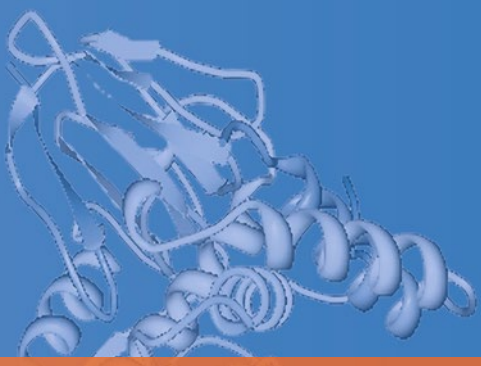
Subcellular Biochemistry 77



Yoji Kato *Editor*

Lipid Hydroperoxide-Derived Modification of Biomolecules

Springer

Lipid Hydroperoxide-Derived Modification of Biomolecules

SUBCELLULAR BIOCHEMISTRY

Recent Volumes in this Series

Volume 57 **Aging Research in Yeast**
Edited by Michael Breitenbach, Michal S. Jazwinski and Peter Laun

Volume 58 **Phosphoinositides I: Enzymes of Synthesis and Degradation**
Edited by Tamas Balla, Matthias Wymann and John D. York

Volume 59 **Phosphoinositides II: The Diverse Biological Functions**
Edited by Tamas Balla, Matthias Wymann and John D. York

Volume 60 **Adherens Junctions: From Molecular Mechanisms to Tissue Development and Disease**
Edited by Tony Harris

Volume 61 **Epigenetics: Development and Disease**
Edited by Kundu and Tapas Kumar

Volume 62 **The Eukaryotic Replisome: a Guide to Protein Structure and Function**
Edited by MacNeill and Stuart

Volume 63 **GPCR Signalling Complexes – Synthesis, Assembly, Trafficking and Specificity**
Edited by Dupré J. Denis, Hébert E. Terence and Jockers Ralf

Volume 64 **Reprogramming Microbial Metabolic Pathways**
Edited by Wang Xiaoyuan, Chen Jian and Quinn Peter

Volume 65 **Protein Aggregation and Fibrillogenesis in Cerebral and Systemic Amyloid Disease**
Edited by Harris J. Robin

Volume 66 **Regulated Proteolysis in Microorganisms**
Edited by David A. Dougan

Volume 67 **Cytochrome P450 2E1: Its Role in Disease and Drug Metabolism**
Edited by Aparajita Dey

Volume 68 **Structure and Physics of Viruses**
Edited by Mauricio G. Mateu

Volume 69 **Peroxisomes and their Key Role in Cellular Signaling and Metabolism**
Edited by Luis A. del Rio

Volume 74 **Proteins and Proteomics of Leishmania and Trypanosoma**
Edited by A.L.S. Santos, M.H. Branquinha, C.M. d'Avila-Levy, L.F. Kneipp and C.L. Sodré

Volume 75 **Carbonic Anhydrase: Mechanism, Regulation, Links to Disease, and Industrial Applications**
Edited by Susan C. Frost and Robert McKenna

For further volumes:
http://www.springer.com/series/6515

Yoji Kato
Editor

Lipid Hydroperoxide-Derived Modification of Biomolecules

Springer

Editor
Yoji Kato
School of Human Science and Environment
University of Hyogo
Himeji, Hyogo, Japan

ISSN 0306-0225
ISBN 978-94-007-7919-8 ISBN 978-94-007-7920-4 (eBook)
DOI 10.1007/978-94-007-7920-4
Springer Dordrecht Heidelberg New York London

Library of Congress Control Number: 2013957711

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

Lipid peroxidation is an important cellular process which can lead to detrimental effects if it is not regulated efficiently. It may also be one of determinants of the shelf life and/or quality of food and as such important in everyday life on a number of levels. Lipid hydroperoxide (ROOH) is formed in an initial step of non-enzymatic lipid peroxidation or it is enzymatically generated by lipoxygenases. Lipid hydroperoxide is also known as a potential source of singlet oxygen.

Generally speaking, harmful aldehydes are formed when the lipid hydroperoxide is degraded. The formed aldehyde has high reactivity against thiol or amine moieties. Therefore, it could act as a signaling molecule, which might induce a changing of gears inside a cell. Among the aldehydes, the reactivity and chemistry of 4-hydroxy-2-nonenal and malondialdehyde have been well researched. On the other hand, recent studies have shown that lipid hydroperoxide or a slightly modified product of the lipid hydroperoxide reacts with biomolecules such as proteins and aminophospholipids, which leads to formation of amide-type adducts. The detailed chemical mechanism which leads to the formation of amide-type conjugates is still unknown. Amide-type adducts could be one of the markers for oxidative stress causing food deterioration and could also be an important player in some diseases. In this book, the chemistry and biochemistry of lipid hydroperoxide along with their conjugates with biomolecules are described.

There are three major parts in this book. Part I, entitled “Lipid Peroxidation and Small Molecule Adducts”, consist of six chapters. This focuses basic studies on generation of singlet oxygen from lipid hydroperoxide, cholesterol oxidation, and some adducts between lipid peroxidation products and biomolecules. The contents of the chapters are so-called molecular-basis studies. As Part II, the relationship between lipid peroxidation and diseases are shown in five chapters. As a pathophysiological consequence, adducts between lipid peroxidation products and biomolecules are formed under a high dose of oxidative stress and might contribute

to the initiation/development/progression of diseases. The final Part III describes the application for diagnosis, including a novel protein chip technology, and also for development of functional food.

I'm deeply indebted to authors in this book for their contributions. I hope that this book lightens some novel aspects on research of lipid peroxidation.

School of Human Science and Environment Yoji Kato
University of Hyogo
Himeji, Hyogo, Japan

Contents

Part I
Lipid Peroxidation and Small Molecule Adducts

Chapter 1
Lipid Hydroperoxides as a Source of Singlet Molecular Oxygen

Sayuri Miyamoto and Paolo Di Mascio

Abstract Lipid hydroperoxides (LOOH) are formed in biological system by enzymatic and non-enzymatic pathways. These hydroperoxides exerts multiple damaging effects on cellular macromolecules and are also important regulators of cellular processes. Several classes of hydroperoxides including fatty acid, phospholipid, cholesterol and cholesteryl ester hydroperoxides have been detected and characterized both *in vitro* and *in vivo*. Although cells are normally endowed with enzymatic defenses capable to reduce LOOH to less reactive hydroxides, LOOH may accumulate in several pathological conditions and attention has been focused on elucidating their pathophysiological role. In the last years we have demonstrated the generation of singlet molecular oxygen (O_2 $^1\Delta_g$ or $^1O_2$) in several reactions involving LOOH. The generation of $^1O_2$ was directly evidenced by spectroscopic detection and characterization of its light emission at 1,270 nm. Moreover, using 18-oxygen labeled hydroperoxides ($L^{18}O^{18}OH$) we could detect the formation of ^{18}O-labeled $^1O_2$ by chemical trapping with anthracene derivatives followed by detection of the corresponding labeled endoperoxides by HPLC coupled to tandem mass spectrometry. The experimental evidences indicate that $^1O_2$ is generated at a yield close to 10 % by the Russell mechanism from LOOH, either free or in membranes, in the presence of biologically relevant oxidants, such as metal ions, peroxynitrite, HOCl and cytochrome c.

Keywords Lipid hydroperoxides • Singlet molecular oxygen • Mass spectrometry • Luminescence

S. Miyamoto (✉) • P. Di Mascio
Departamento de Bioquímica, Instituto de Química,
Universidade de São Paulo, São Paulo, Brazil
e-mail: miyamoto@iq.usp.br

Y. Kato (ed.), *Lipid Hydroperoxide-Derived Modification of Biomolecules*,
Subcellular Biochemistry 77, DOI 10.1007/978-94-007-7920-4_1,

1 Introduction

Lipid hydroperoxides (LOOH) are the primary products of lipid peroxidation and singlet molecular oxygen (O_2 $^1\Delta_g$ or $^1O_2$) mediated oxidation (Girotti 1998). They can also be formed by the action of enzymes such as lipoxygenase and cyclooxygenase (Schneider et al. 2007). Several types of LOOH were characterized, among which the most studied are the hydroperoxides of polyunsaturated fatty acids, phospholipids, cholesterol and cholesteryl esters.

Once formed, LOOH can undergo reactions which reduce and/or increase its toxicity (Girotti 1998).

Usually, most LOOH formed in cells are reduced to the corresponding alcohols by antioxidant enzymes (Ursini and Bindoli 1987), however, in situations where oxidative stress prevails, the LOOH can accumulate and participate in secondary reactions. For example, LOOH reacts with metal ions and other oxidants being converted to peroxyl ($LOO^\bullet$) and/or alkoxyl ($LO^\bullet$) radical intermediates. These radicals can attack other lipids promoting the propagation of lipid peroxidation. Alternatively, they participate in reactions involving cyclizations and fragmentations to generate secondary products such as aldehydes, ketones, and epoxides (Esterbauer et al. 1991). Furthermore, $LOO^\bullet$ may also react with another $LOO^\bullet$ generating $^1O_2$ by means of the Russell mechanism (Russell 1957; Howard and Ingold 1968; Miyamoto et al. 2007) and this aspect is described in details in this chapter.

2 Biological Sources of Singlet Oxygen

Several researchers have investigated the formation $^1O_2$ in chemical and biological system (Kanofsky 1989; Adam et al. 2005; Miyamoto et al. 2007). Among the possible biological sources, we will discuss in details here the generation of $^1O_2$ during phagocytosis, in photosensitized reactions and during lipid peroxidation.

The proposal of $^1O_2$ formation as a microbicidal agent during the process of phagocytosis was first suggested in 1972 based on luminescence studies (Allen et al. 1972). Allen et al. observed the appearance of a luminescence after stimulation of neutrophils with latex and attributed it to the radioactive decay of $^1O_2$ to the ground state. Later studies also observed the appearance of a weak luminescence during phagocytosis (Cheson et al. 1976; Cadenas et al. 1981). Studies conducted by Krinsky (1974) suggested the involvement of $^1O_2$ in bacterial death. They have noted that the mutant white bacteria lineage without carotenoids, *Sarcina lutea*, died during phagocytosis more easily than the normal strains containing carotenoids and attributed this difference to the physical suppression of $^1O_2$ mediated by carotenoids. A similar result was obtained by Tatsuzawa et al. (1999) using strains of *E. coli* transformed to produce lycopene, a very effective physical quencher of $^1O_2$ (Di Mascio et al. 1989). They observed that

lycopene producing strain had a longer survival than the wild strain and concluded that $^1O_2$ might be produced during phagocytosis. Subsequently, Steinbeck et al. (1992) showed the intracellular production of $^1O_2$ in neutrophils using particles coated with 9,10-diphenyl anthracene (DPA), a specific chemical trap for $^1O_2$, yielding the corresponding DPA endoperoxides Using this technique, the authors reported a relatively high conversion rate of 19 % of the oxygen consumed during phagocytosis to $^1O_2$(Steinbeck et al. 1992).

Singlet molecular oxygen production during phagocytosis is primarily attributed to reaction system comprising myeloperoxidase (MPO), H_2O_2 and chloride ions ($MPO/H_2O_2/Cl^-$). MPO catalyzes the reaction of H_2O_2 producing HOCl (1.1), which in turn reacts with H_2O_2 generating $^1O_2$ (1.2). The formation of $^1O_2$ by the $MPO/H_2O_2/Cl^-$ system was first proposed by Rosen and Klebanoff in 1977 (Rosen and Klebanoff 1977), using furan as chemical trap for $^1O_2$. In 1984, Khan confirmed the formation of $^1O_2$ by $MPO/H_2O_2/Cl^-$ system through direct measurements of light emission in the infrared region at 1,270 nm (Khan 1984). Later, Kiryu et al. (1999) also demonstrated the production of $^1O_2$ using physiological concentrations of $MPO/H_2O_2/Cl^-$ by detecting the light emission band centered on 1,270 nm region, thus clearly evidencing the formation of $^1O_2$ by this system.

$$H_2O_2 + Cl^- + H^+ \xrightarrow{MPO} H_2O + HOCl \tag{1.1}$$

$$HOCl + H_2O_2 \rightarrow {}^1O_2 + Cl^- + H_2O + H^+ \tag{1.2}$$

Photosensitized generation of an activated state of oxygen was first suggested by Kautsky (1939). The formation of $^1O_2$ in this process was confirmed by Foote and Wexler (1964; Foote 1968). The photooxidation process (1.3) starts with the absorption of photons by the photosensitizer (^1S) and its activation to a singlet excited state ($^1S^*$). This state is rapidly converted to a longer lifetime species, the triplet excited state ($^3S^*$), which can follow two distinct pathways, called type I and type II. Type I involves electron transfer between the $^3S^*$ and a substrate generating radical or radical ion species. Type II involves energy transfer from the $^3S^*$ to triplet molecular oxygen yielding ^1S and $^1O_2$.

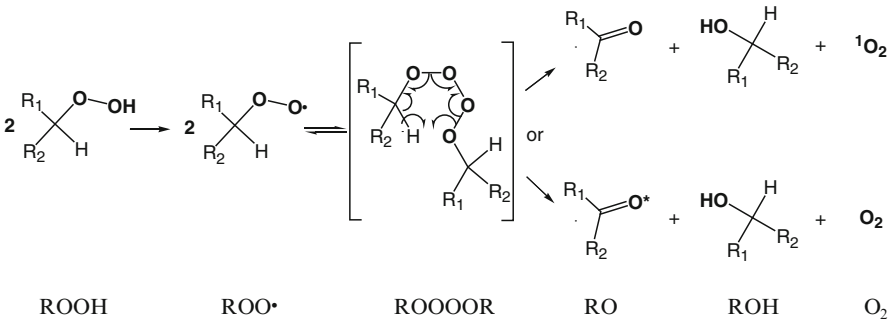
$$\begin{array}{l} {}^1S \xrightarrow{h\nu} {}^1S^* \longrightarrow {}^3S^* \xrightarrow{RH} \text{Radical or radical ions (Type I)} \\ \qquad\qquad\qquad\qquad\quad \xrightarrow{O_2} {}^1S + {}^1O_2 \text{ (Type II)} \end{array} \tag{1.3}$$

Photosensitized reactions in biological systems, usually involving $^1O_2$, occur in certain pathologies. An example is the porphyrias, innate or acquired diseases related to defects in the biosynthesis of heme, which can result in excessive production of porphyrins and its precursors. People with porphyria should not be exposed to the sun, because the action of light on the porphyrins promotes excessive

generation of $^1O_2$ and other reactive species, resulting in ulceration of the skin. Importantly, $^1O_2$ generation can also occur by reactions involving other endogenous photosensitizers such as bilirubin, the flavins (FMN, FAD), and pyridine nucleotides (NADH, NADPH) (Carbonare and Pathak 1992). In contrast to the deleterious effects of uncontrolled photosensitized reactions the controlled photosensitization can be used in certain therapeutic treatments as in photodynamic therapy (PDT). This therapy has been used for the treatment of tumors. Various photosensitizing agents have been developed in order to increase the efficiency and specificity of such PDT treatment. Basically, PDT consists in the application or administration of a photosensitizing agent followed by activation of the agent by light of a specific wavelength. This results in a photochemical sequence of events involving the formation of reactive species such as $^1O_2$ that can promote tumor cell death (Dougherty et al. 1998).

In the search of other endogenous sources of singlet oxygen, studies have shown that lipid peroxidation is usually accompanied by an emission of ultra-weak light (Boveris et al. 1980; Cadenas and Sies 1984). This light emission was attributed to the formation of excited species such as $^1O_2$ and triplet carbonyl species formed during lipid peroxidation process (Nakano et al. 1975). These studies have spurred researchers to investigate the generation of $^1O_2$ from lipid peroxidation and more specifically from lipid hydroperoxides (Hawco et al. 1977; Kanofsky 1986; Timmins et al. 1997).

The mechanism of $^1O_2$ formation during lipid peroxidation was attributed to reactions involving peroxyl radicals. In 1957, Russell proposed a mechanism in which two peroxyl radicals would react to generate a linear tetraoxide (ROOOOR) which undergoes decomposition yielding the corresponding ketone (R═O), alcohol (R–OH) and molecular oxygen as final products (Russell 1957) (1.4). About 10 years later, following the suggestion of Russell, Howard and Ingold reported the formation of $^1O_2$ in the reaction of *sec*-butylhydroperoxide and cerium ions (Ce^{4+}) and attributed it to the termination reaction of peroxyl radicals (Howard and Ingold 1968). Russell drew attention to the fact that the decomposition of the tetraoxide intermediate does not violate the rule of Wigner spin conservation if dioxygen is eliminated in the singlet state, or if the carbonyl product is eliminated in the triplet excited state. Quantitative studies on the relative yields of these two excited species revealed that the yield of excited triplet carbonyl is less than 0.01 % (Mendenhall et al. 1991), while the yield of $^1O_2$ yield is around 10 % (Kanofsky 1986; Niu and Mendenhall 1992). Thus, based on these studies it has been concluded that tetraoxide decomposition via a cyclic mechanism yields $^1O_2$ as the predominant excited specie. Additionally, these studies also demonstrated that $^1O_2$ is generated from primary and secondary peroxyl radicals and not from tertiary peroxyl radicals, indicating that the requirement of α-hydrogen for $^1O_2$ generation.



(1.4)

3 Singlet Molecular Oxygen Generation from Metal Catalyzed Fatty Acid Hydroperoxide Decomposition

Free metals are usually found in extremely low concentrations in the body. However oxidative stress promotes the mobilization of metals from proteins by mechanisms that include oxidation of the Fe-S cluster by $O_2^{\bullet -}$, changes in the redox state of proteins, heme protein oxidative modifications, among others (Imlay 2003). Metals can catalyze the decomposition of LOOH to both alkoxyl ($LO^{\bullet}$) and peroxyl radicals ($LOO^{\bullet}$). The catalysis mediated by metal depends on several factors such as pH, solvent, and the ligands. Generally metals in their lower oxidation state (i.e. Fe^{2+}) leads to the formation of $LO^{\bullet}$ (1.5), while metals in its higher oxidation state (Fe^{3+}) yields $LOO^{\bullet}$ (1.6) (Gardner 1989).

$$LOOH + Fe^{2+} \rightarrow LO^{\bullet} + OH^{-} + Fe^{3+} \quad (1.5)$$

$$LOOH + Fe^{3+} \rightarrow LOO^{\bullet} + H^{+} + Fe^{2+} \quad (1.6)$$

Nakano et al. (1975) were among the first to suggest the formation of $^1O_2$ in the reaction of linoleic acid hydroperoxides (LAOOH) with cerium ion (Ce^{4+}) by measuring emission in the visible region. However they could not detect characteristic emission bands for $^1O_2$. In the same period, Hawco et al. (1977) also obtained results suggesting the formation of $^1O_2$ in the LAOOH reaction with Ce^{4+}, methemoglobin or hematin using diphenylfuran as a chemical trap for $^1O_2$. However, diphenylfuran is a chemical trap with low specificity to $^1O_2$ and their results were subjected to several criticisms. Later, Kanofsky (1986) made more specific measures of $^1O_2$ in the reaction of LAOOH with Ce^{4+} by detecting the $^1O_2$ monomolecular emission at 1,268 nm in the infrared region. In accordance with these findings, additional unequivocal evidences were obtained by our group, in which we confirmed the generation of $^1O_2$ in the reaction of lipid hydroperoxides and metal ions through experiments involving direct detection of $^1O_2$ by luminescence measurements in the visible and near infrared region and by experiments using 18-oxygen labeled linoleic acid hydroperoxides ($LA^{18}O^{18}OH$) (Fig. 1.1) (Miyamoto et al. 2003b). As will be

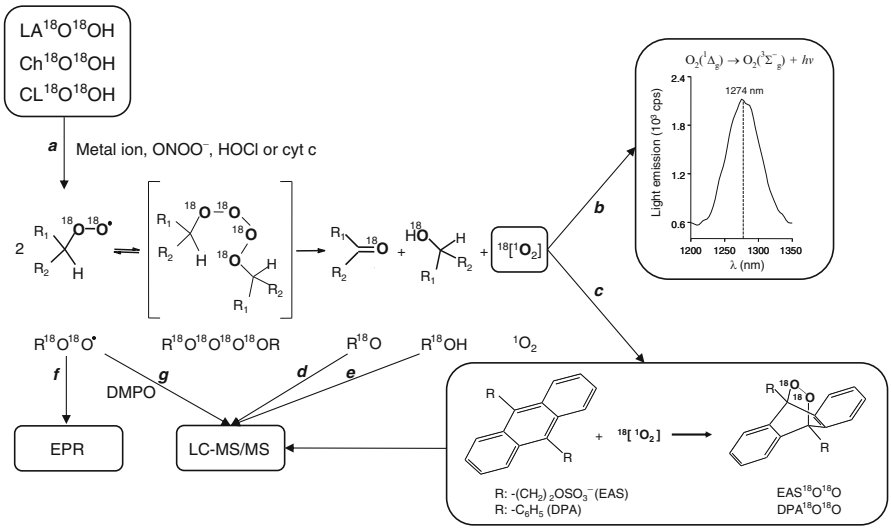


Fig. 1.1 Scheme showing the generation of 18-oxygen labeled singlet molecular oxygen ($^{18}[^1O_2]$) from 18-oxygen hydroperoxides of linoleic acid ($LA^{18}O^{18}OH$), cholesterol ($Ch^{18}O^{18}OH$) and cardiolipin ($CL^{18}O^{18}OH$): (*a*) incubation of labeled hydroperoxides with metal ions, $ONOO^-$, HOCl or cytochrome c (cyt c) generating singlet molecular oxygen by the Russell mechanism; (*b*) singlet molecular oxygen light emission measurements in the near-infrared region, showing a characteristic spectrum with maximum emission at 1,270 nm; (*c*) singlet molecular oxygen chemical trapping with the anthracene derivatives, anthracene-9,10-diyldiethyl disulfate (EAS) or DPA and analysis by LC-MS/MS; (*d*) and (*e*) detection of ketone and alcohols products by LC-MS/MS; and (*f*) and (*g*) analysis of peroxyl radicals directly by EPR or after reaction with DMPO by LC-MS/MS

discussed in the next sections, $^1O_2$ is also formed in reactions involving lipid hydroperoxides and other biologically relevant oxidants, such as, peroxynitrite, HOCl and cytochrome c.

Direct evidences for the formation of $^1O_2$ in the reaction of LAOOH with Ce^{4+} were obtained by detecting both visible ($\lambda > 570$ nm) (1.7) and infrared ($\lambda = 1{,}270$ nm) (1.8) light emission followed by the observation of azide quenching effect (Miyamoto et al. 2003b). Moreover, $^1O_2$ formation was further evidenced by the acquisition of a light emission spectrum in the infrared region showing an emission signal with maximum intensity at 1,270 nm, characteristic for the $^1O_2$ monomolecular decay.

$$2O_2\left(^1\Delta_g\right) \rightarrow 2O_2\left(^3\Sigma_g^-\right) + h\nu\,(\lambda = 634 \text{ and } 703 \text{ nm}) \tag{1.7}$$

$$O_2\left(^1\Delta_g\right) \rightarrow O_2\left(^3\Sigma_g^-\right) + h\nu\,(\lambda = 1{,}270 \text{ nm}) \tag{1.8}$$

Mechanistic aspects of the reactions were investigated using 18-oxygen labeled linoleic hydroperoxides ($LA^{18}O^{18}OH$). These hydroperoxides were synthesized by photooxidation under an atmosphere where 16-dioxygen ($^{16}O_2$) was completely

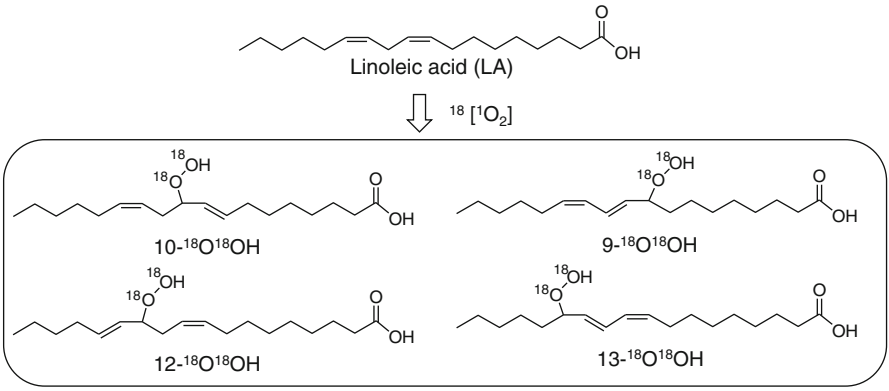


Fig. 1.2 Structures of the four major 18-oxygen labeled linoleic acid hydroperoxides formed during linoleic acid photooxidation in the presence of methylene blue and 18-dioxygen atmosphere

replaced by 18-dioxygen ($^{18}O_2$). Analysis by mass spectrometry (MS) confirmed the successful incorporation of two 18-oxygen atoms in the hydroperoxide (Fig. 1.2) (Miyamoto et al. 2003b, 2004). MS analysis revealed that the labeled hydroperoxide was obtained at purity higher than 99 %. Using this labeled hydroperoxides and DPA as a $^1O_2$ chemical trap we could detect the formation of labeled endoperoxides (DPA^xO^xO, x = 16 or 18) by HPLC coupled to tandem mass spectrometry (HPLC-MS/MS) in the presence of Ce^{4+} or Fe^{2+}.

Relative concentrations of fully labeled endoperoxide ($DPA^{18}O^{18}O$), partially ($DPA^{16}O^{18}O$) and unlabeled endoperoxides ($DPA^{16}O^{16}O$) were affected by the presence of dioxygen present in the reaction media. In fact, under aerated conditions $DPA^{18}O^{18}O$ concentration was relatively low. However, $DPA^{18}O^{18}O$ yield was increased by eightfold when the same reaction was conducted under completely deaerated conditions. Several hypotheses have been raised to explain these results (Miyamoto et al. 2003b). The most plausible ones were the oxygen exchange mechanism between labeled peroxyl radicals and molecular oxygen (Chan et al. 1979) yielding the unlabeled peroxyl radicals and the energy transfer mechanism between 18-oxygen labeled $^1O_2$ ($^{18}[^1O_2]$) and triplet molecular oxygen yielding unlabeled $^1O_2$ ($^{16}[^1O_2]$) (Martinez et al. 2004) (Fig. 1.3). Interestingly the detection of $DPA^{16}O^{18}O$ confirms the occurrence of the first hypothesis leading to the formation of unlabeled peroxy radicals. The partially labeled $^1O_2$ ($^{16-18}[^1O_2]$) is formed by the combination of labeled and unlabeled peroxyl radicals. Despite of these intriguing exchange mechanisms, our data clearly showed that $^1O_2$ is formed from LOOH.

Besides labeled singlet oxygen detection, labeled alcohol and ketone products (Fig. 1.1), the two major products expected to be formed by the mechanism proposed by Russell were also detected by LC-MS/MS (Miyamoto et al. 2003b). Additionally, experiments aiming to detect radical intermediates with the spin trap 5,5-dimethyl-1-pyrroline -oxide (DMPO) showed the formation of DMPO-peroxyl radical adduct by HPLC-MS/MS (Miyamoto 2005). Thus altogether our experiments clearly

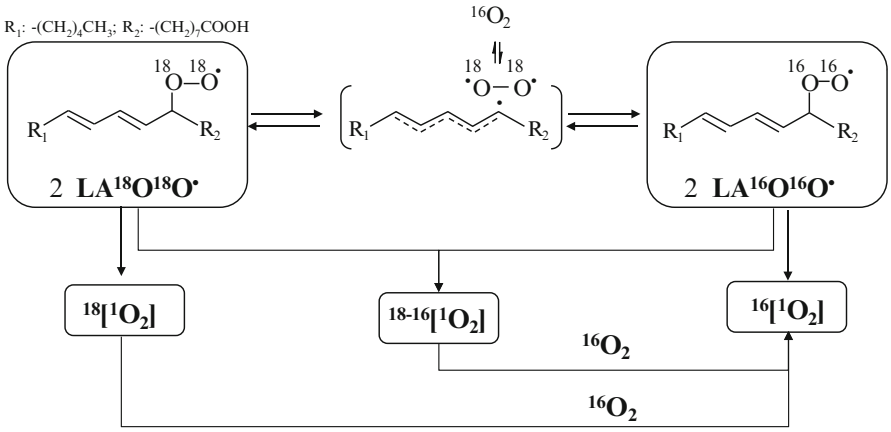


Fig. 1.3 Exchange mechanism between labeled peroxyl radicals ($LA^{18}O^{18}O^{\bullet}$) and triplet state dioxygen ($^{16}O_2$) yielding unlabeled peroxyl radicals ($LA^{16}O^{16}O^{\bullet}$); and energy transfer mechanism between fully and partially labeled singlet molecular oxygen ($^{18}[^1O_2]$ or $^{18\text{-}16}[^1O_2]$) and $^{16}O_2$ yielding unlabeled singlet oxygen ($^{16}[^1O_2]$)

demonstrated the generation of $^1O_2$ in the reaction of fatty acid hydroperoxides with metal ions by a mechanism consistent with the one proposed by Russell.

To test the hypothesis that $^1O_2$ can also be formed from fatty acid hydroperoxides esterified to phospholipids in membranes, we have investigated the generation of $^1O_2$ using liposomes enriched with phosphatidylcholine hydroperoxides. Indeed, luminescence measurements showed a parallel increase in $^1O_2$ formation with increased percentages of hydroperoxides in liposome preparations, indicating that phospholipid hydroperoxides in membranes are also potential sources of $^1O_2$ (Miyamoto 2005).

4 Singlet Molecular Oxygen Generation in the Reaction of Fatty Acid Hydroperoxides with Peroxynitrite

Peroxynitrite ($ONOO^-$) is a powerful oxidant of biological relevance, which is generated in cells or tissues by the fast reaction of NO and $O_2^{\bullet -}$ (Beckman and Koppenol 1996). Peroxynitrite promotes damage in a number of biomolecules, including lipids, carbohydrates, proteins and DNA (Szabo et al. 2007). Their reactions with biomolecules are basically divided in (i) direct reactions, in that peroxynitrite itself or its protonated form reacts directly with the substrate, and (ii) indirect reactions, in which the damaging reactions are mediated by free radicals derived from peroxynitrite homolysis ($HO^{\bullet}$ and $NO_2^{\bullet}$) or from its reaction with CO_2 ($CO_3^{\bullet -}$ and $NO_2^{\bullet}$) (Radi et al. 2001; Augusto et al. 2002).

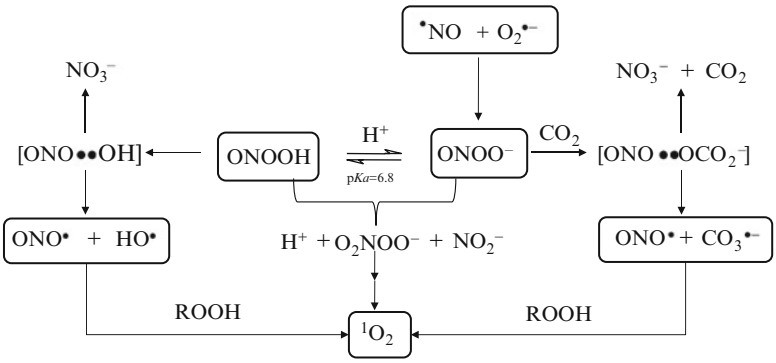


Fig. 1.4 Peroxynitrite formation and decomposition yielding intermediates capable to induce the generation of singlet oxygen

Di Mascio et al. (1994, 1997) were the first to propose the formation of $^1O_2$ in reactions involving $ONOO^-$ and H_2O_2 or *tert*-butyl hydroperoxide. Later, Khan et al. (2000) suggested that $ONOO^-$ decomposition generates $^1O_2$ at stoichiometric values. However, Martinez et al. (2000) and Merenyi et al. (1998) presented various arguments, including thermodynamic calculations and experimental evidences indicating that peroxynitrite could not yield $^1O_2$ at such high yields. The most plausible explanation for the observation of Khan is the reaction of $ONOO^-$ with H_2O_2. Indeed, $ONOO^-$ preparations usually contain relatively high amounts of H_2O_2, and this could be the explanation for the high yields of $^1O_2$ observed by Khan. More recently, studies by our group using a synthetic salt of peroxynitrite, $(CH_3)_4[N]^+[ONOO]^-$ (which was completely free from hydrogen peroxide), showed that $^1O_2$ can be directly formed from $ONOO^-$ under neutral to alkaline pH by a mechanism involving peroxynitrate (O_2NOO^-) formation (Gupta et al. 2009; Miyamoto et al. 2009) (Fig. 1.4). However, again the yield of $^1O_2$ observed for this type of reaction was less than 1 %. In conclusion the amount of $^1O_2$ generated from $ONOO^-$ alone is very low but is largely increased in the presence of H_2O_2 or ROOH.

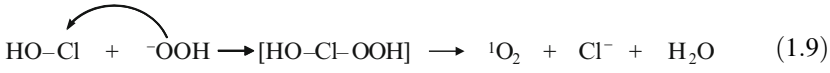
Among different peroxides, lipid hydroperoxides can also react with $ONOO^-$ to generate $^1O_2$ (Miyamoto et al. 2003a). Considering that $ONOO^-$ attacks cell membranes inducing lipid peroxidation and lipid hydroperoxide formation, this reaction could be a potential source of $^1O_2$ in biological media. Similarly to the reactions of LOOH with metal ions, evidences showing the generation of $^1O_2$ were obtained by luminescence measurements in the red spectral region and in the near-infrared region (Miyamoto et al. 2003a). Singlet molecular oxygen formation was unequivocally demonstrated by the acquisition of the emission spectrum with a band centered at 1,270 nm, as well as, by the detection of $^{18}[^1O_2]$ in the reaction of labeled linoleic acid hydroperoxides with $ONOO^-$. The generation of $^1O_2$ in this reaction is mediated by radical intermediates generated from peroxynitrite ($HO^•$, $NO_2^•$ or $CO_3^{•-}$) (Fig. 1.4) which are all thermodynamically capable to abstract a

hydrogen atom from LOOH yielding peroxyl radicals, and subsequently $^1O_2$ by the Russell mechanism (Miyamoto et al. 2003a).

5 Singlet Molecular Oxygen Generation from the Reaction of Fatty Acid Hydroperoxides and HOCl

HOCl is produced by phagocytes in the reaction of Cl^- with H_2O_2 catalyzed by MPO (Hampton et al. 1998; Klebanoff 1999). This enzyme is stored in high concentrations in azurophil granules of neutrophils and is released into the phagosome during phagocytosis. This process is accompanied by the activation of NADPH oxidase with concomitant production of H_2O_2 which is used to produce HOCl by MPO, an agent considered crucial in combating microorganisms (Hampton et al. 1998). However, excessive production of HOCl by MPO can cause tissue damage (Winterbourn and Kettle 2000). HOCl is a highly reactive being capable to promote damage to important biomolecules such as proteins, nucleic acids, lipids and carbohydrates. Studies suggest that this process plays an important role in the development of certain diseases such as atherosclerosis, chronic inflammation and cancer (Podrez et al. 2000).

As mentioned above, HOCl reacts with H_2O_2 to generate stoichiometric quantities form $^1O_2$ (Khan and Kasha 1963). The first evidence of the formation of $^1O_2$ in this reaction was obtained by Seliger in 1960 (Seliger 1960). He observed a flash of red luminescence with emission band centered at 634 nm. In 1963, Khan and Kasha (1963), published a full spectrum for singlet oxygen emission generated from the reaction of H_2O_2 with HOCl, showing emission bands at 634 and 703 nm. The mechanism involved in the reaction of H_2O_2 and HOCl was studied in detail by Cahill and Taube (1952). They conducted experiments using H_2O_2 enriched with 18-oxygen and demonstrated that both oxygens present on $^1O_2$ were derived from H_2O_2 and not from HOCl or water. In 1978, Held et al. (1978) suggested a mechanism involving the nucleophilic attack of peroxide on chlorine atom of HOCl, forming an unstable intermediate that decomposes yielding $^1O_2$ (1.9).



(1.9)

Alternatively to hydrogen peroxide, HOCl produced at inflammatory site can also interact with hydroperoxides derived from lipid peroxidation or as a consequence of the activation of lipoxygenase and cyclooxygenase enzymes. The generation of $^1O_2$ by LAOOH and HOCl was evidenced by measuring both the kinetics of the light emission at 1,270 nm and the spectrum of the light emitted in the near-infrared region (Miyamoto et al. 2006). The capacity of different organic hydroperoxides and hydrogen peroxide to generate $^1O_2$ upon reaction with HOCl was also compared. The highest yield of $^1O_2$ was observed in the reaction of HOCl with H_2O_2, consistent with the stoichiometric generation of $^1O_2$ in this reaction. With LAOOH, the reaction with HOCl yielded

approximately 14 % of $^1O_2$. On the other hand, $^1O_2$ was not formed in the reaction of HOCl with *tertiary* hydroperoxides (*t*-butyl hydroperoxide and cumene hydroperoxide). This result is explained by the fact that *tertiary* hydroperoxides lacks the hydrogen at carbon-α (hydrogen germinal to peroxyl group) that is essential for the generation of $^1O_2$ by the Russell mechanism (Howard and Ingold 1968).

Additional direct evidence for the formation of $^1O_2$ in the reaction of HOCl with LOOH was obtained by studies using 18-oxygen labeled linoleic acid hydroperoxides (Miyamoto et al. 2006). Using LA^{18}O^{18}OH and EAS as a chemical trap for $^1O_2$ we could detect the formation of the corresponding labeled endoperoxides (EAS^{18}O^{18}O and EAS^{18}O^{18}O) by LC-MS/MS analysis, indicating that $^1O_2$ is directly derived from the hydroperoxides (Fig. 1.1). Similar to the results observed with metal ions, the yield of fully labeled endoperoxides increased significantly in the reaction conducted under deaerated conditions, confirming the observations made with metal ions where oxygen exchange and energy transfer mechanisms occur during the course of the reaction. Nonetheless, these data together with the detection of peroxyl radicals by EPR (Miyamoto et al. 2006), indicates that $^1O_2$ is generated from the reaction of lipid hydroperoxides with HOCl by the Russell mechanism (Fig. 1.1).

Studies were also conducted in order to verify $^1O_2$ formation of HOCl in the reaction with hydroperoxides of fatty acids esterified to phospholipids (Miyamoto et al. 2006). Similarly to what we observed with metal ions, $^1O_2$ was generated in the reaction of HOCl with liposomes containing phosphatidylcholine hydroperoxides. This result is important from a biological standpoint, since it indicates that HOCl also reacts with hydroperoxides in membranes yielding $^1O_2$. Additionally, considering that inflammatory processes are usually accompanied by lipid peroxidation (Zhang et al. 2002) it is very likely that lipid hydroperoxides and HOCl are produced at very close sites contributing to the generation of $^1O_2$.

6 Singlet Molecular Oxygen Generation from Cholesterol Hydroperoxides

Cholesterol is one of the major components of biological membranes. It has essential functions in the physical properties of membranes and in the regulation of multiple signaling pathways. The oxidation of cholesterol has been the subject of considerable interest because of its relationship to various pathological conditions such as atherosclerosis (Brown and Jessup 1999). Moreover, studies show a correlation between the incidence of hypercholesterolemia and cardiovascular diseases and neurodegenerative diseases (Casserly and Topol 2004).

The oxidation of cholesterol by reactive species produces various oxidized products, including, hydroperoxides and aldehydes (Smith 1996; Girotti 1998). Its formation may occur by enzymatic or non-enzymatic oxidation mechanisms (Brown and Jessup 1999; Iuliano 2011). The enzymatic oxidation occurs mainly during cholesterol metabolism by enzymes of the cytochrome P450 family (Iuliano

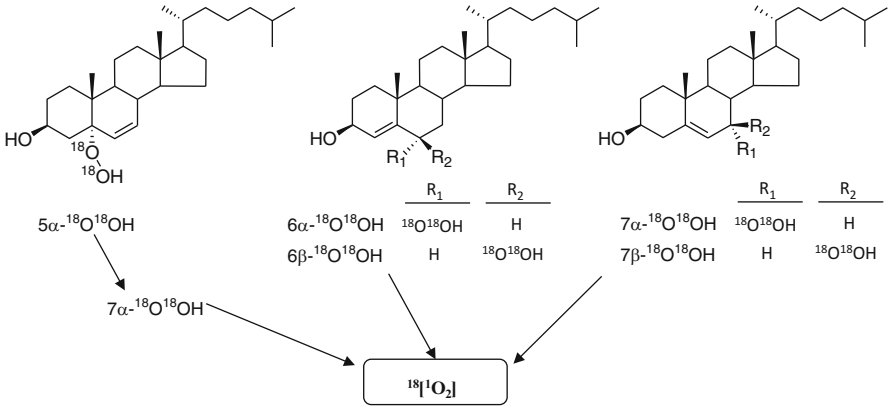


Fig. 1.5 Structures of the 18-oxygen labeled cholesterol hydroperoxides ($Ch^{18}O^{18}OH$) synthesized by photooxidation in the presence of methylene blue and 18-dioxygen atmosphere and the generation of singlet molecular oxygen observed upon reaction with Ce^{4+}

2011). The non-enzymatic oxidation involves the action of free radicals such as, peroxyl and hydroxyl radicals, and non-radical reactive species, such as, ozone and $^1O_2$. Oxidation of cholesterol induced by free radicals yields primarily the 7α-OOH and 7β-OOH products. On the other hand, $^1O_2$ mediated oxidation yields primarily the hydroperoxides, 5α-OOH and 6α-OOH and 6β-OOH (Girotti 1992). In addition to these hydroperoxides, cholesterol oxidation induced by ozone and $^1O_2$ yields two aldehydes, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al and 3β-hydroxy-5β-hydroxy-B-norcholestane-6β-carboxaldehyde (Gumulka and Smith 1983; Wentworth et al. 2003; Brinkhorst et al. 2008; Uemi et al. 2009). Two mechanisms were proposed for the formation of these aldehydes. One is based on the Hock-cleavage of 5α-OOH (Brinkhorst et al. 2008) and the other based on the formation and decomposition of an 1,2-dioxetane intermediate at the Δ5 bond of cholesterol (Uemi et al. 2009).

The possibility of cholesterol hydroperoxide decomposition to generate $^1O_2$ in a similar way to fatty acid hydroperoxides and phospholipid hydroperoxides has been also studied. Singlet molecular oxygen generation was evidenced by light emission measurements at 1,270 nm and by detecting $^1O_2$ in the reaction of each cholesterol hydroperoxide isomer (7α-, 6β-, and 5α-OOH) and Ce^{4+} (Uemi et al. 2011). Again, to unequivocally prove the formation of $^1O_2$ experiments were conducted with 18-oxygen labeled cholesterol hydroperoxides ($Ch^{18}O^{18}OH$) (Fig. 1.5). Using these labeled hydroperoxides and DPA as a chemical trap it was possible to evidence the formation of $^{18}[^1O_2]$, thus clearly evidencing the generation of $^1O_2$. Details of the mechanism were investigated by analyzing the products formed in the reaction by LC-MS/MS. Both 7α- and 6β-OOH yielded the corresponding alcohol and ketone products, characteristic for the Russell mechanism. On the other hand, the 5α-OOH/Ce^{4+} reaction yielded a different pattern of products, including the 5α-OH and the 7α-OH and 7α-keto products. This phenomenon was due to the occurrence of a

rearrangement of the hydroperoxide/peroxyl group from C-5 to C-7 (Beckwith et al. 1989) and explains why the *tertiary* hydroperoxide, 5α-OOH, was able to generate $^1O_2$.

Considering that cholesterol is especially enriched in lipid rafts and caveolaes, the oxidation of cholesterol can yield hydroperoxides that maybe relatively highly concentrated in these regions. Further reaction of these hydroperoxides involving the formation of peroxyl radicals can produce $^1O_2$. This hypothesis is supported by data showing that cholesterol hydroperoxides incorporated to liposomal membranes generates $^1O_2$ (Uemi et al. 2011). In this context, $^1O_2$ derived from cholesterol hydroperoxides may play a role as mediators of protein modifications, in particular by promoting modification in signaling proteins located at raft regions. These hypothesis needs to be further investigated.

7 Singlet Molecular Oxygen Generation from Cardiolipin-Cytochrome c Interaction

Cardiolipin (CL) is a phospholipid found exclusively in the inner mitochondrial membrane (Daum 1985; Schlame et al. 2000). Cardiolipin interacts with several proteins of the mitochondrial inner membrane, including several carriers of the electron transport chain. Among them, the interaction with cytochrome c (cyt c) is the best characterized and studied (Iverson and Orrenius 2004; Kagan et al. 2005). It has been proposed that cardiolipin oxidation and the formation of cardiolipin hydroperoxides (CLOOH) is a critical initial event responsible for the release of cyt c (Shidoji et al. 1999; Petrosillo et al. 2001; Ott et al. 2002; Kagan et al. 2004). It is believed that this release occurs due to the weakening of the hydrophobic/electrostatic resulting from the appearance of a polar hydroperoxide group in the fatty acyl chain of cardiolipin.

The native form of cyt c with its heme group containing the six coordination bonds does not have peroxidase activity. However, partial unfolding of cyt c induced by denaturing agents such as guanidinium chloride, by chemical modification of Met80, and by interaction with anionic phospholipids such as cardiolipin results in increased peroxidase activity (Diederix et al. 2002). This increase in peroxidase activity appears to be due to alteration of protein conformation and a loss of coordination bonds of heme, specifically the interaction Fe-Met80, which increases the accessibility of heme-iron to hydroperoxides (Tuominen et al. 2002).

Recently we have demonstrated that the interaction of CL with cyt c induces the formation of $^1O_2$ by a mechanism dependent on the electrostatic interactions of CL and cyt c (Miyamoto et al. 2012). Evidences for the generation of $^1O_2$ were obtained by luminescence measurements at 1,270 nm and by chemical trapping experiments using EAS. Singlet oxygen was generated at a yield of 15 % for membranes containing tetralinoleoyl CL. This excited specie was not observed when cyt c was incubated with membranes containing monounsaturated or saturated CL

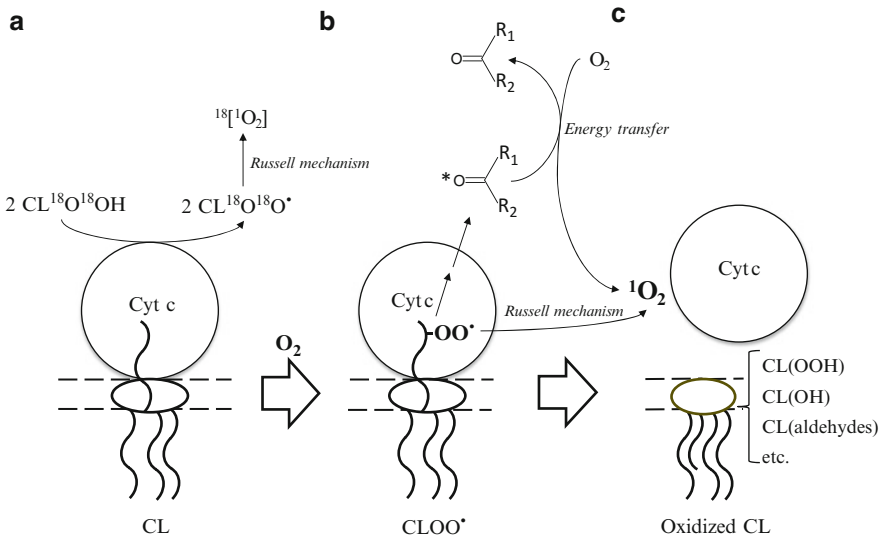


Fig. 1.6 Generation of singlet molecular oxygen by cyt c-CL system. Singlet molecular oxygen can be directly generated from the decomposition of $CL^{18}O^{18}OH$ catalyzed by cyt c-CL complex (**a**). Alternatively, singlet molecular oxygen can also be produced by cyt c promoted oxidation of CL, a process that involves the generation of peroxyl radicals (**b**). Carbonyl compounds (e.g. aldehydes) (**c**). Peroxyl radicals generate singlet molecular oxygen by the Russell mechanism. Carbonyl compounds are further metabolized by cyt c yielding excited triplet carbonyl species (*) that generates singlet molecular oxygen by an energy transfer mechanism

species, suggesting that $^1O_2$ related to fatty acid oxidation promoted by cyt c. Indeed, $^1O_2$ formation was paralleled to CL consumption.

In analogy to the experiments done with fatty acid hydroperoxides, the contribution of CL hydroperoxides for the overall mechanism of $^1O_2$ generation was investigated using liposomes supplemented with 18-oxygen labeled CL hydroperoxides ($CL^{18}O^{18}OH$) (Miyamoto et al. 2012). The generation of $^1O_2$ directly from initially present hydroperoxides was very low as evidenced by the weak luminescence observed in the experiments where liposomes supplemented with 5 and 30 % CLOOH. Indeed, time-dependent analysis of $^1O_2$ by chemical trapping showed the existence of at least two mechanisms leading to the formation of $^1O_2$. One in which $^1O_2$ is directly formed from $CL^{18}O^{18}OH$ decomposition catalyzed by cyt c yielding labeled peroxyl radicals and $^{18}[^1O_2]$ (Fig. 1.6a). The other in which $^1O_2$ was produced after induction of CL peroxidation. Both mechanisms produce peroxyl radicals that react by the Russell mechanism generating $^1O_2$ (Fig. 1.6b, c). In addition to the peroxyl radicals, we also obtained evidences indicating the formation of excited triplet carbonyl species. These species are known to contribute for the generation of $^1O_2$ by an energy transfer mechanism (Fig. 1.6b, c).

Overall, our data indicates that cyt c-CL interaction is a potential source of $^1O_2$ and this excited specie maybe involved in the mechanism leading to cyt c release and induction of cell death.

8 Conclusion

Lipid hydroperoxide toxicity is associated to the production of oxyl radicals and aldehydes capable to induce covalent modifications in proteins and DNA. In addition to these reactive species, LOOH decomposition also generates $^1O_2$. Since the first studies showing an association between lipid peroxidation and ultra-weak light emission, much progress has been made on the identification of the mechanisms responsible for this phenomenon. It is now clear that $^1O_2$ is one of the major species responsible for the emission of light. This excited specie is produced from reactions involving a number of different LOOHs (e.g. fatty acid, phospholipid and cholesterol hydroperoxides) and biologically relevant oxidants, such as, metal ions, $ONOO^-$, HOCl and cyt c. Among the strategies used to demonstrate the generation of $^1O_2$, the use of 18-oxygen labeled lipid hydroperoxides greatly contributed for the characterization of the reaction mechanisms and to unequivocally demonstrate the generation of $^1O_2$.

Acknowledgements The authors acknowledge the Brazilian research funding institutions: FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo; Proc. 2010/50891-0 and Proc. 2012/12663-1), CNPq (Conselho Nacional para o Desenvolvimento Científico e Tecnológico), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), PRONEX/FINEP (Programa de Apoio aos Núcleos de Excelência), PRPUSP (Pro-Reitoria de Pesquisa da Universidade de São Paulo), Instituto do Milênio-Redoxoma, INCT Redoxoma (FAPESP/CNPq/CAPES), NAP Redoxoma (PRPUSP), CEPID Redoxoma (FAPESP; Proc. 2013/07937-8), Fundo Bunka de Pesquisa Banco Sumitomo Mitsui (S.M. Fellowship), L'Oréal-ABC-Unesco (S.M. Fellowship) and John Simon Guggenheim Memorial Foundation (P.D.M. Fellowship).

References

Adam W, Kazakov DV et al (2005) Singlet oxygen chemiluminescence in peroxide reactions. Chem Rev 105:3371–3387

Allen RC, Stjernholm RL et al (1972) Evidence for generation of an electronic excitations state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. Biochem Biophys Res Commun 47:679–684

Augusto O, Bonini MG et al (2002) Nitrogen dioxide and carbonate radical anion: two emerging radicals in biology. Free Radic Biol Med 32:841

Beckman JS, Koppenol WH (1996) Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol Cell Physiol 271:C1424–C1437

Beckwith ALJ, Davies AG et al (1989) The mechanisms of the rearrangements of allylic hydroperoxides: 5[α]-hydroperoxy-3[β]-hydroxycholest-6-ene and 7[α]-hydroperoxy-3[β]-hydroxycholest-5-ene. J Chem Soc Perkin Trans 2:815–824

Boveris A, Cadenas E et al (1980) Organ chemiluminescence: noninvasive assay for oxidative radical reactions. Proc Natl Acad Sci U S A 77:347–351

Brinkhorst J, Nara SJ et al (2008) Hock cleavage of cholesterol 5 alpha-hydroperoxide: an ozone-free pathway to the cholesterol ozonolysis products identified in arterial plaque and brain tissue. J Am Chem Soc 130:12224–12225

Brown AJ, Jessup W (1999) Oxysterols and atherosclerosis. Atherosclerosis 142:1

Cadenas E, Sies H (1984) Low-level chemi-luminescence as an indicator of singlet molecular-oxygen in biological-systems. Methods Enzymol 105:221–231
Cadenas E, Daniele RP et al (1981) Low level chemiluminescence of alveolar macrophages: spectral evidence for singlet oxygen generation. FEBS Lett 123:225–228
Cahill AE, Taube H (1952) The use of heavy oxygen in the study of reactions of hydrogen peroxide. J Am Chem Soc 74:2312–2318
Carbonare MD, Pathak MA (1992) Skin photosensitizing agents and the role of reactive oxygen species in photoaging. J Photochem Photobiol B 14:105–124
Casserly I, Topol E (2004) Convergence of atherosclerosis and Alzheimer's disease: inflammation, cholesterol, and misfolded proteins. Lancet 363:1139–1146
Chan HWS, Levett G et al (1979) The mechanism of the rearrangement of linoleate hydroperoxides. Chem Phys Lipids 24:245–256
Cheson BD, Christensen RL et al (1976) The origin of the chemiluminescence of phagocytosing granulocytes. J Clin Invest 58:789–796
Daum G (1985) Lipids of mitochondria. Biochim Biophys Acta 822:1–42
Di Mascio P, Kaiser S et al (1989) Lycopene as the most efficient biological carotenoid singlet oxygen quencher. Arch Biochem Biophys 274:532–538
Di Mascio P, Bechara EJH et al (1994) Singlet molecular oxygen production in the reaction of peroxynitrite with hydrogen peroxide. FEBS Lett 355:287–289
Di Mascio P, Briviba K et al (1997) The reaction of peroxynitrite with tert-butyl hydroperoxide produces singlet molecular oxygen. Biol Chem 378:1071–1074
Diederix REM, Ubbink M et al (2002) Peroxidase activity as a tool for studying the folding of c-type cytochromes. Biochemistry 41:13067–13077
Dougherty TJ, Gomer CJ et al (1998) Photodynamic therapy. J Natl Cancer Inst 90:889–905
Esterbauer H, Schaur RJ et al (1991) Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 11:81–128
Foote CS (1968) Photosensitized oxygenations and the role of singlet oxygen. Acc Chem Res 1:104–110
Foote CS, Wexler S (1964) Singlet oxygen. A probable intermediate in photosensitized autoxidations. J Am Chem Soc 86:3880–3881
Gardner HW (1989) Oxygen radical chemistry of polyunsaturated fatty acids. Free Radic Biol Med 7:65–86
Girotti AW (1992) Photosensitized oxidation of cholesterol in biological-systems-reaction pathways, cytotoxic effects and defense-mechanisms. J Photochem Photobiol B Biol 13:105–118
Girotti AW (1998) Lipid hydroperoxide generation, turnover, and effector action in biological systems. J Lipid Res 39:1529–1542
Gumulka J, Smith LL (1983) Ozonization of cholesterol. J Am Chem Soc 105:1972–1979
Gupta D, Harish B et al (2009) Peroxynitrate is formed rapidly during decomposition of peroxynitrite at neutral pH. Dalton Trans 29:5730–5736
Hampton MB, Kettle AJ et al (1998) Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood 92:3007–3017
Hawco FJ, O'Brien CR et al (1977) Singlet oxygen formation during hemoprotein catalyzed lipid peroxide decomposition. Biochem Biophys Res Commun 76:354
Held AM, Halko DJ et al (1978) Mechanisms of chlorine oxidation of hydrogen peroxide. J Am Chem Soc 100:5732–5740
Howard JA, Ingold KU (1968) Self-reaction of sec-butylperoxy radicals. Confirmation of Russell mechanism. J Am Chem Soc 90:1056–1058
Imlay JA (2003) Pathways of oxidative damage. Annu Rev Microbiol 57:395–418
Iuliano L (2011) Pathways of cholesterol oxidation via non-enzymatic mechanisms. Chem Phys Lipids 164:457–468
Iverson SL, Orrenius S (2004) The cardiolipin-cytochrome c interaction and the mitochondrial regulation of apoptosis. Arch Biochem Biophys 423:37–46
Kagan VE, Borisenko GG et al (2004) Oxidative lipidomics of apoptosis: redox catalytic interactions of cytochrome c with cardiolipin and phosphatidylserine. Free Radic Biol Med 37:1963–1985

Kagan VE, Tyurin VA et al (2005) Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. Nat Chem Biol 1:223–232
Kanofsky JR (1986) Singlet oxygen production from the reactions of alkylperoxy radicals. Evidence from 1268-nm chemiluminescence. J Org Chem 51:3386–3388
Kanofsky JR (1989) Singlet oxygen production by biological-systems. Chem Biol Int 70:1–28
Kautsky H (1939) Quenching of luminescence by oxygen. Trans Faraday Soc 35:216–219
Khan AU (1984) Myeloperoxidase singlet molecular oxygen generation detected by direct infrared electronic emission. Biochem Biophys Res Commun 122:668–675
Khan AU, Kasha M (1963) Red chemiluminescence of molecular oxygen in aqueous solution. J Chem Phys 39:2105–2106
Khan AU, Kovacic D et al (2000) The decomposition of peroxynitrite to nitroxyl anion (NO^-) and singlet oxygen in aqueous solution. Proc Natl Acad Sci U S A 97:2984–2989
Kiryu C, Makiuchi M et al (1999) Physiological production of singlet molecular oxygen in the myeloperoxidase-$H_2O_2^-$ chloride system. FEBS Lett 443:154–158
Klebanoff SJ (1999) Oxygen metabolites from phagocytes. In: Gallin JI, Snyderman R (eds) Inflammation: basic principles and clinical correlates. Lippincott Williams & Wilkins, Philadelphia
Krinsky NI (1974) Singlet excited oxygen as a mediator of antibacterial action of leukocytes. Science 186:363–365
Martinez GR, Di Mascio P et al (2000) Peroxynitrite does not decompose to singlet oxygen ($^1\Delta_gO_2$) and nitroxyl (NO^-). Proc Natl Acad Sci U S A 97:10307–10312
Martinez GR, Ravanat JL et al (2004) Energy transfer between singlet ($^1\Delta_g$) and triplet ($^3\Sigma_g^-$) molecular oxygen in aqueous solution. J Am Chem Soc 126:3056–3057
Mendenhall GD, Sheng XC et al (1991) Yields of excited carbonyl species from alkoxyl and from alkylperoxyl radical dismutations. J Am Chem Soc 113:8976–8977
Merenyi G, Lind J et al (1998) Peroxynitrous acid homolyzes into OH and.NO_2 radicals. Chem Res Toxicol 11:712–713
Miyamoto S (2005) Lipid hydroperoxides as a biological source of singlet oxygen: studies using isotopic labelling, mass spectrometry and luminescence. PhD thesis, Department of Biochemistry, Institute of Chemistry, University of São Paulo. http://www.teses.usp.br/teses/disponiveis/46/46131/tde-12042007-150108/. Accessed 6 Nov 2012
Miyamoto S, Martinez GR et al (2003a) Direct evidence of singlet molecular oxygen production in the reaction of linoleic acid hydroperoxide with peroxynitrite. J Am Chem Soc 125:4510–4517
Miyamoto S, Martinez GR et al (2003b) Singlet molecular oxygen generated from lipid hydroperoxides by the Russell mechanism: studies using ^{18}O-labeled linoleic acid hydroperoxide and monomol light emission measurements. J Am Chem Soc 125:6172–6179
Miyamoto S, Martinez GR et al (2004) ^{18}O-Labeled lipid hydroperoxides and HPLC coupled to mass spectrometry as valuable tools for studying the generation of singlet oxygen in biological system. Biofactors 22:333–339
Miyamoto S, Martinez GR et al (2006) Linoleic acid hydroperoxide reacts with hypochlorous acid, generating peroxyl radical intermediates and singlet molecular oxygen. Proc Natl Acad Sci USA 103:293–298
Miyamoto S, Ronsein GE et al (2007) Biological hydroperoxides and singlet molecular oxygen generation. IUBMB Life 59:322–331
Miyamoto S, Ronsein GE et al (2009) Direct evidence of singlet molecular oxygen generation from peroxynitrate, a decomposition product of peroxynitrite. Dalton Trans 29:5720–5729
Miyamoto S, Nantes IL et al (2012) Cytochrome c-promoted cardiolipin oxidation generates singlet molecular oxygen. Photochem Photobiol Sci 11:1536–1546
Nakano M, Noguchi T et al (1975) Spectroscopic evidence for the generation of singlet oxygen in the reduced nicotinamide adenine dinucleotide phosphate dependent microsomal lipid peroxidation system. J Biol Chem 250:2404–2406
Niu QJ, Mendenhall GD (1992) Yields of singlet molecular oxygen from peroxyl radical termination. J Am Chem Soc 114:165–172

Ott M, Robertson JD et al (2002) Cytochrome c release from mitochondria proceeds by a two-step process. Proc Natl Acad Sci U S A 99:1259–1263

Petrosillo G, Ruggiero FM et al (2001) Reactive oxygen species generated from the mitochondrial electron transport chain induce cytochrome c dissociation from beef-heart submitochondrial particles via cardiolipin peroxidation. Possible role in the apoptosis. FEBS Lett 509:435–438

Podrez EA, Abu-Soud HM et al (2000) Myeloperoxidase-generated oxidants and atherosclerosis. Free Radic Biol Med 28:1717

Radi R, Peluffo G et al (2001) Unraveling peroxynitrite formation in biological systems. Free Radic Biol Med 30:463–488

Rosen H, Klebanoff SJ (1977) Formation of singlet oxygen by the myeloperoxidase-mediated antimicrobial system. J Biol Chem 252:4803–4810

Russell GA (1957) Deuterium-isotope effects in the autoxidation of aralkyl hydrocarbons – mechanism of the interaction of peroxy radicals. J Am Chem Soc 79:3871–3877

Schlame M, Rua D et al (2000) The biosynthesis and functional role of cardiolipin. Progr Lipid Res 39:257–288

Schneider C, Pratt DA et al (2007) Control of oxygenation in lipoxygenase and cyclooxygenase catalysis. Chem Biol 14:473–488

Seliger HH (1960) A photoelectric method for the measurement of spectra of light sources of rapidly varying intensities. Anal Biochem 1:60–65

Shidoji Y, Hayashi K et al (1999) Loss of molecular interaction between cytochrome c and cardiolipin due to lipid peroxidation. Biochem Biophys Res Commun 264:343–347

Smith LL (1996) Review of progress in sterol oxidations: 1987–1995. Lipids 31:453–487

Steinbeck MJ, Khan AU et al (1992) Intracellular singlet oxygen generation by phagocytosing neutrophils in response to particles coated with a chemical trap. J Biol Chem 267:13425

Szabo C, Ischiropoulos H et al (2007) Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. Nat Rev Drug Discov 6:662–680

Tatsuzawa H, Maruyama T et al (1999) Singlet oxygen ($^1\Delta_gO_2$) as the principal oxidant in myeloperoxidase-mediated bacterial killing in neutrophil phagosome. Biochem Biophys Res Commun 262:647

Timmins GS, dos Santos RE et al (1997) Lipid peroxidation-dependent chemiluminescence from the cyclization of alkylperoxyl radicals to dioxetane radical intermediates. Chem Res Toxicol 10:1090–1096

Tuominen EKJ, Wallace CJA et al (2002) Phospholipid-cytochrome c interaction. Evidence for the extended lipid anchorage. J Biol Chem 277:8822–8826

Uemi M, Ronsein GE et al (2009) Generation of cholesterol carboxyaldehyde by the reaction of singlet molecular oxygen [O_2 ($^1\Delta_g$)] as well as ozone with cholesterol. Chem Res Toxicol 22:875–884

Uemi M, Ronsein GE et al (2011) Cholesterol hydroperoxides generate singlet molecular oxygen [$O_2(^1\Delta_g)$]: near-IR emission, ^{18}O-labeled hydroperoxides, and mass spectrometry. Chem Res Toxicol 24:887–895

Ursini F, Bindoli A (1987) The role of selenium peroxidases in the protection against oxidative damage of membranes. Chem Phys Lipids 44:255–276

Wentworth P Jr, Nieva J et al (2003) Evidence for ozone formation in human atherosclerotic arteries. Science 302:1053–1056

Winterbourn CC, Kettle AJ (2000) Biomarkers of myeloperoxidase-derived hypochlorous acid. Free Radic Biol Med 29:403–409

Zhang R, Brennan M-L et al (2002) Myeloperoxidase functions as a major enzymatic catalyst for initiation of lipid peroxidation at sites of inflammation. J Biol Chem 277:46116–46122

Chapter 2
The Formation of Lipid Hydroperoxide-Derived Amide-Type Lysine Adducts on Proteins: A Review of Current Knowledge

Yoji Kato

Abstract Lipid peroxidation is an important biological reaction. In particular, polyunsaturated fatty acid (PUFA) can be oxidized easily. Peroxidized lipids often react with other amines accompanied by the formation of various covalent adducts. Novel amide-type lipid-lysine adducts have been identified from an in vitro reaction mixture of lipid hydroperoxide with a protein, biological tissues exposed to conditions of oxidative stress and human urine from a healthy person. In this chapter, the current knowledge of amide type adducts is reviewed with a focus on the evaluation of functional foods and diseases with a history of discovery of hexanoyl-lysine (HEL). Although there is extensive research on HEL and other amide-type adducts, the mechanism of generation of the amide bond remains unclear. We have found that the decomposed aldehyde plus peroxide combined with a lysine moiety does not fully explain the formation of the amide-type lipid-lysine adduct that is generated by lipid hydroperoxide. Singlet oxygen or an excited state of the ketone generated from the lipid hydroperoxide may also contribute to the formation of the amide linkage. The amide-adducts may prove useful not only for the detection of oxidative stress induced by disease but also for the estimation of damage caused by an excess intake of PUFA.

Keywords Lipid hydroperoxide • Amide-type adduct • Hexanoyl-lysine • Propanoyl-lysine • Biomarker • Oxidative stress

Abbreviations

PUFA	Polyunsaturated fatty acid
HEL	Hexanoyl-lysine

Y. Kato (✉)
School of Human Science and Environment, University of Hyogo, Himeji, Hyogo, Japan
e-mail: yojikato@shse.u-hyogo.ac.jp

Y. Kato (ed.), *Lipid Hydroperoxide-Derived Modification of Biomolecules*,
Subcellular Biochemistry 77, DOI 10.1007/978-94-007-7920-4_2,

13-HPODE	13-hydroperoxyoctadecadienoic acid
BGK	Benzoyl-glycyl-L-lysine
LC/MS/MS	Liquid chromatography tandem mass spectrometry
SUL	Succinyl-lysine
GLL	Glutary-lysine
AZL	Azelayl-lysine
PRL	Propanoyl-lysine
HHE	4-hydroxy-2-hexenal
DHA	Docosahexaenoic acid
KLH	Keyhole limpet hemocyanin
BSA	Bovine serum albumin
HEEA	Hexanoyl-ethanolamine
8OxodG/8OHdG	8-oxo-deoxyguanosine
MRM	Multiple reaction monitoring
LDL	Low-density lipoprotein
MDA	Malondialdehyde
CML	Carboxymethyllysine
ACR	Acrolein
CPT I	Carnitine palmitoyltransferase I

1 Discovery of Novel Amide-Type Adducts in Lipid Hydroperoxide-Modified Proteins

Lipid peroxidation is initiated by various factors such as metals, light, oxygen, and enzymes accompanied by the subsequent formation of lipid hydroperoxides. The peroxidation process is often described as a chain-reaction because of its characteristics of serial oxidation. Lipid aldehydes, which are highly reactive toward biomolecules, are formed as end-products of the lipid peroxidation process (Esterbauer et al. 1991). Lipid hydroperoxides and aldehydes, including conjugates with proteins and nucleic acids, are often detected in tissues in combination with some diseases. The contribution of lipid peroxidation to the development and initiation of diseases is still relatively unknown. Some lipid-derived oxidation products, especially reactive aldehydes, act as signal molecules that may influence certain processes inside a cell (Levonen et al. 2004). The biological function and formation mechanism of novel amide-type adducts (Fig. 2.1) are not well understood (Kato and Osawa 2010). Thus, in this chapter, I will focus on amide-type adducts and describe the discovery of novel amide-type lipid-lysine conjugates.

1.1 History of HEL Identification as a Marker of n-6 PUFA Oxidation

Originally, in the early 1990s, our group was interested in the formation of aldehyde-independent fluorescent products by reaction of lipid hydroperoxide

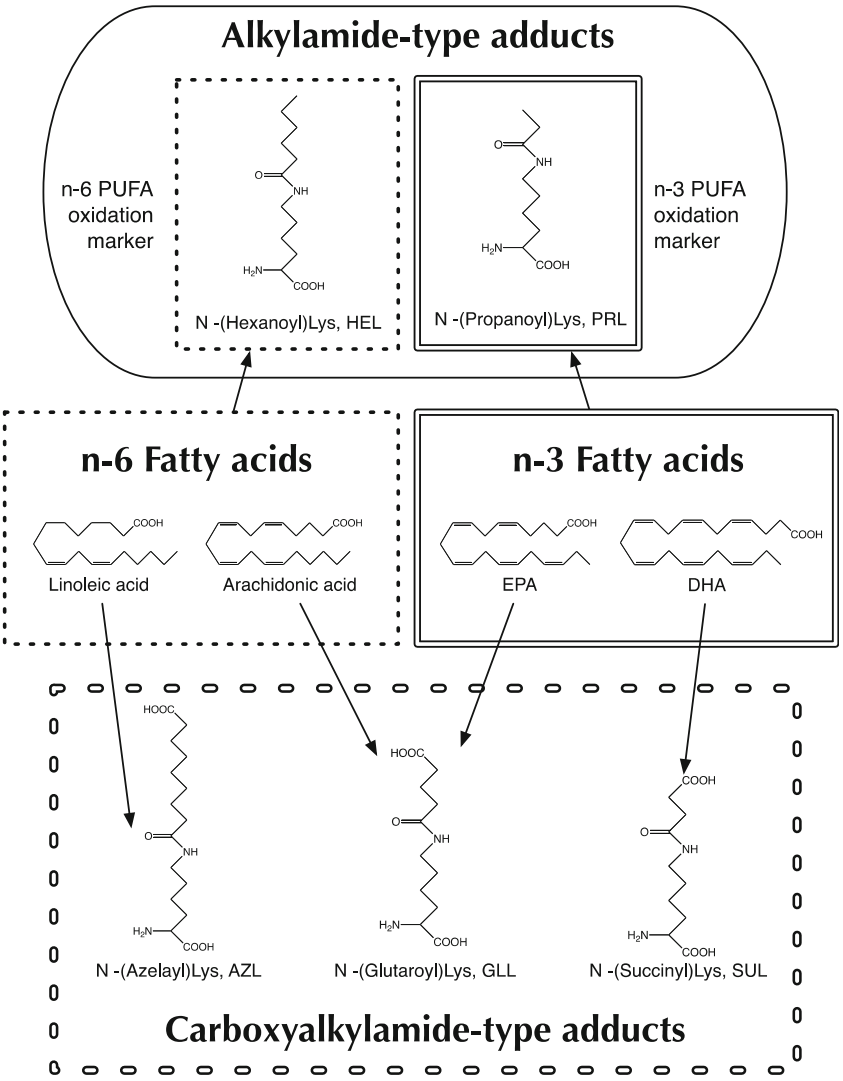


Fig. 2.1 Amide-adducts from n-6 and n-3 PUFA oxidation

with amines such as ethanolamine. However, the amounts of fluorescent products were quite low, making identification of the products difficult. It is noteworthy that a recent study reported the identification and detection of some fluorophores generated by lipid peroxidation (Riazy et al. 2011). To analyze the lipid hydroperoxide-derived specific amine modification, an antibody to the modified amine moiety was prepared. By using linoleic acid hydroperoxide-modified protein as an immunogen, we obtained a polyclonal antibody (Kato et al. 1997) and, similarly, another group prepared an antibody to a lipid hydroperoxide-modified

protein (Kim et al. 1997, 1999). In our group, an antigen was separated from a reaction mixture of lipid hydroperoxide, 13-hydroperoxyoctadecadienoic acid (13-HPODE), and benzoyl-glycyl-L-lysine (BGK), which was used as a model of a peptidyl lysine residue. One of the isolates was identified as a novel type of adduct, *Nε*-hexanoyl-lysine, named HEL, having an amide bond between the lysine epsilon amine and the lipid-derived part. The HEL was detected for the first time in a human atherosclerotic plaque using a specific polyclonal antibody to hexanoylated protein (Kato et al. 1999). The presence of HEL in human urine was also confirmed by liquid chromatography tandem mass spectrometry (LC/MS/MS) using a stable isotope dilution method (Kato et al. 2004). After the discovery of HEL, we identified some other amide-type lysine adducts including succinyl-lysine (SUL), glutary-lysine (GLL), azelayl-lysine (AZL), and propanoyl-lysine (propionyl-lysine, PRL) (Kawai et al. 2003a, b, 2004, 2006). These amide-type adducts can be classified into an alkylamide-type adduct or a carboxyalkylamide-type adduct. The structures and plausible sources are shown in Fig. 2.1. HEL was not recognized by the polyclonal antibody that was prepared in 1997. We then identified the epitope of the polyclonal antibody to linoleic acid hydroperoxide-modified protein as AZL, which contains the carboxy-terminal moiety derived from oxidized linoleic acid (Kawai et al. 2003a). We have also identified 4-hydroxy-2-nonenoyl lysine and used it as an antigen to prepare a polyclonal antibody (unpublished result). In addition, a similar oxo-type conjugate has been recently identified as a 4-oxo-2-nonenal-modified lysine adduct (Zhu and Sayre 2007) and it has been used to prepare a specific antibody (Shibata et al. 2011).

1.2 Identification of PRL as an Oxidation Marker for n-3 Fatty Acid

Ingestion of n-3 polyunsaturated fatty acid (PUFA) is often recommended because it provides some beneficial biological activities (Calder 2012), such as promotion of cardiovascular health and improvement of brain function. These functions are partly due to the fact that the alkyl chain has many double-bonds, thus providing it with flexibility. On the other hand, the double bonds in PUFA are easily oxidized. The formation of an adduct between a reactive aldehyde, 4-hydroxy-2-hexenal (HHE), and histidine residues has been used as a biomarker for n-3 PUFA oxidation (Yamada et al. 2004). Neuroprostane, protectin, and resolvin are also generated from n-3 PUFA by non-enzymatic oxidation or lipoxygenases (Levy 2010; Roberts et al. 1998). An n-3 PUFA-derived alkylamide, PRL has been recently identified in human urine (Hisaka et al. 2009). PRL can be categorized in a similar fashion as other n-3 oxidation biomarkers. PRL is superior to other n-3 oxidation markers in terms of its stability. In contrast, the PRL itself is not chemically reactive compared with aldehydes such as n-3 derived HHE. However, the formation of the PRL

residue might cause the aggregation of protein in vivo, which may lead to neurodegenerative diseases as discussed below.

2 Immunochemical Detection of HEL and Other Amide-Type Adducts

Recently, there has been a gradual increase in reports of the detection of amide-type adducts. In particular, HEL is described as one biomarker for oxidative stress (see Sect. 5). The advantages of HEL as a marker of oxidative stress are its stability and the feasibility of measurement/detection by a specific monoclonal antibody or a commercial ELISA HEL kit. Another alkylamide-type adduct, PRL, has also been detected in rabbit atherosclerotic plaques (Hisaka et al. 2009), the liver of an aminoacetophenone-injected rat (Sun et al. 2012), and the hippocampus region of the aged rat brain (Hisaka et al., manuscript in preparation) by immunological methods. The carboxyalkylamide AZL was also identified in an atherosclerotic plaque (Kawai et al. 2004) and SUL was found in the liver of mice fed with docosahexaenoic acid (DHA) followed by injection of carbon tetrachloride (Kawai et al. 2006). The immunohistochemical detection of lipo-oxidative stress markers in a disease-state or model are discussed in the chapters by Sugiyama and M. Naito, respectively. In these way, immunochemical methods (antibodies) are useful to detect these adducts. In the following Sects. 2.1 and 2.2, the characterization of HEL monoclonal antibody is described in detail. PRL antibody and the antibodies to other carboxyalkylamides are also discussed.

2.1 Preparation and Characterization of Antibodies to HEL and Other Adducts

HEL and PRL residues in a protein can be chemically synthesized using the commercially available anhydrates hexanoyl-anhydrate or propanoyl-anhydrate through a coupling reaction with lysine residues. Hexanoic acid or propionic acid can be also used for the preparation of amide-adducts by combination with carbodiimide and succinimide, which is normally used for the conjugation of a hapten against a carrier protein, such as keyhole limpet hemocyanin (KLH). A polyclonal antibody to HEL was prepared by injection of hexanoylated KLH as the immunogen into a rabbit (Kato and Osawa 2002). The antibody showed high reactivity against HEL and, to a lesser extent, analogs of HEL with different lengths of alkyl chain (Kato et al. 1999). Mouse monoclonal antibodies to HEL and PRL were also prepared (Hisaka et al. 2009; Kato et al. 2000). To obtain a highly specific antibody to an amide adduct, it was necessary to screen the antibodies based on their reactivities toward hexanolyated or propanoylated bovine serum albumin

(BSA) (Kato and Osawa 2009). The monoclonal antibody to the hexanoylated KLH recognized HEL but also some related hexanoyl-compounds at high concentration ranges including hexanoyl-glycine, hexanoyl-ethanolamine (HEEA), hexanoyl-glucosamine, and hexanoyl-sphingosine (Fig. 2.2a, b). Thus, when the HEL monoclonal antibody is used to examine biological specimens, its cross-reactivity against hexanoyl-compounds should be taken into consideration, in particular, HEEA, which is formed from phosphatidyl ethanolamine exposed to n-6 lipid hydroperoxide (Tsuji et al. 2003). However, we have confirmed that the concentration of HEEA in urine, determined by LC/MS/MS, does not interfere with the measurement of urinary HEL. As shown in Fig. 2.3, both free HEL (Nε-HEL) and Bz-Gly-HEL were recognized by the monoclonal antibody but the reactivity of Bz-Gly-HEL was slightly higher than that of free HEL, suggesting that peptidyl-HEL is more antigenic than the free HEL moiety. This is likely because the immunogen was the hexanoylated protein and not the free HEL or HEL-conjugated protein. In a pilot study, we found that urinary HEL tends to increase when some diseases are present (Fig. 2.4). The antibody to HEL and an ELISA kit are commercially available and the detailed information is discussed in the chapter written by Sakai.

The preparation and characterization of PRL antibody, G811, has already been discussed (Hisaka et al. 2009). Research has indicated that the HM6 clone produces more specific antibody to PRL than the G811 clone does. Currently, the antibodies are not commercially available but analysis of urinary PRL is available by using the antibody on a "protein chip". Detailed information on the protein chip is discussed in the chapter written by Hoshino.

SUL, AZL, and GLL are classified as carboxyalkylamide type adducts and they all have a terminal carboxylic acid (COOH). To prepare the antibody to AZL, monomethylazelate was conjugated with a protein using carbodiimide and N-sulfosuccinimide and the methyl moiety was then removed by treatment with a weak alkaline solution (Kawai et al. 2004). The antibody to SUL was prepared using succinate anhydrate (Kawai et al. 2006). We also prepared the antibody to GLL (unpublished result) according to the method for AZL using monomethylglutarate instead of monomethylazelate. Because most lipids are esterified in vivo, a specimen for immunohistochemical analysis has to be treated with alkaline or lipase, which cleaves the ester-bond prior to determination of carboxyalkylamides (Kato and Osawa 1998; Kawai et al. 2003a, 2004).

2.2 Method for Antibody Characterization

The preparation and characterization of a polyclonal antibody was previously reported (Kato and Osawa 2002; Kato et al. 1999). The monoclonal antibody to HEL was characterized as described previously (Kato et al. 2000). In brief, for the competitive indirect ELISA, 50 μl of hexanoylated BSA (5 μg/ml phosphate-buffered saline (PBS)) were pipetted into the wells and kept at 4 °C overnight. At

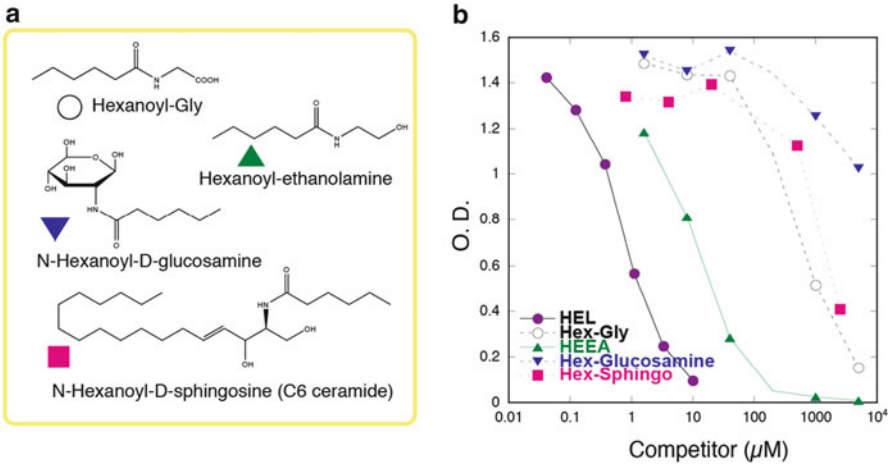


Fig. 2.2 Reactivity of a monoclonal antibody against hexanoyl-lysine (*HEL*) in the presence of hexanoylated compounds. (**a**) Structures of the competitors are indicated by the *symbols*. (**b**) The reactivities were examined by indirect competitive ELISA. The plate was coated with hexanoylated BSA (antigen) and then reacted with the antibody and samples including free HEL. The binding of antibody to the immobilized antigen was evaluated by the use of a secondary antibody conjugated with horseradish peroxidase. A detailed procedure is described in the text

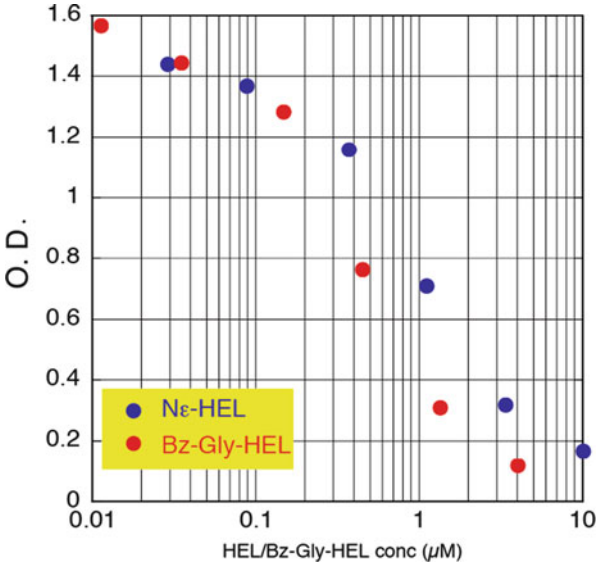


Fig. 2.3 Comparison of the reactivity between free hexanoyl-lysine (*HEL*) and peptidyl HEL (Bz-Gly-HEL). The reactivity was examined as described in the legend for Fig. 2.2. *Blue circle*, free HEL (Nε-HEL); *Red circle*, Bz-Gly-HEL

the same time, 50 μl of antibody (0.5 μg/ml) and 50 μl of sample (competitor) were mixed in a tube and reacted at 4 °C overnight. The plate was washed, blocked with 4 % Block Ace (Dainihon Seiyaku Co., Osaka, Japan), and 90 μl of the reacted solution were then pipetted into a well and further incubated. The binding of the

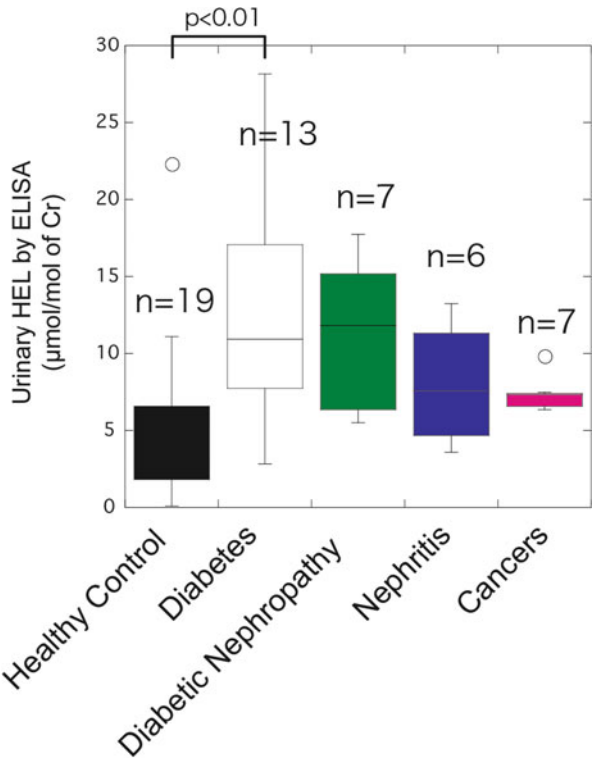


Fig. 2.4 A pilot study of the measurement of urinary HEL as a marker for selected diseases. The HEL was determined by a commercial ELISA kit using Bz-Gly-HEL as a standard. Urinary creatinine was estimated by a creatinine-test WAKO kit

residual antibody on the coated modified BSA was estimated using peroxidase-labeled anti-mouse IgG antibody with *o*-phenylenediamine and hydrogen peroxide.

3 Chemical Analyses of HEL and Other Amide-Type Adducts

To discern the exact amount of the alkylamide-type adducts HEL and PRL we used LC/MS/MS with a stable-isotope dilution method (Hisaka et al. 2009; Kato and Osawa 2009). Both HEL and PRL could be measured simultaneously by this method. It is noteworthy that the chemical standards of HEL and PRL including their stable isotopes for internal standards are not available from commercial sources at the present time. These can be prepared by the conjugation of [$^2d_2$] propionic acid or [$^2d_{11}$] hexanoic acid with lysine derivative as described previously (Kato et al. 2004; Hisaka et al. 2009). The amide adducts from biological sources are often butylated and then analyzed (Hisaka et al. 2009). The derivatization of an amide-adduct improves the separation of contents and also the sensitivity for detection. By using a derivatization method, we found that the excretion of HEL

and PRL into diabetic urine is higher than in healthy urine (Hisaka et al. 2009). The amount of urinary HEL or PRL correlated with that of some oxidative stress biomarkers such as 8-oxo-deoxyguanosine (known as 8OxodG or 8OHdG), isoprostanes, dityrosine, and bromotyrosine. Free PRL in amniotic fluid was also detected at approximately 4 nM (Yoji Kato and Josko Osredkar, unpublished result). Recently, hexanoylation of Lys 91 in a chymotryptic digest of β-lactoglobulin modified with linoleic acid, Fe(II), and ascorbate has been identified (Zhu et al. 2009). This indicates that the hexanoylation of lysine by a lipid hydroperoxide on a protein may not be random.

4 Mechanism of Amide-Adduct Formation

The formation mechanism of the amide-linkage in an amide-adduct has not been confirmed chemically. Baynes's group has identified a hexanoyl-adduct of pyridoxamine and proposed the mechanism of formation (Metz et al. 2003; Onorato et al. 2000). A 4-oxo-nonenal-derived amide adduct was identified but the mechanism could not be adapted to explain HEL generation (Zhu and Sayre 2007). The novel pathway of HEL formation by incubation of hexanal with hydrogen peroxide (or other peroxides) has been reported (Ishino et al. 2008). The proposed mechanism for hexanoylated compounds is shown in Fig. 2.5. In the following Sects. 4.1 and 4.2, data will be presented that should prove useful for obtaining a better understanding of the generation mechanism.

4.1 Mechanism of HEL Formation

Initial studies focused on understanding the formation mechanism of HEL from the reaction of a lipid hydroperoxide with a protein (Kato et al. 1999). Pre-incubation of 13-HPODE before incubation with a protein or lysine resulted in a decrease in the HEL adduct, indicating that decomposed products, such as aldehydes, might be not a major precursor of HEL. We have recently undertaken some experiments that investigate the generation mechanism and the results are shown hereafter. The methods are shown in Sect. 4.2. In the study, the formation of HEL residues in BSA exposed to 13-HPODE was enhanced by the saturation of oxygen gas (O_2) into a buffer and suppressed by the saturation of nitrogen gas (N_2) compared with a control performed in air under normal atmospheric conditions (Fig. 2.6). More interestingly, the formation rate from 0 to 8 h was hyperbolic, suggesting that 13-HPODE was transformed into an unknown precursor, for example, an excited ketone state as discussed later, at an initial stage of the incubation. Although the HEL residues were formed by the incubation of n-hexanal and hydrogen peroxide with peptidyl lysine (Bz-Gly-Lys, BGK) as reported (Ishino et al. 2008), the amount of HEL from the aldehyde plus peroxide system was 47-fold less than that from

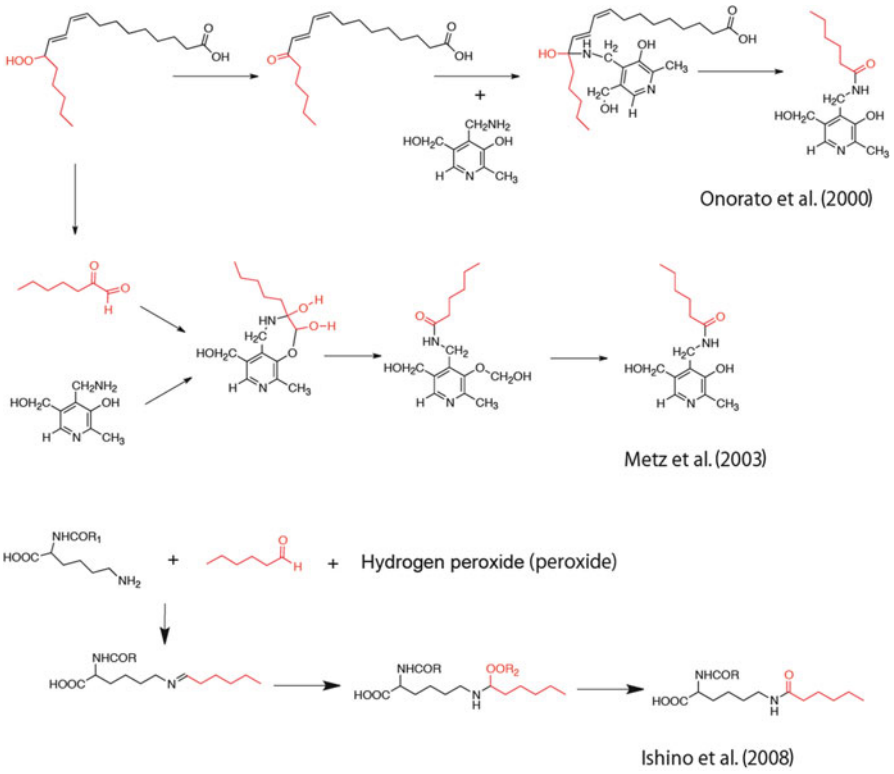


Fig. 2.5 Current proposed mechanisms of hexanoylation to an amine by lipid hydroperoxide or hexanal

13-HPODE at the same concentration, 2.5 mM (Fig. 2.7). A similar result was also obtained when a protein was incubated instead of the peptidyl lysine (data not shown). Moreover, the amount of hexanal generation, which was evaluated by derivatization of the aldehyde with 2,4-dinitrophenylhydrazine, was a few to several μM during incubation of 2.5 mM 13-HPODE (data not shown). Thus, the concentration of hexanal from decomposed 13-HPODE could not account for the amount of HEL generated from the "solo" 13-HPODE system with the lysine residue. Hence, the supplementation of hydrogen peroxide into the 13-HPODE system did not enhance the HEL generation (Fig. 2.7). This might indicate that the aldehyde plus peroxide pathway only minimally accounts for the HEL formation in the reaction, at least, between the lipid hydroperoxide and lysine in vitro. It is noteworthy that the result does not exclude the possibility of HEL generation from hexanal with peroxide in vivo.

It has been reported that singlet oxygen is formed from lipid hydroperoxides and oxidants via a Russell mechanism (Miyamoto et al. 2003) accompanied by the formation of the excited state of the ketone moiety. These species may contribute to the formation of amide-bond between lipid and lysine. Some types of

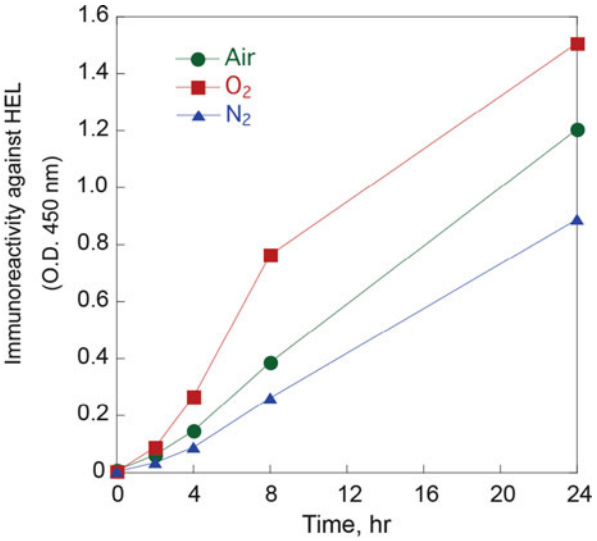


Fig. 2.6 Effect of oxygen on the formation of HEL in a protein exposed to linoleic acid hydroperoxide. Bovine serum albumin (1 mg/ml) was exposed to 2.5 mM of 13-HPODE in 50 mM phosphate buffer under air, O_2, or N_2. The lipo-oxidative modification of BSA was evaluated by ELISA using a specific antibody to HEL. The detailed protocol is described in the text

benzaldehydes with a lysine peptide generate not only a Schiff-base but also amide-type adducts without hydrogen peroxide at physiological conditions (Natsch et al. 2012). The formation mechanism of the amide-bond is still unknown.

4.2 Method of Analyses of HEL Generation In Vitro

1. The effect of atmospheric conditions on HEL generation was examined as follows. BSA (1 mg/ml) was incubated with synthetic 13-HPODE (2.5 mM) in 50 mM phosphate buffer, pH 7.2, in a test tube with a sealing cap. The phosphate buffer was bubbled with oxygen (O_2) or nitrogen (N_2) gas prior to the addition of BSA. After the addition of BSA and 13-HPODE into the buffer, the tubes were saturated with each gas and then sealed tightly. An air sample was reacted under normal atmospheric conditions. After reacting for up to 24 h, the sample was purified by a spin column (Bio-Rad, P-6 microspin column) and the generation of HEL was estimated by ELISA using the polyclonal antibody to HEL.
2. Comparison of HEL formation. The chemical formation of Bz-Gly-HEL from Bz-Gly-Lys was examined by LC/MS/MS using multiple reaction monitoring (MRM). The concentration of chemicals (13-HPODE, H_2O_2, and hexanal) was unified at 2.5 mM each. Bz-Gly-Lys was incubated with the reagents in 50 mM phosphate buffer, pH 7.2, at 37 °C for 24 h. The reacted samples were immediately injected into an HPLC connected with a API3000 tandem mass spectrophotometer. HPLC separation was performed using a Develosil ODS-SR-5 column (2 × 150 mm, Nomura Chemical) at a flow rate of 0.2 ml/min with

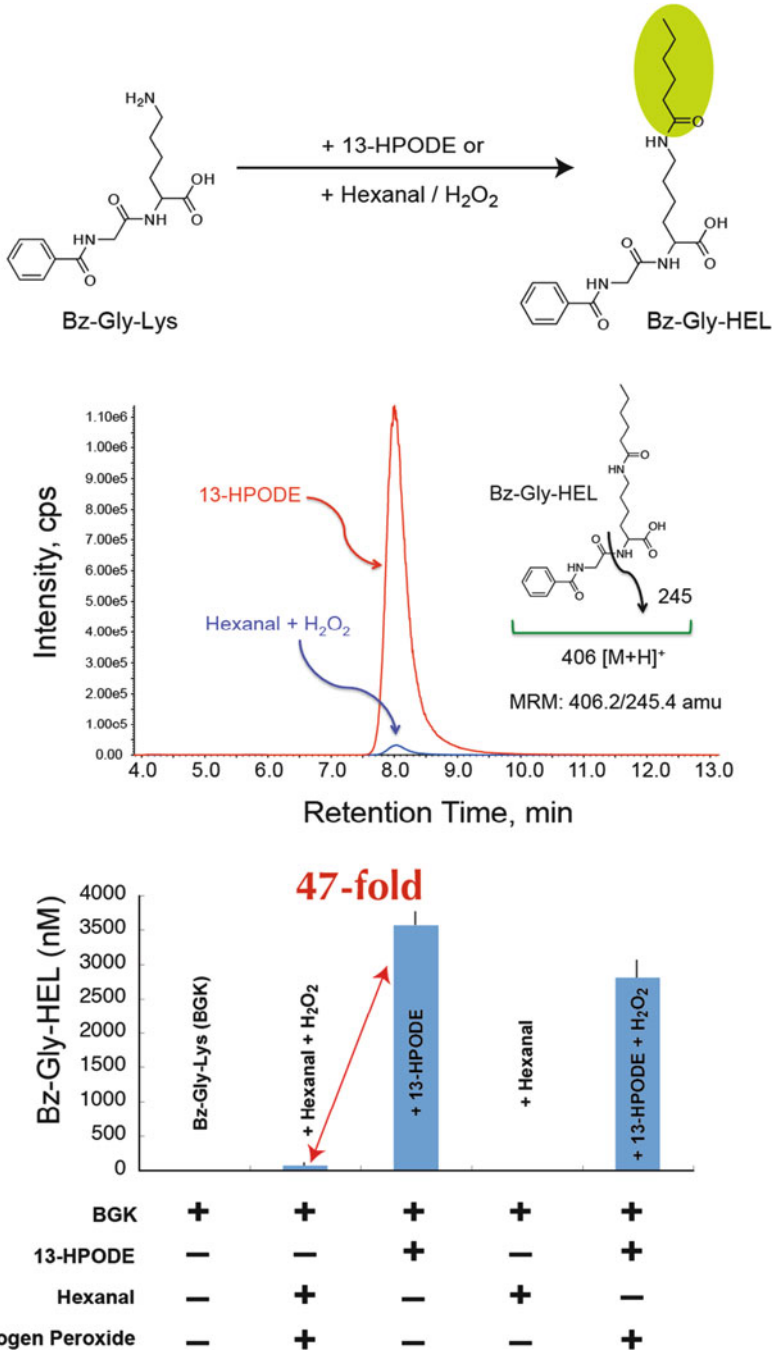


Fig. 2.7 The amount of HEL generation was compared with "solo" lipid hydroperoxide and aldehyde plus peroxide systems. Bz-Gly-Lys was exposed to 13-HPODE, and hexanal with or without hydrogen peroxide. The formation of the hexanylated peptide, Bz-Gly-HEL, was estimated by liquid chromatography tandem mass spectrometry. The detained procedure is described in the text

isocratic elution using 0.1 % acetic acid/CH_3CN (65/35). The formed Bz-Gly-HEL was detected by MRM using the transition of 406/84 and 406/245.

5 Current Topics of Application of Measurements of HEL and Other Markers In Vivo and Vitro

Since the discovery of HEL in 1999, the reports in the literature on HEL have gradually increased (Fukuchi et al. 2008; Okada et al. 2012; Osakabe et al. 2002; Ueno et al. 2002) as shown in Fig. 2.8. We have observed that HEL is stable in cultured medium, and HEL is resistant to digestion by peptidases and proteases (Kato et al. 2000) and proteinase K (unpublished results). Thus, because of its stability in biological specimens, HEL is a valuable oxidative stress marker.

It is believed that atherosclerosis is induced by post-transitional modification, especially oxidation, of low-density lipoprotein (LDL). Thus, inhibition of lipid peroxidation in LDL particles may prevent the development of atherosclerosis. Lipoprotein is one plausible target for adduct formation by lipid peroxidation, amide and aldehyde-derived modifications. Indeed, HEL, PRL, and AZL were immunochemically detected in atherosclerotic lesions (Fukuchi et al. 2008; Hisaka et al. 2009; Kato et al. 1999; Kawai et al. 2004). There are many reports that aldehyde-modified LDL was generated during in vitro oxidative modification of LDL (Palinski et al. 1990; Uchida et al. 1994, 1998). We have also found that HEL was increased with oxidation of LDL in vitro (Kato et al. 1999). We have also compared the frequency of lipo-oxidative modifications using specific antibodies to a 4-hydroxy-2-nonenal (HNE)-derived modification, a malondialdehyde (MDA)-derived modification, carboxymethyllysine (CML) which is a marker of a glyoxal-derived modification, an acrolein (ACR)-derived modification, and HEL. As shown in Fig. 2.9, the formation rate of HEL in copper-oxidized LDL is more rapid than the other aldehyde-derived modifications suggesting that HEL should be an earlier and sensitive marker for oxidative modification of LDL. Taken together, this supports the idea that HEL would be a useful marker for monitoring the development of atherosclerosis and related diseases.

Food is a favorable way to introduce beneficial compounds into our body because we ingest foods daily. An epidemiological study has shown the intake of vegetables including flavonoids reduces the onset of cardiovascular diseases, which is closely related with the development of atherosclerosis (Meir and Leitersdorf 2004). Apolipoprotein-E knock-out mice fed a diet of normal chow have atherosclerosis but the inclusion of cacao polyphenol extract in the diet reduced its development (Weisburger 2001). We found that atherosclerotic lesions were stained by the antibody to HEL and the staining and hence, number of lesions was reduced by intake of cacao polyphenols. A more detailed description of the application of HEL to other markers as a way to characterize functional food is discussed in the chapter by Natsume. The intake of functional food reduced the

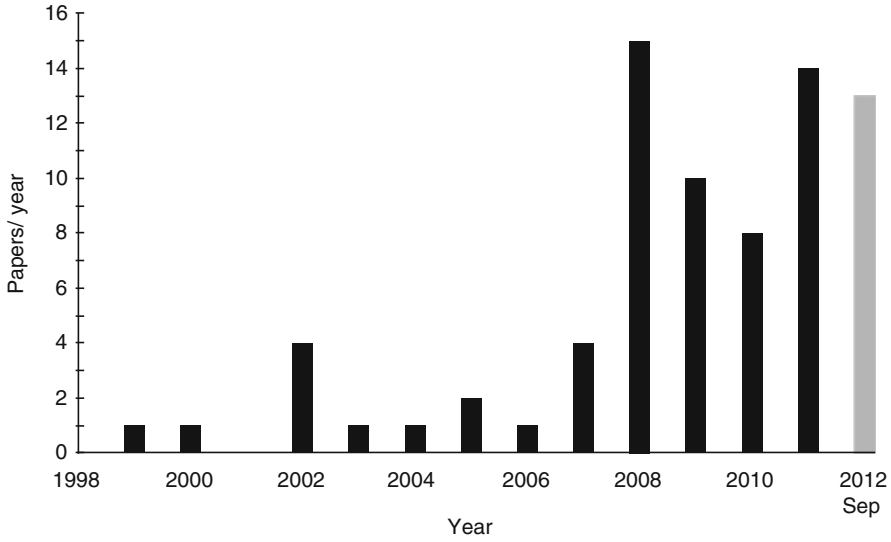


Fig. 2.8 Number of HEL publications per year from PubMed

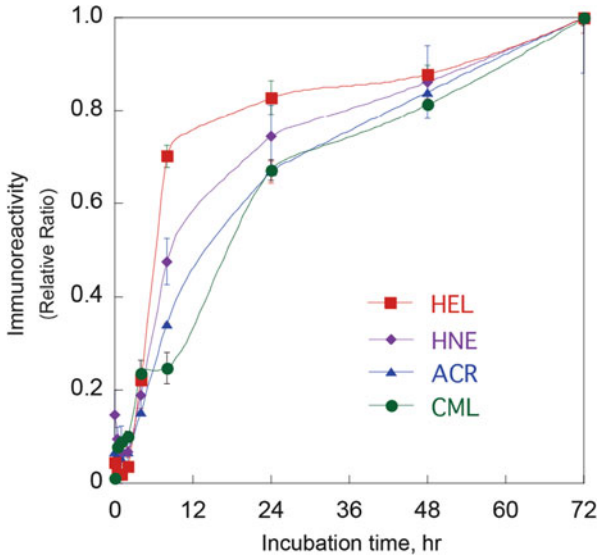


Fig. 2.9 The formation of a lipo-oxidative modification in copper oxidized LDL was evaluated by ELISA. Human LDL (0.25 mg/ml) was incubated with copper ion (50 μM) at 37 °C and the reactions were terminated at the indicated times by the addition of butylated hydroxytoluene and a metal ion chelator. The oxidized LDL was coated onto the plate and then treated with specific antibodies. The color development is shown as the relative ratio because the extent of color development does not indicate the amount of the compounds generated. The O.D. of HEL is the strongest among the four antibodies when using the same antigen, oxidized LDL, but further optimization of the procedure for each antibody should improve the color development

development of lesions, supporting the idea that some food components actually prevent the development of diseases (Duthie and Bellizzi 1999; Weisburger 2001). HEL is also used as a marker of oxidative modification of muscle during exercise (Minato et al. 2003). One of the proteins targeted by peroxidized lipid during exercise was identified as carnitine palmitoyltransferase I (CPT I) and the supplementation of astaxanthin protected the hexanoylation of CPT I (Aoi et al. 2008).

PRL is generated by the reaction between oxidized n-3 PUFA and a lysine residue (Hisaka et al. 2009), but an enzymatic generation pathway is also possible (Garrity et al. 2007). Among the n-3 PUFA, food-derived DHA is accumulated in the brain through the blood brain barrier. The beneficial significance and requirement of DHA in neuronal cells are obvious but, on the other hand, DHA is highly sensitive to oxidative stress. DHA metabolome analysis in neural tumors shows that 17-hydroperoxy-DHA has significant cytotoxic potency (Gleissman et al. 2010). DHA is also known to promote α-synuclein aggregation (De Franceschi et al. 2011). These results suggest that the prevention of DHA peroxidation may suppress neuronal diseases. In addition, resolvins or protectins, which are enzymatically generated from n-3 PUFA, are believed to be DHA-derived signaling molecules (Fredman and Serhan 2011; Levy 2010; Uddin and Levy 2011), indicating that controlled oxidation of DHA is a critical event for living processes.

PRL is not only a possible stable marker for DHA oxidation in vivo, but also may have biological activities. Indeed, a similar type of acylation, the acetylation of lysine in histones has a critical role in DNA replication and repair (Das et al. 2009). Lysine acetylation has also been observed in more than 20 % of total proteins in mitochondria (Kim et al. 2006). Lysine propionylation in histone and non-histone proteins believed to be a novel post-translational modification (Chen et al. 2007; Cheng et al. 2009). Thus, propanoylation (propionylation) of lysine in cellular proteins by enzymatic and also non-enzymatic reactions may have critical biological effects. PRL generation in neurons may play an important role in the pathogenesis of some neurodegenerative diseases (See the chapter by Maruyama).

6 Closing Remarks

The overall scheme for amide-adduct formation is summarized in Fig. 2.10. PUFAs are highly important molecules and have various beneficial functions. However, they are easily oxidized, and the resulting oxidized lipids may react with other biomolecules, such as proteins and aminophospholipids. There is no doubt of the benefits of PUFA ingestion but an excess administration of PUFA, for example, by supplementation, might induce the overproduction of biomolecules which contribute to oxidative stress. Although the generation mechanism of the amide-bond in HEL and related amide adducts remains to be clarified, the identification and characterization of amide-adducts may be useful for quantitating the oxidative stress induced by disease, high amounts of exercise, and excess ingestion of PUFA.

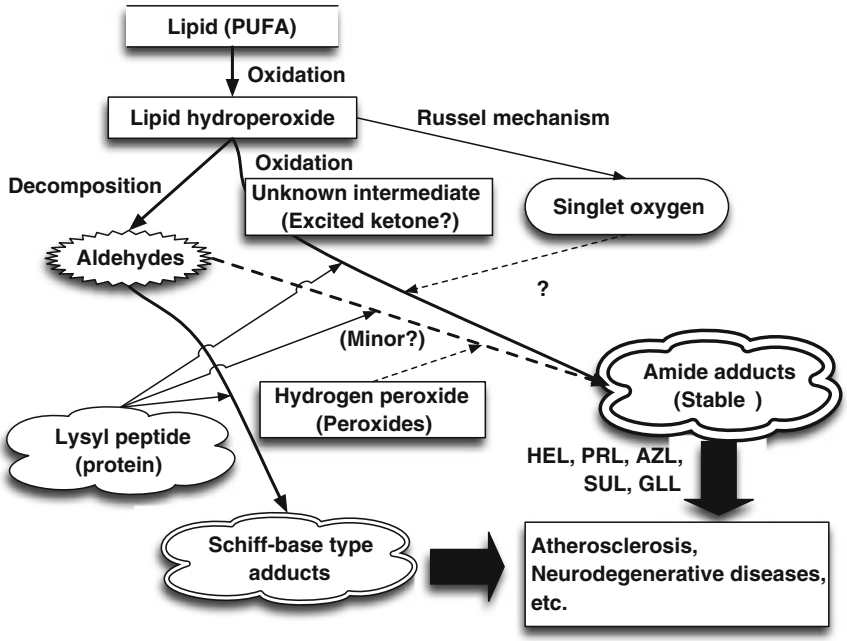


Fig. 2.10 Overall scheme for amide-adduct formation

Acknowledgments I would like to sincerely thank Professor Toshihiko Osawa for his encouragement during this study. I appreciate Professor Sayuri Miyamoto for her valuable comments. I express my thanks to Mr. Akihiro Yoshida (Nakatsugawa Municipal Hospital) and Dr. Michitaka Naito (Sugiyama-Jyogakuen University) for kindly providing the urine samples.

References

Aoi W, Naito Y, Takanami Y, Ishii T, Kawai Y, Akagiri S, Kato Y, Osawa T, Yoshikawa T (2008) Astaxanthin improves muscle lipid metabolism in exercise via inhibitory effect of oxidative CPT I modification. Biochem Biophys Res Commun 366:892–897

Calder P (2012) Mechanisms of action of (n-3) fatty acids. J Nutr 142:592S–599S

Chen Y, Sprung R, Tang Y, Ball H, Sangras B, Kim S, Falck J, Peng J, Gu W, Zhao Y (2007) Lysine propionylation and butyrylation are novel post-translational modifications in histones. Mol Cell Proteomics 6:812–819

Cheng Z, Tang Y, Chen Y, Kim S, Liu H, Li S, Gu W, Zhao Y (2009) Molecular characterization of propionyllysines in non-histone proteins. Mol Cell Proteomics 8:45–52

Das C, Lucia M, Hansen K, Tyler J (2009) CBP/p300-mediated acetylation of histone H3 on lysine 56. Nature 459:113–117

De Franceschi G, Frare E, Pivato M, Relini A, Penco A, Greggio E, Bubacco L, Fontana A, De Laureto PP (2011) Structural and morphological characterization of aggregated species of alpha-synuclein induced by docosahexaenoic acid. J Biol Chem 286:22262–22274

Duthie G, Bellizzi M (1999) Effects of antioxidants on vascular health. Br Med Bull 55:568–577

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

Fredman G, Serhan C (2011) Specialized proresolving mediator targets for RvE1 and RvD1 in peripheral blood and mechanisms of resolution. Biochem J 437:185–197

Fukuchi Y, Miura Y, Nabeno Y, Kato Y, Osawa T, Naito M (2008) Immunohistochemical detection of oxidative stress biomarkers, dityrosine and N(epsilon)-(hexanoyl)lysine, and C-reactive protein in rabbit atherosclerotic lesions. J Atheroscler Thromb 15:185–192

Garrity J, Gardner J, Hawse W, Wolberger C, Escalante-Semerena JC (2007) N-lysine propionylation controls the activity of propionyl-CoA synthetase. J Biol Chem 282:30239–30245

Gleissman H, Yang R, Martinod K, Lindskog M, Serhan C, Johnsen J, Kogner P (2010) Docosahexaenoic acid metabolome in neural tumors: identification of cytotoxic intermediates. FASEB J 24:906–915

Hisaka S, Kato Y, Kitamoto N, Yoshida A, Kubushiro Y, Naito M, Osawa T (2009) Chemical and immunochemical identification of propanoyllysine derived from oxidized n-3 polyunsaturated fatty acid. Free Radic Biol Med 46:1463–1471

Ishino K, Shibata T, Ishii T, Liu Y, Toyokuni S, Zhu X, Sayre L, Uchida K (2008) Protein N-acylation: H_2O_2-mediated covalent modification of protein by lipid peroxidation-derived saturated aldehydes. Chem Res Toxicol 21:1261–1270

Kato Y, Osawa T (1998) Detection of oxidized phospholipid-protein adducts using anti-15-hydroperoxyeicosatetraenoic acid-modified protein antibody: contribution of esterified fatty acid-protein adduct to oxidative modification of LDL. Arch Biochem Biophys 351:106–114

Kato Y, Osawa T (2002) Detection of lipid hydroperoxide-derived protein modification with polyclonal antibodies. Methods Mol Biol 186:37–44

Kato Y, Osawa T (2009) Detection of a lipid-lysine adduct family with an amide bond as the linkage: novel markers for lipid-derived protein modifications. Methods Mol Biol 580:129–141

Kato Y, Osawa T (2010) Detection of lipid-lysine amide-type adduct as a marker of PUFA oxidation and its applications. Arch Biochem Biophys 501:182–187

Kato Y, Makino Y, Osawa T (1997) Characterization of a specific polyclonal antibody against 13-hydroperoxyoctadecadienoic acid-modified protein: formation of lipid hydroperoxide-modified apoB-100 in oxidized LDL. J Lipid Res 38:1334–1346

Kato Y, Mori Y, Makino Y, Morimitsu Y, Hiroi S, Ishikawa T, Osawa T (1999) Formation of Nepsilon-(hexanonyl)lysine in protein exposed to lipid hydroperoxide. A plausible marker for lipid hydroperoxide-derived protein modification. J Biol Chem 274:20406–20414

Kato Y, Miyake Y, Yamamoto K, Shimomura Y, Ochi H, Mori Y, Osawa T (2000) Preparation of a monoclonal antibody to N(epsilon)-(Hexanonyl)lysine: application to the evaluation of protective effects of flavonoid supplementation against exercise-induced oxidative stress in rat skeletal muscle. Biochem Biophys Res Commun 274:389–393

Kato Y, Yoshida A, Naito M, Kawai Y, Tsuji K, Kitamura M, Kitamoto N, Osawa T (2004) Identification and quantification of N(epsilon)-(hexanoyl)lysine in human urine by liquid chromatography/tandem mass spectrometry. Free Radic Biol Med 37:1864–1874

Kawai Y, Kato Y, Fujii H, Makino Y, Mori Y, Naito M, Osawa T (2003a) Immunochemical detection of a novel lysine adduct using an antibody to linoleic acid hydroperoxide-modified protein. J Lipid Res 44:1124–1131

Kawai Y, Saito A, Shibata N, Kobayashi M, Yamada S, Osawa T, Uchida K (2003b) Covalent binding of oxidized cholesteryl esters to protein: implications for oxidative modification of low density lipoprotein and atherosclerosis. J Biol Chem 278:21040–21049

Kawai Y, Fujii H, Kato Y, Kodama M, Naito M, Uchida K, Osawa T (2004) Esterified lipid hydroperoxide-derived modification of protein: formation of a carboxyalkylamide-type lysine adduct in human atherosclerotic lesions. Biochem Biophys Res Commun 313:271–276

Kawai Y, Fujii H, Okada M, Tsuchie Y, Uchida K, Osawa T (2006) Formation of Nepsilon-(succinyl)lysine in vivo: a novel marker for docosahexaenoic acid-derived protein modification. J Lipid Res 47:1386–1398

Kim J, Sabbagh F, Santanam N, Wilcox J, Medford R, Parthasarathy S (1997) Generation of a polyclonal antibody against lipid peroxide-modified proteins. Free Radic Biol Med 23:251–259

Kim J, Taylor W, Parthasarathy S (1999) Demonstration of the presence of lipid peroxide-modified proteins in human atherosclerotic lesions using a novel lipid peroxide-modified anti-peptide antibody. Atherosclerosis 143:335–340

Kim S, Sprung R, Chen Y, Xu Y, Ball H, Pei J, Cheng T, Kho Y, Xiao H, Xiao L, Grishin N, White M, Yang X, Zhao Y (2006) Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. Mol Cell 23:607–618

Levonen A, Landar A, Ramachandran A, Ceaser E, Dickinson D, Zanoni G, Morrow J, Darley-Usmar VM (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

Levy B (2010) Resolvins and protectins: natural pharmacophores for resolution biology. Prostaglandins Leukot Essent Fatty Acids 82:327–332

Meir K, Leitersdorf E (2004) Atherosclerosis in the apolipoprotein-E-deficient mouse: a decade of progress. Arterioscler Thromb Vasc Biol 24:1006–1014

Metz T, Alderson N, Chachich M, Thorpe S, Baynes J (2003) 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

Minato K, Miyake Y, Fukumoto S, Yamamoto K, Kato Y, Shimomura Y, Osawa T (2003) Lemon flavonoid, eriocitrin, suppresses exercise-induced oxidative damage in rat liver. Life Sci 72:1609–1616

Miyamoto S, Martinez G, Medeiros M, Di Mascio P (2003) Singlet molecular oxygen generated from lipid hydroperoxides by the Russell mechanism: studies using 18(O)-labeled linoleic acid hydroperoxide and monomol light emission measurements. J Am Chem Soc 125:6172–6179

Natsch A, Gfeller H, Haupt T, Brunner G (2012) Chemical reactivity and skin sensitization potential for benzaldehydes: can Schiff base formation explain everything? Chem Res Toxicol 25:2203–2215

Okada H, Naito Y, Takagi T, Takaoka M, Oya-Ito T, Fukumoto K, Uchiyama K, Handa O, Kokura S, Nagano Y, Matsui H, Kato Y, Osawa T, Yoshikawa T (2012) Detection of N-(hexanoyl)lysine in the tropomyosin 1 protein in N-methyl-N′-nitro-N-nitrosoguanidine-induced rat gastric cancer cells. J Clin Biochem Nutr 50:47–52

Onorato J, Jenkins A, Thorpe S, Baynes J (2000) Pyridoxamine, an inhibitor of advanced glycation reactions, also inhibits advanced lipoxidation reactions. Mechanism of action of pyridoxamine. J Biol Chem 275:21177–21184

Osakabe N, Yasuda A, Natsume M, Sanbongi C, Kato Y, Osawa T, Yoshikawa T (2002) Rosmarinic acid, a major polyphenolic component of Perilla frutescens, reduces lipopolysaccharide (LPS)-induced liver injury in D-galactosamine (D-GalN)-sensitized mice. Free Radic Biol Med 33:798–806

Palinski W, Yla-Herttuala S, Rosenfeld M, Butler S, Socher S, Parthasarathy S, Curtiss L, Witztum J (1990) Antisera and monoclonal antibodies specific for epitopes generated during oxidative modification of low density lipoprotein. Arteriosclerosis 10:325–335

Riazy M, Lougheed M, Adomat H, Guns E, Eigendorf G, Duronio V, Steinbrecher U (2011) Fluorescent adducts formed by reaction of oxidized unsaturated fatty acids with amines increase macrophage viability. Free Radic Biol Med 51:1926–1936

Roberts LN, Montine T, Markesbery W, Tapper A, Hardy P, Chemtob S, Dettbarn W, Morrow J (1998) Formation of isoprostane-like compounds (neuroprostanes) in vivo from docosahexaenoic acid. J Biol Chem 273:13605–13612

Shibata T, Shimozu Y, Wakita C, Shibata N, Kobayashi M, Machida S, Kato R, Itabe H, Zhu X, Sayre L, Uchida K (2011) Lipid peroxidation modification of protein generates Nepsilon-(4-oxononanoyl)lysine as a pro-inflammatory ligand. J Biol Chem 286:19943–19957

Sun J, Sugiyama A, Hisaka S, Kato Y, Takeuchi T (2012) Immunohistochemical detection of polyunsaturated fatty acid oxidation markers in acetaminophen-induced liver injury in rats. J Vet Med Sci 74:141–147

Tsuji K, Kawai Y, Kato Y, Osawa T (2003) Formation of N-(hexanoyl)ethanolamine, a novel phosphatidylethanolamine adduct, during the oxidation of erythrocyte membrane and low-density lipoprotein. Biochem Biophys Res Commun 306:706–711

Uchida K, Toyokuni S, Nishikawa K, Kawakishi S, Oda H, Hiai H, Stadtman E (1994) Michael addition-type 4-hydroxy-2-nonenal adducts in modified low-density lipoproteins: markers for atherosclerosis. Biochemistry 33:12487–12494

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 U S A 95:4882–4887

Uddin M, Levy B (2011) Resolvins: natural agonists for resolution of pulmonary inflammation. Prog Lipid Res 50:75–88

Ueno Y, Horio F, Uchida K, Naito M, Nomura H, Kato Y, Tsuda T, Toyokuni S, Osawa T (2002) Increase in oxidative stress in kidneys of diabetic Akita mice. Biosci Biotechnol Biochem 66:869–872

Weisburger J (2001) Chemopreventive effects of cocoa polyphenols on chronic diseases. Exp Biol Med (Maywood) 226:891–897

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

Zhu X, Sayre L (2007) Long-lived 4-oxo-2-enal-derived apparent lysine Michael adducts are actually the isomeric 4-ketoamides. Chem Res Toxicol 20:165–170

Zhu X, Tang X, Anderson V, Sayre L (2009) Mass spectrometric characterization of protein modification by the products of nonenzymatic oxidation of linoleic acid. Chem Res Toxicol 22:1386–1397

Chapter 3
Lipid Hydroperoxide-Derived Adduction to Amino-Phospholipid in Biomembrane

Shinsuke Hisaka and Toshihiko Osawa

Abstract Phospholipids such as phosphatidylethanolamine and phosphatidylcholine play crucial roles in the biological system to maintain the cellular environmental condition. Despite that, oxidative stress targets these phospholipids containing polyunsaturated fatty acids and accompanies the oxidized phospholipids. Recent studies have been suggested that oxidized phospholipids have the relationship with inflammation and might induce the atherosclerosis formation by uptake of oxidized LDL through scavenger receptor as ligands. Red blood cells, which have been studied the bilayer model, are also modified by oxidative stress because hemoglobin can mediate and produce the reactive oxygen species, which leads to lipid peroxidation of biomembrane. In these oxidation processes of biomolecules, hexanoylation against phosphatidylethanolamine and phosphatidylserine, which has the primary amine and is the target of this modification, generates the oxidized membrane such as erythrocyte ghosts. This unique structure of phosphatidylethanolamine and phosphatidylserine is possibly the useful biomarker to evaluate the oxidation of biomembrane *in vivo* using liquid chromatography tandem mass spectrometry and monoclonal antibody.

Keywords Oxidation • Lipid peroxidation • Aminophospholipid • Liquid chromatography tandem mass spectrometry • Monoclonal antibody

1 Various Phospholipids in Biomembrane

Phospholipids are major components of cellular membranes which are essential for the structural integrity and function of cells and construction of circulating plasma lipoproteins. A ubiquitous phospholipid in the mammalian plasma membrane,

S. Hisaka (✉)
Department of Pharmacy, Meijo University, Nagyoa, Japan
e-mail: hisakas@meijo-u.ac.jp

T. Osawa
Department of Health and Nutrition, Aichi Gakuin University, Nisshin, Japan

Y. Kato (ed.), *Lipid Hydroperoxide-Derived Modification of Biomolecules*,
Subcellular Biochemistry 77, DOI 10.1007/978-94-007-7920-4_3,

phosphatidylethanolamine (PE) is among the highly abundant molecules in the biological system (Rouser et al. 1971). Phosphatidylcholine (PC) is also major phospholipid in mammalian membrane and is synthesized in the liver via the choline pathway by methylation of PE via phosphatidylethanolamine *N*-methyltransferase (Li et al. 2005). The ratio of PC to PE is important for membrane integrity in the liver and the decrease of this ratio leads to liver failure into progression of steatosis (Li et al. 2006). In contrast with these cationic phospholipids, the negative surface charge of the inner leaflet of the plasma membrane determines the targeting of proteins containing polycationic motifs (Mclaughlin and Murray 2005). Although polyphosphoinositide content contributes to this unique negativity of the plasmalemmal inner leaflet, in part, phosphatidylserine (PS) is the predominant anionic species, which represents 10–20 % of all surface lipid (Vance and Steenbergen 2005). Recent study showed that the negative charge associated with the presence of PS regulates the protein localization by the endocytic pathway (Yeung et al. 2008). Cardiolipins is mainly located in the mitochondrial membrane.

In contrast to these various phospholipids which constructs the bilayer of cellular membrane and maintains the biological behaviors, it has been suggested that apoptotic cells and bodies contain increased levels of these phospholipids oxidation, which shows the oxidative damage targets these phospholipids during apoptotic events (Huber et al. 2002).

2 The Role of Oxidized Phospholipid in the Inflammatory Lesions

Although the head groups of some phospholipids, especially, PEs can be oxidized, which is also "phospholipid oxidation", generally the targets of oxidation in phospholipids are unsaturated fatty acid chains. Therefore, phospholipids containing polyunsaturated fatty acids are susceptible to peroxidation by free radicals, and monounsaturated fatty acyl groups may also be oxidized by non-radical species such as peroxynitrite ($ONOO^-$) and hypochlorous acid (HOCl). Multiple lines show that these oxidized phospholipids and lipid peroxidation are formed in inflammatory diseases such as atherosclerosis, neurodegenerative disease, multiple sclerosis, and systemic lupus erythematosus. In those diseases, most attention has been focused on atherosclerosis because this disease has the relationship with dyslipidemia and chronic inflammation, in addition, the oxidized LDL (oxLDL). LDL oxidation is one of risk factors in the pathogenesis of initiator and progression (Ross 1999). Using electrospray mass spectrometry, oxidized phospholipid species have been analyzed in human LDL (Davis et al. 2008). The major phospholipids oxidation products such as hydroxides, epoxides, isoprostanes, and core aldehydes in human atherosclerotic lesions are reported (Ravandi et al. 2004). A key factor involved in these developmental regions of atherosclerosis was the uncontrolled uptake of oxLDL by macrophages

recognized as possible ligands for scavenger receptor families such as cluster differentiation 36 (CD36), scavenger receptor class A types I and II (SRA-I/II), and class B scavenger receptor. The studies using CD36 and ApoE double-knockout mice showed dramatic reduction in lesion area compared with $ApoE^{-/-}$ mice, both fed on a high-fat diet (Febbraio et al. 2000). In addition, SRA-I/II and CD36 have been shown to be the primary receptors involved in the uptake of modified phospholipids by macrophages (Kunjathoor et al. 2002). Therefore, these reports indicated that oxidized phospholipids in oxLDL could trigger to the development of atherosclerotic plaque through a ligand of SRA-I/II and CD36 receptors. In addition to these reports, the modified phospholipids have been found in other diseases such as diabetes and Alzheimer's disease (Leitinger 2008; Bochkov et al. 2010).

3 The Oxidative Modification of Red Blood Cells Might Lead to Lifestyle Related Diseases

Red blood cells (RBCs) are the main molecules in the blood components which play the role to carry free oxygen around whole tissue. Therefore, RBCs are directly modified by oxidative damage, which is also attributable that RBCs are sensitive to redox balancing. Hemoglobin (Hb) in RBCs has the domain to bind oxygen via ferric ion, concurrently giving an opportunity to produce lipid peroxides. This redox balancing is regulated by including more antioxidative enzymes such as superoxide dismutase and catalase in cell bodies than other cells.

Generally, RBCs limits the life span to approximately 100–120 days because during circulating they undergo senescence with subsequent clearance of the aged RBCs (Lang et al. 2010). The transitions of physical properties such as elasticity of cell membrane are ongoing during ageing. It is reported that aged RBCs included the increasing of their density and enhancement of lipid peroxidation in phospholipids, flip-flop of PS from inside of cell membrane to outside (Linderkamp et al. 1993; Bratosin et al. 1998). Additionally, it suggested the reduction of antioxidative regulators, glutathione reductase, glucose-6 phosphate dehydrogenase, catalase, and glutathione peroxidase (Linderkamp et al. 1993). However, RBCs senescence is under elucidation in detail, Hb might be the most suspicious factor to injure RBCs themselves via production of ROS. The heme iron in Hb (oxHb), which is normally divalent ion and binds oxygen, gives electron to the binding oxygen and then Hb converts metHb (Rachmilewitz 1974). In this processes, oxygen alters superoxide (Scheme 3.1).

$$\mathrm{Hb}\left(\mathrm{Fe}^{2+}\right) + \mathrm{O}_2 \rightarrow \mathrm{Hb}\left(\mathrm{Fe}^{2+}\right)\mathrm{O}_2 \rightarrow \mathrm{Hb}\left(\mathrm{Fe}^{3+}\right)\mathrm{O}^{2-} \qquad \text{(Scheme 3.1)}$$

Hb, drives similar to ferric ion, can mediate Fenton reaction (Sadrzadeh et al. 1984) and cleavage of lipid hydroperoxide (Kim and Sevanian 1991), (Suzuki et al. 1997). Modified lipids including aminophospholipids have been

suggesting that Hb has the relevance of initiator of lipid peroxidation to oxidatively-modified and aged RBCs. In the sickle-cell anemia, one of important factors is the abnormality of Hb, which is more susceptible to oxidative stress and induces to substantially produce of OH radical. Therefore, these capacities of RBCs might also lead to the pathogenesis of lifestyle diseases. For example, in the membrane of RBCs derived from diabetic patients, 9-hydroperoxyoctadecadienoic acid (9-HPODE), one of linoleic acid hydroperoxides, was generated (Inouye et al. 1999). RBCs endocytosed to the arteriosclerosis initiated the lipid peroxidation and enlarged the core of plaques (Lin et al. 2008). Consequently, the aged RBCs, which initiated lipid peroxidation, have the relevance of pathogenesis of disease including lifestyle related diseases and atherosclerosis. This suggestion indicates that it is important to evaluate the generation of oxidized biomembrane such as RBCs.

4 Amino-Phospholipid Is One of Targets to Modification by Lipid Hydroperoxide

Our group has been studied the biomarkers evaluating lipid peroxidation *in vivo*. Previous reports suggested that lysine residue in the proteins was modified by 13-hydroxyperoxyoctadecadienoic acid (13-HPODE), one of linoleic acid hydroperoxides, and accompanied by the formation of hexanoyl moiety at ε-amino group via amide bond (Kato et al. 1999, 2000). This unique structure was also constructed by 15-hydroperoxyeicosatetraenoic acid (15-HPETE), one of arachidonic acid hydroperoxides. This modification had a commonality to have the amide bond structure formed by the reaction between primary amino group, ε-moiety of lysine, and ω-6 lipid hydroperoxide. Therefore, amino-phospholipids, in which PE and PS have primary amino group in the hydrophilic head, can also form the hexanoyl derivative induced by lipid peroxides such as 13-HPODE. To characterize the covalent modifications of PE and PS head group by lipid peroxidation products, we investigated the formation of *N*-hexanoyl-PE (HEPE) and *N*-hexanoyl-PS (HEPS) as plausible hexanoylated compounds with amide bond (Fig. 3.1). Previous studies suggested that the phospholipids oxidation *in vivo* had difficulty to quantify, owing to the large number of different structures of oxidatively modified phospholipids. But by focusing on the hexanoylation derivative induced by oxidative modification and using liquid chromatography tandem mass spectrometry (LC/MS/MS) we can sensitively identify the target oxidized aminophospholipids. By LC/MS/MS method, the hexanoylation of PE and PS was confirmed by the detection of hexanoylethanolamine (HEEA) and hexanoylserine (HESE), which enzymatically hydrolyzed by treatment of the modified PE and PS with phospholipase D (PLD) (Fig. 3.1), in the reaction mixture of egg PE co-incubated with 13-HPODE (Tuji et al. 2003) or erythrocyte ghosts modified 13-HPODE (Hisaka et al. 2010). This is the reasonable method to detect the modification of polar molecules because various fatty acid chains esterified with glycerol did not

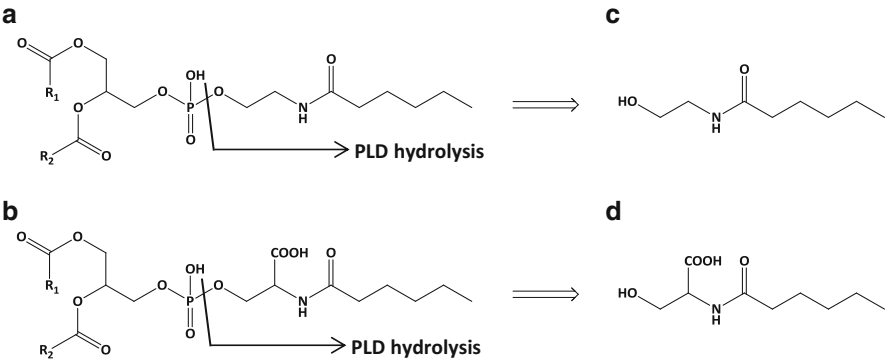


Fig. 3.1 Chemical structure of N-(hexanoyl)phosphatidylethanolamine (**a**), N-(hexanoyl) phophatidylserine (**b**), hexanoylethanolamine (**c**) and hexanoylserine (**d**)

influence the determination of HEEA and HESE (Fig. 3.1) by the detection of LC/MS/MS. Furthermore, hexanoylation derived from the oxidation of ω-6 polyunsaturated fatty acids (PUFAs) such as linoleic acid and arachidonic acid, not from ω-3 PUFAs including docosahexaenoic acid (Tuji et al. 2003), which means the specific marker of modification of ω-6 PUFAs to biomembrane. This monitoring procedure applied to the accurate quantification values of HEPE and HEPS corrected by the stable isotope dilution method. The quantified level of HEPE (HEEA) dose-dependently elevated in the erythrocytes (RBCs) exposed by 13-HPODE (Hisaka et al. 2010). Moreover, HEPE was detectable in human LDL oxidized by Cu^{2+} (Tuji et al. 2003), which indicated that HEPE was possibly a ligand candidate of scavenger receptor in macrophages. Additionally, whereas HEPS (HESE) also increased in this condition, the level of HEPE is about 60-folds higher than that of HEPS (Hisaka et al. 2010). This character of HEPS might derive from less abundant components normally in the inner leaflet of the plasma membrane. The estimation of HEPE and HEPS *in vivo* using LC/MS/MS suggested that hexanoylation against PE and PS was significantly elevated in the oxidative model induced by carbon tetrachloride (CCl_4), in which thiobarbituric acid reactive substances of liver tissue were relatively increasing. These results supported the concept that HEPE and HEPS were the available biomarkers of amino-phospholipids oxidation.

Several lines of evidence suggest that oxidative modifications of proteins, DNA, lipids have been found *in vivo* during aging (Stadtman 1992). Therefore, the level of HEPE derived from the RBCs in the aged rat (24 months old) was analyzed by LC/MS/MS, which showed in a preliminary result that HEPE was elevated compared to control RBCs derived from 6 months old rats (data not shown). This result was obtained from the collaboration with Dr. Wakako Maruyama, department director in National Center of Geriatrics and Gerontology. Because the CD36 receptor, which has been suggested to be crucial role in the progression of atherosclerosis, is a platelet-integral membrane glycoprotein and is highly conserved

between humans and rodents (Febbraio et al. 2001), it is prospective to clear how senescence RBCs, progresses lipid peroxidation and enhanced the generation of HEPE, relate to the pathogenesis of atherosclerosis in the future.

5 Evaluation of HEPE as Biomarkers Using Monoclonal Antibody

To evaluate the target molecule *in vitro* and *in vivo*, monoclonal antibody is a well-known tool. Several studies in humans have been carried out using antibodies raised against oxidized phospholipids, especially one that is reported to be specific for 1-palmitoyl-2-(5'oxo)valery-*sn*-3-glycerophosphocholine in lipoproteins (Tsimikas et al. 2006). Our group has also utilized a monoclonal antibody to detect the unique structures in the biomolecules such as non-enzymatic post-translational modification to proteins (Kawai et al. 2006; Hisaka et al. 2009). Regarding HEPE, we prepared the monoclonal antibody since HEPE was easily generated compared to HEPS and characterized its capacity. In preparing the antibody against HEPE, we made a little ingenuity because HEPE is hard to be conjugated into a protein, which is often used for a carrier of a hapten (immunogen). Then we focused on the liposome to prepare the immunogen. This liposome (Fig. 3.2) containing *N*-(hexanoyl) 1, 2-dipalmitoyl-sn-glycero-3-phosphatidyletanolamine (HEDPPE), which was the model of bilayer modified by lipid peroxidation, was used to immunize mice as antigen with lipid A. Finally, obtained antibody recognized the glycerol-3-phosphate moiety including HEEA structure whereas the structure of HEEA alone slightly influenced the cross-reactivity of this monoclonal antibody. The immunohistochemical analysis using antibody to HEPE showed the validation as the biomarker of HEPE to be detectable the oxidative damage of biomembrane *in vivo*. In the liver derived from oxidative model induced by CCl_4, which was previously described in the analysis using LC/MS/MS (Sect. 4), the antibody to HEPE clearly visualized the presence of that epitope, which did not merge with the loci of hexanoyl-lysine. This result correlated with the result of LC/MS/MS. Hence, monoclonal antibody to HEPE showed the reasonable method to evaluate the oxidative modification to biomembrane *in vivo*.

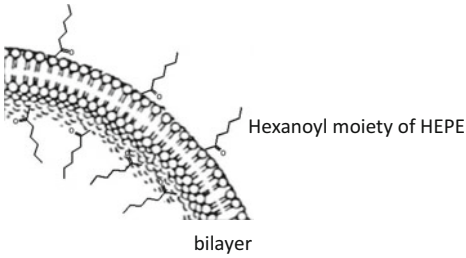


Fig. 3.2 Image of antigen: liposome containing HEDPPE

6 Conclusion

The accumulated studies about phospholipids have been cleared the crucial roles such as membrane integrity and electrostatic interactions with negatively charged molecules in the physiology and biochemistry. Especially, it has been attractive that the oxidized phospholipids might have the relevance of the pathogenesis concerned with inflammation related diseases including atherosclerosis. The red blood cells, which are the main molecules in the blood components, are sensitive to the disruption of redox balance, indicating these attributions might initiate the lipid peroxidation and could lead to atherosclerosis and lifestyle-related diseases. Therefore, HEPE and HEPS are possibly reasonable biomarkers to evaluate the biomembrane oxidation including red blood cells oxidation. In quantification of these biomarkers, antibodies against these unique structures can work for enzyme-linked immunosorbent assay. Finally, the analysis of the unique biomarkers, HEPE and HEPS may lead to the novel understandings of oxidized phospholipids in the future.

References

Bochkov VN, Oskolkova OV, Birukov KG et al (2010) Generation and biological activities of oxidized phospholipids. Antioxid Redox Signal 12:1009–1059

Bratosin D, Mazurier J, Tissier JP et al (1998) Cellular and molecular mechanisms of senescent erythrocyte phagocytosis by macrophages. A review. Biochimie 80:173–195

Davis B, Koster G, Douet LJ et al (2008) Electrospray ionization mass spectrometry identifies substrates and products of lipoprotein-associated phospholipase A2 in oxidized human low density lipoprotein. J Biol Chem 283:6428–6437

Febbraio M, Podrez EA, Smith JD et al (2000) Targeted disruption of the class B scavenger receptor CD36 protects against atherosclerotic lesion development in mice. J Clin Invest 105:1049–1056

Febbraio M, Hajjar DP, Silverstain RL (2001) CD36: a class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism. J Clin Invest 108:785–791

Hisaka S, Kato Y, Kitamoto N et al (2009) Chemical and immunochemical identification of propanoyllysine derived from oxidized n-3 polyunsaturated fatty acid. Free Radic Biol Med 46:1463–1471

Hisaka S, Yamada N, Naito K et al (2010) The immunological and chemical detection of N-(hexanoyl)phosphatidylethanolamine and N-(hexanoyl)phosphatidylserine in an oxidative model induced by carbon tetrachloride. Biochem Biophys Res Commun 393:631–636

Huber J, Vales A, Mitulovic G et al (2002) Oxidized membrane vesicles and blebs from apoptotic cells contain biologically active oxidized phospholipids that induce monocyte-endothelial interactions. Arterioscler Thromb Vasc Biol 22:101–107

Inouye M, Mio T, Sumino K (1999) Formation of 9-hydroxy linoleic acid as a product of phospholipid peroxidation in diabetic erythrocyte membranes. Biochim Biophys Acta 1438:204–212

Kato Y, Mori Y, Makino Y et al (1999) Formation of N^{ε}-(hexanonyl)lysine in protein exposed to lipid hydroperoxide. A plausible marker for lipid hydroperoxide-derived protein modification. J Biol Chem 274:20406–20414

Kato Y, Miyake Y, Yamamoto K et al (2000) Preparation of N^{ε}-(hexanonyl)lysine: application to the evaluation of protective effects of flavonoid supplementation against exercise-induced oxidative stress in rat skeletal muscle. Biochem Biophys Res Commun 274:389–393

Kawai Y, Fujii H, Okada M et al (2006) Formation of Nepsilon-(succinyl)lysine in vivo: a novel marker for docosahexaenoic acid-derived protein modification. J Lipid Res 47:1386–1398

Kim EH, Sevanian A (1991) Hematin- and peroxide-catalyzed peroxidation of phospholipid liposomes. Arch Biochem Biophys 288:324–330

Kunjathoor VV, Febbraio M, Podrez EA et al (2002) Scavenger receptors class A-I/II and CD36 are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages. J Biol Chem 277:49982–499878

Lang F, Gulbins E, Lang PA et al (2010) Ceramide in suicidal death of erythrocyte. Cell Physiol Biochem 26:21–28

Leitinger N (2008) The role of phospholipid oxidation products in inflammatory and autoimmune diseases: evidence from animal models and in humans. Subcell Biochem 49:325–350

Li Z, Aqellon LB, Vance DE (2005) Phosphatidylcholine homeostasis and liver failure. J Biol Chem 280:37798–37802

Li Z, Aquellon LB, Allen TM et al (2006) The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and steatohepatitis. Cell Metab 3:321–331

Lin HL, Xu XS, Lu HX et al (2008) Pathological mechanisms of erythrocyte-induced vulnerability of atherosclerotic plaques. Med Hypotheses 70:105–108

Linderkamp O, Friederichs E, Boehler T et al (1993) Age dependency of red blood cell deformability and density: studies in transient erythroblastopenia of childhood. Br J Haematol 83:125–129

Mclaughlin S, Murray D (2005) Plasma membrane phosphoinositide organization by protein electrostatics. Nature 438:605–611

Rachmilewitz EA (1974) Denaturation of the normal and abnormal hemoglobin molecule. Semin Hematol 11:441–462

Ravandi A, Babaei S, Leung R et al (2004) Phospholipids and oxophospholipids in atherosclerotic plaques at different stages of plaque development. Lipids 39:97–109

Ross R (1999) Atherosclerosis-an inflammatory disease. N Engl J Med 340:115–126

Rouser G, Yamamoto A, Kritchevsky G (1971) Cellular membranes. Structure and regulation of lipid class composition species differences, changes with age, and variations in some pathological states. Arch Intern Med 127:1105–1121

Sadrzadeh SM, Graf E, Panter SS et al (1984) Hemoglobin. A biologic Fenton reagent. J Biol Chem 259:14354–14356

Stadtman ER (1992) Protein oxidation and aging. Science 257:1220–1224

Suzuki H, Kurihara Y, Takeya M et al (1997) A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. Nature 386:292–296

Tsimikas S, Kiechl S, Willeit J et al (2006) Oxidized phospholipids predict the presence and progression of carotid and femoralatherosclerosis and symptomatic cardiovascular disease: five-year prospective results from the Bruneck study. J Am Coll Cardiol 47:2219–2228

Tuji K, Kawai Y, Kato Y et al (2003) Formation of *N*-(hexanoyl)ethanolamine, a novel phosphatidylethanolamine adduct, during the oxidation of erythrocyte membrane and low-density lipoprotein. Biochem Biophys Res Commun 306:706–711

Vance JE, Steenbergen R (2005) Metabolism and functions of phosphatidylserine. Prog Lipid Res 44:207–234

Yeung T, Gilbert GE, Shi J et al (2008) Membrane phosphatidylserine regulates surface charge and protein localization. Science 319:210–213

Chapter 4
Amide-Type Adduct of Dopamine – Plausible Cause of Parkinson Diseases

Xuebo Liu, Naruomi Yamada, and Toshihiko Osawa

Abstract Dopamine is the endogenous neurotransmitter produced by nigral neurons. Dopamine loss can trigger not only prominent secondary morphological changes, but also changes in the density and sensitivity of dopamine receptors; therefore, it is a sign of PD development. The reasons for dopamine loss are attributed to dopamine's molecular instability due to it is a member of catecholamine family, whose catechol structure contributes to high oxidative stress through enzymatic and non-enzymatic oxidation. Oxidative stress in the brain easily leads to the lipid peroxidation reaction due to a high concentration of polyunsaturated fatty acids (PUFA), such as docosahexaenoic acid (DHA, C22:6/ω-3) and arachidonic acid (AA, C18:4/ω-6). Recent studies have shown that lipid hydroperoxides, the primary peroxidative products, could non-specifically react with primary amino groups to form N-acyl-type (amide-linkage) adducts. Therefore, based on the NH_2-teminals in dopamine's structure, the aims of this chapter are to describes the possibility that reactive LOOH species derived from DHA/AA lipid peroxidation may modify dopamine to form amide-linkage dopamine adducts, which might be related to etiology of Parkinson's diseases.

Keywords Docosahexaenoic acid • Arachidonic acid • Lipid hydroperoxide • Dopamine • Amide-type adduct • Parkinson's disease

X. Liu (✉)
The Laboratory of Food and Biodynamics, Graduate School of Bioagricultural Science, Nagoya University, Nagoya 464-8601, Japan

College of Food Science and Engineering, Northwest A&F University, Yangling, China
e-mail: xueboliu@nwsuaf.edu.cn

N. Yamada • T. Osawa
The Laboratory of Food and Biodynamics, Graduate School of Bioagricultural Science, Nagoya University, Nagoya 464-8601, Japan

Y. Kato (ed.), *Lipid Hydroperoxide-Derived Modification of Biomolecules*, Subcellular Biochemistry 77, DOI 10.1007/978-94-007-7920-4_4,

1 Backgrounds

Parkinson's disease (PD) is a neurodegenerative disorder characterized by a dramatic loss of dopaminergic neurons in the substantia nigra, and the subsequent deficiency of dopamine in the brain areas (Galvan and Wichmann 2008). Until now, very little is known about why and how the PD neurodegenerative process begins and progresses; however, an increasing body of evidence suggests that oxidative stress, mitochondrial dysfunction, and impairment of the ubiquitin-proteasome system may be involved in the pathogenesis of PD (Leroy et al. 1998; Schapira 2001; Balaban et al. 2005). Recent studies indicate that there are high levels of basal oxidative stress in the substantia nigra pars compacta (SNc) in the normal brain and this is increased in PD (Jenner 2003).

Oxidative stress in the brain easily leads to the lipid peroxidation reaction due to a high concentration of polyunsaturated fatty acids (PUFA), such as docosahexaenoic acid (DHA, C22:6/ω-3) and arachidonic acid (AA, C18:4/ω-6), which are present in the brain (Porter et al. 1995). The polyunsaturated fatty acids are located almost exclusively in the SN2-position of the phosphoglycerides found in the neural cell membranes. The beneficial physiological effects of DHA and AA have been frequently reported (Simopoulos 1999; Hadders-Algra 2008); however, the fatty acids are highly unsaturated, thus making them particularly susceptible to peroxidation. During the lipid peroxidation reaction, lipid hydroperoxides are generated as primary products. Subsequent decomposition leads to the formation of reactive mediates including aldehydes, which can covalently modify biomolecules. We have recently found that lipid hydroperoxides, the primary peroxidative products, can universally react with primary amino groups to form N-acyl-type (amide-linkage) adducts (Kato et al. 1997, 1999; Kato and Osawa 1998; Kawai et al. 2003, 2004, 2006). In our previous studies, the formation of linoleic acid-derived lysine modification adducts, N-(hexanoyl) lysine and N-(azelayl) lysine, and DHA-derived adducts, N-(succinyl) lysine and N-(propanoyl) lysine, have been identified in vitro or in vivo by LC-MS/MS or immunochemical analysis. In addition, the formation of N-(hexanoyl) lysine also was detected, as well as N-(glutaryl) lysine, during the reaction of oxidized arachidonic acid (AA) with the lysine residue. The N-acyl-type adducts are specific to the peroxidation of polyunsaturated fatty acids, therefore, their formations are the useful markers for the lipid peroxidation, protein modification and related dysfunction that occur in these fatty acids enriched tissues.

Dopamine is the endogenous neurotransmitter produced in nigral neurons. Dopaminergic neuronal loss can trigger not only prominent secondary morphological changes, such as density reduction of the dendritic spines, but also changes in the density and sensitivity of dopamine receptors (Galvan and Wichmann 2008); therefore, it is a sign of PD development. The reasons for dopamine loss are attributed to molecular instability of dopamine. Some possible causes of dopamine are characteristics of dopaminergic neurons (Bove et al. 2005), such as dopamine degradation by monoamine oxidase A (MAO-A) (Gotz et al. 1994) or auto-oxidation (Hald and Lotharius 2005) and the reaction with amino acid cysteine (LaVoie and Hastings 1999).

Dopamine is a member of catecholamine family. The catechol structure of dopamine contributes to vulnerability to oxidative stress. Additionally, the NH_2-teminals in dopamine's structure may represent another reactive spot, however, little experimental evidence have been proven. Based on our previously described reaction between lipid hydroperoxides and NH_2 residues, the possibility that reactive LOOH species derived from lipid peroxidation may modify dopamine to form amide-linkage dopamine adducts was investigated.

2 Chemical Formation of DHA- and AA-Derived Dopamine Adducts

Lipid hydroperoxides, the primary products of lipid peroxidation, could non-specifically react with primary amino groups to form N-acyl-type (amide-linkage) adducts, and also within the chemical structure of dopamine, an amino residue is present. The DHA and AA-derived four amide-linkage dopamine adducts, succinyl dopamine (SUD) and propanoyl dopamine (PRD), hexanoyl dopamine (HED) and glutaroyl dopamine (GLD), were chemically synthesized respectively. The chemical structures of the authentic adducts were identified by NMR. The formation of these dopamine adducts was further confirmed by HPLC-MS/MS analysis. Collision-induced dissociation (CID) of the authentic adducts, SUD (m/z 254), PRD (m/z 210), HED (m/z 252) and GLD (m/z 268), produced the same daughter ions at m/z 91, and 137. SUD, PRD and HED also produced daughter ions at m/z 154, whereas GLD did not. The ion at m/z 137 was detected with the highest peak intensity in the fragments, and this ion was also identified to be derived from the dopamine spectra (Fig. 4.1).

3 In Vitro and In Vivo Detection of Dopamine Adducts

In our previous studies, to determinate the in vitro formation of the dopamine adducts, the reaction of dopamine with DHA- or AA-hydroperoxides were carried out. The results showed that the four adducts including SUD, PRD, HED and GLD were successfully detected by HPLC-MS/MS among the reaction mixtures.

It has been reported that polyunsaturated fatty acids such as DHA and AA are significantly enriched in the brain (Tapiero et al. 2002), and that there are high levels of basal oxidative stress in the normal brain, which increases with aging (Lin and Flint 2006). To investigate whether the DHA- and AA- derived dopamine adducts can be formed in vivo, the brains of 7-week- and 27-week-old male F344/NSIc rats were removed and the homogenates were used. The detection of the dopamine adducts in the homogenates was carried out by HPLC-MS/MS. The whole adducts were detected in the 7- and 27-week rat brains in both the positive

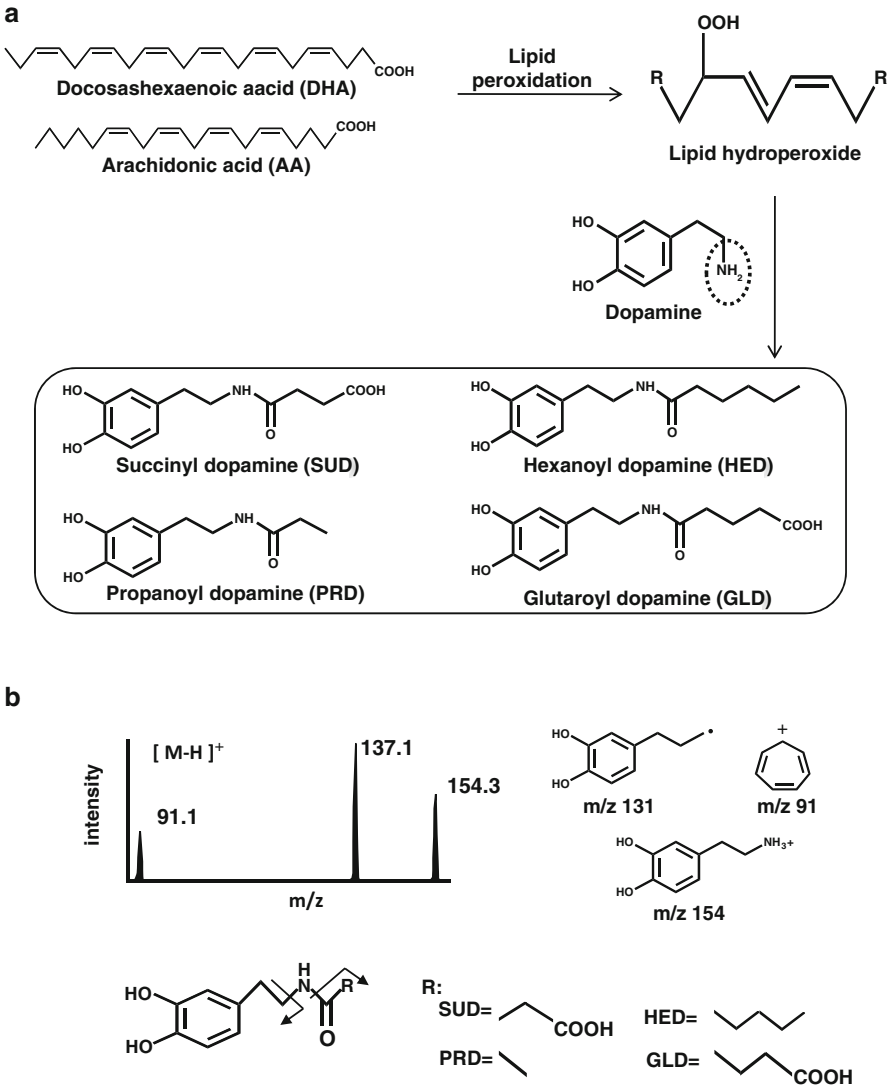


Fig. 4.1 Proposed chemical formation scheme and HPLC-MS/MS analysis of DHA- and AA-derived dopamine adducts. (**a**) Proposed reaction scheme of DHA- and AA-derived dopamine adduct formation. (**b**) The $[MH]^+$ ion m/z 254, 210, 252, and 268 of SUD, PRD, HED, and GLD, respectively, were subjected to CID, and the daughter ions were scanned (*left*, *upper*). The proposed structures of individual ions are shown (*right*, *upper*). The chemical structure composition of the dopamine adducts is proposed by fragmental analysis (*lower*)

ion mode and negative ion mode of LC-MS/MS. The level of HED and PRD, which are derived from the CH_3-teminous of AA and DHA, were more preferentially formed than that of SUD and PRD; however, no significant difference of adduct level was found between the 7- week and 27-week rats.

Dopamine is a natural neurotransmitter in the brain, and its deficiency is a sign of Parkinson's disease (Ang 2006). Although the mechanism of neurodegeneration is not fully understood, some considerations include dopaminergic neuron abnormalities, dopamine degradation by monoamine oxidase A (MAO-A) or auto-oxidation and modification (Gotz et al. 1994; Hald and Lotharius 2005; LaVoie and Hastings 1999). The in vitro and in vivo detections of DHA- and AA-derived dopamine adducts established may indicate an additional clue to the causes of dopamine deficiency in PD. Although the level of the dopamine adducts was not obviously increased in the 27-week-old rat brain compared to the 7-week-old rat brain, 27 weeks represents only middle age for a rat and the level of basal oxidative stress is increased with age (Navarro and Boveris 2007; Forster et al. 1996; Boveris and Navarro 2008); therefore, further study should confirm these adduct formations in the brain using aging model rats such as 1 year age and more and also PD model animals.

4 Identification of HED as a Potent Inducer of Neuronal Apoptosis

In recent years, several dopamine oxidants and dopamine-modified adducts have been reported, such as neuromelanin (Wakamatsu et al. 2003), aminochrome (Graumann et al. 2002), 6-OHDA (Saner and Thoenen 1971) and 5-S-CDA (LaVoie and Hastings 1999), among them 6-OHDA has been generally known as a potent neurotoxin (Pezzella et al. 1997; Izumi et al. 2005; Maharaj et al. 2005). To test that some of these DHA- and AA-derived dopamine adducts could cause neuronal cell death. The effect of these dopamine adducts on the cell viability in SH-SY5Y cells was studied. The results showed that among the tested dopamine adducts, HED and PRD induced about 80 and 30 % of the cell death, respectively. On the other hand, SUD and GLD had almost no influence on the cell viability, suggesting the death of SH-SY5Y cells was induced only by the CH3-terminus-derived adducts, and not by the COOH-terminus-derived adducts. Of interest, two HED analogs, nonanoyl dopamine (NOD) and lauroyl dopamine (LAD), which were synthesized and characterized by more carbons than HED in the methyl terminus, also showed a significant toxicity to SH-SY5Y cells, suggesting that the number of carbon in the CH3-terminus-derived dopamine adducts might be associated with the adduct-induced cell death.

HED was a potent inducer of SH-SY5Y cell death compared to SUD, PRD and GLD. Because apoptosis is suggested to be involved in neurodegeneration, we then characterized whether HED-induced cell death in SH-SY5Y cells is apoptosis or not. The exposure to HED induces to a dose-dependent decrease in the viable cells. Moreover, the fragmented nuclei were found in cells exhibiting the typical morphological features of apoptosis. In addition, the gel electrophoresis of DNA from the SH-SY5Y cells exposed to HED also displayed nucleosomal DNA fragmentation. HED treatment also led to the time- and dose- dependent cleavage of PARP resulting in the accumulation of the 85-kDa fragment and decreasing in the 116-kDa protein, as

well as in the accumulation of the active caspase-3, both which are hallmarks of apoptosis. Moreover, the pretreatment with the caspase-3 inhibitor significantly prevented SH-SY5Y cells from HED-induced DNA fragmentation, providing further evidence that HED induced a caspase-3-mediated apoptotic cell death.

Dopamine-derived metabolites have been reported to inflict damage on neuronal cells (Asanuma et al. 2003). For example, 6-hydroxydopamine (6-OHDA), a hydroxylated analogue of dopamine, has been demonstrated to induce apoptosis in several neuronal cell lines (Hanrott et al. 2006; Chalovich et al. 2006; Jia et al. 2008; Lee et al. 2008). In addition, dopamine autoxidation generating dopamine quinone can react with protein sulfhydryl groups leading to structural modifications of proteins and reduced levels of glutathione (GSH) (Berman and Hastings 1999). HED, an AA-derived dopamine adduct, caused significant cell death in SH-SY5Y cells. Furthermore, the events including DNA fragmentation, chromatin condensation, PARP cleavage and accumulation of active caspase-3 suggest that HED-induced cell death was apoptosis.

5 Regulation of HED-Induced Apoptosis in SH-SY5Y Cells

What might be the signaling mechanism underlying the HED-induced apoptosis is our interests. It is well accepted that reactive oxygen species (ROS) generation is a key contributor to neuronal apoptosis induced by neurotoxin compounds (Chinopoulos and Adam-Vizi 2006). Hence, experiments were first carried out to assess the ROS generation induced by the HED treatment and the possibility that the HED-induced apoptosis is mediated via ROS generation in SH-SY5Y cells. HED led to increased ROS generation in the cells compared to the DMSO-treated cells, whereas the other three dopamine adducts, SUD, PRD and GLD, had a much less effect on the cells. Furthermore, a dose-dependent increase in the ROS generation was found by dichlorofluorescein (DCF) fluorescence staining. The pretreatment with NAC, a potent antioxidant, clearly inhibited the PARP cleavage, indicating that the ROS generation might be critically involved in the HED-induced apoptosis. It is widely accepted that mitochondrial dysfunction may play very important roles in neuronal cell death (Kluck et al. 1997). The cytochrome c release from mitochondria was found in HED-treated cells.

The precise mechanisms regulating apoptotic events in neuronal cells remain largely unclear; however, high levels of ROS generation and the increases in the mitochondrial permeability appear to be common occurrences in many forms of apoptotic neuronal cell death. The finding that HED induced a significant ROS generation and that NAC pretreatment clearly blocked the apoptosis suggests that ROS generation is an essential trigger for HED-induced apoptosis in the SH-SY5Y cells. The source of ROS generation has not been identified, however, the catechol ring is kept in the structure of HED like dopamine and 6-OHDA, therefore, the catechol oxidation might be one of the important causes for the ROS generation in the HED-treated SH-SY5Y cells. The regulation of neuronal apoptosis is generally

characterized by the several signaling mediators such as p-53, Bcl-2 family proteins and cytochrome c release (Gorman et al. 2000). A significant release of cytochrome c from mitochondrial fraction in HED-treated SH-SY5Y cells was found, suggesting that the apoptosis may be critically mediated via a mitochondrial abnormality.

6 Monoamine Transporters Are Important in HED-Induced Apoptosis and ROS Generation

Monoamine transporters including the dopamine transporter (DAT), norepinephrine transporter (NET) and 5-HT transporter (5-HTT), which are of fundamental importance for proper signaling between neurons, have been reported to associate with experimental neurotoxins-induced toxicity (Kita et al. 2003). HED possesses a dopamine-based chemical structure, suggesting that the above-described HED cytotoxicity that occurred in the SH-SY5Y cells might be mediated by uptake of HED by monoamine transporters. The pretreatment with both GBR12909 and Imipramine, the inhibitors of DAT and NET/5-HHT, respectively, clearly inhibited the occurrence of the HED-induced PARP cleavage and active caspase-3 expression in the SH-SY5Y cells. Furthermore, the ROS generation by HED was also found to be suppressed in these two inhibitor-pretreated cells. The result that both monoamine transporter inhibitors showed markedly inhibitive effect on the HED-induced apoptosis and ROS generation suggested that HED might be primarily transported into the SH-SY5Y cells by the monoamine transporters, and inflicted damage on the cells.

To characterize whether the HED-induced cytotoxicity is specific to neuronal cells, our study investigated the effect of HED on apoptotic cell death and ROS generation in mouse embryonic fibroblast NIH-3T3 cells in comparison to that of the SH-SY5Y cells. A dose-dependent analysis revealed that HED led to no apoptotic cell death in the NIH-3T3 cells estimated by Hoechst 33258 and Propidium Iodide (PI) nuclear staining. A further quantitative analysis of the apoptotic cells by flow cytometry also indicated apoptosis in SH-SY5Y cells, whereas not in the NIH-3T3 cells. Moreover, no ROS generation was found in the HED-treated NIH-3T3 cells; on the other hand, the HED analogs, NOD and LAD, also induced only a slight ROS generation in the NIH-3T3 cells. Monoamine transporter is known to be absent in NIH-3T3 cells, which may indicate that the HED-induced cytotoxicity might be specific to neuronal cells.

Monoamine transporters are of fundamental importance for proper signaling between neurons. Plasma membrane transporters, the major subclass of intracellular transporters (Gethe et al. 2006), include the dopamine transporter (DAT), norepinephrine transporter (NET), and 5-HT transporter (5-HTT). In this study, pretreatment with inhibitors of DAT, NET and 5-HTT significantly suppressed ROS generation and apoptosis events induced by HED. In the case of 6-OHDA, similar to HED, a high affinity for several catecholaminergic plasma membrane transporters, such as DAT and NET, is also essential for its entrance into the

neuronal cells to inflict damage. The dependence of monamine transporter is considered to be due to a structural similarity between the HED, dopamine and norepinephrine. The necessity of the monoamine transporter in HED-induced cytotoxicity was further demonstrated by the result that HED could not induce apoptotic cell death and ROS generation in the monoamine transporter-absent NIH-3T3 cells, which also indicates that HED may selectively induce cytotoxicity in different cell lines.

7 Conclusion and Note

Four amide-linkage adducts of dopamine with DHA and AA were synthesized and the *in vivo* formation during the reaction of lipid hydroperoxides with dopamine were also revealed. HED, an AA-derived dopamine adduct, as a potent neurotoxin based on the significant induction of ROS generation and apoptosis in human neuroblastoma SH-SY5Y cells. The mechanism of HED-induced apoptosis has not been fully established in this study; however, it seems to be mediated by ROS generation, mitochondrial abnormalities, and monoamine transporter (Fig. 4.2).

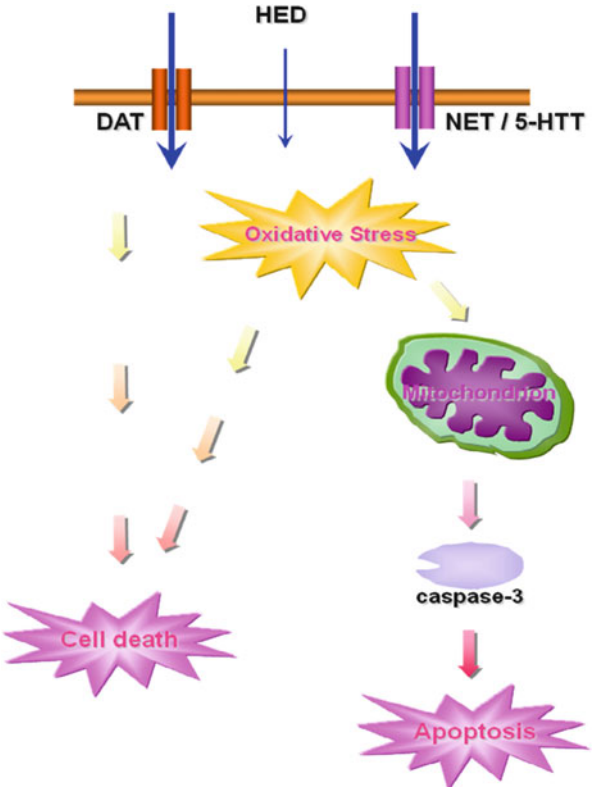


Fig. 4.2 Proposed mechanism of HED-induced apoptosis in SH-SY5Y cells

In fact, either DHA or AA is located almost exclusively in the SN2-position of phosphoglycerides found in the neural cell membranes (Ma et al. 2007; Beermann et al. 2005); however, free fatty acid levels are reported to increase with aging due to an increasing degradation by PLA2 (Rosenberger et al. 2004; Qu et al. 2003; Rapoport 1999), a phospholipase A2, which selectively acts on phosphoglycerides (Diez et al. 1994). DHA is the most enriched polyunsaturated fatty acid in the brain, and it has been implicated that DHA concentration is decreased in AD brain (Bazan et al. 2002); hence, the DHA-derived dopamine adducts formed in this study may be useful biomarkers for not only PD but also AD.

References

Ang SL (2006) Transcriptional control of midbrain dopaminergic neuron development. Development 133:3499–3506

Asanuma M, Miyazaki I, Ogawa N (2003) Dopamine- or L-DOPA-induced Neurotoxicity: the role of dopamine quinone formation and tyrosinase in a model of Parkinson's disease. Neurotox Res 5:165–176

Balaban RS, Nemoto S, Finkel T (2005) Mitochondria, oxidants, and aging. Cell 120:483–495

Bazan NG, Palacios-Pelaez R, Lukiw WJ (2002) Hypoxia signaling to genes: significance in Alzheimer's disease. Mol Neurobiol 26:283–298

Beermann C, Möbius M, Winterling N, Schmitt JJ, Boehm G (2005) sn-position determination of phospholipid-linked fatty acids derived from erythrocytes by liquid chromatography electrospray ionization ion-trap mass spectrometry. Lipids 40:211–218

Berman SB, Hastings TG (1999) Dopamine oxidation alters mitochondrial respiration and induces permeability transition in brain mitochondria: implications for Parkinson's disease. J Neurochem 73:1127–1137

Bove J, Prou D, Perier C, Przedborski S (2005) Toxin-induced models of Parkinson's disease. NeuroRx 2:484–494

Boveris A, Navarro A (2008) Brain mitochondrial dysfunction in aging. IMUBM Life 60:308–314

Buhmann C, Arlt S, Kontush A, Moeller-Bertram T, Sperber S, Oechsner M, Stuerenburg HJ, Beisiegel U (2004) Plasma and CSF markers of oxidative stress are increased in Parkinson's disease and influenced by antiparkinsonian medication. Neurobiol Dis 15:160–170

Chalovich EM, Zhu JH, Caltagarone J, Bowser R, Chu CT (2006) Functional repression of cAMP response element in 6-hydroxydopamine-treated neuronal cells. J Biol Chem 281:17870–17881

Chinopoulos C, Adam-Vizi V (2006) Calcium, mitochondria and oxidative stress in neuronal pathology. Novel aspects of an enduring theme. FEBS J 273:433–450

Dexter DT, Carter CJ, Wells FR, Javoy-Agid F, Agid Y, Lees A, Jenner P, Marsden CD (1989) Basal lipid peroxidation in substantia nigra is increased in Parkinson's disease. J Neurochem 52:381–389

Dexter DT, Holley AE, Filtter WD, Slater TF, Wells FR, Daniel SE, Lees A, Jenner P, Marsden CD (1994) Increased levels of lipid hydroperoxides in the parkinsonian substantia nigra: an HPLC and ESR study. Mov Disord 9:92–97

Diez E, Chilton FH, Stroup G, Mayer RJ, Winkler JD, Fonteh AN (1994) Fatty acid and phospholipid selectivity of different phospholipase A2 enzymes studied by using a mammalian membrane as substrate. Biochem J 301:721–726

Fahn S, Cohen G (1992) The oxidant stress hypothesis in Parkinson's disease: evidence supporting it. Ann Neurol 32:804–812

Forster MJ, Dubey A, Dawson KM, Stutts WA, Lal H, Sohal RS (1996) Age-related losses of cognitive function and motor skills in mice are associated with oxidative protein damage in the brain. Proc Natl Acad Sci USA 93:4765–4769

Galvan A, Wichmann T (2008) Pathophysiology of parkinsonism. Clin Neurophysiol 119:1459–1474

Gethe U, Andersen PH, Larsson OM, Schousboe A (2006) Neurotransmitter transporters: molecular function of important drug targets. Trends Pharmacol Sci 27:375–383

Gorman AM, Ceccatelli S, Orrenius S (2000) Role of mitochondria in neuronal apoptosis. Dev Neurosci 22:348–358

Gotz ME, Kunig G, Riederer P, Youdim MB (1994) Oxidative stress: free radical production in neural degeneration. Pharmacol Ther 63:37–122

Graumann R, Paris I, Martinez-Alvarado P, Rumanque P, Perez-Pastene C, Cardenas SP, Marin P, Diaz-Grez F, Caviedes R, Caviedes P, Segura-Aguilar J (2002) Oxidation of dopamine to aminochrome as a mechanism for neurodegeneration of dopaminergic systems in Parkinson's disease. Possible neuroprotective role of DT-diaphorase. Pol J Pharmacol 54:573–579

Hadders-Algra M (2008) Prenatal long-chain polyunsaturated fatty acid status: the importance of a balanced intake of docosahexaenoic acid and arachidonic acid. J Perinat Med 36:101–109

Hald A, Lotharius J (2005) Oxidative stress and inflammation in Parkinson's disease: is there a causal link? Exp Neurol 176:154–162

Hanrott K, Gudmunsen L, O'Neill MJ, Wonnacott S (2006) 6-hydroxydopamine-induced apoptosis is mediated via extracellular auto-oxidation and caspase 3-dependent activation of protein kinase Cdelta. J Biol Chem 281:5373–5382

Izumi Y, Sawada H, Sakka N, Yamamoto N, Kume T, Katsuki H, Shimohama S, Akaike A (2005) p-Quinone mediates 6-hydroxydopamine-induced dopaminergic neuronal death and ferrous iron accelerates the conversion of p-quinone into melanin extracellularly. J Neurosci Res 79:849–860

Jenner P (2003) Oxidative stress in Parkinson's disease. Ann Neurol 53:S26–S38

Jia Z, Zhu H, Misra HP, Li Y (2008) Potent induction of total cellular GSH and NQO1 as well as mitochondrial GSH by 3H-1,2-dithiole-3-thione in SH-SY5Y neuroblastoma cells and primary human neurons: protection against neurocytotoxicity elicited by dopamine, 6-hydroxydopamine, 4-hydroxy-2-nonenal, or hydrogen peroxide. Brain Res 1197:159–169

Kato Y, Makino Y, Osawa T (1997) Formation of N ε-(hexanonyl) lysine in protein exposed to lipid hydroperoxide A plausible marker for lipid hydroperoxide-derived protein modification. J Lipid Res 38:1334–1346

Kato Y, Mori Y, Makino Y, Morimitsu Y, Hiroi S, Ishikawa T, Osawa T (1999) Formation of N ε-(hexanonyl) lysine in protein exposed to lipid hydroperoxide A plausible marker for lipid hydroperoxide-derived protein modification. J Biol Chem 274:20406–20414

Kato Y, Osawa T (1998) Detection of oxidized phospholipid-protein adducts using anti-15-hydroperoxyeicosatetraenoic acid-modified protein antibody: contribution of esterified fatty acid-protein adduct to oxidative modification of LDL. Arch Biochem Biophys 351:106–114

Kawai Y, Fujii H, Kato Y, Kodama M, Naito N, Uchida K, Osawa T (2004) Esterified lipid hydroperoxide-derived modification of protein: formation of a carboxyalkylamide-type lysine adduct in human atherosclerotic lesions. Biochem Biophys Res Commun 313:271–276

Kawai Y, Fujii H, Okada M, Tsuchie Y, Uchida K, Osawa T (2006) Formation of Nepsilon-(succinyl)lysine in vivo: a novel marker for docosahexaenoic acid-derived protein modification. J Lipid Res 47:1386–1398

Kawai Y, Kato Y, Fujii H, Mkino Y, Mori Y, Naito N, Osawa T (2003) Immunochemical detection of a novel lysine adduct using an antibody to linoleic acid hydroperoxide-modified protein. J Lipid Res 44:1124–1131

Kita T, Wagner GC, Nakashima T (2003) Current research on methamphetamine-induced neurotoxicity: animal models of monoamine disruption. J Pharmacol Sci 92:178–195

Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275:1132–1136

LaVoie MJ, Hastings TG (1999) Peroxynitrite-and nitrite-induced oxidation of dopamine: implications for nitric oxide in dopaminergic cell loss. J Neurochem 73:2546–2554

Lee YM, Park SH, Shin DI, Hwang JY, Park B, Park YJ, Lee TH, Chae HZ, Jin BK, Oh TH, Oh YJ (2008) Oxidative modification of peroxiredoxin is associated with drug-induced apoptotic signaling in experimental models of Parkinson disease. J Biol Chem 283:9986–9998

Leroy E, Boyer R, Auburger G, Leube B, Ulm G, Mezey E, Harta G, Brownstein MJ, Jonnalagada S, Chernova T, Dehejia A, Lavedan C, Gasser T, Steinbach PJ, Wilkinson KD, Polymeropoulos MH (1998) The ubiquitin pathway in Parkinson's disease. Nature 395:451–452

Lin MT, Flint BM (2006) Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature 433:787–795

Liu XB, Shibata T, Hisaka S, Osawa T (2008) DHA hydroperoxides as a potential inducer of neuronal cell death: a mitochondrial dysfunction-mediated pathway. J Clin Biochem Nutr 43:26–33

Long EK, Murphy TC, Leiphon LJ, Watt J, Morrow JD, Milne GL, Howard JR, Picklo MJ Sr (2008) Trans-4-hydroxy-2-hexenal is a neurotoxic product of docosahexaenoic (22:6; n-3) acid oxidation. J Neurochem 105:714–724

Ma QL, Teter B, Ubeda OJ, Morihara T, Dhoot D, Nyby MD, Tuck ML, Frautschy SA, Cole GM (2007) Omega-3 fatty acid docosahexaenoic acid increases SorLA/LR11, a sorting protein with reduced expression in sporadic Alzheimer's disease (AD): relevance to AD prevention. J Neurosci 27:14299–14307

Maharaj H, Mahara DS, Scheepers M, Mokokong R, Daya S (2005) l-DOPA administration enhances 6-hydroxydopamine generation. Brain Res 1063:180–186

Mariani E, Polidori MC, Cherubini A, Mecocci P (2005) Oxidative stress in brain aging, neurodegenerative and vascular diseases: an overview. J Chromatogr B 827:65–75

Maruyama W, Naoi M (2002) Cell death in Parkinson's disease. J Neurol 249(Suppl 2):6–10

Montine KS, Quinn JF, Zhang J, Fessel JP, Roberts LJ 2nd, Morrow JD, Montine TJ (2004) Isoprostanes and related products of lipid peroxidation in neurodegenerative diseases. Chem Phys Lipids 128:117–124

Navarro A, Boveris A (2007) The mitochondrial energy transduction system and the aging process. Am J Physiol Cell Physiol 292:C670–C686

Pezzella A, d'Ischia M, Napolitano A, Misuraca G, Prota G (1997) Iron-mediated generation of the neurotoxin 6-hydroxydopamine quinone by reaction of fatty acid hydroperoxides with dopamine: a possible contributory mechanism for neuronal degeneration in Parkinson's disease. J Med Chem 40:2211–2216

Porter NA, Caldwell SE, Mills KA (1995) Mechanisms of free radical oxidation of unsaturated lipids. Lipids 30:277–290

Qu Y, Chang L, Klaff J, Seeman R, Balbo A, Rapoport SI (2003) Imaging of brain serotonergic neurotransmission involving phospholipase A2 activation and arachidonic acid release in unanesthetized rats. Brain Res Brain Res Protoc 12:16–25

Rapoport SI (1999) In vivo fatty acid incorporation into brain phospholipids in relation to signal transduction and membrane remodeling. Neurochem Res 24:1403–1415

Rosenberger TA, Villacreses NE, Hovda JT, Bosetti F, Weerasinghe G, Wine RN, Harry GJ, Rapoport SI (2004) Rat brain arachidonic acid metabolism is increased by a 6-day intracerebral ventricular infusion of bacterial lipopolysaccharide. J Neurochem 88:1168–1178

Saner A, Thoenen H (1970) Model experiments on the molecular mechanism of action of 6-hydroxydopamine. Mol Pharmacol 7:147–154

Schapira AH (2001) Causes of neuronal death in Parkinson's disease. Adv Neurol 86:155–162

Shamoto-Nagai M, Maruyama W, Kato Y, Isobe K, Tanaka M, Naoi M, Osawa T (2003) An inhibitor of mitochondrial complex I, rotenone, inactivates proteasome by oxidative modification and induces aggregation of oxidized proteins in SH-SY5Y cells. J Neurosci Res 74:589–597

Shibata T, Iio K, Kawai Y, Shibata Y, Kawaguchi M, Toi S, Kobayashi M, Kobayashi M, Yamamoto K, Uchida K (2006) Identification of a lipid peroxidation product as a potential trigger of the p53 pathway. J Biol Chem 281:1196–1204

Siegel SJ, Bieschke J, Powers ET, Kelly JW (2007) The oxidative stress metabolite 4-hydroxynonenal promotes Alzheimer protofibril formation. Biochemistry 46:1503–1510

Simopoulos AP (1999) Essential fatty acids in health and chronic disease. Am J Clin Nutr 70 (Suppl):560–569

Tapiero H, Ba GN, Couvreur P, Tew KD (2002) Polyunsaturated fatty acids (PUFA) and eicosanoids in human health and pathologies. Biomed Pharmacother 56:215–222

Wakamatsu K, Fujikawa K, Zucca FA, Zecca L, Ito S (2003) The structure of neuromelanin as studied by chemical degradative methods. J Neurochem 86:1015–1023

Yoritaka A, Hattori N, Uchida K, Tanaka M, Stadtman ER, Mizuno Y (1996) Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. Proc Natl Acad Sci USA 93:2696–2701

Zecca L, Youdim MBH, Riederer P, Connor JR, Crichton RR (2004) Iron, brain ageing and neurodegenerative disorders. Nat Rev Neurosci 5:863–873

Chapter 5
Determination of HEL (Hexanoyl-Lysine Adduct): A Novel Biomarker for Omega-6 PUFA Oxidation

Kazuo Sakai, Satoko Kino, Aino Masuda, Masao Takeuchi, Tairin Ochi, Josko Osredkar, Barbara Rejc, Ksenija Gersak, Narasimhan Ramarathnam, and Yoji Kato

Abstract Published evidences indicate that reactive oxygen species (ROS) can induce lipid peroxidation, which plays important role in the pathophysiology of numerous diseases including atherosclerosis, diabetes, cancer and aging process. Monitoring of oxidative modification or oxidative damages of biomolecules may therefore be essential for the understanding of aging, and age-related diseases. N-epsilon-Hexanoyl-lysine (HEL) is a novel lipid peroxidation biomarker which is derived from the oxidation of omega-6 unsaturated fatty acid. In this chapter, development of HEL ELISA and its applications are reported. Assay range of HEL ELISA was 2–700 nmol/L, and showed good linearity and reproducibility. Accuracy of this assay was validated by recovery test and absorption test. HEL concentration in human urine was 22.9 ± 15.4 nmol/L and it was suggested that HEL exists as low molecular substances, in a free or in the peptide-attached form. In contrast with the urine sample, serum HEL was suggested to exist in the protein-attached form, and hydrolysis by protease might be essential for the accurate measurement of HEL in protein containing samples such as serum and cultured cells. By sample pretreatment with proteases, HEL was successfully detected in oxidized LDL, oxidized serum, and rat serum. In conclusion, HEL ELISA can be applied to measure urine, serum, and other biological samples independent of the animal species, and may be useful for the assessment of omega-6 PUFA oxidation in the living bodies.

K. Sakai (✉) • S. Kino • A. Masuda • M. Takeuchi • T. Ochi
Japan Institute for the Control of Aging (JaICA), Nikken SEIL Co., Ltd., 710-1 Haruoka, Fukuroi-shi, Shizuoka 437-0122, Japan
e-mail: biotech@jaica.com

J. Osredkar • B. Rejc • K. Gersak
University Medical Centre Ljubljana, Zaloska cesta 2, 1000 Ljubljana, Slovenija

N. Ramarathnam
Genox Corporation, 1414 Key Highway, Suite 300, Baltimore, MD 21230, USA

Y. Kato
School of Human Science and Environment, University of Hyogo, 1-1-12 Shinzaike-honcho, Himeji, Hyogo 670-0092, Japan

Y. Kato (ed.), *Lipid Hydroperoxide-Derived Modification of Biomolecules*, Subcellular Biochemistry 77, DOI 10.1007/978-94-007-7920-4_5,

Keywords Lipid peroxidation • 13-HPODE • Urine • Serum • Tissue • Oxidized LDL • Omega-6 poly unsaturated fatty acid

1 Introduction

Reactive oxygen species (ROS) and/or free radicals may play important roles in aging and age-related diseases such as atherosclerosis, diabetes and cancer (Finkel and Holbrook 2000). Monitoring of oxidative modification or oxidative damages of biomolecules may be essential for the understanding of aging, and age-related diseases. Lipid peroxidation is sometimes a major mechanism of cellular injury mediated by ROS (Comporti 1985), and it has been reported that omega-6 and omega-3 PUFA results in lipid peroxidation and cellular cytotoxicity (Sugihara et al. 1994). Aldehydes such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) have been used as lipid peroxidation markers, but they are late-stage products in multi-steps of lipid peroxidation. Lipid hydroperoxides such as 13-hydroperoxyoctadecadienoic acid (13-HPODE) has been expected to be a good indicator of initial stage of lipid peroxidation (Fig. 5.1). N-epsilon-Hexanoyl-lysine (HEL), which has been initially named as hexanonyl-lysine, is a novel lipid peroxidation biomarker, as reported by Kato et al. (1999). HEL can be detected by LC-MS/MS and has been confirmed that urinary HEL has N-epsilon structure, not alpha structure. HEL is proved to be formed by the attack of lipid hydroperoxide to peptides or proteins, not by the attack of lipid hydroperoxide to free amino acid lysine (Kato et al. 2004). Omega-6 PUFA such as linoleic acid attacked by ROS may form unstable intermediate 13-HPODE. HEL is a stable adduct of 13-HPODE to lysine residue in proteins, and is detectable in urine, serum, and tissue samples. In this chapter the development of HEL ELISA and its applications are reported.

2 Materials and Methods

2.1 Anti HEL Monoclonal Antibody

Anti HEL mouse monoclonal antibody was developed and reported by Kato et al. (2000). Briefly, Balb/c mice were immunized by Hexanoyl-modified keyhole limpet hemocyanine (HEL-KLH), and hybridomas were screened by ELISA coated with hexanoyl-modified BSA. The specificity of this antibody was tested for various length ($n = 0$–9) of alkyl chain in the amide-type adduct (CH_3–$(CH_2)_n$–CO–NH–Lys), and this antibody is highly specific to $n = 4$ length adduct (hexanoyl-lysine). It has also shown that this antibody reacts with 13-HPODE-treated BSA, oxidized linoleic acid-attached BSA, and slightly reacts with 15-hydroperoxyeicosatetraenoic acid (15-HPETE) treated BSA.

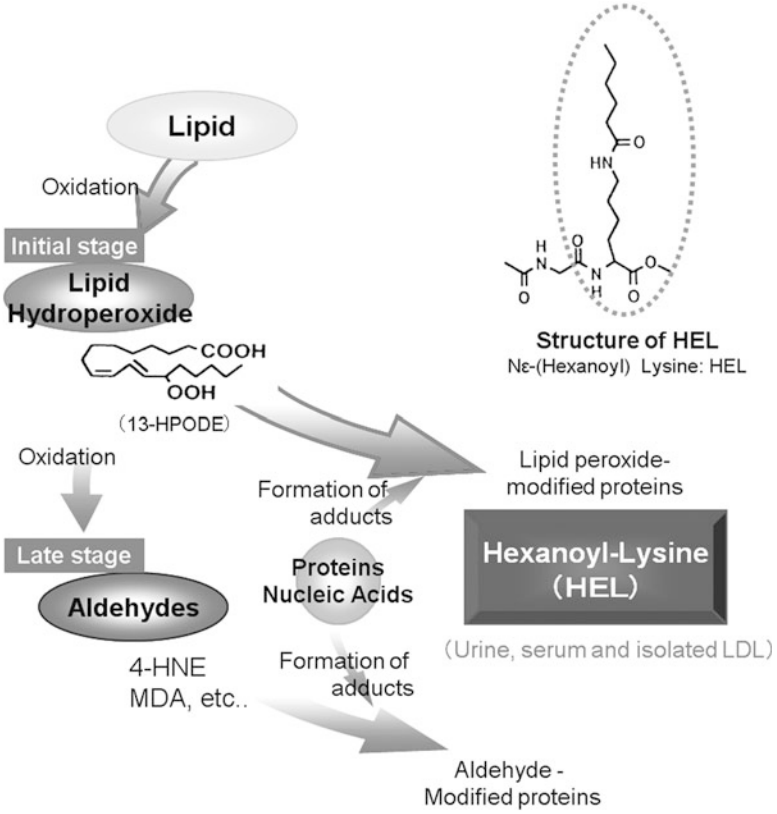


Fig. 5.1 Scheme of HEL formation

2.2 Reagents and Equipment

HEL-BSA conjugate and synthetic HEL antigen (Bz-Gly-HEL) were prepared as reported by Kato et al. (1999). Alpha-chymotrypsin was from Sigma-Aldrich (Code C-4129, alpha-chymotrypsin from bovine pancreas). Ultra filter devices with molecular cut-off 10 kDa were purchased from Millipore (Microcon YM-10) or from Pall Corporation (Nanosep centrifugal device, Code. OD010C34, New York, USA). Microplates were from Thermo Fisher Scientific (Kanagawa, Japan). Human urine samples were collected at the University Medical Centre Ljubljana with the agreement of the ethical committee of University Medical Centre Ljubljana. Animal serum was purchased from Rockland Immunochemicals (Pennsylvania, USA). All other chemicals were purchased from WAKO Pure Chemicals (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan) unless specified. A microplate reader (MPR A4i, Tosoh Corporation, Tokyo, Japan), provided with 450 nm filter, was used for absorbance determination.

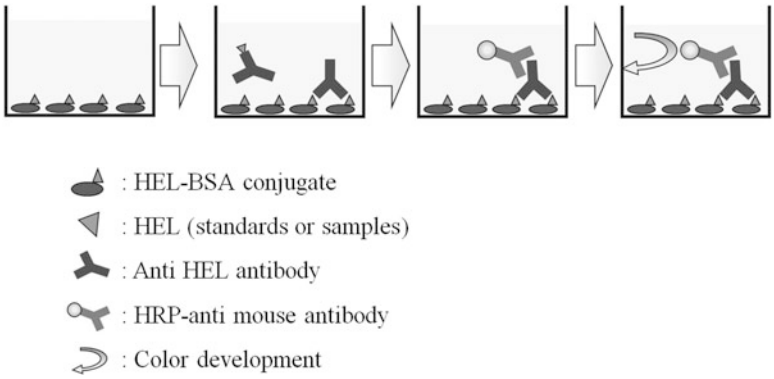


Fig. 5.2 Principle of competitive HEL ELISA

2.3 ELISA Protocol

A competitive ELISA to detect HEL (Fig. 5.2) was performed as follows. Microtitre plates were coated by HEL-conjugated BSA. A synthetic peptide Bz-Gly-HEL was used as the HEL standard. Samples were pretreated and diluted depending on the type of samples. For example, human urine samples were diluted by mixing with three volumes of phosphate buffered saline just before assay. 50 μL of samples or standard solution at concentrations of 2.6, 7.7, 22.7, 69.7, 207 and 624 nmol/L were poured into wells. Anti-HEL antibody solution was added 50 μL to all well, and was incubated at 4–7 °C overnight. The plates were washed three times with 0.05 % Tween 20 in phosphate buffered saline (PBS-tween) and incubated with 100 μL per well of horseradish peroxidase (HRP)-conjugated anti mouse polyclonal antibody solution for 1 h at room temperature. Wells were then washed three times with PBS-tween and incubated with 100 μL per well of 3,3,5,5-tetramethylbenzidine (TMB) for 15 min at room temperature. Color development was stopped by the addition of 100 μL of 1 mol/L phosphoric acid and absorbance at 450 nm was measured using microplate reader. ELISA protocol is summarized in Fig. 5.3.

3 Performance of HEL ELISA

Assay range of HEL ELISA is from 2 to 700 nmol/L. Standard plot was drawn by plotting absorbance vertical axis and log of concentration as the horizontal axis as shown in Fig. 5.4. For the calculation of results, 4-parameter-algorithm, spline algorithm or point-to-point linear algorithm are suitable. In this chapter, point-to-point linear algorithm has been used. The assay response was linear within the assay range. Intraassay variation (C.V.) of standards was below 20 %. This ELISA kit was stable for 24 months if stored at 2–8 °C.

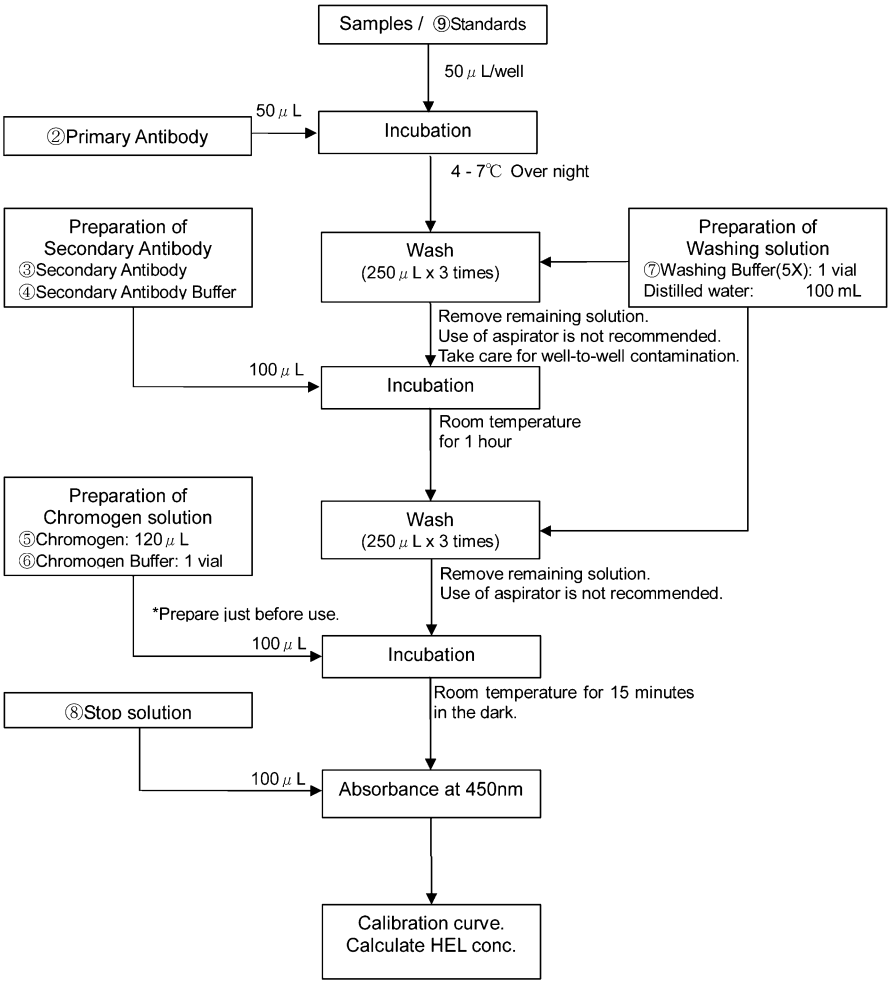


Fig. 5.3 Overview of ELISA procedure

4 Applications of HEL ELISA

4.1 Urine Samples

Human urine samples were collected and stored frozen at −80 °C. Samples were thawed at room temperature for 3 h, centrifuged at 10,000 rpm for 10 min to remove the insoluble materials, were mixed with three volumes (25 % dilution), and applied to HEL ELISA. Mean concentration of urinary HEL was 22.9 ± 15.4 nmol/L. A

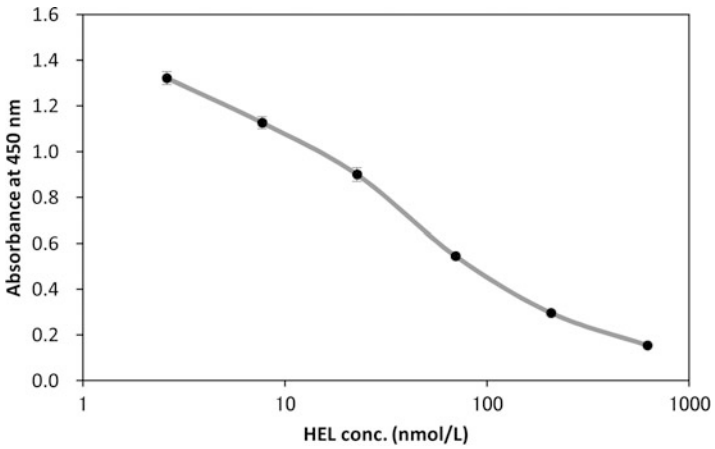


Fig. 5.4 Standard plot of HEL ELISA

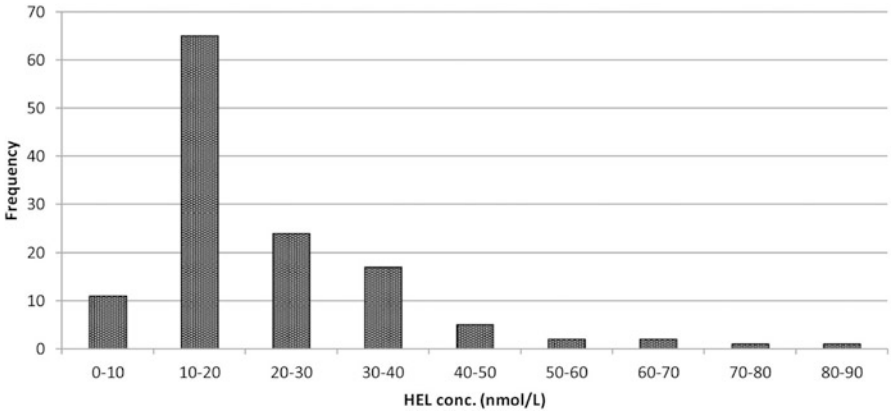


Fig. 5.5 Distribution of HEL concentration in human urine

distribution of HEL concentration is shown in Fig. 5.5. Linearity has been tested by diluting samples (Fig. 5.6). Although good linearity has been observed between 1/10 (10 %) and non-diluted (100 %), ELISA value seems to be unstable at over 1/2 (50 %) dilution. It is recommended to dilute human urine for 1/4 (25 %). The optimum dilution fold may be different depending on the species. For example, 1/4 (25 %) dilution may be suitable for dog urine and 1/10 (10 %) dilution may be suitable for cat urine (data not shown).

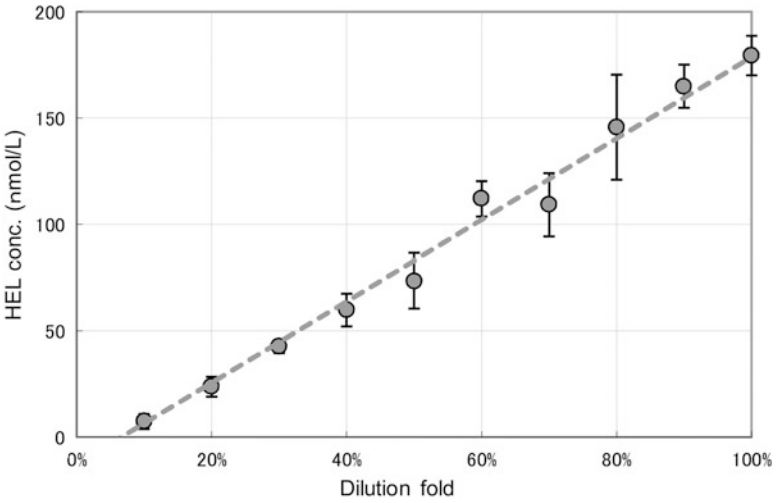


Fig. 5.6 Linearity of HEL assay for human urine

4.2 Recovery Test for Urine Sample

To test the accuracy of this assay, recovery test of standards were performed. Diluted urine samples were spiked with known concentrations and measured by HEL ELISA. The analytical recovery rate was determined by the expected and measured HEL concentrations. The expected levels were calculated as the sum of the measured HEL concentration in the original sample and the spiked HEL amount. The recovery rate for 1/4 diluted urine was 114 % (N = 3).

4.3 Absorption Test for Urine Sample

Specificity of this assay was tested by absorption assay. Urine samples were 1/4 diluted by PBS and mixed with anti HEL monoclonal antibody at final concentration 95 μg/mL. After incubation at 37 °C for 3 h, reaction mixture was filtered by 10 kDa cut-off ultra-filter to remove antibody. HEL value detected by ELISA has been completely absorbed by anti HEL monoclonal antibody (Fig. 5.7).

4.4 Ultrafiltration of Urine Sample

To test whether urinary HEL exists as protein-attached form or free/peptide form, ultrafiltration treatment was performed. Urine samples were applied to 3 or 10 kDa molecular cut-off ultra-filter, and filtrates were applied to HEL ELISA. As a

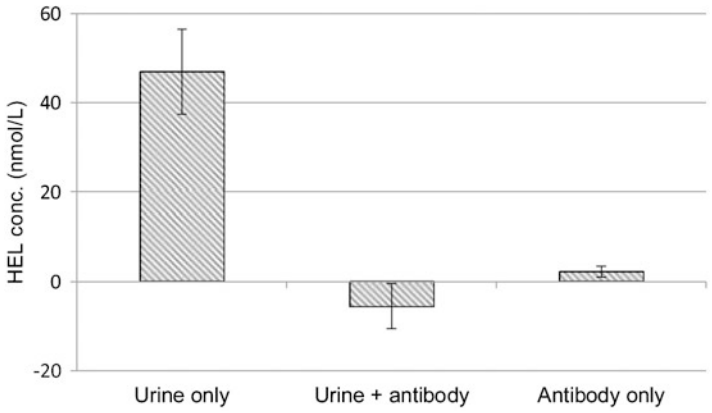


Fig. 5.7 Absorption test for human urine

control, urine sample without ultrafiltration was also measured. No significant difference was observed in HEL value between non-filtered (2.9 nmol/L) and 3 kDa (3.3 nmol/L) or 10 kDa-filtered urine (2.8 nmol/L), and urinary HEL may exist as low molecular weight substances such as free or peptide-attached form.

4.5 Detection of HEL in Oxidized LDL

Low density lipoprotein (LDL) is one of the important targets of oxidation by ROS. Oxidatively modified LDL (ox-LDL) is thought to play important roles in the early development of atherosclerosis (Steinberg 1997), and may be involved in the recruitment of monocyte-macrophages at vessel walls (Hajjar and Haberland 1997). HEL is reported to exist at atherosclerotic lesions (Fukuchi et al. 2008), and in this chapter purified ox-LDL was applied to HEL ELISA. Lysine residue can be observed frequently in proteins, and it would be possible that multiple lysine residues can be modified. To prevent steric effects and to measure accurately, samples were enzymatically hydrolyzed (Taneda and Monnier 1994) before HEL assay. Human LDL and ox-LDL were purified and oxidized as reported (Kato et al. 1999). Native LDL and ox-LDL fractions were dialyzed against PBS, and were concentrated by 30 kDa cut-off ultra-filter. 100 μL of native/ox-LDL fractions were mixed with 100 μL of 40 mg/mL trypsin solution and 2 μL of 100 mmol/L $CaCl_2$ solution, and incubated at 37 °C overnight. Reaction mixtures were applied to 30 kDa cut-off ultra-filter, and the filtrates were applied to HEL ELISA. Results are shown in Fig. 5.8. High concentration of HEL was detected in enzyme-digested ox-LDL fraction. In contrast, only low concentration of HEL was detected for native LDL and ox-LDL without enzymatic digestion. Enzymatic digestion may be essential for HEL detection in protein-containing samples such as ox-LDL.

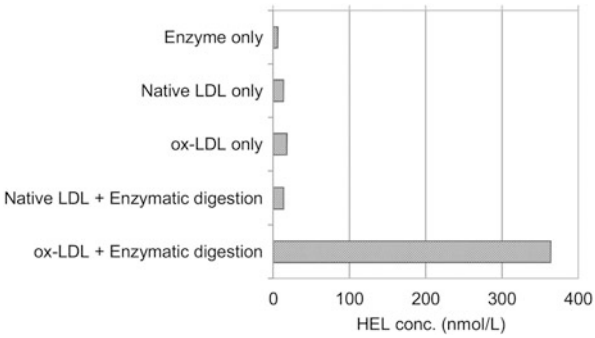


Fig. 5.8 Detection of HEL in native/oxidized-LDL

4.6 Detection of HEL in Oxidized Serum

To assess the possibility to detect serum HEL, serum sample was oxidized and applied to HEL ELISA. One volume of serum sample was mixed with 19 volumes of PBS containing 720 µmol/L of citrate and 300 µmol/L of $CuSO_4$, and incubated for 0, 2, 8 and 24 h at 37 °C. Oxidation reaction was terminated by mixing EDTA-2Na at final concentration 1 mmol/L. Control serum was incubated without $CuSO_4$, and mixed with EDTA-2Na solution at 0, 2, 9 and 24 h. Reaction mixtures were digested by alpha-chymotrypsin, were filtrated by 10 kDa cut-off filter, and applied to HEL ELISA. Results are shown in Fig. 5.9. In comparison with control serum ($CuSO_4(-)$), significantly higher concentration of HEL was detected in $CuSO_4$-oxidized serum in a time-dependent manner. The specificity of this increase in HEL concentration was validated by absorption test by anti HEL monoclonal antibody by the same protocol as described in this chapter. As shown in Fig. 5.10, approximately 87 % of HEL value increased in oxidized serum was absorbed by anti HEL antibody.

4.7 Application to Rat Serum

Rat serum samples were applied to HEL ELISA after hydrolysis by alpha chymotrypsin. Enzyme solution was prepared by dissolving 14 mg of alpha chymotrypsin into 1 mL of PBS. A 150 µL of serum sample was mixed with 150 µL of PBS and 60 µL of enzyme solution, and was incubated at 37 °C overnight. Reaction mixture was applied to 10 kDa cut-off ultra-filter, and the filtrate was further diluted by PBS and applied to ELISA wells. The linearity test has been performed as shown in Fig. 5.11. An absorption test has also been performed for rat serum. A 150 µL of hydrolyzed and filtered serum was mixed with anti HEL monoclonal antibody at final concentration 0.19 mg/mL, and incubated at 37 °C for 3 h. Antibody was removed by ultrafiltration, and was applied to HEL ELISA. In comparison with antibody free control, HEL in hydrolyzed serum was 95 % absorbed by specific antibody. Applications to canine serum (Suzuki et al. 2007) and mouse serum (Kawashima et al. 2012) have been also reported.

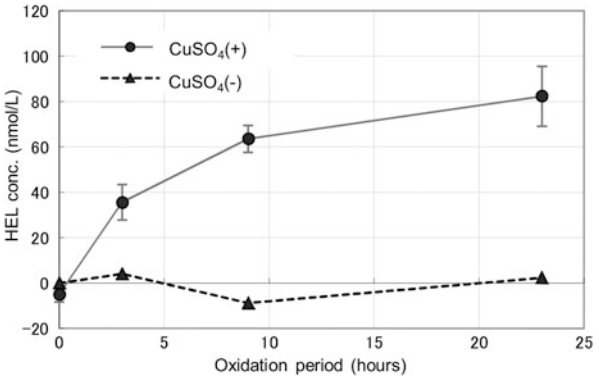


Fig. 5.9 Detection of HEL in oxidized serum

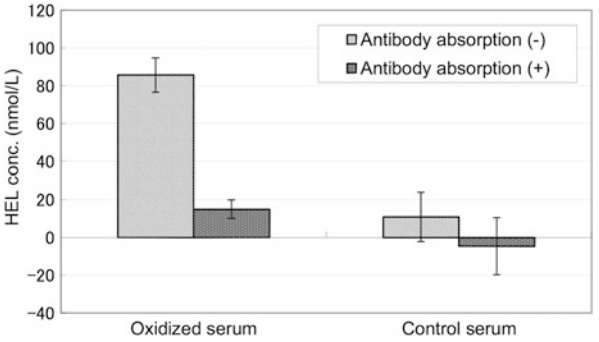


Fig. 5.10 Absorption test for oxidized serum

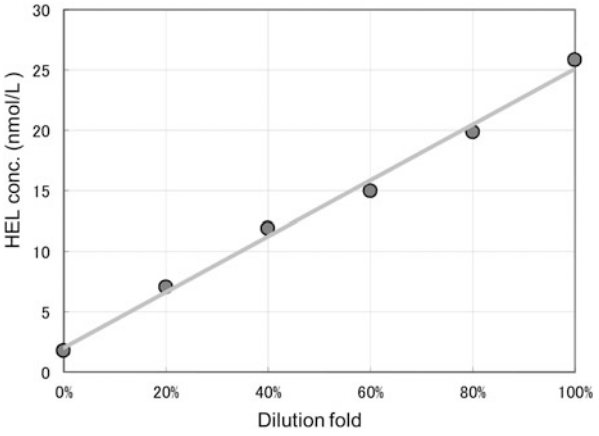


Fig. 5.11 Linearity of HEL assay for rat serum

4.8 Application to Other Biological Samples

HEL concentration in various biological samples has been reported. Rummenie et al. (2008) have shown that HEL concentration in tear samples increased by exposure to cigarette smoke. Ryo et al. (2006) have tried to apply to saliva samples, and have suggested that salivary HEL may be a potential biomarker for Sjogren's syndrome. HEL concentration in seminal plasma has been reported by Sakamoto et al. (2008).

5 Conclusion

Lipid peroxidation, especially oxidation of omega-6 and omega-3 PUFA, may be involved in cytotoxicity by ROS, and lipid oxidation products may be powerful tool for research on ROS-related diseases, aging process, and for the assessment of functional foods to prevent oxidative damage (Kato and Osawa 2010). We have established HEL ELISA as a new biomarker for omega-6 PUFA. HEL ELISA was shown to be applied to urine, serum and other biological samples independent of the animal species, and may be useful for the assessment of omega-6 PUFA oxidation in the living bodies.

Note. HEL ELISA kit is commercially available from Japan Institute for the Control of Aging (JaICA), Nikken SEIL Co., Ltd. (http://www.jaica.com).

References

Comporti M (1985) Lipid peroxidation and cellular damage in toxic liver injury. Lab Invest 53 (6):599–623

Finkel T, Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. Nature 408 (6809):239–247

Fukuchi Y, Miura Y, Nabeno Y et al (2008) Immunohistochemical detection of oxidative stress biomarkers, dityrosine and N(epsilon)-(hexanoyl)lysine, and C-reactive protein in rabbit atherosclerotic lesions. J Atheroscler Thromb 15(4):185–192

Hajjar DP, Haberland ME (1997) Lipoprotein trafficking in vascular cells: molecular Trojan horses and cellular saboteurs. J Biol Chem 272(37):22975–22978

Kato Y, Osawa T (2010) Detection of lipid-lysine amide-type adduct as a marker of PUFA oxidation and its applications. Arch Biochem Biophys 501(2):182–187

Kato Y, Mori Y, Makino Y et al (1999) Formation of N-epsilon-(hexanonyl) lysine in protein exposed to lipid hydroperoxide. A plausible marker for lipid hydroperoxide-derived protein modification. J Biol Chem 274(29):20406–20414

Kato Y, Miyake Y, Yamamoto K et al (2000) Preparation of a monoclonal antibody to N(epsilon)-(hexanonyl)lysine: application to the evaluation of protective effects of flavonoid supplementation against exercise-induced oxidative stress in rat skeletal muscle. Biochem Biophys Res Commun 274(2):389–393

Kato Y, Yoshida A, Naito M et al (2004) Identification and quantification of N(epsilon)-(hexanoyl)lysine in human urine by liquid chromatography/tandem mass spectrometry. Free Radic Biol Med 37(11):1864–1874

Kawashima M, Kawakita T, Inaba T et al (2012) Dietary lactoferrin alleviates age-related lacrimal gland dysfunction in mice. PLoS One 7(3):e33148

Rummenie VT, Matsumoto Y, Dogru M et al (2008) Tear cytokine and ocular surface alterations following brief passive cigarette smoke exposure. Cytokine 43(2):200–208

Ryo K, Yamada H, Nakagawa Y et al (2006) Possible involvement of oxidative stress in salivary gland of patients with Sjogren's syndrome. Pathobiology 73(5):252–260

Sakamoto Y, Ishikawa T, Kondo Y et al (2008) The assessment of oxidative stress in infertile patients with varicocele. BJU Int 101(12):1547–1552

Steinberg D (1997) Low density lipoprotein oxidation and its pathobiological significance. J Biol Chem 272(34):20963–20966

Sugihara N, Tsuruta Y, Date Y et al (1994) High peroxidative susceptibility of fish oil polyunsaturated fatty acid in cultured rat hepatocytes. Toxicol Appl Pharmacol 126(1):124–128

Suzuki T, Kazui T, Yamamoto S, Washiyama N et al (2007) Effect of prophylactically administered edaravone during antegrade cerebral perfusion in a canine model of old cerebral infarction. J Thorac Cardiovasc Surg 133(3):710–716

Taneda S, Monnier VM (1994) ELISA of pentosidine, an advanced glycation end product, in biological specimens. Clin Chem 40(9):1766–1773

Chapter 6
Hexanoyl-Lysine as a Deterioration Marker for Rice During Storage

Ken-ichiro Minato

Abstract N^{ε}-(hexanoyl)lysine (HEL) is known to be an oxidative lipid-decomposition product, and a powerful marker indicating oxidative stress in animal tissue. We investigated whether HEL could be useful as a marker in rice seeds damaged by oxidative stress during storage, as well as animals. We could show an accumulation of HEL in rice stored at high temperature (40 °C). This result significantly corresponded with an accumulation of TBARS. Rice germination deteriorated with non-enzymatic lipid peroxidation during storage at high temperature for a few months. These results suggested that a deterioration of rice germination ability resulted from oxidative damage caused by lipid peroxidation during storage. Moreover, HEL could become a useful marker for oxidative stress induced by lipid peroxidation. In addition, the activities of antioxidant enzymes, catalase and superoxide dismutase, significantly decreased in the rice seeds during storage at 40 °C. The relationship between accumulation of HEL and increases in antioxidant enzymes activities must be further studied. But, these results suggest that HEL might be a useful marker of oxidative stress in rice as well as in animals.

Keywords Antioxidant enzymes • Lipid peroxidation • N^{ε}-(hexanoyl)lysine • Oxidative stress marker • Rice

Abbreviations

HEL	N^{ε}-(hexanoyl)lysine
DT	*o*, *o*-dityrosine
NT	Nitrotyrosine

K. Minato (✉)
Department of Applied Biological Chemistry, Meijo University, 1-501 Shiogamaguchi, Nagoya 468-8502, Japan
e-mail: minato@meijo-u.ac.jp

Y. Kato (ed.), *Lipid Hydroperoxide-Derived Modification of Biomolecules*, Subcellular Biochemistry 77, DOI 10.1007/978-94-007-7920-4_6,

TBARS	Thiobarbituric acid-reactive substance
ELISA	Enzyme-linked immunosorbent assay
AOS	Activated oxygen species
LOX	Lipoxygenase
CAT	Catalase
Mn-SOD	Manganese – superoxide dismutase
Cu, Zn-SOC	Copper, Zinc – superoxide dismutase

1 Introduction

Rice is the staple food in Japan and other East and Southeast Asian countries. Japan is self-sufficient in rice. Its storage stability has been very important in ensuring ongoing high-quality rice availability. High temperature especially is considered to cause rapid deterioration in rice quality and germination ability during storage. Many researchers have studied how to stabilize rice quality during storage (Sharp and Timme 1986; Ramezanzadeh et al. 1999). It has been suggested that lipid degradation is responsible for the deterioration of rice during storage (Takano 1993).

Plants are constantly exposed to the toxicity of activated oxygen species (AOS). The AOS are produced as superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$) during metabolic processes. It has been suggested that these AOS react with lipids, proteins, and nucleic acids in a cell to induce oxidative damage (Rice-Evans et al. 1991). It was suggested that reaction between lipid and molecular oxygen occurred at early stage of oxidative damage. This reaction could take place at the double bond s of unsaturated fatty acids, and be accelerated by singlet oxygen, free radicals, metal ions such as iron, copper, and cobalt, light radiation, elevation of temperature, and enzyme activity such as lipoxygenase which was a transition metal prosthetic group. Lipid peroxidation induced by AOS is considered an important reaction in membrane deterioration in the cells of plants (Shalata and Tal 1998). Moreover, it has been considered to be strongly associated with a decrease in plant viabilities, such as germination ability. In rice, a deterioration in germination ability during storage has been also a serious problem. We hypothesized that this deterioration might result from oxidative damage during storage at high temperatures.

It has been suggested that biomolecules such as proteins and aminolipids are covalently modified by lipid decomposition products such as aldehyde during an even earlier stage of lipid peroxidation in animals (Esterbauer et al. 1991). Kato et al. (1999) identified N^{ε}-(hexanoyl)lysine (HEL) as one of the lipid hydroperoxide-modified lysine residues, considered to be useful markers of early lipid peroxidation-derived protein modification. Some reports suggested that HEL would be a good biomarker for oxidative stress, especially at earlier stage when oxidative damage was occurred by lipid peroxidation than a stage of harmful aldehyde formation (Kato et al. 2000; Ueno et al. 2002). It have been established how to measure this novel oxidative stress biomarker by ELISA antibody detection.

It have been previously reported that HEL accumulates in animal tissue in which oxidative stress was induced (Kato et al. 1999, 2000; Minato et al. 2003). Therefore, we expected that biomolecules in which proteins and/or aminolipids are modified by lipid decomposition products might be a useful oxidative stress marker in rice damaged oxidatively, as well as in animal tissue.

In this chapter, we attempt to establish an estimation of oxidative stress damage in plants by detection of HEL, as well as animals. Hence, we investigated whether the reduction in germination ability resulted from oxidative stress during storage of rice at high temperatures, and whether HEL can be used effectively as an oxidative stress biomarker in rice.

2 A Quality Deterioration in Rice by Oxidative Stress

We investigated rice germination ability as one index of the stability of rice quality. In rice, damage induced by oxidative stress is considered to be accompanied by lipid peroxidation (Suzuki et al. 1996). Hence, we next determined a thiobarbituric acid-reacted substance (TBARS), which is known to be a traditional marker of early-stage lipid peroxidation induced by oxidative stress (Miyake et al. 1998), in the rice seeds before and after storage. We used the rice [*Oryza sativa* (L.)] Sasanishiki, which belongs to the japonica type, with the husk. The rice (300 g) packed in pored polyethylene film bags (350 × 400 mm, 0.03 mm thickness) was stored at 5 °C and 40 °C for 3 months. One hundred grains of stored rice with the husk was placed on filter paper in a 9-cm Petri dish, and 15 ml distilled water was added. The dishes were placed in an incubator at 30 °C, and the number of germinated seeds was counted daily for 4 days. The appearance of a coleoptile about 1 mm in length was considered to correspond to the germination stage. Clearly, its ability to germinate survived during storage for 3 months (Fig. 6.1), and the level was maintained even after 6 months (data not shown). On the other hand, the germination rate of the rice stored at 40 °C was significantly ($p < 0.002$) dampened; it was approximately 70 % in rice stored for 3 months. These results show that high-temperature storage negatively influences the stability of rice germination ability.

Twenty husked rice seeds were picked out and homogenized with extract buffer (50 mM phosphate buffer, pH 7.0) containing 1 % Triton X-100 at 4 °C. The homogenate was centrifuged at 4,500 × g for 15 min, and the supernatant was obtained for measurement of thiobarbituric acid-reactive substance (TBARS). The rice TBARS concentration was expressed as nmol of malondialdehyde per gram of the husked rice seeds (Fig. 6.2). The initial level was 13.9 ± 1.1 $nmol \cdot g^{-1}$. In the rice stored at 5 °C, it did not change. On the other hand, for that stored at 40 °C, the level increased significantly ($p < 0.05$) to 19.1 ± 1.1 $nmol \cdot g^{-1}$ after 3-months storage. These results suggest that lipid peroxidation might be responsible for the reduction in the germination ability of rice during storage at high temperature (40 °C). We surmised that this deterioration in germination ability resulted from oxidative stress.

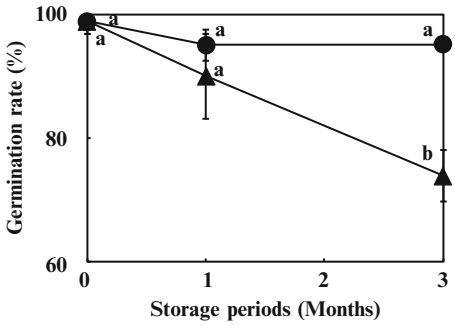


Fig. 6.1 Germination rate of the rice, Sasanishiki, during storage at 5 °C (●) and 40 °C (▲). Each value is presented as the mean ± S.D. of triplicate assays. Statistical analysis was carried out by one-way ANOVA followed by Tukey's HSD test to identify significant difference. Values designated by different letters are significantly different ($p < 0.005$) (This referred to Minato et al. (2005) reprinted by permission of Japan Society for Bioscience, Biotechnology, and Agrochemistry)

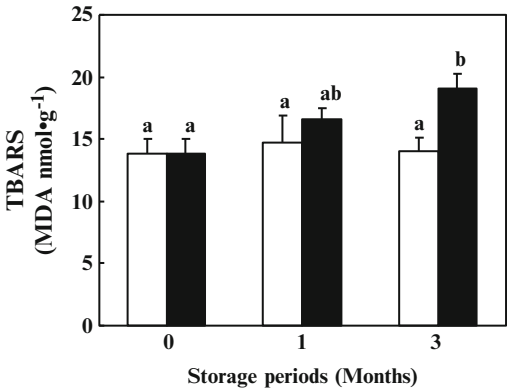


Fig. 6.2 Accumulation of TBARS in the rice during storage at 5 °C (□) and 40 °C (■). Each value is presented as the mean ± S.D. of triplicate assays. Statistical analysis was carried out by one-way ANOVA followed by Tukey's HSD test to identify significant difference. Values designated by different superscript letters are significantly different ($p < 0.005$) (This referred to Minato et al. (2005) reprinted by permission of Japan Society for Bioscience, Biotechnology, and Agrochemistry)

Suzuki et al. (1996) suggested that lipid peroxidation caused by a lipoxygenase (LOX) in rice seeds resulted in oxidative deterioration during storage. Lipoxygenase activity, however, did not significantly increase during storage of rice at high temperatures for 3 months in this study (Fig. 6.3).

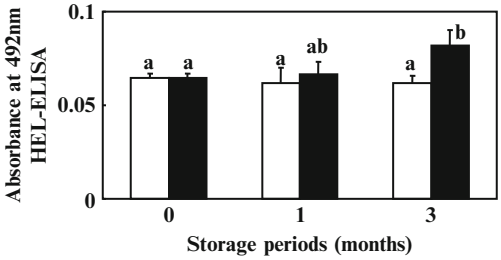


Fig. 6.3 Formation of HEL in the rice during storage at 5 °C (□) and 40 °C (■). Each value is presented as the mean ± S.D. of triplicate assays. Statistical analysis was carried out by one-way ANOVA followed by Tukey's HSD test to identify significant difference. Values designated by different superscript letters are significantly different ($p < 0.005$) (This referred to Minato et al. (2005) reprinted by permission of Japan Society for Bioscience, Biotechnology, and Agrochemistry)

3 Accumulation of HEL in Rice Seed Induced Oxidative Stress Damage

The contents of oxidative stress marker, HEL, in stressed rice was determined by ELISA antibody assay, as described previously (Kato et al. 1999). Briefly, 50 µl of sample solution (10 µg protein · ml^{-1}) was pipetted into the wells and kept at 4 °C for 12 h. Then the plate was incubated with the primary antibodies. After incubation, binding of the antibodies to the modified proteins was evaluated using anti-mouse/rabbit IgG antibody peroxidase labeled with *o*-phenylenediamine and hydrogen peroxide.

Although the level of HEL did not change in the rice during storage at 5 °C, a significant ($p < 0.05$) increase in this biomarker was detected in rice stored at 40 °C for 3 months (Fig. 6.4). This result significantly corresponded with the increase in the TBARS level in rice stored at 40 °C. During lipid peroxidation, proteins and aminolipids can be covalently modified by lipid decomposition products. In the aliphatic aldehydes, such as 1-hexanal or 1-nonanal, the N^{ε}-amino group of the lysine residues in a protein can be modified through the formation of a Schiff base. Moreover, α- and β-unsaturated aldehydes, such as 4-hydroxy-2 nonenal, react with lysine, cysteine, and histidine through a Michael-type addition (Esterbauer et al. 1991; Uchida et al. 1998). N^{ε}-(hexanonyl)lysine (HEL) has been identified as an adduct with an amide bond between the N^{ε}-amino group and lipid-derived part. It has reported that the antibody against this adduct was useful as a biomarker of oxidative stress in animal tissue (Kato et al. 2000; Minato et al. 2003), but it had not yet been reported whether HEL accumulates in plant tissue damaged by oxidative stress. As shown in Figs. 6.3 and 6.4, this HEL indeed accumulated in rice seed that underwent lipid peroxidation. Moreover, the HEL level increased simultaneously with the reduction in germination ability in the rice stored at 40 °C.

These results suggest that detection of HEL formation by ELISA is a useful approach to determining oxidative stress in a plant, as well as in animals, but it

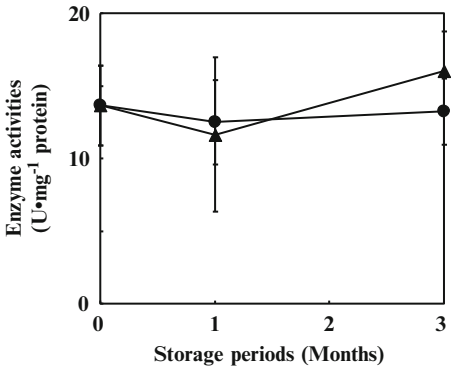


Fig. 6.4 Changes in a lipoxygenase activity in the rice during storage at 5 °C (●) and 40 °C (▲). Lipoxygenase (*LOX*) activity was determined as described by Stevens et al. (1970). One hundred microlitre of linoleic acid was dissolved in 15 ml of absolute alcohol, and water was added to make 25 ml of stock solution. Immediately before use, 5 ml of substrate stock solution were diluted with 25 ml of 0.2 M sodium borate buffer (pH 9.0), and the solution was oxygenated by bubbling gaseous oxygen through it for a few minutes. The enzyme solution to be assayed was also diluted with 0.2 M sodium borate buffer. The reaction mixture contained 50 μl enzyme extract and 0.2 M sodium borate buffer (pH 9.0) to make a final volume of 3 ml. One unit of LOX was defined as a change in A_{234} of 0.01 per min. Each value is presented as the mean ± S.D. of triplicate assays. Statistical analysis was carried out by one-way ANOVA followed by Tukey's HSD test to identify significant difference. There were not significant differences ($p < 0.005$) among each value

remains obscure whether HEL directly influenced the deterioration in rice germination ability. And it was suggested that the oxidative lipid-decomposition product, HEL, accumulated in rice during storage at high temperatures.

4 Antioxidant Enzymes Activities in Oxidative Stress Damaged Rice

All organisms exposed to an aerobic environment must have some protective mechanism against such harmful oxygen species. One of the plant responses to AOS production is an increase in antioxidant enzyme activities which provides protection from oxidative damage induced by several environmental stresses. It is known that antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) play an important role in the active oxygen- scavenging system. In order to clarify the correlation between antioxidant enzyme activity and storage stability as to rice quality, we also measured CAT and SOD activities in rice during storage.

Twenty seeds were homogenized in 10 volumes of 50 mM sodium phosphate buffer (pH 7.4) at 4 °C. And then the supernatant obtained was used for subsequent enzymatical measurement. Total catalase (CAT) activity was determined spectrophotometrically by measuring the decrease in absorbance at 240 nm at 25 °C (Aebi 1983). The reaction mixture contained enzyme extract and 50 mM phosphate buffer

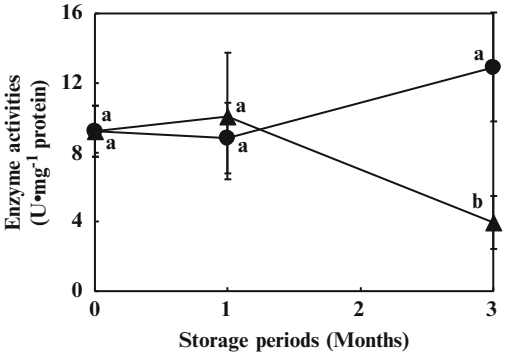


Fig. 6.5 Changes in catalase activity in the rice during storage at 5 °C (●) and 40 °C (▲). Each value is presented as the mean ± S.D. of triplicate assays. Statistical analysis was carried out by one-way ANOVA followed by Tukey's HSD test to identify significant difference. Values designated by different letters are significantly different ($p < 0.005$) (This referred to Minato et al. (2005) reprinted by permission of Japan Society for Bioscience, Biotechnology, and Agrochemistry)

(pH 7.0). The reaction was started by the addition of 45 mM hydrogen peroxide in the reaction buffer. CAT activity was estimated by the decrease in absorbance of H_2O_2 at 240 nm. One unit of CAT was defined as the amount of enzyme dismuting 1 μmole of H_2O_2 per minute. As shown in Fig. 6.5, catalase activity continued during storage at 5 °C, but this activity significantly ($p < 0.025$) decreased in rice stored at 40 °C.

Superoxide dismutase (SOD) activity was determined essentially as described by Spychalla and Desborough (1990). The assay was performed at 25 °C in a cuvette containing 50 mM Na_2CO_3/$NaHCO_3$ (pH 10.2), 0.1 mM EDTA, 0.015 mM ferricytochrome c, and 0.05 mM xanthine. It was initiated by the addition of sufficient xanthine oxidase to produce a basal rate of ferricytochrome c reduction corresponding to an increase in A_{550} of 0.025. Then the amount of xanthine oxidase, defined as the amount of enzyme inhibiting the rate of ferricytochrome c by reduction 50 % was determined. Mn-SOD activity was determined as described above, except that the assay mixture contained 2 mM KCN to inactivate Cu, Zn-SOD. Cu, Zn-SOD was calculated from the difference between total SOD and Mn-SOD activities. Total superoxide dismutase activity also continued at 5 °C storage, whereas it decreased significantly ($p < 0.05$) at 40 °C storage (Fig. 6.6). Moreover, in this study, Mn- and Cu, Zn-SOD activities were determined. Mn-SOD activity did not change during storage at either 5 or 40 °C. But, Cu, Zn-SOD activity fell to one-half its initial level during storage at 40 °C. This result suggests that Cu, Zn-SOD activity might play more important roles in an activated oxygen-scavenging system than Mn-SOD. And, the changes in these antioxidant enzymes activities were well correlated with changes in germination rates and the contents of TBARS and HEL in rice during storage.

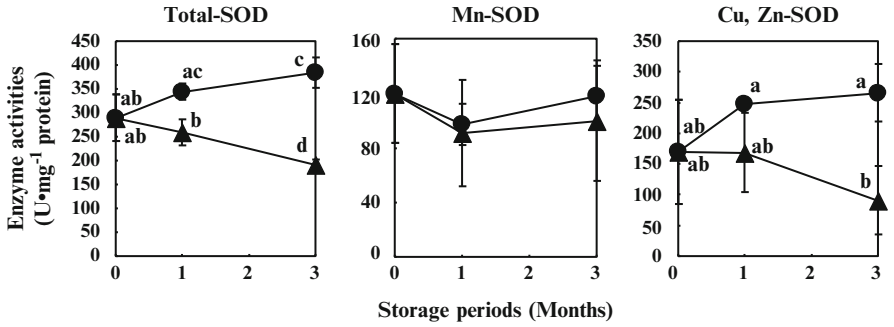


Fig. 6.6 Changes in superoxide dismutase activity in the rice during storage at 5 °C (●) and 40 °C (▲). Each value is presented as the mean ± S.D. of triplicate assays. Statistical analysis was carried out by one-way ANOVA followed by Tukey's HSD test to identify significant difference. Values designated by different letters are significantly different ($p < 0.005$). There were not significant differences ($p < 0.005$) among each value in Mn-SOD activities (This referred to Minato et al. (2005) reprinted by permission of Japan Society for Bioscience, Biotechnology, and Agrochemistry)

Activated oxygen-scavenging enzymes have received a great deal of attention in relation to various kinds of stresses. Higher tolerance to environmental stresses has been associated with higher activities of antioxidant enzymes such as catalase and superoxide dismutase (Shalata and Tal 1998; Olmos et al. 1994; Yu and Rengel 1999; Mehlhorn and Wenzel 1996). In this study, as shown in Figs. 6.4 and 6.5, although the CAT and SOD activities continued in rice stored at 5 °C, their activities significantly decreased with increases in TBARS and HEL levels at 40 °C. These results suggest that these antioxidant enzymes, CAT and SOD, play an important role in the resistance mechanism to oxidative stress in rice seeds during storage. It was suggested that the activity of the activated oxygen-scavenging system due to antioxidant enzymes continued during storage of rice at low temperature (5 °C), thereby preventing oxidative damage in the rice. It was suggested, however, that the decrease in these antioxidant enzymes activities at high temperature (40 °C) might induce the production of activated oxygen species such as H_2O_2 and $O_2^{\bullet -}$. Then oxidative damage would occur as lipid peroxidation.

5 Conclusion

In summary, rice germination deteriorated with non-enzymatic lipid peroxidation during storage at high temperature (40 °C) for a few months. We could show an accumulation of HEL in rice stressed oxidatively at high temperature. This result significantly corresponded with an accumulation of TBARS, a traditional marker of lipid peroxidation. These results suggested that a deterioration of rice germination ability resulted from oxidative stress caused by lipid peroxidation during storage. N^{ε}-(hexanoyl)lysine (HEL) is known to be an oxidative lipid-decomposition

product, and a powerful marker indicating oxidative stress in animal tissue in which lipid peroxidation has been induced. HEL accumulated in rice which oxidative damage was induced by lipid peroxidation. We could show an accumulation of HEL in rice stored at high temperature (40 °C). This result significantly corresponded with an accumulation of TBARS. The activity of antioxidant enzymes, catalase and superoxide dismutase, decreased with accumulation of HEL in rice stored at 40 °C. The relationship between accumulation of HEL and increases in antioxidant enzymes activities must be further studied. But, our findings could show that HEL would be useful as an oxidative stress biomarker in plants as well as in animals.

Acknowledgment I am deeply grateful to Professor Toshihiko Osawa of Aichi-gakuin University and Professor Yoji Kato of University of Hyogo for their guidance and support.

References

Aebi HE (1983) Catalase. In: Bergmeyer HU, Bergmeyer J, Grabl M (eds) Methods of enzymatic analysis, vol III, 3rd edn. Verlag Chemie GmbH, Weinheim, pp 273–286

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

Kato Y, Mori Y, Makino Y, Morimitsu Y, Hiroi S, Ishikawa T, Osawa T (1999) Formation of N^{ε}-(hexanonyl)lysine in protein exposed to lipid hydroperoxide. J Biol Chem 274:20406–20414

Kato Y, Miyake Y, Yamamoto K, Shimomura Y, Ochi H, Mori Y, Osawa T (2000) Preparation of a monoclonal antibody to N^{ε}-(hexanonyl)lysine: application to the evaluation of protective effects of flavonoid supplementation against exercise-induced oxidative stress in rat skeletal muscle. Biochem Biophys Res Commun 274:389–393

Mehlhorn H, Wenzel A (1996) Manganese deficiency enhances ozonetoxicity in bush beans (Phaseolus vulgaris L. cv. Saxa). J Plant Physiol 148:155–159

Minato K, Miyake Y, Fukumoto S, Yamamoto K, Kato Y, Shimomura Y, Osawa T (2003) Lemon flavonoid, eriocitrin, suppresses exercise-induced oxidative damage in rat river. Life Sci 72:1609–1616

Minato K, Gono M, Yamaguchi H, Kato Y, Osawa T (2005) Accumulation of Ne-(Hexanoyl) lysine, an oxidative stress biomarker, in rice seeds during storage. Biosci Biotechnol Biochem 69(9):1806–1810

Miyake Y, Yamamoto K, Tsujihara N, Osawa T (1998) Protective effects of lemon flavonoids on oxidative stress in diabetic rats. Lipids 33:689–695

Olmos E, Hernandez JA, Sevilla F, Hellín E (1994) Induction of several antioxidant enzymes in the selection of a salt tolerant cell line of Pisum sativum. J Plant Physiol 114:594–598

Ramezanzadeh FM, Rao RM, Windhauser M, Prinyawiwatkul W, Marshall WE (1999) Prevention of oxidative rancidity in rice bran during storage. J Agric Food Chem 47:2997–3000

Rice-Evans CA, Diplock AT, Symons MCR (1991) Mechanisms of radical production. In: Burdon RH, van Knippenberg PH (eds) Laboratory techniques in biochemistry and molecular biology, vol 22. Elsevier, Amsterdam, pp 19–50

Shalata A, Tal M (1998) The effects of salt stress on lipid peroxidation and antioxidants in the leaf of the cultivated tomato and its wild salt-tolerant relative Lycopersicon pennellii. Physiol Plant 104:169–174

Sharp RN, Timme LK (1986) Effects of storage time, storage temperature, and packaging methods on shelf life of brown rice. Cereal Chem 63:247–251

Spychalla JP, Desborough SL (1990) Superoxide dismutase, catalase, and α-tocopherol content of stored potato tubers. Plant Physiol 94:1214–1218

Stevens FC, Brown DM, Smith EL (1970) Some properties of soybean lipoxygenase. Arch Biochem Biophys 136:413–421

Suzuki Y, Yasui T, Matsukura U, Terao J (1996) Oxidative stability of bran lipids from rice variety [Oryza sativa (L.)] lacking lipoxygenase-3 in seeds. J Agric Food Chem 44:3479–3483

Takano K (1993) Advances in cereal chemistry and technology in Japan. Cereal Foods World 38:695–698

Uchida K, Kanematsu M, Sasaki 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 U S A 95:4882–4887

Ueno Y, Horio F, Uchida K, Naito M, Nomura H, Kato Y, Tsuda T, Yoyokuni S, Osawa T (2002) Increase in oxidative stress in kidneys of diabetic Akita mice. Biosci Biotechnol Biochem 66(4):869–872

Yu Q, Rengel Z (1999) Micronutrient deficiency influences plant growth and activities of superoxide dismutases in narrow-leafed lupins. Ann Bot 83:175–182

Chapter 7
Cholesterol Hydroperoxides and Their Degradation Mechanism

Junji Terao

Abstract Cholesterol is one of the oxidizable lipids constituting biomembranes and plasma lipoproteins. Cholesterol hydroperoxides (Chol-OOH) are the primary products if cholesterol is subjected to attack by reactive oxygen species. In particular, singlet molecular oxygen reacts with cholesterol to yield cholesterol 5α-hydroperoxide as the major hydroperoxide species. Chol-OOH may accumulate in biological systems because of its resistance to glutathione-dependent enzymatic detoxification reactions. Their degradation products (including hydroxycholesterol and 7-ketocholesterol) participate in the pathophysiological functions of oxysterols. Highly reactive cholesterol 5,6-secosterol present in atherosclerotic lesions can be derived from the degradation of cholesterol 5α-hydroperoxide. Chol-OOH themselves may affect the lipid rafts of biomembranes, thereby leading to the modification of signal transduction pathways.

Keywords Cholesterol hydroperoxides • Singlet molecular oxygen • Cholesterol 5,6-secosterol • Lipid rafts

1 Introduction

Cholesterol is an essential class of lipids constituting biomembranes and one of the major lipid components in plasma lipoproteins. Cholesterol is subjected to lipid peroxidation accompanied with other esterified polyunsaturated fatty acids (PUFAs) such as phospholipids (Niki 2009). However, lipid peroxidation occurring in cholesterol is different from common PUFAs. Similar to PUFAs, cholesterol hydroperoxides (Chol-OOH) are formed as primary products if cholesterol is

J. Terao (✉)
Institute of Health Biosciences, University of Tokushima Graduate School, Tokushima 770-8503, Japan
e-mail: terao@nutr.med.tokushima-u.ac.jp

Y. Kato (ed.), *Lipid Hydroperoxide-Derived Modification of Biomolecules*, Subcellular Biochemistry 77, DOI 10.1007/978-94-007-7920-4_7,

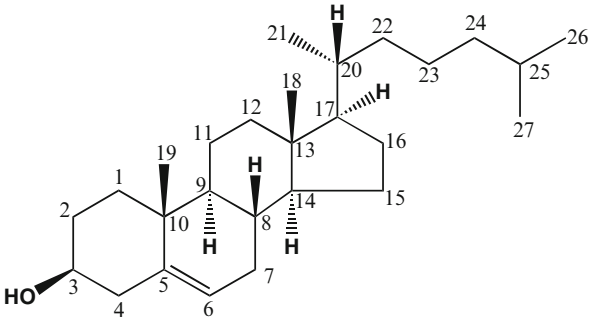


Fig. 7.1 Structure of cholesterol

oxidized by reactive oxygen species (ROS). However, Chol-OOH are known to be resistant to the glutathione-dependent enzymatic detoxification system compared with PUFA hydroperoxides (PUFA-OOH) and their esterified forms (Miyamoto et al. 2010). Therefore, Chol-OOH might accumulate for a long time in biological systems as their native forms. The mechanism for the formation of Chol-OOH can be distinguished to free-radical oxidation and singlet molecular oxygen($O_2(^1\Delta_g)$)-mediated oxygenation. In contrast, cholesterol is oxidized specifically by an enzymatic mechanism to form hydroxycholesterols such as 24-hydroxycholesterol and 27-hydroxy cholesterol. These oxidized cholesterol species (including Chol-OOH) are called as "oxysterols" and their pathophysiological role has attracted much attention in recent years (Brown and Jessup 1999; Otaegui-Arrazola et al. 2010; van Reyk et al. 2006). This chapter is focused on Chol-OOH and their degradation products produced by lipid peroxidation in biomembranes and plasma lipoproteins (Fig. 7.1).

2 Free-Radical Oxidation vs. Singlet Molecular Oxygen-Mediated Oxygenation

From the point of view of the reaction mechanism, non-enzymatic lipid peroxidation is categorized into two types: free-radical chain oxidation reaction and singlet molecular oxygen ($O_2(^1\Delta_g)$)-mediated oxygenation. The reactivity of cholesterol with $O_2(^1\Delta_g)$ is similar to that of PUFAs. The rate constant for cholesterol is $5.7 \times 10^4\ M^{-1}\ S^{-1}$, whereas it is $3 \times 10^4\ M^{-1}\ S^{-1}$ for each non-conjugated *cis*-double bond for C18 PUFAs (Vever-Bizet et al. 1989). This means that $O_2(^1\Delta_g)$ equally attacks cholesterol and the PUFA moiety of phospholipids to form peroxidized cholesterol and peroxidized phospholipids. In contrast, cholesterol is oxidized by free radicals very slowly as compared with PUFAs such as linoleic acid and arachidonic acid. This is because cholesterol lacks doubly allylic hydrogen molecules, which are readily abstracted by free radicals, resulting in carbon-centered radicals (Niki 2005). However, cholesterol has been suggested to be more susceptible to free-radical oxidation than esterified linoleate in cultured cells under oxidative stress (Saito et al. 2007). Yoshida et al. (2007) found that

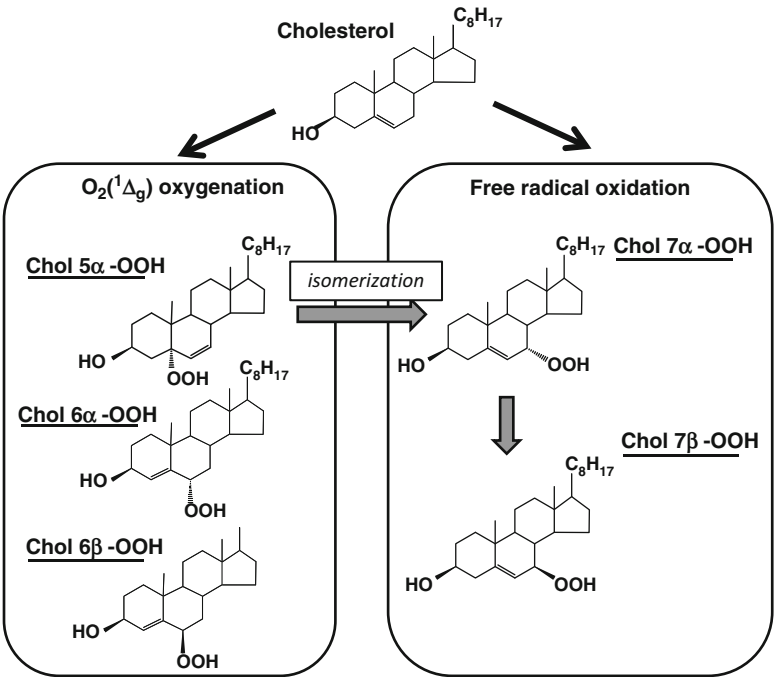


Fig. 7.2 Different pathways of Chol-OOH formation between $O_2(^1\Delta_g)$ oxygenation and free-radical oxidation

cholesterol oxidation products accumulated at unexpectedly high levels (as high as PUFA oxidation products) in human blood. The oxidizability of cholesterol may depend largely on the microenvironment in which lipid peroxidation occurs. For example, free-radical oxidation of cholesterol is suggested to be regulated by "superlattices" in the bilayers of biomembranes (Olsher et al. 2005).

Figure 7.2 shows the different pathways of Chol-OOH formation between $O_2(^1\Delta_g)$ oxygenation and free-radical oxidation. In $O_2(^1\Delta_g)$ oxygenation, cholesterol 5α-hydroperoxide (Chol 5α-OOH), cholesterol 6α-hydoperoxide (Chol 6α-OOH), and cholesterol 6β-hydroperoxide (Chol 6β-OOH) are formed by thee *ene* reaction of electrophilic O_2 ($^1\Delta_g$) with the 5,6-double bond (Freimer 1979), in which the yield of Chol 5α-OOH is superior to that of Chol 6α/β-OOH (Girroti and Korytowski 2000).

In the mechanism of free-radical oxidation, hydrogen abstraction happens exclusively at the C-7 position, resulting in cholesterol 7α-hydroperoxide (Chol 7α-OOH) and cholesterol 7β-hydroperoxide (Chol 7β-OOH). Chol 5α-OOH generated by $O_2(^1\Delta_g)$ oxygenation is isomerized to form Chol 7α-OOH and Chol 7β-OOH during lipid peroxidation. Nevertheless, Chol 5α-OOH has been used frequently as a biomarker of $O_2(^1\Delta_g)$ generation in biological systems. We found recently that Chol 5α-OOH accumulated in the skin of hairless mice, suggesting that $O_2(^1\Delta_g)$ is responsible for lipid peroxidation in mouse skin (Minami et al. 2007). Conversely,

Chol 7α/β-OOH was detected in human skin upon sunlight exposure (Yamazaki et al. 1999). A high-performance (HPLC)-chemiluminescence analysis demonstrated that cholesterol hydroperoxides (Chol 7α-OOH and Chol 7β-OOH) accumulated in rat skeletal muscle and liver tissue after chronic ethanol feeding (Adachi et al. 2000; Ariyoshi et al. 2002). Chol 6β-OOH may be a more reliable marker of $O_2(^1\Delta_g)$ than Chol 5α-OOH because of its higher stability (Korytowski et al. 1992). Different from Chol 5α-OOH,Chol 6β-OOH was shown not to induce membrane damage due to lipid peroxidation (Korytowski et al. 2010).

3 Secondary Products Derived from Chol-OOH

Chol 5α-OOH has been suggested to be a precursor of biologically active cholesterol aldehydes such as cholesterol 5,6-secosterol (Brinkhourt et al. 2008). Ozone has long been suggested to be involved in the formation of cholesterol 5,6-secosterol. However, hock cleavage of Chol 5α-OOH was recently proposed as a mechanism of cholesterol 5,6-secosterol formation (Fig. 7.3). These cholesterol aldehydes can modify proteins covalently, which could have pathophysiological relevance (see Chap. 7). 7-Ketocholesterol (cholest-5-ene-3β-ol-7-one) and 7α/β-hydroxycholesterol (Chol 7α/β-OH; cholest-5-ene-3β-7β-diol, cholest-5-ene-3β-7α-diol) are produced by the one-electron-transfer decomposition of Chol 7α/β-OOH (Fig. 7.4). Chol 7α/β-OOH may be intermediates, and 7-ketocholesterol and 7α/β-hydroxycholesterol accumulate as the final products of cholesterol peroxidation. Chol 7β-OH and 7-ketocholesterol are also formed by the enzymatic reaction of cholesterol with 11β-hydroxysteroid dehydrogenase type 1. Similarly, a reaction of cholesterol with 7α-hydorxylase (CYP7A1) yields Chol 7β-OH.

Cholesterol 5α,6α-epoxide (5α,6α-epoxycholestan-3β-ol) and cholesterol 5β,6-β-epoxide (5β,6β-epoxycholestan-3β-ol) can be generated without the decomposition of Chol-OOH. In this case, an intermediate of lipid peroxidation in PUFAs, lipid peroxyl radicals (LOO·), attach directly to the 5,6-double bond of cholesterol to form α/β epoxides. Their hydrolysis reaction leads to the formation of cholestanetriol (cholestan-3β,5α,6β-triol).

Recently, Uemi et al. found that Chol7α/β-OOH generates $O_2(^1\Delta_g)$ and is accompanied by the formation of Chol 7-α/β OH and 7-ketocholesterol by a cyclic mechanism from a linear tetraoxide intermediate (Uemi et al. 2011). This bimolecular reaction may also participate in the degradation pathway of cholesterol.

4 Detoxification of Cholesterol Hydroperoxides

In human plasma, Chol 7α-OH, Chol 7β-OH and 7-ketocholesterol were detected at the nanomolar level in accordance with the products of the enzymatic oxidation of cholesterol, 24-hydroxycholesterol and 27-hydroxycholesterol (Table 7.1) (Yoshida

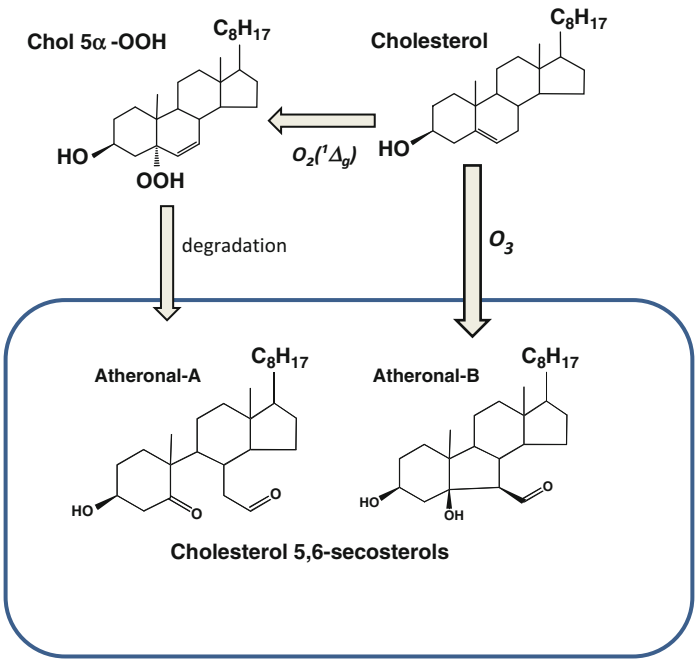


Fig. 7.3 Possible pathways for the formation of cholesterol 5,6-secosterol from the ozolysis of cholesterol and degradation of Chol 5α-OOH

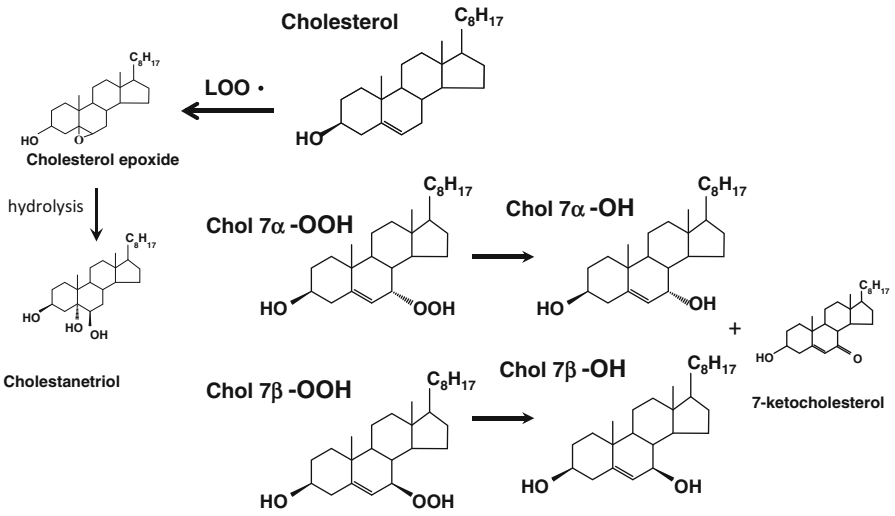


Fig. 7.4 Pathway for the formation of degradation products from Chol 7α/β-OOH and cholesterol epoxides

Table 7.1 Concentration of lipid peroxidation products and antioxidants in human plasma and erythrocytes (Yoshida et al. 2007)

Lipid peroxidation product	Plasma (nmol/L)	Erythrocytes (nmol/packed cell)
Total hydroxyoctadecadienoic acid (tHODE)	203 ± 76	1,917 ± 1,004
Total 8-isoprostaglandin (t8-iso-PGF2α)	0.727 ± 0.604	12.8 ± 7.9
Cholesterol 7α-OH	87.1 ± 50	1,372 ± 461
Cholesterol 7β-OH	156 ± 102	3,854 ± 1,844

The mean value was obtained for plasma and erythrocytes from 44 healthy subjects after reduction and saponification. tHODE is a sum of the free and esterified forms of isomeric hydroxyoctadecadienoic acid. Cholesterol 7α-OH and cholesterol 7β-OH are the sum of esterified forms and free forms of hydroxycholesterol (These data are modified from Yoshida et al. 2007 and are reproduced with permission)

et al. 2007). Among them, Chol 7α-OH and Chol 7β-OH can be produced by the enzymatic detoxification reaction of Chol-OOH by the glutathione-dependent system. In general, lipid hydroperoxides are detoxified to their two-electron-reduction products: lipid hydroxides. This reaction is mediated by at least three enzymes: glutathione peroxidase (GPx), glutathione S-transferase (GST) and peroxiredoxin (PrX) (Miyamoto et al. 2010). Five isoforms of GPx have been identified. GPx4, that is, phospholipid hydroperoxide glutathione peroxidase (PHGPx), has been suggested to be the only enzyme that can detoxify Chol-OOH to yield hydroxycholesterol. However, Chol-OOH was inferior to PUFA hydroperoxides and their esterified forms in the reduction by PHGPx (Korytowski et al. 1996). It seems rational to state that Chol-OOH accumulates at higher levels than PUFA-OOH or its esterified forms (phospholipid hydroperoxides) if Chol-OOH is more resistant to enzymatic detoxification in biological systems. Korytowski et al. (1996) indicated that Chol 5 α-OOH was reduced more slowly than Chol 6β-OOH by the GSH/PHGPx system (5α-OOH < 6α-OOH = 7α/β-OOH < 6β−OOH), resulting in the damage of cells derived from the generation of $O_2(^1\Delta_g)$.

5 Pathophysiological Function of Chol-OOH and Its Related Products

Peroxidation of cholesterol in low-density lipoprotein (LDL) in plasma has been suggested to exert pathological effects such as induction of apoptotic cell death, which can lead to the pathogenesis of atherosclerosis (Lordan et al. 2009). Oxysterols accumulate in human atheroma and exert pro-apoptotic effects to contribute to atherosclerotic development (Larsson et al. 2006). Oxysterols in LDL have been suggested to participate in the progression of atherosclerotic lesions by strongly up-regulating pro-inflammatory factors such as monocyte chemotactic protein-1 (MCP-1) (Leonarduzzi et al. 2005). Sterffen et al. (2006) suggested that the ratio of Chol 7β-OH to 7-ketocholesterol is the crucial determinant for the

cytotoxicity of oxysterol mixtures. Cholesterol 5,6-secosterol has been called as an "atheronal" because it was discovered first in human arterial plaques and suggested to have an effect on the pathogenesis of cardiovascular diseases (Takeuchi et al. 2006). Anticoli et al. (2009) implied that cholesterol 5,6-secosterol possesses a pro-proliferative effect on hepatoblastoma cells at very low concentrations. This finding suggested that this oxysterol can induce the death of liver cells through cell necrosis rather than apoptosis.

Cholesterol is known to be concentrated into microdomains such as lipid rafts in biomembrane structures (Yaqoob 2009). Oxysterols may promote or inhibit the formation of membrane microdomains because oxysterols such as 7-hydroxycholesterol and 7-ketocholesterol transfer spontaneously between biological membranes at rates that are one order of magnitude faster than those of cholesterol (Vila et al. 2001). Thus, oxysterols are postulated to modify the organization of membrane microdomains (Olkkonen and Hynynen 2009; Massey 2006). In fact, Berthier et al. (2004) found that 7-ketocholesterol accumulates preferentially in the lipid-raft domains in plasma membranes. 7-Ketocholesterol modulates the PI3-K/PDK-1/Akt signaling pathway by disorganizing the packaging of cytoplasmic membranes (Vejux et al. 2009). Conversely, an *in vivo* animal experiment demonstrated that intracutaneous injection a mixture of Chol-OOH induces the activity of matrix metalloproteinase-9 in the skin of hairless mice (Minami et al. 2009). It is likely that Chol-OOH also modulates the lipid-raft structure, resulting in the promotion of signaling pathways of matrix metalloproteinases.

Alternatively, Chol-OOH is moved to target sites within the cell to influence cellular functions. Sterol carrier protein-2 (SCP-2) has been suggested to act as a carrier of Chol-OOH to be conveyed to target sites where Chol-OOH reacts with proteins specifically after conversion into their degradation products (Kriska et al. 2010). To investigate the pathophysiological effect of Chol-OOH, *in vivo* studies are required to ascertain the effect of Chol-OOH and its degradation on the modification of lipid rafts in biomembranes and its direct binding to target proteins.

References

Adachi J, Asano M, Ueno Y, Reily M, Mantle D, Peters TJ, Preedy VR (2000) 7α-and 7-β-Hydroperoxycholest-5-ene-3β-ol in muscle as indices of oxidative stress: response to ethanol dosage in rats. Alcohol Clin Exp Res 24:675–681

Anticoli S, Arciello M, Mancinetti A, De Martins M, Ginald L, Iuliano L, Balsano C (2009) 7-ketocholesterol and 5,6-secosterol modulate differently the stress-activated mitogen-activated protein kinase (MAPK) in liver cells. J Cell Physiol 222:586–595

Ariyoshi K, Adachi J, Asano M, Ueno Y, Rejendram R, Preedy V (2002) Effect of chronic ethanol feeding on oxysterol in rat liver. Free Radic Res 36:661–666

Berthier A, Lemaire-Ewing PC, Monier S, Athias A, Bessede G, de Barros J-PP, Laubrier A, Gambert P, Lizard G, Neel D (2004) Involvement of a calcium-dependent dephosphorylation of BAD associated with the localization of Trpc-1 within lipid rafts in 7-ketocholesterol-induced THP-1 cell apoptosis. Cell Death Differ 11:897–905

Brinkhourt J, Nara SJ, Pratt DA (2008) Hock cleavage of cholesterol 5α-hydroperoxides: an ozone-free pathway to the cholesterol ozonolysis products identified in arterial plaque and brain tissue. J Am Chem Soc 130:12224–12225

Brown AJ, Jessup W (1999) Oxysterol and atherosclerosis. Atherosclerosis 142:1–28

Freimer AA (1979) The reaction of singlet oxygen with olefins: the question of mechanism. Chem Rev 79:359–387

Girroti AW, Korytowski W (2000) Cholesterol as a singlet oxygen detector in biological system. Methods Enzymol 319:85–100

Korytowski W, Schmitt JC, Girotti AW (2010) Surprising inability of singlet oxygen-generated 6-hydroperoxycholesterol to induce damaging free radical lipid peroxidation in cell membranes. Photochem Photobiol 86:747–751

Korytowski W, Bachowski G, Girotti A (1992) Photoperoxidation of cholesterol in homogenious solution, isolated membranes and cells: comparison of the 5α-and 6-β-hydroperoxides as indicators of singlet oxygen intermediacy. Photochem Photobiol 56:1–8

Korytowski W, Geiger PG, Girotti AW (1996) Enzymatic reducibility to cytotoxicity for various cholesterol hydroperoxides. Biochemistry 35:8770–8779

Kriska T, Pilat A, Schmitt JC, Girotti AW (2010) Sterolcarrierprotein-2(SCP-2) involvement in cholesterol hydroperoxide cytotoxicity as revealed by SCP-2 inhibitor effects. J Lipid Res 51:3174–3184

Larsson DA, Baird S, Nyhalah JD, Yuan X-M, Li W (2006) Oxysterol mixtures, in atheroma-relevant proportions, display synergistic and proapoptiotic effects. Free Radic Biol Med 41:902–910

Leonarduzzi G, Gamba P, Sottero B, Kadl A, Robbesyn F, Calogero RA, Biasi F, Chiarpotto E, Leitinger N, Sevanian A, Poli G (2005) Oxysterol-induced up-regulation of MCP-1 expression and synthesis in macrophage cells. Free Radic Biol Med 39:1152–1161

Lordan S, Mackrill JJ, O'Brien NM (2009) Oxysterol and mechanism of apoptotic signaling: implications in the pathology of degenerative diseases. J Nutr Biochem 20:321–336

Massey JB (2006) Membrane and protein interactions of oxysterols. Curr Opin Lipidol 17:296–301

Minami Y, Yokoi S, Setoyama M, Bando N, Takeda S, Kawai Y, Terao J (2007) Combination of TLC blotting and gas chromatography–mass spectrometry for analysis of peroxidized cholesterol. Lipids 42:1055–1063

Minami Y, Kawabata K, Kubo Y, Arase S, Hirasaka K, Nikawa T, Bando N, Kawai Y, Terao J (2009) Peroxidized cholesterol-induced matrix metalloproteinase-9 activation and its suppression by dietary β-carotene in photoaging of hairless mouse skin. J Nutr Biochem 20:389–398

Miyamoto S, Arai H, Terao J (2010) Enzymatic antioxidant defenses. In: Aldini G, Yeum K-J, Niki E, Russell RM (eds) Biomarkers for antioxidant defenses and oxidative damage. Principles and practical applications. Wiley-Blackwell Publishing, Hoboken NJ, USA, pp 21–33

Niki E (2005) Lipid peroxidation: mechanisms, inhibition, and biological effects. Biochem Biophys Res Comm 338:668–676

Niki E (2009) Lipid peroxidation: physiological levels and dual biological effects. Free Radic Biol Med 47:469–484

Olkkonen VM, Hynynen R (2009) Interactions of oxysterols with membranes and proteins. Mol Asp Med 30:123–133

Olsher M, Yoon S-I, Chong L-G (2005) Role of sterol superlattice in free radical-induced sterol oxidation in lipid membranes. Biochemistry 44:2080–2087

Otaegui-Arrazola A, Carrno-Nenendez M, Ansorena D, Astiasaran I (2010) Oxysterols: a world to explore. Food Chem Toxicol 48:3289–3303

Saito Y, Yoshida Y, Niki E (2007) Cholesterol is more susceptible to oxidation than linoleate in cultured cells under oxidative stress induced by selenium deficiency and free radicals. FEBS Lett 581:4349–4354

Steffen Y, Wiswedel I, Petyer D, Schewe T, Sies H (2006) Cytotoxicity of myeloperoxidase/nitrite-oxidized low-density lipoprotein toward endothelial cells is due to a high 7β-hydroxy cholesterol to 7-ketocholesterol ratio. Free Radic Biol Med 41:1139–1150

Takeuchi C, Galve R, Nieva J, Witter DP, Wentworth AD, Yroseth RP, Lerner RA, Wentworth P (2006) Proatherogenic effects of the cholesterol ozonolysis products, atheronal-A and atheronal-B. Biochemistry 45:7162–7170

Uemi M, Ronsein GE, Fernanda M, Prado M, Motta FD, Miyamoto S, Medeiros MG, DiMascio PD (2011) Cholesterol hydroperoxides generates singlet molecular oxygen $[O_2{}^1\Delta_g]$: near-IR emission. ^{18}O-labelled hydroperoxides, and mass spectrometry. Chem Res Toxicol 24:887–895

van Reyk D, Brown A, Hult'en LM, Dean R, Jessup W (2006) Oxysterol in biological systems: sources, metabolism and pathophysiological releavance. Redox Rep 11:255–262

Vejux A, Guyot S, Montange T, Riedinger J-M, Kahn E, Lizard G (2009) Phospholipidosis and down-regulation of the PI3-K/PDK-1/Akt signaling pathway are vitamin E inhibitable events associated with 7-ketocholesterol-induced apoptosis. J Nutr Biochem 20:45–61

Vever-Bizet C, Dellinger M, Braut D, Rougee M, Bensasson RV (1989) Singlet molecular oxygen quenching by saturated and unsaturated fatty-acids and by cholesterol. Photochem Photobiol 50:321–325

Vila A, Korytowski W, Girotti AW (2001) Spontaneous intermembrane transfer of various cholesterol-derived hydroperoxide species; kinetic studies with model membranes and cell. Biochemistry 40:14715–14726

Yamazaki S, Ozawa N, Hiratsuka A, Watabe T (1999) Increase in cholesterol 7-hydroperoxides in lipids of human skin by sunlight exposure. Free Radic Biol Med 26:1126–1133

Yaqoob P (2009) The nutritional significance of lipid rafts. Ann Rev Nutr 29:257–282

Yoshida Y, Saito Y, Hayakawa M, Habuchi Y, Imai Y, Sawai Y, Niki E (2007) Level of lipid peroxidation in human plasma and erythrocytes: comparison between fatty acid and cholesterol. Lipids 42:439–449

Part II
Pathophysiological Consequences

Chapter 8
Amide-Adducts in Atherosclerosis

Michitaka Naito

Abstract Too many hypotheses in the etiology of atherosclerosis have been proposed. Classically, lipid insudation hypothesis by Virchow and thrombogenic hypothesis by Rokitansky are famous. However, in the recent progress in the area of atherosclerosis, the response-to-injury hypothesis by Ross (Ross R Glomset JA, N Engl J Med 295:369–377, 420–425, 1976; Ross R, Arteriosclerosis 1:293–311, 1981; Ross R, N Engl J Med 314:488–500, 1986; Ross R, Nature 362:801–809, 1993; Ross R, N Engl J Med 340:115–126, 1999) has been the leading one. In this review, however, the author focuses to the recent debate on the role of oxidative modification of atherogenic lipoproteins.

Keywords Atherosclerosis • Inflammation • Oxidative stress • Amide-adduct

1 Theories of Atherosclerosis

1.1 LDL Oxidation Hypothesis

Steinberg (1997; Steinberg et al. 1989) suggested that oxidative modification of low density lipoprotein (LDL) is important in the pathogenesis of atherosclerosis. LDL can be modified by incubation with endothelial cells, smooth muscle cells, or monocytes/macrophages in the presence of trace amount of transition metals. This biological modification of LDL is mediated by a free radical-induced peroxidation by copper or iron in the absence of cells. The potential atherogenic effects of oxidized LDL include (1) chemotactic activity, which facilitates the recruitment of

M. Naito (✉)
Graduate School of Life Studies, Sugiyama Jogakuen University, Nagoya 464-8662, Japan
e-mail: naito@sugiyama-u.ac.jp

Y. Kato (ed.), *Lipid Hydroperoxide-Derived Modification of Biomolecules*,
Subcellular Biochemistry 77, DOI 10.1007/978-94-007-7920-4_8,

circulating monocytes, (2) the inhibition of the migration of macrophages from the arterial wall back to the circulation, (3) the enhanced uptake by macrophages through scavenger receptors, leading to the formation of foam cells, and (4) cytotoxicity to endothelial cells, that may facilitate the accumulation of LDL and monocytes in the early stage, causing endothelial denudation at a later stage.

1.2 Response-to-Retention Hypothesis

Williams and Tabas (1995, 2005) doubted the Steinberg's LDL oxidation hypothesis, which looks upon the oxidation of LDL as the essential factor in the initiation of atherosclerosis, and proposed the response-to-retention hypothesis. According to their theory, the deposition and retention of lipoprotein particles, particularly LDL, are sufficient for the initiation of atherosclerosis, and the oxidation of LDL is not required for the initial stage of atherosclerosis. LDL accumulated in the arterial intima easily binds to extracellular matrix including proteoglycans, aggregates, and then is taken up by macrophages through scavenger receptors, leading to foam cell formation. Lipolytic enzymes such as lipoprotein lipase and sphingomyelinase may be involved in the intramural retention of lipoproteins. Among the atherogenic lipoproteins, especially lipoprotein(a) is prone to be retained in the intima. Apolipoproteins B100, C-II, and E may also be involved. Among them, apolipoprotein C-II is predisposed to form amyloid, and stimulates inflammatory response of macrophages (Medeiros et al. 2004). In fact, amyloid, macrophages, and apolipoprotein C-II colocalize in human atherosclerotic lesions. Collagen, fibrin, and fibronectin may also be involved in the deposition of lipoproteins.

Although the inflammatory nature of atherosclerosis has been established as will be discussed later, the substances that start inflammation in the artery wall are largely unknown. It is clear that chronic infections yield a higher risk for cardiovascular disease. However, because germ-free animals are also susceptible to atherosclerosis (Wright et al. 2000), it is considered that endogenous substances can also stimulate inflammation. Cholesterol crystal has been reported to be the candidate (Duewell et al. 2010). Small crystals appeared in subendothelial areas that were rich in immune cells as early as 2 weeks after the start of atherogenic diet. A sharp increase in the incidence of cholesterol crystals has been observed in human atherosclerotic lesions as they progress from fatty streaks to more advanced lesions (Rajamäki et al. 2010). Cholesterol crystals activate the NLRP3 inflammasome in phagocytes in vitro in a process that involves phagolysosomal damage. In contrast, cholesteryl esters form droplets rather than crystals and are considered to be a storage form of cholesterol.

1.3 Oxidative Response to Inflammation Hypothesis

In 2004, synthesizing the results obtained from molecular and cellular biology, animal experiments, and clinical and epidemiological studies, Stocker and Keaney (2004) introduced the "oxidative response to inflammation" theory, and proposed that inflammation, but not oxidative stress is the real cause of atherosclerosis, and oxidative stress is merely accompanied as the consequence of inflammation. According to their theory, the primary process of atherosclerosis initiation is not oxidative stress, but inflammation, and oxidative stress is merely a secondary event. Among the evidence related to their theory, atherosclerosis was aggravated in the scavenger receptor deficient animal (de Winther et al. 1999), and the over-expression of scavenger receptor inhibited atherosclerotic process (Whitman et al. 2002). However, the relation between the oxidative stress and inflammation is complex, and it is not easy to differentiate which is the cause and which is the result. Moreover, the view point of atherosclerosis as inflammation is not novel. Indeed, before Ross (1999) proposed the inflammation as the pathogenesis of atherosclerosis, steroidal (Naito et al. 1992) and non-steroidal anti-inflammatory agents (Bailey and Butler 1973) had been reported to inhibit atherosclerosis induced by cholesterol feeding without influencing the plasma cholesterol level.

1.4 Unifying Hypothesis of Atherosclerosis

Atherosclerosis is multifactorial pathology, and the author introduced the unifying theory in 2000 (Naito 2000), putting together various aspects of findings, and pointed out the paramount importance of (1) the insudation, deposition, and denaturalization (including oxidative modification) of plasma components, particularly atherogenic lipoproteins such as LDL and fibrinogen into the subendothelial space, (2) the formation of thrombus, fibrinolysis, and the organization, and (3) the inflammatory response to those events (Fig. 8.1).

Except for the modified version of (inflammatory) response-to-injury hypothesis by Ross, the theories proposed by Steinberg (LDL oxidation hypothesis), Williams and Tabas (response-to-retention hypothesis), and Stocker and Keaney (oxidative response-to-inflammation hypothesis) are all related only to the initiation and early stages of atherosclerosis. After the initiation of atherosclerosis, its development thereafter is explained by Ross's hypothesis. However, even in the modified version of his hypothesis published only several months before his death (Ross 1999), although the importance of chronic inflammation was emphasized, the role of coagulation and fibrinolysis was not mentioned. There is no sufficient evidence to show the participation of mural thrombosis in the initiation of atherosclerosis, however, this mechanism is essential for the growth and development of the lesion.

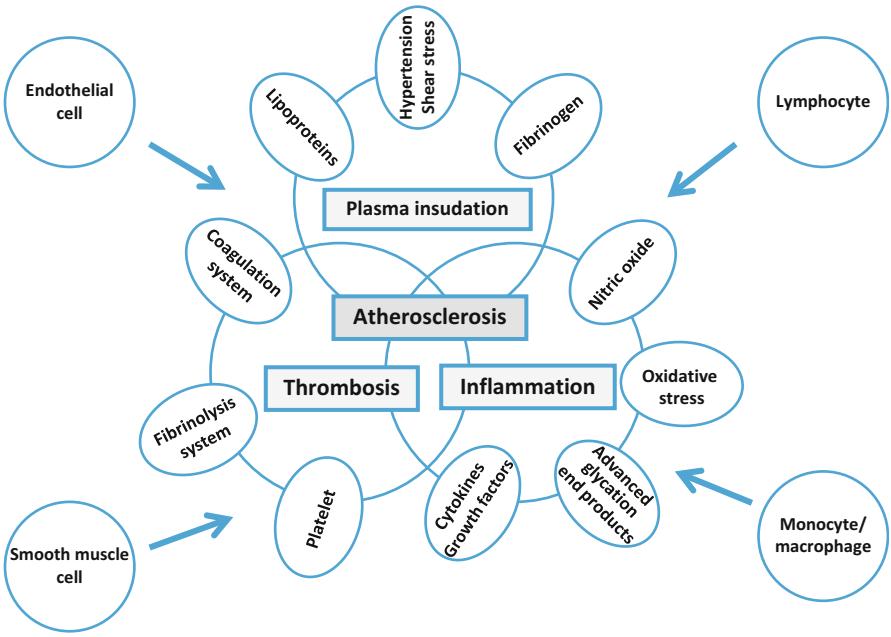


Fig. 8.1 Unifying hypothesis (Revised from Naito 2000)

2 CRP and Atherosclerosis

C-reactive protein (CRP), an acute-phase protein, binds specifically to phosphorylcholine as a component of capsular polysaccharide of many microorganisms. CRP also binds to apoptotic cells, enhancing their clearance. CRP promotes the clearance of CRP-opsonized particles by binding to FCγ receptors (Bharadwaj et al. 1999; Mold et al. 2001). CRP bound to multivalent ligands also activates a classical complement pathway, enhancing phagocytosis (Mold et al. 1999). CRP binds to oxidized LDL but not native LDL, and the binding to oxidized LDL is mediated through recognition of phosphorylcholine. CRP binds oxidized LDL and apoptotic cells by recognition of a phosphorylcholine moiety that becomes exposed and accessible as a result of oxidation of phosphatidylcholine molecule (Chang et al. 2002). These findings indicate that the main biological function of CRP is a first-line innate immune response to oxidized phosphorylcholine-bearing phospholipids within oxidized LDL and on the plasma membrane of apoptotic cells. However, it was also reported that CRP-bound enzymatically modified LDL did not transform macrophages into foam cells (Singh et al. 2008).

CRP is predominantly synthesized by hepatocytes as an acute-phase reactant and is transcriptionally driven by interleukin-6, with synergistic enhancement by interleukin-1. However, some studies showed that CRP may be produced in

atherosclerotic lesion (Yasojima et al. 2001) and by smooth muscle cells (Calabró et al. 2003) and macrophages (Dong and Wright 1996).

CRP is found localized in inflamed tissues, including atherosclerotic lesion (Hatanaka et al. 1995). CRP displays both anti-inflammatory and proinflammatory effects in vitro. The latter includes the ability of ligand-bound CRP to activate the complement system (Volanakis 1982). Activated complements have also been found in human atherosclerotic lesions (Seifert and Kazatchkine 1988).

The expression of vascular CRP is closely colocalized with NAD(P)H oxidase, a crucial enzyme for the origin of reactive oxygen species (ROS) in vessel walls (Kobayashi et al. 2003). It is reported that plaque instability was associated with the expression of CRP in directional coronary atherectomy specimens, and the expression of CRP was colocalized with NAD(P)H oxidase $p22^{phox}$ protein. Incubation of cultured coronary artery smooth muscle cells with CRP resulted in enhanced protein expression of NAD(P)H oxidase $p22^{phox}$ and the generation of H_2O_2. CRP also promotes apoptosis of vascular smooth muscle cells, and may contribute to plaque instability.

CRP increases tissue factor expression in monocytes/macrophages, promotes monocyte chemotaxis and adhesion to endothelial cells, and stimulates the release of ROS, matrix metalloproteinase-1 (MMP-1), CC-chemokine receptor 2 (CCR2), cytokines, and macrophage-colony stimulating factor (M-CSF) (Devaraj et al. 2009). CRP treatment significantly increases the release of MPO from polymorphonuclear cells and monocytes/macrophages and causes nitrotyrosinylation of LDL (Singh et al. 2009). The colocalization of CRP and macrophages has been demonstrated in atherosclerotic lesions in human (Yasojima et al. 2001) and animals (Fukuchi et al. 2008).

3 Lipid Peroxidation and Atherosclerosis

Lipid hydroperoxide-derived modification of protein may serve as one mechanism for the modification of LDL and subsequent foam cell formation in the atherosclerotic lesion. Oxidation of LDL leads to the loss of ε-amino groups from lysine residues in apolipoprotein B100 due to the covalent adduction of the oxidatively decomposed products of polyunsaturated fatty acid esters.

Lipid hydroperoxides are the initial products of lipid peroxidation, which are subsequently decomposed to a variety of products such as aldehydes. It has been shown that reaction of 13-hydroperoxyoctadienoic acid (13-HPODE) with lysine residues resulted in the formation of two major amide-type adducts, N^{ε}-hexanoyl-lysine (HEL) (Kato et al. 1999) and N^{ε}-azelayl-lysine (AZL) (Kawai et al. 2003).

Aldehydes such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), the major lipid peroxidation end-products, are highly reactive to ε-amino groups in proteins. MDA-lysine (Uchida et al. 1997), HNE-lysine (Uchida et al. 1994), and acrolein-lysine adducts (Uchida et al. 1998) are detected in oxidized LDL and atherosclerotic lesion.

In our study, CRP was detected mostly in the macrophage-derived foam cell-rich areas of rabbit fatty lesions. Immunopositive staining of HEL was observed in the foam cell-rich areas, where it almost colocalized with CRP-positive macrophages (Fukuchi et al. 2008). Dityrosine (DY) was also observed in the foam cell-rich areas, essentially similar to the deposition of HEL. HEL and DY were colocalized with CRP-positive macrophages.

HEL is produced in the reaction between linoleic hydroperoxide and lysine moiety, and is an early and stable marker for protein oxidation derived from lipid peroxidation. HEL is shown in oxidized LDL and in human atherosclerotic lesion, using a specific monoclonal antibody to HEL moiety. The hydroperoxide-derived carboxylic adducts, such as AZL, and their esters linked with phospholipids, are also detected in rabbit atherosclerotic lesions (Kawai et al. 2003).

Dityrosine (DY) is formed by the reaction of two tyrosyl radicals and catalyzed by myeloperoxidase in the presence of H_2O_2, but it can also be generated by metal-catalyzed oxidation. DY has been detected immunochemically in lipofuscin of pyramidal neurons of aged human brains (Kato et al. 1998) and in the atherosclerotic lesions of apolipoprotein E-deficient mice (Kato et al. 2000). NAD(P)H oxidase plays a crucial role in the generation of ROS in vascular cells. NAD(P)H oxidase converts oxygen into superoxide (O_2^-), and O_2^-then dismutates into H_2O_2, an oxidizing substrate for myeloperoxidase. Myeloperoxidase, using H_2O_2 generated by this system, forms a DY cross-link from the tyrosine residue of the target protein (Heinecke 2002). Oxidants derived from the phagocyte NAD(P)H oxidase provide one pathway for generating DY cross-links in vivo. Neutrophils markedly increase their content of protein-bound DY when they are activated in wild-type mice; however, this increase fails to occur in mice that are deficient in phagocyte NAD(P)H oxidase (Bhattacharjee et al. 2001). CRP accumulating in atherosclerotic lesion may mediate the production of DY by increasing NAD(P)H oxidase $p22^{phox}$ expression and ROS generation, since DY was produced in LDL oxidized with tyrosyl radical generated by myeloperoxidase-H_2O_2 system (Heinecke 2002).

References

Bailey JM, Butler J (1973) Anti-inflammatory drugs in experimental atherosclerosis. I. Relative potencies for inhibiting plaque formation. Atherosclerosis 17:515–522

Bharadwaj D, Stein MP, Volzer M et al (1999) The major receptor for C-reactive protein on leukocytes is Fcγ receptor II. J Exp Med 190:585–590

Bhattacharjee S, Pennathur S, Byun J (2001) NADPH oxidase of neutrophils elevates o, o'-dityrosine cross-links in proteins and urine during inflammation. Arch Biochem Biophys 395:69–77

Calabró P, Willerson JT, Yeh ET (2003) Inflammatory cytokines stimulated C-reactive protein production by human coronary artery smooth muscle cells. Circulation 108:1930–1932

Chang MK, Binder CJ, Torzewski M et al (2002) C-reactive protein binds to both oxidized LDL and apoptotic cells through recognition of a common ligand: phosphorylcholine of oxidized phospholipids. Proc Natl Acad Sci U S A 99:13043–13048

de Winther MP, Gijbels MJ, van Dijk KW et al (1999) Scavenger receptor deficiency leads to more complex atherosclerotic lesions in APOE3Leiden transgenic mice. Atherosclerosis 144:315–321

Devaraj S, Singh U, Jialal I (2009) The evolving role of C-reactive protein in atherothrombosis. Clin Chem 55:229–238

Dong Q, Wright JR (1996) Expression of C-reactive protein by alveolar macrophages. J Immunol 156:4815–4820

Duewell P, Kono H, Rayner KJ et al (2010) NLRP3 Inflammasomes are required for atherogenesis and activated by cholesterol crystals. Nature 464:1357–1361

Fukuchi Y, Miura Y, Nabeno Y et al (2008) Immunohistochemical detection of oxidative stress biomarkers, dityrosine and N^{ϵ}-(hexanoyl)lysine, and C-reactive protein in rabbit atherosclerotic lesions. J Atheroscler Thromb 15:185–192

Hatanaka K, Li XA, Masuda K et al (1995) Immuno histochemical localization of C-reactive protein-binding sites in human atherosclerotic aortic lesions by a modified streptavidin-biotin-staining method. Pathol Int 45:635–641

Heinecke JW (2002) Tyrosyl radical production by myeloperoxidase: a phagocyte pathway for lipid peroxidation and dityrosine cross-linking of proteins. Toxicology 177:11–22

Kato Y, Maruyama W, Naoi M et al (1998) Immunohistochemical detection of dityrosine in lipofuscin pigments in the aged human brain. FEBS Lett 439:231–234

Kato Y, Mori Y, Makino Y et al (1999) Formation of N^{ϵ}- (hexanonyl)lysine in protein exposed to lipid hydroperoxide. A plausible marker for lipid hydroperoxide-derived protein modification. J Biol Chem 274:20406–20414

Kato Y, Wu X, Naito M et al (2000) Immunochemical detection of protein dityrosine in atherosclerotic lesion of apo-E-deficient mice using a novel monoclonal antibody. Biochem Biophys Res Commun 275:11–15

Kawai Y, Kato Y, Fujii H et al (2003) Immunochemical detection of a novel lysine adduct using an antibody to linoleic acid hydroperoxide-modified protein. J Lipid Res 44:1124–1131

Kobayashi S, Inoue N, Ohashi Y et al (2003) Interaction of oxidative stress and inflammatory response in coronary plaque instability: important role of C-reactive protein. Arterioscler Thromb Vasc Biol 23:1398–1404

Medeiros LA, Khan T, El Khoury JB et al (2004) Fibrillar amyloid protein present in atheroma activates CD36 signal transduction. J Biol Chem 279:10643–10648

Mold C, Gewurz H, Du Clos TW (1999) Regulation of complement activation by C-reactive protein. Immunopharmacology 42:23–30

Mold C, Gresham HD, Du Clos TW (2001) Serum amyloid P component and C-reactive protein mediate phagocytosis through murine FcγRs. J Immunol 166:1200–1205

Naito M (2000) Effects of fibrinogen, fibrin and their degradation products on the behaviour of vascular smooth muscle cells. Nihon Ronen Igakkai Zasshi 37:458–463 [Article in Japanese, abstract in English]

Naito M, Yasue M, Asai K et al (1992) Effects of dexamethasone on experimental atherosclerosis in cholesterol-fed rabbits. J Nutr Sci Vitaminol (Tokyo) 38:255–264

Rajamäki K, Lappalainen J, Oörni K et al (2010) Cholesterol crystals activate the NLRP3 inflammasome in human macrophages: a novel link between cholesterol metabolism and inflammation. PLoS One 23(5):e11765

Ross R (1981) George Lyman Duff memorial lecture. Atherosclerosis: a problem of the biology of arterial wall cells and their interactions with blood components. Arteriosclerosis 1:293–311

Ross R (1986) The pathogenesis of atherosclerosis – an update. N Engl J Med 314:488–500

Ross R (1993) The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 362:801–809

Ross R (1999) Atherosclerosis — an inflammatory disease. N Engl J Med 340:115–126

Ross R, Glomset JA (1976) The pathogenesis of atherosclerosis. N Engl J Med 295:369–377

Seifert PS, Kazatchkine MD (1988) The complement system in atherosclerosis. Atherosclerosis 73:91–104

Singh SK, Suresh MV, Prayther DC et al (2008) C-reactive protein-bound enzymatically modified low-density lipoprotein does not transform macrophages into foam cells. J Immunol 180:4316–4322

Singh U, Devaraj S, Jialal I (2009) C-reactive protein stimulates myeloperoxidase release from polymorphonuclear cells and monocytes: implications for acute coronary syndromes. Clin Chem 55:361–364

Steinberg D (1997) Lewis A. Conner memorial lecture. Oxidative modification of LDL and atherogenesis. Circulation 95:1062–1071

Steinberg D, Parthasarathy S, Carew TE et al (1989) Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. N Engl J Med 320:915–924

Stocker R, Keaney JF Jr (2004) Role of oxidative modifications in atherosclerosis. Physiol Rev 84:1381–1478

Uchida K, Toyokuni S, Nishikawa K et al (1994) Michael addition-type 4-hydroxy-2-nonenal adducts in modified low-density lipoproteins: markers for atherosclerosis. Biochemistry 33:12487–12494

Uchida K, Sakai K, Itakura K et al (1997) Protein modification by lipid peroxidation products: formation of malondialdehyde-derived N^{ε}- (2-propenol)lysine in proteins. Arch Biochem Biophys 346:45–52

Uchida K, Kanematsu M, Sakai K et al (1998) Protein-bound acrolein: potential markers for oxidative stress. Proc Natl Acad Sci U S A 95:4882–4887

Volanakis JE (1982) Complement activation by C-reactive protein complexes. Ann N Y Acad Sci 389:235–250

Whitman SC, Rateri DL, Szilvassy SJ et al (2002) Macrophage-specific expression of class A scavenger receptors in LDL receptor(−/−) mice decreases atherosclerosis and changes spleen morphology. J Lipid Res 43:1201–1208

Williams KJ, Tabas I (1995) The response-to-retention hypothesis of early atherogenesis. Arterioscler Thromb Vasc Biol 15:551–561

Williams KJ, Tabas I (2005) Lipoprotein retention — and clues for atheroma regression. Arterioscler Thromb Vasc Biol 25:1536–1540

Wright SD, Burton C, Hernandez M et al (2000) Infectious agents are not necessary for murine atherogenesis. J Exp Med 191:1437–1442

Yasojima K, Schwab C, McGeer EG et al (2001) Generation of C-reactive protein and complement components in atherosclerotic plaques. Am J Pathol 158:1039–1051

Chapter 9
Oxidative Modification of Lipoproteins

Hirofumi Arai

Abstract Lipoproteins consist of lipids and apolipoproteins that have functional roles in lipid metabolism. It has been suggested that oxidation of lipoproteins by reactive oxygen species (ROS) may be involved in the inception of various diseases. In particular, the relationship between low-density lipoprotein (LDL) oxidation and atherosclerosis has been studied in great detail. The main target molecules of lipoprotein oxidation are polyunsaturated fatty acid residues of lipids and apolipoproteins. Extensive investigations have characterized oxidative modifications of apolipoprotein B100 (apo B100) in LDL. Furthermore, modifications of apo B100 by oxidized lipids have been confirmed in oxidized LDL and atherosclerotic lesions using immunological techniques. In this chapter, characteristics and oxidation mechanisms of lipoproteins by ROS are described from *in vitro* and *in vivo* studies. Oxidative modifications of apo B100 by lipid hydroperoxides, major products of lipid peroxidation at the early stage, are principally reported.

Keywords Lipoprotein • Lipid • Apolipoprotein • Oxidation • Atherosclerosis

1 Characteristics of Lipoproteins

Lipoproteins are particles composed of lipids and apolipoproteins and are mainly classified on the basis of their density. Lipoproteins transport dietary and storage lipids from the small intestine and the liver to peripheral tissues through the circulation system. In this section, lipoprotein structure, classification, metabolism are described in terms of apolipoproteins functions.

H. Arai (✉)
Department of Biotechnology and Environmental Chemistry,
Kitami Institute of Technology, Kitami, Hokkaido 090-8507, Japan
e-mail: araihrfm@mail.kitami-it.ac.jp

Y. Kato (ed.), *Lipid Hydroperoxide-Derived Modification of Biomolecules*,
Subcellular Biochemistry 77, DOI 10.1007/978-94-007-7920-4_9,

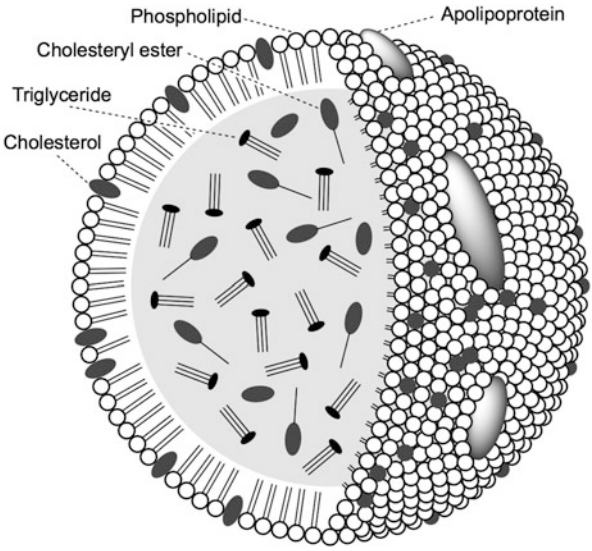


Fig. 9.1 Typical structure of a human lipoprotein. A lipoprotein particle is comprised of a hydrophobic core of triglycerides and cholesterol esters and a hydrophilic shell of phospholipids including apolipoproteins

1.1 Lipoprotein Structure

Lipoprotein particles in human serum contain both lipids and proteins. The general structure of lipoproteins is shown in Fig. 9.1 (Hevonoja et al. 2000). Lipoprotein particles consist of a core of hydrophobic lipids such as triglycerides (TG) and cholesterol esters (CE) covered by a monolayer shell of phospholipids (PL) including apolipoproteins and lipophilic antioxidants such as α-tocopherol. Unesterified cholesterol (UC) is located in both the core (one-third) and the shell (two-thirds). Amphipathic PL and apolipoproteins in the shell are responsible for water-solubility and enable transport of lipoproteins in lymphatic and circulatory systems.

1.2 Lipoprotein Classes

Lipoproteins are generally classified on the basis of their density as shown in Table 9.1 (Berg et al. 2010). Chylomicrons (CM; $d < 0.95$ g/ml), very-low-density lipoproteins (VLDL; $d = 0.95$–1.006), intermediate-density lipoproteins (IDL; $d = 1.006$–1.019), low-density lipoproteins (LDL; $d = 1.019$–1.063), and high-density lipoproteins (HDL; $d = 1.063$–1.210) can be fractionated by ultracentrifugation from blood plasma (Hatch 1968). Lipoprotein density depends on the relative contents of lipid and apolipoprotein and is inversely correlated with lipid contents. The composition of lipoproteins is shown in Table 9.2 (Berg et al. 2010). CM contain large quantities of lipids (98 %, mostly TG), but a very small amount of apolipoproteins (2 %) (Vance and Vance 2008). VLDL include more than 90 %

Table 9.1 Properties of lipoprotein classes

	Density (g/ml)	Diameter (nm)
CM	<0.950	75–1,200
VLDL	0.950–1.006	30–80
IDL	1.006–1.019	15–35
LDL	1.019–1.063	18–25
HDL	1.063–1.210	7.5–20

Abbreviations: *CM* chylomicrons, *VLDL* very-low-density lipoprotein, *IDL* intermediate-density lipoprotein, *LDL* low-density lipoprotein, *HDL* high-density lipoprotein

Table 9.2 Composition of lipoproteins (%)

	Apolipoprotein	TG	CE	PL	UC
CM	2	86	3	8	1
VLDL	8	52	14	18	7
IDL	11	38	30	23	8
LDL	21	10	38	22	8
HDL	33–57	5–10	14–21	19–29	3–7

Abbreviations: *TG* triglyceride, *CE* cholesterol ester, *PL* phospholipid, *UC* unesterified cholesterol

lipids predominantly TG and 8 % apolipoproteins. IDL and LDL, metabolites of VLDL during circulation, have lower lipids and higher apolipoproteins contents than VLDL. The major lipid in LDL is CE (38 %) rather than TG (10 %). HDL contain a higher ratio of apolipoprotein (33–57 %) to lipid than other lipoprotein classes. Although subclasses of LDL have been reported, their classifications are inconsistent. Nonetheless, LDL III and IV (d = 1.043–1.060) are generally considered small dense LDL (sd-LDL) (Hirayama and Miida 2012). In cholesterol-rich LDL, lipoprotein(a) includes apolipoprotein(a) bound covalently to apolipoprotein B100 (apo B100) (Nordestgaard et al. 2010). Notably, serum sd-LDL and lipoprotein(a) concentrations are accepted risk factors for cardiovascular disease.

1.3 Metabolism of Lipoprotein

CM are formed by the exogenous lipids that are absorbed from the epithelium of the small intestine after food intake. As shown in Fig. 9.2, they are secreted into lymph vessels and then veins through the thoracic duct. After TG hydrolysis by lipoprotein lipase (LPL) on the surface of vascular endothelial cells during the circulation, CM provide the resulting fatty acids (FA) for peripheral and adipose tissues. Subsequently, CM become CM remnants and are transported into the liver by remnant receptors.

VLDL are mainly constituted with endogenous lipids in the liver and also supply FA to peripheral and adipose tissues during gradual hydrolysis of TG by LPL in the circulation. VLDL are converted to IDL and then LDL as the density increases by

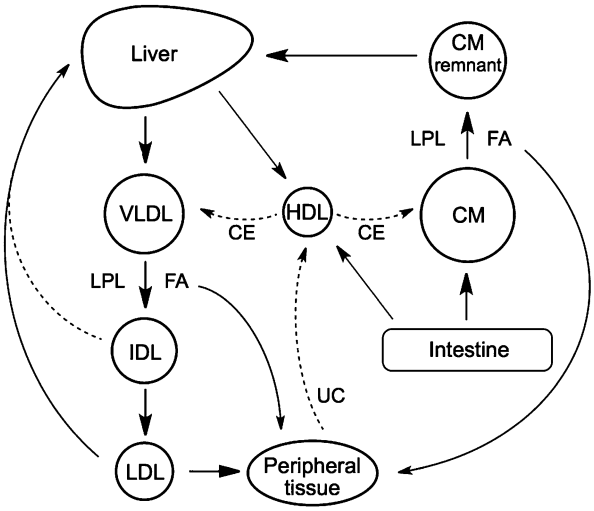


Fig. 9.2 Summary of lipoprotein metabolism. CM are formed in the small intestine and are then secreted into the circulation system. In the circulation, TG in CM are gradually hydrolyzed by lipoprotein lipase (*LPL*) to fatty acids (*FA*), which are absorbed by peripheral tissues. Finally, CM become CM remnants, which are absorbed by the liver. VLDL are produced in the liver, and FA from TG hydrolysis by LPL are then supplied to peripheral tissues. As the density of the lipoprotein particle increases, VLDL turn into IDL and then LDL, which are incorporated by the liver and peripheral tissues

the decrease of TG contents. Consequently, the CE content of LDL is relatively higher than that of TG. LDL particles are finally incorporated into the liver and peripheral tissues.

HDL are synthesized in the liver and the small intestine and are comprised of a relatively higher amount of apolipoproteins than that of other lipoproteins. HDL act to collect UC from peripheral tissues. UC in HDL is esterified to CE by lecithin-cholesterol acyltransferase (LCAT) and the CE is transferred to CM, VLDL, and IDL, which bring it back to the liver.

1.4 Apolipoproteins

Table 9.3 shows the characteristics of major human lipoproteins (Vance and Vance 2008). A function of the apolipoproteins located in the amphipathic PL outer shell of lipoprotein particles is to solubilize lipoproteins including hydrophobic TG and CE in lymph fluid and blood. In fact, apo A1 of HDL has α-helical regions comprised of clusters of both hydrophobic and hydrophilic amino acid residues that contribute to amphipathicity. In addition, some apolipoproteins regulate enzymes of lipoprotein metabolism. For instance, apo A1, apo A4, and apo C1 activate LCAT and apo C2 activates LPL. Apolipoproteins also function

Table 9.3 Characteristics of major human lipoproteins (Vance and Vance 2008)

Apolipoprotein	Molecular weight	Lipoprotein class	Concentration in plasma (mg/dl)	Biosynthesis
A1	28,100	HDL, CM	130	Liver, small intestine
A2	17,400	HDL	40	Liver, small intestine
A4	44,500	CM	15	Liver, small intestine
B48	242,000	CM		Small intestine
B100	512,000	LDL, VLDL	100	Liver
C1	6,600	VLDL, CM, HDL	3	Liver
C2	9,000	VLDL, CM, HDL	12	Liver
C3	9,000	VLDL, CM, HDL	12	Liver
D	22,000	HDL	12	Liver
E	34,200	VLDL, CM, HDL	7	Liver (mainly)

Biosynthesis column was added to the original Table

as ligands for specific lipoprotein receptors and mediate the uptake of lipoproteins by cells. Apo B100, a major apolipoprotein of LDL and VLDL, is a very high molecular weight protein and acts as a ligand for LDL receptors of the liver and peripheral tissues. ApoE of VLDL, HDL, and CM act as a ligand for remnant receptors of the liver.

2 Lipoprotein Oxidation

It has been suggested that reactive oxygen species (ROS) such as hydroxyl radicals directly oxidize amino acid residues of proteins such as arginine, lysine, proline, cysteine, threonine, leucine, and histidine, leading to cleavage of polypeptide chains and formation of cross-linked proteins (Stadtman and Levine 2003). When lipoproteins are exposed to ROS, amino acid residues of apolipoproteins may be oxidized by two pathways, which are direct oxidation by ROS and reactions with oxidized lipids (Fig. 9.3).

LDL, VLDL, and HDL contain a relatively high amount of n-6 polyunsaturated fatty acid residues such as linoleic acid (LA) and arachidonic acid (Hevonoja et al. 2000). Polyunsaturated fatty acids are major targets for ROS-mediated lipid peroxidation, in which lipid hydroperoxides (LOOH) are accumulated by radical chain reactions (Halliwell and Gutteridge 2007). In the presence of transition metal ions such as iron and copper, LOOH are decomposed and a variety of aldehyde compounds are generated as secondary products (Fig. 9.3). Among these, LOOH and aldehydes can induce oxidative modifications of amino acid residues such as lysine, cysteine, and histidine (Kato and Osawa 2010; Sayre et al. 2006). Such oxidative modification leads to protein dysfunctions that may be associated with

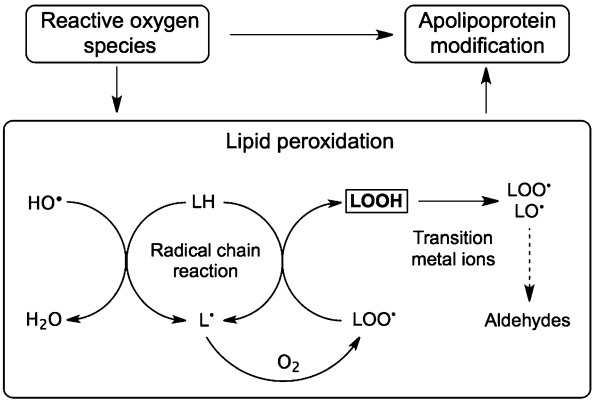


Fig. 9.3 Possible pathways of apolipoproteins oxidation by reactive oxygen species (ROS). Hydrogen of polyunsaturated fatty acids (LH) residues in lipoproteins are abstracted by free radicals such as hydroxyl radicals (HO•). Resulting lipid radicals (L•) react with oxygen molecules and the generated lipid peroxyl radicals (LOO•) react with LH. In this radical chain reaction, lipid hydroperoxides (LOOH) accumulate as the end product. In the presence of transition metal ions, LOOH are decomposed to aldehydes as secondary products. ROS, LOOH, and aldehydes are potential substances to oxidize apolipoproteins

various diseases. This section describes oxidative modifications of apolipoproteins during lipid peroxidation and particularly focuses on reactions between apo B100 in LDL and LOOH.

2.1 Oxidative Modification of Apo B100 in LDL by Lipid Hydroperoxides

It has been suggested that oxidation of LDL may play an important role in the onset of atherosclerosis, although the pathogenic mechanisms remain to be fully elucidated (Steinberg 2009; Yoshida and Kisugi 2010). Excess levels of LDL tend to be accumulated in vascular intima. Under conditions of intima stress, LDL oxidation may be induced by ROS such as superoxide anions (Heinecke et al. 1986; Steinbrecher 1988; Darley-Usmar et al. 1992) and peroxynitrite (Stanbro 2000; Rubbo et al. 2002; Botti et al. 2005), metal ions such as copper (Burkitt 2001; Patel and Darley-Usmar 1999; Ferns et al. 1997) and iron (Yuan and Brunk 1998; Chau 2000; Arai et al. 2005a), and enzymes such as myeloperoxidase (MPO) (Heinecke 1997; Podrez et al. 2000; Carr et al. 2000) and lipoxygenase (LOX) (Harats et al. 1995; Yamashita et al. 1999; Wittwer and Hersberger 2007), although *in vivo* mechanisms of LDL oxidation are unclear. Oxidized LDL (Ox-LDL) particles are ingested by monocyte-derived macrophages through scavenger receptors, which lead to foam cell formation with inflammatory cytokines secretion.

These foam cells can develop into fatty streaks and then fibrous plaques (Halliwell and Gutteridge 2007).

Analytical methods for determining Ox-LDL have been developed in many studies (Yamaguchi et al. 2002). Polyunsaturated fatty acid residues of esterified lipids (CE, PL and TG) and apo B100 are two main targets for LDL oxidation. It is accepted that lipid peroxidation is induced by free radicals and lipid hydroperoxides such as cholesteryl ester hydroperoxides (CE-OOH) and phospholipid hydroperoxides (PL-OOH) are formed (Vance and Vance 2008) as major products by radical chain reactions at the early stage of LDL oxidation. Then oxidized free fatty acids can be released from the *sn*-2 position of PL-OOH by phospholipase A_2 (PLA_2) or Ca^{2+}-independent lipoprotein associated PLA_2 (Levitan et al. 2010). For *in vitro* assay of LDL oxidation induced by free radicals such as azo compounds, LOOH such as CE-OOH and PL-OOH are determined by high performance liquid chromatography (HPLC) with UV detection for conjugated dienes as a general index. More sensitive and specific measurements of LOOH in LDL are achieved using HPLC with chemiluminescence (Miyazawa et al. 1990; Yamaguchi et al. 1999) and electrochemical detection (Thomas et al. 1994). These methods have been applied to estimate LOOH in intact human LDL, which may be derived from minimally oxidized circulating LDL. LOOH are decomposed to aldehydes in the presence of transition metal ions, and this results in the production of thiobarbituric acid reactive substances (TBARS) such as malondialdehyde (MDA). TBARS are also determined as a major index in oxidized human plasma and LDL using absorption photometry or HPLC with fluorescence detection (Bonnefont et al. 1989; Fukunaga et al. 1998).

LDL particles present a single molecule of apo B100 on their surfaces (Segrest et al. 2001). Apo B100 contains a binding site for the LDL receptor, which is rich in positively charged amino acid residues (Boren et al. 1998). Oxidative modification of apo B100 causes electronegativity, fragmentation, and polymerizationas observed by agarose gel and polyacrylamide gel electrophoresis. Protein carbonyls analysis that is based on derivatization with 2,4-dinitrophenylhydrazineis widely used as a marker to detect oxidized proteins (Requena et al. 2003; Levine et al. 2000) and have been applied to Ox-LDL (Hazell et al. 1994). To specifically detect modified apo B100 of LDL oxidized by LOOH and aldehydes, various antibodies against oxidized proteins have been utilized in western blotting (WB) and enzyme-linked immunosorbent assay (Itabe 2009; Kato and Osawa 2010). Itabe et al. developed the monoclonal antibody (mAb) DLH3 that recognizes oxidized phosphatidylcholine-modified proteins, which have been found in human arteriosclerotic lesions using immunohistochemical analyses (Itabe et al. 1994, 1996). In addition, Palinski et al. established the mAb E0 against Ox-LDL and demonstrated its use for immunostaining of human atherosclerotic lesions (Palinski et al. 1996). Kim et al. demonstrated that a polyclonal antibody was raised against LOOH-modified proteins and applied to Ox-LDL and human atherosclerotic lesions (Kim et al. 1997).

Structure analyses of modified apo B100 in Ox-LDL have been conducted using immunological techniques. Kato et al. established polyclonal antibodies against 13-hydroperoxyoctadecadienoic acid (13-HPODE)-modified protein and 15-hydroperoxyeicosatetraenoic acid-modified protein, which recognized modified

Table 9.4 Antibodies against amides adducts and their application to biological samples

Target	Amide-type	Fatty acid	Antibody	Application
HEL	Alkyl	n-6	Polyclonal	Ox-LDL, atherosclerotic lesion
AZL	Carboxyalkyl	n-6	Monoclonal, 19D5	Ox-LDL, atherosclerotic aorta
SUL	Carboxyalkyl	n-3	Monoclonal, 2B12	Oxidized liver
PRL	Alkyl	n-3	Monoclonal, G811	Atherosclerotic lesion

Abbreviations: *HEL* N^{ε}-(hexanoyl)lysine, *AZL* N^{ε}-(azelayl)lysine, *SUL* N^{ε}-(succinyl)lysine, *PRL* N^{ε}-(propanoyl)lysine

apo B100 in LDL that had been oxidized by copper ions or peroxyl radicals generated from azo compound *in vitro* (Kato et al. 1997; Kato and Osawa 1998). Antigenicities of 13-HPODE-modified proteins were significantly enhanced by the alkaline treatments of Ox-LDL, suggesting that oxidized esterified fatty acids covalently bound to apo B100 during LDL oxidation. Osawa and colleagues identified amide-type adducts from the reaction between oxidized polyunsaturated fatty acids and proteins and developed antibodies against them for application to biological samples (Table 9.4). They confirmed that N^{ε}-(hexanoyl)lysine (HEL) and N^{ε}-(azelayl)lysine (AZL) are the recognition moieties of 13-HPODE-modified proteins (Kato et al. 1999; Kawai et al. 2003a). Moreover, HEL was observed in Ox-LDL *in vitro* and in human atherosclerotic lesions using a polyclonal antibody against synthetic hexanoyl proteins. Immunopositive substances against AZL were also detected in Ox-LDL and human atherosclerotic aorta after treatment with alkali or PLA_2 (Kawai et al. 2004). HEL and AZL are specifically derived from the reaction between oxidized n-6 polyunsaturated fatty acids and lysine residues of proteins, whereas N^{ε}-(succinyl)lysine (SUL) and N^{ε}-(propanoyl)lysine (PRL) were determined as major moieties of the protein adducts with oxidized n-3 polyunsaturated fatty acids such as docosahexaenoic acid (Kawai et al. 2006; Hisaka et al. 2009). Although it may be difficult to detect SUL and PRL in Ox-LDL because the concentrations of n-3 polyunsaturated fatty acids in LDL are very low, PRL was recognized in rabbit atherosclerotic plaques using the mAb G811. Taken together, these data indicate that LOOH derived from the oxidation of polyunsaturated fatty acid residues in LDL may be a central pathogenic mechanism in atherosclerosis. Furthermore, aldehydes produced from the decomposition of LOOH can modify protein-based lysine ε-amino group (Uchida 2003). In fact, Uchida et al. developed antibodies against 4-hydroxy-2-nonenal (HNE)-lysine, MDA-lysine, and acrolein-lysine adducts, which were applied to Ox-LDL and atherosclerotic lesions (Uchida et al. 1994, 1997, 1998). Kawai et al. also observed proteins that were modified by 9-oxononanoylcholesterol, a major aldehyde in oxidized CE, in Ox-LDL and atherosclerotic lesions (Kawai et al. 2003b).

2.2 Oxidation of Other Lipoproteins

VLDL are also oxidized by ROS, which may also be a risk factor for atherosclerosis (Keidar et al. 1992; Guha and Gursky 2010). In addition to apo B100, VLDL

particles contain apo E and C. Oxidation of VLDL *in vitro* causes fragmentation and aggregation of these apolipoproteins, which are detected as a smear band by polyacrylamide gel electrophoresis. Nevertheless, HNE and acrolein modified proteins were observed in oxidized VLDL (Ox-VLDL) by WB using anti-HNE- and anti-acrolein-Michael adducts antibodies (Arai et al. 1999, 2005b). Modified apo E of Ox-VLDL lost binding activities to heparin and human hepatocyte, suggesting that functional amino acid residues of apo E were oxidized.

It has been suggested that HDL suppress the development of atherosclerosis, which is related to cholesterol efflux in the reverse cholesterol transport pathway (Wang and Briggs 2004). Various *in vivo* mechanisms of HDL oxidation have been proposed (Francis 2000), and among these myeloperoxidase (MPO)-catalyzed oxidation may be a remarkable process (Malle et al. 2006; Heinecke 2007). Hypochlorite generated by MPO in human atherosclerotic lesions mediates nitration and chlorination of tyrosine residues in apo A1, a major apolipoprotein in HDL. Modification of apo A1 in oxidized HDL impairs their ability to remove excess cellular cholesterol by the ATP-binding cassette transporter ABC1 pathway.

3 Conclusion

ROS-mediated oxidation of lipoproteins such as LDL, VLDL, and HDL is characterized by lipid peroxidation and apolipoproteins modification. In particular, the mechanisms behind oxidative modification of apo B100 in LDL have been studied extensively, and it is suggested that Ox-LDL formation with lipid oxidation products such as LOOH may play an important role in the pathogenesis of atherosclerosis. Immunological techniques have been utilized to detect LOOH-modified proteins and in the future may be used as diagnostic tools for ROS related diseases such as atherosclerosis.

References

Arai H, Kashiwagi S, Nagasaka Y, Uchida K, Hoshii Y, Nakamura K (1999) Oxidative modification of apolipoprotein E in human very-low-density lipoprotein and its inhibition by glycosaminoglycans. Arch Biochem Biophys 367(1):1–8

Arai H, Berlett BS, Chock PB, Stadtman ER (2005a) Effect of bicarbonate on iron-mediated oxidation of low-density lipoprotein. Proc Natl Acad Sci U S A 102(30):10472–10477

Arai H, Uchida K, Nakamura K (2005b) Effect of ascorbate on acrolein modification of very low density lipoprotein and uptake of oxidized apolipoprotein E by hepatocytes. Biosci Biotechnol Biochem 69(9):1760–1762

Berg JM, Tymoczko JL, Stryer L (2010) Biochemistry, 7th edn. W. H. Freeman and Company, New York

Bonnefont D, Legrand A, Peynet J, Emerit J, Delattre J, Galli A (1989) Distribution of thiobarbituric acid-reactive substances in lipoproteins and proteins in serum. Clin Chem 35(10):2054–2058

Boren J, Lee I, Zhu W, Arnold K, Taylor S, Innerarity TL (1998) Identification of the low density lipoprotein receptor-binding site in apolipoprotein B100 and the modulation of its binding activity by the carboxyl terminus in familial defective apo-B100. J Clin Invest 101(5):1084–1093

Botti H, Trostchansky A, Batthyany C, Rubbo H (2005) Reactivity of peroxynitrite and nitric oxide with LDL. IUBMB Life 57(6):407–412

Burkitt MJ (2001) A critical overview of the chemistry of copper-dependent low density lipoprotein oxidation: roles of lipid hydroperoxides, alpha-tocopherol, thiols, and ceruloplasmin. Arch Biochem Biophys 394(1):117–135

Carr AC, McCall MR, Frei B (2000) Oxidation of LDL by myeloperoxidase and reactive nitrogen species: reaction pathways and antioxidant protection. Arterioscler Thromb Vasc Biol 20(7):1716–1723

Chau LY (2000) Iron and atherosclerosis. Proc Natl Sci Counc Repub China B 24(4):151–155

Darley-Usmar VM, Hogg N, O'Leary VJ, Wilson MT, Moncada S (1992) The simultaneous generation of superoxide and nitric oxide can initiate lipid peroxidation in human low density lipoprotein. Free Radic Res Commun 17(1):9–20

Ferns GA, Lamb DJ, Taylor A (1997) The possible role of copper ions in atherogenesis: the Blue Janus. Atherosclerosis 133(2):139–152

Francis GA (2000) High density lipoprotein oxidation: in vitro susceptibility and potential in vivo consequences. Biochim Biophys Acta 1483(2):217–235

Fukunaga K, Yoshida M, Nakazono N (1998) A simple, rapid, highly sensitive and reproducible quantification method for plasma malondialdehyde by high-performance liquid chromatography. Biomed Chromatogr 12(5):300–303

Guha M, Gursky O (2010) Effects of oxidation on structural stability and remodeling of human very low density lipoprotein. Biochemistry 49(44):9584–9593

Halliwell B, Gutteridge JMC (2007) Free radicals in biology and medicine, 4th edn. Oxford University Press, New York

Harats D, Mulkins MA, Sigal E (1995) A possible role for 15-lipoxygenase in atherogenesis. Trends Cardiovasc Med 5(1):29–36

Hatch FT (1968) Practical methods for plasma lipoprotein analysis. Adv Lipid Res 6:1–68

Hazell LJ, van den Berg JJ, Stocker R (1994) Oxidation of low-density lipoprotein by hypochlorite causes aggregation that is mediated by modification of lysine residues rather than lipid oxidation. Biochem J 302(Pt 1):297–304

Heinecke JW (1997) Pathways for oxidation of low density lipoprotein by myeloperoxidase: tyrosyl radical, reactive aldehydes, hypochlorous acid and molecular chlorine. Biofactors 6(2):145–155

Heinecke JW (2007) The role of myeloperoxidase in HDL oxidation and atherogenesis. Curr Atheroscler Rep 9(4):249–251

Heinecke JW, Baker L, Rosen H, Chait A (1986) Superoxide-mediated modification of low density lipoprotein by arterial smooth muscle cells. J Clin Invest 77(3):757–761

Hevonoja T, Pentikainen MO, Hyvonen MT, Kovanen PT, Ala-Korpela M (2000) Structure of low density lipoprotein (LDL) particles: basis for understanding molecular changes in modified LDL. Biochim Biophys Acta 1488(3):189–210

Hirayama S, Miida T (2012) Small dense LDL: an emerging risk factor for cardiovascular disease. Clin Chim Acta 414:215–224

Hisaka S, Kato Y, Kitamoto N, Yoshida A, Kubushiro Y, Naito M, Osawa T (2009) Chemical and immunochemical identification of propanoyllysine derived from oxidized n-3 polyunsaturated fatty acid. Free Radic Biol Med 46(11):1463–1471

Itabe H (2009) Oxidative modification of LDL: its pathological role in atherosclerosis. Clin Rev Allergy Immunol 37(1):4–11

Itabe H, Takeshima E, Iwasaki H, Kimura J, Yoshida Y, Imanaka T, Takano T (1994) A monoclonal antibody against oxidized lipoprotein recognizes foam cells in atherosclerotic

lesions. Complex formation of oxidized phosphatidylcholines and polypeptides. J Biol Chem 269(21):15274–15279
Itabe H, Yamamoto H, Suzuki M, Kawai Y, Nakagawa Y, Suzuki A, Imanaka T, Takano T (1996) Oxidized phosphatidylcholines that modify proteins. Analysis by monoclonal antibody against oxidized low density lipoprotein. J Biol Chem 271(52):33208–33217
Kato Y, Osawa T (1998) Detection of oxidized phospholipid-protein adducts using anti-15-hydroperoxyeicosatetraenoic acid-modified protein antibody: contribution of esterified fatty acid-protein adduct to oxidative modification of LDL. Arch Biochem Biophys 351(1):106–114
Kato Y, Osawa T (2010) Detection of lipid-lysine amide-type adduct as a marker of PUFA oxidation and its applications. Arch Biochem Biophys 501(2):182–187
Kato Y, Makino Y, Osawa T (1997) Characterization of a specific polyclonal antibody against 13-hydroperoxyoctadecadienoic acid-modified protein: formation of lipid hydroperoxide-modified apoB-100 in oxidized LDL. J Lipid Res 38(7):1334–1346
Kato Y, Mori Y, Makino Y, Morimitsu Y, Hiroi S, Ishikawa T, Osawa T (1999) Formation of Nepsilon-(hexanonyl)lysine in protein exposed to lipid hydroperoxide. A plausible marker for lipid hydroperoxide-derived protein modification. J Biol Chem 274(29):20406–20414
Kawai Y, Kato Y, Fujii H, Makino Y, Mori Y, Naito M, Osawa T (2003a) Immunochemical detection of a novel lysine adduct using an antibody to linoleic acid hydroperoxide-modified protein. J Lipid Res 44(6):1124–1131
Kawai Y, Saito A, Shibata N, Kobayashi M, Yamada S, Osawa T, Uchida K (2003b) Covalent binding of oxidized cholesteryl esters to protein: implications for oxidative modification of low density lipoprotein and atherosclerosis. J Biol Chem 278(23):21040–21049
Kawai Y, Fujii H, Kato Y, Kodama M, Naito M, Uchida K, Osawa T (2004) Esterified lipid hydroperoxide-derived modification of protein: formation of a carboxyalkylamide-type lysine adduct in human atherosclerotic lesions. Biochem Biophys Res Commun 313(2):271–276
Kawai Y, Fujii H, Okada M, Tsuchie Y, Uchida K, Osawa T (2006) Formation of Nepsilon-(succinyl)lysine in vivo: a novel marker for docosahexaenoic acid-derived protein modification. J Lipid Res 47(7):1386–1398
Keidar S, Kaplan M, Rosenblat M, Brook GJ, Aviram M (1992) Apolipoprotein E and lipoprotein lipase reduce macrophage degradation of oxidized very-low-density lipoprotein (VLDL), but increase cellular degradation of native VLDL. Metabolism 41(11):1185–1192
Kim JG, Sabbagh F, Santanam N, Wilcox JN, Medford RM, Parthasarathy S (1997) Generation of a polyclonal antibody against lipid peroxide-modified proteins. Free Radic Biol Med 23(2):251–259
Levine RL, Wehr N, Williams JA, Stadtman ER, Shacter E (2000) Determination of carbonyl groups in oxidized proteins. Methods Mol Biol 99:15–24
Levitan I, Volkov S, Subbaiah PV (2010) Oxidized LDL: diversity, patterns of recognition, and pathophysiology. Antioxid Redox Signal 13(1):39–75
Malle E, Marsche G, Panzenboeck U, Sattler W (2006) Myeloperoxidase-mediated oxidation of high-density lipoproteins: fingerprints of newly recognized potential proatherogenic lipoproteins. Arch Biochem Biophys 445(2):245–255
Miyazawa T, Fujimoto K, Oikawa S (1990) Determination of lipid hydroperoxides in low density lipoprotein from human plasma using high performance liquid chromatography with chemiluminescence detection. Biomed Chromatogr 4(3):131–134
Nordestgaard BG, Chapman MJ, Ray K, Boren J, Andreotti F, Watts GF, Ginsberg H, Amarenco P, Catapano A, Descamps OS, Fisher E, Kovanen PT, Kuivenhoven JA, Lesnik P, Masana L, Reiner Z, Taskinen MR, Tokgozoglu L, Tybjaerg-Hansen A (2010) Lipoprotein (a) as a cardiovascular risk factor: current status. Eur Heart J 31(23):2844–2853
Palinski W, Horkko S, Miller E, Steinbrecher UP, Powell HC, Curtiss LK, Witztum JL (1996) Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma. J Clin Invest 98(3):800–814

Patel RP, Darley-Usmar VM (1999) Molecular mechanisms of the copper dependent oxidation of low-density lipoprotein. Free Radic Res 30(1):1–9
Podrez EA, Abu-Soud HM, Hazen SL (2000) Myeloperoxidase-generated oxidants and atherosclerosis. Free Radic Biol Med 28(12):1717–1725
Requena JR, Levine RL, Stadtman ER (2003) Recent advances in the analysis of oxidized proteins. Amino Acids 25(3–4):221–226
Rubbo H, Trostchansky A, Botti H, Batthyany C (2002) Interactions of nitric oxide and peroxynitrite with low-density lipoprotein. Biol Chem 383(3–4):547–552
Sayre LM, Lin D, Yuan Q, Zhu X, Tang X (2006) Protein adducts generated from products of lipid oxidation: focus on HNE and one. Drug Metab Rev 38(4):651–675
Segrest JP, Jones MK, De Loof H, Dashti N (2001) Structure of apolipoprotein B-100 in low density lipoproteins. J Lipid Res 42(9):1346–1367
Stadtman ER, Levine RL (2003) Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. Amino Acids 25(3–4):207–218
Stanbro WD (2000) Modeling the interaction of peroxynitrite with low-density lipoproteins. II: reaction/diffusion model of peroxynitrite in low-density lipoprotein particles. J Theor Biol 205 (3):465–471
Steinberg D (2009) The LDL modification hypothesis of atherogenesis: an update. J Lipid Res 50 (Suppl):S376–S381
Steinbrecher UP (1988) Role of superoxide in endothelial-cell modification of low-density lipoproteins. Biochim Biophys Acta 959(1):20–30
Thomas JP, Kalyanaraman B, Girotti AW (1994) Involvement of preexisting lipid hydroperoxides in Cu(2+)-stimulated oxidation of low-density lipoprotein. Arch Biochem Biophys 315(2):244–254
Uchida K (2003) Histidine and lysine as targets of oxidative modification. Amino Acids 25(3–4):249–257
Uchida K, Toyokuni S, Nishikawa K, Kawakishi S, Oda H, Hiai H, Stadtman ER (1994) Michael addition-type 4-hydroxy-2-nonenal adducts in modified low-density lipoproteins: markers for atherosclerosis. Biochemistry 33(41):12487–12494
Uchida K, Sakai K, Itakura K, Osawa T, Toyokuni S (1997) Protein modification by lipid peroxidation products: formation of malondialdehyde-derived N(epsilon)-(2-propenol)lysine in proteins. Arch Biochem Biophys 346(1):45–52
Uchida K, Kanematsu M, Morimitsu Y, Osawa T, Noguchi N, Niki E (1998) Acrolein is a product of lipid peroxidation reaction. Formation of free acrolein and its conjugate with lysine residues in oxidized low density lipoproteins. J Biol Chem 273(26):16058–16066
Vance DE, Vance JE (2008) Biochemistry of lipids, lipoproteins and membranes, 5th edn. Elsevier, Amsterdam
Wang M, Briggs MR (2004) HDL: the metabolism, function, and therapeutic importance. Chem Rev 104(1):119–137
Wittwer J, Hersberger M (2007) The two faces of the 15-lipoxygenase in atherosclerosis. Prostaglandins Leukot Essent Fatty Acids 77(2):67–77
Yamaguchi Y, Kagota S, Kunitomo M, Haginaka J (1999) High-performance liquid chromatographic assay of hydroperoxide levels in oxidatively modified lipoproteins. J Chromatogr B 731(2):223–229
Yamaguchi Y, Kunitomo M, Haginaka J (2002) Assay methods of modified lipoproteins in plasma. J Chromatogr B 781(1–2):313–330
Yamashita H, Nakamura A, Noguchi N, Niki E, Kuhn H (1999) Oxidation of low density lipoprotein and plasma by 15-lipoxygenase and free radicals. FEBS Lett 445(2–3):287–290
Yoshida H, Kisugi R (2010) Mechanisms of LDL oxidation. Clin Chim Acta 411(23–24):1875–1882
Yuan XM, Brunk UT (1998) Iron and LDL-oxidation in atherogenesis. APMIS 106(9):825–842

Chapter 10
Immunochemical Detection of Lipid Hydroperoxide- and Aldehyde-Modified Proteins in Diseases

Akihiko Sugiyama and Jing Sun

Abstract Polyunsaturated fatty acid (PUFA) is easily peroxidized by free radicals and enzymes. When this occurs, it results in the compromised integrity of cellular membranes and leads to lipid hydroperoxide as a major reaction product, which is decomposed into aldehyde. Lipid hydroperoxide-modified lysine is known to be an early product of the lipid peroxidation process, suggesting that it might be a PUFA-oxidative stress marker during the initial stage of oxidative stress. Lipid hydroperoxides cause or enhance ROS-mediated DNA fragmentation. The α,β-unsaturated aldehydes are end products of PUFA peroxidation. They are highly reactive and readily attack and modify the protein amino acid residues into aldehyde-modified proteins. Lipid peroxidation-derived α,β-unsaturated aldehydes are capable of inducing cellular stress-responsive processes such as cell signaling and apoptosis. The lipid hydroperoxide- and aldehyde-modified proteins have been immunohistochemically detected in diverse pathological situations such as atherosclerosis, Alzheimer's disease, Parkinson's disease, and chemical material-induced liver injury and renal tubular injury in humans and experimental animals. These findings suggest that the expression of the lipid hydroperoxide- and aldehyde-modified proteins is closely associated with the pathogenesis of these diseases in humans and experimental animals.

Keywords Aldehyde • Immunohistochemistry • Lipid hydroperoxide • Oxidation • Polyunsaturated fatty acid

A. Sugiyama (✉) • J. Sun
Course of Veterinary Laboratory Medicine, School of Veterinary Medicine, Faculty of Agriculture, Tottori University, Minami 4-101 m Koyama-cho, Tottori 680-8553, Japan
e-mail: sugiyama@muses.tottori-u.ac.jp

Y. Kato (ed.), *Lipid Hydroperoxide-Derived Modification of Biomolecules*,
Subcellular Biochemistry 77, DOI 10.1007/978-94-007-7920-4_10,

1 Lipid Hydroperoxide- and Aldehyde-Modified Proteins

Polyunsaturated fatty acid (PUFA) is easily peroxidized by free radicals and enzymes. When this occurs, it results in compromised integrity of cellular membranes and leads to lipid hydroperoxide as a major reaction product, which is then decomposed into aldehyde (Kato and Osawa 2010).

Lipid hydroperoxides such as N^{ε}-hexanoyl, N^{ε}-propanoyl, N^{ε}-azelayl, N^{ε}-glutaroyl and N^{ε}-Succinyl can be generated from PUFA peroxidized by free radicals (Kato and Osawa 2010). These lipid hydroperoxides react with lysine to form lipid hydroperoxide-modified proteins such as N^{ε}-hexanoyl-modified lysine (HEL), N^{ε}-propanoyl-modified lysine (PRL), N^{ε}-azelayl-modified lysine (AZL), N^{ε}-glutaroyl-modified lysine (GLL) or N^{ε}-Succinyl-modified lysine (SUL) (Kato and Osawa 2010). Lipid hydroperoxide-modified lysine is an early product of the lipid peroxidation process, suggesting that it might be a PUFA-oxidative stress marker during the initial stage of oxidative stress (Kato and Osawa 2010). HEL and PRL are classified as a group of alkylamide-type adducts, whereas AZL, GLL and SUL are classified as carboxyalkylamide-type adducts (Kato and Osawa 2010). HEL is formed by the reaction of lysine with lipid hydroperoxide derived from linoleic acid or arachidonic acid (Kato et al. 1999). PRL is formed by the reaction of lysine with lipid hydroperoxide derived from docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) or α-linolenic acid (Hisaka et al. 2009). SUL is an N-acyl carboxylic adduct composed of a C4 unit from the COOH terminus of DHA (Kawai et al. 2006). AZL is formed by oxidized linoleic acid and α-linolenic acid (Kato and Osawa 2010), while GLL is formed by oxidized arachidonic acid and EPA (Kato and Osawa 2010). Lipid hydroperoxides cause or enhance ROS-mediated DNA fragmentation (Higuchi 2003).

The aldehydes such as acrolein, 4-hydroxy-2-nonenal (HNE), malondialdehyde (MDA), 4-hydroxyhexenal (HHE), crotonaldehyde (CRA), 4-oxo-2-nonenal (ONE), and glyoxal are end products of the peroxidation of PUFA (Kato and Osawa 2010). They are classified as α,β-unsaturated aldehydes, which are highly reactive and readily attack and modify the protein amino acid residues to protein-bound aldehydes such as acrolein-modified lysine, HHE-modified histidine, CRA-modified lysine and MDA-modified lysine (Kato and Osawa 2010; Uchida 2000). Lipid peroxidation-derived α,β-unsaturated aldehydes are capable of inducing cellular stress-responsive processes such as cell signaling and apoptosis (Lee et al. 2004; Liu et al. 2010). Acrolein is a potent alkylating agent that reacts with matrix tissue or cell surface proteins and alters the structure and function of matrix proteins (Uchida 1999). It also reduces the intracellular GSH level (Uchida 1999). These events activate stress-signaling pathways via protein phosphorylations (Uchida 1999). HNE demonstrates a wide range of biologic activities, including inhibition of protein and DNA synthesis, inactivation of enzymes, stimulation of phospholipase C, reduction of gap junction communication, and stimulation of neutrophil migration (Uchida 1999). HHE induces apoptosis of endothelial cells, which is mediated by the enhancement of apoptotic Bax and the suppression of anti-apoptotic Bcl-2 by peroxynitrite generation (Lee et al. 2004). HHE induces mitochondrial permeability transition (MPT), which

leads to the breakdown of the mitochondrial membrane potential, the inability to synthesize ATP, and finally cell death (Kim et al. 2003; Kristal et al. 1996). HHE also depletes neuronal glutathione (GSH) content and neuronal reactive oxygen species (ROS) in rat cerebral cortical neurons (Long et al. 2008). CRA can penetrate through the cell membrane and bind to GSH without any metabolic activation (Liu et al. 2010). A reduced GSH level leads to imbalance of cellular redox and causes increases of ROS and apoptosis (Liu et al. 2010). CRA-induced apoptosis is mediated via cytochrome c release and caspase cascade (Liu et al. 2010). CRA causes both apoptosis and necrosis, and there is a transition from apoptosis to necrosis corresponding with increasing the CRA concentration (Liu et al. 2010).

These lipid hydroperoxide- and aldehyde-modified proteins have been immunohistochemically detected in diverse diseases in both humans and experimental animals.

2 Atherosclerosis

In humans, atherosclerosis and its complications, i.e., myocardial infarction, stroke, and peripheral vascular diseases, are major causes of morbidity and mortality in the Western world (Grant Maxie and Robinson 2007). Atherosclerosis affects the large elastic arteries (aorta and iliac) and the large and medium muscular arteries (carotid, coronary and femoral). The essential lesion is the atheroma or fibrofatty plaque, which is a focal, raised, intimal plaque with a lipid core (mainly cholesterol and its esters) covered by a fibrous cap (Grant Maxie and Robinson 2007).

Multiple pathogenetic influences can contribute to the development of atherosclerosis (Grant Maxie and Robinson 2007). Oxidised low-density lipoprotein-cholesterol (ox-LDL) plays a major role in the initiation and progression of atherosclerosis (Mitra et al. 2011). Formed by oxidative stress, ox-LDL also triggers the generation of reactive oxygen species (ROS) from a variety of cell types, and contributes to oxidative stress (Mitra et al. 2011). Previous studies have demonstrated many risk factors for atherosclerosis that induce oxidative stress in the vessel wall, including smoking (Bernhard and Wang 2007), diabetes mellitus (Nicolls et al. 2007), dyslipidemia (Mügge et al. 1994), hypertension (Huang et al. 1998), and periodontitis (Ekuni et al. 2009). Lipid peroxidation is involved in the development of atherosclerosis. There is an increase in the levels of serum malondialdehyde in human patients with atherosclerosis and in the rat atherosclerosis model, compared to those of controls (Tamer et al. 2002; Ekuni et al. 2009). There is immunohistochemical evidence of HEL presence in atherosclerotic lesions of humans (Kato et al. 1999), cholesterol-fed rabbits (Fukuchi et al. 2008) and periodontitis model rats (Ekuni et al. 2009). PRL has been immunohistochemically detected in atherosclerotic lesions of hypercholesterolemic rabbits (Hisaka et al. 2009). The presence of AZL was also immunohistochemically detected in the atherosclerotic lesions of humans and rabbits (Kawai et al. 2003, 2004). Immunoexpression of acrolein-modified keyhole limpet hemocyanin (KLH) have

been detected in atherosclerotic lesions from a human aorta (Uchida et al. 1998). It is speculated that HEL, PRL, AZL and acrolein-modified protein may be involved in the pathogenesis of atherosclerosis.

3 Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurologic disorder characterized clinically as a cognitive impairment that includes memory impairment and at least one of the following cognitive disturbances: aphasia, apraxia, agnosia, or disturbance in executive functioning (Zarkovic 2003). The pathology of AD is dominated by neuronal loss and the formation of amyloid-containing neuritic (senile) plaque and neurofibrillary tangles in the frontal cortex and hippocampus (Zarkovic 2003). Immunoreactive intensity of the HNE-histidine adduct in CA2, CA3 and CA4 sectors in the hippocampi was significantly higher in AD patients than in the controls (Fukuda et al. 2009). Strong immunoexpression of acrolein-modified KLH occurred in more than half of the neurofibrillary tangles in AD patients (Calingasan et al. 1999). These results show that pyramidal neurons in these sectors of hippocampi and the neurofibrillary of AD patients are prone to undergo lipid peroxidation. The production of cytotoxic products such as HNE and acrolein may be responsible for the pathogenesis of AD (Fukuda et al. 2009).

4 Parkinson's Disease

Parkinson's disease (PD) is a chronic progressive neurodegenerative movement disorder characterized by a profound and selective loss of nigrostriatal dopaminergic neurons in humans (Jenner 2003). Clinical signs of PD include motor impairments involving resting tremor, a slowing of physical movement, postural instability, gait difficulty, and rigidity (Jomova et al. 2010). The most striking pathological feature of PD is a progressive loss of dopaminergic neurons in the substantia nigra, leading to dopamine deficit in the striatum (Jomova et al. 2010). One of the pathological hallmarks of PD is the presence of intracellular inclusions of Lewy bodies that consist of aggregates of α-synuclein (Jomova et al. 2010). The toxic effects of α-synuclein include impaired endoplasmic reticulum (ER) to Golgi vesicular trafficking and ER stress, Golgi fragmentation, sequestration of anti-apoptotic proteins into aggregates, and the formation of pores on cellular membranes (Cooper et al. 2006).

Oxidative stress has been implicated as one of the important contributors to nigral cell death in PD (Yoritaka et al. 1996). In the previous study, a significantly higher proportion of nigral melanized neurons was positively immunostained for HNE-modified protein in PD than in the control patients (Yoritaka et al. 1996). Immunolocalization of HNE-adduct was demonstrated in Lewy bodies of PD and in

diffuse Lewy body disease (Castellani et al. 2002). The previous study revealed that in the dopamine neurons of the substantia nigra containing neuromelanin obtained from PD patients, acrolein-adduct co-localized with α-synuclein, which was then modified by acrolein with inhibition of proteasome activity (Shamamoto-Nagai et al. 2007). Those previous studies indicate that α,β-unsaturated aldehyde such as HNE and acrolein may be related to the pathogenesis of PD.

5 Carbon Tetrachloride-Induced Liver Injury

Carbon tetrachloride (CCl_4) once was widely used as a solvent, cleaner, and degreaser both for industrial and home use (Weber et al. 2003). Today CCl_4 proves itself useful in experimental models and induces liver injury in many species, including non-human primates (Yoshida et al. 1999), and humans (Weber et al. 2003). The liver is the principal site for CCl_4-induced effects to manifest themselves (Weber et al. 2003). Within hours after the administration of CCl_4, hepatic steatosis and central lobular necrosis are induced (Hartley et al. 1999). Endoplasmic reticulum, plasma membrane, mitochondria, and Golgi apparatus are the main subcellular structures of hepatocytes affected by CCl_4 (Reynolds 1963). CCl_4 metabolism begins with the formation of the trichloromethyl free radical, CCl_3^*, through the action of the mixed function cytochrome P450 oxygenase system of the endoplasmic reticulum (Recknagel et al. 1989; McCay et al. 1984). The CCl_3^* radical reacts with various biologically important substances such as amino acids, nucleotides and fatty acids, as well as proteins, nucleic acids and lipids (Castro 1984). In the presence of oxygen, the CCl_3 radical is converted to the trichloromethyl peroxy radical, CCl_3OO^*. CCl_3OO^* is far more likely than CCl_3^* to abstract a hydrogen from polyunsaturated fatty acids (PUFA) (Forni et al. 1983), thereby initiating the process of lipid peroxidation (Cheeseman et al. 1985). The abstraction of a hydrogen from fatty acid initiates a complex series of reactions that terminate in the complete disintegration of the PUFA molecule with the formation of aldehydes, other carbonyles, and alkanes (Weber et al. 2003). Lipid peroxidation may damage cellular functions in two ways: by compromising membrane function and by covalent binding of reactive intermediates (Weber et al. 2003).

The formation of SUL has been immunohistochemically observed around the portal vein in the liver of mice fed with DHA followed by an intraperitoneal injection of CCl_4, whereas the immunoexpression of SUL was scarcely observed in the control, DHA-alone, and CCl_4-alone groups, using monoclonal antibody (mAb2B12) raised against the SUL adduct (Kawai et al. 2006).

As early as 6 h after oral administration of CCl_4 (1.0 ml/kg), MDA-amine and 4-HNE-sulfhydryl adducts were immunohistochemically detected in hepatocytes localized to zone 2 of the hepatic lobule (Hartley et al. 1999). The density of MDA-adducts-positive hepatocytes and HNE-adducts-positive hepatocytes increased with time up to 36 h post-CCl_4 administration. MDA-adducts- or HNE-adducts-positive hepatocytes were no longer detected by 36–72 h post CCl4 administration

(Hartley et al. 1999). These results demonstrate that HNE-adducts and MDA-adducts in a time-dependent manner, appear to be associated with liver injury-induced CCl_4.

6 Acetaminophen-Induced Liver Injury

Although an overdose of acetaminophen (*N*-acetyl-*p*-aminophenol; APAP) causes liver injury in humans (Larson et al. 2005) and experimental animals (Chen et al. 2009; Saito et al. 2010), APAP is a safe and effective analgesic and antipyretic drug when used at therapeutic levels (Rumack 2004). The initial step in toxicity is the cytochrome P450 metabolism to N-acetyl-p-benzoquinone imine (NAPQI), a highly reactive metabolite (Jaeschke and Bajt 2006; Masubuchi et al. 2005; Nelson 1990). NAPQI depletes the intracellular storage of glutathione (GSH) in the hepatocyte (Bessems and Vermeulen 2001; Jaeschke and Bajt 2006; Nelson 1990). Since GSH is the cofactor for GSH-peroxidase detoxification of peroxides, a main mechanism of peroxide detoxification is compromised in APAP-induced liver injury (Hinson et al. 2010). Thus, GSH depletion could lead to increased intracellular peroxide, and increase oxidative stress via mechanisms of the Fenton reaction (Hinson et al. 2010). This mechanism involves the reduction of peroxide by ferrous ions forming the hydroxyl radical, which leads to oxidation of lipids, proteins and nucleic acids (Hinson et al. 2010).

In the previous study (Sun et al. 2012), the immunoexpression of PUFA oxidation markers PRL, HEL, 4-HHE-histidine and CRA-lysine was examined up to 24 h post-APAP intraperitoneal injection in rats (1 g/kg body weight). Three hr after the post-intraperitoneal injection of APAP in rats, vacuolated hepatocytes were observed in the centrilobular region of the hepatic lobule (Fig. 10.1a); and at 6 h, they increased in number and expanded their distribution to the medzonal region. Apoptotic cells were occasionally observed in hepatocytes in the centrilobular region. Then, at 12 h, coagulative necrosis, single cell necrosis and apoptosis were observed in all regions. At 24 h, necrotic and apoptotic changes became more prominent. Three hour after the post-intraperitoneal injection of APAP, a few vacuolated hepatocytes were positive for TUNEL stain in the centrilobular region (Fig. 10.1b). The TUNEL-positive rate tended to increase at 6 h and showed significant increases at 12 and 24 h. Hepatocytes in the control group were negative for PRL, HEL, HHE-histidine and CRA-lysine. Immunohistochemical expression of these oxidation markers was first detected in the cytoplasm of degenerative hepatocytes in the centrilobular region of the hepatic lobule at 3 h after the injection, earlier than the occurrence of hepatocyte apoptosis (Fig. 10.1b–f). Immunohistochemical expression of these oxidation markers was observed in almost all degenerative hepatocytes 6–24 h post-APAP injection. The results thus suggest that the generation of PRL, HEL, 4-HHE-histidine and CRA-lysine may be the sign of early events preceding the induction of hepatocyte apoptosis, and thus may prove useful for the early detection of oxidative stress-related liver cell injury, and for the detection of PUFA oxidations that may be involved in the pathogenesis of APAP-induced liver injury.

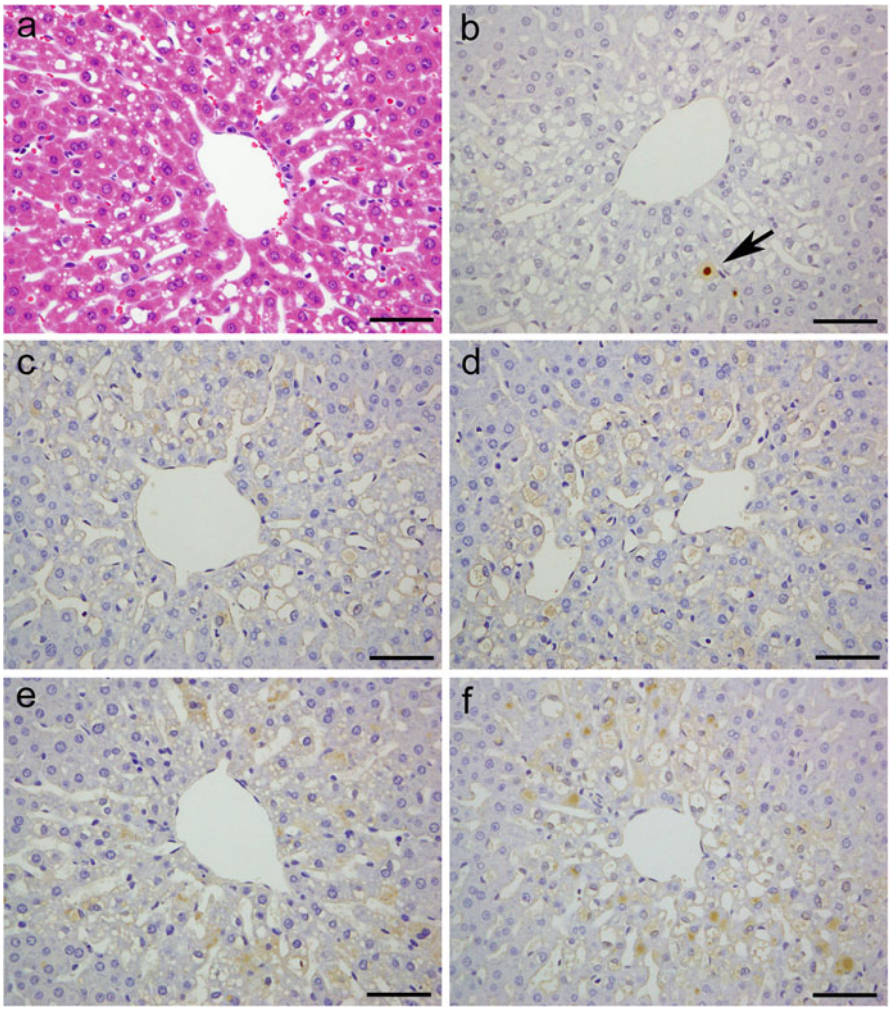


Fig. 10.1 Centrilobular lesions 3 h post-acetaminophen intraperitoneal injection. Bar = 50 μm. (**a**) Vacuolated hepatocytes were observed in the centrilobular region of the hepatic lobule. HE stain. (**b**) A few vacuolated hepatocytes were positive for TUNEL stain in the centrilobular region. (**c**–**f**) Immunohistochemical expression of PRL (**c**), HEL (**d**), HHE (**e**), CRA (**f**) was detected in the cytoplasms of almost all degenerative hepatocytes in the centrilobular region (These figures have been reprinted from the Journal of Veterinary Medical Science, 74, 141–147, 2012. A part of these figures has been revised and used.)

7 Cisplatin-Induced Renal Tubular Injury

Cisplatin [*cis*-diamminedichloroplatinum (II)] is a major anticancer drug used for the treatment of solid tumors in the testis, ovary, head and neck, and elsewhere (Pabla and Dong 2008; Yao et al. 2007). Though the mechanism behind the

anticancer activity of cisplatin is not completely understood, a widely-held view is that cisplatin binds to DNA, leading to the formation inter- and intrastrand cross-links (Pabla and Dong 2008). Cross-linking results in defective DNA templates and arrest of DNA synthesis and replication (Pabla and Dong 2008). The cross-linking can further induce DNA damage in rapidly dividing cells such as neoplastic cells (Pabla and Dong 2008). The chief dose-limiting side effect of cisplatin is nephrotoxicity (Fillastre and Raguenez-Viotte 1989; Pabla and Dong 2008; Yao et al. 2007; Zhou et al. 2004). The kidney accumulates cisplatin to a greater degree than other organs, and the disproportionate accumulation of cisplatin in kidney tissue contributes to cisplatin-induced nephrotoxicity (Aray and Safirstein 2003). Cisplatin-induced nephrotoxicity is characterized by proximal tubular injury and decreased glomerular filtration (Jones et al. 1985; Pabla and Dong 2008). Cisplatin causes tubular injury through multiple mechanisms, including oxidative stress, DNA damage, apoptosis and inflammation (Pabla and Dong 2008; Yao et al. 2007).

Cisplatin causes the generation of oxygen free radicals, such as hydrogen peroxide, superoxide anions and hydroxyl radicals (Kruidering et al. 1997; Masuda et al. 1994). In particular, the hydroxyl radical is highly reactive among oxygen radicals. Once excessive hydroxyl radicals are released, lipid peroxidation, which causes changes in the fluidity and permeability of membranes, is induced (Schmidley 1990). Cisplatin-induced nephrotoxicity is closely associated with increased lipid oxidation markers such as malondialdehyde and 4-HNE in kidney tissue (Chirino et al. 2008; Greggi Antunes et al. 2000; Zhou et al. 2006). In the previous study (Sugiyama et al. 2011), immunoexpression of HEL and acrolein in rat kidneys was examined up to 4 days after cisplatin injection. Cisplatin-induced tubular injury was observed histopathologically on days 2–4 after injection, and became more severe time-dependently. Thus, few histopathological changes were observed in rats at day 1. At days 2 and 3, degenerative changes seen in the S3 segment of the proximal tubule consisted of hydropic degeneration, cytoplasmic vacuolization and tubular dilation. These histopathological changes were more severe at day 3 than day 2. At day 4, many pyknotic nuclei, and the widespread desquamation and necrosis of tubular epithelial cells of the S3 segment of the proximal tubule were observed. There was a significant increase in the number of TUNEL-positive cells at days 3 and 4. Tubular epithelial cells in saline-treated control rats and cisplatin-treated rats at day 1 were negative for HEL or acrolein. The immunohistochemical expression of these oxidation markers was first detected in the cytoplasm of degenerative tubular cells at day 2, preceding the induction of tubular cell apoptosis. On days 3–4, the cytoplasms of damaged proximal tubular cells were immunostained for these oxidation markers. These findings suggest that expression of HEL and acrolein in the S3 segment of the proximal tubule associate closely with pathogenesis of cisplatin-induced renal tubular injury.

8 Conclusion

Lipid hydroperoxide- and aldehyde-modified proteins have been immunohistochemically detected in diverse pathological situations such as atherosclerosis, neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease, carbon tetrachloride- or acetaminophen-induced hepatoxicity and cisplatin-induced nephrotoxicity. These findings suggest that the expression of the lipid hydroperoxide- and aldehyde-modified proteins associate closely with the pathogenesis of these diseases in humans and experimental animals.

References

Aray I, Safirstein RL (2003) Cisplatin nephrotoxicity. Semin Nephrol 23:460–464

Bernhard D, Wang XL (2007) Smoking, oxidative stress and cardiovascular diseases-do anti-oxidative therapies fail? Curr Med Chem 14:1703–1712

Bessems JG, Vermeulen NP (2001) Paracetamol (acetaminophen)-induced toxicity: molecular and biochemical mechanisms, analogues and protective approaches. Crit Rev Toxicol 31:55–138

Calingasan NY, Uchida K, Gibson GE (1999) Protein-bound acrolein: a novel marker of oxidative stress in Alzheimer's disease. J Neurochem 72:751–756

Castellani RJ, Perry G, Siedlak SL et al (2002) Hydroxynonenal adducts indicate a role for lipid peroxidation in neocortical and brainstem Lewy bodies in humans. Neurosci Lett 319:25–28

Castro JA (1984) Mechanistic studies and prevention of free radical cell injury. In: Paton W, Mitchell J, Turner P (eds) Proceedings of IUPHAR 9th International Congress Pharmacol, MacMillan

Cheeseman KH, Albono EF, Tomasi A et al (1985) Biochemical studies on the metabolic activity of halogenated alkanes. Environ Health Perspect 64:85–101

Chen YH, Lin FY, Liu PL et al (2009) Antioxidative and hepatoprotective effects of magnolol on acetaminophen-induced liver damage in rats. Arch Pharm Res 32:221–228

Chirino YI, Sánchez-González DJ, Martínez-Martínez CM et al (2008) Protective effects of apocynin against cisplatin-induced oxidative stress and nephrotoxicity. Toxicology 245:18–23

Cooper AA, Gitler AD, Cashikar A et al (2006) Alpha-synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson's models. Science 313:324–328

Ekuni D, Tomofuji T, Sanbe T et al (2009) Periodontitis-induced lipid peroxidation in rat descending aorta is involved in the initiation of atherosclerosis. J Periodontal Res 44:434–442

Fillastre JP, Raguenez-Viotte G (1989) Cisplatin nephrotoxicity. Toxicol Lett 46:163–175

Forni LG, Packer JE, Slater TF et al (1983) Reaction of the trichloromethyl and halothane-derived peroxy radicals with unsaturated fatty acids: a pulse radiolysis study. Chem Biol Interact 45:171–177

Fukuchi Y, Miura Y, Nabeno Y et al (2008) Immunohistochemical detection of oxidative stress biomarkers, dityrosine and Nε-(hexanoyl)lysine, and c-reactive protein in rabbit atherosclerotic lesions. J Atheroscler Thromb 15:185–192

Fukuda M, Kanou F, Shimada N et al (2009) Elevated levels of 4-hydroxynonenal-histidine Michael adduct in the hippocampi of patients with Alzheimer's disease. Biomed Res 30:227–233

Grant Maxie M, Robinson WF (2007) Cardiovascular system. In: Grant Maxie M (ed) Ubb, Kennedy, and Palmer's pathology of domestic animals, vol 3, 5th edn. Sanders, New York

Greggi Antunes LM, Darin JDC, Biancii MLP (2000) Protective effects of vitamin C against cisplatin-induced nephrotoxicity and lipid peroxidation in adult rats: a dose-dependent study. Pharmacol Res 41:405–411

Hartley DP, Kolaja KL, Reichard J et al (1999) 4-Hydroxynoneal and malondialdehyde hepatic protein adducts in rats treated with carbon tetrachloride: immunohistochemical detection and lobular localization. Toxicol Appl Pharmacol 161:23–33

Higuchi Y (2003) Chromosomal DNA fragmentation in apoptosis and necrosis induced by oxidative stress. Biochem Pharmacol 66:1527–1535

Hinson JA, Roberts DW, James LP (2010) Mechanisms of acetaminophen-induced liver necrosis. Handb Exp Pharmacol 196:369–405

Hisaka S, Kato Y, Kitamoto N et al (2009) Chemical and immunochemical identification of propanoyllysine derived from oxidized n-3 polyunsaturated fatty acid. Free Radic Biol Med 46:1463–1471

Huang A, Sun D, Kaley G et al (1998) Superoxide released to high intra-arteriolar pressure reduces nitric oxide-mediated shear stress-and agonist-induced dilations. Circ Res 83:960–965

Jaeschke H, Bajt ML (2006) Intracellular signaling mechanisms of acetaminophen-induced liver cell death. Toxicol Sci 89:31–41

Jenner P (2003) Oxidative stress in Parkinson's disease. Ann Neurol 53:S26–S36

Jomova K, Vondrakova D, Lawson M et al (2010) Metals, oxidative stress and neurodegenerative disorders. Mol Cell Biochem 345:91–104

Jones TW, Chopra S, Kaufman JS et al (1985) Cis-diamminedichloroplatinum (II)-induced acute renal failure in the rat. Correlation of structural and functional alterations. Lab Invest 52:363–374

Kato Y, Osawa T (2010) Detection of lipid-lysine amide-type adduct as a marker of PUFA oxidation and its applications. Arch Biochem Biophs 501:182–187

Kato Y, Mori Y, Makino Y et al (1999) Formation of Nepsilon-(hexanonyl)lysine in protein exposed to lipid hydroperoxide. A plausible marker for lipid hydroperoxide-derived protein modification. J Biol Chem 274:20406–20414

Kawai Y, Kato Y, Fujii H et al (2003) Immunochemical detection of a novel lysine adduct using an antibody to linoleic acid hydroperoxide-modified protein. J Lipid Res 44:1124–1131

Kawai Y, Fujii H, Kato Y et al (2004) Esterified lipid hydroperoxide-derived modification of protein: formation of a carboxyalkylamide-type lysine adduct in human atherosclerotic lesions. Biochem Biophys Res Commun 313:271–276

Kawai Y, Fujii H, Okada M et al (2006) Formation of N^{ε}-(succinyl)lysine in vivo: a novel marker for docosahexaenoic acid-derived protein modification. J Lipid Res 47:1386–1398

Kim JS, He L, Lemasters JJ (2003) Mitochondrial permeability transition: a common pathway to necrosis and apoptosis. Biochem Biophys Res Commun 304:463–470

Kristal BS, Park BK, Yu BP (1996) 4-Hydroxyhexenal is a potent inducer of the mitochondrial permeability transition. J Biol Chem 271:6033–6038

Kruidering M, Van de Water B, de Heer E (1997) Cisplatin-induced nephrotoxicity in porcine proximal tubular cells: mitochondrial dysfunction by inhibition of complex I to IV of the respiratory chain. J Pharmacol Exp Ther 280:638–649

Larson AM, Polson J, Fontana RJ et al (2005) Acetaminophen-induced acute liver failure: results of a United States multicenter, prospective study. Hepatology 42:1364–1372

Lee JY, Je JH, Kim DH et al (2004) Induction of endothelial apoptosis by 4-hydroxyhexenal. Eur J Biochem 271:1339–1347

Liu XY, Yang ZH, Pan XJ et al (2010) Crotonaldehyde induces oxidative stress and caspase-dependent apoptosis in human bronchial epithelial cells. Toxicol Lett 195:90–98

Long EK, Murphy TC, Leiphon LJ et al (2008) Trans-4-hydroxy-2-hexenal is a neurotoxic product of docosahexaenoic (22:6; n-3) acid oxidation. J Neurochem 105:714–724

Masubuchi Y, Suda C, Horie T (2005) Involvement of mitochondrial permeability transition in acetaminophen-induced liver injury in mice. J Hepatol 42:110–116

Masuda H, Tanaka T, Takahama U (1994) Cisplatin generates superoxide anion by interaction with DNA in a cell-free system. Biochem Biophys Res Commun 203:1175–1180
McCay PB, Lai EK, Poyer JL et al (1984) Oxygen and carbon-centered free radical formation during carbon tetrachloride metabolism. J Biol Chem 259:2135–2143
Mitra S, Goyal T, Mehta JL (2011) Oxidized LDL, LOX-1 and atherosclerosis. Cardiovasc Drug Ther 25:419–429
Mügge A, Brandes RP, Böger RH et al (1994) Vascular release of superoxide radicals is enhanced in hypercholesterolemic rabbits. J Cardiovasc Pharmacol 24:994–998
Nelson SD (1990) Molecular mechanisms of the hepatotoxicity caused by acetaminophen. Semin Liver Dis 10:267–278
Nicolls MR, Haskins K, Flores SC (2007) Oxidant stress, immune dysregulation, and vascular function in type I diabetes. Antioxid Redox Signal 9:879–889
Pabla N, Dong Z (2008) Cisplatin nephrotoxicity: mechanisms and renoprotective strategies. Kidney Int 73:994–1007
Recknagel RO, Glende EA Jr, Dolak JK et al (1989) Mechanisms of carbon tetrachloride toxicity. Pharmacol Ther 43:139–154
Reynolds ES (1963) Liver parenchymal cell injury. I. Initial alterations of the cell following poisoning with carbon tetrachloride. J Cell Biol 19:139–157
Rumack BH (2004) Acetaminophen misconceptions. Hepatology 40:10–15
Saito C, Yan HM, Artigues A et al (2010) Mechanism of protection by metallothionein against acetaminophen hepatotoxicity. Toxicol Appl Pharmacol 242:182–190
Schmidley JW (1990) Free radicals in central nervous system ischemia. Stroke 21:1086–1090
Shamamoto-Nagai M, Maruyama W, Hashizume Y et al (2007) In Parkinsonian substantia nigra, alpha-synuclein is modified by acrolein, a lipid-peroxidation product, and accumulates in the dopamine neurons with inhibition of proteasome activity. J Neural Transm 114:1559–1567
Sugiyama A, Sun J, Nishinohara M et al (2011) Expressions of lipid oxidation markers, N^{ε}-hexanoyl lysine and acrolein in cisplatin-induced nephrotoxicity in rats. J Vet Med Sci 73:821–826
Sun J, Sugiyama A, Hisaka S et al (2012) Immunohistochemical detection of polyunsaturated fatty acid oxidation markers in acetaminophen-induced liver injury in rats. J Vet Med Sci 74:141–147
Tamer L, Sucu N, Polat G et al (2002) Decreased serum total antioxidant status and erythrocyte-reduced glutathione levels are associated with increased serum malondialdehyde in atherosclerotic patients. Arch Med Res 33:257–260
Uchida K (1999) Current status of acrolein as a lipid peroxidation product. Trends Cardiovasc Med 9:109–113
Uchida K (2000) Role of reactive aldehyde in cardiovascular disease. Free Radic Biol Med 28:1685–1696
Uchida K, Kanematsu M, Sakai K et al (1998) Protein-bound acrolein: potential markers for oxidative stress. Proc Natl Acad Sci U S A 95:4882–4887
Weber LW, Boll M, Stampfl A (2003) Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. Crit Rev Toxicol 33:105–136
Yao X, Panichpisal K, Kurtzman N et al (2007) Cisplatin nephrotoxicity: a review. Am J Med Sci 334:115–124
Yoritaka A, Hattori N, Uchida K et al (1996) Immunohistochemical detection of 4-hydroxynoneal protein adducts in Parkinson disease. Proc Natl Acad Sci U S A 93:2696–2701
Yoshida T, Adachi E, Nigi H et al (1999) Changes of sinusoidal basement membrane collagens in early hepatic fibrosis induced with CCl_4 in cynomolgus monkeys. Pathology 31:29–35
Zarkovic K (2003) 4-Hydroxynoneal and neurodegenerative diseases. Mol Aspect Med 24:293–303
Zhou H, Kato A, Yasuda H et al (2004) The induction of cell cycle regulatory and DNA repair proteins in cisplatin-induced acute renal failure. Toxicol Appl Pharmacol 200:111–120
Zhou H, Kato A, Miyaji T et al (2006) Urinary marker for oxidative stress in kidneys in cisplatin-induced acute renal failure in rats. Nephrol Dial Transplant 21:616–623

Chapter 11
Role of Lipid Peroxide in the Neurodegenerative Disorders

Wakako Maruyama, Masayo Shaomoto-Nagai, Yoji Kato, Shinsuke Hisaka, Toshihiko Osawa, and Makoto Naoi

Abstract Nervous system controls all the organs in the living like a symphony. In this chapter, the mechanism of neuronal death in aged is discussed in relation to oxidative stress. Polyunsaturated fatty acid (PUFA) is known to be rich in the membranous component of the neurons and plays an important role in maintaining the neuronal functions. Recent reports revealed that oxidation of omega-3 and omega-6 PUFAs, such as docosahexaenoic acid (DHA) and arachidonic acid (ARA), are potent antioxidant but simultaneously, their oxidation products are potentially toxic. In this chapter, the existence of early oxidation products of PUFA is examined in the samples from neurodegenerative disorders and the cellular model. Accumulation of proteins with abnormal conformation is suggested to induce neuronal death by disturbance of proteolysis and mitochondrial function. The role of lipid peroxide and lipid-derived aldehyde adduct proteins is discussed in relation to brain ageing and age-related neurodegeneration.

Keywords Brain ageing • Lipid peroxidation • Oxidatively modified protein • Parkinson disease • α-synuclein

W. Maruyama (✉) • M. Shaomoto-Nagai
Department of Cognitive Brain Science, National Institute for Geriatrics and Gerontology, 35 Morioka, Obu, Aichi 474-8511, Japan
e-mail: maruyama@ncgg.go.jp

Y. Kato
School of Human Science and Environment, University of Hyogo, Himeji 670-0092, Japan

S. Hisaka
Department of Pharmacognosy, Faculty of Pharmacy, Meijyo University, 150 Yagoto-yama, Tenpaku, Nagoya, Aichi 468-8503, Japan

T. Osawa • M. Naoi
Department of Nutritional Science, Aichi Gakuin University, Nisshin 470-0195, Aichi, Japan

Y. Kato (ed.), *Lipid Hydroperoxide-Derived Modification of Biomolecules*, Subcellular Biochemistry 77, DOI 10.1007/978-94-007-7920-4_11,

1 Importance of Dementia in Ageing Society

In the modern countries, increasing number of aged people induces serious social, medical, and economical problems. Among them, the increase of demented people according to ageing is one of the biggest problems. It is known that the incidence of dementia doubles in the people of 5 years elder, that means the risk of dementia is 10 % in the people over 65 years old, and 25 % in over 85 years old. The three major causes of dementia are Alzheimer disease (AD), Lewy body dementia (LBD), and vascular dementia (VD). In addition, age-related decline of mental activity is closely related to the occurrence of dementia and sometimes it is mixed and impossible to determine the distinct contribution of the diseases and ageing in demented people in individual. To clarify the mechanism of brain ageing is essential to understanding the etiology of the dementia.

2 Implication of Oxidized Polyunsaturated Fatty Acids, Especially DHA to Brain Ageing and Neurodegeneration

Oxidative stress is suggested to be involved in the etiology of both brain ageing and neurodegeneration such as AD and LBD. Because that oxygen consumption is high in the brain neurons where oxidizable unsaturated fatty acid is rich, the level of lipid peroxidation should be continuously high and might injure the cells. Among unsaturated fatty acids, docosahexanoic acid (DHA; 22:6 n-3) is the most abundant omega-3 polyunsaturated fatty acid (PUFA) in the brain. DHA is a component of membrane lipid bilayer and is implicated in the various neuronal functions, such as neuronal plasticity, neural transmission, and signal transduction (Eady et al. 2012; Wurtman et al. 2010). In addition, DHA is known as a potent antioxidant, and *in vitro* and *in vivo* studies proved that application of DHA prevented neuronal damage by oxidative stress (Bazan et al. 2011; Berman et al. 2009; Glozman et al. 1998). However, lipophilic antioxidants such as DHA and beta-carotene are pro-oxidants and when they are oxidized, they become toxic lipid-peroxidation products to make chain reaction of peroxidation (Yavin et al. 2001). Lipid peroxide is the early product of lipid peroxidation compared with the aldehyde (Kato and Osawa 2010) (Fig. 11.1). The distinct chemical reaction of DHA and intermediates are unknown, although it was found that human neuroblastoma SH-SY5Y cells, DHA is metabolized into intermediates, 17-, 14-, 7- or 4-hydoxydocosahexaenoic acid (HDHA) and toxic 17-hydroperoxydocosa-hexaenoic acid (17-HpDHA) (Gleissman et al. 2010). These hydroxyl or cyclic intermediates may be associated with the formation of propanoyl and succinyl to form adduct with lysine residue in the proteins, which is named propanoyl-lysine (PRL) and succinyl-lysine (SUL) respectively. In the same way, oxidation of omega-6 PUFA, arachidonic acid forms hexanoyl-lysine (HEL) and glutaroyl-lysine (GLL) (Fig. 11.1). PRL- and SUL-positive proteins were detected in albumin incubated with oxidized DHA.

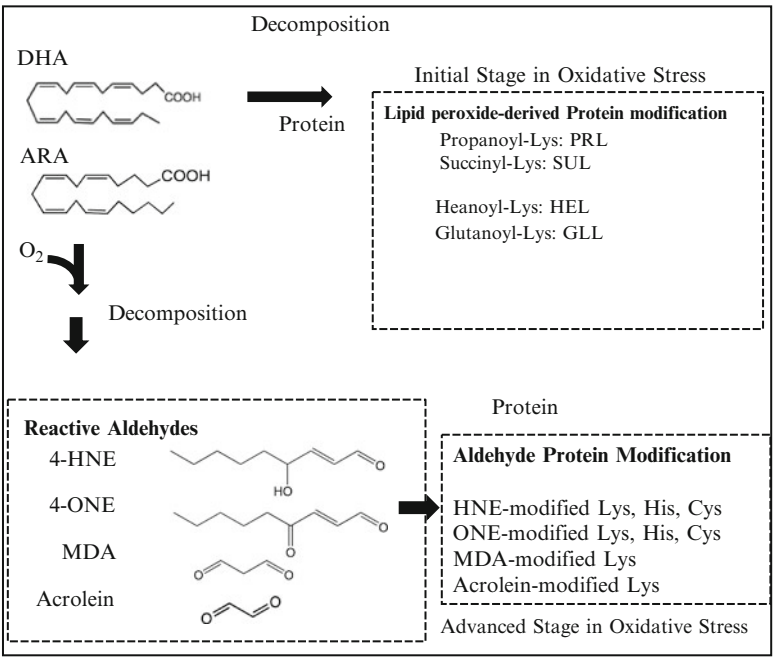


Fig. 11.1 Hypothetic pathway of oxidation pathway of DHA

Furthermore, by animal experiments, the increase of SUL-modified proteins was identified in the liver of mice fed with the diet containing high level of DHA under oxidative stress induced by carbon tetrachloride but not in the mice with high DHA diet only (Kawai et al. 2006). In human, PRL-adduct proteins were found to increase in the urine of diabetic patients (Hisaka et al. 2009). However, little is known about the existence and the role of lipid peroxide derived from DHA in the brain. The study to clarify the involvement of PRL- or SUL-adduct proteins in brain ageing and neurodegeneration has been waited for.

3 LBD and Lipid Peroxidation

One of the common pathological feature of neurodegeneration is accumulation and aggregation of abnormal proteins such as amyloid β protein to make senile plaque in AD and α-synuclein as a main component of Lewy body in LBD. Among neurodegenerative disorders in the aged, increased level of oxidative stress is one of the most important pathogenetic factors of Parkinson disease (PD), the most common phenotype of LBD. In the dopamine neurons of the substantia nigra, neurotransmitter dopamine is easily auto-oxidized to make quinone and produces

superoxide ($O_2^{-\cdot}$) simultaneously. The half-life of superoxide is very short and it reacts with H_2O to produce hydrogen peroxide (H_2O_2). Dopamine is oxidized enzymatically by monoamine oxidase (MAO) and produces H_2O_2 also. In the presence of iron, which is rich in dopamine neurons as a co-factor of tyrosine hydroxylase, H_2O_2 produces reactive and deleterious hydroxyl radical ($OH^{\cdot}$) in the neurons. These reactions are summarized in following lines.

$$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH \quad \text{(Haber-Weiss reaction)}$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH \quad \text{(Fenton reaction)}$$

High level of ROS derived from dopamine metabolism is one of the reason why dopamine neurons are vulnerable in ageing (Carlsson 1978).

Another source of reactive oxygen species (ROS) in the cells is mitochondria. From mitochondrial respiratory chain, ROS is continuously generated and about 1 % of ROS is failed to be quenched by anti-oxidants and anti-oxidizing enzymes to injure the bioactive molecules. Because half-life of ROS is generally short, the most potential target of ROS should be mitochondrial membrane and membrane-bounded proteins. Indeed, we found that in cybrid cells, made by the fusion of the mitochondria-negative cells containing nucleus and another cells containing nucleus without mitochondria, the cells containing mutated mitochondrial genome (mitochondrial encephalopathy with lactic acidosis, MELAS type of mutation) which was established as reported previously (Fujita et al. 2007) was stained by HEL and 4-hydroxy-2-nonenal (HNE) antibodies. By immuno-histochemical observation was done according to Shamoto-Nagai et al. (2007). HEL and HNE positive staining was accumulated in the mitochondria as detected by immunohistochemistry (Fig. 11.2). The presence of modified proteins in mitochondrial fraction was confirmed by Western blotting (Fig. 11.3). Mutation and deletion of mitochondrial genome is causable for mitochondrial cytopathy, in which brain and muscle are commonly disturbed (Morgan-Hughes et al. 1982). Accumulation of functionally impaired mitochondria due to dysfunction of quality control system of mitochondria is now gathering attention as a pathogenesis of some familial type of PD (See Exner et al. (2012) as a review). PD and brain ageing might share the common death pathway such as oxidative stress, mitochondrial dysfunction and lipid peroxidation-derived membrane injury.

4-HNE and acrolein (ACR) are lipid-derived aldehyde and they are reported to increase in the dopamine neurons in the substantia nigra of PD brain (Dalfo et al. 2005; Shamoto-Nagai et al. 2007; Yoritaka et al. 1996) the increased ACR-modified proteins was confirmed by our group also (Fig. 11.4). *In vitro* study revealed that 4-HNE and ACR can make direct adduct with αSyn and produces abnormal oligomer with β-sheet structure or aggregates. Such aldehyde-adduct αSyn shows increased the toxicity to primary cultured neurons and inhibits the proteasome activity *in vitro* and in the cultured neuronal cells (Qin et al. 2007; Shamoto-Nagai et al. 2007). Recently, we investigated the involvement of lipid peroxide such as PRL

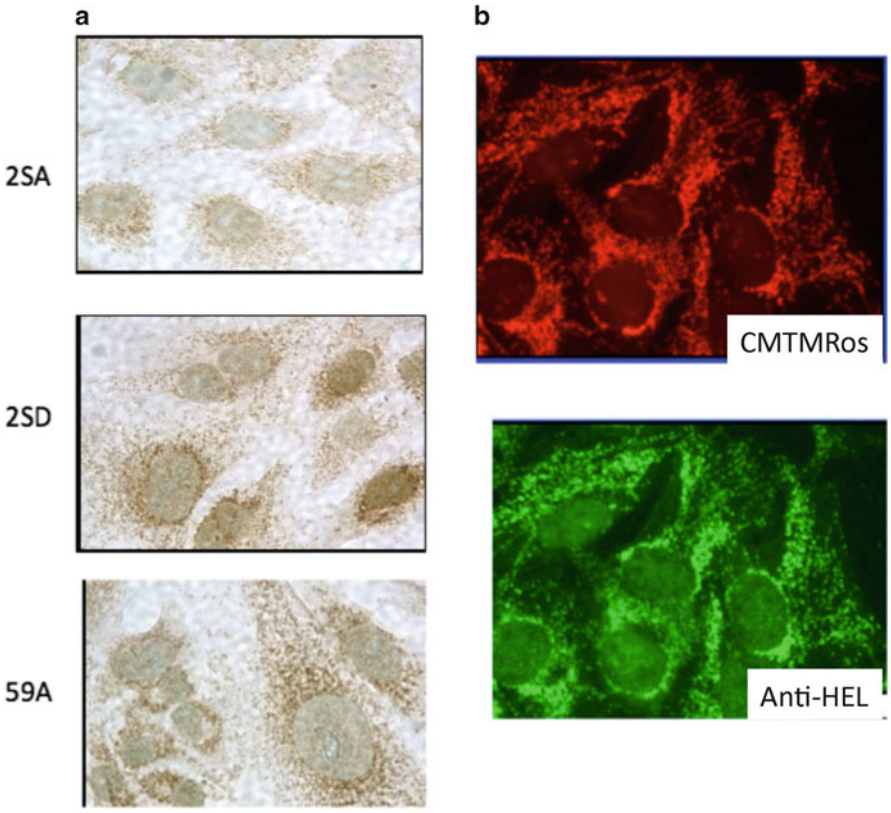


Fig. 11.2 HEL-modification was accumulated in the mutated mitochondrion. (**a**). Immunocytochemical detection of HEL moieties in independent cybrid cell lines. Immunostaining was performed with biotynylated anti-rabbit IgG and peroxidase-conjugated streptavidin and 3, 3'-diaminobenzidine tetrahydrochloride as chromogen. Increased immunoreactivity was detected in cybrid cells fused with mutated mitochondrial DNA (2SA, 2SD, 59A) but not in the cells fused with normal mitochondrial DNA (data not shown). (**b**). 59A cybrid cells were double-stained with CMTMRos, a mitochondrial marker with red fluorescence (*upper*) and also immunohistochemically using anti-HEL antibody and FITC green fluorescence dye (*lower*)

and SUL on the modification of αSyn *in vitro* and in the cellular model of PD. DHA enhanced oligomerization and aggregation of αSyn and in the cells treated with oxidative stress, lipid-peroxide modified aggregates similar to Lewy body was detected (Shamoto-Nagai et al. in preparation). It is consistent with the *in vivo* report that DHA administration enhanced oligomerization of αSyn in the brain of transgenic mice (Yakunin et al. 2012).

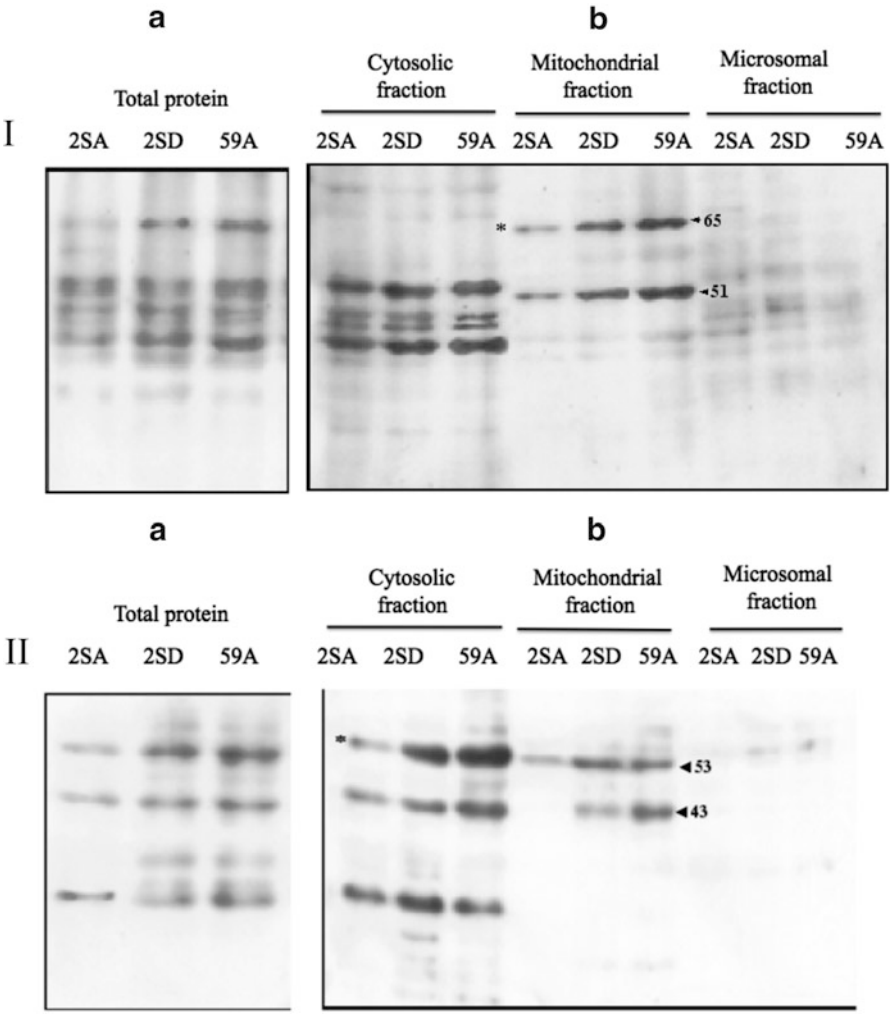


Fig. 11.3 (I) Immunochemical detection of HEL-modified proteins. (**a**). Total protein (10 mg) derived from various cell lines (2SA, 2SD, 59A) was lysed and separated by SDS-PAGE (12 % polyacrylamide) then probed using polyclonal antibody against HEL-conjugated proteins. (**b**). Proteins derived from various cell lines was separated into cytosolic, mitochondrial, microsomal, and nuclear fractions. Each fractions were separated and probed by anti-HEL antibody. HEL-positive proteins were detected in cytosolic and mitochondrial fractions but not in microsomal or nuclear fraction (data not shown). In mitochondrial fraction, two major bands at 51 and 65 kDa were detected. (II) Immunochemical detection of HNE-modified proteins. (**a**). Total protein (10 mg) derived from various cell lines (2SA, 2SD, 59A) was lysed and separated by SDS-PAGE (12 % polyacrylamide) then probed using polyclonal antibody against HNE-conjugated proteins. (**b**). Proteins derived from various cell lines was separated into cytosolic, mitochondrial, microsomal, and nuclear fractions. Each fractions were separated and probed by anti-HNE antibody. HNE-positive proteins were detected in cytosolic and mitochondrial fractions but not in microsomal or nuclear fraction (data not shown). In mitochondrial fraction, two major bands at 45 and 53 kDa were detected

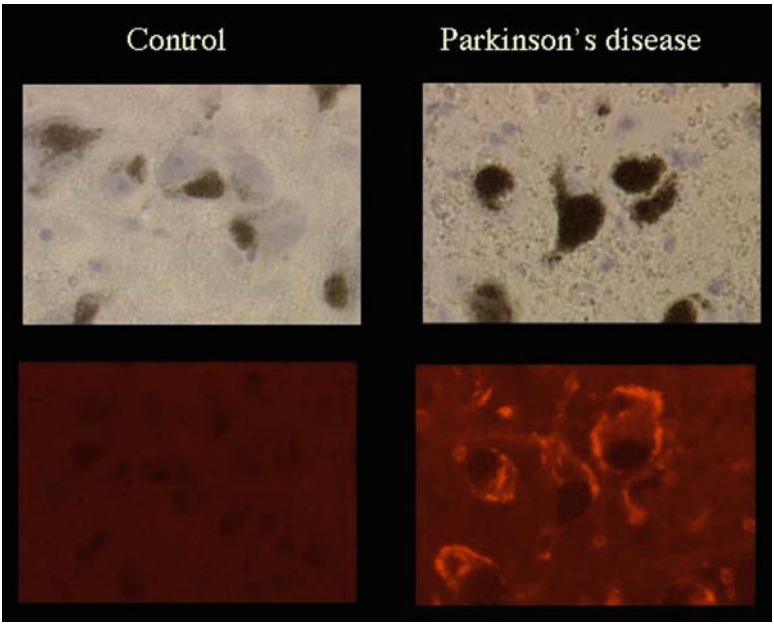


Fig. 11.4 Immunohistochemical detection of HNE-positive moieties using anti-HNE antibody. Control and Parkinsonian substantia nigra was stained with anti-HNE antibody as previously reported (Shamoto-Nagai et al. 2007). The increase of HNE-reactivity in melanine-containing dopaminergic neurons are apparent

4 Can Oxidatively Modified Proteins Be Applicable as a Marker of LBD?

In LBD, not only central nervous system but also peripheral sympathetic and parasympathetic neurons are degenerated with the existence of Lewy body (Orimo et al. 2002). It is indicated that continuous oxidative stress and lipid peroxidation should occur in the degenerating peripheral neurons and there may be oxidatively modified molecules which can be applied as a marker to diagnose or evaluate the progression of the disease.

Recently, neuroprotective or neurorescue therapy is coming to be realistic issue. Several species of natural compounds and drugs have been proposed to be effective to prevent neuronal death in cellular and animal models of PD. Our group reported that the inhibitors of MAO type B (MAO-B), rasagiline and (−) deprenyl protect neuronal cell death induced by various insults (Naoi and Maruyama 2009, 2010). The mechanism of the neuroprotective action is under the investigation. It was found that MAO-B inhibitors and some kind of polyphenols increase the expression of mRNA and protein levels of neuroprotective molecules such as Mn- and Cu/Zn- superoxide oxidase (SOD), brain-derived neurotrophic factor (BDNF) and glial cell line-neurotrophic

factors (GDNF) in the cells, rodents, and primates (Carrillo et al. 2000; Maruyama et al. 2002, 2004; Maruyama and Naoi 2013). In addition, MAO-B inhibitor (−) deprenyl, polyphenol tetrahydrocurcumin and resveratrol prolonged the lifespan of rodents and other animals (Baur 2010; Kitani et al. 2004; Knoll 1988; Rubinsztein et al. 2011; Xiang et al. 2011). Decreased harmful oxidative stress and increased growth factors might be ascribed to the longevity effect of these candidates for neuroprotective drugs (Mattson and Magnus 2006). In neurodegenerative disorders, the symptom and progression of the disease is heterogenous and even though the development of new brain imaging, it is not easy to evaluate the neuroprotective effects of the drugs. Oxidatively modified proteins and humoral factors such as BDNF and GDNF might be applicable to evaluate the efficiency of neuroprotective compounds in human.

5 Conclusion

Lipid peroxidation is involved in the neurodegenerative disorders and brain ageing. Especially, membrane composing PUFA, such as DHA is easily oxidized and produces lipid peroxides and aldehydes which make adducts to proteins with abnormal conformation and are toxic. Lipid peroxide is an early product of lipid peroxidation compared to aldehyde and so, the investigation of lipid peroxide adduct proteins may open a new field of science which clarify the mechanism of neuronal dysfunction and death in the aged people.

Acknowledgement This work was supported by the Research Funding for Longevity Sciences (23–2) from National Center for Geriatrics and Gerontology (NCGG) and Grant-in-Aid for Scientific Research (A) (Grant Number 24248024) from JSPS (W. M.).

References

Baur JA (2010) Resveratrol, sirtuins, and the promise of a DR mimetic. Mech Ageing Dev 131:261–269

Bazan NG, Molina MF, Gordon WC (2011) Docosahexaenoic acid signalolipidomics in nutrition: significance in aging, neuroinflammation, macular degeneration, Alzheimer's, and other neurodegenerative diseases. Annu Rev Nutr 31:321–351

Berman DR, Mozurkewich E, Liu Y, Barks J (2009) Docosahexaenoic acid pretreatment confers neuroprotection in a rat model of perinatal cerebral hypoxia-ischemia. Am J Obstet Gynecol 200(305):e301–e306

Carlsson A (1978) Age-dependent changes in central dopaminergic and other monoaminergic systems. Adv Exp Med Biol 113:1–13

Carrillo MC, Minami C, Kitani K, Maruyama W, Ohashi K, Yamamoto T, Naoi M, Kanai S, Youdim MB (2000) Enhancing effect of rasagiline on superoxide dismutase and catalase activities in the dopaminergic system in the rat. Life Sci 67:577–585

Dalfo E, Portero-Otin M, Ayala V, Martinez A, Pamplona R, Ferrer I (2005) Evidence of oxidative stress in the neocortex in incidental Lewy body disease. J Neuropathol Exp Neurol 64:816–830
Eady TN, Belayev L, Khoutorova L, Atkins KD, Zhang C, Bazan NG (2012) Docosahexaenoic acid signaling modulates cell survival in experimental ischemic stroke penumbra and initiates long-term repair in young and aged rats. PLoS One 7:e46151
Exner N, Lutz AK, Haass C, Winklhofer KF (2012) Mitochondrial dysfunction in Parkinson's disease: molecular mechanisms and pathophysiological consequences. EMBO J 31:3038–3062
Fujita Y, Ito M, Nozawa Y, Yoneda M, Oshida Y, Tanaka M (2007) CHOP (C/EBP homologous protein) and ASNS (asparagine synthetase) induction in cybrid cells harboring MELAS and NARP mitochondrial DNA mutations. Mitochondrion 7:80–88
Gleissman H, Yang R, Martinod K, Lindskog M, Serhan CN, Johnsen JI, Kogner P (2010) Docosahexaenoic acid metabolome in neural tumors: identification of cytotoxic intermediates. FASEB J 24:906–915
Glozman S, Green P, Yavin E (1998) Intraamniotic ethyl docosahexaenoate administration protects fetal rat brain from ischemic stress. J Neurochem 70:2484–2491
Hisaka S, Kato Y, Kitamoto N, Yoshida A, Kubushiro Y, Naito M, Osawa T (2009) Chemical and immunochemical identification of propanoyllysine derived from oxidized n-3 polyunsaturated fatty acid. Free Radic Biol Med 46:1463–1471
Kato Y, Osawa T (2010) Detection of lipid-lysine amide-type adduct as a marker of PUFA oxidation and its applications. Arch Biochem Biophys 501:182–187
Kawai Y, Fujii H, Okada M, Tsuchie Y, Uchida K, Osawa T (2006) Formation of Nepsilon-(succinyl)lysine in vivo: a novel marker for docosahexaenoic acid-derived protein modification. J Lipid Res 47:1386–1398
Kitani K, Yokozawa T, Osawa T (2004) Interventions in aging and age-associated pathologies by means of nutritional approaches. Ann N Y Acad Sci 1019:424–426
Knoll J (1988) The striatal dopamine dependency of life span in male rats. Longevity study with (−)deprenyl. Mech Ageing Dev 46:237–262
Maruyama W, Naoi M (2013) "70th Birthday Professor Riederer" Induction of glial cell line-derived and brain-derived neurotrophic factors by rasagiline and (−)deprenyl: a way to a disease-modifying therapy? J Neural Transm 120:83–89
Maruyama W, Akao Y, Carrillo MC, Kitani K, Youdium MB, Naoi M (2002) Neuroprotection by propargylamines in Parkinson's disease: suppression of apoptosis and induction of prosurvival genes. Neurotoxicol Teratol 24:675–682
Maruyama W, Nitta A, Shamoto-Nagai M, Hirata Y, Akao Y, Yodim M, Furukawa S, Nabeshima T, Naoi M (2004) N-Propargyl-1 (R)-aminoindan, rasagiline, increases glial cell line-derived neurotrophic factor (GDNF) in neuroblastoma SH-SY5Y cells through activation of NF-kappaB transcription factor. Neurochem Int 44:393–400
Mattson MP, Magnus T (2006) Ageing and neuronal vulnerability. Nat Rev Neurosci 7:278–294
Morgan-Hughes JA, Hayes DJ, Clark JB, Landon DN, Swash M, Stark RJ, Rudge P (1982) Mitochondrial encephalomyopathies: biochemical studies in two cases revealing defects in the respiratory chain. Brain 105(Pt 3):553–582
Naoi M, Maruyama W (2009) Functional mechanism of neuroprotection by inhibitors of type B monoamine oxidase in Parkinson's disease. Expert Rev Neurother 9:1233–1250
Naoi M, Maruyama W (2010) Monoamine oxidase inhibitors as neuroprotective agents in age-dependent neurodegenerative disorders. Curr Pharm Des 16:2799–2817
Orimo S, Oka T, Miura H, Tsuchiya K, Mori F, Wakabayashi K, Nagao T, Yokochi M (2002) Sympathetic cardiac denervation in Parkinson's disease and pure autonomic failure but not in multiple system atrophy. J Neurol Neurosurg Psychiatry 73:776–777
Qin Z, Hu D, Han S, Reaney SH, Di Monte DA, Fink AL (2007) Effect of 4-hydroxy-2-nonenal modification on alpha-synuclein aggregation. J Biol Chem 282:5862–5870
Rubinsztein DC, Marino G, Kroemer G (2011) Autophagy and aging. Cell 146:682–695
Shamoto-Nagai M, Maruyama W, Hashizume Y, Yoshida M, Osawa T, Riederer P, Naoi M (2007) In Parkinsonian substantia nigra, alpha-synuclein is modified by acrolein, a lipid-peroxidation

product, and accumulates in the dopamine neurons with inhibition of proteasome activity. J Neural Transm 114:1559–1567

Shamoto-Nagai M, Hisaka S, Akao Y, Osawa T, Naoi M, Maruyama W (in preparation) A membrane-component, docosahexaenoic acid induces neuronal cell death with accumulation of abnormal-synuclein modified by lipid-peroxides: its relevance to the pathogenesis of Parkinson disease

Wurtman RJ, Cansev M, Sakamoto T, Ulus I (2010) Nutritional modifiers of aging brain function: use of uridine and other phosphatide precursors to increase formation of brain synapses. Nutr Rev 68(Suppl 2):S88–S101

Xiang L, Nakamura Y, Lim YM, Yamasaki Y, Kurokawa-Nose Y, Maruyama W, Osawa T, Matsuura A, Motoyama N, Tsuda L (2011) Tetrahydrocurcumin extends life span and inhibits the oxidative stress response by regulating the FOXO forkhead transcription factor. Aging (Albany NY) 3:1098–1109

Yakunin E, Loeb V, Kisos H, Biala Y, Yehuda S, Yaari Y, Selkoe DJ, Sharon R (2012) Alpha-synuclein neuropathology is controlled by nuclear hormone receptors and enhanced by docosahexaenoic acid in a mouse model for Parkinson's disease. Brain Pathol 22:280–294

Yavin E, Glozman S, Green P (2001) Docosahexaenoic acid accumulation in the prenatal brain: prooxidant and antioxidant features. J Mol Neurosci 16:229–235; discussion 279–284

Yoritaka A, Hattori N, Uchida K, Tanaka M, Stadtman ER, Mizuno Y (1996) Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. Proc Natl Acad Sci USA 93:2696–2701

Chapter 12
Lipid Hydroperoxide-Derived Modification of Proteins in Gastrointestinal Tract

Yuji Naito, Tomohisa Takagi, Osamu Handa, and Toshikazu Yoshikawa

Abstract Role of lipid peroxidation in the pathogenesis of gastrointestinal diseases has been evaluated by measuring the tissue levels of lipid peroxides as thiobarbituric acid-reactive substances in the animal models as well as human. Recently, N^{ε}-(hexanoyl)lysine (HEL) and 4-hydroxy-2-nonenal (HNE) are recognized as reliable and sensitive biomarkers for the early phase and the late phase of lipid peroxidation, respectively. The presence of HNE- and HEL-modified proteins has been demonstrated in *in vivo* models of several gastrointestinal diseases. In the present review, we introduced HNE-modification of TRPV1 channel in esophageal epithelial cells, HEL-modification of tropomyosin 1 (TMP1) in gastric cancer cells, and HEL-modification of gastrokine 1 in the healing of gastric ulcer.

Keywords Gastrokine • N^{ε}-(Hexanoyl)lysine • 4-Hydroxy-2-nonenal • Tropomyosin • TRPV1

1 Introduction

Oxidative stress is well known to be involved in the pathogenesis of gastrointestinal diseases. During oxidative stress, the oxidation of cellular components results in the modification of DNA, proteins, lipids, and carbohydrates. Among these biomolecules, we have demonstrated that lipid peroxidation plays a significant role in the pathogenesis of gastrointestinal diseases, including ischemia-reperfusion-induced gastric injury (Yoshikawa et al. 1989), indomethacin-induced gastric and intestinal

Y. Naito (✉) • T. Takagi • O. Handa • T. Yoshikawa
Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, 465 Kajiicho, Kamigyo-ku, Kyoto 602-8566, Japan
e-mail: ynaito@koto.kpu-m.ac.jp

Y. Kato (ed.), *Lipid Hydroperoxide-Derived Modification of Biomolecules*, Subcellular Biochemistry 77, DOI 10.1007/978-94-007-7920-4_12,

injuries (Yoshikawa et al. 1993), gastric ulcers (Naito et al. 1995), and chemical colitis induced by dextran sodium sulfate (Naito et al. 2003) and trinitrobenzoic acid (Yoshikawa et al. 1997a). The significance of lipid peroxidation has been revealed by measuring the tissue levels of thiobarbituric acid (TBA)-reactive substances as well as by evaluating the effects of several antioxidants on their levels (Yoshikawa et al. 1997b; Naito et al. 1998, 2002). When the unsaturated fatty acid is exposed to oxidative stress, a lipid peroxidation response progresses like a chain reaction, and various kinds of degradation products by lipid peroxidation responses are generated. Previous many studies showed that presence of the aldehyde molecules, which is an end-product of lipid peroxidation, has been implicated as causative agents in cytotoxic processes initiated by the exposure of biological systems to oxidizing agents. Recently, some of the lipid peroxidation products could exhibit a facile reactivity with proteins, generating a variety of intra- and intermolecular covalent adducts.

Among the reactive aldehydes, 4-hydroxy-2-nonenal (HNE), is believed to be largely responsible for the cytopathological effects observed during oxidative stress (Fig. 12.1). The most common and reliable approach for detection of HNE adducts is the use of antibodies, which recognize HNE bound to amino acid side-chains of proteins. In 1995, Toyokuni et al. (1995) raised a monoclonal antibody directed to the HNE-modified protein. This monoclonal antibody has been attested to be specific for the HNE-histidine Michael adduct. The development of specific antibodies against protein-bound HNE has made it possible for us to obtain highly probable evidence for the occurrence of lipid peroxidation *in vivo* as a late phase or footprint marker. It has been demonstrated the presence of HNE-modified proteins *in vivo* in the iron-nitrolotriacetate (Fe^{3+}-NTA)-induced renal carcinogenesis (Toyokuni et al. 1995), hyperglycemia injury of pancreatic β-cells (Ihara et al. 1999), ischemia-reperfusion injury (Yamagami et al. 2000), carbon tetrachloride-induced liver injury (Hartley et al. 1997), inflammatory bowel disease (Nair et al. 2006), chronic hepatitis type C (Kageyama et al. 2000), non-alcoholic steatohepatitis (Seki et al. 2002; Serviddio et al. 2008), atherosclerotic lesion (Uchida et al. 1994), Alzheimer's disease (Lauderback et al. 2001), Parkinson's disease (Qin et al. 2007), amyotrophic lateral sclerosis (Perluigi et al. 2005), and exercise-induced muscular injury (Aoi et al. 2003).

N^{ε}-(Hexanoyl)lysine (HEL) has been found in the reaction between linoleic hydroperoxide and lysine moiety of proteins (Fig. 12.1). It has been shown that the formation of HEL is a good marker for oxidative modification by oxidized *m*-6 fatty acids such as linoleic acid and arachidonic acid as an early marker of lipid peroxidation (Kato et al. 1999). By the immunostaining using monoclonal and polyclonal antibodies against HEL, it has been reported the HEL-positivity in muscular tissues (Kato et al. 2000), foam cell-rich areas in atherosclerotic lesion (Fukuchi et al. 2008), and liver treated with D-galactosamine (Osakabe et al. 2002).

In this session, we have introduced our recent data derived from the analysis for HNE or HEL-modified proteins in gastrointestinal tract.

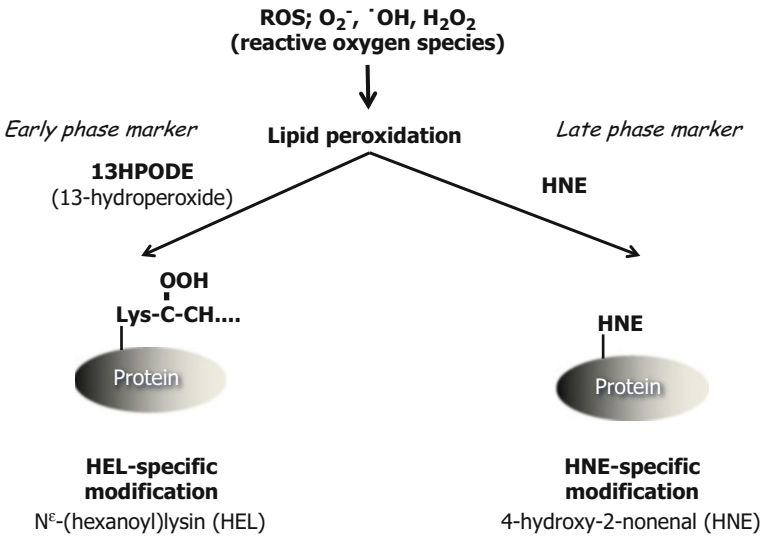


Fig. 12.1 Reactive molecules derived from lipid peroxidation and the modification of proteins by N^{ε}-(hexanoyl)lysine (HEL) and 4-hydroxy-2-nonenal (HNE), which are recognized as reliable and sensitive biomarkers for the early phase and the late phase of lipid peroxidation, respectively

2 HNE-Modification of TRPV1 in Esophageal Epithelial Cells

Transient receptor potential vanilloid subtype 1 (TRPV1) is a calcium-permeable nonselective cation channel that is activated by capsaicin, heat, protons, and endogenous lipids. The expression of TRPV1 in sensory neurons is well known. The presence of functional TRPV1 receptors has been reported in non-neuronal cells such as keratinocytes of the epidermis, human hair follicles, human bronchial epithelial cells, the bladder urothelium, and rat gastric epithelial cells (Avelino et al. 2002; Bodo et al. 2005; Kato et al. 2003; Southall et al. 2003). Recent reports indicated that TRPV1 is expressed not only on nerves but also on epithelial cells of cat esophageal mucosa, and that HCl-activated epithelial TRPV1 could initiate the induction of inflammation (Cheng et al. 2009). We have also confirmed that TRPV1 is expressed in human esophageal epithelial cell line Het1A and human esophageal epithelial cells as well as in rat esophageal mucosa (Kishimoto et al. 2011), and demonstrated that interleukin-8 (IL-8) is produced from the capsaicin-stimulated Het1A cells via the activation of TRPV1. Our data are supported by previous findings that TRPV1 mediates inflammatory responses in various cells stimulated with certain types of particular materials. These raise the possibility that TRPV1 plays an important role on inflammatory response at the cellular level against noxious stimuli, including gastric HCl, when expressed not only on nerves but also in human esophageal epithelial cells.

In addition, our results have shown that the levels of reactive oxygen species (ROS)-modified and HNE-modified proteins are increased in Het1A cells following treatment with capsaicin, and that these increases are blocked by a TRPV1 antagonist. Stimulation of Het1A cells with capsaicin has resulted in the induction of several HNE-modified proteins in a dose-dependent manner, as assessed by Western blot using a polyclonal anti-HNE antibody. To determine whether TRPV1 is modified with HNE in these cells, the TRPV1 proteins was immunoprecipitated from synthetic HNE-treated cells using an anti-TRPV1 antibody and immunoblotted with a monoclonal anti-HNE antibody. As shown in Fig. 12.2, the TRPV1 protein is modified by HNE in synthetic HNE-treated cells but not in control cells. Moreover, double-immunofluorescence staining demonstrated that HNE-modified TRPV1 is more strongly stained in HNE-treated cells than in control cells, and that merged images have revealed the colocalization of HNE-modified protein and TRPV1 in HNE-treated cells.

We have hypothesized that HNE-modification of TRPV1 protein might affect the function or the sensitivity of the receptor. Although low concentration of HNE (1 μM) alone did not induce IL-8 production from Het1A cells, the pretreatment with 1 μM HNE significantly enhanced IL-8 production in capsaicin-stimulated cells, indicating the possibility of the interaction between HNE and TRPV1 proteins. TRPV1 contains several histidine and cysteine residues that could potentially be involved in redox modulation. Because the monoclonal anti-HNE antibody used in our study recognizes HNE-modified amino acid residues such as histidine, HNE might bind to extracellular or intracellular specific amino acid sites of TRPV1 and modify the function of TRPV1. Although it remains to be investigated whether oxidative stress-induced modification of TRPV1 raises the hypersensitivity of the esophagus, defining these mechanisms may help in the development of novel therapies for gastroesophageal reflux disease.

3 HEL-Modification of Tropomyosin 1 in Gastric Cancer Cells

Several *in vivo* cancer models support the hypothesis that lipid peroxidation plays a critical role in experimental carcinogenesis (Kawai et al. 2003; Murugan et al. 2007). Lipid peroxidation under excessive oxidative stress may play a crucial role in oxidative modification in the process of carcinogenesis, because reactive lipid-decomposition products trigger secondary modification of proteins and nucleic acids. Modification of DNA bases and proteins by reactive aldehydes, such as HNE, malondialdehyde, and acrolein, is thought to contribute to the mutagenic and carcinogenic effects associated with oxidative stress-induced lipid peroxidation (Kawai et al. 2003). Shimokawa et al. (2008) recently succeeded in the establishment of a novel, transformed cell line (RGK-1) derived from a normal gastric mucosal cell line (RGM-1) of Wistar rats after treatment with the alkylating

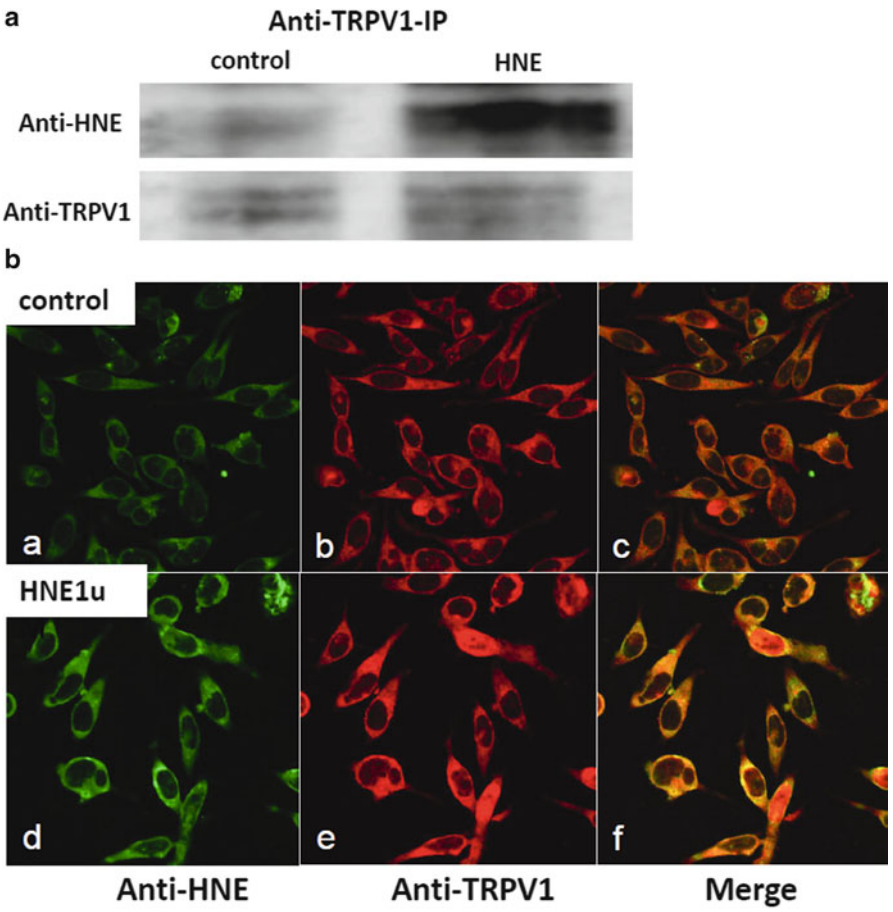


Fig. 12.2 Synthetic HNE modified TRPV1 in human esophageal epithelial Het1A cells. (**a**): Cell lysates of Het1A cells incubated with synthetic HNE (10 μM) for 20 min were centrifuged, and the supernatants were immunoprecipitated with a polyclonal anti-TRPV1 antibody (Anti-TRPV1-IP). Immunoprecipitates were analyzed by Western blot analysis using a monoclonal anti-HNE and an anti-TRPV1 antibody. (**b**): Double-immunofluorescence staining with a monoclonal anti-HNE antibody (AlexaFluoro 488, **a** and **d**) and an anti-TRPV1 antibody (AlexaFluoro 647, **b** and **e**). The merged images (*yellow color*) show the co-localization of TRPV1 with HNE-modified proteins (**c** and **f**) (Reprinted with permission from Kishimoto et al. 2011)

carcinogen *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG). This cell line showed signs of neoplasia and transformation, in that it lost contact inhibition and formed tumors in nude mice. RGK-1 cells are the first MNNG-induced neoplastic mutant cells derived from a noncancerous, nonembryonic gastric epithelial cell line (RGM-1). This establishment of RGK-1 cells has made it possible to investigate gastric carcinogenesis using two paired cell lines: RGM-1 and RGK-1 cells. We have succeeded to detect HEL-modified proteins by two-dimensional

polyacrylamide gel electrophoresis (2D-PAGE) and Western blotting using a novel monoclonal antibody against HEL, and determined by peptide mass fingerprinting using MALDI-TOF MS and the MASCOT search engine by comparing protein spots derived from RGM-1 and transformed RGK-1 cells (Okada et al. 2012).

To detect HEL-modified proteins in RGM-1 and RGK-1 cells, whole proteins from cells were separated by SDS-PAGE or 2-DE, and analyzed by Western blotting using anti-HEL polyclonal antibody. HEL-modified proteins were detected in RGM-1 and RGK-1 cells, and the protein band between 30 and 40 kDa was increased in RGK-1 cells compared with RGM-1 cells. To identify the two HEL-modified protein spots in RGK-1 cells, the protein spots were excised from 2-DE gel stained with CBB, and analyzed by mass spectrometry. The data obtained using mass spectrometry (Fig. 12.3) were searched using the MASCOT search engine, and both protein spots were identified as the TPM1 protein with a MASCOT score of 65 and 39 % sequence coverage (Okada et al. 2012). To confirm the presence of HEL-modified TPM1 in RGM-1 and RGK-1 cells, we detected TPM1 and HEL using Western blotting of SDS-PAGE-separated or 2-DE-separated proteins. As shown Fig. 12.3, the protein band between 30 and 40 kDa reacted with both anti-TPM1 antibody and anti-HEL antibody. The TPM1 protein expression level was increased in RGK-1 cells compared with RGM-1 cells and was modified by HEL in RGK-1 cells. Moreover, as shown in Fig. 12.3, the HEL-modified protein spots reacted with anti-TPM1 antibody. To further confirm the presence of HEL-modified TPM1, we detected TPM1 and HEL by Western blotting in the protein sample obtained by immunoprecipitation with anti-TPM1 antibody. To the best of our knowledge, TPM1 is the first protein for which HEL modification has been determined by the 2D-PAGE and MS.

Although TPM isoforms have been known to function in the regulation of muscle contraction, the functional significance of the multiple TPM isoforms present in non-muscle cells, especially in cancer cells, remains largely unknown. The most striking observation from our study was that HEL-modified TPM1 was identified even though the total protein levels in transformed gastric cancer cells were higher than those in normal gastric cells. It has been reported that the regulation of TPM1 expression appears to be mediated by TPM1 promoter methylation, histone deacetylation (Bharadwaj and Prasad 2002), and microRNA-21 (Zhu et al. 2008). In addition to these regulations, the present study indicates the possibility that post-translational modification of the TPM1 protein under excessive oxidative stress may affect its function. Thus, in addition to epigenetic and translational regulations, TPM1 is also regulated by post-translational modification. It is our expectation that the molecular mechanism of HEL modification and the functional influence of this modification on the TPM1 protein will be identified in the near future.

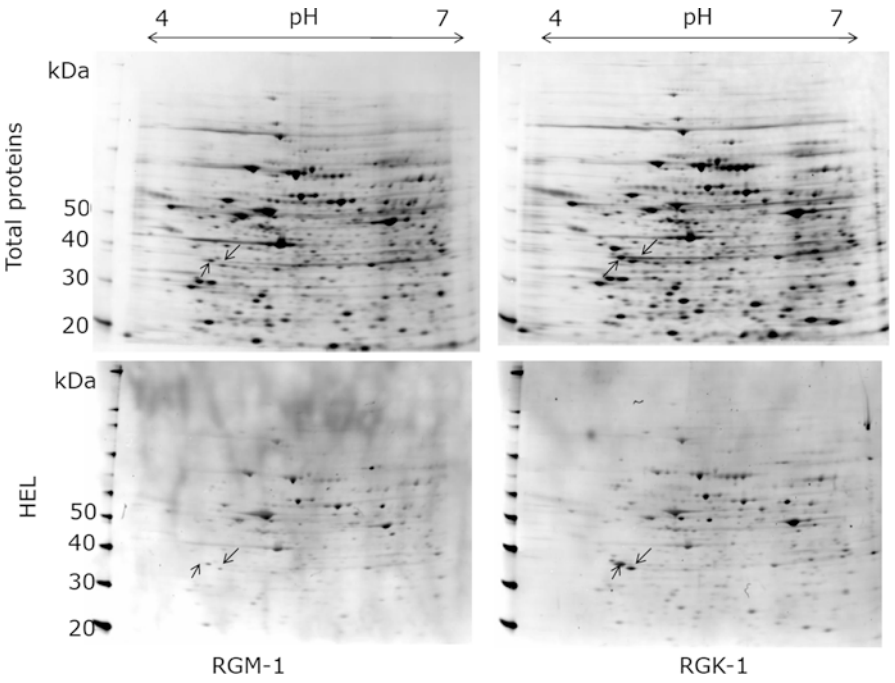


Fig. 12.3 Detection of HEL-modified proteins from RGM-1 and RGK-1 cells by 2-DE. Proteins extracts were separated using 2-DE. The 2-DE gels were transferred onto nitrocellulose membranes and stained with Deep Purple Total Stain to detect total proteins. The membranes were subjected to Western blotting with anti-HEL antibody (Reprinted with permission from Okada et al. 2012)

4 Lipid Peroxidation in the Development of Gastric Ulcer

Although much attention has been focused on the role of lipid peroxidation in precipitating acute gastric mucosal injuries induced by several kinds of stress or ischemia-reperfusion (Yoshikawa et al. 1986, 1989, 1993), these injuries are multiple shallow erosions and are limited to the gastric mucosa, which are apparently different from a solitary human gastric ulcer penetrating the muscularis mucosa. In the following study, we have investigated the role of lipid peroxidation in the development and the healing process of gastric ulcer using a murine model of gastric ulcer. We have succeeded to produce a solitary gastric ulcer penetrating into the muscular layer in rats by the local injection into the gastric submucosa of ferrous and ascorbic acid (Fe/ASA) solution that is known to generate active oxygen species *in vitro* (Naito et al. 1995). With a local injection of saline alone or ascorbic acid alone in to the gastric mucosa of rats, no ulcers or erythema were observed 24 h after the injection. Although slightly erythematous areas were observed on the mucosal surface at the site of the local injection of $FeSO_4$ alone, no ulcer formed. In the Fe/ASA group, a round solitary ulcer formed on the mucosa at the site of local

injection of the Fe/ASA solution 24 after the injection in a dose-dependent manner. Histological studies revealed that the base of the ulcer extended beyond the proper muscle layers, and in some cases the ulcers had penetrated to the liver as well. The concentration of TBA-reactive substances in the gastric mucosa 24 h after the injection significantly increased in rats treated with the Fe/ASA solution, but did not increase in rats injected with saline, ascorbic acid, or $FeSO_4$. To clarify the cause-effect relationship of ulcer formation and lipid peroxidation, the time-course changes in these parameters in the gastric mucosa were investigated for up to 24 h after the injection of the Fe/ASA solution at a dose of 400 μM. Interestingly, TBA-reactive substances in the gastric mucosa increased significantly 1 h after the injection with the Fe/ASA solution, and remained elevated up to 24 h after the injection. These increases in TBA-reactive substances preceded grossly evident gastric ulcer. These results together indicate that lipid peroxidation plays a crucial role in the pathogenesis of the gastric ulcer induced by the Fe/ASA solution, as well as acute superficial injuries produced by burn shock, water-immersion restraint stress, indomethacin, and ischemia-reperfusion.

In addition, simultaneous administration of CuZn-SOD significantly reduced the area of the gastric ulcer and significantly inhibited the increase in TBA-reactive substances in the gastric mucosa 24 h after injection of the Fe/ASA solution. These findings suggest that lipid peroxidation mediated by superoxide radicals generated by the Fe/ASA system plays an important role in the development of these ulcers. Although the detailed of the mechanism of ascorbate autoxidation are extremely complex, only native but not apo- or heated SOD reduced mucosal injury in our *in vivo* experiment, indicating that superoxide radicals directly, or reactive oxygen species produced by the reduction of superoxide, are involved in the formation of gastric ulcers.

5 HEL-Modification of Gastrokine in the Healing of Gastric Ulcer

Recently, we have confirmed that Rikkunshito, a Japanese traditional herbal medicine, enhances the healing process of gastric ulcer induced by acetic acid in mice (Horie et al. 2012). The model of gastric ulcer by acetic acid has been well mimicked to human ulcers in terms of both pathological features and healing process. As it has already been demonstrated that the area of the ulcer lesions reached a maximum on day 2 or 3 after the induction of acetic gastric ulcer, to focus on the effects of mucosal healing by the treatment with Rikkunshito, all mice were administered either placebo or Rikkunshito (100 mg/kg/day) starting on day 2 after the induction of acetic gastric ulcer until day 7. The area of gastric ulcer on day 7 significantly decreased in mice treated with Rikkunshito by approximately 70 % of the initial value observed on mice without the treatment with Rikkunshito. As well as Rikkunshito has been used to treat various gastrointestinal tract disorders,

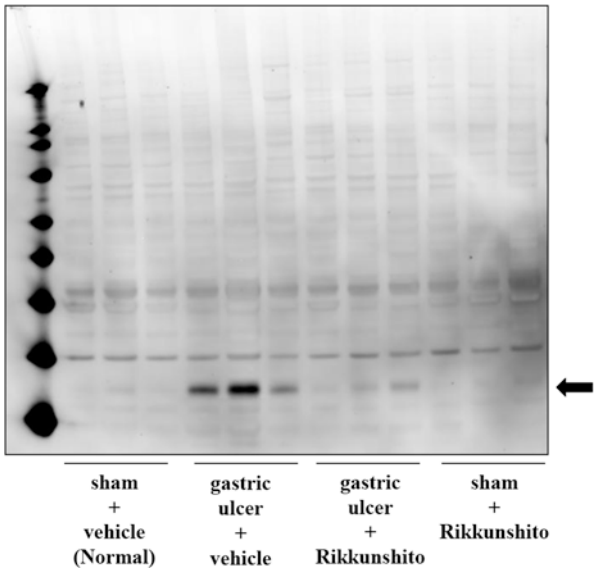


Fig. 12.4 Detection of HEL-modified proteins in gastric mucosa of mice with gastric ulcer. HEL-modified proteins in gastric mucosa of mice were evaluated by western blotting using anti-HEL monoclonal antibody

such as functional dyspepsia, gastroesophageal reflux, dyspeptic symptoms of post-gastrointestinal surgery, and chemotherapy-induced nausea, our data firstly confirmed that Rikkunshito has a therapeutic potential against gastric ulcers.

The purified cytosolic fractions of mucosal homogenates from murine gastric mucosa were separated by SDS-PAGE or 2-DE, and analyzed by western blotting using anti-HEL monoclonal antibody. As several HEL-modified proteins were detected in gastric mucosa, the protein band between 20 and 30 kDa was increased in gastric mucosa 7 days after the induction of acetic gastric ulcer in the SDS-PAGE analysis, and the increased band was diminished by the treatment with Rikkunshito (Fig. 12.4). To identify the HEL-modified protein spot, the protein spot was excised from 2DE-gel stained with Deep Purple Total Protein Stain, and analyzed by mass spectrometry. The data obtained using mass spectrometry was searched using the MASCOT search engine and the protein spot was identified as gastrokine-1 (GKN1) protein. The expression of GKN1 proteins and mRNA in gastric mucosa did not change between sham-treated mice and mice with gastric ulcer induced by acetic acid. Furthermore, the administration of Rikkunshito did not affect the expression of GKN1 proteins and mRNA compared to vehicle treatment mice in both groups that were sham-treated mice and mice treated with acetic acid.

GKN1 is abundantly and specifically expressed in superficial gastric epithelium in all areas of stomach (Oien et al. 2004), and protects gastric mucosa and promotes healing after gastric mucosal injuries through the facilitating restitution and proliferation of gastric epithelial cells (Martin et al. 2003). Consistently, we confirmed that GKN1 was expressed in gastric epithelial cell in normal gastric mucosa and in regenerated mucosal cells around ulcer margin. Furthermore, the expression of GKN1 has be reportedly decreased in gastric mucosa of the subjects with aspirin

treatment patients (Martin et al. 2008) and in that of the patients infected with *Helicobacter pylori* (Nardone et al. 2008). In the present study, the expression of GKN1 protein and mRNA did not change between sham-treated mice and mice with gastric ulcer induced by acetic acid. These results indicate that HEL-modified GKN1 may be a candidate to explain the properties of GKN1; the enhancement of ulcer healing.

6 Conclusion

As shown in our previous studies, lipid peroxidation plays a significant role in the pathogenesis of gastrointestinal injuries, inflammation, and carcinogenesis. Recent studies using more specific and reliable markers including HNE- and HEL-modification have confirmed our hypothesis. In the above pathological conditions, the function of target proteins would be regulated by their post-translational modification. It is our expectation that the molecular mechanism of HNE/HEL-modifications and the functional influence of these modifications on the target protein will be identified in the near future. It will be more important to investigate the relationship between the function and the modification compared with the detection of modified proteins.

References

Aoi W, Naito Y, Sakuma K et al (2003) Astaxanthin limits exercise-induced skeletal and cardiac muscle damage in mice. Antioxid Redox Signal 5:139–144

Avelino A, Cruz C, Nagy I, Cruz F (2002) Vanilloid receptor 1 expression in the rat urinary tract. Neuroscience 109:787–798

Bharadwaj S, Prasad GL (2002) Tropomyosin-1, a novel suppressor of cellular transformation is downregulated by promoter methylation in cancer cells. Cancer Lett 183:205–213

Bodo E, Biro T, Telek A et al (2005) A hot new twist to hair biology: involvement of vanilloid receptor-1 (VR1/TRPV1) signaling in human hair growth control. Am J Pathol 166:985–998

Cheng L, de la Monte S, Ma J et al (2009) HCl-activated neural and epithelial vanilloid receptors (TRPV1) in cat esophageal mucosa. Am J Physiol Gastrointest Liver Physiol 297:G135–G143

Fukuchi Y, Miura Y, Nabeno Y et al (2008) Immunohistochemical detection of oxidative stress biomarkers, dityrosine and N(epsilon)-(hexanoyl)lysine, and C-reactive protein in rabbit atherosclerotic lesions. J Atheroscler Thromb 15:185–192

Hartley DP, Kroll DJ, Petersen DR (1997) Prooxidant-initiated lipid peroxidation in isolated rat hepatocytes: detection of 4-hydroxynonenal- and malondialdehyde-protein adducts. Chem Res Toxicol 10:895–905

Horie H, Naito Y, Takagi T et al (2012) Rikkunshito, a Japanese traditional herbal medicine, promotes murine gastric ulcer healing through the inhibition of the oxidative modification to proteins. Gastroenterology 140:S315–S316

Ihara Y, Toyokuni S, Uchida K et al (1999) Hyperglycemia causes oxidative stress in pancreatic beta-cells of GK rats, a model of type 2 diabetes. Diabetes 48:927–932

Kageyama F, Kobayashi Y, Kawasaki T et al (2000) Successful interferon therapy reverses enhanced hepatic iron accumulation and lipid peroxidation in chronic hepatitis C. Am J Gastroenterol 95:1041–1050

Kato Y, Mori Y, Makino Y et al (1999) Formation of Nepsilon-(hexanonyl)lysine in protein exposed to lipid hydroperoxide. A plausible marker for lipid hydroperoxide-derived protein modification. J Biol Chem 274:20406–20414

Kato Y, Miyake Y, Yamamoto K et al (2000) Preparation of a monoclonal antibody to N(epsilon)-(Hexanonyl)lysine: application to the evaluation of protective effects of flavonoid supplementation against exercise-induced oxidative stress in rat skeletal muscle. Biochem Biophys Res Commun 274:389–393

Kato S, Aihara E, Nakamura A et al (2003) Expression of vanilloid receptors in rat gastric epithelial cells: role in cellular protection. Biochem Pharmacol 66:1115–1121

Kawai Y, Furuhata A, Toyokuni S, Aratani Y, Uchida K (2003) Formation of acrolein-derived 2′-deoxyadenosine adduct in an iron-induced carcinogenesis model. J Biol Chem 278:50346–50354

Kishimoto E, Naito Y, Handa O et al (2011) Oxidative stress-induced posttranslational modification of TRPV1 expressed in esophageal epithelial cells. Am J Physiol Gastrointest Liver Physiol 301:G230–G238

Lauderback CM, Hackett JM, Huang FF et al (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

Martin TE, Powell CT, Wang Z et al (2003) A novel mitogenic protein that is highly expressed in cells of the gastric antrum mucosa. Am J Physiol Gastrointest Liver Physiol 285:G332–G343

Martin G, Wex T, Treiber G, Malfertheiner P, Nardone G (2008) Low-dose aspirin reduces the gene expression of gastrokine-1 in the antral mucosa of healthy subjects. Aliment Pharmacol Ther 28:782–788

Murugan RS, Mohan KV, Uchida K et al (2007) Modulatory effects of black tea polyphenols on oxidant-antioxidant profile and expression of proliferation, apoptosis, and angiogenesis-associated proteins in the rat forestomach carcinogenesis model. J Gastroenterol 42:352–361

Nair J, Gansauge F, Beger H et al (2006) Increased etheno-DNA adducts in affected tissues of patients suffering from Crohn's disease, ulcerative colitis, and chronic pancreatitis. Antioxid Redox Signal 8:1003–1010

Naito Y, Yoshikawa T, Yoneta T et al (1995) A new gastric ulcer model in rats produced by ferrous iron and ascorbic acid injection. Digestion 56:472–478

Naito Y, Yoshikawa T, Matsuyama K et al (1998) Neutrophils, lipid peroxidation, and nitric oxide in gastric reperfusion injury in rats. Free Rad Biol Med 24:494–502

Naito Y, Takagi T, Ishikawa T et al (2002) alpha-Phenyl-N-tert-butylnitrone provides protection from dextran sulfate sodium-induced colitis in mice. Antioxid Redox Signal 4:195–206

Naito Y, Takagi T, Handa O et al (2003) Enhanced intestinal inflammation induced by dextran sulfate sodium in tumor necrosis factor-alpha deficient mice. J Gastroenterol Hepatol 18:560–569

Nardone G, Martin G, Rocco A et al (2008) Molecular expression of Gastrokine 1 in normal mucosa and in Helicobacter pylori-related preneoplastic and neoplastic gastric lesions. Cancer Biol Ther 7:1890–1895

Oien KA, McGregor F, Butler S et al (2004) Gastrokine 1 is abundantly and specifically expressed in superficial gastric epithelium, down-regulated in gastric carcinoma, and shows high evolutionary conservation. J Pathol 203:789–797

Okada H, Naito Y, Takagi T et al (2012) Detection of N-(hexanoyl)lysine in the tropomyosin 1 protein in N-methyl-N′-nitro-N-nitrosoguanidine-induced rat gastric cancer cells. J Clin Biochem Nutr 50:47–52

Osakabe N, Yasuda A, Natsume M et al (2002) Rosmarinic acid, a major polyphenolic component of Perilla frutescens, reduces lipopolysaccharide (LPS)-induced liver injury in D-galactosamine (D-GalN)-sensitized mice. Free Radic Biol Med 33:798–806

Perluigi M, Fai Poon H, Hensley K et al (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

Qin Z, Hu D, Han S et al (2007) Effect of 4-hydroxy-2-nonenal modification on alpha-synuclein aggregation. J Biol Chem 282:5862–5870

Seki S, Kitada T, Yamada T et al (2002) In situ detection of lipid peroxidation and oxidative DNA damage in non-alcoholic fatty liver diseases. J Hepatol 37:56–62

Serviddio G, Bellanti F, Tamborra R et al (2008) Uncoupling protein-2 (UCP2) induces mitochondrial proton leak and increases susceptibility of non-alcoholic steatohepatitis (NASH) liver to ischaemia-reperfusion injury. Gut 57:957–965

Shimokawa O, Matsui H, Nagano Y et al (2008) Neoplastic transformation and induction of H+, K + −adenosine triphosphatase by N-methyl-N′-nitro-N-nitrosoguanidine in the gastric epithelial RGM-1 cell line. In vitro Cell Dev Biol 44:26–30

Southall MD, Li T, Gharibova LS et al (2003) Activation of epidermal vanilloid receptor-1 induces release of proinflammatory mediators in human keratinocytes. J Pharmacol Exp Ther 304:217–222

Toyokuni S, Miyake N, Hiai H et al (1995) The monoclonal antibody specific for the 4-hydroxy-2-nonenal histidine adduct. FEBS Lett 359:189–191

Uchida K, Toyokuni S, Nishikawa K et al (1994) Michael addition-type 4-hydroxy-2-nonenal adducts in modified low-density lipoproteins: markers for atherosclerosis. Biochemistry 33:12487–12494

Yamagami K, Yamamoto Y, Kume M et al (2000) Formation of 8-hydroxy-2′-deoxyguanosine and 4-hydroxy-2-nonenal-modified proteins in rat liver after ischemia-reperfusion: distinct localization of the two oxidatively modified products. Antioxid Redox Signal 2:127–136

Yoshikawa T, Miyagawa H, Yoshida N, Sugino S, Kondo M (1986) Increase in lipid peroxidation in rat gastric mucosal lesions induced by water-immersion restraint stress. J Clin Biochem Nutr 1:271–277

Yoshikawa T, Ueda S, Naito Y et al (1989) Role of oxygen-derived free radicals in gastric mucosal injury induced by ischemia or ischemia-reperfusion in rats. Free Rad Res Commun 7:285–291

Yoshikawa T, Naito Y, Kishi A et al (1993) Role of active oxygen, lipid peroxidation, and antioxidants in the pathogenesis of gastric mucosal injury induced by indomethacin in rats. Gut 34:732–737

Yoshikawa T, Yamaguchi T, Yoshida N et al (1997a) Effect of Z-103 on TNB-induced colitis in rats. Digestion 58:464–468

Yoshikawa T, Minamiyama Y, Ichikawa H et al (1997b) Role of lipid peroxidation and antioxidants in gastric mucosal injury induced by the hypoxanthine-xanthine oxidase system in rats. Free Radic Biol Med 23:243–250

Zhu S, Wu H, Wu F et al (2008) MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. Cell Res 18:350–359

Part III
Applications for Diagnosis and Development of Functional Food

Chapter 13
Low-Cost and Easy-to-Use "on-Chip ELISA" for Developing Health-Promoting Foods

Fumihiko Hoshino, Osamu Watanabe, Xiaohong Wu, Yosuke Takimoto, and Toshihiko Osawa

Abstract We have determined that a biological molecule can be physically immobilized on a polymer containing an azobenzene (azopolymer) using irradiating light. We immobilized antibodies and antigens on the surface of an azopolymer coated glass slide (antibody array) to establish "on-chip ELISAs". The assays used the flat-surface of a glass slide and could be applied to both sandwich and competitive ELISAs. The sensitivity and accuracy of the on-chip ELISA were similar to a conventional ELISA using a polystyrene plate. Using the assay system, we proved that representative oxidative-biomarkers could be simultaneously measured from uL of urine. That should realize low-cost study on animal or human, and accelerate development of health-promoting foods. So, this new concept antibody array has promising applications in proteomic studies, and could be used to examine biomarkers to investigate health-promoting food.

Keywords Antibody • ELISA • Hexanoyl lysine • 8-OHdG

1 Principles of Molecular-Imprinting and Immobilization

We found that the surface of a polymer containing an azobenzene (azopolymer) can transform along with the surface of an attached object by means of irradiating blue light (Kawata et al. 1999; Ikawa et al. 2000, 2001, 2006; Hasegawa et al. 2001). Figure 13.1 shows the chemical structure of a typical azopolymer. When exposed to

F. Hoshino (✉) • O. Watanabe
TOYOTA Central R&D Labs., Inc., Nagoya, Aichi, Japan
e-mail: hoshino77@mosk.tytlabs.co.jp

X. Wu • Y. Takimoto
Healthcare Systems, Inc., Nagoya, Aichi, Japan

T. Osawa
Department of Health and Nutrition, Aichi Gakuin University, Nisshin, Aichi, Japan

Y. Kato (ed.), *Lipid Hydroperoxide-Derived Modification of Biomolecules*,
Subcellular Biochemistry 77, DOI 10.1007/978-94-007-7920-4_13,

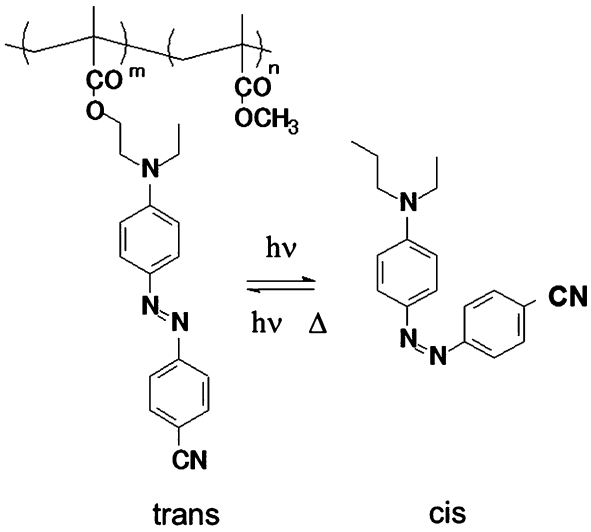


Fig. 13.1 The chemical structure of a typical azopolymer. The azobenzene moiety isomerizes between the trans and cis form. Isomeric transition of the azobenzene moiety arises during photo-irradiation

light of a certain wavelength, the trans form can be isomerized to the cis form. Cis-trans back-isomerization can occur thermally and/or photochemically and isomeric transition of the azobenzene moiety arises during photo-irradiation. This property of the azobenzene moiety leads to a drastic change in the physical properties of the polymer matrix. Azopolymer is a photoinduced deformable polymer: it deforms slowly during light irradiation and stays deformed after light irradiation has ceased.

Figure 13.2 shows a scanning electron microscope (SEM) image of an azopolymer surface after irradiation and partial removal of the polystyrene nanosphere by sonication. The polystyrene nanospheres are tightly immobilized on the azopolymer surface: the area where the polystyrene nanospheres were removed is deformed in a circle. Similar to polystyrene nanospheres, an antibody, 15 nm in size, can be immobilized on the azopolymer surface (Fig. 13.3) (Ikawa et al. 2006). In contrast with the smooth surface of the azopolymer (Fig. 13.3a), small particles similar in size to an antibody could be observed on the azopolymer surface where antibodies were immobilized (Fig. 13.3b), and small dimples similar in size to an antibody could be seen on the surface when the immobilized antibodies were removed by denaturing and washing with surfactant SDS (Fig. 13.3c).

2 Antibody Array Using the Photoimmobilization Method

We applied the photoimmobilization method to obtaining protein chips and, more specifically, an antibody array. An enzyme-linked immuno-sorbent assay (ELISA) is commonly used to detect small amounts of protein in sample solutions, such as serum. Although an ELISA is an excellent method for the detection of proteins,

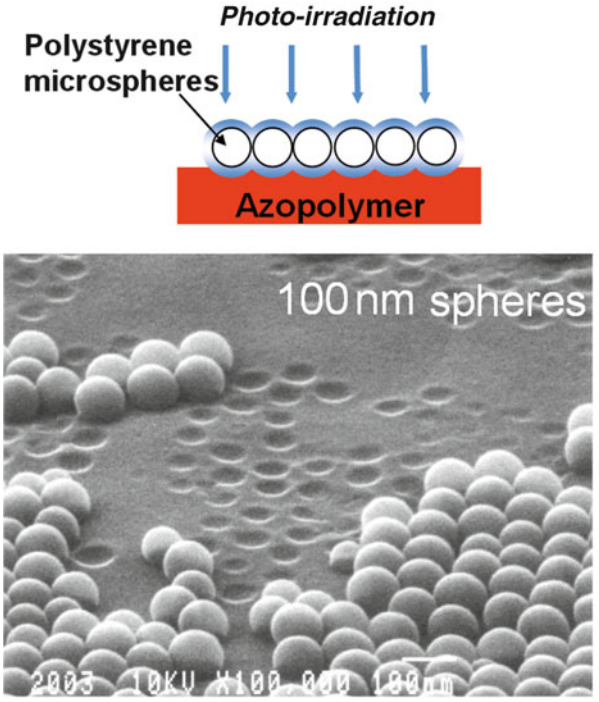


Fig. 13.2 Schematic illustration of the photoimmobilization of microspheres on the surface of an azopolymer. The surface of the azopolymer is deformed to the round shape of microspheres after photo-irradiation

there are disadvantages: it is comparatively expensive, and it is difficult to detect proteins when using small quantities of the sample solution. We considered that an antibody array could act as a micro ELISA system with the capability of dealing with small quantities of sample solutions and simultaneous measurements of several biomarkers in one test sample.

2.1 Preparation of an Antibody Array

For the antibody array, we prepared a spin-coated azopolymer film, 40 nm in thickness, on a standard glass slide. Antibody solution was applied by contact using pins or non-contact using a dispenser device, such as an ink-jet nozzle or micro syringe. Spotted drops were dried naturally and the surface was then irradiated with incoherent light of 470 nm wavelength and 20 mW/cm^2 optical power density from a 5 × 10 array of bluelight-emitting diodes (LEDs) for 30 min (Fig. 13.4). Finally, the glass slide was washed with 0.01 % Tween 20 PBS (TPBS).

We initially examined the specific reactions of photoimmobilized antibodies on azopolymer surfaces for the antibody array application. Solutions of anti-goat IgG (left-hand side) and anti-rabbit IgG (right-hand side) were spotted onto an azopolymer surface at different concentrations, the layout of which is shown in Fig. 13.5a. After

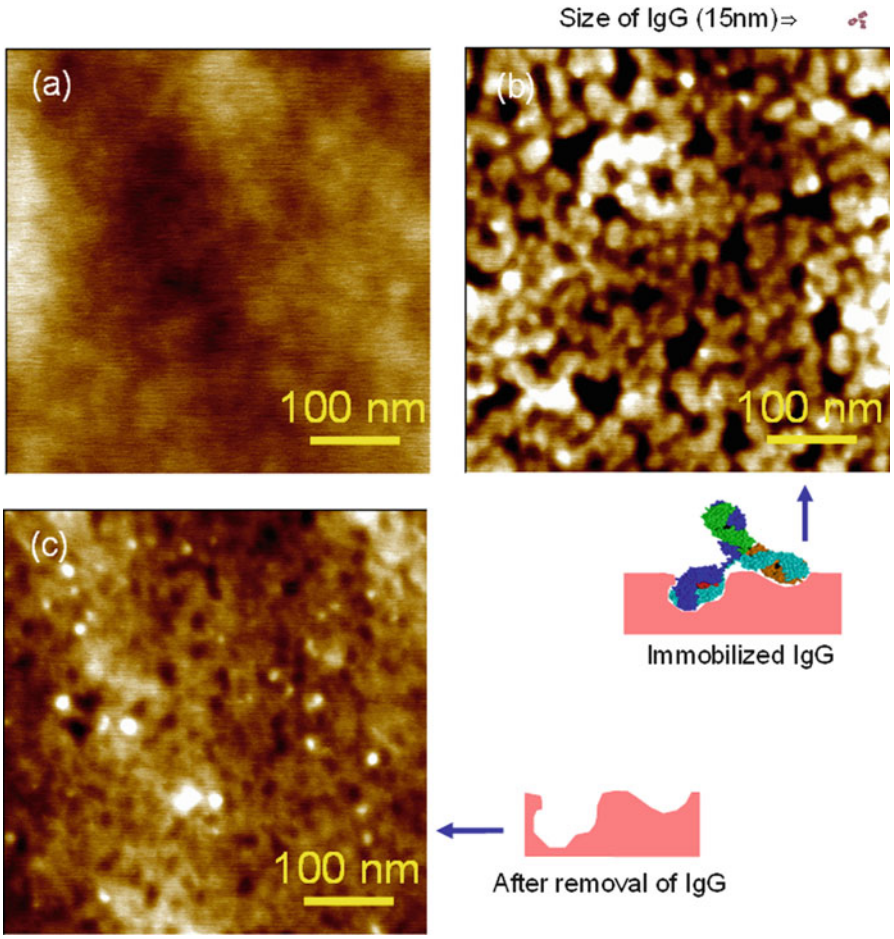


Fig. 13.3 Azopolymer surface deformation with antibody moiety. (**a**) Control showing the bare azopolymer surface after irradiation. (**b**) Tapping-mode atomic force microscope image of the azopolymer surface covered with antibodies. (**c**) Same image taken after removal of the antibodies by washing with PBS containing 2 % SDS

photoimmobilization and washing, the samples were incubated separately with Cy5-labeled antigens (goat IgG and rabbit IgG). The anti-goat IgG antibody recognized goat IgG when Cy5-labeled goat IgG was introduced onto the sample, and anti-rabbit IgG recognized rabbit IgG when Cy5-labeled rabbit IgG was introduced, as shown in Fig. 13.5b, c. The specific reactivity of the antibodies was realized by fixing photoimmobilized antibodies on the surface of the azopolymer.

We also examined the stability of the photoimmobilized antibodies. The reactivity of the antibodies decreased over a period of 10 days when stored at room temperature, however, the stability was maintained for about 2 months when stored at 4 °C (data not shown). This result is acceptable in terms of commercial viability, though further increases in stability would be preferable.

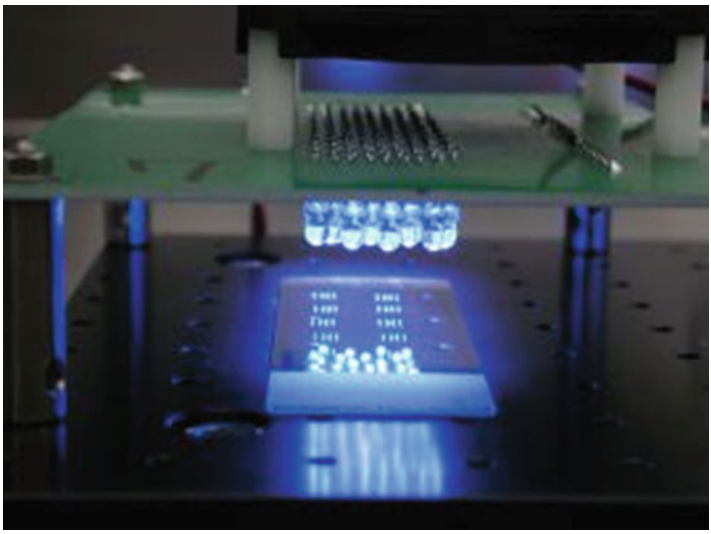


Fig. 13.4 Irradiation of an azopolymer slide after spotting and drying of protein solution

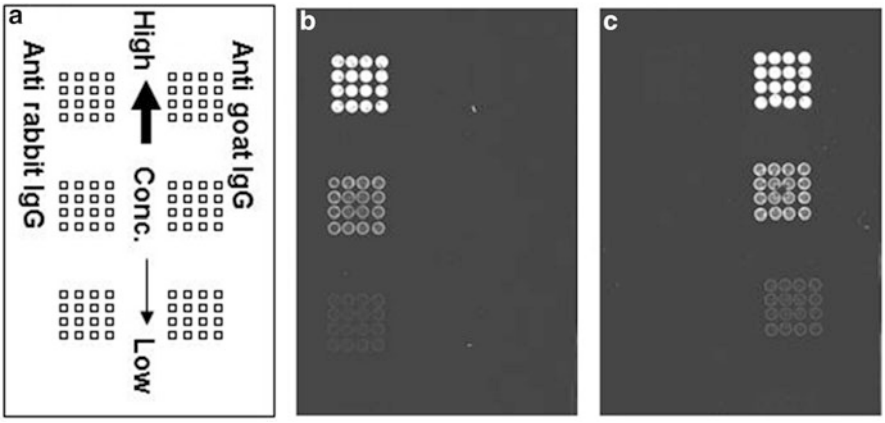


Fig. 13.5 (**a**) Layout of a slide spotted and immobilized with rabbit anti-goat IgG antibody and goat anti-rabbit IgG antibody. (**b**) Fluorescent image after incubation with a Cy5-labeled goat IgG. (**c**) Fluorescent image after incubation with a Cy5-labeled rabbit IgG

2.2 Sandwich ELISA of Antibody Array

We established a sandwich ELISA method for the antibody array. Figure 13.6 illustrates the sandwich immunoassay system to measure mouse adiponectin (Acrp30) in serum. We photoimmobilized anti-mouse Acrp30 antibody (R&D Systems Inc. AF119) onto the azopolymer surface, after which mouse Acrp30, biotin-labeled anti-mAcrp30 antibody and alkaline phosphatase-labeled streptavidin were

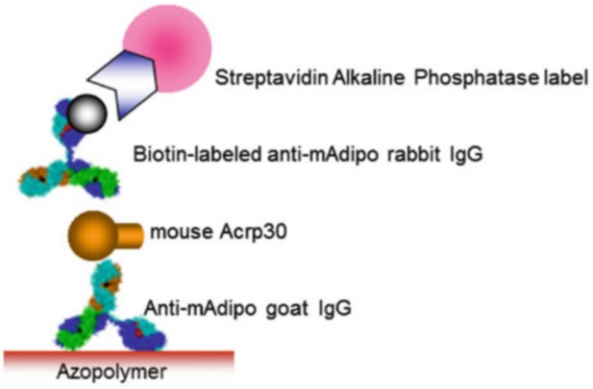


Fig. 13.6 Illustration of a sandwich ELISA using antibody array

sequentially bound, as in a conventional ELISA. The details of the procedure are as follows:

1. First antibody-antigen reaction
   1 μL of standard dilutions of Acrp30, or diluted serum samples, were dispensed independently onto the azopolymer surface where the antibody was immobilized.
2. Second antibody-antigen reaction
   40 μL of biotin-labeled anti-mAcrp30 antibody solution (Otsuka Pharmaceutical Co., Ltd.) was applied to the reaction and immediately covered with a gap cover glass which has spacers along the long side of the cover glass, about 10 μm thickness (Matsunami Glass Ind., Ltd.).
3. Alkaline phosphatase-labeled streptavidin reaction
   40 μL of alkaline phosphatase-labeled streptavidin solution was then applied to the reaction and covered with a gap cover glass.
4. Chemiluminescent detection
   40 μL of CDP-star Emerald II solution was applied before being covered with a gap cover glass. Luminescence was measured, by exposure for 5–15 min, with a CCD detector (AISIN, LV-400).

Adiponectin is a biologically active agent excreted from adipose cells, and prevents arteriosclerosis. Figure 13.7 shows the calibration curve obtained using low concentrations of adiponectin, and it illustrates that a linear relationship exists between luminescent intensity and concentration. We detected adiponectin in a sample solution down to a concentration of at least 0.1 ng/ml, which is almost the same sensitivity as that obtained with a conventional ELISA. We compared the conventional ELISA system and an antibody array system using mouse adiponectin from culture supernatant. Figure 13.8 shows the correlation between the antibody array and conventional ELISA. A high degree of correlation was observed ($r^2 = 0.97$, $p < 0.01$), indicating that the use of an antibody array with an azopolymer film is a promising candidate for practical use.

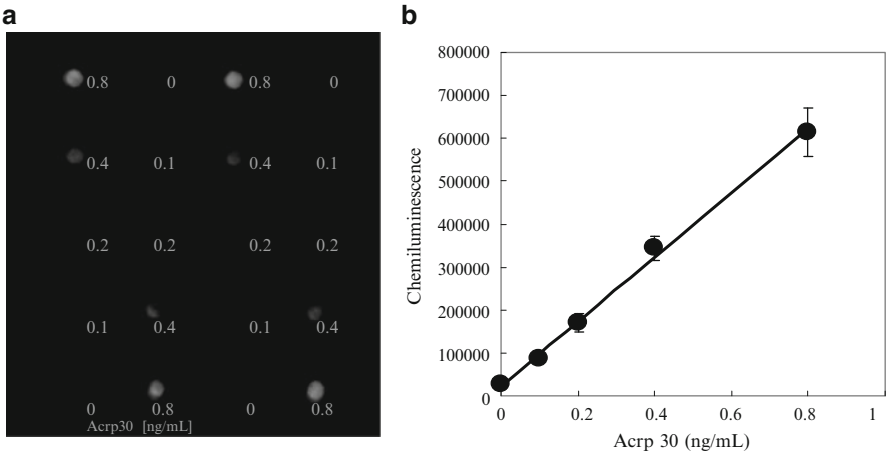


Fig. 13.7 Quantification of mouse adiponectin. (**a**) Chemiluminescent image. (**b**) The calibration curves for the Acrp30. Each error *bar* indicates the standard deviation for each data point

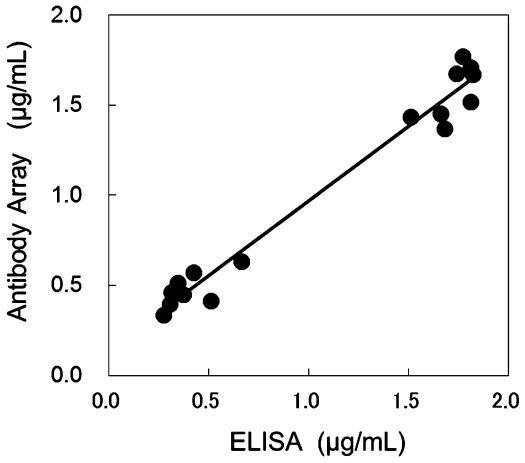


Fig. 13.8 Correlation between a conventional ELISA and the antibody array in quantifying mouse adiponectin in cell culture medium. Significant correlation was demonstrated ($r = 0.987$, $p < 0.01$)

2.3 Competitive ELISA of Antibody Array

We then established a competitive method to measure biomarkers of low molecular weight. Figure 13.9 shows a competitive immunoassay system to measure hexanoyl lysine (HEL). A hexanoyl lysine adduct is formed through oxidative modification of omega-6 fatty acids, such as linoleic acid, the predominant polyunsaturated fatty acid. We photoimmobilized the HEL-BSA conjugate to the azopolymer surface

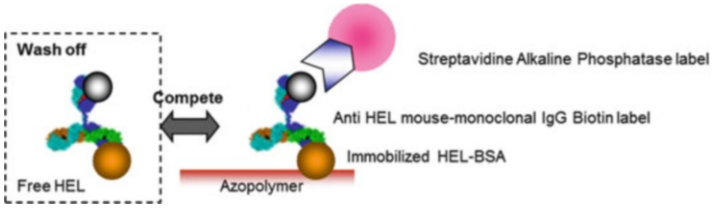


Fig. 13.9 Illustration of a competitive ELISA using the antibody array

before the competitive reaction between immobilized HEL and free HEL using biotin-labeled anti-HEL antibody. The anti-HEL monoclonal antibody was raised by Kato et al. (1999, 2000). Finally, alkaline phosphatase-labeled streptavidin was sequentially bound to the immobilized HEL-BSA conjugate. The details of the procedure are as follows:

1. Mixing
   5 μL of standard dilutions of free HEL or diluted samples were mixed with 5 μL of biotin-labeled anti-HEL antibody solution.
2. Competitive reaction
   The mixtures were immediately and independently dispensed onto the area with immobilized HEL-BSA conjugate.
3. Alkaline phosphatase-labeled streptavidin reaction
   40 μL of alkaline phosphatase-labeled streptavidin solution was applied before being covered with a gap cover glass.
4. Chemiluminescent detection
   40 μL of CDP-star Emerald II solution was applied before being covered with a gap cover glass. Luminescence was measured for 15 min with a CCD detector (AISIN, LV-400).

3 Antibody Array System for Simultaneous Measurement of Oxidative Stress Markers

Our array was able to measure two or more biomarkers using small droplets of real samples. The upper part of Fig. 13.10 shows the array format for HEL and 8-OHdG (8-hydroxydeoxyguanosine) which represents oxidative stress occurring in the body. One separated droplet covers four spots of two biomarkers. Each antibody reacted specifically to defined spots (lower part of Fig. 13.10), and the resulting chemiluminescence of the spots did not affect the measurement value of the biomarkers at the same droplet. Figure 13.10 shows a chemiluminescent image of a standard glass slide containing 40 regions of test samples in which four spots of HEL and 8-OHdG conjugates are immobilized. A calibration curve using the strength of each spot within a “standard” region in a chemiluminescent image was obtained. We observed

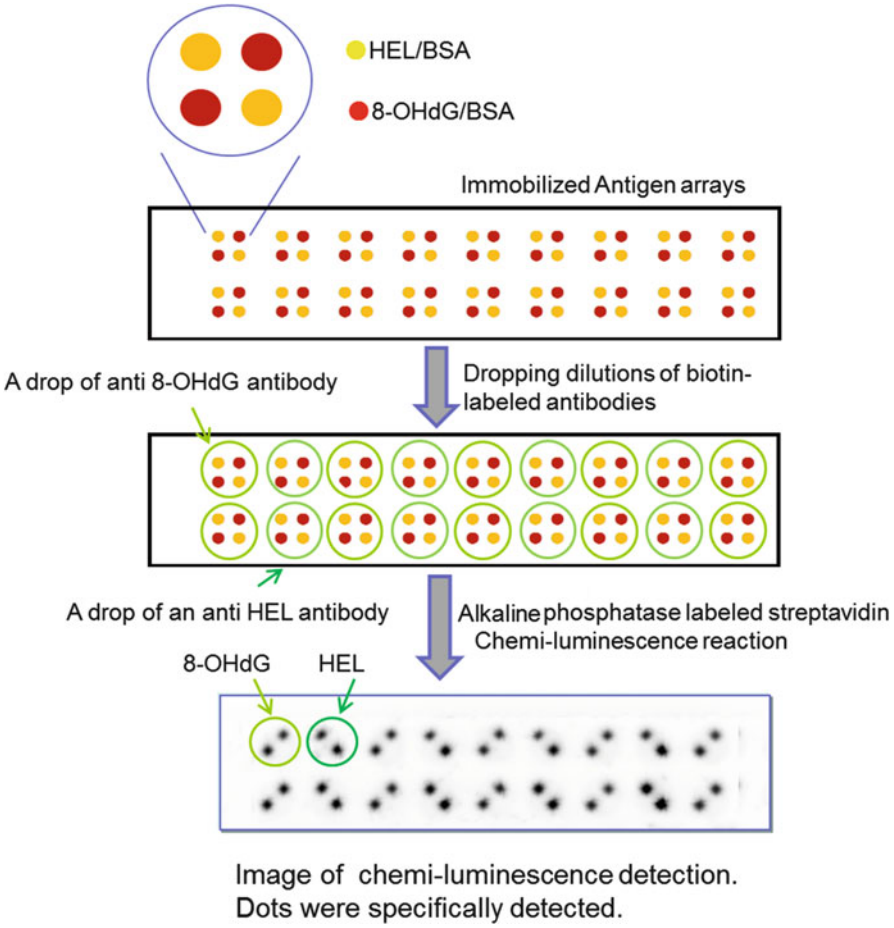


Fig. 13.10 Antibody array system for simultaneous measurement of oxidative stress markers. Each antibody reacted specifically to defined spots

a linear relationship between luminescent intensity and the concentration of both markers. We detected HEL from 2.5 to 249.5nM and 8-OHdG from 1 to 100 ng/ml (Fig. 13.11). The detectable ranges of both biomarkers were equal to those in a conventional ELISA system.

4 Conclusion

We have introduced the principles of a newly created photoimmobilization technology using photoresponsive azopolymers, and its application to on-chip ELISAs. An antibody array was assessed to quantify biomarkers using small sample

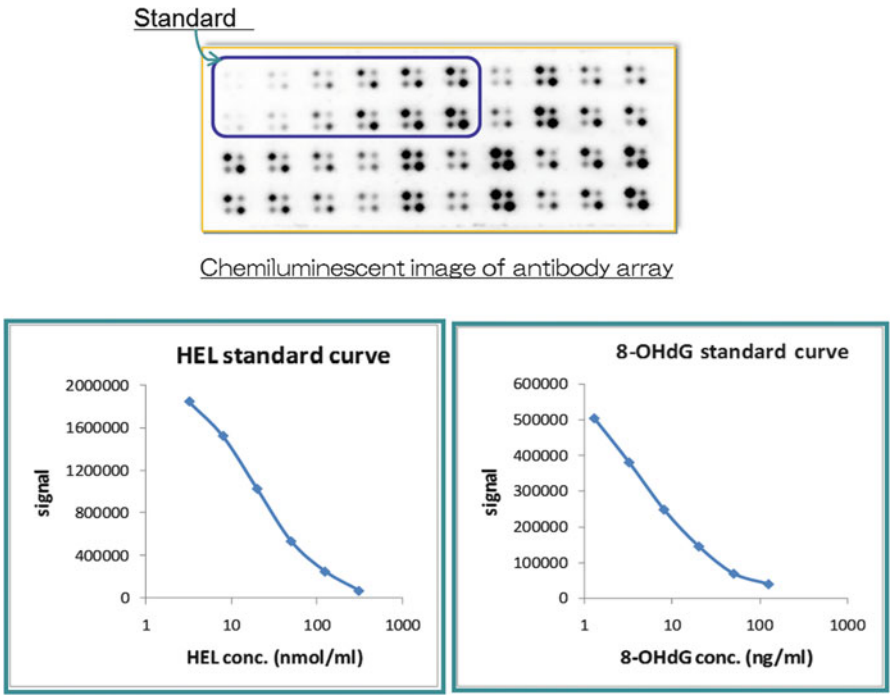


Fig. 13.11 Chemiluminescent image of an antibody array for simultaneous measurement in order to quantify oxidative biomarkers (HEL and 8-OHdG), and the calibration curves to determine the biomarkers in test samples

volumes. We found the on-chip ELISA had a similar sensitivity to conventional ELISAs, however, much lower volumes can be used in on-chip ELISAs. In order to estimate oxidative stress with ease and low cost, we established an antibody array simultaneously measuring two biomarkers, HEL and 8-OHdG. This antibody array system could be used to investigate health-promoting food and it would be applicable to use other biomarkers and various biological fluids at low volumes that could not be previously measured by conventional methods.

References

Hasegawa M, Ikawa T, Tsuchimori M, Watanabe O, Kawata Y (2001) Topographical nanostructure patterning on the surface of a thin film of polyurethane containing azobenzene moiety using the optical near field around polystyrene spheres. Macromolecules 34:7471–7476

Ikawa T, Mitsuoka T, Hasegawa M, Tsuchimori M, Watanabe O, Kawata Y, Egami C, Sugihara O, Okamoto N (2000) Optical near field induced change in viscoelasticity on an azobenzene-containing polymer surface. J Phys Chem B 104:9055–9058

Ikawa T, Mitsuoka T, Hasegawa M, Tsuchimori M, Watanabe O, Kawata Y (2001) Azobenzene polymer surface deformation due to the gradient force of the optical near field of monodispersed polystyrene spheres. Phys ReV B 64(19):5408

Ikawa T, Hoshino F, Matsuyama T, Takahashi H, Watanabe O (2006) Molecular-shape imprinting and immobilization of biomolecules on a polymer containing azo dye. Langmuir 22:2747–2753

Kato Y, Mori Y, Makino Y, Morimitsu Y, Hiroi S, Ishikawa T, Osawa T (1999) Formation of Nepsilon-(hexanonyl)lysine in protein exposed to lipid hydroperoxide. A plausible marker for lipid hydroperoxide-derived protein modification. J Biol Chem 274(29):20406–20414

Kato Y, Miyake Y, Yamamoto K, Shimomura Y, Ochi H, Mori Y, Osawa T (2000) Preparation of a monoclonal antibody to N(epsilon)-(Hexanonyl)lysine: application to the evaluation of protective effects of flavonoid supplementation against exercise-induced oxidative stress in rat skeletal muscle. Biochem Biophys Res Commun 274(2):389–393

Kawata Y, Egami O, Nakamura O, Sugihara N, Okamoto M, Tsuchimori M, Watanabe O (1999) Non-optically probing near-field microscopy. Opt Commun 161:6–12

Chapter 14
Hexanoyl-Lysine as an Oxidative-Injured Marker – Application of Development of Functional Food

Ken-ichiro Minato and Yoshiaki Miyake

Abstract We could proposed that N^{ε}-(hexanoy)lysine, HEL, become a useful biomarker for detection of oxidative stress damage occurred by exhaustive exercise. We examined the preventive effect of flavonoid compound, eriocitrin, against exercise-induced oxidative damage in rat liver. Eriocitrin administration prior to exercise significantly suppressed the increases in thiobarbituric acid-reactive substance caused by lipid peroxidation during exhaustive exercise. The increase in the contents of HEL in rat liver was also abolished by eriocitrin administration. The concentration of oxidized glutathione was significantly increased by exercise, but the eriocitrin administration suppressed this increase. These results suggested that eriocitrin administration prior to exercise prevented oxidative damages caused by exhaustive exercise-induced oxidative stress. Therefore, it was suggested that HEL could be a good biomarker for oxidative stress, especially at earlier stage when oxidative damage was occurred by lipid peroxidation than a stage of harmful aldehyde formation. Moreover, it was suggested that eriocitrin metabolites, eriodictyol and 3, 4 – dihydroxyhydrocinnamic, might scavenge free radicals and reactive oxygen species, resulting in suppression of lipid peroxidation and reactive proteins with radicals to form HEL. These findings implied that eriocitrin might be useful as an anti-oxidative compound to protect oxidative stress damages.

Keywords Eriocitrin • ELISA • Exercise-induced oxidative stress • N^{ε}-(hexanoyl) lysine • Lemon flavonoid

K. Minato (✉)
Department of Applied Biological Chemistry, Meijo University,
1-501 Shiogamaguchi, Nagoya 468-8502, Japan
e-mail: minato@meijo-u.ac.jp

Y. Miyake
Department of Human Wellness, Tokaigakuen University, 21-233,
Nishinohora, Ukigai-cho, Miyoshi, Aichi 470-0207, Japan

Y. Kato (ed.), *Lipid Hydroperoxide-Derived Modification of Biomolecules*,
Subcellular Biochemistry 77, DOI 10.1007/978-94-007-7920-4_14,

Abbreviations

HEL	N^{ε}-(hexanonyl)lysine
DT	*o*,*o*-dityrosine
NT	Nitrotyrosine
TBARS	Thiobarbituric acid-reactive substance
ROS	Reactive oxygen species
ELISA	Enzyme-linked immunosorbent assay
GSH	Reduced glutathione
GSSG	Oxidized glutathione

1 Introduction

Physical exhaustive exercise results in injury to fibers in the active muscles. It had been considered that clinical symptoms by exhaustive exercise was associated with the pathology including elevation of plasma enzymes activities such as creatine kinase, myoglobin, and protein metabolites in injured muscles, as well as the structural damage. By the way, it has been suggested that strenuous physical exercise with dramatically increased oxygen uptake was associated with the generation of free radicals and ROS, resulting in oxidative damages in body (Li 1995). Most of the oxygen is utilized in the mitochondria for substrate metabolism and ATP production and is reduced to water. However, a small portion of oxygen is considered to be converted univalently into several radical intermediates such as $O_2^{\bullet -}$, H_2O_2, and $^{\bullet}OH$. It is considered that $O_2^{\bullet -}$, and $^{\bullet}OH$ are free radicals by definition because they contain an unpaired electron in their atomic structure, whereas H_2O_2 is not. They subsequently leak out of the electron transport chain, collectively, these molecule species are classified as the reactive oxygen species (ROS). Much attention has been focused on involvement of oxidative stress caused by free radicals and reactive oxygen species (ROS) in disease and aging. The oxidative stress has been considered to play a causal role in the senescence of various organisms and to cause the accrual of irreparable damages to lipids, proteins, and DNA, which may result in diseases (Stadtman 1992; Ames et al. 1993; Yu 1996). A powerful approach to studying oxidative damage is the analyses of the diseased tissues for specific markers. It has been suggested that the products formed by oxidation with free radical and ROS accumulate as modified compounds in tissues during the process of oxidative stress (Kato et al. 1998, 1999; Leeuwenburgh et al. 1999; Frost et al. 2000). The production of ROS is considered to be the underlying mechanism for a series of biochemical and physiological changes that occur during aerobic exercise. It could be indicative of oxidative stress. The excessive production of ROS is thought to cause cellular damage *in vivo*. There has been strong evidence that free radicals were indeed involved in tissue and cell damage by exhaustive exercise. Davies et al. (1982) suggested that enhancement of free radical signals were measured in liver of rat, which exhaustively exercised by acute bout of treadmill running, using by the

electron paramagnetic resonance (EPR) spectroscopy method. It has been considered that such increase in free radical signals could cause a series of cellular disorders. However, a direct relationship between free radical generation and these deleterious processes has yet become clear, because an EPR method and an associated technic were difficult, and an availability of them was limited. These processes might be recognized as oxidative damage rather than free radical damage. Therefore, it was desired that alternative methods, which were more simply and exactly, could be developed to measure oxidative damage by using other reasonable marker.

Biomolecules such as proteins and aminolipids are covalently modified by lipid decomposition products such as aldehyde during an even earlier stage of lipid peroxidation induced by oxidative damage (Esterbauer et al. 1991; Fruebis et al. 1992). When polyunsaturated fatty acids on the biomembrane are attacked by free radicals in the presence of molecular oxygen, a chain of peroxidative reactions occurs. And then, it was lead to the formation of hydro carbon gases and aldehydes. It has been suggested that byproducts in lipid peroxidation were the most frequently studied markers in oxidative damaged tissue. It had been shown that pentane was increased in the expired gas of human subjects after long-term exercise (Dillard et al. 1978). It was also reported that expired pentane levels increased proportionally during exercise in humans (Kanter et al. 1993).

However, the validity of using expired hydrocarbons as a maker of lipid peroxidation *in vivo* has not confirmed yet. Therefore, it was considered that malondialdehyde (MDA) could become a useful biomarker as thiobarbituric acid-reactive substance (TBARS) for detection of oxidative stress damage in various tissues. Its content has been found to increase during exercise in a variety of tissues, and the extent of lipid peroxidation also appears to depend on exercise intensity (Allessio 1993). Although assessment of MDA accumulation in overall tissue lipid peroxidation has been assisted by several additional methods that measure intermediary compounds of lipid peroxidation chain reaction, such as conjugated dienes and lipid peroxides, it could be a one of reasonable methods for oxidative stress detection.

There is a consensus that multiple indexes are required to verify the occurrence of lipid peroxidation during exercise. Therefore, it have been already reported that N^{ε}-(hexanoy)lysine (named HEL) was identified as one of the lipid hydroperoxide-modified lysine residues, which is considered to be a useful marker for the lipid peroxidation, as well as MDA (Kato et al. 1999). It has also been suggested that this biomarker exists in skeletal muscle after exercise (Kato et al. 2000). It was also suggested that HEL accumulate in biological fluids and tissues during exhaustive exercise (Kato et al. 1998; Leeuwenburgh et al. 1999; Kato et al. 2000). It have been already reported that ELISA (enzyme linked immunosorbent assay) was useful for the measurement of oxidative stress markers (Kato et al. 2000).

Flavonoids in citrus fruits known as bioflavonoids or vitamin P exhibit beneficial effects on capillary permeability and fragility (Rusznyak and Szent-Gyorgyi 1936). Subsequent studies have reported their physiological functions such as anti-inflammatory, anticarcinogenic, and antitumor activities (Bracke et al. 1994; Middleton and Kandaswami 1994; Attaway 1994). A lemon fruit among citrus fruits contains many functional flavonoids, *e.g.* hesperidin, diosmin, and *C*-glucosyldiosmin (Miyake

et al. 1997a, 1998a). We have previously isolated a strong antioxidative flavonoid glycoside from lemon fruit and identified it as eriocitrin (eriodictyol 7-*O*-rutinoside) (Fig. 16.1, Miyake et al. 1997b). It was shown that the antioxidant activity of eriocitrin is stronger than the activities of the other citrus flavonoids such as hesperidin and naringin (Miyake et al. 1997a). Moreover, it was reported that eriocitrin shows a significant suppressive effect against oxidative stress caused by diabetes in rats, suggesting that it may be useful in preventing oxidative damages (Miyake et al. 1998b). On the other hand, it was reported that the supplementation of eriocitrin significantly suppressed the exercise-induced increase in HEL in rat skeletal muscle (Kato et al. 2000). In this chapter, it will be shown that an increase in HEL as well as MDA in liver, especially, of rat during acute exercise, and discussed a protective effect of eriocitrin administration against the accumulation of these markers. Moreover, we determined the changes in glutathione levels considered to protect cells against oxidative damage.

2 Anti-oxidative Lemon Flavonoids

Lemon is used as various food materials, and it is one of the most popular citrus fruits in the world. Lemon fruit contains a number of nutrients such as citric acid, ascorbic acid, minerals, and flavonoids. It has been recognized that flavonoid compounds were widespread in the plants, and comprised a large group of naturally occurring compounds which were found in all vascular plants. They were contained in Citrus as well as in other fruits and vegetables. It had been shown that the typical western diet contained about 1 g of mixed flavonoids (Kuhnan 1976). We have already reported that eriocitrin, a major lemon flavonoid, shows the antioxidative activity which inhibits lipid peroxidation *in vitro* (Miyake et al. 1997b). Eriocitrin has been identified as the flavanone glycoside, eriodictyol 7-O-β-rutinoside. It had a stronger antioxidant than the other citrus flavonoid compounds. It was suggested that this flavonoid compound was metabolized to eriodictyol of its aglycone by *Bacteroides distasonis* or *Bacteroides uniformis* of intestinal bacteria. And then, eriodictyol was converted to 3, 4 – dihydroxyhydrocinnamic (DHCA), and phloroglucinol was produced as a byproduct by *Clostridium butyricum* (Fig. 14.1, Miyake et al. 2000). These metabolites were detected in large intestines and plasma following administration of eriocitrin, but not in serosal side. These results suggested that eriodictyol and 3, 4-DHCA were absorbed from the intestine after eriocitrin was hydrolyzed to these metabolites by intestinal bacteria. These eriocitrin metabolites showed a strong capability of DPPH radical scavenging in our previous study (data not shown). Moreover, it seemed that eriodictyol was absorbed slowly because it is absorbed in the hydrolysis by intestinal bacteria. And then, it seemed to be metabolized to homoeriodictyol and hesperetin by methoxylation in the liver.

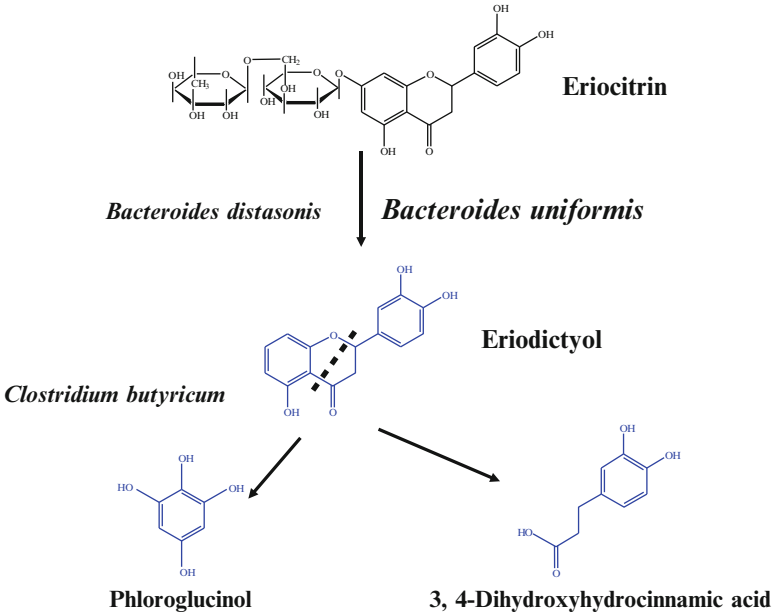


Fig. 14.1 Eriocitrin has been identified as the flavanone glycoside, eriodictyol 7-O-β-rutinoside. It was suggested that this flavonoid compound was metabolized to eriodictyol of its aglycone by *Bacteroides distasonis* or *Bacteroides uniformis* of intestinal bacteria. And then, eriodictyol was divided into phloroglucinol and 3, 4 – dihydroxyhydrocinnamic (DHCA) by *Clostridium butyricum*

3 Effect of Eriocitrin on Thiobarbituric Acid-Reactive Substance (TBARS) Level in Liver of Exercised Rat

3.1 Animals and Experimental Design

Experimental Rats (male Wistar rats, 10 weeks old, Japan SLC) were randomly divided into a control group and an eriocitrin group. Rats in each group were subdivided into a sedentary group and an exercise group. We prepared eriocitrin from lemon peel extract as described previously (Miyake et al. 1997b). An eriocitrin solution (600 mg eriocitrin·kg^{-1} of body weight) was administered intragastrically to rats in the eriocitrin group. The same volume of saline was given to rats in the control group in the same manner. Four hours after administration, the animals in the exercise group were exercised for 35 min according to the method of Armstrong et al. (1983) using a motor-driven treadmill (running down a 16° incline at a speed of 20 m·min^{-1}). Immediately after running, the rats were anesthetized and liver was obtained.

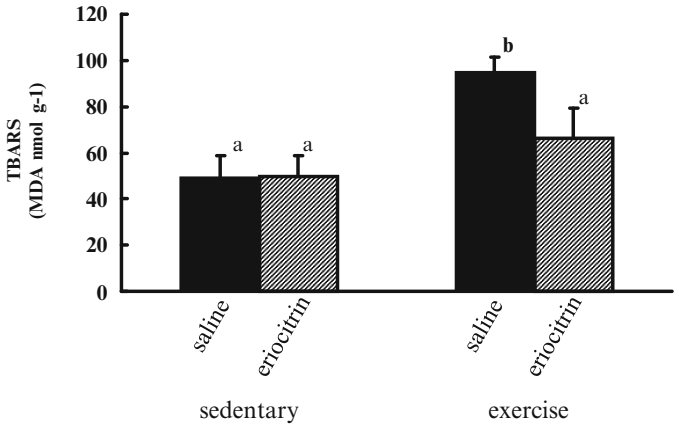


Fig. 14.2 The suppressive effect of eriocitrin on an increase in TBARS by exercise-induced oxidative stress. Values are means ± SD (n = 7). Values designated by different superscript letters are significantly different ($p < 0.05$) by Two-Way ANOVA followed by Turkey's HSD test (This referred to Minato et al. 2003 reprinted by permission of Elsevier Science Inc.)

3.2 Measurement of TBARS in Exhaustive Exercised Rat

The livers obtained from exercised rats were homogenized in ten volumes of 1.15 % KCl at 4 °C. The homogenate was centrifuged at 4,500 × g for 15 min, and the supernatant was obtained for the TBARS measurement. The TBARS concentration was measured as described previously (Miyake et al. 1998b). The effect of eriocitrin on TBARS production was examined in the liver of exercised rat. As shown in Fig. 14.2, acute exercise significantly increased the TBARS level in rat liver, and this increase was significantly suppressed by eriocitrin administration. It was reported that exercise increases oxygen utilization and causes formation of free radical and reactive oxygen species (Wetzstein et al. 1998). It has been also revealed that eriocitrin suppresses an increase in TBARS in rat liver (Miyake et al. 1998b).

4 Effect of Eriocitrin on the Formation of Hexanoyl-Lysine (HEL) in the Exercised Rat

4.1 Enzyme-Linked Immunosorbent Assay (ELISA) for Measurement of HEL

The livers obtained were also homogenized in ten volumes of 50 mM sodium phosphate buffer (pH 7.4) at 4 °C for measurement of HEL contents by enzyme-linked immunosorbent assay (ELISA). The homogenate was centrifuged at

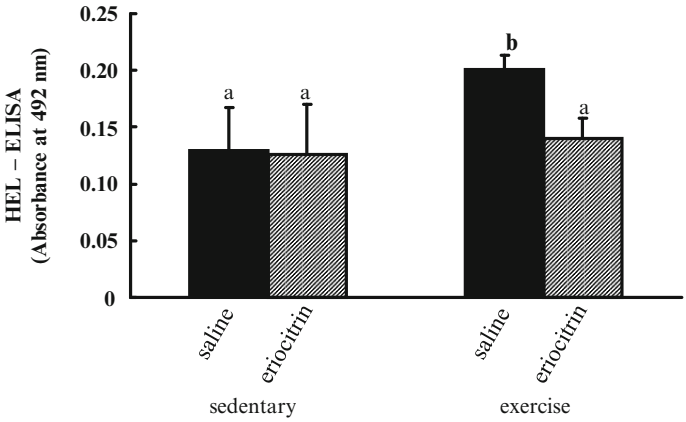


Fig. 14.3 The preventive effect of eriocitrin on an increase in HEL of oxidative modified protein in liver by exercise. Values are means ± SD (n = 7). Values designated by different superscript letters are significantly different ($p < 0.05$) by Two-Way ANOVA followed by Turkey's HSD test (This referred to Minato et al. 2003 reprinted by permission of Elsevier Science Inc.)

4,500 × g for 15 min, and the supernatant obtained was used for the following biochemical measurement. The noncompetitive ELISA was performed for measurement of HEL as described previously (Kato et al. 2000). Briefly, 50 μl of sample solution (10 μg protein·ml^{-1}) were pipetted into the wells and kept at 4 °C overnight. Then the plate was incubated with the primary antibodies against HEL. After incubation, the binding of the antibodies to the antigens were evaluated by using the anti-mouse/rabbit IgG antibody peroxidase-labeled with *o*-phenylenediamine and hydrogen peroxide.

4.2 HEL as an Oxidative Damaged Marker

HEL accumulation in liver of rat was determined as the makers of exercise-induced oxidative damage. The formation of HEL was significantly increased by acute exercise, and the increases were suppressed by eriocitrin administration (Fig. 14.3). Strenuous aerobic exercise is associated with oxidative stress and generates oxygen free radicals (Li 1995). N^{ε}-(hexanoyl)lysine, designated HEL, were identified as a novel lipid hydroperoxide-modified lysine residue. And it is considered to become a good marker for oxidative damage in early stage (Kato et al. 1999; Ueno et al. 2002). In our previous study, eriocitrin administration inhibited increases in HEL as well as TBARS levels, suggesting that exercise-induced lipid peroxidation in rat liver was suppressed by the administration. And, it was suggested that eriocitrin could inhibit HEL accumulation in skeletal muscle in exercise-trained rats. It was suggested that the HEL moiety was formed through

the reaction of lipid hydroperoxidation with protein. A detection of HEL could estimate the protein modification by lipid peroxidation in early stage. Moreover, Kato et al. observed the positive staining by HEL antibody in human atherosclerotic lesions. They also suggested that HEL could be a useful biomarker for the lipid hydroperoxide of biomolecules. These reports suggested that HEL would be a good biomarker for oxidative stress, especially at earlier stage when oxidative damage was occurred by lipid peroxidation than a stage of harmful aldehyde formation.

5 The Changes in Glutathione Level During Exercise

5.1 Action of Glutathione in the Tissue Induced Oxidative Stress

It is known that glutathione is related to a variety of antioxidant processes (Vina 1990). It was suggested that exhaustive physical exercise induced to increase formations of oxygen free radicals in various tissues. The redox cycle of glutathione could be a major antioxidant system that prevented from accumulating toxic hydroperoxides (Fig. 14.4). Thus, reduced glutathione (GSH) could protect cells against oxidative stress inducing damage by free radicals. Oxidative stress has been defined as a disruption in a balance between prooxidant and antioxidant. Aerobic cells, such as muscle cells, could be interesting for oxidative stress study. An increase in oxidized glutathione (GSSG) level was considered to be able to become as a characteristic index of oxidative stress. It has been reported that total glutathione and glutathione disulfide significantly increased in plasma of rat exercised by running (Lew et al. 1985). And a ratio of reduced GSH to GSSG decreased in liver and in skeletal muscle as well as in plasma after exhaustive physical exercise. These results suggested that glutathione antioxidant system could be depleted during exercise leading to exhaustion.

5.2 The Change in Level of Glutathione After Exercise

Measurements of total glutathione (GSH + GSSG) and oxidized glutathione (GSSG) were carried out as described previously (Pyke et al. 1986). The total glutathione level was expressed in GSH equivalents. The GSH level was calculated by subtracting GSSG from total glutathione.

In Table 14.1, the levels of glutathiones were determined in exercised rat liver. Total glutathione content did not change or tended to decrease by an acute exercise as compared with control rat (sedentary, saline administration). However, oxidized

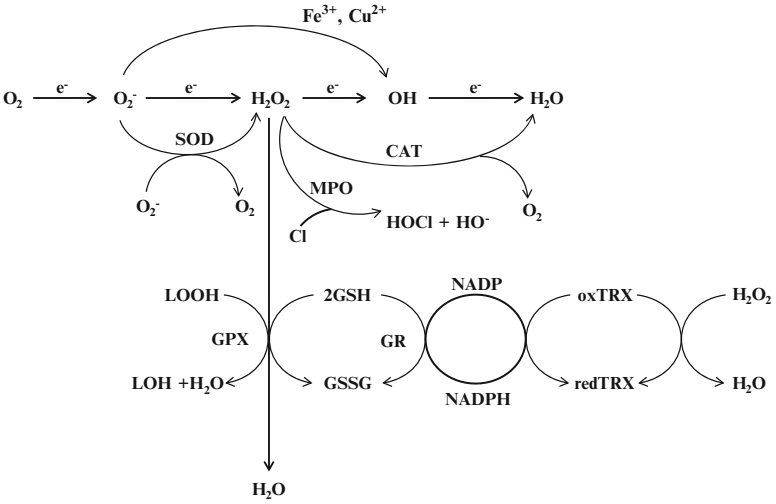


Fig. 14.4 The redox cycle of antioxidant enzymes and glutathione could be a major antioxidant system. It was suggested to prevent from accumulating toxic hydroperoxides. *CAT* catalase, *GPX* glutathione peroxidase, *GR* glutathione reductase, *GSSG* oxidized glutathione, *GSH* reduced glutathione, *MPO* myeloperoxidase, *SOD* superoxide dismutase, *TRX* thioredoxin

glutathione (GSSG) significantly increased by 3.3-folds as compared with control rat, and reduced glutathione (GSH) tended to decrease in exercised rat liver. On the other hand, it was shown that an increase in GSSG level was significantly suppressed by 1.8-folds as compared with control rat, and a level of GSH was maintained in liver by an eriocitrin administration after exercise. It was considered that the primary preventative antioxidant system which circumvents the harmful accumulation of hydroperoxides and superoxide was dependent on glutathione and glutathione peroxidase (Gohil et al. 1988). It was reported previously that levels of total glutathione in liver might fall to relatively low values when the animal was subjected to exhaustive exercise (Pyke et al. 1986). Also, it was shown that glutathione disulfide increased significantly in rat which was exercised to exhaustion by running (Sastre et al. 1992). In our previous study, eriocitrin administration was very effective for suppression of an increase in GSSG and a depletion of GSH (Table 14.1). However, it is obscure how eriocitrin metabolites influence for the glutathione antioxidant system during exercise-induced oxidative stress. It might be suggested that eriocitrin metabolites could scavenge the radicals generated by oxidative stress, and prevent the formation of superoxide and hydroperoxide. The primary preventative antioxidant pathway for hydroperoxide removal involves action of glutathione peroxidase in cytosol and mitochondria, and catalase in peroxisome. Therefore, excessive depletion of glutathione might be prevented in antioxidant system, and this matter would prevent the increases in TBARS and the level of HEL during strenuous exercise-induced oxidative stress.

Table 14.1 The protect effect of eriocitrin on depletion of glutathione during exercise-induced oxidative stress

	Sedentary		Exercise	
	Saline	Eriocitrin	Saline	Eriocitrin
Total glutathione (GSH eq), μmol · g^{-1}	1.79 ± 0.15	1.69 ± 0.14	1.55 ± 0.13	1.65 ± 0.15
GSH, μmol · g^{-1}	1.77 ± 0.15[a]	1.68 ± 0.14[a,b]	1.50 ± 0.12[b]	1.63 ± 0.15[a,b]
GSSG, μmol · g^{-1}	0.015 ± 0.003[a]	0.012 ± 0.004[a]	0.051 ± 0.008[b]	0.028 ± 0.006[c]

Values are means ± SD (n = 7). Values designated by different superscript letters are significantly different ($p < 0.05$) by Two-Way ANOVA followed by Turkey's HSD test. This referred to Minato et al. 2003 reprinted by permission of Elsevier Science Inc.
GSH reduced glutathione, *GSSG* oxidized glutathione

6 Conclusion

It was clearly showed that acute exercise-induced increases in TBARS and oxidative liver damages analyzed by oxidative stress markers were suppressed by eriocitrin administration prior to exercise. Moreover, we could proposed that N^{ε}-(hexanoy)lysine, HEL, could become a useful biomarker for detection of oxidative stress damage occurred by exhaustive exercise. It was suggested that eriocitrin metabolites, eriodictyol and 3, 4 – dihydroxyhydrocinnamic, may scavenge free radicals, reactive oxygen species, and reactive nitrogen species, resulting in suppression of lipid peroxidation and reactive proteins with radicals to form HEL. Furthermore, it was also suggested that eriocitrin metabolites could play a role in suppression of glutathione oxidation caused by exercise in the liver. These findings imply that eriocitrin may be useful as an anti-oxidative compound to protect oxidative stress damages. Thus, it was suggested that absorbed eriocitrin metabolites scavenged the free radical/reactive oxygen species that occurred by exercise and inhibited lipid peroxidation.

Acknowledgment This work was supported by a grant aid from the Japan Science and Technology Corporation (JST). We are deeply grateful to Professor Toshihiko Osawa of Aichi- Gakuin University for his guidance.

References

Allessio HM (1993) Exercise-induced oxidative stress. Med Sci Sports Exerc 25:218–224
Ames BN, Shigenaga MK, Hagen TM (1993) Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci U S A 90:7915–7922
Armstrong RB, Ogilvie RW, Schwane JA (1983) Eccentric exercise-induced injury to rat skeletal muscle. J Appl Physiol 54:80–93

Attaway JA (1994) Citrus juice flavonoids with anticarcinogenic and antitumor properties. In: Huang MT, Osawa T, Ho CT, Rosen RT (eds) Food phytochemicals for cancer prevention I, fruits and vegetables. American Chemical Society, Washington, DC, pp 240–248

Bracke ME, Bruyneel EA, Vermeulen SJ, Vennekens KI, Marck VV, Mareel MM (1994) Citrus flavonoid effect on tumor invasion and metastasis. Food Technol 48:121–124

Davies KJA, Quintanilha TA, Brooks GA, Packer L (1982) Free radical and tissue damage produced by exercise. Biochem Biophys Res Commun 107:1198–1205

Dillard CJ, Litov RE, Savin WM, Dumclin EE, Tapple AL (1978) Effect of exercise, vitamin E, and ozone on pulmonary function and lipid peroxidation. J Appl Physiol 45:927–932

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

Frost MT, Halliwell B, Moore KP (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

Fruebis J, Parthasarathy S, Steinberg D (1992) Evidence for a concerted reaction between lipid hydroperoxides and polypeptides. Proc Natl Acad Sci U S A 89:10588–10592

Gohil K, Viguie C, Stanley WC, Brooks GA, Packer L (1988) Blood glutathione oxidation during human exercise. J Appl Physiol 64:115–119

Kanter MM, Nolte LA, Holloszy JO (1993) Effect of an antioxidant vitamin mixture on lipid peroxidation at rest and postexercise. J Appl Physiol 74:965–969

Kato Y, Maruyama W, Naoi M, Hashizume Y, Osawa T (1998) Immunohistochemical detection of dityrosine in lipofuscin pigments in the aged human brain. FEBS Lett 439:231–234

Kato Y, Mori Y, Makino Y, Morimitsu Y, Hiroi S, Ishikawa T, Osawa T (1999) Formation of Nepsilon-(hexanonyl)lysine in protein exposed to lipid hydroperoxide. A plausible marker for lipid hydroperoxide-derived protein modification. J Biol Chem 274:20406–20414

Kato Y, Miyake Y, Yamamoto K, Shimomura Y, Ochi H, Mori Y, Osawa T (2000) Preparation of a monoclonal antibody to N(epsilon)-(Hexanonyl)lysine: application to the evaluation of protective effects of flavonoid supplementation against exercise-induced oxidative stress in rat skeletal muscle. Biochem Biophys Res Commun 274:389–393

Kuhnan J (1976) The flavonoids. A class of semi-essential food components; their role in human nutrition. World Rev Nutr Diet 24:117–191

Leeuwenburgh C, Hansen PA, Holloszy JO, Heinecke JW (1999) Oxidized amino acids in the urine of aging rats: potential markers for assessing oxidative stress in vivo. Am J Physiol 274 (Regul Integr Comp Physiol 45): R128–R135

Lew H, Pyke S, Quintanilha A (1985) Changes in the glutathione status of plasma, liver, and muscle following exhaustive exercise in rats. FEBS Lett 185:262–266

Li LJ (1995) Oxidative stress during exercise: implication of antioxidant nutrients. Free Radic Biol Med 18:1079–1086

Middleton E, Kandaswami C (1994) Potential health-promoting properties of citrus flavonoids. Food Technol 48:115–119

Minato K, Miyake Y, Fukumoto S, Yamamoto K, Kato Y, Shimomura Y, Osawa T (2003) Lemon flavonoid, eriocitrin, suppresses exercise-induced oxidative damage in rat liver. Life Sci 72:1609–1616

Miyake Y, Yamamoto K, Morimitsu Y, Osawa T (1997a) Isolation of C-glucosyl flavone from lemon peel and antioxidative activity of flavonoid compounds in lemon fruit. J Agric Food Chem 45:4619–4623

Miyake Y, Yamamoto K, Osawa T (1997b) Isolation of eriocitrin (epiodictyol 7-rutinoside) from lemon fruit (C*itrus limon* Burm. f.) and its antioxidative activity. Food Sci Technol Int 3:84–89

Miyake Y, Yamamoto K, Morimitsu Y, Osawa T (1998a) Characterization of antioxidative flavonoids glycosides in lemon fruit. Food Sci Technol Int 4:48–53

Miyake Y, Yamamoto K, Tsujihara N, Osawa T (1998b) Protective effects of lemon flavonoids on oxidative stress in diabetic rats. Lipids 33:689–695

Miyake Y, Shimoi K, Kumazawa S, Yamamoto K, Kinae N, Osawa T (2000) Identification and antioxidant activity of flavonoid metabolites in plasma and urine of eriocitrin-treated rats. J Agric Food Chem 48:3217–3224

Pyke S, Lew H, Quintaniha A (1986) Severe depletion in liver glutathione during physical exercise. Biochem Biophys Res Commun 139:926–931

Rusznyak S, Szent-Gyorgyi A (1936) Vitamin nature of flavones. Nature 128:1172–1178

Sastre J, Asensi M, Gasco E, Pallardo FV, Ferrero JA, Furukawa T, Vina J (1992) Exhaustive physical exercise causes oxidation of glutathione status in blood: prevention by antioxidant administration. Am J Physiol 263 (Regul Integr Comp Physiol 32): R992–R995

Stadtman ER (1992) Protein oxidation and aging. Science 257:1220–1224

Ueno Y, Horio F, Uchida K, Naito M, Nomura H, Kato Y, Ysuda T, Toyokuni S, Osawa T (2002) Increase in oxidative stress in kidneys of diabetic Akita mice. Biosci Biotechnol Biochem 66 (4):869–872

Vina J (1990) Glutathione, metabolism and physiological functions. CRC, Boca Raton

Wetzstein CJ, Shern-Brewer RA, Santanam N, Green NR, White-Welkley JE, Parthasarathy S (1998) Does acute exercise affect the susceptibility of low density lipoprotein to oxidation? Free Radic Biol Med 24:679–682

Yu BP (1996) Aging and oxidative stress: modulation by dietary restriction. Free Radic Biol Med 21:651–668

Chapter 15
Potential Role of Oxidative Protein Modification in Energy Metabolism in Exercise

Wataru Aoi, Yuji Naito, and Toshikazu Yoshikawa

Abstract Exercise leads to the production of reactive oxygen species (ROS) via several sources in the skeletal muscle. In particular, the mitochondrial electron transport chain in the muscle cells produces ROS along with an elevation in the oxygen consumption during exercise. Such ROS generated during exercise can cause oxidative modification of proteins and affect their functionality. Many evidences have been suggested that some muscle proteins, i.e., myofiber proteins, metabolic signaling proteins, and sarcoplasmic reticulum proteins can be a targets modified by ROS generated due to exercise. We detected the modification of carnitine palmitoyltransferase I (CPT I) by *N*ε-(hexanoyl)lysine (HEL), one of the lipid peroxides, in exercised muscles, while the antioxidant astaxanthin reduced this oxidative stress-induced modification. Exercise-induced ROS may diminish CPT I activity caused by HEL modification, leading to a partly limited lipid utilization in the mitochondria. This oxidative protein modification may be useful as a potential biomarker to examine the oxidative stress levels, antioxidant compounds, and their possible benefits in exercise.

Keywords Skeletal muscle • Lipid metabolism • Mitochondria • Carnitine palmitoyltransferase I • Nε-(hexanoyl)lysine

W. Aoi (✉)
Laboratory of Health Science, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, 1-5 Hangi-cho Shimogamo, Sakyo-ku, Kyoto 606-8522, Japan
e-mail: waoi@kpu.ac.jp

Y. Naito • T. Yoshikawa
Department of Molecular Gastroenterology and Hepatology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan

Y. Kato (ed.), *Lipid Hydroperoxide-Derived Modification of Biomolecules*, Subcellular Biochemistry 77, DOI 10.1007/978-94-007-7920-4_15,

1 Generation of Reactive Oxygen Species in Exercise

Exercise leads to the production of reactive oxygen species (ROS), mainly via the mitochondrial electron transport chain, xanthine oxidase, and phagocytes (Fig. 15.1). A small percent of the oxygen utilized in the mitochondria is converted to superoxide during the electron transport chain reaction. It is known that oxygen consumption during aerobic exercise is elevated 10 to 20-fold in the whole body and over 100-fold in the skeletal muscle, which causes ROS generation that correlates with the duration and intensity of exercise (Blomstrand et al. 1997; Ferguson et al. 2001). In addition, xanthine oxidase is activated via the ischemia-reperfusion process during exercise, a process known as "blood flow redistribution," resulting in the production of ROS by the capillary endothelium in contracting muscles (Duarte et al. 1993; Flamm et al. 1990). Oxidative stress generated during exercise also leads to further ROS production due to the invasion of phagocytes into the muscles after exercise via the redox-sensitive inflammatory cascade (Aoi et al. 2004).

Previously, studies have shown elevated levels of ROS in response to exercise by using various methods. Electron-spin-resonance (ESR) studies have demonstrated that the ESR signal is markedly increased in muscle homogenates obtained immediately after exercise (Ashton et al. 1999; Davies et al. 1982). Other studies have shown a decrease in diet-derived antioxidants, and a reduced level of glutathione in tissues and blood (Aikawa et al. 1984; Liu et al. 2000; Rietjens et al. 2007), caused by its usage for ROS elimination. As in the above antioxidant capacity, ROS act as oxidative stressors and oxidize cell components such as proteins, lipids, and DNA in tissues such as skeletal muscle, blood, and internal

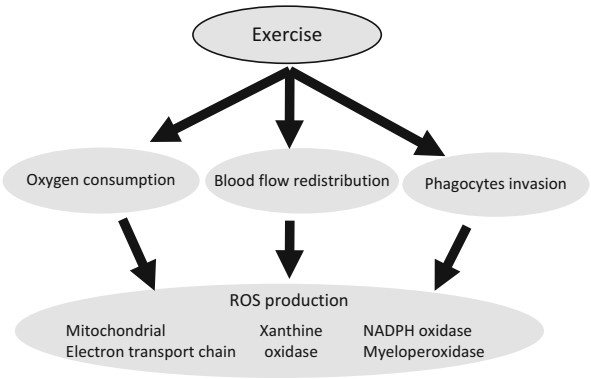


Fig. 15.1 Exercise leads to ROS production via mitochondrial electron transport chain, endothelium xanthine oxidase, and phagocytes in muscle tissues. Exercise elevates oxygen consumption over 100-fold in skeletal muscle, leading to production of reactive oxygen species (ROS) via mitochondrial electron transport chain. Xanthine oxidase is activated via the ischemia-reperfusion process during exercise, a process known as "blood flow redistribution," resulting in the production of ROS by the capillary endothelium in contracting muscles. Following exercise, further ROS production is increased by an invasion of phagocytes into the muscles

organs, and oxidation results in various oxidative products. Specifically, peroxidation of polyunsaturated fatty acids such as arachidonic acid, which are a large constituent of the membranes around cells and their organelles, occurs via a radical chain reaction and has been used as a useful marker of exercise-induced oxidative stress. Numerous previous animal and human studies have shown that acute exercise such as running, cycling, and resistance exercise, markedly elevates thiobarbituric acid reactive substances (TBARS), one of the oldest and most frequently used methods for measuring the peroxidation of fatty acids in the skeletal muscle immediately after exercise and on the next day after the exercise (Aoi et al. 2004; Liao et al. 2010; Miyazaki et al. 2001). In addition, this elevation in TBARS is also shown in high-intensity exercise training (Chang et al. 2007; Couillard et al. 2003). On the other hand, dietary supplementation with antioxidants such as vitamin E, vitamin C, carotenoids, and polyphenols can decrease oxidative damage induced by acute and chronic exercises (Aoi et al. 2003, 2004; Bryer and Goldfarb 2006; Goldfarb et al. 2011). These observations clearly demonstrate that exercise induces oxidative stress and causes the oxidation of cellular components. Such oxidative stress is not always negative, and many evidences suggest that moderate oxidative stress is necessary for beneficial adaptations induced by dietary exercise, as mentioned below. The effect of oxidative stress on bodily functions in living body would be due to the extent of this stress, and whether it acts a damaging factor or a protective factor is dependent on the intensity, duration, frequency, and habits of exercise.

2 Involvement of Oxidative Stress on Fatigue and Damage in Muscle

The possibility that oxidative stress induced by exercise causes a health disorder is a debatable matter. However, it has been demonstrated that the excess ROS generated by exercise are involved in muscle fatigue and muscle damage. It is well established that ROS have important influences on force production in the skeletal muscle. A study using both spin traps and vitamin E in animals demonstrated that scavenging ROS in muscles during exercise delays the onset of muscular fatigue (Novelli et al. 1990). Moreover, many reports have shown that administration of the antioxidant *N*-acetylcysteine (NAC), which acts as a reduced thiol donor supporting glutathione re-synthesis, delays muscular fatigue during a variety of submaximal exercise tasks such as electrically stimulated fatigue of the muscle, cycling exercise, and repetitive handgrip exercise (Cobley et al. 2011; Matuszczak et al. 2005; McKenna et al. 2006; Medved et al. 2004; Reid et al. 1994). In animal studies, NAC administration has also been shown to delay fatigue in both *in vitro* and *in situ* muscle preparations (Kobzik et al. 1995; Perkins et al. 1997). Studies using excised muscle fiber bundles also revealed that force production during submaximal tetanic contractions is decreased by nitric oxide (NO) donors

(Morrison et al. 1998; Richmonds and Kaminski 2001) and increased by nitric oxide synthase (NOS) inhibitors and NO scavengers (Joneschild et al. 1999; Kobzik et al. 1994).

Unaccustomed and strenuous exercise can cause muscle damage that presents clinically as muscular pain and involves protein degradation and ultrastructural changes. As such, muscle damage usually occurs sometime after exercise and not during or immediately after exercise; this is called as "delayed-onset muscle damage." Previous studies have demonstrated that delayed-onset muscle damage is mainly induced by mechanical stress, especially eccentric muscle contraction (Komulainen et al. 1998; Proske and Morgan 2001), and disturbances in calcium homeostasis (Chen et al. 2007; Gissel and Clausen 2001). In addition, we demonstrated that delayed-onset muscle damage induced by prolonged exercise is partly related to inflammation via phagocyte infiltration caused by ROS generated during exercise (Aoi et al. 2004). In an *in vitro* study using myotube cells, addition of H_2O_2 induced the translocation of p65, a component of the redox-sensitive transcription nuclear factor-kappa B (NF-κB), into the nucleus and subsequently increased the expression of cytokine-induced neutrophil chemoattractant-1 (CINC-1) and monocyte chemoattractant protein-1 (MCP-1). Prolonged acute exercise caused an increase in the amount of nuclear p65, a constitutive protein of NF-κB, in rat gastrocnemius muscles at 1 h after exercise, which was similar to the *in vitro* results, and it caused muscle damage with neutrophil invasion on the next day. Therefore, delayed-onset muscle damage after prolonged exercise is related to inflammation secondary to phagocyte infiltration caused by ROS generated during exercise. In contrast, dietary antioxidants such as vitamin E and carotenoids can partly attenuate delayed-onset muscle damage along with inflammatory changes (Aoi et al. 2003, 2004; Rosa et al. 2009), though several studies reported that supplementation of dietary antioxidants does not have this preventative effect.

3 Oxidative Protein Modification in the Muscle Induced by Exercises

Numerous posttranslational modifications have been characterized as resulting either from direct modification of amino acid residues or through the formation of reactive intermediates by the oxidation of other cellular components (Naito and Yoshikawa 2009). The modification of 20 different of amino acids plays an important role in the manifestation of the function of many proteins. The modification can be subdivided into two general forms: reversible modification and irreversible modifications. Some of the lipid peroxidation products exhibit a facile reactivity with proteins, generating a variety of intra- and intermolecular covalent adducts. In addition, the oxidation of cysteine to sulfenic, sulfinic, and sulfonic

Table 15.1 Non-enzymatic oxidative protein modification induced by exercise/muscle contraction

Products	Target proteins	Function	Reference
*N*ε-(hexanoyl) lysine	Carnitine palmitoyltransferase I	Lipid metabolism	Aoi et al. (2008)
	Whole lysate proteins	–	Kato et al. (2000)
4-hydroxy-2-nonenal	Insulin receptor substrate I	Insulin sensitivity	Aoi et al. (2012)
	Mitochondrial proteins	–	Sahlin et al. (2010)
Carbonyl	Myosin heavy chain	Force production	Yamada et al. (2007)
	Whole lysate proteins	–	Magherini et al. (2012)
			Veskoukis et al. (2008)
3-nitro-tyrosine	SR calcium-dependent ATPase	Contractile activity	Viner et al. (2000)
	Whole lysate proteins	–	Vassilakopoulos et al. (2003)

acids has been shown to occur frequently, and these sulfenic and sulfinic acids can often be enzymatically reduced. Nitration by reactive nitrogen species, chlorination by hypochlorous acid, and bromination by hypobromous acid of the target protein are also the frequently detected modifications. Many evidences suggest that such protein modifications can be associated with the onset of various common diseases, including cancer, inflammation, and metabolic disorders (Bidasee et al. 2004; Hill and Bhatnagar 2012; Oya-Ito et al. 2011; Sultana and Butterfield 2009). Some muscular proteins are also targets modified by exercise-induced ROS or nitrogen oxide species (Aoi et al. 2003; Kato et al. 2000; Magherini et al. 2012; Veskoukis et al. 2008; Vassilakopoulos et al. 2003; Barreiro and Hussain 2010) (Table 15.1).

The influence of oxidative stress on the sarcoplasmic reticulum (SR), a subcellular organelle, which controls the contractile state of the muscle by regulating the calcium concentration in the cytosol, has been studied extensively in the skeletal muscle, and is associated with the oxidative modification of the membrane proteins (Anzueto et al. 1992; Salama et al. 1992; Xia et al. 2003). Muscle contraction is performed by increasing intracellular calcium concentrations, which are released from the SR via the ryanodine receptor (RyR) calcium-release channel following active potentials during the excitation-contraction coupling process. Afterwards, calcium is immediately taken into the SR via SR calcium-dependent ATPase (SERCA), which relaxes the muscle. It has been known that the responsive proteins in the SR are sensitive to redox modulation (Sun et al. 2001; Zhang et al. 1999). The RyR appears to be in close association with the NADP(H) oxidase(s) found in the SR, and locally generated superoxide appears to be the major ROS capable of influencing this channel (Xia et al. 2003). Each subunit of this large tetrameric protein contains a small number of regulatory cysteines. ROS and NO oxidize thiol residues on neighboring cysteines to form disulfide bonds, which induce channel opening. Disulfide formation is reversed by reducing agents, providing a mechanism for direct redox modulation of channel activity. The SERCA, another

potential target, contains a small number of critical sulfhydryls near the SERCA active site, which have been shown to slow the reuptake of calcium into the SR (Daiho and Kanazawa 1994; Xu et al. 1997; Gutierrez-Martin et al. 2004). Exposure to elevated NO also inhibits SERCA activity via thiol oxidation and nitration of tyrosine residues (Viner et al. 1997, 2000). Consequently, oxidation of SR proteins tends to increase cytosolic calcium levels, which causes prevention of muscle relaxation. Therefore, the modification of calcium transport proteins in the SR can causes excess or chronic muscle contraction via increasing intracellular calcium levels, leading to muscle fatigue.

Muscle myofilaments are also sensitive to direct redox modification (Fedorova et al. 2009). Myosin heavy chains contain several sulfhydryl residues, which are useful sites for protein labeling; however, thiol modification generally does not dramatically alter myosin function (Crowder and Cooke 1984). On the other hand, Yamada et al. (2007) reported that a force reduction in the soleus muscle of hyperthyroid rat is associated with carbonylation of the myosin heavy chain. In addition, it has been suggested that the myosin heavy chain is easily glycosylated, which changes the structural and functional properties of the protein (Haus et al. 2007). However, the involvement of these modifications on exercise-induced fatigue is unclear. In contrast, myosin light chains, actin, and tropomyosin appear less sensitive to redox modulation (Liu et al. 1990; Williams and Swenson 1982).

In delayed-onset muscle damage, protein modification is also observed. We reported the elevation of 4-hydroxy-2-nonenal (4-HNE), a lipid peroxidation product that covalently modifies the proteins on cysteine, histidine, and lysine residues, in the damaged muscle obtained from mice on the next day after acute running (Aoi et al. 2003). Liu et al. (2005) reported a positive correlate between 4-HNE and creatine kinase activity in blood following strenuous exercise in humans. Recently, insulin receptor substrate-1 (IRS-1) was detected as a 4-HNE-targeted protein (Aoi et al. 2012). IRS-1 is upstream in the PI3K/Akt-dependent insulin-signaling pathway in muscle cells and regulates glucose uptake via glucose transporter 4. In the damaged muscle after strenuous exercise, insulin-stimulated glucose uptake is decreased along with a reduction of insulin signal transduction, which suggests that 4-HNE modification of IRS-1 is involved in the transient impairment in insulin sensitivity. Sahlin et al. (2010) showed a marked elevation of 4-HNE modification of mitochondrial protein after acute endurance exercise. In addition, Kato et al. (2000) have reported that *N*ε-(hexanoyl)lysine (HEL), which is generated from the reaction between the lysine moiety and 13-hydroperoxyoctadecadienoic acid (13-HPODE), is increased in the muscle obtained from rats, which have performed high-intensity exercise training for 3 weeks. In contrast, the administration of eriocitrin, a flavonoid contained in lemon and lime fruits, to exercise-trained rats suppressed the formation of HEL.

4 HEL-Modification of CPT-I and Lipid Metabolism in Exercise

A major source of ROS production during exercise is the mitochondrial electron transport. Thus, mitochondrial proteins could be a major target of posttranslational oxidative modification during exercise. The HEL moiety is a novel adduct formed by the reaction of linoleic acid hydroperoxide and lysine, and is a marker of lipid peroxidation-derived protein modification in the early stages after oxidative stress (Kato et al. 1999; Osawa and Kato 2005), and therefore may be useful in detecting oxidative modification of mitochondrial protein during acute exercise (Fig. 15.2). Thus, we hypothesized that proteins on the mitochondrial membrane are easily modified by lipid peroxide generated on the membrane. In an analysis of mouse gastrocnemius muscle obtained immediately after running, the modification of carnitine palmitoyltransferase I (CPT I) by HEL was detected (Aoi et al. 2008). CPT I is located on the mitochondrial membrane and is a rate-limiting step in fatty acyl-CoA entry into the mitochondria in the muscle (McGarry and Brown 1997). In contrast, astaxanthin, an antioxidant in the mitochondrial membrane, limits the modification of CPT I by HEL after exercise, suggesting that astaxanthin directly traps the oxygen radicals generated by exercise, and/or that astaxanthin promotes the activation of a defense mechanism such as the induction of antioxidative enzymes, and/or that astaxanthin furthers the excretion of HEL from the skeletal muscle by activation of the proteasome. We previously reported that orally administered astaxanthin is absorbed in the intestine and accumulates in the muscle tissues in mice (Aoi et al. 2008). In addition, Manabe et al. (2008) have demonstrated that astaxanthin accumulates in the mitochondrial fraction and inhibits the oxidative modification of mitochondrial proteins in mesangial cells. Thus, the mitochondria are considered to be a major target of astaxanthin.

Several studies (Campbell et al. 2004; Holloway et al. 2006) have shown that the fatty acid translocase/cluster of differentiation 36 (FAT/CD36) is associated with CPT I on the mitochondrial membrane and increases its function. We found that the interaction between CPT I and FAT/CD36 in the muscle during exercise was facilitated by astaxanthin (Aoi et al. 2008). Thus, modification of CPT I by HEL may alter the colocalization of CPT I with FAT/CD36 by changing the CPT I structure, which could lead to the regulation of lipid metabolism during exercise. Lipolysis in the body is important during exercise to facilitate lipid utilization in the muscle rather than release it from the adipose tissue. The utilization ratio of carbohydrates and lipids for energy generation is almost equal when exercise is of low to moderate intensity. A possible factor influencing the utilized ratio of these energy substrates is the colocalization of CPT I with FAT/CD36. Exercise-induced ROS may partly limit the utilization of fatty acids via diminishing the CPT I activity caused by HEL modification (Fig. 15.3). Indeed, we and another group found that inhibition of this modification by dietary astaxanthin increased fat utilization during exercise as compared with mice on a normal diet and prolonged the running time to

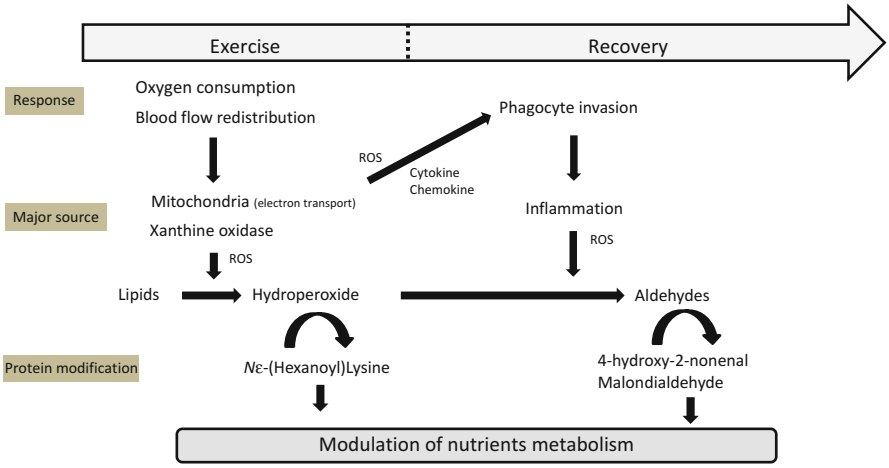


Fig. 15.2 Relationship among ROS, oxidative protein modification, and nutrient metabolism. ROS are generated via mitochondria and xanthine oxidase, and then oxidizes lipids during exercise, which results in elevation of *N*ε-(hexanoyl)lysine, an early stage lipid peroxidation marker. In recovery period following exercise, invasion of phagocytes into muscle tissues occurs, which progresses muscle damage via inflammation. In this process, lipid peroxide reaction is further progressed and aldehyde including 4-hydroxy-2-nonenal is produced. Such lipid peroxides modify particular metabolic protein posttranslationally, leading to modulation of nutrient metabolism

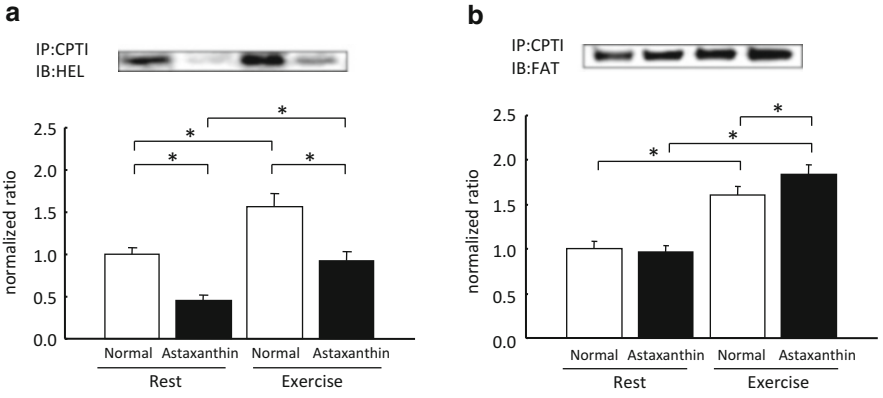


Fig. 15.3 HEL modification of CPT-I on mitochondrial membrane. Amount of FAT/CD36 that coimmunoprecipitated with CPT I (A) and HEL-modified CPT I (B) in skeletal muscle of ICR mice. A single bout of exercise was performed at 30 m/min for 30 min. Lysate protein from the muscle collected immediately after running was immunoprecipitated with CPT I antibody. Immunoprecipitates were separated by SDS-PAGE and membranes probed for FAT/CD36 (A) or HEL (B). Values are the mean ± SE. *, significant difference at the level of $P < 0.05$ (Data are from Aoi et al. 2008)

exhaustion (Aoi et al. 2008; Ikeuchi et al. 2006). Therefore, HEL-modification of CPT I can partly suppress lipid metabolism during exercise, which would affect the endurance performance and efficiency of adipose tissue reduction with training.

5 Prospective

Many evidences have indicated that oxidative stress has both positive and negative effects. A moderate grade of oxidative stress enhances muscle force production, nutrient metabolism, and antioxidant enzymes (Ristow et al. 2009; Gomez-Cabrera et al. 2008; Powers and Jackson 2008), although excess ROS functions as a damaging factor in cellular components. In contrast, dietary vitamin C and E cancel many exercise-induced adaptive benefits, such as improvements in insulin sensitivity, blood pressure, and endurance capacity, which are caused by suppressing the expression of redox-sensitive proteins, including PPAR gamma coactivator-1 alpha, AMP activated kinase, and superoxide dismutase 2. Therefore, the dietary intake of antioxidants for exercise therapy for the treatment and prevention of diseases and for training to improve the athletic performance is a debatable matter. On the other hand, some antioxidants accelerate energy metabolism and insulin sensitivity induced by exercise via the elevation of key modulators (Aoi et al. 2008; Richards et al. 2010; Henriksen 2006; Dolinsky et al. 2012). Therefore, we cannot group all antioxidants together, and we should consider the respective properties of each antioxidant individually but not their absolute antioxidative capacity. This may be responsible for the beyond-antioxidant properties that each compound has specifically. Oxidative protein modification during early-stage oxidative damage, e.g. HEL, may be useful as a potential biomarker to examine the benefit of antioxidant compounds in the sporting scene as well as in regulating oxidative stress levels, although a study focusing on the relationship between protein modification and physiological function in exercise is underdevelopment. Further research is required regarding the functional analysis of modified proteins along with whether this can be a biomarker of athletic performance levels, fatigue, and health benefits obtained from dietary exercise.

References

Aikawa KM, Quintanilha AT, de Lumen BO, Brooks GA, Packer L (1984) Exercise endurance training alters vitamin E tissue level and red blood cell hemolysis in rodents. Biosci Rep 4:253–257

Anzueto A, Andrade FH, Maxwell LC, Levine SM, Lawrence RA, Gibbons WJ, Jenkinson SG (1992) Resistive breathing activates the glutathione redox cycle and impairs performance of rat diaphragm. J Appl Physiol 72:529–534

Aoi W, Naito Y, Sakuma K, Kuchide M, Tokuda H, Maoka T, Toyokuni S, Oka S, Yasuhara M, Yoshikawa T (2003) Astaxanthin limits exercise-induced skeletal and cardiac muscle damage in mice. Antioxid Redox Signal 5:139–144

Aoi W, Naito Y, Takanami Y, Kawai Y, Sakuma K, Ichikawa H, Yoshida N, Yoshikawa T (2004) Oxidative stress and delayed-onset muscle damage after exercise. Free Radic Biol Med 37:480–487

Aoi W, Naito Y, Takanami Y, Ishii T, Kawai Y, Akagiri S, Kato Y, Osawa T, Yoshikawa T (2008) Astaxanthin improves muscle lipid metabolism in exercise via inhibitory effect of oxidative CPT I modification. Biochem Biophys Res Commun 366:892–897

Aoi W, Naito Y, Tokuda H, Tanimura Y, Oya-Ito T, Yoshikawa T (2012) Exercise-induced muscle damage impairs insulin signaling pathway associated with IRS-1 oxidative modification. Physiol Res 61:81–88

Ashton T, Young IS, Peters JR, Jones E, Jackson SK, Davies B, Rowlands CC (1999) Electron spin resonance spectroscopy, exercise, and oxidative stress: an ascorbic acid intervention study. J Appl Physiol 87:2032–2036

Barreiro E, Hussain SN (2010) Protein carbonylation in skeletal muscles: impact on function. Antioxid Redox Signal 12:417–429

Bidasee KR, Zhang Y, Shao CH, Wang M, Patel KP, Dincer UD, Besch HR Jr (2004) Diabetes increases formation of advanced glycation end products on sarco(endo)plasmic reticulum Ca^{2+}-ATPase. Diabetes 53:463–473

Blomstrand E, Rådegran G, Saltin B (1997) Maximum rate of oxygen uptake by human skeletal muscle in relation to maximal activities of enzymes in the Krebs cycle. J Physiol 501:455–460

Bryer SC, Goldfarb AH (2006) Effect of high dose vitamin C supplementation on muscle soreness, damage, function, and oxidative stress to eccentric exercise. Int J Sport Nutr Exerc Metab 16:270–280

Campbell SE, Tandon NN, Woldegiorgis G, Luiken JJ, Glatz JF, Bonen A (2004) A novel function for fatty acid translocase (FAT)/CD36: involvement in long chain fatty acid transfer into the mitochondria. J Biol Chem 279:36235–36241

Chang CK, Huang HY, Tseng HF, Hsuuw YD, Tso TK (2007) Interaction of vitamin E and exercise training on oxidative stress and antioxidant enzyme activities in rat skeletal muscles. J Nutr Biochem 18:39–45

Chen W, Ruell PA, Ghoddusi M, Kee A, Hardeman EC, Hoffman KM, Thompson MW (2007) Ultrastructural changes and sarcoplasmic reticulum Ca^{2+} regulation in red vastus muscle following eccentric exercise in the rat. Exp Physiol 92:437–447

Cobley JN, McGlory C, Morton JP, Close GL (2011) N-Acetylcysteine's attenuation of fatigue after repeated bouts of intermittent exercise: practical implications for tournament situations. Int J Sport Nutr Exerc Metab 21:451–461

Couillard A, Maltais F, Saey D, Debigaré R, Michaud A, Koechlin C, LeBlanc P, Préfaut C (2003) Exercise-induced quadriceps oxidative stress and peripheral muscle dysfunction in patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 167:1664–1669

Crowder MS, Cooke R (1984) The effect of myosin sulphydryl modification on the mechanics of fibre contraction. J Muscle Res Cell Motil 5:131–146

Daiho T, Kanazawa T (1994) Reduction of disulfide bonds in sarcoplasmic reticulum Ca^{2+}-ATPase by dithiothreitol causes inhibition of phosphoenzyme isomerization in catalytic cycle. This reduction requires binding of both purine nucleotide and Ca^{2+} to enzyme. J Biol Chem 269:11060–11064

Davies KJ, Quintanilha AT, Brooks GA, Packer L (1982) Free radicals and tissue damage produced by exercise. Biochem Biophys Res Commun 107:1198–1205

Dolinsky VW, Jones KE, Sidhu RS, Haykowsky M, Czubryt MP, Gordon T, Dyck JR (2012) Improvements in skeletal muscle strength and cardiac function induced by resveratrol during exercise training contribute to enhanced exercise performance in rats. J Physiol 590:2783–2799

Duarte JA, Appell HJ, Carvalho F, Bastos ML, Soares JM (1993) Endothelium-derived oxidative stress may contribute to exercise-induced muscle damage. Int J Sports Med 14:440–443

Fedorova M, Kuleva N, Hoffmann R (2009) Reversible and irreversible modifications of skeletal muscle proteins in a rat model of acute oxidative stress. Biochim Biophys Acta 1792:1185–1193

Ferguson RA, Ball D, Krustrup P, Aagaard P, Kjaer M, Sargeant AJ, Hellsten Y, Bangsbo J (2001) Muscle oxygen uptake and energy turnover during dynamic exercise at different contraction frequencies in humans. J Physiol 536:261–271

Flamm SD, Taki J, Moore R et al (1990) Redistribution of regional and organ blood volume and effect on cardiac function in relation to upright exercise intensity in healthy human subjects. Circulation 81:1550–1559

Gissel H, Clausen T (2001) Excitation-induced Ca^{2+} influx and skeletal muscle cell damage. Acta Physiol Scand 171:327–334

Goldfarb AH, Garten RS, Cho C, Chee PD, Chambers LA (2011) Effects of a fruit/berry/vegetable supplement on muscle function and oxidative stress. Med Sci Sports Exerc 43:501–508

Gomez-Cabrera MC, Domenech E, Romagnoli M, Arduini A, Borras C, Pallardo FV, Sastre J, Viña J (2008) Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance. Am J Clin Nutr 87:142–149

Gutierrez-Martin Y, Martin-Romero FJ, Inesta-Vaquera FA, Gutierrez-Merino C, Henao F (2004) Modulation of sarcoplasmic reticulum Ca^{2+}-ATPase by chronic and acute exposure to peroxynitrite. Eur J Biochem 271:2647–2657

Haus JM, Carrithers JA, Trappe SW, Trappe TA (2007) Collagen, cross-linking, and advanced glycation end products in aging human skeletal muscle. J Appl Physiol 103:2068–2076

Henriksen EJ (2006) Exercise training and the antioxidant alpha-lipoic acid in the treatment of insulin resistance and type 2 diabetes. Free Radic Biol Med 40:3–12

Hill BG, Bhatnagar A (2012) Protein S-glutathiolation: redox-sensitive regulation of protein function. J Mol Cell Cardiol 52:559–567

Holloway GP, Bezaire V, Heigenhauser GJ, Tandon NN, Glatz JF, Luiken JJ, Bonen A, Spriet LL (2006) Mitochondrial long chain fatty acid oxidation, fatty acid translocase/CD36 content and carnitine palmitoyltransferase I activity in human skeletal muscle during aerobic exercise. J Physiol 571:201–210

Ikeuchi M, Koyama T, Takahashi J, Yazawa K (2006) Effects of astaxanthin supplementation on exercise-induced fatigue in mice. Biol Pharm Bull 29:2106–2110

Joneschild ES, Chen LE, Seaber AV, Frankel ES, Urbaniak JR (1999) Effect of a NOS inhibitor, L-NMMA, on the contractile function of reperfused skeletal muscle. J J Reconstr Microsurg 15:55–60

Kato Y, Mori Y, Makino Y, Morimitsu Y, Hiroi S, Ishikawa T, Osawa T (1999) Formation of Nε-(hexanonyl)lysine in protein exposed to lipid hydroperoxide. A plausible marker for lipid hydroperoxide-derived protein modification. J Biol Chem 274:20406–20414

Kato Y, Miyake K, Yamamoto Y, Shimomura H, Ochi Y, Mori T, Osawa T (2000) Preparation of a monoclonal antibody to Nε-(hexanonyl)lysine: application to the evaluation of protective effects of flavonoid supplementation against exercise-induced oxidative stress in rat skeletal muscle. Biochem Biophys Res Commun 274:389–393

Kobzik L, Reid MB, Bredt DS, Stamler JS (1994) Nitric oxide in skeletal muscle. Nature 372:546–548

Kobzik L, Stringer B, Balligand JL, Reid MB, Stamler JS (1995) Endothelial type nitric oxide synthase in skeletal muscle fibers: mitochondrial relationships. Biochem Biophys Res Commun 211:375–381

Komulainen J, Takala TE, Kuipers H, Hesselink MK (1998) The disruption of myofibre structures in rat skeletal muscle after forced lengthening contractions. Pflugers Arch 436:735–741

Liao P, Zhou J, Ji LL, Zhang Y (2010) Eccentric contraction induces inflammatory responses in rat skeletal muscle: role of tumor necrosis factor-α. Am J Physiol Regul Integr Comp Physiol 298: R599–R607

Liu DF, Wang D, Stracher A (1990) The accessibility of the thiol groups on G- and F-actin of rabbit muscle. Biochem J 266:453–459

Liu J, Yeo HC, Overvik-Douki E, Hagen T, Doniger SJ, Chyu DW, Brooks GA, Ames BN (2000) Chronically and acutely exercised rats: biomarkers of oxidative stress and endogenous antioxidants. J Appl Physiol 89:21–28

Liu JF, Chang WY, Chan KH, Tsai WY, Lin CL, Hsu MC (2005) Blood lipid peroxides and muscle damage increased following intensive resistance training of female weightlifters. Ann N Y Acad Sci 1042:255–261

Magherini F, Abruzzo PM, Puglia M, Bini L, Gamberi T, Esposito F, Veicsteinas A, Marini M, Fiorillo C, Gulisano M, Modesti A (2012) Proteomic analysis and protein carbonylation profile in trained and untrained rat muscles. J Proteomics 75:978–992

Manabe E, Handa O, Naito Y, Mizushima K, Akagiri S, Adachi S, Takagi T, Kokura S, Maoka T, Yoshikawa T (2008) Astaxanthin protects mesangial cells from hyperglycemia-induced oxidative signaling. J Cell Biochem 103:1925–1937

Matuszczak Y, Farid M, Jones J, Lansdowne S, Smith MA, Taylor AA, Reid MB (2005) Effects of N-acetylcysteine on glutathione oxidation and fatigue during handgrip exercise. Muscle Nerve 32:633–638

McGarry JD, Brown NF (1997) The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur J Biochem 244:1–14

McKenna MJ, Medved I, Goodman CA, Brown MJ, Bjorksten AR, Murphy KT, Petersen AC, Sostaric S, Gong X (2006) N-acetylcysteine attenuates the decline in muscle Na^+, K^+-pump activity and delays fatigue during prolonged exercise in humans. J Physiol 576:279–288

Medved I, Brown MJ, Bjorksten AR, McKenna MJ (2004) Effects of intravenous N-acetylcysteine infusion on time to fatigue and potassium regulation during prolonged cycling exercise. J Appl Physiol 96:211–217

Miyazaki H, Oh-ishi S, Ookawara T, Kizaki T, Toshinai K, Ha S, Haga S, Ji LL, Ohno H (2001) Strenuous endurance training in humans reduces oxidative stress following exhausting exercise. Eur J Appl Physiol 84:1–6

Morrison RJ, Miller CC 3rd, Reid MB (1998) Nitric oxide effects on force-velocity characteristics of the rat diaphragm. Comp Biochem Physiol A Mol Integr Physiol 119:203–209

Naito Y, Yoshikawa T (2009) Oxidative stress-induced posttranslational modification of proteins as a target of functional food. Forum Nutr 61:39–54

Novelli GP, Bracciotti G, Falsini S (1990) Spin-trappers and vitamin E prolong endurance to muscle fatigue in mice. Free Radic Biol Med 8:9–13

Osawa T, Kato Y (2005) Protective role of antioxidative food factors in oxidative stress caused by hyperglycemia. Ann N Y Acad Sci 1043:440–451

Oya-Ito T, Naito Y, Takagi T, Handa O, Matsui H, Yamada M, Shima K, Yoshikawa T (2011) Heat-shock protein 27 (Hsp27) as a target of methylglyoxal in gastrointestinal cancer. Biochim Biophys Acta 1812:769–781

Perkins WJ, Han YS, Sieck GC (1997) Skeletal muscle force and actomyosin ATPase activity reduced by nitric oxide donor. J Appl Physiol 83:1326–1332

Powers SK, Jackson MJ (2008) Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. Physiol Rev 88:1243–1276

Proske U, Morgan DL (2001) Muscle damage from eccentric exercise: mechanism, mechanical signs, adaptation and clinical applications. J Physiol 537:333–345

Reid MB, Stokic DS, Koch SM, Khawli FA, Leis AA (1994) N-acetylcysteine inhibits muscle fatigue in humans. J Clin Invest 94:2468–2474

Richards JC, Lonac MC, Johnson TK, Schweder MM, Bell C (2010) Epigallocatechin-3-gallate increases maximal oxygen uptake in adult humans. Med Sci Sports Exerc 42:739–744

Richmonds CR, Kaminski HJ (2001) Nitric oxide synthase expression and effects of nitric oxide modulation on contractility of rat extraocular muscle. FASEB J 15:1764–1770

Rietjens SJ, Beelen M, Koopman R, VAN Loon LJ, Bast A, Haenen GR (2007) A single session of resistance exercise induces oxidative damage in untrained men. Med Sci Sports Exerc 39:2145–2151

Ristow M, Zarse K, Oberbach A, Klöting N, Birringer M, Kiehntopf M, Stumvoll M, Kahn CR, Blüher M (2009) Antioxidants prevent health-promoting effects of physical exercise in humans. Proc Natl Acad Sci U S A 106:8665–8670

Rosa EF, Ribeiro RF, Pereira FM, Freymüller E, Aboulafia J, Nouailhetas VL (2009) Vitamin C and E supplementation prevents mitochondrial damage of ileum myocytes caused by intense and exhaustive exercise training. J Appl Physiol 107:1532–1538

Sahlin K, Shabalina IG, Mattsson CM, Bakkman L, Fernström M, Rozhdestvenskaya Z, Enqvist JK, Nedergaard J, Ekblom B, Tonkonogi M (2010) Ultraendurance exercise increases the production of reactive oxygen species in isolated mitochondria from human skeletal muscle. J Appl Physiol 108:780–787

Salama G, Abramson JJ, Pike GK (1992) Sulphydryl reagents trigger Ca^{2+} release from the sarcoplasmic reticulum of skinned rabbit psoas fibres. J Physiol 454:389–420

Sultana R, Butterfield DA (2009) Proteomics identification of carbonylated and HNE-bound brain proteins in Alzheimer's disease. Methods Mol Biol 566:123–135

Sun J, Xu L, Eu JP, Stamler JS, Meissner G (2001) Classes of thiols that influence the activity of the skeletal muscle calcium release channel. J Biol Chem 276:15625–15630

Vassilakopoulos T, Deckman G, Kebbewar M, Rallis G, Harfouche R, Hussain SN (2003) Regulation of nitric oxide production in limb and ventilatory muscles during chronic exercise training. Am J Physiol Lung Cell Mol Physiol 284:L452–L457

Veskoukis AS, Nikolaidis MG, Kyparos A, Kokkinos D, Nepka C, Barbanis S, Kouretas D (2008) Effects of xanthine oxidase inhibition on oxidative stress and swimming performance in rats. Appl Physiol Nutr Metab 33:1140–1154

Viner RI, Krainev AG, Williams TD, Schoneich C, Bigelow DJ (1997) Identification of oxidation-sensitive peptides within the cytoplasmic domain of the sarcoplasmic reticulum Ca^{2+}-ATPase. Biochemistry 36:7706–7716

Viner RI, Williams TD, Schoneich C (2000) Nitric oxide-dependent modification of the sarcoplasmic reticulum Ca-ATPase: localization of cysteine target sites. Free Radic Biol Med 29:489–496

Williams DL Jr, Swenson CA (1982) Disulfide bridges in tropomyosin effect on ATPase activity of actomyosin. Eur J Biochem 127:495–499

Xia R, Webb JA, Gnall LL, Cutler K, Abramson JJ (2003) Skeletal muscle sarcoplasmic reticulum contains a NADH-dependent oxidase that generates superoxide. Am J Physiol Cell Physiol 285:C215–C221

Xu KY, Zweier JL, Becker LC (1997) Hydroxyl radical inhibits sarcoplasmic reticulum Ca^{2+}-ATPase function by direct attack on the ATP binding site. Circ Res 80:76–81

Yamada T, Mishima T, Sakamoto M, Sugiyama M, Matsunaga S, Wada M (2007) Myofibrillar protein oxidation and contractile dysfunction in hyperthyroid rat diaphragm. J Appl Physiol 102:1850–1855

Zhang JZ, Wu Y, Williams BY, Rodney G, Mandel F, Strasburg GM, Hamilton SL (1999) Oxidation of the skeletal muscle Ca^{2+} release channel alters calmodulin binding. Am J Physiol Cell Physiol 276:C46–C53

Chapter 16
Suppressive Effects of Cacao Polyphenols on the Development of Atherosclerosis in Apolipoprotein E-Deficient Mice

Midori Natsume and Seigo Baba

Abstract Previous studies in humans have shown that the cacao polyphenols, (−)-epicatechin and its oligomers, prevent *in vitro* and *ex vivo* low-density lipoprotein oxidation mediated by free radical generators and metal ions and also reduce plasma LDL-cholesterol levels. The aim of this study was to examine the effects of cacao polyphenols on the development of atherosclerosis in apolipoprotein E-deficient (−/−) mice. Mice aged 8 weeks (n = 90) were randomized into three groups, and fed either normal mouse chow (controls) or chow supplemented with 0.25 or 0.40 % cacao polyphenols for 16 weeks. The mean plaque area in cross-sections of the brachiocephalic trunk was measured and found to be lower in the 0.25 % cacao polyphenol group than in the control group ($p < 0.05$). Pathological observations showed that accumulation of cholesterol crystals in the plaque area was greater in the control group compared with the 0.40 % cacao polyphenol group ($p < 0.05$). Immunochemical staining in the 0.25 and 0.40 % groups showed that expression of the cell adhesion molecules (VCAM-1 and ICAM-1) and production of oxidative stress markers (4-hydroxynonenal, hexanoyl-lysine, and dityrosine) were reduced in cross-sections of the brachiocephalic trunk. These results suggest that cacao polyphenols inhibit the development of atherosclerosis in apolipoprotein E-deficient (−/−) mice by reducing oxidative stress and inflammatory responses.

Keywords Atherosclerosis • Cacao polyphenols • Apolipoprotein E-deficient (−/−) mice

M. Natsume (✉)
Functional Evaluation Department, Food Science Research Laboratories, R&D Division, Meiji Co., Ltd., 540 Naruda, Odawara, Kanagawa, 250-0862, Japan
e-mail: midori.natsume.ba@meiji.com

S. Baba
Clinical Research and Scientific Information, Product Development Department, Healthcare and Nutritionals Division, Meiji Co., Ltd., 2-10 Shinsuna 1-chome, Koto-ku, Tokyo 136-8908, Japan

Y. Kato (ed.), *Lipid Hydroperoxide-Derived Modification of Biomolecules*, Subcellular Biochemistry 77, DOI 10.1007/978-94-007-7920-4_16,

1 Polyphenolic Substances in Cocoa and Their Antioxidative Activity

Cacao beans contain various polyphenolic substances including epicatechin and its oligomers linked by C4 → C8 bonds such as procyanidin B2 (dimer), procyanidin C1 (trimer), and cinnamtannin A2 (tetramer). The chemical structure of these compounds is shown in Fig. 16.1 (Natsume et al. 2000; Hatano et al. 2002). These compounds have been shown to be the major antioxidative components of cocoa and chocolate (Natsume et al. 2000). Their effect on the susceptibility of human low density lipoprotein (LDL) to oxidation has been demonstrated (Lotito et al. 2000; Osakabe et al. 2002). LDL oxidation is known to be a major cause of atherosclerosis, with antioxidative substances thought to suppress the onset and development of atherosclerosis.

2 Human Studies

Studies we carried out on healthy human subjects also showed that intake of dairy cocoa powder enhanced the resistance of LDL to oxidation (Baba et al. 2007a, b; Osakabe et al. 2001). To further delineate the role of cocoa powder in protection against atherogenesis we examined the effects of cocoa intake on plasma concentrations of oxidized LDL and lipids and the urinary oxidative stress marker, 8-oxo-7,8-dihydro-2-deoxyguanosine, and lipid hydroperoxide-derived protein modification in normocholesterolemic and mildly hypercholesterolemic human subjects. Hollenberg et al. also reported that the Kuna Indians of Panama who frequently drink cocoa have a reduced risk of cardiovascular disease (Hollenberg et al. 2009). In addition, meta-analyses of randomized, controlled trials have been performed to examine the effect of cocoa on cardiovascular risk and hypertension (Desch et al. 2010; Hooper et al. 2008, 2012).

3 The Effect of Cacao Polyphenol Supplementation on Atherogenesis in Apolipoprotein E-Deficient (−/−) Mice

A clinical study in healthy volunteers demonstrated that daily intake of cocoa powder decreased the susceptibility of LDL to oxidation. Intake of polyphenolic-rich fractions derived from cocoa powder was also shown to suppress the formation of atherosclerosis in hypercholesterolemic rabbits (Osakabe et al. 2000) and KHC rabbits (Kurosawa et al. 2005a, b). In this chapter, we describe a study on the anti-atherosclerotic effect of 24 week of cacao polyphenol administration in apolipoprotein E-deficient (−/−) mice.

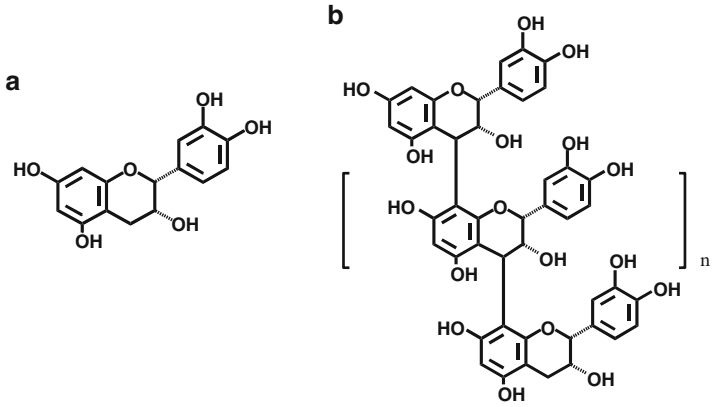


Fig. 16.1 Structure of the cacao polyphenols (**a**) (-)-epicatechin and (**b**) procyanidins (n = 0–2)

3.1 Body Weight and Plasma Analyses

The mice received a diet based on the composition of AIN-93G. The apolipoprotein E (+/+) mice fed a normal diet. The apolipoprotein E-deficient (−/−) mice in the 0.25 or 0.40 % cacao procyanidin groups received a diet, respectively. The polyphenol composition of catechin, epicatechin, procyanidin B2, procyanidin B5, procyanidin C1, and cinnamtannin A2 in the cacao polyphenols was analyzed as epicatechin equivalents. The quantities of the constituents were 2.43 % catechin, 5.77 % epicatechin, 3.85 % procyanidin B2, 1.27 % procyanidin B5, 1.71 % procyanidin C1, and 0.995 % cinnamtannin A2.

Body weight and plasma total cholesterol, LDL-cholesterol and triglyceride levels in the three groups at the beginning and end of the study are shown in Table 16.1. Weight at the end of the 26-week experiment was similar in the three groups. There was also no significant difference in total-cholesterol, HDL-cholesterol and triglyceride levels in the three groups. There is evidence that apolipoprotein E-deficient (−/−) mice have elevated plasma cholesterol and increased lipid peroxidation in the arterial wall. In the present study, dietary supplementation of cacao polyphenols in apolipoprotein E-deficient (−/−) mice did not affect plasma total cholesterol, HDL cholesterol, or triglyceride levels. These findings are similar to those observed in apolipoprotein E-deficient (−/−) mice fed tea catechins (Miura et al. 2001).

3.2 Atherosclerotic Lesion Area in Apolipoprotein E-Deficient (−/−) Mice

Atherosclerotic lesions in the proximal aorta of the mice were determined using a previously described method (Bentzon et al. 2001). The percentage of

Table 16.1 Body weight and plasma lipids in the four groups of mice

		Apolipoprotein E-deficient (−/−) mice			Apolipoprotein E (+/+) mice
		Control	0.25 % cacao polyphenols	0.40 % cacao polyphenols	Normal
Body weight	Initial (8w)	32.5 ± 3.7	31.8 ± 3.2	32.0 ± 2.8	22.0 ± 1.0
	Final (24w)	50.1 ± 8.4	48.0 ± 7.1	47.4 ± 7.0	26.9 ± 1.6
Total cholesterol (mg/dL)	Initial (8w)	428 ± 120	421 ± 143	378 ± 89	52.8 ± 5.5
	Final (24w)	440 ± 115	431 ± 100	429 ± 92	52.9 ± 4.2
HDL cholesterol (mg/dL)	Initial (8w)	104 ± 25	102 ± 27	105 ± 26	38.4 ± 4.1
	Final (24w)	112 ± 26	94 ± 19	93 ± 23	37.9 ± 6.9
Triglyceride (mg/dL)	Initial (8w)	251 ± 175	243 ± 115	236 ± 96	87.5 ± 16.3
	Final (24w)	215 ± 109	197 ± 95	190 ± 64	59.4 ± 11.2

atherosclerotic lesions in the aortic root was measured in 10 mice and the cross section of the brachiocephalic trunk measured in a further 10 mice. The area of atherosclerotic lesions in the aortic root and brachiocephalic trunk stained with oil red was measured using computer assisted image analysis.

The atherosclerotic lesion area in each aortic section (entire aorta, arch and thoracic plus abdominal) is shown in Fig. 16.2. In the normal diet group, 33 % of the entire aortic area was covered with fatty plaque, while 35 and 29 % were covered in the 0.25 and 0.4 % cacao polyphenol diet groups, respectively. Lesions of comparable morphology were found in all the aortic sites examined.

The atherosclerotic lesion area in the cross-section of the brachiocephalic trunk is shown in Fig. 16.3. Mean plaque area was found to be lower in the 0.25 % cacao polyphenol group than in the control group ($p < 0.05$). These data indicated that cacao polyphenols reduce formation of atherosclerotic lesions in the aortic sinus. Proanthocyanidins in wine, tea catechins and flavonoids have also been shown to reduce the extent of these lesions in the aorta (Miura et al. 2001; Chyu et al. 2004; Hayek et al. 1997).

3.3 Pathological Observations of the Brachiocephalic Trunk

Cross sections of the brachiocephalic trunk were cut from the aortic sinus of apolipoprotein E-deficient (−/−) mice fed either the control diet or 0.25 or 0.4 % cacao polyphenols. Morphological features including atheroma formation, foam cells, and cholesterol crystals were evaluated. The pathological classification of atheroma formation, form cells and cholesterol crystals in plaque was evaluated as either no change, slight, moderate, or marked. All evaluations were conducted in a double-blind fashion by Fuji Biomedix Co., Ltd. (Saitama Japan).

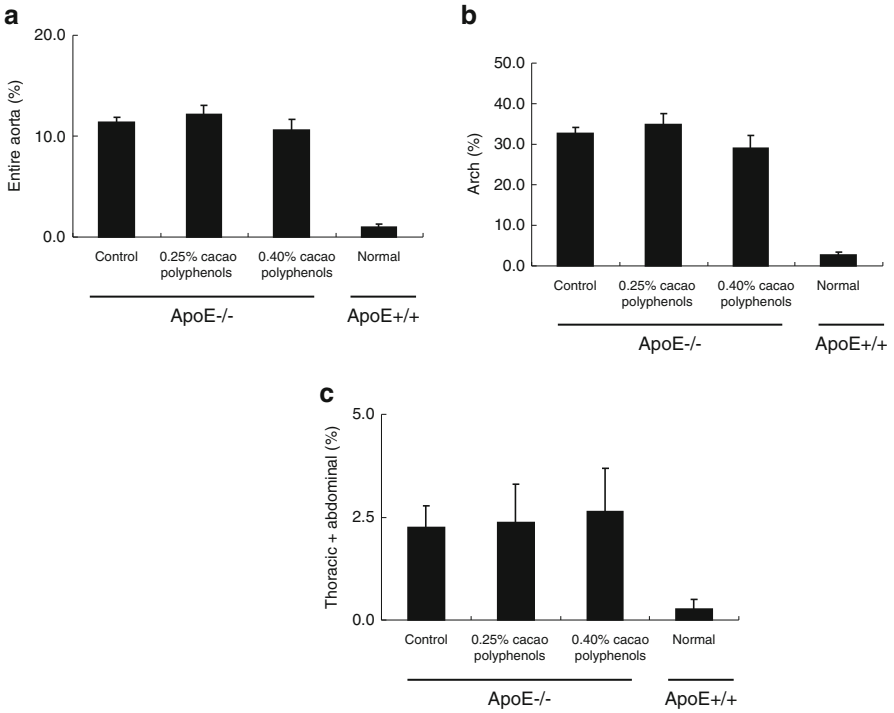


Fig. 16.2 Effects of cacao polyphenols on the size of aortic atherosclerotic lesions in apolipoprotein E-deficient (−/−) mice. The aortic lesion size of (**a**) the entire aorta, (**b**) arch, and (**c**) thoracic + abdominal was measured by the same person using computer-assisted image analysis. The percentages of lesion area relative to the entire aorta are expressed as mean ± SE of 10 mice

Atheroma formation, foam cells, and cholesterol crystals in the lesions are shown in Fig. 16.4 and the results of the pathological observation summarized in Table 16.2. The degree of cholesterol crystal accumulation in the plaque area was higher in the control group than in the 0.40 % cacao polyphenol group ($p < 0.05$). These data indicate that cacao polyphenols reduce cholesterol accumulation in the aorta.

3.4 Immunohistochemical Staining with Individual Antibodies

Cross sections of aortic sinuses were stained with antibodies against macrophages, VCAM-1 (Biogenesis) and ICAM-1 (Anti-CD36 antibody produced in rabbit; SIGMA), 4-hydroxynonenal (HNE) (Anti-HNE-Michael Adducts, Calibochem), hexanoyl-lysine (HEL) (Kato et al. 2000a) and dityrosine (DT) (Kato et al. 2000b). Typical results are shown in Fig. 16.5. The production and

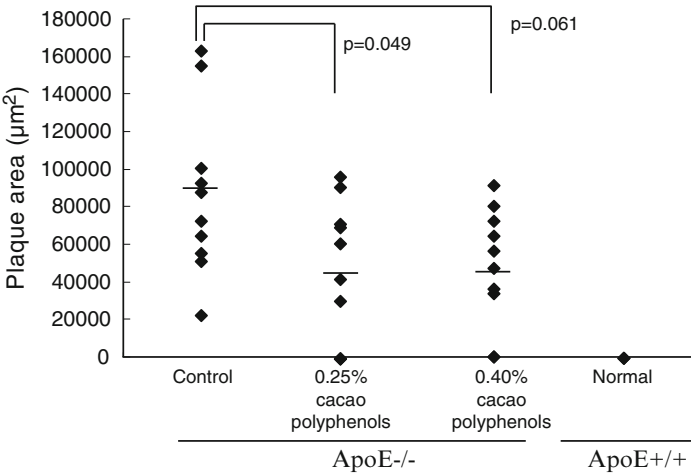


Fig. 16.3 Effects of cacao polyphenols on the size of aortic atherosclerotic area in cross sections of the brachiocephalic trunk in apolipoprotein E-deficient (−/−) mice. The cross sections were cut from the aortic sinus from apolipoprotein E-deficient (−/−) mice fed either a control diet or 0.25 and 0.40 % cacao polyphenols for 26 weeks and from apolipoprotein E (+/+) mice fed a normal diet. The aortic lesions were analyzed as described in the Methods section. Each point represents the measured lesion size in each mouse. Results are expressed as the mean ± SE of 10 mice

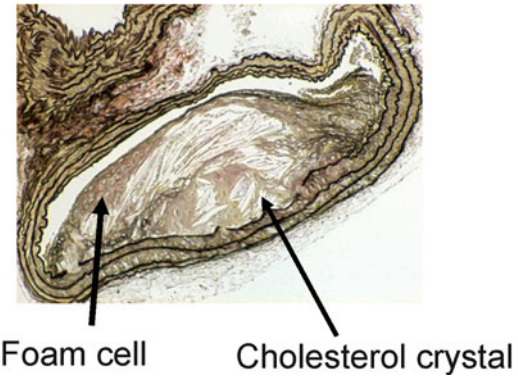


Fig. 16.4 Pathological observation of the brachiocephalic trunk. Cross sections of the brachiocephalic trunk illustrating a fatty streak (foam cells only) located distally in the artery and a mature plaque containing cholesterol crystals

localization of oxidative stress markers are co-localized with macrophages in the atherosclerotic lesions. Cacao polyphenols reduced staining intensity in sections treated with antibodies against macrophages, VCAM-1 and ICAM-1, HNE, HLE, and DT. Fukuchi et al. reported that HEL and DT produce and localize oxidative stress markers in atherosclerotic lesions (Fukuchi et al. 2008). HEL is considered to be an earlier and stable marker of lipid peroxidation-derived products in human atherosclerotic plaque, while HNE is formed by lipid peroxidation of omega-6 unsaturated fatty acids. HNE is also associated with various diseases. The sections were stained with antibodies against macrophage, VCAM-1 and ICAM-1. Noh

Table 16.2 Pathoanatomical observations

	No change	Slight	Moderate	Marked	
Atheroma formation					
Apolipoprotein E-deficient (−/−) mice					
Control	0/10	1/10	5/10	4/10	
0.25 % cacao polyphenols	3/10	3/10	4/10	0/10	$p = 0.011$ (vs. control)
0.40 % cacao polyphenols	2/10	1/10	7/10	0/10	$p = 0.059$ (vs. control)
Apolipoprotein E (+/+) mice					
Normal	10/10	0/10	0/10	0/10	
Foam cells in plaque					
Apolipoprotein E-deficient (−/−) mice					
Control	3/10	4/10	2/10	0/10	
0.25 % cacao polyphenols	3/10	2/10	3/10	2/10	
0.40 % cacao polyphenols	4/10	0/10	4/10	2/10	
Apolipoprotein E (+/+) mice					
Normal	10/10	0/10	0/10	0/10	
Cholesterol crystals in plaque					
Apolipoprotein E-deficient (−/−) mice					
Control	2/10	4/10	3/10	1/10	
0.25 % cacao polyphenols	6/10	3/10	1/10	0/10	$p = 0.090$ (vs. control)
0.40 % cacao polyphenols	9/10	0/10	1/10	0/10	$p = 0.010$ (vs. control)
Apolipoprotein E (+/+) mice					
Normal	10/10	0/10	0/10	0/10	

et al. reported that polyphenols from Phellinus baumii reduced the level of aortic mRNA expression of ICAM-1 and VCAM-1 in apolipoprotein E- deficient (−/−) mice (Noh et al. 2011). The development of atherosclerosis is associated with monocyte adhesion, with ICAM-1 and VCAM-1 being induced by tumor necrosis factor-α (TNF-α) and oxidized LDL, resulting in monocyte adhesion. Furthermore, atherosclerosis is a chronic inflammatory disease initiated by the interaction of circulating monocytes (Ross 1999). There is evidence that cacao polyphenols reduce the production of inflammatory mediators such as lipoxygenase and myeloperoxidase (Sies et al. 2005). These observations suggest that reduction of oxidative stress and adhesion factors in the arterial wall may contribute to the anti-atherogenic effect of cacao polyphenols.

4 Conclusion

As shown in Fig. 16.6, cacao polyphenols reduced the numerous loci of atherosclerosis. Oxidative stress caused by reactive oxygen species, oxidation of plasma and LDL, and chronic inflammation are associated with monocyte recruitment in the arterial wall.

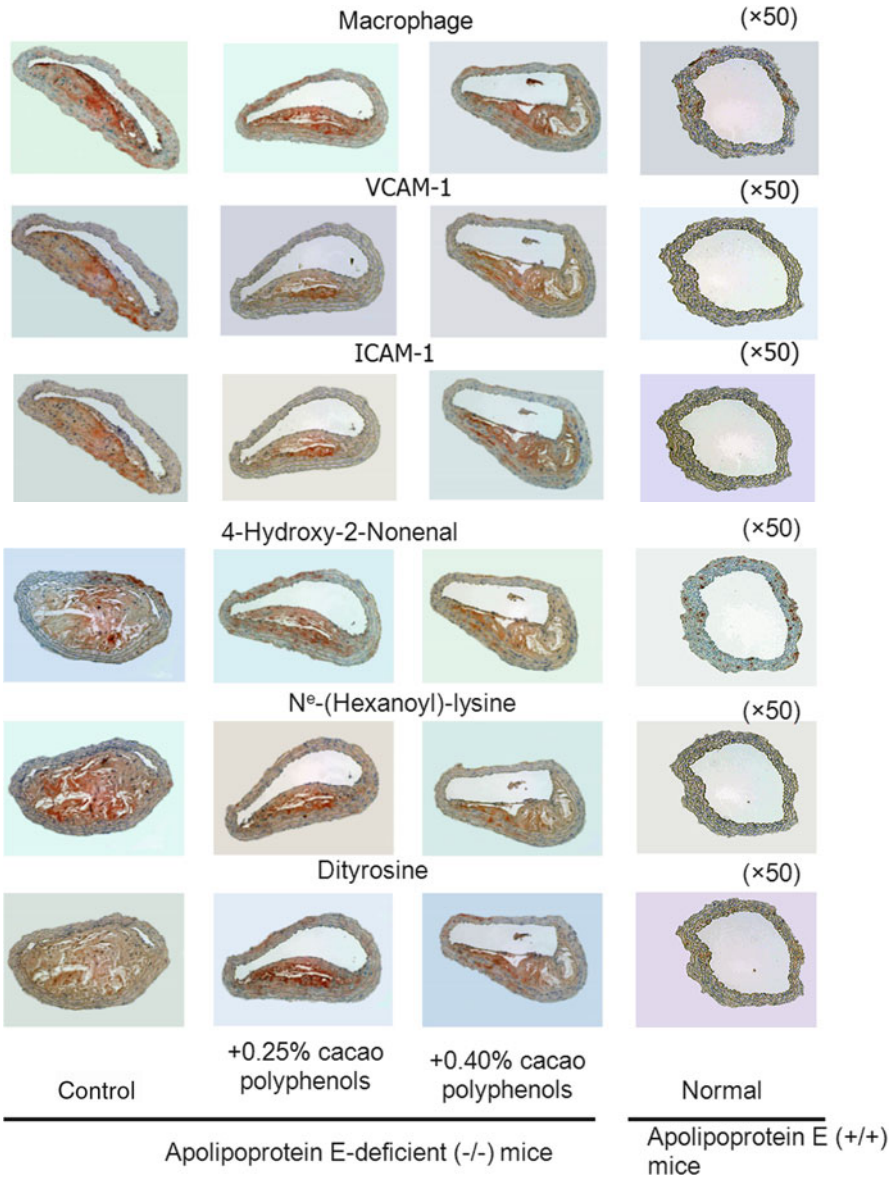


Fig. 16.5 Immunohistochemical detection of antibodies in cross sections of aortic sinuses. The cross sections of the aortic sinuses were stained with antibodies against macrophages, VCAM-1 and ICAM-1, 4-hydroxy-2-nonenal, N^{e}-(hexanoyl)lysine and dityrosine

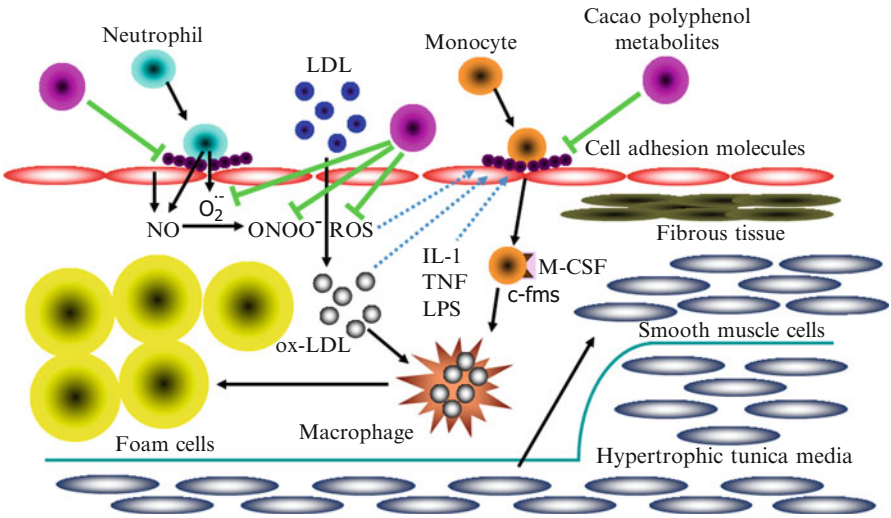


Fig. 16.6 Schematic diagram showing a possible mechanism by which cacao polyphenols prevent the development of atherosclerosis

Cacao polyphenols have properties that are well suited to the maintenance of healthy blood vessels as they inhibit platelet aggregation, regulate vasodilatory activity by controlling the levels of eicosanoids, NO and cytokines, and also inhibit oxidation of LDL. These effects may also contribute to the anti-atherosclerotic effect observed in the present study.

Acknowledgments We thank Dr. T Kodama (The University of Tokyo) for procurement of apolipoprotein E- deficient (−/−) mice. We also thank Dr. H Takano (Kyoto University) for analysis using computer assisted image analysis and Dr Y Kato (University of Hyogo) for procurement of hexanoyl-lysine (HEL) and dityrosine (DT).

References

Baba S, Natsume M, Yasuda A et al (2007a) Plasma LDL and HDL cholesterol and oxidized LDL concentrations are altered in normo- and hypercholesterolemic humans after intake of different levels of cocoa powder. J Nutr 137:1436–1441

Baba S, Osakabe N, Kato Y et al (2007b) Continuous intake of polyphenolic compounds containing cocoa powder reduces LDL oxidative susceptibility and has beneficial effects on plasma HDL-cholesterol concentrations in humans. Am J Clin Nutr 85:709–717

Bentzon JF, Skovenborg E, Hansen C et al (2001) Red wine does not reduce mature atherosclerosis in apolipoprotein E- deficient mice. Circulation 103:1681–1687

Chyu KY, Babbidge SM, Zhao X et al (2004) Differential effects of green tea-derived catechin on developing versus established atherosclerosis in apolipoprotein E-null mice. Circulation 109:2448–2453

Desch S, Schmidt J, Kobler D et al (2010) Effect of cocoa products on blood pressure: systematic review and meta-analysis. Am J Hypertens 23:97–103

Fukuchi Y, Miura Y, Nabeno Y et al (2008) Immunohistochemical detection of oxidative stress biomarkers, dityrosine and N(epsilon)-(hexanoyl)lysine, and C-reactive protein in rabbit atherosclerotic lesions. J Atheroscler Thromb 15:185–192

Hatano T, Miyatake H, Natsume M et al (2002) Proanthocyanidin glycosides and related polyphenols from cacao liquor and their antioxidant effects. Phytochemistry 59:749–758

Hayek T, Fuhrman B, Vaya J et al (1997) Reduced progression of atherosclerosis in apolipoprotein E-deficient mice following consumption of red wine, or its polyphenols quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and aggregation. Arterioscler Thromb Vasc Biol 17:2744–2752

Hollenberg NK, Fisher ND, McCullough ML (2009) Flavanols, the Kuna, cocoa consumption, and nitric oxide. J Am Soc Hypertens 3:105–112

Hooper L, Kroon PA, Rimm EB et al (2008) Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials. Am J Clin Nutr 88:38–50

Hooper L, Kay C, Abdelhamid A et al (2012) Effects of chocolate, cocoa, and flavan-3-ols on cardiovascular health: a systematic review and meta-analysis of randomized trials. Am J Clin Nutr 95:740–751

Kato Y, Miyake Y, Yamamoto K et al (2000a) Preparation of a monoclonal antibody to N (epsilon)-(Hexanonyl)lysine: application to the evaluation of protective effects of flavonoid supplementation against exercise-induced oxidative stress in rat skeletal muscle. Biochem Biophys Res Commun 274:389–393

Kato Y, Wu X, Naito M et al (2000b) Immunochemical detection of protein dityrosine in atherosclerotic lesion of apo-E-deficient mice using a novel monoclonal antibody. Biochem Biophys Res Commun 275:11–15

Kurosawa T, Itoh F, Nozaki A et al (2005a) Suppressive effects of cacao liquor polyphenols (CLP) on LDL oxidation and the development of atherosclerosis in Kurosawa and Kusanagi-hypercholesterolemic rabbits. Atherosclerosis 179:237–246. Epub 2005 Jan 19

Kurosawa T, Itoh F, Nozaki A et al (2005b) Suppressive effect of cocoa powder on atherosclerosis in Kurosawa and Kusanagi-hypercholesterolemic rabbits. J Atheroscler Thromb 12:20–28

Lotito SB, Actis-Goretta L, Renart ML et al (2000) Influence of oligomer chain length on the antioxidant activity of procyanidins. Biochem Biophys Res Commun 276:945–951

Miura Y, Chiba T, Tomita I et al (2001) Tea catechins prevent the development of atherosclerosis in apoprotein E-deficient mice. J Nutr 131:27–32

Natsume M, Osakabe N, Yamagishi M et al (2000) Analyses of polyphenols in cacao liquor, cocoa, and chocolate by normal- phase and reversed-phase HPLC. Biosci Biotechnol Biochem 64:2581–2587

Noh JR, Lee IK, Kim YH et al (2011) Antiatherogenic effect of antioxidant polyphenols from Phellinus baumii in apolipoprotein E-deficient mice. Ann Nutr Metab 59:145–153

Osakabe N, Natsume M, Adachi T et al (2000) Effects of cacao liquor polyphenols on the susceptibility of low-density lipoprotein to oxidation in hypercholesterolemic rabbits. J Atheroscler Thromb 7:164–168

Osakabe N, Baba S, Yasuda A et al (2001) Daily cocoa intake reduces the susceptibility of low-density lipoprotein to oxidation as demonstrated in healthy human volunteers. Free Radic Res 34:93–99

Osakabe N, Yasuda A, Natsume M et al (2002) Catechins and their oligomers linked by C4 –> C8 bonds are major cacao polyphenols and protect low-density lipoprotein from oxidation in vitro. Exp Biol Med (Maywood) 227:51–56

Ross R (1999) Atherosclerosis–an inflammatory disease. N Engl J Med 340:115–126

Sies H, Schewe T, Heiss C et al (2005) Cocoa polyphenols and inflammatory mediators. Am J Clin Nutr 81:304S–312S

Index

Y. Kato (ed.), *Lipid Hydroperoxide-Derived Modification of Biomolecules*, Subcellular Biochemistry 77, DOI 10.1007/978-94-007-7920-4,