

Chapter 3

Lipid Hydroperoxide-Derived Adduction to Amino-Phospholipid in Biomembrane

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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,

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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

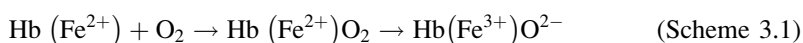
Although the head groups of some phospholipids, especially, PEs can be oxidized, which is also “phospholipid oxidation”, generally the targets of oxidation in phospholipids are unsaturated fatty acid chains. Therefore, phospholipids containing polyunsaturated fatty acids are susceptible to peroxidation 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 methHb (Rachmilewitz 1974). In this processes, oxygen alters superoxide (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-hydroperoxyoctadecadienoic 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 hexanylethanolamine (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

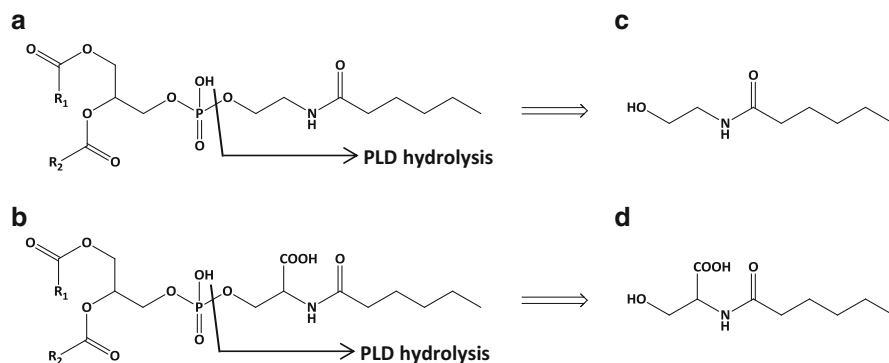


Fig. 3.1 Chemical structure of N-(hexanoyl)phosphatidylethanolamine (a), N-(hexanoyl)phosphatidylserine (b), hexanoylethanolamine (c) and hexanoylserine (d)

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 CCl₄, 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*.

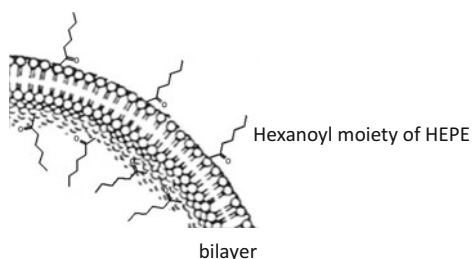


Fig. 3.2 Image of antigen: liposome containing HEDPPE

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.

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