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Reduction of Lipid Hydroperoxides by Apolipoprotein B-100

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We have previously isolated two proteins which can reduce phosphatidylcholine hydroperoxide (PC-OOH) from human blood plasma and identified one of the proteins as apolipoprotein A-I (Mashima, R., *et al.* (1998) *J. Lipid Res.* 39, 1133–1140). In the present study we have identified the other protein as apolipoprotein B-100 (apo B-100) by amino acid sequence analysis of its tryptic peptides. The reactivity of lipid hydroperoxides with apo B-100 decreased in the order of PC-OOH > linoleic acid hydroperoxide > cholesteryl ester hydroperoxide under our experimental conditions. Pretreatment of apo B-100 with chloramine T, an oxidant of methionine, diminished the PC-OOH-reducing activity, indicating that some of 78 methionines are responsible for the reduction of PC-OOH. Despite the presence of 6 methionines in albumin, albumin was inactive to reduce PC-OOH. Free methionine was also inactive. These data suggest that the accessibility and binding of lipid hydroperoxides to the protein methionine residues are crucial for reduction of lipid hydroperoxides.

Key Words: **apolipoprotein B-100; phosphatidylcholine hydroperoxide; phosphatidylcholine hydroxide; methionine; methionine sulfoxide; low density lipoprotein.**

Oxygen radicals are reputed to have important roles in aging and in the progression of degenerative diseases such as heart failure, diabetes, and cancer (1). Antioxidants are the first defense and convert oxygen radicals to stable products to prevent free radical-mediated chain oxidation of biomolecules such as lipids. Once that lipid peroxidation is initiated, however, at least one molecule of lipid hydroperoxide is formed even if a lipid peroxyl radical is directly scavenged by an antioxidant molecule. These lipid hydroperoxides need to be eliminated since metal-catalyzed decomposition of lipid hydroperoxides give rise to oxygen radicals which may further initiate the chain oxidation of biomolecules. Glutathione peroxidases serve this purpose as preventive antioxidant enzymes. In addition to glutathione peroxidases, glutathione transferase (2) and thioredoxin reductase (3) are also active in the reduction of hydroperoxides.

In our examination of hydroperoxide-reducing proteins in human plasma, we have recently isolated two endogenous proteins other than glutathione peroxidase, which can reduce phosphatidylcholine hydroperoxide to its stable hydroxide (4). One of the proteins has been characterized as apolipoprotein A-I (apo A-I) by its N-terminal amino acid sequence analysis (4). Furthermore, Garner *et al.* (5, 6) have independently observed that apo A-I and apolipoprotein A-II reduce cholesteryl ester hydroperoxide to its corresponding hydroxide. Here we report that the remaining hydroperoxide-reducing protein previously isolated by us is now identified as apolipoprotein B-100 (apo B-100).

EXPERIMENTAL

Materials. Cholesteryl linoleate, 1-palmitoyl-2-linoleoyl phosphatidylcholine, linoleic acid, and methionine were purchased from Sigma (Tokyo). Trypsin was obtained from Wako Pure Chemicals (Osaka). Chloramine T was purchased from Nacalai Tesque (Kyoto). ProSpin was obtained from Applied Biosystems (Foster City, CA).

Both 1-palmitoyl-2-linoleoyl-phosphatidylcholine hydroperoxide (PLPC-OOH) and linoleic acid hydroperoxide (18:2-OOH) were prepared by an enzymatic oxidation with soybean lipoxidase as de-

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Abbreviations used: apo A-I, apolipoprotein A-I; apo B-100, apolipoprotein B-100; Ch18:2-OH, cholesteryl linoleate hydroxide; Ch18:2-OOH, cholesteryl linoleate hydroperoxide; HPLC, high performance liquid chromatography; LDL, low density lipoprotein; 18:2-OH, linoleic acid hydroxide; 18:2-OOH, linoleic acid hydroperoxide; PBS, phosphate-buffered saline; PLPC-OH, 1-palmitoyl-2-linoleoyl-phosphatidylcholine hydroxide; PLPC-OOH, 1-palmitoyl-2-linoleoyl-phosphatidylcholine hydroperoxide; PAGE, polyacrylamide gel electrophoresis.

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scribed previously (4). Cholesteryl linoleate hydroperoxide (Ch18:2-OOH) was prepared by the following procedure. To remove oxidation products, cholesteryl linoleate (100 mg) was purified on a preparative octadecylsilyl column (Superiorex ODS, 20 x 250 mm, Shiseido, Tokyo) using 2-propanol/methanol (3/1, v/v) as the mobile phase delivered at a flow rate of 8 ml/min. The purified cholesteryl linoleate was mixed with α-tocopherol (5 mole % of cholesteryl linoleate) and was autoxidized at room temperature under aerobic conditions for 3 days. α-Tocopherol was added to reduce the formation of *trans, trans*-hydroperoxides and to accelerate autoxidation (7). The oxidation products were separated by using the same HPLC conditions described above and further purified by semipreparative chromatography on a silica-gel column (Supelcosil LC-Si, 5 μm, 10 x 250 mm, Supelco, Tokyo) using hexane/2-propanol (500/1, v/v) as the mobile phase at a flow rate of 4 ml/min. One of the isomers, 13-hydroperoxy-9-*cis*, 11-*trans*-cholesteryl linoleate, was dissolved in methanol/*tert*-butyl alcohol (1/1, v/v) and used for further experiments.

Peptide mapping and amino acid sequence analysis. The procedures for the purification of PLPC-OOH-reducing proteins have been described previously (4). The purified protein (1.8 nmol) was hydrolyzed by trypsin (substrate/enzyme = 100/1) in 2 ml of a 20 mM sodium phosphate buffer (pH 8) containing 1 M NaCl and 1 mM EDTA for 12 h at 37°C. The hydrolyzed peptides (450 pmol) were isolated by reversed-phase HPLC on a polymer-based octyl column (C8P 4E-50, 4.6 x 250 mm, Shodex, Tokyo) at 40°C with UV detection at 215 nm. Two mobile phases were used in stepwise linear gradient: 10 mM ammonium hydrogen carbonate in water (pH 8, solvent A) and 10 mM ammonium hydrogen carbonate in acetonitrile/water (80/20, v/v, pH 8, solvent B). The flow rate of mobile phase was 0.8 ml/min and the compositions of the solvent B were 0% (0-5 min), 0-50% (5-45 min), 50-100% (45-50 min), and 100% (50-55 min). Component peptides were manually collected and concentrated on a ProSpin poly(vinylidene fluoride) membrane by centrifugation at 5000 rpm until dry. The amino acid sequences of the isolated peptides were analyzed using a gas-phase sequencer (type PSSQ-21, Shimadzu, Kyoto).

Reduction of lipid hydroperoxides by apo B-100. The hydroperoxide-reducing protein previously isolated (4) is identified as apo B-100. Hereafter we refer to this protein as apo B-100 since no significant contaminant was observed as shown in Fig. 1. The reduction of PLPC-OOH by apo B-100 was carried out at 37°C under aerobic conditions. 2.5 μl of methanolic PLPC-OOH (100 μM) was added to 50 μl of 10 mM phosphate-buffered saline (PBS, pH 7.4) containing 1 mM EDTA and apo B-100 (final concentration: 24-96 nM). At the desired time points, reduction reactions were terminated by placing the sample on ice and adding 200 μl of methanol. After centrifugation at 12000 rpm for 3 min, a 100 μl sample of aqueous methanol phase was analyzed by HPLC for PLPC-OOH and 1-palmitoyl-2-linoleoyl-phosphatidylcholine hydroxide (PLPC-OH). HPLC analyses were carried out by a reported method (8) on an ODS column (Capcell Pak ODS, 5 μm, 4.6 x 250 mm, Shiseido, Tokyo) using acetonitrile/methanol/water (100/99/1, v/v/v) containing 10 mM choline chloride as the mobile phase at a flow rate of 2 ml/min. PLPC-OOH and PLPC-OH which eluted at 8.0 and 8.5 min, respectively, and their concentrations were determined photometrically by absorbance at 234 nm.

The reduction of 5 μM 18:2-OOH by 48 nM apo B-100 was carried out similarly. The same ODS analytical column was employed for the separation of 18:2-OOH and linoleic acid hydroxide (18:2-OH) using acetonitrile/water/acetic acid (600/400/1, v/v/v) as the mobile phase at a flow rate of 1 ml/min. Their retention times were 14 and 12 min, respectively.

The reduction of 5 μM Ch18:2-OOH by 48 nM apo B-100 was conducted similarly with the exception that methanol/*tert*-butyl alcohol (1/1, v/v) was used as a solvent for Ch18:2-OOH and 2-propanol was used to terminate the reaction. Ch18:2-OOH and Ch18:2-OH were separated on the same ODS analytical column using acetonitrile/2-propanol/water (44/54/2, v/v/v) as the mobile phase at a flow rate of 1 ml/min (9); Ch18:2-OOH and Ch18:2-OH eluted at 10 and 12 min, respectively.

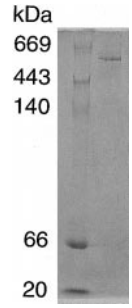


FIG. 1. SDS-polyacrylamide gel (5%) electrophoresis of a PLPC-OOH-reducing protein isolated from human plasma (4). Protein and molecular weight markers were visualized using Coomassie Brilliant Blue R-250.

Chloramine T pretreatment. Apo B-100 (96 nM) and chloramine T (20 nM–200 μM) were dissolved in PBS containing 1 mM EDTA. Equal volumes (25 μl) were mixed and preincubated for 30 min at 37°C. Then, 2.5 μl of methanolic PLPC-OOH (100 μM) was added and the reaction mixture was incubated for another 60 min. Concentrations of PLPC-OOH and PLPC-OH were measured as described above.

Incubation of PLPC-OOH with methionine or albumin. PLPC-OOH ($\sim$20 μM) in 50 mM Tris buffer (pH 7.4) containing 150 mM NaCl and 100 μM EDTA was incubated in the presence or absence of 600 μM methionine at 37°C under aerobic conditions. Concentration of PLPC-OOH was measured as described above. Next, we substituted 600 μM methionine with 100 μM albumin (total methionine residues = 600 μM) and aliquots of sample (5 μl) were injected onto HPLC equipped with a hydroperoxide-specific, chemiluminescence detection system (10). PLPC-OOH was separated on a silica gel column (Supelcosil LC-Si, 5 μm, 4.6 x 250 mm, Supelco, Tokyo) using methanol/40 mM monobasic sodium phosphate (9/1, v/v) as an eluant at a flow rate of 1 ml/min.

RESULTS AND DISCUSSION

We have isolated two hydroperoxide-reducing protein fractions from human plasma by a sequential purification scheme, comprising an ammonium sulfate precipitation followed by sequential chromatography on anion exchange, hydrophobic interaction, and heparin columns (4). One of the proteins was previously identified as apo A-I by N-terminal amino acid sequence analysis (4). Figure 1 shows that the unidentified PLPC-OOH-reducing protein migrated as a single band corresponding to a molecular weight of 500 kDa on SDS-polyacrylamide gel electrophoresis (PAGE). The reduction of this protein with 2-mercaptoethanol did not change its electrophoretic migration position (data not shown), suggesting that this protein is a monomer without intermolecular disulfide bonding.

We next trypsinized this protein and the resultant peptides were separated by reversed-phase HPLC as

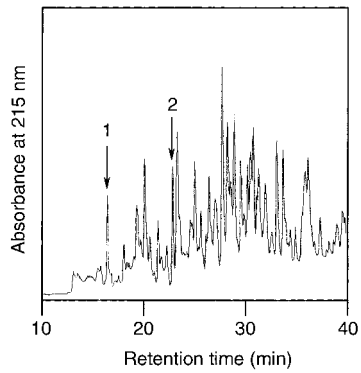


FIG. 2. Reversed-phase HPLC profile of tryptic peptides of the 500 kDa PLPC-OOH-reducing protein isolated from human blood plasma. The purified protein (1.8 nmol) was digested with trypsin (substrate/enzyme = 100/1) in 20 mM sodium phosphate (pH 8) containing 1 M NaCl and 1 mM EDTA at 37°C for 12 h. The hydrolyzed peptides (450 pmol) was separated by a reversed-phase HPLC. HPLC conditions are described in materials and methods. Peaks 1 and 2 were isolated and analyzed by amino acid sequencing.

shown in Fig. 2. Two peptides, **1** and **2,** were isolated and their N-terminal amino acid sequences were determined to be DFSAEYEEDG and DLKVE, respectively. These sequences are consist with the amino acid residues in apo B-100 at the position from 3926 to 3935 and from 2668 to 2672, respectively (11, 12). Apo B-100 is reported to be a monomeric protein of 512 kDa and this is also consistent with our SDS-PAGE results described above. Hereafter we refer to this hydroperoxide-reducing protein as apo B-100. Apo B-100 is a major apoprotein in low density lipoprotein (LDL).

Figure 3A shows that apo B-100 caused the reduction of PLPC-OOH to PLPC-OH in a time- and dose-dependent manner. Not only PC-OOH, but also Ch18:

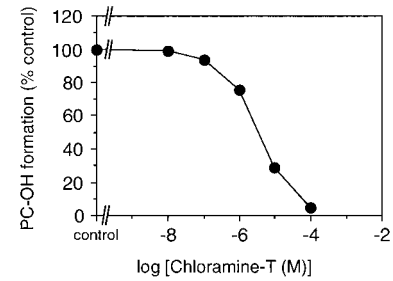


FIG. 4. Inhibition of PLPC-OOH-reducing activity of apo B-100 by chloramine T. 48 nM apo B-100 (total methionine residues = 3.7 μM) was treated with various concentration (10 nM–100 μM) of chloramine T in PBS containing 1 mM EDTA at 37°C for 30 min. Then, PLPC-OOH (final concentration: 5 μM) was added and incubated for another 60 min. 0.36 μM PLPC-OH was formed in the control experiment without chloramine T pretreatment.

2-OOH and 18:2-OOH were reduced by apo B-100, and the rates of substrate reduction decreased in the order of PLPC-OOH > 18:2-OOH > Ch18:2-OOH as shown in Fig. 3B. These results may be due to the fact that both PLPC-OOH and apo B-100 are located at the surface of LDL. We believe that this is the first report indicating that apo B-100 has a lipid hydroperoxides-reducing activity. This is consistent with the observation that LDL reduces cholesteryl ester hydroperoxide to its hydroxide (13).

Given that chloramine T is known to oxidize methionine to methionine sulfoxide (14) and that the reduction of cholesteryl ester hydroperoxide by apo A-I is methionine dependent (6), we examined the effect of pretreating 48 nM apo B-100 with various concentrations of chloramine T (10 nM–100 μM) on its PLPC-OOH-reducing activity. Figure 4 shows that chloramine T dose-dependently inhibited the PLPC-OOH-reducing activity of apo B-100, with 100 μM of

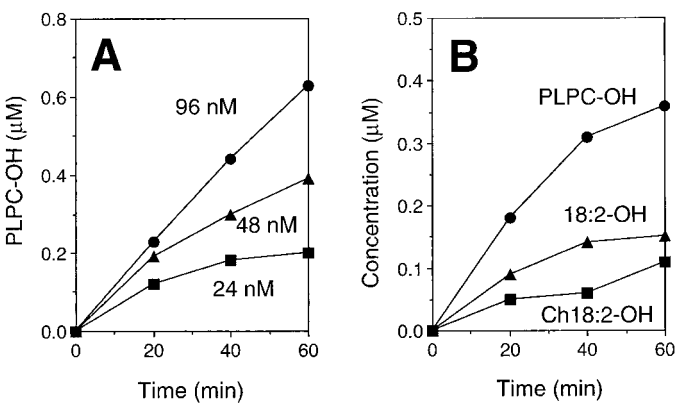


FIG. 3. Reduction of lipid hydroperoxides by apo B-100: (A) PLPC-OOH (5 μM) was incubated with 24 (■), 48 (▲), and 96 nM (●) apo B-100, and (B) 48 nM apo B-100 was incubated with 5 μM PLPC-OOH (●), 18:2-OOH (▲), and Ch18:2-OOH (■); all reactions were measured in PBS containing 1 mM EDTA at 37°C under aerobic conditions.

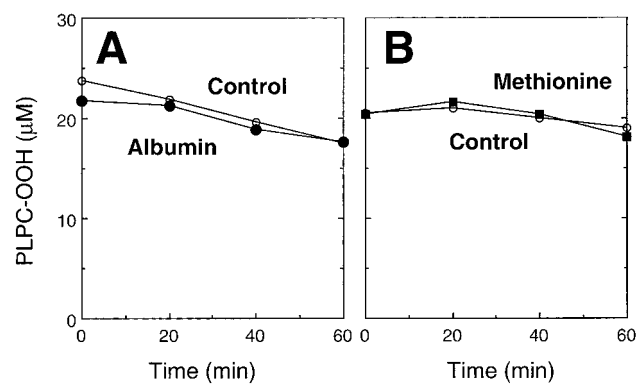
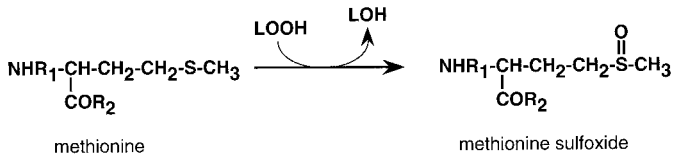


FIG. 5. Changes in the level of PLPC-OOH: (A) PLPC-OOH (23.8 μM) was incubated without (○) or with (●) 100 μM albumin (total methionine residues = 600 μM), and (B) PLPC-OOH (20.5 μM) incubated without (○) or with (■) 600 μM methionine in 50 mM Tris buffer (pH 7.4) containing 150 mM NaCl and 100 μM EDTA at 37°C under aerobic conditions.



SCHEME 1. Reduction of lipid hydroperoxides by methionine residues in polypeptides.

chloramine T almost completely inhibiting the protein reactivity. These data provide evidence that methionines in apo B-100 are involved in the reduction of PLPC-OOH by apo B-100.

Albumin is the most abundant protein (about 650 μM) in human plasma (15) and has 6 methionine residues (16). Figure 5A shows that albumin (100 μM; total methionine residues = 600 μM) does not reduce PLPC-OOH (20 μM), despite the high ratio of methionine to PLPC-OOH. Moreover, free methionine (600 μM) does not reduce PLPC-OOH (20 μM), either (Fig. 5B). These results indicate that not all protein methionines are reactive to lipid hydroperoxides and that the accessibility and binding of lipid hydroperoxides to protein methionines should be very important factors. Indeed, the treatment of high density lipoprotein with SDS diminished the hydroperoxide-reducing activity of apo A-I (6).

Lipid hydroperoxide-induced oxidation product of methionine in apo A-I is methionine sulfoxide (5). We also confirmed the formation of methionine sulfoxide in the reaction of methionine-containing synthetic peptides (LSPLGEEMR and WQEEMELYR) with linoleic acid hydroperoxide by gas chromatography/mass spectrometry after digestion with carboxypeptidase Y and derivatization with *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluroacetamide (data not shown). Moreover, methionine-free synthetic peptides (LSPLGEELR and WQEELELYR) did not reduce lipid hydroperoxides (data not shown). These data indicate the reaction of lipid hydroperoxide and methionine is stoichiometric as shown in Scheme 1.

Apo B-100 contains 78 methionines in a single polypeptide (11, 12). 200 nM PLPC-OH were produced during 60 min of incubation with 24 nM apo B-100 as shown in Fig. 3A, indicating that at least 8 (200/24 $\approx$ 8.3) methionines are reactive to PLPC-OOH. The level of apo B-100 in human plasma is about 2 μM (17). Therefore, apo B-100 in human plasma has the capacity to reduce at least 16 (= 8 x 2) μM PC-OOH.

The significance of apo B-100-dependent reduction of lipid hydroperoxides *in vivo* would be very convincing if we could detect methionine sulfoxide in apo B-100 isolated from human plasma, LDL, or arteriosclerotic lesion. However, this is not easy since a method with appropriate sensitivity for detecting small concentrations of protein methionine sulfoxide is not available, that the apo B-100 moleclue is too large to detect minor changes in a polypeptides after oxidation, and that methionine sulfoxide may be reduced by endogenous reductase occurring in blood and other tissues (18). Nevertheless, the presence of oxidized forms of apo A-I and apolipoprotein A-II in human atherosclerotic plaques has been suggested (6).

Oxidation of LDL is suggested to play a significant role in atherogenesis (19). Therefore, apo B-100-dependent reduction of lipid hydroperoxides can be an important defense mechanism since lipid hydroperoxides may produce oxygen radicals to initiate further oxidation and/or may activate 15-lipoxygenase and myeloperoxidase which are also believed to play important roles in atherogenesis (19).

In summary, we provide the first demonstration that apo B-100 reduces lipid hydroperoxides. Methionine residues in apo B-100 are suggested to be involved in this reduction.

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