a low-grade oxidative stress, such as during ischemic preconditioning or following neurohormonal-coupled free radical production is not injurious. Indeed oxidative stress under such circumstances seems to be an integral component of the signaling network response that follows these interventions. Thus the term oxidative *stress* may generally be inappropriate, as it is uncertain that any harm, dysfunction, or injury actually accompanies the majority of pro-oxidative events that occur in the everyday life of cells and tissues.

In contrast, the oxidative stress that progressively occurs during ischemia and is heightened at reperfusion occurs at a time when the heart is compromised in many ways (severe energy depletion, cell and mitochondrial swelling, elevated intracellular calcium, etc.). Obviously oxidative stress at this time may be enough to push the heart "over the edge," ultimately resulting in necrosis. Clearly, a "fixed amount of oxidative stress" will likely cause more injury to a severely ischemic heart than one that has endured limited or no ischemia.

Extensive laboratory animal studies have demonstrated that prevention of oxidative stress during both ischemia and reperfusion with antioxidants offers significant improvement in functional recovery. In stark contrast, the evidence for the therapeutic benefits of antioxidant treatment in human cardiac diseases is largely yet to be established (Tardif, 2003). A study into the administration of SOD in acute myocardial infarction patients undergoing PTCA found no benefit (Flaherty et al., 1994), while a large trial in smokers with acute myocardial infarction failed to show any cardiovascular benefit of long-term vitamin E and beta-carotene treatment (Rapola et al., 1997). A number of recent clinical trials (HOPE, GISSI) based on the theory that combating damaging oxidative stress with antioxidants should bring about an improvement in the health of heart disease patients have also failed to show any benefit (1999; Yusuf et al., 2000). It has been suggested that antioxidant clinical trials have failed, as a combination of antioxidants is required for clinical effectiveness. The idea here is that both

lipid-soluble and hydrophilic antioxidants work as a network for the removal of oxidative stress and that only a synergistic combination of these agents would prove effective.

However, despite the wealth of data from laboratory studies, it remains possible that oxidative stress may not be a major mediator of injury in the complex setting of human cardiovascular disease. Antioxidants given in this way may prevent the damaging effects of oxidants, but at the same time they may detrimentally interfere with normal cellular events, such as redox signaling. We have seen that oxidative stress can affect protein function in a variety of manners and at low dose also activate cardioprotective pathways.

Clearly, the one-size-fits-all approach is largely not as effective when it comes to antioxidant supplementation of heart disease. It is likely then that much more targeted approaches to the treatment or manipulation of oxidative stress may be more successful. A fuller understanding of specific protein modifications would permit focused therapies aimed at altering oxidative modifications of specific proteins. For this therapy to be possible, we not only need to identify proteins that become modified during conditions of oxidative stress, but we also have to demonstrate that this has some biological relevance. Advances in proteomic methods have allowed the detection of a number of proteins oxidatively modified in a range of tissues, including the heart, and as techniques and analytical methods improve, we will undoubtedly identify more targets. However, in parallel, it is essential to establish whether there is a true correlate between redox state, functional activity, and physiological performance.

One example of a drug that has potential to manipulate the redox-dependent activity of a protein in a selective way is disulfiram. This thiuram disulfide compound has known anticancer properties (Irving et al., 1983), and it has been shown to differentially activate/inactivate PKC isozymes (Chu and O'Brian, 2005). The redox regulation of the kinase likely involves the drug undergoing disulfide exchange reactions with the protein, leading to the formation of PKC that is *S*-thiolated by disulfiram. The fact that this drug can activate one PKC isozyme but inhibit another at the very same concentration offers significant promise for drugs of this type. Once proteins are discovered that are directly redox regulated, the development of specific or selective compounds that oxidize or reduce functionally crucial amino acids may offer significant therapeutic potential.

It is clear that redox homeostasis is pivotal in the context of both health and disease, and not just during cardiac ischemia-reperfusion. A fuller understanding of the targets of oxidative stress and how and when they are regulated by oxidants is a logical first step in the development of more effective, rationally designed, therapies.

ACKNOWLEDGMENTS

Research in the authors' laboratory is supported by grants from The Wellcome Trust, The UK Biotechnology and Biological Sciences Research Council, and The British Heart Foundation.

LIST OF ABBREVIATIONS

$[K]_o$, extracellular potassium
ANT, adenine nucleotide translocase
ASK, apoptosis signaling-regulated kinase
ATP, adenosine triphosphate
Cys, cysteine
DCF, dichlorodihydrofluorescein
eNOS, endothelial nitric oxide synthase
ESR, electron spin resonance
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
GSH, reduced glutathione
GSSG, glutathione disulfide
H_2O_2, hydrogen peroxide;
HNE, 4-hydroxynonenal
HSP, heat-shock protein
JNK, c-Jun *N*-terminal kinase
K_{ATP}, ATP-sensitive potassium channel
LOOH, lipid hydroperoxides
MAPK, mitogen activated protein kinase
MAPKAPK2, MAPK-activated protein kinase 2
MDA, malondialdehyde
MPG, *N*-2-mercaptopropionyl glycine
NAC, *N*-acetylcysteine
NAD^+, nicotinamide adenine dinucleotide
NADH, reduced NAD
NADPH, reduced nicotinamide adenine dinucleotidephosphate
NF-κB, nuclear factor κB
NO, nitric oxide
PBN, *N*-tert-butyl-alpha-phenylnitrone
PKC, protein kinase C
PSO_2H, sulfinic acid
PSO_3H, sulfonic acid
PSOH, sulfenic acid
PTCA, percutaneous transluminal coronary angioplasty
RLOP, reactive lipid oxidation products
RNS, reactive nitrogen species
ROS, reactive oxygen species
SERCA, sarco/endoplasmic reticulum calcium (Ca^{2+}) ATPase

SOD, superoxide dismutase
TNF, tumor necrosis factor
VDAC, voltage-dependent anion channel
VF, ventricular fibrillation
VPB, ventricular premature beat
VT, ventricular tachycardia

REFERENCES

Abbate A., Biondi-Zoccai G.G., Baldi A. (2002). Pathophysiologic role of myocardial apoptosis in post-infarction left ventricular remodeling. *J Cell Physiol* 193: 145–153.

Adachi T., Weisbrod R.M., Pimentel D.R., Ying J., Sharov V.S., Schoneich C., Cohen R.A. (2004). *S*-Glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. *Nat Med* 10: 1200–1207.

Aksenov M.Y., Aksenova M.V., Butterfield D.A., Geddes J.W., Markesbery W.R. (2001). Protein oxidation in the brain in Alzheimer's disease. *Neuroscience* 103: 373–383.

Allen R.G., Tresini M. (2000). Oxidative stress and gene regulation. *Free Radic Biol Med* 28: 463–499.

Amrani M., Chester A.H., Jayakumar J., Schyns C.J., Yacoub M.H. (1995). L-Arginine reverses low coronary reflow and enhances postischaemic recovery of cardiac mechanical function. *Cardiovasc Res* 30: 200–204.

Anzai K., Ogawa K., Ozawa T., Yamamoto H. (2000). Oxidative modification of ion channel activity of ryanodine receptor. *Antioxid Redox Signal* 2: 35–40.

Aota M., Matsuda K., Isowa N., Wada H., Yodoi J., Ban T. (1996). Protection against reperfusion-induced arrhythmias by human thioredoxin. *J Cardiovasc Pharmacol* 27: 727–732.

Arduini A., Mezzetti A., Porreca E., Lapenna D., DeJulia J., Marzio L., Polidoro G., Cuccurullo F. (1988). Effect of ischemia and reperfusion on antioxidant enzymes and mitochondrial inner membrane proteins in perfused rat heart. *Biochim Biophys Acta* 970: 113–121.

Arroyo C.M., Kramer J.H., Leiboff R.H., Mergner G.W., Dickens B.F., Weglicki W.B. (1987). Spin trapping of oxygen and carbon-centered free radicals in ischemic canine myocardium. *Free Radic Biol Med* 3: 313–316.

Baines C.P., Goto M., Downey J.M. (1997). Oxygen radicals released during ischemic preconditioning contribute to cardioprotection in the rabbit myocardium. *J Mol Cell Cardiol* 29: 207–216.

Balabanli B., Kamisaki Y., Martin E., Murad F. (1999). Requirements for heme and thiols for the nonenzymatic modification of nitrotyrosine. *Proc Natl Acad Sci USA* 96: 13136–13141.

Balshaw D.M., Xu L., Yamaguchi N., Pasek D.A., Meissner G. (2001). Calmodulin binding and inhibition of cardiac muscle calcium release channel (ryanodine receptor). *J Biol Chem* 276: 20144–20153.

Barrett W.C., DeGnore J.P., Keng Y.F., Zhang Z.Y., Yim M.B., Chock P.B. (1999). Roles of superoxide radical anion in signal transduction mediated by reversible regulation of protein-tyrosine phosphatase 1B. *J Biol Chem* 274: 34543–34546.

Becker L.B., vanden Hoek T.L., Shao Z.H., Li C.Q., Schumacker P.T. (1999). Generation of superoxide in cardiomyocytes during ischemia before reperfusion. *Am J Physiol* 277: H2240–2246.

Beckman J.S. (1996). Oxidative damage and tyrosine nitration from peroxynitrite. *Chem Res Toxicol* 9: 836–844.

Bernier M., Hearse D.J., Manning A.S. (1986). Reperfusion-induced arrhythmias and oxygen-derived free radicals. Studies with "anti-free radical" interventions and a free radical-generating system in the isolated perfused rat heart. *Circ Res* 58: 331–340.

Bersohn M.M., Philipson K.D., Fukushima J.Y. (1982). Sodium-calcium exchange and sarcolemmal enzymes in ischemic rabbit hearts. *Am J Physiol* 242: C288–C295.

Biteau B., Labarre J., Toledano M.B. (2003). ATP-dependent reduction of cysteine-sulphinic acid by *S. cerevisiae* sulphiredoxin. *Nature* 425: 980–984.

Blasig I.E., Shuter S., Garlick P., Slater T. (1994). Relative time-profiles for free radical trapping, coronary flow, enzyme leakage, arrhythmias, and function during myocardial reperfusion. *Free Radic Biol Med* 16: 35–41.

Bolli R. (1991). Oxygen-derived free radicals and myocardial reperfusion injury: An overview. *Cardiovasc Drugs Ther* 5: 249–268.

Bolli R., Marban E. (1999). Molecular and cellular mechanisms of myocardial stunning. *Physiol Rev* 79: 609–634.

Bolli R., Patel B.S., Jeroudi M.O., Lai E.K., McCay P.B. (1988). Demonstration of free radical generation in "stunned" myocardium of intact dogs with the use of the spin trap alpha-phenyl *N*-tert-butyl nitrone. *J Clin Invest* 82: 476–485.

Bolli R., Zhu W.X., Hartley C.J., Michael L.H., Repine J.E., Hess M.L., Kukreja R.C., Roberts R. (1987). Attenuation of dysfunction in the postischemic "stunned" myocardium by dimethylthiourea. *Circulation* 76: 458–468.

Braunwald E., Kloner R.A. (1982). The stunned myocardium: Prolonged, postischemic ventricular dysfunction. *Circulation* 66: 1146–1149.

Brennan J.P., Wait R., Begum S., Bell J.R., Dunn M.J., Eaton P. (2004). Detection and mapping of widespread intermolecular protein disulfide formation during cardiac oxidative stress using proteomics with diagonal electrophoresis. *J Biol Chem* 279: 41352–41360.

Brown J.M., Terada L.S., Grosso M.A., Whitman G.J., Velasco S.E., Patt A., Harken A.H., Repine J.E. (1988). Hydrogen peroxide mediates reperfusion injury in the isolated rat heart. *Mol Cell Biochem* 84: 173–175.

Cahuana G.M., Tejedo J.R., Jimenez J., Ramirez R., Sobrino F., Bedoya F.J. (2004). Nitric oxide-induced carbonylation of Bcl-2, GAPDH and ANT precedes apoptotic events in insulin-secreting RINm5F cells. *Exp Cell Res* 293: 22–30.

Campbell D.L., Stamler J.S., Strauss H.C. (1996). Redox modulation of L-type calcium channels in ferret ventricular myocytes: Dual mechanism regulation by nitric oxide and *S*-nitrosothiols. *J Gen Physiol* 108: 277–293.

Canton M., Neverova I., Menabo R., Van Eyk J., Di Lisa F. (2004). Evidence of myofibrillar protein oxidation induced by postischemic reperfusion in isolated rat hearts. *Am J Physiol Heart Circ Physiol* 286: H870–H877.

Cappel R.E., Gilbert H.F. (1989). The effects of mevinolin on the thiol/disulfide exchange between 3-hydroxy-3-methyglutaryl-coenzyme A reductase and glutathione. *J Biol Chem* 264: 9180–9187.

Cargnoni A., Ceconi C., Gaia G., Agnoletti L., Ferrari R. (2002). Cellular thiols redox status: A switch for NF-kappaB activation during myocardial post-ischaemic reperfusion. *J Mol Cell Cardiol* 34: 997–1005.

Cassina A.M., Hodara R., Souza J.M., Thomson L., Castro L., Ischiropoulos H., Freeman B.A., Radi R. (2000). Cytochrome c nitration by peroxynitrite. *J Biol Chem* 275: 21409–21415.

Chai Y.C., Ashraf S.S., Rokutan K., Johnston R.B., Jr., Thomas J.A. (1994). *S*-Thiolation of individual human neutrophil proteins including actin by stimulation of the respiratory burst: Evidence against a role for glutathione disulfide. *Arch Biochem Biophys* 310: 273–281.

Chen W., Gabel S., Steenbergen C., Murphy E. (1995). A redox-based mechanism for cardioprotection induced by ischemic preconditioning in perfused rat heart. *Circ Res* 77: 424–429.

Cheung P.Y., Danial H., Jong J., Schulz R. (1998). Thiols protect the inhibition of myocardial aconitase by peroxynitrite. *Arch Biochem Biophys* 350: 104–108.

Chi N.C., Karliner J.S. (2004). Molecular determinants of responses to myocardial ischemia/ reperfusion injury: Focus on hypoxia-inducible and heat shock factors. *Cardiovasc Res* 61: 437–447.

Choi H., Kim S., Mukhopadhyay P., Cho S., Woo J., Storz G., Ryu S. (2001). Structural basis of the redox switch in the OxyR transcription factor. *Cell* 105: 103–113.

Chong K.Y., Lai C.C., Lille S., Chang C., Su C.Y. (1998). Stable overexpression of the constitutive form of heat shock protein 70 confers oxidative protection. *J Mol Cell Cardiol* 30: 599–608.

Chu F., Chen L.H., O'Brian C.A. (2004). Cellular protein kinase C isozyme regulation by exogenously delivered physiological disulfides—Implications of oxidative protein kinase C regulation to cancer prevention. *Carcinogenesis* 25: 585–596.

Chu F., O'Brian C.A. (2005). PKC sulfhydryl targeting by disulfiram produces divergent isozymic regulatory responses that accord with the cancer preventive activity of the thiuram disulfide. *Antioxid Redox Signal* 7: 855–862.

Chu F., Ward N.E., O'Brian C.A. (2001). Potent inactivation of representative members of each PKC isozyme subfamily and PKD via *S*-thiolation by the tumor-promotion/progression antagonist glutathione but not by its precursor cysteine. *Carcinogenesis* 22: 1221–1229.

Chu F., Ward N.E., O'Brian C.A. (2003). PKC isozyme *S*-cysteinylation by cystine stimulates the pro-apoptotic isozyme PKC delta and inactivates the oncogenic isozyme PKC epsilon. *Carcinogenesis* 24: 317–325.

Claiborne A., Yeh J.I., Mallett T.C., Luba J., Crane E.J. III, Charrier V., Parsonage D. (1999). Protein-sulfenic acids: diverse roles for an unlikely player in enzyme catalysis and redox regulation. *Biochemistry* 38: 15407–15416.

Clark C., Foreman M.I., Kane K.A., McDonald F.M., Parratt J.R. (1980). Coronary artery ligation in anesthetized rats as a method for the production of experimental dysrhythmias and for the determination of infarct size. *J Pharmacol Methods* 3: 357–368.

Collison M.W., Thomas J.A. (1987). *S*-Thiolation of cytoplasmic cardiac creatine kinase in heart cells treated with diamide. *Biochim Biophys Acta* 928: 121–129.

Costantini P., Belzacq A.S., Vieira H.L., Larochette N., de Pablo M.A., Zamzami N., Susin S.A., Brenner C., Kroemer G. (2000). Oxidation of a critical thiol residue of

the adenine nucleotide translocator enforces Bcl-2-independent permeability transition pore opening and apoptosis. *Oncogene* 19: 307–314.

Crompton M., Costi A. (1988). Kinetic evidence for a heart mitochondrial pore activated by Ca^{2+}, inorganic phosphate and oxidative stress: A potential mechanism for mitochondrial dysfunction during cellular Ca^{2+} overload. *Eur J Biochem* 178: 489–501.

Csonka C., Szilvassy Z., Fulop F., Pali T., Blasig I.E., Tosaki A., Schulz R., Ferdinandy P. (1999). Classic preconditioning decreases the harmful accumulation of nitric oxide during ischemia and reperfusion in rat hearts. *Circulation* 100: 2260–2266.

Cumming R.C., Andon N.L., Haynes P.A., Park M., Fischer W.H., Schubert D. (2004). Protein disulfide bond formation in the cytoplasm during oxidative stress. *J Biol Chem* 279: 21749–21758.

Daiho T., Yamasaki K., Saino T., Kamidochi M., Satoh K., Iizuka H., Suzuki H. (2001). Mutations of either or both Cys876 and Cys888 residues of sarcoplasmic reticulum Ca^{2+}-ATPase result in a complete loss of Ca^{2+} transport activity without a loss of Ca^{2+}-dependent ATPase activity. Role of the CYS876–CYS888 disulfide bond. *J Biol Chem* 276: 32771–32778.

Dalle-Donne I., Giustarini D., Rossi R., Colombo R., Milzani A. (2003). Reversible *S*-glutathionylation of Cys374 regulates actin filament formation by inducing structural changes in the actin molecule. *Free Radic Biol Med* 34: 23–32.

Das D.K. (2001). Redox regulation of cardiomyocyte survival and death. *Antioxid Redox Signal* 3: 23–37.

Das D.K. (2004). Thioredoxin regulation of ischemic preconditioning. *Antioxid Redox Signal* 6: 405–412.

De Nigris F., Lerman L.O., Condorelli M., Lerman A., Napoli C. (2001). Oxidation-sensitive transcription factors and molecular mechanisms in the arterial wall. *Antioxid Redox Signal* 3: 1119–1130.

Delaunay A., Pflieger D., Barrault M.B., Vinh J., Toledano M.B. (2002). A thiol peroxidase is an H_2O_2 receptor and redox-transducer in gene activation. *Cell* 111: 471–481.

Depre C., Fierain L., L. Hue (1997). Activation of nitric oxide synthase by ischaemia in the perfused heart. *Cardiovasc Res* 33: 82–87.

Downey J.M., Hearse D.J., Yellon D.M. (1988). The role of xanthine oxidase during myocardial ischemia in several species including man. *J Mol Cell Cardiol* 20 Suppl 2: 55–63.

Dulhunty A., Haarmann C., Green D., Hart J. (2000). How many cysteine residues regulate ryanodine receptor channel activity? *Antioxid Redox Signal* 2: 27–34.

Eaton P., Bell R.M., Cave A.C., M.J. Shattock (2005). Ischemic preconditioning: A potential role for protein *S*-thiolation? *Antioxid Redox Signal* 7: 882–888.

Eaton P., Byers H.L., Leeds N., Ward M.A., Shattock M.J. (2002a). Detection, quantitation, purification, and identification of cardiac proteins *S*-thiolated during ischemia and reperfusion. *J Biol Chem* 277: 9806–9811.

Eaton P., Fuller W., Shattock M.J. (2002b). *S*-Thiolation of HSP27 regulates its multimeric aggregate size independently of phosphorylation. *J Biol Chem* 277: 21189–21196.

Eaton P., Hearse D.J., Shattock M.J. (2001). Lipid hydroperoxide modification of proteins during myocardial ischaemia. *Cardiovasc Res* 51: 294–303.

Eaton P., Jones M.E., McGregor E., Dunn M.J., Leeds N., Byers H.L., Leung K.Y., Ward M.A., Pratt J.R., Shattock M.J. (2003). Reversible cysteine-targeted oxidation of proteins during renal oxidative stress. *J Am Soc Nephrol* 14: S290–S296.

Eaton P., Li J.M., Hearse D.J., Shattock M.J. (1999). Formation of 4-hydroxy-2-nonenal-modified proteins in ischemic rat heart. *Am J Physiol* 276: H935–H943.

Eaton P., Shattock M.J. (2002). Purification of proteins susceptible to oxidation at cysteine residues: Identification of malate dehydrogenase as a target for *S*-glutathiolation. *Ann NY Acad Sci* 973: 529–532.

Eaton P., Wright N., Hearse D.J., Shattock M.J. (2002c). Glyceraldehyde phosphate dehydrogenase oxidation during cardiac ischemia and reperfusion. *J Mol Cell Cardiol* 34: 1549–1560.

Eddy L.J., Stewart J.R., Jones H.P., Engerson T.D., McCord J.M., Downey J.M. (1987). Free radical-producing enzyme, xanthine oxidase, is undetectable in human hearts. *Am J Physiol* 253: H709–H711.

Ernest M.J., Kim K.H. (1974). Regulation of rat liver glycogen synthetase D: Role of glucose 6-phosphate and enzyme sulfhydryl groups in activity and glycogen binding. *J Biol Chem* 249: 5011–5018.

Esposito F., Ammendola R., Faraonio R., Russo T., Cimino F. (2004). Redox control of signal transduction, gene expression and cellular senescence. *Neurochem Res* 29: 617–628.

Fan H., Sun B., Gu Q., Lafond-Walker A., Cao S., Becker L.C. (2002). Oxygen radicals trigger activation of NF-kappaB and AP-1 and upregulation of ICAM-1 in reperfused canine heart. *Am J Physiol Heart Circ Physiol* 282: H1778–H1786.

Ferrari R., Ceconi C., Curello S., Guarnieri C., Caldarera C.M., Albertini A., Visioli O. (1985). Oxygen-mediated myocardial damage during ischaemia and reperfusion: Role of the cellular defences against oxygen toxicity. *J Mol Cell Cardiol* 17: 937–945.

Finkel T. (2001). Reactive oxygen species and signal transduction. *IUBMB Life* 52: 3–6.

Flaherty J.T., Pitt B., Gruber J.W., Heuser R.R., Rothbaum D.A., Burwell L.R., George B.S., Kereiakes D.J., Deitchman D., Gustafson N. (1994). Recombinant human superoxide dismutase (h-SOD) fails to improve recovery of ventricular function in patients undergoing coronary angioplasty for acute myocardial infarction. *Circulation* 89: 1982–1991.

Fleet W.F., Johnson T.A., Graebner C.A., Gettes L.S. (1985). Effect of serial brief ischemic episodes on extracellular K^+, pH, and activation in the pig. *Circulation* 72: 922–932.

Florens E., Salvi S., Peynet J., Elbim C., Mallat Z., Bel A., Nguyen A., Tedgui A., Pasquier C., Menasche P. (2001). Can statins reduce the inflammatory response to cardiopulmonary bypass? A clinical study. *J Card Surg* 16: 232–239.

Forman H.J., Torres M., Fukuto J. (2002). Redox signaling. *Mol Cell Biochem* 234–235: 49–62.

Fratelli M., Demol H., Puype M., Casagrande S., Eberini I., Salmona M., Bonetto V., Mengozzi M., Duffieux F., Miclet E., Bachi A., Vandekerckhove J., Gianazza E., Ghezzi P. (2002). Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. *Proc Natl Acad Sci USA* 99: 3505–3510.

Froede H.C., Geraci G., Mansour T.E. (1968). Studies on heart phosphofructokinase: Thiol groups and their relationship to activity. *J Biol Chem* 243: 6021–6029.

Frostegard J., Kjellman B., Gidlund M., Andersson B., Jindal S., Kiessling R. (1996). Induction of heat shock protein in monocytic cells by oxidized low density lipoprotein. *Atherosclerosis* 121: 93–103.

Fryer R.M., Patel H.H., Hsu A.K., Gross G.J. (2001). Stress-activated protein kinase phosphorylation during cardioprotection in the ischemic myocardium. *Am J Physiol Heart Circ Physiol* 281: H1184–H1192.

Galang N., Sasaki H., Maulik N. (2000). Apoptotic cell death during ischemia/reperfusion and its attenuation by antioxidant therapy. *Toxicology* 148: 111–118.

Ganote C.E., Sims M., Safavi S. (1982). Effects of dimethylsulfoxide (DMSO) on the oxygen paradox in perfused rat hearts. *Am J Pathol* 109: 270–276.

Gao W.D., Atar D., Liu Y., Perez N.G., Murphy A.M., Marban E. (1997). Role of troponin I proteolysis in the pathogenesis of stunned myocardium. *Circ Res* 80: 393–399.

Garlick P.B., Davies M.J., Hearse D.J., Slater T.F. (1987). Direct detection of free radicals in the reperfused rat heart using electron spin resonance spectroscopy. *Circ Res* 61: 757–760.

Georgiou G. (2002). How to flip the (redox) switch. *Cell* 111: 607–610.

Gilbert H.F. (1982). Biological disulfides: the third messenger? Modulation of phosphofructokinase activity by thiol/disulfide exchange. *J Biol Chem* 257: 12086–12091.

Gilbert H.F. (1990). Molecular and cellular aspects of thiol-disulfide exchange. *Adv Enzymol Relat Areas Mol Biol* 63: 69–172.

Giraldez R.R., Panda A., Xia Y., Sanders S.P., Zweier J.L. (1997). Decreased nitric-oxide synthase activity causes impaired endothelium-dependent relaxation in the postischemic heart. *J Biol Chem* 272: 21420–21426.

Gopalakrishna R., Jaken S. (2000). Protein kinase C signaling and oxidative stress. *Free Radic Biol Med* 28: 1349–1361.

Gow A.J., Buerk D.G., Ischiropoulos H. (1997). A novel reaction mechanism for the formation of *S*-nitrosothiol in vivo. *J Biol Chem* 272: 2841–2845.

Graumann J., Lilie H., Tang X., Tucker K.A., Hoffmann J.H., Vijayalakshmi J., Saper M., Bardwell J.C., Jakob U. (2001). Activation of the redox-regulated molecular chaperone Hsp33—A two-step mechanism. *Structure (Camb)* 9: 377–387.

Grill H.P., Zweier J.L., Kuppusamy P., Weisfeldt M.L., Flaherty J.T. (1992). Direct measurement of myocardial free radical generation in an in vivo model: Effects of postischemic reperfusion and treatment with human recombinant superoxide dismutase. *J Am Coll Cardiol* 20: 1604–1611.

Grune T., Reinheckel T., Davies K.J. (1997). Degradation of oxidized proteins in mammalian cells. *Faseb J* 11: 526–534.

Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. (1999). Dietary supplementation with *n*-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: Results of the GISSI-Prevenzione trial. *Lancet* 354: 447–455.

Guarnieri C., Flamigni F., Rossoni-Caldarera C. (1979). Glutathione peroxidase activity and release of glutathione from oxygen-deficient perfused rat heart. *Biochem Biophys Res Commun* 89: 678–684.

Gunter T.E., Pfeiffer D.R. (1990). Mechanisms by which mitochondria transport calcium. *Am J Physiol* 258: C755–C786.

Guyton K.Z., Liu Y., Gorospe M., Xu Q., Holbrook N.J. (1996). Activation of mitogen-activated protein kinase by H_2O_2: Role in cell survival following oxidant injury. *J Biol Chem* 271: 4138–4142.

Haddad J.J. (2002). Antioxidant and prooxidant mechanisms in the regulation of redox(y)-sensitive transcription factors. *Cell Signal* 14: 879–897.

Haddock P.S., Shattock M.J., Hearse D.J. (1995). Modulation of cardiac Na^+-K^+ pump current: Role of protein and nonprotein sulfhydryl redox status. *Am J Physiol* 269: H297–H307.

Halestrap A.P., Brennerb C. (2003). The adenine nucleotide translocase: A central component of the mitochondrial permeability transition pore and key player in cell death. *Curr Med Chem* 10: 1507–1525.

Haramaki N., Stewart D.B., Aggarwal S., Ikeda H., Reznick A.Z., Packer L. (1998). Networking antioxidants in the isolated rat heart are selectively depleted by ischemia-reperfusion. *Free Radic Biol Med* 25: 329–339.

Harris A.S., Bisheni A., Russall R.A., Brigham J.C., Fireston J.E. (1954). Excitatory factors in ventricular tachycardia resulting from myocardial ischemia: Potassium a major excitant. *Science* 119: 200–203.

Hashimoto K., Satoh H., Shibuya T., Imai S. (1982). Canine-effective plasma concentrations of antiarrhythmic drugs on the two-stage coronary ligation arrhythmia. *J Pharmacol Exp Ther* 223: 801–810.

Hausladen A., Privalle C.T., Keng T., DeAngelo J., Stamler J.S. (1996). Nitrosative stress: Activation of the transcription factor OxyR. *Cell* 86: 719–729.

Hayashi Y., Sawa Y., Ohtake S., Fukuyama N., Nakazawa H., Matsuda H. (2001). Peroxynitrite formation from human myocardium after ischemia-reperfusion during open heart operation. *Ann Thorac Surg* 72: 571–576.

Hearse D.J. (1990). Ischemia, reperfusion, and the determinants of tissue injury. *Cardiovasc Drugs Ther* 4: 767–776.

Hearse D.J. (1992). Myocardial injury during ischemia and reperfusion: Concepts and controversies. In: Yellon D.M., Jennings R.B. (eds.), *Myocardial Protection: The Pathophysiology of Reperfusion and Reperfusion Injury*, Raven Press, New York, pp. 13–32.

Hearse D.J. (1994). Myocardial ischaemia: Can we agree on a definition for the 21st century? *Cardiovasc Res* 28: 1737–1744: discussion 1745–1746.

Hearse D.J., Bolli R. (1992). Reperfusion induced injury: Manifestations, mechanisms, and clinical relevance. *Cardiovasc Res* 26: 101–108.

Hearse D.J., Kusama Y., Bernier M. (1989). Rapid electrophysiological changes leading to arrhythmias in the aerobic rat heart: Photosensitization studies with rose bengal-derived reactive oxygen intermediates. *Circ Res* 65: 146–153.

Hein S., Scheffold T., Schaper J. (1995). Ischemia induces early changes to cytoskeletal and contractile proteins in diseased human myocardium. *J Thorac Cardiovasc Surg* 110: 89–98.

Hein S., Scholz D., Fujitani N., Rennollet H., Brand T., Friedl A., Schaper J. (1994). Altered expression of titin and contractile proteins in failing human myocardium. *J Mol Cell Cardiol* 26: 1291–1306.

Hernandez L.A., Grisham M.B., Twohig B., Arfors K.E., Harlan J.M., Granger D.N. (1987). Role of neutrophils in ischemia-reperfusion-induced microvascular injury. *Am J Physiol* 253: H699–H703.

Heyndrickx G.R., Millard R.W., McRitchie R.J., Maroko P.R., Vatner S.F. (1975). Regional myocardial functional and electrophysiological alterations after brief coronary artery occlusion in conscious dogs. *J Clin Invest* 56: 978–985.

Hidalgo C., Aracena P., Sanchez G., Donoso P. (2002). Redox regulation of calcium release in skeletal and cardiac muscle. *Biol Res* 35: 183–193.

Holmberg S.R., Cumming D.V., Kusama Y., Hearse D.J., Poole-Wilson P.A., Shattock M.J., Williams A.J. (1991). Reactive oxygen species modify the structure and function of the cardiac sarcoplasmic reticulum calcium-release channel. *Cardioscience* 2: 19–25.

Iliou J.P., Thollon C., Robin F., Cambarrat C., Guillonneau C., Regnier G., Lenaers A., Vilaine J.P. (1993). Protective effect of S12340 on cardiac cells exposed to oxidative stress. *Eur J Pharmacol* 248: 263–272.

Inagaki K., Hahn H.S., Dorn G.W. II, Mochly-Rosen D. (2003). Additive protection of the ischemic heart ex vivo by combined treatment with delta-protein kinase C inhibitor and epsilon-protein kinase C activator. *Circulation* 108: 869–875.

Irving C.C., Daniel D.S., Murphy W.M. (1983). The effect of disulfiram on the carcinogenicity of *N*-butyl-*N*-(3-carboxypropyl)nitrosamine in the rat. *Carcinogenesis* 4: 617–620.

Ishida H., Ichimori K., Hirota Y., Fukahori M., Nakazawa H. (1996). Peroxynitrite-induced cardiac myocyte injury. *Free Radic Biol Med* 20: 343–350.

Jekabsone A., Ivanoviene L., Brown G.C., Borutaite V. (2003). Nitric oxide and calcium together inactivate mitochondrial complex I and induce cytochrome c release. *J Mol Cell Cardiol* 35: 803–809.

Jennings R.B., Reimer K.A. (1991). The cell biology of acute myocardial ischemia. *Ann Rev Med* 42: 225–246.

Jensen A.G., Espersen F., Skinhoj P., Frimodt-Moller N. (1998). Bacteremic *Staphylococcus aureus* spondylitis. *Arch Intern Med* 158: 509–517.

Jolly S.R., Kane W.J., Bailie M.B., Abrams G.D., Lucchesi B.R. (1984). Canine myocardial reperfusion injury: Its reduction by the combined administration of superoxide dismutase and catalase. *Circ Res* 54: 277–285.

Kardesch M., Hogancamp C., Bing R. (1958). The effect of complete ischemia on the intracellular electrical activity of the whole mammalian heart. *Circ Res* 6: 715–720.

Keira N., Tatsumi T., Matoba S., Shiraishi J., Yamanaka S., Akashi K., Kobara M., Asayama J., Fushiki S., Fliss H., Nakagawa M. (2002). Lethal effect of cytokine-induced nitric oxide and peroxynitrite on cultured rat cardiac myocytes. *J Mol Cell Cardiol* 34: 583–596.

Khaliulin I., Schwalb H., Wang P., Houminer E., Grinberg L., Katzeff H., Borman J.B., Powell S.R. (2004). Preconditioning improves postischemic mitochondrial function and diminishes oxidation of mitochondrial proteins. *Free Radic Biol Med* 37: 1–9.

Kim M.S., Akera T. (1987). O_2 free radicals: Cause of ischemia-reperfusion injury to cardiac Na^+-K^+-ATPase. *Am J Physiol* 252: H252–H257.

Kim S.O., Merchant K., Nudelman R., Beyer W.F. Jr., T. Keng, DeAngelo J., Hausladen A., Stamler J.S. (2002). OxyR: A molecular code for redox-related signaling. *Cell* 109: 383–396.

Kleber A.G. (1983). Resting membrane potential, extracellular potassium activity, and intracellular sodium activity during acute global ischemia in isolated perfused guinea pig hearts. *Circ Res* 52: 442–450.

Knight R.J., Kofoed K.F., Schelbert H.R., Buxton D.B. (1996). Inhibition of glyceraldehyde-3-phosphate dehydrogenase in post-ischaemic myocardium. *Cardiovasc Res* 32: 1016–1023.

Koeck T., Fu X., Hazen S.L., Crabb J.W., Stuehr D.J., Aulak K.S. (2004). Rapid and selective oxygen-regulated protein tyrosine denitration and nitration in mitochondria. *J Biol Chem* 279: 27257–27262.

Kubler W., Spieckermann P.G. (1970). Regulation of glycolysis in the ischemic and the anoxic myocardium. *J Mol Cell Cardiol* 1: 351–377.

Kukreja R.C. (2002). NFkappaB activation during ischemia/reperfusion in heart: Friend or foe? *J Mol Cell Cardiol* 34: 1301–1304.

Kukreja R.C., Weaver A.B., Hess M.L. (1990). Sarcolemmal Na^+-K^+-ATPase: Inactivation by neutrophil-derived free radicals and oxidants. *Am J Physiol* 259: H1330–H1336.

Kulanthaivel P., Simon B.J., Leibach F.H., Mahesh V.B., Ganapathy V. (1990). An essential role for vicinal dithiol groups in the catalytic activity of the human placental Na^+-H^+ exchanger. *Biochim Biophys Acta* 1024: 385–389.

Lacampagne A., Duittoz A., Bolanos P., Peineau N., Argibay J.A. (1995). Effect of sulfhydryl oxidation on ionic and gating currents associated with L-type calcium channels in isolated guinea-pig ventricular myocytes. *Cardiovasc Res* 30: 799–806.

Lee W.H., Gounarides J.S., Roos E.S., Wolin M.S. (2003). Influence of peroxynitrite on energy metabolism and cardiac function in a rat ischemia-reperfusion model. *Am J Physiol Heart Circ Physiol* 285: H1385–H1395.

Leonarduzzi G., Robbesyn F., Poli G. (2004). Signaling kinases modulated by 4-hydroxynonenal. *Free Radic Biol Med* 37: 1694–1702.

Lesnefsky E.J., Repine J.E., Horwitz L.D. (1989). Oxidation and release of glutathione from myocardium during early reperfusion. *Free Radic Biol Med* 7: 31–35.

Li C., Kao R.L., Ha T., Kelley J., Browder I.W., Williams D.L. (2001). Early activation of IKKbeta during in vivo myocardial ischemia. *Am J Physiol Heart Circ Physiol* 280: H1264–H1271.

Lin K.M., Lin B., Lian I.Y., Mestril R., Scheffler I.E., Dillmann W.H. (2001). Combined and individual mitochondrial HSP60 and HSP10 expression in cardiac myocytes protects mitochondrial function and prevents apoptotic cell deaths induced by simulated ischemia-reoxygenation. *Circulation* 103: 1787–1792.

Lind C., Gerdes R., Hamnell Y., Schuppe-Koistinen I., von Lowenhielm H.B., Holmgren A., Cotgreave I.A. (2002). Identification of *S*-glutathionylated cellular proteins during oxidative stress and constitutive metabolism by affinity purification and proteomic analysis. *Arch Biochem Biophys* 406: 229–240.

Linder N., Rapola J., Raivio K.O. (1999). Cellular expression of xanthine oxidoreductase protein in normal human tissues. *Lab Invest* 79: 967–974.

Linke K., Jakob U. (2003). Not every disulfide lasts forever: Disulfide bond formation as a redox switch. *Antioxid Redox Signal* 5: 425–434.

Liu P., Hock C.E., Nagele R., Wong P.Y. (1997). Formation of nitric oxide, superoxide, and peroxynitrite in myocardial ischemia-reperfusion injury in rats. *Am J Physiol* 272: H2327–H2336.

Liu W., Akhand A.A., Kato M., Yokoyama I., Miyata T., Kurokawa K., Uchida K., Nakashima I. (1999). 4-Hydroxynonenal triggers an epidermal growth factor receptor-linked signal pathway for growth inhibition. *J Cell Sci* 112: 2409–2417.

Lubbe W.F., Daries P.S., Opie L.H. (1978). Ventricular arrhythmias associated with coronary artery occlusion and reperfusion in the isolated perfused rat heart: A model for assessment of antifibrillatory action of antiarrhythmic agents. *Cardiovasc Res* 12: 212–220.

MacFarlane N.G., Miller D.J. (1992). Depression of peak force without altering calcium sensitivity by the superoxide anion in chemically skinned cardiac muscle of rat. *Circ Res* 70: 1217–1224.

Mackay K., Mochly-Rosen D. (2001). Localization, anchoring, and functions of protein kinase C isozymes in the heart. *J Mol Cell Cardiol* 33: 1301–1307.

Mahadev K., Zilbering A., Zhu L., Goldstein B.J. (2001). Insulin-stimulated hydrogen peroxide reversibly inhibits protein-tyrosine phosphatase 1b in vivo and enhances the early insulin action cascade. *J Biol Chem* 276: 21938–21942.

Mallis R.J., Buss J.E., Thomas J.A. (2001). Oxidative modification of H-ras: *S*-thiolation and *S*-nitrosylation of reactive cysteines. *Biochem J* 355: 145–153.

Marber M.S., Latchman D.S., Walker J.M., Yellon D.M. (1993). Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation* 88: 1264–1272.

Marber M.S., Mestril R., Chi S.H., Sayen M.R., Yellon D.M., Dillmann W.H. (1995). Overexpression of the rat inducible 70-kD heat stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury. *J Clin Invest* 95: 1446–1456.

Martinez-Ruiz A., Lamas S. (2004). *S*-Nitrosylation: A potential new paradigm in signal transduction. *Cardiovasc Res* 62: 43–52.

Matsumoto A., Comatas K.E., Liu L., Stamler J.S. (2003). Screening for nitric oxide-dependent protein-protein interactions. *Science* 301: 657–661.

Maupoil V., Rochette L., Tabard A., Clauser P., Harpey C. (1990). Evolution of free radical formation during low-flow ischemia and reperfusion in isolated rat heart. *Cardiovasc Drugs Ther* 4: 791–795.

McDonough J.L., Arrell D.K., Van Eyk J.E. (1999). Troponin I degradation and covalent complex formation accompanies myocardial ischemia/reperfusion injury. *Circ Res* 84: 9–20.

McStay G.P., Clarke S.J., Halestrap A.P. (2002). Role of critical thiol groups on the matrix surface of the adenine nucleotide translocase in the mechanism of the mitochondrial permeability transition pore. *Biochem J* 367: 541–548.

Mehlen P., Hickey E., Weber L.A., Arrigo A.P. (1997). Large unphosphorylated aggregates as the active form of hsp27 which controls intracellular reactive oxygen species and glutathione levels and generates a protection against TNFalpha in NIH-3T3-ras cells. *Biochem Biophys Res Commun* 241: 187–192.

Meldrum D.R. (1998). Tumor necrosis factor in the heart. *Am J Physiol* 274: R577–R595.

Mihm M.J., Bauer J.A. (2002). Peroxynitrite-induced inhibition and nitration of cardiac myofibrillar creatine kinase. *Biochimie* 84: 1013–1019.

Miki S., Ashraf M., Salka S., Sperelakis N. (1988). Myocardial dysfunction and ultrastructural alterations mediated by oxygen metabolites. *J Mol Cell Cardiol* 20: 1009–1024.

Miller R.M., Sies H., Park E.M., Thomas J.A. (1990). Phosphorylase and creatine kinase modification by thiol-disulfide exchange and by xanthine oxidase-initiated *S*-thiolation. *Arch Biochem Biophys* 276: 355–363.

Milzani A., DalleDonne I., Colombo R. (1997). Prolonged oxidative stress on actin. *Arch Biochem Biophys* 339: 267–274.

Mohr S., Stamler J.S., Brune B. (1996). Posttranslational modification of glyceraldehyde-3-phosphate dehydrogenase by *S*-nitrosylation and subsequent NADH attachment. *J Biol Chem* 271: 4209–4214.

Morishita R., Sugimoto T., Aoki M., Kida I., Tomita N., Moriguchi A., Maeda K., Sawa Y., Kaneda Y., Higaki J., Ogihara T. (1997). In vivo transfection of cis element "decoy" against nuclear factor-kappaB binding site prevents myocardial infarction. *Nat Med* 3: 894–899.

Morita-Fujimura Y., Fujimura M., Yoshimoto T., Chan P.H. (2001). Superoxide during reperfusion contributes to caspase-8 expression and apoptosis after transient focal stroke. *Stroke* 32: 2356–2361.

Murphy A.M., Kogler H., Georgakopoulos D., McDonough J.L., Kass D.A., Van Eyk J.E., Marban E. (2000). Transgenic mouse model of stunned myocardium. *Science* 287: 488–491.

Murry C.E., Jennings R.B., Reimer K.A. (1986). Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 74: 1124–1136.

Murry C.E., Richard V.J., Jennings R.B., Reimer K.A. (1988). Preconditioning with ischaemia: Is the protective effect mediated by free radical-induced myocardial stunning? (Abstract). *Circulation* 78: 77.

Myers M.L., Bolli R., Lekich R.F., Hartley C.J., Roberts R. (1985). Enhancement of recovery of myocardial function by oxygen free-radical scavengers after reversible regional ischemia. *Circulation* 72: 915–921.

Myers M.L., Bolli R., Lekich R.F., Hartley C.J., Roberts R. (1986). *N*-2-Mercapto-propionylglycine improves recovery of myocardial function after reversible regional ischemia. *J Am Coll Cardiol* 8: 1161–1168.

Nakamura H., Vaage J., Valen G., Padilla C.A., Bjornstedt M., Holmgren A. (1998). Measurements of plasma glutaredoxin and thioredoxin in healthy volunteers and during open-heart surgery. *Free Radic Biol Med* 24: 1176–1186.

Nakano A., Baines C.P., Kim S.O., Pelech S.L., Downey J.M., Cohen M.V., Critz S.D. (2000). Ischemic preconditioning activates MAPKAPK2 in the isolated rabbit heart: Evidence for involvement of p38 MAPK. *Circ Res* 86: 144–151.

Nakashima K., Horecker B.L., Pontremoli S. (1970). Regulatory sulfhydryl groups and activation by homocystine in liver fructose diphosphatase. *Arch Biochem Biophys* 141: 579–587.

Nakaya H., Takeda Y., Tohse N., Kanno M. (1992). Mechanism of the membrane depolarization induced by oxidative stress in guinea-pig ventricular cells. *J Mol Cell Cardiol* 24: 523–534.

Okubo S., Wildner O., Shah M.R., Chelliah J.C., Hess M.L., Kukreja R.C. (2001). Gene transfer of heat-shock protein 70 reduces infarct size in vivo after ischemia/reperfusion in the rabbit heart. *Circulation* 103: 877–881.

Pantke, U., T. Volk, M. Schmutzler, W.J. Kox, N. Sitte and T. Grune (1999). Oxidized proteins as a marker of oxidative stress during coronary heart surgery. *Free Radic Biol Med* 27: 1080–1086.

Park Y., Kanekal S., Kehrer J.P. (1991). Oxidative changes in hypoxic rat heart tissue. *Am J Physiol* 260: H1395–H1405.

Pike M.M., Kitakaze M., Marban E. (1990). ^{23}Na-NMR measurements of intracellular sodium in intact perfused ferret hearts during ischemia and reperfusion. *Am J Physiol* 259: H1767–H1773.

Ping P., Murphy E. (2000). Role of p38 mitogen-activated protein kinases in preconditioning: A detrimental factor or a protective kinase? *Circ Res* 86: 921–922.

Poole L.B., Karplus P.A., Claiborne A. (2004). Protein sulfenic acids in redox signaling. *Ann Rev Pharmacol Toxicol* 44: 325–347.

Poston J.M., Parenteau G.L. (1992). Biochemical effects of ischemia on isolated, perfused rat heart tissues. *Arch Biochem Biophys* 295: 35–41.

Powell S.R., Gurzenda E.M., Wahezi S.E. (2001). Actin is oxidized during myocardial ischemia. *Free Radic Biol Med* 30: 1171–1176.

Premaratne S., Limm W., Mugiishi M.M., Piette L.H., McNamara J.J. (1993). Quantitative assessment of free-radical generation during ischemia and reperfusion in the isolated rabbit heart. *Coron Artery Dis* 4: 1093–1096.

Prieto-Alamo M.J., Jurado J., Gallardo-Madueno R., Monje-Casas F., Holmgren A., Pueyo C. (2000). Transcriptional regulation of glutaredoxin and thioredoxin pathways and related enzymes in response to oxidative stress. *J Biol Chem* 275: 13398–13405.

Przyklenk K., Whittaker P., Kloner R.A. (1990). In vivo infusion of oxygen free radical substrates causes myocardial systolic, but not diastolic dysfunction. *Am Heart J* 119: 807–815.

Putkey J.A., Dotson D.G., Mouawad P. (1993). Formation of inter- and intramolecular disulfide bonds can activate cardiac troponin C. *J Biol Chem* 268: 6827–6830.

Rapola J.M., Virtamo J., Ripatti S., Huttunen J.K., Albanes D., Taylor P.R., Heinonen O.P. (1997). Randomised trial of alpha-tocopherol and beta-carotene supplements on incidence of major coronary events in men with previous myocardial infarction. *Lancet* 349: 1715–1720.

Ravichandran V., Seres T., Moriguchi T., Thomas J.A., Johnston, R.B. Jr. (1994). *S*-Thiolation of glyceraldehyde-3-phosphate dehydrogenase induced by the phagocytosis-associated respiratory burst in blood monocytes. *J Biol Chem* 269: 25010–25015.

Reddy S., Jones A.D., Cross C.E., Wong P.S., van der Vliet A. (2000). Inactivation of creatine kinase by S-glutathionylation of the active-site cysteine residue. *Biochem J* 347: 821–827.

Reimer K.A., Murry C.E., Yamasawa I., Hill M.L., Jennings R.B. (1986). Four brief periods of myocardial ischemia cause no cumulative ATP loss or necrosis. *Am J Physiol* 251: H1306–H1315.

Reznick A.Z., Kagan V.E., Ramsey R., Tsuchiya M., Khwaja S., Serbinova E.A., Packer L. (1992). Antiradical effects in L-propionyl carnitine protection of the heart against ischemia-reperfusion injury: The possible role of iron chelation. *Arch Biochem Biophys* 296: 394–401.

Rigobello M.P., Bindoli A. (1993). Effect of pyruvate on rat heart thiol status during ischemia and hypoxia followed by reperfusion. *Mol Cell Biochem* 122: 93–100.

Romson J.L., Hook B.G., Kunkel S.L., Abrams G.D., Schork M.A., Lucchesi BR. (1983). Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. *Circulation* 67: 1016–1023.

Rozanski G.J., Xu Z. (2002). Sulfhydryl modulation of K^+ channels in rat ventricular myocytes. *J Mol Cell Cardiol* 34: 1623–1632.

Rubin Y., Navon G. (1993). Inhibition of sodium influx and improved preservation of rat hearts during hypothermic ischemia by furosemide and bumetanide: a ^{23}Na- and ^{31}P-NMR study. *J Mol Cell Cardiol* 25: 1403–1411.

Sabri A., Hughie H.H., Lucchesi P.A. (2003). Regulation of hypertrophic and apoptotic signaling pathways by reactive oxygen species in cardiac myocytes. *Antioxid Redox Signal* 5: 731–740.

Saitoh M., Nishitoh H., Fujii M., Takeda K., Tobiume K., Sawada Y., Kawabata M., Miyazono K., Ichijo H. (1998). Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *Embo J* 17: 2596–2606.

Sato H., Hori M., Kitakaze M., Iwai K., Takashima S., Kurihara H., Inoue M., Kamada T. (1993). Reperfusion after brief ischemia disrupts the microtubule network in canine hearts. *Circ Res* 72: 361–375.

Saurin A., Pennington D., Raat N., Latchman D., Owen M., Marber M. (2002). Targeted disruption of the protein kinase C epsilon gene abolishes the infarct size reduction that follows ischaemic preconditioning of isolated buffer-perfused mouse hearts. *Cardiovasc Res* 55: 672.

Saurin A.T., Martin J.L., Heads R.J., Foley C., Mockridge J.W., Wright M.J., Wang Y., Marber M.S. (2000). The role of differential activation of p38-mitogen-activated protein kinase in preconditioned ventricular myocytes. *Faseb J* 14: 2237–2246.

Saurin A.T., Neubert H., Brennan J.P., Eaton P. (2004). Widespread sulfenic acid formation in tissues in response to hydrogen peroxide. *Proc Natl Acad Sci USA* 101: 17982–17987.

Schulz R., Cohen M.V., Behrends M., Downey J.M., Heusch G. (2001). Signal transduction of ischemic preconditioning. *Cardiovasc Res* 52: 181–198.

Schwalb H., Olivson A., Li J., Houminer E., Wahezi S.E., Opie L.H., Maulik D., Borman J.B., Powell S.R. (2001). Nicorandil decreases postischemic actin oxidation. *Free Radic Biol Med* 31: 607–614.

Sethuraman M., McComb M.E., Huang H., Huang S., Heibeck T., Costello C.E., Cohen R.A. (2004). Isotope-coded affinity tag (ICAT) approach to redox proteomics: Identification and quantitation of oxidant-sensitive cysteine thiols in complex protein mixtures. *J Proteome Res* 3: 1228–1233.

Shattock M.J., Matsuura H. (1993). Measurement of Na^+-K^+ pump current in isolated rabbit ventricular myocytes using the whole-cell voltage-clamp technique: Inhibition of the pump by oxidant stress. *Circ Res* 72: 91–101.

Shioji K., Kishimoto C., Nakamura H., Masutani H., Yuan Z., Oka S., Yodoi J. (2002). Overexpression of thioredoxin-1 in transgenic mice attenuates adriamycin-induced cardiotoxicity. *Circulation* 106: 1403–1409.

Souza J.M., Radi R. (1998). Glyceraldehyde-3-phosphate dehydrogenase inactivation by peroxynitrite. *Arch Biochem Biophys* 360: 187–194.

Stamler J.S., Lamas S., Fang F.C. (2001). Nitrosylation: The prototypic redox-based signaling mechanism. *Cell* 106: 675–683.

Stokoe D., Engel K., Campbell D.G., Cohen P., Gaestel M. (1992). Identification of MAPKAP kinase 2 as a major enzyme responsible for the phosphorylation of the small mammalian heat shock proteins. *FEBS Lett* 313: 307–313.

Stoyanovsky D., Murphy T., Anno P.R., Kim Y.M., Salama G. (1997). Nitric oxide activates skeletal and cardiac ryanodine receptors. *Cell Calcium* 21: 19–29.

Suzuki Y.J., Ford G.D. (1999). Redox regulation of signal transduction in cardiac and smooth muscle. *J Mol Cell Cardiol* 31: 345–353.

Taegtmeyer H., Goodwin G.W., Doenst T., Frazier O.H. (1997). Substrate metabolism as a determinant for postischemic functional recovery of the heart. *Am J Cardiol* 80: 3A–10A.

Tanaka M., Fujiwara H., Yamasaki K., Sasayama S. (1994). Superoxide dismutase and MPG attenuate infarct sze limitation effect of ischaemic preconditioning in the rabbit. *Cardiovasc Res* 28: 980–986.

Tardif J.-C. (2003). Oxidative stress and coronary heart disease. *Cardiology Rounds* 7.

Tennant R., Wiggers C. (1935). The effect of coronary occlusion on myocardial contraction. *Am J Physiol* 112: 351–361.

Terada L.S., Rubinstein J.D., Lesnefsky E.J., Horwitz L.D., Leff J.A., Repine J.E. (1991). Existence and participation of xanthine oxidase in reperfusion injury of ischemic rabbit myocardium. *Am J Physiol* 260: H805–H810.

Thannickal V.J., Fanburg B.L. (2000). Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol* 279: L1005–L1028.

Thollon C., Iliou J.P., Cambarrat C., Robin F., Vilaine J.P. (1995). Nature of the cardiomyocyte injury induced by lipid hydroperoxides. *Cardiovasc Res* 30: 648–655.

Timoshin A.A., Lakomkin V.L., Ruuge E.K. (1993). (Free radical centers in isolated rat heart tissue in a normal state, in ischemia, and reperfusion). *Biofizika* 38: 179–186.

Timoshin A.A., Tskitishvili O.V., Serebryakova L.I., Kuzmin A.I., Medvedev O.S., Ruuge E.K. (1994). Microdialysis study of ischemia-induced hydroxyl radicals in the canine heart. *Experientia* 50: 677–679.

Tokube K., Kiyosue T., Arita M. (1998). Effects of hydroxyl radicals on K_{ATP} channels in guinea-pig ventricular myocytes. *Pflugers Arch* 437: 155–157.

Tritto I., D'Andrea D., Eramo N., Scognamiglio A., De Simone C., Violante A., Esposito A., Chiariello M., Ambrosio G. (1997). Oxygen radicals can induce preconditioning in rabbit hearts. *Circ Res* 80: 743–748.

Tritto I., Duilio C., Santoro G., Elia P.P., Cirillo P., De Simone C., Chiariello M., Ambrosio G. (1998). A short burst of oxygen radicals at reflow induces sustained release of oxidized glutathione from postischemic hearts. *Free Radic Biol Med* 24: 290–297.

Turoczi T., Chang V.W., Engelman R.M., Maulik N., Ho Y.S., Das D.K. (2003). Thioredoxin redox signaling in the ischemic heart: An insight with transgenic mice overexpressing Trx1. *J Mol Cell Cardiol* 35: 695–704.

Valen G., Yan Z.Q., Hansson G.K. (2001). Nuclear factor kappa-B and the heart. *J Am Coll Cardiol* 38: 307–314.

van den Dobbelsteen D.J., Nobel C.S., Schlegel J., Cotgreave I.A., Orrenius S., Slater A.F. (1996). Rapid and specific efflux of reduced glutathione during apoptosis induced by anti-Fas/APO-1 antibody. *J Biol Chem* 271: 15420–15427.

van Emous J.G., Nederhoff M.G., Ruigrok T.J., van Echteld C.J. (1997). The role of the Na^+ channel in the accumulation of intracellular Na^+ during myocardial ischemia: consequences for post-ischemic recovery. *J Mol Cell Cardiol* 29: 85–96.

Van Eyk J.E., Powers F., Law W., Larue C., Hodges R.S., Solaro R.J. (1998). Breakdown and release of myofilament proteins during ischemia and ischemia/reperfusion in rat hearts: Identification of degradation products and effects on the pCa-force relation. *Circ Res* 82: 261–271.

Vanden Hoek T.L., Becker L.B., Shao Z., Li C., Schumacker P.T. (1998). Reactive oxygen species released from mitochondria during brief hypoxia induced preconditioning in cardiomyocytes. *J Biol Chem* 273: 18092–18098.

Vanden Hoek T.L., Becker L.B., Shao Z.-H., Li C.-Q., Schumacker P.T. (2000). Preconditioning in cardiomyocytes protects by attenuating oxidant stress at reperfusion. *Circ Res* 86: 541–548.

Vanden Hoek T.L., Li C., Shao Z., Schumacker P.T., Becker L.B. (1997a). Significant levels of oxidants are generated by isolated cardiomyocytes during ischemia prior to reperfusion. *J Mol Cell Cardiol* 29: 2571–2583.

Vanden Hoek T.L., Shao Z., Li C., Schumacker P.T., Becker L.B. (1997b). Mitochondrial electron transport can become a significant source of oxidative injury in cardiomyocytes. *J Mol Cell Cardiol* 29: 2441–2450.

von Harsdorf R., Li P.F., Dietz R. (1999). Signaling pathways in reactive oxygen species-induced cardiomyocyte apoptosis. *Circulation* 99: 2934–2941.

Walker M.J., Curtis M.J., Hearse D.J., Campbell R.W., Janse M.J., Yellon D.M., Cobbe S.M., Coker S.J., Harness J.B., Harron D.W., et al. (1988). The Lambeth Conventions: guidelines for the study of arrhythmias in ischaemia infarction, and reperfusion. *Cardiovasc Res* 22: 447–455.

Wang H., Mao Y., Chen A.Y., Zhou N., LaVoie E.J., Liu L.F. (2001). Stimulation of topoisomerase II-mediated DNA damage via a mechanism involving protein thiolation. *Biochemistry* 40: 3316–3323.

Wang P., Zweier J.L. (1996). Measurement of nitric oxide and peroxynitrite generation in the postischemic heart: Evidence for peroxynitrite-mediated reperfusion injury. *J Biol Chem* 271: 29223–29230.

Ward N.E., Pierce D.S., Chung S.E., Gravitt K.R., O'Brian C.A. (1998). Irreversible inactivation of protein kinase C by glutathione. *J Biol Chem* 273: 12558–12566.

Weinstein D.M., Mihm M.J., Bauer J.A. (2000). Cardiac peroxynitrite formation and left ventricular dysfunction following doxorubicin treatment in mice. *J Pharmacol Exp Ther* 294: 396–401.

Westfall M.V., Solaro R.J. (1992). Alterations in myofibrillar function and protein profiles after complete global ischemia in rat hearts. *Circ Res* 70: 302–313.

Woo H.A., Chae H.Z., Hwang S.C., Yang K.S., Kang S.W., Kim K., Rhee S.G. (2003). Reversing the inactivation of peroxiredoxins caused by cysteine sulfinic acid formation. *Science* 300: 653–656.

Woo H.A., Jeong W., Chang T.S., Park K.J., Park S.J., Yang J.S., Rhee S.G. (2005). Reduction of cysteine sulfinic acid by sulfiredoxin is specific to 2-Cys peroxiredoxins. *J Biol Chem* 280: 3125–3128.

Yasmin W., Strynadka K.D., Schulz R. (1997). Generation of peroxynitrite contributes to ischemia-reperfusion injury in isolated rat hearts. *Cardiovasc Res* 33: 422–432.

Yellon D.M., Marber M.S. (1994). Hsp70 in myocardial ischaemia. *Experientia* 50: 1075–1084.

Yoshizumi M., Tsuchiya K., Tamaki T. (2001). Signal transduction of reactive oxygen species and mitogen-activated protein kinases in cardiovascular disease. *J Med Invest* 48: 11–24.

Yusuf S., Dagenais G., Pogue J., Bosch J., Sleight P. (2000). Vitamin E supplementation and cardiovascular events in high-risk patients: The Heart Outcomes Prevention Evaluation Study Investigators. *N Engl J Med* 342: 154–160.

Zahradnikova A., Minarovic I., Venema R.C., Meszaros L.G. (1997). Inactivation of the cardiac ryanodine receptor calcium release channel by nitric oxide. *Cell Calcium* 22: 447–454.

Zhao Z.Q. (2004). Oxidative stress-elicited myocardial apoptosis during reperfusion. *Curr Opin Pharmacol* 4: 159–165.

Zheng M., Aslund F., Storz G. (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* 279: 1718–1721.

Zweier J.L., Fertmann J., Wei G. (2001). Nitric oxide and peroxynitrite in postischemic myocardium. *Antioxid Redox Signal* 3: 11–22.

Zweier J.L., Kuppusamy P., Williams R., Rayburn B.K., Smith D., Weisfeldt M.L., Flaherty J.T. (1989). Measurement and characterization of postischemic free radical generation in the isolated perfused heart. *J Biol Chem* 264: 18890–18895.

Zweier J.L., Samouilov A., Kuppusamy P. (1999). Non-enzymatic nitric oxide synthesis in biological systems. *Biochim Biophys Acta* 1411: 250–262.

20

PROTEOME ANALYSIS OF OXIDATIVE STRESS: GLUTATHIONYL HEMOGLOBIN IN DIABETIC AND UREMIC PATIENTS

TOSHIMITSU NIWA

20.1 INTRODUCTION

20.1.1 Oxidative Stress

Oxidative stress occurs when there is excessive free radical production in defective antioxidant defenses. Oxidative stress produces profound alterations to cellular membrane lipids, proteins, and nucleic acids, impairing cell metabolism and viability. Oxidative stress has been considered to be involved in aging (Stadtman, 1992) and such diseases as diabetes mellitus (Son et al., 2004), uremia (Vaziri, 2004), atherosclerosis (Stocker and Keaney, 2004), hyperlipidemia (Warnholtz et al., 2001), rheumatoid arthritis (Hitchon and El-Gabalawy, 2004), adult respiratory distress syndrome (Chow et al., 2003), reoxygenation injury (Janero, 1995), human immunodeficiency virus infection (Bautista, 2001), cystic fibrosis (van der Vliet and Cross, 2000), and Friedreich's ataxia (Cooper and Schapira, 2003). Oxidative stress corresponds to an imbalance between the production of reactive oxygen species, mainly the superoxide anion (O_2^-), hydroxyl radical ($^{\bullet}OH$), peroxyl radicals ($LOO^{\bullet}$), and hydrogen peroxide (H_2O_2), and protective mechanisms. Several enzymatic systems can detoxify reactive oxygen species: superoxide dismutase catalyzes the conversion of O_2^- to H_2O_2 and works concomitantly with catalases and a selenoprotein, glutathione peroxidase. The level

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

of reduced glutathione (GSH) is a limiting factor in this enzymatic process, which requires the maintenance of a high reduced-to-oxidized glutathione (GSH/GSSG) ratio as achieved by glutathione reductase. In addition some reducing agents act as free radical scavengers to nonenzymatically detoxify reactive oxygen species: GSH, vitamin E, and vitamin C.

20.1.2 Glutathione

The tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine) is the major intracellular nonprotein thiol compound, and plays a major role in the protection of cells and tissue structures from oxidative injury. Glutathione can be reduced (GSH), oxidized (GSSG), or bound to proteins. GSH inhibits free radical mediated injury by eliminating reactive oxygen species, and protects protein thiol groups from oxidation by serving as a biological redox agent. Intracellular and blood concentrations of GSH are in millimolar range, while plasma concentration is in the micromolar range and accounts for approximately 0.4% of total blood GSH (Michelet et al., 1995; Richie et al., 1996). Erythrocytes play an important role in the delivery of GSH to tissues with high rate of GSH utilization, including lung, heart, gut, and brain. Both the liver and kidney normally release substantial amounts of GSH in the form of precursor amino acids that are extracted by erythrocytes, resynthesized into GSH, and then transported to the tissues.

20.1.3 Diabetes Mellitus and Oxidative Stress

Oxidative stress has been proposed as a pathogenic factor for diabetic complications (Giugliano et al., 1995; Mullarkey et al., 1990; Tesfamariam et al., 1994). Under diabetic conditions the Maillard reaction facilitates the production of reactive oxygen species, and antioxidant defense systems are impaired, including decreased activity of superoxide dismutase and low GSH levels in the erythrocytes (Tesfamariam et al., 1994; Murakami et al., 1989; Yoshida et al., 1995; Thornalley et al., 1996). In fact the presence of diabetic complications correlate negatively with the concentration of GSH in erythrocytes (Thornalley et al., 1996).

20.1.4 Uremia and Oxidative Stress

Oxidative stress has also been reported in uremic patients undergoing hemodialysis (Descamps-Latscha, 1988; Loughrey et al., 1994). Hemodialysis is a major cause of oxidative stress due to the activation of polymorphonuclear neutrophils through the contact of blood with dialysis membranes (Westhuyzen et al., 1995; Luciak and Trznadel, 1991). Oxidative stress is considered to be responsible for shortened life span of erythrocytes (Jacob et al., 1975; Naets, 1975). The extent of oxidative stress is exacerbated by a decreased efficiency in the antioxidant system. In fact GSH levels are low in whole blood and erythrocytes accompanied by a decreased GSH/GSSG ratio (Costagliola et al., 1989; Vanella et al., 1983; Hassan et al., 1995; Pasaoglu et al., 1996; Ross et al., 1997; Canestrari

et al., 1994, 1995; Ceballos-Picot et al., 1996; Yung et al., 1991), and a decreased activity of glutathione-dependent enzymes such as glutathione *S*-transferase, glutathione reductase, and glutathione peroxidase (Canestrari et al., 1994) have been observed in the erythrocytes of hemodialysis patients. Further, superoxide dismutase activity and erythrocyte vitamin E are low, and plasma malondialdehyde (MDA), an end-product of lipid peroxidation, is elevated in dialysis patients (Vanella et al., 1983; Hassan et al., 1995; Pasaoglu et al., 1996; Canestrari et al., 1995; Paul et al., 1993; Cristol et al., 1997).

20.1.5 Markers of Oxidative Stress

Many studies have investigated the markers of oxidative stress. Among these markers are MDA and thiobarbituric acid reactive substances, antioxidant defense systems such as superoxide dismutase, glutathione peroxidase activity, and free radical scavengers (e.g., GSH, vitamin E, and vitamin C). Although reactive oxygen species have been directly detected in vitro by electron spin resonance with or without spin-trapping reagents or by chemiluminescence, these direct detection methods are not yet applicable for clinical examination because of the instability of the reactive oxygen species and the need for expensive equipments. Erythrocyte GSH has been measured by using the enzyme-recycling method (Griffith, 1980), the spectrophotometric assay (Canestrari et al., 1995; Cristol et al., 1997), or high-performance liquid chromatography (HPLC) with derivatization and fluorescence detection (Michelet et al., 1995; Ross et al., 1997). Except for MDA, these methods are not used for routine clinical examination, since they require complicated sample preparation and elaborate techniques. We measured plasma MDA levels by using the thiobarbituric acid method (Yagi, 1976) in diabetic patients, hemodialysis patients, diabetic hemodialysis patients, and healthy subjects. In contrast to the previous reports (Canestrari et al., 1995; Cristol et al., 1997; Mol et al., 1997), plasma MDA levels were not increased in diabetic patients (1.79 ± 0.05 nmol/ml, mean $\pm$ SE, $n = 18$) nor in hemodialysis patients (pre-HD: 2.00 ± 0.07 nmol/ml, $n = 11$), but were increased only in diabetic hemodialysis patients (3.14 ± 0.21 nmol/ml, $n = 12$, $p < 0.0001$, Fisher's PLSD test) as compared with healthy subjects (1.90 ± 0.25 nmol/ml, $n = 20$). We therefore believe that MDA is not so sensitive as to be used as a marker of oxidative stress. Further these markers have not yet been recognized as useful clinical markers of oxidative stress. Consequently, we have demonstrated that glutathionyl Hb is elevated in such diseases as diabetes and uremia and can be used as a sensitive marker of oxidative stress (Niwa et al., 2000; Naito et al., 1999, 2000; Takayama et al., 2001).

20.2 GLUTATHIONYL Hb AS A MARKER OF OXIDATIVE STRESS

20.2.1 Pretreatment of Blood Samples

Blood samples were obtained from 37 diabetic patients (type 2), 17 hyperlipidemic patients, 30 hemodialysis patients, 10 patients on continuous ambulatory peritoneal

dialysis (CAPD), and 20 healthy subjects. Blood samples were drawn using EDTA as an anticoagulant. Whole blood samples (15 μl) were immediately diluted in distilled water (485 μl). The mixture was subsequently centrifuged at 12,000 g for 10 minutes. The supernatant was kept at −40°C until liquid chromatography–electrospray ionization–mass spectrometry (LC–ESI–MS) analysis.

20.2.2 Liquid Chromatography–Electrospray Ionization–Mass Spectrometry (LC–ESI–MS)

LC-ESI-MS was performed using a triple-stage quadrupole mass spectrometer (TSQ7000; Thermoquest, San Jose, CA, USA) equipped with a reversed phase column (TSKgel Phenyl-5PW RP 4.6 mm i.d. × 7.5 cm). A mobile phase consisting of solution (A) (2% acetonitrile in 0.2% acetic acid) and solution (B) (90% acetonitrile in 0.2% acetic acid) was delivered at a flow rate of 0.5 ml/min at ambient temperature. The mobile phase was linearly programmed from 15% of solution (B) to 45% of solution (B) in 30 minutes. The conditions for ESI-MS were as follows: electric field 4.5 kV, nitrogen sheath gas 70 psi, auxiliary gas 15 units, and capillary temperature 275°C. Samples (10 μl) were diluted with solvent A (90 μl), and subsequently were subjected to LC-ESI-MS. Molecular weights of proteins were determined by deconvoluted mass spectra of their peaks. The levels of glutathionyl Hbβ were expressed as percentages of the peak height ratios to intact Hbβ.

20.2.3 Glutathionyl Hb in Diabetic Patients

Figures 20.1a and 20.2a show the reconstructed ion chromatograms of Hb fraction from a normal subject and a diabetic subject, demonstrating the separation of the Hbα and Hbβ chains. Figures 20.1b and 20.2b show the deconvoluted mass spectra of Hbα (peak 1), and those of the glycated Hbα, but no glutathionyl Hbα could be detected. Figures 20.1c and 20.2c show the deconvoluted mass spectra of the Hbβ chain (peak 2). The Hbβ chain shows a molecular weight of 15,868 Da. The glycated β chain was detected at 16,030 Da (15,868 + 162), while the glutathionyl β chain was detected at 16,173 Da (15,868 + 305). The peak at 16,173 Da was identified as a glutathionyl β chain, based on the following findings: (1) the peak disappeared when the sample was reduced with 1 M dithiothreitol in distilled water, and was accompanied by the simultaneous appearance of a peak of GSH at m/z 308, $(M + H)^+$ (Fig. 20.3), and (2) the peak could be detected by incubating Hb (15 mg/ml) (Sigma Chemical Co., St. Louis, MO, USA) with 1 mM GSH (Sigma Chemical Co.) in distilled water at 37°C for 14 days. Notably the synthesis of glutathionyl Hb was enhanced by adding H_2O_2 (1 mM) to the incubation solution.

The level of the glutathionyl Hbβ chain is expressed as a percentage of the intact Hbβ chain, and it markedly increased in diabetic patients ($7.9 \pm 0.5\%$, mean ± SE, $p < 0.001$) and hyperlipidemic patients ($8.1 \pm 0.8\%$, $p < 0.001$) as compared with healthy subjects ($3.7 \pm 0.3\%$) (Fig. 20.4) (Niwa et al., 2000). The oral administration of vitamin E (tocopherol nicotinate) at a dose of 600 mg/day

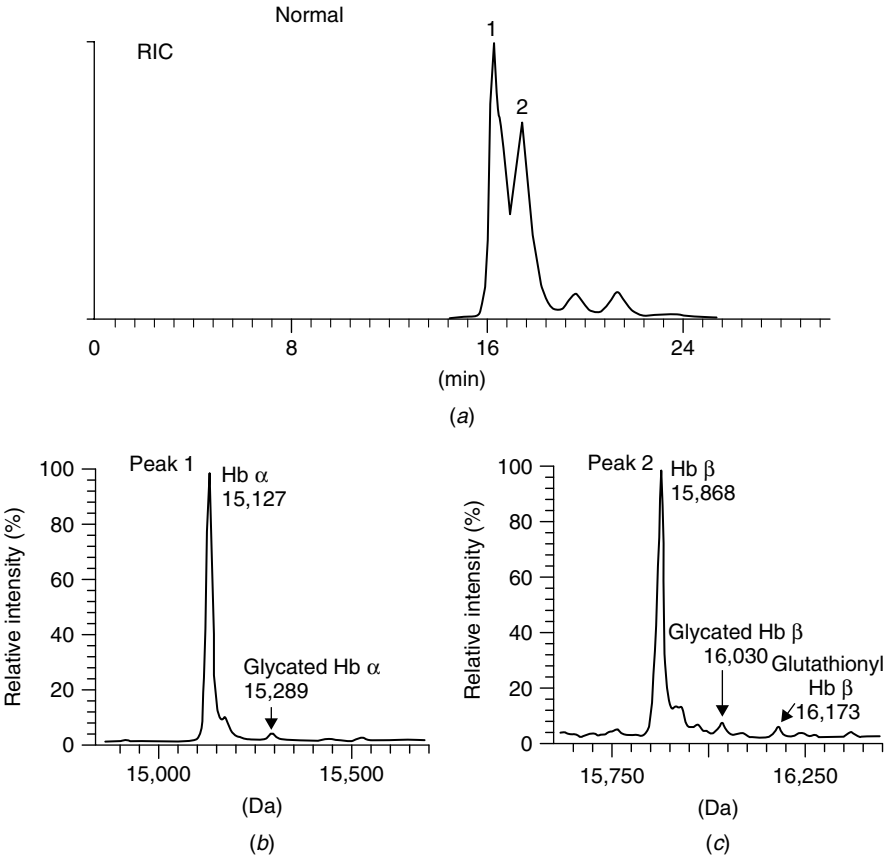


FIGURE 20.1 Reconstructed ion chromatogram (RIC) of Hb from a normal subject (*a*), and deconvoluted ESI mass spectra of peak 1 (*b*) and peak 2 (*c*) in the RIC chromatogram. Glycated Hbα and Hbβ could be detected, whereas glutathionyl Hbβ but no glutathionyl Hbα could be detected.

for 8 weeks to 10 diabetic patients markedly reduced the level of glutathionyl Hb (before vitamin E: $10.2 \pm 0.8\%$, mean $\pm$ SE; after vitamin E: $4.1 \pm 0.4\%$, $p < 0.001$), whereas it did not change Hb_{A1c} (glycohemoglobin) levels at all (before vitamin E: $7.6 \pm 0.3\%$; after vitamin E: $7.6 \pm 0.4\%$) (Figs. 20.5 and 20.6) (Naito et al., 2000).

20.2.4 Glutathionyl Hb in Uremic Patients

Figure 20.7 shows the deconvoluted mass spectra of the Hbα fraction and the Hbβ fraction from a hemodialysis patient. The glutathionyl Hb levels were measured in hemodialysis and continuous ambulatory peritoneal dialysis (CAPD) patients and normal subjects by calculating the peak height ratios of glutathionyl Hbβ to intact

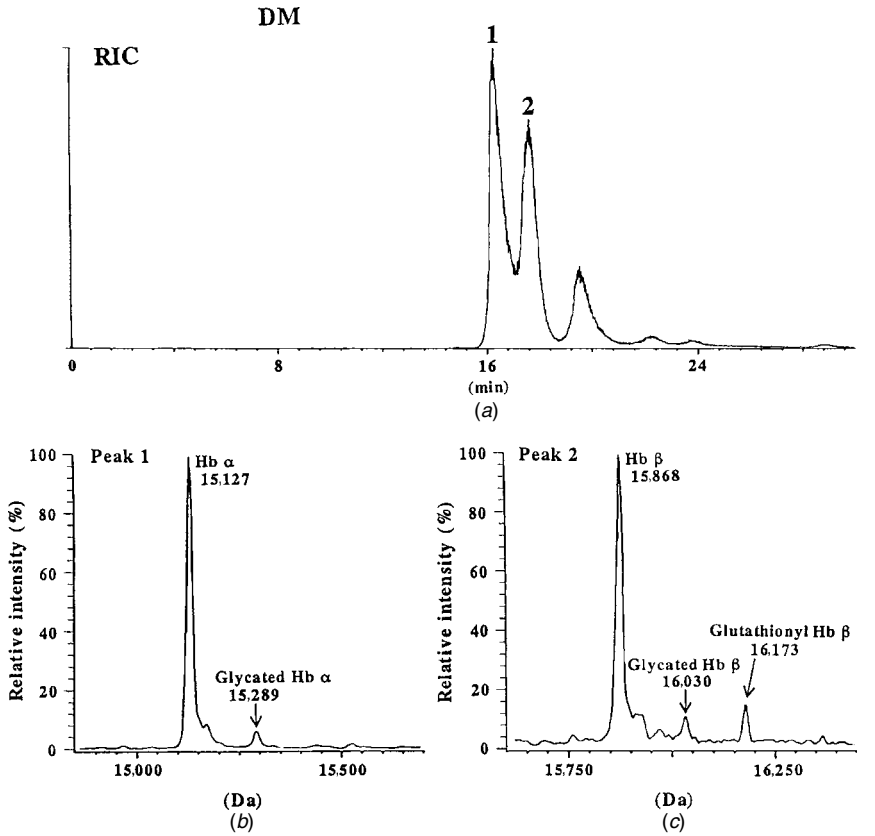


FIGURE 20.2 Reconstructed ion chromatogram (RIC) of Hb from a diabetic patient (*a*), and deconvoluted ESI mass spectra of peak 1 (*b*) and peak 2 (*c*) in the RIC chromatogram. Glycated Hbα and Hbβ could be detected, whereas glutathionyl Hbβ but no glutathionyl Hbα could be detected. (DM: diabetes mellitus)

Hbβ. The glutathionyl Hb levels in hemodialysis patients ($8.0 \pm 3.6\%$; $n = 30$; $p < 0.0001$) and continuous ambulatory peritoneal dialysis patients ($5.9 \pm 2.7\%$; $n = 10$; $p < 0.05$) were significantly elevated as compared with normal subjects ($3.0 \pm 1.6\%$; $n = 20$) (Fig. 20.8) (Takayama et al., 2001). However, there were no significant differences in glutathionyl Hb between hemodialysis patients and continuous ambulatory peritoneal dialysis patients. There were also no significant differences in the glutathionyl Hb levels between before ($8.7 \pm 3.2\%$; $n = 12$) and after HD ($8.7 \pm 2.8\%$; $n = 12$). Glutathionyl Hb levels before hemodialysis were correlated well with those after hemodialysis ($r^2 = 0.925$, $p < 0.0001$).

20.2.5 Glutathionyl Hb in Patients with Friedreich's Ataxia

Friedreich's ataxia is a neurodegenerative disease due to a GAA expansion in a gene coding for a mitochondrial protein (frataxin), implicated in the regulation of

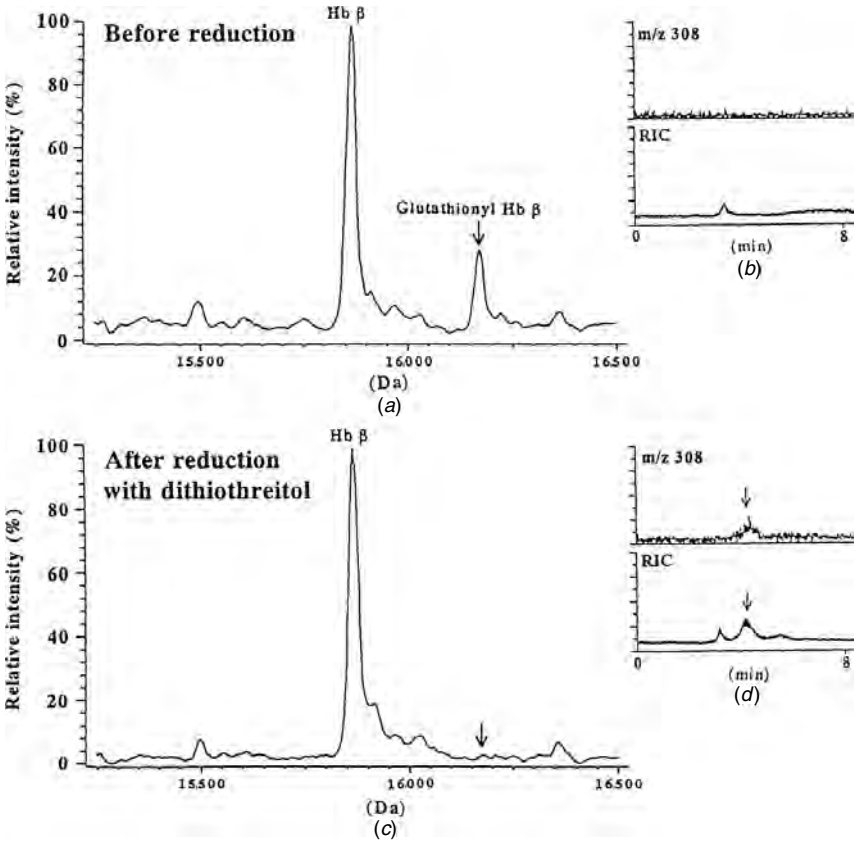


FIGURE 20.3 Deconvoluted ESI mass spectra of Hbβ (*a*, *c*) and mass chromatograms at m/z 308, $(M + H)^+$, and reconstructed ion chromatograms (RIC) of GSH (*b*, *d*) before and after its reduction with dithiothreitol (1 M) for 15 minutes at room temperature. After reduction, the solution was centrifuged at 6,000 g for 5 minutes, and the supernatant solution was subjected to ESI/LC-MS.

iron metabolism. Oxidative stress and mitochondrial dysfunction have long been considered to play a role in Friedreich's ataxia. Piemonte et al. (2001) studied glutathione metabolism in the blood of 14 patients with Friedreich's ataxia by measuring total, free, and protein-bound glutathione concentrations. Total and free glutathione concentrations were determined by reverse phase liquid chromatography with fluorescence detection. They found a reduction of free glutathione levels in the blood of patients with Friedreich's ataxia, a total glutathione concentration comparable to the controls. Glutathionyl Hb in erythrocytes was measured by ESI-MS. Piemonte and colleagues found a significant increase in glutathionyl Hb in the patients with Friedreich's ataxia as compared with healthy subjects (15 ± 1.5 vs. $8 \pm 1.8\%$, $p < 0.05$). Thus, this study also proves that glutathionyl Hb is useful as a clinical marker for oxidative stress in Friedreich's ataxia; it

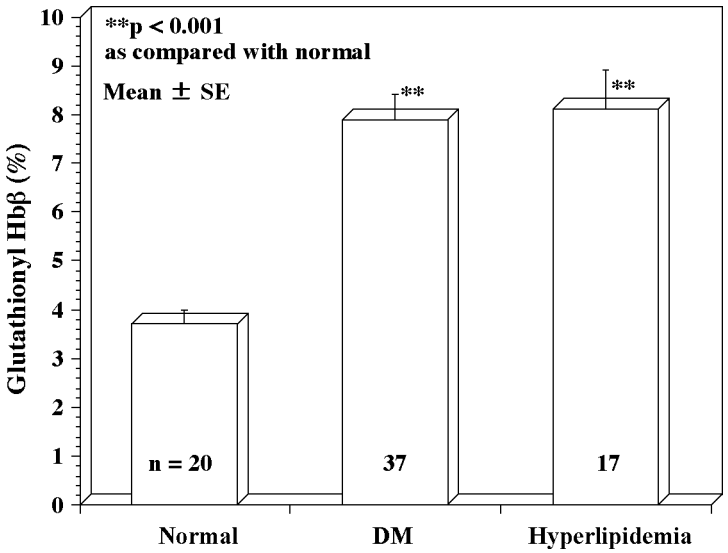


FIGURE 20.4 Levels of glutathionyl Hbβ in patients with diabetes mellitus (DM) and patients with hyperlipidemia.

suggests that free radical cytotoxicity has a big role in the pathophysiology of neurodegeneration.

20.2.6 Oxygen Affinity of Glutathionyl Hb

Glutathionyl Hb prepared in vitro, by incubating Hb with GSH, shows a marked increase in oxygen affinity and a marked decrease in a Hill coefficient as compared to Hb incubated without GSH. The increase in oxygen affinity was similarly observed in Hb treated with other sulfhydryl reagents such as *N*-ethylmaleimide (Riggs, 1961; Benesch et al., 1961; Imai, 1973). The high oxygen affinity of glutathionyl Hb, and also probably those of the other *S*-modified Hb described above, is ascribed to the perturbation of the tertiary structure of β chain and the α_1-β_2 contacts in the T state of Hb (Craescu et al., 1986), leading to the shift of the allosteric equilibrium toward the high-affinity R-state. The increased levels of glutathionyl Hb with high oxygen affinity and low cooperativity in diabetes and uremia may lead to reduced tissue oxygen delivery.

20.2.7 Formation of Glutathionyl Hb

Human adult Hb (HbA) can react in vitro with GSH with disulfide bond formation between Cys-β93 and the cysteine of GSH (Garel et al., 1986). The glutathione adduct formation is associated with the β chain but not the α chain because Cys-β93 provides the only accessible thiol group at the surface of the Hb molecule. The glutathionyl Hb produced in vitro shows increased oxygen affinity, a reduced

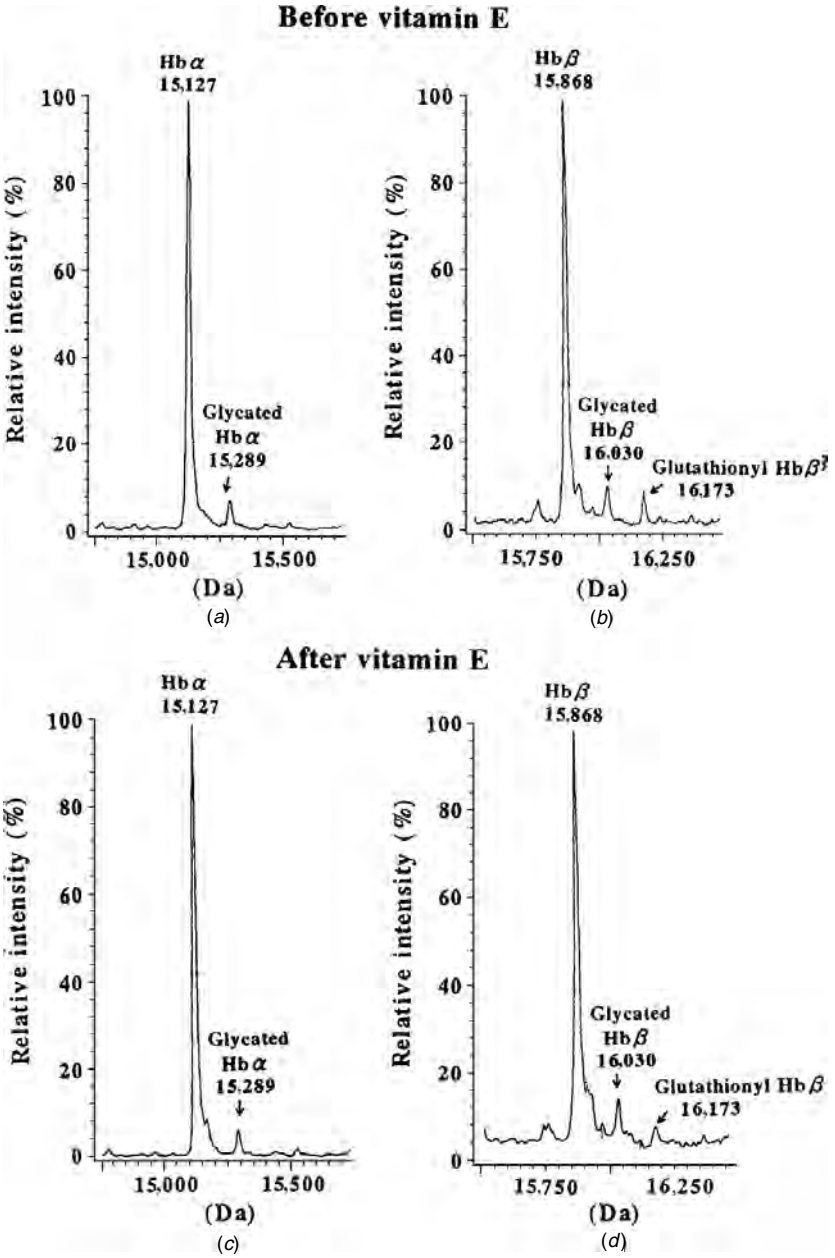


FIGURE 20.5 Deconvoluted ESI mass spectra of peak 1 (*a*) and peak 2 (*b*) in the RIC chromatogram of Hb from a diabetic patient before vitamin E administration, and of their respective peaks (*c*, *d*) in the same patient 8 weeks after administration of vitamin E. Glycated Hbα and Hbβ could be detected, whereas glutathionyl Hbβ but no glutathionyl Hbα could be detected. The peak height of glutathionyl Hbβ 8 weeks after administration of vitamin E was decreased as compared with that before vitamin E administration.

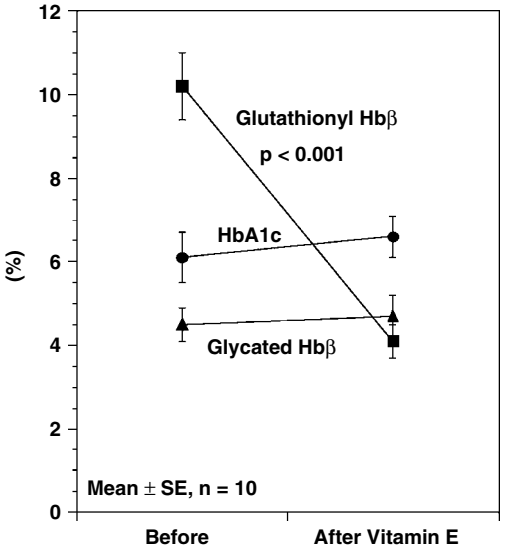


FIGURE 20.6 Levels of glutathionyl Hbβ, Hb_{A1c}, and glycated Hbβ before and after administration of vitamin E (600 mg/day) for 8 weeks in diabetic patients.

cooperativity, and a reduced alkaline Bohr effect (Craescu et al., 1986). Glutathionyl Hb was produced in vitro by a thiol–disulfide exchange between the mixed disulfides of Hb and GSH in the study of its anti-sickling effect. It is possible to bind most of the intracellular GSH to Hb by a two-step reaction: the formation of a mixed disulfide, followed by a thiol–mixed disulfide exchange. By this method up to 25% of intracellular Hb could be obtained in the glutathionyl Hb form. However, glutathionyl Hb could not be detected in normal erythrocytes by electrophoresis (Garel et al., 1982), because GSSG, which forms a mixed disulfide with Hb, is present at a very low concentration (Ceballos-Picot et al., 1996), and unlike many disulfides, it reacts very slowly with Hb (Garel et al., 1982). The glutathionyl Hb level in normal erythrocytes is so low that we could only detect it by using highly sensitive and specific LC/ESI-MS. In diabetes and uremia, however, the increased oxidative stress leads to increased levels of erythrocyte GSSG, which then forms a disulfide with Hbβ to produce glutathionyl Hb.

Murakami et al. (2003) studied the biochemical consequences of Hb oxidation in intact human erythrocytes. Incubation of the washed erythrocyte with 1 mM *tert*-butylhydroperoxide induced an increase in glutathionyl Hb. The formation of glutathionyl Hb occurred linearly for 10 minutes in parallel with the formation of metHb after exhaustion of GSH. Thus they demonstrate that metHb, but not normal Hb, reacts with GSSG to form glutathionyl Hb.

Glutathione-modified α-crystalline at Cys131 and Cys142 was detected in the lens of uremic patients (Smith et al., 1995). The formation of glutathionyl crystalline is also considered to be due to oxidative stress associated with uremia, and

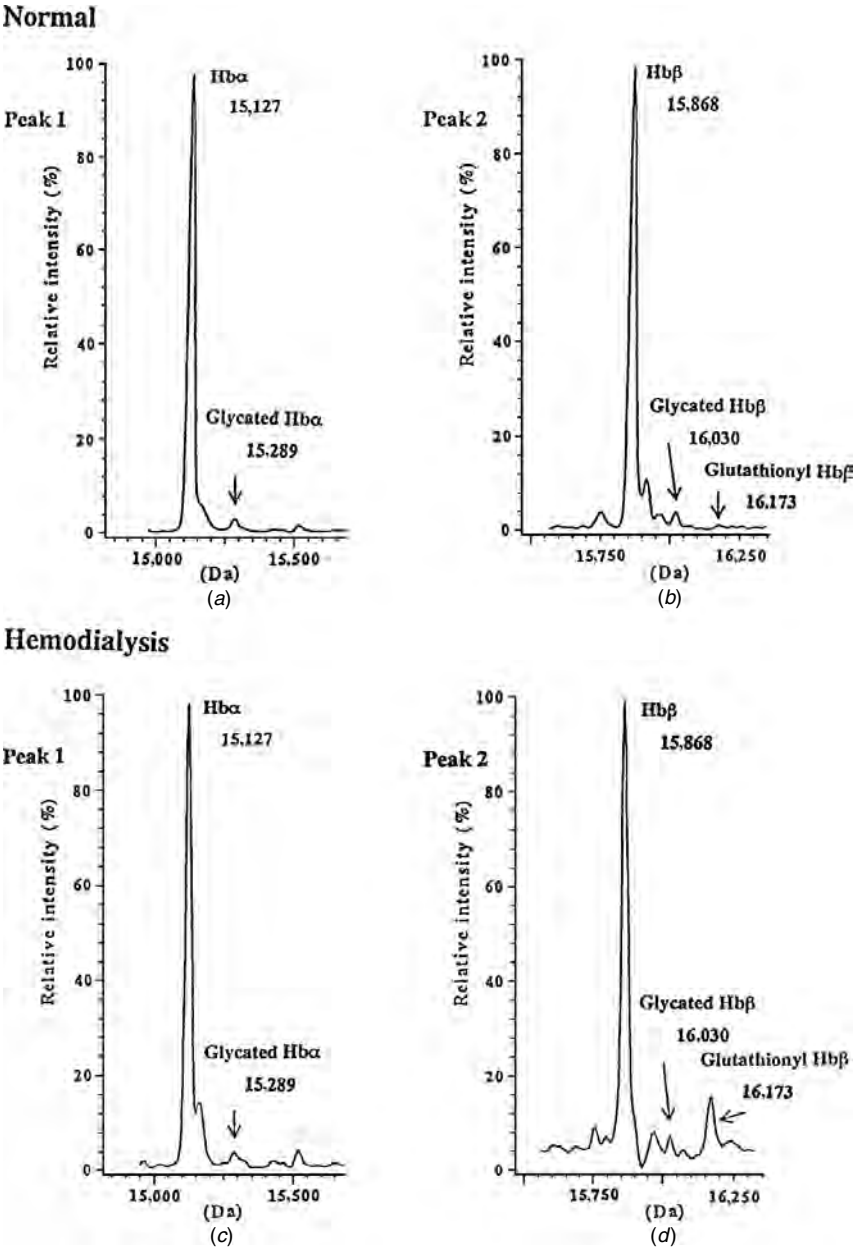


FIGURE 20.7 Deconvoluted mass spectra of peak 1 (*a*) and peak 2 (*b*) from a normal subject and peak 1 (*c*) and peak 2 (*d*) from a hemodialysis patient. Hbα was detected at a molecular weight of 15,127 Da, and glycated Hbα at 15,289 Da (15,127 + 162). Hbβ was detected at 15,868 Da, and glycated Hbβ at 16,030 Da (15,868 + 162). Glutathionyl Hbβ was detected at 16,173 Da (15,868 + 305). Interestingly glutathionyl Hbβ was markedly increased in the HD patient as compared with the normal subject.

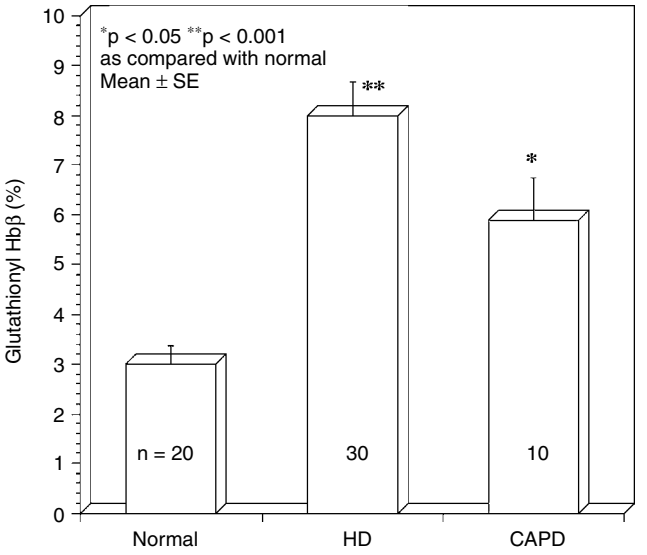


FIGURE 20.8 Levels of glutathionyl Hbβ in patients on hemodialysis (HD) and patients on continuous ambulatory peritoneal dialysis (CAPD).

this may be involved in the development of cataract. Glutathionyl Hb, which is more easily examined by using blood, may represent the glutathione modification of proteins in lens.

20.2.8 Matrix-Assisted Laser Desorption Ionization–Time-of-Flight Mass Spectrometry (MALDI–TOF–MS)

Biroccio et al. (2005) reported a quantitative application of linear MALDI-TOF-MS for the determination of glutathionyl Hb in blood samples from 184 individuals, and found a bimodal distribution of glutathionyl Hb. In fact 65.22% of screened individuals had glutathionyl Hb levels lower than 0.50%, while 34.78% had glutathionyl Hb levels higher than 0.50%. A semiautomatic robotic procedure using MALDI-TOF-MS was developed for fast analysis of a large number of samples, allowing fast screening of glutathionyl Hb.

20.2.9 High-Performance Liquid Chromatography (HPLC) of Glutathionyl Hb

Glutathionyl Hb has been suggested as a clinical marker of oxidative stress. To be widely used, a simple method such as HPLC should be developed for the measurement of glutathionyl Hb. Pastore et al. (2003) reported a simple method to measure glutathionyl Hb level in erythrocytes, using cation-exchange HPLC with UV detection. The glutathionyl Hb level was measured in erythrocytes of

healthy subjects, with a mean value of $2.58 \pm 0.7\%$, calculated as the percentage of its peak area ratio to that of total Hb. The availability of a simple and reproducible method to detect the glutathionyl Hb concentration in blood could be useful in monitoring oxidative stress, and for investigating the efficacy of antioxidant therapies in clinical trials.

Rossi et al. (2002) reported that oxidation of GSH to form GSSG occurs during sample preparation. To minimize the artifact oxidation of GSH to form glutathionyl Hb during sample preparation, *N*-ethylmaleimide, which passes freely thorough cytoplasm membrane and quenches SH-groups, should be added immediately to blood, followed by centrifugation and hemolysis procedures for HPLC analysis. EDTA, a metal chelator that is able to decrease the oxidation process, should be used as an anticoagulant.

20.3 CONCLUSION

Glutathionyl Hb levels are markedly increased in diabetic patients, hyperlipidemic patients, uremic patients, and patients with Friedreich's ataxia. The enhanced oxidative stress in these diseases accounts for the increased glutathionyl Hb, based on the finding that the administration of vitamin E has proved to reduce the glutathionyl Hb levels in diabetic patients, and the addition of H_2O_2 in the incubation of Hb with GSH enhanced the synthesis of glutathionyl Hb. The increased glutathionyl Hb may lead to reduced oxygen supply to peripheral tissues, because of the increased oxygen affinity for Hb. A simple HPLC system that detects glutathionyl Hb, similar to that used for Hb_{A1c}, should result in glutathionyl Hb being widely used as a clinical marker for oxidative stress.

LIST OF ABBREVIATIONS

CAPD, continuous ambulatory peritoneal dialysis
GSH, glutathione
GSSG, oxidized glutathione
HbA, human adult Hb
Hb_{A1c}, glycohemoglobin
HPLC, high-performance liquid chromatography
LC-ESI-MS, liquid chromatography–electrospray ionization–mass spectrometry
MALDI-TOF-MS, matrix-assisted laser desorption ionization–time-of-flight –mass spectrometry
MDA, malondialdehyde

REFERENCES

Bautista A.P. (2001). Free radicals, chemokines, and cell injury in HIV-1 and SIV infections and alcoholic hepatitis. *Free Radic Biol Med* 31: 1527–1532.

Benesch R., Benesch R.E. (1961). The chemistry of the Bohr effect. *J Biol Chem* 236: 405–410.

Biroccio A., Urbani A., Massoud R., di Ilio C., Sacchetta P., Bernardini S., Cortese C., Federici G. (2005). A quantitative method for the analysis of glycated and glutathionylated hemoglobin by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Anal Biochem* 336: 279–288.

Canestrari F., Galli F., Giorgini A., Albertini M.C., Galiotta P., Pascucci M., Bossu M. (1994). Erythrocyte redox state in uremic anemia: Effects of hemodialysis and relevance of glutathione metabolism. *Acta Haematol* 91: 187–193.

Canestrari F., Buoncristiani U., Galli F., Giorgini A., Albertini M.C., Carobi C., Pascucci M., Bossu M. (1995). Redox state, antioxidative activity and lipid peroxidation in erythrocytes and plasma of chronic ambulatory peritoneal dialysis patients. *Clin Chim Acta* 234: 127–136.

Ceballos-Picot I., Witko-Sarsat V., Merad-Boudia M., Nguyen A.T., Thevenin M., Jaudon M.C., Zingraff J., Verger C., Jungers P., Descamps-Latscha B. (1996). Glutathione antioxidant system as a marker of oxidative stress in chronic renal failure. *Free Radic Biol Med* 21: 845–853.

Chow C.W., Herrera Abreu M.T., Suzuki T., Downey G.P. (2003). Oxidative stress and acute lung injury. *Am J Respir Cell Mol Biol* 29: 427–431.

Cooper J.M., Schapira A.H. (2003). Friedreich's ataxia: Disease mechanisms, antioxidant and Coenzyme Q10 therapy. *Biofactors* 18: 163–171.

Costagliola C., Romano L., Sorice P., Di Benedetto A. (1989). Anemia and chronic renal failure: the possible role of the oxidative state of glutathione. *Nephron* 52: 11–14.

Craescu C.T., Poyart C., Schaeffer C., Garel M.C., Kister J., Beuzard Y. (1986). Covalent binding of glutathione to hemoglobin. II. Functional consequences and structural changes reflected in NMR spectra. *J Biol Chem* 261: 14710–14716.

Cristol J.P., Bosc J.Y., Badiou S., Leblanc M., Lorrho R., Descomps B., Canaud B. (1997). Erythropoietin and oxidative stress in haemodialysis: Beneficial effects of vitamin E supplementation. *Nephrol Dial Transplant* 12: 2312–2317.

Descamps-Latscha B. (1988). Phagocyte oxidative metabolism in hemodialysis. *Contrib Nephrol* 62: 132–139.

Garel M.C., Beuzard Y., Thillet J., Domenget C., Martin J., Galacteros F., Rosa J. (1982). Binding of 21 thiol reagents to human hemoglobin in solution and in intact cells. *Eur J Biochem* 123: 513–519.

Garel M.C., Domenget C., Caburi-Martin J., Prehu C., Galacteros F., Beuzard Y. (1986). Covalent binding of glutathione to hemoglobin. I. Inhibition of hemoglobin S polymerization. *J Biol Chem* 261: 14704–14709.

Giugliano D., Ceriello A., Paolisso G. (1995). Diabetes mellitus, hypertension, and cardiovascular disease: Which role for oxidative stress? *Metabolism* 44: 363–368.

Griffith O.W. (1980). Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal Biochem* 106: 207–212.

Hassan M.Q., Hussain S.A., Zaki M.A., Alsharif N.Z., Stohs S.J. (1995). Protective effects of antioxidants against uraemia-induced lipid peroxidation and glutathione depletion in humans. *Pharmacol Toxicol* 77: 407–411.

Hitchon C.A., El-Gabalawy H.S. (2004). Oxidation in rheumatoid arthritis. *Arthritis Res Ther* 6: 265–278.

Imai K. (1973). Analyses of oxygen equilibria of native and chemically modified human adult Hbs on the basis of Adair's stepwise oxygenation. Theory and the allosteric model of Monod, Wyman, and Changeux. *Biochemistry* 2: 798–808.

Jacob H.S., Eaton J.W., Yawata Y. (1975). Shortened red blood cell survival in uremic patients: Beneficial and deleterious effects of dialysis. *Kidney Int* (Suppl 2): 139–143.

Janero D.R. (1995). Ischemic heart disease and antioxidants: Mechanistic aspects of oxidative injury and its prevention. *Crit Rev Food Sci Nutr* 35: 65–81.

Loughrey C.M., Young I.S., Lightbody J.H., McMaster D., McNamee P.T., Trimble E.R. (1994). Oxidative stress in haemodialysis. *QJM* 87: 679–683.

Luciak M., Trznadel K. (1991). Free oxygen species metabolism during haemodialysis with different membranes. *Nephrol Dial Transpl* 6: 66–70.

Michelet F., Gueguen R., Leroy P., Wellman M., Nicolas A., Siest G. (1995). Blood and plasma glutathione measured in healthy subjects by HPLC: Relation to sex, aging, biological variables, and life habits. *Clin Chem* 41: 1509–1517.

Mol M.J., de Rijke Y.B., Demacker P.N., Stalenhoef A.F. (1997). Plasma levels of lipid and cholesterol oxidation products and cytokines in diabetes mellitus and cigarette smoking: Effects of vitamin E treatment. *Atherosclerosis* 129: 169–176.

Mullarkey C.J., Edelstein D., Brownlee M. (1990). Free radical generation by early glycation products: A mechanism for accelerated atherogenesis in diabetes. *Biochem Biophys Res Commun* 173: 932–939.

Murakami K., Kondo T., Ohtsuka Y., Fujiwara Y., Shimada M., Kawakami Y. (1989). Impairment of glutathione metabolism in erythrocytes from patients with diabetes mellitus. *Metabolism* 38: 753–758.

Murakami K., Mawatari S. (2003). Oxidation of hemoglobin to methemoglobin in intact erythrocyte by a hydroperoxide induces formation of glutathionyl hemoglobin and binding of alpha-hemoglobin to membrane. *Arch Biochem Biophys* 417: 244–250.

Naets J.P. (1975). Hematologic disorders in renal failure. *Nephron* 14: 181–194.

Naito C., Kajita M., Niwa T. (1999). Determination of glutathionyl hemoglobin in hemodialysis patients using electrospray ionization liquid chromatography–mass spectrometry. *J Chromatogr* 731: 121–124.

Naito C., Niwa T. (2000). Analysis of glutathionyl hemoglobin levels in diabetic patients by electrospray ionization liquid chromatography–mass spectrometry: Effect of vitamin E administration. *J Chromatogr* 746: 91–94.

Niwa T., Naito C., Mawjood A.H.M., Imai K. (2000). Increased glutathionyl hemoglobin in diabetes mellitus and hyperlipiemia demonstrated by liquid chromatography/electrospray ionization-mass spectrometry. *Clin Chem* 46: 82–88.

Pasaoglu H., Muhtaroglu S., Gunes M., Utas C. (1996). The role of the oxidative state of glutathione and glutathione-related enzymes in anemia of hemodialysis patients. *Clin Biochem* 29: 567–572.

Pastore A., Mozzi A.F., Tozzi G., Gaeta L.M., Federici G., Bertini E., Lo Russo A., Mannucci L., Piemonte F. (2003). Determination of glutathionyl-hemoglobin in human erythrocytes by cation-exchange high-performance liquid chromatography. *Anal Biochem* 312: 85–90.

Paul J.L., Sall N.D., Soni T., Poignet J.L., Lindenbaum A., Man N.K., Moatti N., Raichvarg D. (1993). Lipid peroxidation abnormalities in hemodialyzed patients. *Nephron* 64: 106–109.

Piemonte F., Pastore A., Tozzi G., Tagliacozzi D., Santorelli F.M., Carrozzo R., Casali C., Damiano M., Federici G., Bertini E. (2001). Glutathione in blood of patients with Friedreich's ataxia. *Eur J Clin Invest* 31: 1007–1011.

Richie J.P. Jr, Skowronski L., Abraham P., Leutzinger Y. (1996). Blood glutathione concentrations in a large-scale human study. *Clin Chem* 42: 64–70.

Riggs A. (1961). The binding of *N*-ethylmaleimide by human Hb and its effect upon the oxygen equilibrium. *J Biol Chem* 236: 1948–1954.

Ross E.A., Koo L.C., Moberly J.B. (1997). Low whole blood and erythrocyte levels of glutathione in hemodialysis and peritoneal dialysis patients. *Am J Kidney Dis* 30: 489–494.

Rossi R., Milzani A., Dalle-Donne I., Giustarini D., Lusini L., Colombo R., Di Simplicio P. (2002). Blood glutathione disulfide: in vivo factor or in vitro artifact? *Clin Chem* 48: 742–753.

Smith J.B., Shun-Shin G.A., Sun Y., Miesbauer L.R., Yang Z., Yang Z., Zhou X., Schwedler J., Smith D.L. (1995). Glutathione adducts, not carbamylated lysines, are the major modification of lens alpha-crystallins from renal failure patients. *J Protein Chem* 14: 179–188.

Son S.M., Whalin M.K., Harrison D.G., Taylor W.R., Griendling K.K. (2004). Oxidative stress and diabetic vascular complications. *Curr Diab Rep* 4: 247–252.

Stadtman E.R. (1992). Protein oxidation and aging. *Science* 257: 1220–1224.

Stocker R., Keaney J.F. Jr. (2004). Role of oxidative modifications in atherosclerosis. *Physiol Rev* 84: 1381–1478.

Takayama F., Tsutsui S., Horie M., Shimokata K., Niwa T. (2001). Glutathionyl hemoglobin in uremic patients undergoing hemodialysis and continuous ambulatory peritoneal dialysis. *Kidney Int* 59(Suppl 78): S155–S158.

Tesfamariam B. (1994). Free radicals in diabetic endothelial cell dysfunction. *Free Radic Biol Med* 16: 383–391.

Thornalley P.J., McLellan A.C., Lo T.W., Benn J., Sonksen P.H. (1996). Negative association between erythrocyte reduced glutathione concentration and diabetic complications. *Clin Sci* 91: 575–582.

Vanella A., Geremia E., Pinturo R., Tiriolo P., Liuzzo G., Tiriolo C., Custorella A., Condorelli G., Giglio A. (1983). Superoxide dismutase activity and reduced glutathione content in erythrocytes of uremic patients on chronic dialysis. *Acta Haematol* 70: 312–315.

van der Vliet A., Cross C.E. (2000). Phagocyte oxidants and nitric oxide in cystic fibrosis: New therapeutic targets? *Curr Opin Pulm Med* 6: 533–539.

Vaziri N.D. (2004). Oxidative stress in uremia: nature, mechanisms, and potential consequences. *Semin Nephrol* 24: 469–473.

Warnholtz A., Mollnau H., Oelze M., Wendt M., Munzel T. (2001). Antioxidants and endothelial dysfunction in hyperlipidemia. *Curr Hypertens Rep* 3: 53–60.

Westhuyzen J., Adams C.E., Fleming S.J. (1995). Evidence for oxidative stress during in vitro dialysis. *Nephron* 70: 49–54.

Yagi K. (1976). A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem Med* 15: 212–216.

Yeung J.H. (1991). Effects of glycerol-induced acute renal failure on tissue glutathione and glutathione-dependent enzymes in the rat. *Meth Find Exp Clin Pharmacol* 13: 23–28.

Yoshida K., Hirokawa J., Tagami S., Kawakami Y., Urata Y., Kondo T. (1995). Weakened cellular scavenging activity against oxidative stress in diabetes mellitus: regulation of glutathione synthesis and efflux. *Diabetologia* 38: 201–210.

21

GLYCO-OXIDATIVE BIOCHEMISTRY IN DIABETIC RENAL INJURY

TOSHIO MIYATA

21.1 PRESENCE OF LOCAL, BUT NOT GENERALIZED, OXIDATIVE STRESS IN DIABETES

The existence of oxidative stress in diabetes, its possible localization and relation to hyperglycemia, and its cause remain disputed. The increased cellular NADH/NAD^+ ratio led Williamson et al. (1993) to suggest that diabetes is a state of reductive stress and pseudohypoxia rather than oxidative stress. The presence of oxidative stress in diabetes rests on indirect evidence, including increased ratios of $NADP^+$/NADPH and of oxidized to reduced glutathione, ascorbate, and albumin (Bravi et al., 1997; Jennings et al., 1987; Suzuki et al., 1992). However, such imbalances in intracellular redox systems may be induced by nonoxidative mechanisms (e.g., the polyol pathway) and cause secondary alterations of sulfhydryl homeostasis, thus mimicking oxidative stress (Miyata et al., 1999a). There is no a priori reason to conclude that these alterations necessarily indicate oxidative stress.

The issue of oxidative stress in diabetes has been recently revisited in studies using new methodologies to assess modifications of protein. The group of Baynes was the first to argue against the presence of generalized oxidative stress in diabetes (Wells-Knecht et al., 1997). These authors measured two oxidized amino acids in skin collagen, *ortho*-tyrosine and methionine sulfoxide. They demonstrated an age-dependent increase in oxidative damage in skin collagen, pointing out that aging is indeed an oxidative stress–dependent phenomenon. Surprisingly, however, the age-adjusted levels of oxidized amino acids in collagen

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

proved virtually identical in diabetics and in nondiabetics. They thus concluded that diabetes is not associated with generalized oxidative stress.

However, we analyzed the protein oxidative modifications induced by carbohydrates and lipids, and demonstrated the existence of local oxidative stress in diabetic nephropathy (Horie et al., 1997). Advanced glycation end-products (AGEs) include a variety of molecular structures generated by different mechanisms. The formation of two well-known AGEs, pentosidine (Sell and Monnier, 1989) and N^{ε}-carboxymethyllysine (CML) (Ahmed et al., 1986), depends not only on glycation but also to a greater extent on oxidation (Miyata et al., 1997). By contrast, pyrraline, another AGE, is formed only through a glucose-dependent but an oxidative stress–independent mechanism (Miyata and Monnier, 1992). If tissue AGE formation depended only on hyperglycemia, all AGE structures would be detected in diabetic glomerular lesions. The results indicate that pentosidine and CML are identified in early mesangial expansion as well as in advanced nodular deposits, together with protein modifications derived from oxidation of lipids, malondialdehyde-lysine, and 4-hydroxynonenal adduct (Horie et al., 1997; Suzuki et al., 1999). By contrast, pyrraline is absent in diabetic glomeruli but readily identified in the interstitium. These findings implicate oxidative stress rather than hyperglycemia in the formation of AGEs within diabetic glomerular lesions.

The existence of local oxidative stress has been confirmed in diabetic vascular lesions by the group of Heinecke (Pennathur et al., 2001). In streptozotocin diabetic monkey, vascular levels of *ortho*-tyrosine, *meta*-tyrosine, and di-tyrosine, all of which are protein modifications induced by hydroxyl and tyrosyl radicals, are significantly higher in diabetics than nondiabetics. Interestingly these levels are correlated with those of hemoglobin A1c, suggesting a link between hyperglycemia and oxidative stress. Altogether, diabetes is associated not with generalized, but rather with local oxidative stress, at least in glomerular and vascular lesions.

21.2 OXIDATIVE PROTEIN DAMAGE IN VIVO

The oxidative protein damage present in vivo in diabetic nephropathy is a complex phenomenon. It involves many factors and pathways as illustrated in Figure 21.1. Hyperglycemia-induced glyco-oxidation generates some AGEs (e.g., pentosidine and CML). Reactive oxygen species, hydroxyl and tyrosyl radicals, produce *ortho*-tyrosine, *meta*-tyrosine, and di-tyrosine. Lipid peroxidation generates the advanced lipoxidation end-products (ALEs) such as malondialdehyde-lysine, 4-hydroxy-nonenal adduct, and acrolein adduct (Miyata et al., 1999a; Requena et al., 1997; Uchida et al., 1998). Inflammatory cell-derived myeloperoxidase and hydrogen peroxide produce 3-chlorotyrosine (Hazen et al., 1996). Nitric oxide reacts with superoxide and generates peroxynitrite, which produces 3-nitrotyrosine (Ischiropoulos and al-Mehdi, 1995). Available evidence suggests that different oxidative pathways prevail in diabetic glomerular and vascular damage: 3-nitrotyrosine is

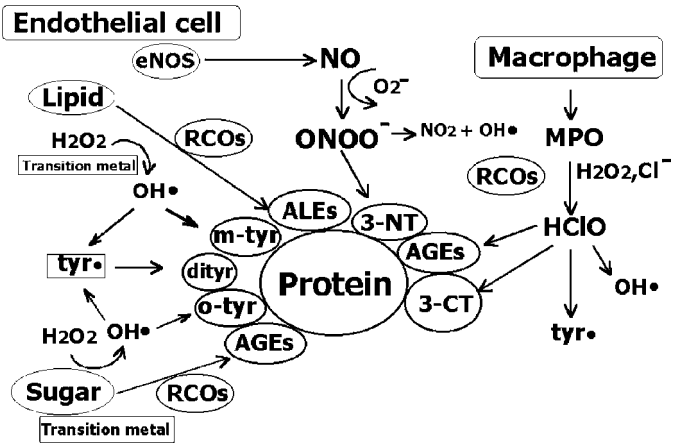


FIGURE 21.1 Schema of various oxidative protein modifications proposed in vivo in diabetic renal injury.

detected by immunochemistry in glomerular lesions of rats given streptozotocin (Onozato et al., 2002) but is absent by chemical analysis in vascular lesions of monkey given streptozotocin (Pennathur et al., 2001). Identification of oxidative pathways actually implicated in human diabetic nephropathy should prove therefore of particular interest.

21.3 ANTIOXIDATIVE PROPERTIES OF MEDICAL AGENTS

Can these oxidative pathways be modified by therapeutic agents? Here again, protein modifications may serve as useful markers and help us ascertain the mechanism of action of the medical agents. Further several medical agents currently in clinical use for various diseases interfere with oxidative protein damage. They act through different mechanisms characteristic of their chemical structures.

Two well-known antihypertensive agents, nifedipine (a calcium channel blocker) and valsartan (an angiotensin receptor blocker, ARB), share an antioxidative potential though their mechanisms of action differ (Fig. 21.2). Valsartan is a potent scavenger for hydroxyl and tyrosyl radicals and an inhibitor for glyco-oxidation reaction. Valsartan inhibits the in vitro formation of AGEs (pentosidine and CML) (Miyata et al., 2002), *ortho*-tyrosine, *meta*-tyrosine, and di-tyrosine (our unpublished observations). Nifedipine, on the other hand, is a potent scavenger for peroxynitrite and inhibits in vitro the formation of 3-nitrotyrosine. It does not react with reactive oxygen species. Valsartan and nifedipine thus act at different steps of oxidative protein damage.

Whether these in vitro findings can be extrapolated to in vivo models is as yet unknown. Nevertheless, they have already opened up an exciting prospect for new therapeutic interventions.

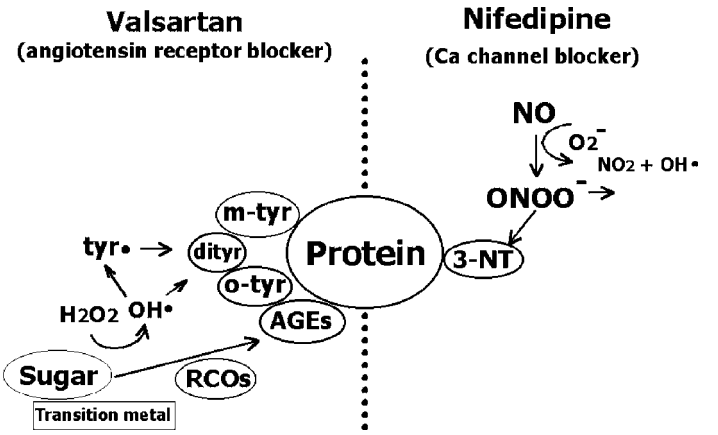


FIGURE 21.2 Schema of different anti-oxidative mechanisms of two well-known anti-hypertensive agents: valsartan (angiotensine receptor blocker) and nifedipine (calcium channel blocker). The oxidative protein damages inhibited by each agent are shown.

21.4 THERAPEUTIC PERSPECTIVES FOR AGE INHIBITORS

The development of effective drugs that ameliorate oxidative protein damage has become a major therapeutic goal for several pharmaceutical companies. Inhibitors of the glyco-oxidation reaction and AGE formation (the so-called AGE inhibitors) have been of particular interest.

21.4.1 Aminoguanidine and Other Hydrazine Derivatives

Aminoguanidine, the first AGE inhibitor discovered in 1986 (Brownlee et al., 1986), was followed by a more effective compound, (±)-2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide (OPB-9195) (Nakamura et al., 1997). Both are hydrazine-derivatives. In vitro, OPB-9195 inhibits the formation of pentosidine and CML from a variety of individual precursors including ribose, glucose, and ascorbate, as well as that of two ALE moieties, malondialdehyde-lysine and 4-hydroxynonenal adduct, from arachidonate (Miyata et al., 2000a). It also inhibits pentosidine generation from diabetic and uremic plasma (Miyata et al., 1998) or from conventional glucose-based peritoneal dialysis fluid fortified with bovine serum albumin (Miyata et al., 2000b).

The mechanism of this inhibitory effect on AGE formation is complex. The hydrazine nitrogen atom of OPB-9195 reacts with the reactive carbonyl compound (RCO) precursors for AGEs, directly or via the free base, upon hydrolysis to form eventually hydrazone (Miyata et al., 2000a). This mechanism is similar to that proposed for aminoguanidine (aminoguanidine has in addition a guanidine group able to bind carbonyl precursors).

OPB-9195 corrects several biological effects associated with AGE formation. In murine thymocyte and fibroblast cultures, it inhibits the phosphorylation of

tyrosine residues of a number of intracellular proteins induced by cell surface Schiff-base formation (Akhand et al., 1999). In the experimental diabetic animal, such as the Otsuka-Long-Evans-Tokushima fatty (OLETF) rat, it reduces urinary albumin excretion and improves glomerular morphology (Nakamura et al., 1997). Given to rats, after balloon injury of their carotid arteries, it effectively reduces neointima proliferation in arterial walls (Miyata et al., 1999b).

Unfortunately, clinical benefits of this compound given to diabetic patients have been hampered by side effects, for example, related to the characteristic trapping of pyridoxal with an attendant vitamin B_6 deficiency (Taguchi et al., 1998).

21.4.2 Angiotensin II Receptor Blockers (ARB) and Angiotensin Converting Enzyme Inhibitors (ACEI)

We therefore investigated the AGE inhibitory potential of other drugs whose tolerance has been demonstrated in clinical conditions. Unexpectedly, we found that both ARB and ACEI, well-known antihypertensive agents with clinically evident renoprotection, lower the in vitro formation of AGEs (Miyata et al., 2002). Inhibition is more striking for valsartan than for aminoguanidine at the tested concentrations. The effect on AGE formation is common to the other six tested ARB (e.g., olmesartan, candesartan, irbesartan, losartan, termisartan) and their common core structure, 5-(4′ methylbiphenyl-2-yl)-1H-tetrazol, suggesting that this is a class effect. A similar but milder effect on AGE formation was observed with 4 ACEI tested at similar concentrations (e.g., temocaprilat, enalaprilat, captopril, and perindprilat). However, no common core structure was identified in the tested ACEI.

The AGE lowering mechanism of ARB and ACEI differs markedly from that of aminoguanidine and OPB-9195 (Miyata et al., 2002). In contrast to the two previous drugs, neither ARB nor ACEI entraps and lowers in vitro the concentration of RCOs or that of pyridoxal. Rather, they decrease RCO production and thereby AGE formation probably as a result of their potent ability to scavenge hydroxyl radicals and to chelate transition metals necessary for the Fenton reaction (Fig. 21.3).

21.5 AGE INHIBITION AND RENOPROTECTION

We assessed the anti-oxidative stress and AGE lowering effect of ARB experimentally in a unique type 2 diabetic rat model with nephropathy, SHR/NDmcr-cp (Nangaku et al., 2003). This model has the same genetic background as spontaneously hypertensive rats (SHR) and their original strain, the Wistar-Kyoto rat (WKY). Like SHR, SHR/NDmcr-cp became hypertensive. Systemic blood pressure was much lower in SHR/NDmcr-cp than in the SHR rats. In SHR/NDmcr-cp, the hypertension was accompanied by a unique metabolic derangement. In SHR, the metabolic markers such as body weight and plasma levels of glucose, insulin, and lipids were normal. In SHR/NDmcr-cp, by contrast, the genetic mutation

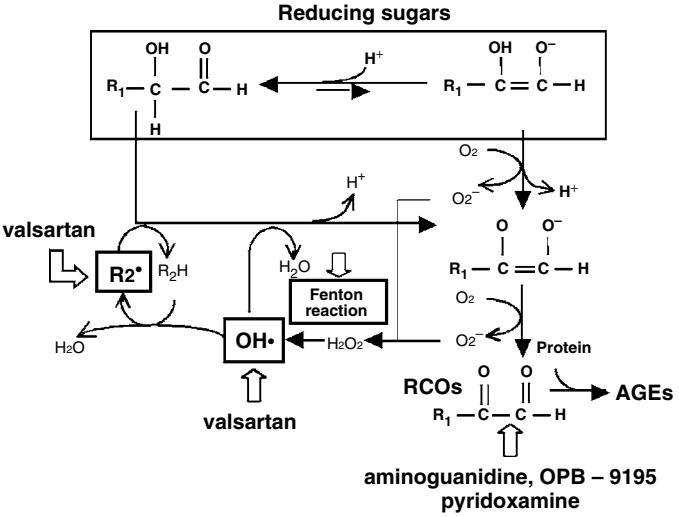


FIGURE 21.3 Schema of different AGE lowering mechanisms.

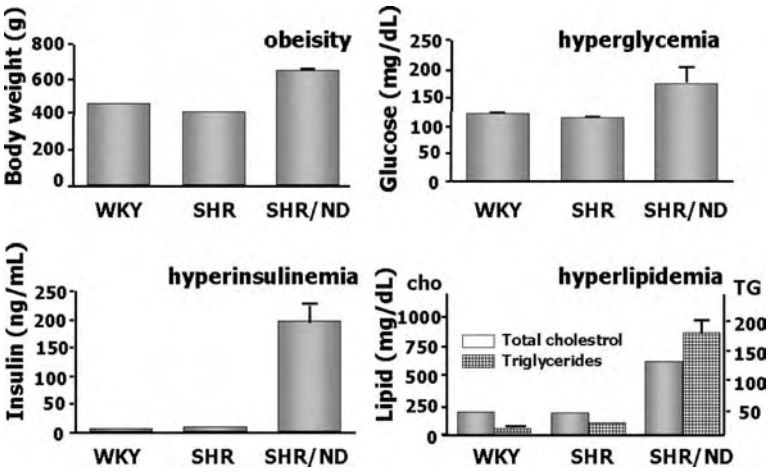


FIGURE 21.4 Metabolic syndrome in a type 2 diabetic rat model, SHR/NDmcr-cp. Metabolic values at 33 weeks are shown. In addition to hypertension characteristic to SHR, SHR/NDmcr-cp exhibits obesity, hyperglycemia, hyperinsulinemia, and hyperlipidemia, all characteristics to human type 2 diabetes.

of the leptin receptor generated obesity, hyperglycemia, hyperinsulinemia, and hyperlipidemia, all characteristics of human type 2 diabetes (Fig. 21.4).

The respective contributions of hypertension and metabolic derangements to diabetic renal damage can be assessed by comparing SHR/NDmcr-cp with SHR. Hypertension in SHR increased proteinuria and induced mild glomerular

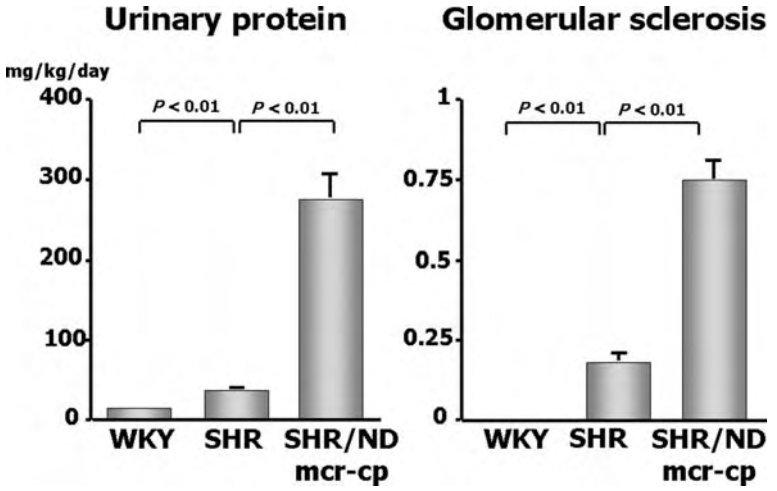


FIGURE 21.5 Renal damage associated with hypertension and/or metabolic syndrome. Hypertension in SHR increases proteinuria and induces mild glomerular sclerosis. In SHR/NDmcr-cp with severe metabolic syndrome, although hypertension is less severe, proteinuria and glomerular sclerosis become much more severe.

sclerosis. In SHR/NDmcr-cp, although hypertension was less severe, proteinuria and glomerular sclerosis were much more severe (Fig. 21.5). These observations point to the critical role played by metabolic derangements in addition to hypertension in diabetic renal damage.

Are these renal disorders preventable? The SHR/NDmcr-cp rats were given three different types of antihypertensive agents: valsartan (an ARB), nifedipine (a calcium channel blocker), and atenolol (a beta blocker). They normalized systolic blood pressure to the same extent. However, ARB alone successfully decreased proteinuria, whereas nifedipine and atenolol were not effective (our unpublished observations). This finding fits with our clinical experience that ARB (and ACEI also) is endowed with additional renoprotective properties beyond their blood pressure lowering effects (Brenner et al., 2001; Lewis et al., 2001; Parving et al., 2001; Viberti and Wheeldon, 2002). Renoprotection by ARB has been linked to the inhibition of the renin-angiotensin system (RAS): ARB reduces glomerular hypertension and also exhibits numerous nonhemodynamic, pleiotropic effects on mesangial cells, podocytes, and tubular cells, all of which express on their surfaces angiotensin II receptor (Taal and Brenner, 2000).

We were interested to know whether ARB is endowed with additional renoprotective properties beyond blood pressure lowering and RAS inhibition. The SHR/NDmcr-cp rats were given high doses of ARB (valsartan and olmesartan), exceeding the level necessary for angiotensin II receptor saturation. Beyond 80 mg/kg/day for valsartan and 50 mg/kg/day for olmesartan, they failed to further reduce blood pressure, suggesting the complete blockade of angiotensin II receptor in the vascular system. Nevertheless, renoprotection witnessed by

proteinuria progressed continuously in a dose-dependent manner (our unpublished observations). The renoprotective action at high doses of ARB is obviously independent of blood pressure lowering and also appears independent of the RAS inhibition.

Similar data have been obtained in human clinical studies by Weinberg (Weinberg et al., 2004). He administered supramaximal doses of candesartan, another ARB, to subjects with heavy proteinuria, surprisingly up to 96 mg/day, which is far above the approved dose for this drug (12 mg/day in Japan). He reported that beyond 30 mg/day candesartan failed to reduce blood pressure. Nevertheless, the high doses further reduced proteinuria in a dose-dependent fashion without concomitant additional blood pressure lowering. Thus there is no optimal dose for renoprotection of ARB. Once the receptor blockade is maximally saturated, the additional, very linear dose-dependent renoprotection by high doses of ARB suggests a chemical contribution rather than a biological action on angiotensin II inhibition.

The question then arises: Is the chemical structure of ARB itself renoprotective? Among the chemical properties of ARB, we are focusing on their potent ability to inhibit oxidative stress and AGE formation. In vivo, the AGE content in the rat kidney is not elevated in SHR, and this is similar to WKY (Nangaku et al., 2003). Therefore, hypertension itself does not enhance AGE genesis. In SHR/NDmcr-cp, by contrast, the renal AGE content is markedly elevated in association with the metabolic syndrome. Importantly, administration of ARB, but not nifedipine nor atenolol, could markedly reduce renal AGE content without improvement of the metabolic syndrome (Nangaku et al., 2003; our unpublished observations). The renal AGE content significantly correlates with proteinuria in our diabetic rats given several types of antihypertensive agents (Nangaku et al., 2003). Taken together, the renoprotective effect of ARB depends not only on blood pressure lowering and RAS inhibition but also, at least in part, on the local inhibition of oxidative stress and AGE formation.

21.6 FUTURE PROSPECTS

Hudson and colleagues have recently identified a new class of AGE inhibitors, pyridoxamine (Booth et al., 1996). Subsequently the group of Baynes has demonstrated that pyridoxamine inhibits the modification of lysine residues in RNase and low-density lipoprotein during metal-catalyzed oxidation reactions, and inhibits the formation of AGEs (CML and CEL) as well as ALEs (malondialdehyde-lysine and hydroxynonenal-lysine) (Onorato et al., 2000). Unlike aminoguanidine and OPB-9195, pyridoxamine does not trap pyridoxal. Its preclinical efficacy has been proved in animal models of diabetic nephropathy (Degenhardt et al., 2002). A phase 2 clinical trial for pyridoxamine has been completed successfully in the United States (McGill et al., 2004).

We also have characterized the AGE lowering profile of several thousands of chemical compounds, including currently used medical drugs. By random screening utilizing a combination of several in vitro assays (e.g., pentosidine,

CML, transition metal chelation, hydroxyl radical scavenging), three classes of novel structures (not pyridoxamine and hydrazine-derivatives like aminoguanidine) have been identified that efficiently inhibit in vitro AGE formation. Like ARB, they inhibit AGE formation probably as a result of their potent ability to scavenge hydroxyl radicals and to chelate transition metals. They have no affinity to angiotensin II receptor. Several lead compounds are now under investigation. Some of them decrease proteinuria efficiently in SHR/NDmcr-cp without correction of hypertension and the metabolic syndrome (our unpublished observations).

An issue of particular interest is the identification of proteins whose functions are influenced by in vivo administration of AGE inhibitors, and this promises to be a fruitful topic of future proteomics research. The present findings are nevertheless in good agreement with the recent therapeutic concept on diabetic nephropathy that multiple risk factor interventions are critical, including antihypertensive treatment by agents with RAS inhibition, correction of obesity, and conection of the metabolic syndrome. Our studies further implicate a therapeutic potential of inhibition of oxidative stress and AGE formation. More effective oxidative stress and AGE inhibitors should expand our therapeutic options in diabetic renal injury.

ACKNOWLEDGMENTS

This study was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research (OPSR) in Japan.

LIST OF ABBREVIATIONS

ACEI, angiotensin converting enzyme inhibitors
ARB, angiotensin II receptor blockers
AGEs, advanced glycation end-products
ALEs, advanced lipoxidation end-products
CML, N^{ε}-carboxymethyllysine
OLETF, Otsuka-Long-Evans-Tokushima fatty rat
OPB-9195, (±)-2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide
RAS, renin-angiotensin system
RCO, reactive carbonyl compounds
SHR, spontaneous hypertensive rat
WKY, Wistar Kyoto rat

REFERENCES

Ahmed M.U., Thorpe S.R., Baynes J.W. (1986). Identification of N^{ε}-carboxymethyllysine as a degradation product of fructoselysine in glycated protein. *J Biol Chem* 261: 4889–4894.

Akhand A.A., Kato M., Suzuki H., Liu W., Du J., Hamaguchi M., Miyata T., Kurokawa K., Nakashima I. (1999). Carbonyl compounds cross-link cellular proteins and activate protein-tyrosine kinase p60c-Src. *J Cell Biochem* 72: 1–7.

Booth A.A., Khalifah R.G., Hudson B.G. (1996). Thiamine pyrophosphate and pyridoxamine inhibit the formation of antigenic advanced glycation end-products: Comparison with aminoguanidine. *Biochem Biophys Res Commun* 220: 113–119.

Bravi M.C., Pietrangeli P., Laurenti O., Basili S., Cassone-Faldetta M., Ferri C., De Mattia G. (1997). Polyol pathway activation and glutathione redox status in non-insulin-dependent diabetic patients. *Metabolism* 46: 1194–1198.

Brenner B.M., Cooper M.E., de Zeeuw D., Keane W.F., Mitch W.E., Parving H.H., Remuzzi G., Snapinn S.M., Zhang Z., Shahinfar S. RENAAL Study Investigators (2001). Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *N Engl J Med* 345: 861–869.

Brownlee M., Vlassara H., Kooney A., Ulrich P., Cerami A. (1986). Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. *Science* 232: 1629–1632.

Degenhardt T.P., Alderson N.L., Arrington D.D., Beattie R.J., Basgen J.M., Steffes M.W., Thorpe S.R., Baynes J.W. (2002). Pyridoxamine inhibits early renal disease and dyslipidemia in the streptozotocin-diabetic rat. *Kidney Int* 61: 939–950.

Hazen S.L., Hsu F.F., Mueller D.M., Crowley J.R., Heinecke J.W. (1996). Human neutrophils employ chlorine gas as an oxidant during phagocytosis. *J Clin Invest* 98: 1283–1289.

Horie K., Miyata T., Maeda K., Miyata S., Sugiyama S., Sakai H., van Ypersole de Strihou C., Monnier V.M., Witztum J.L., Kurokawa K. (1997). Immunohistochemical colocalization of glycoxidation products and lipid peroxidation products in diabetic renal glomerular lesions. *J Clin Invest* 100: 2995–3004.

Ischiropoulos H., al-Mehdi A.B. (1995). Peroxynitrite-mediated oxidative protein modifications. *FEBS Lett* 364: 279–282.

McGill J.B., Williams M.E., Dengenhardt T.P., Szabo J.R., Schotzinger R.J., PYR-205/207 study group. (2004). A phase 2 clinical investigation of pyridoxamine (Pyridorin™) in type 1 and type 2 diabetic patients with overt diabetic nephropathy (PYR-205/207) (abstract). *J Am Soc Nephrol* 15: 565A.

Jennings P.E., Chirico S., Jones A.F., Lunec J., Barnett A.H. (1987). Vitamin C metabolites and microangiopathy in diabetes mellitus. *Diabetes Res* 6: 151–154.

Lewis E.J., Hunsicker L.G., Clarke W.R., Berl T., Pohl M.A., Lewis J.B., Ritz E., Atkins R.C., Rohde R., Raz I. Collaborative Study Group (2001). Renoprotective effect of the angiotensin-receptor antagonist irbesartan in patients with nephropathy due to type 2 diabetes. *N Engl J Med* 345: 851–860.

Miyata S., Monnier V.M. (1992). Immunohistochemical detection of advanced glycosylation end products in diabetic tissues using monoclonal antibody to pyrraline. *J Clin Invest* 89: 1102–1112.

Miyata T., Maeda K., Kurokawa K., van Ypersele de Strihou C. (1997). Oxidation conspires with glycation to generate noxious advanced glycation end products in renal failure. *Neprohl Dial Transplant* 12: 255–258.

Miyata T., Ueda Y., Yamada Y., Izuhara Y., Wada T., Jadoul M., Saito A., Kurokawa K., van Ypersele de Strihou C. (1998). Accumulation of carbonyls accelerates the formation of pentosidine, an advanced glycation end product: carbonyl stress in uremia. *J Am Soc Nephrol* 9: 2349–2356.

Miyata T., van Ypersele de Strihou C., Kurokawa K., Baynes J.W. (1999a). Alterations in nonenzymatic biochemistry in uremia: Origin and significance of "carbonyl stress" in long-term uremic complications. *Kidney Int* 55: 389–399.

Miyata T., Ishikawa S., Asahi K., Inagi R., Suzuki D., Horie K., Tatsumi K., Kurokawa K. (1999b). 2-Isopropylidenehydrazono-4-oxo-thiazolidin-5ylacetanilide (OPB-9195) inhibits the neointima proliferation of rat carotid artery following balloon injury: Role of glycoxidation and lipoxidation reactions in vascular tissue damage. *FEBS Lett* 445: 202–206.

Miyata T., Ueda Y., Asahi K., Izuhara Y., Inagi R., Saito A., Van Ypersele de Strihou C., Kurokawa K. (2000a). Mechanism of the inhibitory effect of OPB-9195 [(±)-2-isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide] on advanced glycation end product and advanced lipoxidation end product formation. *J Am Soc Nephrol* 11: 1719–1725.

Miyata T., Horie K., Ueda Y., Fujita Y., Izuhara Y., Hirano H., Uchida K., Saito A., van Ypersele de Strihou C., Kurokawa K. (2000b). Advanced glycation and lipoxidation of the peritoneal membrane: Respective roles of serum and peritoneal fluid reactive carbonyl compounds. *Kidney Int* 58: 425–435.

Miyata T., van Ypersele de Strihou C., Ueda Y., Ichimori K., Inagi R., Onogi H., Ishikawa N., Nangaku M., Kurokawa K. (2002). Angiotensin II receptor antagonists and angiotensin converting enzyme inhibitors lower in vitro the formation of advanced glycation end products: Biochemical mechanisms. *J Am Soc Nephrol* 13: 2478–2487.

Nakamura S., Makita Z., Ishikawa S., Yasumura K., Fujii W., Yanagisawa K., Kawata T., Koike T. (1997). Progression of nephropathy in spontaneous diabetic rats is prevented by OPB-9195, a novel inhibitor of advanced glycation. *Diabetes* 46: 895–899.

Nangaku M., Miyata T., Sada T., Mizuno M., Inagi R., Ueda Y., Ishikawa N., Yuzawa H., Koike H., van Ypersele de Strihou C., Kurokawa K. (2003). Anti-hypertensive agents inhibit in vivo the formation of advanced glycation end products and improve renal damage in a type 2 diabetic nephropathy rat model. *J Am Soc Nephrol* 14: 1212–1222.

Onorato J.M., Jenkins A.J., Thorpe S.R., Baynes J.W. (2000). Pyridoxamine, an inhibitor of advanced glycation reactions, also inhibits advanced lipoxidation reactions: Mechanism of action of pyridoxamine. *J Biol Chem* 275: 21177–21184.

Onozato M.L., Tojo A., Goto A., Fujita T., Wilcox C.S. (2002). Oxidative stress and nitric oxide synthase in rat diabetic nephropathy: Effects of ACEI and ARB. *Kidney Int* 61: 186–194.

Parving H.H., Lehnert H., Brochner-Mortensen J., Gomis R., Andersen S., Arner P. Irbesartan in Patients with Type 2 Diabetes and Microalbuminuria Study Group (2001). The effect of irbesartan on the development of diabetic nephropathy in patients with type 2 diabetes. *N Engl J Med* 345: 870–878.

Pennathur S., Wagner J.D., Leeuwenburgh C., Litwak K.N., Heinecke J.W. (2001). A hydroxyl radical-like species oxidizes cynomolgus monkey artery wall proteins in early diabetic vascular disease. *J Clin Invest* 107: 853–860.

Requena J.R., Fu M.X., Ahmed M.U., Jenkins A.J., Lyons T.J., Baynes J.W., Thorpe S.R. (1997). Quantification of malondialdehyde and 4-hydroxynonenal adducts to lysine residues in native and oxidized human low-density lipoprotein. *Biochem J* 322: 317–325.

Sell D.R., Monnier V.M. (1989). Structure elucidation of a senescence cross-link from human extracellular matrix. *J Biol Chem* 264: 21597–21602.

Suzuki E., Yasuda K., Takeda N., Sakata S., Era S., Kuwata K., Sogami M., Miura K. (1992). Increased oxidized form of human serum albumin in patients with diabetes mellitus. *Diabetes Res Clin Pract* 18: 153–158.

Suzuki D., Miyata T., Saotome N., Horie K., Inagi R., Yasuda Y., Uchida K., Izuhara Y., Yagame M., Sakai H., Kurokawa K. (1999). Immunohistochemical evidence for an increased oxidative stress and carbonyl modification of proteins in diabetic glomerular lesions. *J Am Soc Nephrol* 10: 822–832.

Taal M.W., Brenner B.M. (2000). Renoprotective benefits of RAS inhibition: from ACEI to angiotensin II antagonists. *Kidney Int* 57: 1803–1817.

Taguchi T., Sugiura M., Hamada Y., Miwa I. (1998). In vivo formation of a Schiff base of aminoguanidine with pyridoxal phosphate. *Biochem Pharmacol* 55: 1667–1671.

Uchida K., Kanematsu M., Sakai K., Matsuda T., Hattori N., Mizuno Y., Suzuki D., Miyata T., Noguchi N., Niki E., Osawa T. (1998). Protein-bound acrolein: Potential markers for oxidative stress. *Proc Natl Acad Sci USA* 95: 4882–4887.

Viberti G., Wheeldon N.M. MicroAlbuminuria Reduction With VALsartan (MARVAL) Study Investigators (2002). Microalbuminuria reduction with valsartan in patients with type 2 diabetes mellitus: A blood pressure-independent effect. *Circulation* 106: 672–678.

Weinberg A.J., Zappe D.H., Ashton M., Weinberg M.S. (2004). Safety and tolerability of high-dose Angiotensin receptor blocker therapy in patients with chronic kidney disease: A pilot study. *Am J Nephrol* 24: 340–345.

Wells-Knecht M.C., Lyons T.J., McCance D.R., Thorpe S.R., Baynes J.W. (1997). Age-dependent increase in *ortho*-tyrosine and methionine sulfoxide in human skin collagen is not accelerated in diabetes: Evidence against a generalized increase in oxidative stress in diabetes. *J Clin Invest* 100: 839–846.

Williamson J.R., Chang K., Frangos M., Hasan K.S., Ido Y., Kawamura T., Nyengaard J.R., van den Enden M., Kilo C., Tilton R.G. (1993). Hyperglycemic pseudohypoxia and diabetic complications. *Diabetes* 42: 801–813.

22

QUANTITATIVE SCREENING OF PROTEIN GLYCATION, OXIDATION, AND NITRATION ADDUCTS BY LC-MS/MS: PROTEIN DAMAGE IN DIABETES, UREMIA, CIRRHOSIS, AND ALZHEIMER'S DISEASE

PAUL J. THORNALLEY

22.1 INTRODUCTION: DERIVATIZATION FREE DETECTION WITH APPLICATION TO MODIFIED PROTEINS AND AMINO ACIDS

Proteins are susceptible to spontaneous modification by glycation, oxidation, and nitration in the physiological setting. Many different adducts are formed from amino acid residues at different sites within many different proteins. It is a major technical challenge to quantify the multiple adducts of these different types of protein damage in small amounts of protein. The best technique available currently to meet this challenge is liquid chromatography with positive ion electrospray ionization and tandem mass spectrometric detection (LC-MS/MS), calibrated with stable isotope substituted standards. Protein substrates are hydrolyzed and the glycation, oxidation, and nitration adducts are then quantified. Although LC-MS/MS analysis has been used for the determination of amino acids in congenital metabolic disorders and elsewhere for a decade or more (Rashed et al., 1995), recent advances in understanding of important physiological processes of protein glycation—particularly, the modification of

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

proteins by arginine-derived hydroimidazolone adducts in physiological systems (Thornalley, 1999)—has required new technical approaches. First, hydroimidazolones analytes are unstable in acid hydrolysis and hence a procedure for exhaustive enzymatic hydrolysis of proteins substrates was developed (Ahmed et al., 2002). Steps were taken to avoid oxidative degradation of protein adducts residues during enzymatic hydrolysis—addition of the antioxidant thymol and incubation under nitrogen or carbon monoxide (the latter to exclude oxygen and inactivate heme in hemoglobin digests) (Ahmed et al., 2002; Thornalley et al., 2003). After an initial step with pepsin under acidic conditions (replaced by collagenase for analysis of collagen) (Dobler et al., 2004), antibiotics were included in the enzymatic digest to prevent bacterial growth in the amino acid solution being produced (Ahmed et al., 2002). These procedures give acceptable analyte stabilities and exhaustive proteolysis that proceeds to near completion for proteins modified minimally by glycation, oxidation, and nitration. Proteolysis is inefficient for proteins modified highly by glycation adducts—such as in some experimental preparations of proteins highly modified by advanced glycation endproducts (AGEs) and also some proteins in thermally processing food. This may be because high modification of arginine and lysine residues blocks cleavage sites for proteolysis and also because some glycation adducts inhibit proteases (Ahmed and Thornalley, 2002; Oste et al., 1987). Exhaustive proteolysis is often thought to be inefficient, but this impression could have been gained by failing to protect against bacterial growth with consequent decreased yield of amino acids.

During method development for the LC-MS/MS quantitative screening assay of protein glycation, oxidation, and nitration adducts, we found that *N*-terminal derivatization procedures for modification of exhaustive hydrolysates led to overestimation of protein oxidation analytes, loss of hydroimidazolone analytes, and also compromised the use of stable isotope-substituted standard calibration. Hydroimidazolones have moderate stability at physiological pH with half-lives of two to six weeks (Ahmed et al., 2002), degrading to form mainly the precursor α-oxoaldehyde and arginine. During *N*-terminal derivatization at high pH and temperature—even as used in the relatively mild conditions of 6-aminoquinolyl-*N*-hydroxysuccimidylcarbamate (AQC) derivatization procedures (Ahmed et al., 2002)—the α-oxoaldehyde precursor-related moieties of hydroimidazolones underwent arginine residue exchange from arginine to added [$^{15}N_2$]arginine, thereby compromising the stable isotope internal calibration. It became clear that because of sample oxidations and hydroimidazolone instability, a procedure using a chromatographic stationary phase that would retain analytes without derivatization was required. This was achieved with a graphitic stationary phase, as in the Hypercarb™ columns (Thermohypersil, Bellefonte, PA). The use of two columns in series (2.1 × 50 mm and 2.1 × 250 mm) with switching facilitated the retention and sequential elution of analytes of diverse hydrophobicity and column washing (Thornalley et al., 2003). The method was reported initially for N_ε-fructosyl-lysine (FL) and 12 AGEs, two oxidation markers, and the nitration marker 3-nitrotyrosine (3-NT). Further analytes have been added since (Table 22.1 and Figs. 22.1 and 22.2). Alternative procedures using octadecyl

silica columns have short retention times of analytes and do not provide for initial diversion of nonvolatile salts to waste (Mottaran et al., 2002); this can be improved with ion pair reagents (Piraud et al., 2003).

Analysis of analytes in exhaustive digests of protein gives the amounts of protein glycation, oxidation, and nitration adducts residues normalized to amount protein (pmol analyte per mg protein) or to corresponding unmodified amino acid (mmol analyte per mol unmodified amino acid). Analysis of analytes in undigested ultrafiltrates (12 kDa cutoff membrane filter) of plasma, urine, or other physiological fluid gives the concentrations of glycation, oxidation, and nitration free adducts (glycated, oxidized, and nitrated amino acids). The release of further free adducts by enzymatic digestion of the ultrafiltrate gives the amount of protein glycation, oxidation, and nitration adducts residues in low molecular mass polypeptides that are called "glycated, oxidized, and nitrated peptides." Other investigators used immunoassay techniques to determine AGEs in peripheral venous plasma fractionated by gel filtration and called the low molecular mass fraction "AGE peptides," failing to appreciate the presence of AGE free adducts (Makita et al., 1994). From quantitative LC-MS/MS analysis we have been able to detect AGE (and oxidation adduct) enrichment on low molecular mass peptides in vivo only in portal venous plasma of human subjects and not in peripheral venous plasma (Thornalley et al., 2003; Ahmed et al., 2004b). Immunoassay of "AGE peptides," therefore, probably detected AGE free adducts. We now classify protein damage in three molecular forms: glycation, oxidation, and nitration adducts present as residues in proteins (>12 kDa), residues in peptides (<12 kDa), and related free adducts (Fig. 22.3). Protein glycation, oxidation, and nitration free adducts have been detected previously: for example, plasma pentosidine in uremia as an indicator of dialysis efficiency (Friedlander et al., 1996), and urinary dityrosine and 3-NT as markers of oxidative stress (Leeuwenburgh et al., 1999; Schwemmer et al., 2000).

The major restriction of access to this technique is the availability of isotope-substituted standards for a comprehensive range of analytes, since most are not available commercially. The overwhelming advantage of this technique is that it can provide a relatively comprehensive and quantitative analysis of protein glycation, oxidation, and nitration adduct residues and free adducts using a small amount of sample (50–100 μg protein and 50–100 μl of ultrafiltrate). The current range of detection of glycation adducts, FL and 11 AGEs, three oxidation markers, and 3-NT includes the most important glycation, oxidation, and nitration adducts. For example, for the glycation of albumin to minimal extent as found in vivo (Thornalley et al., 2000a; Ahmed et al., 2004b), more than 90% of total glycation adducts derived from glucose and more than 99% derived from methylglyoxal were detected in this quantitative screening assay (Ahmed et al., 2002, 2005b; Thornalley et al., 2003). We include in the quantitative screening assay major markers of oxidative damage to methionine, tyrosine, and tryptophan—methionine sulfoxide (MetSO), dityrosine, *N*-formylkynurenine (NFK) (Brot and Weissbach, 1983; Giulivi and Davies, 2001; Geibauf et al., 1996), and the nitration marker 3-NT (Hurst, 2002). Other glycation, oxidation,

TABLE 22.1 Chromatographic Retention Times and Mass Spectrometric Multiple Reaction Monitoring Detection of Protein Glycation, Oxidation, and Nitration Adducts

Analyte Group	Analyte	Parent Ion (Da)	Fragment Ion (Da)	CE (eV)	Neutral Fragment Loss(es)	Internal Standard (IS)
Amino acids	Arg	175.2	70.3	15	H_2CO_2, $NH_2C(=NH)NH_2$	[$^{15}N_2$]-Arg
	Lys	147.1	84.3	15	H_2CO_2, NH_3	[$^{13}C_6$]-Lys
	Met	150.0	104.2	11	H_2CO_2	[*methyl*-$^{2}H_3$]-Met
	Tyrosine	182.1	136.2	13	H_2CO_2	[*ring*-$^{2}H_4$]-Tyrosine
	Valine	118.0	72.1	15	H_2CO_2	[$^{2}H_8$]-val
	Tryptophan	205.0	159.1	15	H_2CO_2	[$^{15}N_2$]-Trp
Fructosamines	FL	291.0	84.3	31	H_2CO_2, fructosylamine	[$^{2}H_4$]-FL
	Fructosylvaline	280.2	198.4	20	H_2CO_2, $2H_2O$	[$^{2}H_8$]-fructosylvaline
Hydroimidazolones[a]	G-H1	215.0	100.2	14	$NH_2CH(CO_2H)CH_2CH{=}CH_2$	[$^{15}N_2$]-G-H1
	MG-H1	229.2	114.3	14	$NH_2CH(CO_2H)CH_2CH{=}CH_2$	[$^{15}N_2$]-MG-H1
	3DG-H	319.1	114.8	20	$NH_2CH(CO_2H)CH_2CH{=}CH_2$	[$^{15}N_2$]-3DG-H
Monolysyl AGEs	CEL	219.2	130.1	13	$NH_2CH(CH_3)CO_2H$	[$^{13}C_6$]-CEL
	CML	204.9	130.1	12	$NH_2CH_2CO_2H$	[$^{13}C_6$]-CML
	Pyrraline	255.2	84.3	23	2-CHO-5-HOCH$_2$-pyrrole, H_2CO_2	[$^{2}H_4$]-pyrraline

Imidazolium AGEs	GOLD	327.1	198.3	21	$NH_2CH(CO_2H)CH_2CH_2CH{=}CH_2$	$[^2H_8]$-GOLD
	MOLD	341.2	212.3	21	$NH_2CH(CO_2H)CH_2CH_2CH{=}CH_2$	$[^2H_8]$-MOLD
	DOLD	431.2	302.4	26	$NH_2CH(CO_2H)CH_2CH_2CH{=}CH_2$	$[^2H_7]$-DOLD
Fluorescent AGEs	Argpyrimidine	255.3	140.3	17	$NH_2CH(CO_2H)CH_2CH{=}CH_2$	$[^{15}N_2]$-Argpyrimidine
	Pentosidine	379.3	250.4	22	$NH_2CH(CO_2H)CH_2CH_2CH{=}CH_2$	$[^{13}C_6]$-Pentosidine
Oxidation adducts	MetSO	166.1	102.2	14	$CH_3{-}SOH$	[methyl-$^2H_3$]-MetSO
	Dityrosine	361.2	315.3	15	H_2CO_2	[*ring*-$^2H_6$]-Dityrosine
	NFK	237.0	191.0	12	H_2CO_2	$[^2N_{15}]$-NFK
Nitration adduct	3-NT	227.1	181.2	13	H_2CO_2	[*ring*-$^2H_3$]-3-NT

Source: From Thornalley et al. (2003).

[a]For hydroimidazolones, structural isomer-1 is denoted, although for 3DG-H detection, structural isomers 3DG-H1, 3DG-H2 and 3DG-H3 are all detected.

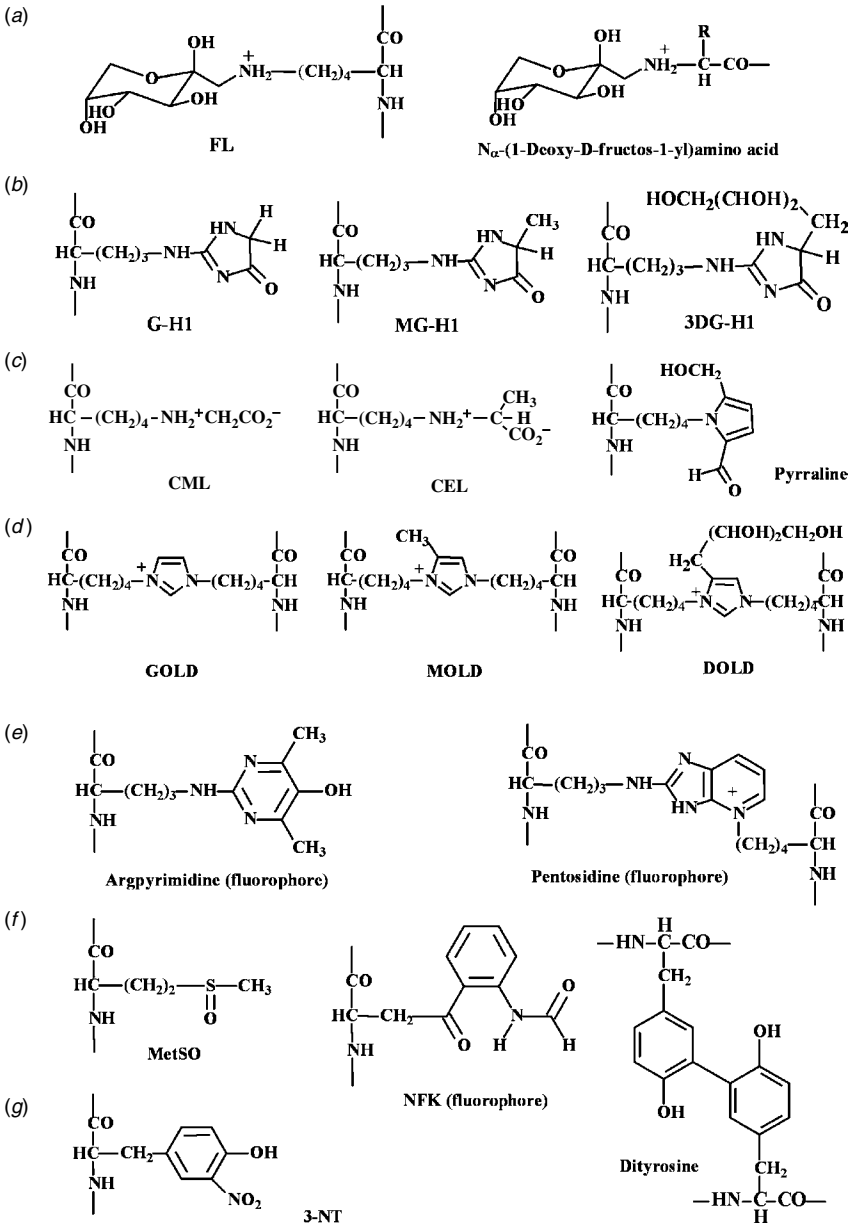


FIGURE 22.1 Protein glycation, oxidation, and nitration adduct residues in physiological systems. (*a*) Early glycation adducts: FL and N_{α}-(1-deoxy-D-fructos-1yl)amino acid residues. Advanced glycation end-products: (*b*) hydroimidazolones, (*c*) monolysyl AGEs, (*d*) imidazolium cross-links, (*e*) fluorophores, (*f*) oxidation adducts, and (*g*) nitration adduct. Protein glycation, oxidation, and nitration adduct residues are shown. For the corresponding free adducts at physiological pH, the *N*-terminal amino group is protonated $-NH_3^+$ and the *C*-terminal carbonyl is a carboxylate $-CO_2^-$ moiety.

and nitration adducts will be added in future studies. Overall, we obtain a fingerprint of the quantitative damage to cellular and extracellular proteins in physiological systems and related proteolytic debris—protein glycation, oxidation, and nitration free adducts. This way we are not likely to overlook important types of protein damage. The levels of one or a combination of these adducts may provide a critical marker for disease diagnosis and progression monitoring. This has been applied to screening protein glycation, oxidation, and nitration markers in plasma, red blood cells (RBCs) and leukocytes, tissue proteins, urine, and cerebrospinal fluid (CSF) (Thornalley et al., 2003; Ahmed et al., 2004a).

Protein glycation and nitration adduct residues are often detected by immunoassay (Ikeda et al., 1996; Makita et al., 1994; Ceriello et al., 2004). Quantifying such adducts by immunoassay is beset with doubts over reliability

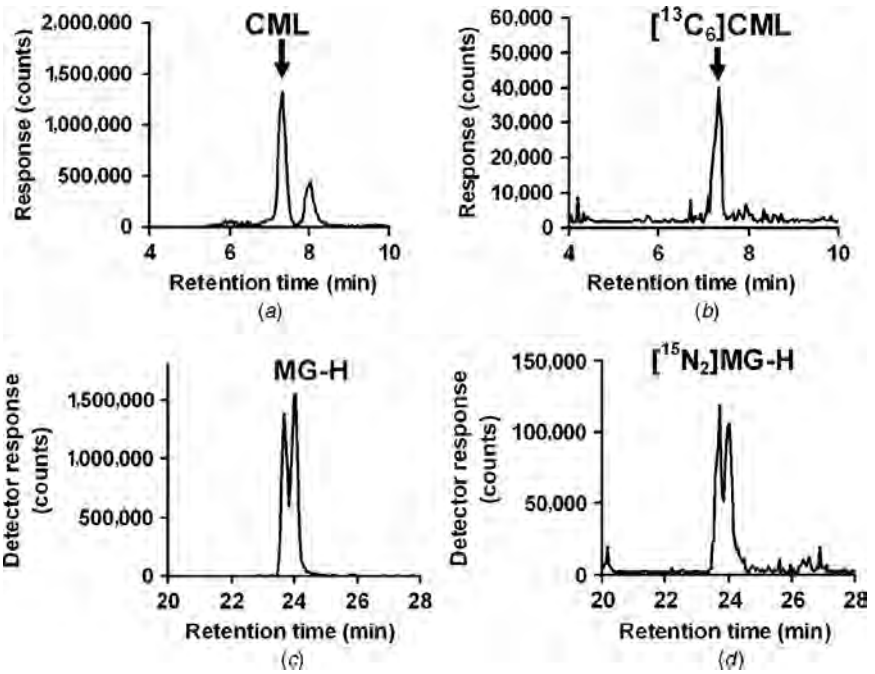


FIGURE 22.2 Detection of protein glycation, oxidation, and nitration adduct residues and free adducts in health and disease. CML free adduct in urine of normal, healthy human subject: (*a*) CML and (*b*) 10 pmol [$^{13}C_6$]CML standard. Methylglyoxal-derived hydroimidazolone residues in human lens protein of an elderly human subject: (*c*) MG-H1 and (*d*) [$^{15}N_2$]MG-H1 (50 pmol) in human lens protein (50 μg equivalent). Oxidation free adduct in plasma filtrate of a type 1 diabetic patient: (*e*) MetSO and (*f*) 50 pmol [$^{2}H_3$]MetSO standard. Nitration adduct residues in hemoglobin of a normal healthy human subject: (*g*) 3-NT and (*h*) [$^{2}H_3$]3-NT standard (10 pmol). Data were collected in multiple reaction monitoring mode under the conditions given in Table 22.1 from Thornalley et al. (2003) and Ahmed et al. (2003, 2005a).

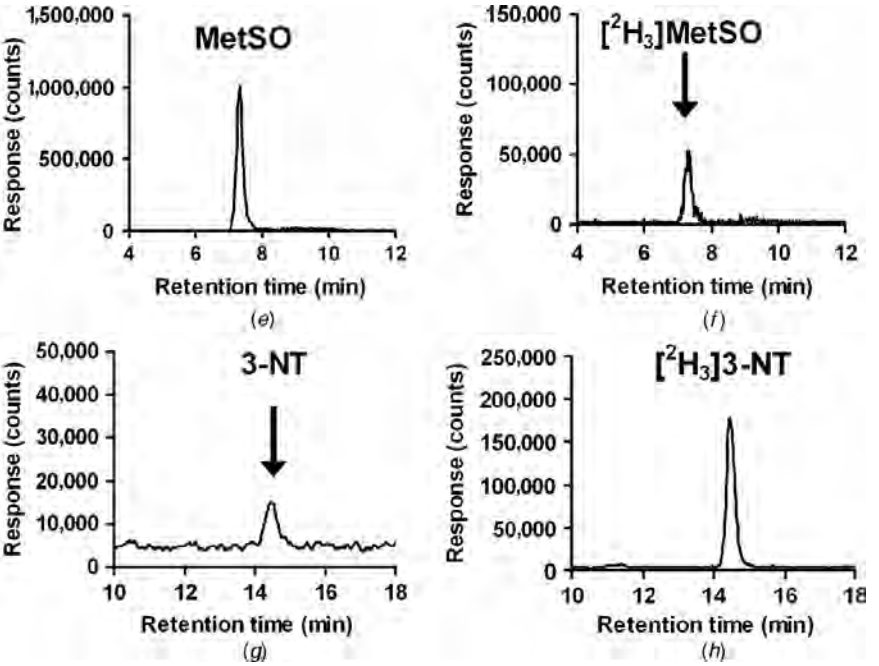


FIGURE 22.2 (*continued*)

of the data obtained. This is largely because of antibody adsorption onto AGE epitopes in proteins used to block nonspecific antibody binding, uncertain epitope specificity of antibodies employed—particularly, for many anti-AGE antibodies—and because of the use of highly modified standard antigens dissimilar to the minimally modified antigens in physiological samples. For these reasons immunoassay often does not provide absolute concentration or amounts of analyte but rather arbitrary units with or without normalization to a reference glycated, oxidized, or nitrated protein standard. Major disparities in immunoassay detection of analytes of protein damage have been the use of the monoclonal antibody to detect N_{ε}-carboxymethyl-lysine (CML) residues that was later found to detect N_{ε}-carboxyethyl-lysine (CEL) as well (Ikeda et al., 1996; Koito et al., 2004) and the 50- to 100-fold discrepancies of detection of 3-NT residues in plasma protein by immunoassay and LC-MS/MS (Ahmed et al., 2002, 2005a; Gaut et al., 2002; Ahmed et al., 2005a). AGEs have also been determined by "total AGE fluorescence," measuring fluorescence with excitation and emission wavelengths of 350 nm and 450 nm, respectively (Sebekova et al., 2001). However, fluorescence detection of NFK interferes with this measurement (Fukunaga et al., 1982). Chromatographic techniques with electrochemical detection have been used to quantify some analytes, but they are susceptible to interference and have led to overestimation (Tohgi et al., 1999; Hensley et al., 1998; Ahmed et al., 2004a). For the future it will be important to

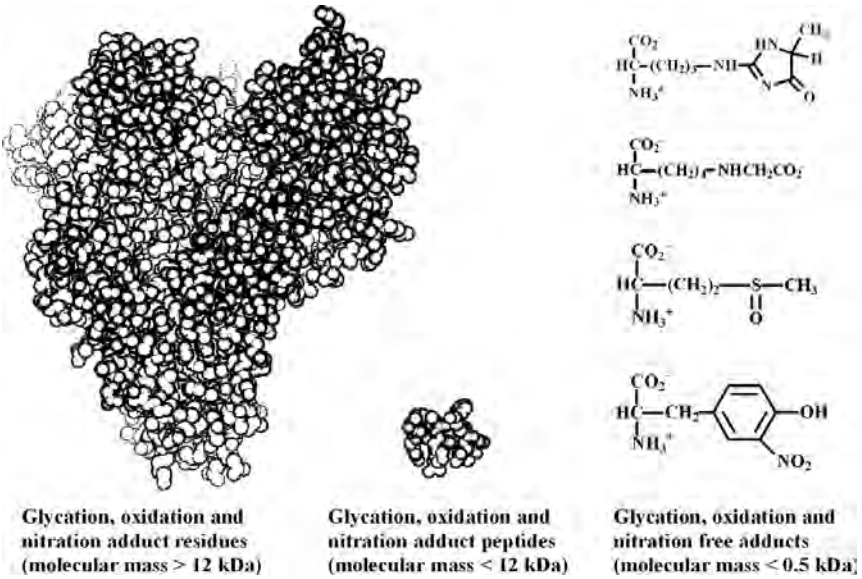


FIGURE 22.3 Classification of protein glycation, oxidation, and nitration adduct residues, peptides, and free adducts.

corroborate immunoassays to the LC-MS/MS "gold standard" method for each sample assay matrix to be used before deployment in clinical studies.

22.2 PHYSIOLOGICAL SOURCES OF GLYCATED, OXIDIZED, AND NITRATED AMINO ACID RESIDUES AND FREE ADDUCTS

Glycation of proteins is a complex series of parallel and sequential reactions collectively called the Maillard reaction. It occurs in all tissues and body fluids. Early stage reactions in glycation lead to the formation of fructosyl-lysine (FL) and *N*-terminal amino acid residue-derived fructosamines. Later stage reactions form stable end-stage adducts, called advanced glycation end-products (AGEs) (Thornalley, 1999). FL degrades slowly to form AGEs. Glyoxal, methylglyoxal, and 3-deoxyglucosone are also potent glycating agents. They are formed by the degradation of glycated proteins, glycolytic intermediates, and lipid peroxidation. They react with proteins to form AGEs directly (Fig. 22.4*a*). Important AGEs quantitatively are hydroimidazolones derived from arginine residues modified by glyoxal, methylglyoxal, and 3-deoxyglucosone: N_δ-(5-hydro-4-imidazolon-2-yl)ornithine (G-H1), N_δ-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1), and N_δ-(5-hydro-5-(2,3,4-trihydroxybutyl)-4-imidazolon-2-yl)ornithine and related structural isomers (3DG-H). Monolysyl AGEs widely studied are CML, CEL, and pyrraline. Protein cross-links are also formed, and glyoxal, methylglyoxal, and 3-deoxyglucosone-derived bis(lysyl) cross-links, GOLD, MOLD,

and DOLD, and pentosidine are the most often studied (Thornalley et al., 2003). Glycation damage to proteins is decreased by enzymatic prevention and repair mechanisms that constitute the enzymatic defense against glycation (Thornalley, 2003b): glyoxalase I catalyzes the detoxification of glyoxal and methylglyoxal (Thornalley, 2003a), and aldehyde reductases and dehydrogenases detoxify 3-deoxyglucosone and other α-oxoaldehydes (Niwa, 1999). FL residues are deglycated by the action of fructosamine 3-phosphokinase (Szwergold et al., 2001). Hydroimidazolones have relatively moderate half-lives (2–6 weeks) and slow dynamic reversibility; therefore protein content of hydroimidazolones can be decreased if the concentrations of the precursor α-oxoaldehydes are decreased. Where hydroimidazolones accumulate with donor age, the increase is related to decreased activity of glyoxalase I and increased methylglyoxal concentration (Ahmed et al., 2003; Haik et al., 1994).

Protein oxidation is promoted by reactive oxygen species—superoxide, hydrogen peroxide, and the hydroxyl radicals (ROS), peroxynitrite and hypochlorite (Berlett and Stadtman, 1997). Susceptible amino acid residues are cysteine, methionine, tyrosine, and tryptophan. The initial oxidation products are cystine, MetSO, dityrosine, and NFK, respectively. ROS, peroxynitrite, and hypochlorite will produce all of these protein oxidation adducts. Enzymatic and nonenzymatic antioxidant defenses (glutathione [GSH], superoxide dismutase, catalase, GSH peroxidase, and others) suppress the levels of oxidants and thereby prevent oxidative damage to proteins. Cystine is rapidly repaired by the action of GSH, glutaredoxin, and thioredoxin (Cumming et al., 2004), and MetSO is also repaired by MetSO reductase (Weissbach et al., 2002). Protein nitration is promoted by peroxynitrite and nitryl chloride (Reiter et al., 2000; Eiserich et al., 1998). 3-NT has major urinary metabolite 3-nitro-4-hydroxyphenylacetic acid (Ohshima et al., 1990) (Fig. 22.4*b*).

Protein glycation was viewed originally as a post-translational modification that accumulated mostly on extracellular proteins. Specifically, AGEs were thought to be formed slowly throughout life, and the concentrations of AGEs found to represent a lifelong accumulation of the glycation adduct. Stable oxidation markers were thought to accumulate similarly. This applies to chemically stable AGEs and oxidation adducts formed on long-lived proteins. For example, CML, CEL, pentosidine, and *ortho*-tyrosine residue accumulation on skin collagen (Verzijl et al., 2000; Wells-Knecht et al., 1997). FL and some AGEs (e.g., hydroimidazolones) have relatively short chemical half-lives under physiological conditions (2–6 weeks). Their concentration depends on the balance of the rates of formation and decomposition. Moreover protein glycation, oxidation, and nitration adduct residues are also formed on cellular and short-lived extracellular proteins. The turnover of these proteins by cellular proteolysis releases glycation, oxidation, and nitration free glycation adducts (Thornalley et al., 2003). Protein damage by glycation, oxidation, and nitration is implicated in protein misfolding. Misfolded proteins are degraded by the proteasome to ensure the high quality of intracellular proteins (Goldberg, 2003); the median half-life of cellular proteins was 32 hours (Gerner et al., 2002).

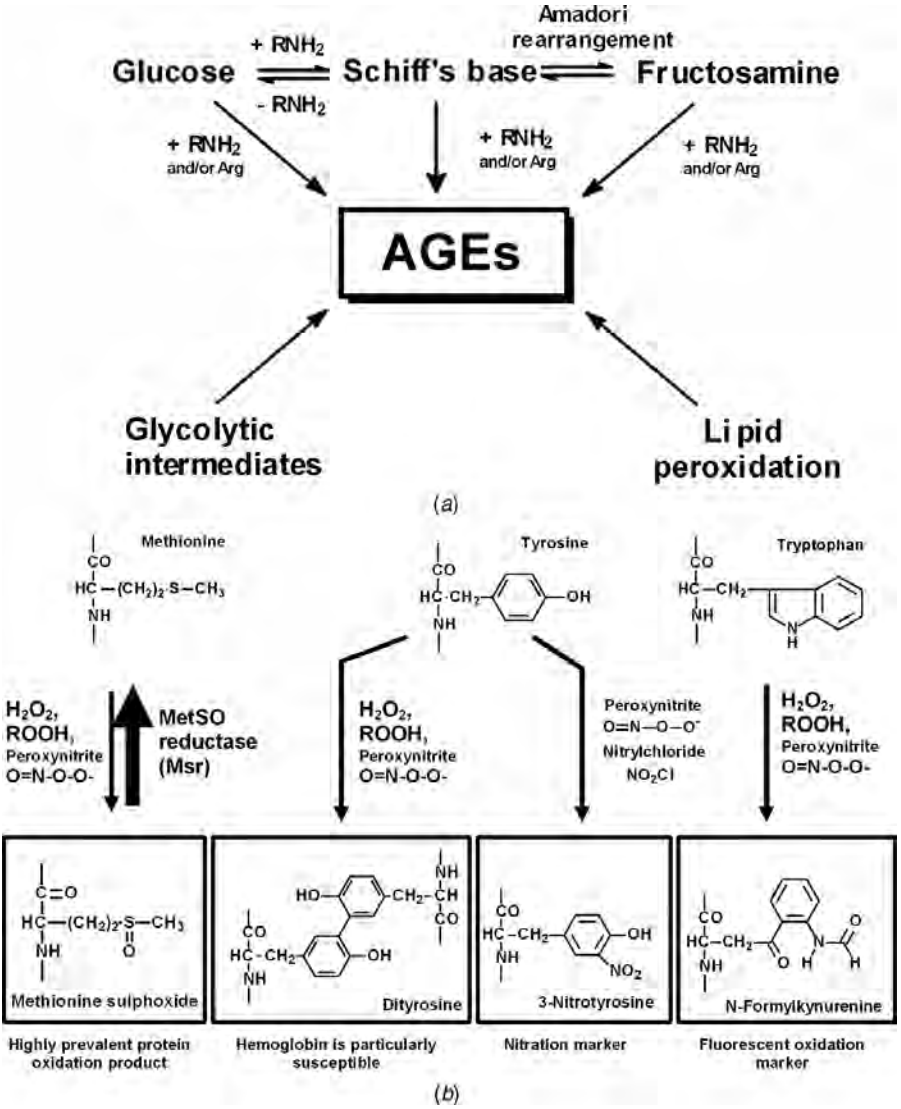


FIGURE 22.4 (*a*) Pathways for the formation of advanced glycation end-products (AGEs). (*b*) Pathways for the formation of protein oxidation and nitration adducts.

Protein glycation, oxidation, and nitration free glycation adducts are therefore the excreted debris of cellular proteolysis of damaged proteins. This appears to be an efficient process, since there has been little evidence of low molecular mass damaged peptides in peripheral venous plasma. Additional sources of free adducts are direct glycation of lysine and arginine, oxidation of methionine, tyrosine, and tryptophan, and nitration of tyrosine. These probably make a minor contribution

because of the relatively low concentration of free amino acids compared to the concentration of corresponding amino acid residues in proteins (Thornalley et al., 2003).

Saccharide-rich and thermally processed foods are a good source of FL, AGEs, and oxidation adduct residues (Henle, 2003). Our recent research suggests, however, that there is a low bioavailability of AGE residues in proteins of ingested foods such that <10% is absorbed (Ahmed et al., 2005c). This may be because proteins glycated highly by FL and AGEs are resistant to proteolysis (Ahmed and Thornalley, 2002) and some AGEs inhibit intestinal proteases (Oste et al., 1987). The highest concentration of absorbed food AGEs is expected in portal venous plasma where we found the hydroimidazolone MG-H1 and MetSO residues enriched by 10-fold and 6-fold, respectively, in peptides (Ahmed et al., 2004b). AGEs and oxidation adducts are therefore probably absorbed from food as both AGE free adducts and AGE-rich peptides; the latter appear to be degraded efficiently after absorption. Major glycation free adducts and irreparable oxidation free adducts had high renal clearances (Thornalley et al., 2003) (Table 22.2 and Fig. 22.5). This suggests that as long as renal function is normal, protein glycation and oxidation adducts absorbed from food may pose little threat, although this remains controversial (see Section 22.7).

Proteins in tissues, plasma, and extracellular matrix in vivo have high FL residue content (0.1–1.8 mmol/mol lys) and a wide range of AGE residue contents (0.001–15 mmol/mol amino acid modified), depending on the location and type of AGE. Hydroimidazolones were the most important AGEs quantitatively both as protein residues and free adducts—particularly, methylglyoxal derived

TABLE 22.2 Advanced Glycation End-Products in Blood Plasma and Renal Clearance in Normal Healthy Human Subjects

Analyte	Plasma Protein (mmol/mol arg, lys, met or tyr (nM))	Urine (nM)	Renal Clearance (ml/min)
CML	0.021 ± 0.005 (1,109)	$1{,}607 \pm 473$	75 ± 22
CEL	0.011 ± 0.005 (581)	$2{,}761 \pm 879$	93 ± 18
G-H1	0.057 ± 0.028 (962)	$1{,}653 \pm 426$	44 ± 15
MG-H1	0.921 ± 0.120 (15,540)	$5{,}281 \pm 3{,}273$	38 ± 6
3DG-H	0.351 ± 0.083 (5,922)	$4{,}356 \pm 2{,}738$	35 ± 6
MOLD	10.0008 ± 0.0002 (42)	48 ± 28	4.2 ± 1.9
Argpyrimidine	<0.03 (<500)	24 ± 4	0.90 ± 0.62
Pentosidine	0.0056 ± 0.0016 (474)	14 ± 2	22 (7 − 67)
FL	0.767 ± 0.139 (40,504)	$3{,}956 \pm 1{,}962$	>30
MetSO	0.762 ± 0.151 (27,500)	48 ± 24	2.9 ± 0.8
3-NT	0.0018 ± 0.0014 (34)	38 ± 9	8.3 ± 2.8

Source: From Thornalley et al. (2003).

Note: The creatinine clearance was 108 ± 15 ml/min, the concentration of plasma protein 64.4 ± 7.4 mg/ml ($n = 5$). Pentosidine residue concentration in plasma protein is given as mmol/mol lys. Data are mean ± SD or median (minimum − maximum); $n = 5$–6.

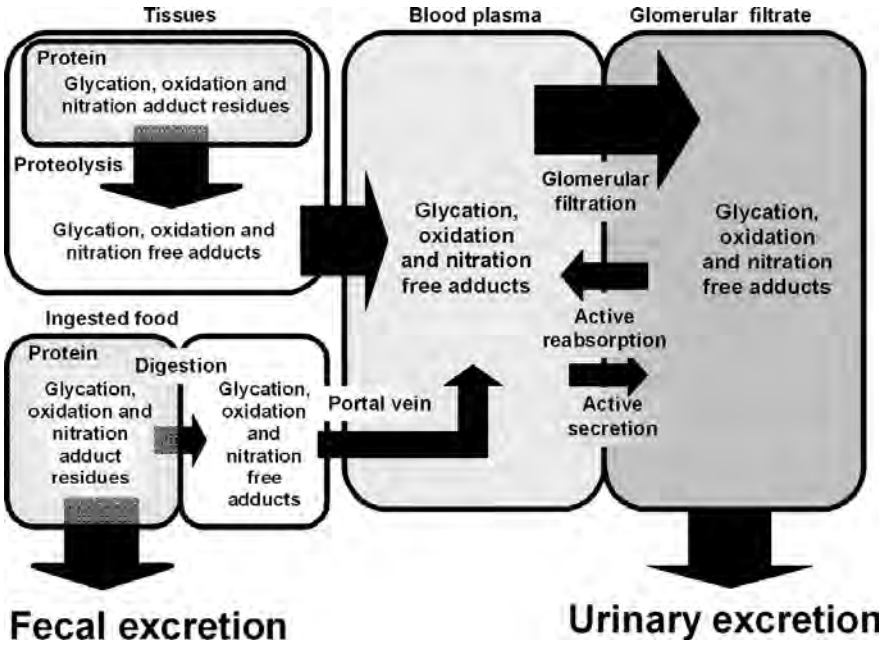


FIGURE 22.5 Biodistribution scheme illustrating flows of formation and removal of protein glycation, oxidation, and nitration free adducts.

hydroimidazolone MG-H1, CML, and CEL were typically at levels 5- to 10-fold lower, and pentosidine and imidazolium cross-links at levels 100- to 1000-fold lower than hydroimidazolones. Hydroimidazolone AGEs are found in highest concentrations in lens protein of elderly human subjects; MG-H1 residue content was 1% to 2% of total arginine residues. MetSO and NFK residues were major oxidation markers, whereas 3-NT is typically 0.001 to 0.01 mmol/mol tyrosine—a minor marker quantitatively of protein damage (Degenhardt et al., 1998; Thornalley et al., 2003; Ahmed et al., 2003; Thornalley, 2005). The glycation, oxidation, and nitration adduct residue contents and molecular mass measurements of proteins in plasma of normal human subjects in vivo indicated that serum albumin was modified minimally by glycation, oxidation, and nitration adduct residues in vivo (Thornalley et al., 2000a); that is, less than 10% of the proteins have one glycation, oxidation, and nitration adduct residue. Stable AGEs such as CML accumulate on lens capsule, skin, and cartilage collagen with age but reached almost 6 mmol/mol lys in cartilage in old age (Ahmed et al., 1997; Verzijl et al., 2000).

Serum albumin modified minimally by glycation was not extracted preferentially from the circulation (Johnson et al., 1991), and endogenous glycated protein did not undergo hepatic extraction in human subjects (Ahmed et al., 2004b). Glycated human serum albumin is probably processed in tubular epithelial cells as is unglycated albumin (Russo et al., 2002). Formation of 3-NT residues in

human serum albumin was found to lead to preferential endothelial transcytosis (Predescu et al., 2002). MetSO residues are relatively high in plasma protein of normal human subjects (0.76 mmol/mol Met), probably because there is no extracellular MetSO reductase. It is conceivable, however, that MetSO residues are repaired during endothelial transcytosis of albumin.

AGE free adducts are exported from cells, leak into plasma, and are excreted in the urine. Major quantitative glycation adducts, FL, hydroimidazolones, CML, and CEL, have high renal clearances, 35 to 93 ml/min in normal human subjects, but this declines in uremia (Thornalley et al., 2003) (Table 22.2). AGE free adducts are the major form by which glycation adducts are eliminated—taking into account AGEs in both urinary albumin and protein fragments (Greive et al., 2001). MetSO and 3-NT both had low renal clearances (<10 ml/min) (Thornalley et al., 2003). This reflects the efficient renal reduction of MetSO by MetSO reductase (Weissbach et al., 2002) and renal re-uptake and/or metabolism of 3-NT (Ohshima et al., 1990).

The physiological importance of protein glycation, oxidation, and nitration remains under intensive investigation. Particularly damaging effects are produced by covalent cross-linking of proteins, which confers resistance to proteolysis (DeGroot et al., 2001) and also oxidation of cysteine thiols in the active sites of enzyme—protein tyrosine phosphatases, for example (Denu and Tanner, 1998). I propose that protein modification is also damaging when amino acid residues are located in sites of protein–protein interaction, enzyme–substrate interaction, and protein–DNA interaction (for transcription factors). A bioinformatics analysis of receptor binding domains indicated that arginine residues have the highest probability of being located in such sites (19.6%) (Gallet et al., 2000). The major modification of arginine residues is by hydroimidazolone AGEs. Their formation causes structural distortion, loss of side chain charge, and functional impairment (Ahmed et al., 2005b). These and other protein modifications are expected to impair functional interactions and may be involved in biochemical dysfunction in disease.

22.3 PROTEIN GLYCATION AND OXIDATION IN DIABETES: DAMAGE TO CELLULAR AND EXTRACELLULAR PROTEINS

22.3.1 Sources of Increased Protein Glycation, Oxidation, and Nitration in Diabetes

Glycation of protein is increased in diabetes associated with hyperglycemia. High-plasma glucose increases the glycation of plasma proteins and extracellular matrix proteins of arterial and vascular basement membrane and skin. Glycation of cellular proteins is also increased in cells with facilitated diffusion uptake of glucose via the glucose transporter GLUT1. This includes vascular endothelial cells and pericytes—particularly in the kidney, retina, and peripheral endoneural microvessels, peripheral neurons, and Schwann cells, lens fiber cells, and red blood cells. In these cells there is increased cellular glucose concentration giving

rise to increased formation of FL and increased flux through glycolysis and the polyol pathway (Myint et al., 1995; Brownlee, 2001). Increased flux through glycolysis gives rise to increased flux of methylglyoxal (Thornalley, 1988). This is exacerbated by oxidative stress, which is driven mainly by increased formation of superoxide by mitochondrial dysfunction, activation of vascular NADPH oxidase, and oxidative uncoupling of vascular nitric oxide synthase (Nishikawa et al., 2000; Hink et al., 2001; Du et al., 1999). Oxidative stress leads to activation of poly(ADP-ribose) polymerase (PARP), poly(ADP) ribosylation, and inhibition of glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) (Du et al., 2003). This exacerbates the accumulation of triosephosphates, increasing further the formation of methylglyoxal, electron flux into mitochondria via the glycerophosphate shuttle, and formation of diacylglycerol (DAG) for activation of protein kinase C and thereby NADPH oxidase (Fig. 22.6). Increased ROS formation leads to increased protein oxidation and increased peroxynitrite formation leads to increased protein nitration. Increased expression of endothelial nitric oxide synthase (eNOS) (Koo and Vaziri, 2003). Protein glycation adducts in albumin and hemoglobin are employed diagnostically as indicators of glycemic control and damage to proteins by glycation, oxidation, and nitration are implicated in the development of the chronic vascular complications of diabetes—nephropathy, retinopathy, peripheral neuropathy (microvascular complications), cardiovascular disease, and cerebrovascular disease (macrovascular complications) (Brownlee, 2001).

22.3.2 Protein Glycation, Oxidation, and Nitration Adducts in Experimental Diabetes

Application of the LC-MS/MS quantitative screening assay for protein glycation, oxidation, and nitration adducts to experimental and clinical diabetes in vivo has revealed increased damage to cellular proteins by glycation and extracellular proteins by glycation, oxidation, and nitration. In streptozotocin (STZ) induced diabetic rats, glycation adducts in cytosolic proteins of the renal glomeruli, retina, and peripheral nerve had increased FL and AGE residue contents. FL residue concentration was increased markedly in renal glomeruli (6-fold), retina (3-fold), and sciatic nerve (7-fold) (Thornalley et al., 2003). The marked increases of FL residues at all these sites was suggested previously by immunoblotting detection (Myint et al., 1995). There were significant increases of CML residues—renal glomeruli (86%), retina (164%), and sciatic nerve (216%), and CEL residues—renal glomeruli (115%), and sciatic nerve (351%). The highest concentration of AGE residues in rat tissues was found for the hydroimidazolones; MG-H1 and 3DG-H residues were found generally at much higher concentration than G-H1. There were tissue-specific increases in hydroimidazolone residue contents of diabetic rats: G-H1 residue content was increased in the retina (152%) and sciatic nerve (136%); MG-H1 residue content was increased in renal glomeruli (195%), retina (279%), and sciatic nerve (111%); and 3DG-H was increased in renal glomeruli (51%), retina (110%), and sciatic

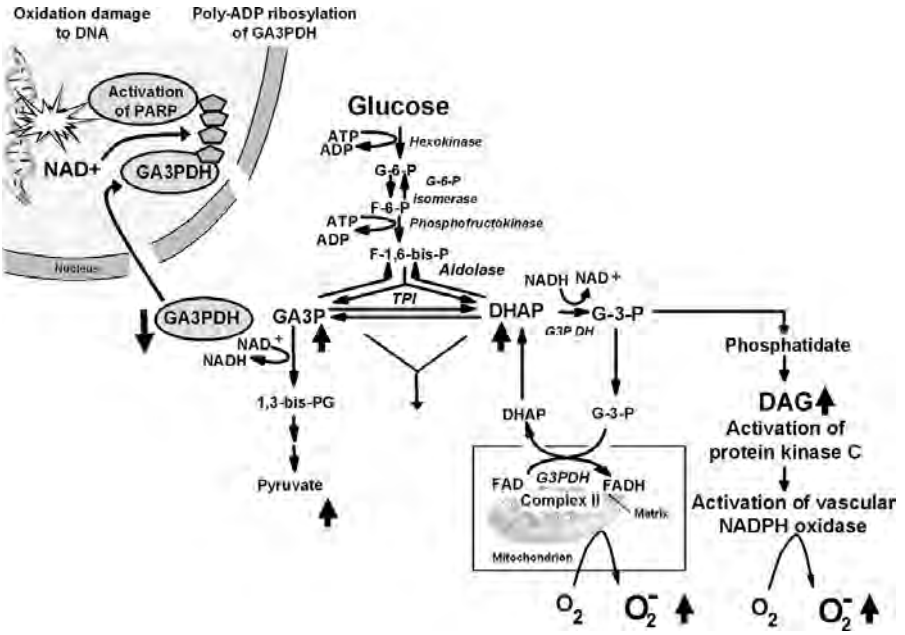


FIGURE 22.6 Mechanism of increased formation of methylglyoxal, mitochondrial dysfunction, and activation of NADPH oxidase by depletion of cytosolic glyceral dehyde-3-phosphate dehydrogenase in response to DNA damage.

nerve (50%). Surprisingly, there was no significant change in MetSO residue concentration in diabetic rats, although some tissues had high levels of MetSO residues in the normal control rats—particularly, the retina and nerve (Thornalley et al., 2003) (Table 22.3). Mild oxidative stress was present in the diabetic rats—plasma protein thiol concentration was decreased by 31%, with respect to controls (Babaei-Jadidi et al., 2003), but it was not sufficient to overwhelm MetSO reductase activity. Therapeutic intervention with high-dose thiamine and Benfotiamine decreased the AGE accumulation by reversing multiple pathways of biochemical dysfunction—leading to decreased α-oxoaldehyde concentrations—and prevented diabetic nephropathy and retinopathy without change in FL (Babaei-Jadidi et al., 2003; Hammes et al., 2003). Overall, the quantitative screening of glycation adducts in STZ diabetic rats was consistent with a role of advanced glycation in the development of vascular complications of diabetes.

22.3.3 Protein Glycation Adducts as Markers of Glycemic Control in Clinical Diabetes

Glycated plasma protein and glycated hemoglobin HbA_{1c} measurements in clinical diabetes reflect glycemic control in the 15 to 30 days and 60 to 120 days prior

TABLE 22.3 Advanced Glycation End-Products in Tissues, Blood Plasma, and Urine of Control and Streptozotocin-Induced Diabetic Rats

Analyte	Group	Renal Glomeruli	Retina	Sciatic Nerve	Skeletal Muscle	Plasma Protein
CML	Control	0.269 ± 0.111	0.172 ± 0.051	0.151 ± 0.087	0.188 ± 0.093	0.033 ± 0.004
(mmol/mol lys)	Diabetic	0.501 ± 0.186*	0.451 ± 0.291*	0.437 ± 0.077**	NS	0.062 ± 0.008***
CEL	Control	0.329 ± 0.102	0.339 ± 0.091	0.115 ± 0.069	0.050 ± 0.026	0.008 ± 0.003
(mmol/mol lys)	Diabetic	0.706 ± 0.047***	NS	0.519 ± 0.286**	0.102 ± 0.042*	0.017 ± 0.006**
G-H1	Control	0.044 ± 0.029	0.552 ± 0.103	0.517 ± 0.238	0.119 ± 0.061	0.275 ± 0.041
(mmol/mol arg)	Diabetic	NS	1.39 ± 0.89**	1.22 ± 0.55*	NS	0.565 ± 0.206**
MG-H1	Control	2.30 ± 0.25	1.88 ± 0.51	4.75 ± 2.74	1.70 ± 0.77	1.45 ± 0.39
(mmol/mol arg)	Diabetic	6.79 ± 0.19***	5.24 ± 2.34***	10.03 ± 0.66**	NS	2.24 ± 0.38**
3DG-H	Control	3.23 ± 0.90	0.20 ± 0.09	2.85 ± 1.24	2.11 ± 1.00	2.26 ± 0.89
(mmol/mol arg)	Diabetic	4.87 ± 0.32**	0.42 ± 0.15*	5.73 ± 0.72**	NS	NS
FL	Control	0.233 ± 0.015	0.72 ± 0.21	0.49 ± 0.09	0.515 ± 0.290	1.77 ± 0.36
(mmol/mol lys)	Diabetic	0.974 ± 0.098***	2.59 ± 1.23**	3.69 ± 0.72***	0.962 ± 0.305*	7.35 ± 1.59***

Source: Data from Thornalley et al. (2003).

Note: Significance: *, **, *** and NS indicate $p < 0.05$, < 0.01, < 0.001, and > 0.05, respectively, with respect to normal healthy controls.

to blood sampling, respectively (Sacks et al., 2002). In a study of type 1 diabetic patients, we found the FL residue content of plasma protein was increased 172% and the glycated hemoglobin HbA_{1c} was increased 42%, with respect to normal controls. The lower increase of HbA_{1c} may be due to cellular repair of glycated hemoglobin by fructosamine 3-phosphokinase (Delpierre et al., 2004). In plasma, the FL residue of human serum albumin is mainly located at lys-525 (Iberg and Fluckiger, 2002), and in hemoglobin, glycated hemoglobin HbA_{1c} is a mixture of mainly N_{α}-valyl-fructosamine at β-val-1 (60%) and α-lys-61 (40%). HbA_{1c} is routinely determined by affinity or cation exchange chromatography where glycated subunit exchange leads to around a 2-fold underestimation of the percentage of glycated hemoglobin compared to mass spectrometric measurement (Zhang et al., 2001). Roberts and co-workers have validated the use of electrospray mass spectrometry for the analysis of glycated hemoglobin in assessment of glycemic control (Roberts et al., 2001). Similar approaches have been used by Lapolla and co-workers to measure AGEs in hemoglobin (Lapolla et al., 2004), but the LC-MS/MS technique has the advantage of unequivocal quantitation by reference to stable isotope substituted standards (Thornalley et al., 2003; Ahmed et al., 2005a). HbA_{1c} is typically increased from 5% of total hemoglobin in normal controls to less than or equal to 7% (good glycemic control), 7% to 10% (moderate control), and more than 10% (poor control). In contrast, there is typically no significant increase of fructosamine and glycated hemoglobin in pre-diabetic impaired glucose tolerance. Glycated plasma protein and HbA_{1c} are also modifiable risk factors for the development of vascular complications of diabetes, reflecting the link of diabetic complications to hyperglycemia (although the association is weaker for macrovascular complications than for microvascular complications) (Diabetes Control and Complications Trial Research Group, 1993; Stratton et al., 2002).

22.3.4 Protein Glycation, Oxidation, and Nitration Adducts and Protein Damage in Clinical Diabetes

Clinical diabetes differs from experimental diabetes in that control of hyperglycemia, hypertension, and dyslipidemia is improved by therapeutic interventions. Nevertheless, we discovered profound increases in protein glycation and oxidation free adducts in a study of type 1 diabetic patients with moderate glycemic control (HbA_{1c} 7.8%) (Ahmed et al., 2005a). CML residues in plasma protein and hemoglobin were not increased significantly. Indeed studies showing increases of CML residues have evaluated patients with poor glycemic control (HbA_{1c} up to 20%) (Schleicher et al., 1997); there was a positive correlation of CML residues in plasma protein and hemoglobin with HbA_{1c}. The remarkable finding was, however, that concentration of CML free adduct in plasma was increased 4-fold in diabetic patients and the urinary excretion increased 2-fold. The MG-H1 residues in plasma protein and hemoglobin were increased 3-fold and 31%, respectively—the lower increase in hemoglobin consistent with red blood cell glyoxalase I activity protecting against glycation, and the concentration of MG-H1 free adduct increased 10-fold and the urinary excretion increased

15-fold. These marked increases in glycation free adducts in clinical diabetes suggest that the diabetic state is associated with increased protein damage by glycation and increased proteolysis of glycated proteins (Fig. 22.7).

Protein oxidation markers are also typically changed in clinical diabetes: cysteine residue thiols in plasma protein are decreased typically by 10% to 20% (Jennings et al., 1991). We determined the MetSO residue contents in plasma protein and hemoglobin, which were increased by 4-fold and 47%, respectively. Similarly there was a 2-fold increase in NFK residue content in plasma protein and no significant increase of NFK residue content in hemoglobin, reflecting the increased oxidative environment of plasma and the mainly preserved reducing environment of red blood cells in diabetes. In plasma, there was a 5-fold increase in MetSO free adduct and 4-fold, 6-fold, and 2-fold increases in urinary excretion of MetSO, NFK, and dityrosine free adducts, although the absolute levels of MetSO excretion were small because of efficient reduction by renal MetSO

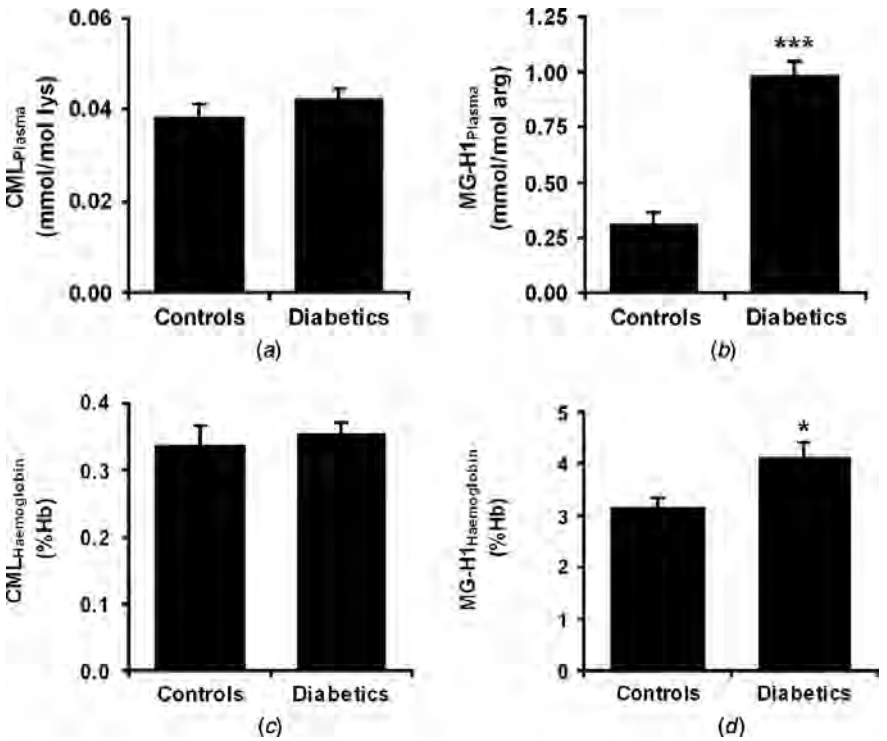


FIGURE 22.7 Effect of moderately controlled type 1 diabetes on the concentration of advanced glycation end-product residues and free adducts in plasma, red blood cells, and urine. Concentrations of CML and MG-H1 residues in plasma protein (*a, b*) and hemoglobin (*c*, *d*). Concentrations of CML and MG-H1 free adducts in plasma (*e*, *f*) Urinary excretion of CML and MG-H1 free adducts (*g*, *h*). Data are mean ± SD. From Ahmed et al. (2005a).

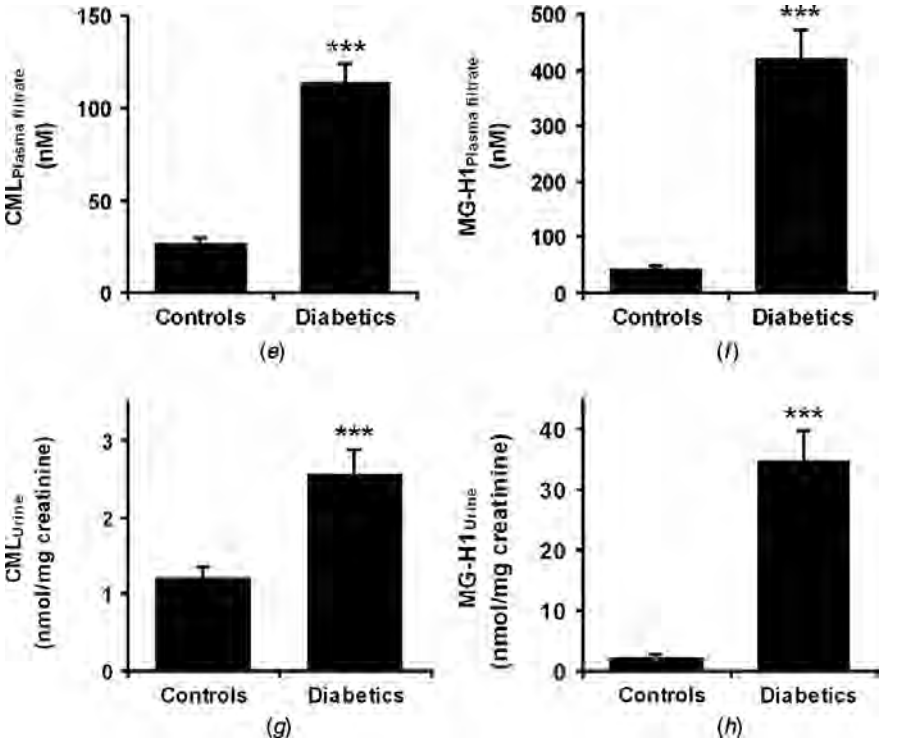


FIGURE 22.7 (*continued*)

reductase. These marked increases in oxidation adduct residues and free adducts in clinical diabetes suggest that the diabetic state is associated with increased oxidative damage by glycation and increased proteolysis of proteins damaged oxidatively.

Nitration of plasma protein has been claimed to be a marker of oxidative stress in clinical diabetes, but immunoassay measurements with commercial ELISA often overestimate the level of 3-NT residues by 10- to 50-fold (Ceriello et al., 2002; Gaut et al., 2002). Our estimate by LC-MS/MS of 3-NT residue content in plasma protein of control subjects was 0.0006 mmol/mol tyr, similar to those of artifact free detection (Gaut et al., 2002). This was increased 2-fold in diabetes (Ahmed et al., 2005a). For the first time we found 3-NT residues in hemoglobin (0.029% hemoglobin), which surprisingly decreased in diabetic patients. The decrease of 3-NT residue concentration in hemoglobin and increase in plasma protein of diabetic subjects, with respect to controls, may relate to the compartmentalization of hemoglobin and peroxynitrite formation. Peroxynitrite forms inside RBCs with nitric oxide (NO) originating outside RBCs and superoxide originating from intra- and/or extra-RBC sources. Increased peroxynitrite formation in diabetes is linked to the increased production of superoxide

in vascular cells (Soriano et al., 2001). As NO crosses from the endothelium through plasma, it is intercepted by superoxide with increased efficiency in diabetes, increasing the formation of peroxynitrite and 3-NT in plasma proteins and matrix proteins (Soriano et al., 2001). Less NO then enters RBCs to form 3-NT in hemoglobin. The effects of peroxynitrite, as well as NO (Gladwin et al., 2003), are compartmentalized in blood physiologically. A similar effect of compartmentalized formation of pentosidine precursor may explain the decreased pentosidine residues of hemoglobin in diabetes (Fig. 22.8).

22.3.5 Protein Damage in the Diabetic State and Future Therapeutics

Overall, the diabetic state is associated with increases in protein damage by glycation and oxidation with modest increase in 3-NT. Glycation damage was particularly marked in renal glomeruli, retina, and peripheral nerve in experimental diabetes. Increases in MG-H1 free adduct concentration in plasma and

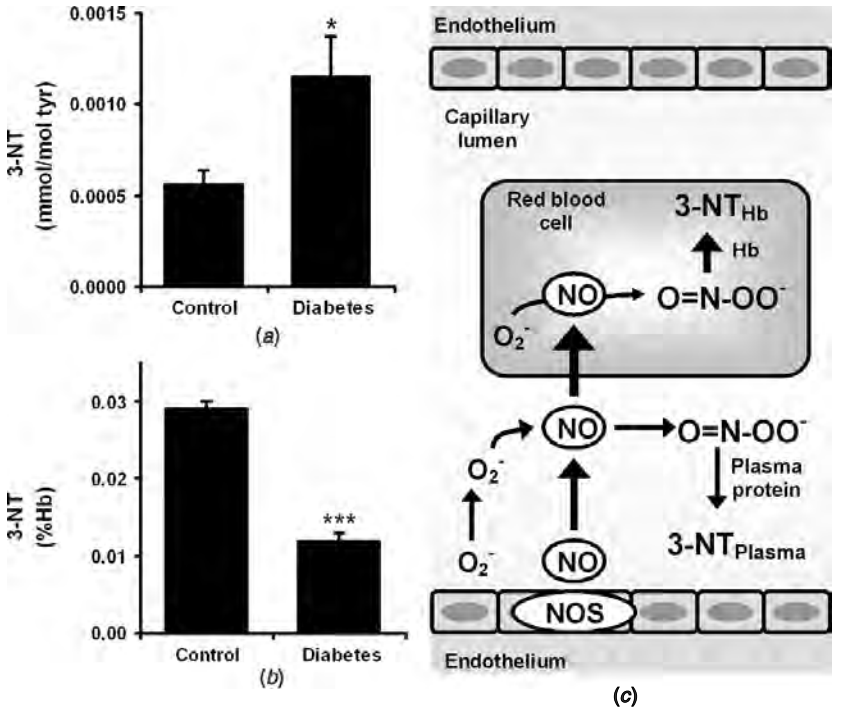


FIGURE 22.8 Evidence for compartmentalization of the formation of 3-nitrotyrosine in blood and effect of diabetes. (*a*, *b*) Concentration of 3-NT residues in plasma protein and hemoglobin, respectively. Data are Mean ± SEM. Significance: * and ***; $p < 0.05$ and $p < 0.001$ with respect to control subjects (t-test). (*c*) Schematic diagram showing a cross section through a blood capillary lumen illustrating the flows of nitric oxide from the endothelium into the red blood cells and formation of peroxynitrite and 3-NT-residues in plasma protein and hemoglobin. From Ahmed et al. (2005a).

urinary were particularly profound in clinical diabetes. Increased protein glycation and oxidative damage is linked to the development of diabetic complications (McCance et al., 1993; McLellan et al., 1994; Brownlee, 2001). Intensive control of blood glucose, blood pressure, and dyslipidemia may partly correct this damage. The emerging strategy of high-dose thiamine therapy was also effective in decreasing α-oxoaldehyde concentrations and associated proteins glycation (Babaei-Jadidi et al., 2003, 2004; Hammes et al., 2003).

22.4 PROFOUND MISHANDLING OF GLYCATED, OXIDIZED, AND NITRATED AMINO ACIDS IN UREMIA

Protein glycation, oxidation, and nitration free adducts released from proteins by cellular proteolysis and absorbed from food are normally excreted efficiently in the urine. They are a class of uremic toxic. With a moderate decline in renal function (without the need to renal replacement therapy or dialysis), there is decreased renal clearance of glycation and oxidation free adducts such that their plasma concentrations increase without increase in 24 hour urinary excretion (Fig. 22.9*a,b*). With further decline in renal function to end-stage renal disease (ESRD) and implementation of hemodialysis or peritoneal dialysis therapy, high concentrations of protein glycation, oxidation, and nitration free adducts are maintained or increased further. In ESRD patients, plasma glycation free adducts were increased up to 18- fold on peritoneal dialysis (PD) and up to 40-fold on hemodialysis (HD). Glycation free adduct concentrations in peritoneal dialysate increased over 2 to 12 hours dwell time, exceeding the plasma levels markedly (Fig. 22.9*c, d*), suggesting that there may be formation of protein glycation and oxidation adducts in the peritoneal cavity and active secretion of these adducts across the capillary endothelium into the peritoneal cavity during a dialysis dwell time. The high concentrations of glucose osmolyte (74–214 mM) and α-oxoaldehydes formed during heat sterilization (up to 100–200 μM) sustain increased glycation in the peritoneal cavity (Friedlander et al., 1996; Nakamura et al., 2001; Honda et al., 1999; Owen et al., 1993; Mortier et al., 2004). Glycation adduct formation in the peritoneal cavity was decreased by use of low α-oxoaldehyde-containing PD fluid (Mortier et al., 2004). Plasma glycation free adducts equilibrated rapidly with dialysate of HD patients, with both plasma and dialysate concentrations decreasing during a 4 hour dialysis session (Figs. 22.9*e, f*). Protein glycation, oxidation, and nitration adduct residues increase in uremia as a consequence of increased concentrations of α-oxoaldehyde glycating agents and oxidative stress associated with the inflammatory response to uremic toxins and interaction with dialysis membranes and PD fluids. Changes in the turnover and plasma concentration of albumin may also affect the content of glycation, oxidation, and nitration adduct residues in plasma protein in ESRD patients. Although protein glycation, oxidation, and nitration free adducts are eliminated by dialysis, the plasma concentrations of the inflammatory mediator monocyte chemotactic protein-1 and transforming growth factor-β were maintained by dialysis therapy. The inflammation of dialysis and associated increased

glycation, oxidation, and nitration may be linked, but there was no immediate benefit of clearance of protein glycation, oxidation, and nitration free adducts in a dialysis session. As practiced currently, however, neither PD nor HD normalize the plasma concentrations of protein glycation, oxidation, and nitration free adducts. PD fluids with improved biocompatibility and increased frequency of hemodialysis with higher flux may improve the elimination of glycation free adducts.

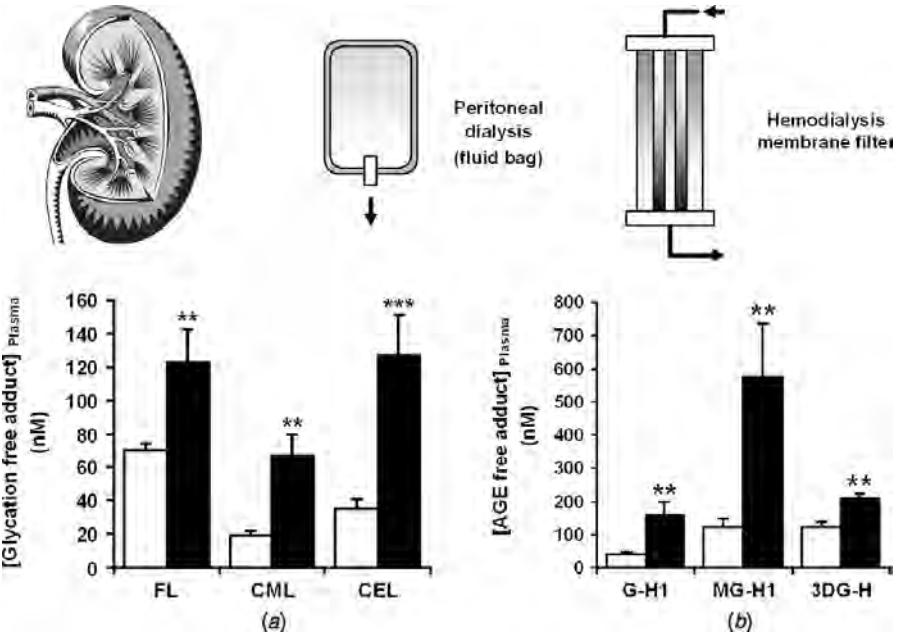


FIGURE 22.9 Accumulation of protein glycation free adducts in chronic renal failure and end-stage renal disease and their elimination by peritoneal dialysis and hemodialysis. (*a*, *b*) Concentrations of glycation free adducts in control subjects (white bars, $n = 7$; plasma creatinine $= 98 \pm 16$ μM) and chronic renal failure subjects (black bars, $n = 7$; plasma creatinine $= 137 \pm 62$ μM). Concentrations of glycation free adducts in peritoneal dialysate and effect of dialysate dwell time in the peritoneal cavity: (*c*) CML and (*d*) MG-H1 ($n = 8$; plasma creatinine $= 639 \pm 130$ μM). Concentrations of glycation free adducts in plasma and hemodialysate before and after a 2 hour hemodialysis session. (*e*) CML and (*f*) MG-H1. Key: Con, control; P(0) and HD(0), plasma and hemodialysate at the start of the dialysis session; P(4) and HD(4), plasma and hemodialysate at the end of the dialysis session (ESRD patients: $n = 8$; plasma creatinine $= 881 \pm 194$ μM and 347 ± 111 μM before and after dialysis). *Symbols*: *, significance with respect to control plasma (plasma samples only); ∘, significance with respect to before dialysis (plasma and dialysate); and +, significance of dialysate sample with respect to corresponding plasma. One, two, and three symbols represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. From Agalou et al. (2005).

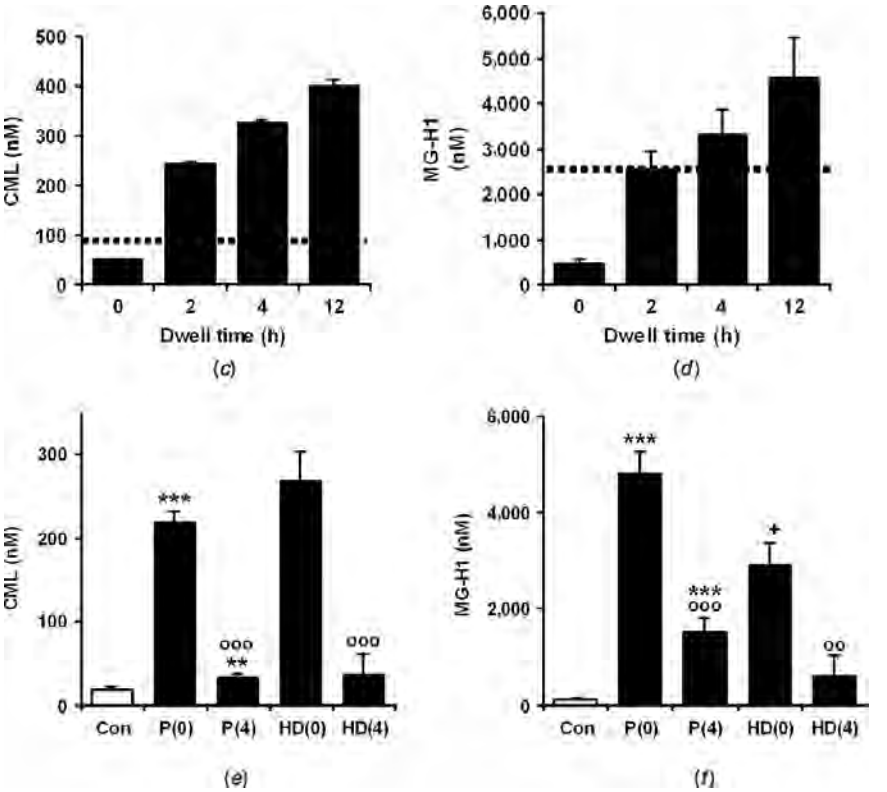


FIGURE 22.9 (*continued*)

22.5 INCREASED GLYCATED AND OXIDIZED AMINO ACIDS OF BLOOD PLASMA IN LIVER CIRRHOSIS—A SIGNATURE OF HEPATIC OXIDATIVE STRESS

22.5.1 Cirrhosis

Cirrhosis is a family of chronic liver diseases characterized by a severe decline in liver function, fibrosis, and nodular regeneration leading to complications of liver failure, portal hypertension, and hepatoma. Major causes of cirrhosis are chronic alcohol abuse (50%) and hepatitis C infection (40%). Infection with hepatitis B and D are common causes of cirrhosis globally (Burroughs and McNamara, 2003; Lavanchy, 2004; Hutin et al., 2004; Pouletty, 2002). In the early stages of cirrhosis, pro-inflammatory processes are associated with hepatic oxidative stress and lipid peroxidation, leading to protein modification by malondialdehyde and other carbonyl compounds (Willis et al., 2002; Kharbanda et al., 2001; Sebekova et al., 2002; Bianchi et al., 1997; Paradis et al., 1997). This is associated with increased proteasomal activity with the expectation that free adducts derived

from aldehyde-modified protein will be released into the systemic circulation (Fataccioli et al., 1999).

22.5.2 Increased Protein Glycation, Oxidation, and Nitration Adduct Residues and Free Adducts of Plasma in Cirrhosis

In recent studies we determined the concentrations of protein glycation, oxidation, and nitration adduct residues and free adducts in plasma of cirrhotic patients (Ahmed et al., 2004b). Blood samples were collected from cirrhotic patients (undergoing the transjugular intrahepatic portosystemic shunt procedure) and normal healthy controls. The concentrations of the major glycation adduct residues, FL, G-H1, MG-H1, 3DG-H, and CML in plasma protein were increased in cirrhotic subjects, confirming a previous report of increased CML residues determined by immunoassay (Sebekova et al., 2002). The reason for this is probably a decrease in the rate of albumin degradation associated with decreased plasma albumin concentration in cirrhosis (Sterling, 1951), although glycation may be increased in cirrhosis by impairment of the enzymatic defense against glycation in oxidative stress (Thornalley, 2003b). The concentrations of CML, CEL, MetSO, and 3-NT residues in plasma protein were increased with increasing severity of cirrhosis, as assessed by the Child-Pugh index (Fig. 22.10*a*).

In both portal and hepatic venous plasma of cirrhotic subjects, there was a remarkable 14- to 16-fold increase of glyoxal-derived hydroimidazolone G-H1 free adduct, with respect to hepatic and peripheral venous plasma in normal controls (Fig. 22.10*b*). Increased formation of G-H1 probably arises from increased glycation of proteins by glyoxal followed by proteolysis. In cirrhosis, increased formation of glyoxal may be associated with lipid peroxidation stimulated in oxidative stress-associated pro-inflammatory responses in the liver (Willis et al., 2002; Kharbanda et al., 2001) and possibly a previously unrecognized metabolite of ethanol (Terelius et al., 2003). Other hydroimidazolone free adducts were also increased in cirrhosis less markedly (MG-H1, +58%; 3DG-H, 2- to 3-fold). MetSO free adduct was increased 75% to 104%. The high levels of G-H1 free adduct may therefore be a "signature" of severe lipid peroxidation, protein modification, and increased proteasomal activity in the cirrhotic liver. Therapeutic strategies to decrease protein glycation, particularly by glyoxal, may be beneficial.

22.5.3 Countering Glycation in Cirrhosis

Protein glycation by glyoxal may be prevented by scavenging glyoxal with a therapeutic agent such as aminoguanidine (Thornalley, 2003c), increasing the detoxification of glyoxal by glyoxalase I by thiol antioxidants to increase the concentration of GSH—the cofactor of glyoxalase I (Thornalley, 2003a)—and preventing glyoxal formation by maintaining cellular reducing status. The latter effect is probably achieved by high-dose therapy with thiamine (Babaei-Jadidi et al., 2003).

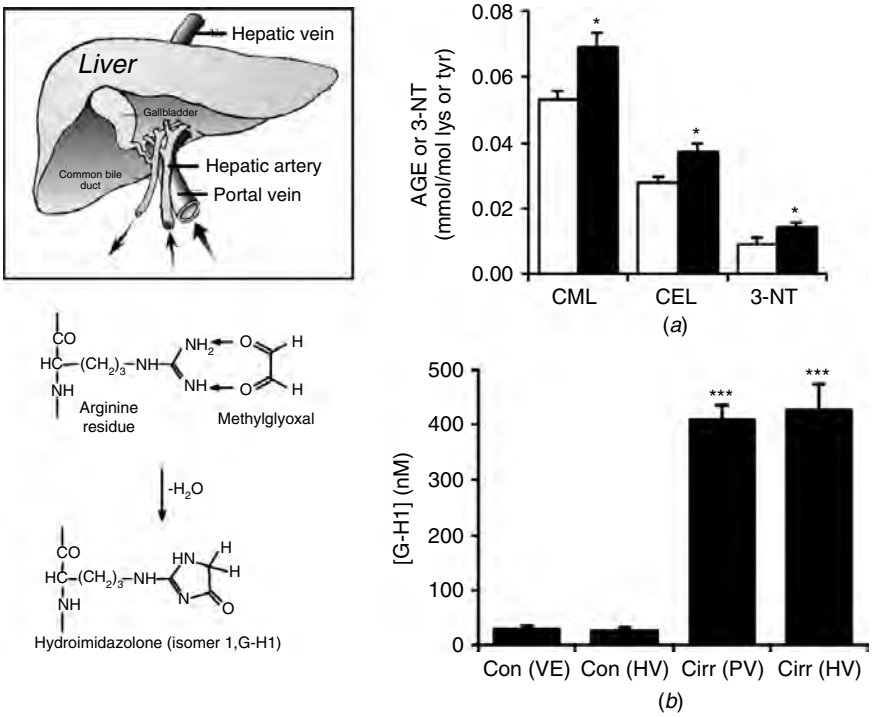


FIGURE 22.10 Increased glyoxal hydroimidazolone G-H1 free adduct in blood plasma of subjects with cirrhosis and association of protein damage with disease severity. (*a*) Increased plasma protein AGE and nitration adduct residues with increased severity of cirrhosis. *Key*: White bars, Child-Pugh index A (mild cirrhosis); black bars, B & C (moderate and severe cirrhosis). (*b*) Increased glyoxal-derived hydroimidazolone G-H1 free adduct in cirrhosis. Concentration of G-H1 free adduct in plasma of peripheral venous plasma and hepatic venous plasma of subjects with normal liver function and portal and hepatic venous plasma of cirrhotic patients. *Key*: Con, subjects with normal liver function; Cirr, patients with cirrhosis; VE, peripheral venous plasma; PV, portal venous plasma; HV, hepatic venous plasma. *Significance*: *** indicates $P < 0.001$, with respect to normal controls. Data are mean ± SEM. From Ahmed et al. (2004b).

Aminoguanidine is a scavenger of glyoxal, methylglyoxal, and 3-deoxyglucosone (Thornalley et al., 2000b). It decreased the severity of damage and improved survival in a thioacetamide-induced model of acute hepatic failure in the rat (Rahman and Hodgson, 2003), an effect that may be related to both its antiglycation activity and its potent inhibition of inducible nitric oxide synthase (Thornalley, 2003c). The thiol antioxidant *N*-acetylcysteine gave some improvement in the treatment of hepatitis (Le Moine et al., 2000). High-dose thiamine therapy decreased the concentrations of glyoxal, methylglyoxal, and 3-deoxyglucosone in experimental diabetes, probably by suppressing the dicarbonyl formation and maintaining high activities of dicarbonyl metabolizing enzymes

(Babaei-Jadidi et al., 2003). Thiamine deficiency is prevalent in alcoholic and nonalcoholic cirrhosis (Levy et al., 2002), and thiamine repletion decreases cirrhotic hyperglycemia (Hassan et al., 1991). Hyperglycemia in cirrhosis is linked to insulin resistance of muscle and an inadequate response of the β-cells to appropriately secrete insulin (Petrides et al., 1994). Our study of increased glycation adducts in cirrhosis may provide support for thiamine supplementation in cirrhosis, regardless of the cause (Levy et al., 2002), to decrease hyperglycemia and AGE formation.

22.6 INCREASED METHYLGLYOXAL-DERIVED HYDROIMIDAZOLONE AND 3-NITROTYROSINE FREE ADDUCTS IN CEREBROSPINAL FLUID OF SUBJECTS WITH ALZHEIMER'S DISEASE—A SIGNATURE OF NEURONAL DAMAGE

Alzheimer's disease (AD) is a leading cause of dementia in old age. Apart from specific genetic factors in some cases of AD [mutations of amyloid precursor protein (APP); presenilin 1; and presenilin 2 genes—accounting for nearly 5% of AD; and the apoE4 allele], advancing age is overwhelmingly the most important known risk factor for AD (Casserly and Topol, 2004). The brains of subjects with AD have two characteristic proteinaceous lesions: extracellular amyloid or senile plaques (SPs) and intracellular neurofibrillary tangles (NFTs) of hyperphosphorylated tau-protein (P-tau) in the cerebral cortex and hippocampus. SPs contain insoluble aggregates of small toxic cleavage products of APP, amyloid-beta (Aβ), Aβ40, and Aβ42. Plaque density does not correlate well with the severity of dementia, and although the density of NFTs does, they emerge relatively late in the development of AD. Focus is now turning to early stage damage of neuronal synapses and dendritic cells in which the toxicity of small soluble protofibrils and oligomers of Aβ are implicated (Bossy-Wetzel et al., 2004). It is still unclear how Aβ causes toxicity. It may be through binding to receptors on neuronal and glial cells: the α7-nicotinic acetylcholine receptor, neurotrophin p75 receptor, the *N*-methyl-D-aspartate receptor, the receptor for advanced glycation end-products (RAGE), and others (Verdier and Penke, 2004). Increased production of ROS and peroxynitrite, catalyzed by redox active metal ions and inducing oxidative and nitrosative stress, is also involved, leading to oxidation and nitration of proteins (Smith et al., 1996, 1997). Protein glycation is also increased by potentiation of oxidative glycation processes in oxidative stress and increased accumulation of potent dicarbonyl glycating agents (Smith et al., 1994). Protein damage by glycation, oxidation, and nitration induces misfolding and proteasomal degradation, liberating corresponding free adducts. As neuronal cell damage and glial cell activation increase with progression of AD, increasing concentrations of these modified amino acids may drain in the CSF for eventual renal excretion (Fig. 22.11*a*). Increased concentrations of glycating, oxidizing, and nitrating agents in neuronal tissue of AD subjects may leak into the CSF and increase glycation, oxidation, and nitration of CSF proteins. Quantitative screening of protein glycation, oxidation, and nitration adducts in the CSF may provide

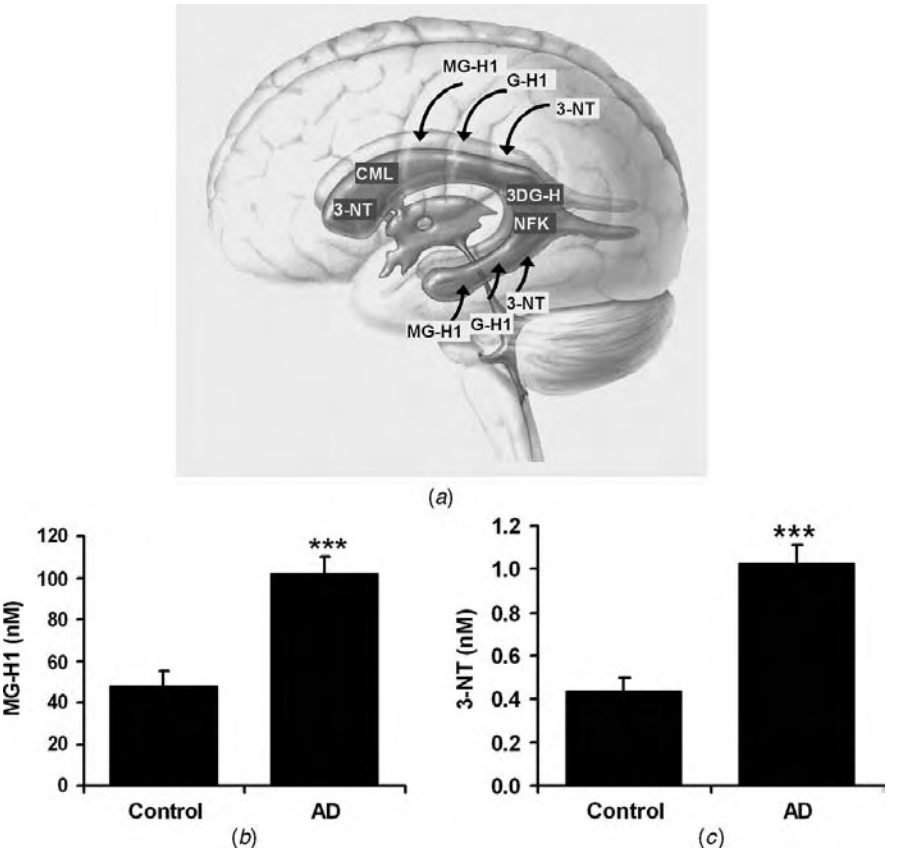


FIGURE 22.11 Protein glycation, oxidation, and nitration residues and free adducts increased in Alzheimer's disease. (*a*) Proposed leaking of protein glycation, oxidation, and nitration free adducts from damaged neurones into the ventricles and formation of adduct residues in CSF protein. Cutaway view of the brain showing ventricles containing CSF. *Abbreviations key*: Black text/white background, protein glycation, oxidation, and nitration free adducts; white text/dark gray background, protein glycation, oxidation, and nitration residues in CSF protein in the ventricles. Protein glycation and nitration free adducts in CSF of subjects with Alzheimer's disease and normal healthy controls and correlation with cognitive impairment. (*b*) MG-H1 and (*c*) 3-NT. Data are mean ± SEM. From Ahmed et al. (2004a).

a novel diagnostic indicators for AD and a signature of protein damage in the cortex and hippocampus.

3-NT and dityrosine has been studied in CSF for diagnostic utility. Increased 3-NT and dityrosine residues in postmortem brain tissue were found in several neurological disorders other than AD including Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis (Good et al., 1998; Cross et al., 1998; Sasaki et al., 2000). The quantitative levels of 3-NT adduct of the CSF in these

diseases are uncertain and remain controversial with suspected overestimates reported (Tohgi et al., 1999; Hensley et al., 1998; Ryberg et al., 2004; Calabrese et al., 2002).

Quantitative screening of glycation, oxidation, and nitration adducts in CSF by LC-MS/MS showed increased concentrations of 3-NT, CML, 3DG-H, and NFK residues in CSF protein of AD patients and increased concentrations of 3-NT, MG-H1, and G-H1 hydroimidazolone free adducts. The Mini-Mental State Examination (MMSE) score correlated negatively with 3-NT residue concentration. Multiple linear regression gave a negative regression model of MMSE score on 3-NT, FL, and CEL residues (Ahmed et al., 2004a). The increased 3-NT residues and free adducts in CSF of AD subjects probably reflect increased leakage of protein-nitrating agents and increased protein nitration in brain tissue, respectively (Good et al., 1996; Smith et al., 1997; Luth et al., 2002). Several targets of protein nitration in AD have been identified (Castegna et al., 2003). Low levels of 3-NT residues and free adducts are expected because the protein nitrating agents react preferentially with other amino acid residues—cysteine, methionine, tryptophan, and FL residues (Quijano et al., 1997; Pryor et al., 1994; Murray et al., 2003; Nagai et al., 2002).

The concentrations of 3-NT and MG-H1 free adducts were increased with highest significance in AD patients ($p < 0.001$) (Fig. 22.11*b, c*). These free adducts are candidate indicators for AD diagnosis appropriate for further study. MG-H1 and G-H1 residue and free adduct concentrations reflect protein glycation by methylglyoxal and glyoxal, respectively (Thornalley, 1999). These dicarbonyls are substrates of glyoxalase I, which was found recently to have increased expression in postmortem brain tissue of subjects with AD (Chen et al., 2004). Increased glyoxalase I expression was associated previously with induction by chronic exposure clinically or pharmacologically to high levels of methylglyoxal and glyoxal (McLellan et al., 1994; Ueno et al., 1991). Neuronal dysfunction in AD subjects may lead to chronic exposure to increased levels of these dicarbonyls. Hence leakage of increased levels of MG-H1 and G-H1 free adducts in CSF is expected. It was reported that CML residue concentration in CSF protein (determined by competitive ELISA) was increased in AD and not in vascular dementia, and the concentration of pentosidine residues was increased in vascular dementia but not in AD (Bar et al., 2003). We confirmed the increase in CML residue concentration in CSF protein of AD subjects but CML residues were overestimated 4-fold and pentosidine 2-fold in this previous report (Ahmed et al., 2004a).

We found a significant negative correlation of MMSE score with FL residues (Ahmed et al., 2004a). A previous study using immunoassay to determine fructosamines (predominantly FL residues) suggested FL residues increased with increasing severity of AD (Shuvaev et al., 2001). Increased formation of FL residues in AD is not expected, since there is no increase in the glucose concentration in the CSF. A probable explanation for increased FL residues with increasing severity of AD is decreased turnover of CSF protein. The production of CSF decreases with subject age and declines further in subjects with AD (May

et al., 1990; Silverberg et al., 2001). The decline in cognitive function in AD subjects was linked to FL residue concentration (a surrogate indicator of CSF protein turnover), protein nitration, and protein glycation by methylglyoxal. Interpolation on regression of MMSE on the [FL] × [3-NT] × [CEL] variable for the normal controls (mean product variable = 0.000893) gave a predicted MMSE of 23. This was similar to the standard cutoff MMSE score of nearly less than 24, which was insensitive (63%) but had a good specificity (96%) for dementia in a population with cognitive complaints (Kukull et al., 1994). This variable is a novel biochemical indicator of cognitive function in AD. These findings indicate that protein glycation, oxidation, and nitration adduct residues and free adducts were increased in CSF of subjects with Alzheimer's disease. A combination of nitration and glycation adduct estimates of CSF may provide an indicator for the diagnosis of Alzheimer's disease.

22.6.1 Importance of the Early Diagnosis of Alzheimer's Disease

The impact of AD may be decreased by the use of existing and emerging drugs (acetylcholinesterase inhibitors, statins, β-sheet breakers, β- and γ-secretase inhibitors, anti-amyloid vaccine, anti-inflammatory drugs, etc.) (Mattson, 2004). The greatest therapeutic advantage will be gained, however, if the earliest stages of dementia, pre-dementia or mild cognitive impairment (MCI) can be detected and therapy commenced promptly (Blennow, 2004b; Nestor et al., 2004). Advances in neuropsychological and neuroimaging techniques have improved diagnosis of AD, but they are unreliable in MCI (Nestor et al., 2004). Assay of biomarkers in CSF is a current investigational strategy for diagnosis of AD. Most effort focuses on decreased levels of Aβ42 and increased levels of P-tau and total tau protein as an indicator of AD. CSF Aβ42 and total tau protein combination measurements gave good clinical specificity for distinguishing AD from nondemented, depressed, and Parkinson's disease subjects but poor specificity against vascular dementia and dementia with Lewy bodies (Blennow, 2004a). Variables linked to protein nitration, oxidation, and glycation adducts in CSF may be valuable clinical indicators for the early diagnosis and assessment of progression of AD.

22.7 GLYCATION ADDUCTS IN FOOD AND BEVERAGES

Saccharide-rich foods are a source of FL and AGE residues and free adducts, particularly, bakery products with up to 2 mmol AGE consumed per day (Henle, 2003). The presence of glycation adducts contributes to flavor, antioxidant, and antimutagenic effects (Chuyen, 2001). There are major glycation adducts in food that are similar to, and others different from, those found widespread in human tissues. Distinctive glycation adducts in food are found where atypical saccharide substrates and high-temperature processing are involved in the glycation process (Fig. 22.12).

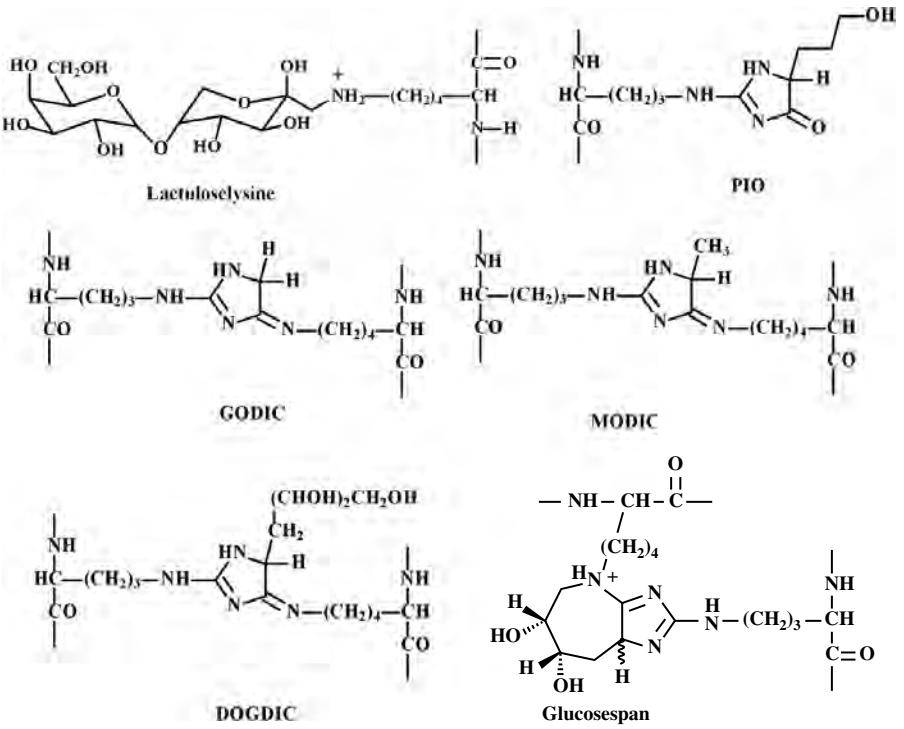


FIGURE 22.12 Glycation adduct residues found in food.

FL and related products of the Amadori rearrangement, collectively called Amadori products, have been quantified in food by acid hydrolysis and conversion to furosine, or by chromatographic detection of individual Amadori products. N_{ε}-Lactuloselysine residues were a major type of Amadori product residue formed from lactose in bovine milk (Henle et al., 1991). In raw milk the concentration of Amadori products residues was 9.7 ± 0.2 mg furosine equivalents per 100 g protein, equivalent to approximately 30 μM Amadori products. The concentration of Amadori product residues increased 14-fold during sterilization (Baptista and Carvalho, 2004). Electrospray and MALDI mass spectrometry proteomics studies showed that most Amadori product residues were on α s_1-casein and β-casein—major proteins of milk. The lysine residues modified preferentially therein were lys-7, lys-34, lys-83, lys-103, lys-105, lys-132, and lys-193 in α s_1-casein; and lys-32, lys-48, lys-107, lys-113, and lys-176 in β-casein (Scaloni et al., 2002). Assessment of metabolic transit sowed that 60% to 80% of ingested FL free adduct was excreted in urine and 3% to 10% of FL residues of ingested protein. The remainder was degraded by intestinal bacteria systemically after absorption or excreted in feces (Faist and Erbersdobler, 2001).

AGEs have been assayed in food and beverages by immunoassay procedures where the antibodies used detected CML and possibly other AGEs (Koschinsky

et al., 1997; Goldberg et al., 2004). In this application of AGE immunoassay, the sample matrix is markedly diverse from that usually employed in validation studies—plasma or serum protein. This may have led to unreliable assessments. For example, with LC-MS/MS, we were unable to corroborate a claim of high levels of AGEs in cola drinks suggested by AGE immunoassay (Koschinsky et al., 1997); we found only trace levels of AGE free adducts (Ahmed et al., 2005c). LC-MS/MS techniques have been employed in few studies only for analysis of food and beverages. AGE residues and AGE free adducts were analyzed in raw bovine milk and after pasteurization and sterilization. The CML residue content of protein in raw milk was 337 ± 94 nM and increased 3-fold during pasteurization and 6-fold during sterilization. The concentration of CML free adduct in raw milk was 147 ± 2 nM and increased 46% during pasteurization and 71% during sterilization. The CEL residue content of protein in raw milk was 662 ± 234 nM and increased 3-fold during pasteurization and sterilization. The concentration of CEL free adduct in raw milk was 147 ± 4 nM and was not increased significantly during pasteurization but was increased 14% during sterilization. The MG-H1 residue content of protein in raw milk was 765 ± 207 nM and increased 3-fold during pasteurization and sterilization. The concentration of MG-H1 free adduct in raw milk was 51 ± 5 nM and was not increased significantly during pasteurization but was decreased 85% during sterilization. Henle and co-workers detected a further hydroimidazolone derivative, N_{δ}-(5-(3-hydroxypropyl)imidazol-4-on-2-yl)ornithine (PIO) that may be a major arginine-derived AGE in milk (Mavric et al., 2004). The selective accumulation of AGE residues with only minor increases (or decrease) in AGE free adducts in milk during pasteurization and sterilization is probably due to the high concentration of arginine and lysine residues in milk protein, relative to the concentration of free arginine and lysine (Gabris and Duran, 1983), and to the formation of AGEs residues from the degradation of saccharide and lipid moieties attached to milk protein (Birlouez-Aragon et al., 2004).

MG-H1 residues were present in high concentration in bread crust, accounting for up to 20% to 30% of total arginine residues (Henle et al., 1994). Arginine- and lysine-derived cross-links formed by glycation of proteins by glyoxal, methylglyoxal, 3-deoxyglucosone, and glucose were also identified (Fig. 22.12): 2-ammonio-6-({2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4,5-dihydro-1*H*-imidazol-5-ylidene}amino)hexanoate(GODIC), 2-ammonio-6-({2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4-methyl-4,5-dihydro-1*H*-imidazol-5-ylidene}amino)hexanoate (MODIC), 2-ammonio-6-({2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4-(2,3,4-trihydroxybutyl)-4,5-dihydro-1*H*-imidazol-5-ylidene}amino) hexanoate (DOGDIC), and 2-ammonio-6-{2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo[4,5-b]azepin-4-yl}hexanoate (glucosespan) (Biemel et al., 2001). They were detected by LC-MS in bakery products and cooked egg white in the concentration range 7 to 151 mg/kg (Biemel et al., 2001). They were also detected in protein plasma and human lens where glucosespan is a major glycation cross-link (Biemel et al., 2002).

Diets containing high levels of AGEs gave increased urinary excretion of AGE free adducts. This has been found for both dietary-rich CML and pyrraline (Liardon et al., 1987; Foerster and Henle, 2003). Laboratory rodent food was a rich source of AGE residues. Measurement of glycation adducts in 24 hour urine samples of normal and diabetic rats indicated that $<10\%$ of glycation adduct residue consumption was excreted (Ahmed et al., 2005c). Low bioavailability diminishes the bioactivity of dietary AGEs and may be attributed to resistance to proteolysis of highly glycated proteins (Ahmed and Thornalley, 2002), glycation adduct inhibition of intestinal proteases (Oste et al., 1987), and pre-systemic metabolism by intestinal bacteria (Wynne et al., 2002). Positron emission tomography of 4-fluorobenzoyl derivatives of CML and CEL free adducts injected intravenously into rats were retained temporarily in the liver (Bergmann et al., 2001), but the derivatization probably affected the tissue partitioning of these AGEs significantly. Since AGE free adducts have high renal clearance and low plasma concentrations, AGEs absorbed from food are expected to have low toxicity in subjects with normal renal function.

Glycation adducts in food have been termed "glycotoxins" (Koschinsky et al., 1997). After ingestion of thermally processing food and beverages rich in AGEs, immunossay of AGEs indicated only a minor fraction (30%) of the ingested AGEs was excreted and the elimination of AGEs was decreased in diabetic nephropathy (Koschinsky et al., 1997). Extracts of dietary AGEs induced depletion of glutathione and induction of glutathione peroxidase in endothelial cells in vitro (Cai et al., 2002) and changed functional activity of low-density lipoprotein (Cai et al., 2004). Diets rich in glycotoxins were associated with increased inflammatory mediators in diabetic subjects (Vlassara et al., 2002), and in patients on PD and HD, serum CML correlated with CML content of ingested food (Uribarri et al., 2003). It is not clear currently, however, if the assay of AGE excretion in these studies was reliable, if incubation of vascular cells in culture with glycotoxins reflected a physiologically relevant glycation insult, and if it was possible to produce diets rich in AGEs experimentally without change in essential micronutrients (e.g., thermally labile vitamins). Studies with AGE-rich food prepared with radiolabeled glucose have shown absorbed radioactivity in rats with decreased tissue retention in the presence of the α-oxoaldehyde scavenger, aminoguanidine (He et al., 1999). The effects of aminoguanidine in these studies suggests, however, that α-oxoaldehyde compounds such as glyoxal, methylglyoxal or 3-deoxyglucosone, or precursors of them (glucose and fructose moieties), attached reversibly to ingested protein were absorbed from the diet rather than AGEs (Thornalley et al., 1999; Thornalley, 2003c). The importance of dietary precursors of AGEs, such as methylglyoxal, is diminished by the relative high endogenous formation in human tissues. The flux of methylglyoxal formation in human cells is high compared to the amount in ingested foodstuffs (Thornalley, 1988). The metabolic flux of methylglyoxal of a 70 kg human subject is estimated to be around 8.4 mmol/day. This is the same amount as in about 24 kg of toast or about 1440 liters of brewed coffee (Nagao et al., 1986; Hayashi and Shibamoto, 1985). Normal dietary intake of methylglyoxal therefore may represent a minor

contribution to the total body pool of this metabolite. High concentrations of exogenous saccharides and glycation adducts may pose a significant glycation challenge to tissues locally, however: dietary glycating agents and AGEs may activate and damage intestinal epithelial cells, leading to RAGE-activated inflammation, mutagenesis, and carcinogenesis (Zill et al., 2003; Zhang et al., 1993; Dragsted et al., 2002); and PD fluids containing relatively high concentrations of glucose and degradation products of glucose produced during heat sterilization (α-oxoaldehydes) posed a significant glycation challenge to the peritoneal membrane and mesothelium (Mortier et al., 2004).

AGE-rich foods may also have beneficial effects. Rats fed on diets rich in CML residues showed an induction of glutathione transferase (GST) activity in the kidney (86% increased activity) and fed on a diet rich in FL residues an induction of GST activity in intestinal mucosa (64% increased activity) (Wenzel et al., 2002). The expression of GST is regulated by the transcription factor nuclear factor-erythroid 2 p45-related factor-2 (nrf2) by binding to the antioxidant response element (ARE) of glutathione transferase gene promoter (Hayes et al., 2005). Lipid aldehydes are known to activate nrf2 and induce ARE-linked gene expression (Ishii et al., 2004). It is conceivable that some aldehydes formed in glycation, or glycation-stimulated lipid peroxidation, mediate the induction of GST. Similarly CML-modified albumin induced the expression of γ-glutamylcysteine ligase—also ARE-linked gene expression—and increased the cellular concentration of glutathione. These responses may explain, in part, the antimutagenic and antioxidant effects of some AGE-rich foods.

Research on "glycotoxins" has stimulated debate on the contribution of dietary AGEs to total AGE exposure. If endogenous formation of AGEs is relatively high, as in diabetes and ESRD subjects on dialysis (Ahmed et al., 2005a; Agalou et al., 2005), the contribution of exogenous dietary AGEs is probably relatively low. The contributions of AGEs absorbed from ingested food to total AGE exposure is likely to be greatest when the endogenous formation of AGEs is low and renal clearance is impaired—as in nondiabetic subjects with mild uremia. Hence in mild renal failure without dialysis but decreased renal clearance of AGE free adducts, the concentration of AGEs is increased (Agalou et al., 2005) and AGEs from food probably make their greatest impact to the total AGE exposure in this conditions. The toxicity may be mediated mainly by effects of AGE free adducts.

22.8 CONCLUDING REMARKS: PHYSIOLOGICAL FORMATION AND PROTEOLYTIC PROCESSING OF GLYCATED, OXIDIZED, AND NITRATED PROTEINS IN DISEASE PROCESSES—THE IMPORTANCE OF MEASURING "DAMAGE AND DEBRIS"

The proteome is under continual chemical attack by glucose and reactive dicarbonyl glycating agents, ROS, and reactive nitrogen species. This leads to the formation of protein glycation, oxidation, and nitration adduct residues—the damage to the proteome. Both cellular and extracellular proteins are susceptible, but degradation and replacement is more accessible to cellular proteins. The

dynamic nature of the turnover of cellular proteins implies, however, that protein damage may accumulate when the rate of cellular proteolysis is decreased. There are enzymatic defenses against glycation and oxidative damage and when these and/or proteasomal activity fail, viability declines or aging ensures (Thornalley, 2003a,b; Sitte et al., 2000). Sites of damage to the proteome that cause most harm are probably those susceptible to covalent cross-links and sites of protein–protein interaction, enzyme-substrate interaction, and protein-DNA interaction (for transcription factors). Cellular proteolysis releases protein glycation, oxidation, and nitration free adducts for excretion—the debris of protein damage. This may provide an indirect but accessible measurement in plasma and urine of proteome damage in remote, inaccessible human tissues and a "fingerprint" of tissue protein damage in disease. Little is known of the toxicity of protein glycation, oxidation, and nitration free adducts, but failure to excrete them, as occurs in uremia, may lead to toxicity (Deuther-Conrad et al., 2001; Yoshihara et al., 2001). We can now recognize that protein damage may produce adverse effects by at least four different mechanisms:

1. Activation of cell responses by the specific cell surface receptors—as for AGE receptors (Thornalley, 1998, 2004).
2. Impairment of protein–protein, enzyme–substrate, and protein–DNA interactions (Ahmed et al., 2005b).
3. Resistance to proteolysis of extracellular matrix proteins (Kuzuya et al., 2001).
4. Cellular responses induced by glycation, oxidation, and nitration free adducts.

ACKNOWLEDGMENTS

I thank the Wellcome Trust (UK), Juvenile Diabetes Research Foundation International (USA), Diabetes UK (UK), Cancer Research UK (UK), Baxter Healthcare (USA), and Food Standards Agency (UK) for support of my research.

LIST OF ABBREVIATIONS

Aβ, amyloid-beta
AD, Alzheimer's disease
AGEs, advanced glycation endproducts
APP, amyloid precursor protein
AQC, 6-aminoquinolyl-*N*-hydroxysuccimidylcarbamate
ARE, antioxidant response element
1,3-bis-PG, 1,3-bisphosphoglycerate
CEL, N_{ε}-carboxyethyl-lysine

CML, N_{ε}-carboxymethyl-lysine

CSF, cerebrospinal fluid

DAG, diacylglycerol

3DG-H, N_{δ}-(5-hydro-5-(2,3,4-trihydroxybutyl)-4-imidazolon-2-yl)ornithine and related structural isomers

DOGDIC, 2-ammonio-6-({2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4-(2,3,4-trihydroxybutyl)-4,5-dihydro-1*H*-imidazol-5-ylidene}amino) hexanoate

DOLD, 3-deoxyglucosone-derived bis(lysyl) cross-link

ESRD, end-stage renal disease

FL, N_{ε}-fructosyl-lysine

F-1,6-bis-P, fructose-1,6-bis-phosphate

GA3PDH, glyceraldehyde-3-phosphate dehydrogenase

G-H1, N_{δ}-(5-hydro-4-imidazolon-2-yl)ornithine

glucosespan, 2-ammonio-6-{2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-6,7-dihydroxy-4,5,6,7,8,8a-hexahydroimidazo[4,5-b]azepin-4-yl}hexanoate

GODIC, 2-ammonio-6-({2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4,5-dihydro-1*H*-imidazol-5-ylidene}amino)hexanoate

GOLD, glyoxal-derived bis(lysyl) cross-link

G6P, glucose-6-phosphate

G3P, glycerol-3-phosphate

GA3P, glyceraldehyde-3-phosphate

GSH, glutathione

GST, glutathione transferase

HD, hemodialysis

LC-MS/MS, liquid chromatography with tandem mass spectrometric detection

MCI, mild cognitive impairment

MetSO, methionine sulfoxide

MG-H1, N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine

MMSE, Mini–Mental State Examination

MODIC, 2-ammonio-6-({2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4-methyl-4,5-dihydro-1*H*-imidazol-5-ylidene}amino)hexanoate

MOLD, methylglyoxal-derived bis(lysyl) cross-links

NFTs, neurofibrillary tangles

NFK, *N*-formylkynurenine

nrf2, nuclear factor-erythroid 2 p45-related factor-2

3-NT, 3-nitrotyrosine

PARP, poly(ADP-ribose) polymerase

PD, peritoneal dialysis

PIO, N_{δ}-(5-(3-hydroxypropyl)imidazol-4-on-2-yl)ornithine

P-tau, hyperphosphorylated tau-protein

RAGE, receptor for advanced glycation end-products
RBCs, red blood cells
ROS, reactive oxygen species
SP, senile plaque
STZ, streptozotocin
TPI, triosephosphate isomerase

REFERENCES

Agalou S., Ahmed N., Babaei-Jadidi R., Dawnay A., Thornalley P.J. (2005). Profound mishandling of protein glycation degradation products in uremia and dialysis. *J Am Soc Nephrol* 16: 1471–1485.

Ahmed M.U., Brinkmann Frye E., Degenhardt T.P., Thorpe S.R., Baynes J.W. (1997). N^{ε}-(Carboxyethyl)lysine, a product of chemical modification of proteins by methylglyoxal, increases with age in human lens proteins. *Biochem J* 324: 565–570.

Ahmed N., Ahmed U., Thornalley P.J., Hager K., Fleischer G.A., Munch G. (2004a). Protein glycation, oxidation and nitration marker residues and free adducts of cerebrospinal fluid in Alzheimer's disease and link to cognitive impairment. *J Neurochem* 92: 255–263.

Ahmed N., Argirov O.K., Minhas H.S., Cordeiro C.A., Thornalley P.J. (2002). Assay of advanced glycation endproducts (AGEs): Surveying AGEs by chromatographic assay with derivatisation by aminoquinolyl-*N*-hydroxysuccimidyl-carbamate and application to N_{ε}-carboxymethyl-lysine- and N_{ε}-(1-carboxyethyl)lysine-modified albumin. *Biochem J* 364: 1–14.

Ahmed N., Babaei-Jadidi R., Howell S.K., Beisswenger P.J., Thornalley P.J. (2005a). Degradation products of proteins damaged by glycation, oxidation and nitration in clinical type 1 diabetes. *Diabetologia* 48: 1590–1603.

Ahmed N., Dobler D., Dean M., Thornalley P.J. (2005b). Peptide mapping identifies hotspot site of modification in human serum albumin by methylglyoxal involved in ligand binding and esterase activity. *J Biol Chem* 280: 5724–5732.

Ahmed N., Mirshekar-Syahkal B., Kennish L., Karachalias N., Babaei-Jadidi R., Thornalley P.J. (2005c). Assay of advanced glycation endproducts in selected beverages and food by liquid chromatography with tandem mass spectrometric detection. *Molec Nutrit Food Res* 49: 691–699.

Ahmed N., Thornalley P.J. (2002). Chromatographic assay of glycation adducts in human serum albumin glycated in vitro by derivatisation with aminoquinolyl-*N*-hydroxysuccimidyl-carbamate and intrinsic fluorescence. *Biochem J* 364: 15–24.

Ahmed N., Thornalley P.J., Dawczynski J., Franke S., Strobel J., Stein G., Haik G.M. Jr. (2003). Methylglyoxal-derived hydroimidazolone advanced glycation endproducts of human lens proteins. *Invest Ophthalmol Vis Sci* 44: 5287–5292.

Ahmed N., Thornalley P.J., Luthen R., Haussinger D., Sebekova K., Schinzel R., Voelker W., Heidland A. (2004b). Processing of protein glycation, oxidation and nitrosation adducts in the liver and the effect of cirrhosis. *J Hepatol* 41: 913–919.

Babaei-Jadidi R., Karachalias N., Ahmed N., Battah S., Thornalley P.J. (2003). Prevention of incipient diabetic nephropathy by high dose thiamine and Benfotiamine. *Diabetes* 52: 2110–2120.

Babaei-Jadidi R., Karachalias N., Kupich C., Ahmed N., Thornalley P.J. (2004). High dose thiamine therapy counters dyslipidaemia in streptozotocin-induced diabetic rats. *Diabetologia* 47: 2235–2246.

Baptista J.A.B., Carvalho R.C.B. (2004). Indirect determination of Amadori compounds in milk-based products by HPLC/ELSD/UV as an index of protein deterioration. *Food Res Internat* 37: 739–747.

Bar K.J., Franke S., Wenda B., Muller S., Kientsch-Engel R., Stein G., Sauer H. (2003). Pentosidine and *N*-epsilon-(carboxymethyl)-lysine in Alzheimer's disease and vascular dementia. *Neurobiol Aging* 24: 333–338.

Bergmann R., Helling R., Heichert C., Scheunemann W., Mading P., Wittrisch H., Johannsen B., Henle T. (2001). Radiofluorination and positron emission tomography (PET) as a new approach to study the in vivo distribution and elimination of the advanced glycation endproducts Nε-carboxymethyllysine (CML) and Nε-carboxyethyllysine (CEL). *Nahrung-Food* 45: 182–188.

Berlett B.S., Stadtman E.R. (1997). Protein oxidation in aging, disease and oxidative stress. *J Biol Chem* 272: 20313–20316.

Bianchi G., Marchesini G., Fabbri A., Ronchi M., Chianese R., Grossi G. (1997). Lipoperoxide plasma levels in patients with liver cirrhosis. *Hepato-Gastroenterology* 44: 784–788.

Biemel K.M., Buhler H.P., Reihl O., Lederer M.O. (2001). Identification and quantitative evaluation of the lysine- arginine crosslinks GODIC, MODIC, DODIC, and glucosepan in foods. *Nahrung-Food* 45: 210–214.

Biemel K.M., Friedl D.A., Lederer M.O. (2002). Identification and quantification of major Maillard cross-links in human serum albumin and lens protein—Evidence for glucosepane as the dominant compound. *J Biol Chem* 277: 24907–24915.

Birlouez-Aragon I., Pischetsrieder M., Leclere J., Morales F.J., Hasenkopf K., Kientsch-Engel R., Ducauze C.J., Rutledge D. (2004). Assessment of protein glycation markers in infant formulas. *Food Chem* 87: 253–259.

Blennow K. (2004a). Cerebrospinal fluid markers for Alzheimer's disease. *NeuroRx* 1: 213–225.

Blennow K. (2004b). CSF markers for mild cognitive impairment. *J Intern Med* 256: 224–234.

Bossy-Wetzel E., Schwarzenbacher R., Lipton S.A. (2004). Molecular pathways to neurodegeneration. *Nat Med* 10: S2–S9.

Brot N., Weissbach H. (1983). Biochemistry and physiological role of methionine sulfoxide reductase in proteins. *Arch Biochem Biophys* 253: 271–281.

Brownlee M. (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature* 414: 813–820.

Burroughs A., McNamara D. (2003). Liver disease in Europe. *Alimentary Pharmacol Therapeut* 18: 54–59.

Cai W., He J.C., Zhu L., Peppa M., Lu C., Uribarri J., Vlassara H. (2004). High levels of dietary advanced glycation end products transform low-density lipoprotein into a potent redox-sensitive mitogen-activated protein kinase stimulant in diabetic patients. *Circulation* 110: 285–291.

Cai W.J., Gao Q.D., Zhu L., Peppa M., He C.J., Vlassara H. (2002). Oxidative stress-inducing carbonyl compounds from common foods: Novel mediators of cellular dysfunction. *Mol Med* 8: 337–346.

Calabrese V., Scapagnini G., Ravagna A., Bella R., Foresti R., Bates T.E., Stella A.M.G., Pennisi G. (2002). Nitric oxide synthase is present in the cerebrospinal fluid of patients with active multiple sclerosis and is associated with increases in cerebrospinal fluid protein nitrotyrosine and *S*-nitrosothiols and with changes in glutathione levels. *J Neurosci Res* 70: 580–587.

Casserly I., Topol E. (2004). Convergence of atherosclerosis and Alzheimer's disease: inflammation, cholesterol, and misfolded proteins. *Lancet* 363: 1139–1146.

Castegna A., Thongboonkerd V., Klein J.B., Lynn B., Markesbery W.R., Butterfield D.A. (2003). Proteomic identification of nitrated proteins in Alzheimer's disease brain. *J Neurochem* 85: 1394–1401.

Ceriello A., Quagliaro L., Catone B., Pascon R., Piazzola M., Bais B., Marra G., Tonutti L., Taboga C., Motz E. (2002). Role of hyperglycemia in nitrotyrosine postprandial generation. *Diabetes Care* 25: 1439–1443.

Ceriello A., Quagliaro L., Piconi L., Assaloni R., Da Ros R., Maier A., Esposito K., Giugliano D. (2004). Effect of postprandial hypertriglyceridemia and hyperglycemia on circulating adhesion molecules and oxidative stress generation and the possible role of simvastatin treatment. *Diabetes* 53: 701–710.

Chen F., Wollmer M.A., Hoerndli F., Munch G., Kuhla B., Rogaev E.I., Tsolaki M., Papassotiropoulos A., Gotz J. (2004). Role for glyoxalase I in Alzheimer's disease. *Proc Natl Acad Sci USA* 101: 7687–7692.

Chuyen N.V. (2001). Maillard reaction and food processing. Application aspects. In: Shahidi S. (ed.), *Process-Induced Chemical Changes in Food*, Plenum Press, New York, pp. 213–235.

Cross A.H., Manning P.T., Keeling R.M., Schmidt R.E., Misko T.P. (1998). Peroxynitrite formation within the central nervous system in active multiple sclerosis. *J Neuroimmunol* 88: 45–56.

Cumming R.C., Andon N.L., Haynes P.A., Park M., Fischer W.H., Schubert D. (2004). Protein disulfide bond formation in the cytoplasm during oxidative stress. *J Biol Chem* 279: 21749–21758.

Degenhardt T.P., Thorpe S.R., Baynes J.W. (1998). Chemical modification of proteins by methylglyoxal. *Cell Molec Biol* 44: 1139–1145.

DeGroot J., Verzijl N., Wenting-Van Wijk M.J.G., Bank R.A., Lafeber F.P.J.G., Bijlsma J. W.J., TeKoppele J.M. (2001). Age-related decrease in susceptibility of human articular cartilage to matrix metalloproteinase-mediated degradation—The role of advanced glycation end products. *Arthritis Rheum* 44: 2562–2571.

Delpierre G., Vertommen D., Communi D., Rider M.H., Van Schaftingen E. (2004). Identification of fructosamine residues deglycated by fructosamine-3-kinase in human hemoglobin. *J Biol Chem* 279: 27613–27620.

Denu J.M., Tanner K.G. (1998). Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: Evidence for a sulfenic acid intermediate and implications for redox regulation. *Biochemistry* 37: 5633–5642.

Deuther-Conrad W., Franke S., Sommer M., Henle T., Stein G. (2001). Differences in the modulating potential of advanced glycation (AGE) peptides versus AGE proteins. *Kidney Internat* 59: S63–S66.

Dobler D., Ahmed N., Thornalley P.J. (2004) Peptide mapping of type IV collagen modified minimally by methylglyoxal in vitro. *Ann N Y Acad Sci* 1043: 906.

Dragsted L.O., Daneshvar B., Vogel U., Autrup H.N., Wallin H., Risom L., Moller P., Molck A.M., Hansen M., Poulsen H.E., Loft S. (2002). A sucrose-rich diet induces mutations in the rat colon. *Cancer Res* 62: 4339–4345.

Du X., Matsumura T., Edelstein D., Rosetti L., Zsengeller Z., Szabo C., Brownlee M. (2003). Inhibition of GAPDH activity by poly(ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells. *J Clin Invest* 112: 1049–1057.

Du X., Stockklauser-Farber K., Rosen P. (1999). Generation of reactive oxygen intermediates, activation of NF-kB and induction of apoptosis in human endothelial cells by glucose: Role of nitric oxide synthase? *Free Radic Biol Med* 27: 752–763.

Eiserich J.P., Hristova M., Cross C.E., Jones A.D., Freeman B.A., Halliwell B., van der Vliet A. (1998). Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 391: 393–397.

Faist V., Erbersdobler H. (2001). Metabolic transit and in vivo effects of melanoidins and precursor compounds deriving from the Maillard reaction. *Ann Nutr Met* 45: 1–12.

Fataccioli V., Andraud E., French S.W., Rouach H. (1999). Effects of chronic ethanol administration on rat liver proteasome activities: relationship with oxidative stress. *Hepatology* 29: 14–20.

Foerster A., Henle T. (2003). Glycation in food and metabolic transit of dietary AGEs (advanced glycation end-products): Studies on the urinary excretion of pyrraline. *Biochem Soc Trans* 31: 1383–1385.

Friedlander M.A., Wu Y.W., Elgawish A., Monnier V.M. (1996). Early and advanced glycosylation end products: Kinetics of formation and clearance in peritoneal dialysis. *J Clin Invest* 97: 728–735.

Fukunaga Y., Katsuragi Y., Izumi T., Sakiyama F. (1982). Fluorescence characteristics of kynurenine and N′-formylkynurenine: Their use as reporters of the environment of tryptophan 62 in hen egg-white lysozyme. *J Biochem* 92: 129–141.

Gabris J., Duran A. (1983). Level of free amino-acids in the milk of cows in relation to their levels in arterial and venous blood. *Vet Med* 28: 465–471.

Gallet X., Charloteaux B., Thomas A., Braseur R. (2000). A fast method to predict protein interaction sites from sequences. *J Mol Biol* 302: 917–926.

Gaut J.P., Byun J., Tran H.D., Heinecke J.W. (2002). Artifact-free quantitation of free 3-chlorotyrosine, 3-bromotyrosine, and 3-nitrotyrosine in human plasma by electron capture-negative chemical ionization gas chromatography mass spectrometry and liquid chromatography–electrospray ionization tandem mass spectrometry. *Anal Biochem* 300: 252–259.

Geibauf A., van Wickern B., Simat T., Steinhart H., Esterbauer H. (1996). Formation of *N*-formylkynurenine suggests the involvement of apolipoprotein B-100 centered tryptophan radicals in the initiation of LDL lipid peroxidation. *FEBS Lett* 389: 136–140.

Gerner C., Vejda S., Gelbmann D., Bayer E., Gotzmann J., Schulte-Hermann R., Mikulitis W. (2002). Concomitant determination of absolute values of cellular protein amounts, synthesis rates, and turnover rates by quantitative proteome profiling. *Mol Cell Proteomics* 1: 528–537.

Giulivi C., Davies K.J.A. (2001). Mechanism of the formation and proteolytic release of H_2O_2-induced dityrosine and tyrosine oxidation products in hemoglobin and red blood cells. *J Biol Chem* 276: 24129–24136.

Gladwin M.T., Lancaster J.R., Freeman B.A., Schechter A.N. (2003). Nitric oxide's reactions with hemoglobin: A view through the SNO-storm. *Nat Med* 9: 496–500.

Goldberg A.L. (2003). Protein degradation and protection against misfolded or damaged proteins. *Nature* 426: 895–899.

Goldberg T., Cai W.J., Peppa M., Dardaine V., Baliga B.S., Uribarri J., Vlassara H. (2004). Advanced glycoxidation end products in commonly consumed foods. *J Am Dietetic Assoc* 104: 1287–1291.

Good P.F., Hsu A., Werner P., Perl D.P., Olanow C.W. (1998). Protein nitration in Parkinson's disease. *J Neuropathol Exp Neurol* 57: 338–342.

Good P.F., Werner P., Hsu A., Olanow C.W., Perl D.P. (1996). Evidence for neuronal oxidative damage in Alzheimer's disease. *Am J Pathol* 149: 21–28.

Greive K.A., Balazs N.D.H., Comper W.D. (2001). Protein fragments in urine have been considerably underestimated by various protein assays. *Clin Chem* 47: 1717–1719.

Haik JR G.M., Lo T.W.C., Thornalley P.J. (1994). Methylglyoxal concentration and glyoxalase activities in human lens. *Exp Eye Res* 59: 497–500.

Hammes H.-P., Du X., Edelstein D., Taguchi T., Matsumura T., Ju Q., Lin J., Bierhaus A., Nawroth P., Hannak D., Neumaier M., Bergfeld R., Giardino I., Brownlee M. (2003). Benfotiamine blocks three major pathways of hyperglycemic damage and prevents experimental diabetic retinopathy. *Nat Med* 9: 294–299.

Hassan R., Qureshi H., Zuberi S.J. (1991). Effect of thiamine on glucose-utilization in hepatic cirrhosis. *J Gastroenterol Hepatol* 6: 59–60.

Hayashi T., Shibamoto T. (1985). Analysis of methylglyoxal in foods and beverages. *J Agric Food Chem* 33: 1090–1093.

Hayes J.D., Flanagan J.U., Jowsey I.R. (2005). Glutathione transferases. *Ann Rev Pharmacol Toxicol* 45: 51–88.

He C., Sabol J., Mitsuhashi T., Vlassara H. (1999). Dietary glycotoxins. Inhibition of reactive products by aminoguanidine facilitates renal clearance and reduced tissue sequestration. *Diabetes* 48: 1308–1315.

Henle T. (2003). AGEs in foods: Do they play a role in uremia? *Kidney Int* 63: S145–S147.

Henle T., Walter A., Haeβner R., Klostermeyer H. (1994). Detection and identification of a protein-bound imidazolone resulting from the reaction of arginine residues and methylglyoxal. *Z Lebensm Unters Forsch* 199: 55–58.

Henle T., Walter H., Klostermeyer H. (1991). Evaluation of the extent of the early Maillard-reaction in milk products by direct measurement of the Amadori-product lactuloselysine. *Z Lebensm Unters Forsch* 193: 119–122.

Hensley K., Maidt M.L., Yu Z., Sang H., Markesbery W.R., Floyd R.A. (1998). Electrochemical analysis of protein nitrotyrosine and dityrosine in the Alzheimer brain indicates region-specific accumulation. *J Neurosci* 18: 8126–8132.

Hink U., Li H., Mollnau H., Oelze M., Matheis E., Hartmann M., Skatchkov M., Thaiss F., Stahl R.A.K., Warnholtz A., Meinertz T., Griendling K., Harison D.G., Fostermann U., Munzel T. (2001). Mechanisms underlying endothelial dysfunction in diabetes mellitus. *Circ Res* 88: 14–22.

Honda K., Nitta K., Horita S., Yumura W., Nihei H., Nagai R., Ikeda K., Horiuchi S. (1999). Accumulation of advanced glycation end products in the peritoneal vasculature of continuous ambulatory peritoneal dialysis patients with low ultra-filtration. *Nephrol Dialysis Transplant* 14: 1541–1549.

Hurst J.K. (2002). Whence nitrotyrosine. *J Clin Invest* 109: 1287–1289.

Hutin Y., Kitler M.E., Dore G.J., Perz J.F., Armstrong G.L., Dusheiko G., Ishibashi H., Grob P., Kew M., Marcellin P., Seeff L.B., Beutels P., Nelson C., Stein C., Zurn P., Clifford G., Vranckx R., Alberti A., Hallaj Z.S., Hadler S., Lavanchy D. (2004). Global burden of disease (GBD) for hepatitis C. *J Clin Pharmacol* 44: 20–29.

Iberg N., Fluckiger R. (2002). Nonenzymatic glycosylation of albumin in vivo: Identification of multiple glycosylation sites. *J Biol Chem* 261: 13542–13545.

Ikeda K., Higashi T., Sano H., Jinnouchi Y., Yishida M., Araki T., Ueda S., Horiuchi S. (1996). N_{ε}-(Carboxymethyl)lysine protein adduct is a major immunological epitope in proteins modified with advanced glycation end products of the Maillard reaction. *Biochemistry* 35: 8075–8083.

Ishii T., Itoh K., Ruiz E., Leake D.S., Unoki H., Yamamoto M., Mann G.E. (2004). Role of Nrf2 in the regulation of CD36 and stress protein expression in murine macrophages: activation by oxidatively modified LDL and 4-hydroxynonenal. *Circ Res* 94: 609–616.

Jennings P.E., McLaren M., Scott N.A., Saniabadi A.R., Belch J.J.F. (1991). The relationship of oxidative stress to thrombotic tendency in type-1 diabetic patients with retinopathy. *Diabetic Medicine* 8: 860–865.

Johnson R.N., Easdale R.W., Tatnell M., Baker J.R. (1991). Significance of variation in turnover of glycated albumin on indexes of diabetic control. *Clin Chim Acta* 198: 229–238.

Kharbanda K.K., Todero S.L., Shubert K.A., Sorrell M.F., Tuma D.J. (2001). Malondialdehyde-acetaldehyde-protein adducts increase secretion of chemokines by rat hepatic stellate cells. *Alcohol* 25: 123–128.

Koito W., Araki T., Horiuchi S., Nagai R. (2004). Conventional antibody against N_{ε}-(carboxymethyl)lysine (CML) shows cross-reaction to N_{ε}-(carboxyethyl)lysine (CEL): Immunochemical quantification of CML with a specific antibody. *J Biochem* 136: 831–837.

Koo J.R., Vaziri N.D. (2003). Effects of diabetes, insulin and antioxidants on NO synthase abundance and NO interaction with reactive oxygen species. *Kidney Int* 63: 195–201.

Koschinsky T., He C.-J., Mitsuhashi T., Bucala R., Liu C., Buenting C., Heitman K., Vlassara H. (1997). Orally absorbed reactive glycation products (glycotoxins): An environmental risk factor in diabetic nephropathy. *Proc Natl Acad Sci USA* 94: 6474–6479.

Kukull W., Larson E., Teri L., Bowen J., McCormick W., Pfanschmidt M.L. (1994). The Mini-Mental State Examination score and the clinical diagnosis. *J Clin Epidemiol* 47: 1061–1067.

Kuzuya M., Asai T., Kanda S., Maeda K., Cheng X.W., Iguchi A. (2001). Glycation crosslinks inhibit matrix metalloproteinase-2 activation in vascular smooth muscle cells cultured on collagen lattice. *Diabetologia* 44: 436.

Lapolla A., Tubaro M., Reitano R., Arico N.C., Ragazzi E., Seraglia R., Vogliardi S., Traldi P., Fedele D. (2004). The complexity of non-enzymatic glycation product sets of human globins. *Diabetologia* 47: 1712–1715.

Lavanchy D. (2004). Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepatitis* 11: 97–107.

Le Moine O., Evrard S., Eisendrath P., Descamps C., Deviere J. (2000). Treatment of alcoholic hepatitis with intravenous n-acetylcysteine (NAC): A pilot trial. *Gastroenterology* 118: 1069.

Leeuwenburgh C., Hansen P.A., Holloszy J.O., Heinecke J.W. (1999). Oxidized amino acids in the urine of aging rats: Potential markers for assessing oxidative stress in vivo. *Am J Physiol-Regulatory Integrative and Comparative Physiol* 276: R128–R135.

Levy S., Herve C., Delacoux E., Erlinger S. (2002). Thiamine deficiency in hepatitis C virus and alcohol-related liver diseases. *Digestive Dis and Sci* 47: 543–548.

Liardon R., de Weck-Gaudard D., Philipossian G., Finot P.-A. (1987). Identification of N_{ε}-carboxymethyllysine: A new Maillard reaction product, in rat urine. *J Agric Food Chem* 35: 427–431.

Luth H.J., Munch G., Arendt T. (2002). Aberrant expression of NOS isoforms in Alzheimer's disease is structurally related to nitrotyrosine formation. *Brain Res* 953: 135–143.

Makita Z., Radoff S., Rayfield E.J., Friedman E.A., Kaufman A.M., Korbet S.M., Barth R.H., Winston J.A., Fuh H., Manogue K.R., Cerami A., Vlassara H. (1994). Reactive glycosylation endproducts in diabetic uraemia and treatment of renal failure. *Lancet* 343: 1519–1522.

Mattson M.P. (2004). Pathways towards and away from Alzheimer's disease. *Nature* 430: 631–639.

Mavric E., Kumpf Y., Schuster K., Kappenstein O., Scheller D., Henle T. (2004). A new imidazolinone resulting from the reaction of peptide-bound arginine and oligosacccharides with 1,4-glycosidic linkages. *Eur Food Res Technol* 218: 213–218.

May C., Kaye J.A., Atack J.R., Schapiro M.B., Friedland R.P., Rapoport S.I. (1990). Cerebrospinal fluid production is reduced in healthy aging. *Neurology* 40: 500–503.

McCance D.R., Dyer D.G., Dunn J.A., Baiue K.E., Thorpe S.R., Baynes J.W., Lyons T.J. (1993). Maillard reaction products and their relation to complications in insulin-dependent diabetes mellitus. *J Clin Invest* 91: 2470–2478.

McLellan A.C., Thornalley P.J., Benn J., Sonksen P.H. (1994). The glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications. *Clin Sci* 87: 21–29.

Mortier S., Faict D., Schalkwijk C.G., Lameire N.H., De Vriese A.S. (2004). Long-term exposure to new peritoneal dialysis solutions: Effects on the peritoneal membrane. *Kidney Int* 66: 1257–1265.

Mottaran E., Stewart S.F., Rolla R., Vay D., Cipriani V., Moretti M., Vidali M., Sartori M., Rigamonti C., Day C.P., Albano E. (2002). Lipid peroxidation contributes to immune reactions associated with alcoholic liver disease. *Free Radic Biol Med* 32: 38–45.

Murray J., Taylor S.W., Zhang B., Ghosh S.S., Capaldi R.A. (2003). Oxidative damage to mitochondrial complex I due to peroxynitrite. *J Biol Chem* 278: 37223–37230.

Myint T., Hoshi S., Ookawara T., Miyazawa N., Keiichiro M., Suzuki K., Taniguchi N. (1995). Immunological detection of glycated proteins in normal and streptozotocin-induced diabetic rats using anti hexitol-lysine IgG. *Biochim Biophys Acta* 1272: 73–79.

Nagai R., Unno Y., Hayashi M.C., Masuda S., Hayase F., Kinae N., Horiuchi S. (2002). Increased 3-NT dityrosine residues in post-mortem brain tissue was found in several neurological disorders including Parkinson's disease, multiple sclerosis and amyotrophic lateral sclerosis of Amadori product and generation of glucosone and glyoxal from glucose. *Diabetes* 51: 2833–2839.

Nagao M., Fujita Y., Sugimura T., Kosuge T. (1986). Methylglyoxal in beverages and foods its mutagenicity and carcinogenicity. *IARC Sci Publ* 70: 283–291.

Nakamura S., Miyazaki S., Sakai S., Morita T., Hirasawa Y., Niwa T. (2001). Localization of imidazolone in the peritoneum of CAPD patients: A factor for a loss of ultrafiltration. *Am J Kidney Dis* 38: S107–S110.

Nestor P.J., Scheltens P., Hodges J.R. (2004). Advances in the early detection of Alzheimer's disease. *Nat Med* 10: S34–S41.

Nishikawa T., Edelstein D., Liang Du X., Yamagishi S., Matsumura T., Kaneda Y., Yorek M.A., Beede D., Oates P.J., Hammes H.-P., Giardino I., Brownlee M. (2000). Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemia damage. *Nature* 404: 787–790.

Niwa T. (1999). 3-Deoxyglucosone: Metabolism, analysis, biological activity, and clinical implication. *J Chromatogr B* 731: 23–36.

Ohshima H., Friesen M., Brouet I., Bartsch H. (1990). Nitrotyrosine as a new marker for endogenous nitrosation and nitration of proteins. *Food Chem Toxicol* 28: 647–652.

Oste R.E., Miller R., Sjostrom H., Noren O. (1987). Effect of Maillard reaction-products on protein digestion—Studies on pure compounds. *J Agric Food Chem* 35: 938–942.

Owen W.F., Lew N.L., Liu Y., Lowrie E.G., Lazarus J.M. (1993). The urea reduction ratio and serum albumin concentration as predictors of mortality in patients undergoing hemodialysis. *New Engl J Med* 329: 1001–1006.

Paradis V., Kollinger M., Fabre M., Holstege A., Poynard T., Bedossa P. (1997). In situ detection of lipid peroxidation by-products in chronic liver diseases. *Hepatology* 26: 135–142.

Petrides A.S., Vogt C., Schulzeberge D., Matthews D., Strohmeyer G. (1994). Pathogenesis of glucose-intolerance and diabetes-mellitus in cirrhosis. *Hepatology* 19: 616–627.

Piraud M., Vianey-Saban C., Petritis K., Elfakir C., Steghens J.P., Morla A., Bouchu D. (2003). ESI-MS/MS analysis of underivatised amino acids: A new tool for the diagnosis of inherited disorders of amino acid metabolism. Fragmentation study of 79 molecules of biological interest in positive and negative ionisation mode. *Rapid Commun Mass Spectrom* 17: 1297–1311.

Pouletty P. (2002). Drug addictions: Towards socially accepted and medically treatable diseases. *Nat Rev Drug Discov* 1: 731–736.

Predescu D., Predescu S., Malik A.B. (2002). Transport of nitrated albumin across continuous vascular endothelium. *Proc Natl Acad Sci USA* 99: 13932–13937.

Pryor W.A., Jin X., Squadrito G.L. (1994). One electron and two electron oxidations of methionine by peroxynitrite. *Proc Natl Acad Sci USA* 91: 11173–11177.

Quijano C., Alvarez B., Gatt R.M., Augusto O., Radi R. (1997). Pathways of peroxynitrite oxidation of thiol groups. *Biochem J* 322: 167–173.

Rahman T.M. Hodgson H.J.F. (2003). The effects of early and late administration of inhibitors of inducible nitric oxide synthase in a thioacetamide-induced model of acute hepatic failure in the rat. *J Hepatology* 35: 583–590.

Rashed M.S., Ozand P.T., Bucknall M.P., Little D. (1995). Diagnosis of inborn-errors of metabolism from blood spots by acylcarnitines and amino-acids profiling using automated electrospray tandem mass-spectrometry. *Pediatric Res* 38: 324–331.

Reiter C.D., Teng R.J., Beckman J.S. (2000). Superoxide reacts with nitric oxide to nitrate tyrosine at physiological pH via peroxynitrite. *J Biol Chem* 275: 32460–32466.

Roberts N.B., Amara A.B., Morris M., Green B.N. (2001). Long-term evaluation of electrospray ionization mass spectrometric analysis of glycated hemoglobin. *Clin Chem* 47: 316–321.

Russo L.M., Bakris G.L., Comper W.D. (2002). Renal handling of albumin: A critical review of basic concepts and perspective. *Am J Kidney Dis* 39: 899–919.

Ryberg H., Soderling A.S., Davidsson P., Blennow K., Caidahl K., Persson L.I. (2004). Cerebrospinal fluid levels of free 3-nitrotyrosine are not elevated in the majority of patients with amyotrophic lateral sclerosis or Alzheimer's disease. *Neurochem Internat* 45: 57–62.

Sacks D.B., Bruns D.E., Goldstein D.E., Maclaren N.K., McDonald J.M., Parrott M. (2002). Guidelines and recommendations for laboratory analysis in the diagnosis and management of diabetes mellitus. *Clin Chem* 48: 436–472.

Sasaki S., Shibata N., Komori T., Iwata M. (2000). iNOS and nitrotyrosine immunoreactivity in amyotrophic lateral sclerosis. *Neurosci Lett* 291: 44–48.

Scaloni A., Perillo V., Franco P., Fedele E., Froio R., Ferrara L., Bergamo P. (2002). Characterization of heat-induced lactosylation products in caseins by immunoenzymatic and mass spectrometric methodologies. *Biochim Biophys Acta-Proteins and Proteomics* 1598: 30–39.

Schleicher E.D., Wagner E., Nerlich A.G. (1997). Increased accumulation of the glycoxidation product N_{ε}-(carboxymethyl)lysine in human tissues in diabetes and aging. *J Clin Invest* 99: 457–468.

Schwemmer M., Fink B., Kockerbauer R., Bassenge E. (2000). How urine analysis reflects oxidative stress—Nitrotyrosine as a potential marker. *Clin Chim Acta* 297: 207–216.

Sebekova K., Kupcova V., Schinzel R., Heidland A. (2002). Markedly elevated levels of plasma advanced glycation end products in patients with liver cirrhosis—Amelioration by liver transplantation. *J Hepatology* 36: 66–71.

Sebekova K., Podracka L., Blazicek P., Syrova D., Heidland A., Schinzel R. (2001). Plasma levels of advanced glycation end products in children with renal disease. *Pediatric Nephrol* 16: 1105–1112.

Shuvaev V.V., Laffont I., Serot J.-M., Fujii J., Taniguchi N., Siest G. (2001). Increased protein glycation in cerebrospinal fluid of Alzheimer's disease. *Neurobiol Aging* 22: 397–402.

Silverberg G.D., Heit G., Huhn S., Jaffe R.A., Chang S.D., Bronte-Stewart H., Rubenstein E., Possin K., Saul T.A. (2001). The cerebrospinal fluid production rate is reduced in dementia of the Alzheimer's type. *Neurology* 57: 1763–1766.

Sitte N., Merker K., von Zglinicki T., Davies K.J.A., Grune T. (2000). Protein oxidation and degradation during cellular senescence of human BJ fibroblasts. Part II: Aging of non-dividing cells. *FASEB J* 14: 2503–2510.

Smith M.A., Perry G., Sayre L.M., Anderson V.E., Beal M.F., Kowall N. (1996). Oxidative damage in Alzheimer's disease. *Nature* 382: 120–121.

Smith M.A., Richey Harris P.L., Sayre L.M., Beckman J.S., Perry G. (1997). Widespread peroxynitrite-mediated damage in Alzheimer's disease. *J Neurosci* 17: 2653–2657.

Smith M.A., Taneda S., Richey P.L., Miyata S., Yan S.-D., Stern D., Sayre L.M., Monnier V.M., Perry G. (1994). Advanced Maillard reaction end products are associated with Alzheimer disease pathology. *Proc Natl Acad Sci USA* 91: 5710–5714.

Soriano F.G., Virag L., Jagtap P., Szabo E., Mabley J.G., Liaudet L., Marton M., Hoyt D.G., Murthy K.G.K., Salzman A.L., Southan G.J., Szabo C. (2001). Diabetic endothelial dysfunction: The role of poly(ADP-ribose) polymerase activation. *Nat Med* 7: 108–113.

Sterling K. (1951). Serum albumin turnover in Laennec's cirrhosis as measured by ^{131}I-tagged albumin. *J Clin Invest* 30: 1238–1246.

Stratton I.M., Adler A.I., Neil H.A.W., Matthews D.R., Manley S.E., Cull C.A., Hadden D., Turner R.C., Holman R.R. (2002). Association of glycaemic with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study. *BMJ* 321: 405–412.

Szwergold B.S., Howell S., Beisswenger P.J. (2001). Human fructosamine-3-kinase. Purification, sequencing, substrate specificity, and evidence of activity in vivo. *Diabetes* 50: 2139–2147.

Terelius Y., Norsten-Hoog C., Cronholm T., Ingelman-Sundberg M. (2003). Acetaldehyde as a substrate for ethanol-inducible cytochrome P450 (CYP2E1). *Biochem Biophys Res Commun* 179: 689–694.

The Diabetes Control and Complications Trial Research Group (1993). The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *New Engl J Med* 327: 977–986.

Thornalley P.J. (1988). Modification of the glyoxalase system in human red blood cells by glucose in vitro. *Biochem J* 254: 751–755.

Thornalley P.J. (1998). Cell activation by glycated proteins. AGE receptors, receptor recognition factors and functional classification of AGEs. *Cell Mol Biol* 44: 1013–1023.

Thornalley P.J. (1999). Clinical significance of glycation. *Clin Lab* 45: 263–273.

Thornalley P.J. (2003a). Glyoxalase I—Structure, function and a critical role in the enzymatic defence against glycation. *Biochem Soc Trans* 31: 1343–1348.

Thornalley P.J. (2003b). The enzymatic defence against glycation in health, disease and therapeutics: a symposium to examine the concept. *Biochem Soc Trans* 31: 1343–1348.

Thornalley P.J. (2003c). Use of aminoguanidine (Pimagedine) to prevent the formation of advanced glycation endproducts. *Arch Biochem Biophys* 419: 31–40.

Thornalley P.J. (2004). Glycation, receptor-mediated cell activation and vascular complications of diabetes. *Diabetes Vac Dis Res* 1: 21–22.

Thornalley P.J. (2005) Dicarbonyl intermediates in the Maillard reaction. *Ann NY Acad Sci* 1043: 111–117.

Thornalley P.J., Argirova M., Ahmed N., Mann V.M., Argirov O.K., Dawnay A. (2000a). Mass spectrometric monitoring of albumin in uraemia. *Kidney Internat* 58: 2228–2234.

Thornalley P.J., Battah S., Ahmed N., Karachalias N., Agalou S., Babaei-Jadidi R., Dawnay A. (2003). Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry. *Biochem J* 375: 581–592.

Thornalley P.J., Langborg A., Minhas H.S. (1999). Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. *Biochem J* 344: 109–116.

Thornalley P.J., Yurek-George A., Argirov O.K. (2000b). Kinetics and mechanism of the reaction of aminoguanidine with the α-oxoaldehydes, glyoxal, methylglyoxal and 3-deoxyglucosone under physiological conditions. *Biochem Pharmacol* 60: 55–65.

Tohgi H., Abe T., Yamazaki K., Murata T., Ishizaki E., Isobe C. (1999). Alterations of 3-nitrotyrosine concentration in the cerebrospinal fluid during aging and in patients with Alzheimer's disease. *Neurosci Lett* 269: 52–54.

Ueno H., Segawa T., Hasegawa T., Nakamuro K., Maeda H. (1991). Subchronic oral toxicity of glyoxal via drinking water in rats. *Fundam Appl Toxicol* 16: 763–772.

Uribarri J., Peppa M., Cai W.J., Goldberg T., Lu M., Baliga S., Vassalotti J.A., Vlassara H. (2003). Dietary glycotoxins correlate with circulating advanced glycation end product levels in renal failure patients. *Am J Kidney Dis* 42: 532–538.

Verdier Y. Penke B. (2004). Binding sites of amyloid beta-peptide in cell plasma membrane and implications for Alzheimer's disease. *Curr Protein Peptide Sci* 5: 19–31.

Verzijl N., DeGroot J., Thorpe S.R., Bank R.A., Shaw J.N., Lyons T.J., Bijlsma J.W.J., Lafeberi F.P.J.G., Baynes J.W., TeKoppele J.M. (2000). Effect of collagen turnover on the accumulation of advanced glycation endproducts. *J Biol Chem* 275: 39027–39031.

Vlassara H., Cai W.J., Crandall J., Goldberg T., Oberstein R., Dardaine V., Peppa M., Rayfield E.J. (2002). Inflammatory mediators are induced by dietary glycotoxins, a major risk factor for diabetic angiopathy. *Proc Natl Acad Sci USA* 99: 15596–15601.

Weissbach H., Etienne F., Hoshi T., Heinemann S.H., Lowther W.T., Matthews B., St John G., Nathan C., Brot N. (2002). Peptide methionine sulfoxide reductase: Structure, mechanism of action, and biological function. *Arch Biochem Biophys* 397: 172–178.

Wells-Knecht M.C., Lyons T.J., McCance D.R., Thorpe S.R., Baynes J.W. (1997). Age-dependent increases in ortho-tyrosine and methionine sulfoxide in human skin collagen is not accelerated in diabetes. *J Clin Invest* 100: 839–846.

Wenzel E., Tasto S., Erbersdobler H.F., Faist V. (2002). Effect of heat-treated proteins on selected parameters of the biotransformation system in the rat. *Ann Nutrit Metab* 46: 9–16.

Willis M.S., Klassen L.W., Tuma D.J., Thiele G.M. (2002). Malondialdehyde-acetaldehyde-haptenated protein Induces cell death by induction of necrosis and apoptosis in immune cells. *Internat Immunopharmacol* 2: 519–535.

Wynne A., Sauter C., Gibson G.R., Ames J.M. (2002). Microbial degradation of heated gluten-glucose systems and the implications for human gut health. *Maillard Reaction in Food Chemistry and Medical Science: Update for the Postgenomic Era* 1245: 481–482.

Yoshihara K., Okumura T., Yoshida T., Beppu M. (2001). Inhibitory effect of peptide-free forms of advanced glycation end products on the proliferation and extracellular matrix protein production of cultured cells. *J Health Sci* 47: 296–301.

Zhang X., Medzihradszhy K.F., Cunningham J., Lee P.D.K., Rognerud C.L., Ou C.-N., Harmatz P., Witkowska H.E. (2001). Characterization of glycated hemoglobin in diabetic patients: usefulness of electrospray mass spectrometry in monitoring the extent and distribution of glycation. *J Chromatogr B* 759: 1–15.

Zhang X.M., Chan C.C., Stamp D., Minkin S., Archer M.C., Bruce W.R. (1993). Initiation and promotion of colonic aberrant crypt foci in rats by 5-hydroxymethyl-2-furaldehyde in thermolyzed sucrose. *Carcinogenesis* 14: 773–775.

Zill H., Bek S., Hofmann. T., Huber J., Frank O., Lindenmeier M., Weigle B., Erbersdobler H.F., Scheidler S., Busch A.E., Faist V. (2003). RAGE-mediated MAPK activation by food-derived AGE and non-AGE products. *Biochem Biophys Res Commun* 300: 311–313.

23

PROTEIN TARGETS AND FUNCTIONAL CONSEQUENCES OF TYROSINE NITRATION IN VASCULAR DISEASE

LAURA M. S. BAKER, BRUCE A. FREEMAN, AND MUTAY ASLAN

23.1 ASSOCIATION OF VASCULAR DISEASE WITH INCREASED PRODUCTION OF REACTIVE OXYGEN/NITROGEN SPECIES AND ACCUMULATION OF NITRATED PROTEINS

The vasculature is lined with endothelial cells that historically were considered physiologically inert, with their sole function being to form a membrane around blood vessels and to help maintain vessel wall permeability (Cines et al., 1998). However, dramatic progress in biomedical research and modern medicine has highlighted the essential functions of the endothelium in vascular homeostasis and inflammation, including pivotal roles in vasodilation, coagulation, and cell adherence and growth. One of the primary mechanisms by which endothelium modulates these effects is by the generation and release of nitric oxide (nitrogen monoxide, $^{\bullet}$NO). Nitric oxide is a simple gaseous molecule, yet it has diverse biological functions ranging from neurotransmission, modulation of vascular tone, host defense, and cell signaling. One of its main physiological roles is to bind soluble, heme-containing guanylate cyclases to stimulate production of cyclic guanosine 3′,5′-monophosphate (cGMP), in part accounting for its vasoactive and anti-inflammatory effects. Impairment of $^{\bullet}$NO production can lead to endothelial dysfunction and vascular disease (Cooke et al., 2000). Moreover, $^{\bullet}$NO bioavailability is decreased when $^{\bullet}$NO combines with reactive oxygen

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

metabolites to form reactive nitrogen species (RNS), such as nitrogen dioxide ($^{\bullet}NO_2$) and peroxynitrite ($ONOO^-$), which also contribute to vascular cell signaling and pathology. Loss of endothelial barrier function, adhesion of platelets, and abnormal vasoregulation can all result from endothelial injury derived from ischemia/reperfusion, inflammation, xenobiotic metabolism, hyperoxic exposure, and other diseases when the redox chemical environment tips toward the consumption of $^{\bullet}NO$.

Physiological studies have demonstrated that superoxide ($O_2^{\bullet-}$) diverts the reactivities of $^{\bullet}NO$ and inhibits $^{\bullet}NO$-dependent vascular function. The addition of superoxide dismutase (SOD), an antioxidant enzyme that consumes $O_2^{\bullet-}$, prevents the loss of $^{\bullet}NO$ (Gryglewski et al., 1986), thereby indirectly implicating reactive oxygen species (ROS) in vascular dysfunction. Both toxic and cytoprotective effects are observed as a consequence of $^{\bullet}NO$ reactions with ROS, and increasingly, RNS have been shown to play a role in vascular pathological processes that were previously attributed solely to the excess production of ROS (Rubbo et al., 1996). Alone, $^{\bullet}NO$ is a weak reducing agent, but in combination with other reactive oxygen intermediates, the potential of $^{\bullet}NO$ to damage biomolecules and tissues is markedly increased. Superoxide and $^{\bullet}NO$ react at a diffusion-limited rate in aqueous solutions to form $ONOO^-$, a species that can oxidize DNA and proteins and cause lipid peroxidation, while simultaneously diminishing immediate $^{\bullet}NO$ availability and its beneficial effects on vascular function (Beckman, 1996b). Nitrosation, the addition of a nitroso (–NO) group, is one key chemical modification mediated by RNS in vivo. An example of this phenomenon is the reaction of $ONOO^-$ with polyhydroxy compounds or thiols, yielding a nitrito derivative (RO-NO) and *S*-nitrosothiols (RS-NO), respectively. A second modification caused by RNS is nitration, the addition of a nitro ($–NO_2$) group to biomolecules. The covalent addition of $–NO_2$ to free and protein-bound tyrosine residues at either of the equivalent ortho positions of the aromatic ring creates the 3-nitro-L-tyrosine (NO_2Tyr) adduct. Because detection of RNS is hampered by the short half-lives of intermediates and fast reactions with other biomolecules, measurement of NO_2Tyr serves as an indirect biomarker for nitrating species derived from $^{\bullet}NO$ and is a reflection of extent of in vivo oxidant production during physiological and pathological conditions.

Nitration of protein Tyr residues has garnered much interest during the investigation of redox cell signaling and oxidative inflammatory injury, due to the fact that nitration has been shown to alter protein function, including modulation of catalytic activity, cell signaling, and cytoskeletal organization (Schopfer et al., 2003). Additionally NO_2Tyr levels have been shown to be elevated during acute and chronic disease states whose progression is associated with oxidative stress with these levels now being revealed to be clinically correlated with disease risk. Depending on the disease and the tissue, previous assessments of NO_2Tyr formation during disease processes showed increases in both protein NO_2Tyr levels (2–10-fold) and free NO_2Tyr (1.5–2-fold) (Greenacre and Ischiropoulos, 2001). Earlier measurements of NO_2Tyr concentrated on the total cellular NO_2Tyr content, but recently more examples of specific proteins with

selective Tyr modifications have been the focus of many studies (Greenacre and Ischiropoulos, 2001; Turko and Murad, 2002). Accumulation of the NO_2Tyr modification is linked increasingly with vascular diseases, and specific examples of proteins modified by NO_2Tyr have been discovered where functional changes (usually in a negative manner) are a reflection of disease progression (Turko and Murad, 2002). Accordingly, recent clinical studies have noted an increase in circulating levels of plasma and serum protein NO_2Tyr in patients with coronary artery disease, providing a new marker of inflammation and risk for cardiovascular disease (Shishehbor et al., 2003). NO_2Tyr production can also be induced by myeloperoxidase (MPO), an enzyme enriched in the granules of monocytes, which catalyzes the formation of $^{\bullet}$NO- and nitrite (NO_2^-)-derived oxidants. Additional clinical studies have shown that circulating MPO levels can predict the risk for subsequent cardiovascular events, thereby further linking NO_2Tyr production to vascular disease progression (Baldus et al., 2003).

The prevalence of NO_2Tyr as a post-translational alteration indicates that it is a reflection of the nature and degree of tissue redox reactions and a significant modulator of protein function and structure. Herein, we provide examples of protein nitration detected under physiological conditions in various models of vascular disease or in clinically-derived tissues and discuss the impact that this post-translational protein modification can have on cell and organ function.

23.2 PRODUCTION OF REACTIVE OXYGEN AND NITROGEN SPECIES IN THE VASCULATURE

Protein Tyr nitration is a result of the combined, simultaneous production of RNS and ROS, and their interaction with each other leads to a complex biochemical milieu. The generation of one species invariably modulates the production of the other by altering bioavailability and through the creation of new, more reactive molecules with the potential to alter downstream signaling events. It is at this intersection of ROS and RNS production that the chemical potency and complexity of inflammatory free radical production is realized with the generation of $ONOO^-$ and other reactive species derived from the simultaneous and sometimes co-localized production of $^{\bullet}$NO, $O_2^{\bullet-}$, hydrogen peroxide (H_2O_2), and their secondary products. Because of important cell signaling roles of $^{\bullet}$NO in the vasculature, this compartment can be a key locus for inflammatory-derived reactions that yield NO_2Tyr. Transient reactive species required for NO_2Tyr production (e.g., $O_2^{\bullet-}$ and $^{\bullet}$NO) must diffuse together in the same compartment to yield stable nitration products or create secondary nitrating intermediates that are much more reactive and powerful oxidants than their precursors. The immunohistochemical detection of NO_2Tyr in atherosclerotic lesions from human coronary arteries first established the presence of NO_2Tyr production in vascular tissues (Beckman et al., 1994), and more current studies have focused on linking reactive species production and endothelial dysfunction in mammalian vascular tissues (van der Loo et al., 2000).

23.2.1 Sources of Nitric Oxide

Nitric oxide is enzymatically generated by nitric oxide synthase (NOS), which catalyzes the oxidation of the terminal guanidino nitrogen of L-arginine, converting the precursor amino acid substrate to L-citrulline. There are three NOS isoforms: neuronal (nNOS, NOS1), inducible (iNOS, NOS2), and endothelial (eNOS, NOS3), all of which require NADPH and O_2 as co-substrates. All three isoforms bind calmodulin, but iNOS works independently of Ca^{2+} concentration, unlike eNOS and nNOS, which require Ca^{2+} for activity. Because of its role in producing $^{\bullet}NO$ for the regulation of vaso-function and its wide distribution throughout the vascular tissue of the kidney, liver, and spleen, eNOS may be considered a good candidate as the source of $^{\bullet}NO$ involved in RNS generation (Drew and Leeuwenburgh, 2002).

Yet, it is iNOS-derived $^{\bullet}NO$ production that is probably the most relevant to vascular disease. This is because its transcription is upregulated by inflammatory processes and infection. Therefore iNOS is viewed to be responsible for increased RNS production under these circumstances. In response to inflammatory stimuli such as cytokines and bacterial endotoxins, iNOS can generate $^{\bullet}NO$ at substantially greater rates for a longer amount of time, compared with the other NOS isoforms (Zweier et al., 2001). This sustained production is beneficial when $^{\bullet}NO$ is involved in host defense against invading pathogens; however, the development of cardiovascular disease states has also been correlated with excess $^{\bullet}NO$ production (Drew and Leeuwenburgh, 2002; Turko and Murad, 2002). The enzymatic production of $^{\bullet}NO$ is also modulated by tetrahydrobiopterin availability, eNOS translocation to caveolae, substrate concentration, and increased NOS expression.

The structurally simple $^{\bullet}NO$ molecule, with its single unpaired electron, is relatively small, neutrally charged, and hydrophobic. It is thus readily capable of transversing membranes and diffusing to adjacent cells. The molecule's diffusibility is due to a relatively low reactivity with organic molecules, and $^{\bullet}NO$ is not typically associated with direct initiation of free radical reactions. The reactivity of $^{\bullet}NO$ with ROS leads to a diversity of both toxic and cytoprotective effects. Nitric oxide is more likely to act as a pro-oxidant when the concentration of $O_2^{\bullet-}$ is greater than or equal to that of $^{\bullet}NO$, via the facile radical reaction of these species to form the potent oxidant $ONOO^-$ (Patel et al., 1999).

23.2.2 Sources of Superoxide

Superoxide is produced enzymatically by a variety of oxidases, including the NADPH oxidase complex of activated phagocytes, the NADPH oxidase-like Nox and Duox isoforms present in diverse noninflammatory cells, and by autooxidation of electron transport components of the mitochondrial respiratory chain where ($\sim$1%) of all oxygen consumption is diverted to the generation of $O_2^{\bullet-}$ and its dismutation product, H_2O_2 (Ischiropoulos and Beckman, 2003). If the electron transport chain becomes uncoupled, there can also be a significant increase in $O_2^{\bullet-}$ production. Phagocytic cell NADPH oxidase-dependent production of $O_2^{\bullet-}$ contributes to increases in the production of ROS upon activation

of the immune responses that also promote elevations in $^{\bullet}NO$ levels. Xanthine oxidase, an important source of increased reactive species production in tissue injury, generates $O_2^{\bullet-}$ as a by-product of purine catabolism. Increases in xanthine oxidase plasma levels have been shown to mediate endothelial dysfunction (Houston et al., 1999) and enhance injury following ischemia reperfusion (Xia and Zweier, 1995).

As an oxidant, $O_2^{\bullet-}$ is not exceptionally cytotoxic, particularly because high levels of cellular antioxidants such as SOD rapidly convert $O_2^{\bullet-}$ to H_2O_2. While also not an extremely potent oxidant itself, H_2O_2 can react with reduced transition metals via the Fenton reaction to form a far more reactive product, the hydroxyl radical ($^{\bullet}OH$). The hydroxyl radical is not a selective oxidant and typically reacts indiscriminately with most biomolecules with a rate constant that approaches the diffusion limit ($\sim 10^9\ M^{-1}s^{-1}$), abrogating targeted reactions. Additionally $^{\bullet}OH$ rarely participates in free radical propagation; instead, addition reactions with $^{\bullet}OH$ predominate (Radi et al., 2001), which diminishes the role of $^{\bullet}OH$ in free radical-mediated oxidant toxicity. MPO, a highly abundant enzyme in neutrophils, is oxidized by H_2O_2 to a ferryl-oxo complex, which oxidizes Cl^- to hypochlorous acid (HOCl), a strongly oxidizing or chlorinating agent.

The vasculature provides an environment where all the components required for the production of ROS and RNS are abundant. Leukocytes and other reticuloendothelial cells that promote inflammation are globally distributed throughout the vasculature. Monocytes produce $^{\bullet}NO$ through the upregulation of iNOS when stimulated with various cytokines and vasoactive hormones (Ischiropoulos et al., 1992). Additionally, stimulated leukocytes will generate $O_2^{\bullet-}$ and H_2O_2 via the respiratory burst. Thus, the vasculature is an area of increased reactive species production due to its proximity to leukocytes and to $^{\bullet}NO$ generation by iNOS and eNOS in endothelial cells, providing a physiological environment that has the potential to support ROS and RNS production, especially under inflammatory conditions.

To begin to assess the role ROS and RNS play in physiological and pathological conditions, relevant biomarkers of these species often need to be analyzed because of the inherent difficulty in directly detecting evanescent species in the face of efficient endogenous scavenging mechanisms. Proteins are readily available for oxidative modification due to their high intracellular concentration ($\sim$1 M) and are relatively stable after undergoing oxidative/nitrosative insults, with thiols being the primary target for post-translational modification (Dalle-Donne et al., 2005). The less reactive aromatic amino acid Tyr presents itself as a unique amino acid target of redox reactions, with Tyr modifications depending on the type of oxidative or nitrosative stress (Heinecke, 1999). There is an abundance of Tyr ($\sim$4 mol%) in most proteins, with Tyr capable of modification to a variety of stable end-products. For example, Tyr undergoes post-translational modification by phosphorylation, sulfation, iodination, chlorination, bromination, hydroxylation, and nitration, with each modification representing a response to a unique cell signaling stimuli and milieu of reactive inflammatory mediators (Greenacre and Ischiropoulos, 2001). Quantification of NO_2Tyr in proteins

provides a key strategy for assessing the extent of protein damage attributed to RNS, because Tyr nitration occurs at increased rates in the presence of a variety of reactive inflammatory mediators. Although other related Tyr modifications are also produced during exposure to RNS (e.g., di-Tyr, 3,4-dihydroxy phenylalanine, and its corresponding quinone), these products are formed to a lesser extent in vivo.

23.3 TYROSINE NITRATION MECHANISMS

Post-translational modification of Tyr to 3-NO_2Tyr occurs via multiple pathways and involves a variety of reactive species. To ultimately nitrate Tyr, nitrogen dioxide ($^{\bullet}NO_2$) reacts with the tyrosyl radical (Tyr$^{\bullet}$) at diffusion-limited rates (3×10^9 $M^{-1}s^{-1}$) (Schopfer et al., 2003) in a concerted, two-step mechanism, whereby initially Tyr$^{\bullet}$ is generated on free or protein-bound Tyr via reaction with one electron oxidants, including the carbonate anion radical ($^{\bullet}CO_3^-$):

$$\text{Tyr} + {}^{\bullet}\text{OH}, {}^{\bullet}\text{CO}_3^- \text{ or } {}^{\bullet}\text{NO}_2 \rightarrow \text{Tyr}^{\bullet} + \text{OH}^-, \text{CO}_3^{2-} \text{ or } \text{NO}_2^- \qquad (1)$$

Next the tyrosyl radical reacts with nitrogen dioxide to give nitrotyrosine:

$$\text{Tyr}^{\bullet} + {}^{\bullet}\text{NO}_2 \rightarrow \text{NO}_2\text{Tyr} \qquad (2)$$

The limiting step in this reaction is the formation of $^{\bullet}NO_2$, which occurs both enzymatically and nonenzymatically via three primary pathways described below, but it can be easily inferred that nitration of proteins always stems indirectly from $^{\bullet}$NO production or the presence of dietary nitrite (NO_2^-) (Greenacre and Ischiropoulos, 2001; Kissner et al., 1997). Indeed NO_2Tyr-generating pathways invariably include Tyr$^{\bullet}$, $^{\bullet}NO_2$, and $^{\bullet}CO_3^-$ radicals, so for the most part, NO_2Tyr formation generally proceeds as outlined above (Radi, 2004). Glutathione, cysteine, ascorbate, and other reductants exist in high concentrations intracellularly and can thus inhibit the accumulation of initiating radicals to limit NO_2Tyr formation. Spatial sequestration of key mediators of Tyr nitration into hydrophobic environments such as the interior of cell membranes and hydrophobic regions of proteins may provide the protection necessary to propagate these reactions (Zweier et al., 2001). An imbalance in antioxidant to oxidant ratios especially during phagocytic immune responses can also produce situations that are pro-oxidative.

23.3.1 Nonenzymatic Nitrogen Dioxide Formation

Generation of Peroxynitrite The most widely studied nitrating intermediate is $ONOO^-$, which is formed from the radical–radical reaction of $^{\bullet}$NO with $O_2^{\bullet-}$. Superoxide readily undergoes a free-radical termination reaction with $^{\bullet}$NO because the rate constant for this reaction is three times faster (1.9×10^{10} M^{-1} s^{-1}) (Kissner et al., 1997) than the reaction rate of $O_2^{\bullet-}$ with SOD. The rate

constant for the formation of $ONOO^-$ has been determined in three independent experiments, and each approaches the diffusion limited rate of 10^{10} M^{-1} s^{-1} (Radi et al., 2001). This extremely fast reaction for $^{\bullet}NO$ with $O_2^{\bullet-}$ and the high concentrations of $^{\bullet}NO$ make the reaction to form $ONOO^-$ an extremely likely pathway to direct $O_2^{\bullet-}$ and $^{\bullet}NO$ toxicity (Beckman, 1996b). At neutral pH, $O_2^{\bullet-}$ is anionic ($pK_a = 4.8$) and not as readily diffusible across biological membranes as $^{\bullet}NO$, so $ONOO^-$ formation may be more likely to occur proximally to sites of $O_2^{\bullet-}$ production (Alvarez et al., 1999).

Peroxynitrite can directly nitrate thiols (Radi et al., 1991a) and lipids (Radi et al., 1991b), but it does not react directly with Tyr. When free Tyr was added to a solution of $ONOO^-$, the rate of $ONOO^-$ decomposition was not increased (Alvarez et al., 1999), indicating that $ONOO^-$ is intermediate to the ultimate oxidizing and nitrating species. Peroxynitrite is in fast, dynamic equilibrium with its conjugated acid, peroxynitrous acid (ONOOH, pKa 6.8), which can either rapidly undergo homolytic cleavage (~35%) to yield $^{\bullet}NO_2$ and $^{\bullet}OH$ (Schopfer et al., 2003) or isomerize to nitrate (NO_3^-, ~65%). Tyrosines will then react with $^{\bullet}OH$ and $^{\bullet}NO_2$ derived from $ONOO^-$ to form $Tyr^{\bullet}$, which recombines with $^{\bullet}NO_2$ to produce NO_2Tyr. Because ONOOH is less stable than $ONOO^-$ at physiological pH and decays rapidly (0.9 s^{-1} at 37°C, pH 7.4), most $ONOO^-$ (>99%) in vivo will react with other cellular components such as thiols and CO_2 before homolyzing to yield NO_2Tyr-forming radical intermediates (Alvarez and Radi, 2003). The low yield of NO_2Tyr from this pathway is most likely due to the greater ease of adding $^{\bullet}OH$ to the Tyr phenolic ring than abstracting a hydrogen atom from it (Alvarez and Radi, 2003).

Although synthetic $ONOO^-$ is relatively stable and can be stored at −80°C for several months (Beckman et al., 1994), the in vivo half-life of $ONOO^-$ is 10 to 20 ms (Denicola et al., 1998), making this transient species extremely difficult to measure directly. In fact the biological effects of $ONOO^-$ remain difficult to deconvolute from the direct effects of its precursor radicals, $O_2^{\bullet-}$ and $^{\bullet}NO$ and other $^{\bullet}NO$-derived radicals (Radi et al., 2001). The problems poised by the specificity and sensitivity of $ONOO^-$ and NO_2Tyr detection and the complications imposed by competing free radical reactions have sparked some debate as to whether or not $ONOO^-$ robustly participates in protein Tyr nitration in vivo.

Studies conducted in vitro with synthetic $ONOO^-$ support that $ONOO^-$ has considerable potential to nitrate free and protein-associated Tyr residues (Beckman and Koppenol, 1996; Ischiropoulos et al., 1992). In contrast, other reports suggested that continuous, equimolar generation of $^{\bullet}NO$ and $O_2^{\bullet-}$ did not induce Tyr nitration to the same degree as a bolus addition of synthetic $ONOO^-$ (Pfeiffer and Mayer, 1998). This study was erroneous in suggesting that $ONOO^-$ only contributes to NO_2Tyr formation under very high, nonphysiological fluxes of $O_2^{\bullet-}$ and $^{\bullet}NO$ (Pfeiffer et al., 2000). A central problem with these studies was that they were conducted in homogeneous, aqueous systems, and NO_2Tyr formation was hampered by uric acid consumption of $ONOO^-$ in the xanthine/xanthine oxidase system used to generate $O_2^{\bullet-}$. Also the oxidative products formed will autoinactivate the $O_2^{\bullet-}$-generating system, and purine substrate depletion is expected

to occur during long incubation periods. It has also been proposed from in vitro studies that steady-state production of low fluxes of $^{\bullet}NO$ and $O_2^{\bullet-}$ may not result in NO_2Tyr formation because Tyr competes with $Tyr^{\bullet}$ for $^{\bullet}NO_2$, thereby limiting the extent of NO_2Tyr formation (Goldstein et al., 2000). Additional studies employing shorter reaction times for the co-generation of $^{\bullet}NO$ and $O_2^{\bullet-}$, more sensitive NO_2Tyr detection techniques, and XO substrates that do not readily scavenge $ONOO^-$ have resolved these discrepancies, and it is now more generally accepted that in situ generation of $ONOO^-$ (in the absence of competing radicals) will induce Tyr nitration (Reiter et al., 2000).

A major concern regarding $ONOO^-$ as a primary contributor to oxidative/nitrosative damage in vivo is that $ONOO^-$ generation requires the simultaneous production of $O_2^{\bullet-}$ and $^{\bullet}NO$ in the same subcellular locale, a condition that is difficult to predict or measure in a non-homogeneous, compartmentalized biological system (Grisham et al., 1999). In vitro studies show that equivalent rates of $^{\bullet}NO$ and $O_2^{\bullet-}$ production in a 1:1 ratio are optimal for $ONOO^-$ formation (Miles et al., 1996), suggesting that obtaining favorable NO_2Tyr yields might necessitate an equivalent ratio of radicals because a surplus of either $^{\bullet}NO$ or $O_2^{\bullet-}$ can react additionally with $Tyr^{\bullet}$ and $^{\bullet}NO_2$ and divert these radicals down other reaction pathways away from NO_2Tyr formation. Recent studies reveal that the direct reactions of $ONOO^-$ are not affected by excess $^{\bullet}NO$ or $O_2^{\bullet-}$ (Jourd'heuil et al., 2001), but NO_2Tyr formation relies on the protonation of $ONOO^-$ to ONOOH and homolytic scission to yield $^{\bullet}OH$ and $^{\bullet}NO_2$, which can still react with excess $^{\bullet}NO$ and $O_2^{\bullet-}$ to attenuate NO_2Tyr production. Examination of a biological model (activated murine peritoneal macrophages) for $ONOO^-$ production argued against a role for $ONOO^-$ in protein Tyr nitration in vivo, since $^{\bullet}NO$ and $O_2^{\bullet-}$ were produced on different time scales, and protein Tyr nitration that did occur was attributed to a $ONOO^-$-independent mechanism (Pfeiffer et al., 2001). Similarly it was observed that $ONOO^-$ did not cause nitration of green fluorescent protein in an in vivo system (Espey et al., 2002). While additional peroxidase-catalyzed pathways for NO_2Tyr formation exist in vivo and in vitro, they do not necessarily negate the contribution of $ONOO^-$ to this phenomenon in vivo. In support of this, recent in vivo studies have ascribed a prominent role for endogenous $ONOO^-$ in mediating NO_2Tyr formation in vascular smooth muscle cells exposed to cytokines and lipopolysaccharide (Fries et al., 2003) and in the motor neurons undergoing apoptosis (Estevez et al., 1999), where both processes are $O_2^{\bullet-}$-dependent, NO_2Tyr-yielding pathways that also depend on the bioavailability of enzymatically produced $^{\bullet}NO$.

Homolytic Cleavage of Nitrosoperoxocarbonate While the direct reactions of $ONOO^-$ and its conjugate acid, ONOOH, to produce in vivo Tyr nitration may be limited, transformation of the oxidative chemistry of $ONOO^-$ by reaction with carbon dioxide/bicarbonate (CO_2/HCO_3^{2-}) potently changes the reaction rates, product yields, and product distribution of $ONOO^-$-mediated reactions, with these changes significantly increasing protein Tyr nitration yields. The changes in $ONOO^-$ reactivity upon exposure to CO_2 were first noticed in studies

examining the chemiluminescence of luminal in the presence of $ONOO^-$, which can oxidize luminol to the luminol radical, a precursor to the unstable luminol endoperoxide that decomposes by a light emitting pathway (Radi et al., 1993). In the presence of bicarbonate buffers, but not in phosphate-based buffers, an increase in $ONOO^-$-mediated luminol oxidation occurred, indicating that a more reactive oxidizing species was being created. To explain this phenomena, the formation of nitrosoperoxocarbonate ($ONOOCO_2^-$) and its decay into $^\bullet CO_3^-$ was proposed (Radi et al., 1993).

Peroxynitrite reacts with CO_2 at a reaction rate of $4.6 \times 10^4\ M^{-1}\,s^{-1}$ at pH 7.4 and 37°C to produce $ONOOCO_2^-$ (Denicola et al., 1996), a short-lived radical species (<1 μs), whereby nearly 30% decomposes to give $^\bullet NO_2$ and $^\bullet CO_3^-$ and nearly 70% of $ONOOCO_2^-$ decomposes by hydrolysis to give NO_3^- and CO_2 (Fig. 23.1). A mechanism for Tyr nitration was advanced involving one electron oxidation by $ONOOCO_2^-$, rather than direct nitration of the Tyr aromatic ring by a $ONOOCO_2^-$ intermediate (Lymar et al., 1996). This free radical mechanism proposed that $^\bullet CO_3^-$ is an extremely facile oxidant for Tyr and preferentially generates $Tyr^\bullet$ by abstracting protons from the phenolic ring of Tyr in a manner more efficient than the $ONOO^-$-mediated route (Alvarez and Radi, 2003); the $^\bullet NO_2$ radical then adds to $Tyr^\bullet$ to give NO_2Tyr (Fig. 23.1). This mechanism was confirmed using continuous fast flow EPR to detect $^\bullet CO_3^-$ formed during $ONOOCO_2^-$ decomposition (Bonini et al., 1999). Compared to the homolysis of protonated $ONOO^-$, which occurs at a slow rate ($0.9\ s^{-1}$), it is kinetically more likely that the formation of NO_2Tyr is mediated by the faster reaction of $ONOO^-$ with CO_2. Additionally an increase in nitration reactions in response to $ONOOCO_2^-$ can be attributed to elevated $^\bullet NO_2$ generation due

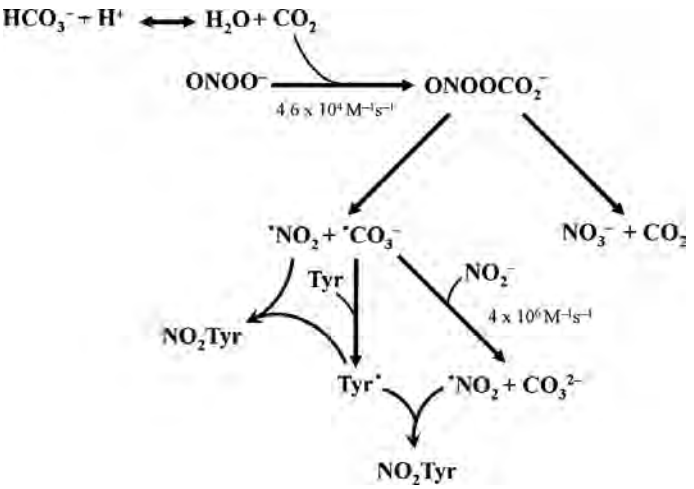


FIGURE 23.1 Pathways of tyrosine nitration resulting from nitrosoperoxocarbonate decay.

to rapid electron transfer between $^{\bullet}CO_3^-$ and NO_2^- to form CO_3^{2-} and $^{\bullet}NO_2$ ($k = 4 \times 10^6\ M^{-1}\ s^{-1}$) (Schopfer et al., 2003) (Fig. 23.1). Since CO_2 levels are present in high concentrations both intracellularly and extracellularly (>1 mM) and since the reaction of CO_2 with $ONOO^-$ is one of the fastest known reactions of $ONOO^-$ (Lymar and Hurst, 1995), $ONOOCO_2^-$ may be a more physiologically relevant nitrating agent (Dalle-Donne et al., 2005; Radi et al., 1993).

Further studies confirmed the oxidant-enhancing effect of HCO_3^{2-}/CO_2 on $ONOO^-$-mediated reactions, and it was shown that, in the presence of HCO_3^{2-}, free Tyr nitration increased twofold in vitro in a pH-dependent manner (van der Vliet et al., 1994; Lymar et al., 1996; Denicola et al., 1996). In contrast, other in vitro studies placed more emphasis on further reactions of $^{\bullet}NO$ and $O_2^{\bullet-}$ with $ONOOCO_2^-$ and heralded an increase in nitrosation, rather than Tyr nitration, in the presence of bicarbonate buffer (Jourd'heuil et al., 1999). However, this precept has not been substantiated in vivo. To understand the reactivity of $ONOOCO_2^-$ toward more complex biomolecules such as proteins, studies on human plasma proteins (Gow et al., 1996a), on glutamine synthetase, and on BSA (Tien et al., 1999) showed that NO_2Tyr formation increased in a pH-dependent manner in the presence of CO_2. The acute effect of $ONOOCO_2^-$ on cellular environments undergoing inflammatory conditions has shown that $ONOO^-$-directed oxidative reactions such as protein nitration were also elevated in vivo. From a similar clinical perspective, treatment of acute respiratory distress syndrome involves ventilation of inflamed lungs in a manner that increases CO_2 tensions to create hypercapnic conditions (15% CO_2). This reduced ventilation rate and tidal volume is done in the hopes of reducing mechanical stress (barotrauma) to the alveolar air–blood barrier. However, epithelial barrier function is actually impaired in model systems due to the increase in inflammatory responses secondary to the increase in CO_2 levels. Alveolar epithelial cells under hypercapnic conditions also express elevated levels of $^{\bullet}NO$ and iNOS that are concomitant with an increase in cellular NO_2Tyr content formed from presumed reactions of $ONOOCO_2^-$-derived species (Lang et al., 2000). Along the same lines, hypercapnic conditions caused an increase in lung inflammation and oxidative injury in an anesthetized, ventilated rabbit model of acute respiratory distress syndrome, which correlated with an increase in lung iNOS expression and NO_2Tyr formation (Lang et al., 2005). The effects of hypercapnia appear in these cases to be injurious in a setting of inflammation rather than protective. These studies demonstrate that cell structure and function can be negatively impacted under conditions of elevated CO_2 due to apparently more potent actions of $ONOOCO_2^-$ compared with $ONOO^-$, in oxidative and nitrosative inflammatory responses.

Metal-Catalyzed, Peroxynitrite-Dependent Nitrotyrosine Formation There are several reports that metalloproteins, such as the heme-containing MPO and Cu,ZnSOD, are catalysts of nitration reactions by promoting the decomposition of $ONOO^-$ to $^{\bullet}NO_2$ (Ischiropoulos et al., 1992; Floris et al., 1993). Additionally, nonprotein metal centers undergo some of the fastest reactions known with $ONOO^-$ (Alvarez and Radi, 2003). A Lewis acid (LA) adduct is formed when

$ONOO^-$ combines with the metal center to yield $^{\bullet}NO_2$ and a corresponding radical intermediate ($^{\bullet}O–LA^-$), which can also rearrange so that the metal center becomes oxidized (oxo-compound, $O{=}LA^{\bullet-}$) (Radi et al., 2000). These metal-catalyzed decomposition reactions of $ONOO^-$ parallel the decomposition of ONOOH and $ONOOCO_2^-$ to $^{\bullet}NO_2$ and a one electron oxidant. The secondary oxidizing species at the metal center of a protein can react with proximal amino acids such as Tyr so that nitration is favored and enzyme activity is altered. For example, $ONOO^-$-treated MnSOD displays a loss of enzymatic activity, with mass spectrometric analysis identifying a catalytically critical Tyr34 residue located near the Mn center as a primary target of Tyr nitration (Yamakura et al., 1998). The presence of transition metal ions can enhance the nitration of this Tyr in MnSOD (Quijano et al., 2001).

23.3.2 Enzymatic Formation of Nitrogen Dioxide by Heme Peroxidases

Not only do metalloproteins react directly with $ONOO^-$ to promote protein Tyr nitration, but heme peroxidases, such as MPO and eosinophil peroxidase (EPO), contribute to Tyr nitration in the presence of H_2O_2 by converting NO_2^-, the primary metabolic end-product of $^{\bullet}NO$, to $^{\bullet}NO_2$ (van der Vliet et al., 1997; Brennan et al., 2002; Baldus et al., 2002; Wu et al., 1999; Pfeiffer et al., 2001). Like $ONOO^-$, the heme peroxidase-mediated generation of $^{\bullet}NO_2$ leading to protein nitration relies on inflammatory and infectious conditions that promote greater $^{\bullet}NO$ production. MPO-dependent NO_2Tyr formation relies on the bioavailability of NO_2^-, which is created endogenously in the vasculature by (1) the direct reaction of $^{\bullet}NO$ with molecular oxygen, (2) the reaction of methemoglobin (Hb^{3+}) with $^{\bullet}NO$ forming a complex (Hb–NO) that releases NO_2^- through hydrolysis, (3) the decomposition of ONOOH, and (4) dietary sources. Therefore increased NO_2^- levels frequently reflect an elevation in $^{\bullet}NO$ production. Nevertheless, the accrual of NO_2^- in vivo is prevented by its rapid oxidation to NO_3^- by oxymyoglobin, oxyhemoglobin or catalase.

The MPO/H_2O_2 system of oxidation employs a complex mechanism and involves three different enzyme redox states. When fully reduced, the ferric heme (Fe^{3+}) can be converted by H_2O_2 oxidation to the two-electron oxidized ferryl heme (Fe^{4+}) with an associated cation radical, a state termed compound I, that proceeds by the following reaction:

$$\text{Peroxidase } (Fe^{3+}) + H_2O_2 \rightarrow \text{Compound I } (Fe^{4+} + \text{cation radical}) \quad (3)$$

The strongly oxidizing compound I converts NO_2^- to $^{\bullet}NO_2$ through a one electron oxidation reaction, giving compound II and retaining the ferryl heme according to the following

$$\text{Compound I} + NO_2^- \rightarrow \text{Compound II } (Fe^{4+}) + {}^{\bullet}NO_2 \quad (4)$$

Reduction by Tyr, ascorbate or chloride converts MPO compound II back to the ground state. EPO follows an identical pathway, except that EPO's compound I

is more specific for a bromide ion, rather than chloride, oxidizing this substrate to hypobromous acid (HOBr). Initial in vitro investigations of NO_2^- oxidation by the MPO/H_2O_2 system indicated that $^{\bullet}NO_2$ was thought to be the likely intermediate product responsible for the formation of NO_2Tyr by a two-step mechanism (van der Loo et al., 2000). In the first step, Tyr is oxidized to Tyr$^{\bullet}$, and a subsequent $^{\bullet}NO_2$ can then combine with Tyr$^{\bullet}$ to form NO_2Tyr. This reliance on the one electron oxidation by $^{\bullet}NO_2$ in two different instances to form NO_2Tyr may not always result in nitration, since $^{\bullet}NO_2$ can quickly oxidize a wide variety of biomolecular targets (Beckman, 1996a). Direct oxidation of Tyr to Tyr$^{\bullet}$ by the MPO/H_2O_2 system can substitute for the free radical oxidation of Tyr by $^{\bullet}NO_2$; the effective one electron oxidation of Tyr to Tyr$^{\bullet}$ by MPO is not inhibited by chloride, which was thought to be the main physiological substrate of MPO compound I (Marquez and Dunford, 1995).

While other metalloproteins such as microperoxidase oxidize NO_2^- to $^{\bullet}NO_2$ (Ricoux et al., 2001), the heme peroxidases, MPO and EPO, contribute primarily to protein Tyr nitration in vivo (Schopfer et al., 2003). Under inflammatory conditions there is a large influx of leukocytes, including neutrophils and eosinophils, with the most abundant proteins stored in the azurophilic granules of these immune cells being MPO and EPO. These peroxidases are released into the vasculature during degranulation where they can reduce H_2O_2, organic peroxides, and multiple other substrates to generate microbicidal hypohalous acids from plasma levels of chloride and bromide. In addition RNS are generated by the leukocyte peroxidases that contribute to NO_2Tyr formation. Myeloperoxidase/EPO knockout mice and several in vivo models of acute inflammation and infection were utilized to demonstrate that NO_2^- was converted by MPO/EPO to form $^{\bullet}NO_2$, and that NO_2Tyr formation correlated with peroxidase activity in some cases (Brennan et al., 2002). Because NO_2Tyr formation was not increased in each model of inflammation, it was proposed that a two-electron oxidation of NO_2^- could lead to the generation of $ONOO^-$ that was transiently bound to the peroxidase heme. Release of metal-catalyzed $ONOO^-$ decomposition products was not observed directly, but this proposal agrees with the observations that active metal centers can catalyze NO_2Tyr production from $ONOO^-$. Additionally HOCl may be reacting with NO_2^- to form the potent chlorinating, nitrating, and oxidizing agent NO_2Cl, which has been demonstrated in vitro to nitrate Tyr residues in BSA (Eiserich et al., 1996) and LDL (Panasenko et al., 1997). Nitration by HOCl-derived products is MPO-dependent, limiting NO_2Tyr formation to inflammatory conditions involving polymorphonuclear leukocytes and macrophages with the NO_2Cl reaction less viable in vivo than in vitro (Whiteman et al., 2003).

Additional lines of evidence support the role of leukocyte peroxidases in protein Tyr nitration. Upon release into the vasculature by activated neutrophils, MPO can enter endothelial cells, following binding to endothelial glycosaminoglycans. After internalization, MPO can be trafficked to the sub-endothelial space and, in the presence of H_2O_2, catalyze the formation of NO_2Tyr. The potential of MPO to diffuse away from areas of direct neutrophil infiltration and cause

protein nitration was demonstrated using immunohistochemical techniques that illustrated the co-localization of MPO with areas of NO_2Tyr formation (Baldus et al., 2002). Fibronectin and other cytoskeletal proteins were abundantly nitrated along the vascular lumen and the alveolar epithelium in a variety of diseased tissues (coronary arteries of a patient with coronary artery disease, liver and lung from a sickle cell patient, and a lung biopsy from a lung transplant patient undergoing rejection). The formation of NO_2Tyr during inflammatory conditions in an MPO-dependent manner indicates that NO_2Tyr provides a viable marker of oxidative processes resulting from leukocyte infiltration. However, like HOCl formation, nitration by MPO or EPO necessitates the presence of a particular enzyme, which limits this mechanism of NO_2Tyr formation, conditions involving inflammatory cells likely to predominate in the vasculature (Radi et al., 2001).

23.3.3 Other Biologically Relevant Reactions of Nitric Oxide Leading to Nitrotyrosine Formation

Aside from the addition of $^{\bullet}NO_2$ to $Tyr^{\bullet}$ to form NO_2Tyr, $Tyr^{\bullet}$ can also undergo direct attack by $^{\bullet}NO$, forming a nitroso-Tyr, which upon further oxidation by $^{\bullet}NO$ results in the formation of NO_2Tyr. The direct nitration of $Tyr^{\bullet}$ by $^{\bullet}NO$ in a $ONOO^-$-independent manner has been observed in prostaglandin H synthase-2 (Gunther et al., 1997) and ribonucleotide reductase (Lepoivre et al., 1994), which rely on a catalytic metal center to direct $^{\bullet}NO$ reactivity toward the $Tyr^{\bullet}$. Another NO_2Tyr pathway that is independent of $ONOO^-$ depends on the acidification of NO_2^- to nitrous acid (HNO_2) under low pH conditions (pH < 3.0), such as those found in the gastric system and phagosomes and lysosomes. Tyrosine nitration of albumin and β-casein in vitro was achieved at low pH after long incubations in high concentrations of NO_2^- (25 mM) (Natake and Ueda, 1986). However, cysteine residues were predominantly converted to nitroso-cysteine over other residues in the presence of acidified NO_2^- (Simon et al., 1996), and so this pathway may only result in a minor route for the slow nitration of Tyr in conditions unfavorable to $ONOO^-$ formation. Additionally this reaction most likely leads to the formation of artifacts during chromatographic analysis of NO_2Tyr from samples extracted under acidic conditions (Yi et al., 2000).

The wide spectrum of reactions that promote NO_2Tyr formation in biological tissues indicates that NO_2Tyr cannot be used simply as a footprint for $ONOO^-$. Instead, NO_2Tyr modifications are a sign that there is an amplification in $^{\bullet}NO$ production and its reactions with oxygen or ROS with the source of nitration being a specific function of the type of disease, the progression of disease, the physiological process that mediates RNS or ROS upregulation, and the types and amounts of antioxidants present (Greenacre and Ischiropoulos, 2001). Because of the complexity of cellular systems, the nitration of proteins is likely to be the product of the multiple $^{\bullet}NO_2$-generating pathways that operate simultaneously. However, during conditions of chronic inflammation, such as atherosclerosis, where NO_2Tyr formation is concomitant with heme peroxidase activity and leukocyte infiltration, the MPO/H_2O_2 pathway is most likely going

to predominate (Ischiropoulos, 1998). In summary, the identification of specific nitrating agents in clinical inflammatory processes and models of inflammatory diseases is complex and multifaceted.

23.4 METHODS FOR DETECTING NITROTYROSINE

Nitrotyrosine and its deaminated, decarboxylated metabolite, 3-nitro-4-hydroxyphenylacetic acid, were first detected in human urine from sepsis patients using GS coupled to a thermal energy analyzer (Ohshima et al., 1990). Because the incidence of NO_2Tyr has been estimated to occur as infrequently as 1 in 10^6 in biological samples (Khan et al., 1998; Shigenaga et al., 1997), assays detecting NO_2Tyr must meet the requirements of precision, accuracy, and allow for a low limit of detection (Duncan, 2003). Due to the reactivity of Tyr with adventitious NO_2^-, these techniques additionally need to incorporate methods that can detect any artifactual generation of NO_2Tyr during sample processing and analysis, such as the inclusion of isotopomers prior to quantitative analysis.

Initially methods development centered on strategies that quantified global NO_2Tyr formation in biological samples or on those that allowed for definitive NO_2Tyr identification in tissues or complex mixtures. Increasingly procedures relied on the immunoreactivity of the NO_2Tyr adduct to antibodies raised against artificially nitrated proteins. Anti–NO_2Tyr antibodies have yielded extensive support for the ubiquity of Tyr nitration in biological systems, but their application has frequently not excluded the formation of other oxidized and nitrated biomolecules and the artifactual generation of NO_2Tyr during sample processing (Beckman, 1996a). Results from Western blotting experiments employing the anti–NO_2Tyr antibodies also have been confounded by signal dissipation due to the processing of biological samples in the presence of heme and reducing conditions, which leads to the conversion of NO_2Tyr to amino-Tyr, an adduct not detected by anti–NO_2Tyr antibodies (Balabanli et al., 1999). Assessment of total cellular NO_2Tyr was initially accomplished using the semiquantitative, yet high-throughput enzyme-linked immunosorbent assay (ELISA) (Khan et al., 1998). Past studies employing ELISA did not define the detection limits or the assay linearity, and nor did they account for the varying affinity of different NO_2Tyr antigens on proteins different from the originating antigen, making the quantitative data obtained in these studies suspect (Duncan, 2003).

To evaluate the levels of protein nitration using spectrophotometric methods, the pH-dependent peak absorbance for NO_2Tyr at 430 nm was used with relatively pure protein samples. At pH>9, the extinction coefficient for NO_2Tyr was 4,400 M^{-1} cm^{-1}, but the absorbance at 430 nm disappeared at lower pH. Therefore determination of the difference between the A_{430} at pH 10 and pH 5.5 gave a reasonable way to quantify NO_2Tyr levels in pure proteins exposed in vitro to bolus additions of $ONOO^-$ (Crow and Ischiropoulos, 1996). However, quantification of NO_2Tyr levels in biological samples requires more sensitive approaches that involve chromatography linked to spectrophotometric or electrochemical detection. The majority of the high-pressure liquid chromatography

(HPLC) methods have been shown to be too insensitive to detect the low levels of NO_2Tyr in biological tissues when coupled with ultraviolet or fluorescence based detection (Duncan, 2003). Additionally the good separation and high sensitivity of NO_2Tyr using HPLC methods coupled with electrochemical detection have been complicated by peaks from biological samples with similar retention times (Kaur et al., 1998).

To more incisively detect biological NO_2Tyr levels, isotope dilution gas chromatography (GC) joined with mass spectrometry (MS) detection methods on chemically hydrolyzed tissue and fluid samples has been employed. Typically, after the addition of isotope-labeled internal standards to a delipidated sample, the protein is hydrolyzed with acid, and the resulting amino acids are isolated from the hydrolysate by chromatography on a solid phase extraction column prior to derivatization (Heinecke, 1999). These methods have considerable drawbacks, however, because the high temperatures and chemical derivatization strategies employed to give the sample volatility for GC analysis often degraded amino acids. Protein hydrolysis with hydrochloric acid (HCl) also cause artifactual NO_2Tyr generation due to the frequent presence of adventitious NO_2^- that can nitrate Tyr under acidic conditions (Shigenaga et al., 1997). Consequently methods involving alkaline hydrolysis were developed instead (Frost et al., 2000). Unfortunately, analysis of low levels of NO_2Tyr in complex biological mixtures remains problematic because GC–MS methods still do not reach the required sensitivity (Duncan, 2003).

HPLC, in line with tandem MS (HPLC/MS/MS) of protein and tissue homogenates after chemical or enzymatic hydrolysis, has proved to be a more simple and sensitive approach than GC-based methods. This is because the use of soft ionization techniques such as electrospray ionization (ESI) precludes the need for sample derivatization and high temperatures. Studies using this technique often involve the addition of stable isotope internal standards to protein fractions obtained from in vivo samples to monitor intrapreparative nitration, prior to preseparation of the reaction mixture by HPLC (Brennan et al., 2002). Separated analytes ionized in the ESI chamber are detected in the multiple reaction monitoring (MRM) scan mode of a triple quadrupole tandem mass spectrometer. Only those ions with mass to charge (m/z) ratios corresponding to NO_2Tyr or stable isotope-labeled NO_2Tyr internal standards are evaluated. Measurement of chromatographic peak areas of select product ions taken from the full-scan total ion chromatogram routinely allow the detection of NO_2Tyr at levels of 10 fmol, improving the sensitivity fivefold over the lower limit of GC–MS based methods (50 fmol) (Radi et al., 2001). For now, the accurate assessment of NO_2Tyr levels in biological samples relies on expensive tandem MS instrumentation that is not widely accessible and may limit experimental opportunities for some laboratories.

To begin to link the phenomena of inflammatory-induced increases in protein NO_2Tyr derivatives to protein dysfunction and consequent pathological conditions, evaluation of discrete NO_2Tyr modifications on specific proteins must be undertaken. Mass spectrometric, proteomics-based strategies allow for the identification of all individual proteins that are nitrated by separating a tissue

homogenate using 2-D gel electrophoresis, detecting the nitrated proteins using an anti-NO_2Tyr antibody, and then identifying the peptides generated during an in-gel proteolytic digest using matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) MS. Because nitration increases the molecular weight of the protein by 45 Da, peptides containing the NO_2Tyr modification can be identified. However, if other post-translational modifications occur, molecular weight interpretation may not be straightforward, and proteolytically digested peptide fragments have to be identified using the unique absorption properties of NO_2Tyr. This method was successful in identifying more than 40 NO_2Tyr-immunopositive proteins modified during in vivo inflammatory conditions (Aulak et al., 2001). However, this MS method was not used to identify the exact sites of specific protein Tyr modifications.

In order to fully understand the functional alterations induced by NO_2Tyr, identification of specific sites of NO_2Tyr modifications, rather than overall levels of NO_2Tyr, provides powerful insight into the implications of protein nitration. Techniques that employ affinity purification of the nitrated protein of interest and subsequent tandem MS analysis to identify nitration sites have begun to give a clearer picture of the specificity of nitration (Jiao et al., 2001; Aslan et al., 2003; Viner et al., 1999; Roberts et al., 1998; Schmidt et al., 2003; Ischiropoulos et al., 1992). To determine the location of exclusively nitrated Tyr, modified peptides from tryptic digests were analyzed in the positive ion mode using MALDI-TOFMS where the expected molecular ion for the side chain of NO_2Tyr is observed in addition to decomposition products involving the loss of one and two oxygens (-16 and -32 Da, respectively). Reduction of peptides to the amino-Tyr adduct forms two new decomposition species with two hydrogens each (-14 and -30 Da, respectively) that can be used to identify NO_2Tyr-containing peptides even in complex mixtures (Sarver et al., 2001). Alternatively, ESI-MS can be used to analyze nitration sites using a similar strategy where the mass difference between the NO_2Tyr and amino-Tyr peptides is used to identify peptides containing NO_2Tyr. After alkylating free cysteines, the masses of peptides separated by HPLC can also be analyzed by MALDI-TOFMS and then compared to database sequences for identification (Aslan et al., 2003). Collision-induced dissociation (CID) sequencing can also be used to identify nitration sites. One drawback to these methods is that accurate quantification of the NO_2Tyr modification can be hampered by variable peptide release from the whole protein during tryptic digest. To overcome this problem, the native reference peptide (NRP) method can be used in LC–MS based techniques, which allows for the peak area of an unmodified peptide from the same tryptic digest to be used as an internal standard and compared to the peak area of the NO_2Tyr-containing peptide (Willard et al., 2003).

The "holy grail" of NO_2Tyr identification and quantification is a method that traps and labels all NO_2Tyr-containing proteins in vivo and allows for the selective removal of nitrated proteins from complex mixtures for mass analysis and identification. One approach for the detection and characterization of low abundance NO_2Tyr-containing proteins is to chemically modify NO_2Tyr residues to

amino-Tyr and then specifically label the modified proteins with a cleavable affinity tag such as biotin. This way the tagged proteins can be captured on an affinity column (streptavidin) and released from the column (after washing off nonspecifically bound proteins) with a cleavage agent that releases the tag leaving a sulfhydryl-containing modified residue that has a unique and reliable MS signature (Nikov et al., 2003). However, the use of this approach does not take into consideration endogenously produced Tyr amination, which may lead to overestimation of nitration sites and has shown limited success when applied to complex biological samples (personal communication H. Ischiropoulos, 2004).

Because many of these techniques rely on expensive MS instrumentation that requires a highly-trained operator, they may not be widely accessible. Development of a small molecule that can trap NO_2Tyr modifications without further derivatization and can display a unique spectral signal could possibly enhance NO_2Tyr in vivo detection and allow for detailed study of the subsequent metabolism of NO_2Tyr-containing proteins. Furthermore development of antibodies specific for the nitrated form of the proteins that do not detect the nonnitrated form would also aid in the selective analysis of nitrated proteins. While sometimes technically challenging, the analytical MS techniques evaluating NO_2Tyr-containing proteins to date may provide the most accurate information regarding the location and magnitude of NO_2Tyr formation in vivo, especially when used in conjunction with immunohistochemical approaches (Greenacre and Ischiropoulos, 2001).

23.5 SELECTIVITY OF TYROSINE NITRATION

During pathological processes, nitration of proteins does not appear to occur in all cellular locations or in all tissue substructures. Instead, electron microscopy of tissues under various disease states treated with the anti–NO_2Tyr antibodies reveals that nitration is localized to specific areas of the cell (van der Loo et al., 2000; Bolan et al., 2000; Giasson et al., 2000). In the same way, the nitration of proteins in these subcellular locales is a selective process that is not directed by a specific Tyr-containing signature in the primary sequence nor is the occurrence of nitration simply due to the relative abundance of a particular protein or the total amount of Tyr (Souza et al., 1999). This speaks to the selectivity of the process, which is due to the many factors including the local environment in which the Tyr residue resides and the proximity of the protein to the generation of nitrating agents (Ischiropoulos, 2003). So far selective nitration of Tyr has been demonstrated in proteins diverse as cytochrome c, cytochrome P450, actin, histone, MnSOD, Cu,ZnSOD, α-synuclein, albumin, and angiotensin II (Schopfer et al., 2003). Additionally proteomic studies routinely show that only certain proteins are nitrated in selective tissue extracts (Aulak et al., 2001; Ara et al., 1998; Kanski et al., 2005).

Those proteins directly positioned near the source of nitrating agents are more susceptible to nitration than other proteins in the same subcellular environment.

In numerous clinical disorders associated with an upregulation in oxidative stress, Tyr nitration has been limited to certain cell types and to selective sites of injury, indicating that the targets of nitration may simply be due to immediate spatial association with sites of RNS and ROS production (Ischiropoulos, 2003). This is aptly illustrated in studies mapping the relative distribution of MPO and NO_2Tyr formation, where it was shown that nitrated fibronectin, an extracellular matrix protein, was spatially associated with MPO distribution (Baldus et al., 2001). In the same manner cellular location of proteins can influence extents of NO_2Tyr formation. For example, when Tyr are located in a hydrophilic environment (e.g., the cytosol), they are more readily ionized, so MPO-catalyzed Tyr nitration reactions that rely on Tyr protonation are more likely to occur (Schopfer et al., 2003). Likewise the hydrophobic environments of membrane-bound proteins may promote Tyr nitration because $^{\bullet}NO_2$ has a longer half-life in membranes and partitions easily into hydrophobic environments (Zhang et al., 2001). It was further demonstrated that increases in $ONOO^-$ mediated nitration of membrane protein Tyr residues was facilitated by the increasing depth of the Tyr residue inside of lipid bilayers (Zhang et al., 2003).

Unlike other Tyr modifications such as phosphorylation and sulfation, the selectivity of Tyr nitration does not appear to be a function of the immediate primary sequence (Ischiropoulos, 1998) and instead is directed by local electrostatic and structural environments in a folded protein. For instance, the presence of a proximal, negatively-charged residue (e.g., Glu or Asp) promotes the selective nitration of a Tyr provided there is an absence of Cys and Met in the local environment, as these residues will preferentially react with nitrating agents (Alvarez et al., 1999). It is important to consider that the immediate electrostatic environment may also play a role in modulating the local concentration of charged nitrating species, which can also influence protein nitration (Ischiropoulos, 2003). Tyr residues are more likely to be surface exposed because of their lower hydrophobicity packing order compared to other amino acids (Ischiropoulos, 2003). In support of this precept, it has been demonstrated that nitration occurs predominantly on solvent accessible Tyr, even though not all surface exposed Tyr are nitrated (Berlett et al., 1996). Despite the influence of hydrophobic environments such as the lipid bilayer on Tyr nitration in model studies, Tyr located in the hydrophobic interior of the protein are less vulnerable to nitration (Schopfer et al., 2003). Even the secondary structure of the protein can influence Tyr nitration, with Tyr nitration in β-sheets and α-helices occurring less predominantly than nitration of Tyr in loops (Souza et al., 1999). While not all nitrated amino acids are located in loop structures, the lack of steric hindrance upon nitration of surface-exposed, protruding residues may facilitate this post-translational event, especially when mediated by a $^{\bullet}CO_3^-$ radical (Alvarez and Radi, 2003).

Finally, the presence or absence of enzymic metal cofactors proximal to a Tyr residue is expected to confer specificity to nitration due to the metal-catalyzed formation of $^{\bullet}NO_2$ from $ONOO^-$. For example, specific nitration of Tyr430 in prostacyclin synthase isolated from bovine aortic microsomes treated with $ONOO^-$ occurred at a Tyr proximally located to the active site heme (Schmidt

et al., 2003). Similarly, selective nitration of Tyr positioned closely to active site metal centers was observed in cytochrome $P450_{BM-3}$ (Daiber et al., 2000), in MnSOD (Yamakura et al., 1998), and in cytochrome c (Cassina et al., 2000). However, the critical Tyr122 radical in the small subunit of ribonucleotide reductase that was adjacent to the catalytic iron center was not extensively nitrated after $ONOO^-$ treatment, most likely due to its restricted accessibility in the interior of the protein, indicating that the mechanisms directing Tyr nitration specificity are complex (Guittet et al., 2000). In summary, protein abundance, total number of Tyr residues, and primary sequence do not play predominate roles in the selectivity of Tyr nitration. Alternatively, other factors including the relative Tyr protein surface/solvent availability, the absence of steric hindrance, and local electrostatic features constitute structural requirements that play more operative roles in guiding protein Tyr nitration (Ischiropoulos, 2003).

23.6 MECHANISTIC CONSEQUENCES OF NITROTYROSINE FORMATION: PROTEIN NITRATION IN VIVO AND VASCULAR DISEASE

The impact of the nitration of a single Tyr residue often has broad implications on the activity of biologically critical proteins, which has become increasingly related to pathological conditions. NO_2Tyr-induced mechanistic changes alter protein structure and function due to the creation of a bulky, anionic adduct on Tyr, which triggers changes in local protein conformations and electrostatic environments. Depending on the local environment, the addition of $–NO_2$ to Tyr can lower the pK_a of the phenolic –OH by two to three units from 10.1 to 7.2, which in return imparts a net negative charge to half of the nitrated residues at physiological pH (Schopfer et al., 2003). These alterations induced by Tyr nitration give rise to many different functional changes, including gain of protein function. For example, the addition of peroxidase activity in cytochrome c (Cassina et al., 2000), accelerated clot formation with fibrinogen (Vadseth et al., 2004), and translocation of protein kinase C ε (Balafanova et al., 2002) all occur after nitration of a critical Tyr. What is found most commonly, however, is that Tyr nitration is associated with the reduction or loss of essential enzyme activity, leading to cellular dysfunction and disease progression.

The nitration of Tyr causes conformational changes that impede iNOS dimerization (Lanone et al., 2002), displace heme ligands in cytochrome c (Cassina et al., 2000), block substrate accessibility in MnSOD (Yamakura et al., 1998), and disrupt stabilizing hydrogen bonding interactions in cytochrome P450 (Lin et al., 2003). In these proteins structural changes induced by either Tyr nitration or concomitant oxidation of other amino acid residues lead to abolition or decreases in enzyme activity. Additionally, the steric hindrance and protein structural distortions caused by Tyr nitration were shown to interfere with other post-translational modifications, including the phosphorylation of Tyr in the cell cycle kinase CDC2, leading to disruptions in cell signaling (Kong et al., 1996).

Charge–charge interactions can also be affected by nitration, as evidenced by the altered contacts between the mammalian aldolase A carboxyl-terminal region and its substrate, causing a decline in activity when Tyr363 was selectively nitrated by $ONOO^-$ (Koeck et al., 2004b). Increasingly NO_2Tyr adducts have been found in structural proteins, affecting their assembly and association with other proteins. The introduction of the electronegative $–NO_2$ group into actin has been shown to reduce the dynamic assembly/disassembly process of actin filaments (Aslan et al., 2003), while the incorporation of free NO_2Tyr into α tubulin significantly alters microtubule formation and structure (Eiserich et al., 1999). A growing list of nitrated proteins correlates the negative effects of protein nitration with their accumulation in a wide variety of diseases related to oxidative stress (Greenacre and Ischiropoulos, 2001).

23.6.1 Actin Nitration in Sickle Cell Disease

Increased plasma and tissue protein NO_2Tyr derivatives in an animal model of sickle cell disease (SCD) and clinical samples obtained from SCD patients (Aslan et al., 2003) reinforce that oxidative inflammatory pathways are operative and are mediating pathogenic tissue responses that lead to the post-translational nitration of structurally and functionally important target molecules. The occurrence of xanthine oxidase-derived $O_2^{\bullet -}$ production in SCD (Aslan et al., 2001) and elevated expression of iNOS in kidney and liver of SCD mice and humans (Aslan et al., 2003) leads to enhanced production of $ONOO^-$. Similarly increased tissue levels of CO_2 that arise as a consequence of impaired vascular function occur in SCD (Maitre et al., 2000) and create a setting for increased formation of the secondary nitrating species, $ONOOCO_2^-$ (Radi et al., 1999). Additionally MPO and other heme proteins abundantly present in SCD can oxidize NO_2^- (Eiserich et al., 1998, 2002), a $^{\bullet}NO$ metabolite shown to be elevated in SCD (Rees et al., 1995) that serves as a precursor for the nitrating species $^{\bullet}NO_2$. Finally, the acidotic conditions present in poorly perfused tissue compartments may promote protonation of NO_2^-, conferring a chemistry that can also result in HNO_2-mediated Tyr nitration (Knowles et al., 1974).

Immunoprecipitation and MALDI-TOFMS assisted identification of actin as the predominant nitrated protein in the liver and kidney of SCD mouse provided critical insight into pathogenic events to be expected from this inflammatory milieu. Actin, one of the most abundant proteins in eukaryotic cells, constitutes 5% or more of cell protein (Sheterline and Sparrow, 1994) and serves with other cytoskeletal proteins such as tubulin (Eiserich et al., 1999) as a critical target for nitration-induced functional impairment. MALDI-TOFMS and MS/MS analysis of NO_2Tyr-enriched actin fractions from SCD mouse liver and kidney homogenates revealed nitration of Tyr91, Tyr198, and Tyr240 (Aslan et al., 2003). As shown in Figure 23.2*a*, the MS/MS spectrum of the peptide corresponding to actin residues 85–95 (^{85}IWHHTFYNELR^{95}) reflected a mass increase of +45 in y_{10}, y_9, y_8, y_7, and y_6 daughter ions indicative of Tyr91 nitration. Likewise the MS/MS spectrum of the peptide corresponding to residues

197–206 (^{197}GYSFTTTAER^{206}) showed a b_2 ion with an increase of +45 mass units, identifying Tyr198 as the nitrated residue (Fig. 23.2*b*). The MS/MS spectrum of the tryptic fragment ^{239}SYELPDGQVITIGNER^{254} also revealed a b_2 ion that shifted +45 mass units, indicative of Tyr240 nitration (Fig. 23.2*c*).

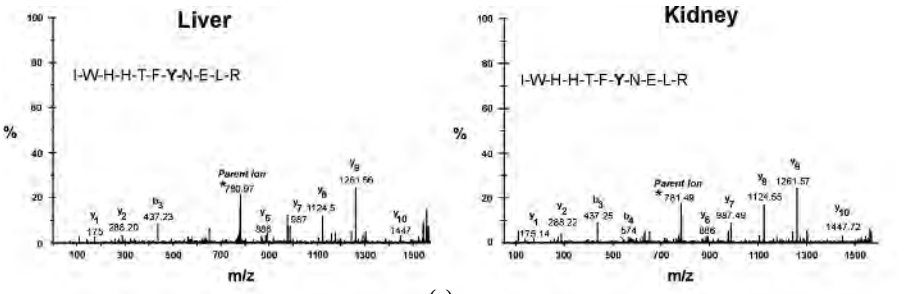
Confocal microscopy images of tissue actin distribution and morphology strongly affirm the influence of Tyr nitration on actin polymerization properties by reflecting a disorganized actin assembly in regions of both mouse and human SCD kidney where NO_2Tyr-containing actin is localized (Aslan et al., 2003). Indeed, owing to the cooperative nature of actin subunit assembly (Erickson, 1989), the functional consequences of Tyr nitration on actin dynamics is profound. The introduction of an electronegative NO_2 group onto Tyr198 and Tyr240, located at the "pointed" end of actin (Fig. 23.2*d*), leads to the formation of ionic bonds with cationic residues located at the barbed end of a growing filament. This interaction stabilizes both actin nucleus and filament formation, as evidenced by the shortened lag phase and accelerated filament elongation. Depolymerization of the actin filament is twofold slower for nitrated actin as compared to native actin, which is consistent with the higher affinity of nitrated monomeric actin for the actin filament (Aslan et al., 2003).

A dynamic network of cytoskeletal actin is required for cell function by compartmentalizing metabolic pathways (Hennessey et al., 1993), promoting intracellular motility (Weeds et al., 1991), and maintaining a dynamic cytoskeleton (Way and Weeds, 1990). Organization of actin filaments is also necessary for a direct physical link between the extracellular matrix and the cytoskeleton (Maniotis et al., 1997). Importantly, multiple stimuli for actin filament depolymerization will induce apoptosis (Martin and Leder, 2001; Re et al., 1994; Suarez-Huerta et al., 2000). The ability of actin Tyr nitration to alter actin polymerization thus also links actin nitration with enhanced apoptosis observed in regions of NO_2Tyr immunoreactivity in the liver and kidney of SCD mouse and human (Aslan et al., 2003).

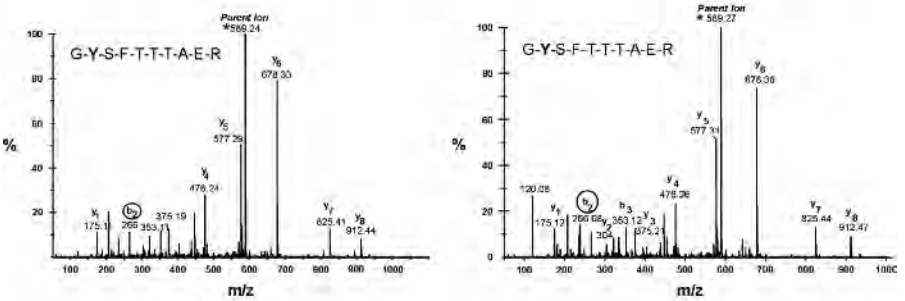
In summary, an oxidative inflammatory milieu exists in the vasculature, kidney, and liver of SCD patients with $^{\bullet}$NO-mediated nitration reactions catalyzing the post-translational modification and functional impairment of a key cell cytoskeletal protein, actin. In addition to adversely affecting vascular function, the selective nitration of liver and kidney actin Tyr can also lead to apoptotic cell death and loss of organ function observed in SCD.

23.6.2 Inducible Nitric Oxide Synthase Nitration in Sepsis

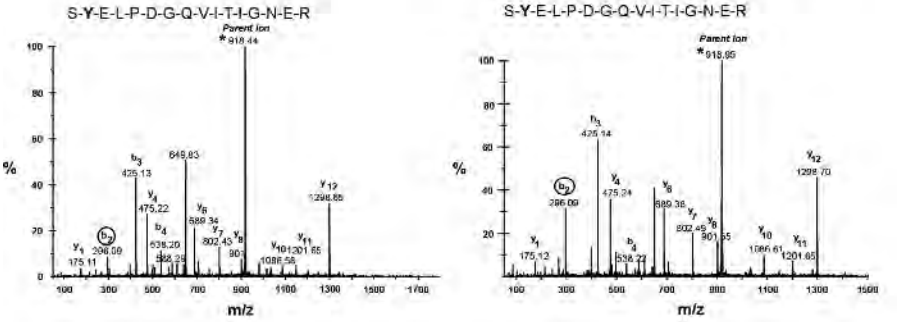
Excessive amounts of $^{\bullet}$NO production under conditions of oxidative stress results in $ONOO^-$ formation and elevated tissue NO_2^- and NO_3^- levels (Schopfer et al., 2003). In pathological conditions like sepsis, this phenomenon leads to nitration of target proteins, one of which is reported to be iNOS (Lanone et al., 2002). Inducible NOS nitration is documented in *rectus abdominis* muscle biopsy specimens obtained from septic patients undergoing surgical treatment related to intra-abdominal infection (Lanone et al., 2002). Appreciating that sepsis is also


Liver
I-W-H-H-T-F-Y-N-E-L-R
Parent Ion
*780.97
m/z
Kidney
I-W-H-H-T-F-Y-N-E-L-R
Parent Ion
*781.49
m/z

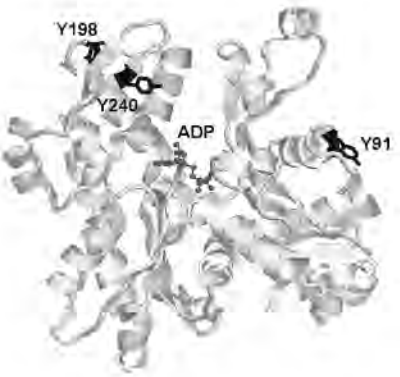
(a)


G-Y-S-F-T-T-T-A-E-R
Parent Ion
*568.24
m/z
G-Y-S-F-T-T-T-A-E-R
Parent Ion
* 569.27
m/z

(b)


S-Y-E-L-P-D-G-Q-V-I-T-I-G-N-E-R
Parent Ion
*918.44
m/z
S-Y-E-L-P-D-G-Q-V-I-T-I-G-N-E-R
Parent Ion
*918.95
m/z

(c)


Y198
Y240
ADP
Y91

(d)

associated with ongoing inflammation, MPO-dependent oxidation of NO_2^- to the nitrating species $^{\bullet}NO_2$ might be considered as a mechanism leading to protein nitration in septic patients (Eiserich et al., 1998, 2002). However, while non–neutrophil-associated MPO distribution was not considered, the possibility of a peroxidase-dependent nitration was excluded in this reported study because no inflammatory cell infiltration was observed in patient tissues examined. This seems a reasonable conclusion, as uncoupled iNOS can also serve as a source of $O_2^{\bullet -}$ and consequently $ONOO^-$.

Muscle homogenates from 16 sepsis patients and 21 controls were analyzed for protein nitration and iNOS expression via Western blot analysis. Immunoblotting with an anti–NO_2Tyr antibody revealed a single 130 kDa NO_2Tyr-containing protein in septic patients that was not present in controls. Similarly, Western blot analysis with an anti–iNOS antibody showed a single 130 kDa protein band present only in patient samples. From these observations the investigators concluded that the 130 kDa NO_2Tyr-containing protein was iNOS (Lanone et al., 2002). MALDI-TOFMS identified nitration of 4 of the 31 Tyr residues present in human iNOS. Nitration of Tyr299 and Tyr366 was observed in the three septic patients examined, and additionally Tyr446 and Tyr698 nitration were observed separately in two of the patients. The analysis of the four nitrated Tyr sites revealed that these residues belonged to a sequence motif described as Asp/Glu/Gln-Xaa_{3-6}-Ly*S*-Xaa_{0-1}-Tyr-Xaa_{4-7}-Asp/Glu, where Xaa is an indeterminate amino acid residue and the numerical subscripts describe the number of undefined residues between neighboring residues in the sequence motif. In the reported study (Lanone et al., 2002), in vitro Tyr nitration of human recombinant iNOS with $ONOO^-$ led to nitration of the same four Tyr residues that were nitrated in samples obtained from septic patients. In vitro nitration of iNOS also resulted in a significant decrease in enzyme activity. Thus it was suggested that Tyr nitration induces a conformational change in the protein that impacts on dimerization and catalytic function. This presumed auto-inactivation of iNOS may thus represent a tissue adaptive response to inflammation.

23.6.3 Myofibrillar Creatine Kinase Nitration in Heart Failure

Myofibrillar isoform of creatine kinase (MM-CK) is a protein found in myofibrils and functions in energy producing and utilizing pathways (Ventura-Clapier et al., 1998). Understanding the basics of heart muscle contraction and energy

FIGURE 23.2 MS/MS identification and representation of in vivo nitrated actin residues. (*a*) MS/MS spectrum of the tryptic fragment ^{85}IWHHTFYNELR^{95} $[M + 2H]^{2+}$ (m/z 781). (*b*) MS/MS spectrum of the tryptic fragment ^{197}GYSFTTTAER^{206} $[M + 2H]^{2+}$ (m/z 589). (*c*) MS/MS spectrum of the tryptic fragment ^{239}SYELPDGQVITIGNER^{254} $[M + 2H]^{2+}$ (m/z 918). (*d*) Ribbon representation of actin [PDB Id: 1J6Z] produced using Rasmol version 2.7. Nitrated tyrosine residues are illustrated as black sticks and are labeled with the three-letter amino acid code. (Modified from Aslan et al., 2003, by permission of the American Society for Biochemistry and Molecular Biology).

metabolism is required to appreciate the consequences of MM-CK nitration on cardiac function, and thus will be briefly reviewed. Myofibrils are contractile elements in heart muscle and are formed from repeated units of sarcomeres. Sarcomeres contain an ordered array of thin and thick filaments composed of actin and myosin II, respectively (Cooke, 1986). At low cytosolic Ca^{2+} concentrations, accessory proteins bound to actin filaments, troponins and tropomyosin, block the interaction between actin and myosin. Nerve impulse-triggered release of Ca^{2+} from the sarcoplasmic reticulum leads to Ca^{2+}-troponin C binding, which allows myosin to slide past the actin filament. During cardiac contraction, the myosin heads bind and hydrolyze ATP, providing energy for filament movement (Holmes, 1997).

Energy is an absolute requirement for cardiac function. Heart muscle produces 90% of its energy from mitochondrial respiration (Ingwall and Weiss, 2004). Thus there is a stringent relationship between in vivo oxygen consumption and cardiac work that occurs at constant cellular ATP. The presence of high-energy phosphotransfer systems is an essential feature of cardiac energy metabolism. Creatine phosphate present in cardiac muscle serves as a reservoir of high-potential phosphoryl groups that can be readily used to regenerate ATP from ADP during strenuous exercise (Ingwall and Weiss, 2004). This reaction, as shown below, is catalyzed by creatine kinase (CK) at a rate nearly 10 times faster than the rate of ATP synthesis by oxidative phosphorylation (Bittl and Ingwall, 1985). Thus under conditions when ATP demand exceeds ATP supply, as in acute ischemic cardiac failure, use of creatine phosphate via the CK reaction is important to maintain high levels of cellular ATP:

$$\text{Creatine phosphate} + \text{ADP} + \text{H}^+ \leftrightarrow \text{ATP} + \text{Creatine} \tag{5}$$

Creatine kinase exists as dimers composed of two subunits, M and B, giving three isoenzymes, MM, BB, and MB (Ventura-Clapier et al., 2004). A fourth octomeric isoenzyme found in the mitochondria (mi-CK) represents 20% to 40% of all CK activity in cardiac cells (Wyss et al., 1992). Myofibrillar MM-CK can use creatine phosphate to re-phosphorylate all of the ADP produced by myosin ATPase and thus provides enough energy for maximal force and normal contractile kinetics (Ventura-Clapier et al., 1994). Myofibrillar MM-CK is also strongly bound to the sarcoplasmic reticulum membranes where it is functionally coupled to the Ca^{2+}-ATPase, and ensures efficient energy provision for calcium uptake (Ventura-Clapier et al., 1994).

The ability of the heart to increase cardiac muscle performance in response to demand is known as contractile reserve, which is dependent on energy reserves supplied from the transfer of phosphoryl groups from creatine phosphate to regenerate ATP (Ingwall and Weiss, 2004). Indeed decreasing energy reserve by chemically inhibiting CK activity has been shown to limit contractile reserve in isolated rat hearts (Tian and Ingwall, 1996; Hamman et al., 1995). Studies on creatine kinase activity, isozyme composition, and total creatine content have been performed on left ventricular myocardium biopsy samples and myocardium

homogenates obtained from human subjects (Ingwall et al., 1985; Nascimben et al., 1996). Data from these studies have shown that a decrease in creatine kinase activity and creatine content occur in both left ventricular hypertrophy and cardiac failure.

Although impairment of creatine kinase activity has been documented in heart failure, the mechanism by which this occurs remains to be fully elucidated. A recent study has suggested that nitration caused inactivation of MM-CK in experimental heart failure (Mihm et al., 2001a). The reported study was conducted on rats that have undergone left coronary artery ligation and showed Tyr nitration of MM-CK immunoprecipitated from the left myocardium. This finding was accompanied by observations of decreased CK activity (Mihm et al., 2001a). Similarly atrial myofibrillar fractions obtained from atrial fibrillation patients demonstrated significant elevation of protein nitration that was inversely correlated with MM-CK activity (Mihm et al., 2001b). In conclusion, present studies provide evidence that altered myofibrillar energetics contribute to contractile dysfunction and that protein nitration of MM-CK may play an important role in this condition by impairing catalytic activity of the enzyme. Further studies are needed to identify sites of Tyr nitration and establish the mechanism of nitration-induced inactivation of MM-CK and rule in or out the contribution of other potentially oxidized amino acids.

23.6.4 Prostacyclin Synthase Nitration in Atherosclerosis and Hyperglycemia

Prostaglandins (PG) are potent lipid-derived, receptor-dependent signaling molecules that are important in cell proliferation, differentiation and apoptosis. The committed step of PG synthesis is the conversion of arachidonic acid to prostaglandin H_2 (PGH_2) (Fig. 23.3). This reaction is catalyzed by prostaglandin endoperoxide synthase [PGHS; cyclooxygenase (COX)], an enzyme present in the endoplasmic reticulum and nuclear membrane of many cell types (Funk, 2001). PGHS has cyclooxygenase and peroxidase activities that are catalyzed by different active sites in the enzyme (Smith et al., 2000). The cyclooxygenase activity is responsible for incorporating two molecules of O_2 into arachidonic acid, forming prostaglandin hydroperoxy endoperoxide (PGG_2), which is then converted to prostaglandin H_2 (PGH_2) by the peroxidase activity (Mayes, 1996) (Fig. 23.3). Once synthesized, PGH_2 can subsequently be metabolized to prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) by prostacyclin and thromboxane synthase, respectively (Helliwell et al., 2004). Prostacyclin synthase (PGI_2 synthase) is a membrane-bound heme protein that is widely expressed in human tissues and is particularly found in endothelial cells. Prostacyclin is a vasodilator and inhibitor of platelet aggregation (Helliwell et al., 2004).

Prostaglandin H_2 can also undergo spontaneous or enzymatic transformation to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin E_2 (PGE_2), and prostaglandin D_2 (PGD_2) (Fig. 23.3). The predominance of relaxing prostanoids, such as PGI_2, or contracting prostanoids, such as PGH_2 and $PGF_{2\alpha}$, released by endothelial cells will primarily depend on the secondary pathways that yield the different

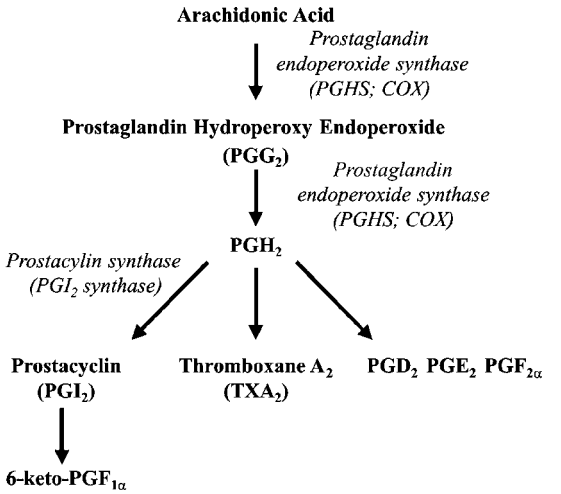


FIGURE 23.3 Diagram of the pathway involved in prostacyclin synthesis. The enzymes involved in each step are depicted in italics.

prostanoids (Helliwell et al., 2004). The release of PGI_2 can be measured by levels of its degradation product 6-keto-$PGF_{1\alpha}$, while PGI_2 synthase activity is measured by the conversion of PGH_2 into 6-keto-$PGF_{1\alpha}$ (Helliwell et al., 2004) (Fig. 23.3).

Nitration of PGI_2 synthase has been documented in atherosclerotic bovine coronary arteries (Zou et al., 1999). In the reported study, protein homogenates from normal and atherosclerotic bovine arteries were immunoprecipitated with either an anti–PGI_2 synthase or an anti–NO_2Tyr antibody. Precipitated proteins were then separated via SDS-PAGE and immunostained with either anti–NO_2Tyr or anti–PGI_2 synthase antibodies. Staining of Western blots with anti–NO_2Tyr showed selective nitration of PGI_2 synthase, while electrophoretic separation and silver staining of proteins following immunoprecipitation with anti–NO_2Tyr revealed a single 52 kDa band, which was identified as PGI_2 synthase via immunoblotting. Nitration of PGI_2 synthase was further confirmed via immunostaining arteries for both NO_2Tyr and PGI_2 synthase. Nitrotyrosine staining was prevalent in atherosclerotic lesions and co-localized with PGI_2 synthase.

Nitration of PGI_2 synthase in atherosclerotic vessels was accompanied by an altered prostanoid biosynthetic pattern and defective relaxation (Zou et al., 1999). Stimulation of atherosclerotic vessels with arachidonic acid caused decreased 6-keto-$PGF_{1\alpha}$ production and increased PGE_2 levels, suggesting an impaired PGI_2 synthase activity and reorientation of PGH_2 metabolism toward PGE_2. Isometric measurement of tension in bovine coronary arteries also revealed altered PGI_2-dependent relaxation in atherosclerotic vessels supporting the phenomenon of decreased PGI_2 synthase activity and accumulation of the potent vasoconstrictor PGH_2.

In vitro nitration of PGI_2 synthase with 25 μM $ONOO^-$ revealed specific nitration of Tyr430, as determined by Fourier transform–ion cyclotron resonance (FT-ICR) MS (Schmidt et al., 2003). HPLC analysis of $ONOO^-$-treated PGI_2 synthase, after extensive digestion with thermolysin, showed a distinct peak at 365 nm with a retention time of 31 minutes (Fig. 23.4*b*). A small peak with similar retention time was also observed in the untreated enzyme (Fig. 23.4*a*), indicative of a small amount of basal nitration. ESI/FT-ICRMS of the HPLC-isolated peptide yielded a single major protonated molecular ion at m/z 582.29073 (Fig. 23.4*c*), corresponding to a nitrated tetrapeptide (^{427}LKNY^{430}). Decreased catalytic activity of PGI_2 synthase following $ONOO^-$ treatment has led investigators to speculate that $-NO_2$ added to Tyr430 could cause steric hindrance and block access to the active site.

Prostacyclin synthase nitration has also been observed in human aortic cells exposed to high glucose (Zou et al., 2002). In the reported study, Tyr nitration of PGI_2 synthase was determined by positive NO_2Tyr immunoreactivity observed in Western blots of cell lysates immunoprecipitated with anti–PGI_2 synthase. Selective nitration of PGI_2 synthase in hyperglycemic cells was also confirmed after immunoprecipitation with anti–NO_2Tyr antibodies. Nitration of PGI_2 synthase was accompanied by decreased enzyme activity, as determined by decreased 6-keto-$PGF_{1\alpha}$ (Zou et al., 2002). In summary, PGI_2 synthase nitration has been documented in both atherosclerotic lesions and in hyperglycemic aortic cells. Nitration of PGI_2 synthase is accompanied by decreased enzyme activity, which could contribute to endothelial dysfunction observed in both atherosclerotic and diabetic patients.

23.6.5 Succinyl-CoA:3 Oxoacid CoA-Transferase Nitration in Diabetes

Succinyl-CoA:3 oxoacid CoA-transferase (SCOT) is localized in the mitochondrial matrix and catalyzes the formation of acetoacetyl-CoA from acetoacetate (Laffel, 1999). Acetoacetate is a ketone body formed by the liver from free fatty acids. During periods of glucose deficiency such as starvation or prolonged exercise, acetoacetate is released into the blood and converted into acetoacetyl-CoA in the heart, kidney, brain, and skeletal muscle. Once formed, acetoacetyl-CoA can enter the citric acid cycle to produce energy (Laffel, 1999). SCOT-catalyzed formation of acetoacetyl-CoA in extrahepatic organs is the rate determining step in ketolysis, which is the process of ketone body conversion into energy (Mitchell et al., 1995).

Altered myocardial energy substrate use has been implicated in diabetes-associated cardiomyopathies (Avogaro et al., 2004). Indeed decreased rates of ketone-body oxidation and decreased activity of SCOT have been shown in heart mitochondria of diabetic rats (Grinblat et al., 1986). Although molecular mechanisms leading to the impairment of SCOT activity in diabetes remain to be fully determined, a recent study has described that SCOT undergoes Tyr nitration, which is associated with decreased catalytic activity in diabetic heart (Turko et al., 2001). In the reported study, diabetes was induced in rats via a single intraperitoneal injection (60 mg/kg) of streptozotocin (STZ) and heart mitochondrial fractions were analyzed for SCOT Tyr nitration. Tyr nitration of SCOT in

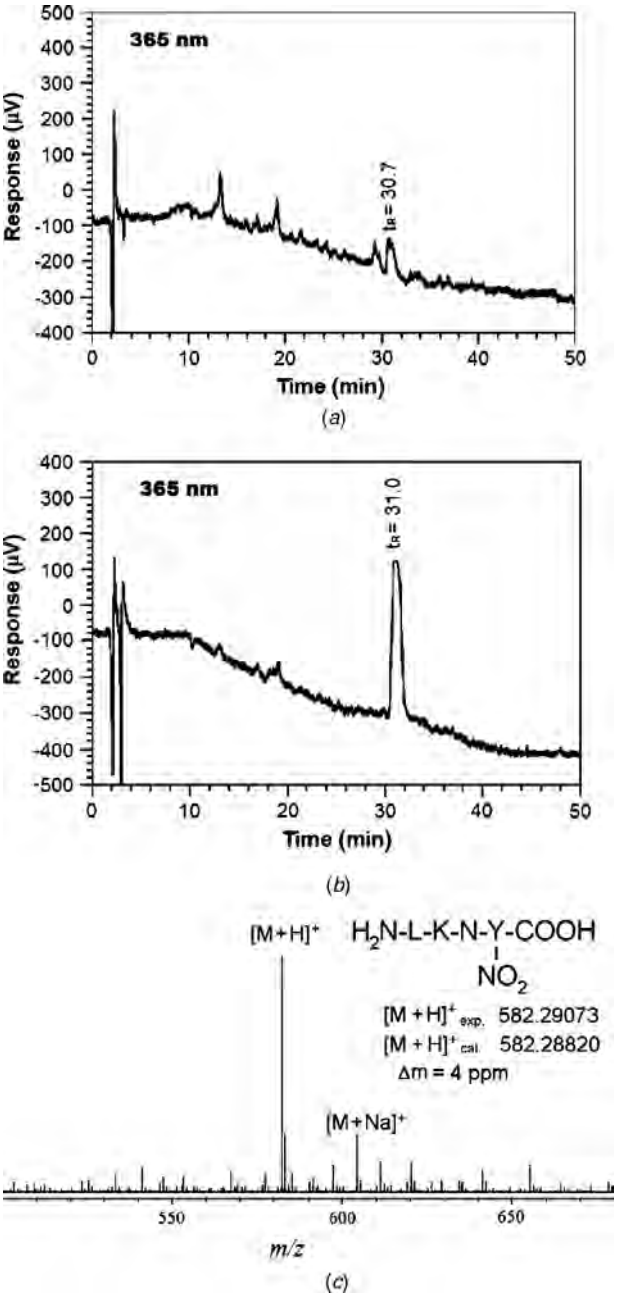


FIGURE 23.4 Identification of PGI_2 synthase nitration site. HPLC of (*a*) untreated and (*b*) $ONOO^-$-treated PGI_2 synthase digested with thermolysin. (*c*) ESI- FT-ICR MS spectrum of the HPLC-isolated peptide. (Modified from Schmidt et al., 2003, with permission of the American Society for Biochemistry and Molecular Biology).

diabetic rat hearts was determined by positive SCOT immunoreactivity observed in Western blots of solubilized mitochondria immunoprecipitated with anti–NO_2 Tyr antibodies. An increase in nitration observed at four weeks after STZ treatment was increased further after eight weeks and was accompanied by a progressive decrease in enzymatic activity (Turko et al., 2001).

In summary, SCOT is reported to be Tyr nitrated in diabetic rat hearts. Nitrated SCOT exhibits decreased catalytic activity, which may be an important contributing factor in altered myocardial energy use reported in diabetes. Further studies are needed to identify sites of Tyr nitration and establish the mechanism of nitration-induced inhibition of SCOT activity.

23.6.6 Alpha-1-Protease Inhibitor and Ceruloplasmin Nitration in Acute Respiratory Distress Syndrome

The main function of the lungs is gas exchange that occurs in the alveoli. Alveoli are small air sacs that have thin walls surrounded by capillary. These anatomic structures provide lungs with a large surface area to exchange oxygen and carbon dioxide (Fig. 23.5) (Matthay et al., 1993). The alveolar wall has a basement membrane positioned between capillary endothelial cells and epithelial cells that line the alveolar space.

Acute respiratory distress syndrome (ARDS) is pathophysiologically characterized by diffuse alveolar damage, resulting in the breakdown of both barrier and gas exchange functions of the lung (Petty, 1996). In the early stages, the syndrome presents all features of acute lung inflammation characterized by neutrophilic alveolitis and resultant leakage of protein-rich fluid into the interstitium and alveolar space (Garber et al., 1996). Elevated levels of neutrophil-derived products such as neutrophil elastase (Lee et al., 1981) and collagenase (Christner et al., 1985) have been observed in the alveolar space of patients with ARDS. These proteolytic enzymes have been implicated to play a role in progressive

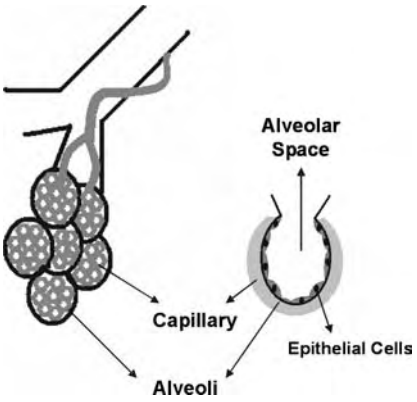


FIGURE 23.5 Diagram of alveoli in the lung. A cross section of a single alveolus is depicted on the right.

parenchymal damage associated with the fibrotic late phase of ARDS (McGuire et al., 1982).

Alpha-1-protease inhibitor (α_1-PI) is regarded as the main physiological inhibitor of neutrophil elastase and is found to be significantly elevated in bronchoalveolar lavage (BAL) fluid from patients with ARDS (Luisetti et al., 1992). Although α_1-PI can be secreted locally by inflammatory cells (Venembre et al., 1994), most of the enzyme reaching the pulmonary air space is considered to be derived from the circulation (Sallenave et al., 1999). Indeed α_1-PI recovered in BAL has the same molecular weight and glycosylation pattern as the enzyme produced in the liver (Sallenave et al., 1999).

Elevated levels of ceruloplasmin (CP) have also been detected in BAL fluid from patients with ARDS (Krsek-Staples et al., 1992). Although CP can be locally produced in the lungs (Yang et al., 1996), the main source of the enzyme found in BAL fluid of patients with ARDS is plasma exudation (Baker et al., 2000). Ceruloplasmin is a serum ferroxidase that utilizes copper to oxidize ferrous iron (Fe^{+2}) into the ferric (Fe^{+3}) product, which is subsequently incorporated into apotransferrin (Hellman and Gitlin, 2002). Ceruloplasmin reduces lipid peroxidation induced by metal ions and thus decreases cellular damage induced by toxic peroxidation products (Broadley and Hoover, 1989).

Increased α_1-PI and CP levels observed in patients with ARDS are not associated with protection from inflammatory injury (Sallenave et al., 1999; Baker et al., 2000). In fact the reported increases are strongly associated with worsening hypoxia and increased BAL protein content in patients with ARDS (Rocker et al., 1989). One explanation for this adverse consequence may be the extent of plasma exudation and alveolar damage, but one should also take into consideration the possibility of protein inactivation due to proteolytic cleavage (Kingston et al., 1977) and/or post-translational modifications (Gole et al., 2000).

Tyr nitration of α_1-PI and CP has been observed in patients with ARDS and associated with a significant loss of activity (Gole et al., 2000). In the reported study, plasma from 12 ARDS patients was used for analyses of nitrated proteins. Plasma proteins immunoprecipitated with polyclonal anti–NO_2Tyr antibodies were probed independently with specific antibodies against CP and α_1-PI. Nitration of CP and α_1-PI was associated with 81% and 44% reduction of catalytic activity, respectively. The reported post-translational Tyr nitration and catalytic inhibition of CP and α_1-PI may thus play an important role in alveolar tissue destruction observed in patients with ARDS.

23.6.7 Fibrinogen Nitration in Coronary Artery Disease

Vascular endothelium has multifunctional roles including maintenance of vascular tone, trafficking of blood cells, control of blood fluidity, and dynamic regulation of coagulation and fibrinolysis. Under normal physiological conditions the endothelium expresses anticoagulant properties, but can acquire clot-promoting properties whereby blood coagulation is initiated and propagated on the cell surface (Cines et al., 1998). Functional changes of the endothelium may

occur in the total absence of morphological alterations, leading to the concept of "endothelial cell perturbation" as a fundamental pathogenic mechanism in a number of diseases (Gertler and Abbott, 1992). Endothelial perturbation creates a prothrombotic microenvironment via enhancing the synthesis of tissue factor and plasminogen activator inhibitor-1, and downregulating the expression of anti–thrombotic substances such as thrombomodulin and heparin-sulfate (Gertler and Abbott, 1992). This imbalance promotes the formation of blood clots by generating fibrin and dampening fibrinolysis, thus leading to persistent fibrin accumulation (Gertler and Abbott, 1992; Bombeli et al., 1997).

Fibrin is made from the soluble plasma protein fibrinogen through a proteolytic reaction catalyzed by the serine protease thrombin (Pechik et al., 2004). Fibrinogen comprises 2% to 3% of plasma protein and elevated levels in the plasma can predict cardiovascular disease (Wilhelmsen et al., 1984). Fibrinogen is a 340 kDa glycoprotein consisting of three pairs of nonidentical protein chains α, β, and γ that are linked together by 29 disulfide bonds to form a hexamer (Yang et al., 2000) (Fig. 23.6*a*). During coagulation, the soluble fibrinogen is converted to fibrin, a process initiated by thrombin, which catalyzes the cleavage of *N*-terminal fibrinopeptides α and β. Upon release of the fibrinopeptides, the fibrin monomers aggregate spontaneously to form fibrin polymers (Murthy et al., 2000) (Fig. 23.6*b*).

In vitro Tyr nitration of fibrinogen has been reported to generate a pro-thrombotic state via accelerating the lag phase of fibrin polymerization (Vadseth et al., 2004). The observed increase in fibrin polymerization is not due to accelerated thrombin cleavage, since fibrinopeptide α and β release is found to be the same between control and nitrated fibrinogen. Clots formed by nitrated fibrinogen were composed of large bundles of twisted thin fibrin fibers with decreased stiffness

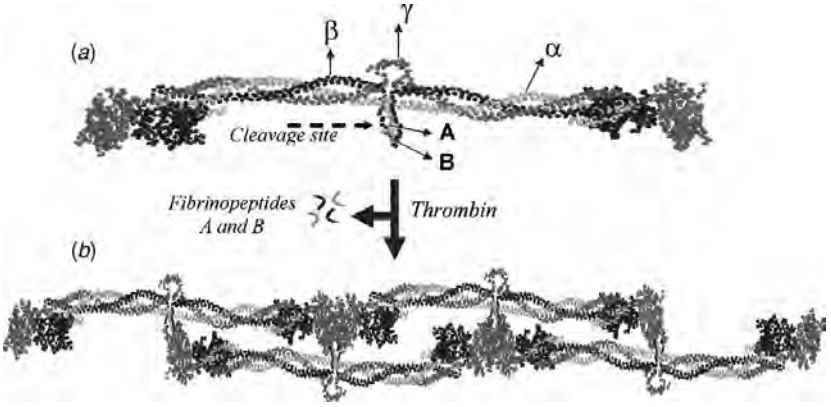


FIGURE 23.6 (*a*) Spacefill representation of fibrinogen [PDB Id:1EI3] produced using Rasmol version 2.7. Fibrinogen chains are represented as follows: light gray, α; black, β; and gray, γ. (*b*) Protofibril formed by fibrin monomers ($\alpha_2\beta_2\gamma_2$) created by proteolytic cleavage of thrombin at the fibrin cleavage site.

and increased permeation, suggesting that they could easily be deformed by mechanical stress. Tyr nitration of fibrinogen had no effect on plasmin-induced fibrinolysis and fibrin-platelet aggregation (Vadseth et al., 2004).

In vivo nitration of fibrinogen was assessed in the plasma of 30 coronary artery disease patients and 26 age- and gender-matched controls by quantifying fibrinogen NO_2Tyr levels (Vadseth et al., 2004) using HPLC with online ESI-MS/MS. The extent of fibrinogen nitration was significantly greater (30%) in patients with clinically documented coronary artery disease compared to age-matched controls. Similar to in vitro findings, thrombin-induced polymerization of fibrinogen isolated from coronary artery disease patients showed a shorter lag phase, a rapid rise in the initial velocity, and increased final turbidity (Vadseth et al., 2004). In summary, increased Tyr nitration of fibrinogen in coronary artery disease patients can alter the polymerization kinetics of fibrin monomers and lead to an imbalance in the dynamic equilibrium of coagulation and fibrinolysis.

23.6.8 Apolipoprotein A-1 Nitration in Cardiovascular Disease

Apolipoprotein A-1 (apoA-I) is a key constituent of high-density lipoprotein (HDL), and it plays an important role in the function of HDL (Frank and Marcel, 2000). The globular *N*-terminal domain of apoA-1 has 43 amino acids, while the lipid-binding *C*-terminal domain (residues 44–243) consists of eight 22- and two 11-tandem amino acid residue sequence repeats that form an almost continuous amphipathic α helix (Borhani et al., 1997) (Fig. 23.7*c*). The formation of amphipathic α-helix allows the interaction of apoA-1 with phospholipids through its hydrophobic face while the hydrophilic face interacts with the aqueous milieu. Studies have shown that two apoA-I monomers surround small units of phospholipid bilayers and form a discoidal, nascent HDL particle (Jonas et al., 1990).

Epidemiologic studies have demonstrated that a decreased level of plasma HDL is a risk factor for atherosclerosis (Gordon et al., 1977). The cardioprotective effect of HDL is attributed to its role in reverse cholesterol transport, a pathway that transports excess cholesterol from peripheral cells back to the liver for excretion in the bile. Cholesterol efflux from the periphery back to the liver is initiated by lipid-poor HDL particles that contain apoA-I and phospholipids (von Eckardstein et al., 2001).

Understanding of the molecular mechanisms of cholesterol transfer from peripheral cells to HDL came from studies of Tangier disease patients. Patients with this rare genetic disorder have mutations in the gene encoding the ATP-binding cassette transporter A1 (ABCA1) and accumulate cholesterol esters in macrophages (Brooks-Wilson et al., 1999; Bodzioch et al., 1999; Rust et al., 1999). ATP-binding cassette transporter A1 is a 240 kDa protein belonging to a large family of transmembrane proteins that transport a wide variety of substrates, including ions, drugs, peptides, and lipids across cell membranes (Croop, 1998). Binding and chemical cross-linking studies have provided evidence for a direct interaction between ABCA1 and apoA-1 (Wang et al., 2000). This interaction is

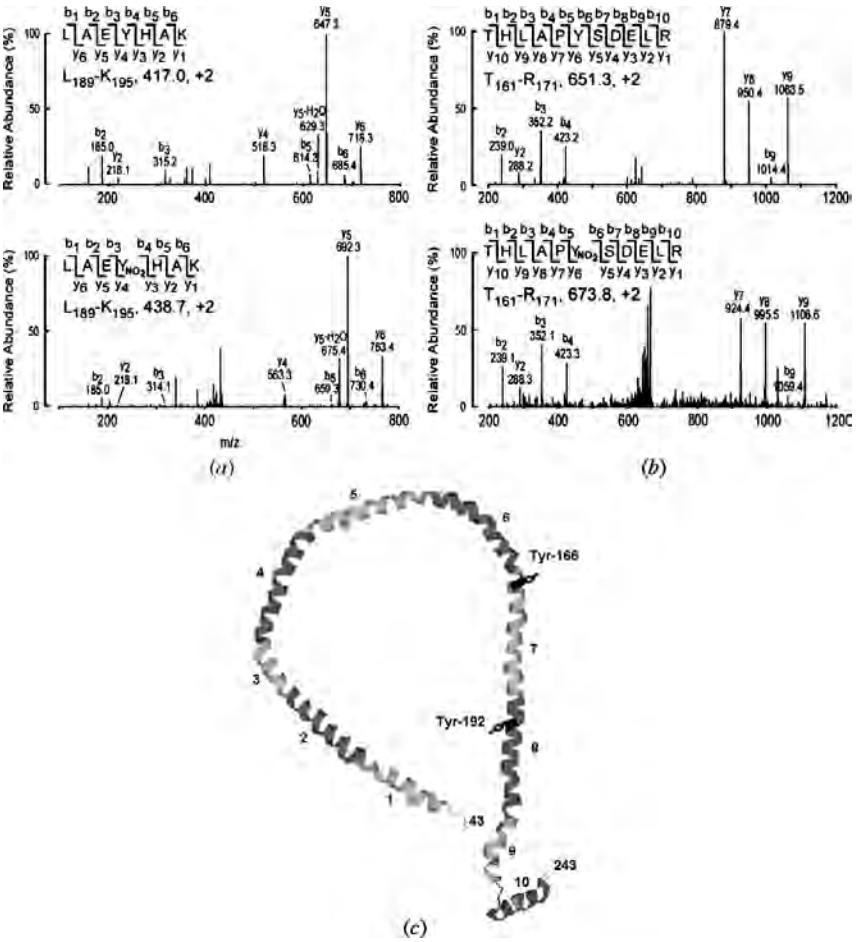


FIGURE 23.7 MS/MS spectrum of unmodified and in vivo nitrated apoA-1. (*a*) Tyr192 nitration is demonstrated by a mass increase of +45 Da in y_4, y_5, y_6, b_5, and b_6 daughter ions. (*b*) Tyr166 nitration is shown by a mass increase of +45 Da in y_7, y_8, y_9, and b_9 daughter ions. (*c*) Ribbon representation of the apoA-1 monomer Δ(1–42) (with the globular *N*-terminal domain absent) [PDB Id: 1AV1] produced using Rasmol version 2.7. The lipid binding *C*-terminal domain (residues 43–243) is composed of α-helices 1–10, which are numerically labeled. The in vivo nitrated Tyr residues are illustrated as black sticks labeled with the three-letter amino acid code. (Modified from Zheng et al., 2005, by permission of the American Society for Biochemistry and Molecular Biology).

not only critical for reverse cholesterol transport; it appears to be required for the initial formation of nascent HDL particles. Tangier patients have extremely low levels of HDL suggesting that lipid-free apoA-1 is rapidly cleared from plasma unless it becomes associated with lipids via the ABCA1 transporter (Schaefer et al., 1980; Horowitz et al., 1993).

The molecular basis for the interaction between ABCA1 and apoA-1 has been studied extensively via mutagenesis of apoA-1. Helix 10 truncation mutants (Δ 220-243) of apoA-1 significantly reduce ABCA1-mediated cholesterol efflux compared to wild-type (Gillotte et al., 1999; Panagotopulos et al., 2002). Point mutations of apoA-1 that alter the charge on the polar face of helix 10 also reduce levels of apolipoprotein-mediated cholesterol efflux (Panagotopulos et al., 2002). The properties of synthetic peptides that correspond to each of the predicted apoA-1 helical segments have been examined and it has been shown that only peptides corresponding to helix 1 (44–65) and helix 10 (220–241) have significant lipid binding affinity (Palgunachari et al., 1996). Helix swap mutants, in which helix 10 is replaced with helix 1 of apoA-1, demonstrate the reduced rate of lipid clearance relative to wild-type apoA-1 (Panagotopulos et al., 2002), which also supports the critical role of helix 10 in cholesterol efflux.

Once incorporated in the HDL particle, cholesterol is esterified by lecithin-cholesterol acyltransferase (LCAT), a plasma enzyme associated with HDL (Jonas, 1991). Although LCAT can bind lipids directly, optimum lipid affinity of the enzyme requires the presence of an apoprotein (Jonas, 1991). Apolipoprotein A-1 is the first described and most potent activator of LCAT (Jonas, 1991; Fielding et al., 1972). It is clearly established that residues 144–186, which allow presentation of cholesterol to the enzyme, is the LCAT activator domain of apoA-1 (Dhoest et al., 1997; Frank et al., 1998). Mutations in the LCAT activating domain of apoA-1 result in reduced HDL-cholesterol levels, but are not necessarily associated with cardiovascular disease (Frank and Marcel, 2000).

ApoA-1 nitration and functional impairment has been documented in patients with cardiovascular disease (Zheng et al., 2004). In the reported study, serum samples from three controls and three patients with cardiovascular disease were separated by SDS-PAGE and probed with monoclonal NO_2Tyr antibodies. Among the numerous immunoreactive bands observed in both control and cardiovascular disease patients, the 29 kDa protein band corresponding to apoA-1 was selected and analyzed by MS/MS following enzymatic in-gel digestion. The identification of apoA-1 nitration was further confirmed by anti–NO_2Tyr affinity chromatography. Plasma from one patient with cardiovascular disease was eluted from an affinity matrix composed of polyclonal anti–NO_2Tyr antibodies, and the recovered sample was separated by two-dimensional SDS-PAGE. The presence of apoA-1 was confirmed by Western blot analysis using an antibody against apoA-1.

ApoA-1 nitration was also observed in HDL particles isolated from human atherosclerotic lesions obtained from autopsy samples (Zheng et al., 2004). HDL particles recovered from atherosclerotic aorta and femoral artery of 12 subjects were pooled and subjected to SDS-PAGE with Western blot analysis using either anti–NO_2Tyr or anti–apoA-1 antibodies. Immunoblotting with anti–NO_2Tyr antibody revealed multiple NO_2Tyr-containing proteins with one immunoreactive band being similar in molecular mass to a band observed when using apoA-1 antibodies.

The NO_2Tyr content of apoA-1, quantified via stable isotope dilution LC-ESI-MS/MS, was 1.4-fold greater in cardiovascular disease patient serum and 6-fold greater in atherosclerotic aortic tissues compared to controls (Zheng et al., 2004). Post-translational Tyr nitration of apoA-1 was accompanied by Tyr chlorination, observed in both arterial and serum samples of controls and cardiovascular disease patients (Zheng et al., 2004). Appreciating that MPO is the only known source of 3-chlorotyrosine, studies were conducted to demonstrate the association between MPO and apoA-1. Plasma from both control and cardiovascular disease patients were immunoprecipitated with either anti–MPO or anti–apoA-1 antibodies and subsequently analyzed via apoA-1 and MPO Western blots, respectively. Obtained data showed that MPO co-immunoprecipitated with apoA-1 in cardiovascular disease patients but not in controls (Zweier et al., 2001).

In vitro nitrated Tyr in apoA-1 were mapped after exposure of HDL to $ONOO^-$- and MPO-generated nitrating oxidants (Shao et al., 2004; Zheng et al., 2005). Conflicting results were obtained from the reported data. One study showed that Tyr192 and Tyr166 were nitrated in HDL-associated apoA-1 incubated with $MPO/NO_2^-/H_2O_2$, (Zheng et al., 2005), while another study revealed that Tyr236, Tyr100, Tyr29, and Tyr18 were nitrated in HDL-bound apoA-1 treated under similar conditions (Shao et al., 2004). The second study also reported that MPO-mediated Tyr192 nitration only occurred in lipid free apoA-1. The reported $ONOO^-$-mediated nitration sites of HDL-associated apoA-1 were also conflicting. The first study showed $ONOO^-$-mediated nitration of apoA-1 at Tyr18, Tyr166, and Tyr236 (Zheng et al., 2005), while the second study documented similar nitration to occur at Tyr18, Tyr29, Tyr115, Tyr166, Tyr192, and Tyr236 (Shao et al., 2004). In vivo nitration of apoA-1 at Tyr192 and Tyr166 was documented in HDL isolated from human atheroma (Zheng et al., 2005). The MS/MS spectrum of unmodified and nitrated peptides corresponding to apoA-1 residues 189–195 (^{189}LAEYHAK^{195}) and 161–171 (^{161}THLAPYSDELR^{171}) are shown in Figure 23.7*a* and *b*, respectively. A mass increase of +45 in y_4, y_5, y_6, b_5, and b_6 daughter ions are indicative of Tyr192 nitration (Fig. 23.7*a*). Similarly Tyr166 nitration is demonstrated by a mass increase of +45 in y_7, y_8, y_9, and b_9 daughter ions (Fig. 23.7*b*). The reported in vivo nitration of apoA-1 at Tyr192 contradicts data that shows nitration of this residue only in lipid-free apoA-1 (Shao et al., 2004).

Descriptions of the impact of nitration on HDL-mediated cholesterol efflux have also been conflicting. Incubation of either HDL or lipid-free apoA-1 with the MPO-generated nitrating system is reported to cause a significant decrease (Zheng et al., 2005) or minimally effect (Shao et al., 2004) the ABCA1-dependent cholesterol efflux. In this context, it is important to note that nitration of apoA-1 in human atheroma occurs at Tyr192 and Tyr166 (Zheng et al., 2005), both of which are located outside of helix 10 (residues 220–243) critical in ABCA1 mediated cholesterol efflux (Panagotopulos et al., 2002). In summary, apoA-1 is clearly a target for Tyr nitration in cardiovascular disease. These findings also support studies that show an association between systemic levels of NO_2Tyr and risk of coronary artery disease (Shishehbor et al., 2003).

23.6.9 MnSOD in Angiotensin II-Induced Hypertension

Previous in vitro studies have demonstrated that mitochondrial MnSOD becomes nitrated specifically at Tyr34 by $ONOO^-$ and then inactivated (Yamakura et al., 1998). The molecular masses of native and $ONOO^-$- inactivated MnSOD differ by 45 mass units, suggesting the addition of a single nitro group. Figure 23.8*a* shows the HPLC chromatogram of the native (1) and $ONOO^-$-treated (2) MnSOD digested with a serine protease. Among the nine major peptide peaks identified, peptide 5 has a different elution position between the two samples. Upon further digestion of this peptide with lysylendopeptidase to identify the exact position of the nitrated Tyr residue, the same sequence was obtained for each peptide except for the fragment corresponding to ^{30}HHAA(Y/X)VNNLNVTEE^{43}. The elution profile of this fragment shows a Tyr at the fifth amino acid position in the native peptide (Fig. 23.8*b*-1) that differs in the $ONOO^-$-treated MnSOD (Fig. 23.8*b*-2). The new peak observed in the $ONOO^-$-treated peptide elutes at the same position as authentic NO_2Tyr (Fig. 23.8*b*-3). From observation of the crystal structure of human MnSOD (Borgstahl et al., 1992) (Fig. 23.8*c*), one can comprehend that Tyr34 nitration, located near the active metal center, likely occurs via Mn-catalyzed decomposition of $ONOO^-$ to $^{\bullet}NO_2$.

In vivo nitration and inactivation of MnSOD was initially observed during chronic rejection of human kidney transplants (MacMillan-Crow et al., 1996). Nitration-induced reduction of SOD activity was suggested to progressively increase cellular levels of ROS, leading to oxidative tissue injury resulting in chronic rejection of renal allografts. New studies have shown that angiotensin-II-induced hypertension in rat kidney caused an increase in NO_2Tyr formation in MnSOD as assessed by HPLC-UV detection of immunoprecipitated MnSOD (Guo et al., 2003). The progression of hypertension due to an upregulation in the angiotensin-renin system is correlated with increased ROS and RNS production, which is thought to lead to the increased generation of nitrating agents in hypertensive tissues (Onozato et al., 2002; Rajagopalan et al., 1996; Wang et al., 2001).

23.7 METABOLISM, REVERSIBILITY, AND STABILITY OF THE NITRATED TYROSINE

The accumulation of NO_2Tyr on specific proteins has been increasingly correlated with pathological processes, and protein nitration has been typically viewed as a static pathway leading to cellular damage and dysfunction with few options for cellular repair of NO_2Tyr formation beyond antioxidant defenses against ROS and RNS. However, recent evidence suggests that the physiological nitration of protein Tyr represents a dynamic and selective process, rather than an irreversible, random event, presenting the possibility of Tyr nitration being a cell signaling event rather than just a physiologically detrimental process (Aulak et al., 2001). Bolus injections of NO_2Tyr are rapidly removed from plasma (Tabrizi-Fard et al., 1999) and the deaminated, and decarboxylated metabolites of NO_2Tyr

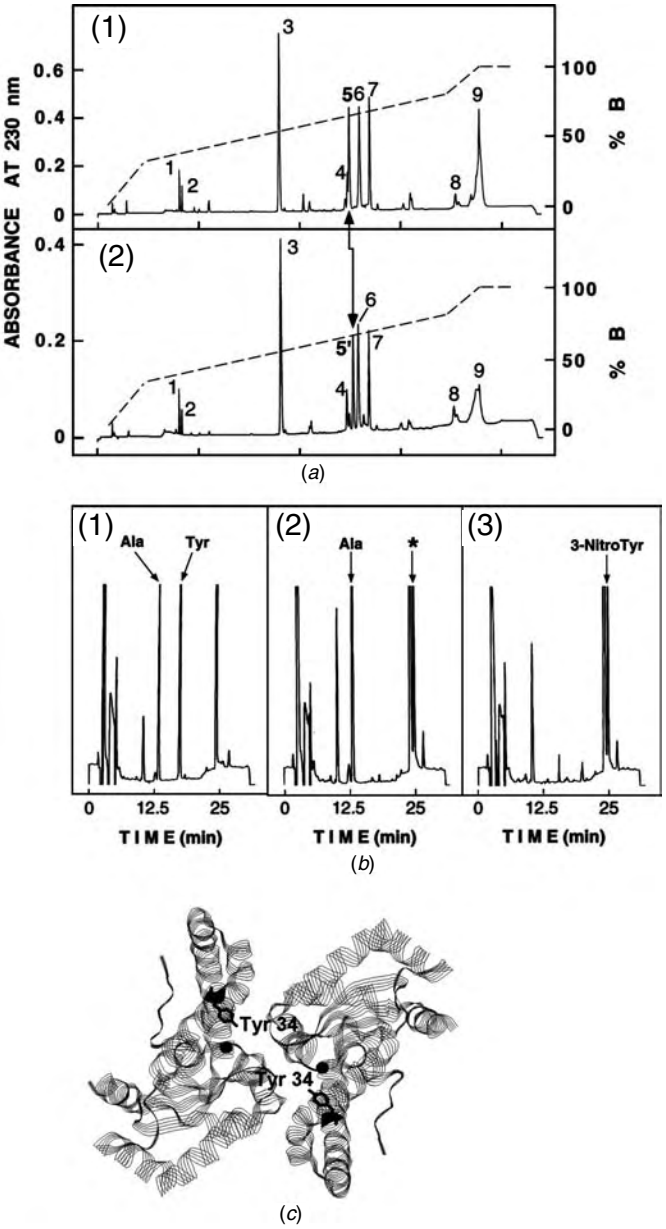


FIGURE 23.8 (*a*) HPLC chromatogram of protease-digested, native (1) and $ONOO^-$-treated (2) Mn-SOD. (*b*) Elution profile of the fifth peptide peak digested with lysylendopeptidase. (*c*) Structure of human MnSOD homodimer [PDB Id: 1N0J] produced by using Rasmol version 2.7. Manganese ions are represented as gray balls. Nitrated Tyr residues are illustrated as black sticks and are labeled with the three-letter amino acid code. (Modified from Yamakura et al., 1998, with permission of the American Society for Biochemistry and Molecular Biology).

are eliminated by renal clearance (Ohshima et al., 1990), indicating that NO_2Tyr is readily metabolized in vivo.

Current data reveal that cumulative protein nitration can contribute to inflammatory disease progression, as a consequence of the increased generation of ROS and RNS and an imbalance in the tissue antioxidant capacity. Post-translationally modified proteins have been shown to be targets for proteolytic degradation and removal, which suggests that the proteolysis and/or repair of nitrated proteins may become impaired during times of oxidative stress (Ischiropoulos, 2003). How then does the cell protect itself from nitration? One route is the direct reduction of ROS or RNS responsible for Tyr nitration by antioxidant enzymes and small molecules. For example, it has been shown that glutathione peroxidase can protect against NO_2Tyr formation in human fibroblast lysates, most likely through the direct reduction of $ONOO^-$ (Sies et al., 1997). Additionally cells can accelerate the removal of damaged proteins through specific proteolytic pathways, or $–NO_2$ could be selectively and reversibly removed from Tyr or reduced to amino-Tyr. These are areas that require further investigation but are beginning to indicate that NO_2Tyr post-translational modifications are reversible and may be repaired.

One mechanism for NO_2Tyr clearance from cellular proteins is denitration, an enzymatic activity that specifically removes $–NO_2$ from Tyr without degrading the rest of the protein, thereby regenerating proteins to their nonmodified state. Putative denitrase activity initially was demonstrated in a number of rat tissues, in particular, lung and spleen (Kamisaki et al., 1998), and in brain and heart (Kuo et al., 1999), whereby nitrated BSA exposed to tissue extracts displayed a decreasing affinity for the anti–NO_2Tyr antibody over time. Observations that denitrase activity was heat labile, trypsin sensitive, and was retained after membrane filtration indicated a role for a macromolecule in the removal of $–NO_2$ from Tyr (Kamisaki et al., 1998). Similar observations of selective and rapid protein denitration were made in samples of respiring mitochondria subjected to hypoxia-anoxia, where protein nitration decreased from basal levels under physiological conditions in the presence of protease inhibitors (Aulak et al., 2004; Koeck et al., 2004b). Renitration of proteins was observed in an $^{\bullet}NO$-dependent manner when mitochondrial samples were reoxygenated, suggesting that denitration becomes activated under conditions of low oxygen (Aulak et al., 2004). In these cases protein nitration and denitration may beneficially regulate and selectively protect mitochondrial performance during periods of ischemia-reperfusion (Koeck et al., 2004b). Validation of this activity awaits identification and isolation of the protein(s) and/or cofactor(s) responsible for protein NO_2Tyr denitrase activity. Additionally, direct structural evaluation of Tyr during these processes would clarify that the removal of $–NO_2$ results in the reformation of unmodified Tyr rather than amino-Tyr or some other modification that is not detected by the anti–NO_2Tyr-based analytical strategies. Further studies may reveal that the denitrase activity protects the cell from oxidative stress, while also playing a role in cell function by modulating signal transduction (*vide infra*) (Ischiropoulos, 2003).

Mechanisms for the clearance for NO_2Tyr adducts from tissues include the proteolytic degradation of proteins by proteasome-dependent pathways, which have been shown to readily recognize and degrade oxidized proteins in a selective manner (Grune et al., 1996). It was suggested that protein nitration can act as a signal for the upregulated removal of NO_2Tyr-modified proteins when nitrated BSA is hydrolyzed more efficiently than untreated BSA incubated in red blood cell lysates (Gow et al., 1996b) with $ONOO^-$ treatment, increasing the potential of protein becoming ubiquitinated and escorted to the 26S proteasome (Buchczyk et al., 2000). Susceptibility to proteolytic elimination was shown to vary according to the degree of protein exposure to oxidative conditions (Grune et al., 1998), and further studies demonstrated that nitration of a single Tyr can dramatically enhance the removal of NO_2Tyr from post-translationally modified proteins by the 20S proteasome, the proteolytic core of the 26S complex (Souza et al., 2000). In the same manner, Tyr nitration of the transferrin receptor enhanced its proteolytic removal in a $^{\bullet}$NO-dependent manner (Kotamraju et al., 2003). Degradation in the 20S proteasome relies on multiple peptidase activities, including a chymotrypsin-like activity. Proteolytic degradation, unexpectedly, can be retarded by a single nitrated Tyr (Souza et al., 2000), although it was not determined if other proteolytic activities such as trypsin are similarly affected. This NO_2Tyr-mediated susceptibility to degradation most likely provides one route for cellular protection from oxidative stress and the negative effects of NO_2Tyr on protein function.

Further consideration needs to be given to the immune system for its role in the removal of nitrated proteins. The wide availability of anti–NO_2Tyr antibodies indicates that the presence of the NO_2Tyr adducts promotes an antigenic response from the immune system, which is capable of generating antibodies that selectively recognize the nitrated versus the nonnitrated protein (Giasson et al., 2000). A differential innate response to proteins selectively modified by nitration could very well serve as an additional pathway for NO_2Tyr removal (Greenacre and Ischiropoulos, 2001). With these removal pathways in effect, the reasons for NO_2Tyr accumulation during disease states may be due to the decreasing effectiveness of clearance as well as modulations in the antioxidant capacity of the organism.

23.8 TYROSINE NITRATION AS A CELL SIGNALING EVENT

Many of the effects of protein nitration have been examined according to the potential of NO_2Tyr to abrogate protein function. However, Tyr nitration has diverse biological effects beyond enzyme activation or inactivation. Recent data suggest that protein Tyr nitration may also be involved in cellular signal transduction, in a manner analogous to Tyr phosphorylation (Schopfer et al., 2003; Irani, 2000). It has already been demonstrated that low levels of ROS and RNS are operative in redox-dependent signal transduction pathways (Irani, 2000) and that $^{\bullet}$NO has a prominent role in cellular signaling based on its redox chemistry (Deora and Lander, 2000). The co-generation of $^{\bullet}$NO and $O_2^{\bullet-}$ activates many signaling processes, with the mechanisms leading to Tyr nitration also potentially regulating

Tyr phosphorylation events in a $ONOO^-$-dependent manner (Monteiro, 2002). These observations support a larger role for protein nitration as a cellular signaling mechanism, but in order for protein nitration to be considered a signaling event, four basic criteria must be satisfied: (1) a controlled rate of formation, (2) selectivity in the modification of the target residue, (3) activity alterations of target protein and cell function, and (4) reversible removal of the signal without target protein degradation (Schopfer et al., 2003).

It has been convincingly demonstrated that Tyr nitration displays a degree of selectivity because not all proteins with accessible Tyr are nitrated, and the specificity of Tyr nitration seems to follow distinct structural requirements, analogous to the prerequisites for other post-translational Tyr modifications (Ischiropoulos, 2003). During nitration events, protein function is altered in some, but not all, proteins, as discussed above, also indicating selectivity in the nitration process. Nitrotyrosine formation is a relatively rapid physiological process that requires selective conditions involving multiple enzymatic reactions (Gow et al., 2004). Basal NO_2Tyr formation occurs under normal physiological conditions (Koeck et al., 2004a) and may be important in the regulation of mitochondrial function, axonal transport, synaptic transmission, and gene expression (Greenacre and Ischiropoulos, 2001). Along the same lines, preliminary reports of a Tyr denitrase activity are beginning to indicate that NO_2Tyr may be reversible without having to sacrifice the host molecule to degradation, giving NO_2Tyr the potential to play a more dynamic role in cell physiology (Aulak et al., 2004). Interestingly the total cellular level of NO_2Tyr (0.01–0.1 mol%) quantitatively compares with the levels of Tyr phosphorylation, suggesting that low levels of the modification are satisfactory for participation in cellular signaling events (Greenacre and Ischiropoulos, 2001). In aggregate, there is sufficient evidence that protein Tyr nitration does not occur randomly and is a normal participant in cellular function.

Information affirming the involvement of Tyr nitration in cell signaling is thus emerging. One way that NO_2Tyr affects signaling is by simply blocking the ability of Tyr to become phosphorylated, and thereby disrupting protein Tyr phosphorylation-mediated events (Monteiro, 2002). The nitro group is added to the carbon adjacent to the phenoxyl group targeted for phosphorylation, but the NO_2Tyr adduct is large enough and negative enough to cause steric hindrance and electrostatic repulsion of phospho-group addition. Modulation or prevention of phosphorylation by $ONOO^-$-mediated nitration was observed in purified cdc2, a cell cycle kinase (Kong et al., 1996), and in bovine pulmonary artery endothelial cells (Gow et al., 1996b). Additional examples of NO_2Tyr interference in Tyr phosphorylation include the nitration of p130Cas, a protein found as part of adhesion complexes (Saeki and Maeda, 1999), and the nitration of p85, the regulatory subunit of PI 3-kinase (Hellberg et al., 1998), events that could have implications on focal adhesion complex assembly and PI 3-kinase signaling. Further studies are needed to substantiate a direct effect on cellular activity in vivo, but the blockage of Tyr phosphorylation may be one way that NO_2Tyr exerts its effects on signal transduction pathways.

The impact of NO_2Tyr formation on cell signaling is not limited to simply blocking Tyr phosphorylation. In some cases the nitration of certain signaling molecules has been shown to selectively activate Src family members of the protein Tyr kinases (Monteiro, 2002). Thus nitration of proteins and peptides may mimic phosphorylation due to the retention of a similar electrostatic environment with nitration, where a negative charge is added to Tyr. This property may provide a mechanism by which nitration might even substitute for phosphorylation events. This method of activation by nitration was demonstrated using peptides with SH2 binding affinity, where nitrated Tyr activated Src kinases in the same manner as phosphorylated peptides (Mallozzi et al., 2001). If this observation is established in vivo, then nitration could affect Tyr kinase-regulated protein–protein interactions and signaling. Interestingly nitration and phosphorylation of critical Tyr residues in Src kinases isolated from human pancreatic adenocarcinoma cells were elevated compared to normal tissues, in conjunction with Src kinase activity and protein–protein interactions, suggesting that nitration may enhance Src signaling pathways (MacMillan-Crow et al., 2000).

Importantly, the protein functional changes induced by nitration occur under conditions that also favor the formation of other amino acid oxidative modifications. Therefore, it is a present challenge to begin to ascribe specific causal connections among redox-dependent protein modifications, alterations in protein structure and function, and the consequent alterations in cell and tissue function. In most instances reviewed herein, it has not been incisively defined if other critical amino acids are also affected by the elevated ROS and RNS production that leads to Tyr nitration. The identification of specific nitrated proteins, the presence of other concurrent amino acid modifications, the participation of NO_2Tyr in novel signaling events, and the isolation of factors involved in denitrase activity will thus better define relationships between protein nitration and cell signaling pathways.

23.9 SUMMARY

Considering the low yield of NO_2Tyr formation in proteins, this post-translational modification might at first glance be seen as just another marker of oxidative stress with other more facile (e.g., thiol oxidation) oxidative modifications more likely responsible for the loss of protein function. Potential protein nitration reversibility may also impede the contribution of this post-translational protein modification to protein dysfunction. However, the selectivity of Tyr nitration and the fact that endogenous levels of Tyr nitration are sufficient to frequently alter enzyme activity and structural protein function indicates that this process is biologically significant at least during inflammatory events. The alteration of protein turnover due to variations in proteasome and protease activities and the induction of immune responses with protein nitration will also significantly impact physiological outcome following protein nitration. Given these considerations, however, the relevance of NO_2Tyr modifications still needs to be better defined in the context of other biomolecular alterations caused by RNS, such as oxidation of protein

thiol and methionine residues, damage to iron-sulfur clusters, and oxidation of transition metal centers, processes that may be more efficient in disturbing normal physiological processes (Radi, 2004).

Is this focused examination of one protein modification a revealing approach to examine the effects of oxidative and nitrosative stress? If conditions prevail where oxidative stress induces Tyr nitration, then other oxidative modifications are occurring as well. Probably the most effective way to begin to ascribe a molecular process to a protein post-translational modification would be to examine these modifications in aggregate by MS. Instead of focusing solely on one specific modification on one type of amino acid residue—a necessary first step in studying post-translational protein modifications—it is now timely to determine protein modifications on a wider scale. Therefore redox studies examining the "nitro-proteome" or the "over-oxidized thiol proteome" are revealing but may underestimate the impact on cell protein structure and function induced by ROS and RNS. Recent studies have begun to apply a more global approach to the analysis of redox-dependent, biomolecular modifications. For example, while other post-translational modifications on histone 2B were examined, it was determined that Tyr98 and Tyr42 were the only two principal residues modified in vivo, indicating that other oxidative modifications did not take precedence in this particular target (Haqqani et al., 2002). When using a proteomics approach to identify specific amino acid residues that are modified, it is also possible to detect other amino acid alterations resulting from RNS or ROS exposure. Evaluation of MS data after proteolytic digestion can allow for the identification of peptide fragments altered by post-translational modifications because of the increase (or decrease) in the expected mass of the unmodified peptide. For example, searching protein databases via LC–MS/MS data derived from tissue extracts using an algorithm that allows for the specification of certain oxidative modifications resulted in the identification of a subset of proteins with a unique tryptophan oxidation that was compared to the overall methionine oxidative content (Taylor et al., 2003). Mass evaluation of all peptides from the protein of interest, rather than only those peptides containing the residues of interest, would begin to facilitate observations of proteins containing multiple types of post-translational modifications. The ability of MS to provide additional data beyond the narrow scope of the original investigation makes this technique such a powerful tool in analyzing post-translational protein modifications.

Because of a lack of evidence demonstrating that nitration, rather than the oxidation of other residues, is responsible for inducing changes in protein structure and function, NO_2Tyr is, at the very least, a biomarker of oxidative and nitrosative stress but may have the potential to serve as a mediator of cellular dysfunction. Indeed rising levels of ROS and RNS are not often the cause of many human diseases, but instead are an indirect result of the disease process (Dalle-Donne et al., 2005). In order to more clearly define the biological role of specific proteins that are post-translationally modified by nitration and other oxidative modifications, more detailed analysis of tissue protein properties must be accomplished in the context of magnitudes of specific NO_2Tyr modifications.

LIST OF ABBREVIATIONS

ABCA1, ATP-binding cassette transporter A1
α_1-PI, alpha-1-protease inhibitor
apoA-I, apolipoprotein A-1
ARDS, acute respiratory distress syndrome
BAL, bronchoalveolar lavage
$^{\bullet}CO_3^{-}$, carbonate anion radical
CP, ceruloplasmin
EPO, eosinophil peroxidase
ESI, electrospray ionization
GC, gas chromatography
HCO_3^{2-}, bicarbonate
HDL, high-density lipoprotein
HNO_2, nitrous acid
H_2O_2, hydrogen peroxide
HOCl, hypochlorous acid
HPLC, high/pressure liquid chromatography
$^{\bullet}OH$, hydroxyl radical
LCAT, lecithin-cholesterol acyltransferase
MALDI-TOF, matrix-assisted laser desorption ionization–time of flight
MM-CK, myofibrillar creatine kinase
MPO, myeloperoxidase
MS, mass spectrometry
NO_3^{-}, nitrate
$^{\bullet}NO$, nitric oxide
NO_2^{-}, nitrite
$^{\bullet}NO_2$, nitrogen dioxide
NOS, nitric oxide synthase
NO_2Tyr, 3-nitro-L-tyrosine
$O_2^{\bullet-}$, superoxide
$ONOO^{-}$, peroxynitrite
$ONOOCO_2^{-}$, nitrosoperoxocarbonate
ONOOH, peroxynitrous acid
PG, prostaglandin
PGHS, prostaglandin endoperoxide synthase
PGI_2, prostacyclin
RNS, reactive nitrogen species
ROS, reactive oxygen species

SCD, sickle cell disease
SCOT, succinyl-CoA:3 oxoacid CoA-transferase
SOD, superoxide dismutase
Tyr•, tyrosyl radical

REFERENCES

Alvarez B., Ferrer-Sueta G., Freeman B.A., Radi R. (1999). Kinetics of peroxynitrite reaction with amino acids and human serum albumin. *J Biol Chem* 274: 842–848.

Alvarez B., Radi R. (2003). Peroxynitrite reactivity with amino acids and proteins. *Amino Acids* 25: 295–311.

Ara J., Przedborski S., Naini A.B., Jackson-Lewis V., Trifiletti R.R., Horwitz J., Ischiropoulos H. (1998). Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Proc Natl Acad Sci USA* 95: 7659–7663.

Aslan M., Ryan T.M., Adler B., Townes T.M., Parks D.A., Thompson J.A., Tousson A., Gladwin M.T., Patel R.P., Tarpey M.M., Batinic-Haberle I., White C.R., Freeman B.A. (2001). Oxygen radical inhibition of nitric oxide-dependent vascular function in sickle cell disease. *Proc Natl Acad Sci USA* 98: 15215–15220.

Aslan M., Ryan T.M., Townes T.M., Coward L., Kirk M.C., Barnes S., Alexander C.B., Rosenfeld S.S., Freeman B.A. (2003). Nitric oxide-dependent generation of reactive species in sickle cell disease: Actin tyrosine induces defective cytoskeletal polymerization. *J Biol Chem* 278: 4194–4204.

Aulak K.S., Koeck T., Crabb J.W., Stuehr D.J. (2004). Dynamics of protein nitration in cells and mitochondria. *Am J Physiol Heart Circ Physiol* 286: H30–H38.

Aulak K.S., Miyagi M., Yan L., West K.A., Massillon D., Crabb J.W., Stuehr D.J. (2001). Proteomic method identifies proteins nitrated in vivo during inflammatory challenge. *Proc Natl Acad Sci USA* 98: 12056–12061.

Avogaro A., Vigili K., Negut C., Tiengo A., Scognamiglio R. (2004). Diabetic cardiomyopathy: A metabolic perspective. *Am J Cardiol* 93: 13A–16A.

Baker C.S., Evans T.W., Haslam P.L. (2000). Measurement of ceruloplasmin in the lungs of patients with acute respiratory distress syndrome: Is plasma or local production the major source? *Respiration* 67: 533–538.

Balabanli B., Kamisaki Y., Martin E., Murad F. (1999). Requirements for heme and thiols for the nonenzymatic modification of nitrotyrosine. *Proc Natl Acad Sci USA* 96: 13136–13141.

Balafanova Z., Bolli R., Zhang J., Zheng Y., Pass J.M., Bhatnagar A., Tang X.L., Wang O., Cardwell E., Ping P. (2002). Nitric oxide (NO) induces nitration of protein kinase Cepsilon (PKCepsilon), facilitating PKCepsilon translocation via enhanced PKCepsilon-RACK2 interactions: a novel mechanism of NO-triggered activation of PKCepsilon. *J Biol Chem* 277: 15021–15027.

Baldus S., Eiserich J.P., Brennan M.L., Jackson R.M., Alexander C.B., Freeman B.A. (2002). Spatial mapping of pulmonary and vascular nitrotyrosine reveals the pivotal role of myeloperoxidase as a catalyst for tyrosine nitration in inflammatory diseases. *Free Radic Biol Med* 33: 1010–1019.

Baldus S., Eiserich J.P., Mani A., Castro L., Figueroa M., Chumley P., Ma W., Tousson A., White C.R., Bullard D.C., Brennan M.L., Lusis A.J., Moore K.P., Freeman B.A. (2001). Endothelial transcytosis of myeloperoxidase confers specificity to vascular ECM proteins as targets of tyrosine nitration. *J Clin Invest* 108: 1759–1770.

Baldus S., Heeschen C., Meinertz T., Zeiher A.M., Eiserich J.P., Munzel T., Simoons M.L., Hamm C.W. (2003). Myeloperoxidase serum levels predict risk in patients with acute coronary syndrome. *Circulation* 108: 1440–1445.

Beckman J.S. (1996a). Oxidative damage and tyrosine nitration from peroxynitrite. *Chem Res Toxicol* 9: 836–844.

Beckman J.S. (1996b). The physiological and pathological chemistry of nitric oxide. In: Lancaster J.R., Jr. (ed). *Nitric Oxide Principles and Actions*, Academic Press, San Diego, pp. 1–82.

Beckman J.S., Chen J., Ischiropoulos H., Crow J.P. (1994). Oxidative chemistry of peroxynitrite. *Methods Enzymol* 233: 229–240.

Beckman J.S., Koppenol W.H. (1996). Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and ugly. *Am J Physiol* 271: C1424–C1437.

Beckman J.S., Ye Y.Z., Anderson P.G., Chen J., Accavitti M.A., Tarpey M.M., White C.R. (1994). Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. *Biol Chem Hoppe Seyler* 375: 81–88.

Berlett B.S., Friguet B., Yim M.B., Chock P.B., Stadtman E.R. (1996): Peroxynitrite-mediated nitration of tyrosine residues in *Escherichia coli* glutamine synthetase mimics adenylylation: Relevance to signal transduction. *Proc Natl Acad Sci USA* 93: 1776–1780.

Bittl J.A., Ingwall J.S. (1985). Reaction rates of creatine kinase and ATP synthesis in the isolated rat heart. A ^{31}P NMR magnetization transfer study. *J Biol Chem* 260: 3512–3517.

Bodzioch M., Orso E., Klucken J., Langmann T., Bottcher A., Diederich W., Drobnik W., Barlage S., Buchler C., Porsch-Ozcurumez M., Kaminski W.E., Hahmann H.W., Oette K., Rothe G., Aslanidis C., Lackner K.J., Schmitz G. (1999). The gene encoding ATP-binding Cassette transporter 1 is mutated in Tangier disease. *Nat Genet* 22: 347–351.

Bolan E.A., Gracy K.N., Chan J., Trifiletti R.R., Pickel V.M. (2000). Ultrastructural localization of nitrotyrosine within the caudate-putamen nucleus and the globus pallidus of normal rat brain. *J Neurosci* 20: 4798–4808.

Bombeli T., Mueller M., Haeberli A. (1997). Anticoagulant properties of the vascular endothelium. *Thromb Haemost* 77: 408–423.

Bonini M.G., Radi R., Ferrer-Sueta G., Ferreira A.M., Augusto O. (1999). Direct EPR detection of the carbonate radical anion produced from peroxynitrite and carbon dioxide. *J Biol Chem* 274: 10802–10806.

Borgstahl G.E., Parge H.E., Hickey M.J., Beyer W.F., Jr., Hallewell R.A., Tainer J.A. (1992). The structure of human mitochondrial manganese superoxide dismutase reveals a novel tetrameric interface of two 4-helix bundles. *Cell* 71: 107–118.

Borhani D.W., Rogers D.P., Engler J.A., Brouillette C.G. (1997). Crystal structure of truncated human apolipoprotein A-I suggests a lipid-bound conformation. *Proc Natl Acad Sci USA* 94: 12291–12296.

Brennan M.L., Wu W., Fu X., Shen Z., Song W., Frost H., Vadseth C., Narine L., Lenkiewicz E., Borchers M.T., Lusis A.J., Lee J.J., Lee N.A., Abu-Soud H.M.,

Ischiropoulos H., Hazen S.L. (2002). A tale of two controversies: Defining both the role of peroxidases in nitrotyrosine formation in vivo using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. *J Biol Chem* 277: 17415–17427.

Broadley C., Hoover R.L. (1989). Ceruloplasmin reduces the adhesion and scavenges superoxide during the interaction of activated polymorphonuclear leukocytes with endothelial cells. *Am J Pathol* 135: 647–655.

Brooks-Wilson A., Marcil M., Clee S.M., Zhang L.H., Roomp K., van Dam M., Yu L., Brewer C., Collins J.A., Molhuizen H.O., Loubser O., Ouelette B.F., Fichter K., Ashbourne-Excoffon K.J., Sensen C.W., Scherer S., Mott S., Denis M., Martindale D., Frohlich J., Morgan K., Koop B., Pimstone S., Kastelein J.J., Hayden M.R. (1999). Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat Genet* 22: 336–345.

Buchczyk D.P., Briviba K., Hartl F.U., Sies H. (2000). Responses to peroxynitrite in yeast: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a sensitive intracellular target for nitration and enhancement of chaperone expression and ubiquitination. *Biol Chem* 381: 121–126.

Cassina A.M., Hodara R., Souza J.M., Thomson L., Castro L., Ischiropoulos H., Freeman B.A., Radi R. (2000). Cytochrome c nitration by peroxynitrite. *J Biol Chem* 275: 21409–21415.

Christner P., Fein A., Goldberg S., Lippmann M., Abrams W., Weinbaum G. (1985). Collagenase in the lower respiratory tract of patients with adult respiratory distress syndrome. *Am Rev Respir Dis* 131: 690–695.

Cines D.B., Pollak E.S., Buck C.A., Loscalzo J., Zimmerman G.A., McEver R.P., Pober J.S., Wick T.M., Konkle B.A., Schwartz B.S., Barnathan E.S., McCrae K.R., Hug B.A., Schmidt A.M., Stern D.M. (1998). Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood* 91: 3527–3561.

Cooke J.P., Mont-Reynaud R., Tsao P.S., Maxwell A.J. (2000). Nitric oxide and vascular disease. In: Ignarro L.J. (ed.), *Nitric Oxide Biology and Pathobiology*, Academic Press, San Diego, pp. 759–783.

Cooke R. (1986). The mechanism of muscle contraction. *CRC Crit Rev Biochem* 21: 53–118.

Croop J.M. (1998). Evolutionary relationships among ABC transporters. *Methods Enzymol* 292: 101–116.

Crow J.P., Ischiropoulos H. (1996). Detection and quantitation of nitrotyrosine residues in proteins: In vivo marker of peroxynitrite. *Methods Enzymol* 269: 185–194.

Daiber A., Herold S., Schoneich C., Namgaladze D., Peterson J.A., Ullrich V. (2000). Nitration and inactivation of cytochrome P450BM-3 by peroxynitrite: Stopped-flow measurements prove ferryl intermediates. *Eur J Biochem* 267: 6729–6739.

Dalle-Donne I., Scaloni A., Giustarini D., Cavarra E., Tell G., Lungarella G., Colombo R., Rossi R., Milzani A. (2005). Proteins as biomarkers of oxidative/nitrosative stress in diseases: The contribution of redox proteomics. *Mass Spectrom Rev* 24: 55–99.

Denicola A., Freeman B.A., Trujillo M., Radi R. (1996). Peroxynitrite reaction with carbon dioxide/bicarbonate: Kinetics and influence on peroxynitrite-mediated oxidations. *Arch Biochem Biophys* 333: 49–58.

Denicola A., Souza J.M., Radi R. (1998). Diffusion of peroxynitrite across erythrocyte membranes. *Proc Natl Acad Sci USA* 95: 3566–3571.

Deorra A.A., Lander H.M. (2000). Role of nitric oxide and other radicals in signal transduction. In: Ignarro L.J. (ed.), *Nitric Oxide Biology and Pathobiology*, Academic Press, San Diego, pp. 251–264.

Dhoest A., Zhao Z., De Geest B., Deridder E., Sillen A., Engelborghs Y., Collen D., Holvoet P. (1997). Role of the Arg123-Tyr166 paired helix of apolipoprotein A-I in lecithin:cholesterol acyltransferase activation. *J Biol Chem* 272: 15967–15972.

Drew B., Leeuwenburgh C. (2002). Aging and the role of reactive nitrogen species. *Ann NY Acad Sci* 959: 66–81.

Duncan M.W. (2003). A Review of approaches to the analysis of 3-nitrotyrosine. *Amino Acids* 25: 351–361.

Eiserich J.P., Baldus S., Brennan M.L., Ma W., Zhang C., Tousson A., Castro L., Lusis A.J., Nauseef W.M., White C.R., Freeman B.A. (2002). Myeloperoxidase, a leukocyte-derived vascular NO oxidase. *Science* 296: 2391–2394.

Eiserich J.P., Cross C.E., Jones A.D., Halliwell B., van der Vliet A. (1996). Formation of nitrating and chlorinating species by reaction of nitrite with hypochlorous acid: A novel mechanism for nitric oxide-mediated protein modification. *J Biol Chem* 271: 19199–19208.

Eiserich J.P., Estevez A.G., Bamberg T.V., Ye Y.Z., Chumley P.H., Beckman J.S., Freeman B.A. (1999). Microtubule dysfunction by posttranslational nitrotyrosination of alpha-tubulin: A nitric oxide-dependent mechanism of cellular injury. *Proc Natl Acad Sci USA* 96: 6365–6370.

Eiserich J.P., Hristova M., Cross C.E., Jones A.D., Freeman B.A., Halliwell B., van der Vliet A. (1998). Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 391: 393–397.

Erickson H.P. (1989). Co-operativity in protein–protein association: The structure and stability of the actin filament. *J Mol Biol* 206: 465–474.

Espey M.G., Xavier S., Thomas D.D., Miranda K.M., Wink D.A. (2002). Direct real-time evaluation of nitration with green fluorescent protein in solution and within human cells reveals the impact of nitrogen dioxide vs. peroxynitrite mechanisms. *Proc Natl Acad Sci USA* 99: 3481–3486.

Estevez A.G., Crow J.P., Sampson J.B., Reiter C., Zhuang Y., Richardson G.J., Tarpey M.M., Barbeito L., Beckman J.S. (1999). Induction of nitric oxide-dependent apoptosis in motor neurons by zinc-deficient superoxide dismutase. *Science* 286: 2498–2500.

Fielding C.J., Shore V.G., Fielding P.E. (1972). A protein cofactor of lecithin:Cholesterol acyltransferase. *Biochem Biophys Res Commun* 46: 1493–1498.

Floris R., Piersma S.R., Yang G., Jones P., Wever R. (1993). Interaction of myeloperoxidase with peroxynitrite: A comparison with lactoperoxidase, horseradish peroxidase and catalase. *Eur J Biochem* 215: 767–775.

Frank P.G., Marcel Y.L. (2000). Apolipoprotein A-I: Structure-function relationships. *J Lipid Res* 41: 853–872.

Frank P.G., N'Guyen D., Franklin V., Neville T., Desforges M., Rassart E., Sparks D.L., Marcel Y.L. (1998). Importance of central alpha-helices of human apolipoprotein A-I in the maturation of high-density lipoproteins. *Biochemistry* 37: 13902–13909.

Fries D.M., Paxinou E., Themistocleous M., Swanberg E., Griendling K.K., Salvemini D., Slot J.W., Heijnen H.F., Hazen S.L., Ischiropoulos H. (2003). Expression of inducible nitric-oxide synthase and intracellular protein tyrosine nitration in vascular smooth muscle cells: Role of reactive oxygen species. *J Biol Chem* 278: 22901–22907.

Frost M.T., Halliwell B., Moore K.P. (2000). Analysis of free and protein-bound nitrotyrosine in human plasma by a gas chromatography/mass spectrometry method that avoids nitration artifacts. *Biochem J* 345(Pt 3): 453–458.

Funk C.D. (2001). Prostaglandins and leukotrienes: Advances in eicosanoid biology. *Science* 294: 1871–1875.

Garber B.G., Hebert P.C., Yelle J.D., Hodder R.V., McGowan J. (1996). Adult respiratory distress syndrome: A systemic overview of incidence and risk factors. *Crit Care Med* 24: 687–695.

Gertler J.P., Abbott W.M. (1992). Prothrombotic and fibrinolytic function of normal and perturbed endothelium. *J Surg Res* 52: 89–95.

Giasson B.I., Duda J.E., Murray I.V., Chen Q., Souza J.M., Hurtig H.I., Ischiropoulos H., Trojanowski J.Q., Lee V.M. (2000). Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. *Science* 290: 985–989.

Gillotte K.L., Zaiou M., Lund-Katz S., Anantharamaiah G.M., Holvoet P., Dhoest A., Palgunachari M.N., Segrest J.P., Weisgraber K.H., Rothblat G.H., Phillips M.C. (1999). Apolipoprotein-mediated plasma membrane microsolubilization: Role of lipid affinity and membrane penetration in the efflux of cellular cholesterol and phospholipid. *J Biol Chem* 274: 2021–2028.

Goldstein S., Czapski G., Lind J., Merenyi G. (2000). Tyrosine nitration by simultaneous generation of nitric oxide and superoxide under physiological conditions: How radicals do the job. *J Biol Chem* 275: 3031–3036.

Gole M.D., Souza J.M., Choi I., Hertkorn C., Malcolm S., Foust R.F., III, Finkel B., Lanken P.N., Ischiropoulos H. (2000). Plasma proteins modified by tyrosine nitration in acute respiratory distress syndrome. *Am J Physiol Lung Cell Mol Physiol* 278: L961–L967.

Gordon T., Castelli W.P., Hjortland M.C., Kannel W.B., Dawber T.R. (1977). High density lipoprotein as a protective factor against coronary heart disease: The Framingham study. *Am J Med* 62: 707–714.

Gow A., Duran D., Thom S.R., Ischiropoulos H. (1996a). Carbon dioxide enhancement of peroxynitrite-mediated protein tyrosine nitration. *Arch Biochem Biophys* 333: 42–48.

Gow A.J., Duran D., Malcolm S., Ischiropoulos H. (1996b). Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation. *FEBS Lett* 385: 63–66.

Gow A.J., Farkouh C.R., Munson D.A., Posencheg M.A., Ischiropoulos H. (2004). Biological significance of nitric oxide-mediated protein modifications. *Am J Physiol Lung Cell Mol Physiol* 287: L262–L268.

Greenacre S.A., Ischiropoulos H. (2001): Tyrosine nitration: localisation, quantification, consequences for protein function and signal transduction. *Free Radic Res* 34: 541–581.

Grinblat L., Pacheco Bolanos L.F., Stoppani A.O. (1986). Decreased rate of ketone-body oxidation and decreased activity of D-3-hydroxybutyrate dehydrogenase and succinyl-CoA:3-oxo-acid CoA-transferase in heart mitochondria of diabetic rats. *Biochem J* 240: 49–56.

Grisham M.B., Jourd'heuil D., Wink D.A. (1999). Nitric oxide. I. Physiological chemistry of nitric oxide and its metabolites: implications in inflammation. *Am J Physiol* 276: G315–G321.

Grune T., Blasig I.E., Sitte N., Roloff B., Haseloff R., Davies K.J. (1998). Peroxynitrite increases the degradation of aconitase and other cellular proteins by proteasome. *J Biol Chem* 273: 10857–10862.

Grune T., Reinheckel T., Davies K.J. (1996). Degradation of oxidized proteins in K562 human hematopoietic cells by proteasome. *J Biol Chem* 271: 15504–15509.

Gryglewski R.J., Palmer R.M., Moncada S. (1986). Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature* 320: 454–456.

Guittet O., Decottignies P., Serani L., Henry Y., Le Marechal P., Laprevote O., Lepoivre M. (2000). Peroxynitrite-mediated nitration of the stable free radical tyrosine residue of the ribonucleotide reductase small subunit. *Biochemistry* 39: 4640–4648.

Gunther M.R., Hsi L.C., Curtis J.F., Gierse J.K., Marnett L.J., Eling T.E., Mason R.P. (1997). Nitric oxide trapping of the tyrosyl radical of prostaglandin H synthase-2 leads to tyrosine iminoxyl radical and nitrotyrosine formation. *J Biol Chem* 272: 17086–17090.

Guo W., Adachi T., Matsui R., Xu S., Jiang B., Zou M.H., Kirber M., Lieberthal W., Cohen R.A. (2003). Quantitative assessment of tyrosine nitration of manganese superoxide dismutase in angiotensin II-infused rat kidney. *Am J Physiol Heart Circ Physiol* 285: H1396–H1403.

Hamman B.L., Bittl J.A., Jacobus W.E., Allen P.D., Spencer R.S., Tian R., Ingwall J.S. (1995). Inhibition of the creatine kinase reaction decreases the contractile reserve of isolated rat hearts. *Am J Physiol* 269: H1030–H1036.

Haqqani A.S., Kelly J.F., Birnboim H.C. (2002). Selective nitration of histone tyrosine residues in vivo in mutatect tumors. *J Biol Chem* 277: 3614–3621.

Heinecke J.W. (1999). Mass spectrometric quantification of amino acid oxidation products in proteins: insights into pathways that promote LDL oxidation in the human artery wall. *FASEB J* 13: 1113–1120.

Hellberg C.B., Boggs S.E., Lapetina E.G. (1998). Phosphatidylinositol 3-kinase is a target for protein tyrosine nitration. *Biochem Biophys Res Commun* 252: 313–317.

Helliwell R.J., Adams L.F., Mitchell M.D. (2004). Prostaglandin synthases: recent developments and a novel hypothesis. *Prostaglandins, Leukotrienes and Essential Fatty Acids* 70: 101–113.

Hellman N.E., Gitlin J.D. (2002). Ceruloplasmin metabolism and function. *Ann Rev Nutr* 22: 439–458.

Hennessey E.S., Drummond D.R., Sparrow J.C. (1993). Molecular genetics of actin function. *Biochem J* 291(Pt 3): 657–671.

Holmes K.C. (1997). The swinging lever-arm hypothesis of muscle contraction. *Curr Biol* 7: R112–R118.

Horowitz B.S., Goldberg I.J., Merab J., Vanni T.M., Ramakrishnan R., Ginsberg H.N. (1993). Increased plasma and renal clearance of an exchangeable pool of apolipoprotein A-I in subjects with low levels of high density lipoprotein cholesterol. *J Clin Invest* 91: 1743–1752.

Houston M., Estevez A., Chumley P., Aslan M., Marklund S., Parks D.A., Freeman B.A. (1999). Binding of xanthine oxidase to vascular endothelium. Kinetic characterization and oxidative impairment of nitric oxide-dependent signaling. *J Biol Chem* 274: 4985–4994.

Ingwall J.S., Kramer M.F., Fifer M.A., Lorell B.H., Shemin R., Grossman W., Allen P.D. (1985). The creatine kinase system in normal and diseased human myocardium. *N Engl J Med* 313: 1050–1054.

Ingwall J.S., Weiss R.G. (2004). Is the failing heart energy starved? On using chemical energy to support cardiac function. *Circ Res* 95: 135–145.

Irani K. (2000). Oxidant signaling in vascular cell growth, death, and survival: A review of the roles of reactive oxygen species in smooth muscle and endothelial cell mitogenic and apoptotic signaling. *Circ Res* 87: 179–183.

Ischiropoulos H. (1998). Biological tyrosine nitration: A pathophysiological function of nitric oxide and reactive oxygen species. *Arch Biochem Biophys* 356: 1–11.

Ischiropoulos H. (2003). Biological selectivity and functional aspects of protein tyrosine nitration. *Biochem Biophys Res Commun* 305: 776–783.

Ischiropoulos H., Beckman J.S. (2003). Oxidative stress and nitration in neurodegeneration: cause, effect, or association? *J Clin Invest* 111: 163–169.

Ischiropoulos H., Zhu L., Beckman J.S. (1992). Peroxynitrite formation from macrophage-derived nitric oxide. *Arch Biochem Biophys* 298: 446–451.

Jiao K., Mandapati S., Skipper P.L., Tannenbaum S.R., Wishnok J.S. (2001). Site-selective nitration of tyrosine in human serum albumin by peroxynitrite. *Anal Biochem* 293: 43–52.

Jonas A. (1991). Lecithin-cholesterol acyltransferase in the metabolism of high-density lipoproteins. *Biochim Biophys Acta* 1084: 205–220.

Jonas A., Wald J.H., Toohill K.L., Krul E.S., Kezdy K.E. (1990). Apolipoprotein A-I structure and lipid properties in homogeneous, reconstituted spherical and discoidal high density lipoproteins. *J Biol Chem* 265: 22123–22129.

Jourd'heuil D., Jourd'heuil F.L., Kutchukian P.S., Musah R.A., Wink D.A., Grisham M.B. (2001). Reaction of superoxide and nitric oxide with peroxynitrite: Implications for peroxynitrite-mediated oxidation reactions in vivo. *J Biol Chem* 276: 28799–28805.

Jourd'heuil D., Miranda K.M., Kim S.M., Espey M.G., Vodovotz Y., Laroux S., Mai C.T., Miles A.M., Grisham M.B., Wink D.A. (1999). The oxidative and nitrosative chemistry of the nitric oxide/superoxide reaction in the presence of bicarbonate. *Arch Biochem Biophys* 365: 92–100.

Kamisaki Y., Wada K., Bian K., Balabanli B., Davis K., Martin E., Behbod F., Lee Y.C., Murad F. (1998). An activity in rat tissues that modifies nitrotyrosine-containing proteins. *Proc Natl Acad Sci USA* 95: 11584–11589.

Kanski J., Behring A., Pelling J., Schoneich C. (2005). Proteomic identification of 3-nitrotyrosine-containing rat cardiac proteins: Effects of biological aging. *Am J Physiol Heart Circ Physiol* 288: H371–H381.

Kaur H., Lyras L., Jenner P., Halliwell B. (1998). Artefacts in HPLC detection of 3-nitrotyrosine in human brain tissue. *J Neurochem* 70: 2220–2223.

Khan J., Brennand D.M., Bradley N., Gao B., Bruckdorfer R., Jacobs M. (1998). 3-nitrotyrosine in the proteins of human plasma determined by an ELISA method. *Biochem J* 330 (Pt 2): 795–801.

Kingston I.B., Kingston B.L., Putnam F.W. (1977). Chemical evidence that proteolytic cleavage causes the heterogeneity present in human ceruloplasmin preparations. *Proc Natl Acad Sci USA* 74: 5377–5381.

Kissner R., Nauser T., Bugnon P., Lye P.G., Koppenol W.H. (1997). Formation and properties of peroxynitrite as studied by laser flash photolysis, high-pressure stopped-flow technique, and pulse radiolysis. *Chem Res Toxicol* 10: 1285–1292.

Knowles M.E., McWeeny D.J., Couchman L., Thorogood M. (1974). Interaction of nitrite with proteins at gastric pH. *Nature* 247: 288–289.

Koeck T., Fu X., Hazen S.L., Crabb J.W., Stuehr D.J., Aulak K.S. (2004a). Rapid and selective oxygen-regulated protein tyrosine denitration and nitration in mitochondria. *J Biol Chem* 279: 27257–27262.

Koeck T., Levison B., Hazen S.L., Crabb J.W., Stuehr D.J., Aulak K.S. (2004b). Tyrosine nitration impairs mammalian aldolase A activity. *Mol Cell Proteomics* 3: 548–557.

Kong S.K., Yim M.B., Stadtman E.R., Chock P.B. (1996). Peroxynitrite disables the tyrosine phosphorylation regulatory mechanism: lymphocyte-specific tyrosine kinase fails to phosphorylate nitrated cdc2(6–20) NH_2 peptide. *Proc Natl Acad Sci USA* 93: 3377–3382.

Kotamraju S., Tampo Y., Keszler A., Chitambar C.R., Joseph J., Haas A.L., Kalyanaraman B. (2003). Nitric oxide inhibits H_2O_2-induced transferrin receptor-dependent apoptosis in endothelial cells: role of ubiquitin-proteasome pathway. *Proc Natl Acad Sci USA* 100: 10653–10658.

Krsek-Staples J.A., Kew R.R., Webster R.O. (1992). Ceruloplasmin and transferrin levels are altered in serum and bronchoalveolar lavage fluid of patients with the adult respiratory distress syndrome. *Am Rev Respir Dis* 145: 1009–1015.

Kuo W.N., Kanadia R.N., Shanbhag V.P., Toro R. (1999). Denitration of peroxynitrite-treated proteins by "protein nitratases" from rat brain and heart. *Mol Cell Biochem* 201: 11–16.

Laffel L. (1999). Ketone bodies: a review of physiology, pathophysiology and application of monitoring to diabetes. *Diabetes Metab Res Rev* 15: 412–426.

Lang J.D., Jr., Chumley P., Eiserich J.P., Estevez A., Bamberg T., Adhami A., Crow J., Freeman B.A. (2000). Hypercapnia induces injury to alveolar epithelial cells via a nitric oxide-dependent pathway. *Am J Physiol Lung Cell Mol Physiol* 279: L994–L1002.

Lang J.D., Figueroa M., Sanders K.D., Aslan M., Liu Y., Chumley P., Freeman B.A. (2005). Hypercapnia via reduced rate and tidal volume contributes to lipopolysaccharide-induced lung injury. *Am J Respir Crit Care Med* 171: 147–157.

Lanone S., Manivet P., Callebert J., Launay J.M., Payen D., Aubier M., Boczkowski J., Mebazaa A. (2002). Inducible nitric oxide synthase (NOS2) expressed in septic patients is nitrated on selected tyrosine residues: Implications for enzymic activity. *Biochem J* 366: 399–404.

Lee C.T., Fein A.M., Lippmann M., Holtzman H., Kimbel P., Weinbaum G. (1981). Elastolytic activity in pulmonary lavage fluid from patients with adult respiratory-distress syndrome. *N Engl J Med* 304: 192–196.

Lepoivre M., Flaman J.M., Bobe P., Lemaire G., Henry Y. (1994). Quenching of the tyrosyl free radical of ribonucleotide reductase by nitric oxide: Relationship to cytostasis induced in tumor cells by cytotoxic macrophages. *J Biol Chem* 269: 21891–21897.

Lin H.L., Kent U.M., Zhang H., Waskell L., Hollenberg P.F. (2003). Mutation of tyrosine 190 to alanine eliminates the inactivation of cytochrome P450 2B1 by peroxynitrite. *Chem Res Toxicol* 16: 129–136.

Luisetti M., Piccioni P.D., Donnetta A.M., Bulgheroni A., Peona V. (1992). Protease-antiprotease imbalance: Local evaluation with bronchoalveolar lavage. *Respiration* 59 Suppl 1: 24–27.

Lymar S.V., Hurst J.K. (1995). Rapid reaction between peroxynitrite ion and carbon dioxide: Implications for biological activity. *J Am Chem Soc* 117: 8867–8868.

Lymar S.V., Jiang Q., Hurst J.K. (1996). Mechanism of carbon dioxide-catalyzed oxidation of tyrosine by peroxynitrite. *Biochemistry* 35: 7855–7861.

MacMillan-Crow L.A., Crow J.P., Kerby J.D., Beckman J.S., Thompson J.A. (1996). Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. *Proc Natl Acad Sci USA* 93: 11853–11858.

MacMillan-Crow L.A., Greendorfer J.S., Vickers S.M., Thompson J.A. (2000). Tyrosine nitration of c-Src tyrosine kinase in human pancreatic ductal adenocarcinoma. *Arch Biochem Biophys* 377: 350–356.

Maitre B., Habibi A., Roudot-Thoraval F., Bachir D., Belghiti D.D., Galacteros F., Godeau B. (2000). Acute chest syndrome in adults with sickle cell disease. *Chest* 117: 1386–1392.

Mallozzi C., Di Stasi A.M., Minetti M. (2001). Nitrotyrosine mimics phosphotyrosine binding to the SH2 domain of the Src family tyrosine kinase Lyn. *FEBS Lett* 503: 189–195.

Maniotis A.J., Chen C.S., Ingber D.E. (1997). Demonstration of mechanical connections between integrins, cytoskeletal filaments, and nucleoplasm that stabilize nuclear structure. *Proc Natl Acad Sci USA* 94: 849–854.

Marquez L.A., Dunford H.B. (1995). Kinetics of oxidation of tyrosine and dityrosine by myeloperoxidase compounds I and II: Implications for lipoprotein peroxidation studies. *J Biol Chem* 270: 30434–30440.

Martin S.S., Leder P. (2001). Human MCF10A mammary epithelial cells undergo apoptosis following actin depolymerization that is independent of attachment and rescued by Bcl-2. *Mol Cell Biol* 21: 6529–6536.

Matthay M.A., Folkesson H.G., Campagna A., Kheradmand F. (1993). Alveolar epithelial barrier and acute lung injury. *New Horiz* 1: 613–622.

Mayes P.A. (1996). Metabolism of unsaturated fatty acids and eicosanoids. In: Murray R.K., Granner D.K., Mayes P.A. and Rodwell V.W. eds. *Harper's Biochemistry*, Appleton Lange, Stanford, CT, pp. 236–244.

McGuire W.W., Spragg R.G., Cohen A.B., Cochrane C.G. (1982). Studies on the pathogenesis of the adult respiratory distress syndrome. *J Clin Invest* 69: 543–553.

Mihm M.J., Coyle C.M., Schanbacher B.L., Weinstein D.M., Bauer J.A. (2001a). Peroxynitrite induced nitration and inactivation of myofibrillar creatine kinase in experimental heart failure. *Cardiovasc Res* 49: 798–807.

Mihm M.J., Yu F., Carnes C.A., Reiser P.J., McCarthy P.M., Van Wagoner D.R., Bauer J.A. (2001b). Impaired myofibrillar energetics and oxidative injury during human atrial fibrillation. *Circulation* 104: 174–180.

Miles A.M., Bohle D.S., Glassbrenner P.A., Hansert B., Wink D.A., Grisham M.B. (1996). Modulation of superoxide-dependent oxidation and hydroxylation reactions by nitric oxide. *J Biol Chem* 271: 40–47.

Mitchell G.A., Kassovska-Bratinova S., Boukaftane Y., Robert M.F., Wang S.P., Ashmarina L., Lambert M., Lapierre P., Potier E. (1995). Medical aspects of ketone body metabolism. *Clin Invest Med* 18: 193–216.

Monteiro H.P. (2002). Signal transduction by protein tyrosine nitration: competition or cooperation with tyrosine phosphorylation-dependent signaling events? *Free Radic Biol Med* 33: 765–773.

Murthy S.N., Wilson J.H., Lukas T.J., Veklich Y., Weisel J.W., Lorand L. (2000). Transglutaminase-catalyzed crosslinking of the alpha and gamma constituent chains in fibrinogen. *Proc Natl Acad Sci USA* 97: 44–48.

Nascimben L., Ingwall J.S., Pauletto P., Friedrich J., Gwathmey J.K., Saks V., Pessina A.C., Allen P.D. (1996). Creatine kinase system in failing and nonfailing human myocardium. *Circulation* 94: 1894–1901.

Natake M., Ueda M. (1986). Changes in food proteins reacted with nitrite at gastric pH. *Nutr Cancer* 8: 41–45.

Nikov G., Bhat V., Wishnok J.S., Tannenbaum S.R. (2003). Analysis of nitrated proteins by nitrotyrosine-specific affinity probes and mass spectrometry. *Anal Biochem* 320: 214–222.

Ohshima H., Friesen M., Brouet I., Bartsch H. (1990). Nitrotyrosine as a new marker for endogenous nitrosation and nitration of proteins. *Food Chem Toxicol* 28: 647–652.

Onozato M.L., Tojo A., Goto A., Fujita T., Wilcox C.S. (2002). Oxidative stress and nitric oxide synthase in rat diabetic nephropathy: effects of ACEI and ARB. *Kidney Int* 61: 186–194.

Palgunachari M.N., Mishra V.K., Lund-Katz S., Phillips M.C., Adeyeye S.O., Alluri S., Anantharamaiah G.M., Segrest J.P. (1996). Only the two end helixes of eight tandem amphipathic helical domains of human apo A-I have significant lipid affinity: Implications for HDL assembly. *Arterioscler Thromb Vasc Biol* 16: 328–338.

Panagotopulos S.E., Witting S.R., Horace E.M., Hui D.Y., Maiorano J.N., Davidson W.S. (2002). The role of apolipoprotein A-I helix 10 in apolipoprotein-mediated cholesterol efflux via the ATP-binding cassette transporter ABCA1. *J Biol Chem* 277: 39477–39484.

Panasenko O.M., Briviba K., Klotz L.O., Sies H. (1997). Oxidative modification and nitration of human low-density lipoproteins by the reaction of hypochlorous acid with nitrite. *Arch Biochem Biophys* 343: 254–259.

Patel R.P., McAndrew J., Sellak H., White C.R., Jo H., Freeman B.A., Darley-Usmar V.M. (1999). Biological aspects of reactive nitrogen species. *Biochim Biophys Acta* 1411: 385–400.

Pechik I., Madrazo J., Mosesson M.W., Hernandez I., Gilliland G.L., Medved L. (2004). Crystal structure of the complex between thrombin and the central "E" region of fibrin. *Proc Natl Acad Sci USA* 101: 2718–2723.

Petty T.L. (1996). Acute respiratory distress syndrome: consensus, definitions, and future directions. *Crit Care Med* 24: 555–556.

Pfeiffer S., Lass A., Schmidt K., Mayer B. (2001). Protein tyrosine nitration in mouse peritoneal macrophages activated in vitro and in vivo: Evidence against an essential role of peroxynitrite. *FASEB J* 15: 2355–2364.

Pfeiffer S., Mayer B. (1998). Lack of tyrosine nitration by peroxynitrite generated at physiological pH. *J Biol Chem* 273: 27280–27285.

Pfeiffer S., Schmidt K., Mayer B. (2000). Dityrosine formation outcompetes tyrosine nitration at low steady-state concentrations of peroxynitrite: Implications for tyrosine modification by nitric oxide/superoxide in vivo. *J Biol Chem* 275: 6346–6352.

Quijano C., Hernandez-Saavedra D., Castro L., McCord J.M., Freeman B.A., Radi R. (2001). Reaction of peroxynitrite with Mn-superoxide dismutase: Role of the metal center in decomposition kinetics and nitration. *J Biol Chem* 276: 11631–11638.

Radi R. (2004). Nitric oxide, oxidants, and protein tyrosine nitration. *Proc Natl Acad Sci USA* 101: 4003–4008.

Radi R., Beckman J.S., Bush K.M., Freeman B.A. (1991a). Peroxynitrite oxidation of sulfhydryls: The cytotoxic potential of superoxide and nitric oxide. *J Biol Chem* 266: 4244–4250.

Radi R., Beckman J.S., Bush K.M., Freeman B.A. (1991b). Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys* 288: 481–487.

Radi R., Cosgrove T.P., Beckman J.S., Freeman B.A. (1993). Peroxynitrite-induced luminol chemiluminescence. *Biochem J* 290 (Pt 1): 51–57.

Radi R., Denicola A., Alvarez B., Ferrer-Sueta G., Rubbo H. (2000). The biological chemistry of peroxynitrite. In: Ignarro L.J. (ed.), *Nitric Oxide Biology and Pathobiology*, Academic Press, San Diego, pp. 57–82.

Radi R., Denicola A., Freeman B.A. (1999). Peroxynitrite reactions with carbon dioxide-bicarbonate. *Methods Enzymol* 301: 353–367.

Radi R., Peluffo G., Alvarez M.N., Naviliat M., Cayota A. (2001). Unraveling peroxynitrite formation in biological systems. *Free Radic Biol Med* 30: 463–488.

Rajagopalan S., Kurz S., Munzel T., Tarpey M., Freeman B.A., Griendling K.K., Harrison D.G. (1996). Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation: Contribution to alterations of vasomotor tone. *J Clin Invest* 97: 1916–1923.

Re F., Zanetti A., Sironi M., Polentarutti N., Lanfrancone L., Dejana E., Colotta F. (1994). Inhibition of anchorage-dependent cell spreading triggers apoptosis in cultured human endothelial cells. *J Cell Biol* 127: 537–546.

Rees D.C., Cervi P., Grimwade D., O'Driscoll A., Hamilton M., Parker N.E., Porter J.B. (1995). The metabolites of nitric oxide in sickle-cell disease. *Br J Haematol* 91: 834–837.

Reiter C.D., Teng R.J., Beckman J.S. (2000). Superoxide reacts with nitric oxide to nitrate tyrosine at physiological pH via peroxynitrite. *J Biol Chem* 275: 32460–32466.

Ricoux R., Boucher J.L., Mansuy D., Mahy J.P. (2001). Microperoxidase 8 catalyzed nitration of phenol by nitrogen dioxide radicals. *Eur J Biochem* 268: 3783–3788.

Roberts E.S., Lin H., Crowley J.R., Vuletich J.L., Osawa Y., Hollenberg P.F. (1998). Peroxynitrite-mediated nitration of tyrosine and inactivation of the catalytic activity of cytochrome P450 2B1. *Chem Res Toxicol* 11: 1067–1074.

Rocker G.M., Wiseman M.S., Pearson D., Shale D.J. (1989). Diagnostic criteria for adult respiratory distress syndrome: Time for reappraisal. *Lancet* 1: 120–123.

Rubbo H., Darley-Usmar V., Freeman B.A. (1996). Nitric oxide regulation of tissue free radical injury. *Chem Res Toxicol* 9: 809–820.

Rust S., Rosier M., Funke H., Real J., Amoura Z., Piette J.C., Deleuze J.F., Brewer H.B., Duverger N., Denefle P., Assmann G. (1999). Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. *Nat Genet* 22: 352–355.

Saeki M., Maeda S. (1999). p130cas is a cellular target protein for tyrosine nitration induced by peroxynitrite. *Neurosci Res* 33: 325–328.

Sallenave J.M., Donnelly S.C., Grant I.S., Robertson C., Gauldie J., Haslett C. (1999). Secretory leukocyte proteinase inhibitor is preferentially increased in patients with acute respiratory distress syndrome. *Eur Respir J* 13: 1029–1036.

Sarver A., Scheffler N.K., Shetlar M.D., Gibson B.W. (2001). Analysis of peptides and proteins containing nitrotyrosine by matrix-assisted laser desorption/ionization mass spectrometry. *Am Soc Mass Spec* 12: 439–448.

Schaefer E.J., Zech L.A., Schwartz D.E., Brewer H.B., Jr. (1980). Coronary heart disease prevalence and other clinical features in familial high-density lipoprotein deficiency (Tangier disease). *Ann Intern Med* 93: 261–266.

Schmidt P., Youhnovski N., Daiber A., Balan A., Arsic M., Bachschmid M., Przybylski M., Ullrich V. (2003). Specific nitration at tyrosine 430 revealed by high resolution mass spectrometry as basis for redox regulation of bovine prostacyclin synthase. *J Biol Chem* 278: 12813–12819.

Schopfer F.J., Baker P.R., Freeman B.A. (2003). NO-dependent protein nitration: A cell signaling event or an oxidative inflammatory response? *Trends Biochem Sci* 28: 646–654.

Shao B., Bergt C., Fu X., Green P., Voss J.C., Oda M.N., Oram J.F., Heinecke J.W. (2004). Tyrosine 192 in apolipoprotein A-I is the major site of nitration and chlorination by myeloperoxidase, but only chlorination markedly impairs ABCA1-dependent cholesterol transport. *J Biol Chem* 280: 5983–5993.

Sheterline P., Sparrow J.C. (1994). Actin. *Protein Profile* 1: 1–121.

Shigenaga M.K., Lee H.H., Blount B.C., Christen S., Shigeno E.T., Yip H., Ames B.N. (1997). Inflammation and NO(X)-induced nitration: assay for 3-nitrotyrosine by HPLC with electrochemical detection. *Proc Natl Acad Sci USA* 94: 3211–3216.

Shishehbor M.H., Aviles R.J., Brennan M.L., Fu X., Goormastic M., Pearce G.L., Gokce N., Keaney J.F., Jr., Penn M.S., Sprecher D.L., Vita J.A., Hazen S.L. (2003). Association of nitrotyrosine levels with cardiovascular disease and modulation by statin therapy. *JAMA* 289: 1675–1680.

Sies H., Sharov V.S., Klotz L.O., Briviba K. (1997). Glutathione peroxidase protects against peroxynitrite-mediated oxidations: A new function for selenoproteins as peroxynitrite reductase. *J Biol Chem* 272: 27812–27817.

Simon D.I., Mullins M.E., Jia L., Gaston B., Singel D.J., Stamler J.S. (1996). Polynitrosylated proteins: characterization, bioactivity, and functional consequences. *Proc Natl Acad Sci USA* 93: 4736–4741.

Smith W.L., DeWitt D.L., Garavito R.M. (2000). Cyclooxygenases: structural, cellular, and molecular biology. *Ann Rev Biochem* 69: 145–182.

Souza J.M., Choi I., Chen Q., Weisse M., Daikhin E., Yudkoff M., Obin M., Ara J., Horwitz J., Ischiropoulos H. (2000). Proteolytic degradation of tyrosine nitrated proteins. *Arch Biochem Biophys* 380: 360–366.

Souza J.M., Daikhin E., Yudkoff M., Raman C.S., Ischiropoulos H. (1999). Factors determining the selectivity of protein tyrosine nitration. *Arch Biochem Biophys* 371: 169–178.

Suarez-Huerta N., Mosselmans R., Dumont J.E., Robaye B. (2000). Actin depolymerization and polymerization are required during apoptosis in endothelial cells. *J Cell Physiol* 184: 239–245.

Tabrizi-Fard M.A., Maurer T.S., Fung H.L. (1999). In vivo disposition of 3-nitro-L-tyrosine in rats: Implications on tracking systemic peroxynitrite exposure. *Drug Metab Dispos* 27: 429–431.

Taylor S.W., Fahy E., Murray J., Capaldi R.A., Ghosh S.S. (2003). Oxidative post-translational modification of tryptophan residues in cardiac mitochondrial proteins. *J Biol Chem* 278: 19587–19590.

Tian R., Ingwall J.S. (1996). Energetic basis for reduced contractile reserve in isolated rat hearts. *Am J Physiol* 270: H1207–H1216.

Tien M., Berlett B.S., Levine R.L., Chock P.B., Stadtman E.R. (1999). Peroxynitrite-mediated modification of proteins at physiological carbon dioxide concentration: pH Dependence of carbonyl formation, tyrosine nitration, and methionine oxidation. *Proc Natl Acad Sci USA* 96: 7809–7814.

Turko I.V., Marcondes S., Murad F. (2001). Diabetes-associated nitration of tyrosine and inactivation of succinyl-CoA:3–oxoacid CoA-transferase. *Am J Physiol Heart Circ Physiol* 281: H2289–H2294.

Turko I.V., Murad F. (2002). Protein nitration in cardiovascular diseases. *Pharmacol Rev* 54: 619–634.

Vadseth C., Souza J.M., Thomson L., Seagraves A., Nagaswami C., Scheiner T., Torbet J., Vilaire G., Bennett J.S., Murciano J.C., Muzykantov V., Penn M.S., Hazen S.L., Weisel J.W., Ischiropoulos H. (2004). Pro-thrombotic state induced by post-translational modification of fibrinogen by reactive nitrogen species. *J Biol Chem* 279: 8820–8826.

van der Loo B., Labugger R., Skepper J.N., Bachschmid M., Kilo J., Powell J.M., Palacios-Callender M., Erusalimsky J.D., Quaschning T., Malinski T., Gygi D., Ullrich V., Luscher T.F. (2000). Enhanced peroxynitrite formation is associated with vascular aging. *J Exp Med* 192: 1731–1744.

van der Vliet A., Eiserich J.P., Halliwell B., Cross C.E. (1997). Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite: A potential additional mechanism of nitric oxide-dependent toxicity. *J Biol Chem* 272: 7617–7625.

van der Vliet A., O'Neill C.A., Halliwell B., Cross C.E., Kaur H. (1994). Aromatic hydroxylation and nitration of phenylalanine and tyrosine by peroxynitrite: Evidence for hydroxyl radical production from peroxynitrite. *FEBS Lett* 339: 89–92.

Venembre P., Boutten A., Seta N., Dehoux M.S., Crestani B., Aubier M., Durand G. (1994). Secretion of alpha 1-antitrypsin by alveolar epithelial cells. *FEBS Lett* 346: 171–174.

Ventura-Clapier R., Veksler V., Hoerter J.A. (1994). Myofibrillar creatine kinase and cardiac contraction. *Mol Cell Biochem* 133–134: 125–144.

Ventura-Clapier R., Kaasik A., Veksler V. (2004). Structural and functional adaptations of striated muscles to CK deficiency. *Mol Cell Biochem* 256–257: 29–41.

Ventura-Clapier R., Kuznetsov A., Veksler V., Boehm E., Anflous K. (1998). Functional coupling of creatine kinases in muscles: species and tissue specificity. *Mol Cell Biochem* 184: 231–247.

Viner R.I., Ferrington D.A., Williams T.D., Bigelow D.J., Schoneich C. (1999). Protein modification during biological aging: selective tyrosine nitration of the SERCA2a isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase in skeletal muscle. *Biochem J* 340(Pt 3): 657–669.

von Eckardstein A., Nofer J.R., Assmann G. (2001). High density lipoproteins and arteriosclerosis. Role of cholesterol efflux and reverse cholesterol transport. *Arterioscler Thromb Vasc Biol* 21: 13–27.

Wang H.D., Xu S., Johns D.G., Du Y., Quinn M.T., Cayatte A.J., Cohen R.A. (2001). Role of NADPH oxidase in the vascular hypertrophic and oxidative stress response to angiotensin II in mice. *Circ Res* 88: 947–953.

Wang N., Silver D.L., Costet P., Tall A.R. (2000). Specific binding of apoA-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1. *J Biol Chem* 275: 33053–33058.

Way M., Weeds A. (1990). Actin-binding proteins. Cytoskeletal ups and downs. *Nature* 344: 292–294.

Weeds A.G., Gooch J., Hawkins M., Pope B., Way M. (1991). Role of actin-binding proteins in cytoskeletal dynamics. *Biochem Soc Trans* 19: 1016–1020.

Whiteman M., Siau J.L., Halliwell B. (2003). Lack of tyrosine nitration by hypochlorous acid in the presence of physiological concentrations of nitrite: Implications for the role of nitryl chloride in tyrosine nitration in vivo. *J Biol Chem* 278: 8380–8384.

Wilhelmsen L., Svardsudd K., Korsan-Bengtsen K., Larsson B., Welin L., Tibblin G. (1984). Fibrinogen as a risk factor for stroke and myocardial infarction. *N Engl J Med* 311: 501–505.

Willard B.B., Ruse C.I., Keightley J.A., Bond M., Kinter M. (2003). Site-specific quantitation of protein nitration using liquid chromatography/tandem mass spectrometry. *Anal Chem* 75: 2370–2376.

Wu W., Chen Y., Hazen S.L. (1999). Eosinophil peroxidase nitrates protein tyrosyl residues: Implications for oxidative damage by nitrating intermediates in eosinophilic inflammatory disorders. *J Biol Chem* 274: 25933–25944.

Wyss M., Smeitink J., Wevers R.A., Wallimann T. (1992). Mitochondrial creatine kinase: A key enzyme of aerobic energy metabolism. *Biochim Biophys Acta* 1102: 119–166.

Xia Y., Zweier J.L. (1995). Substrate control of free radical generation from xanthine oxidase in the postischemic heart. *J Biol Chem* 270: 18797–18803.

Yamakura F., Taka H., Fujimura T., Murayama K. (1998). Inactivation of human manganese-superoxide dismutase by peroxynitrite is caused by exclusive nitration of tyrosine 34 to 3-nitrotyrosine. *J Biol Chem* 273: 14085–14089.

Yang F., Friedrichs W.E., deGraffenried L., Herbert D.C., Weaker F.J., Bowman B.H., Coalson J.J. (1996). Cellular expression of ceruloplasmin in baboon and mouse lung during development and inflammation. *Am J Respir Cell Mol Biol* 14: 161–169.

Yang Z., Mochalkin I., Veerapandian L., Riley M., Doolittle R.F. (2000). Crystal structure of native chicken fibrinogen at 5.5-Å resolution. *Proc Natl Acad Sci USA* 97: 3907–3912.

Yi D., Ingelse B.A., Duncan M.W., Smythe G.A. (2000). Quantification of 3-nitrotyrosine in biological tissues and fluids: Generating valid results by eliminating artifactual formation. *J Am Soc Mass Spectrom* 11: 578–586.

Zhang H., Bhargava K., Keszler A., Feix J., Hogg N., Joseph J., Kalyanaraman B. (2003). Transmembrane nitration of hydrophobic tyrosyl peptides: Localization, characterization, mechanism of nitration, and biological implications. *J Biol Chem* 278: 8969–8978.

Zhang H., Joseph J., Feix J., Hogg N., Kalyanaraman B. (2001). Nitration and oxidation of a hydrophobic tyrosine probe by peroxynitrite in membranes: Comparison with nitration and oxidation of tyrosine by peroxynitrite in aqueous solution. *Biochemistry* 40: 7675–7686.

Zheng L., Nukuna B., Brennan M.L., Sun M., Goormastic M., Settle M., Schmitt D., Fu X., Thomson L., Fox P.L., Ischiropoulos H., Smith J.D., Kinter M., Hazen S.L. (2004). Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease. *J Clin Invest* 114: 529–541.

Zheng L., Settle M., Brubaker G., Schmitt D., Hazen S.L., Smith J.D., Kinter M. (2005). Localization of nitration and chlorination sites on apolipoprotein A-I catalyzed by myeloperoxidase in human atheroma and associated oxidative impairment in ABCA1-dependent cholesterol efflux from macrophages. *J Biol Chem* 280: 38–47.

Zou M.H., Leist M., Ullrich V. (1999). Selective nitration of prostacyclin synthase and defective vasorelaxation in atherosclerotic bovine coronary arteries. *Am J Pathol* 154: 1359–1365.

Zou M.H., Shi C., Cohen R.A. (2002). High glucose via peroxynitrite causes tyrosine nitration and inactivation of prostacyclin synthase that is associated with thromboxane/prostaglandin H(2) receptor-mediated apoptosis and adhesion molecule expression in cultured human aortic endothelial cells. *Diabetes* 51: 198–203.

Zweier J.L., Fertmann J., Wei G. (2001). Nitric oxide and peroxynitrite in postischemic myocardium. *Antioxid Redox Signal* 3: 11–22.

24

OXIDATION OF ARTERY WALL PROTEINS BY MYELOPEROXIDASE: A PROTEOMICS APPROACH

TOMAS VAISAR AND JAY W. HEINECKE

24.1 OXIDATIVE STRESS IN ATHEROSCLEROSIS

In humans, a chronically elevated serum level of C-reactive protein (CRP), a marker of inflammation, is an independent risk factor for cardiovascular disease. This observation suggests that inflammatory proteins might play a role in atherosclerosis, which can be regarded as a chronic inflammatory disorder.

Many lines of evidence support the view that inflammation is of central importance in atherosclerosis (Ross, 1999). A key early event in atherogenesis is the recruitment of circulating monocytes—a major cellular effector of both the innate and acquired immune response—into the intima of the artery wall. Monocytes differentiate into macrophages that subsequently become lipid-laden foam cells, the cellular hallmark of the early atherosclerotic lesion. Foam cells are also abundant at later stages of lesion development, and macrophages have been implicated as a major source of proteinases and other inflammatory mediators that may play critical roles in atherogenesis.

Reactive oxygen and nitrogen species produced by phagocytic white blood cells may also be atherogenic, although they normally play important roles in host defense mechanisms of the innate immune system. Investigations of cultured cells have shown that all the major cell types found in atherosclerotic lesions—endothelial cells, smooth muscle cells, and monocyte/macrophages—are capable of producing such reactive intermediates (Witztum and Steinberg, 1991). Moreover high

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

levels of oxidized lipids and proteins have been detected in atherosclerotic lesions (Heinecke, 1999).

One important mechanism by which phagocytes generate reactive species involves myeloperoxidase (MPO), a secreted heme enzyme that converts hydrogen peroxide to a variety of strong oxidants, including hypochlorous acid and nitrogen dioxide radical. Localization of MPO to regions of atherosclerotic plaque rich in macrophages and in the necrotic core (Daugherty et al., 1994) indicate that the enzyme might play an important role in both the development of atherosclerotic lesions and their rupture, the major cause of both acute myocardial infarction and sudden death in humans.

Low-density lipoprotein (LDL), which transports cholesterol to cells, may be an important target for reactive species generated by MPO (Heinecke, 1999). Although an elevated level of LDL is a major risk factor for atherosclerotic vascular disease, LDL in its native form fails to convert macrophages into foam cells. In contrast, oxidized LDL that binds to pattern recognition receptors on macrophages can be taken up in an unregulated manner. Moreover oxidized LDL has been detected in human and animal atherosclerotic lesions, and lipid-soluble antioxidants retard atherosclerosis in hypercholesterolemic animals.

In contrast to LDL, high-density lipoprotein (HDL) appears to protect the artery wall from atherosclerosis (Miller, 1980). One important mechanism involves the removal of excess cellular cholesterol by a pathway involving apolipoprotein A-I (apoA-I), the major protein in HDL (Bergt et al., 2004a,b). ApoA-I interacts with ABCA1, a membrane-associated transport system, to promote cholesterol efflux from macrophages. However, recent studies indicate that HDL is oxidized in patients with established atherosclerosis. Such alteration may limit the ability of apoA-I to participate in reverse cholesterol transport.

Lipoproteins and their associated proteins are not the only potential targets of reactive intermediates in the artery wall. Inflammation alters the expression of many proteins whose functionality can potentially be modulated by oxidative modification. For example, matrix metalloproteinases (MMPs), such as gelatinase B (MMP-9) and matrilysin (MMP-7), have been proposed to play a critical role in the generation of inflammatory mediators and the destruction of structural proteins of the artery wall. The observation that oxidants regulate the activity of MMP-1, MMP-7, and MMP-9 in vitro has raised the possibility that oxidative stress is important for triggering plaque rupture and controlling the turnover of matrix proteins in atherosclerotic tissue (Weiss et al., 1985; Peppin and Weiss, 1986; Fu et al., 2001). It is noteworthy that MMP-7 co-localizes with MPO in human atherosclerotic lesions (Fu et al., 2001).

The oxidative pathways that damage biomolecules in vivo are difficult to identify because toxic intermediates are short-lived and difficult to measure directly. One approach to assessing the importance of oxidative pathways that operate in vivo is to analyze normal and pathological tissues for stable end-products of oxidative reactions that have been identified through in vitro studies (Heinecke, 1999). For example, oxidation may generate abnormal forms of the amino acid tyrosine. Such products serve as fingerprints for specific pathways. In this chapter

we describe sensitive and quantitative mass spectrometric methods that have enabled us to determine the relative amounts of oxidized amino acids in proteins and lipoproteins oxidized in vitro. We also used this approach to quantify levels of modified amino acids in atherosclerotic lesions, inflammatory tissues, and tissue proteins of aging animals. Using tandem mass spectrometric analysis, we investigated the potential role of site-specific protein oxidation in atherogenesis. Our observations indicate that mass spectrometry is a powerful tool for identifying the pathways and mechanisms that oxidatively modify proteins in vitro and in vivo.

24.2 POTENTIAL ROLE OF REDOX-ACTIVE METAL IONS AND GLUCOSE IN OXIDATIVE STRESS

Redox-active metal ions, either alone or in conjunction with glucose, are potential agents for protein and lipoprotein oxidation. Using the strategy described above, we identified distinct patterns of oxidized amino acids that appear when proteins are oxidized by redox-metal ions in vitro (Heinecke, 1999), and we looked for those patterns in vivo.

24.2.1 Redox-Active Metal Ions

Redox-active transition metal ions are commonly used to oxidize LDL in vitro. For instance, increasing concentrations of iron or copper modify LDL in the presence of smooth muscle cells (Heinecke et al., 1984). Oxidation also occurs in the absence of cells when sufficiently high concentrations of metal ions are used (Heinecke et al., 1984; Steinbrecher et al., 1984; Heinecke et al., 1986). Moreover metal chelators inhibit LDL oxidation by cultured cells of the artery wall (Heinecke et al., 1984; Steinbrecher et al., 1984; Morel et al., 1984). Protein-bound metal ions in ceruloplasmin (Ehrenwald et al., 1994; Ehrenwald and Fox, 1996) and hemin (Balla et al., 1991) promote LDL oxidation, although the mechanisms may differ from those involving free metal ions.

Evidence for the presence of free redox-active metal ions (or low-molecular-weight chelates of metal ions) in vivo is limited. Redox-active metal ions were detected in tissue homogenates of atherosclerotic tissue, but their concentrations in normal aortic tissue subjected to the same homogenization procedure were not reported (Smith et al., 1992; Swain and Gutteridge, 1995; Lamb et al., 1995). However, recent studies using noninvasive methods suggest that the concentration of redox-active metal ions is elevated in human atherosclerotic tissue (Dean et al., 1997; Stadler et al., 2004). Several lines of evidence suggest that metal ions are effectively sequestered by metal-chelating proteins in blood. Thus, the high-affinity binding sites for iron and copper on transferrin, the major iron carrier in plasma, are only partly saturated in normal individuals (Aasa et al., 1963). Moreover low concentrations of albumin, the most abundant protein in plasma, inhibit metal ion-dependent LDL oxidation (Thomas, 1992) and bind free copper avidly (Peters and Blumnestock, 1967). Consequently, extracellular free metal ions are unlikely to be present in normal arterial tissue.

One strategy for investigating the oxidation of proteins by free redox-active metal cations is to use surrogate markers of their action, such as the hydroxyl radical ($HO^{\bullet}$), an extremely reactive agent produced by metal ion-dependent reactions (Fridovich, 1978). For example, hydroxyl radical forms when a reduced metal ion, such as Fe^{2+} or Cu^{1+}, reacts with hydrogen peroxide (H_2O_2):

$$M^{n+} + H_2O_2 \rightarrow HO^{\bullet} + HO^{-} + M^{n+-1} \quad (1)$$

When model proteins are exposed to hydroxyl radical generated by metal ions, their content of two unnatural isomers of tyrosine—*ortho*-tyrosine and *meta*-tyrosine—rises significantly (Huggins et al., 1993; Leeuwenburgh et al., 1997a). Hydroxyl radical also generates low levels of *o*,*o*′-dityrosine, a cross-linked form of tyrosine. LDL oxidized by copper accumulates large amounts of *ortho*-tyrosine and *meta*-tyrosine (Leeuwenburgh et al., 1997a), suggesting that these unusual tyrosine isomers might be useful markers of damage inflicted by metal ions in vivo.

24.2.2 Redox-Active Metal Ions plus Glucose

In vitro studies have shown that glucose promotes glycoxidation reactions and the formation of *ortho*-tyrosine (Baynes and Thorpe, 1999; Pennathur et al., 2005). Additional glucose oxidation can generate advanced glycation end (AGE) products, which are much more abundant in diabetic subjects than in people without diabetes. AGEs bind metal ions, an interaction that might promote tissue damage (Saxena et al., 1999). Moreover autoxidation of glucose generates α-dicarbonyl compounds, which have been implicated in the formation of cross-linked proteins and AGE products in vitro (Yim et al., 1995; Lee et al., 1998). Collectively these observations suggest that hyperglycemia might promote protein oxidation by mechanisms that operate only under features unique to the diabetic artery wall, perhaps by a pathway involving redox-active metal ions.

24.3 POTENTIAL ROLE OF CELLULAR PATHWAYS IN OXIDATIVE STRESS

Lipid-laden foam cells derived from macrophages are the cellular hallmark of the early atherosclerotic lesion. Because arterial cells in culture convert LDL to a form that binds to scavenger receptors (Goldstein et al., 1979; Fogelman et al., 1980; Henriksen et al., 1981), they might also promote foam cell formation in vivo. Investigations of cultured endothelial cells, smooth muscle cells, and monocyte/macrophages—the major cell types in the artery wall—have shown that oxidative reactions can render LDL atherogenic. A number of possible mechanisms have emerged. Cellular products that can oxidize lipoproteins in vitro include reactive nitrogen species and oxidants generated by MPO.

24.3.1 Reactive Nitrogen Species

Nitric oxide produced by the constitutive form of nitric oxide synthase is essential to normal physiology because it regulates vasomotor tone (Beckman et al., 1994a; Moncada et al., 1991). The larger amounts of nitric oxide produced by the inducible isoform of the enzyme help inflammatory cells kill microbial organisms and tumor cells. Under pathological conditions, however, nitric oxide ($NO^{\bullet}$) might injure normal tissue. For example, it can react with superoxide ($O_2^{\bullet -}$), a product of activated phagocytes. The reaction generates peroxynitrite ($ONOO^-$), a powerful oxidizing intermediate (Beckman et al., 1990):

$$NO^{\bullet} + O_2^{\bullet -} \rightarrow ONOO^- \tag{2}$$

Peroxynitrite oxidizes lipoprotein lipids to hydroperoxides, converting the lipoprotein into an atherogenic form that promotes the transformation of macrophages into foam cells (Graham et al., 1993). This reactive species is also a potent nitrating reagent that converts tyrosine to 3-nitrotyrosine in vitro (Beckman et al., 1994a). Peroxynitrite also hydroxylates phenylalanine to *ortho*- and *meta*-tyrosine, and converts two tyrosines to *o*,*o*′-dityrosine, although these reactions occur less readily than tyrosine nitration when the oxidant is added as a bolus to a reaction mixture (Beckman et al., 1994a; Leeuwenburgh et al., 1997a). Nitric oxide might also protect host tissues because it inhibits LDL oxidation by both murine macrophages and copper (Jessup et al., 1992; Yates et al., 1992; Hogg et al., 1993; Rubbo et al., 1995; Hayashi et al., 1995). It might suppress lipoprotein oxidation by inhibiting heme-containing enzymes, scavenging superoxide, reacting with lipid radicals, or nitrosylating important cellular proteins. Studies in hypercholesterolemic rabbits and mice have yielded conflicting results regarding its role in fatty streak formation (Cooke and Tsao, 1994; Aji et al., 1997; Kuhlencordt et al., 2001; Knowles et al., 2000).

24.3.2 Myeloperoxidase

Phagocytes also secrete the heme protein MPO, which interacts with hydrogen peroxide to generate antimicrobial toxins (Klebanoff, 1980; Hurst and Barette, 1989). These cells produce their own hydrogen peroxide by generating superoxide ($O_2^{\bullet -}$), using a membrane-associated NADPH oxidase that directly reduces molecular oxygen (Fig. 24.1):

$$NADPH + 2O_2 \rightarrow 2O_2^{\bullet -} + NADP^+ + H^+ \tag{3}$$

Superoxide dismutates into hydrogen peroxide (H_2O_2):

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \tag{4}$$

In contrast to the many oxidation reactions that have been studied in vitro, this pathway does not require free metal ions.

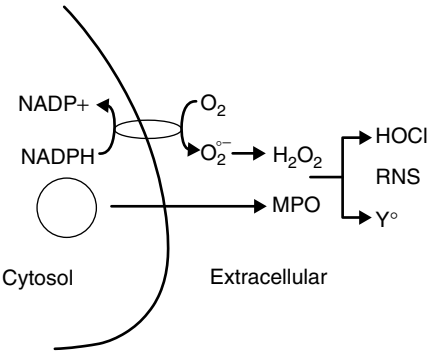


FIGURE 24.1 Pathways for the generation of reactive intermediates by MPO.

The hydrogen peroxide generated this way can be used by MPO to oxidize chloride ion to hypochlorous acid (HOCl; Harrison and Schultz, 1976):

$$H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O \quad (5)$$

Hypochlorous acid is a potent bactericide that may inadvertently damage host proteins at sites of inflammation in vivo. We and others have shown that it chlorinates the phenolic amino acid tyrosine to 3-chlorotyrosine and that 3-chlorotyrosine is found only in proteins oxidized by an MPO pathway (Kettle, 1996; Hazen et al., 1996; Hazen and Heinecke, 1997; Gaut et al., 2001).

MPO can also convert L-tyrosine to tyrosyl radical (Heinecke et al., 1993a; McCormick et al., 1998), which can initiate peroxidation of LDL lipid (Savenkova et al., 1994). This process bears remarkable biochemical similarities to the enzymatic peroxidation of polyunsaturated fatty acids by cyclooxygenase, a reaction that might also involve tyrosyl radical (Karthein et al., 1988). Proteins are damaged when tyrosyl radical converts their tyrosyl residues to *o*,*o*′-dityrosine (Heinecke et al., 1993b; Francis et al., 1993). Plasma concentrations of tyrosine greatly enhance MPO's ability to form *o*,*o*′-dityrosine cross-links in proteins (Savenkova et al., 1994; Heinecke et al., 1993b). Therefore dityrosine, an intensely fluorescent and acid-stable compound, might serve as a marker for proteins that have been oxidatively damaged by activated phagocytes (Bhattacharjee et al., 2001).

Lipoproteins and other model proteins exposed to free tyrosyl radical generated by MPO in vitro become markedly enriched in *o*,*o*′-dityrosine but not in *ortho*-tyrosine, one of the markers of protein oxidation by metal ions (Leeuwenburgh et al., 1997a; Heinecke et al., 1993b). The mechanism involves a recombination reaction between protein-bound tyrosyl radical and either free tyrosyl radical or another protein-bound tyrosyl radical (Heinecke et al., 1993b; Francis et al., 1993).

MPO can also generate nitrating species that could oxidize tyrosine in vivo (Eiserich et al., 1996, 1998). For example, the HOCl it generates can react

with nitrite (NO_2^-), whose levels in plasma increase markedly during acute and chronic inflammation. One potential product is nitryl chloride (NO_2Cl) (Eiserich et al., 1996, 1998; Byun et al., 1999a):

$$HOCl + NO_2^- \rightarrow NO_2Cl + HO^- \quad (6)$$

MPO can also generate nitrogen dioxide radical ($NO_2^\bullet$), through direct one-electron oxidation of NO_2^- by compound I, a complex of MPO and H_2O_2 (Eiserich et al., 1996, 1998; Byun et al., 1999a):

$$NO_2^- + \text{compound I} + H^+ \rightarrow NO_2^\bullet + H_2O + \text{compound II} \quad (7)$$

These reactions might be physiologically relevant because human neutrophils can use the MPO–H_2O_2–NO_2^- system to chlorinate and nitrate tyrosine analogues and nucleobases (Eiserich et al., 1998; Byun et al., 1999a). Moreover, reactive nitrogen species produced by MPO promote peroxidation of lipoprotein lipid (Byun et al., 1999b). Recent studies with acutely inflamed, MPO-deficient mice have demonstrated the enzyme's central role in the chlorination and nitration of tyrosine (Gaut et al., 2001, 2002), strongly supporting the proposal that the enzyme is a physiologically relevant source of chlorinating and nitrating intermediates.

The involvement of such species in atherosclerosis was suggested by the detection of MPO activity and protein in human atherosclerotic tissue (Daugherty et al., 1994; Sugiyama et al., 2001). In intermediate lesions, the enzyme co-localizes with macrophages; in advanced lesions, it associates closely with cholesterol clefts in extracellular lipid deposits (Daugherty et al., 1994). A similar pattern of immunostaining of protein-bound lipid oxidation products has been seen in rabbit atherosclerotic lesions (Rosenfeld et al., 1990). Moreover, LDL exposed in vitro to HOCl, a major product of MPO, aggregates and promotes the transformation of macrophages into foam cells (Hazell and Stocker, 1993). Thus MPO might be one important agent for lipoprotein oxidation in vivo.

24.4 EVIDENCE FOR OXIDATIVE MODIFICATION OF LDL IN THE HUMAN ARTERY WALL

24.4.1 Redox-Active Metal Ions

We demonstrated that isotope dilution GC–MS can readily measure levels of *ortho*-tyrosine and *meta*-tyrosine in human LDL (Leeuwenburgh et al., 1997b). Using this approach, we detected similar levels of oxidation products in plasma LDL and LDL isolated from atherosclerotic lesions. Significantly levels of the two markers in fatty streaks—the earliest lesion of atherosclerosis—were similar to those in normal aortic tissue. Thus reactions catalyzed by metal ions are unlikely to account for LDL oxidation early in atherogenesis. In contrast, we observed a two- to threefold increase in levels of *ortho*-tyrosine and *meta*-tyrosine

in advanced atherosclerotic lesions, although the increase was not statistically significant (Leeuwenburgh et al., 1997b). This raises the possibility that redox-active metal ions, perhaps released from necrotic or dysfunctional cells, might promote LDL oxidation late in the atherosclerotic process.

24.4.2 Glucose

Experimentally induced diabetes accelerates atherosclerosis in diabetic *cynomolgus* monkeys (Litwak et al., 1998). However, two other important risk factors for vascular disease—blood cholesterol and triglyceride concentrations—remain essentially normal in euglycemic and diabetic animals. Thus, the effects of hyperglycemia on atherosclerosis can be isolated in this primate model. We have used isotope dilution gas chromatography–mass spectrometry (GC–MS) to investigate pathways that oxidatively damage aortic proteins in normal and diabetic *cynomolgus* monkeys.

Our experiments showed that even short-term hyperglycemia elevates levels of *ortho*-tyrosine, *meta*-tyrosine, and *o*,*o*′-dityrosine in aortic proteins (Pennathur et al., 2001). We also found a striking correlation between serum levels of glycated hemoglobin, a measure of glycemic control, and aortic tissue levels of both *ortho*-tyrosine and *meta*-tyrosine. These observations strongly suggest a link between hyperglycemia and oxidative damage to the artery wall. Thus, hyperglycemia appears to promote the formation of *ortho*-tyrosine and *meta*-tyrosine early in diabetic macrovascular disease. In vitro, these unnatural tyrosine isomers arise through oxidation of phenylalanine residues of proteins by hydroxyl radical (Huggins et al., 1993; Leeuwenburgh et al., 1997a).

24.4.3 Reactive Nitrogen Species

Studies of LDL oxidized by a variety of oxidation systems in vitro indicate that 3-nitrotyrosine is a specific marker of damage by reactive nitrogen species (Beckman et al., 1994a; Leeuwenburgh et al., 1997b); *o*-tyrosine and *o*,*o*′-dityrosine are minor products of the reaction pathway. Immunohistochemical studies have detected 3-nitrotyrosine in human atherosclerotic lesions, suggesting that reactive nitrogen species might promote LDL oxidation in vivo (Beckman et al., 1994b).

To explore the possibility that reactive nitrogen species could oxidize LDL in the human artery wall, we quantified 3-nitrotyrosine in LDL isolated from atherosclerotic lesions, using isotope dilution GC–MS (Leeuwenburgh et al., 1997b). This lesion LDL contained 80 times more 3-nitrotyrosine than circulating LDL (Fig. 24.2). This observation raises the possibility that nitric oxide can render LDL atherogenic.

Studies suggest that nitric oxide also inhibits lipid peroxidation by reacting with radical intermediates. The relative balance between its pro- and antioxidant effects may depend on the relative rates at which nitric oxide and superoxide are produced. It is therefore possible that the detection of elevated levels of 3-nitrotyrosine in lesion LDL indicate that nitric oxide inhibits lipid peroxidation in the artery wall.

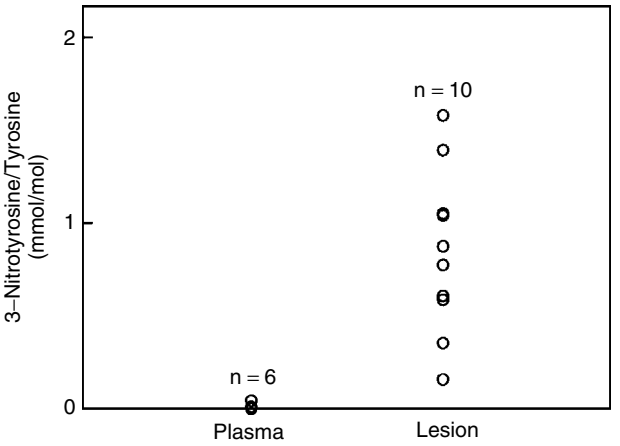


FIGURE 24.2 Levels of protein-bound 3-nitrotyrosine in LDL isolated from plasma and human atherosclerotic tissue (lesion). Atherosclerotic lesions harvested at surgery were pulverized in liquid N_2. LDL was isolated from the tissue extract and from plasma by density gradient ultracentrifugation. ^{13}C-Labeled internal standards were added, and the delipidated LDL protein was subjected to hydrolysis in HBr supplemented with phenol and benzoic acid. Amino acids were isolated by solid phase extraction on a C18 column, derivatized, and subjected to isotope dilution GC/MS analysis (Leeuwenburgh et al., 1997).

24.4.4 Myeloperoxidase

To determine whether tyrosyl radical contributes to LDL oxidation in vivo, we quantified *o*,*o*′-dityrosine levels, using isotope dilution GC–MS (Leeuwenburgh et al., 1997b). The level of *o*,*o*′-dityrosine in LDL isolated from atherosclerotic lesions was 100-fold higher than that in circulating LDL. In striking contrast, there was no evidence of *ortho*-tyrosine enrichment. Tissue *o*,*o*′-dityrosine levels were also markedly elevated in fatty streaks (the earliest lesion of atherosclerosis) and advanced atherosclerotic lesions. These results suggest that tyrosyl radical, perhaps generated in part by MPO, contributes to LDL oxidation both early and late in the disease process.

We obtained further evidence that MPO oxidizes artery wall constituents by quantifying levels of 3-chlorotyrosine. Chlorinated biomolecules should be specific markers of oxidative damage by activated phagocytes because, at plasma concentrations of halide, MPO is the only human enzyme known to generate hypochlorous acid (Harrison and Schultz, 1976; Weiss et al., 1986). In vitro, 3-chlorotyrosine forms in the protein component of LDL when the lipoprotein is oxidized by the MPO-peroxide-chloride system (Hazen and Heinecke, 1997). In contrast, 3-chlorotyrosine is undetectable when LDL is oxidized by hydroxyl radical, copper, iron, horseradish peroxidase, hemin, glucose, or peroxynitrite (Hazen and Heinecke, 1997).

Using isotope dilution GC–MS, we detected a marked increase in the levels of 3-chlorotyrosine in LDL isolated from human atherosclerotic tissue and in atherosclerotic lesions harvested at surgery (Hazen and Heinecke, 1997). By using MPO-deficient mice, we also obtained evidence that the MPO system of normal neutrophils chlorinates tyrosine residues in vivo (Gaut et al., 2001). These results provide strong evidence that oxidative damage to LDL—and perhaps to other macromolecules involved in plaque formation—can result from the action of MPO in the human artery wall. The recent demonstration that elevated blood levels of MPO associate strongly with increased risk of coronary artery disease (Zhang et al., 2001) supports this hypothesis.

Reactive nitrogen species produced by MPO promote peroxidation of lipoprotein lipid and consequently enhance the recognition of peroxidized LDL by macrophage scavenger receptors (Byun et al., 1999b; Podrez et al., 1999). Also both 3-chlorotyrosine and 3-nitrotyrosine are found at abnormally high levels in human atherosclerotic tissue (Leeuwenburgh et al., 1997b; Hazen and Heinecke, 1997). Therefore, reactive nitrogen species produced by MPO might contribute to inflammatory tissue injury and the pathogenesis of human disease.

24.5 OXIDATIVE MODIFICATION OF HDL

24.5.1 Oxidized HDL in Humans

Several epidemiologic studies have demonstrated an inverse relationship between plasma HDL levels and cardiovascular disease risk, establishing low HDL levels as a strong independent marker for atherosclerosis (Miller, 1980; Gordon et al., 1986; Gordon et al., 1977). HDL and its major protein, apolipoprotein A-I (apo A-I), are not generally associated with inflammation. However, HDL is one of the major blood components that bind to bacterial lipopolysaccharide (LPS), and high HDL levels protect animals from LPS-induced septic shock (Wu et al., 2004). Studies suggest that HDL also has potent anti-inflammatory and antioxidant properties (Barter et al., 2004; Navab et al., 2004), although the mechanisms are incompletely understood.

The rates at which apoA-I is produced in the liver and degraded in the periphery can influence plasma levels of HDL. The extent to which each mechanism contributes to low HDL levels during inflammation is unclear, however. Interestingly a low HDL level is one of the hallmarks of the metabolic syndrome (Grundy et al., 2004) that significantly increases the risk of cardiovascular disease. Furthermore HDL is remodeled to a considerable extent during inflammation (Cabana et al., 1996). Under these conditions it is possible that HDL and apoA-I become important targets for oxidative modification. Indeed inflammation has been proposed to significantly change the anti-atherogenic properties of HDL (Barter et al., 2004; Navab et al., 2004).

We recently showed that circulating HDL is oxidized in humans (Bergt et al., 2004a; Pennathur et al., 2004). Using isotope dilution GC–MS, we found that HDL isolated from the blood of patients with acute coronary artery disease contains markedly elevated levels of both 3-chloro- and 3-nitrotyrosine. Thus, apoA-I

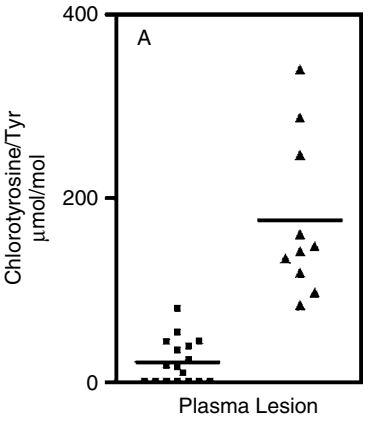


FIGURE 24.3 3-Chlorotyrosine content of HDL isolated from plasma and atherosclerotic human carotid tissue. Levels of the oxidized amino acid were determined by isotope dilution GC/MS (Bergt et al., 2004).

may be an important target for oxidative modification in cardiovascular disease. Chlorinated HDL (Fig. 24.3) and nitrated HDL also appear in human atherosclerotic lesions (Bergt et al., 2004a; Pennathur et al., 2004; Zheng et al., 2004, 2005) and in vitro experiments indicate that these abnormal forms of the lipoprotein arise when MPO oxidizes apoA-I (Bergt et al., 2004b; Pennathur et al., 2004). ApoA-I that is chlorinated in vitro loses its ability to promote cholesterol efflux by the ABCA1 pathway, suggesting that HDL oxidation might contribute to the formation of macrophage foam cells in the artery wall. Involvement of MPO in the formation of 3-chlorotyrosine and 3-nitrotyrosine in HDL can be further inferred from in vivo studies because protein chlorination and nitration are impaired in mice deficient in MPO (Gaut et al., 2001, 2002).

24.5.2 Site-Specific Modification of ApoA-I

Little is known about the factors that control the site-specific chlorination of tyrosine residues in proteins. ApoA-I is composed of 10 amphipathic helices with essential roles in lipid binding, lipoprotein stability, and reverse cholesterol transport. Five of the seven tyrosine residues in apo A-I are located in amphipathic helices. Using tandem mass spectrometric (MS/MS) analysis (Bergt et al., 2004b), we demonstrated that tyrosine residue 192 (Tyr192), which resides in helix 8 of apoA-I, is the single major site of chlorination when the protein is exposed to HOCl or the MPO-H_2O_2-chloride system in vitro (Fig. 24.4). Tyr192 resides in an YXXK motif (Y = tyrosine, K = lysine, X = unreactive amino acid), and molecular modeling demonstrates that these tyrosine and lysine residues are adjacent on the same face of an amphipathic α-helix in apoA-I (Bergt et al., 2004b). HOCl reacts rapidly with the ε amino group of lysine to form long-lived chloramines. Studies with synthetic peptides demonstrate that

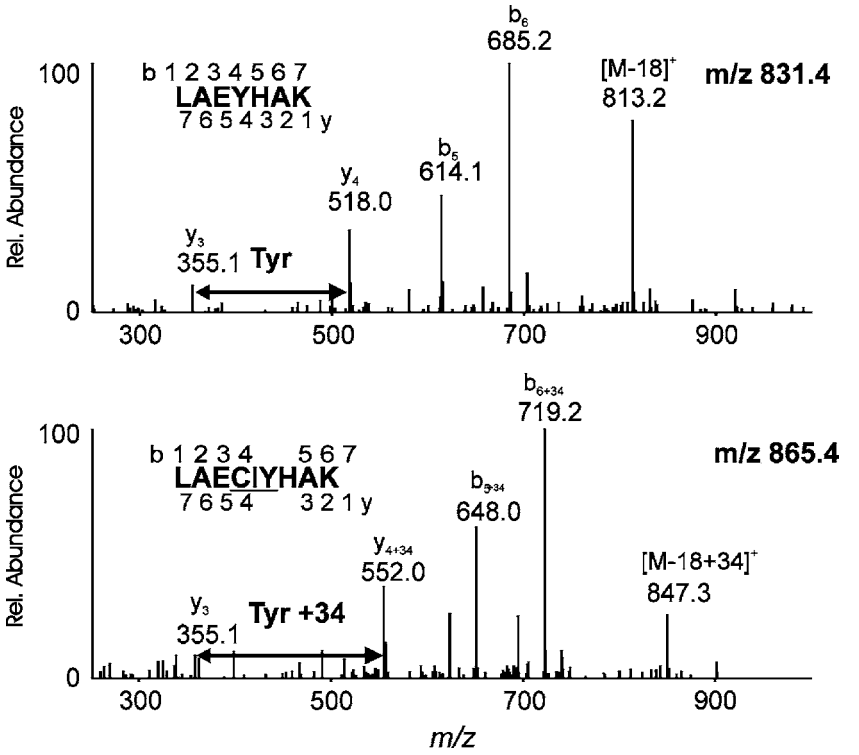


FIGURE 24.4 MS/MS identification of the major site of tyrosine chlorination in apolipoprotein A-I exposed to HOCl. MS/MS analysis of $[LAEYHAK + H]^+$ (m/z 831.4) and $[LAEClYHAK + H]^+$ (m/z 865.4) in HDL oxidized with HOCl. HDL was exposed to HOCl (80:1, mol/mol, oxidant/HDL particle) for 120 minutes at 37°C in PBS (pH 7.4). After the reaction was terminated with L-methionine, the HDL proteins were digested with trypsin, and the tryptic digest peptides were subjected to analysis by LC-ESI-MS/MS (Bergt et al., 2004b).

lysine residues can direct the regiospecific chlorination of tyrosine residues by a reaction pathway involving chloramine formation, suggesting that the site-specific chlorination of apoA-I requires the participation of a nearby lysine residue (Bergt et al., 2004b).

24.6 OXIDATIVE REGULATION OF MATRIX METALLOPROTEINASES

Oxidative modification by MPO is unlikely to be limited to lipoproteins in atherosclerotic lesions. Other possible target is the matrix metalloproteinases (MMPs), a structurally related family of proteinases that catalyze a wide variety

of normal and pathological processes involved in tissue remodeling (Woessner and Nagase, 2000; Parks et al., 2001; Birkedal-Hansen et al., 1993; D'Armiento, 2002; Galis and Khatri, 2002). In vitro, MMPs degrade virtually all structural and basement membrane components of extracellular matrix found in vascular tissue. At least five MMPs are clearly overexpressed in human atherosclerosis at both the protein and mRNA level (D'Armiento, 2002; Galis and Khatri, 2002; Halpert et al., 1996; Thompson et al., 1995; McMillan et al., 1995; Freestone et al., 1995): MMP-2 (72 kDa gelatinase), MMP-3 (stromelysin), MMP-7 (matrilysin), MMP-9 (92 kDa gelatinase), and MMP-12 (macrophage metalloelastase). Whereas a variety of cell types in lesions express MMP-2 and MMP-3, only human macrophages express high levels of MMP-7, MMP-9, and MMP-12. Immunohistochemical studies have revealed extensive degradation and fragmentation of collagen in the atherosclerotic intima (Sukhova et al., 1999), and MMPs and cathepsin K are the only known agents that initiate collagen cleavage (Woessner and Nagase, 2000). Collectively these observations indicate that MMPs likely play critical roles in inflammation and matrix degradation.

Because of the need to carefully control proteolytic activity in tissue, MMPs are highly regulated at both the transcriptional and post-translational levels (Van Wart and Birkedal-Hansen, 1990). They are synthesized as inactive zymogens, which contain a prodomain that shields the active site. This prodomain contains a highly conserved cysteine residue called the "cysteine switch." Along with three histidine residues at the active site, the cysteine switch coordinates with the zinc cation in the catalytic domain. When the cysteine's thiol group stops interacting with the zinc ion, the active site becomes unmasked (Van Wart and Birkedal-Hansen, 1990).

A key unresolved issue is the nature of the biochemical events that activate MMP zymogens in vivo. Various agents have been proposed. Their common feature is the ability to disrupt the coordination between the cysteine residue and the zinc ion.

The most widely studied model for MMP activation involves proteolytic cleavage of the pro-domain because removing this part of the protein separates the cysteine switch from the zinc ion. Although about one-third of known mammalian MMPs are activated intracellularly by furin-like proprotein convertases, the physiological relevance of proteolytic activation of the other members, including the MMPs found in atherosclerotic lesions, is not clearly established. Another protease, plasmin, is widely regarded as a key activator of pro-MMP, and reducing levels of urokinase-type plasminogen activator protected against media destruction and aneurysm formation in a genetic model of atherosclerosis (Carmeliet et al., 1997). These observations suggest that plasmin activates pro-MMPs in vivo. In contrast, other studies demonstrated that loss of plasminogen greatly accelerated intimal lesion formation in hypercholesterolemic animals (Xiao et al., 1997; Lijnen et al., 1998). Thus the exact mechanisms that convert pro-MMPs into MMPs in vivo are unclearal, although mechanisms not involving plasmin are likely to exist.

24.6.1 Site-Specific Oxidative Activation of MMP-7

An alternative mechanism for activating latent MMPs might be to oxidatively modify, rather than remove, the cysteine switch. Oxidation of this cysteine's thiol group dramatically changes its chemical properties and effectively eliminates its ability to coordinate the zinc cation in the catalytic domain.

Thiol groups of proteins are readily oxidized by a wide range of reactive intermediates, including H_2O_2, $ONOO^-$, and HOCl (Winterbourn, 1985). Thus oxidants generated by macrophages and other cells in the artery wall—such as those produced by MPO—might be important regulators of MMP activity and elastin degradation in vivo.

To explore the potential role of oxidants in MMP activation, we used MS/MS to determine the nature and specific locations of the oxidative modifications that HOCl, a specific product of MPO, creates in MMP-7. To determine whether HOCl can convert pro-MMP-7 to an active form by oxygenating the cysteine switch, we exposed the zymogen to oxidant and determined its proteolytic activity. Pro-MMP-7 exposed to increasing concentrations of HOCl increased its proteolytic activity, which became optimal at nearly a 50:1 mole ratio of HOCl to protein (Fu et al., 2001). In contrast, pro-MMP-7 failed to display proteolytic activity during exposure to increasing concentrations of H_2O_2 (Fig. 24.5). Model studies with cysteine switch peptide showed that two major products were observed both with the H_2O_2/MPO system and with HOCl corresponding to formation of a disulfide (RSSR) and sulfinic acid (RSO_2H). A minor production of sulfonic acid (RSO_3H) was also detected (Fu et al., 2001). Compared to HOCl, hydrogen peroxide reacted with MMP-7 at a much slower rate, and its reaction produced exclusively disulfide (RSSR) rather than sulfinic acid (RSO_2H) (Fu et al., 2001). Thus HOCl, but not H_2O_2, can convert pro-MMP-7 to an enzymatically active form (Fig. 24.5).

To investigate the mechanism, we exposed the proenzyme to HOCl, digested it with trypsin, and analyzed the mixture with liquid chromatography–electrospray ionization MS (Fu et al., 2001). A new peptide that appeared in the proteolytic digest in a dose-dependent manner had an m/z of 821.2, corresponding to the protonated peptide $[C(SO_2H)GVPDVAE + H]^+$, which is located in the pro-MMP-7 prodomain and includes the cysteine switch residue. It should be noted that the CGVPDVAE peptide is not found in the tryptic digest of the unoxidized proenzyme and that the *C*-terminal Glu residue corresponds to the formation of the $C(SO_2H)GVPDVAE$ peptide via autolytic cleavage of Glu77-Tyr78 bond resulting in activation of the enzyme. Collision-induced dissociation of this ion produced a fragmentation pattern consistent with that peptide's sequence and indicated the addition of 32 amu to the cysteine residue. These results show that HOCl converts the thiol residue of the cysteine switch of pro-MMP-7 to the sulfinic acid derivative (RSO_2H; M + 32 mass units) by adding two oxygen atoms (Fig. 24.6).

We studied macrophages in human atherosclerotic lesions to determine whether MPO produces oxidants that might interact with MMP-7 in vivo. Immunohistochemistry of serial sections of atherosclerotic arteries detected both MMP-7 and

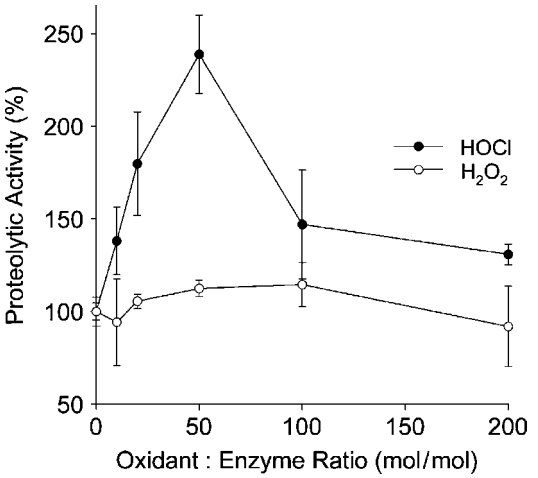


FIGURE 24.5 Proteolytic activity of pro-MMP-7 exposed to H_2O_2 or HOCl. Pro-MMP-7 (1.5 μM) was exposed to H_2O_2 or HOCl at the indicated mole ratio for 30 minutes at 37°C in buffer A. Reactions were initiated by adding oxidant and terminated by adding 500 μM L-methionine. The proteolytic activity of pro-MMP-7 was then assessed by monitoring the hydrolysis of Mca peptide. Results shown are the means ± SD of three determinations and are representative of the findings of three independent experiments (Fu et al., 2001).

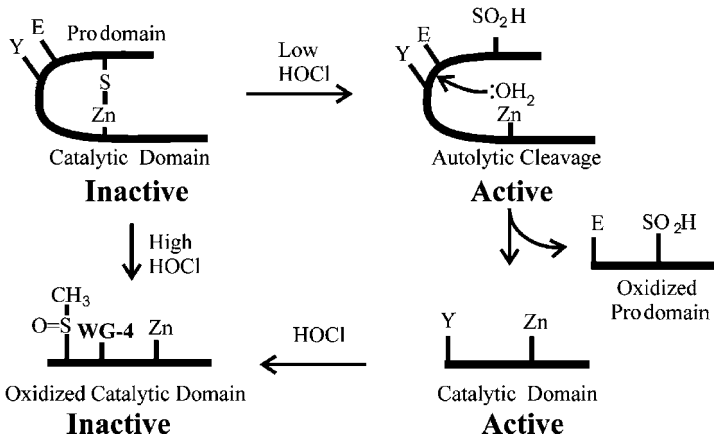


FIGURE 24.6 Scheme of oxidative-mediated activation and inactivation of pro-MMP (Fu et al., 2003).

MPO in foamy macrophages at the border of the lipid core, a region of the lesion prone to plaque rupture (Fu et al., 2001). This co-localization suggests that HOCl generated by MPO might regulate MMP-7 in vivo.

24.6.2 Site-Specific Oxidative Inactivation of MMP-7

We found that active MMP-7 can be inactivated in vitro if it is exposed to oxidants for a prolonged period or if oxidant concentration is high (Fu et al., 2001, 2003, 2004). Others have reported similar observations for MMP-2, MMP-8, and MMP-9 (Rajagopalan et al., 1996; Weiss et al., 1985; Peppin and Weiss, 1986; Michaelis et al., 1992; Vissers and Winterbourn, 1987). Thus generation of oxidants by phagocytes could provide one mechanism for regulating both the activation and inactivation of MMPs in vivo.

To determine how oxidants inactivate MMP-7, we incubated the enzyme with HOCl or H_2O_2. Exposure to increasing concentrations of HOCl destroyed proteolytic activity, as assessed with a fluorescent peptide substrate or casein zymography (Fu et al., 2003). To investigate the molecular basis for this inactivation, we exposed MMP-7 to H_2O_2 or HOCl and used high-performance liquid chromatography–MS (HPLC–MS) to analyze the tryptic digest. We found a strong linear correlation between loss of a single peptide (VVWGTADIMIGFAR, which is located in the catalytic domain) and MMP-7 inactivation, suggesting that oxidation of this peptide is likely involved in enzyme inactivation. The tryptophan and methionine residues of this peptide are particularly vulnerable to oxidation by HOCl. We used MS and MS/MS (tandem MS) to characterize the oxidized peptide (Fu et al., 2003). Unexpectedly, the peptide's methionine residue had not been oxidized. Rather, the peptide's mass was 4 amu less than that of the parent peptide. The collision-induced dissociation spectrum of this peptide unequivocally indicated that this loss resulted from changes on adjacent tryptophan and glycine residues. We therefore termed the product peptide WG–4 (Fig. 24.7).

To determine the structure of WG–4, we synthesized a peptide that mimicked this region of MMP-7, oxidized it in vitro, and isolated the oxidized peptide by HPLC. The structure of the oxidized peptide was elucidated using high-resolution ^{1}H NMR analysis, total correlated spectroscopy, nuclear Overhauser effect spectroscopy, and proton-detected heteronuclear multiple quantum coherence analysis (Fu et al., 2004). These studies demonstrated that HOCl oxidizes adjacent tryptophan and glycine residues into an unusual cross-linked, aromatic compound, introducing a new ring structure into the protein backbone (Fig. 24.8). This moiety profoundly affects the backbone's secondary structure, causing it to bend in a manner that likely changes the protein's three-dimensional structure (Fu et al., 2004).

Our findings suggest that oxidants can both activate and inactivate MMPs, depending on their concentration and persistence (Fig. 24.6). High concentrations of oxidants could appear on the cell surface of phagocytes, which store MMPs in secretory compartments and produce H_2O_2 in their outer membrane (Segal, 1989; Hurst and Barrette, 1989; Witztum and Steinberg, 1991). Because of its high reactivity, the thiol of the cysteine switch would be the preferred target in the latent MMP, and its oxygenation would lead to rapid activation (Fu et al., 2001). Once the thiol residue of the prodomain was oxidized or when oxidant levels were high, additional sites in the MMP would be modified, and the enzyme would become inactive (Fu et al., 2001, 2003, 2004). Thus oxidants could sequentially

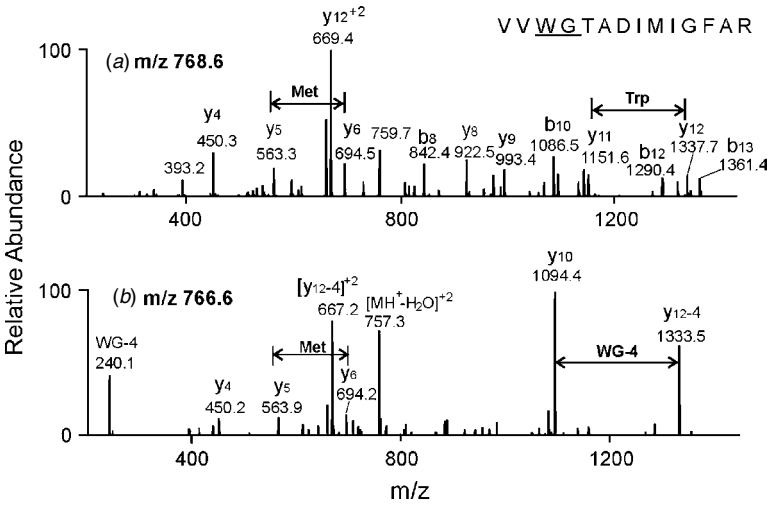


FIGURE 24.7 MS/MS identification of the oxidative modification of Trp-Gly moiety in the catalytic domain of MMP-7 exposed to HOCl. MS/MS analysis of [VVWGTADIMIGFAR + 2H]$^{2+}$ (*m/z* 768.6) (a) and peptide WG-4 (*m/z* 766.6) (b) from tryptic digest of HOCl treated MMP-7. MMP-7 was incubated with HOCl (50:1, mol/mol, oxidant/protein) for 30 minutes at 37°C in HEPES buffer pH 7.4, before quenching with 10-fold molar excess of methionine and trypsin digestion (Fu et al., 2003).

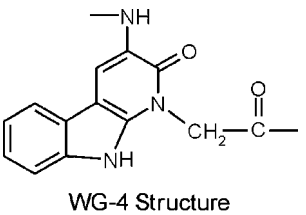


FIGURE 24.8 Structure of WG-4 moiety in MMP-7 inactivated by HOCl (50:1, mol/mol, oxidant/protein, for 30 minutes at 37°C in HEPES buffer pH 7.4) (Fu et al., 2003).

activate pro-MMPs and inactivate MMPs. They might also inactivate MMPs that have been activated by other pathways.

Our suggestion that oxidants inactivate MMP-7 provides an alternative mechanism to the ones typically ascribed to protein inactivators, such as TIMPs (Woessner and Nagase, 2000; Visse and Nagase, 2003). Moreover previous studies have shown that MMP-2, MMP-8, and MMP-9 are also inactivated by oxidants in vitro (Rajagopalan et al., 1996; Weiss et al., 1985; Peppin and Weiss, 1986; Michaelis et al., 1992; Vissers and Winterbourn, 1987). However, the WG motif does not appear in these proteins, indicating that other mechanisms are responsible for oxidative inactivation. In vivo, oxidants

generated by phagocytes could confine MMP activity to bursts of pericellular proteolysis that are highly regulated in space and time.

24.7 CONCLUSIONS

Isotope dilution GC–MS analyses of tissue and lipoproteins have provided strong evidence regarding the physiological relevance of specific pathways in oxidizing LDL and HDL in the human artery wall. ESI-MS/MS analysis of proteins oxidized in vitro has demonstrated that specific sites in apoA-I and MMP-7 are targeted for oxidation. Moreover site-specific oxidation appears to alter the biological activity of these proteins. These observations support the proposal that oxidants play important roles in atherogenesis, and demonstrate the power of mass spectrometry for investigating the oxidative post-translational modification of proteins.

LIST OF ABBREVIATIONS

AGEs, advanced glycation end products
apoA-I, apolipoprotein A-I
CRP, C-reactive protein
ESI-MS/MS, electrospray ionization tandem mass spectrometry
GC–MS, isotope dilution gas chromatography mass spectrometry
HDL, high-density lipoprotein
HOCl, hypochlorous acid
HPLC–MS, high-performance liquid chromatography–mass spectrometry
LDL, low-density lipoprotein
LPS, lipopolysaccharide
MMP-7, matrilysin
MMP-9, gelatinase B
MMPs, metalloproteinases
MPO, myeloperoxidase
$ONOO^-$, peroxynitrite
RSO_2H, sulfinic acid
RSO_3H, sulfonic acid
RSSR, disulfide

REFERENCES

Aasa R., Malmstrom B.G., Saltman P., Vanngard T. (1963). The specific binding of iron (III) and copper (II) to transferrin and conalbumin. *Biochem Biophys Acta* 75: 203–209.

Aji W., Ravalli S., Szabolcs M., Jiang X.-C., Sciacca R.R., Michler R.E., Canon P.J. (1997). L-Arginine prevents xanthoma development and inhibits atherosclerosis in LDL receptor knockout mice. *Circulation* 95: 430–437.

Balla G., Eaton J.W., Belcher J.D., Vercellotti G.M. (1991). Hemin: A possible physiological mediator of low density lipoprotein oxidation and endothelial injury. *Arterioscler Thromb* 11: 1700–1711.

Barter P.J., Nicholls S., Rye K.A., Anantharamaiah G.M., Navab M., Fogelman A.M. (2004). Antiinflammatory properties of HDL. *Circ Res* 95: 764–772.

Baynes J.W., Thorpe S.R. (1999). Role of oxidative stress in diabetic complications: A new perspective on an old paradigm. *Diabetes* 48: 1–9.

Beckman J.S., Beckman T.W., Chen J., Marshall P.A., Freeman B.A. (1990). Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 87: 1620–1623.

Beckman J.S., Chen J., Ischiropoulos H., Crow J.P. (1994a). Oxidative chemistry of peroxynitrite. *Methods Enzymol* 233: 229–240.

Beckman J.S., Ye Y.Z., Anderson P.G., Chen J., Accavitti M.A., Tarpey M.M., White C.R. (1994b). Extensive nitration of protein tyrosines in human atherosclerosis detected by immunohistochemistry. *Biol Chem Hoppe-Seyler* 375: 81–88.

Bergt C., Pennathur S., Fu X., Byun J., O'Brien K., McDonald T.O., Singh P., Anantharamaiah G.M., Chait A., Brunzell J., Geary R.L., Oram J.F., Heinecke J.W. (2004a). The myeloperoxidase product hypochlorous acid oxidizes HDL in the human artery wall and impairs ABCA1-dependent cholesterol transport. *Proc Natl Acad Sci USA* 101: 13032–13037.

Bergt C., Fu X., Huq N.P., Kao J., Heinecke J.W. (2004b). Lysine residues direct the chlorination of tyrosines in YXXK motifs of apolipoprotein A-I when hypochlorous acid oxidizes high density lipoprotein. *J Biol Chem* 279: 7856–7866.

Bhattacharjee S., Pennathur S., Byun J., Crowley J., Mueller D., Gischler J., Hotchkiss R.S., Heinecke J.W. (2001). NADPH Oxidase of neutrophils elevates *o*,*o*′-dityrosine cross-links in proteins and urine during inflammation. *Arch Biochem Biophys* 395: 69–77.

Birkedal-Hansen H., Moore W.G.I., Bodden H.K., Windsor L.I., Birkedal-Hansen B., DeCarlo A., Engler J.A. (1993). Matrix metalloproteinases: A review. *Crit Rev Oral Biol Med* 4: 197–250.

Byun J., Henderson J.P., Mueller D.M., Heinecke J.W. (1999a). 8-Nitro-2′-deoxyguanosine, a specific marker of oxidation by reactive nitrogen species, is generated by the MPO-hydrogen peroxide-nitrite system of activated human phagocytes. *Biochemistry* 38: 2590–2600.

Byun J., Mueller D.M., Fabjan J.S., Heinecke J.W. (1999b). Nitrogen dioxide radical generated by the MPO-hydrogen peroxide-nitrite system promotes lipid peroxidation of low density lipoprotein. *FEBS Lett* 455: 243–246.

Cabana V.G., Lukens J.R., Rice K.S., Hawkins T.J., Getz G.S. (1996). HDL content and composition in acute phase response in three species: Triglyceride enrichment of HDL a factor in its decrease. *J Lipid Res* 37: 2662–2674.

Carmeliet P., Moons L., Lijnen R., Baes M., Lemaitre V., Tipping P., Drew A., Eeckhout Y., Shapiro S., Lupu F., Collen D. (1997). Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. *Nat Genet* 17: 439–444.

Cooke J.P., Tsao P.S. (1994). Is NO an endogenous antiatherogenic molecule? *Arterioscler Thromb* 14: 653–655.

D'Armiento J. (2002). Matrix metalloproteinase disruption of the extracellular matrix and cardiac dysfunction. *Trends Cardiovasc Med* 12: 97–101.

Daugherty A., Rateri D.L., Dunn J.L., Heinecke J.W. (1994). Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. *J Clin Invest* 94: 437–444.

Dean R.T., Fu S., Stocker R., Davies M.J. (1997). Biochemistry and pathology of radical-mediated protein oxidation. *Biochem J* 324: 1–18.

Ehrenwald E., Chisolm G.M., Fox P.L. (1994). Intact ceruloplasmin oxidatively modifies low density lipoprotein. *J Clin Invest* 93: 1493–1501.

Ehrenwald E., Fox P.L. (1996). Role of endogenous ceruloplasmin in low density lipoprotein oxidation by human U937 monocytic cells. *J Clin Invest* 97: 884–890.

Eiserich J.P., Cross C.E., Jones A.D., Halliwell B., van der Vliet A. (1996). Formation of nitrating and chlorinating species by reaction of nitrite with hypochlorous acid: A novel mechanism for nitric oxide-mediated protein modification. *J Biol Chem* 271: 19199–19208.

Eiserich J.P., Hristova M., Cross C.E., Jones A.D., Freeman B.A., Halliwell B., van der Vliet A. (1998). Formation of nitric oxide-derived inflammatory oxidants by MPO in neutrophils. *Nature* 391: 393–395.

Fogelman A.M., Shechter I., Seager J., Hokom M., Child J.S., Edwards P.A. (1980). Malondialdehyde alteration of low density lipoproteins leads to cholesteryl ester accumulation in human monocyte-macrophages. *Proc Natl Acad Sci USA* 77: 2214–2217.

Francis G.A., Mendez A.J., Bierman E.L., Heinecke J.W. (1993). Oxidative tyrosylation of high density lipoprotein by peroxidase enhances cholesterol removal from cultured fibroblasts and macrophage foam cells. *Proc Natl Acad Sci USA* 90: 6631–6634.

Freestone T., Turner R.J., Coady A., Higman D.J., Greenhalgh R.M., Powell J.T. (1995). Inflammation and matrix metalloproteinases in the enlarging abdominal aortic aneurysm. *Arterioscler Thromb Vasc Biol* 15: 1145–1151.

Fridovich I. (1978). The biology of oxygen radicals. *Science* 201: 875–880.

Fu X., Kao J.L., Bergt C., Kassim S.Y., Huq N.P., d'Avignon A., Parks W.C., Mecham R.P., Heinecke J.W. (2004). Oxidative cross-linking of tryptophan to glycine restrains matrix metalloproteinase activity: Specific structural motifs control protein oxidation. *J Biol Chem* 279: 6209–6212.

Fu X., Kassim S.Y., Parks W.C., Heinecke J.W. (2003). Hypochlorous acid generated by myeloperoxidase modifies adjacent tryptophan and glycine residues in the catalytic domain of matrix metalloproteinase-7 (matrilysin): An oxidative mechanism for restraining proteolytic activity during inflammation. *J Biol Chem* 278: 28403–28409.

Fu X., Kassim S.Y., Parks W.C., Heinecke J.W. (2001). Hypochlorous acid oxygenates the cysteine switch domain of pro-matrilysin (MMP-7). A mechanism for matrix metalloproteinase activation and atherosclerotic plaque rupture by myeloperoxidase. *J Biol Chem* 276: 41279–41287.

Galis Z.S., Khatri J.J. (2002). Matrix metalloproteinases in vascular remodeling and atherogenesis: The good, the bad, and the ugly. *Circ Res* 90: 251–262.

Gaut J.P., Yeh G.C., Tran H.D., Byun J., Henderson J.P., Richter G.M., Brennan M.L., Lusis A.J., Belaaouaj A., Hotchkiss R.S., Heinecke J.W. (2001). Neutrophils employ

the MPO system to generate antimicrobial brominating and chlorinating oxidants during sepsis. *Proc Natl Acad Sci USA* 98: 11961–11966.

Gaut J.P., Byun J., Tran H.D., Lauber W.M., Carroll J.A., Hotchkiss R.S., Belaaouaj A., Heinecke J.W. (2002). Myeloperoxidase produces nitrating oxidants in vivo. *J Clin Invest* 109: 1311–1319.

Goldstein J.L., Ho Y.K., Basu S.K., Brown M.S. (1979). Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc Natl Acad Sci USA* 76: 333–336.

Gordon D.J., Knoke J., Probstfield J.L., Superko R., Tyroler H.A. (1986). High-density lipoprotein cholesterol and coronary artery disease in hypercholesterolemic men: The Lipid Research Clinics Coronary Primary Prevention Trial. *Circulation* 73: 1217–1225.

Gordon T., Castelli W.P., Hjortland, M.C., Kannel, W.B., Dawber T.R. (1977). High density lipoproteins as a protective factor against coronary heart disease: The Framingham study. *Am J Med* 62: 707–714.

Graham A.N., Hogg N., Kalyanaraman B., O'Leary V., Darley-Usmar V., Moncada S. (1993). Peroxynitrite modifications of LDL leads to recognition by the macrophage scavenger receptor. *FEBS Lett* 330: 181–185.

Grundy S.M., Brewer H.B. Jr., Cleeman J.I., Smith S.C. Jr., Lenfant C. (2004). Definition of metabolic syndrome: Report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. *Arterioscler Thromb Vasc Biol* 24: e13–18.

Halpert I., Roby J.D., Sires U.I., Potter-Perigo S., Wight T.N., Welgus H.G., Shapiro S.D., Wickline S.A., Parks W.C. (1996). Matrilysin is expressed by lipid-laden macrophages at sites of potential rupture in atherosclerotic lesions and localizes to areas of versican deposition, a proteoglycan substrate for the enzyme. *Proc Natl Acad Sci USA* 93: 9748–9753.

Harrison J.E., Schultz J. (1976). Studies on the chlorinating activity of MPO. *J Biol Chem* 251: 1371–1376.

Hayashi K., Noguchi N., Niki E. (1995). Action of nitric oxide as an antioxidant against oxidation of soybean phosphatidylcholine liposomal membranes. *FEBS Lett* 370: 37–40.

Hazell L.J., Stocker R. (1993). Oxidation of low-density lipoprotein with hypochlorite causes transformation of lipoprotein into a high-uptake form for macrophages. *Biochem J* 290: 165–172.

Hazen S.L., Heinecke J.W. (1997). 3-Chlorotyrosine, a specific marker of MPO-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. *J Clin Invest* 99: 2075–2081.

Hazen S.L., Hsu F.F., Mueller D.M., Crowley J.R., Heinecke J.W. (1996). Human neutrophils employ chlorine gas as an oxidant during phagocytosis. *J Clin Invest* 98: 1283–1289.

Heinecke J.W., Baker L., Rosen H., Chait A. (1986). Superoxide-mediated modification of low density lipoprotein by arterial smooth muscle cells. *J Clin Invest* 77: 757–761.

Heinecke J.W., Li W., Daehnke H.L., Goldstein J.A. (1993a). Dityrosine, a specific marker of oxidation, is synthesized by the MPO-hydrogen peroxide system of human neutrophils and macrophages. *J Biol Chem* 268: 4069–4077.

Heinecke J.W., Li W., Francis G.A., Goldstein J.A. (1993b). Tyrosyl radical generated by MPO catalyzes the oxidative cross-linking of proteins. *J Clin Invest* 91: 2866–2872.

Heinecke J.W., Rosen H., Chait A. (1984). Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *J Clin Invest* 74: 1890–1895.

Heinecke J.W. (1999). Mass spectrometric quantification of amino acid oxidation products in proteins: Insights into pathways that promote LDL oxidation in the human artery wall. *FASEB J* 13: 1113–1120.

Henriksen T., Mahoney E.M., Steinberg D. (1981). Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: Recognition by receptors for acetylated low density lipoproteins. *Proc Natl Acad Sci USA* 78: 6499–6503.

Hogg N., Kalyanaraman B., Joseph J., Struck A., Parthasarathy S. (1993). Inhibition of low-density lipoprotein oxidation by nitric oxide: Potential role in atherogenesis. *FEBS Lett* 334: 170–174.

Huggins T.G., Wells-Knecht M.C., Detorie N.A., Baynes J.W., Thorpe S.R. (1993). Formation of o-tyrosine and dityrosine in proteins during radiolytic and metal-catalyzed oxidation. *J Biol Chem* 268: 12341–12347.

Hurst J.K., Barette W.C. (1989). Leukocytic oxygen activation and microbicidal oxidative toxins. *CRC Critical Reviews Biochem Mol Biol* 24: 271–328.

Jessup W., Mohr D., Gieseg S.P., Dean R.T., Stocker R. (1992). The participation of nitric oxide in cell free and its restriction of macrophage-mediated oxidation of low-density lipoprotein. *Biochim Biophys Acta* 1180: 73–82.

Karthein R., Dietz R., Nastainczyk W., Ruff H. (1988). Higher oxidation states of prostaglandin H synthase. *Eur J Biochem* 171: 313–320.

Kettle A.J. (1996). Neutrophils convert tyrosyl residues in albumin to chlorotyrosine. *FEBS Lett* 379: 103–106.

Klebanoff S.F. (1980). Oxygen metabolism and the toxic properties of phagocytes. *Ann Int Med* 93: 480–489.

Knowles J.W., Reddick R.L., Jennette J.C., Shesely E.G., Smithies O., Maeda N. (2000). Enhanced atherosclerosis and kidney dysfunction in eNOS(-/-)Apoe(-/-) mice are ameliorated by enalapril treatment. *J Clin Invest* 105: 451–458.

Kuhlencordt P.J., Chen J., Han F., Astern J., Huang P.L. (2001). Genetic deficiency of inducible nitric oxide synthase reduces atherosclerosis and lowers plasma lipid peroxides in apolipoprotein E-knockout mice. *Circulation* 103: 3099–3104.

Lamb D., Mitchinson M.J., Leake D.S. (1995). Transition metals within human atherosclerotic lesions can catalyze the oxidation of low density lipoprotein by macrophages. *FEBS Lett* 374: 12–16.

Lee C., Yim M.B., Boon Chock P., Yim H., Kang S. (1998). Oxidation-reduction properties of methylglyoxal-modified protein in relation to free radical generation. *J Biol Chem* 273: 25272–25278.

Leeuwenburgh C., Rasmussen J.E., Hsu F.F., Mueller D.M., Pennathur S., Heinecke J.W. (1997a). Mass spectrometric quantification of markers for protein oxidation by tyrosyl radical, copper, and hydroxyl radical in low density lipoprotein isolated from human atherosclerotic plaques. *J Biol Chem* 272: 3520–3526.

Leeuwenburgh C., Hardy M.M., Hazen S.L., Wagner P., Oh-ishi S., Steinbrecher U.P., Heinecke J.W. (1997b). Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerosis. *J Biol Chem* 272: 1433–1436.

Lijnen H.R., Silence J., Lemmens G., Frederix L., Collen D. (1998). Regulation of gelatinase activity in mice with targeted inactivation of components of the plasminogen/plasmin system. *Thromb Haemost* 79: 1171–1176.

Litwak K.N., Cefalu W.T., Wagner J.D. (1998). Chronic hyperglycemia increases arterial low-density lipoprotein metabolism and atherosclerosis in *cynomolgus* monkeys. *Metabolism* 47: 947–954.

McCormick M.L. Gaut J.P., Lin T., Britigan B.E., Buettner G.R., Heinecke J.W. (1998). Electron paramagnetic resonance detection of free tyrosyl radical generated by MPO, lactoperoxidase, and horseradish peroxidase. *J Biol Chem* 273: 32030–32037.

Mcmillan W.D., Patterson B.K., Keen R.R., Shively V.P., Cipollone M., Pearce W.H. (1995). In situ localization and quantification of mRNA for 92-kD type IV collagenase and its inhibitor in aneurysmal, occlusive, and normal aorta. *Arterioscler Thromb Biol* 15: 1139–1144.

Michaelis J., Vissers M.C., Winterbourn C.C. (1992). Different effects of hypochlorous acid on human neutrophil metalloproteinases: Activation of collagenase and inactivation of collagenase and gelatinase. *Arch Biochem Biophys* 292: 555–562.

Miller G.J. (1980). HDL and atherosclerosis. *Ann Rev Med* 31: 97–108.

Moncada S., Palmer R.M., Higgs E.A. (1991). Nitric oxide: Physiology, pathophysiology and pharmacology. *Pharmacol Rev* 43: 109–142.

Morel D.W., DiCorleto P.E., Chisolm G.M. (1984). Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation. *Arterioscler* 4: 357–364.

Navab M., Ananthramaiah G.M., Reddy S.T., Van Lenten B.J., Ansell B.J., Fonarow G.C., Vahabzadeh K., Hama S., Hough G., Kamranpour N., Berliner J.A., Lusis A.J., Fogelman A.M. (2004). The oxidation hypothesis of atherogenesis: the role of oxidized phospholipids and HDL. *J Lipid Res* 45: 993–1007.

Parks W.C., Lopez-Boado Y.S., Wilson C.L. (2001). Matrilysin in epithelial repair and defense. *Chest* 120: 36S–41S.

Pennathur S., Wagner J.D., Leeuwenburgh C., Litwak K.N., Heinecke J.W. (2001). A hydroxyl radical-like species oxidizes *cynomolgus* monkey artery wall proteins in early diabetic vascular disease. *J Clin Invest* 107: 853–860.

Pennathur S., Bergt C., Shao B., Byun J., Kassim S.Y., Singh P., Green P.S., McDonald T.O., Brunzell J., Chait A., Oram J.F., O'Brien K., Geary R.L., Heinecke J.W. (2004). Human atherosclerotic intima and blood of patients with established coronary artery disease contain high density lipoprotein damaged by reactive nitrogen species. *J Biol Chem* 279: 42977–42983.

Pennathur S., Ido Y., Heller J.I., Byun J., Danda R., Pergola P., Williamson J.R., Heinecke J.W. (2005). Reactive carbonyls and polyunsaturated fatty acids produce a hydroxyl radical-like species: A potential pathway for oxidative damage of retinal proteins in diabetes. *J Biol Chem* 280: 22706–22714.

Peppin G.J., Weiss S.J. (1986). Activation of the endogenous metalloproteinase, gelatinase, by triggered human neutrophils. *Proc Natl Acad Sci USA* 83: 4322–4326.

Peters T. Jr., Blumenstock F.A. (1967). Copper-binding properties of bovine serum albumin and its amino-terminal peptide fragment. *J Biol Chem* 242: 1574–1580.

Podrez E.A., Schmitt D., Hoff H.F., Hazen S.L. (1999). Myeloperoxidase-generated reactive nitrogen species convert LDL into an atherogenic form in vitro. *J Clin Invest* 103: 1547–1560.

Rajagopalan S., Meng X.P., Ramasamy S., Harrison D.G., Galis Z.S. (1996). Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro: Implications for atherosclerotic plaque stability. *J Clin Invest* 98: 2572–2579.

Rosenfeld M.E., Palinski W., Yla-Herttuala S., Butler S., Witztum J.L. (1990). Distribution of oxidation specific lipid-protein adducts and apolipoprotein-B in atherosclerotic lesions of varying severity from WHHL rabbits. *Arterioscler* 10: 336–349.

Ross R. (1999). Atherosclerosis—An inflammatory disease. *N Engl J Med* 14: 115–126.

Rubbo H., Parthasarathy S., Barnes S., Kirk M., Kalyanaraman B., Freeman B.A. (1995). Nitric oxide inhibition of lipoxygenase-dependent liposome and low-density lipoprotein oxidation: Termination of radical chain propagation reactions and formation of nitrogen-containing oxidized lipid derivatives. *Arch Biochem Biophys* 324: 15–25.

Savenkova M.I., Mueller D.M., Heinecke J.W. (1994). Tyrosyl radical generated by MPO is a physiological catalyst for initiation of lipid peroxidation in low density lipoprotein. *J Biol Chem* 269: 20394–20400.

Saxena A.K., Saxena P., Wu X., Obrenovich M., Weiss M.F., Monnier V.M. (1999). Protein aging by carboxymethylation of lysines generates sites for divalent metal and redox active copper binding: Relevance to diseases of glycoxidative stress. *Biochem Biophys Res Commun* 260: 332–338.

Segal A.W. (1989). The electron transport chain of the microbicidal oxidase of phagocytic cells and its involvement in the molecular pathology of chronic granulomatous disease. *J Clin Invest* 83: 1785–1793.

Smith C., Mitchinson M.J., Aruoma O.I., Halliwell B. (1992). Stimulation of lipid peroxidation and hydroxyl radical generation by the contents of human atherosclerotic lesions. *Biochem J* 286: 901–905.

Stadler N., Lindner R.A., Davies M.J. (2004). Direct detection and quantification of transition metal ions in human atherosclerotic plaques: Evidence for the presence of elevated levels of iron and copper. *Arterioscler Thromb Vasc Biol* 24: 949–954.

Steinbrecher U.P., Parthasarathy S., Leake D.S., Witztum J.L., Steinberg D. (1984). Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc Natl Acad Sci USA* 81: 3883–3886.

Sugiyama S., Okada Y., Sukhova G.K., Virmani R., Heinecke J.W., Libby P. (2001). Macrophage MPO regulation by granulocyte macrophage colony-stimulating factor in human atherosclerosis and implications in acute coronary syndromes. *Am J Pathol* 158: 879–891.

Sukhova G.K., Schonbeck U., Rabkin E., Schoen F.J., Poole A.R., Billinghurst R.C., Libby P. (1999). Evidence for increased collagenolysis by interstitial collagenases-1 and -3 in vulnerable human atheromatous plaques. *Circulation* 99: 2503–2509.

Swain J., Gutteridge J.M.C. (1995). Prooxidant iron and copper, with ferroxidase and xanthine oxidase activity in human atherosclerotic material. *FEBS Lett* 368: 513–515.

Thomas C.E. (1992). The influence of medium components on Cu-dependent oxidation of low density lipoproteins and its sensitivity to superoxide dismutase. *Biochim Biophys Acta* 1128: 50–57.

Thompson R.W., Holmes D.R., Mertens R.A., Liao S., Botney M.D., Mecham R.P., Welgus H.G., Parks W.C. (1995). Production and localization of 92-kilodalton gelatinase in abdominal aortic aneurysms: An elastolytic metalloproteinase expressed by aneurysm-infiltrating macrophages. *J Clin Invest* 96: 318–326.

Van Wart H.E., Birkedal-Hansen H. (1990). The cysteine switch: A principle of regulation of metalloproteinase activity with potential applicability to the matrix metalloproteinase gene family. *Proc Natl Acad Sci USA* 87: 5578–5582.

Vissers M.C., Winterbourn C.C. (1987). Myeloperoxidase-dependent oxidative inactivation of neutrophil proteinases and microbicidal enzymes. *Biochem J* 245: 277–280.

Visse R., Nagase H. (2003). Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 92: 827–839.

Weiss S.J., Peppin G., Ortiz X., Ragsdale C., Test S.T. (1985). Oxidative autoactivation of latent collagenase by human neutrophils. *Science* 227: 747–749.

Weiss S.J., Test S.T., Eckmann C.M., Ross D., Regiani S. (1986). Brominating oxidants generated by human eosinophils. *Science* 234: 200–202.

Winterbourn C.C. (1985). Comparative reactivities of various biological compounds with myeloperoxidase-hydrogen peroxide-chloride, and similarity of the oxidant to hypochlorite. *Biochim Biophys Acta* 840: 204–210.

Witztum J.L., Steinberg D. (1991). Role of oxidized low density lipoprotein in atherogenesis, *J Clin Invest* 88: 1785–1792.

Woessner J.F., Nagase H. (2000). *Matrix Metalloproteinases and TIMPs*, Oxford University Press, Oxford.

Wu A., Hinds C.J., Thiemermann C. (2004). High-density lipoproteins in sepsis and septic shock: metabolism, actions, and therapeutic applications. *Shock* 21: 210–221.

Xiao Q., Danton M.J., Witte D.P., Kowala M.C., Valentine M.T., Bugge T.H., Degen J.L. (1997). Plasminogen deficiency accelerates vessel wall disease in mice predisposed to atherosclerosis. *Proc Natl Acad Sci USA* 94: 10335–10340.

Yates M.T., Lambert L.E., Whitten J.P., MacDonald I., Mano M., Ku G., Mao S.J.T. (1992). A protective role for nitric oxide in the oxidative modification of low density lipoprotein by mouse macrophages. *FEBS Lett* 309: 135–138.

Yim H., Kang S., Hah Y., Boon Chock P., Yim M.B. (1995). Free radicals generated during the glycation reaction of amino acids by methylglyoxal. *J Biol Chem* 270: 28228–28233.

Zhang R., Brennan M.L., Fu X., Aviles R.J., Pearce G.L., Penn M.S., Topol E.J., Sprecher D.L., Hazen S.L. (2001). Association between MPO levels and risk of coronary artery disease. *JAMA* 286: 2136–2142.

Zheng L., Nukuna B., Brennan M.L., Sun M., Goormastic M., Settle M., Schmitt D., Fu X., Thomson L., Fox P.L., Ischiropoulos H., Smith J.D., Kinter M., Hazen S.L. (2004). Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease. *J Clin Invest* 114: 529–541.

Zheng L., Settle M., Brubaker G., Schmitt D., Hazen S.L., Smith J.D., Kinter M. (2005). Localization of nitration and chlorination sites on apolipoprotein A-I catalyzed by myeloperoxidase in human atheroma and associated oxidative impairment in ABCA1-dependent cholesterol efflux from macrophages. *J Biol Chem* 280: 38–47.

25

OXIDATIVE STRESS AND PROTEIN OXIDATION IN PRE-ECLAMPSIA

MAARTEN T. M. RAIJMAKERS, WILBERT H. M. PETERS, CHRISTIANNE J. DE GROOT, AND ERIC A. P. STEEGERS

25.1 INTRODUCTION

The preferred definition of pre-eclampsia is a diagnosis of pregnancy-induced hypertension (diastolic blood pressure >90 mmHg) occurring after the twentieth week of gestation with proteinuria (either ≥300 mg protein/day or an urinary protein/creatinine ratio ≥30 mg/mmol) according to the criteria of the International Society of the Study of Hypertension in Pregnancy (Brown et al., 2001). When patients have liver dysfunction, thrombocytopenia, and hemolysis, they are classified as suffering from the HELLP syndrome (i.e., hemolysis, elevated liver enzymes, low platelets) (Curtin and Weinstein, 1999). While the definitions focus on these relatively simple clinical parameters, it should be realized that pre-eclampsia is a multisystem disorder, which variably may affect essential organs such as brain, lungs, kidney, and liver. Its etiology, however, remains largely unknown. This disorder affects between 0.5% and 3% of all pregnancies. It is associated with the highest maternal and fetal morbidity and mortality of all pregnancy complications (Villar et al., 2003). Poor placentation is an important predisposing factor. The proposed "two-stage model" (Roberts and Hubel, 1999), where reduced placental perfusion (stage 1) leads to the maternal syndrome (stage 2), provides a simple but probably largely accurate description of the origin of severe, early onset disease (Villar et al., 2003). However, this model may be less relevant for late onset, mild pre-eclamptic disease (Redman and Sargent,

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

2000). Several theories have been proposed to explain how the placenta may be associated with the maternal syndrome (Hubel, 1999; Redman and Sargent, 2003; Levine et al., 2004). The one in which increased generation of placental superoxide leads to oxidative stress is increasingly recognized (Hubel, 1999; Raijmakers et al., 2004a, 2005). Free radicals not only may initiate lipid peroxidation and oxidative damage of other biomolecules but may have deleterious effects on (redox-sensitive) gene expression, thereby compromising cellular function. It has recently been proposed that a combination of these processes may initiate maternal vascular endothelial dysfunction and leucocyte activation that are both features of this disorder (Raijmakers et al., 2004a). This chapter focuses on recent investigations on oxidative stress in women with pre-eclampsia and its role in the etiology of the syndrome.

25.2 OXIDATIVE STRESS AND PRE-ECLAMPSIA

25.2.1 Sources of Oxidative Stress during Pregnancy

The generation of free radicals in the human placenta can occur, as in most other tissues, through mitochondrial electron "escape" and local formation of superoxide. The recent focus on the role of oxidative stress in pre-eclampsia and the finding that placental tissue of women with pre-eclampsia produces high levels of superoxide as compared to placentas of normotensive women (Sikkema et al., 2001; Wang and Walsh, 2001) has led to a revival of interest in enzymatic pathways, including xanthine oxidase and NAD(P)H oxidase, of free radical generation in the human placenta (Poston and Raijmakers, 2004).

Xanthine oxidoreductase exists in two interconvertible forms: xanthine dehydrogenase, the predominant form, and xanthine oxidase (Harrison, 2002). These enzymes convert hypoxanthine to xanthine and xanthine to urate, respectively. Xanthine oxidase-mediated conversion of purines is coupled to the generation of superoxide, but its contribution to superoxide synthesis compared to that of the NAD(P)H oxidases is relatively minor (Harrison, 2002). The expression of xanthine oxidase has been described in placenta by Many and colleagues in 1996 (Many et al., 1996), who later showed by immunohistochemical staining that xanthine oxidase expression was very modest at 18 weeks' gestation in normal trophoblasts and slightly greater by 33 weeks' gestation (Many et al., 2000). More recently Hung and colleagues have shown that in vitro exposure of normal term placenta to a period of hypoxia followed by reperfusion (the stimulus for an increase of xanthine oxidase activity) leads to an increase in nitrotyrosine staining in trophoblast and activation of apoptotic pathways (Hung et al., 2002). Both were more intense than that observed with a hypoxia stimulus alone, and a role for free radicals was implied by the observation that the free radical scavenger desferrioxamine prevented the apoptotic response. Therefore occlusion of a vessel by thrombus generation and subsequent dissolution, which may occur in pre-eclampsia, could hypothetically result in chronic hypoxia/reperfusion insults that are potent stimuli for the conversion of the enzyme complex into xanthine oxidase.

NAD(P)H oxidases are multimeric enzymes composed of several subunits and are a major source of superoxide in neutrophils, vascular endothelial cells, vascular smooth muscle cells, and trophoblast (Griendling et al., 2000). NAD(P)H oxidases are closely related as many components (e.g., p22phox, p47phox, p67phox, and rac) are homologous. Moreover the major catalytic subunit, gp91phox, is identical in neutrophils, endothelial cells, fibroblasts, and vascular smooth muscle cells (Bayraktutan et al., 2000; Li and Shah, 2002; Touyz et al., 2002). Superoxide generation by NAD(P)H oxidase is increased either by activation of the enzyme complex or increased subunit expression in response to hormones, the best characterized stimulus being angiotensin II (AII). However, the platelet-derived growth factor, thrombin, cytokines (e.g., tumor necrosis factor-α), hemodynamic forces, and local metabolic changes (Griendling et al., 2000) are all known activators. The placental NAD(P)H oxidase has a different sensitivity to pH and temperature than the well-characterized neutrophil NAD(P)H oxidase, which may indicate that a difference in subunit composition contributes to enzyme activity (Manes, 2001). Recently Dechend et al. have reported the presence of the p22phox, p47phox, and p67phox subunits of NAD(P)H oxidase in cytotrophoblast cells and the stem villus arteries of term human placenta (Dechend et al., 2003). These authors also showed that stimulation with AII increased subunit expression and that the AII response was similar to that observed in a preparation of human vascular smooth muscle. We have recently evaluated NAD(P)H oxidase-derived superoxide generation by lucigenin chemiluminescence in placental tissues from normal pregnancies obtained at different stages of gestation. In contrast to the overall weak expression of xanthine oxidase observed by Many et al. (2000) we observed the highest NAD(P)H oxidase activity in samples obtained at 7 to 13 weeks' gestation (data unpublished).

Free radical generation in the placenta may induce shedding of syncytiotrophoblast microvesicles that, as in vitro evidence suggests, may lead to activation of maternal neutrophils (Redman and Sargent, 2003). Therefore placental oxidative stress, by this mechanism, may directly or indirectly lead to systemic oxidative stress in the maternal circulation. Maternal neutrophils may be locally activated either during passage of maternal blood through the placenta or by placental factors in the circulation. This results in the so-called neutrophil respiratory burst of superoxide release, which is mainly mediated by NAD(P)H oxidase (Lee et al., 2003a). Subsequently synthesis of cytokines, mediated by activated neutrophils, could contribute to maternal endothelial cell activation with subsequent adhesion of leucocytes, and hence to further neutrophil activation. Isolated neutrophils from women with pre-eclampsia synthesize more superoxide upon activation with either receptor or non–receptor-mediated stimuli (*N*-formyl-methionyl-leucyl-phenylalanine and phorbol 12-myristate 13-acetate, respectively) than neutrophils of normotensive pregnant women (Tsukimori et al., 1993; Crocker et al., 1999; Lee et al., 2003b). However, not all reports have described this effect using non–receptor-mediated stimulation (Tsukimori et al., 1993; Zusterzeel et al., 2001b). NAD(P)H oxidase-mediated superoxide generation is closely related to the release and activation of cytokines.

25.2.2 Evidence of Placental Oxidative Stress in Pre-Eclampsia

Numerous independent biomarkers of oxidative stress indicate the presence of placental oxidative stress in women with pre-eclampsia and have mainly focused on lipid peroxidation (Raijmakers et al., 2005). Several reports have shown higher placental levels of the lipid peroxidation products malondialdehyde (MDA) and isoprostanes (Gülmezoglu et al., 1996; Gratacos et al., 1998; Walsh et al., 2000) in women with pre-eclampsia, or demonstrated higher production and secretion rates of isoprostanes in vitro as compared with placentas from normal pregnancies (Walsh et al., 2000). Oxidative modification of proteins has been investigated to a lesser extent. Proteins may be modified either by direct oxidative attack or indirectly by lipid peroxidation products. This may result in a structural change in amino acid side chains and in the formation of protein carbonyls, which have been shown elevated in the placenta of women with pre-eclampsia (Zusterzeel et al., 2001a). In addition the increased expression of endothelial nitric oxide synthase, leading to an elevated production of NO in the fetal-placental vasculature in pre-eclampsia (Myatt et al., 1997), in combination with an enhanced placental superoxide generation, could lead to the increased formation of peroxynitrite. Peroxynitrite is a relatively stable and powerful oxidant that is able to nitrate aromatic amino acids such as tyrosine. It is therefore not surprising that both placental vascular smooth muscle and villous trophoblasts show elevated nitrotyrosine staining in women with pre-eclampsia as compared to normotensive pregnant women (Myatt et al., 1996; Many et al., 2000).

In placental tissue the transcription of the genes encoding for the important enzymatic antioxidants Cu-Zn SOD and GPX is downregulated (Wang and Walsh, 1996; Walsh, 1998), resulting in lower protein levels in women with pre-eclampsia (Poranen et al., 1996; Wang and Walsh, 1996). In addition the level of GSTPi, the major GST isoform in placenta, and that of the nonenzymatic antioxidant vitamin E were lower in placental tissue of women with pre-eclampsia (Wang and Walsh, 1996; Zusterzeel et al., 1999). Most studies have shown that placental antioxidant capacity is decreased in pre-eclampsia (Gratacos et al., 1998; Zusterzeel et al., 2001a). In contrast, some studies have reported an elevation of placental antioxidant capacity in women with pre-eclampsia, which has been pointed out to be a response to increased levels of oxidative stress (Gülmezoglu et al., 1996; Wang and Walsh, 1996; Knapen et al., 1999). In general, the elevation of lipid peroxidation markers and the downregulation of the antioxidant system are clear indications for the presence of placental oxidative stress in women with pre-eclampsia.

25.2.3 Evidence of Maternal Oxidative Stress in Pre-Eclampsia

Although reports of oxidative stress in the maternal circulation have shown variable results, there is consensus on elevated levels of reactive oxygen species, oxidative damage, and diminished antioxidant capacity (Hubel, 1999; Roggensack et al., 1999; Kharb, 2000c; Raijmakers et al., 2004a). Superoxide generation could be one of the factors initiating or maintaining oxidative stress in the maternal

circulation in pre-eclampsia. Recently it was shown that superoxide production by NAD(P)H oxidase was elevated in cultured lymphoblasts of women with pre-eclampsia; however, NAD(P)H oxidase subunit expression was not changed as compared to control lymphoblasts (Lee et al., 2003a). NAD(P)H oxidase-mediated superoxide generation has been associated with the expression of cytokines, adhesion molecules, and interleukines (Griendling et al., 2000), possibly mediating the activation of neutrophils. Indeed the respiratory burst observed in women with pre-eclampsia (Clark et al., 1998) provides direct evidence of increased superoxide generation in these women.

Pre-eclampsia is a state of dyslipidemia with serum free fatty acids, triglycerides, and very low density lipoproteins being higher. Concentrations of cholesterol, lipoprotein (a), and the other lipoproteins (Hubel et al., 1996; Sattar et al., 2000; Wetzka et al., 1999) are reported to be similar, whereas a decrease in particle size of low-density lipoprotein (LDL) as compared to normal pregnant women could be noticed (Hubel et al., 1998). These changes in lipid profile make LDL more susceptible to oxidative modification (Hubel et al., 1998). Indeed both increased levels of antibodies against an epitope on the oxidized form of LDL (oxLDL) (Hubel, 1999; Wakatsuki et al., 2000), and a higher oxLDL to native LDL antibody ratio have been found in women with pre-eclampsia (Branch et al., 1994; Uotila et al., 1998). MDA, a major breakdown product of lipid peroxides, was one of the first biomarkers suggesting elevated lipid peroxidation in women with pre-eclampsia (Hubel et al., 1989). To date, numerous studies using either MDA or thiobarbituric acid reactive substances, which mainly consist of MDA, have confirmed these original findings (Bayhan et al., 2000; Kharb, 2000c; Madazli et al., 1999; Mutlu-Turkoglu et al., 1998; Takacs et al., 2001; Yanik et al., 1999). Only one study could not demonstrate higher MDA concentrations in pre-eclampsia, but the ratio of MDA over total antioxidant capacity was much larger in women with pre-eclampsia, indicating the presence of an oxidizing environment in these women (Davidge et al., 1992).

Another consequence of lipid peroxidation is bond migration in the hydrocarbon chain of the unsaturated fatty acid leading to the formation of conjugated dienes. It has been proposed that these could be good lipid peroxidation biomarkers (Hubel et al., 1989). Conjugated dienes in either plasma or platelets are elevated in women with pre-eclampsia as compared to normotensive pregnant women (Hubel et al., 1989; Garzetti et al., 1993; Uotila et al., 1993). Direct free radical attack on arachidonic acid results in the generation of stable products called iso-prostanes; however, the reported results on these seem ambiguous. In plasma of women with pre-eclampsia higher levels of 8-isoprostane were detected (Barden et al., 1996; Barden et al., 2001), whereas in urine no differences were found (Regan et al., 2001). This different finding in plasma and urine might be explained by impaired renal clearance in women with pre-eclampsia (Barden et al., 1996). Only recently the noxious effects of oxidative stress on carbohydrates, amino acids, proteins, and other biomolecules have been studied in more detail, and it was shown that the resulting protein carbonyl groups are elevated in plasma of women with pre-eclampsia (Zusterzeel et al., 2000).

The presence of a reactive –SH group in proteins and (amino) thiols not only predisposes these molecules to oxidative modification but also confers potent antioxidant properties. The assessment of total or reduced, oxidized, and protein-bound thiols could both provide an estimate of the antioxidant potential, and could also provide insight into the redox-status. Thiol status in pre-eclampsia has predominantly been investigated in whole blood or blood plasma. The total amount of—SH-containing molecules or the amount of specific thiols has been reported to be lower in plasma (Chen et al., 1994; Hubel et al., 1997; Uotila et al., 1994; Nemeth et al., 2001) or erythrocyte lysate (Kharb, 2000a) of women with pre-eclampsia as compared to the corresponding values in women with normotensive pregnancy.

As each amino thiol (glutathione, homocysteine, and cysteine) may play a distinct role in vascular metabolism, the separate quantification of each thiol has the preference over quantification of total –SH content. Most studies have reported lower plasma glutathione levels (Chen et al., 1994; Kharb, 2000b) or whole blood glutathione:hemoglobin ratios in women with pre-eclampsia compared to normotensive control women (Knapen et al., 1998). In contrast, one study showed that women with pre-eclampsia tended to higher oxidized levels of glutathione in the erythrocyte, although differences did not reach significance (Spickett et al., 1998). Homocysteine concentrations in pre-eclampsia are of specific interest because of the striking similarities between pre-eclampsia and cardiovascular diseases, for which homocysteine is an independent risk factor (Gratacos, 2000). Plasma homocysteine is consistently reported to be higher in women with pre-eclampsia compared to normotensive pregnant women (Rajkovic et al., 1997; Powers et al., 1998; Hogg et al., 2000; Raijmakers et al., 2000; Lopez-Quesada et al., 2003), and it has been found to be higher as early as 15 weeks of gestation (Cotter et al., 2001). Although various explanations have been proposed, including hormonal influences (Hogg et al., 2000), changes in plasma volume (Raijmakers et al., 2000), altered albumin concentrations (Walker et al., 1999), and nutritional or genetic defects including the methylenetetrahydrofolate reductase (MTHFR) polymorphism (Rajkovic et al., 1997), the reason for increased homocysteine levels in plasma of women with pre-eclampsia remains unclear (Cotter et al., 2001; Hogg et al., 2000; Powers et al., 1998; Rajkovic et al., 1997). Concentrations of cysteine, the most important –SH-containing amino acid, may not only reflect antioxidant power, but changes in cysteine status may also be a manifestation of glutathione turnover or protein synthesis rates. In parallel with homocysteine, cysteine concentrations have been associated with coronary artery diseases (El Khairy et al., 2003). Cysteine has hardly been investigated in pre-eclampsia; one study showed that plasma cysteine levels are elevated in pre-eclampsia (Raijmakers et al., 2000).

Oxidation of thiols may occur when the free –SH group reacts with free radicals, leading to the formation of (mixed) disulfides. As a result the free-to-oxidized ratio will decrease. We have previously shown that during pregnancy the free-to-oxidized ratios for cysteine, cysteinylglycine, and homocysteine are lower in women with pre-eclampsia as compared to normal pregnancies (Raijmakers

et al., 2001), whereas the ratio for homocysteine remains lower until six months after pregnancy. Additionally nonpregnant women with previous pre-eclampsia showed higher total plasma homocysteine concentrations than women with an uncomplicated obstetrical record (Raijmakers et al., 2004b). Interestingly approximately 40% of the subsequent pregnancies after pre-eclampsia remained normotensive, suggesting the presence of a predisposing disturbance in the redox balance of homocysteine in favor of the oxidized state in women with a history of pre-eclampsia (Raijmakers et al., 2004b).

The determination of the levels of specific antioxidants or the total antioxidant capacity offers other measures of oxidative stress in pre-eclampsia. However, the measurement of antioxidants only provides an indirect estimation and is therefore less reliable than the assessment of the products of oxidative damage. A clear example is the "total" antioxidant capacity assay "total peroxyl radical-trapping antioxidative parameter" (TRAP) that was found to be higher in women with pre-eclampsia (Uotila et al., 1994). However, the major component in this assay is uric acid, the breakdown product of purines, which is elevated in women with pre-eclampsia (Poranen et al., 1996; Kharb, 2000c; Zusterzeel et al., 2002). In line with this, it is not surprising that the ORAC, which is based on direct quenching of free radicals and does not include all available antioxidants (Cao and Prior, 1998), was found to be similar in women with pre-eclampsia and controls (Zusterzeel et al., 2002). Other studies reported that the overall antioxidant capacity, based on inhibition of the spontaneous auto-oxidation of brain homogenates, was lower in women with pre-eclampsia (Davidge et al., 1992).

As vitamin E is transported by lipoproteins and pre-eclampsia is characterized by hyperlipoproteinemia (Brigelius-Flohe et al., 2002), vitamin E concentration should preferably be expressed as a vitamin E/lipid ratio. This adjustment is not always performed and consequently results on vitamin E levels are ambiguous. The nonadjusted vitamin E levels have been reported to be either lower (Mikhail et al., 1994; Madazli et al., 1999; Sagol et al., 1999; Yanik et al., 1999; Kharb, 2000d), similar (Uotila et al., 1994; Valsecchi et al., 1999), or higher (Schiff et al., 1996; Hubel et al., 1997; Zusterzeel et al., 2002) in pre-eclampsia as compared to normotensive pregnancy. However, when corrected for the lipid profile, no differences were found (Hubel et al., 1997; Zhang et al., 2001; Chappell et al., 2002). Vitamin C is claimed to exert a synergistic effect on the activity of vitamin E. Most studies reported lowered vitamin C levels in pre-eclampsia (Petit Clerc and Solberg, 1987; Mikhail et al., 1994; Madazli et al., 1999; Rao et al., 1999; Sagol et al., 1999; Kharb, 2000d; Chappell et al., 2002; Zhang et al., 2002), whereas some found unchanged levels (Uotila et al., 1994; Mutlu-Turkoglu et al., 1998; Zusterzeel et al., 2002). In summary, in pre-eclampsia it seems that although lipid-adjusted vitamin E concentrations are not altered, impairment of vitamin E regeneration due to the low vitamin C levels could be a major determinant for the free radical scavenging capacity of vitamin E.

In contrast to the nonenzymatic antioxidants, in pre-eclampsia the presence and activity of enzymatic oxidants have hardly been studied, and contradictory findings have been reported in the few studies performed so far. The level of SOD

expression in the vasculature of women with pre-eclampsia is lower (Roggensack et al., 1999), as is the SOD enzyme activity in erythrocytes (Chen et al., 1994; Mutlu-Turkoglu et al., 1998). Kumar and Das reported a lower erythrocyte enzyme activity of both SOD and catalase, but a higher than normal GPX activity in erythrocytes of patients with pre-eclampsia as compared to controls (Kumar and Das, 2000). These authors do not discuss their findings, but it can be hypothesized that the expression of GPX, being a protective enzyme, is induced in order to prevent excessive lipid peroxidation due to the low SOD and catalase enzyme activities. In another study no differences were found for erythrocyte SOD enzyme activity, whereas the higher GPX activity was confirmed (Diedrich et al., 2001). Similar erythrocyte catalase enzyme activities in women with pre-eclampsia and controls were reported in two other papers (Bayhan et al., 2000; Loverro et al., 1996). In blood plasma, however, it is reported that there is no difference in the enzyme activity of GPX between women with pre-eclampsia and normotensive pregnancy (Diedrich et al., 2001; Karsdorp et al., 1998).

It is widely acknowledged that oxidative stress is implicated in the pathophysiology of pre-eclampsia. To date, the best evidence for this are the elevated levels of markers for lipid peroxidation, which might indirectly tell us something about cellular function. In contrast, oxidative protein damage has hardly been subject of investigation. Oxidative stress could lead to oxidative modification, thereby inhibiting protein function, as well as to altered gene expression. The currently rapid development of new and exciting technologies will provide us with a more complete insight into the effects of oxidative stress in pre-eclampsia in the near future.

25.3 PROTEOMICS

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) play important physiological functions and can cause extensive cellular damage. Although the mechanism is unknown, an imbalance between the generation of reactive oxygen and nitrogen species and the antioxidant defense capacity of the body has been associated with human diseases. There is no doubt that proteins are major targets for radicals and other oxidants formed in vivo. Furthermore proteins can retain the fingerprint of the initial oxidative/nitrosative insult that mediates damage (Dalle-Donne et al., 2005). Thus a major advantage of proteins is the possibility to reveal specific polypeptide modifications at cellular level of a particular disease or stressing condition.

Recently the Human Genome Project has yielded a description of almost the complete human genome (DNA). Less advanced are approaches for monitoring cellular proteomes, partly due to the complexity of processes such as alternative mRNA splicing and post-translational modifications. Conceivably more than one million distinct protein forms in all human tissues will be possible (Kabuyama et al., 2004).

Proteome analyses, providing researchers with a general approach to describe all protein components, are an ideal choice for revealing all polypeptide

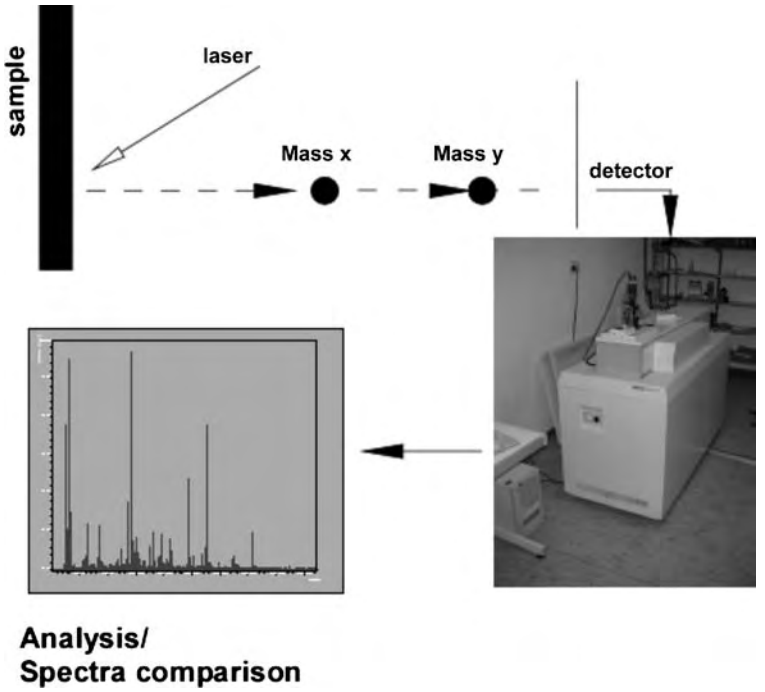


FIGURE 25.1 Protein analysis by mass spectrometry. The principle of mass spectrometry is that the sample is ionized and transferred to mass analyzer and detector according to the mass to charge ratio. The result is a peptide mass fingerprint.

modifications. For this approach accurate resolution of thousands of proteins is a requirement. Two-dimensional (2D) electrophoresis approaches have widely been used to separate proteins according to their molecular weight and isoelectric point. Detection of differently expressed or modified proteins is achieved by comparing 2D electrophoretic maps of samples of patients with corresponding controls. Identification of specific proteins is possible by detection with specific antibodies or by separation by HPLC or GLC, coupled to detection by mass spectrometry. A newly developed analytical strategy for the detection of oxidation products in isolated proteins is based on mass spectrometry (Fig. 25.1) (Bischoff et al., 2004). In general, the principle of mass spectrometry is based on the observation that reactions are determined by a specific molecular variation. These molecular variations are detected by their mass measurements. Improved software allows peptide sequencing via data in which ions are automatically selected and fragmented by MS/MS. Higher coverage of proteomes is observed with this technique as compared to the 2D electrophoresis as described above (Spirin, 2004). A critical issue for the latest approach is quantifying peptide and protein abundances, such as ion suppression due to charge competition among peptides within a mixture of peptides. One solution to this problem involves differential isotope or mass tag labeling of peptides. We recently reported the first

promising steps in the comparison of peptide patterns in relative low numbers (approximately 125) of laser capture microdissected trophoblast or stroma cells obtained from frozen placenta sections (de Groot et al., 2005). By the analysis by MALDI–TOF mass spectrometry, specific peptide patterns that consist on average of 35 peptides for trophoblast cells and surrounding villous stroma cells could be obtained. Furthermore we found exclusive discriminating peptide patterns among trophoblast and surrounding villous stroma cells. In the future this method could be a potentially suitable instrument for characterization of specific peptides and identification of proteins related to the pathogenesis of trophoblast-related pregnancy diseases such as pre-eclampsia.

Although no proteomic strategy is capable at present of fully surveying an entire proteome (Greef vd et al., 2004; Mayr et al., 2004), several studies have provided a useful description of the mechanisms in cell regulation. The level of innovation in this rapidly advancing field is noteworthy, and new technologies in proteomics may soon allow comprehensive proofing of protein expression and their interactions so that proteomics may unravel the complexity of biological systems.

LIST OF ABBREVIATIONS

GLC, gas liquid chromatography
GPX, glutathione peroxidase
GST, glutathione-*S*-transferase
HELLP, hemolysis, elevated liver enzymes, low platelets
HPLC, high-performance liquid chromatography
LDL, low-density lipoprotein
MALDI-TOF, matrix-assisted laser desorption—ionization time of flight
MDA, malondialdehyde
MS/MS, tandem mass spectrometry
MTHFR, methylenetetrahydrofolate reductase
oxLDL, oxidized LDL
RNS, reactive nitrogen species
ROS, reactive oxygen species
SOD, superoxide dismutase
TRAP, total peroxyl radical-trapping antioxidative parameter

REFERENCES

Barden A., Beilin L.J., Ritchie J., Croft K.D., Walters B.N., Michael C.A. (1996). Plasma and urinary 8-iso-prostane as an indicator of lipid peroxidation in pre-eclampsia and normal pregnancy. *Clin Sci* 91: 711–718.

Barden A., Ritchie J., Walters B., Michael C., Rivera J., Mori T., Croft K., Beilin L. (2001). Study of plasma factors associated with neutrophil activation and lipid peroxidation in preeclampsia. *Hypertension* 38: 803–808.

Bayhan G., Atamer Y., Atamer A., Yokus B., Baylan Y. (2000). Significance of changes in lipid peroxides and antioxidant enzyme activities in pregnant women with preeclampsia and eclampsia. *Clin Exp Obstet Gynecol* 27: 142–146.

Bayraktutan U., Blayney L., Shah A.M. (2000). Molecular characterization and localization of the NAD(P)H oxidase components gp91-phox and p22-phox in endothelial cells. *Arterioscler Thromb Vasc Biol* 20: 1903–1911.

Bischoff R., Luider T.M. (2004). Methodological advances in the discovery of protein and peptide disease markers. *J Chromatogr* 803: 27–40.

Branch D.W., Mitchell M.D., Miller E., Palinski W., Witztum J.L. (1994). Pre-eclampsia and serum antibodies to oxidised low-density lipoprotein. *Lancet* 343: 645–646.

Brigelius-Flohe R., Kelly F.J., Salonen J.T., Neuzil J., Zingg J.M., Azzi A. (2002). The European perspective on vitamin E: Current knowledge and future research. *Am J Clin Nutr* 76: 703–716.

Brown M.A., Lindheimer M.D., de Swiet M., van Assche A., Moutquin J.M. (2001). The classification and diagnosis of the hypertensive disorders of pregnancy: Statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP). *Hypertens Pregnancy* 20: ix–xiv.

Cao G., Prior R.L. (1998). Comparison of different analytical methods for assessing total antioxidant capacity of human serum. *Clin Chem* 44: 1309–1315.

Chappell L.C., Seed P.T., Briley A., Kelly F.J., Hunt B.J., Charnock-Jones D.S., Mallet A.I., Poston L. (2002). A longitudinal study of biochemical variables in women at risk of preeclampsia. *Am J Obstet Gynecol* 187: 127–136.

Chen G., Wilson R., Cumming G., Walker J.J., Smith W.E., McKillop J.H. (1994). Intracellular and extracellular antioxidant buffering levels in erythrocytes from pregnancy-induced hypertension. *J Hum Hypertens* 8: 37–42.

Clark P., Boswell F., Greer I.A. (1998). The neutrophil and preeclampsia. *Semin Reprod Endocrinol* 16: 57–64.

Cotter A.M., Molloy A.M., Scott J.M., Daly S.F. (2001). Elevated plasma homocysteine in early pregnancy: A risk factor for the development of severe preeclampsia. *Am J Obstet Gynecol* 185: 781–785.

Crocker I.P., Wellings R.P., Fletcher J., Baker P.N. (1999). Neutrophil function in women with pre-eclampsia. *Br J Obstet Gynaecol* 106: 822–828.

Curtin W.M., Weinstein L. (1999). A review of HELLP syndrome. *J Perinatol* 19: 138–143.

Dalle-Donne I., Scaloni A., Giustarini D., Cavarra E., Tell G., Lungarella G., Colombo R., Rossi R., Milzani A. (2005). Proteins as biomarkers of oxidative/nitrosative stress in diseases: The contribution of redox proteomics. *Mass Spectrom Rev* 24: 55–99.

Davidge S.T., Hubel C.A., Brayden R.D., Capeless E.C., Mclaughlin M.K. (1992). Sera antioxidant activity in uncomplicated and preeclamptic pregnancies. *Obstet Gynecol* 79: 897–901.

Dechend R., Viedt C., Muller D.N., Ugele B., Brandes R.P., Wallukat G., Park J.K., Janke J., Barta P., Theuer J., Fiebeler A., Homuth V., Dietz R., Haller H., Kreuzer J., Luft F.C. (2003). AT1 receptor agonistic antibodies from preeclamptic patients stimulate NADPH oxidase. *Circulation* 107: 1632–1639.

Diedrich F., Renner A., Rath W., Kuhn W., Wieland E. (2001). Lipid hydroperoxides and free radical scavenging enzyme activities in preeclampsia and HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome: No evidence for circulating primary products of lipid peroxidation. *Am J Obstet Gynecol* 185: 166–172.

El Khairy L., Vollset S.E., Refsum H., Ueland P.M. (2003). Plasma total cysteine, pregnancy complications, and adverse pregnancy outcomes: The Hordaland Homocysteine Study. *Am J Clin Nutr* 77: 467–472.

Garzetti G.G., Tranquilli A.L., Cugini A.M., Mazzanti L., Cester N., Romanini C. (1993). Altered lipid composition, increased lipid peroxidation, and altered fluidity of the membrane as evidence of platelet damage in preeclampsia. *Obstet Gynecol* 81: 337–340.

Gratacos E. (2000). Lipid-mediated endothelial dysfunction: A common factor to preeclampsia and chronic vascular disease. *Eur J Obstet Gynecol Reprod Biol* 92: 63–66.

Gratacos E., Casals E., Deulofeu R., Cararach V., Alonso P.L., Fortuny A. (1998). Lipid peroxide and vitamin E patterns in pregnant women with different types of hypertension in pregnancy. *Am J Obstet Gynecol* 178: 1072–1076.

Griendling K.K., Sorescu D., Ushio-Fukai M. (2000). NAD(P)H oxidase: Role in cardiovascular biology and disease. *Circ Res* 86: 494–501.

de Groot C.J.M., Steegers-Theunissen R.P., Güzel C., Steegers E.A.P., Luider T.M. (2005). Peptide patterns of laser capture dissected human trophoblasts of placenta tissue analyzed by MALDI-TOF mass spectrometry. *Proteomics* 5: 597–607.

Gülmezoglu A.M., Oosthuizen M.M.J., Hofmeyr G.J. (1996). Placental malondialdehyde and glutathione levels in a controlled trial of antioxidant treatment in severe preeclampsia. *Hypertens Pregnancy* 15: 287–295.

Greef vd J., Stroobant P., Heijden vd R. (2004). The role of analytical sciences in medical systems biology. *Curr Opin Chem Biol* 8: 559–65.

Harrison R. (2002). Structure and function of xanthine oxidoreductase: Where are we now? *Free Radic Biol Med* 33: 774–797.

Hogg B.B., Tamura T., Johnston K.E., Dubard M.B., Goldenberg R.L. (2000). Second-trimester plasma homocysteine levels and pregnancy-induced hypertension, preeclampsia, and intrauterine growth restriction. *Am J Obstet Gynecol* 183: 805–809.

Hubel C.A. (1999). Oxidative stress in the pathogenesis of preeclampsia. *Proc Soc Exp Biol Med* 222: 222–235.

Hubel C.A., Kagan V.E., Kisin E.R., Mclaughlin M.K., Roberts J.M. (1997). Increased ascorbate radical formation and ascorbate depletion in plasma from women with preeclampsia: Implications for oxidative stress. *Free Radic Biol Med* 23: 597–609.

Hubel C.A., Mclaughlin M.K., Evans R.W., Hauth B.A., Sims C.J., Roberts J.M. (1996). Fasting serum triglycerides, free fatty acids, and malondialdehyde are increased in preeclampsia, are positively correlated, and decrease within 48 hours post partum. *Am J Obstet Gynecol* 174: 975–982.

Hubel C.A., Roberts J.M., Taylor R.N., Musci T.J., Rogers G.M., Mclaughlin M.K. (1989). Lipid peroxidation in pregnancy: New perspectives on preeclampsia. *Am J Obstet Gynecol* 161: 1025–1034.

Hubel C.A., Shakir Y., Gallaher M.J., Mclaughlin M.K., Roberts J.M. (1998). Low-density lipoprotein particle size decreases during normal pregnancy in association with triglyceride increases. *J Soc Gynecol Investig* 5: 244–250.

Hung T.H., Skepper J.N., Charnock-Jones D.S., Burton G.J. (2002). Hypoxia-reoxygenation: A potent inducer of apoptotic changes in the human placenta and possible etiological factor in preeclampsia. *Circ Res* 90: 1274–1281.

Kabuyama Y., Resing K.A., Ahn N.G. (2004). Applying proteomics to signaling networks. *Curr Opin Genet Dev* 14: 492–498.

Karsdorp V.H.M., Dekker G.A., Bast A., van Kamp G.J., Bouman A.A., van Vugt J.M., van Geijn H.P. (1998). Maternal and fetal plasma concentrations of endothelin, lipidhydroperoxides, glutathione peroxidase and fibronectin in relation to abnormal umbilical artery velocimetry. *Eur J Obstet Gynecol Reprod Biol* 80: 39–44.

Kharb S. (2000d). Vitamin E and C in preeclampsia. *Eur J Obstet Gynecol Reprod Biol* 93: 37–39.

Kharb S. (2000a). Altered thiol status in preeclampsia. *Gynecol Obstet Invest* 50: 36–38.

Kharb S. (2000b). Low whole blood glutathione levels in pregnancies complicated by preeclampsia and diabetes. *Clin Chim Acta* 294: 179–183.

Kharb S. (2000c). Total free radical trapping antioxidant potential in pre-eclampsia. *Int J Gynaecol Obstet* 69: 23–26.

Knapen M.F.C.M., Mulder T.P.J., Van Rooij I.A.L.M., Peters W.H.M., Steegers E.A.P. (1998). Low whole blood glutathione levels in pregnancies complicated by preeclampsia or the hemolysis, elevated liver enzymes, low platelets syndrome. *Obstet Gyn* 92: 1012–1015.

Knapen M.F.C.M., Peters W.H.M., Mulder T.P.J., Merkus H.M.W.M., Jansen J.B.M.J., Steegers E.A.P. (1999). Glutathione and glutathione-related enzymes in decidua and placenta of controls and women with pre-eclampsia. *Placenta* 20: 541–546.

Kumar C.A., Das U.N. (2000). Lipid peroxides, anti-oxidants and nitric oxide in patients with pre- eclampsia and essential hypertension. *Med Sci Monit* 6: 901–907.

Lee V.M., Quinn P.A., Jennings S.C., Ng L.L. (2003b). Neutrophil activation and production of reactive oxygen species in pre-eclampsia. *J Hypertens* 21: 395–402.

Lee V.M., Quinn P.A., Jennings S.C., Ng L.L. (2003a). NADPH oxidase activity in preeclampsia with immortalized lymphoblasts used as models. *Hypertension* 41: 925–931.

Levine R.J., Maynard S.E., Qian C., Lim K.H., England L.J., Yu K.F., Schisterman E.F., Thadhani R., Sachs B.P., Epstein F.H., Sibai B.M., Sukhatme V.P., Karumanchi S.A. (2004). Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med* 350: 672–683.

Li J.M., Shah A.M. (2002). Intracellular localization and preassembly of the NADPH oxidase complex in cultured endothelial cells. *J Biol Chem* 277: 19952–19960.

Lopez-Quesada E., Vilaseca M.A., Lailla J.M. (2003). Plasma total homocysteine in uncomplicated pregnancy and in preeclampsia. *Eur J Obstet Gynecol Reprod Biol* 108: 45–49.

Loverro G., Greco P., Capuano F., Carone D., Cormio G., Selvaggi L. (1996). Lipoperoxidation and antioxidant enzymes activity in pregnancy complicated with hypertension. *Eur J Obstet Gynecol Reprod Biol* 70: 123–127.

Madazli R., Benian A., Gumustas K., Uzun H., Ocak V., Aksu F. (1999). Lipid peroxidation and antioxidants in preeclampsia. *Eur J Obstet Gynecol Reprod Biol* 85: 205–208.

Manes C. (2001). Human placental NAD(P)H oxidase: Solubilization and properties. *Placenta* 22: 58–63.

Many A., Hubel C.A., Fisher S.J., Roberts J.M., Zhou Y. (2000). Invasive cytotrophoblasts manifest evidence of oxidative stress in preeclampsia. *Am J Pathol* 156: 321–331.

Many A., Westerhausen-Larson A., Kanbour-Shakir A., Roberts J.M. (1996). Xanthine oxidase/dehydrogenase is present in human placenta. *Placenta* 17: 361–365.

Mayr M., Mandal K., Xu Q. (2004). Pitfalls of proteomics. *Circulation* 110: e316.

Mikhail M.S., Anyaegbunam A., Garfinkel D., Palan P.R., Basu J., Romney S.L. (1994). Preeclampsia and antioxidant nutrients: Decreased plasma levels of reduced ascorbic acid, alpha-tocopherol, and beta-carotene in women with preeclampsia. *Am J Obstet Gynecol* 171: 150–157.

Mutlu-Turkoglu U., Ademoglu E., Ibrahimoglu L., Aykac-Toker G., Uysal M. (1998). Imbalance between lipid peroxidation and antioxidant status in preeclampsia. *Gynecol Obstet Invest* 46: 37–40.

Myatt L., Eis A.L., Brockman D.E., Greer I.A., Lyall F. (1997). Endothelial nitric oxide synthase in placental villous tissue from normal, pre-eclamptic and intrauterine growth restricted pregnancies. *Hum Reprod* 12: 167–172.

Myatt L., Rosenfield R.B., Eis A.L., Brockman D.E., Greer I., Lyall F. (1996). Nitrotyrosine residues in placenta: Evidence of peroxynitrite formation and action. *Hypertension* 28: 488–493.

Nemeth I., Orvos H., Boda D. (2001). Blood glutathione redox status in gestational hypertension. *Free Radic Biol Med* 30: 715–721.

Petit Clerc C., Solberg H.E. (1987). International federation of clinical chemistry: Scientific committee, clinical section expert panel on theory of reference values (EPTRV). Approved recommendation (1987) on the theory of reference values. Part 2. Selection of individuals for the production of reference values. *Clin Chim Acta* 170: S3–S12.

Poranen A.-K., Ekblad U., Uotila P., Ahotupa M. (1996). Lipid peroxidation and antioxidants in normal and pre-eclamptic pregnancies. *Placenta* 17: 401–405.

Poston L., Raijmakers M.T.M. (2004). Trophoblast oxidative stress, antioxidants and pregnancy outcome. *Placenta* S25: S72–S78.

Powers R.W., Evans R.W., Majors A.K., Ojimba J.I., Ness R.B., Crombleholme W.R., Roberts J.M. (1998). Plasma homocysteine concentration is increased in preeclampsia and is associated with evidence of endothelial activation. *Am J Obstet Gynecol* 179: 1605–1611.

Raijmakers M.T.M., Dechend R., Poston L. (2004a). Oxidative stress and pre-eclampsia: The rationale for antioxidant clinical trials. *Hypertension* 44: 1–7.

Raijmakers M.T.M., Peters W.H.M., Steegers E.A.P., Poston L. (2005). Amino thiols, detoxification and oxidative stress in pre-eclampsia and other disorders of pregnancy. *Curr Pharm Des* 11: 711–734.

Raijmakers M.T.M., Roes E.M., Zusterzeel P.L.M., Steegers E.A.P., Peters W.H.M. (2004b). Thiol status and antioxidant capacity in women with a history of severe pre-eclampsia. *BJOG* 111: 207–212.

Raijmakers M.T.M., Zusterzeel P.L.M., Roes E.M., Steegers E.A.P., Mulder T.P.J., Peters W.H.M. (2001). Oxidized and total whole blood thiols in women with preeclampsia. *Obstet Gynecol* 97: 272–276.

Raijmakers M.T.M., Zusterzeel P.L.M., Steegers E.A.P., Hectors M.P.C., Demacker P.N.M., Peters W.H.M. (2000). Plasma thiol status in preeclampsia. *Obstet Gynaecol* 95: 180–184.

Rajkovic A., Catalano P.M., Malinow M.R. (1997). Elevated homocyst(e)ine levels with preeclampsia. *Obstet Gyn* 90: 168–171.

Rao G.N., Katki K.A., Madamanchi N.R., Wu Y., Birrer M.J. (1999). JunB forms the majority of the AP-1 complex and is a target for redox regulation by receptor tyrosine kinase and G protein-coupled receptor agonists in smooth muscle cells. *J Biol Chem* 274: 6003–6010.

Redman C.W.G., Sargent I.L. (2000). Placental debris, oxidative stress and pre-eclampsia. *Placenta* 21: 597–602.

Redman C.W.G., Sargent I.L. (2003). Pre-eclampsia, the placenta and the maternal systemic inflammatory response—A review. *Placenta* 24: S21–S27.

Regan C.L., Levine R.J., Baird D.D., Ewell M.G., Martz K.L., Sibai B.M., Rokach J., Lawson J.A., Fitzgerald G.A. (2001). No evidence for lipid peroxidation in severe preeclampsia. *Am J Obstet Gynecol* 185: 572–578.

Roberts J.M., Hubel C.A. (1999). Is oxidative stress the link in the two-stage model of pre-eclampsia? *Lancet* 354: 788–789.

Roggensack A.M., Zhang Y., Davidge S.T. (1999). Evidence for peroxynitrite formation in the vasculature of women with preeclampsia. *Hypertension* 33: 83–89.

Sagol S., Ozkinay E., Ozsener S. (1999). Impaired antioxidant activity in women with pre-eclampsia. *Int J Gynaecol Obstet* 64: 121–127.

Sattar N., Clark P., Greer I.A., Shepherd J., Packard C.J. (2000). Lipoprotein (a) levels in normal pregnancy and in pregnancy complicated with pre-eclampsia. *Atherosclerosis* 148: 407–411.

Schiff E., Friedman S.A., Stampfer M., Kao L., Barrett P.H., Sibai B.M. (1996). Dietary consumption and plasma concentrations of vitamin E in pregnancies complicated by preeclampsia [see comments]. *Am J Obstet Gynecol* 175: 1024–1028.

Sikkema J.M., van Rijn B.B., Franx A., Bruinse H.W., de Roos R., Stroes E.S.G., van Faassen E.E. (2001). Placental superoxide is increased in pre-eclampsia. *Placenta* 22: 304–308.

Spickett C.M., Reglinski J., Smith W.E., Wilson R., Walker J.J., McKillop J. (1998). Erythrocyte glutathione balance and membrane stability during preeclampsia. *Free Radic Biol Med* 24: 1049–1055.

Spirin A.S. (2004). High-throughput cell-free systems for synthesis of functionally active proteins. *Trends Biotechnol* 22: 538–545.

Takacs P., Kauma S.W., Sholley M.M., Walsh S.W., Dinsmoor M.J., Green K. (2001). Increased circulating lipid peroxides in severe preeclampsia activate NF-kappaB and upregulate ICAM-1 in vascular endothelial cells. *FASEB J* 15: 279–281.

Touyz R.M., Chen X.., Tabet F., Yao G., He G., Quinn M.T., Pagano P.J., Schiffrin E.L. (2002). Expression of a functionally active gp91phox-containing neutrophil-type NAD(P)H oxidase in smooth muscle cells from human resistance arteries: regulation by angiotensin II. *Circ Res* 90: 1205–1213.

Tsukimori K., Maeda H., Ishida K., Nagata H., Koyanagi T., Nakano H. (1993). The superoxide generation of neutrophils in normal and preeclamptic pregnancies. *Obstet Gynecol* 81: 536–540.

Uotila J., Solakivi T., Jaakkola O., Tuimala R., Lehtimaki T. (1998). Antibodies against copper-oxidised and malondialdehyde-modified low density lipoproteins in pre-eclampsia pregnancies. *Br J Obstet Gynaecol* 105: 1113–1117.

Uotila J.T., Kirkkola A.L., Rorarius M., Tuimala R.J., Metsa-Ketela T. (1994). The total peroxyl radical-trapping ability of plasma and cerebrospinal fluid in normal and preeclamptic parturients. *Free Radic Biol Med* 16: 581–590.

Uotila J.T., Tuimala R.J., Aarnio T.M., Pyykko K.A., Ahotupa M.O. (1993). Findings on lipid peroxidation and antioxidant function in hypertensive complications of pregnancy. *Br J Obstet Gynaecol* 100: 270–276.

Valsecchi L., Cairone R., Castiglioni M.T., Almirante G.M., Ferrari A (1999). Serum levels of alpha-tocopherol in hypertensive pregnancies. *Hypertens Pregnancy* 18: 189–195.

Villar K., Say L., Gülmezoglu A.M., Merialdi M., Lindheimer M.D., Betran A.P., Piaggio G. (2003). Eclampsia and pre-eclampsia: A health problem for 2000 years. In: Critchley H., MacLean A.B., Poston L., Walker J.J. (eds.), *Pre-eclampsia*. RCOG Press, London, pp. 189–207.

Wakatsuki A., Ikenoue N., Okatani Y., Shinohara K., Fukaya T. (2000). Lipoprotein particles in preeclampsia: Susceptibility to oxidative modification. *Obstet Gynecol* 96: 55–59.

Walker M.C., Smith G.N., Perkins S.L., Keely E.J., Garner P.R. (1999). Changes in homocysteine levels during normal pregnancy. *Am J Obstet Gynecol* 180: 660–664.

Walsh S.W. (1998). Maternal-placental interactions of oxidative stress and antioxidants in preeclampsia. *Semin Reprod Endocrinol* 16: 93–104.

Walsh S.W., Vaughan J.E., Wang Y., Roberts L.J. (2000). Placental isoprostane is significantly increased in preeclampsia. *FASEB J* 14: 1289–1296.

Wang Y., Walsh S.W. (2001). Increased superoxide generation is associated with decreased superoxide dismutase activity and mRNA expression in placental trophoblast cells in pre-eclampsia. *Placenta* 22: 206–212.

Wang Y., Walsh S.W. (1996). Antioxidant activities and mRNA expression of superoxide dismutase, catalase, and glutathione peroxidase in normal and preeclamptic placentas. *J Soc Gynecol Investig* 3: 179–184.

Wetzka B., Winkler K., Kinner M., Friedrich I., Marz W., Zahradnik H.P. (1999). Altered lipid metabolism in preeclampsia and HELLP syndrome: Links to enhanced platelet reactivity and fetal growth. *Semin Thromb Hemost* 25: 455–462.

Yanik F.F., Amanvermez R., Yanik A., Celik C., Kokcu A. (1999). Pre-eclampsia associated with increased lipid peroxidation and decreased serum vitamin E levels. *Int J Gynaecol Obstet* 64: 27–33.

Zhang C., Williams M.A., King I.B., Dashow E.E., Sorensen T.K., Frederick I.O., Thompson M.L., Luthy D.A. (2002). Vitamin C and the risk of preeclampsia—Results from dietary questionnaire and plasma assay. *Epidemiology* 13: 409–416.

Zhang C., Williams M.A., Sanchez S.E., King I.B., Ware-Jauregui S., Larrabure G., Bazul V., Leisenring W.M. (2001). Plasma concentrations of carotenoids, retinol, and tocopherols in preeclamptic and normotensive pregnant women. *Am J Epidemiol* 153: 572–580.

Zusterzeel P.L.M., Mulder T.P.J., Peters W.H.M., Wiseman S.A., Steegers E.A.P. (2000). Plasma protein carbonyls in nonpregnant, healthy pregnant and preeclamptic women. *Free Radic Res* 33: 471–476.

Zusterzeel P.L.M., Peters W.H.M., De Bruyn M.A., Knapen M.F.C.M., Merkus H.W.J.M., Steegers E.A.P. (1999). Glutathione *S*-transferase isoenzymes in decidua and placenta of preeclamptic pregnancies. *Obstet Gynecol* 94: 1033–1038.

Zusterzeel P.L.M., Rutten H., Roelofs H.M.J., Peters W.H.M., Steegers E.A.P. (2001a). Protein carbonyls in decidua and placenta of pre-eclamptic women as markers for oxidative stress. *Placenta* 22: 213–219.

Zusterzeel P.L.M., Steegers Theunissen R.P.M., Harren F.J.M., Stekkinger E., Kateman H., Timmerman B.H., Berkelmans R., Nieuwenhuizen A., Peters W.H.M., Raijmakers M.T.M., Steegers E.A.P. (2002). Ethene and other biomarkers of oxidative stress in hypertensive disorders of pregnancy. *Hypertens Pregnancy* 21: 39–49.

Zusterzeel P.L.M., Wanten G.J.A., Peters W.H.M., Merkus H.M.W.M., Steegers E.A.P. (2001b). Neutrophil oxygen radical production in pre-eclampsia with HELLP syndrome. *Eur J Obstet Gynecol Reprod Biol* 99: 213–218.

26

INVOLVEMENT OF OXIDANTS IN THE ETIOLOGY OF CHRONIC AIRWAY DISEASES: PROTEOMIC APPROACHES TO IDENTIFY REDOX PROCESSES IN EPITHELIAL CELL SIGNALING AND INFLAMMATION

ALBERT VAN DER VLIET, NIKI L. REYNAERT, PETER F. BOVE, KARINA CKLESS, ANNE-KATRIN GREUL, MILENA HRISTOVA, AND YVONNE M. JANSSEN-HEININGER

26.1 INTRODUCTION

Over the past several decades it has been well established that diverse chronic inflammatory diseases of the respiratory tract are commonly associated with an increasingly pro-oxidant environment, due to increased production of reactive oxygen and nitrogen species (ROS and RNS) and decreases in local antioxidant status. Based on such observations, it is generally assumed that oxidative or nitrosative stress actively contributes to disease pathology, by affecting specific cell signaling pathways and by covalent oxidative modifications of critical cellular and/or extracellular targets that are expected to have direct functional or structural consequences. Despite our increased appreciation of the potential significance of oxidative protein modifications in relation to cellular dysfunction or disease development, our current understanding of the precise involvement of specific protein oxidations in biology and disease is still rather limited, partly

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

due to incomplete characterization of specific protein modifications and their relation to functional changes, and partly due to incomplete understanding of the major critical protein targets for oxidation by ROS or RNS. Other factors that add to the complexity of this research area are represented by the knowledge that biological oxidants comprise diverse reactive intermediates with highly variable reactivity. Consequently a wide array of possible protein modifications, which include both reversible and irreversible amino acid modifications, may contribute to various possible biological outcomes. While many past efforts have focused on quantitating irreversible protein modifications as potential biomarkers of disease, reversible protein modifications by biological oxidants are believed to be more important in their contribution to regulation of cellular signaling, gene expression, and alterations in cell function. Hence, characterization of cellular targets for such reversible modifications is receiving rapidly increasing attention, and strategies are being developed and employed to characterize protein targets for such reversible modifications, in an attempt to link the production of biological oxidants to functional changes that may mediate disease processes. This chapter will briefly discuss the current evidence in support of oxidative stress in chronic airway diseases such as asthma, and describe recent efforts to determine how oxidative stress may impact on important local cell signaling pathways that modulate inflammation. Finally, we will discuss new strategies that are being employed to characterize and identify targets for reversible oxidation in the airways, strategies that address the importance of biological oxidants in the development and/or progression of chronic airway diseases such as asthma.

26.2 CHRONIC AIRWAY INFLAMMATION: CONDITIONS ASSOCIATED WITH OXIDATIVE AND NITROSATIVE STRESS

26.2.1 Asthma as a Paradigm for Chronic Airway Inflammation

It is by now unambiguously demonstrated that oxidative stress is a feature of several chronic inflammatory lung diseases, such as asthma, chronic pulmonary obstructive disease, and cystic fibrosis. Yet the degree to which oxidative stress contributes to disease pathology is less clearly defined, as are the potential biochemical mechanisms that are involved. It would be beyond the scope of this review to exhaustively review the current evidence in support of oxidative stress in various lung diseases, and this has been addressed in several recent publications (van der Vliet et al., 1997; Macnee and Rahman, 1999; Boots et al., 2003). Hence we will restrict our discussion of current evidence of oxidative stress mechanisms primarily to asthma, although it should be emphasized that many of these events are also relevant to other chronic inflammatory airway diseases. Asthma is a chronic respiratory disease of rapidly increasing incidence in the industrialized world, and it affects millions of individuals throughout their life spans. The hallmarks of asthma include airway inflammation and increased responsiveness to allergens and other environmental factors (O'Bryne and Postma, 1999). Its symptoms may range in severity from mild to life threatening and include episodic

occurrences of cough, wheezing, shortness of breath, or tightness in the chest, either alone or in combination. While a number of therapies are available for the treatment of asthma symptoms, especially of acute exacerbations, effective treatments targeted at the causes of asthma remain absent. One of the main characteristic features of asthma is the presence of elevated inflammatory responses in the lung, orchestrated by $CD4^+$ Th2 lymphocytes and elevated levels of Th2 proinflammatory cytokines, such as the interleukins IL-4, IL-5, and IL-13. These cytokines promote the infiltration and activation of various leukocytes and primarily eosinophils, which are increased in over 75% of the reported cases of allergic airways disease. These cytokines appear to contribute substantially to the pathobiology of asthma as they are associated with epithelial desquamation, airway smooth muscle perturbation, and airway remodeling. Both genetic and environmental factors, such as environmental tobacco smoke or nitrogen dioxide ($NO_2^{\bullet}$) in photochemical smog, are known to contribute to asthma development and exacerbations (Chauhan et al., 1998; von Mutius, 1998; McConnell et al., 1999; Gold, 2000; Strachan, 2000).

26.2.2 Inflammatory Oxidants: NADPH Oxidases and Peroxidases

Because asthma, like other chronic lung diseases, is largely characterized by ongoing inflammatory-immune processes, this condition is associated with increased production of inflammatory oxidants by activated respiratory burst oxidases, thus resulting in a pro-oxidant environment within the asthmatic airways. One primary line of evidence for such increased oxidant production is the detection of elevated levels of H_2O_2 in exhaled breath condensates from patients with asthma, which have been found to correlate with the extent of inflammation and with asthma severity (Antczak et al., 1997; Emelyanov et al., 2001). While the biological sources of this oxidant production most likely represent resident and infiltrating inflammatory cells (macrophages, neutrophils, and eosinophils, which generate large amounts of superoxide anion [$O_2^{\bullet-}$] upon activation of NADPH oxidase), the recent identification of novel NADPH oxidase homologues in nonphagocytic cells, such as the dual oxidases Duox1 and 2 in the airway epithelium (Geiszt et al., 2003; Forteza et al., 2005; Shao and Nadel, 2005), points to an additional source of oxidants that potentially participates in innate mucosal host defense, and perhaps also in the local regulation of inflammatory-immune processes. These novel NADPH oxidase homologues have also been implicated in cellular responses to various growth factors or cytokines (Finkel, 2003). In addition, various cell stimuli can induce changes in oxidant production from mitochondria, which in turn may specifically regulate cellular pathways leading to inflammatory gene expression or apoptotic cell death (Werner and Werb, 2002). Although the principal product of NADPH oxidases is $O_2^{\bullet-}$, spontaneous or enzyme-catalyzed dismutation (by superoxide dismutases; SOD) converts it to H_2O_2. As discussed below, reaction of H_2O_2 with peroxidases of, for example, neutrophils or eosinophils gives rise to the generation of secondary oxidants, including hypohalous acids such as hypochlorous acid (HOCl) or hypobromous acid (HOBr). Indirect evidence for the increased formation of such secondary

oxidants, for example, in asthmatic airways has been obtained in the form of specific reaction products with proteins.

26.2.3 Nitric Oxide and Its Metabolism

In addition to increases in NADPH oxidase-derived oxidants, inflammatory conditions are commonly characterized by increased local production of nitric oxide ($NO^{\bullet}$), resulting from induction and/or activation of $NO^{\bullet}$ synthases (Andreadis et al., 2003). Notably type 2 $NO^{\bullet}$ synthase (NOS2, also referred to as inducible NOS, or iNOS) is induced within asthmatic airways, both within the epithelium and in resident or extravasated inflammatory-immune cells, and it has been linked to increased levels of $NO^{\bullet}$ in the expired breath of asthmatic subjects (Gaston et al., 1994; Guo et al., 2000). While an important biological function of NOS2 induction may be its contribution to antimicrobial activity against invading pathogens (Fang, 1997; Nathan, 1997), many studies also implicate $NO^{\bullet}$ in inflammatory cell signaling and injury, thereby actively participating in inflammatory-immune processes (Idriss et al., 1999; Raychaudhuri et al., 1999; Thomassen and Kavuru, 2001). Through pharmacological NOS inhibition and also through targeted deletion of individual NOS isozymes, it has been demonstrated that $NO^{\bullet}$ has many diverse effects on lung inflammation and injury with indications of both pro- and anti-inflammatory properties (Akaike et al., 1996; Feder et al., 1997; Kristof et al., 1998; De Sanctis et al., 1999; Xiong et al., 1999; Kleeberger et al., 2001; Okamoto et al., 2004).

An important factor that contributes to the diversity in $NO^{\bullet}$-mediated effects on inflammation is the variability in its oxidative catabolism, which depends on the degree and location of $NO^{\bullet}$ production and the local presence of oxidative processes. First, $NO^{\bullet}$ is well known to react rapidly with superoxide anion ($O_2^{\bullet -}$), the primary product of NADPH oxidases, to form the strong oxidant peroxynitrite ($ONOO^-$). Hence, its biological activity is dramatically affected in conditions where $O_2^{\bullet -}$ is produced simultaneously (Beckman and Koppenol, 1996). Second, $NO^{\bullet}$ can also be metabolized by heme peroxidases such as myeloperoxidase (MPO) or eosinophil peroxidase (EPO), which are activated in inflammatory conditions that involve neutrophils and/or eosinophils (van der Vliet et al., 1999; Eiserich et al., 2002). In addition to reducing $NO^{\bullet}$ levels and interfering with its biological function, such oxidative catabolism results in intermediate formation of reactive nitrogen species ($ONOO^-$ or $NO_2^{\bullet}$), which themselves may have biological function by oxidizing and/or nitrating target molecules. Indeed 3-nitrotyrosine, a characteristic molecular footprint for RNS (van der Vliet et al., 1999), has been detected in increased amounts in lung tissues, secretions, and exhaled breath condensates from patients with asthma (Saleh et al., 1998; Kaminsky et al., 1999; Hanazawa et al., 2000; MacPherson et al., 2001), illustrating formation of RNS by such oxidative $NO^{\bullet}$ catabolism within the lungs of these subjects.

26.2.4 Alterations in Antioxidant Defenses

Coupled to the enhanced production of ROS and RNS in inflammatory lung diseases such as asthma are several observations of altered antioxidant defenses in

these conditions (Andreadis et al., 2003; Caramori and Papi, 2004). Notably the activity of superoxide dismutase (SOD), which catalyzes the conversion of superoxide to H_2O_2, is reduced in asthmatic lungs compared to controls, and it decreases further in asthmatic individuals following segmental allergen challenge (Andreadis et al., 2003). Moreover, it was recently determined that Mn-SOD obtained from bronchial specimens of asthmatic subjects contains multiple oxidative aromatic amino acid modifications, including 3-nitrotyrosine and 3-bromotyrosine, which could have been causally related to its inactivation (Comhair et al., 2005; Janssen-Heininger et al., 2005). On the other hand, local levels of extracellular GSH peroxidase appear to be increased in asthmatic airways compared to normal subjects, which presumably reflects its upregulation in response to increased oxidative stress (Andreadis et al., 2003). Similarly, bronchoalveolar lavage levels of GSH were found to be increased in asthmatic subjects (Smith et al., 1993), presumably reflecting upregulated GSH synthesis in response to oxidative stress. In addition to changes in antioxidant enzymes, concentrations of antioxidant micronutrients, such as ascorbate (vitamin C) and α-tocopherol (vitamin E), within lung lining fluids are lowered in patients with asthma (Kelly et al., 1999). Similarly, levels of GSH in exhaled breath condensates were found to be decreased in association with asthma exacerbations (Corradi et al., 2003). Further support for the significance of such changes in local lung antioxidant status comes from several studies that have demonstrated beneficial effects of antioxidant supplementation. For instance, administration of α-tocopherol (vitamin E) has been found to inhibit IgE responses to allergic stimuli in animals (Zheng et al., 1999), an observation that is consistent with epidemiologal data that associate vitamin E intake with reduced frequency of allergen sensitization (Fogarty et al., 2000). Furthermore, administration of the synthetic catalytic antioxidant AEOL 10113, which possesses both SOD and catalase mimetic activity, was found to inhibit airway inflammation and hyperreactivity in experimental models of asthma (Chang and Crapo, 2002). Similar studies using the GSH-peroxidase mimetic ebselen or the cysteine pro-drug L-2-oxothiazolidine-4-carboxylic acid (OTC) also demonstrated marked inhibition of inflammation and hyperreactivity in a similar model (Zhang et al., 2002; Lee et al., 2004b), implicating involvement of reactive oxygen species in the development of allergic airways disease.

In summary, there is ample evidence for increased production of ROS and RNS within asthmatic airways, in association with some alterations in local antioxidant status, and a strong case can be made for involvement of ROS and RNS in chronic inflammatory lung diseases such as asthma. A secondary question is then how such reactive intermediates contribute to disease pathology, which conceivably involves the oxidation of critical biological constituents, resulting in direct functional changes or production of secondary mediators that affect cellular pathways. Indeed, one type of oxidative modification concerns the oxidative degradation of lipids, as demonstrated by increased levels of stable products of biological lipid oxidation in airway secretions and/or exhaled breath condensates (Montuschi et al., 1999; Caramori and Papi, 2004) that may themselves have secondary effects. These include malondialdehyde, ethane, or more specific end-products

of nonenzymatic lipid oxidation such as F_2-isoprostanes. Similarly, analysis of exhaled breath condensates, airway secretions, tracheal biopsies, or postmortem lung tissues have also revealed increased levels of stable oxidation products in DNA (8-hydrodeoxyguanosine), which may reflect a mechanism by which oxidative stress can contribute to mutagenesis. While the oxidation of biological lipids or DNA could certainly be expected to have major biological consequences, the high overall abundance of proteins in biological systems, and the fact that proteins are primarily responsible for most functional processes within cells, has led to a strong bias in characterizing oxidative protein modifications in attempts to link these to functional changes. The strong emphasis on protein oxidation is also based on known reaction rate constants of oxidation of major classes of biomolecules within cells, and their relative abundance, which suggest that the majority of generated ROS and RNS (50–75%) can be expected to react with proteins (Davies et al., 1999). Indeed there is ample evidence for increased oxidative modifications in proteins (e.g., protein carbonyls, 3-nitrotyrosine, 3-bromotyrosine) in asthmatic subjects (Kaminsky et al., 1999; Montuschi et al., 1999; Wu et al., 2000; Duguet et al., 2001; Brennan et al., 2002; Schock et al., 2003; Caramori and Papi, 2004), and it has been speculated that such oxidative modifications may directly contribute to disease pathology. Recent technical advances to detect specific oxidative modifications in proteins and their application to proteomic research have paved the way for future approaches to further address the functional significance of protein oxidation in disease, and this chapter will review these aspects specifically in the context of pulmonary diseases such as asthma.

26.3 BIOLOGICAL SIGNIFICANCE OF PROTEIN OXIDATION

26.3.1 Protein Oxidation: Biomarker of Disease or Mediator of Inflammation and Injury?

As mentioned above, there is strong evidence for the presence of increased levels of stable products of protein oxidation in asthmatic airways or secretions. These include protein carbonyls (a general marker of protein oxidation by a variety of mechanisms) and specific products that are more characteristic of selected oxidant mechanisms, such as 3-nitrotyrosine or 3-bromotyrosine. For example, characteristic oxidation products such as 3-bromotyrosine reflect the presence of specific oxidant mechanisms by activation of eosinophil peroxidase (EPO) (Wu et al., 2000). As noted previously, the formation of 3-nitrotyrosine reflects oxidative $NO^{\bullet}$ metabolism by various oxidative mechanisms, but the observation of markedly attenuated levels of 3-nitrotyrosine in EPO-deficient animals strongly implicates the involvement of this enzyme in this post-translational protein modification, by intermediate formation of $NO_2^{\bullet}$ (Duguet et al., 2001; MacPherson et al., 2001). Importantly, it was recently demonstrated that eosinophils actively contribute to airway hyperreactivity in experimental models of asthma (Lee et al., 2004a), which raises the intriguing possibility that EPO-derived oxidants may contribute to disease pathology by oxidizing critical substrates. Even though

studies with EPO-deficient mice have so far failed to reveal a contribution of this enzyme to allergic airway inflammation (Denzler et al., 2001), differences in eosinophil properties and degranulation between mice and men do not allow firm conclusions regarding the role of EPO in human asthma.

The analysis of protein oxidation products in relation to disease conditions such as asthma generally serves two separate purposes. A first purpose is its potential use as a biomarker of disease and response to treatment, which may reflect the contribution of specific biochemical mechanisms, even though the significance of the protein oxidation per se may be unknown. A second objective would be to identify specific protein oxidation events within specific target proteins, in an attempt to provide a functional link between ROS or RNS generation and changes in lung function, for example. In order to function as suitable biomarkers of oxidative modifications in relation to disease, it is critical that such oxidation products are stable, can accumulate to detectable levels, reflect specific oxidation pathways, and correlate with disease severity, so that they can be used as a diagnostic tool. For these reasons, analysis of 3-nitrotyrosine (a stable marker for $NO^{\bullet}$-derived oxidants), halogenated products such as 3-Cl-tyrosine or 3-Br-tyrosine, or unnatural isomers of tyrosine (e.g., *o*-Tyr as an indicator of $OH^{\bullet}$) has been performed in various cases, and several diverse methods have been developed for such measurements (van der Vliet et al., 2000; Brennan et al., 2002). Consistent with the potential application as a biomarker, analysis of such stable oxidation products has shown correlations with the degree of eosinophilic inflammation, for example, and with overall disease severity (Saleh et al., 1998), suggesting that such oxidation products are useful biomarkers of disease status.

The secondary purpose, to link specific oxidative protein modifications to changes in function, is considerably more challenging. In the traditional view, irreversible oxidative modifications in proteins (carbonylation, nitration, oxidative cross-linking, etc.) are believed to be associated with a permanent loss of function, and damaged proteins are eliminated by protein degradation pathways or accumulate in the form of protein aggregates (e.g., lipofuscins). In this view, extensive irreversible protein oxidation may have significant functional consequences by causing metabolic stress due to excessive protein degradation or accumulation of protein aggregates. Nevertheless, the question whether irreversible oxidative protein modifications, such as the nitration of tyrosine residues in specific proteins contribute significantly to their functional alteration still remains to be adequately addressed. Although many biochemical studies have demonstrated that nitration of specific tyrosine residues can result in the inactivation of various proteins or enzymes, it is questionable whether this occurs to a sufficient extent in vivo, even in severe pathological conditions. Studies using purified glutathione *S*-transferase have shown that its inactivation by RNS parallels the overall degree of tyrosine nitration; however, the quantitative extent of nitration of critical tyrosine residues appeared insufficient to fully account for the functional effects (Wong et al., 2001; Wong and van der Vliet, 2002). In fact most commonly measured stable oxidative amino acid modifications (e.g., 3-nitrotyrosine, *o*-tyrosine, dityrosine, carboxymethyllysine) (Table 26.1) typically affect only

TABLE 26.1 Summary of Major Reversible and Irreversible Oxidative Protein Modifications

Amino Acid	Oxidation Product	ROS/RNS	Method of Detection
Phenylalanine	2-OH-phenylalanine (*o*-tyrosine)	$OH^{\bullet}$	HPLC, GC/LC-MS
	3-OH-phenylalanine (*m*-tyrosine)	$OH^{\bullet}$	HPLC, GC/LC-MS
Tyrosine	3,4-diOH-phenylalanine (DOPA)	$OH^{\bullet}$	HPLC, GC/LC-MS
	3,3′-dityrosine (*o*,*o*-dityrosine)	$OH^{\bullet}$, Fe(V)=O[a]	HPLC, GC/LC-MS
	3-NO_2-tyrosine	$NO_2^{\bullet}$, $ONOO^-$	HPLC, GC/LC-MS, IC[b]
	3-Cl-tyrosine	HOCl, Cl_2	HPLC, GC/LC-MS
	3-Br-tyrosine	HOBr, Br_2	GC/LC-MS
Tryptophan	3-, 4-, 5-, 6- or 7-OH tryptophan	$OH^{\bullet}$	HPLC
	5-nitrotryptophan	ONOO–, NO2$^{\bullet}$	HPLC
	N-formyl-kynurenine	$OH^{\bullet}$	HPLC
Histidine	2-oxohistidine	$OH^{\bullet}$	HPLC, GC/LC-MS
Methionine	Methionine sulfoxide (Met-S=O)	H_2O_2, HOCl, $ONOO^-$	GC/LC-MS
	Methionine sulfone (Met-SO_2)	H_2O_2, HOCl, $ONOO^-$	GC/LC-MS
Cysteine	Cysteine sulfenic acid (Cys-S–OH)	H_2O_2, HOCl, ONOO–	IC[b]
	Cysteine sulfinic acid (Cys-SO_2H)	H_2O_2, HOCl, ONOO–	GC/LC-MS
	Cysteine sulfonic acid (Cys-SO_3H)	H_2O_2, HOCl, ONOO–	GC/LC-MS
	S-nitrosocysteine (Cys-S–NO)	N_2O_3, HNO_2	IC,[b] LC-MS

Note: The major oxidation products listed are recognized as stable markers of protein oxidation, and are implicated in functional protein modifications. For each modification, the responsible ROS/RNS are given as well as the major available methods of detection.

[a] Fe(V)=O refers to activated heme peroxidases (compound I).

[b] Immunochemical detection.

a minor fraction (<0.1%) of the parent amino acid even in severe conditions (Leeuwenburgh et al., 1997; Wells-Knecht et al., 1997; Ahmed and Thornalley, 2003). So protein nitration may be insignificant in explaining functional loss, although the combined impact of multiple concordant oxidative modifications

within a protein may be functionally more significant. In contrast, the possibility of acquired new functionality by such irreversible protein modifications has not been rigorously addressed. In this regard recent studies that link protein nitration to enhanced immune responses to either autologous or airborne environmental proteins (Birnboim et al., 2003) are intriguing as they may lead to similar additional causal relationships between irreversible protein oxidations and disease development.

In contrast to the relative low abundance of such irreversible tyrosine modifications, even in conditions of severe oxidative stress, other amino acid residues (primarily cysteine and methionine) are much more susceptible to oxidation and are quantitatively more extensively oxidized. For example, the relative extent of biological methionine oxidation may range from 2% to 10% (Wells-Knecht et al., 1997; Stadtman et al., 2005), and this type of oxidation is therefore more likely of functional importance. An additional, and perhaps much stronger, argument for the biological significance of cysteine or methionine oxidation by ROS or RNS is the existence of several elaborate enzymatic systems that actively regulate and reverse such modifications, such as the thioredoxin/thioredoxin reductase (Trx/Trx-red) system and several isoforms of glutaredoxin (Grx1/2) or methionine sulfoxide reductase (MsrA/B). Hence, the majority of changes in cell function or cell signaling in response to oxidative stress is most likely mediated by reversible modifications in redox-sensitive cell targets that, in addition to transition metal ions in, such as heme proteins, primarily involve reactive protein cysteine or methionine residues. Since enzymatic reversal of protein modifications can restore protein activity, reversible protein modifications are thought to be critical in protein function regulation or to protect critical cysteine residues from irreversible oxidation. In addition to being susceptible to reversible oxidation, reactive protein cysteine residues are susceptible to *S*-nitrosylation (a presumed mechanism involved in cell signaling by $NO^{\bullet}$), and to alkylation by electrophilic end-products of enzymatic or nonenzymatic lipid oxidation. This way such cysteine modifications may serve as central mediators of cellular responses to a broad range of reactive biological mediators from various origins.

Because of the biological reversibility of such oxidative protein modifications, and a current relative lack of available specific and sensitive methodology to detect them, our understanding of the relative importance of oxidative modifications in regulating specific cellular pathways is still limited. Recent methodological advances have made analyses of reversible oxidative protein modifications more applicable to proteomic approaches, thereby allowing assessment of the relative importance of reversible modifications in specific proteins and identification of critical proteins that are involved in overall cellular responses to oxidative or nitrosative stress. Since some of these advances are discussed in other chapters of this volume, the main emphasis of the present chapter is the application of newly developed strategies in studies of lung inflammation and disease. In this context we first briefly review the current concepts of redox regulation of cell signaling pathways that are involved in inflammation, with a special focus on redox regulation of the nuclear factor NF-κB, a central regulator of inflammatory

responses to many diverse stimuli. We then summarize the proteomic strategies that have been used to determine protein modifications and responses to oxidative stress in the lung, and finally discuss some newly developed approaches that are used to more specifically address reversible oxidations of critical targets within the lung epithelium.

26.3.2 Redox Signaling: Cysteine Oxidation, Thiolation, and Nitrosylation

Following the traditional view that ROS and RNS cause cell injury and death by oxidizing several classes of biological molecules, in more recent years it has become appreciated that these agents can also participate in diverse biological processes, and regulate normal cell growth, induction of cell transformation, and cellular senescence or apoptosis. The discovery of several homologues of NADPH oxidases in various nonphagocytic cells has spurred much interest in the potential role of ROS in cellular signaling pathways that control these various events, thereby being somewhat analogous to the various signaling events elicited by $NO^{\bullet}$ or RNS produced by several $NO^{\bullet}$ synthases. A question that arises, however, is how a seemingly simple increase in oxidant production can cause such divergent responses. One explanation is that different biological outcomes reflect differences in the level or duration of oxidant production. The recently developed "floodgate hypothesis," which predicts that H_2O_2-induced signaling only occurs when peroxiredoxin (Prx) is overoxidized as H_2O_2 reaches critical concentrations (Wood et al., 2003), is consistent with this view. Alternatively, the different biological effects of ROS may be related to their formation at distinct cellular compartments, and to differences in the nature of oxidative protein modifications. For example, at levels that stimulate cell growth or transformation, biologically produced ROS or RNS may target selected signaling pathways that are highly susceptible to redox regulation. Higher concentrations of ROS/RNS, or the formation of more reactive ROS/RNS, may result in more extensive and/or more irreversible oxidation of biological targets. Furthermore, the spectrum of oxidative modifications may increase when biological systems are exposed to higher oxidant concentrations, because of increased degrees of oxidation of less susceptible targets and depletion of cellular antioxidant defenses. A main question that remains is whether the contributing effects of oxidants in disease are primarily related to irreversible oxidative modifications that are associated with higher levels of ROS and RNS production or to more pronounced or prolonged reversible modifications of more susceptible targets. Based on rapidly accumulating evidence of regulation of redox-sensitive cysteine residues in several target proteins in association with cell growth, transcription factor activation, and/or apoptosis, it is increasingly appreciated that deregulation of such redox signaling pathways may also underlie the involvement of inflammatory oxidants in disease.

What Are the Main Targets in Redox Signaling? Based on observations that cellular effects of NADPH oxidase derived oxidants are usually prevented by catalase, the presumed principal mediator involved in NADPH oxidase dependent signaling is H_2O_2. The question then becomes what are the main cellular targets

that "sense" H_2O_2, and respond to changes in H_2O_2 production and mediate H_2O_2-dependent cellular effects. Kinetic data predict that H_2O_2 will react primarily with ferric heme proteins (heme peroxidases: e.g., catalase, cytochrome c peroxidase, myeloperoxidase), selenocysteine residues (e.g., in GSH peroxidase), or thiolate forms of cysteine residues (e.g., in peroxiredoxins) (Stone, 2004). Because cysteine residues are often involved in the activity of cellular proteins, including various metabolic enzymes, protein kinases and phosphatases, ion channels, proteases, and transcription factors, it is assumed that many of these proteins can also be regulated by H_2O_2, and many studies have demonstrated direct or indirect evidence for redox regulation of these proteins (reviewed in Finkel, 2003; Cross and Templeton, 2004; Forman et al., 2004; Stone, 2004; den Hertog et al., 2005). However, to what extent redox modifications in specific proteins are related to changes in cell function is less clear. The reactivity of protein cysteine residues to H_2O_2 is highly variable, and depends largely on their pK_a, which determines the extent to which they are present in the reactive thiolate form. While kinetic data can be used as a guideline to predict the sensitivity of various biological targets to H_2O_2 (Forman et al., 2004; Stone, 2004), this can be misleading since kinetic data do not address the significance of compartmentalization and direct target interactions with NADPH oxidases, which would allow H_2O_2 to reach sufficient local concentrations to oxidize substrate cysteines before it is being removed by kinetically favored reactions with peroxidases such as Prx or glutathione peroxidases (GPX). Alternatively, other as yet unidentified systems may be present to catalyze kinetically less favorable oxidation of protein cysteine residues.

Among the best-studied targets for redox regulation are protein tyrosine phosphatases (PTP's), which contain a highly conserved thiolate cysteine in the CX5R motif that participates in the dephosphorylation reaction. Oxidation of cysteine (RS^-) by H_2O_2 will initially yield sulfenate (RSO^-), which is the ionized form of a sulfenic acid (RSOH). Although formation of sulfenic acids can in some cases be demonstrated (Poole et al., 2004), in biological systems they are generally unstable and react rapidly with another cysteine (most likely from GSH) to form a mixed disulfide (RSSR' or RSSG). Such mixed disulfide formation with GSH is generally referred to as *S*-glutathionylation, and this modification has been observed in a growing number of proteins (Cotgreave and Gerdes, 1998; Barrett et al., 1999). The precise physiological significance of such protein *S*-glutathionylation is still unclear. *S*-Glutathionylation may either directly regulate protein function or enzyme activity or serve to prevent further oxidation, thereby protecting protein cysteines from irreversible oxidation, or both. Other secondary reactions of sulfenic acids with neighboring protein cysteine residues will result in intra- or intermolecular disulfides, and such reactions with secondary "resolving" cysteine residues may similarly affect enzymatic function by introducing structural changes, or serve to prevent further cysteine oxidation to less reversible products such as sulfinic or sulfonic acids (RSO_2H and RSO_3H, respectively). Indeed intramolecular disulfide formation is the principal mechanism involved in the catalytic activities of thioredoxin (Trx), glutaredoxins (Grx),

and peroxizedoxins (Prx), which can all be recycled by enzymatic reducing mechanisms (Trx reductase, GSH reductase, etc.). In this sense protein glutathionylation is often viewed as a similar protective mechanism that prevents overoxidation of critical cysteines, and can be reversed, such as by Grx. Nevertheless, while cysteine thiolate oxidation may result in decreases in enzymatic activity, the exact oxidative modification that is responsible for functional alterations is generally not known and may even require secondary reactions. An intriguing example of this is PTP1B, in which oxidation of the critical cysteine thiolate to a sulfenic acid is rapidly followed by its reaction with the main chain N atom of the adjacent residue to form a sulphenyl-amide intermediate, thereby inducing a significant conformational change in the protein that affects substrate binding and facilitates reactivation by biological thiols (Salmeen et al., 2003; van Montfort et al., 2003).

Redox Signaling by $NO^{\bullet}$: A Special Role for S-Nitrosylation An additional post-translational modification that is associated with oxidative metabolism of $NO^{\bullet}$ is the reaction with redox-sensitive protein cysteine residues to form *S*-nitrosothiols. Such *S*-nitrosylation (or *S*-nitrosation, as it is often referred to based on presumed chemical mechanisms) can also induce structural and/or functional alterations (Stamler et al., 2001), and it has been implicated as an important additional signaling mechanism related to $NO^{\bullet}$ activity (Hess et al., 2005). Indeed a large number of proteins have to date been identified as putative targets for *S*-nitrosylation, including channels/transporters, structural proteins, metabolic enzymes, signaling proteins, proteolytic enzymes, and transcription factors. The precise biological mechanisms that regulate protein *S*-nitrosylation, or that mediate its reversal, still remain poorly characterized and are most likely multifactorial. They may involve Trx, low-molecular-weight thiols, metal-containing proteins, and a recently described GSNO reductase (Nikitovic and Holmgren, 1996; van der Vliet et al., 1998; Broillet, 1999; Ji et al., 1999; Nedospasov et al., 2000; Spencer et al., 2000; Liu et al., 2001; Hogg, 2002; Liu et al., 2004). Overall, the various biochemical/enzymatic pathways that may control *S*-nitrosylation are indicative of its tight regulation, and this can be viewed as further evidence for *S*-nitrosylation as an important signaling pathway associated with $NO^{\bullet}$. The fact that *S*-nitrosothiols can also react with other thiols to form mixed disulfides within or between proteins illustrates the potential overlap between the redox biology of H_2O_2 and $NO^{\bullet}$, which might even regulate similar biological pathways by inducing structural or functional changes such as due to inter- or intramolecular disulfide formation through different initiating mechanisms, either by oxidation to *S*-OH or by *S*-nitrosylation.

Despite significant technical challenges to detect *S*-nitrosothiols in intact cells or biological systems and the reversible nature of *S*-nitrosation, the presence of *S*-nitrosothiols has been demonstrated in intact biological systems such as plasma (Gaston et al., 1994; Marley et al., 2000), bronchoalveolar lavage fluids, or tracheal aspirates (Gaston et al., 1998; Grasemann et al., 1999; Dweik et al., 2001), and *S*-nitrosothiol levels were found to change in various disease conditions. For instance, while *S*-nitrosothiol levels were found to increase during

pneumonia, *S*-nitrosothiol levels in tracheal aspirates or bronchoalveolar lavage fluids were markedly decreased in patients with asthma or cystic fibrosis (Gaston et al., 1998; Grasemann et al., 1999; Dweik et al., 2001). These changes most likely reflect accelerated degradation of *S*-nitrosothiols in these latter conditions, or altered oxidative $NO^{\bullet}$ metabolism that would limit its ability to *S*-nitrosylate proteins (Trujillo et al., 1998; van der Vliet et al., 1998; Fang et al., 2000). The observation of elevated protein tyrosine nitration in these conditions (Kaminsky et al., 1999; van der Vliet et al., 2000; Dweik et al., 2001; MacPherson et al., 2001) illustrates enhanced oxidative $NO^{\bullet}$ catabolism. Of note, therapeutic strategies using, for example, aerosolized *S*-nitrosothiols have been proposed to alleviate symptoms of asthma or cystic fibrosis, with some preliminary evidence of benefit (Richardson and Benjamin, 2002; Snyder et al., 2002). However, in analogy to other cysteine modifications (oxidation, alkylation), it is still a matter of controversy to what extent *S*-nitrosylation of certain protein cysteine residues is involved in normal physiological aspects of $NO^{\bullet}$ biology or nitrosative stress that is associated with overproduction of $NO^{\bullet}$ and RNS such as in inflammatory conditions. More reliable methodology that can be applied to proteomic analysis is needed to address these issues, and recent advances over the past few years will be discussed next.

26.3.3 Redox Regulation of Inflammation: A Central Role for NF-κB

The NF-κB Signaling Pathway: Targets for Redox Regulation One of the hallmarks of chronic lung diseases such as asthma is the persistent activation of inflammatory-immune processes. Perhaps the main initial event in the inflammatory response to inhaled microbes or allergens is the activation of the transcription factor Nuclear Factor kappa-B (NF-κB), which controls a diversity of functional outcomes, including inflammation, survival, and proliferation. NF-κB can be activated by over 150 different stimuli, and this in turn regulates transcription of over 150 different genes. So NF-κB has been termed the central mediator of the human immune response (Pahl, 1999). The activity of NF-κB is tightly controlled by the inhibitory protein IκBα, which forms complexes with NF-κB dimers within the cytoplasm. Exposure to NF-κB activating stimuli (e.g., cytokines, bacterial products, viruses) will result in carefully orchestrated phosphorylation, ubiquitination, and degradation of IκB, resulting in liberated NF-κB dimers with unmasked nuclear localization sequences, which can translocate into the nucleus, bind to responsive elements in the DNA called κB motifs, and regulate the transcription of the target genes (Baeuerle, 1998; Janssen-Heininger et al., 2000). The phosphorylation of the inhibitory protein IκB occurs at specific serine residues by the IκB Kinase (IKK) complex, a 700 to 900 kDa complex composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ (DiDonato et al., 1996; Maniatis, 1997; Zandi and Karin, 1999). Since IKK serves as a point of convergence for both positive and negative NF-κB regulators (Delhase et al., 1999), it presents a central regulator of NF-κB activation. The activation of NF-κB causes enhanced expression of genes encoding inflammatory cytokines, chemokines, acute phase proteins, immunoreceptors, and so forth, which are important in

the recruitment of neutrophils and eosinophils (Dunn et al., 1994; Driscoll et al., 1995; Roebuck et al., 1995; Sanceau et al., 1995; Harant et al., 1996; Hein et al., 1997). Thus, the induction of NF-κB by pro-inflammatory stimuli appears to be a critical signal in evoking an inflammatory response in lung (Barnes et al., 1998). Furthermore, various recent studies using genetic approaches have indicated that activation of NF-κB within the airway epithelium is critical in the inflammatory response to inhaled lipopolysaccharide or allergen challenge (Poynter et al., 2003, 2004; Sadikot et al., 2003).

In addition to activated inflammatory-immune processes, another characteristic of asthma as well as other acute and chronic lung diseases include injury to the airway epithelium, as manifested by reduced epithelial barrier function and/or increased apoptotic or necrotic cell death. Important signaling pathways that control epithelial survival and death are the mitogen-activated protein kinases (MAPK) or stress-activated protein kinases, of which c-Jun *N*-terminal kinase (JNK) represents a major pathway involved in the programmed cell death in response to stress stimuli. Each of these MAPK pathways has been shown to be regulated by ROS at various levels. It is of interest to the topic discussed in this chapter that ROS/RNS can disrupt the balance of IKK and JNK activation, by inhibiting, for example, tumor necrosis factor (TNF)α-induced IKK activation and simultaneously activating JNK. This disruption of IKK and JNK activity appears to depend on the nature of the oxidant and on specific cytokine/death receptors. For example, H_2O_2 appears to signal via TNF-receptors, while $ONOO^-$ was found to promote Fas-dependent JNK activation and cell death, possibly by direct oxidative modification of Fas (Pantano et al., 2003; Shrivastava et al., 2004).

Although several oxidant-producing stimuli have been shown to be capable of activating the NF-κB cascade (Janssen-Heininger et al., 2000), it has become increasingly apparent that various steps within the NF-κB cascade are also susceptible to redox regulation, and can be inhibited by oxidation, *S*-nitrosylation, or glutathionylation of critical protein cysteine residues. For example, the p50 subunit of NF-κB, which is critical for transcriptional activation, contains a redox-sensitive cysteine residue and oxidative modification (involving *S*-nitrosylation or glutathionylation), and it has been found to prevent DNA binding activity (Peng et al., 1995; Marshall et al., 2000; Marshall and Stamler, 2001; Pineda-Molina et al., 2001). With regard to such negative regulation by $NO^{\bullet}$, this is thought to represent a feedback mechanism resulting from NOS2 induction in response to NF-κB activation, in order to avoid prolonged NF-κB activation and inflammation. More recently it has also become apparent that the IKK complex, which is an important mediator in the positive and negative regulation of NF-κB, is subject to redox regulation (Delhase et al., 1999; Zandi and Karin, 1999). Specifically, the IKKβ subunit, which has a major role in responding to many pro-inflammatory stimuli, has been found to be susceptible to inactivation by either oxidation or alkylation of a critical cysteine residue within the transactivation loop (Kapahi et al., 2000; Rossi et al., 2000; Ji et al., 2001; Korn et al., 2001; Valacchi et al., 2005).

Multifactorial Regulation of NF-κB Signaling by NO• In several studies using cultured human bronchial epithelial cells (HBE1) or the mouse alveolar type II cell line C10, we have demonstrated that cell exposure to NO• donor compounds such as *S*-nitrosoglutathione (GSNO) and *S*-nitroso-*N*-acetyl-penicillamine (SNAP) can prevent the activation of NF-κB by the pro-inflammatory cytokine TNF-α. By analysis of several consecutive events in the NF-κB activation cascade, we have observed that *S*-nitrosothiols can inhibit TNFα-induced phosphorylation of IκB, nuclear translocation of RelA, NF-κB binding activity to DNA, and expression of NF-κB regulated genes such as matrix metalloproteinase (MMP)-9 (Okamoto et al., 2002; Reynaert et al., 2004). These inhibitory effects were independent of cGMP production, augmented by depletion of cellular GSH, and associated with increases in protein *S*-nitrosothiol levels, strongly suggesting that such inhibition was mediated by *S*-nitrosylation of specific proteins involved in the NF-κB signaling cascade. Exposure of C10 cells that were transfected with a NF-κB luciferase reporter construct to *S*-nitrosocysteine (CSNO) dose-dependently inhibited NF-κB-derived luciferase activity (Fig. 26.1), which parallels with decreased phosphorylation of IκBα (Reynaert et al., 2004), indicating inhibitory effects on an upstream event in the NF-κB cascade, such as IKK activation. In support of this notion, *S*-nitrosothiols were found to inhibit the enzymatic activity of activated IKK in vitro, and also to suppress TNFα-induced IKK activation in intact cells. Similarly inhibition of NOS activity with L-NMMA enhanced basal and TNFα-stimulated IKK activity in Jurkat T cells (Reynaert et al., 2004). However, this was not observed in C10 or HBE1 cells (Fig. 26.1), perhaps because they do not express inducible NOS. Nevertheless, the studies implicate the IKK complex as an important direct target for NO•-dependent inhibition of NF-κB, most likely through *S*-nitrosylation, in addition to other regulatory mechanisms by *S*-nitrosylation of alternative targets within the NF-κB pathway, such as the p50 subunit of NF-κB itself (Matthews et al., 1996; Marshall and Stamler, 2002; Hess et al., 2005).

Although NO• has been shown to be capable of inhibiting NF-κB activation and gene expression, it can also stimulate NF-κB activation. Various mechanisms have been implicated in NO•-mediated NF-κB activation, including cGMP-dependent mechanisms through activation of guanylyl cyclase, activation of p21Ras by *S*-nitrosylation, or activation of more indirect mechanisms such as nitration of IκB, which would prevent its degradation, and nitration of p65, which was linked to inactivation of NF-κB (Lander et al., 1997; Connelly et al., 2001, 2003; Thomassen and Kavuru, 2001; Matata and Galinanes, 2002; Park et al., 2005). In several studies we could demonstrate NO•-mediated activation of NF-κB and of IKK within airway epithelial cells (Fig. 26.1). As shown, addition of low concentrations of NO• donors potentiates IKK activation by TNFα in C10 cells. Moreover, in HBE1 cells that were stably transfected with NOS2, inhibition of NOS activity slightly suppressed IKK activation by TNFα, whereas no such inhibition was found in untransfected HBE1 cells. Collectively, the effects of NO• on regulating NF-κB activation are diverse and may vary in different cell types. Many factors, including the localization and extent of NOS activation in relation to the activation of NF-κB signaling pathways, as well as variations in NO•

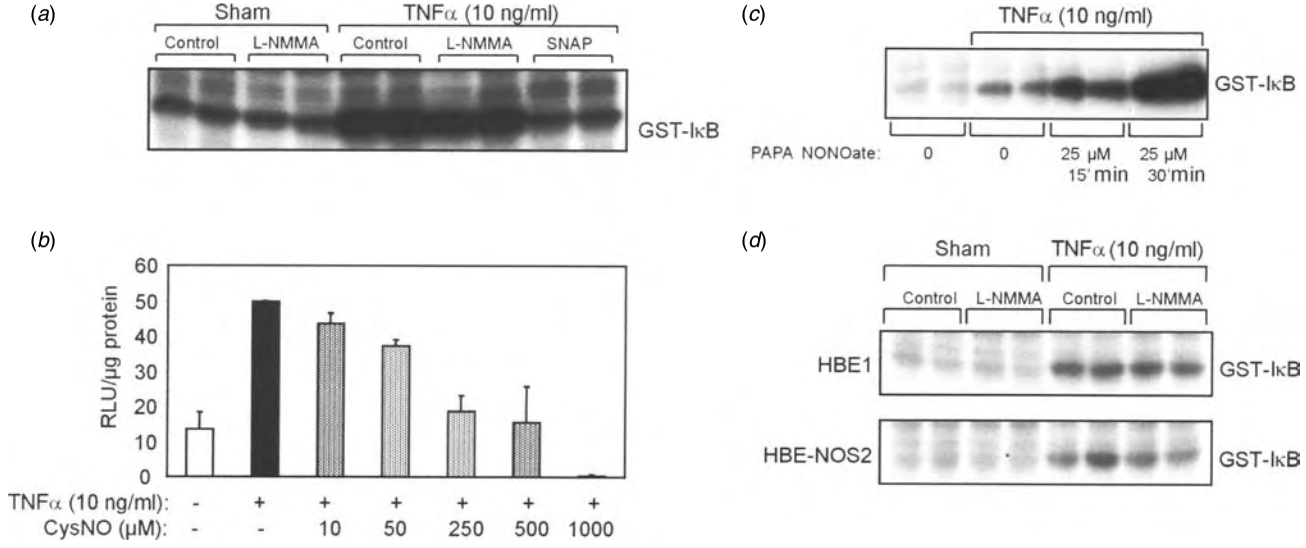


FIGURE 26.1 NO• donors can both inhibit and activate IKK and NF-κB-mediated gene expression in airway epithelial cells. Exposure of airway or alveolar epithelial cells to *S*-nitrosocysteine (CysNO) or *S*-nitroso-*N*-acetylpenicillamine (SNAP) results in decreased activation of IKK and NF-κB by TNFα in accordance with our previous results (Okamoto et al., 2002; Reynaert et al., 2004). Effects of endogenous NOS activity or exogenous putative NO• donor compounds (SNAP, 500 μM; CSNO; PAPA NONOate) were assessed in either HBE1 cells (*a, d*) or in C10 cells (*b, c*), in vitro by kinase assays of IKK activity using GST-IkB as a substrate (*a, c, d*), or by analysis of NF-κB-mediated gene expression in C10 cells transfected with a NF-κB luciferase reporter construct (*b*). While *S*-nitrosothiols are inhibitory, the NO• donor PAPA NONOate (which spontaneously decays by releasing NO• with a half-life of 30 min) as well as endogenous NOS2 activity in transfected HBE1 cells (HBE-NOS2) (Okamoto et al., 2002) appear to activate IKK.

metabolism that affect its overall bioactivity, will determine the ultimate effects of $NO^{\bullet}$ on this important signaling pathway. The notion that NF-κB is involved in various aspects of inflammation, in addition to mediating its initiation (Kupfner et al., 2001; Lawrence et al., 2001; Fujihara et al., 2002; Poynter et al., 2002), presents an additional complicating factor, since NOS activation and/or $NO^{\bullet}$ metabolism are highly variable during the inflammatory process. In order to more clearly establish mechanisms that control the dynamic regulation of NF-κB by $NO^{\bullet}$, quantitative approaches are needed to determine global protein targets, such as for *S*-nitrosylation or tyrosine nitration, and such approaches will be discussed in the next sections.

26.4 PROTEOMIC APPROACHES TO STUDY PROTEIN OXIDATION IN AIRWAY DISEASE

26.4.1 General Concepts and Current Applications

When compared to the diversity of the human genome, comprising an estimated number of about 30,000 protein-encoding genes, the number of different proteins, due to multiple splice variants and many post-translational modifications, is dramatically greater and is probably close to 1 million. This illustrates the enormous challenges that can be expected in analysis of overall protein profiles and changes therein in relation to cell activation and/or disease. Further complicating factors include the large diversity among cells (at least 250 different human cell types), the large range in protein concentrations (a single cell can contain between 1 and more than 100,000 copies of a single protein), diversities in physical properties of proteins (membrane-bound or cytosolic, differences in hydrophobicity and ionic charge), and oftentimes dramatic variations in protein levels or modifications over time or in response to extracellular factors. Thus, while the field of genomics research has substantially matured over the past decades, the proteomics era is still in an early stage, with many remaining technical challenges still to be resolved. Nevertheless, there have been substantial recent technical improvements and innovations that have improved the ability to separate, detect, and identify diverse proteins and their specific modifications from complex mixtures. Similarly, recent advances have been made to more global analyses of specific oxidative protein modifications, and these are expected to contribute importantly to our understanding the role of oxidative stress and protein oxidation in disease.

The main technical development that has revolutionalized proteomics research is the increased availability and improvement of mass spectrometry (MS) technology, which has enabled identification and sequencing of proteins and peptides. The separation of proteins from complex mixtures for identification largely relies on traditional approaches of one- or two-dimensional gel electrophoresis, and such approaches have been substantially refined to broaden their applicability and scope, allowing separation of highly ionic as well as hydrophobic proteins (Wildgruber et al., 2000; Herbert et al., 2001; Rabilloud, 2002; Lehner et al., 2003; Luche et al., 2004). A major technical advance has been the development

of two-dimensional HPLC procedures to separate complex mixtures after limited proteolytic digestion before online MS analysis, termed multidimensional protein identification technology (MudPIT) (Link et al., 1999; Whitelegge, 2002). Data analysis tools such as SEQUEST or SALSA (scoring algorithm for spectral analysis) are commonly used to identify proteins and peptides and their modifications (Link et al., 1999; Liebler, 2002), and novel strategies incorporating peptide accurate mass tags (AMTs) or isotope-coded affinity tags (ICATs) have been developed to allow more quantitative analysis of protein mixtures (Gygi et al., 1999; Smith et al., 2002). Another recently developed strategy that avoids the complexity and limited sensitivity of 2D electrophoresis-based approaches is surface-enhanced laser desorption/ionization (SELDI) combined with MS (Issaq et al., 2002; Kwapiszewska et al., 2004). This involves application of the sample on specifically designed ProteinChips with different surface areas to selectively collect either hydrophobic, cationic, or anionic proteins, or specific ligands (e.g., when specific antibodies or receptors are used). Compared to more classic 2D electrophoresis, SELDI offers the advantage of increased sample throughput, and allows detection of small or hydrophobic proteins (Wu et al., 2005). It is beyond the scope of this chapter to provide a detailed review of current MS technology in proteomics research, and many of these developments are addressed in other chapters of this volume. We therefore focus on current strategies to determine protein targets for specific oxidative modifications and their applicability to research of inflammatory lung diseases, such as asthma.

To date, the application of proteomics to lung disease has primarily focused on analysis of bronchoalveolar lavage (BAL) fluids, or of related specimens obtained by less invasive methods, such as blood plasma (to measure specific lung proteins) or exhaled breath condensates (for a comprehensive overview, the reader is referred to a recent review by Hirsch et al., 2004). Proteomics approaches have been used to detect global changes in overall protein profiles in BAL fluids from experimental animals, smoking subjects, or patients with lung disease (Wattiez et al., 2000, 2003; Ghafouri et al., 2002), or in lung tissues or alveolar macrophages from experimental animals with chronic airway inflammation (Roh et al., 2004; Wu et al., 2005). Moreover, some studies have aimed to identify modifications in specific BAL proteins in relation to lung disease (Bai et al., 2004). However, despite some recent developments to apply proteomic strategies to determine profiles of specific protein oxidation products such as protein carbonyls or protein nitrotyrosine (e.g., Aulak et al., 2001; Conrad et al., 2001; Yoo and Regnier, 2004), application of such methods to characterize oxidative stress in lung disease has so far been limited. Nevertheless, recent identifications of tyrosine nitrated proteins in cells subjected to oxidative stress (Aulak et al., 2001; Xiao et al., 2005) and of dynamic changes in protein nitration in subcellular compartments (Koeck et al., 2004) may set the stage for similar analyses in the future in relation to in vivo lung disease.

26.4.2 Redox Proteomics: Definitions and Approaches

The term "redox proteomics" broadly includes strategies to analyze protein changes in relation to oxidative stress or changes in cellular redox status. This could, on one hand, be defined as the identification of changes in overall protein expression profiles and/or post-translational protein modifications in response to oxidative stress. On the other hand, a more narrow definition of redox proteomics would be the study of that part of the proteome that is directly targeted by specific oxidative modifications. For our purposes here we will primarily adhere to the latter definition and limit our discussion to current strategies that are used to identify targets for oxidative modifications.

Several past studies have attempted to characterize protein targets for irreversible protein modifications (protein carbonyls, nitrotyrosine) using two-dimensional gel electophoresis (2DE). In such procedures, protein spots of interest are typically identified using immunological procedures, such as antibodies against nitrotyrosine, carbonyl hydrazide derivatization for labeling with biotin or dinitrophenyl (DNP) to enable their detection with streptavidin or α-DNP antibodies (Aulak et al., 2001; Conrad et al., 2001; Yoo and Regnier, 2004). Application of these approaches to lung disease have yielded some insights into the biochemical oxidative mechanisms. More recent substantial emphasis has been on developing procedures for global analysis of functionally more important reversible protein oxidations. Several investigators have applied such strategies with success to cell studies of oxidative stress, but their application to in vivo models of lung disease or to analysis of altered redox signaling in the airways of patients with chronic lung diseases is still problematic, and this is hindered by technical challenges related to detection sensitivity and selectivity. In this section we briefly review the current approaches to detect cysteine oxidation and their application to proteomics research. Because some of these approaches have recently been more extensively reviewed (Ghezzi and Bonetto, 2003; Dalle-Donne et al., 2005) and are covered more fully in other chapters of this volume, we will center our discussion primarily on advances from our laboratories in applying and improving such methodology for analysis of redox-dependent events in the airway epithelium. Our discussion will also describe our recently developed strategies to detect *S*-nitrosylated proteins in airway epithelial cells, and their application to in vivo studies.

Direct and Indirect Approaches to Detect Cysteine Oxidation and Thiolation

The major challenge in both qualitative and quantitative analysis of various cysteine oxidation products in cells or tissues, in addition to their reversibility and potential instability during tissue homogenization, for example, is the general difficulty in directly detecting these products and the lack of sensitivity or specificity of such direct technological approaches. Direct cysteine oxidation or thiolation in purified proteins can be easily characterized by MS approaches. For example, we have recently characterized the Cys residue within creatine kinase that is susceptible to *S*-glutathionylation (Reddy et al., 2000). However, direct analysis is more difficult in proteins in more complex mixtures, and

most approaches therefore rely on selective labeling of protein thiols with fluorescent, biotinylated, or radiolabeled probes in order to increase detection sensitivity. Analysis of differences in thiol labeling is then used to detect modification/oxidation of specific cysteine residues during oxidizing conditions. A large number of diverse thiol labeling agents have been developed for such purposes, some of which are summarized in a recent review (Ghezzi and Bonetto, 2003). Examples of such approaches to label reactive thiols include (4-iodobutyl)triphenylphosphonium (IBTP), an agent that accumulates in mitochondria and thus labels mitochondrial protein thiols with relative selectivity (Taylor et al., 2003), and 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM), which has been used recently to identify hyperreactive cysteines in ryanodine receptors (Voss et al., 2004). Changes in the extent of thiol labeling of specific proteins during conditions of oxidative stress will reflect oxidation/modification of cysteine residues within these proteins. The use of isotope-coded affinity tags (ICATs) has recently been applied to more quantitatively identify protein cysteine oxidation, by using light (^{12}C) ICAT reagents on control samples and heavy (^{13}C) ICAT on oxidized samples (Sethuraman et al., 2004a,b).

Thiol Labeling after Selective Reduction of Reversible Cysteine Modifications

A downside of direct thiol labeling is that the labeling technologies also label thiol-containing proteins that may not be subject to redox regulation. Moreover, redox-sensitive proteins may be present in relatively low abundance, so, coupled with the expected partial modifications of their critical cysteines, such general thiol labeling strategies become unsuitable for detection of minor changes. These strategies also do not address the type of cysteine modification that has occurred. For these reasons differential labeling approaches have been developed in which unreacted cysteines are derivatized first by thiolation or alkylation, and after that the reversibly oxidized cysteines are subjected to various selective reducing agents in an attempt to selectively reduce specific oxidative states of cysteine residues. The reduced cysteines are then labeled in a second derivatization step with various detectable thiol reagents. For example, labeling of oxidized cysteines has been performed using 5-iododacetamidofluorescein (IAF), following initial alkylation of reduced cysteines with *N*-ethyl-maleimide (NEM) and reduction of oxidized cysteines with DTT (Baty et al., 2002). A variant of this approach involves initial carbamidomethylation of reduced cysteines with the thiol-modifying agent iodoacetamide (IAM), followed by reduction of oxidized cysteines (disulfides, sulfenic acids, etc.) with DTT and their subsequent labeling with [^{14}C]IAM (Leichert and Jakob, 2004). Separation of proteins by 2DE and fluorescence scanning or autoradiography then allows detection of proteins in which cysteines are subject to oxidation during oxidative stress. An added advantage of cysteine labeling strategies with biotin, for example, is that they can also be used to selectively capture cysteinyl peptides by way of streptavidin matrices and thus significantly simplify the peptide mixture before MS analysis (Spahr et al., 2000). As an alternative, quaternary amine tagging of cysteine

residues has been proposed as a tool to selectively purify cysteinyl peptides from more complex mixtures (Ren et al., 2004).

The big challenge in these approaches is to distinguish among the various reversible cysteine modifications (sulfenic acids, disulfides between vicinal thiols, mixed disulfides, or *S*-nitrosothiols), and several selective reduction strategies have been used for this purpose. Although sulfenic acids are generally unstable in proteins and often undergo thiolation or overoxidation, an approach to selectively detect sulfenic acids in target proteins has involved alkylation of reduced cysteines with maleimide followed by selective reduction of sulfenic acids using arsenite (Poole et al., 2004). Labeling of the reduced cysteines with biotin-maleimide then allows the selective detection of sulfenic acid-containing proteins, and this can be verified by a sample pre-treatment with dimedone to eliminate the sulfenic acids (Saurin et al., 2004). By this procedure various proteins in cardiac tissues have been found to be oxidized to sulfenic acids in response to exposure to H_2O_2, including cytosolic and mitochondrial metabolic enzymes, as well as cytoskeletal and myofilament proteins (Saurin et al., 2004). Analogous strategies have been successful in detecting *S*-nitrosylated proteins, in which ascorbate is used as a selectively reducing agent (Jaffrey et al., 2001a). We have adapted and refined strategies to detect *S*-nitrosylated proteins in our recent studies, as will be discussed in the next paragraphs.

Approaches to Distinguish among Various Reversible Cysteine Modifications
In addition to oxidation or nitrosylation, cysteine modification can also involve mixed disulfide formation, such as with GSH to form *S*-glutathionylated proteins. Although such adducts can be detected directly in purified proteins or peptides by MS strategies (Reddy et al., 2000), more indirect approaches have been developed to characterize targets for protein *S*-glutathionylation such as in cultured cells. One such approach involves labeling of cell GSH pools with ^{35}S, by brief preincubation with [^{35}S]cysteine, prior to cell exposure to oxidants, after which the formation of *S*-glutathionylated proteins can be identified by protein separation under nonreducing conditions (e.g., 2DE) and autoradiography (Fratelli et al., 2002, 2003). An alternative indirect approach to identify targets for protein glutathionylation involves the use of *S*-nitrosoglutathione-Sepharose beads, which can be used to selectivily capture redox sensitive proteins that can undergo *S*-thiolation (Klatt et al., 2000). Using such approaches, several metabolic enzymes and transcription factors (e.g., NF-κB p50) have been identified as potential targets for *S*-glutathionylation. Another approach to detect *S*-glutathionylated proteins relies on thiol derivatization following specific reduction of mixed disulfides with Grx (Lind et al., 2002). In addition to *S*-glutathionylation, protein cysteines can also undergo intermolecular disulfide formation with other proteins, and a diagonal electrophoresis approach was recently developed to identify proteins that undergo such interprotein interactions. Briefly, samples are first separated on linear gels by non-reducing electrophoresis, after which each lane with resolved proteins is laid horizontally on a second gel, and subjected to reducing electrophoresis. While most proteins will appear on a diagonal band, proteins that were engaged in intermolecular disulfide formation

will migrate faster on the secondary gel, and can then be identified by MS approaches (Brennan et al., 2004).

26.4.3 Analysis of Protein *S*-Nitrosylation: Direct and Indirect Approaches

Direct Analysis by ESI-MS Because of the unique involvement of *S*-nitrosylation as a post-translational modification associated with NOS activation (Hess et al., 2005), there has been considerable interest in methodology to specifically detect such modification. The chemical stability of *S*-nitrosothiol bonds varies dramatically, depending on the local environment of the cysteine residue, but *S*-nitrosylated proteins are sometimes sufficiently stable for direct analysis and localization of *S*-nitrosothiol bond within proteins by MS. The first direct demonstration by MS of a signaling protein that is *S*-nitrosylated on a single cysteine residue was perhaps p21ras. Electrospray ionization–mass spectrometry (ESI-MS) analysis of $NO^{\bullet}$ exposed p21ras showed the characteristic mass increase of 30, consistent with addition of one $NO^{\bullet}$ molecule (Mirza et al., 1995). Subsequent studies demonstrated the specificity of *S*-nitrosylation on one specific Cys residue (Cys^{118}), and as well as its stimulatory effects on guanine nucleotide exchange (Williams et al., 2003). Other proteins for which *S*-nitrosylation of a critical cysteine was demonstrated by direct MS include caspase-3 and hemoglobin (Ferranti et al., 1997; Zech et al., 1999). Following our observations of regulation of IKK activation by $NO^{\bullet}$ at the level of a redox-sensitive Cys^{179} residue within IKKβ (Reynaert et al., 2004), we attempted to use EI-MS to directly demonstrate *S*-nitrosylation of the corresponding Cys residue in a synthetic oligomeric peptide that encompasses the active site sequence of IKKβ, because the full-length protein is difficult to obtain in sufficient quantities and contains many variable post-translational modifications. As shown in Figure 26.2, the molecular mass of the peptide increases by 30 (indicative of addition of $NO^{\bullet}$) after exposure to GSNO or SNAP, and this was reversed by treatment with DTT (not shown), consistent with *S*-nitrosylation of this cysteine residue.

Chemiluminescence Analysis of $NO^{\bullet}$ after Chemical Reduction of S-Nitrosothiols Direct MS strategies to detect *S*-nitrosylation have significant limitations. MS is not easily applicable to large proteins that are highly heterogeneous (e.g., due to other posttranslational modifications), and the chemical stability of *S*-nitrosothiols is compromised by the harsh conditions used to partially digest proteins for peptide analysis. So the more usual approach to detect *S*-nitrosothiols in proteins has been to immunopurify the protein of interest and then analyze the $NO^{\bullet}$ content after chemical reduction by way of ozone-enhanced chemiluminescence analysis. Although this detection procedure is specific for $NO^{\bullet}$, whether $NO^{\bullet}$ originates from *S*-nitrosothiols depends on the reduction chemistry that is used, which often can also reduce nitrite (NO_2^-) or other nitrosoadducts to proteins; then secondary approaches are needed to assure specificity (Fang et al., 1998; Bryan et al., 2004; Reynaert et al., 2004). Further, this procedure is not easily applicable to global proteomic screening of *S*-nitrosylation in protein mixtures.

For example, chemiluminescence-based analysis of *S*-nitrosylation has involved caspases that are implicated in NO•-mediated regulation of cellular apoptosis. Specifically, mitochondrial forms of caspases are found to be *S*-nitrosylated, and Fas-induced apoptosis is associated with denitrosylation (Mannick et al., 1999, 2001). Importantly, chemiluminescence detection of NO• allows quantitative assessment of the extent of *S*-nitrosylation, and consequently mitochondrial forms of caspases were found to contain near stoichiometric amounts of *S*-nitrosothiols, which is consistent with a lack of activity (Mannick et al., 2001). Utilizing a similar approach to determine whether the IKKβ subunit is *S*-nitrosylated under conditions in which exogenous NO•-donors inhibit IKK activity, we have recently shown that IKKβ that was immunoprecipitated from CSNO-treated Jurkat T cells have increased *S*-nitrosothiol content compared to IKKβ obtained from untreated cells. The specific presence of *S*-nitrosothiol was confirmed by the disappearance of the NO• signal after sample treatment with $HgCl_2$, which selectively decomposes *S*-nitrosothiols (Reynaert et al., 2004).

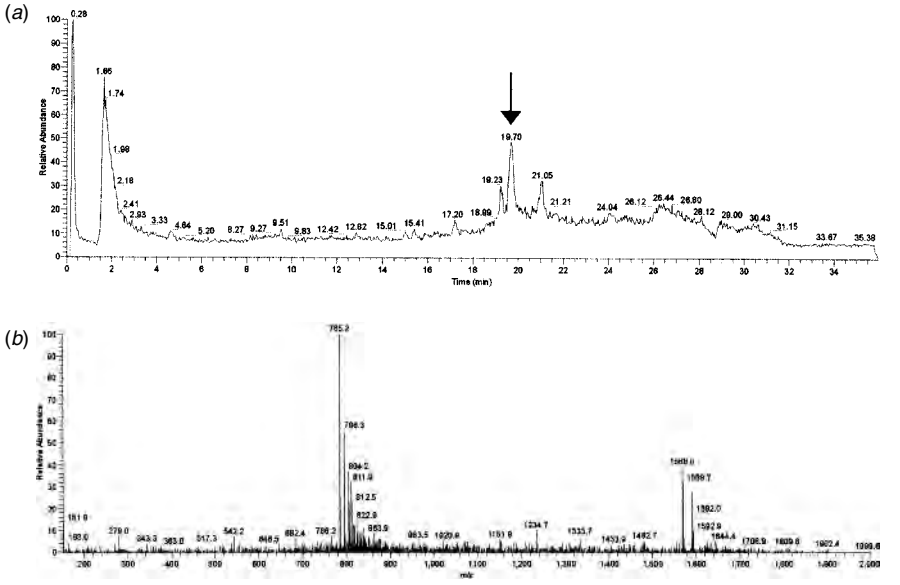


FIGURE 26.2 Demonstration of *S*-nitrosylation of a synthetic 15-residue peptide of the primary 173–187 residue sequence (including Cys^{179}) of mouse IKKβ by ESI-LCMS. Peptide solutions (0.5 mg/ml) were reacted with CysNO or GSNO for 30 minutes and analyzed by LCMS (Thermo-Finnigan DECA-XPplus LCQ). (*a*) Total ion (mass range of *m*/*z* 150–2000) chromatogram (TIC) of unreacted peptide solution, showing a major peak eluting at 19.7 minutes (*arrow*) with major molecular ions of 1568.6 and 785.2 (*b*), representing the expected *m*/*z* of the single double-ionized peptide. (*c*) Similar TIC of peptide following reaction with CysNO, and (*d*) MS of the major product eluting at 21.65 minutes (*arrow*), with *m*/*z* 1598.4 and 799.6, indicating addition of one molecule of NO• (+30). Reversal of this NO• addition with dithiothreitol indicates its addition to a cysteine residue (not shown).

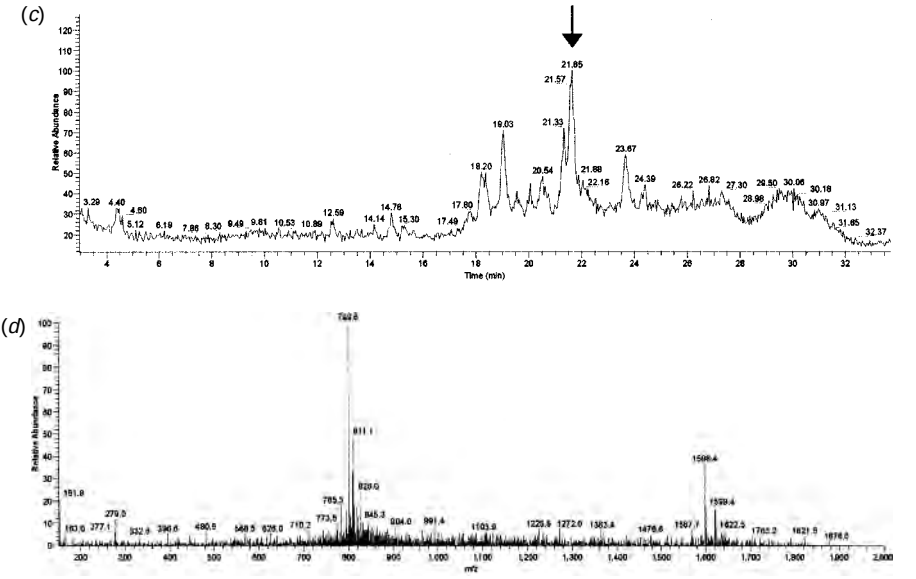


FIGURE 26.2 (*continued*)

Thiol Labeling Strategies after Selective Reduction of S-Nitrosothiols To overcome the limitations of direct approaches, in particular, their lack sensitivity and their uselessness in more global proteomic analyses of *S*-nitrosothiols, strategies have recently been advanced that are based on thiol labeling after selective *S*-nitrosothiol reduction. Jaffrey and co-workers first reported a derivation procedure that involves an initial thiolation of reduced protein cysteines using methyl methanethiosulfonate (MMTS), followed by selective reduction of *S*-nitrosothiols with ascorbate, after which they are labeled by thiol-specific biotinylating agents such as *N*-[6-(biotinamido)hexyl]-3′-(2′pyridyldithio) propionamide (biotin-HPDP) (Jaffrey et al., 2001a). This procedure to detect *S*-nitrosylated proteins is now commonly referred to as "biotin switch." Subsequently other approaches have used alternative thiol blocking agents (e.g., *N*-ethyl-maleimide) and alkylating biotinylating agents such as *N*-(3-malemidylpropionyl)biocytin (MPB) or radiolabeled reagents such as [^{35}S]-2-amino-3-(2-pyridyldithio)-proprionate (APDP). In these approaches Western blotting with streptavidin, affinity-purification using avidin-sepharose, or autoradiography is then used to detect and identify the *S*-nitrosylated proteins. Western blotting has led to the identification of different cell targets that are *S*-nitrosylated in relation to NOS activity (Jaffrey et al., 2001a, 2002). More recently some similar derivatization procedures were applied to more global proteomic analyses of *S*-nitrosylation targets using 2DE (Foster and Stamler, 2004; Kuncewicz et al., 2004; Martinez-Ruiz and Lamas, 2004; Rhee et al., 2005).

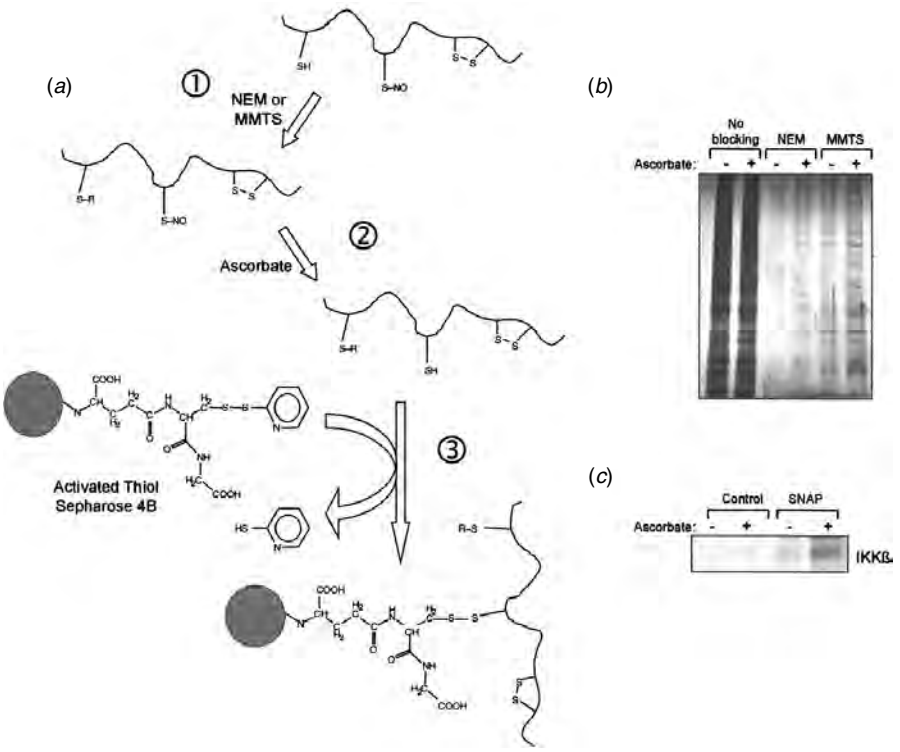


FIGURE 26.3 Application of Activated Thiol Sepharose to collect *S*-nitrosylated proteins in cell lysates. (*a*) Schematic of derivatization procedure to collect *S*-nitrosylated proteins. Step 1: Alkylation or thiolation of reduced cysteines. Step 2: Selective reduction of *S*-nitrosothiols with ascorbate. Step 3: Binding of reduced cysteines to Activated Thiol Sepharose beads for purification. NEM: *N*-ethyl-maleimide; MMTS: methyl methanethiosulfonate. (*b*) Effect of blocking of unreacted cysteines and ascorbate reduction on recovery of proteins with Activated Thiol Sepharose, analyzed by SDS-PAGE and silver stain. (*c*) Demonstration of IKKβ as a target for *S*-nitrosylation using Activated Thiol Sepharose and Western blotting with an antibody against IKKβ (Santa Cruz).

In our attempt to selectively capture *S*-nitrosylated proteins from complex mixtures for proteomic analysis, we used a strategy based on selective *S*-nitrosothiol reduction and capture using Activated Thiol Sepharose® 4B (a mixed disulfide between 2,2′-dipyridyl disulfide and GSH coupled to CNBr-activated Sepharose 4B), from which the thiols can be eluted by reducing agents. An advantage of this procedure is that it eliminates the need for biotin-streptavidin systems to collect proteins, and therefore may improve specificity. Figure 26.3 illustrates the chemical principle of Activated Thiol Sepharose reduction to collect *S*-nitrosylated proteins, and its application to lysates from $NO^{\bullet}$-exposed cells. We have applied several of derivatization strategies to establish whether IKKβ is a direct target for *S*-nitrosylation. Using anti-biotin antibodies or streptavidin-conjugated detection

systems, we could confirm that IKKβ is *S*-nitrosylated in Jurkat cells in response to CSNO and that as a consequence endogenous NOS activity occurs (Reynaert et al., 2004). Similarly, we could recover large amounts of IKKβ from the lysates of the SNAP-treated HBE1 cells, after selective *S*-nitrosothiol reduction and collection with Activated Thiol Sepharose (Fig. 26.3), which indicates increased *S*-nitrosylation of IKKβ.

26.4.4 Visualization of Cysteine Oxidation and Nitrosylation in Intact Cells and Tissues

Besides the development in identification of specific protein targets, several of the above-summarized strategies have been used to elucidate cysteine oxidations in intact cells and tissues. Some researchers took the opportunity to investigate these redox events in association with co-localized NADPH oxidases or NOS isozymes. For example, protein *S*-glutathionylation was observed in cells undergoing oxidant stress using anti–GSH antibodies, and this indicates that *S*-glutathionylation is localized primarily to membrane blebs, nuclear areas, and areas of punctate staining in the perinuclear region (Soderdahl et al., 2003).

Until very recently the only way to study *S*-nitrosylated proteins in intact cells or tissues was to utilize immunohistochemistry using an antibody against *S*-nitrosocysteine (Gow et al., 2002; Atochina et al., 2004). Because of concerns regarding the specificity of such antibodies, we and others sought an alternative approach. We adapted the biotin switch procedure for detection of *S*-nitrosylated cysteines in intact cells, by applying streptavidin-fluorescein isothiocyanate (FITC) to detect biotinylated proteins using confocal microscopy. We were able to demonstrate basal levels of *S*-nitrosylated protein in C10 cells, which increase following NOS activation or exposure to nitrosothiols, and can be suppressed by NOS inhibition (Ckless et al., 2004). Surprisingly, in our studies we found that *S*-nitrosylation is largely associated with nuclear compartments, and this was confirmed by detection of *S*-nitrosylation of specific nuclear proteins and increased nuclear localization of *S*-nitrosylated proteins following inhibition of nuclear export (Ckless et al., 2004). Using a comparable labeling procedure with Alexa568-conjugated maleimide, we further observed *S*-nitrosothiol staining in HBE1 cells that were stimulated with interferon (IFN)-γ to induce NOS2, and this was prevented by NOS inhibition (Fig. 26.4). In a similar, recently published approach, *S*-nitrosylation was found in endothelial cells exposed to exogenous $NO^{\bullet}$ donors, by selective labeling of *S*-nitrosocysteines with a thiol-alkylation reagent conjugated to Texas Red (Yang and Loscalzo, 2005). In this study *S*-nitrosylation was largely localized to mitochondria, but we should note that methanol was used for cell fixation rather than paraformaldehyde (PFA), which may be less suitable for determination of subcellular localization and could have preclude detection of nuclear proteins. Conversely, cell fixation with PFA could have resulted in some cross-linking of small soluble *S*-nitrosothiols to proteins, thereby potentially overestimating the protein *S*-nitrosylation. Collectively, these studies illustrate that *S*-nitrosylated proteins can be localized within cells,

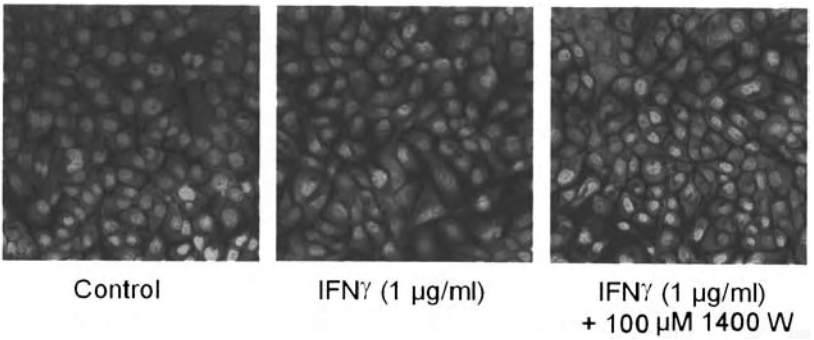


FIGURE 26.4 Visualization of *S*-nitrosylated proteins in airway epithelial cells following induction of NOS2. Confluent monolayers of HBE1 cells were stimulated with IFNγ in the absence or presence of the NOS2 inhibitor 1,400 W, and after 24 hours cells were fixed and analyzed for *S*-nitrosothiol content essentially as described earlier (Ckless et al., 2004), except that maleimide-Alexa568 was used to label cysteines for visualization.

although the influence of different labeling or cell fixation strategies still needs to be worked out.

In addition to our studies of cultured cells, we were able to adapt the in situ technique to detect *S*-nitrosylated proteins in intact lung tissues. Analysis of lung tissues from mice that were exposed to GSNO by intratracheal instillation (50 μl of a 5 mM stock solution) revealed increased biotinylation that was localized primarily to airway epithelial cells (Ckless et al., 2004). The initial findings thus showed that modified biotin switch labeling procedures can be used successfully to detect *S*-nitrosothiols in intact lung tissues. These findings will be useful for our future studies on temporal and local changes in *S*-nitrosylation during airway inflammation.

26.5 TISSUE PROTEOMICS AND APPLICATION TO STUDY PROTEIN OXIDATION

26.5.1 Approaches to Tissue Proteomics

The application of proteomics approaches to detect overall changes in protein profiles or specific protein oxidation patterns in relation to lung disease has primarily involved the analysis of homogenized lung tissues from experimental animals or of BAL fluids or serum from patients (Hirsch et al., 2004). The main advantage is that the procedure is relatively noninvasive. Analysis of BAL fluids is also useful because these fluids reflect local phenomena within the airway lumen, including released factors from airway epithelial and inflammatory cells, as well as proteins that originate from circulation and may have diffused into the extracellular airway compartment (Wattiez et al., 2000). Nevertheless, analysis of extracellular proteins is limited as it obviously ignores the potential changes of cellular proteins that regulate intracellular signaling mechanisms. As noted in

earlier sections, the cells of the airway epithelium are critical in orchestrating inflammatory responses to inhaled pathogens or allergens, and they are major sites where both NOS and NADPH oxidase homologues are expressed. Likely it is these cells that are primarily targeted by oxidative modifications that regulate redox-dependent cell signaling events. While such redox-dependent signaling mechanisms can be relatively conveniently explored in cultured epithelial cells, it is ultimately critical to establish the significance of such events in the context of airway inflammation in vivo.

At the present time, there are relatively few published studies that aim to identify specific protein oxidative modifications in the context of inflammatory lung diseases using global proteomic strategies, and such attempts have primarily sought to analyze whole lung homogenates. The obvious limitation is that lung samples can contain proteins from about 40 different known cell types within the lung, not taking into account the highly variable contribution of infiltrated inflammatory cells depending on the degree of inflammation. Hence, it is highly unsuitable to explore direct relationships between protein oxidation and signaling pathways that are cell specific and are largely confined to the epithelial compartment. One approach to analyze protein profiles in relation to tissue localization is by using recently developed procedures for direct tissue profiling and imaging mass spectrometry (Chaurand et al., 2004). For these purposes, tissue sections are directly placed on an appropriate surface and coated with MALDI matrix, after which the tissue can be scanned by MALDI for imaging of proteins with selected masses, or profiling of protein patterns within selected tissue areas (Chaurand et al., 2004). However, despite the enormous potential of this approach there is a significant limitation in the application to redox proteomics, which requires higher specificity and sensitivity.

26.5.2 Approaches to Collect Airway Epithelial Proteins for Proteomic Analysis

As mentioned in the previous sections, the methodology used to selectively derivatize specific cysteine modifications and apply fluorescence imaging allows characterization of spatial location of these events within tissues and cells, but does not allow identification of protein targets. Nevertheless, attempts to localize cysteine oxidation events are helpful in identifying the primarily cell type(s) within tissues that is subject to such modifications, which will guide alternative approaches to collect selected populations of cells from tissue sections for proteomic analysis. Indeed our recent studies have illustrated that protein *S*-nitrosylation after intratracheal instillation of GSNO occurs primarily within the airway epithelium. Hence, the optimal approaches to collect airway epithelial cells from lung tissues would be instrumental in establishing the importance of *S*-nitrosylation of epithelial proteins in airway inflammation. Two strategies that allow relatively selective collection of airway epithelial cell material from lung tissues are discussed in the next paragraphs.

Laser Capture Microdissection (LCM) The first established procedure to collect selected cell types from tissue sections is laser capture microdissection (LCM), which was introduced by Liotta and co-workers (Emmert-Buck et al., 1996). LCM allows for direct selection of cell types without the need for enzymatic processing or growth in culture. In this approach tissue sections are visualized under an inverted microscope, and a cap coated with thermolabile film is placed in contact with the tissue section. Laser beams of 7.5 to 30 μm are used to locally melt this film on selected areas, and material that is fused to the cap can then be specifically removed. Several thousand "shots" can be captured on a single cap, thereby allowing one to concentrate on desired cell types. This way some 50,000 to 100,000 cells of a specific phenotype can be collected within a reasonable time span. For the past several years LCM has been used successfully for cell-specific analysis of gene expression, which is facilitated by the ability to amplify genetic material using PCR. While such amplification is not applicable to proteins, LCM has also been successfully used in proteomic analysis, the main application being the analysis of cancer tissues in comparison to normal tissue (Wulfkuhle et al., 2001; Craven et al., 2002; Jain, 2002; Li et al., 2004; Roberts et al., 2004). In one recent study LCM was applied to an analysis of specific changes in the airway epithelium in response to exposure to particulate air pollution (Roberts et al., 2004). In this case the captured proteins were analyzed by reverse phase protein microarrays (Paweletz et al., 2001) to demonstrate the activation of NF-κB and MAPK pathways. While LCM can be successfully combined with proteomic studies, and is applicable to banked tissue sections from animal studies or human tissue specimens, its suitability for redox proteomics may be more limited, as it concerns reversible modifications in only a subset of proteins. Thus, the ability to obtain sufficient material for analysis may be insufficient, given the estimated yield of cellular protein that can be obtained by LCM (among the maximally collected 50,000–100,000 cells only ng quantities of total protein are present). For example, detection of specific *S*-nitrosylated proteins using biotin switch strategies has estimated to require mg quantities of total protein in each sample (Jaffrey et al., 2001b), suggesting that tissue collection by LCM for such studies is impractical. A second concern with LCM is that the heat and radiation of the laser may destroy labile protein modifications and alter the oxidation status of reactive cysteines, which would make LCM less applicable to redox proteomics.

Lysis Lavage To avoid some of the limitations of LCM, an alternative procedure to collect airway epithelial proteins was recently developed by Wheelock and co-workers. These investigators developed and refined a strategy using lung lavage with a detergent solution to rapidly collect airway epithelial proteins with relative selectivity (Wheelock et al., 2004). In this approach the lungs are initially instilled with a 0.75% agarose solution at a volume that reflects 50% of the total lung capacity, followed by an equal volume of 5% dextrose solution. This procedure causes the agarose to solidify within the parenchymal regions of the lungs, and allows selective access of subsequently instilled lysis buffers to the proximal airway regions. The composition of the lysis buffer can be customized to

optimally solubilize proteins (detergents and urea) and preserve the redox status of proteins (e.g., using thiol-alkylating agents). Analysis of the marker proteins specific for airway epithelial cells or smooth muscle cells, and analysis of lung tissues by histopathology and scanning electron microscopy, have demonstrated that this procedure allows relatively selective collection of airway epithelial proteins without causing major destruction of the underlying lung structures (Wheelock et al., 2004). Furthermore, mg protein quantities can be obtained from one mouse lung, which is sufficient for identification of less abundant proteins or of quantitatively less abundant oxidative protein modifications. However, while lysis lavage may be suitable for redox proteomic studies in airway epithelial cells in studies with mouse models of lung disease, such a procedure is obviously not applicable to human studies. Moreover, although lysis lavage may allow relatively selective collection of airway epithelial proteins, proteins from other cell types are recovered as well, including some smooth muscle cells and inflammatory cells. Thus, while epithelial cell proteins may be recovered with relative purity from healthy mice, this may not be the case in conditions of active lung inflammation, even though extravasated inflammatory cells can be removed by pre-lavage.

We applied the lysis lavage procedure to our analysis of *S*-nitrosylation of epithelial proteins from mice, following the procedure of Wheelock et al. First, we confirmed that lavage with 400 μl lysis buffer (composition: 2 M thiourea, 7 M urea, 4% w/v CHAPS, 0.4% w/v Triton X-100, and 2% protease inhibitor cocktail III [Calbiochem]) allows retrieval of substantially more protein (6.6 ± 2.3 mg/ml; 210 ± 35 μl total recovered volume; $n = 8$) than a corresponding control lavage with 5% dextrose (0.2 ± 0.1 mg/ml protein; $n = 4$). Approximately 1.5 mg of airway epithelial protein could be recovered from each mouse lung by this procedure. The relative purity of lysis lavage proteins was verified by Western blot analysis of cytochrome P450 2F2 (CYP2F2) and Clara cell 26kD protein (CC26), two specific marker proteins for airway epithelial cells (Stripp et al., 2002; Wheelock et al., 2004), and of the smooth muscle marker α-actin. As shown in Figure 26.5, CYP2F2 and CC26 were abundant in lysis lavage samples, which were relatively devoid of α-actin, thus illustrating the presence of primarily epithelial cell proteins. Western blot analysis of IKKβ indicated that we also recovered large amounts of this cellular protein. We also confirmed that this lysis lavage procedure can be applied to the analysis of *S*-nitrosothiols in epithelial proteins by using selective reduction with ascorbate and collection of thiol-containing proteins with Activated Thiol Sepharose. As illustrated in Figure 26.5, several *S*-nitrosylated proteins were detected in the lysis lavage returns from normal C57Bl6 mice, and the abundance of *S*-nitrosylated proteins was increased when mice were first subjected to intranasal instillation of GSNO (50 μl; 10 mM GSNO in PBS) one hour prior to lysis lavage. Our future studies are being aimed at identifying these proteins in order to gain better insight into *S*-nitrosylation targets within the airway epithelium, and learn whether changes in *S*-nitrosylation of specific proteins can be linked to lung inflammation.

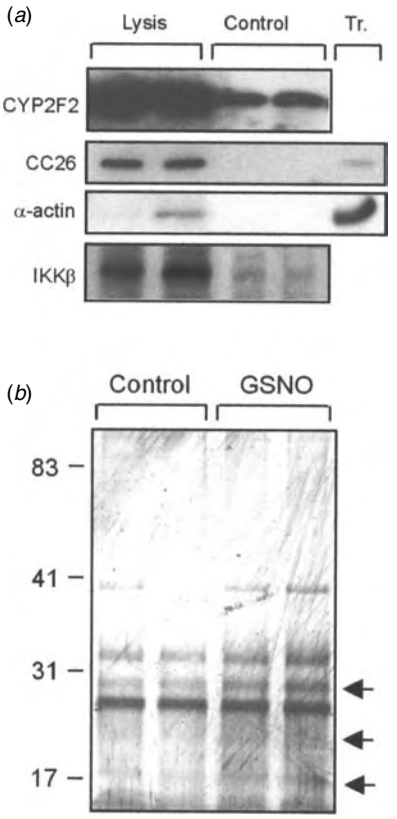


FIGURE 26.5 Collection of airway epithelial proteins from mice by lysis lavage. C57Bl6 mice were subjected to lysis lavage as described in detail by Wheelock et al. (2004), and lysis lavage samples were analyzed for various marker proteins, in comparison to control lavages with dextrose, or analysis of tracheal homogenates (Tr.). (*a*) Lysis lavage samples contained substantially increased amounts of the epithelial marker proteins CYP2F2 or CC26, as analyzed by SDS-PAGE and Western blotting (Stripp et al., 2002; Wheelock et al., 2004), while the smooth muscle marker α-actin was relatively absent. (*b*) Analysis of *S*-nitrosylated proteins in lysis lavage samples from untreated C57Bl6 mice (control) or mice that were instilled intratracheally with GSNO (10 mM, 50 μl). *S*-Nitrosylated proteins were analyzed using Activated Thiol Sepharose and silver staining, as in Figure 26.3.

26.6 SUMMARY AND CONCLUSIONS

Although it is by now well accepted that acute and chronic inflammatory conditions, including those of the respiratory tract, are associated with increased oxidative stress, the question to what extent and how oxidative stress contributes to disease pathology still remains largely unresolved. Despite multiple lines of evidence of oxidative protein modifications in association with other markers of

inflammation, the exact contribution of protein oxidation to functional changes is unclear, and the main targets for these oxidative modifications are mostly unknown. The recent development of proteomic strategies to elucidate protein oxidation targets is expected to contribute importantly to resolving these issues, and proteomic approaches are already beginning to be applied to animal models of lung disease, for example, and to specimens from human subjects. A more important recent development may even be the increased understanding gained of oxidants in cell signaling pathways, which largely involve reversible modifications of reactive protein cysteine residues. Hence proteomic strategies to detect protein targets for reversible oxidative modifications will continue to be developed and refined, allowing their application to in vivo studies of inflammation. Improved procedures to obtain sufficient quantities of selected cell populations for such analysis will be essential in these endeavors. Although much of this technology is in its infancy, we are at an exciting stage in this research field, and we can look forward to many developments and new applications as well as innovative analytical strategies to further our understanding of inflammatory lung diseases and of disease mechanisms in general.

ACKNOWLEDGMENTS

The authors thank NIH for research support, and Drs. John Heim and Dwight Matthews for their assistance with the MS analysis.

LIST OF ABBREVIATIONS

AMT, accurate mass tags
APDP, 2-amino-3-(2-pyridyldithio)-proprionate
BAL, bronchoalveolar lavage
Biotin-HPDP, *N*-[6-(biotinamido)hexyl]-3′-(2′pyridyldithio) propionamide
CC26, Clara cell 26kD protein
CPM, 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin
CSNO, *S*-nitrosocysteine
CYP2F2, cytochrome P450 2F2
2DE, two-dimensional electrophoresis
DNP, dinitrophenyl
DTT, dithiothreitol
ESI, electrospray ionization
EPO, eosinophil peroxidase
FITC, fluorescein isothiolcyanate
GPx, GSH peroxidase
Grx, glutaredoxin
GSNO, *S*-nitrosoglutathione

HPLC, high-performance liquid chromatography
IAF, 5-iododacetamidofluorescein
IAM, iodoacetamide
IBTP, (4-iodobutyl)triphenylphosphonium
ICAT, isotope-coded affinity tags
IFN, interferon
I-κB, inhibitor of nuclear factor kappaB
IKK, I-kappaB kinase
JNK, c-Jun-*N*-terminal kinase
LCM, laser capture microdissection
MALDI, matrix-assisted laser desorption/ionization
MAPK, mitogen-activated protein kinase
MMP, matrix metalloproteinase
MMTS, methyl methanethiosulfonate
MPB, *N*-(3-malemidylpropionyl)biocytin
MPO, myeloperoxidase
MS, mass spectrometry
NEM, *N*-ethylmaleimide
NF-κB, nuclear factor kappaB
NOS, nitric oxide synthase
PFA, paraformaldehyde
Prx, peroxiredoxin
PTP, protein tyrosine phosphatase
RNS, reactive nitrogen species
ROS, reactive oxygen species
SELDI, surface-enhanced laser desorption/ionization
SNAP, *S*-nitroso-*N*-acetylpennicilamine
TNF, tumor necrosis factor
TOF, time of flight
Trx, thioredoxin

REFERENCES

Ahmed N., Thornalley P.J. (2003). Quantitative screening of protein biomarkers of early glycation, advanced glycation, oxidation and nitrosation in cellular and extracellular proteins by tandem mass spectrometry multiple reaction monitoring. *Biochem Soc Trans* 31: 1417–1422.

Akaike T., Noguchi Y., Ijiri S., Setoguchi K., Suga M., Zheng Y.M., Dietzschold B., Maeda H. (1996), Pathogenesis of influenza virus-induced pneumonia: Involvement of both nitric oxide and oxygen radicals. *Proc Natl Acad Sci USA* 93: 2448–2453.

Andreadis A.A., Hazen S.L., Comhair S.A., Erzurum S.C. (2003). Oxidative and nitrosative events in asthma. *Free Radic Biol Med* 35: 213–225.

Antczak A., Nowak D., Shariati B., Krol M., Piasecka G., Kurmanowska Z. (1997). Increased hydrogen peroxide and thiobarbituric acid-reactive products in expired breath condensate of asthmatic patients. *Eur Respir J* 10: 1235–1241.

Atochina E.N., Beers M.F., Hawgood S., Poulain F., Davis C., Fusaro T., Gow A.J. (2004). Surfactant protein-D, a mediator of innate lung immunity, alters the products of nitric oxide metabolism. *Am J Respir Cell Mol Biol* 30: 271–279.

Aulak K.S., Miyagi M., Yan L., West K.A., Massillon D., Crabb J.W., Stuehr D.J. (2001). Proteomic method identifies proteins nitrated in vivo during inflammatory challenge. *Proc Natl Acad Sci USA* 98: 12056–12061.

Baeuerle P.A. (1998). IkappaB-NF-kappaB structures: at the interface of inflammation control. *Cell* 95: 729–731.

Bai Y., Galetskiy D., Damoc E., Paschen C., Liu Z., Griese M., Liu S., Przybylski M. (2004). High resolution mass spectrometric alveolar proteomics: Identification of surfactant protein SP-A and SP-D modifications in proteinosis and cystic fibrosis patients. *Proteomics* 4: 2300–2309.

Barnes P., Chung K., Page C. (1998). Inflammatory mediators of asthma: An update. *Pharmacol Rev* 50: 515–596.

Barrett W.C., DeGnore J.P., Konig S., Fales H.M., Keng Y.F., Zhang Z.Y., Yim M.B., Chock P.B. (1999). Regulation of PTP1B via glutathionylation of the active site cysteine 215. *Biochemistry* 38: 6699–6705.

Baty J.W., Hampton M.B., Winterbourn C.C. (2002). Detection of oxidant sensitive thiol proteins by fluorescence labeling and two-dimensional electrophoresis. *Proteomics* 2: 1261–1266.

Beckman J.S., Koppenol W.H. (1996). Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and ugly. *Am J Physiol* 271: C1424–C1437.

Birnboim H.C., Lemay A.M., Lam D.K., Goldstein R., Webb J.R. (2003). Cutting edge: MHC class II-restricted peptides containing the inflammation-associated marker 3-nitrotyrosine evade central tolerance and elicit a robust cell-mediated immune response. *J Immunol* 171: 528–532.

Boots A.W., Haenen G.R., Bast A. (2003). Oxidant metabolism in chronic obstructive pulmonary disease. *Eur Respir J Suppl* 46: 14s–27s.

Brennan J.P., Wait R., Begum S., Bell J.R., Dunn M.J., Eaton P. (2004). Detection and mapping of widespread intermolecular protein disulfide formation during cardiac oxidative stress using proteomics with diagonal electrophoresis. *J Biol Chem* 279: 41352–41360.

Brennan M.L., Wu W., Fu X., Shen Z., Song W., Frost H., Vadseth C., Narine L., Lenkiewicz E., Borchers M.T., Lusis A.J., Lee J.J., Lee N.A., Abu-Soud H.M., Ischiropoulos H., Hazen S.L. (2002). A tale of two controversies: Defining both the role of peroxidases in nitrotyrosine formation in vivo using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. *J Biol Chem* 277: 17415–17427.

Broillet M.C. (1999). S-nitrosylation of proteins. *Cell Mol Life Sci* 55: 1036–1042.

Bryan N.S., Rassaf T., Maloney R.E., Rodriguez C.M., Saijo F., Rodriguez J.R., Feelisch M. (2004). Cellular targets and mechanisms of nitros(yl)ation: An insight into their nature and kinetics in vivo. *Proc Natl Acad Sci USA* 101: 4308–4313.

Caramori G., Papi A. (2004). Oxidants and asthma. *Thorax* 59: 170–173.

Chang L.Y., Crapo J.D. (2002). Inhibition of airway inflammation and hyperreactivity by an antioxidant mimetic. *Free Radic Biol Med* 33: 379–386.

Chauhan A., Krishna M., Frew A., Holgate S. (1998). Exposure to nitrogen dioxide (NO_2) and respiratory disease risk. *Rev Environ Health* 13: 73–90.

Chaurand P., Sanders M.E., Jensen R.A., Caprioli R.M. (2004). Proteomics in diagnostic pathology: Profiling and imaging proteins directly in tissue sections. *Am J Pathol* 165: 1057–1068.

Ckless K., Reynaert N.L., Taatjes D.J., Lounsbury K.M., van der Vliet A., Janssen-Heininger Y. (2004). In situ detection and visualization of *S*-nitrosylated proteins following chemical derivatization: Identification of Ran GTPase as a target for *S*-nitrosylation. *Nitric Oxide* 11: 216–227.

Comhair S.A., Xu W., Ghosh S., Thunnissen F.B., Almasan A., Calhoun W.J., Janocha A.J., Zheng L., Hazen S.L., Erzurum S.C. (2005). Superoxide dismutase inactivation in pathophysiology of asthmatic airway remodeling and reactivity. *Am J Pathol* 166: 663–674.

Connelly L., Jacobs A.T., Palacios-Callender M., Moncada S., Hobbs A.J. (2003). Macrophage endothelial nitric-oxide synthase autoregulates cellular activation and pro-inflammatory protein expression. *J Biol Chem* 278: 26480–26487.

Connelly L., Palacios-Callender M., Ameixa C., Moncada S., Hobbs A.J. (2001). Biphasic regulation of NF-kappa B activity underlies the pro- and anti-inflammatory actions of nitric oxide. *J Immunol* 166: 3873–3881.

Conrad C.C., Choi J., Malakowsky C.A., Talent J.M., Dai R., Marshall P., Gracy R.W. (2001). Identification of protein carbonyls after two-dimensional electrophoresis. *Proteomics* 1: 829–834.

Corradi M., Folesani G., Andreoli R., Manini P., Bodini A., Piacentini G., Carraro S., Zanconato S., Baraldi E. (2003). Aldehydes and glutathione in exhaled breath condensate of children with asthma exacerbation. *Am J Respir Crit Care Med* 167: 395–399.

Cotgreave I.A., Gerdes R.G. (1998). Recent trends in glutathione biochemistry–glutathione-protein interactions: A molecular link between oxidative stress and cell proliferation? *Biochem Biophys Res Commun* 242: 1–9.

Craven R.A., Totty N., Harnden P., Selby P.J., Banks R.E. (2002). Laser capture microdissection and two-dimensional polyacrylamide gel electrophoresis: Evaluation of tissue preparation and sample limitations. *Am J Pathol* 160: 815–822.

Cross J.V., Templeton D.J. (2004). Thiol oxidation of cell signaling proteins: Controlling an apoptotic equilibrium. *J Cell Biochem* 93: 104–111.

Dalle-Donne I., Scaloni A., Giustarini D., Cavarra E., Tell G., Lungarella G., Colombo R., Rossi R., Milzani A. (2005). Proteins as biomarkers of oxidative/nitrosative stress in diseases: the contribution of redox proteomics. *Mass Spectrom Rev* 24: 55–99.

Davies M.J., Fu S., Wang H., Dean R.T. (1999). Stable markers of oxidant damage to proteins and their application in the study of human disease. *Free Radic Biol Med* 27: 1151–1163.

De Sanctis G., MacLean J., Hamada K., Mehta S., Scott J., Jiao A., Yandava C., Kobzik L., Wolyniec W., Fabian A., Venugopal C., Grasemann H., Huang P., Drazen J. (1999). Contribution of nitric oxide synthases 1, 2, and 3 to airway hyperresponsiveness and inflammation in a murine model of asthma. *J Exp Med* 189: 1621–1630.

Delhase M., Hayakawa M., Chen Y., Karin M. (1999). Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation. *Science* 284: 309–313.

den Hertog J., Groen A., van der Wijk T. (2005). Redox regulation of protein-tyrosine phosphatases. *Arch Biochem Biophys* 434: 11–15.

Denzler K.L., Borchers M.T., Crosby J.R., Cieslewicz G., Hines E.M., Justice J.P., Cormier S.A., Lindenberger K.A., Song W., Wu W., Hazen S.L., Gleich G.J., Lee J.J., Lee N.A. (2001). Extensive eosinophil degranulation and peroxidase-mediated oxidation of airway proteins do not occur in a mouse ovalbumin-challenge model of pulmonary inflammation. *J Immunol* 167: 1672–1682.

DiDonato J., Mercurio F., Rosette C., Wu-Li J., Suyang H., Ghosh S., Karin M. (1996). Mapping of the inducible IkappaB phosphorylation sites that signal its ubiquitination and degradation. *Mol Cell Biol* 16: 1295–1304.

Driscoll K., Hassenbein D., Howard B., Isfort R., Cody D., Tindal M., Suchanek M., Carter J. (1995). Cloning, expression, and functional characterization of rat MIP-2: A neutrophil chemoattractant and epithelial cell mitogen. *J Leukoc Biol* 58: 359–364.

Duguet A., Iijima H., Eum S.Y., Hamid Q., Eidelman D.H. (2001). Eosinophil peroxidase mediates protein nitration in allergic airway inflammation in mice. *Am J Respir Crit Care Med* 164: 1119–1126.

Dunn S., Coles L., Lang R., Gerondakis S., Vadas M., Shannon M. (1994). Requirement for nuclear factor (NF)-kappa B p65 and NF-interleukin-6 binding elements in the tumor necrosis factor response region of the granulocyte colony-stimulating factor promoter. *Blood* 83: 2469–2479.

Dweik R.A., Comhair S.A., Gaston B., Thunnissen F.B., Farver C., Thomassen M.J., Kavuru M., Hammel J., Abu-Soud H.M., Erzurum S.C. (2001). NO chemical events in the human airway during the immediate and late antigen-induced asthmatic response. *Proc Natl Acad Sci USA* 98: 2622–2627.

Eiserich J.P., Baldus S., Brennan M.L., Ma W., Zhang C., Tousson A., Castro L., Lusis A.J., Nauseef W.M., White C.R., Freeman B.A. (2002). Myeloperoxidase, a leukocyte-derived vascular NO oxidase. *Science* 296: 2391–2394.

Emelyanov A., Fedoseev G., Abulimity A., Rudinski K., Fedoulov A., Karabanov A., Barnes P.J. (2001). Elevated concentrations of exhaled hydrogen peroxide in asthmatic patients. *Chest* 120: 1136–1139.

Emmert-Buck M.R., Bonner R.F., Smith P.D., Chuaqui R.F., Zhuang Z., Goldstein S.R., Weiss R.A., Liotta L.A. (1996). Laser capture microdissection. *Science* 274: 998–1001.

Fang F.C. (1997). Perspectives series: Host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. *J Clin Invest* 99: 2818–2825.

Fang K., Johns R., Macdonald T., Kinter M., Gaston B. (2000). *S*-Nitrosoglutathione breakdown prevents airway smooth muscle relaxation in the guinea pig. *Am J Physiol Lung Cell Mol Physiol* 279: L716–L721.

Fang K., Ragsdale N.V., Carey R.M., MacDonald T., Gaston B. (1998). Reductive assays for *S*-nitrosothiols: Implications for measurements in biological systems. *Biochem Biophys Res Commun* 252: 535–540.

Feder L.S., Stelts D., Chapman R.W., Manfra D., Crawley Y., Jones H., Minnicozzi M., Fernandez X., Paster T., Egan R.W., Kreutner W., Kung T.T. (1997). Role of nitric oxide on eosinophilic lung inflammation in allergic mice. *Am J Respir Cell Mol Biol* 17: 436–442.

Ferranti P., Malorni A., Mamone G., Sannolo N., Marino G. (1997). Characterisation of *S*-nitrosohaemoglobin by mass spectrometry. *FEBS Lett* 400: 19–24.

Finkel T. (2003). Oxidant signals and oxidative stress. *Curr Opin Cell Biol* 15: 247–254.

Fogarty A., Lewis S., Weiss S., Britton J. (2000). Dietary vitamin E, IgE concentrations, and atopy. *Lancet* 356: 1573–1574.

Forman H.J., Fukuto J.M., Torres M. (2004). Redox signaling: thiol chemistry defines which reactive oxygen and nitrogen species can act as second messengers. *Am J Physiol Cell Physiol* 287: C246–C256.

Forteza R., Salathe M., Miot F., Conner G.E. (2005). Regulated hydrogen peroxide production by duox in human airway epithelial cells. *Am J Respir Cell Mol Biol* 32: 462–469.

Foster M.W., Stamler J.S. (2004). New insights into protein S-nitrosylation. Mitochondria as a model system. *J Biol Chem* 279: 25891–25897.

Fratelli M., Demol H., Puype M., Casagrande S., Eberini I., Salmona M., Bonetto V., Mengozzi M., Duffieux F., Miclet E., Bachi A., Vandekerckhove J., Gianazza E., Ghezzi P. (2002). Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. *Proc Natl Acad Sci USA* 99: 3505–3510.

Fratelli M., Demol H., Puype M., Casagrande S., Villa P., Eberini I., Vandekerckhove J., Gianazza E., Ghezzi P. (2003). Identification of proteins undergoing glutathionylation in oxidatively stressed hepatocytes and hepatoma cells. *Proteomics* 3: 1154–1161.

Fujihara S., Ward C., Dransfield I., Hay R., Uings I., Hayes B., Farrow S., Haslett C., Rossi A. (2002). Inhibition of nuclear factor-kB activation un-masks the ability of TNF-α to induce human eosinophil apoptosis. *Eur J Immunol* 32: 457–466.

Gaston B., Drazen J.M., Loscalzo J., Stamler J.S. (1994). The biology of nitrogen oxides in the airways. *Am J Respir Crit Care Med* 149: 538–551.

Gaston B., Sears S., Woods J., Hunt J., Ponaman M., McMahon T., Stamler J.S. (1998). Bronchodilator *S*-nitrosothiol deficiency in asthmatic respiratory failure. *Lancet* 351: 1317–1319.

Geiszt M., Witta J., Baffi J., Lekstrom K., Leto T.L. (2003). Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense. *Faseb J* 17: 1502–1504.

Ghafouri B., Stahlbom B., Tagesson C., Lindahl M. (2002). Newly identified proteins in human nasal lavage fluid from non-smokers and smokers using two-dimensional gel electrophoresis and peptide mass fingerprinting. *Proteomics* 2: 112–120.

Ghezzi P., Bonetto V. (2003). Redox proteomics: identification of oxidatively modified proteins. *Proteomics* 3: 1145–1153.

Gold D.R. (2000). Environmental tobacco smoke, indoor allergens, and childhood asthma. *Environ Health Perspect* 108: 643–651.

Gow A.J., Chen Q., Hess D.T., Day B.J., Ischiropoulos H., Stamler J.S. (2002). Basal and stimulated protein *S*-nitrosylation in multiple cell types and tissues. *J Biol Chem* 277: 9637–9640.

Grasemann H., Gaston B., Fang K., Paul K., Ratjen F. (1999). Decreased levels of nitrosothiols in the lower airways of patients with cystic fibrosis and normal pulmonary function. *J Pediatr* 135: 770–772.

Guo F.H., Comhair S.A., Zheng S., Dweik R.A., Eissa N.T., Thomassen M.J., Calhoun W., Erzurum S.C. (2000). Molecular mechanisms of increased nitric oxide (NO) in asthma:

Evidence for transcriptional and post-translational regulation of NO synthesis. *J Immunol* 164: 5970–5980.

Gygi S.P., Rist B., Gerber S.A., Turecek F., Gelb M.H., Aebersold R. (1999). Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* 17: 994–999.

Hanazawa T., Kharitonov S.A., Barnes P.J. (2000). Increased nitrotyrosine in exhaled breath condensate of patients with asthma. *Am J Respir Crit Care Med* 162: 1273–1276.

Harant H., de Martin R., Andrew P.J., Foglar E., Dittrich C., Lindley I.J.D. (1996). Synergistic activation of interleukin-8 gene transcription by all-trans-retinoic acid and tumor necrosis factor-alpha involves the transcription factor NF-kappaB. *J Biol Chem* 271: 26954–26961.

Hein H., Schluter C., Kulke R., Christophers E., Schroder J., Bartels J. (1997). Genomic organization, sequence, and transcriptional regulation of the human eotaxin gene. *Biochem Biophys Res Comm* 237: 537–542.

Herbert B., Galvani M., Hamdan M., Olivieri E., MacCarthy J., Pedersen S., Righetti P.G. (2001). Reduction and alkylation of proteins in preparation of two-dimensional map analysis: why, when, and how? *Electrophoresis* 22: 2046–2057.

Hess D.T., Matsumoto A., Kim S.O., Marshall H.E., Stamler J.S. (2005). Protein *S*-nitrosylation: Purview and parameters. *Nat Rev Mol Cell Biol* 6: 150–166.

Hirsch J., Hansen K.C., Burlingame A.L., Matthay M.A. (2004). Proteomics: current techniques and potential applications to lung disease. *Am J Physiol Lung Cell Mol Physiol* 287: L1–L23.

Hogg N. (2002). The biochemistry and physiology of *S*-nitrosothiols. *Annu Rev Pharmacol Toxicol* 42: 585–600.

Idriss S.D., Gudi T., Casteel D.E., Kharitonov V.G., Pilz R.B., Boss G.R. (1999). Nitric oxide regulation of gene transcription via soluble guanylate cyclase and type I cGMP-dependent protein kinase. *J Biol Chem* 274: 9489–9493.

Issaq H.J., Veenstra T.D., Conrads T.P., Felschow D. (2002). The SELDI-TOF MS approach to proteomics: Protein profiling and biomarker identification. *Biochem Biophys Res Commun* 292: 587–592.

Jaffrey S.R., Erdjument-Bromage H., Ferris C.D., Tempst P., Snyder S.H. (2001). Protein *S*-nitrosylation: A physiological signal for neuronal nitric oxide. *Nat Cell Biol* 3: 193–197.

Jaffrey S.R., Fang M., Snyder S.H. (2002). Nitrosopeptide mapping: A novel methodology reveals *S*-nitrosylation of dexras1 on a single cysteine residue. *Chem Biol* 9: 1329–1335.

Jain K.K. (2002). Application of laser capture microdissection to proteomics. *Methods Enzymol* 356: 157–167.

Janssen-Heininger Y., Ckless K., Reynaert N., van der Vliet A. (2005). SOD inactivation in asthma: Bad or no news? *Am J Pathol* 166: 649–652.

Janssen-Heininger Y.M., Poynter M.E., Baeuerle P.A. (2000). Recent advances towards understanding redox mechanisms in the activation of nuclear factor kappaB. *Free Radic Biol Med* 28: 1317–1327.

Ji C., Kozak K.R., Marnett L.J. (2001). IkappaB kinase, a molecular target for inhibition by 4-hydroxy-2-nonenal. *J Biol Chem* 276: 18223–18228.

Ji Y., Akerboom T.P., Sies H., Thomas J.A. (1999). *S*-Nitrosylation and *S*-glutathiolation of protein sulfhydryls by S- nitroso glutathione. *Arch Biochem Biophys* 362: 67–78.

Kaminsky D.A., Mitchell J., Carroll N., James A., Soultanakis R., Janssen Y. (1999). Nitrotyrosine formation in the airways and lung parenchyma of patients with asthma. *J Allergy Clin Immunol* 104: 747–754.

Kapahi P., Takahashi T., Natoli G., Adams S.R., Chen Y., Tsien R.Y., Karin M. (2000). Inhibition of NF-kappa B activation by arsenite through reaction with a critical cysteine in the activation loop of Ikappa B kinase. *J Biol Chem* 275: 36062–36066.

Kelly F.J., Mudway I., Blomberg A., Frew A., Sandstrom T. (1999). Altered lung antioxidant status in patients with mild asthma. *Lancet* 354: 482–483.

Klatt P., Pineda Molina E., Perez-Sala D., Lamas S. (2000). Novel application of *S*-nitrosoglutathione-Sepharose to identify proteins that are potential targets for *S*-nitrosoglutathione-induced mixed-disulphide formation. *Biochem J* 349: 567–578.

Kleeberger S.R., Reddy S.P., Zhang L.Y., Cho H.Y., Jedlicka A.E. (2001). Toll-like receptor 4 mediates ozone-induced murine lung hyperpermeability via inducible nitric oxide synthase. *Am J Physiol Lung Cell Mol Physiol* 280: L326–L333.

Koeck T., Fu X., Hazen S.L., Crabb J.W., Stuehr D.J., Aulak K.S. (2004). Rapid and selective oxygen-regulated protein tyrosine denitration and nitration in mitochondria. *J Biol Chem* 279: 27257–27262.

Korn S.H., Wouters E.F., Vos N., Janssen-Heininger Y.M. (2001). Cytokine-induced activation of nuclear factor-kappa B is inhibited by hydrogen peroxide through oxidative inactivation of ikappa B kinase. *J Biol Chem* 276: 35693–35700.

Kristof A.S., Goldberg P., Laubach V., Hussain S.N. (1998). Role of inducible nitric oxide synthase in endotoxin-induced acute lung injury. *Am J Respir Crit Care Med* 158: 1883–1889.

Kuncewicz T., Sheta E.A., Goldknopf I.L., Kone B.C. (2004). Proteomic analysis reveals novel protein targets of *S*-nitrosylation in mesangial cells. *Contrib Nephrol* 141: 221–230.

Kupfner J.G., Arcaroli J.J., Yum H.K., Nadler S.G., Yang K.Y., Abraham E. (2001). Role of NF-kappaB in endotoxemia-induced alterations of lung neutrophil apoptosis. *J Immunol* 167: 7044–7051.

Kwapiszewska G., Meyer M., Bogumil R., Bohle R.M., Seeger W., Weissmann N., Fink L. (2004). Identification of proteins in laser-microdissected small cell numbers by SELDI-TOF and Tandem MS. *BMC Biotechnol* 4: 30.

Lander H.M., Hajjar D.P., Hempstead B.L., Mirza U.A., Chait B.T., Campbell S., Quilliam L.A. (1997). A molecular redox switch on p21(ras): Structural basis for the nitric oxide-p21(ras) interaction. *J Biol Chem* 272: 4323–4326.

Lawrence T., Gilroy D.W., Colville-Nash P.R., Willoughby D.A. (2001). Possible new role for NF-kappaB in the resolution of inflammation. *Nat Med* 7: 1291–1297.

Lee J.J., Dimina D., Macias M.P., Ochkur S.I., McGarry M.P., O'Neill K.R., Protheroe C., Pero R., Nguyen T., Cormier S.A., Lenkiewicz E., Colbert D., Rinaldi L., Ackerman S.J., Irvin C.G., Lee N.A. (2004a). Defining a link with asthma in mice congenitally deficient in eosinophils. *Science* 305: 1773–1776.

Lee Y.C., Lee K.S., Park S.J., Park H.S., Lim J.S., Park K.H., Im M.J., Choi I.W., Lee H.K., Kim U.H. (2004b). Blockade of airway hyperresponsiveness and inflammation in a murine model of asthma by a prodrug of cysteine, L-2-oxothiazolidine-4-carboxylic acid. *Faseb J* 18: 1917–1919.

Leeuwenburgh C., Rasmussen J.E., Hsu F.F., Mueller D.M., Pennathur S., Heinecke J.W. (1997). Mass spectrometric quantification of markers for protein oxidation by tyrosyl radical, copper, and hydroxyl radical in low density lipoprotein isolated from human atherosclerotic plaques. *J Biol Chem* 272: 3520–3526.

Lehner I., Niehof M., Borlak J. (2003). An optimized method for the isolation and identification of membrane proteins. *Electrophoresis* 24: 1795–1808.

Leichert L.I., Jakob U. (2004). Protein thiol modifications visualized in vivo. *PLoS Biol* 2: e333.

Li C., Hong Y., Tan Y.X., Zhou H., Ai J.H., Li S.J., Zhang L., Xia Q.C., Wu J.R., Wang H.Y., Zeng R. (2004). Accurate qualitative and quantitative proteomic analysis of clinical hepatocellular carcinoma using laser capture microdissection coupled with isotope-coded affinity tag and two-dimensional liquid chromatography mass spectrometry. *Mol Cell Proteomics* 3: 399–409.

Liebler D.C. (2002). Proteomic approaches to characterize protein modifications: new tools to study the effects of environmental exposures. *Environ Health Perspect* 110: 3–9.

Lind C., Gerdes R., Hamnell Y., Schuppe-Koistinen I., von Lowenhielm H.B., Holmgren A., Cotgreave I.A. (2002). Identification of *S*-glutathionylated cellular proteins during oxidative stress and constitutive metabolism by affinity purification and proteomic analysis. *Arch Biochem Biophys* 406: 229–240.

Link A.J., Eng J., Schieltz D.M., Carmack E., Mize G.J., Morris D.R., Garvik B.M., Yates J.R. III (1999). Direct analysis of protein complexes using mass spectrometry. *Nat Biotechnol* 17: 676–682.

Liu L., Hausladen A., Zeng M., Que L., Heitman J., Stamler J.S. (2001). A metabolic enzyme for *S*-nitrosothiol conserved from bacteria to humans. *Nature* 410: 490–494.

Liu L., Yan Y., Zeng M., Zhang J., Hanes M.A., Ahearn G., McMahon T.J., Dickfeld T., Marshall H.E., Que L.G., Stamler J.S. (2004). Essential roles of *S*-nitrosothiols in vascular homeostasis and endotoxic shock. *Cell* 116(4): 617–628.

Luche S., Diemer H., Tastet C., Chevallet M., Van Dorsselaer A., Leize-Wagner E., Rabilloud T. (2004). About thiol derivatization and resolution of basic proteins in two-dimensional electrophoresis. *Proteomics* 4: 551–561.

Macnee W., Rahman I. (1999). Oxidants and antioxidants as therapeutic targets in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 160: S58–S65.

MacPherson J.C., Comhair S.A., Erzurum S.C., Klein D.F., Lipscomb M.F., Kavuru M.S., Samoszuk M.K., Hazen S.L. (2001). Eosinophils are a major source of nitric oxide-derived oxidants in severe asthma: characterization of pathways available to eosinophils for generating reactive nitrogen species. *J Immunol* 166: 5763–5772.

Maniatis T. (1997). Catalysis by a multiprotein IkappaB kinase complex. *Science* 278: 818–819.

Mannick J.B., Hausladen A., Liu L., Hess D.T., Zeng M., Miao Q.X., Kane L.S., Gow A.J., Stamler J.S. (1999). Fas-induced caspase denitrosylation. *Science* 284: 651–654.

Mannick J.B., Schonhoff C., Papeta N., Ghafourifar P., Szibor M., Fang K., Gaston B. (2001). *S*-Nitrosylation of mitochondrial caspases. *J Cell Biol* 154: 1111–1116.

Marley R., Feelisch M., Holt S., Moore K. (2000). A chemiluminescense-based assay for *S*-nitrosoalbumin and other plasma S- nitrosothiols. *Free Radic Res* 32: 1–9.

Marshall H.E., Merchant K., Stamler J.S. (2000). Nitrosation and oxidation in the regulation of gene expression. *Faseb J* 14: 1889–1900.

Marshall H.E., Stamler J.S. (2001). Inhibition of NF-kappa B by *S*-nitrosylation. *Biochemistry* 40: 1688–1693.

Marshall H.E., Stamler J.S. (2002). Nitrosative stress-induced apoptosis through inhibition of NF-kappa B. *J Biol Chem* 277: 34223–34228.

Martinez-Ruiz A., Lamas S. (2004). Detection and proteomic identification of *S*-nitrosylated proteins in endothelial cells. *Arch Biochem Biophys* 423: 192–199.

Matata B.M., Galinanes M. (2002). Peroxynitrite is an essential component of cytokines production mechanism in human monocytes through modulation of nuclear factor-kappa B DNA binding activity. *J Biol Chem* 277: 2330–2335.

Matthews J.R., Botting C.H., Panico M., Morris H.R., Hay R.T. (1996). Inhibition of NF-kappaB DNA binding by nitric oxide. *Nucleic Acids Res* 24: 2236–2242.

McConnell R., Berhane K., Gilliland F., London S.J., Vora H., Avol E., Gauderman W.J., Margolis H.G., Lurmann F., Thomas D.C., Peters J.M. (1999). Air pollution and bronchitic symptoms in Southern California children with asthma. *Environ Health Perspect* 107: 757–760.

Mirza U.A., Chait B.T., Lander H.M. (1995). Monitoring reactions of nitric oxide with peptides and proteins by electrospray ionization–mass spectrometry. *J Biol Chem* 270: 17185–17188.

Montuschi P., Corradi M., Ciabattoni G., Nightingale J., Kharitonov S.A., Barnes P.J. (1999). Increased 8-isoprostane, a marker of oxidative stress, in exhaled condensate of asthma patients. *Am J Respir Crit Care Med* 160: 216–220.

Nathan C. (1997). Inducible nitric oxide synthase: What difference does it make? *J Clin Invest* 100: 2417–2423.

Nedospasov A., Rafikov R., Beda N., Nudler E. (2000). An autocatalytic mechanism of protein nitrosylation. *Proc Natl Acad Sci USA* 97: 13543–13548.

Nikitovic D., Holmgren A. (1996). *S*-Nitrosoglutathione is cleaved by the thioredoxin system with liberation of glutathione and redox regulating nitric oxide. *J Biol Chem* 271: 19180–19185.

O'Bryne P., Postma D. (1999). The many faces of airway inflammation: Asthma and chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 159: S41–S66.

Okamoto T., Gohil K., Finkelstein E.I., Bove P., Akaike T., van der Vliet A. (2004). Multiple contributing roles for NOS2 in LPS-induced acute airway inflammation in mice. *Am J Physiol Lung Cell Mol Physiol* 286: L198–L209.

Okamoto T., Valacchi G., Gohil K., Akaike T., van der Vliet A. (2002). *S*-Nitrosothiols inhibit cytokine-mediated induction of matrix metalloproteinase-9 in airway epithelial cells. *Am J Respir Cell Mol Biol* 27: 463–473.

Pahl H.L. (1999). Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* 18: 6853–6866.

Pantano C., Shrivastava P., McElhinney B., Janssen-Heininger Y. (2003). Hydrogen peroxide signaling through tumor necrosis factor receptor 1 leads to selective activation of c-Jun N-terminal kinase. *J Biol Chem* 278: 44091–44096.

Park S.W., Huq M.D., Hu X., Wei L.N. (2005). Tyrosine nitration on p65: A novel mechanism to rapidly inactivate nuclear factor-{kappa}B. *Mol Cell Proteomics* 4: 300–309.

Paweletz C.P., Charboneau L., Bichsel V.E., Simone N.L., Chen T., Gillespie J.W., Emmert-Buck M.R., Roth M.J., Petricoin I.E., Liotta L.A. (2001). Reverse phase

protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front. *Oncogene* 20: 1981–1989.

Peng H.B., Libby P., Liao J.K. (1995). Induction and stabilization of I kappa B alpha by nitric oxide mediates inhibition of NF-kappa B. *J Biol Chem* 270: 14214–14219.

Pineda-Molina E., Klatt P., Vazquez J., Marina A., Garcia de Lacoba M., Perez-Sala D., Lamas S. (2001). Glutathionylation of the p50 subunit of NF-kappaB: a mechanism for redox-induced inhibition of DNA binding. *Biochemistry* 40: 14134–14142.

Poole L.B., Karplus P.A., Claiborne A. (2004). Protein sulfenic acids in redox signaling. *Ann Rev Pharmacol Toxicol* 44: 325–347.

Poynter M., Irvin C.G., Janssen-Heininger Y.M. (2002). Rapid activation of nuclear factor kappa-B in airway epithelium in a murine model of allergic airway inflammation. *Am J Pathol* 160: 1325–1334.

Poynter M.E., Cloots R., van Woerkom T., Butnor K.J., Vacek P., Taatjes D.J., Irvin C.G., Janssen-Heininger Y.M. (2004). NF-kappa B activation in airways modulates allergic inflammation but not hyperresponsiveness. *J Immunol* 173: 7003–7009.

Poynter M.E., Irvin C.G., Janssen-Heininger Y.M. (2003). A prominent role for airway epithelial NF-kappa B activation in lipopolysaccharide-induced airway inflammation. *J Immunol* 170: 6257–6265.

Rabilloud T. (2002). Two-dimensional gel electrophoresis in proteomics: Old, old fashioned, but it still climbs up the mountains. *Proteomics* 2: 3–10.

Raychaudhuri B., Dweik R., Connors M.J., Buhrow L., Malur A., Drazba J., Arroliga A.C., Erzurum S.C., Kavuru M.S., Thomassen M.J. (1999). Nitric oxide blocks nuclear factor-kappaB activation in alveolar macrophages. *Am J Respir Cell Mol Biol* 21: 311–316.

Reddy S., Jones A.D., Cross C.E., Wong P.S., van der Vliet A. (2000). Inactivation of creatine kinase by *S*-glutathionylation of the active-site cysteine residue. *Biochem J* 347: 821–827.

Ren D., Julka S., Inerowicz H.D., Regnier F.E. (2004). Enrichment of cysteine-containing peptides from tryptic digests using a quaternary amine tag. *Anal Chem* 76: 4522–4530.

Reynaert N.L., Ckless K., Korn S.H., Vos N., Guala A.S., Wouters E.F., van der Vliet A., Janssen-Heininger Y.M. (2004). Nitric oxide represses inhibitory kappaB kinase through *S*-nitrosylation. *Proc Natl Acad Sci USA* 101: 8945–8950.

Rhee K.Y., Erdjument-Bromage H., Tempst P., Nathan C.F. (2005). S-nitroso proteome of *Mycobacterium tuberculosis*: Enzymes of intermediary metabolism and antioxidant defense. *Proc Natl Acad Sci USA* 102: 467–472.

Richardson G., Benjamin N. (2002). Potential therapeutic uses for *S*-nitrosothiols. *Clin Sci (Lond)* 102: 99–105.

Roberts E., Charboneau L., Espina V., Liotta L., Petricoin E., Dreher K. (2004). Application of laser capture microdissection and protein microarray technologies in the molecular analysis of airway injury following pollution particle exposure. *J Toxicol Environ Health A* 67: 851–861.

Roebuck K.A., Rahman A., Lakshminarayanan V., Janakidevi K., Malik A.B. (1995). H_2O_2 and tumor necrosis factor-alpha activate intercellular adhesion molecule 1 (ICAM-1) gene transcription through distinct cis-regulatory elements within the ICAM-1 promoter. *J Biol Chem* 270: 18966–18974.

Roh G.S., Shin Y., Seo S.W., Yoon B.R., Yeo S., Park S.J., Cho J.W., Kwack K. (2004). Proteome analysis of differential protein expression in allergen-induced asthmatic mice lung after dexamethasone treatment. *Proteomics* 4: 3318–3327.

Rossi A., Kapahi P., Natoli G., Takahashi T., Chen Y., Karin M., Santoro M.G. (2000). Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IkappaB kinase. *Nature* 403: 103–108.

Sadikot R.T., Han W., Everhart M.B., Zoia O., Peebles R.S., Jansen E.D., Yull F.E., Christman J.W., Blackwell T.S. (2003). Selective I kappa B kinase expression in airway epithelium generates neutrophilic lung inflammation. *J Immunol* 170: 1091–1098.

Saleh D., Ernst P., Lim S., Barnes P.J., Giaid A. (1998). Increased formation of the potent oxidant peroxynitrite in the airways of asthmatic patients is associated with induction of nitric oxide synthase: Effect of inhaled glucocorticoid. *Faseb J* 12: 929–937.

Salmeen A., Andersen J.N., Myers M.P., Meng T.C., Hinks J.A., Tonks N.K., Barford D. (2003). Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate. *Nature* 423: 769–773.

Sanceau J., Kaisho T., Hirano T., Wietzerbin J. (1995). Triggering of the human interleukin-6 gene by interferon-gamma and tumor necrosis factor-alpha in monocytic cells involves cooperation between interferon regulatory factor-1, NF kappa B, and Sp1 transcription factors. *J Biol Chem* 270: 27920–27931.

Saurin A.T., Neubert H., Brennan J.P., Eaton P. (2004). Widespread sulfenic acid formation in tissues in response to hydrogen peroxide. *Proc Natl Acad Sci USA* 101: 17982–17987.

Schock B.C., Young I.S., Brown V., Fitch P.S., Shields M.D., Ennis M. (2003). Antioxidants and oxidative stress in BAL fluid of atopic asthmatic children. *Pediatr Res* 53: 375–381.

Sethuraman M., McComb M.E., Heibeck T., Costello C.E., Cohen R.A. (2004a). Isotope-coded affinity tag approach to identify and quantify oxidant-sensitive protein thiols. *Mol Cell Proteomics* 3: 273–278.

Sethuraman M., McComb M.E., Huang H., Huang S., Heibeck T., Costello C.E., Cohen R.A. (2004b). Isotope-coded affinity tag (ICAT) approach to redox proteomics: Identification and quantitation of oxidant-sensitive cysteine thiols in complex protein mixtures. *J Proteome Res* 3: 1228–1233.

Shao M.X., Nadel J.A. (2005). Dual oxidase 1-dependent MUC5AC mucin expression in cultured human airway epithelial cells. *Proc Natl Acad Sci USA* 102: 767–772.

Shrivastava P., Pantano C., Watkin R., McElhinney B., Guala A., Poynter M.L., Persinger R.L., Budd R., Janssen-Heininger Y. (2004). Reactive nitrogen species-induced cell death requires Fas-dependent activation of c-Jun *N*-terminal kinase. *Mol Cell Biol* 24: 6763–6772.

Smith L.J., Houston M., Anderson J. (1993). Increased levels of glutathione in bronchoalveolar lavage fluid from patients with asthma. *Am Rev Respir Dis* 147: 1461–1464.

Smith R.D., Anderson G.A., Lipton M.S., Pasa-Tolic L., Shen Y., Conrads T.P., Veenstra T.D., Udseth H.R. (2002). An accurate mass tag strategy for quantitative and high-throughput proteome measurements. *Proteomics* 2: 513–523.

Snyder A., McPherson M., Hunt J., Johnson M.A., Stamler J., Gaston B. (2002). Acute effects of aerosolized *S*-nitrosoglutathione in cystic fibrosis. *Am J Respir Crit Care Med* 165: 922–926.

Soderdahl T., Enoksson M., Lundberg M., Holmgren A., Ottersen O.P., Orrenius S., Bolcsfoldi G., Cotgreave I.A. (2003). Visualization of the compartmentalization of glutathione and protein-glutathione mixed disulfides in cultured cells. *Faseb J* 17: 124–126.

Spahr C.S., Susin S.A., Bures E.J., Robinson J.H., Davis M.T., McGinley M.D., Kroemer G., Patterson S.D. (2000). Simplification of complex peptide mixtures for proteomic analysis: Reversible biotinylation of cysteinyl peptides. *Electrophoresis* 21: 1635–1650.

Spencer N.Y., Zeng H., Patel R.P., Hogg N. (2000). Reaction of *S*-nitrosoglutathione with the heme group of deoxyhemoglobin. *J Biol Chem* 275: 36562–36567.

Stadtman E.R., Van Remmen H., Richardson A., Wehr N.B., Levine R.L. (2005) Methionine oxidation and aging. *Biochim Biophys Acta* 1703: 135–140.

Stamler J.S., Lamas S., Fang F.C. (2001). Nitrosylation: The prototypic redox-based signaling mechanism. *Cell* 106: 675–683.

Stone J.R. (2004). An assessment of proposed mechanisms for sensing hydrogen peroxide in mammalian systems. *Arch Biochem Biophys* 422: 119–124.

Strachan D.P. (2000). The role of environmental factors in asthma. *Br Med Bull* 56: 865–882.

Stripp B.R., Reynolds S.D., Boe I.M., Lund J., Power J.H., Coppens J.T., Wong V., Reynolds P.R., Plopper C.G. (2002). Clara cell secretory protein deficiency alters clara cell secretory apparatus and the protein composition of airway lining fluid. *Am J Respir Cell Mol Biol* 27: 170–178.

Taylor E.R., Hurrell F., Shannon R.J., Lin T.K., Hirst J., Murphy M.P. (2003). Reversible glutathionylation of complex I increases mitochondrial superoxide formation. *J Biol Chem* 278: 19603–19610.

Thomassen M.J., Kavuru M.S. (2001). Human alveolar macrophages and monocytes as a source and target for nitric oxide. *Int Immunopharmacol* 1: 1479–1490.

Trujillo M., Alvarez M.N., Peluffo G., Freeman B.A., Radi R. (1998). Xanthine oxidase-mediated decomposition of *S*-nitrosothiols. *J Biol Chem* 273: 7828–7834.

Valacchi G., Pagnin E., Phung A., Nardini M., Schock B.C., Cross C.E., van der Vliet A. (2005). Inhibition of NFkappaB activation and IL-8 expression in human bronchial epithelial cells by acrolein. *Antioxid Redox Signal* 7: 25–31.

van der Vliet A., Eiserich J.P., Marelich G.P., Halliwell B., Cross C.E. (1997). Oxidative stress in cystic fibrosis: Does it occur and does it matter? *Adv Pharmacol* 38: 491–513.

van der Vliet A., Eiserich J.P., Shigenaga M.K., Cross C.E. (1999). Reactive nitrogen species and tyrosine nitration in the respiratory tract: Epiphenomena or a pathobiologic mechanism of disease? *Am J Respir Crit Care Med* 160: 1–9.

van der Vliet A., Hoen P.A., Wong P.S., Bast A., Cross C.E. (1998). Formation of *S*-nitrosothiols via direct nucleophilic nitrosation of thiols by peroxynitrite with elimination of hydrogen peroxide. *J Biol Chem* 273: 30255–30262.

van der Vliet A., Nguyen M.N., Shigenaga M.K., Eiserich J.P., Marelich G.P., Cross C.E. (2000). Myeloperoxidase and protein oxidation in cystic fibrosis. *Am J Physiol Lung Cell Mol Physiol* 279: L537–L546.

van Montfort R.L., Congreve M., Tisi D., Carr R., Jhoti H. (2003). Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B. *Nature* 423: 773–777.

von Mutius E. (1998). Air pollution and asthma—fact or artifact? A plea for inclusion of objective measures in environmental epidemiology. *Pediatr Pulmonol* 25: 297–298.

Voss A.A., Lango J., Ernst-Russell M., Morin D., Pessah I.N. (2004). Identification of hyperreactive cysteines within ryanodine receptor type 1 by mass spectrometry. *J Biol Chem* 279: 34514–34520.

Wattiez R., Hermans C., Cruyt C., Bernard A., Falmagne P. (2000). Human bronchoalveolar lavage fluid protein two-dimensional database: Study of interstitial lung diseases. *Electrophoresis* 21: 2703–2712.

Wattiez R., Noel-Georis I., Cruyt C., Broeckaert F., Bernard A., Falmagne P. (2003). Susceptibility to oxidative stress: Proteomic analysis of bronchoalveolar lavage from ozone-sensitive and ozone-resistant strains of mice. *Proteomics* 3: 658–665.

Wells-Knecht M.C., Lyons T.J., McCance D.R., Thorpe S.R., Baynes J.W. (1997). Age-dependent increase in ortho-tyrosine and methionine sulfoxide in human skin collagen is not accelerated in diabetes: Evidence against a generalized increase in oxidative stress in diabetes. *J Clin Invest* 100: 839–846.

Werner E., Werb Z. (2002). Integrins engage mitochondrial function for signal transduction by a mechanism dependent on Rho GTPases. *J Cell Biol* 158: 357–368.

Wheelock A.M., Zhang L., Tran M.U., Morin D., Penn S., Buckpitt A.R., Plopper C.G. (2004). Isolation of rodent airway epithelial cell proteins facilitates in vivo proteomics studies of lung toxicity. *Am J Physiol Lung Cell Mol Physiol* 286: L399–L410.

Whitelegge J.P. (2002). Plant proteomics: BLASTing out of a MudPIT. *Proc Natl Acad Sci USA* 99: 11564–11566.

Wildgruber R., Harder A., Obermaier C., Boguth G., Weiss W., Fey S.J., Larsen P.M., Gorg A. (2000). Towards higher resolution: Two-dimensional electrophoresis of *Saccharomyces cerevisiae* proteins using overlapping narrow immobilized pH gradients. *Electrophoresis* 21: 2610–2616.

Williams J.G., Pappu K., Campbell S.L. (2003). Structural and biochemical studies of p21Ras *S*-nitrosylation and nitric oxide-mediated guanine nucleotide exchange. *Proc Natl Acad Sci USA* 100: 6376–6381.

Wong P.S., Eiserich J.P., Reddy S., Lopez C.L., Cross C.E., van der Vliet A. (2001). Inactivation of glutathione *S*-transferases by nitric oxide-derived oxidants: Exploring a role for tyrosine nitration. *Arch Biochem Biophys* 394: 216–228.

Wong P.S., van der Vliet A. (2002). Quantitation and localization of tyrosine nitration in proteins. *Methods Enzymol* 359: 399–410.

Wood Z.A., Poole L.B., Karplus P.A. (2003). Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* 300: 650–653.

Wu H.M., Jin M., Marsh C.B. (2005). Toward functional proteomics of alveolar macrophages. *Am J Physiol Lung Cell Mol Physiol* 288: L585–L595.

Wu W., Samoszuk M.K., Comhair S.A., Thomassen M.J., Farver C.F., Dweik R.A., Kavuru M.S., Erzurum S.C., Hazen S.L. (2000). Eosinophils generate brominating oxidants in allergen-induced asthma. *J Clin Invest* 105: 1455–1463.

Wulfkuhle J.D., McLean K.C., Paweletz C.P., Sgroi D.C., Trock B.J., Steeg P.S., Petricoin E.F. III (2001). New approaches to proteomic analysis of breast cancer. *Proteomics* 1: 1205–1215.

Xiao G.G., Nel A.E., Loo J.A. (2005). Nitrotyrosine-modified proteins and oxidative stress induced by diesel exhaust particles. *Electrophoresis* 26: 280–292.

Xiong Y., Karupiah G., Hogan S.P., Foster P.S., Ramsay A.J. (1999). Inhibition of allergic airway inflammation in mice lacking nitric oxide synthase 2. *J Immunol* 162: 445–452.

Yang Y., Loscalzo J. (2005). *S*-Nitrosoprotein formation and localization in endothelial cells. *Proc Natl Acad Sci USA* 102: 117–122.

Yoo B.S., Regnier F.E. (2004). Proteomic analysis of carbonylated proteins in two-dimensional gel electrophoresis using avidin-fluorescein affinity staining. *Electrophoresis* 25: 1334–1341.

Zandi E., Karin M. (1999). Bridging the gap: composition, regulation, and physiological function of the IkappaB kinase complex. *Mol Cell Biol* 19: 4547–4551.

Zech B., Wilm M., van Eldik R., Brune B. (1999). Mass spectrometric analysis of nitric oxide-modified caspase-3. *J Biol Chem* 274: 20931–20936.

Zhang M., Nomura A., Uchida Y., Iijima H., Sakamoto T., Iishii Y., Morishima Y., Mochizuki M., Masuyama K., Hirano K., Sekizawa K. (2002). Ebselen suppresses late airway responses and airway inflammation in guinea pigs. *Free Radic Biol Med* 32: 454–464.

Zheng K., Adjei A.A., Shinjo M., Shinjo S., Todoriki H., Ariizumi M. (1999). Effect of dietary vitamin E supplementation on murine nasal allergy. *Am J Med Sci* 318: 49–54.

27

SEQUESTERING AGENTS OF INTERMEDIATE REACTIVE ALDEHYDES AS INHIBITORS OF ADVANCED LIPOXIDATION END-PRODUCTS (ALEs)

MARINA CARINI, GIANCARLO ALDINI, AND
ROBERTO MAFFEI FACINO

27.1 INTRODUCTION

Lipoxidation (oxidation of polyunsaturated fatty acids, PUFAs) generates a burst of intermediate reactive carbonyl species (RCS), mainly unsaturated aldehydes, such as 4-hydroxy-*trans*-2-nonenal (HNE), acrolein (ACR), and malondialdehyde (MDA), which have been implicated as causative agents in cytotoxic processes initiated by the exposure of biological systems to oxidizing agents and free radicals (Esterbauer et al., 1991). These RCS are now thought to be involved in the onset and progression of several pathologies such as cardiovascular (atherosclerosis, long-term complications of diabetes) and neurodegenerative diseases, Alzheimer's disease (AD), Parkinson's disease (PD), cerebral ischemia, rheumatoid arthritis, post-ischemic reoxygenation injury, and aging (Uchida, 2000; Poli and Schaur, 2000). Accumulation in serum of reactive carbonyl toxins derived from lipids (and carbohydrates) and the subsequent carbonyl modifications of proteins have been defined for the first time by Miyata et al., (1999a) as "carbonyl stress."

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases,
Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield

Compared to free radicals, RCS are stable and can diffuse within or even escape from the cell and attack targets far from the site of the original formation. Therefore they are not only end-products of lipoxidation processes, they also act as "second cytotoxic messengers." Once formed or activated, RCS induce different aspects of cellular stress, including peroxide formation (oxidative stress), chromosomal aberrations, sister chromatid exchanges, point mutations, and cell mortality. It has been also demonstrated that RCS induce a specific program of gene expression (cellular stress response), including activation of transcription factors such as *c-fos* and *c-jun*, whose gene products are involved in the cellular defense against genotoxic agents (Uchida, 2000). In addition recent studies point to a relationship between oxidative stress (and RCS formation) and immune activation and recruitment in a variety of diseases processes (Simpson et al., 2004).

Most biological effects of intermediate aldehydes are attributed to their capacity to react with the nucleophilic sites of proteins and to form covalently modified biomolecules and advanced lipoxidation end-products (ALEs). Glycation is another spontaneous protein modification, starting from the binding of reducing sugars to free amino groups, a reaction recognized as the initial step of a very complex process (the Maillard cascade) that involves the formation of several end-point products that are responsible for protein modification. Reactive oxygen species (ROS) are generated during the process, and complex interactions between oxidation and glycation (the glycoxidation process) are considered to play a key role in chemical and functional protein alteration (Thorpe and Baynes, 2003). In contrast to glycation/glycoxidation, the process of lipoxidation may follow nonlinear kinetics and depend on specific factors, such as the lipid environment and turnover, the rate of oxidant production, and the status of antioxidant defense mechanisms. As a result lipoxidation may occur sporadically at locations yet to be identified or unattainable to current drug therapies. While advanced glycation steps have been extensively studied (Singh et al., 2001), less is known about protein modification by lipoxidation-derived RCS. Already, however, a large body of evidence indicates that many of the effects of vascular dysfunction on cardiovascular diseases are mediated by lipid-derived RCS. Unsaturated aldehyde levels are consistently elevated in plasma of patients with congestive heart failure, associated with impairment of left ventricular contractility (Mak et al., 2000); carbonyl stress has been implicated in cardiac reperfusion injury, since the reperfusion-induced mitochondrial dysfunction is mediated in part by modification of specific mitochondrial proteins by RCS, a process that has been shown to increase in an age-dependent manner (Lucas and Szweda, 1998; Eaton et al., 1999). The sequence of toxic events that results can compromise tissue survival during ischemia and recovery on reperfusion.

Both diabetes and atherosclerosis are diseases that are closely linked to one another: the common link is that the chemical modification of proteins is primarily the result of protein carbonylation. The emerging role of lipid-derived RCS in hyperglycemia and in development of complications in diabetes is now well documented (Januszewski et al., 2003): hyperglycemia exacerbates the chemical modification of proteins by lipids, and lipid-derived, rather than carbohydrate-derived,

RCS are considered to be the immediate and major source of chemical modifications leading to tissue damage, pro-inflammatory processes, and chronic complications in diabetes. Thus, while severe hyperlipidemia may be sufficient to induce lipoxidative damage, hyperlipidemia combined with hyperglycemia, and possibly an increase in oxidative stress in diabetes and obesity, exacerbates the chemical modification of proteins by lipids in diabetes. These observations indicate that lipid peroxidation and the consequent lipoxidative modification of proteins play a key role in the development of cardiovascular and renal disease in diabetes. Carbonyl stress has been implicated in the progression of renal diseases, and increased plasma levels of RCS or RCS adducts have been determined in patients with chronic renal failure, those on chronic hemodialysis, in long-term complications associated to hemodialysis, such as dialysis-related amyloidosis, and in uremic subjects (Miyata et al., 2000a, 2001a,b; Siems et al., 2002).

The involvement of toxic RCS as products and propagators of oxidative damage in neurodegenerative diseases, mainly AD, is becoming elucidated (Liu et al., 2003a) since elevated levels of carbonyl species have been determined in plasma and in different brain regions of AD patients (Picklo et al., 2002): free HNE, but not MDA, is elevated in plasma (McGrath et al., 2001) and in cerebrospinal fluid and ACR in the amygdala and hippocampus/parahippocampal gyrus of the AD brain compared with age-matched control subjects (Lovell et al., 2001). MDA accumulation (mainly in cytoplasm of astrocytes and neurons) has been immunohistochemically detected in the brain in both normal aging and AD, and the extent of deposition is similar in both conditions (Dei et al., 2002). Increased concentrations of MDA (plasma and serum) and of HNE (plasma and cerebrospinal fluid) of patients with Parkinson's disease have been also reported (Selley, 1998). HNE levels are significantly elevated in the sera and spinal fluid of ALS (amyotrophic lateral sclerosis) patients compared with control population, and positively correlate with the extent of the disease but not with the rate of progression, to suggest HNE as a possible biomarker of the disease (Simpson et al., 2004).

By considering the emerging functional/toxicological role of aldehyde/protein adducts in several human diseases, a new potential therapeutic strategy, based on the inhibition of ALE formation, has been developed in the last years. This review sheds focus on the state of the art of ALE's inhibitors, starting from fundamental studies on protein/aldehyde adducts formation and detection in biological matrices, with particular emphasis to HNE-, ACR-, and MDA-modified proteins, and providing an overview of the recently developed approaches for the treatment of many chronic diseases, the most promising based on aldehyde-sequestering agents. Opportunities exist for therapeutic intervention in these pathological states by use of certain water-soluble, low-molecular-weight drugs that contain thiols or primary amine groups. Such agents, administered orally, can form stable derivatives with toxic carbonyls and thus protect cellular components. The more deeply investigated drug candidates (sulfur compounds, cysteine- and histidine-containing peptides, aminoguanidine, pyridoxamine, hydrazinophthalazines), even if belong to different chemical classes, are nucleophilic compounds that react with carbonyls at a faster rate than do cell macromolecules. For all these compounds several aspects,

including reaction mechanisms, structure elucidation, and detection of conjugated products, and biological effects will be considered and discussed. Future studies of such carbonyl-trapping agents may include their use in combination with other classes of drugs, such as antioxidants, anti-inflammatory products, or neuroactive agents. This conceptually simple approach may offer new opportunities for the design of a new clinical management of many chronic disease states.

27.2 LIPOXIDATION-DERIVED REACTIVE ALDEHYDES

27.2.1 Mechanisms of Formation, Biological Effects, and Protein Adduction

The key feature of lipoxidation is the breakdown of polyunsaturated fatty acids (PUFAs) to yield a broad array of RCS (smaller fragments, three to nine carbons in length), the most reactive and cytotoxic being α,β-unsaturated aldehydes (HNE and ACR), di-aldehydes (MDA and glyoxal, GO), and keto-aldehydes (4-oxo-*trans*-2-nonenal, ONE) (Fig. 27.1). Among other saturated and unsaturated aldehydes, 2-hydroxyheptanal was found to be a major aldehydic product of lipid peroxidation of ω-6 PUFAs (linoleic acid, arachidonic acid), while 2-hydroxyhexanal (HHE) is generated in much lower yield. 2-Hydroxyalkanales with 8, 9, 10, and 11 C-atoms are derived from hydroperoxides of oleic acid (Loidl-Stahlhofen and Spiteller, 1994).

RCS such as GO and ACR are also produced during the myeloperoxidase-catalyzed metabolism of amino acids, and because the generation of reactive aldehydes by myeloperoxidase is nearly quantitative at plasma concentrations of L-serine, L-threonine, and chloride, phagocyte-mediated formation of these products may be of central importance in promoting tissue injury at sites of inflammation (Anderson et al., 1997). ACR has been shown to be produced from the oxidative deamination of spermine and spermidine catalyzed by polyamine oxidase (Sakata et al., 2003a, 2003b). ACR occurs also as an ubiquitous pollutant

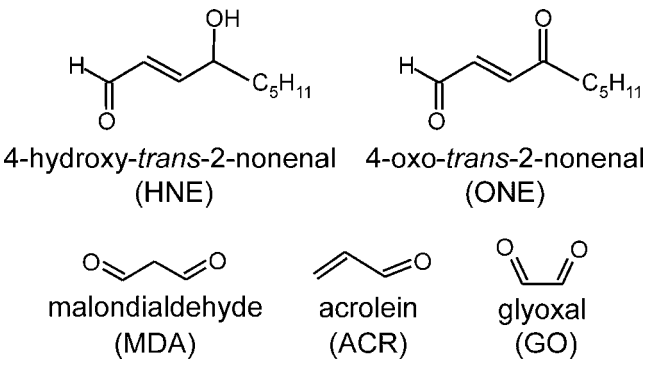


FIGURE 27.1 Structures of the most reactive lipoxidation-derived aldehydes.

in the environment (a by-product of overheating organic matter, formed during the incomplete combustion of petrol, coal, wood, and plastic material), and it is present in the gas phase of cigarette smoke in large amounts (25 to 140 μg/cigarette). Moreover ACR is a toxic metabolite formed during biotransformation of allyl compounds (allyl alcohol, allylamine) and of the widely used anticancer agent cyclophosphamide (Al-Rawithi et al., 1998).

4-Hydroxy-trans-2-nonenal (HNE) Among α,β-unsaturated aldehydes, HNE is one of the most abundant and toxic lipid-derived compound, generated through the β-cleavage of hydroperoxides from ω-6 PUFAs, through a mechanism recently proposed by Schneider et al., (2001). Because there are several excellent review articles published in recent years on the biological relevance of HNE (see Zarkovic, 2003 for a comprehensive list), starting in 1990 and 1991 with those done by Hermann Esterbauer (Esterbauer et al., 1990, 1991), who discovered the aldehyde, here we briefly summarize the key features of its reactivity toward nucleophilic target sites on proteins.

HNE reactivity is due to the conjugation of the double bond with the aldehyde function, which makes the C-3 carbon a strong electrophilic center, highly reactive with cellular nucleophiles via 1,2- and 1,4-Michael addition and, in particular, with the sulfhydryl groups of proteins leading to the formation of thioether adducts that further undergo cyclization to form cyclic hemiacetals (Esterbauer et al., 1991; Petersen and Doorn, 2004; Carini et al., 2004) (Fig. 27.2). Such Michael adducts have also been observed with peptides containing thiol groups such as glutathione (GSH), whose reaction with HNE is a well recognized pathway of detoxification in those biological systems (e.g., erythrocytes, liver tissue) where GSH concentrations are particularly high (Srivastava et al., 2000; Laurent et al., 2000). Anyway, the facile reactivity of HNE with thiols, which makes it an ideal substrate for GSH conjugation, results in depletion of cellular GSH and disruption of cellular redox status.

Beside cysteine (Cys) residues, HNE can also form Michael adducts with the imidazole moiety of the histidine (His) residues (Uchida and Stadtman, 1992) and the ε-amino group of the lysine (Lys) residues, which further undergo cyclization between the aldehyde moiety and the C-4 position of HNE to form a hemiacetal structure (Szweda et al, 1993; Nadkarni and Sayre, 1995) (Fig. 27.2). HNE reactivity has been also demonstrated with the ε-amino group of Lys (1,2-addition) to form a carbinolamine intermediate that rearranges and loses water to yield a Schiff base. Although the physiological relevance of such adduct is uncertain, because of the reversibility of the reaction, the Schiff base can eventually lead to the formation of a 2-pentylpyrrole derivative through an enammine intermediate (Sayre et al., 1993) (Fig. 27.2) and, to a lesser extent, to fluorescent cross-linking products.

Furthermore, since HNE is a difunctional molecule (electrophilic C-3 and carbonyl group), it can form intra- and intermolecular protein cross-links through Michael addition at C-3 followed by Schiff base formation (Cohn et al., 1996). Figure 27.3*a* exemplifies the cross-link formation between HNE and two nucleophilic residues (Hys and Lys). Although Cys is the most reactive residue toward

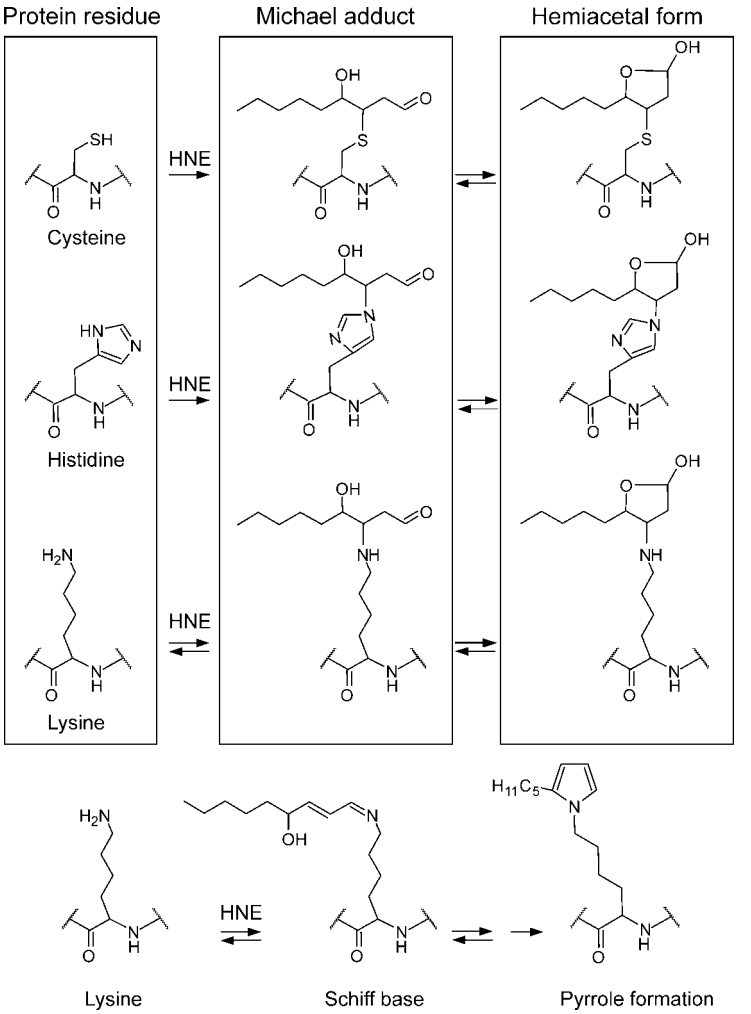


FIGURE 27.2 Interaction of HNE with the nucleophilic amino acid residues Cys, His, and Lys in peptides/proteins. (Reprinted from Carini et al., 2004, by permission of John Wiley and Sons.)

HNE if compared to His and Lys, the thiol adduct has been postulated to be less stable (Uchida, 2003) than His and Lys conjugates, due to a typical retro-Michael reaction (thiol is a better leaving group than His).

4-Oxo-trans-2-nonenal (ONE) The group of Blair (Rindgen et al., 1999; Lee and Blair, 2000) demonstrated for the first time that the iron-induced decomposition of 13-hydroperoxy-octadecadienoic acid (HPODE) results in the formation of a novel and unique end-product of lipoxidation, 4-oxo-2-*trans*-nonenal (ONE), a particularly potent electrophilic genotoxin that induces novel modifications to

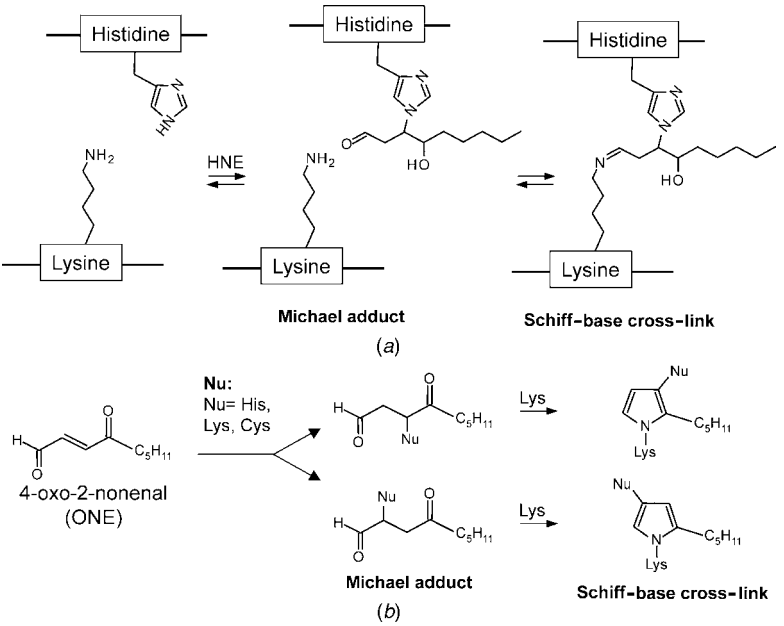


FIGURE 27.3 Proposed mechanisms for the formation of cross-linked protein by HNE (*a*) and ONE (*b*). (Panel *a* adapted from Carini et al., 2004, by permission of John Wiley and Son.)

free nucleobases and DNA (ONE should also form from arachidonic acid.) The functional group at C4 position makes the compound more reactive toward nucleophiles than HNE because the ketone moiety is potentially an additional target for nucleophilic species. Although the potential toxicological consequence of ONE protein adduction could be extensive protein cross-linking and aggregation, until now this potential has not yet been demonstrated. The reactions of ONE with nucleophilic sites in proteins has been recently investigated by Zhang et al., (2003) and Doorn and Petersen (2003). The predominant initial reaction appears to involve Michael addition to the central ONE double bond, more at C3 than at C2, to give substituted 4-oxo-nonanals. These adducts may then condense with the ε-amino group of Lys to give substituted pyrrole cross-links (Fig. 27.3*b*). In contrast to HNE reactivity, ONE was found to modify also peptides containing Arg residues (besides His, Lys, Cys) to give stable, covalent adducts. While the order potency for amino acid adduction is similar for HNE and ONE (Cys, His, Lys, Arg), the difference in absolute potency (i.e., rate constants) between the two aldehydes is remarkable, with ONE being much more reactive than HNE toward thiol and amine nucleophiles. The group of Sayre (Liu et al., 2003b) recently investigated the modification of proteins (myoglobin and apomyoglobin) induced by HNE and 4-oxo-2-nonenal ONE using mass spectrometric approaches.

Malondialdehyde (MDA) MDA in peroxidized biological samples results mainly from the oxidative degradation of PUFAs with more than two unconjugated double bonds: in mammalian tissues arachidonic acid (20:4) and docosahexaenoic acid (22:6), while oleic and linoleic acids are considered to be weak precursors. The mechanism of formation, proposed by Pryor and Stanley in 1975, involves the formation of bicycle-endoperoxides as intermediates, which subsequently break down to free MDA by thermal or acid-catalyzed reactions. In certain tissues MDA can also be formed by enzymatic processes, for example, by human platelet thromboxane synthetase from prostaglandins (PGH_2, PGH_3, and PGG_2) or by renal polyamine oxidase from spermine.

MDA cannot be considered as a highly reactive compound under physiological conditions, but at lower pH, when the β-hydroxyacrolein becomes the predominant species, its electrophilic character and reactivity is significantly increased. This makes the molecule able to react with nucleophiles in a Michael-type 1,4-addition reaction, similarly to other α,β-unsaturated aldehydes such as HNE or ACR. The primary 1:1 reaction product may further react to give 1:2 adducts (Fig. 27.4). Reaction of MDA with several amino acids has been fully investigated by Nair et al., (1981), and the reaction products characterized by different analytical approaches: under physiological conditions His, Tyr, and Arg react exclusively at the α-amino group to form the corresponding enaminals (1:1 adducts), and no 1:2 adducts are formed even at prolonged incubation times. Reaction of Cys with MDA at neutral pH leads to the formation of an adduct containing two Cys residues and three MDA molecules (Fig. 27.4).

Free MDA is removed from the bloodstream faster, with a half-life of two hours in rats (Slatter et al., 2004), but a significant proportion of MDA has a

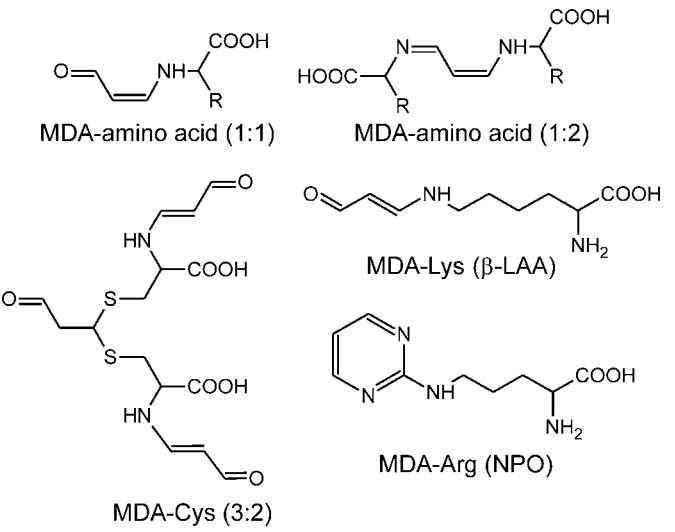


FIGURE 27.4 Structures of the reaction products of MDA with amino acids. In brackets the stoichiometric ratios.

longer half-life in vivo because it binds to protein amine groups (at least 80% MDA in tissues is bound reversibly to proteins in vivo). Lys or Arg are the only amino acids whose side chains react with MDA to give adducts, the semi-stable N^{ε}-β-lysiyl-amino-acrolein (β-LAA) (Slatter et al., 1998) and the stable N^{δ}-(2-pyrimidyl)-L-ornithine (NPO) (Slatter et al., 1999) (Fig. 27.4). The latter has been isolated from collagenous Arg reacted with MDA, and detected in human skin from a diabetic subject (Slatter et al., 2000). Compared with free amino acids, proteins are much more readily modified by MDA under physiological conditions. MDA reacts rapidly with apolipoprotein B of circulating low-density lipoproteins (LDL); it has been shown to cross-link bovine serum albumin to form dimers and also modifies ribonuclease A (RNase A), crystalline, hemoglobin, and collagen (Slatter et al., 2000). Such modified proteins, less susceptible to proteases, are formed much faster when protein is already glycated by carbohydrates.

Acrolein (ACR) Among the α,β-unsaturated aldehydes, ACR is by far the strongest electrophile and therefore shows the highest reactivity with nucleophiles such as thiols and amines (ACR reacts more than 100 times faster with GSH than HNE). The mechanism of reaction with GSH (and thiol groups in proteins) is the same as described for HNE, but in this case the 1:1 adduct does not cyclize and the free aldehyde function may undergo secondary reaction (i.e., Schiff's base formation). In proteins, ACR reacts preferentially with Cys, Lys, and His residues: of these Lys generates the most stable product. The reaction of ACR with Lys results in β-substituted propanals (R–NH–CH_2–CH_2–CHO) and Schiff's bases (R–NH–CH_2–CH_2–CH=N–R), but the major adduct formed on reaction with protein is the N^{ε}-(3-formyl-3,4-dehydropiperidino)lysine adduct (FDP-Lys) (Fig. 27.5*a*), whose mechanism of formation has been elucidated by Uchida (1999). By amino acid analysis Uchida et al., (1998a) demonstrated the presence of the adduct not only in the ACR-treated low-density lipoproteins (LDL) but also in LDL exposed to metal-catalyzed oxidation. Furuhata et al., (2003), using oxidized B chain of insulin as a model peptide, were able to characterize a novel ACR-Lys adduct, N^{ε}-(3-methylpyridinium)lysine (MP-Lys). This condensation adduct, recognized as an intrinsic epitope of a monoclonal antibody 5F6 raised against ACR-modified protein, represents the major antigenic product generated in ACR-modified protein. Figure 27.5*a* reports the proposed mechanisms for the formation of FDP-Lys and MP-Lys.

ACR quickly depletes cellular GSH levels (Haenen et al., 1998), and it has been postulated to inactivate the reductase responsible for reducing vitamin E radicals, leading to exacerbation of lipoxidation processes. Kehrer and Biswal (2000), in reviewing the available literature on the molecular effects of ACR, evidenced that the mechanism of such effects may be related to the ability of ACR to deplete cellular thiols, and/or to effects on gene activation, either directly or subsequently to effects on transcription factors, particularly those that are redox regulated. The acute effects of ACR have been extensively investigated in several experimental models (Tsakadze et al., 2003) and the cytotoxic effects have been mainly attributed to its alkylating properties and high reactivity toward

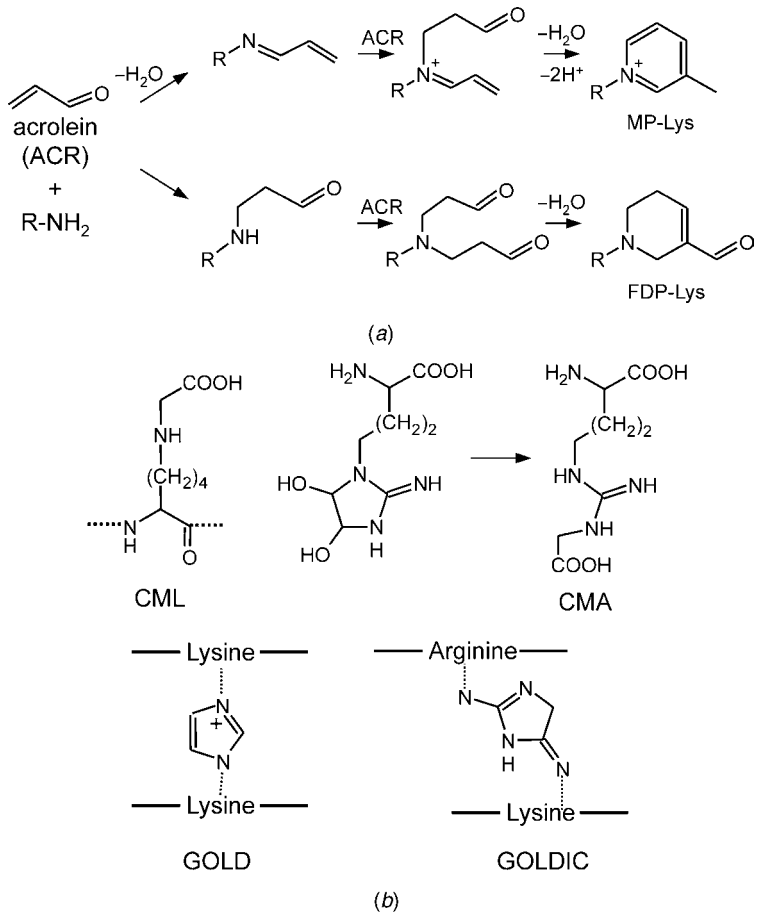


FIGURE 27.5 Proposed mechanisms of FDP-lysine and MP-lysine formation (*a*) and products of the reaction of GO with protein amino acids (*b*). (Panel *a* adapted from Furuhata et al., 2003, by permission of the American Society for Biochemistry and Molecular Biology.)

nucleophiles. ACR causes chromosomal aberrations, sister chromatid exchanges and point mutations, and reduces the colony-forming efficiency of mammalian cells (Ghilarducci and Tjeerdema, 1995).

Glyoxal (GO) GO, a well-established α-ketoaldehyde intermediate formed by oxidative degradation of glucose and degradation of glycated proteins, and ascorbate autoxidation, has been demonstrated to be also a product of lipoxidation reactions (Mlakar and Spiteller, 1994; Fu et al., 1996). GO formation has been indeed observed during UV irradiation of PUFAs, during oxidation of linolenic acid (in an iron-ascorbate model system), and autoxidation of arachidonic acid.

GO reacts with the free amino groups of proteins forming Schiff bases (by a nonenzymatic reaction called glycation) that undergo rearrangement to form relatively stable ketoamines (Amadori products). The glycated biomolecules undergo further dehydration, cyclization, oxidation, and rearrangement to generate advanced glycation end-products (AGEs). GO reacts with Lys to form N^{ε}-(carboxymethyl)lysine (CML) (Fig. 27.5*b*), and with Arg to give N^{7}-carboxymethylarginine CMA (Fig. 27.5*b*) (Glomb and Lang, 2001). Dihydroxyimidazolidine adducts have been recently recognized as the primary products of modification of RNase A by GO, Arg39, and Arg85 (which are closest to the active site of the enzyme) being the primary sites of modification (Cotham et al., 2004). The dicarbonyl structure of GO makes it able to react with two Lys residues to form protein imidazolium cross-links (i.e., the GO-Lys dimer, GOLD) and imidazole cross-links (i.e., the GO-Lys-Arg dimer, GODIC) (Wells-Knecht et al., 1995; Lederer and Klaiber, 1999) (Fig. 27.5*b*). The ability of GO to cross-link proteins has been widely reported and elevated levels of GOLD have been measured in serum of uremic and hemodialysis patients (Odani et al., 1998). Although it has been established that a free amino group (either as the *N*-terminus or as a Lys residue), but not Arg, is essential for cross-linking (Meade et al., 2003; Miller et al., 2003), many of the GO cross-links likely occurring in vivo remain uncharacterized, because the molecular mechanisms of the crosslinking reaction need to be fully elucidated.

The biological and toxicological effects of GO have been recently reviewed (Shangari et al., 2003; Shangari and O'Brien, 2004): GO induces cell damage and accelerates the rate of glycation, leading to the formation of advanced glycation end-products (AGEs). Oxidative stress induced by GO seems to play a key role in the damaging effect, but the mechanism of cytotoxicty has not been definitively elucidated.

Isoketals (IsoK) The most interesting products resulting from arachidonic acid oxidation *via* the H2-isoprostane pathway are the highly reactive γ-ketoaldehydes, first identified by Zagorski and Salomon in 1982 as rearrangement and fragmentation products of the bicyclic endoperoxide prostaglandin H_2 (levuglandin E_2 and D_2), and now termed isoketals (IsoK) (Fig. 27.6*a*). The reactivity of IsoK (compared to that of α,β-unsaturated aldehydes) toward nucleophilic sites of proteins, as well as the rapidity of adduction have been recently reviewed by Davies et al., (2004). IsoK react with Lys residues by the same mechanism described for α,β-unsaturated aldehydes, with formation of a reversible imine (Schiff base). In contrast with α,β-unsaturated aldehydes, the remaining carbonyl function can undergo intramolecular nucleophilic attack by the amine to give an unstable intermediate pyrrolidine adduct that undergoes dehydration to give an irreversible pyrrole adduct (Fig. 27.6*b*). Further oxidation of the pyrrole adducts makes IsoK more prone to protein–protein cross-links. The review by Davies et al., (2004) covers many other important aspects, including the effects of IsoK on cellular functions, in vivo detection, localization and quantitation of IsoK adducts, and identification of IsoK adducted proteins.

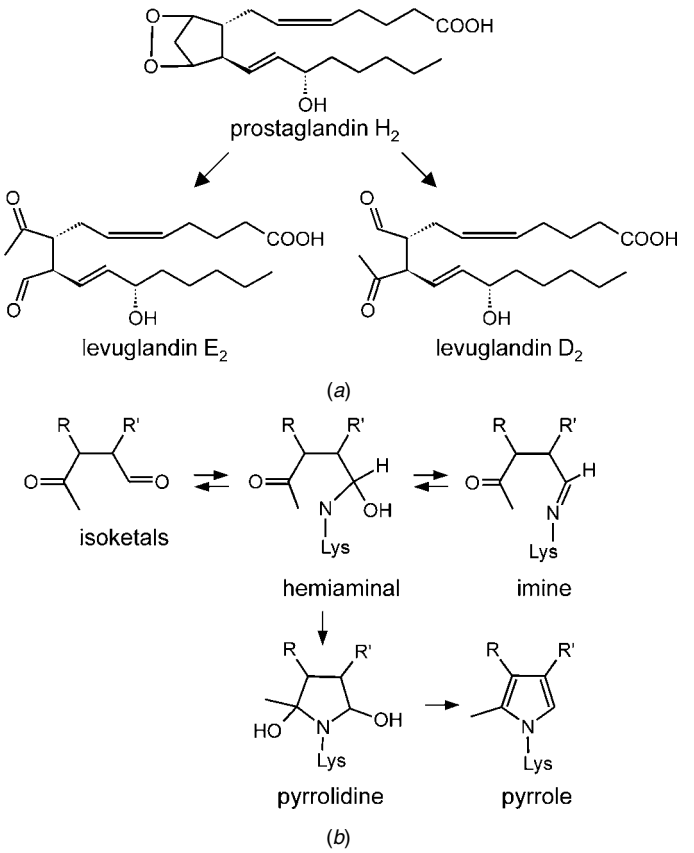


FIGURE 27.6 Isoketals formation (*a*) and proposed mechanism of reaction with lysine (*b*). (Adapted from Davies et al., 2004, by permission of Elsevier.)

27.2.2 Protein-Aldehyde Adducts: Occurrence and Detection in Biological Matrices

Methods for Detection Immunologic detection is a powerful tool that can be used to evaluate the presence of a desired target and its subcellular localization. Major advantages of this technique over the chemical approaches are the evaluation of small numbers of cells or archival tissues that may otherwise not be subject to analysis. The development of specific antibodies against protein-bound HNE has made it possible to obtain highly probable evidence for the occurrence of oxidative stress in vivo. In particular, antisera raised against HNE, polyclonal antibodies, allowed the immunochemical detection of HNE-protein conjugates in rabbit and human atherosclerotic lesions, human renal carcinoma cells, hepatocytes of carbon tetrachloride treated rats, and renal proximal tubes of iron/nitrilotriacetate treated rats (Uchida, 2003).

These results first demonstrated the causal involvement of HNE in the lipid peroxidation pathology and gave conclusive evidences for aldehyde modified proteins in the peroxidized biological samples. However, polyclonal antibodies, even if purified by affinity chromatography, exhibit a relatively broad specificity and are therefore of limited value for unequivocal characterization of HNE-positive epitopes in complex biological samples, such as cells, subcellular fractions or lipoproteins. Following independently the same line of research, Esterbauer and Uchida's groups (Waeg et al., 1996; Uchida et al., 1995) prepared anti–HNE-histidyl peptide antibodies, which were found to be highly selective for HNE bound to His (HNE-His) since cross-reaction with HNE bound to Lys or Cys was negligible.

The use of anti–HNE monoclonal antibodies has recently reached a great popularity and wide applications both in immunohistochemistry and immunoblot analyses, since it is highly suitable to confirm HNE involvement in several physiopathological conditions such as atherosclerotic lesions (Uchida et al., 1995), nigral neurons in Parkinson's disease (Yoritaka et al., 1996), actinic elastosis (Tanaka et al., 2001), diabetes (Traverso et al., 1998), pancreatites (Reinheckel et al., 1998), liver diseases (Li et al., 1997), and Alzheimer's disease, particularly in neurofibrillary tangles (the major diagnostic lesion in AD brain) and in senile plaques (Sayre et al., 1997).

Although the unequivocal evidence given by the immunological analysis, this approach does not give information on the chemical structure of the aldehydes-protein adducts. In the last decade the increases in the accessible mass ranges of modern instruments and advances in ionization methods have made possible a fundamental improvement in the analysis of protein-HNE adducts by mass spectrometry, and in particular by matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) tandem mass spectrometry, new focus was given to bioanalysis. The data so far available in the literature (Carini et al., 2004) have shown that MS-based techniques play a key role for the study of the kinetics and mechanisms of HNE-protein interaction: the first applications were focused on measuring the molecular masses of the reaction products between HNE and proteins/peptides, leading to estimate the stoichiometry of the reaction and giving data consistent with modification via addition rather than condensation processes. Complete structure assignment of protein-HNE adducts was obtained by trypsin digestion followed by peptide mass mapping, mainly by HPLC-ESI-MS/MS techniques. Until now the MS approaches have been applied, with few exceptions, to characterize HNE adducts in vitro in systems (incubation of the target peptide/protein with HNE in phosphate buffer), often forcing the reaction with large and nonphysiopathological HNE concentrations, and only recently to detect peptide-HNE adducts as metabolites (GSH and histidine containing dipeptides as conjugating agents) directly in crude biological matrices as cellular and extracellular media and tissues extracts (rat skeletal muscle and rat retina) (Carini et al., 2004), but the rapid technical developments in the areas of ionization techniques, mass analyzers, and data analysis software will ensure that MS will play an even greater role in this biological/toxicological field in the near future.

HNE, ACR, and MDA Protein Adducts For what concerns protein-bound aldehydes and cardiovascular disease, the adduction of aldehydes to apolipoprotein B in LDLs has been implicated as the mechanism by which LDL is converted to an atherogenic form that is recognized by macrophage scavenger receptors far more readily than normal LDL, leading to the formation of cholesterol-engorged foam cells. Thus, if less than 15% of Lys side chains are modified, the LDL is still recognizable to its receptor; if more than 15% are modified, however, only the scavenger receptor can recognize LDL (Slatter et al., 2000). The clinical relevance of the reaction between MDA and proteins is highlighted in atherosclerosis: MDA-LDL, in addition to oxidized LDL, mediates several pro-inflammatory and pro-atherogenic processes, all of which ultimately lead to foam cell generation. Atherosclerotic lesions of varying severity from human aorta contain indeed material recognized by antibodies raised against specific aldehyde adducts other than HNE-His, such as MDA-Lys and ACR-Lys (FDP-Lys) (Uchida, 2000) in which intense positivity is generally associated with cells, primarily macrophages.

The extent and time course for the formation of HNE-modified proteins during ischemia and ischemia plus reperfusion were evaluated in isolated rat hearts using an antibody to HNE-Cys/His/Lys and densitometry of Western blots (Eaton et al., 1999). The increased HNE immunofluorescence was primarily localized to the sarcolemma, suggesting that the membrane is indeed the major site of formation of HNE-modified proteins, but HNE immunofluorescence also showed longitudinal striations with possible co-localization with the myofibrils, suggesting possible modification of contractile proteins. Considerable evidence now suggests that the myofilaments are also an important target for oxidant stress during ischemia-reperfusion, and thus myofilament dysfunction may be related to the formation of HNE adducts with key contractile proteins. Using ventricular myocytes isolated from adult male rats, it was recently demonstrated that HNE inhibits cardiac myocyte contraction (Aberle et al., 2004). In this context we have indeed recently demonstrated, by a combined mass spectrometric and computational approach (Aldini et al., 2005), that actin isolated from rabbit skeletal muscle, in both monomeric (G) and polymeric form (F), readily reacts with HNE, with the formation of a Cys374 Michael adduct.

The importance of lipid-derived MDA in diabetes mellitus has been highlighted by Slatter et al., (2000), because the initial modification of collagen by sugar adducts forms a series of glycation products, which then stimulate breakdown of the lipids to MDA and hence further cross-linking by MDA of the already modified collagen (formation of pyridyl cross-links). This inter-and intramolecular cross-linking of collagen by MDA, leading to a change of the macromolecule properties and to modified cell–matrix interactions, is particularly important in the late complications of diabetes mellitus because it contributes to the stiffening of the cardiovascular tissue. The status of oxidative stress in the serum of type 2 diabetes mellitus has been confirmed in human subjects by measuring serum HNE-modified albumin by the use of a specific monoclonal antibody (HNEJ-2) against HNE-histidine adducts, as well as an antibody against human serum albumin (Toyokuni et al., 2000); the results revealed significantly higher

levels of HNE-modified albumin in serum of type 2 diabetes outpatients than in the matched nondiabetics.

The monoclonal antibody to acrolein-modified protein (mAb5F6), developed by Uchida et al., (1998b), is specific to the FDP-Lys and clearly able to distinguish the ACR-modified bovine serum albumin (BSA) and oxidized LDL from native BSA and LDL. The specificity of mAb5F6 has been confirmed in fatty streak lesions of human arterial tissue. Protein-bound ACR has been immunohistochemically detected in plaque deposits in atherosclerosis (Uchida, 1998b), in serum of patients (end-stage renal disease) receiving hemodialysis, where levels were found to be significantly elevated compared to those of healthy individuals (Noiri et al., 2002), and in AD (Calingasan et al., 1999). The levels of protein-bound ACR in human plasma have been recently measured by an immunoassay system. In this way it was possible demonstrate that the extracellular concentrations of protein-bound ACR in vivo could reach submillimolar range (Sakata et al., 2003a, b).

Protein adduction of HNE or ACR creates new epitopes whose formation has led to the development of immunohistochemical methods to identify cell types and subcellular compartments affected by carbonyl stress in AD brain. Most of the HNE-protein adducts are localized to neuronal cytoplasm and neurofibrillary tangles, but not neuritic plaques (Picklo et al., 2002), while ACR has been found to be incorporated into proteins to give adducts that co-localize with neurofibrillary tangles and distrophic neuritis surrounding senile plaques, but no adducts were observed in the amyloid core of plaques (Calingasan et al, 1999). This may be explained by the fact that ACR preferentially reacts with Lys residues, which are prominent components of tau, the primary constituent of neurofibrillary tangles. Lipoxidation adducts are not only increased in neuronal cell bodies of AD cases, but are also present in axons in the white matter of different aged cases: immunoblotting analysis of axonal proteins indicated that the major species displaying HNE adducts were 200 kDa and 150 kDa proteins (heavy and medium subunits of neurofilament) (Wataya et al., 2002). This indicates that modification of specific macromolecules, rather than aspecific oxidation, is involved in the disease.

The identification of specific proteins that may be more susceptible to carbonyl modification has been investigated by Aksenov et al., (2001): β-actin, β-tubulin, and creatine kinase BB were identified, by reaction with dinitrophenylhydrazine followed by two-dimensional electrophoresis, as putative proteins susceptible to RCS attack.

Protein-bound ACR, besides the MDA-Lys and HNE-protein adduct, have been also detected in human renal tissues (glomerular lesions) from patients with diabetic nephropathy (Suzuki and Miyata, 1999), and distinct deposition of MDA and HNE adducts has been immunohistochemically demonstrated in kidney (especially in the glomerular epithelial cells) of patients with congenital nephritic syndrome, a human model disease of proteinuria, with a reciprocal local decrease of phospholipids hydroperoxide glutathione peroxidase (PHGPx),

the major enzyme system protecting against lipid peroxidation in glomeruli (Solin et al., 2001).

More recently ACR-Lys adducts have been detected in plasma LDL and in the aorta of cyclophosphamide-treated animals by agarose gel electrophoresis, immunoblot, and immunohistochemical methods (Arikketh et al., 2004). High levels of HNE-modified mitochondrial proteins were also found in hearts from old rats, when compared with those from young rats (Moreau et al., 2003), and very recently the same research group (Moreau et al., 2005) found elevated CML levels in serum, aorta, and heart proteins from old F344 rats (serum albumin, transferrin, and immunoglobulins were most prominently adducted by both CML and HNE).

The status of oxidative stress and its relationship to the degree of prematurity and clinical condition in neonates have been recently evaluated by Tsukahara et al., (2004), by studying three groups of subjects: healthy term neonates, clinically stable preterm neonates requiring no supplemental oxygen, and clinically sick preterm neonates requiring supplemental oxygen and ventilator support. In the sick preterm group, neonates developing active retinopathy showed significantly higher urinary levels of ACR-Lys adducts than the other neonates without retinopathy.

The presence of hepatic MDA- and HNE-modified proteins has been documented in different animal models of chronic alcohol ingestion and in humans with advanced stages of chronic liver disease (Kono et al., 2001; Niemela et al., 1999, 2003; Paradis et al., 1997a, 1997b; Sampey et al., 2003).

Formation of α,β-unsaturated aldehydes from ultraviolet-induced peroxidation of epidermis and dermis lipid constituents has been recently proposed as a key factor in the oxidative-dependent skin damage. Both ACR and HNE have been associated with actinic elastosis, which in photodamaged skin of aged individuals is characterized by the accumulation of fragmented elastic fibers in the sun-exposed areas. Using antibodies against ACR and HNE in human skin specimens obtained from sun-damaged areas, it was demonstrated (Tanaka et al., 2001) that both antibodies react with the accumulations of elastic material. Double immunofluorescence labeling demonstrated that ACR/elastin and HNE/elastin were co-localized in the actinic elastosis.

Other Reactive Aldehydes/Protein Adducts The peroxidation of ω-3 PUFAs, such as docosahexaenoic acid and eicosapentaenoic acid, generates a HNE-related compound, 4-hydroxy-2-hexenal (HHE), but until now, the endogenous production of HHE in vivo has not been determined. Although long-chain ω-3 PUFAs are usually consumed in small quantities, they are readily incorporated into atherosclerotic-plaque lipids (Thies et al., 2003). Thus it is likely that the incorporation of ω-3 PUFAs into plaque lipids results in the enhanced production of HHE and its protein adducts in the lesions. In a recent study by Yamada et al., (2004), using a monoclonal antibody (MAb) directed to the protein-bound HHE as an index of the peroxidation of ω-3 PUFAs, it was confirmed that human atheromatous lesions contain protein-bound HHE, co-localizing mainly with foamy

macrophages. By immunohistochemical and enzyme-linked immunosorbent assay (ELISA) techniques, Shibata et al., (2004) have demonstrated that protein-bound HHE was prominent in the entire gray matter of spinal cords obtained at autopsy from 10 sporadic ALS (amyotrophic lateral sclerosis) patients, and localized in the neurons, reactive astrocytes, microglial cells, and the surrounding neuropil, while the immunoreactivity was obscure or undetectable in the control cases. This finding suggests that the selective vulnerability of motor neurons to HHE mediates the pathomechanism of this disease.

Using a monoclonal antibody directed to a trihydropyridinone structure, formed from Lys and 2-hydroxyheptanal, another reactive aldehyde species generated during the peroxidation of ω-6 PUFAs, it was demonstrated (Itakura et al., 2003) that the antigenic structure was present in atherosclerotic lesions of the human aorta.

27.3 INTERVENTION AGAINST LIPOXIDATION-DERIVED CARBONYL STRESS: ALE INHIBITORS

27.3.1 Advanced Lipoxidation End-Products (ALEs)

Carbonyl stress can be considered the imbalance of RCS production and carbonyl scavenging mechanisms that originate from a multitude of mechanistically related pathways, like glycation, autooxidation of sugars, lipid peroxidation, and UV damage (Rahbar and Figarola, 2003). As discussed above, decomposition of lipid peroxides initiates chain reactions that lead to the formation of a variety of RCS, among which MDA, HNE, and ACR, by reacting with Cys, His, and Lys residues in proteins, generate characteristic ALEs. Most of them, MDA-Lys (Schiff base adduct), HNE-Lys (Michael adduct), HNE-Lys (pyrrole derivative), FDP-Lys, levuglandin adducts (pyrrole derivatives), and N^{ε}-(hexanoyl)lysine (hexanoic acid amides), have been detected in oxidized lipoproteins and/or atherosclerotic lesions by immunohistochemical or chemical assays (Baynes and Thorpe, 2000; Baynes, 2003; Thorpe and Baynes, 2003). It must be emphasized that the residual aldehyde groups in some ALEs (Schiff base and Michael adducts) can further react to give protein cross-links and fluorescent products that are very similar to AGEs. The poorly characterized lipofuscin, the nondegradable intralysosomal fluorescent pigment that accumulates with age in postmitotic cells, is a recognized hallmark of aging occurring with a rate inversely related to longevity that is now considered also to be the residual debris from lipooxidation reactions, since its formation proceeds through iron-catalyzed oxidation/polymerization of protein and lipid residues (Terman and Brunk, 2004).

Unlike lipoxidation, reaction of carbohydrates with proteins leading to Amadori adducts takes place under anaerobic conditions, as well as their further rearrangement to give more reactive dicarbonyl compounds such as GO or methylglyoxal (MGO) that react with Lys or Arg residues in protein to give AGEs. Some of these, such as pentosidine (Arg-Lys cross-link) and di-Lys cross-links, which require oxygen for their formation (glycoxidation products), have been detected by instrumental and immunohistochemical techniques in aged and diabetic tissue protein (Tessier et al., 1999).

Other compounds have been detected and quantified in tissue protein that, since they can originate from either carbohydrates or lipids, are collectively termed as EAGLEs (either advanced glycation or lipoxidation end-products), the most important biomarkers being CML and N^{ε}-(carboxyethyl)lysine (CEL). Fu et al., (1996) reported for the first time that CML, a well-known AGE product formed on protein by combined nonenzymatic glycation and oxidation (glycoxidation) reactions, is also formed during metal-catalyzed oxidation of PUFAs (arachidonic and linoleic acids) in the presence of protein. For this reason CML may be a general marker of oxidative stress and long-term damage to protein in aging, AD, atherosclerosis, and diabetes (Girones et al., 2004). For comprehensive reviews on these marker compounds, see Baynes and Thorpe (2000), Baynes (2003), and Thorpe and Baynes (2003). The fact that CML and CEL, among the major chemical modifications of tissue proteins, are derived from both carbohydrates and lipid precursors emphasizes the intersection between carbohydrate and lipid chemistry. All these species—ALEs, AGEs, and EAGLEs, and their precursors, the soluble reactive intermediates HNE or MDA—are not only cytotoxic per se, but they also behave as mediators and propagators of oxidative stress and tissue damage.

Oxidative stress and oxidative damage to tissues are common end points of chronic diseases, such as atherosclerosis, diabetes, neurodegenerative diseases, and rheumatoid arthritis. It has been proposed (Baynes and Thorpe, 1999) that the increased chemical modification of proteins by carbohydrates and lipids in chronic diseases is the result of overload on metabolic pathways involved in detoxification of RCS, leading to a general increase in steady state levels of these compounds formed by both oxidative and nonoxidative reactions (actually in both diabetes and uremia, not only glycoxidation and lipoxidation products are increased but also the products of reaction of proteins with dicarbonyl compounds formed by nonoxidative mechanisms). The increase in glycoxidation and lipoxidation of tissue proteins may therefore be viewed as the result of increased carbonyl stress. According to this view, and considering the complexity of pathways and reactions involved in ALE, AGE, and EAGLE formation, the therapeutic approaches to inhibit the nonenzymatic modification of proteins should be mainly based on the efficient control of the sequence of events at the earliest possible stage.

27.3.2 Aldehyde-Sequestering Agents

A recently developed strategy to attenuate damage by reactive aldehydes focuses on endogenous and exogenous compounds that can deactivate or degrade cytotoxic carbonyls. These are the "aldehyde-sequestering agents" that, in behaving as sacrificial nucleophiles, spare cell macromolecules and thereby slow disease progression (Shapiro, 1998; Burcham et al., 2002). Overwhelming evidence indicates that trapping of reactive carbonyl intermediates is a useful strategy for inhibiting or restraining carbonyl stress-associated pathologies. Even if this strategy does not completely abolish or contain the oxidative stress, it can reduce the toxicological consequences of RCS attack and slow down the progression of pathological events.

Several drug candidates have been proposed to inhibit the formation of AGEs in diabetes, including aminoguanidine, pyridoxamine, OPB-9195, nucleophilic compounds able to trap RCS intermediates in AGE formation. Although no specific drugs have been designed to inhibit ALE formation, growing evidences indicate that the AGE inhibitors so far studied are also able to restrain the chemical modifications of proteins induced by lipoxidation products, due to the general role of intermediate reactive carbonyl species in AGE, ALE, and EAGLE formation. A detailed description of these drug candidates, including their mechanism of reaction with ALEs and their pharmacodynamic profiles, is provided in the following paragraphs.

Glutathione (GSH) and Sulfur Compounds Metabolism of reactive intermediate aldehydes to less reactive molecules is a prerequisite to ensure cell surviving and normal functioning. Conjugation with GSH to give Michael adducts, catalyzed by GSH-S-transferases (GSTs), is a well-recognized pathway of detoxification in living cells, and HNE metabolism has been extensively studied in different biological systems (erythrocytes, liver slices, smooth muscle cells, enterocytes, endothelial cells, synovial fibroblasts, isolated perfused heart, liver, intestine, kidney, urine, and bile). Comprehensive reviews on the intracellular metabolism and on the metabolic fate of HNE in vivo have been recently published by Siems and Grune (2003), Alary et al., (2003), and Petersen and Doorn (2004). The aldehydic function in the GSH-HNE Michael adduct can further undergo to oxidation/reduction enzymatic processes to give the corresponding alcohol and carboxylic derivatives. In vivo, GSH conjugates are further metabolized to mercapturic acid (MA) derivatives, and the metabolic pathway for the renal transformation of GSH conjugates to MA involves cleavage of γ-glutamyl moiety by γ-glutamyltranspeptidase, followed by removal of glycine by dipeptidase and N-acetylation by cysteine conjugate-N-acetyltransferase.

Since epidermal cells are potential targets of HNE generated from UV-induced peroxidation of epidermis and dermis lipids, we have recently investigated its metabolic fate in a human keratinocytes cell line (Aldini et al., 2003). The main conjugated metabolites with GSH were identified, to confirm a key role of the GSH-dependent detoxification pathway in the skin. The evidence is that accumulation of carbonyl compounds in photodamaged skin is consequent to a metabolic impairment of the defense mechanism (GSH depletion and/or inactivation of enzyme systems).

Because GSH depletion impairs the detoxification of RCS and potentiates the formation of AGEs and ALEs, repletion of the endogenous GSH pool may prove useful by supplementation with GSH precursors, N^{α}-acetyl-L-cysteine (NAC) or Cys. Moreover, on the basis of the well-documented facile reactivity of thiols with unsaturated aldehydes, another simple approach to afford pharmacological protection against lipoxidation-derived carbonyl stress is based on thiol scavengers, selective for HNE and ACR, such as NAC. Neely et al., (2000) compared the potency of NAC and some lipophilic congeners (N^{α}-acetyl-L-cysteine methylester and N^{α}-pentanoyl-L-cysteine) to that of aminoguanidine (AG, a well-known AGE

inhibitor, see the aminoguanidine paragraph), to entrap HNE and to prevent some toxic effects of HNE in multiple biological models. Both NAC and AG had comparable chemical reactivity with HNE, but only NAC and its congeners have been shown to block HNE-protein adduct formation in vitro and in neuronal cultures. Moreover NAC and its congeners, but not AG, effectively protected brain mitochondrial respiration and neuronal microtubule structure from deleterious effects of HNE contributing to neurodegeneration in AD. For these reasons NAC and its lipophilic derivatives could be considered drug candidates as neuroprotectants. In fact, NAC has been already employed in human subjects to treat central nervous system diseases other than AD (Selwa, 1999).

In a long-term comparative trial of NAC, oxerutin, and taurine in experimental diabetes, induced by streptozocin (STZ) in rats, it was found that the combined NAC plus taurine treatment resulted in reduced accumulation of collagen-linked fluorescence in skin (Odetti et al., 2003). All treatments except taurine reduced also glomerular accumulation of EAGLEs (CML) and protected against the increase in glomerular volume typical of diabetes.

Very recently (Hsu et al., 2004) it has been demonstrated that the intake of NAC and other hydrophilic cysteine-containing compounds naturally present in *Allium* plants (garlic and onion), such as *S*-allyl cysteine, *S*-ethyl cysteine, *S*-methyl cysteine, and *S*-propyl cysteine, markedly improved diabetic complications, and significantly reduced lipid oxidation (MDA levels) in diabetic mice.

An alternative pharmacological approach to restrain the deleterious effects of RCS is to increase the levels of those enzyme systems massively involved in their detoxification, and several sulfur compounds have been explored as potential therapeutic agents for their ability to increase antioxidant response element (ARE)-inducible genes (the promoters for the genes of GSTs contain ARE). Some examples are represented by naturally occurring isothiocyanates and sulforophane (Nakamura et al., 2000).

In a systematic study performed by Wondrak et al., (2002), based on the development of a new screening method for selective identification of α-dicarbonyl scavengers (exclusion of any antioxidant/metal chelation effect), it was found that the cysteine derivative 3,3-dimethyl-D-cysteine (D-penicillamine) is a potent carbonyl trapping agent, being able to protect human skin keratinocytes and fibroblasts against GO- and MGO-induced carbonyl toxicity. The mechanism of reaction has been investigated under physiological conditions and the structure of the reaction products between MGO and phenylglyoxal (but not GO) elucidated: D-penicillamine readily reacts with the aldehyde group forming thiazolidine derivatives. Although severe systemic toxicity upon long-term administration has been described in patients taking high doses of D-penicillamine (Levy et al., 1983)—the drug is used in copper storage disease (Wilson's disease), cystinuria, heavy metal-ion intoxication, and as a second-order medication for rheumatoid arthritis—the irreversible reactivity toward electrophilic α-dicarbonyls and the expected micromolar tissue concentrations of the target α-dicarbonyls may qualify this drug and derivatives as potential protective agents against carbonyl stress.

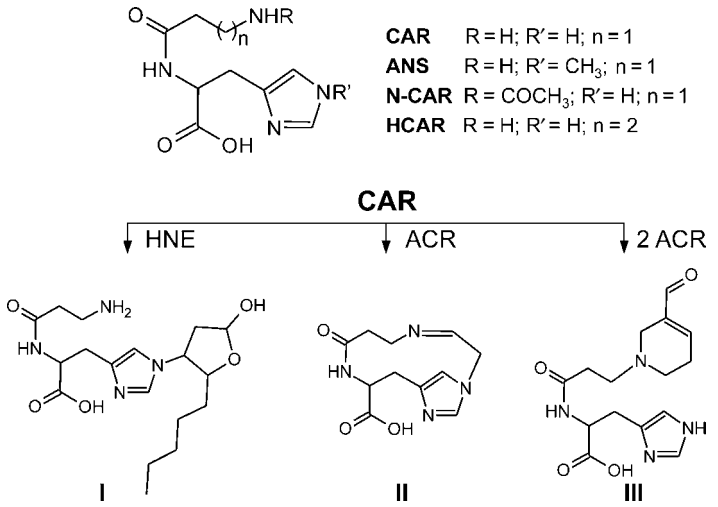


FIGURE 27.7 Histidine-containing dipeptides and structures of the adducts between carnosine (CAR) and HNE or ACR.

Other thiol compounds showed inhibitory activity, but even at the highest concentration tested (10 mM), L-cysteine-*O*-methylester, L-Cys, and NAC achieved only 50% inhibition in the screen (better inhibition was observed with the thiol cysteamine and the mercaptoimidazole derivative L-ergothioneine). On the basis of these results, the α-amino-β-mercapto-β,β-dimethylethane moiety was identified as the structural requirement for effective α-dicarbonyl scavenging (by 2-acyl-5,5-dimethyl-thiazolidine formation), a promising pharmacophore for development of related α-dicarbonyl scavengers as potential therapeutic agents.

Histidine Containing Peptides: Carnosine and Analogues The dipeptide carnosine (β-alanyl-L-histidine, CAR) is the archetype of a variety of aminoacyl histidine dipeptides (HD) such as homocarnosine (γ-amino-butyryl-histidine, HCAR), and anserine (β-alanyl-L-1-methylhistidine, ANS) (Fig. 27.7), widely distributed in vertebrate organisms and particularly abundant in excitable tissues (Crush, 1970) such as nervous system (Bonfanti et al., 1999) and skeletal muscles (Stuerenburg, 2000). CAR is a very water soluble compound, biosynthesized from β-alanine and histidine by carnosine synthase (Horinishi et al., 1978), and degraded by a specific metal-ion dependent homodimeric dipeptidase (carnosinase), recently characterized by Teufel et al., (2003). The activity and location of these anabolic and catabolic enzymes regulate the tissue distribution of CAR and related dipeptides.

The biological function(s) of HD still remain enigmatic, although for HCAR and CAR several hypotheses have been proposed. In the nervous system, HCAR was suggested to act as a γ-aminobutyric acid (GABA) reservoir, through the

control of one or several carnosinases (Petroff et al., 2001), while CAR meets many criteria as a neurotransmitter and/or neuromodulator, in particular, in the olfactory pathway (Margolis, 1974). In skeletal muscles, since present in a mM concentration range, CAR seems to play a crucial role as cytosolic buffer to neutralize lactic acid (Abe, 2000). Moreover several observations and experimental data suggest that CAR may exert important roles as an anti-aging molecule since (1) it can delay senescence in cultured human fibroblasts, (2) it can reverse the senescent phenotype in cultured human cells, (3) it is present in long-lived mammalian tissues at surprisingly high concentrations, which seem to decline with age, and (4) the intramuscular concentrations of CAR appear to directly correlate with species maximal life and span in mammals (Hipkiss and Brownson, 2000).

Hipkiss first considered that the anti-aging effect of CAR could be due, at least in part, to its ability to react with electrophilic aldehydes and ketones thus to prevent protein-peptide carbonylation (Hipkiss, 1998). In particular CAR is able to inhibit protein cross-linking induced by a range of potential toxic aldehydes including aldose and ketose sugars, trioses, MDA, acetaldehyde, and formaldehyde, presumably acting as a sacrificial nucleophile (Hipkiss et al., 1994, 1997; Hipkiss and Chana, 1998). The role of CAR as a naturally occurring anti-glycating agent is reinforced by the fact that carnosine's structure mimics the preferred protein glycation sites, that is, a target amino group with proximal imidazole and carboxyl groups (Hipkiss, 1998). In addition CAR was found to quench lipoxidation end-products such as nonenal and HNE (Zhou and Decker, 1999). Moreover Hipkiss and co-workers reported that CAR can react with proteins bearing carbonyls to form protein-carbonyl-carnosine adducts ("carnosylated" proteins) that can undergo different metabolic fates, such as formation of inert lipofuscin and proteolysis via proteosoma activation pathway (Hipkiss et al., 2002).

We recently demonstrated the carbonyl trapping ability of CAR toward HNE and defined the mechanism of reaction by a combined NMR and ESI-MS/MS approach (Aldini et al., 2002a). CAR reacts with HNE through a mechanism involving both the amino group of β-alanine and the Nτ atom of the imidazole ring of the L-histidyl residue, giving the Michael adduct **I** (Fig. 27.7), a reaction product also reported by the group of Sayre (Liu et al., 2003c).

CAR and HCAR react also with acrolein (Carini et al., 2003): the quenching mechanism involves a sequential addition of 1, 2, and 3 moles of ACR/mole dipeptide to both the β-alanine and histidine residues, with formation of several intermediates and final products (Fig. 27.7), as the 14-membered macrocyclic derivative **II** and the N^{β}-(3-formyl-3,4-dehydropiperidino) derivative (**III**) arising from the Michael addition of two ACR molecules followed by an aldol condensation and dehydration. The reaction of HCAR with ACR follows the same pathway, giving rise to the formation of homologous adducts.

CAR and related dipeptides are able to react with α,β-unsaturated aldehydes in a biological milieu, as demonstrated by the formation of the HNE Michael adducts (compound **I**) in tissues exposed to oxidative/carbonylation damage (Aldini et al., 2002a,b). This through the application of a sensitive and specific analytical LC-ESI-MS/MS method, which was also suitable to detect GSH, GSH–HNE Michael

conjugate, and the corresponding oxidized and reduced metabolites (Aldini et al., 2003, 2004; Orioli et al., 2005). These findings well demonstrate that CAR and related dipeptides react in tissues with HNE generated from the lipid peroxidation, and unequivocally indicate the existence in skeletal muscle (which is highly susceptible to peroxidative attack) of a histidine dipeptide-dependent detoxification pathway against cytotoxic HNE, it being alternative/concomitant to that involving thiol-containing peptides. This additional pathway is substantiated by recent findings of Ált et al., (2004), who demonstrated that the relative increase in AGEs/ALEs in muscle protein (total skeletal muscle and myofibril protein) in diabetes was less than that observed in skin collagen, to indicate that muscle protein is partially protected by endogenous agents. In addition, the protective effect of CAR has been demonstrated for the first time in a cellular model (human keratinocyte cell line NCT2544) that mimics the skin damage induced by UV radiation (sequential exposure to UVB and HNE). By LC-MS/MS analysis the ability of keratinocytes to detoxify HNE was demonstrated with formation of hydrophilic and unreactive phase I and phase II metabolites, the latter arising by HNE conjugation with GSH followed by enzymatic oxidation/reduction reactions on the aldehyde function (Aldini et al., 2003). When keratinocytes were exposed to HNE plus UVB radiation, a significant impairment of the metabolic biotransformation was observed, accompanied by a cytotoxic effect that were completely reversed in the presence of carnosine: the cytoprotective mechanism is due to the capacity of the dipeptide to trap the cytotoxic aldehyde and to inhibit its intracellular diffusion (Carini et al., 2002).

Dietary CAR appears to be readily absorbed intact, primarily in the jejenum by a carrier-mediated transport system (Ferraris et al., 1988), but most of the dipeptide is hydrolyzed in the plasma by carnosinase, a specific serum hydrolases that cleaves the β-alanine-histidine peptidic bond. By using a selective and sensitive LC-ESI-MS/MS method, recently developed and validated to quantitate CAR and HD derivatives in biological matrices (Aldini et al., 2004), we confirmed the rapid hydrolysis of CAR in serum in both in vitro (human) and ex vivo experiments (rat).

To our knowledge, no pharmacological studies have been up to now designed to evince the carbonyl quenching activity of CAR and related dipeptides. However, CAR was found to be effective in preventing several diseases that are supposed to be dependent on the carbonylation process, such as aging, diabetes-related diseases, and ischemia damage, implying the involvement of CAR as a carbonyl trapping agent. In particular, Hipkiss et al., (2001) found that CAR is effective in suppressing diabetes-associated increase in blood pressure in fructose-fed rats. CAR has been implicated as an anti-aging agent (Gallant et al., 2000) because it can influence age-associated changes and average life span (mean life span 20% longer in 100 mg/Kg CAR-treated animals) in senescence-accelerated mice, an anti-aging effect that could be associated to a detoxification effect of CAR toward reactive aldehydes acting as cross-linking agents. CAR has been also found to be effective in preventing/treating cataract formation, an age-related eye disease that is dependent on protein oxidation and carbonylation (Boscia et al.,

2000), and to be effective as an anti-ischemic agent in both brain and heart, as reviewed by Stvolinsky et al., (2000). In experimental cerebral ischemia, CAR decreased mortality and improved the neurological conditions of the animals. In cardiac ischemia, CAR protected cardiomyocytes from damage and improved the contractility of the heart. The pronounced anti-ischemic effect of CAR in the brain and heart could be due to a combination of antioxidant and membrane-protecting functions, its proton buffering capacity, its formation of complexes with transition metals, and its regulation of macrophage activity. Moreover the anti-ischemic activity of CAR could be in part due to its effectiveness in carbonyl trapping, considering that ischemia causes a major increase in the carbonyl-protein adduct, which can lead to a toxic sequence of events that can compromise tissue survival during ischemia and reperfusion (Eaton et al., 1999).

Recall from our discussion above that CAR is rapidly hydrolyzed in blood. This limits its application as a detoxifying agent for that reactive aldehydes acting as cytotoxic metabolites in blood and CNS and involved in the pathogenic mechanisms of atherosclerosis, ischemia, and neurodegenerative disorders. Nevertheless, CAR can be used as a model for developing long-lasting drugs that maintain the trapping activity and safety of the parent compound but are resistant to the enzymatic hydrolysis catalyzed by peptidases and, in particular, by serum carnosinases.

Among CAR derivatives, *N*-acetylcarnosine (*N*-CAR) (Fig. 27.7) has been found to be suitable for the nonsurgical prevention and treatment of cataracts. This molecule protects the crystalline lens from oxidative stress-induced damage, and in a recent clinical trial it produced a safe and effective long-term improvement in vision, proving to be effective in the management of age-related cataract reversal and prevention both in the human and canine eye. When administered topically to the eye, *N*-CAR, which is resistant to hydrolysis catalyzed by carnosinase, functions as a time-release prodrug of L-carnosine (Babizhayev et al., 2004).

We recently found that β-alanyl-D-histidine (D-CAR) fulfills the above-mentioned requirements. We observed a superimposable quenching ability of the two isomers toward HNE and ACR, indicating that the change of chiral carbon configuration of CAR does not affect its reactivity (unpublished data). Although the carbonyl-trapping effectiveness of CAR and D-CAR was found to be similar, their bioavailability and plasma stability, studied in male rats treated *by gavage* with the two isomers at a dose of 200 mg kg^{-1}, was significantly different. The pharmacokinetic profile of the two isomers (LC-ESI-MS/MS analysis) indicates that while CAR disappeared in plasma within 120 minutes, D-CAR was still detectable up to 8 hours, suggesting greater metabolic stability as a consequence of its resistance to the hydrolytic effect of carnosinase. From these data it is evident that D-CAR acts as a potential protective agent in the blood compartment toward cytotoxic carbonyl compounds. Studies are in progress to evaluate the effects of a long-term administration of D-CAR (compared to CAR) in a nondiabetic animal model characterized by obesity and hyperlipidemia (Zucker rats), where ALE formation plays a key role in the development of renal and cardiac dysfunction (Alderson et al., 2003).

Aminoguanidine Aminoguanidine (hydrazinecarboximidamide, AG) is a nucleophilic, highly water-soluble compound characterized by two key reaction centers: the nucleophilic hydrazine function and the guanidine group (Fig. 27.8). AG is a hydrazine compound characterized by several biological effects: (1) it decreases the formation of AGEs that occur during chronic hyperglycemia (the effective concentration ranges from 3 to 200 μM); (2) it is a potent inhibitor of the inducible form of nitric oxide synthase (iNOS), $IC_{50} = 31$ μM, and a weaker inhibitor of neuronal NOS (nNOS; $IC_{50} = 170$ μM) and endothelial NOS (eNOS; $IC_{50} = 330$ μM); (3) it is a potent and irreversible inhibitor of semicarbazide-sensitive amine oxidase, with a *Ki* value for the aortal enzyme of 1 to 2 μM (benzylamine as substrate); (4) at high concentrations (7 mM), AG reacts with other carbonyl compounds, such as pyruvate, and forms a corresponding hydrazone adduct (Thornalley, 2003).

AG is a prototype scavenging agent of α,β-dicarbonyls that prevents the formation of AGEs from α,β-dicarbonyl precursors such as GO, MGO, and 3-deoxyglucosone (Thornalley et al., 2000) and of α,β-dicarbonyl moieties of glycated proteins, to form 3-amino-1,2,4-triazine derivatives (Thornalley et al., 2000; Hirsch et al., 1992), as shown in Figure 27.8. The first report describing the preventive effect of AG on AGE formation was the demonstration of inhibition of diabetes-induced arterial wall protein cross-linking (Brownlee et al., 1986). Later, several ex vivo experiments demonstrated the ability of AG to inhibit AGEs/ALEs and AGE/ALE-mediated modification of proteins. In particular, in animal models of diabetes, AG decreased AGE formation in tissues such as aorta (Forbes et al., 2004), lens, and kidney (Thornalley, 2003). Moreover a direct evidence of oxoaldehyde scavenging by AG in vivo has been given by the detection of the triazine products (Thornalley, 2003).

AG was also found to prevent apo B Lys modification and to dose-dependently inhibit the oxidatively induced increase in subsequent macrophage uptake, to indicate that AG inhibits oxidative modifications of LDL protein in large part by binding reactive aldehydes formed during lipid peroxidation and preventing their subsequent conjugation to apo B (Picard et al., 1992). The trapping ability of AG toward lipid-derived aldehydes has been investigated by Al-Abed and Bucala (1997): AG is an efficient scavenger of α,β-unsaturated aldehydes when compared to nucleophilic amino acids (Cys, Lys, His). In particular, AG reacts with HNE in physiological conditions to yield a stable adduct, consistent with the structure of a resonance-stabilized Schiff base whose formation has been furtherly confirmed (NMR studies) by Neely et al., (2000). AG reacts also with MDA, giving as a final product a 5-hydroxy-2-pyrazoline-1-carboxamidine derivative (Fig. 27.8).

The pharmacological activity of AG has been extensively studied in several carbonyl-related diseases such as aging and diabetes, and it has been recently reviewed by Thornalley (2003). In STZ-induced diabetic rats, AG was found to prevent the formation of AGEs and ALEs such as CML and CEL and to inhibit several diabetic-related complications such as nephropathy, retinopathy, neuropathy, and diabetes-accelerated atherosclerosis and vascular diseases (Thornalley, 2003). More recently long-term AG administration has been shown to markedly

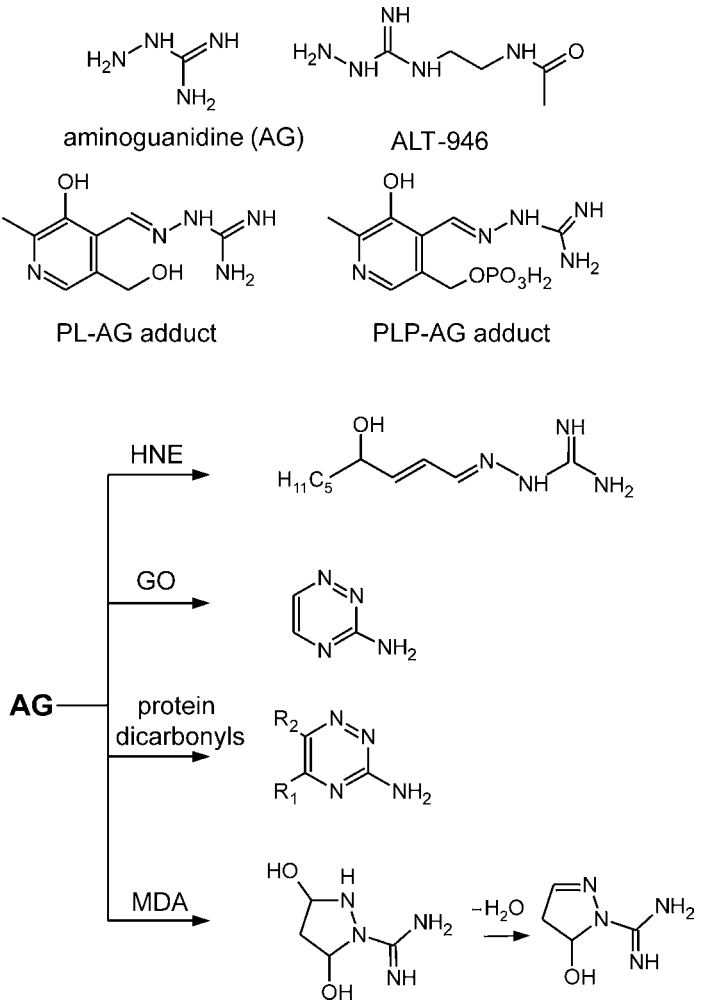


FIGURE 27.8 Structures of aminoguanidine (AG) and derivatives and reaction of AG with HNE, GO, protein di-carbonyls, and MDA.

reduce diabetes-accelerated atherosclerosis in STZ-induced diabetic apolipoprotein E-deficient (apoE-/-) mice, since a sixfold increase in plaque area with diabetes was attenuated 40% in AG-treated mice (Forbes et al., 2004).

In aged rats, AG treatment was found to significantly decrease age-related diseases such as increased blood pressure, decline in glomerular filtration rate, glomerulosclerosis, nephron loss, proteinuria, cardiac hypertrophy, and aorta stiffness (Thornalley, 2003; Chang et al., 2004). Regarding the reaction mechanism, it should be considered that besides the pharmacological effects of AG observed in the above-mentioned animal models, as is attributed to its ability in trapping

carbonyl and di-carbonyl compounds, there is the ability of AG to inhibit iNOS. Because modifications of native proteins are recognized as markers as well as contributors of the aging process, nonenzymatic glycoxidation and lipoxidation of proteins continues to stimulate great interest in geriatric research. The cardiovascular system represents an ideal system to evaluate the extent of glycoxidation and lipoxidation of native proteins (its constituents are slowly renewed, it is metabolically aerobic, and derives energy from glucose and fatty acids, the precursors of AGEs and ALEs). Moreau et al., (2005) have recently studied the potential usefulness of AG in normal aging, which is considered to share pathophysiological attributes with diabetes and dyslipidemia. By different methodological approaches (ELISA for detection of CML, Western blotting for HNE-modified proteins in serum and heart mitochondria, and nano-LC-ESI MS/MS to identity serum polypeptides modified by CML and HNE), it was demonstrated that a three months administration of AG to 344 old Fischer rats substantially lowered their CML content, by an average of 15%, 44%, and 28% in serum, aorta, and heart, respectively. In addition AG treatment lessened the accumulation of CML- and HNE-modified proteins (mainly albumin and heavy and short chains components of immunoglobulins) in the serum of old rats and/or the extent of their formation. For these reasons the use of AG as a pharmacological tool to prevent some diseases associated with aging is a matter for further investigations.

Finally, AG Clinical Trials in Overt (type 1 and 2) Diabetic Nephropathy (ACTION) were performed: ACTION I was conducted in patients with type 1 diabetes mellitus and ACTION II in patients with type 2 diabetes mellitus. The primary end points in both ACTIONs I and II was the time to the doubling of the baseline serum creatinine. In particular, ACTION I was a randomized, double-masked, placebo-controlled study performed on 690 patients with type 1 diabetes mellitus, nephropathy, and retinopathy (Bolton et al., 2004). The patients received twice daily dosing with placebo, AG 150 mg, or AG 300 mg for two to four years. The study did not demonstrate a statistically significant beneficial effect of AG on the progression of overt nephropathy resulting from type 1 diabetes. ACTION II (Freedman et al., 1999) was a randomized, double-blind, placebo-controlled trial comparing two dose levels of AG with placebo on the progression of nephropathy in 599 type 2 diabetic patients with renal disease from 84 centers in the United States and Canada. The External Safety Monitoring Committee recommended the early termination of the ACTION II trial because of safety concerns and apparent lack of efficacy. Among the AG adverse reactions: gastrointestinal symptoms, abnormalities in liver function tests, antinuclear antibody-associated lupus-like illness, flu-like syndromes, and, rarely, anti-neutrophil cytoplasmic antibody-associated vasculitis in both healthy volunteers and diabetic patients.

Aminoguanidine Derivatives Taguchi et al., (1998) demonstrated that the hepatic pyridoxal phosphate (PLP) content of mice given AG was significantly decreased compared with control mice, and that a Schiff base adduct between AG and PLP (PLP-AG) was formed in AG-treated mouse livers. Based on these

results and on the consideration that a deficiency of vitamin B6 is a plausible molecular basis of various diseases, including carpal tunnel syndrome, diabetic retinopathy, arteriosclerosis, and myocardial infarction, an aminoguanidine-pyridoxal Schiff base adduct (PL-AG) (Fig. 27.8) was suggested as a safer and more promising compound than AG (Taguchi et al., 1999). PL-AG was found to dose-dependently inhibit AGE formation with a potency similar to AG and to be at least in part absorbed from the gastrointestinal tract, without decreasing pyridoxal and its phosphate levels in tissues (Taguchi et al., 1999). More recently, in a model of diabetic nephropathy, PL-AG significantly improved urinary albumin excretion and also had a better preventive effect on progression of renal pathology than AG. Inhibition of glycation by both drugs was similar, but the antioxidant activity of the pyridoxal adduct was superior (Myoshi et al., 2002). In particular, PL-AG was found to be more potent than AG and reference compounds (pyridoxal and pyridoxamine) as a scavenger of hydrogen peroxide, hydroxyl radical, superoxide radical, and as an LDL-oxidation inhibitor. In addition, unlike AG, PL-AG did not show pro-oxidant activity (Chen et al., 2003). In vivo, the inhibitory effect of PL-AG against lipid peroxidation in diabetic rats was higher than that of AG. Also AG dramatically decreased the pyridoxal phosphate level in the diabetic rat liver, whereas PL-AG only moderated affected it (Chen et al., 2003). More recently (Chen et al., 2004) it was found that the PL-AG adduct is superior to AG in preventing diabetic neuropathy and cataracts in STZ-diabetic rats, at least partly because of the higher antioxidant activity of PL-AG. In particular, the adduct, but not AG, significantly improved motor nerve conduction velocity and the time to develop cataract was longer in adduct-treated rats than in untreated and AG-treated rats. The increase in opacification of lenses in culture medium containing high glucose levels (55.5 mM) was more efficiently attenuated by the adduct than by AG. The adduct and AG similarly lowered glycated hemoglobin levels, but the level of urinary 8-hydroxy-2′-deoxyguanosine, a marker of oxidative DNA damage, and the level of liver MDA plus 4-hydroxy-2-alkenals, both of which were elevated by diabetes, were significantly reduced by the PL-AG but not by AG (Chen et al., 2004).

N-(2-acetamidoethyl) hydrazinecarboximidamide hydrochloride (ALT-946) (Fig. 27.8) is a more potent inhibitor of AGE-derived protein modification than AG. It is characterized by a poor inhibition effect on nitric oxide synthase and by a lower toxic effect than AG (Forbes et al., 2001). In STZ-induced diabetic rats, ALT-946 was found to inhibit AGE-protein cross-linking and to reproduce the renoprotective effect of AG. The protective effect of ALT-946 was then confirmed in the diabetic transgenic (mREN-2)27 rat where it was found to reduce glomerulosclerosis and cortical tubular degeneration (Wilkinson-Berka et al., 2002).

Pyridoxamine and Analogues Pyridoxamine, 3-pyridinemethanol,4-(aminomethyl)-5-hydroxy-6-methyl- (PM) is a water soluble, bioavailable, and low-toxic compound (5–7.5 g Kg^{-1} oral LD_{50} in rodents) (Degenhardt et al., 2002) characterized by a 2-methyl-3-hydroxyl-5-hydroxymethyl pyridinic ring substituted in position 4 by a nucleophilic aminomethyl moiety (Fig. 27.9*a*). PM and the

related pyridine compounds, pyridoxine (PN) and pyridoxal (PL), are naturally occurring forms of vitamin B6, which, during metabolic conversion, become phosphorylated at the 5-hydroxymethyl substituent. Although both pyridoxamine-5′-phosphate and pyridoxal-5′-phosphate interconvert as coenzyme form, PLP is the coenzyme form that participates in the large number of B6-dependent enzyme reactions, including the metabolism of amino acids and glycogen, the synthesis of nucleic acids, hemoglobin, sphingomyelin, and other sphingolipids, and the synthesis of the neurotransmitters serotonin, dopamine, norepinephrine, and GABA (Burtis and Ashwood, 1999).

Booth and colleagues introduced PM, a novel and effective post-Amadori inhibitor that acts by reducing the conversion of intermediates in protein glycation reactions (Amadori compounds) to antigenic AGE products (Booth et al., 1996, 1997). The reaction mechanism was then proposed by Voziyan et al., (2003), who gave evidences that PM inhibits post-Amadori glycoxidation reactions by binding to redox metal ions and interfering with their catalytic role. Onorato

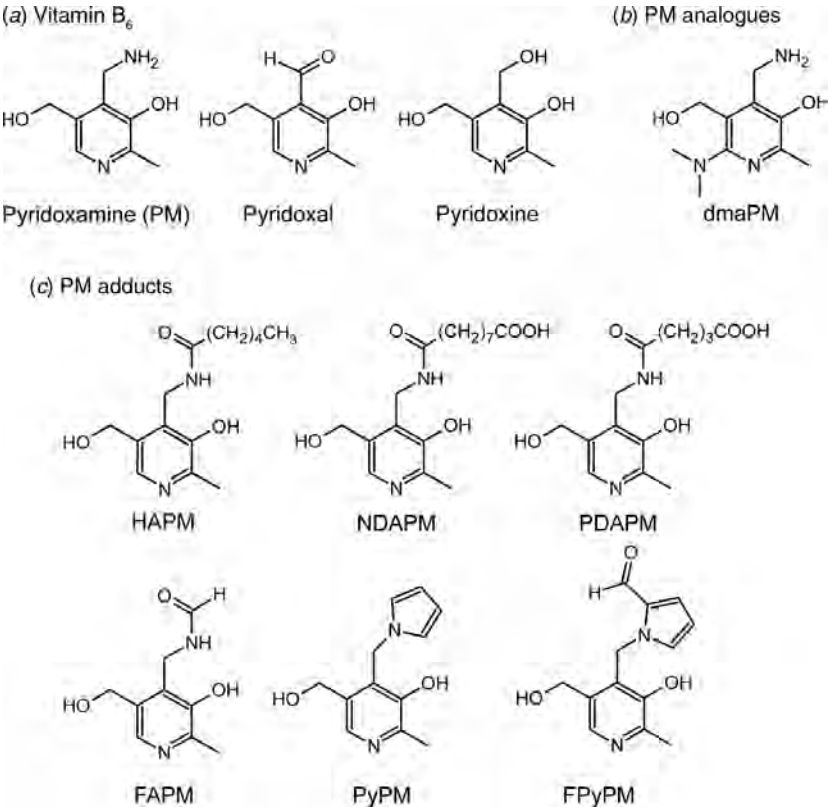


FIGURE 27.9 Structures of pyridoxamine (PM) and other vitamins: B6 (*a*), PM analogues (*b*), and PM adducts (*c*).

et al., (2000) found that PM, besides acting as an AGE inhibitor, inhibits the chemical modification of proteins during lipid peroxidation reactions in vitro, and thus provided evidence that PM traps reactive intermediates during lipid peroxidation. In particular, PM is a potent inhibitor of modification of Lys residues of proteins by peroxidizing lipids, both in a model protein-lipid system (arachidonate with the model protein ribonuclease A, RNase A) and during copper-catalyzed oxidation of LDL. In this experimental model, PM was found to prevent the formation of ALEs such as CML, CEL, MDA-Lys, and HNE-Lys. The major products formed on reaction of linoleic acid (LA) with PM under autoxidative conditions were identified as the hexanoic acid amide (*N*-hexanoyl-PM, HAPM) and nonanedioic acid monoamide (*N*-nonanedioyl-PM, NDAPM) derivatives of PM (Onorato et al., 2000). Later these results were extended (Metz et al., 2003a), by identification of 12 PM adducts formed in vitro during incubation of PM with LA and arachidonic acid (AA) (Fig. 27.9*c*). Moreover six of these compounds were then detected in the urine of diabetic and hyperlipidemic rats treated with PM: HAPM, NDAPM, *N*-pentanedioyl-PM (PDAPM), *N*-formyl-PM (FAPM), *N*-pyrrolo-PM (PyPM), and *N*-(2-formyl)-pyrrolo-PM (FPyPM). This is the first direct demonstration of the ability of PM to trap intermediates of lipid peroxidation reactions in vivo. Moreover the paper by Metz et al., (2003a), besides focusing on the mechanism of action of PM, clearly demonstrated the major role for lipid peroxidation and lipoxidative modification of proteins in the development of chronic complications in diabetes. The elevated levels of PM adducts with the intermediates of lipid peroxidation in the urine of diabetic as well as of hyperlipidemic rats suggest a major role for lipid peroxidation and lipoxidative modification of proteins in the development of chronic complications in diabetes. In particular, the protective effects of PM are consistent with its lipid-lowering activity and with its ability to trap intermediates in lipoxidation reactions.

Voziyan et al., (2002) studied the reaction mechanism of PM with glycolaldehyde (GLA) and GO (the latter a carbonyl compound deriving from the autoxidation of sugars as well as of PUFAs). PM was found to inhibit the modification of Lys residues and loss of enzymatic activity of RNase A in the presence of GO and GLA, and inhibited the formation of CML during reaction of GO and GLA with bovine serum albumin. The trapping ability of PM has been recently extended to 1,4-dicarbonyls (Amarnath et al., 2004), a class of reactive compounds such as 2,5-hexanedione and the recently discovered endogenous 4-ketoaldehydes (levuglandins, isoketals, and neuroketals) that exhibits severe toxicity and marked proclivity to form pyrroles on proteins. PM was found to react extremely rapidly, with a second-order rate constant at physiological pH, being nearly 2,300 times faster than that of N^{α}-acetyllysine. The extreme reactivity of PM was unique to 1,4-dicarbonyls, as its reactions with MGO and HNE were much slower and only slightly faster than with N^{α}-acetyllysine.

The ability of PM to delay the development of diabetic complications has been well documented in animal models. Treatment of STZ-induced diabetic rats with PM was found to be effective in reducing the development of renal disease (Degenhardt et al., 2002; Alderson et al., 2004), of retinopathy (Stitt et al., 2002),

and of peripheral neuropathy (Metz et al., 2003b). PM was also found effective in preventing the development of nephropathy in a nondiabetic animal model, using Zucker obese (*fa*/*fa*) rats (Alderson et al., 2003). This animal is characterized by obesity, hyperlipidemia, hypertension, and insulin resistance, and it may be considered a model of the metabolic syndrome (syndrome X) in humans. As in the STZ-diabetic rats, PM protected against renal disease in Zucker rats, by inhibiting the development of both albuminuria and creatinemia, preventing the increase in systolic blood pressure and mean arterial pressure and inhibiting thickening of the aortic wall and the walls of small arteries in the heart and kidney, as well as the increase in AGE/ALE formation. In detail, PM treatment significantly inhibits (1) the increase in CML, CEL, and MDA-Lys, but not pentosidine in the skin collagen of diabetic rats (Alderson et al., 2004); (2) the accumulation of CML in neural retina of diabetic rats (Stitt et al., 2002); and (3) the formation of AGEs/ALEs in skin collagen of Zucker rats (Alderson et al., 2003). Another important pharmacological effect of long-term administration of PM, evinced in both the animal models (characterized by a severe hyperlipidemia) is the lipid-lowering effect. PM induced a more than 50% reduction in triglycerides and total cholesterol, which correlated significantly with the improvement in renal function and the decrease in AGEs, without affecting overall weight gain, glycemia, or insulin resistance (Degenhardt et al., 2002; Alderson et al., 2003). Overall, with the exception that PM did not affect blood pressure in the STZ-diabetic rat, the beneficial effects of PM in the Zucker rat were similar to those observed in the diabetic model (Alderson et al., 2003).

The biological effects of PM, and in particular, the inhibition of renal and vascular disease in both diabetic and Zucker rats, could be primarily attributed to its activity as inhibitor of AGE/ALE formation through a carbonyl-trapping mechanism. This hypothesis is unequivocally confirmed by the detection of adducts between PM and reactive carbonyl or di-carbonyl precursors of AGE/ALE in the urine of diabetic and Zucker rats treated with PM. Beside this hypothesis Alderson et al., (2003), by considering that both dyslipidemia and hyperlipidemia are recognized as independent risk factors for the development of renal disease, suggested as an alternative hypothesis that the beneficial effect of PM could be secondary to its lipid-lowering effect.

To better understand the mechanism of action of PM, and in general, the biological effect of AGE/ALE inhibitors, the ability of PM to restrain AGE/ALE formation and nephropathy development in STZ-diabetic rats was recently compared to that of the ACE (angiotensin converting enzyme) inhibitor enalapril, the antioxidant vitamin E, and the thiol compound lipoic acid (Alderson et al., 2003). The results of the study indicated that the maximal protective effect was achieved with PM, which also limited dyslipidemia and AGE/ALE formation, and that limited protection of renal function can occur in the absence of effects on AGE/ALE formation. Thus, for optimal protection of renal function, it would be beneficial to select drugs whose mechanism of action includes inhibition of AGE/ALE formation. In summary, PM appears to inhibit the crucial steps that lead to chemical modification of proteins by trapping reactive carbonyl compounds derived from

both sugars and lipids, in addition to its inhibition of the formation of AGEs derived from Amadori adducts, through a metal-ions chelating mechanism. This protective mechanism, together with the lipid-lowering effect, should explain the biological effects of PM, and in particular, its ability to prevent the progression of renal and vascular diseases in both diabetic and Zucker rats.

PM has a weak hydrogen-donating ability, making it only a marginally effective antioxidant (Onorato et al., 2000). In consideration of this limitation Culbertson et al., (2003a) proposed 6-dimethylaminopyridoxamine (dmaPM) (Fig. 27.9*b*), a PM derivative designed to act both as carbonyl- and radical-trapping agent. This novel derivative was obtained by inserting a strong electron-donating group, the dimethylamino moiety, in para position to the phenolic OH group, which, by decreasing the O–H bond dissociation enthalpy, increases the radical trapping capacity without lowering the ionization potential to the point that there is a direct reaction with oxygen. The radical trapping ability of dmaPM was then studied in an in vitro model, based on ABAP (2,2′-azobis(2-amidinopropane) dihydrochloride) as radical inducer and on the fluorescent protein allophycocyanin as substrate: dmaPM was significantly effective, with a potency similar to that of the well-known radical scavenger Trolox, whereas PM had the opposite effect, and thus increased the oxidation rate of the substrate. This new carbonyl-trapping and radical-trapping agent, also owing to its metal-ion chelating properties, showed excellent inhibition of AGE formation in vitro (Culbertson et al., 2003b). Hence dmaPM as a multifunctional agent seems to have great promise, but further studies are required to demonstrate its efficacy in vivo.

Hydrazinophthalazines Hydralazine (1(2H)-phathalazinone, hydrazone, HY) (Fig. 27.10*a*) is a vasodilating antihypertensive agent characterized by a strong nucleophilic hydrazine group. HY causes direct relaxation of arteriolar smooth muscle, and its vasodilating effect is associated with powerful stimulation of the sympathetic nervous system, which results in increased heart rate and contractility, increased plasma renin activity, and fluid retention. Due to the strong nucleophilic group, HY readily scavenges several biological keto compounds in humans, such as pyruvate, through the formation of the corresponding hydrazone.

Burcham et al., (2000) first reported HY as an efficient ACR scavenger and a powerful inhibitor of ACR-mediated toxicity in hepatocytes (Burcham et al., 2002). The compound afforded strong cytoprotection at concentrations that were several orders of magnitude lower than those of the other scavengers tested, including AG, CAR, and PY. The reaction products between HY and ACR have been recently isolated and characterized as the hydrazone derivatives (Kaminskas et al., 2004a). The cytoprotective effects of hydrazinophthalazines were then studied in isolated hepatocytes exposed to allyl and crotyl alcohols, which are converted to the corresponding aldehydes by alcohol dehydrogenase (Kaminskas et al., 2004b). Micromolar concentrations of hydralazine and di-hydralazine (Fig. 27.10*a*) afforded clear, concentration-dependent protection; di-hydralazine, bearing two hydrazine substituents, was approximately twice as potent in respect to hydralazine. The cytoprotective effect due to the trapping mechanism toward

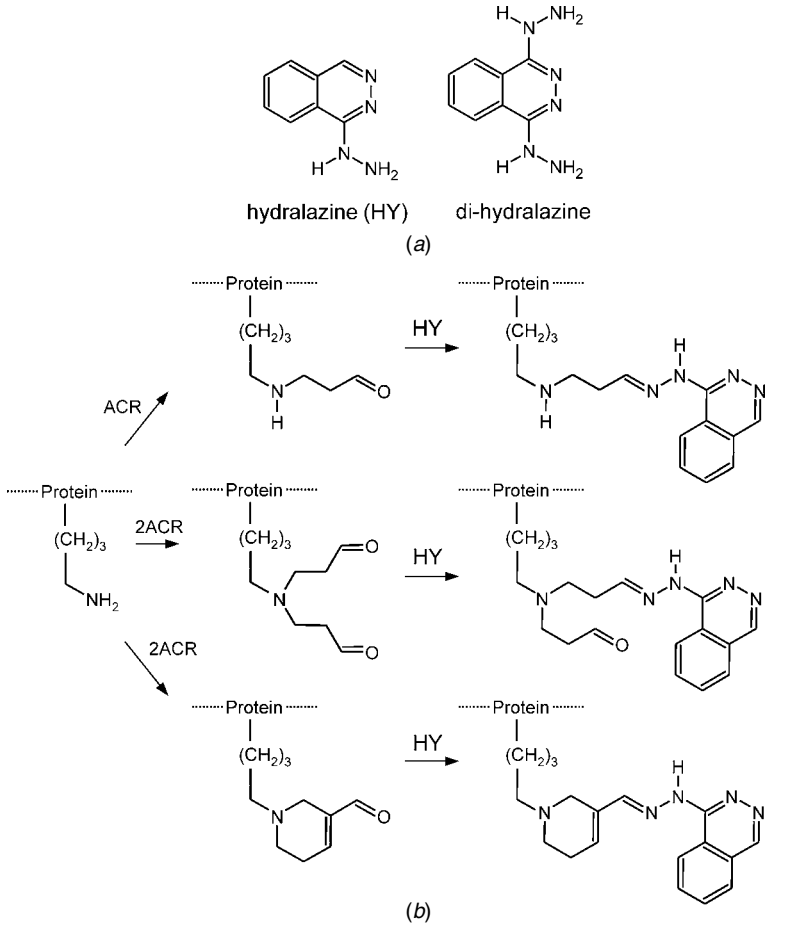


FIGURE 27.10 Structures of hydralazine (HY) and derivatives (*a*) and reaction of HY with ACR-modified lysine-containing peptides (*b*). (Adapted from Kaminskas et al., 2004b, with permission of The American Society for Pharmacology and Experimental Therapeutics.)

α,β-unsaturated aldehydes was confirmed by detecting in cellular media the corresponding hydrazones. Moreover it was recently found that HY, besides scavenging free aldehydes, is also able to react extensively with ACR-derived protein adducts, forming the corresponding hydrazone derivatives (Burcham et al., 2004). An antibody raised against such HY-stabilized ACR adducts (in a Western blotting experiment) allowed intense adduct-trapping to be detected in allyl alcohol-preloaded hepatocytes exposed to low micromolar concentrations of HY (Burcham et al., 2004). Direct evidence of this mechanism was obtained by ESI-MS for an ACR-adducted model peptide. In particular, the ability of HY to react with the carbonyl group of different ACR-peptide adducts was

demonstrated. Among these were mono- and di-Michael adducts and the cyclized FDP-Lys adduct, forming the corresponding peptide-hydrazone derivatives, as shown in Figure 27.10*b*.

In regard to the pharmacological effects of HY, which are secondary to its carbonyl-trapping mechanism, it was initially found that HY is able to protect rats against the cardiovascular toxicity of allylamine, a syndrome involving ACR formation via vascular amine oxidase (Lalich and Paik, 1974). More recently Kaminskas et al., (2004b) reported that HY affords strong hepatoprotection when administered in the early stages of allyl alcohol intoxication in mice, an effect accompanied by intense, dose-dependent trapping of a broad range of carbonylated proteins, to indicate that HY inactivates reactive carbonyl-retaining protein adducts formed by ACR, thereby preventing secondary reactions that trigger cellular death.

Notwithstanding the well-documented carbonyl-trapping ability, we must keep in mind that HY is an antihypertensive drug (one of the first orally active antihypertensive drugs to be marketed in the United States) characterized by two types of side effects: the former are consequent to the vasodilating effect (headache, nausea, hypotension, palpitation, tachicardia, dizziness, and angina pectoris); the latter are linked to immunological reactions, of which the lupus syndrome is the most common. Administration of HY can also result in illness that resembles serum sickness, haemolytic anemia, vasculitis, and rapidly progressive glomerulonephritis. The mechanism of these autoimmune reactions is unknown, but HY has been shown to inhibit methylation of DNA and induce self-reactivity in T cells (Oates and Brown, 2001).

Other Compounds Several other compounds have been developed and/or reinvestigated for their efficacy as trapping agents for RCS, among which the most representative are OPB-9195, tenilsetam, metformin, and 2,3-diaminophenazine (DAP), whose structures are reported in Figure 27.11.

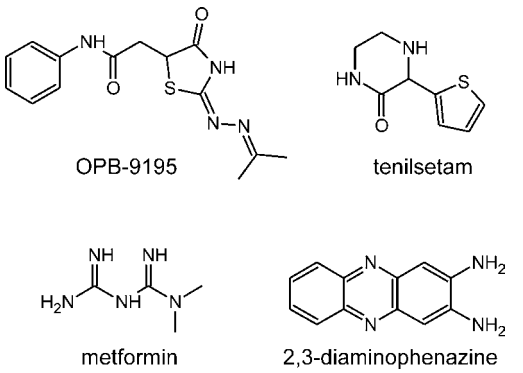


FIGURE 27.11 Structures of other carbonyl-trapping compounds.

OPB-9195, (+/−)-2-Isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide, belongs to a group of thiazolidine derivatives originally developed in Otsuka Pharmaceutical (Japan) as an insulin sensitizer, and further recognized as a novel inhibitor of advanced glycation given its strong inhibitory effect on the formation of AGE, by trapping dicarbonyl compounds and reducing AGE accumulation in tissues. For this reason OPB-9195 has been proposed as a candidate drug to prevent the progression of diabetic nephropathy. Nakamura et al., (1997) first demonstrated, by enzyme-linked immunosorbent assay (ELISA) and SDS-PAGE, the in vitro inhibitory effects of OPB-9195 on AGE formation and AGE-derived cross-linking, and its preventive effect on the progression of diabetic glomerular sclerosis in OLETF rats (a model of non–insulin-dependent diabetes mellitus), by lowering serum levels of AGEs and attenuating AGE deposition in the glomeruli. Later, in a study designed to evaluate histologically the role of CML in the development of diabetic nephropathy (using the above-described experimental model), it was found (Nakamura et al., 2003) that inhibition of CML accumulation by OPB-9195 is associated directly with the prevention of glomerular lesions. The majority of studies on OPB-9195 has been carried out, starting from 1998, by the group of Miyata (Miyata et al., 1998a,b; Miyata and van Ypersele de Strihou, 2003), who demonstrated that the drug is a potent inhibitor of both glycoxidation and lipoxidation reactions. Long-term oral administration of the drug has been shown to prevent the development of vascular lesions (neointima proliferation in arterial walls) in rats following balloon injury of carotid arteries (Miyata et al., 1999b), an early step in development of atherosclerotic lesions, and to inhibit the rise of serum AGEs and the in situ expression of immunoreactive AGEs in the peripheral nerve in diabetic rats (Wada et al., 2001). The mechanism of action of OPB-9195 has been elucidated by delineating the AGE and ALE precursors targeted by this drug (Miyata et al., 2000b). Inhibition of CML and pentosidine formation by OPB-9195 was more efficient than by aminoguanidine, while the efficiency in blocking the processes involved in the formation of two ALEs (MDA-Lys and HNE-protein adduct) was similar to that of aminoguanidine. The reaction product of OBP-9195 with RCS was proposed by Miyata et al., (2000b) to be a hydrazone derivative, but no definitive structure has been assigned to the reaction product(s), due to lack of suitable analytical data, nor has the mechanism of reaction been elucidated. Further, although the therapeutic use of OPB-9195 seems to be promising, currently the clinical efficacy of OPB-9195 remains to be demonstrated. Also a systematic study specifically designed to define the ability of this compound to trap different lipid-derived carbonyl species has yet to be performed.

Tenilsetam [(±)-3-(2-thienyl)-2-piperazinone], first introduced as an antiischemic molecule, and then as a cognition-enhancing drug, is now successfully used for treatment of patients suffering from Alzheimer's disease. The drug has been demonstrated to inhibit protein cross-linking by AGEs in vitro (Munch et al., 1994, 1997) and to inhibit the Maillard reaction in vivo, as demonstrated in STZ-induced diabetic rats chronically treated with 50 mg/kg·day. Tenilsetam administration suppresses to control levels the formation of two different types of fluorescence characteristic of AGEs in the renal cortex and aorta of diabetic

rats (Shoda et al., 1997). According to the proposed mechanism, Tenilsetam acts via covalent attachment to the glycated proteins, thus blocking the reactive sites from further polymerization reactions. This mechanism of reaction has not yet been established, however, nor has its ability to trap lipid-derived RCS.

Metformin (dimethylbiguanide) was introduced into clinical practice in 1957 as an oral antihyperglycemic agent for the management of non–insulin-dependent diabetes mellitus. Metformin can prevent diabetic complications not only by lowering glycemia but also by inhibiting AGE formation and by stimulating antioxidant defenses (Bonnefont-Rousselot, 2003; Tanaka et al., 1997; Rahbar et al., 2000). The United Kingdom Prospective Diabetes Study (UKPDS) found that metformin reduces macrovascular complications in type 2 diabetic patients, and Mamputu et al., (2003), by investigating the mechanisms involved, found that metformin inhibits AGE-induced monocyte adhesion to endothelial cells and expression of endothelial cell adhesion molecules, as well as foam cell formation induced by minimally modified LDL. These results suggest new mechanisms by which metformin may reduce the risk of vascular complications in patients with type 2 diabetes. The drug, structurally related to aminoguanidine, has been also investigated for its ability to trap dicarbonyl compounds (Ruggiero-Lopez et al., 1999): the compound strongly reacts with GO (and MGO), forming original guanidine-dicarbonyl adducts.

2,3-Diaminophenazine (DAP) was developed as an AGE inhibitor that, unlike aminoguanidine, does not inhibit NO synthase. The compound inhibits AGE formation both in vitro and in vivo (Soulis et al., 1999). Treatment of diabetic rats with DAP resulted in an attenuation of mesenteric vascular hypertrophy and reduction of AGE formation, as measured by RIA and immunohistochemistry in these vessels, and the same reduction was observed in the kidney. No information is available on the efficacy of DAP as a lipid-derived aldehyde quencher.

Finally, in re-investigating the mechanism of action of ascorbic acid, Sowell et al., (2004) recently demonstrated that ascorbate acts as a nucleophile, and forms Michael-type conjugates with electrophilic lipoxidation (LPO) products. Several ascorbyl-LPO product conjugates, resulting from the interaction of ascorbic acid with hydroperoxy octadecadienoic acid in vitro, were identified by tandem mass spectrometry, including ascorbyl conjugates of HNE, ONE, and 12-oxo-9-hydroxy-10-dodecenoic acid (the same ascorbyl-LPO conjugates were detected by mass spectrometry in human plasma from healthy subjects in a μM range). Sowell et al., (2004) confirmed that ascorbate functions as a one-electron donor to HPODE, inducing the formation of the alkoxy radical that undergoes α,β-carbon-carbon bond cleavage with generation of HNE and other LPO products (as previously observed by Lee et al., 2001). However, Sowell et al., (2004) also demonstrated that ascorbate subsequently acts as a detoxifying agent against HNE, functioning as a Michael donor, as was previously unrecognized. Hence vitamin C conjugation can be considered a biologically relevant pathway for the elimination of reactive aldehydes and may have implications for the prevention and treatment of chronic inflammatory diseases, as well as the development of novel biomarkers of oxidative stress.

27.4 CONCLUSIONS AND FUTURE PERSPECTIVE

A large body of evidence implicates the formation and accumulation of lipoxidation-derived reactive RCS and of advanced lipoxidation end-products as key factors in the development and progression of a variety of chronic diseases and in the physiological aging process. Functional impairment of structural proteins and enzymes by covalent modification (cross-linking), triggering of key cell signaling systems, and enzyme inactivation are now well recognized signs of cell and tissue damage induced by these compounds.

Among the therapeutic strategies to prevent or restrain the RCS-induced damage, trapping of lipid-derived reactive aldehydes (identified as the chemical intermediaries between hyperglycemia/hyperlipidemia and their complications) has received great attention in the last few years, and several nucleophilic compounds have been tested in both in vitro and in vivo studies for their ability to inhibit protein carbonylation and ALE and EAGLE formation. A first conclusion that can be drawn from this survey of data is that the aldehyde specificity of the nucleophilic compounds here considered, even not fully investigated, differs significantly, with some compounds (e.g., pyridoxamine) being able to preferentially react with "early" carbonyl intermediates of the lipoxidation process. Unfortunately, for many of the compounds developed or recognized first as AGE and then as ALE inhibitors, the mechanism of action has not been extensively investigated, and it remains to demonstrate their ability to trap and deactivate highly reactive intermediate RCS other than GO and MGO, such as α,β-unsaturated aldehydes HNE and ACR. Greater effort must also be garnered to confirm the carbonyl-trapping capacities of these compounds in humans in vivo and the mechanism involved in the tissue protection afforded by these agents. The knowledge of the mechanism of action is important not only for better understanding the roles of RCS and ALEs in the pathogenesis of age-related disease but also for developing more specific, effective, and safe potential therapeutic agents.

In conclusion, as new compounds are designed and already available ALE inhibitors modified to improve their bioavailability and efficacy, careful attention must be given to the drug candidates to ensure that they fulfill some basic requirements: (1) Highly nucleophilic centers showing high rate constants for reaction with RCS thus to interact with endogenously generated aldehydes at a faster rate than do cell macromolecules. For example, AG is effective in inhibiting cross-linking and preventing inactivation of RNase A (Miller et al., 2003) because it reacts with α-dicarbonyls (GO and MGO) at a much faster rate than they do with proteins. However, this appears not always to be the case with 3,5-dimethylpyrazole-1-carboxamidine (DMPC), which does not react with MGO quickly enough to prevent it from reacting with the protein. (2) High selectivity toward lipoxidation-derived aldehydes to prevent cross reactivity with physiologically relevant aldehydes; this to avoid side effects due to their depletion, as reported for aminoguanidine. Hence activity screening tests in vitro, besides HNE, ACR, or GO, should routinarily include pyridoxal as a safety marker, and above all provide elucidation of the adducts' structure and evaluate their stability in physiological conditions and in biological matrices. In particular, for

different drug-aldehyde adducts, sensitive assays (based again on mass spectrometric techniques) should be developed and applied for analysis of body fluids from drug-treated animals, to confirm an effective carbonyl trapping in vivo. Reaction products must be unreactive, safe, and easily excreted. (3) Satisfactory hydrophobic properties are required to ensure high bioavailability. A suitable lipophilic character allows the molecules to pass through the blood brain barrier (for neuroprotective drug candidates) and through cell membranes to inhibit intracellular ALE formation. Moreover, compounds with short plasma or tissue half-life should not be considered as candidates, because the mechanism of action involves direct interaction of the pharmacological agent with the target aldehyde. (4) Not promiscuous activity, to guarantee safety. The failure of AG to achieve a successful clinical application stems from its side effects, mainly due its inhibitory action on inducible NO-synthase. Administration of lower doses to restrain side effects greatly reduces the efficacy of the compound as carbonyl-trapping agent in vivo, and high concentrations are required for direct interaction. Another example is represented by hydrazinophthalazine, whose antihypertensive effect can hamper its generalized use as an ALE inhibitor. (5) Because transition metal chelators appear to be excellent therapeutic agents in the management of long-term complications of diabetes and neurodegenerative disorders, mainly Alzheimer's disease, a potentially successful ALE inhibitor should combine at least metal chelating with aldehyde-sequestering properties. This is the case with PM, which has been suitably modified into the multifunctional derivative dmaPM (carbonyl-, radical-, and metal-ion-trapping agent).

Another important approach, well exemplified by the recently discovered detoxifying role of ascorbic acid and carnosine, could be a detailed reinvestigation on the mechanism of action of other endogenous compounds or micronutrients which are known to be implicated in protection against oxidative stress-induced damage.

ALE inhibitors aside, the new emerging strategies of potential therapeutic interest in atherosclerosis have been limited to date to the experimental animal. Several studies have shown that immunization of hypercholesterolemic animals with native or oxLDL leads to a significant reduction of atherosclerosis development. Also the ability to induce an atheroprotective immunity by active or passive immunization against oxLDL epitopes has been clearly established in experimental animals (Palinsky, 1995). Using a library of MDA-modified polypeptides covering the complete amino acid sequence of human apoB-100, a large number of epitopes recognized by antibodies present in human plasma have been recently identified (Fredrikson, 2003a). The levels of several of these antibodies show an inverse association with plasma oxLDL, suggesting that antibodies are involved in the clearance of these particles. Immunization of apoE-/- mice with corresponding human apoB peptides was found to reduce plaque formation and to stabilize a plaque phenotype, as indicated by the increased collagen content (Fredrikson, 2003b). The antibodies recently found to inhibit atherosclerosis were human IgG1 specific for MDA-modified human apoB-100 sequences (Schiopu et al., 2004). Three weekly doses of these antibodies were injected into male apoE-/-

mice, and one of the IgG1 antibodies significantly and dose-dependently reduced the extent of atherosclerosis as well as the plaque content of oxLDL epitopes and macrophages. Thus passive immunization against MDA-modified apoB-100 peptide sequences may represent a future therapeutic approach for the prevention and treatment of cardiovascular disease. Nevertheless, much still remains to be determined as to whether similar atheroprotective immunity can be induced in humans.

ACKNOWLEDGMENT

Financial support from MIUR (Cofinanziamento Programma Nazionale 2004) is gratefully acknowledged.

LIST OF ABBREVIATIONS

AA, arachidonic acid
ACR, acrolein
AD, Alzheimer's disease
AG, aminoguanidine
AGEs, advanced glycation end-products
ALEs, advanced lipoxidation end-products
ALS, amyotrophic lateral sclerosis
ANS, anserine
BSA, bovine serum albumin
CAR, carnosine
CEL, N^{ε}-(carboxyethyl)lysine
CMA, N^{7}-carboxymethylarginine
CML, N^{ε}-(carboxymethyl)lysine
DAP, 2,3-diaminophenazine
dmaPM, 6-dimethylaminopyridoxamine
EAGLEs, advanced glycation or lipoxidation end-products
ELISA, enzyme-linked immunosorbent assay
ESI-MS, electrospray ionization mass spectrometry
FDP-Lys, N^{ε}-(3-formyl-3,4-dehydropiperidino)lysine adduct
GABA, γ-aminobutyric acid
GLA, glycolaldehyde
GO, glyoxal
GODIC, GO-Lys-Arg dimer
GOLD, GO–Lys dimer
GSH, glutathione

GSTs, GSH S-transferases
HCAR, homocarnosine
HD, histidine-containing dipeptides
HHE, 4-hydroxy-2-hexenal
HNE, 4-hydroxy-*trans*-2-nonenal
HPLC, high performance liquid chromatography
HPODE, hydroperoxy-octadecadienoic acid
HY, hydralazine
NOS, nitric oxide synthase
IsoK, isoketals
LA, linoleic acid
LC, liquid chromatography
LDL, low-density lipoprotein
MALDI, matrix-assisted laser desorption/ionization
MDA, malondialdehyde
MGO, methylglyoxal
MP-Lys, N^{ε}-(3-methylpyridinium)lysine
MS, mass spectrometry
NAC, N^{α}-acetyl-L-cysteine
N-CAR, *N*-acetylcarnosine
NPO, N^{δ}-(2-pyrimidyl)-L-ornithine
ONE, 4-oxo-*trans*-2-nonenal
PD, Parkinson's disease
PL, pyridoxal
PL-AG, aminoguanidine-pyridoxal adduct
PLP-AG, aminoguanidine-pyridoxal phosphate adduct
PM, pyridoxamine
PN, pyridoxine
PUFAs, polyunsaturated fatty acids
RNase A, ribonuclease A
RCS, reactive carbonyl species
ROS, reactive oxygen species
SDS-PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis
STZ, streptozocin
UV, ultraviolet radiation

REFERENCES

Abe H. (2000). Role of histidine-related compounds as intracellular proton buffering constituents in vertebrate muscle. *Biochemistry (Mosc)* 65: 757–765.

Aberle N.S. 2nd, Picklo M.J. Sr, Amarnath V., Ren J. (2004). Inhibition of cardiac myocyte contraction by 4-hydroxy-trans-2-nonenal. *Cardiovasc Toxicol* 4: 21–28.

Aksenov M.Y., Aksenova M.V., Butterfield D.A., Geddes J.W., Markesbery W.R. (2001). Protein oxidation in the brain in Alzheimer's disease. *Neuroscience* 103: 373–383.

Al-Abed Y., Bucala R. (1997). Efficient scavenging of fatty acid oxidation products by aminoguanidine. *Chem Res Toxicol* 10: 875–879.

Alary J., Gueraud F., Cravedi J.P. (2003). Fate of 4-hydroxynonenal in vivo: Disposition and metabolic pathways. *Mol Aspects Med* 24: 177–187.

Alderson N.L., Chachich M.E. Youssef N.N., Beattie R.J., Nachtigal M., Thorpe S.R., Baynes J.W. (2003). The AGE inhibitor pyridoxamine inhibits lipemia and development of renal and vascular disease in Zucker obese rats. *Kidney Int* 63: 2123–2133.

Alderson N.L., Chachich M.E., Frizzell N., Canning P., Metz T.O., Januszewski A.S., Youssef N.N., Stitt A.W., Baynes J.W., Thorpe S.R. (2004). Effect of antioxidants and ACE inhibition on chemical modification of proteins and progression of nephropathy in the streptozotocin diabetic rat. *Diabetologia* 47: 1385–1395.

Aldini G., Carini M., Beretta G., Bradamante S., Facino R.M. (2002a) Carnosine is a quencher of 4-hydroxy-nonenal: Through what mechanism of reaction? *Biochem Biophys Res Commun* 298: 699–706.

Aldini G., Granata P., Carini M. (2002b). Detoxification of cytotoxic α,β-unsaturated aldehydes by carnosine: Characterization of conjugated adducts by electrospray ionization tandem mass spectrometry and detection by liquid chromatography/mass spectrometry in rat skeletal muscle. *J Mass Spectrom* 37: 1219–1228.

Aldini G., Granata P., Orioli M., Santaniello E., Carini M. (2003). Detoxification of 4-hydroxynonenal (HNE) in keratinocytes: Characterization of conjugated metabolites by liquid chromatography/elecytrospray ionization tandem mass spectrometry. *J Mass Spectrom* 38: 1160–1168.

Aldini G, Orioli M, Carini M, Maffei Facino R. (2004). Profiling histidine-containing dipeptides in rat tissues by liquid chromatography/electrospray ionization tandem mass spectrometry. *J Mass Spectrom* 39: 1417–1428.

Aldini G., Dalle-Donne I., Vistoli G., Maffei Facino R., Carini M. (2005). Covalent modification of actin by 4-hydroxy-trans-2-nonenal (HNE): LC-ESI-MS/MS evidence for cys374 Michael adduction. *J Mass Spectrom* 40: 946–954.

Al-Rawithi S., El-Yazigi A., Ernst P., Al-Fiar F., Nicholls P.J. (1998). Urinary excretion and pharmacokinetics of acrolein and its parent drug cyclophosphamide in bone marrow transplant patients. *Bone Marrow Transplant* 22: 485–490.

Alt N., Carson J.A., Alderson N.L., Wang Y., Nagai R., Henle T., Thorpe S.R., Baynes J.W. (2004). Chemical modification of muscle protein in diabetes. *Arch Biochem Biophys* 425: 200–206.

Amarnath V., Amarnath K., Amarnath K., Davies S., Roberts L.J. 2nd. (2004). Pyridoxamine: An extremely potent scavenger of 1,4-dicarbonyls. *Chem Res Toxicol* 173: 410–415.

Anderson M.M., Hazen S.L., Hsu F.F., Heinecke J.W. (1997). Human neutrophils employ the myeloperoxidase-hydrogen peroxide-chloride system to convert hydroxy-amino acids into glycolaldehyde, 2-hydroxypropanal, and acrolein: A Mechanism for the generation of highly reactive α-hydroxy and α,β-unsaturated aldehydes by phagocytes at sites of inflammation. *J Clin Invest* 9: 424–432.

Arikketh D., Niranjali S., Devaraj H. (2004). Detection of acrolein-lysine adducts in plasma low-density lipoprotein and in aorta of cyclophosphamide-administered rats. *Arch Toxicol* 78: 397–401.

Babizhayev M.A., Deyev A.I., Yermakova V.N., Brikman I.V., Bours J. (2004). Lipid peroxidation and cataracts: *N*-Acetylcarnosine as a therapeutic tool to manage age-related cataracts in human and in canine eyes. *Drugs RD* 5: 125–139.

Baynes J.W., Thorpe S.R. (1999). Role of oxidative stress in diabetic complications: A new perspective on an old paradigm. *Diabetes* 48: 1–9.

Baynes J.W., Thorpe S.R. (2000). Glycoxidation and lipoxidation in atherogenesis. *Free Radic Biol Med* 28: 1708–1716.

Baynes J.W. (2003). Chemical modification of proteins by lipids in diabetes. *Clin Chem Lab Med* 41: 1159–1165.

Bolton W.K., Cattran D.C., Williams M.E., Adler S.G., Appel G.B., Cartwright K., Foiles P.G., Freedman B.I., Raskin P., Ratner R.E., Spinowitz B.S., Whittier F.C., Wuerth J.P. (2004). ACTION I Investigator GroupRandomized trial of an inhibitor of formation of advanced glycation end products in diabetic nephropathy. *Am J Nephrol* 24: 32–40.

Bonfanti L., Peretto P., De Marchis S., Fasolo A. (1999). Carnosine-related dipeptides in the mammalian brain. *Prog Neurobiol* 59: 333–353.

Bonnefont-Rousselot D. (2003). Advanced glycation end products: Production and pathophysiological significance. *Cahiers de Nutrition et de Dietetique* 38: 122–129.

Booth A.A., Khalifah R.G., Hudson B.G. (1996). Thiamine pyrophosphate and pyridoxamine inhibit the formation of antigenic advanced glycation end-products: Comparison with aminoguanidine. *Biochem Biophys Res Commun* 20: 113–119.

Booth A.A., Khalifah R.G., Todd P., Hudson B.G. (1997). In vitro kinetic studies of formation of antigenic advanced glycation end products (AGEs): Novel inhibition of post-Amadori glycation pathways. *J Biol Chem* 272: 5430–5437.

Boscia F., Grattagliano I., Vendemiale G., Micelli-Ferrari T., Altomare E. (2000). Protein oxidation and lens opacity in humans. *Invest Ophthalmol Vis Sci* 41: 2461–2465.

Brownlee M., Vlassara H., Kooney A., Ulrich P., Cerami A. (1986). Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. *Science* 232: 1629–1632.

Burcham P.C., Kerr P.G., Fontaine F. (2000). The antihypertensive hydralazine is an efficient scavenger of acrolein. *Redox Rep* 5: 47–49.

Burcham P.C., Kaminskas L.M., Fontaine F.R., Petersen D.R., Pyke S.M. (2002). Aldehyde-sequestering drugs: tools for studying protein damage by lipid peroxidation products. *Toxicology* 181–182: 229–236.

Burcham P.C., Fontaine F.R., Kaminskas L.M., Petersen D.R., Pyke S.M. (2004). Protein adduct-trapping by hydrazinophthalazine drugs: Mechanisms of cytoprotection against acrolein-mediated toxicity. *Mol Pharmacol* 65: 655–664.

Burtis C.A., Ashwood E.R. (1999). *Tietz Textbook of Clinical Chemistry*, 3rd ed., W.B. Saunders, Philadelphia.

Calingasan N.Y., Uchida K., Gibson G.E. (1999). Protein-bound acrolein: a novel marker of oxidative stress in Alzheimer's disease. *J Neurochem* 72: 751–756.

Carini M., Aldini G, Maffei Facino R, Bozzetto L, Ghirardini A. (2002). Detoxification of cytotoxic unsaturated aldehydes in keratinocytes is a GSH-dependent pathway: Inhibition by UVB and protective effect of histidine and cysteine-containing peptides. Preprints of the 22nd IFSCC, Edinburgh, 23–26 September 2002, vol.1 pp. 129–143.

Carini M., Aldini G., Beretta G., Arlandini E., Facino R.M. (2003). Acrolein-sequestering ability of endogenous dipeptides: Characterization of carnosine and homocarnosine/acrolein adducts by electrospray ionization tandem mass spectrometry. *J Mass Spectrom* 38: 996–1006.

Carini M., Aldini G., Maffei Facino R. (2004). Mass spectrometry for detection of 4-hydroxy-trans-2-nonenal (HNE) adducts with peptides and proteins. *Mass Spectrom Rev* 23: 281–305.

Chang K.C., Hsu K.L., Chou T.F., Lo H.M., Tseng Y.Z. (2004). Aminoguanidine prevents age-related deterioration in left ventricular-arterial coupling in Fisher 344 rats. *Br J Pharmacol* 142: 1099–1104.

Chen A.S., Taguchi T., Aoyama S. Sugiura M., Haruna M., Wang M.W., Miwa I. (2003). Antioxidant activity of a Schiff base of pyridoxal and aminoguanidine. *Free Radic Biol Med* 35: 1392–1403.

Chen A.S., Taguchi T., Sugiura M., Wakasugi Y., Kamei A., Wang M.W., Miwa I. (2004). Pyridoxal-aminoguanidine adduct is more effective than aminoguanidine in preventing neuropathy and cataract in diabetic rats. *Horm Metab Res* 36: 183–187.

Cohn J.A., Tsai L., Friguet B., Szweda L.I. (1996). Chemical characterization of a protein-4-hydroxy-2-nonenal cross-link: Immunochemical detection in mitochondria exposed to oxidative stress. *Arch Biochem Biophys* 328: 158–164.

Cotham W.E., Metz T.O., Ferguson P.L., Brock J.W., Hinton D.J., Thorpe S.R., Baynes J.W., Ames J.M. (2004). Proteomic analysis of arginine adducts on glyoxal-modified ribonuclease. *Mol Cell Proteomics* 3: 1145–1153.

Crush K.G. (1970). Carnosine and related substances in animal tissues. *Comp Biochem Physiol* 34: 3–30.

Culbertson S.M., Enright G.D., Ingold K.U. (2003a). Synthesis of a novel radical trapping and carbonyl group trapping anti-AGE agent: A pyridoxamine analogue for inhibiting advanced glycation (AGE) and lipoxidation (ALE) end products. *Org Lett* 5: 2659–2662.

Culbertson S.M., Vassilenko E.I., Morrison L.D., Ingold K.U. (2003b). Paradoxical impact of antioxidants on post-Amadori glycoxidation. *J Biol Chem* 278: 38384–38394.

Davies S.S., Amarnath V., Roberts L.J. 2nd. (2004). Isoketals: highly reactive gamma-ketoaldehydes formed from the H2-isoprostane pathway. *Chem Phys Lipids* 128: 85–99.

Degenhardt T.P., Alderson N.L., Arrington D.D., Beattie R.J., Basgen J.M., Steffes M.W., Thorpe S.R., Baynes J.W. (2002). Pyridoxamine inhibits early renal disease and dyslipidemia in the streptozotocin-diabetic rat. *Kidney Int* 61: 939–950.

Dei R., Takeda A., Niwa H., Li M., Nakagomi Y., Watanabe M., Inagaki T., Washimi Y., Yasuda Y., Horie K., Miyata T., Sobue G. (2002). Lipid peroxidation and advanced glycation end products in the brain in normal aging and in Alzheimer's disease. *Acta Neuropathol* 104: 113–122.

Doorn J.A., Petersen D.R. (2003). Covalent adduction of nucleophilic amino acids by 4-hydroxynonenal and 4-oxononenal. *Chem Biol Interact* 143–144: 93–100.

Eaton P., Li J.M., Hearse D.J., Shattock M.J. (1999). Formation of 4-hydroxy-2-nonenal-modified proteins in ischemic rat heart. *Am J Physiol* 276: H935–H943.

Esterbauer H., Zollner H., Schaur R.J. (1990). Aldehydes formed by lipid peroxidation: mechanisms of formation, occurrence, and determination. In: Vigo-Pelfrey C. (ed.), *Membrane lipid oxidation, Vol. 1*, CRC Press, Boca Raton, FL, pp. 239–268.

Esterbauer H., Schaur R.J., Zollner H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* 11: 81–128.

Ferraris R.P., Diamond J., Kwan W.W. (1988). Dietary regulation of intestinal transport of the dipeptide carnosine. *Am J Physiol* 255: G143–G150.

Forbes J.M., Soulis T., Thallas V., Panagiotopoulos S., Long D.M., Vasan S., Wagle D., Jerums G., Cooper M.E. (2001). Renoprotective effects of a novel inhibitor of advanced glycation. *Diabetologia* 44: 108–114.

Forbes J.M., Yee L.T., Thallas V., Lassila M., Candido R., Jandeleit-Dahm K.A., Thomas M.C., Burns W.C., Deemer E.K., Thorpe S.M., Cooper M.E., Allen T.J. (2004). Advanced glycation end product interventions reduce diabetes-accelerated atherosclerosis. *Diabetes* 53: 1813–1823.

Fredrikson G.N., Hedblad B. Berglund G., Alm R., Ares M., Cercek B., Chyu K.Y., Shah P.K., Nilsson J. (2003a). Identification of immune responses against aldehyde-modified peptide sequences in apoB associated with cardiovascular disease. *Arterioscler Thromb Vasc Biol* 23: 872–878.

Fredrikson G.N., Söderberg I., Lindholm M., Dimayuga P., Chyu K.Y., Shah P.K., Nilsson J. (2003b). Inhibition of atherosclerosis in apoE-null mice by immunization with apoB-100 peptide sequences. *Arterioscler Thromb Vasc Biol* 23: 879–884.

Freedman B.I., Wuerth J.P., Cartwright K., Bain R.P., Dippe S., Hershon K., Mooradian A.D., Spinowitz B.S. (1999). Design and baseline characteristics for the Aminoguanidine Clinical Trial in Overt Type 2 Diabetic Nephropathy (ACTION II). *Control Clin Trials* 20: 493–510.

Fu M.X., Requena J.R., Jenkins A.J., Lyons T.J., Baynes J.W., Thorpe S.R. (1996). The advanced glycation end product, N^{ε}-(carboxymethyl)lysine, is a product of both lipid peroxidation and glycoxidation reactions. *J Biol Chem* 271: 9982–9986.

Furuhata A., Ishii T., Kumazawa S., Yamada T., Nakayama T., Uchida K. (2003). N^{ε}-(3-methylpyridinium)lysine, a major antigenic adduct generated in acrolein-modified protein. *J Biol Chem* 278: 48658–48665.

Gallant S., Semyonova M., Yuneva M. (2000). Carnosine as a potential anti-senescence drug. *Biochemistry (Mosc)* 65: 866–868.

Ghilarducci D.P., Tjeerdema R.S. (1995). Fate and effects of acrolein. *Rev Environ Contam Toxicol* 144: 95–146.

Girones X., Guimera A., Cruz-Sanchez C.Z., Ortega A., Sasaki N., Makita Z., Lafuente J.V., Kalaria R., Cruz-Sanchez F.F. (2004). N^{ε}-Carboxymethyllysine in brain aging, diabetes mellitus, and Alzheimer's disease. *Free Radic Biol Med* 36: 1241–1247.

Glomb M.A., Lang G. (2001). Isolation and characterization of glyoxal-arginine modifications. *J Agric Food Chem* 49: 1493–1501.

Haenen G.R., Vermeulen N.P., Tai Tin Tsoi J.N., Ragetli H.M., Timmerman H., Blast A. (1988). Activation of the microsomal glutathione-*S*-transferase and reduction of the glutathione dependent protection against lipid peroxidation by acrolein. *Biochem Pharmacol* 37: 1933–1938.

Hipkiss A.R., Michaelis J., Syrris P., Kumar S., Lam Y. (1994). Carnosine protects proteins against in vitro glycation and cross-linking. *Biochem Soc Trans* 22: 399S.

Hipkiss A.R., Preston J.E., Himswoth D.T., Worthington V.C., Abbot N.J. (1997). Protective effects of carnosine against malondialdehyde-induced toxicity towards cultured rat brain endothelial cells. *Neurosci Lett* 238: 135–138.

Hipkiss A.R. (1998). Carnosine, a protective, anti-aging peptide? *Int J Biochem Cell Biol* 30: 863–868.

Hipkiss A.R., Chana H. (1998). Carnosine protects proteins against methylglyoxal-mediated modifications. *Biochem Biophys Res Commun* 248: 28–32.

Hipkiss A.R., Brownson C. (2000). A possible new role for the anti-aging peptide carnosine. *Cell Mol Life Sci* 57: 747–753.

Hipkiss A.R., Brownson C., Carrier M.J. (2001). Carnosine, the anti-aging, anti-oxidant dipeptide, may react with protein carbonyl groups. *Mech Aging Dev* 122: 1431–1445.

Hipkiss A.R., Brownson C., Bertani M.F., Ruiz E., Ferro A. (2002). Reaction of carnosine with aged proteins: Another protective process? *Ann NY Acad Sci* 959: 285–294.

Hirsch J., Petrakova E., Feather M.S. (1992). The reaction of some dicarbonyl sugars with aminoguanidine. *Carbohydr Res* 232: 125–130.

Horinishi H., Grillo M., Margolis F.L. (1978). Purification and characterization of carnosine synthetase from mouse olfactory bulbs. *J Neurochem* 31: 909–919.

Hsu C.C., Yen H.F., Yin M.C., Tsai C.M., Hsieh C.H. (2004). Five cysteine-containing compounds delay diabetic deterioration in Balb/cA mice. *J Nutr* 134: 3245–3249.

Itakura K., Furuhata A., Shibata N., Kobayashi M., Uchida K. (2003). Maillard reaction-like lysine modification by a lipid peroxidation product: immunochemical detection of protein-bound 2-hydroxyheptanal in vivo. *Biochem Biophys Res Commun* 308: 452–457.

Januszewski A.S., Alderson N.L., Metz T.O., Thorpe S.R., Baynes J.W. (2003). Role of lipids in chemical modification of proteins and development of complications in diabetes. *Biochem Soc Trans* 31: 1413–1416.

Kaminskas L.M., Pyke S.M., Burcham P.C. (2004a). Reactivity of hydrazinophthalazine drugs with the lipid peroxidation products acrolein and crotonaldehyde. *Org Biomol Chem* 2: 2578–2584.

Kaminskas L.M., Pyke S.M., Burcham P.C. (2004b). Strong protein adduct trapping accompanies abolition of acrolein-mediated hepatotoxicity by hydralazine in mice. *J Pharmacol Exp Ther* 310: 1003–1010.

Kehrer J.P., Biswal S.S. (2000). The molecular effects of acrolein. *Toxicol Sci* 57: 6–15.

Kono H., Arteel G.E., Rusyn I., Sies H., Thurman R.G. (2001). Ebselen prevents early alcohol-induced liver injury in rats. *Free Radic Biol Med* 30: 403–411.

Lalich J.J., Paik W.C. (1974). Influence of hydralazine consumption on allylamine induced myocardial fibrosis and hypertrophy in rats. *Exp Mol Pathol* 21: 29–39.

Laurent A., Perdu-Durand E., Alary J., Debrauwer L., Cravedi J.P. (2000). Metabolism of 4-hydroxynonenal, a cytotoxic product of lipid peroxidation, in rat precision-cut liver slices. *Toxicol Lett* 114: 203–214.

Lederer M.O., Klaiber R.G. (1999). Cross-linking of proteins by Maillard processes: Characterization and detection of lysine-arginine cross-links derived from glyoxal and methylglyoxal. *Bioorg Med Chem* 7: 2499–2507.

Lee S.H., Blair I.A. (2000). Characterization of 4-oxo-2-nonenal as novel product of lipid peroxidation. *Chem Res Toxicol* 13: 698–702.

Lee S.H., Oe T., Blair I.A. (2001). Vitamin C-induced decomposition of lipid hydroperoxides to endogenous genotoxins. *Science* 292: 2083–2086.

Levy R.S., Fisher M., Alter J.N. (1983). Penicillamine: Review and cutaneous manifestations. *J Am Acad Dermatol* 8: 548–558.

Li C.J., Nanji A.A., Siakotos A.N., Lin R.C. (1997). Acetaldehyde-modified and 4-hydroxynonenal-modified proteins in the livers of rats with alcoholic liver disease. *Hepatology* 26: 650–657.

Liu Q., Raina A.K., Smith M.A., Sayre L.M., Perry G. (2003a). Hydroxynonenal, toxic carbonyls, and Alzheimer disease. *Mol Aspects Med* 24: 305–313.

Liu Z., Minkler P.E., Sayre L.M. (2003b). Mass spectroscopic characterization of protein modification by 4-hydroxy-2-(E)-nonenal and 4-oxo-2-(E)-nonenal. *Chem Res Toxicol* 16: 901–911.

Liu Y., Xu G., Sayre L. (2003c). Carnosine inhibits (E)-4-hydroxy-2-nonenal-induced protein cross-linking: Structural characterization of carnosine-HNE adducts. *Chem Res Toxicol* 16: 1589–1597.

Loidl-Stahlhofen A., Spiteller G. (1994). Alpha-hydroxyaldehydes, products of lipid peroxidation. *Biochim Biophys Acta* 1211: 156–160.

Lovell M.A., Xie C., Markesbery W.R. (2001). Acrolein is increased in Alzheimer's disease brain and is toxic to primary hippocampal cultures. *Neurobiol Aging* 22: 187–194.

Lucas D.T., Szweda L.I. (1998). Cardiac reperfusion injury: Aging, lipid peroxidation, and mitochondrial dysfunction. *Proc Natl Acad Sci USA* 95: 510–514.

Mak S., Lehotay D.C., Yazdanpanah M., Azevedo E.R., Liu P.P., Newton G.E. (2000). Unsaturated aldehydes including 4-OH-nonenal are elevated in patients with congestive heart failure. *J Card Fail* 6: 108–114.

Mamputu J.C., Wiernsperger N., Renier G. (2003). Metformin inhibits monocyte adhesion to endothelial cells and foam cell formation. *Brit J Diab Vasc Dis* 3: 302–310.

Margolis F.L. (1974). Carnosine in the primary olfactory pathway. *Science* 184: 909–911.

McArdle F., Rhodes L.E., Parslew R.A., Close G.L., Jack C.I., Friedmann P.S., Jackson M.J. (2004). Effects of oral vitamin E and β-carotene supplementation on ultraviolet radiation-induced oxidative stress in human skin. *Am J Clin Nutr* 80: 1270–1275.

McGrath L.T., McGleenon B.M., Brennan S., McColl D., McILroy S., Passmore A.P. (2001). Increased oxidative stress in Alzheimer's disease as assessed with 4-hydroxynonenal but not malondialdehyde. *Q J Med* 94: 485–490.

Meade S.J., Miller A.G., Gerrard J.A. (2003). The role of dicarbonyl compounds in non-enzymatic crosslinking: a structure-activity study. *Bioorg Med Chem* 11: 853–862.

Metz T.O., Alderson N.L., Chachich M.E., Thorpe S.R., Baynes J.W. (2003a). Pyridoxamine traps intermediates in lipid peroxidation reactions in vivo: Evidence on the role of lipids in chemical modification of protein and development of diabetic complications. *J Biol Chem* 278: 42012–42019.

Metz T.O., Alderson N.L., Thorpe S.R., Baynes J.W. (2003b). Pyridoxamine, an inhibitor of advanced glycation and lipoxidation reactions: A novel therapy for treatment of diabetic complications. *Arch Biochem Biophys* 419: 41–49.

Miller A.G., Meade S.J., Gerrard J.A. (2003). New insights into protein crosslinking via the Maillard reaction: Structural requirements, the effect on enzyme function, and predicted efficacy of crosslinking inhibitors as anti-aging therapeutics. *Bioorg Med Chem* 11: 843–852.

Miyata T., Inagi R., Asahi K., Yamada Y., Horie K., Sakai H., Uchida K., Kurokawa K. (1998a). Generation of protein carbonyls by glycoxidation and lipoxidation reactions with autoxidation products of ascorbic acid and polyunsaturated fatty acids. *FEBS Lett* 437: 24–28.

Miyata T., Ueda Y., Yamada Y., Izuhara Y., Wada T., Jadoul M., Saito A., Kurokawa K., van Ypersele de Strihou C. (1998b). Accumulation of carbonyls accelerates the formation of pentosidine, an advanced glycation end product: Carbonyl stress in uremia. *J Am Soc Nephrol* 9: 2349–2356.

Miyata T., vanYpersele de Strihou C., Kurokawa K, Baynes J.W. (1999a). Alterations in nonenzymatic biochemistry in uremia: Origin and significance of "carbonyl stress" in long-term uremic complications. *Kidney Int* 55: 389–399.

Miyata T., Ishikawa S., Asahi K., Inagi R., Suzuki D., Horie K., Tatsumi K., Kurokawa K. (1999b). 2-Isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide (OPB-9195) treatment inhibits the development of intimal thickening after balloon injury of rat carotid artery: Role of glycoxidation and lipoxidation reactions in vascular tissue damage. *FEBS Lett* 445: 202–206.

Miyata T., Kurokawa K., Van Ypersele De Strihou C. (2000a). Advanced glycation and lipoxidation end products: Role of reactive carbonyl compounds generated during carbohydrate and lipid metabolism. *J Am Soc Nephrol* 11: 1744–1752.

Miyata T., Ueda Y., Asahi K., Izuhara Y., Inagi R., Saito A., van Ypersele De Strihou C., Kurokawa K. (2000b). Mechanism of the inhibitory effect of OPB-9195 [(+/−)-2-isopropylidenehydrazono-4-oxo-thiazolidin-5-yla cetanilide] on advanced glycation end product and advanced lipoxidation end product formation. *J Am Soc Nephrol* 11: 1719–1725.

Miyata T., Sugiyama S., Saito A., Kurokawa K. (2001a). Reactive carbonyl compounds related uremic toxicity ("carbonyl stress"). *Kidney Int Suppl* 78: S25–S31.

Miyata T., Saito A., Kurokawa K., van Ypersele de Strihou C. (2001b). Advanced glycation and lipoxidation end products: Reactive carbonyl compounds-related uraemic toxicity. *Nephrol Dial Transplant* 16 (Suppl 4): 8–11.

Miyata T., van Ypersele de Strihou C. (2003). Angiotensin II receptor blockers and angiotensin converting enzyme inhibitors: Implication of radical scavenging and transition metal chelation in inhibition of advanced glycation end product formation. *Arch Biochem Biophys* 419: 50–54.

Miyoshi H., Taguchi T., Sugiura M., Takeuchi M., Yanagisawa K. Watanabe Y., Miwa I., Makita Z., Koike T. (2002). Aminoguanidine pyridoxal adduct is superior to aminoguanidine for preventing diabetic nephropathy in mice. *Horm Metab Res* 34: 371–377.

Mlakar A., Spiteller G. (1994). Reinvestigation of lipid peroxidation of linolenic acid. *Biochim Biophys Acta* 1214: 209–220.

Moreau R., Heath S.H., Doneanu C.E., Lindsay J.G., Hagen T.M. (2003). Age-related increase in 4-hydroxynonenal adduction to rat heart alpha-ketoglutarate dehydrogenase does not cause loss of its catalytic activity. *Antioxid Redox Sign* 5: 517–527.

Moreau R., Nguyen B.T., Doneanu C.E., Hagen T.M. (2005). Reversal by aminoguanidine of the age-related increase in glycoxidation and lipoxidation in the cardiovascular system of Fischer 344 rats. *Biochem Pharmacol* 69: 29–40.

Munch G., Taneli Y., Schraven E., Schindler U., Schinzel R., Palm D., Riederer P. (1994). The cognition-enhancing drug tenilsetam is an inhibitor of protein crosslinking by advanced glycosylation. *J Neural Transm Park Dis Demen Sect* 8: 193–208.

Munch G., Mayer S., Michaelis J., Hipkiss A.R., Riederer P., Muller R., Neumann A., Schinzel R., Cunningham A.M. (1997). Influence of advanced glycation end-products and AGE-inhibitors on nucleation-dependent polymerization of beta-amyloid peptide. *Biochim Biophys Acta* 1360: 17–29.

Nadkarni D.V., Sayre L.M. (1995). Structural definition of early lysine and histidine adduction chemistry of 4-hydroxynonenal. *Chem Res Toxicol* 8: 284–291.

Nair V., Vietti D.E., Cooper C.S. (1981). Degenerative chemistry of malondialdehyde: Structure, stereochemistry, and kinetics of formation of enaminals from reaction with amino acids. *J Am Chem Soc* 103: 3030–3096.

Nakamura S., Makita Z., Ishikawa S., Yasumura K., Fujii W., Yanagisawa K., Kawata T., Koike T. (1997). Progression of nephropathy in spontaneous diabetic rats is prevented by OPB-9195, a novel inhibitor of advanced glycation. *Diabetes* 45: 895–899.

Nakamura Y., Morimitsu Y., Uzu T., Ohigashi H., Murakami A., Naito Y., Nakagawa Y., Osawa T., Uchida K. (2000). A glutathione S-transferase inducer from papaya: Rapid screening, identification and structure-activity relationship of isothiocyanates. *Cancer Lett* 157: 193–200.

Nakamura S., Tachikawa T., Tobita K., Aoyama I., Takayama F., Enomoto A., Niwa T. (2003). An inhibitor of advanced glycation end product formation reduces N^{ε}-(carboxymethyl)lysine accumulation in glomeruli of diabetic rats. *Am J Kidney Dis* 41(Suppl 1): S68–S71.

Neely M.D., Zimmerman L., Picklo M.J., Ou J.J., Morales C.R., Montine K.S., Amaranth V., Montine T.J. (2000). Congeners of *N*(alpha)-acetyl-L-cysteine but not aminoguanidine act as neuroprotectants from the lipid peroxidation product 4-hydroxy-2-nonenal. *Free Radic Biol Med* 29: 1028–1036.

Niemela O., Parkkial S., Pasanen M., Viitala K., Vilianueva J.A., Halsted C.H. (1999). Induction of cytochrome P450 enzymes and generation of protein-aldehyde adducts are associated with sex-dependent sensitivity to alcohol-induced liver disease in micropigs. *Hepatology* 30: 1011–1017.

Niemela O., Parkkila S., Worrall S., Emery P.W., Preedy V.R. (2003). Generation of aldehyde-derived protein modifications in ethanol-exposed heart. *Alcohol Clin Exp Res* 27: 1987–1992.

Noiri E., Yamada S., Nakao A., Tsuchiya M., Masaki I., Fujino K. (2002). Serum protein acrolein adducts: Utility in detecting oxidant stress in hemodialysis patients and reversal using a vitamin E-bonded hemodialyzer. *Free Radic Biol Med* 33: 1651–1656.

Oates J.A., Brown N.J. (2001). Antihypertensive agents and the drug therapy of hypertension. In: Hardman J.G. and Limbird L.E. (eds.), *Goodman and Gilman's the Pharmacological Basis of Therapeutics*, 10th ed., McGraw-Hill, New York, pp. 871–900.

Odani H., Shinzato T., Usami J., Matsumoto Y., Brinkmann Frye E., Baynes J.W., Maeda K. (1998). Imidazolium crosslinks derived from reaction of lysine with glyoxal and methylglyoxal are increased in serum proteins of uremic patients: evidence for increased oxidative stress in uremia. *FEBS Lett* 427: 381–385.

Odetti P., Pesce C., Traverso N., Menini S., Maineri E.P., Cosso L., Valentini S., Patriarca S., Cottalasso D., Marinari U.M., Pronzato M.A. (2003). Comparative trial of *N*-acetyl-cysteine, taurine, and oxerutin on skin and kidney damage in long-term experimental diabetes. *Diabetes* 52: 499–505.

Onorato J.M., Jenkins A.J., Thorpe S.R., Baynes J.W. (2000). Pyridoxamine, an inhibitor of advanced glycation reactions, also inhibits advanced lipoxidation reactions. Mechanism of action of pyridoxamine. *J Biol Chem* 275: 21177–21184.

Orioli M., Aldini G., Beretta G., Maffei Facino R., Carini M. (2005). LC-ESI-MS/MS determination of 4-hydroxy-*trans*-2-nonenal Michael adducts with cysteine and histidine-containing peptides as early markers of oxidative stress in excitable tissues *J Chromatogi B* 827: 109–118.

Palinski W., Miller E., Witztum J.L. (1995). Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis. *Proc Natl Acad Sci USA* 92: 821–825.

Paradis V., Kollinger M., Fabre M., Holstege A., Poynard T., Bedossa P. (1997a). In situ detection of lipid peroxidation by-products in chronic liver diseases. *Hepatology* 26: 135–142.

Paradis V., Mathurin P., Kollinger M., Imbert-Bismut F., Piton A., Opolon P., Holstege A., Poynard T., Bedossa P. (1997b). In situ detection of lipid peroxidation in chronic hepatitis C: correlation with pathological features. *J Clin Pathol* 50: 401–406.

Petersen D.R., Doorn J.A. (2004). Reactions of 4-hydroxynonenal with proteins and cellular targets. *Free Radic Biol Med* 37: 937–945.

Petroff O.A., Hyder F., Rothman D.L., Mattson R.H. (2001). Homocarnosine and seizure control in juvenile myoclonic epilepsy and complex partial seizures. *Neurology* 56: 709–715.

Picard S., Parthasarathy S., Fruebis J., Witztum J.L. (1992). Aminoguanidine inhibits oxidative modification of low density lipoprotein protein and the subsequent increase in uptake by macrophage scavenger receptors. *Proc Natl Acad Sci USA* 89: 6876–6880.

Picklo M.J., Montine T.J., Amarnath V., Neely M.D. (2002). Carbonyl toxicology and Alzheimer's disease. *Toxicol Appl Pharmacol* 184: 187–197.

Poli G., Schaur J. (2000). 4-Hydroxynonenal in the pathomechanisms of oxidative stress. *IUBMB Life* 50: 315–321.

Pryor A.W., Stanley P. (1975). A suggested mechanism for the production of malondialdehyde during the autooxidation of polyunsaturated fatty acids: Nonenzymatic production of prostaglandin endoperoxides during autoxidation. *J Org Chem* 40: 3615–3617.

Rahbar S., Natarajan R., Yerneni K., Scott S., Gonzales N., Nadler J.L. (2000). Evidence that pioglitazone, metformin and pentoxifylline are inhibitors of glycation. *Clin Chim Acta* 301: 65–77.

Rahbar S., Figarola J.L. (2003). Novel inhibitors of advanced glycation end products. *Arch Biochem Biophys* 419: 63–79.

Reinheckel T., Nedelev B., Prause J., Augustin W., Schulz H.U., Lippert H., Halangk W. (1998). Occurrence of oxidatively modified proteins: An early event in experimental acute pancreatitis. *Free Radic Biol Med* 24: 393–400.

Rindgen D., Nakajima M., Wehrli S., Xu K., Blair I.A. (1999). Covalent modifications to 2′-deoxyguanosine by 4-oxo-2-nonenal, a novel product of lipid peroxidation. *Chem Res Toxicol* 12: 1195–1204.

Ruggiero-Lopez D., Lecomte M., Moinet G., Patereau G., Lagarde M., Wiernsperger N. (1999). Reaction of metformin with dicarbonyl compounds: Possible implication in the inhibition of advanced glycation end product formation. *Biochem Pharmacol* 58: 1765–1773.

Sakata K., Kashiwagi K., Sharmin S., Ueda S., Irie Y., Murotani N., Igarashi K. (2003a). Increase in putrescine, amine oxidase, and acrolein in plasma of renal failure patients. *Biochem Biophys Res Commun* 305: 143–149.

Sakata K., Kashiwagi K., Sharmin S., Ueda S., Igarashi K. (2003b). Acrolein produced from polyamines as one of the uraemic toxins. *Biochem Soc Trans* 31: 371–374.

Sampey B.P., Korourian S., Ronis M.J., Badger T.M., Petersen D.R. (2003). Immunohistochemical characterization of hepatic malondialdehyde and 4-hydroxynonenal modified proteins during early stages of ethanol-induced liver injury. *Alcohol Clin Exp Res* 27: 1015–1022.

Sayre L.M., Arora P.K., Iyer R.S., Salomon R.G. (1993). Pyrrole formation from 4-hydroxynonenal and primary amines. *Chem Res Toxicol* 6: 19–22.

Sayre L.M., Zelasko D.A., Harris P.L.R., Perry G., Salomon R.G., Smith M.A. (1997). 4-hydroxynonenal-derived advanced lipid peroxidation end products are increased in Alzheimer's disease. *J Neurochem* 68: 2092–2097.

Schiopu A., Bengtsson J., Soderberg I., Janciauskiene S., Lindgren S., Ares M., Shah P.K., Carlsson R., Nilsson J., Fredrikson G.N. (2004). Recombinant human antibodies against aldehyde-modified apolipoprotein B-100 peptide sequences inhibit atherosclerosis. *Circulation* 110: 2047–2052.

Schneider C., Tallman K.A., Porter N.A., Brash A.R. (2001). Two distinct pathways of formation of 4-hydroxynonenal: Mechanisms of nonenzymatic transformation of the 9- and 13-hydroperoxides of linoleic acid to 4-hydroxyalkenals. *J Biol Chem* 276: 20831–20838.

Selley M.L. (1998). (E)-4-hydroxy-2-nonenal may be involved in the pathogenesis of Parkinson's disease. *Free Radic Biol Med* 25: 169–174.

Selwa L.M. (1999). *N*-Acetylcysteine therapy for Unverricht-Lundborg disease. *Neurology* 52: 426–427.

Shangari N., Bruce W.R., Poon R., O'Brien P.J. (2003). Toxicity of glyoxals—Role of oxidative stress, metabolic detoxification and thiamine deficiency. *Biochem Soc Trans* 31: 1390–1393.

Shangari N., O'Brien P.J. (2004). The cytotoxicity mechanism of glyoxal involves oxidative stress. *Biochem Pharmacol* 668: 1433–1442.

Shapiro H.K. (1998). Carbonyl-trapping therapeutic strategies. *Am J Ther* 5: 323–353.

Shibata N., Yamada S., Uchida K., Hirano A., Sakoda S., Fujimura H., Sasaki S., Iwata M., Toi S., Kawaguchi M., Yamamoto T., Kobayashi M. (2004). Accumulation of protein-bound 4-hydroxy-2-hexenal in spinal cords from patients with sporadic amyotrophic lateral sclerosis. *Brain Res* 1019: 170–177.

Shoda H., Miyata S., Liu B.F., Yamada H., Ohara T., Suzuki K., Oimomi M., Kasuga M. (1997). Inhibitory effects of tenilsetam on the Maillard reaction. *Endocrinology* 138: 1886–1892.

Siems W., Carluccio F., Grune T., Jakstadt M., Quast S., Hampl H., Sommerburg O. (2002). Elevated serum concentration of cardiotoxic lipid peroxidation products in chronic renal failure in relation to severity of renal anemia. *Clin Nephrol* 58 (Suppl 1): S20–S25.

Siems W., Grune T. (2003). Intracellular metabolism of 4-hydroxynonenal. *Mol Aspects Med* 24: 167–175.

Simpson E.P., Henry Y.K., Henkel J.S., Smith R.G., Appel S.H. (2004). Increased lipid peroxidation in sera of ALS patients. *Neurobiol* 62: 1758–1765.

Singh R., Burden A, Mori T., Bellin L. (2001). Advanced glycation end-products. *Diabetologia* 44: 129–146.

Slatter D.A., Murray M., Bailey A.J. (1998). Formation of a dihydropyridine derivative as a potential cross-link from malondialdehyde in physiological systems. *FEBS Letts* 421: 180–184.

Slatter D.A., Paul R.G., Murray M., Bailey A.J. (1999). Reactions of lipid-derived malondialdehyde with collagen. *J Biol Chem* 274: 19661–19669.

Slatter D.A., Bolton C.H., Bailey A.J. (2000). The importance of lipid-derived malondialdehyde in diabetes mellitus. *Diabetologia* 43: 550–557.

Slatter D.A., Avery N.C., Bailey A.J. (2004). Identification of a new cross-link and unique histidine adduct from bovine serum albumin incubated with malondialdehyde. *J Biol Chem* 279: 61–69.

Solin M.L., Ahola H., Haltia A., Ursini F., Montine T., Roveri A., Kerjaschki D., Holthofer H. (2001). Lipid peroxidation in human proteinuric disease. *Kidney Int* 59: 481–487.

Soulis T., Sastra S., Thallas V., Mortensen S.B., Wilken M., Clausen J.T., Bjerrum O.J., Petersen H., Lau J., Jerums G., Boel E., Cooper M.E. (1999). A novel inhibitor of advanced glycation end-product formation inhibits mesenteric vascular hypertrophy in experimental diabetes. *Diabetologia* 42: 472–479.

Sowell J., Frei B., Stevens J.F. (2004). Vitamin C conjugates of genotoxic lipid peroxidation products: Structural characterization and detection in human plasma. *Proc Natl Acad Sci USA* 101: 17964–17969.

Srivastava S., Dixit B.L., Cai J., Sharma S., Hurst H.E., Bhatnagar A., Srivastava S.K. (2000). Metabolism of lipid peroxidation product, 4-hydroxynonenal (HNE) in rat erythrocytes: Role of aldose reductase. *Free Radic Biol Med* 29: 642–651.

Stitt A., Gardiner T.A., Alderson N.L., Canning P., Frizzell N., Duffy N., Boyle C., Januszewski A.S., Chachich M. Baynes J.W., Thorpe S.R., Anderson N.L. (2002). The AGE inhibitor pyridoxamine inhibits development of retinopathy in experimental diabetes. *Diabetes* 51: 2826–2832.

Stuerenburg H.J. (2000). The roles of carnosine in aging of skeletal muscle and in neuromuscular diseases. *Biochemistry (Mosc)* 65: 862–865.

Stvolinsky S.L., Dobrota D. (2000). Anti-ischemic activity of carnosine. *Biochemistry (Mosc)* 65: 849–855.

Suzuki D., Miyata T. (1999). Carbonyl stress in the pathogenesis of diabetic nephropathy. *Intern Med* 38: 309–314.

Szweda L.I., Uchida K., Tsai L., Statdman E.R. (1993). Inactivation of glucose-6-phosphate dehydrogenase by 4-hydroxy-2-nonenal. *J Biol Chem* 268: 3342–3347.

Taguchi T., Sugiura M., Hamada Y., Miwa I. (1998). In vivo formation of a Schiff base of aminoguanidine with pyridoxal phosphate. *Biochem Pharmacol* 55: 1667–1671.

Taguchi T., Sugiura M., Hamada Y., Miwa I. (1999). Inhibition of advanced protein glycation by a Schiff base between aminoguanidine and pyridoxal. *Eur J Pharmacol* 378: 283–289.

Tanaka Y., Iwamoto H., Onuma T., Kawamori R. (1997). Inhibitory effect of metformin on formation of advanced glycation end products. *Curr Ther Res* 58: 693–697.

Tanaka N., Tajima S., Ishibashi A., Uchida K., Shigematsu T. (2001). Immunohistochemical detection of lipid peroxidation products, protein-bound acrolein and 4-hydroxynonenal protein adducts, in actinic elastosis of photodamaged skin. *Arch Dermatol Res* 293: 363–367.

Terman A., Brunk U.T. (2004). Lipofuscin. *Int J Biochem Cell Biol* 36: 1400–1404.

Tessier F., Obrenovich M., Monnier V.M. (1999). Structure and mechanism of formation of human lens fluorophore LM-1: Relationship to vesperlysine A and the advanced Maillard reaction in aging, diabetes, and cataractogenesis. *J Biol Chem* 274: 20796–20804.

Teufel M., Saudek V., Ledig J.P., Bernhardt A., Boularand S., Carreau A., Cairns N.J., Carter C., Cowley D.J., Duverger D., Ganzhorn A.J., Guenet C., Heintzelmann B., Laucher V., Sauvage C., Smirnova T. (2003). Sequence identification and characterization of human carnosinase and a closely related non-specific dipeptidase. *J Biol Chem* 278: 6521–6531.

Thies F., Garry J.M., Yaqoob P., Rerkasem K., Williams J., Shearman C.P., Gallagher P.J., Calder P.C., Grimble R.F. (2003). Association of *n*-3 polyunsaturated fatty acids with stability of atherosclerotic plaques: A randomised controlled trial. *Lancet* 361: 477–485.

Thornalley P.J., Yurek-George A., Argirov O.K. (2000). Kinetics and mechanism of the reaction of aminoguanidine with the alpha-oxoaldehydes glyoxal, methylglyoxal, and 3-deoxyglucosone under physiological conditions. *Biochem Pharmacol* 60: 55–65.

Thornalley P.J. (2003). Use of aminoguanidine (Pimagedine) to prevent the formation of advanced glycation end products. *Arch Biochem Biophys* 419: 31–40.

Thorpe S.R., Baynes J.W. (2003). Maillard reaction products in tissue proteins: New products and new perspectives. *Amino Acids* 25: 275–281.

Toyokuni S., Yamada S., Kashima M., Ihara Y., Yamada Y., Tanaka T., Hiai H., Seino Y., Uchida K. (2000). Serum 4-hydroxy-2-nonenal-modified albumin is elevated in patients with type 2 diabetes mellitus. *Antioxid Redox Sign* 2: 681–685.

Traverso N., Menini S., Cosso L., Odetti P., Albano E., Pronzato M.A., Marinari U.M. (1998). Immunological evidence for increased oxidative stress in diabetic rats. *Diabetologia* 41: 265–270.

Tsakadze N.L., Srivastava S., Awe S.O., Adeagbo A.S., Bhatnagar A., D'Souza S.E. (2003). Acrolein-induced vasomotor responses of rat aorta. *Am J Physiol Heart Circ Physiol* 285: H727–H734.

Tsukahara H., Jiang M.Z., Ohta N., Sato S., Tamura S., Hiraoka M., Maeda M., Mayumi M. (2004). Oxidative stress in neonates: Evaluation using specific biomarkers. *Life Sci* 75: 933–938.

Uchida K., Stadtman E.R. (1992). Modification of histidine residues in proteins by reaction with 4-hydroxynonenal. *Proc Natl Acad Sci USA* 89: 4544–4548.

Uchida K., Itakura K., Kawakishi S., Hiai H., Toyokuni S., Stadtman E.R. (1995). Characterization of epitopes recognized by 4-hydroxy-2-nonenal specific antibodies. *Arch Biochem Biophys* 324: 241–248.

Uchida K., Kanematsu M., Morimitsu Y., Osawa T., Noguchi N., Niki E. (1998a). Acrolein is a product of lipid peroxidation reaction. Formation of acrolein and its conjugate with lysine residues in oxidized low-density lipoproteins. *J Biol Chem* 273: 16058–16066.

Uchida K., Kanematsu M., Sakai K., Matsuda T., Hattorisd N., Mizuno Y., Suzuki D., Miyata T., Noguchi N., Niki E. (1998b). Protein-bound acrolein: Potential markers for oxidative stress. *Proc Natl Acad Sci USA* 95: 4882–4887.

Uchida K. (1999). Current status of acrolein as lipid peroxidation product. *Trends Cardiovasc Mol* 9: 109–113.

Uchida K. (2000). Role of reactive aldehyde in cardiovascular diseases. *Free Radic Biol Med* 28: 1685–1696.

Uchida K. (2003). 4-Hydroxy-2-nonenal: A product and mediator of oxidative stress. *Prog Lipid Res* 42: 318–343.

Uchida K., Kumagai T. (2003). 4-hydroxy-2-nonenal as a COX-2 inducer. *Mol Aspects Med* 24: 213–218.

Voziyan P.A., Metz T.O., Baynes J.W., Hudson B.G. (2002). A post–Amadori inhibitor pyridoxamine also inhibits chemical modification of proteins by scavenging carbonyl intermediates of carbohydrate and lipid degradation. *J Biol Chem* 277: 3397–3403.

Voziyan P.A., Khalifah R.G., Thibaudeau C., Yildiz A., Jacob J., Serianni A.S., Hudson B.G. (2003). Modification of proteins in vitro by physiological levels of glucose: Pyridoxamine inhibits conversion of Amadori intermediate to advanced glycation endproducts through binding of redox metal ions. *J Biol Chem* 278: 46616–46624.

Wada R., Nishizawa Y., Yagihashi N., Takeuchi M., Ishikawa Y., Yasumura K., Nakano M., Yagihashi S. (2001). Effects of OPB-9195, anti-glycation agent, on experimental diabetic neuropathy. *Eur J Clin Invest* 31: 513–520.

Waeg G., Dimsity G., Esterbauer H. (1996). Monoclonal antibodies for detection of 4-hydroxynonenal modified proteins. *Free Radic Res* 25: 149–159.

Wataya T., Nunomura A., Smith M.A., Siedlak S.L., Harris P.L., Shimohama S., Szweda L.I., Kaminski M.A., Avila J., Price D.L., Cleveland D.W., Sayre L.M., Perry G. (2002). High molecular weight neurofilament proteins are physiological substrates of adduction by the lipid peroxidation product hydroxynonenal. *J Biol Chem* 277: 4644–4648.

Wells-Knecht K.J., Brinkmann E., Baynes J.W. (1995). Characterization of an imidazolium salt formed from glyoxal and N^{α}-hippuryllysine: a model for Maillard reaction crosslinks in proteins. *J Org Chem* 60: 6246–6247.

Wilkinson-Berka J.L., Kelly D.J., Koerner S.M., Jaworski K., Davis B., Thallas V., Cooper M.E. (2002). ALT-946 and aminoguanidine, inhibitors of advanced glycation, improve severe nephropathy in the diabetic transgenic (mREN-2)27 rat. *Diabetes* 51: 3283–3289.

Wondrak G.T., Cervantes-Laurean D., Roberts M.J., Qasem J.G., Kim M., Jacobson E.L., Jacobson M.K. (2002). Identification of alpha-dicarbonyl scavengers for cellular protection against carbonyl stress. *Biochem Pharmacol* 63: 361–373.

Yamada S., Funada T., Shibata N., Kobayashi M., Kawai Y., Tatsuda E., Furuhata A., Uchida K. (2004). Protein-bound 4-hydroxy-2-hexenal as a marker of oxidized *n*-3 polyunsaturated fatty acids. *J Lipid Res* 45: 626–634.

Yoritaka A., Hattori N., Uchida K., Tanaka M., Stadtman E.R., Mizuno Y. (1996). Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. *Proc Natl Acad Sci USA* 93: 2696–2701.

Zagorski M.G., Salomon R.G. (1982). Prostaglandin endoperoxides. Carboxylate catalysis and the effects of proton donors on the decomposition of 2,3-dioxabicyclo [2.2.1] heptane. *J Am Chem Soc* 104: 3498–3503.

Zarkovic N. (2003). 4-hydroxynonenal as a bioactive marker of pathophysiological processes. *Mol Aspects Med* 24: 281–291.

Zhang W.H., Liu J., Xu G., Yuan Q., Sayre L.M. (2003). Model studies on protein side chain modification by 4-oxo-2-nonenal. *Chem Res Toxicol* 16: 512–523.

Zhou S., Decker E.A. (1999). Ability of carnosine and other skeletal muscle components to quench unsaturated aldehydic lipid oxidation products. *J Agric Food Chem* 47: 51–55.

INDEX

Redox Proteomics: from Protein Modifications to Cellular Dysfunction and Diseases, Edited by Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield