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Review

Detection of oxidized high-density lipoprotein

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Abstract

This paper reviews working procedures for the separation and detection of oxidized high-density lipoproteins (ox-HDL) and their constituents. It begins with an introductory overview of structural alterations of the HDL particle and its constituents generated during oxidation. The main body of the review delineates various procedures for the isolation and detection of ox-HDL as well as the purification and separation of phosphatidylcholine metabolites and denatured apolipoproteins in the particle. The useful methods published more recently are picked up and the utility of the separation techniques is described. The last section covers a clinical evaluation of changes in these factors in ox-HDL as well as future directions of ox-HDL research.

Keywords: Reviews; High-density lipoproteins

Contents

1. Introduction

High-density lipoprotein (HDL) includes a heterogeneous class of lipoproteins, which generally have a high density ($1.063 < d < 1.21$ g/ml) and a small size (Stoke's diameter 5–17 nm) [1]. HDL

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particles freshly isolated from healthy volunteers are composed of lipids, apolipoproteins, enzymes, and lipid transfer proteins. Numerous potential mechanisms for the oxidation of lipoproteins in vivo have been proposed [2]. In addition, HDL oxidation can be initiated in vitro either by incubation with copper salt [3–6], or with endothelial cells [4], or by irradiation with ultraviolet [7,8]. Several alterations of constituent factors are observed in the HDL particle caused by these oxidative events.

Phosphatidylcholine (PC), 1,2-diacyl-*sn*-glycerol-3-phosphorylcholine, is the main constituent of biological membranes. In addition to having this important role for the structural and functional properties of the cell membrane of all living organisms, the PC also plays a main role in signal processing and as a precursor for many other biologically important molecules. PC is converted into lysophosphatidylcholine (lyso-PC) and fatty acid by treatment with phospholipase A. PC, which is generally known to localize in lipoprotein particles, is eliminated and lyso-PC increases by turn in the HDL particles during oxidation as observed in low-density lipoprotein (LDL) [9,10]. These pieces of evidence demonstrate that increased formation of lyso-PC may be one of the most reasonable indicators observed in HDL particles during oxidation. It has been shown that each lipoprotein possesses a characteristic fluid state, which is determined by the specific concentrations of the various constituents of the particles, i.e., protein, cholesterol, phospholipids, and triacylglycerol [11]. Schroeder et al. have hypothesized that regulation of plasma lipoprotein metabolism is partially determined by the fluid state of the particles [12]. Ferretti et al. have reported that fluorescent polarization of exogenous PC derivatives incorporated into HDL particles was altered during HDL oxidation, suggesting that the fluidity at the lipoprotein surface decreases in oxidized HDL (ox-HDL) [13]. Moreover, it has been suggested that the oxidative modification of HDL might lead to a loss of the cholesterol effluxing capacity, resulting from the rigidification through the disturbance of optimal fluidity [14,15]. Thus, peroxidative incidents seem to cause physicochemical modifications in the lipidic structure. Although some modifications in cellular membranes have been already described by spectroscopic techniques such as fluorescence polarization and electron spin resonance [16,17], they are mainly identified as a progressive rigidification of the lipidic moiety. This change in rigidification is partly due to double bond breakages on the oxygenated free radical attack and to a further reorganization of the lipids.

The level of thiobarbituric acid-reactive substance in HDL is also higher than in LDL from patients without coronary artery spasm [18]. HDL lipids are oxidized in preference to those in LDL when human plasma is exposed to aqueous peroxyl radicals [19,20]. The degree of lipid peroxidation in ox-HDL particles can be determined by monitoring the formation of conjugated dienes and thiobarbituric acid-reactive substances (TBARS). TBARS and conjugated dienes are associated with the differential fluorescence, which reflects the formation of iminopropene groups between aldehydes and amino groups of the protein moiety of HDL [21,22]. However, the actual compounds of lipid peroxides are not detected in these methods.

At present, little is known about the stability of apolipoprotein–lipid association in oxidatively modified lipoproteins. It is suggested that alteration in protein–lipid binding can affect the biological function of these lipoproteins. It has been postulated that hinged regions of the amphipathic helix of apolipoproteins in HDL are involved in the interaction with the cell membrane [23]. It is likely that cross-linking of apolipoproteins may alter such segments of the amphipathic helix and thus affect the ability of HDL to bind to HDL receptors on the cell surface. Sakai et al. demonstrated that the significant loss of the ligand activity of HDL toward the HDL receptor upon Cu^{2+}-mediated oxidation is due to cross-linking of HDL apolipoproteins, particularly of apolipoprotein A-I (apoA-I) [24]. Previous studies indicate that the cross-linking of HDL apoproteins by treatment with tetranitromethane [25,26] and chemical cross-linkers [27,28] is important in the loss of its ligand activity. Thus, it must be noted that the ability of HDL to interact with its binding site has been shown to be markedly inhibited after cross-linking of apoA-I [29].

The decrease in residues of lysine (Lys) and tryptophan (Trp) might also be involved in the diminution of the binding affinity and in the reduced activity of the particle to remove cholesterol from the

cells. Indeed, Duell et al. has recently demonstrated the importance of the Lys residues in the HDL receptor-dependent efflux of intracellular cholesterol [30]. The marked disappearance of Lys derivative in HDL by oxidative modification is photometrically determined from the decreased amount of ε-dinitrophenyl lysine produced by reaction with 2,4,6-trinitrobenzenesulfonic acid [31]. This is suggested to be based on modification of some of the amino group of Lys residues in apolipoproteins during the oxidation process. Few studies have addressed the changes in hydrophobic amino acids such as Trp, which can serve as indicator of the stability of apolipoprotein–lipid interactions under potentially denatured conditions [5,8,32]. This change appears to be due to partial destruction of the indole ring of Trp residues in apolipoproteins during oxidation of HDL with Cu^{2+} [5]. Trp has an intrinsic ultraviolet fluorescence, which is quite sensitive to solvent polarity in addition to its high quantum yield. Shoukry et al. demonstrated that oxidation of Trp in HDL is associated with an increase in maximum emission wavelength that is detectable, suggesting that this increase in wavelength may be indicative of changes in the Trp environment consistent with conformational changes in apolipoproteins in HDL [33]. These properties seem to make the detection of Lys and Trp residue a useful built-in probe to study apolipoprotein structure in HDL. Moreover, the oxidation of specific methionine (Met) residues on apoA-I has also been detected in isolated human HDL using a reversed-phase high-performance liquid chromatography (HPLC) [34].

Thus, many investigators have recently reported the structural alterations of constituents in the HDL particle during oxidation. These discoveries are thought to be due to the development of separation technology in recent years. This paper describes a comprehensive approach to provide a critical review on separation methodologies for the analysis of ox-HDL itself and its constituents.

2. HDL isolation from plasma and detection of ox-HDL particles

Lipoproteins have a lower hydrated density than other proteins in serum, because lipoproteins are composed of several apolipoproteins and many lipid molecules such as phospholipid, triglyceride, and cholesterol. Therefore, sequential flotation has been proposed as the principal method used for the isolation and classification of lipoproteins. HDL has been traditionally separated ultracentrifugally from plasma by discontinuous stepwise increases in solvent density [35,36]. Lipoprotein of lower density is in turn separated from the plasma with the solvent using high concentrations of solid salts, potassium bromide, or sodium bromide. An advantage of this method is the ability to process large volumes of plasma. Because numerous amounts of salts are present in HDL fractions, considerable time is consumed for extensive dialysis in order to perform the following experiment. It must be noted that protease inhibitors and antioxidants add to the plasma sample in the isolation step and that the isolated HDL is kept in the dark at 4 °C without freezing, to prevent from further oxidation and disruption of the particles [34].

Some investigators have proposed the rapid separation method of HDL particles from plasma using column chromatography [37–39]. Haginaka et al. have recently developed an anion-exchange HPLC method using a ProtEx-diethylaminoethyl (DEAE) column for sensitive separation of lipoproteins [40]. In this system, each lipoprotein is in turn separated by stepwise elution of both solvent A (20 m*M* sodium phosphate buffer, pH 7.0, containing 1 m*M* ethylenediaminetetraacetic acid) and solvent B (500 m*M* sodium chloride containing 1 m*M* ethylenediaminetetraacetic acid) at a flow-rate of 1.0 ml/min. After post-column reaction with an enzymatic cholesterol reagent (20 m*M* sodium phosphate buffer, pH 7.0 containing 0.2% Triton X-100, 5 μg/ml cholesteryl ester hydrolase, 20 μg/ml cholesterol oxidase, 50 μg/ml peroxidase, and 500 mg/ml homovanillic acid) by alkalization by a sodium hydroxide solution, lipoproteins are fluorometrically detected (excitation wave length, 325 nm; emission wave length, 420 nm). The separation pattern by this method is shown in Fig. 1. HDL, LDL, and VLDL are stepwisely eluted by increasing the concentration of solvent B to 25, 40, and 100%, respectively. The quantitated limit of cholesterol amount in lipoproteins is about 5 μg/ml. Because the gels of ProtEx-DEAE are composed of hydrophobic matrices and hydrophilic cross-linked surface layers as well as

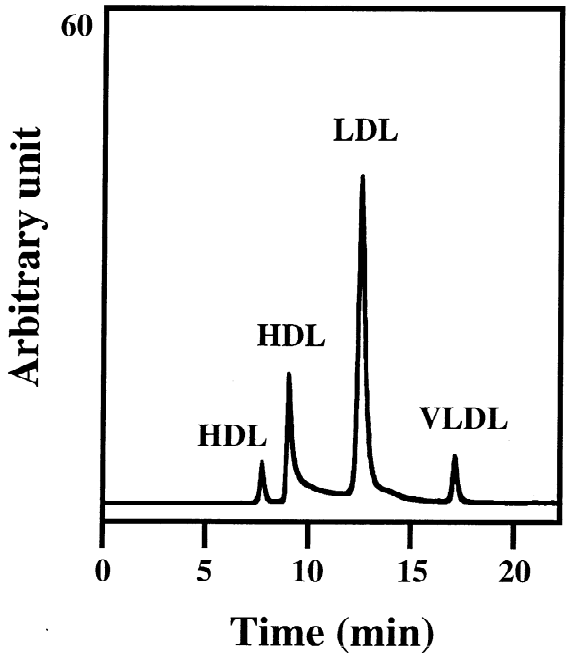

Fig. 1. Chromatogram of human plasma sample on a ProtEx–DEAE column. A 10-μl aliquot of plasma sample was injected (from Ref. [40]).

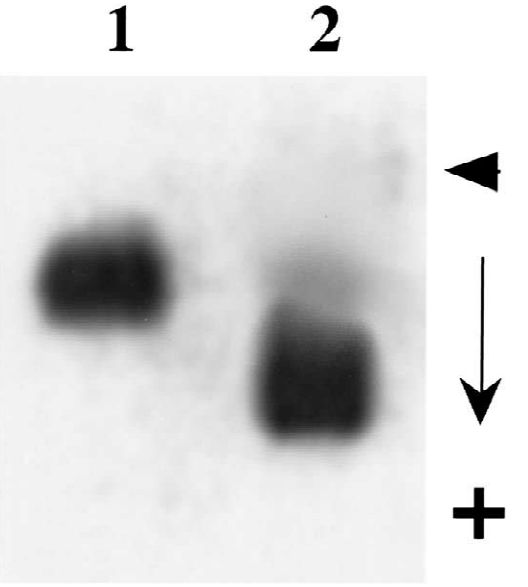

Fig. 2. Agarose gel electrophoresis of ox-HDL. Ten μg each of native HDL (lane 1) and ox-HDL (prepared by incubation at 37 °C for 16 h in the presence of 10 μ*M* Cu^{2+}) (lane 2). The arrowhead shows the origin and the arrow shows the direction.

DEAE–glucomannan, each protein appears to separate clearly without serious hydrophobic interactions with lipoproteins. If the performance condition is further improved, this method may be useful for sensitive detection of ox-HDL as well as rapid separation of HDL.

Generally, the electrophoretic method using an agarose gel has been recognized as a method of detection of the oxidatively modified form of HDL [41]. Because several kits are commercially available, agarose gel electrophoresis for detection of ox-HDL is one of the most widely performed tools. As shown in Fig. 2, difference of electrophoretic mobility between native HDL and ox-HDL is due to an increase in the total negative charges of the particle by oxidative modification. Little structural information is however obtained from this separation. Musanti and Ghiselli have reported the detection of the peak derived from ox-HDL on a molecular-sieve chromatography [42].

3. Detection of lyso-PC

3.1. Lipid extraction procedures

The general procedures for the isolation of lyso-PC from HDL particles are virtually the same as for all glycerophospholipids containing PC. Lipids present in HDL particles are associated with proteins as a complex characterized by dipole or hydrogen bonding interactions involving the polar and nonpolar moieties of lipid–protein complexes. To effectively disrupt the noncovalent bonding requires the use of a combination of polar and nonpolar solvents for complete recovery of the polar lipid from HDL.

Both methods using chloroform–methanol–water [43,44] and hexane–isopropanol mixtures [45] as the extracting solvent are well used. The procedure of the former is as follows: a mixture of 10 ml of chloroform, 20 ml of methanol, and 8 ml of water is added to 200 mg of HDL solution at 4 °C. The mixing for 30 s follows this after the addition of 10 ml of chloroform. Then, 10 ml of water are added

and the mixture is stirred for 30 s. The chloroform–methanol–water solvent ratios for the initial and final stages should be maintained at 1:2:0.8 and 2:2:1.8, respectively. After the chloroform layer is separated, the extraction procedure should be repeated with the water layer by the addition of a fresh aliquot of chloroform to ensure quantitative recovery of lipids. The separated chloroform solution is shaken with 10 ml of methanol–water (10:9) to remove non-lipid impurities. Evaporation of the chloroform extract under a N_2 stream yields the lipid mixture.

The method using the hexane–isopropanol mixture is environmentally more acceptable than the method described above. HDL solution (200 mg) and hexane–isopropanol (3:2) are mixed and shaken for 30 s. After the water layer is removed, the hexane layer is thoroughly rinsed with fresh extraction solvent. The mixture is stirred for 1 min with 12 ml of 1% sodium sulfate. Removal of the solvent from the upper layer gives the crude lipid residues.

3.2. Purification procedures

Despite the importance of good methods for phospholipid analyses, relatively little attention has up to now been paid to analysis of the molecular species of the different phospholipid classes. The reason for this is probably that separation and detection of these compounds have been relative complicated and time consuming. Thus, the method of choice was for many years to use thin-layer chromatography for class separation, followed by extraction and hydrolysis of the phospholipids and the use of gas chromatography [45] or HPLC [46] for identification of covalently modified derivatives. Although two-dimensional thin-layer chromatography (TLC) for phospholipid detection is well performed according to the method reported by Hanahan [47], high sensitivity and reproducibility are needed for detection of a trace amount of lyso-PC in vital samples. Analysis for phospholipid in HDL particles is also performed on a gas chromatograph equipped with a butanediol–succinate-packed capillary column and a flame ionization detector [48]. Despite their high analytical sensitivity, these methods require laborious sample derivatization pre-treatment, such as methylation with a methanol solution containing 2% sulfuric acid and 0.2% benzene. Therefore, a simple and efficient analytical method is needed for lyso-PC analysis. Several sensitive procedures using fast protein liquid chromatography with Mono-Q column, HPLC with light-scattering detection, and capillary electrophoresis have been developed [42,49].

Recently, the sensitive detection of lyso-PC in lipids extracted from HDL using an HPLC–electrospray ionization-mass spectrometry (LC–ESI-MS) has been reported [10,50,51]. In this system, a normal-phase HPLC with silica column (4.6×250 mm) is connected to a mass spectrometer, equipped with a nebulizer-assisted electrospray ionization interface. The column is eluted with a linear gradient of chloroform–methanol–30% ammonium hydroxide (80:19.5:0.5) to chloroform–methanol–water–30% ammonium hydroxide (60:34:5.5:0.5) in 14 min, and held for 10 min, at a flow-rate of 1 ml/min and the eluent is admitted directly into the mass spectrometer. The capillary exit voltage is set at 150 V, with the electron multiplier at 1795 V. Positive and negative spectra are examined in the mass range 450–1100 and 250–1100 u. As shown in Fig. 3, some PC peroxides (PC-OOH and PC-CHO) as well as lyso-PCs are sensitively detected in ox-HDL prepared by treatment for 6 h with a peroxynitrite donor compared with those in native HDL. Although the equipment in this system is costly, this is an applicable tool which can sensitively detect some changes in lipids caused by certain events.

3.3. Other methods

We have developed a sensitive sandwich enzyme-linked immunosorbent assay (ELISA) method using a specific monoclonal antibody 9F5-3a against lyso-PC present in ox-HDL particles, but not PC and lyso-PC alone [9]. A microtiter plate is coated with 1 μg/ml antibody 9F5-3a in 50 m*M* carbonate buffer, pH 9.5, and kept at 4 °C overnight. The plate is washed five times with phosphate-buffered saline containing 0.1% Triton X-100 after blocking. One hundred μl of lipoprotein solution are added to the well and then incubated for 1.5 h at 37 °C. After

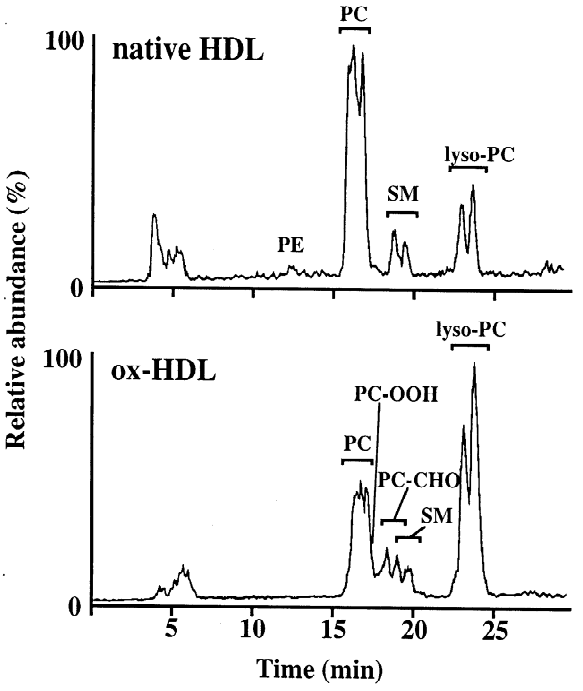

Fig. 3. LC–ESI-MS analysis of phospholipids in ox-HDL. Ox-HDL was prepared by incubation at 37 °C for 6 h with 1 m*M* 3-morpholinosydnonimine. Eluted peaks were identified by relative retention times of standards and the molecular mass of the components as follows: PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; lyso-PC, lysophosphatidylcholine; PC-OOH, phosphatidylcholine hydroperoxides; PC-CHO, phosphatidylcholine core aldehyde (from Ref. [10]).

washing the plate, a peroxidase-conjugated monoclonal antibody against apoA-I is added to the well and incubated for 1.5 h at 37 °C. After washing, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and hydrogen peroxide in 100 m*M* citrate buffer, pH 4.0, is added to the well, and the remaining peroxidase activity is then estimated by spectrometric measurement at 415 nm. The detection limit of this ELISA method is about 100 ng/ml of ox-HDL. This only defect is that the collection and further investigation of the detected particles is very difficult. The advantages of this method are follows: (1) peroxidase can be detected with improved sensitivity; and (2) ox-HDL particles can be used directly without complicated extraction of lipids. Therefore, this ELISA may be one of the most useful methods for the detection of ox-HDL.

4. Detection of lipid peroxides

The content of lipid peroxides was commonly estimated by determination of TBARS in samples. However, fluorescent detection of lipid peroxides using diphenyl-1-pyrenylphosphine (DPPP) has been recently developed, and the method has been successfully applied as a fluorescent probe for lipid peroxidation of culture cells [52]. Since this fluorescent reaction is simple and sensitive for the detection of lipid peroxide [53], it appears to be applicable to the detection of a trace in culture cells and tissues. Peroxides of PC and cholesterol in HDL are sensitively detected on HPLC analysis using a chemiluminescence system as determined by Sattler et al. [39].

A blotting technique has been developed to specifically detect lipid hydroperoxides in thin-layer chromatography as reported by Terao et al. [54]. A lipid solution extracted from chloroform–methanol (2:1) is charged onto a TLC plate and developed with the solvent hexane–diethylether–acetic acid (70:30:1). After the TLC plate is thoroughly dried, the plate is immersed in a solvent consisting of isopropanol–0.2% calcium chloride–methanol (40:20:7) and 0.01% DPPP. The plate is placed onto a glass fiber and then covered with a polyvinylidene difluoride (PVDF) membrane, a polytrifluoroethylene membrane, and finally a grass fiber onto the layer. After pressing of the TLC sandwich for 60 s at 180 °C, PVDF membrane is illuminated with a 150-W projection lamp for 20 min at room temperature and the lipid peroxide onto the membrane is fluorometrically determined. The limit of fluorescence detection of peroxides of PC and cholesterol with this method is in the range of 0.1–0.5 nmol. In the previous TLC analysis of lipid peroxide, detection by spraying with a reagent such as potassium iodide–starch, ammonium thiocyanate–ferrous sulfate, and dimethylaminoaniline, was commonly used, however, these spraying procedures seem to reduce the sensitivity and reproducibility. Because the detectable range of iodometry using potassium iodide and

methylene blue, as determined by Meditsh [55], is up to 1.0 nmol, the TLC blotting method for lipid peroxide using DPPP seems to be a useful method.

5. Detection of oxidized apolipoproteins

5.1. Pretreatment of HDL

Since contamination of lipid with apolipoproteins lowers the ability of separation, lipids should be removed on ice from HDL particles using the chloroform–methanol methods as described in Section 4. Finally, the water layer is collected and dried under a N_2 stream.

5.2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Ten μg of nondenatured apolipoproteins are applied onto an SDS–polyacrylamide gradient gel (5–15% slab gel) and electrophoresed in Tris–glycine buffer for 1 h at a constant electric current of 25 mA according to the method reported by Laemmli [56]. After washing sufficiently, the gel is stained with Coomassie brilliant blue. The patterns of SDS–PAGE of native HDL and ox-HDL are shown in Fig. 4. As revealed by monoclonal antibodies directed against apoA-I, native HDL displays one band of 28 rel. mol. mass units. Incubation of HDL with 10 μ*M* Cu^{2+} resulted in partial polymerization of apoA-I, as demonstrated by the appearance of bands of a higher molecular mass.

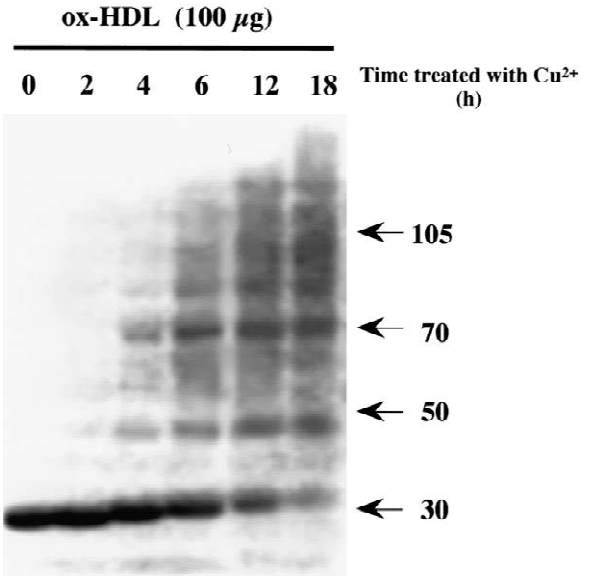

Fig. 4. SDS–PAGE of ox-HDL. Ox-HDL was prepared by incubation at 37 °C in the presence of 10 μ*M* Cu^{2+} for different time intervals. Ten μg of each sample was electrophoresed (5–15% gradient slab gel). Molecular masses of protein standards are indicated in kDa. Lanes are as follows: (1) untreated, (2) 1 h, (3) 2 h, (4) 4 h, (5) 8 h, and (6) 16 h.

5.3. HPLC separation

Some investigators have developed a reliable, sensitive, and relatively rapid method for the quantitation of oxidized apolipoproteins present in HDL using reversed-phase HPLC [57–60]. Aliquots of the proteins are dissolved in 6 *M* guanidine hydrochloride to disrupt the interaction of proteins. Samples are taken for analytical reversed-phase HPLC as described by Anantharamaiah et al. [34]. Preparative reversed-phase HPLC equipped with reversed-phase C_{18} column (22×250 mm) with a C_{18} guard column was performed. The solvent gradient is given by mixing solvent A (water containing 0.1% trifluoroacetic acid) and solvent B (90% acetonitrile containing 0.1% trifluoroacetic acid). The column is equilibrated with 75% of solvent A at a flow-rate of 1 ml/min. Ten mg of proteins in 6 *M* guanidine hydrochloride are injected into the system, and the solvent equilibrating the column is allowed to flow for 15 min. The peak of salts is detected at approximately 14 min and this time is assumed to be the void volume delay. The apoA-I containing oxidized Met residues is easily detected by this method because of the differential hydrophobic interaction between the HPLC matrix and the oxidized apoA-I. As shown in Fig. 5, oxidized forms of apoA-I and apoA-II were not detected in freshly isolated HDL. Concomitant with the loss of unoxidized apoA-I and apoA-II, some peaks were newly detected. Increased peaks eluting with a retention time of 0.81 and 0.94 relative to apoA-I shows apoA-I_{+32} (containing the oxidized form of Met^{112} and Met^{148}) and apoA-I_{+16}

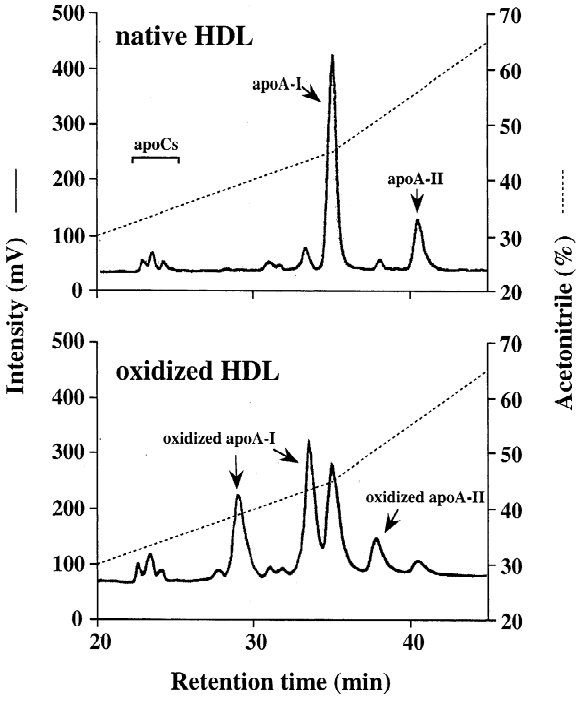

Fig. 5. HPLC pattern of apolipoproteins in ox-HDL. Ox-HDL was prepared by incubation at 37 °C for 16 h in the presence of 10 μM Cu^{2+}. Eluted peaks were identified by relative retention times of standards and the molecular mass of the components.

(containing one oxidized Met), respectively, as reported by Garner et al. [59]. In addition, another peak newly detected between apoA-I and apoA-II is known to contain one of two Met^{26} residues in an apoA-II dimer as Met sulfoxide [34]. The proposed mechanism for the formation of apolipoproteins containing oxidized Met in the presence of lipid hydroperoxides is shown in Fig. 6.

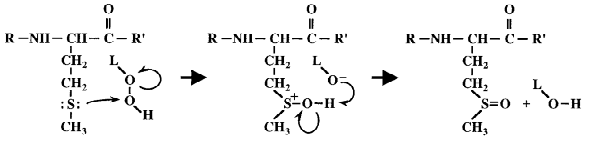

Fig. 6. Proposed mechanism for the reduction of HDL-associated lipid hydroperoxides by human apoA-I and apoA-II. Lipid hydroperoxide (LOOH) is reduced to the corresponding lipid hydroxide (LOH) via direct two-electron transfer from the sulfide of thioethers of the oxidation-prone Met^{112} and Met^{148} residues of human apoA-I and Met^{26} of apoA-II, resulting in the formation of their respective oxidized Met (from Ref. [60]).

6. Clinical evaluation and future directions of ox-HDL research

HDL has been considered as an anti-atherogenic lipoprotein for its reverse cholesterol transport activity [61–63]. Binding of HDL to its receptor initiates a translocation of cholesterol from intracellular pools to the cell membrane as the phenomenon, which precedes cholesterol efflux [64]. The role of HDL is thus to mediate reverse cholesterol transport to the liver for excretion into the bile. It has been reported that HDL may have a direct effect on the release of endothelium-derived relaxing factor or may have an indirect effect on endothelial proliferation to preserve normal endothelial function [65–68]. As another protective function, HDL has been reported to have anti-thrombotic effects [69] and prostacyclin-stabilizing activity [70,71].

Recently, there is growing evidence that oxidative modification of HDL could occur in vivo followed by alteration of its conformation biologically and chemically [3,72–74]. Clinical investigations have reported that the low levels of apoA-I, a major apoprotein in HDL, observed in patients with variant angina could be discriminated from normal control subjects or patients with coronary artery disease [75,76]. Immunohistochemical analysis using Cu^{2+}-ox-HDL-specific 9F5-3a antibody indicated the presence of ox-HDL in the intima of atheromatous plaques in the human abdominal aorta [77]. Western blot analysis using the 9F5-3a antibody has also detected smaller ox-HDL in HDL solution isolated from patients with chronic renal failure [78]. Ohmura et al. demonstrated that HDL from patients with coronary artery spasm has a higher susceptibility to lipid peroxidative modification than LDL [18]. Moreover, in vitro studies showed that HDL is more resistant to oxidation than LDL [79,80].

Cu^{2+}-mediated oxidation of HDL reduced its capacity to enhance cholesterol efflux from macrophage foam cells [3] and fibroblasts [72] and to bind to its specific receptor. The HDL receptor has been characterized [81–83] and cloned [84], and oxidative modification of HDL particles reduces the ligand activity toward these reported HDL receptors [24,30]. Although it was well known that ox-LDL and ox-HDL are recognized and endocytosed by the same scavenger receptor on the macrophage [85,86],

Parthasarathy et al. reported that ox-HDL did not apparently behave as a ligand for the scavenger receptor [4]. We have recently reported on the localization of a 130-rel. mol. mass unit protein, which binds to ox-HDL on the surface of endothelial cells on a radioimmunoassay using ^{125}I-labeled ox-HDL [77]. Ox-HDL reduces its beneficial properties such as stimulation of cholesterol efflux from foam cells [3], endothelium-dependent vasoreactivity [87], and anti-oxidative activity [4,88–90]. It is moreover reported that ox-HDL converts itself into a cytotoxic particle such as ox-LDL [7,19]. Ox-HDL is cytotoxic to lymphoblastoid cells, but less so than ox-LDL [7]. It has also been reported that ox-HDL and their lipid peroxides inhibit the secretion of tumor necrosis factor-α by macrophages, suggested that ox-HDL contributes to the modulation of the inflammatory response by macrophages as observed for ox-LDL [91]. These results suggested that oxidized lipoproteins including both HDL and LDL might contribute to the genesis of coronary artery spasm, while the possibility should also be considered that generation of oxidized lipoproteins could reflect an oxidative stress state predisposing the patient to coronary artery spasm.

HDL has also been demonstrated to play an important role in preventing the generation of ox-LDL [4,88–90]. This fact therefore suggests that reduced levels of HDL might facilitate the generation of ox-LDL. Ohta et al. reported that HDL particles containing apoA-I but not apoA-II had a strong inhibitory effect on the Cu^{2+}-mediated oxidation of LDL, suggesting the possibility that HDL might inhibit generation of atherogenic lipoproteins in vivo [92]. Since apolipoproteins in HDL are reported by Oram et al. to contribute to the removal of excess intracellular cholesterol from cells [93], it might be speculated that the decrease in cholesterol efflux observed with Cu^{2+}-ox-HDL is correlated to the polymerization of apoA-I. Disturbances in transferring phospholipids onto apoA-I in the HDL generation process are suggested to have a tight association with the progression of Tangier disease [94].

Several investigators demonstrated that the factors protecting against formation of lipid hydroperoxides in plasma appear to be apoA-I [59,60,95] and the enzyme paraoxonase (PON) [96–102]. The probable mechanism for the formation of lyso-PC under oxidative conditions is shown in Fig. 7. PON, which is a calcium-dependent HDL-associated ester hydrolase that catalyzes the hydrolysis of organophosphates, aromatic carboxylic acid ester, and carbamates, is tightly associated with apoA-I in HDL [103]. Incubation of phospholipid with PON in the presence of apoA-I generates lyso-PC [50], and PON arylesterase activity is modulated by apoA-I in the presence of lipids [104]. Thus, the formation of lyso-PC appears to be mainly caused by interaction of apoA-I and PON in HDL particles. It has been recently demonstrated that PON activity in HDL decreased during oxidation, suggested that PON in HDL may be a major contributor to the anti-atherogenicity of HDL [10,102,105].

HDL can accept oxidized cholesteryl esters from LDL, a process mediated by cholesteryl ester transfer protein [106]. This fact is of potential physiological significance as lipid oxidation products derived from LDL can impair the interaction of HDL with

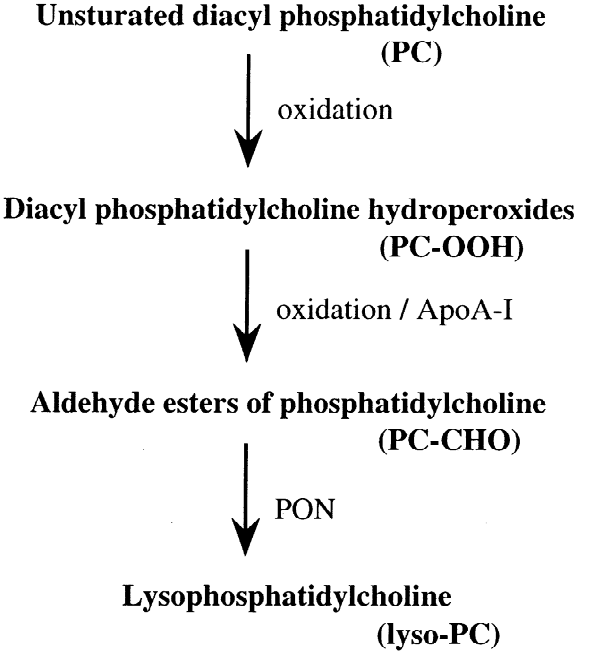

Fig. 7. Proposed mechanism for the oxidation of PC. PC is oxidized by treatment with oxygen radicals or oxidant donors and converted into PC-OOH. The fatty acyl group in PC-OOH is hydrolyzed by an additional oxidation in the presence of apoA-I, and, as the major end products, lyso-PC is formed by treatment of the generated PC-CHO with PON.

lecithin–cholesterol acyltransferase [107], and increase the clearance of HDL from plasma [108,109]. Ox-LDL or the increased content of lyso-PC in ox-LDL particles has been suggested to play a crucial role in alteration of endothelium-derived arterial relaxation [87,110,111] and to potentiate agonist-induced hyper-responsiveness of vasoconstriction by direct action on the vascular smooth muscle cells [112].

Epidemiological studies have identified an inverse relationship between the plasma level of HDL and the occurrence of arteriosclerosis [113]. Although individuals with type 2 diabetes often have low HDL levels, type 1 diabetics develop equally severe atherosclerosis despite normal or even elevated HDL levels [114]. HDL apoproteins in the serum of hyperglycemic individuals are more highly glycated than in normoglycemic individuals [115] and the glycation of HDL apolipoproteins causes a marked decrease in high-affinity binding to the HDL receptor on cultured cells [116]. Advanced glycation end products (AGEs) could conceivably impair endothelium-dependent relaxation by several mechanisms [117,118] that involve the glycosylation and oxidative modification of LDL, which in turn disrupts the formation and action of NO [119], as well as the direct quenching of nitric oxide (NO) by AGEs [120]. Furthermore, glycated LDL may be atherogenic, since it is cleared from serum more slowly than LDL, has less affinity for the LDL receptor, and is more avidly internalized by human monocyte-derived macrophages [121]. Galle et al. reported that glycated LDL enhances the inactivation of NO via increased production in endothelial cells [122]. Moreover, Lyons has suggested that increased glycation of LDL may play a role in accelerated development of atherosclerosis in diabetics [123]. The possibility that HDL can occur in vivo is suggested by the studies of Nishigaki et al. in which increased levels of lipid peroxides were found in plasma from diabetic patients, with the greatest increase found in the HDL fraction [124]. It was also reported that glycation of HDL increases its susceptibility to oxidation through reduced paraoxonase activity by glucose exposure [125], and lyso-PC stimulates superoxide anion production in endothelial cells through a NADH/NADPH oxidase-dependent mechanism [126]. PON has been identified as an independent, genetic risk factor for vascular disease, particularly in diabetic patients [127–130]. There are many reports of lower activity of PON in patients with both type-1 and type-2 diabetes mellitus [131–134]. This decrease on PON activity in HDL is suggested to be associated with additional oxidation during the HDL glycation process.

Thus, localization of ox-HDL has been a gradually obvious incident in the plasma from patients with atherosclerosis or diabetes mellitus. The role of the constituents of ox-HDL particles have also became clear by continuous investigations of numerous researchers. However, the method covered on this review does not go very far in having measured ox-HDL prepared in vivo. Therefore, it is an extremely important subject to try to detect ox-HDL particles and their components in the plasma from patients with some vascular diseases using these methods. Because oxidative modification of HDL makes the clearance of the particles shorter [108,109] and the amounts are found in vivo, analytical methods which are capable of detecting more simply and sensitively must be developed through further research. The important role of ox-HDL as a critical indicator of oxidative stress-associated vascular diseases and as a contributor to future medical development is expected to be confirmed.

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