Evidence that lipid hydroperoxides inhibit plasma lecithin:cholesterol acyltransferase activity

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Abstract The oxidation of low density lipoproteins (LDL) has been implicated in the development of atherosclerosis. Recently, we found that polar lipids isolated from minimally oxidized LDL produced a dramatic inhibition of lecithin: cholesterol acyltransferase (LCAT) activity, suggesting that HDL-cholesterol transport may be impaired during early atherogenesis. In this study, we have identified molecular species of oxidized lipids that are potent inhibitors of LCAT activity. Treatment of LDL with soybean lipoxygenase generated small quantities of lipid hydroperoxides (20 ± 4 nmol/mg LDL protein, n = 3); but when lipoxygenase-treated LDL (1 mg protein/ml) was recombined with the d > 1.063 g/ml fraction of human plasma, LCAT activity was rapidly inhibited (25 ± 4 and 65 ± 16% reductions by 1 and 3 h, respectively). As phospholipid hydroperoxides (PL-OOH) are the principal oxidation products associated with lipoxygenase-treated LDL, we directly tested whether PL-OOH inhibited plasma LCAT activity. Detailed dose–response curves revealed that as little as 0.2 and 1.0 mole % enrichment of plasma with PL-OOH produced 20 and 50% reductions in LCAT activity by 2 h, respectively. To gain insight into the mechanism of LCAT impairment, the enzyme's free cysteines (Cys31 and Cys184) and active site residues were "capped" with the reversible sulfhydryl compound, DTNB, during exposure to either minimally oxidized LDL or PL-OOH. Reversal of the DTNB "cap" after such exposures revealed that the enzyme was completely protected from both sources of peroxidized phospholipids. We, therefore, conclude that PL-OOH inhibited plasma LCAT activity by modifying the enzyme's free cysteine and/or catalytic residues. These studies are the first to suggest that PL-OOH may accelerate the atherogenic process by impairing LCAT activity.—Bielicki, J. K., and T. M. Forte. **Evidence that lipid hydroperoxides inhibit plasma lecithin:cholesterol acyltransferase activity. *J. Lipid Res.* 1999.** 40: **948–954.**

Supplementary key words phospholipid hydroperoxides • lecithin:cholesterol acyltransferase • high density lipoproteins • minimally oxidized LDL • atherogenesis

Lecithin:cholesterol acyltransferase (LCAT) plays an important role in vascular lipoprotein metabolism by catalyzing the esterification of cholesterol on high density lipoproteins (HDL). This esterification reaction is both necessary and sufficient for catalyzing HDL particle maturation whereby nascent discoidal HDL are converted into mature spherical particles containing a cholesteryl ester core (1–3). The critical importance of LCAT in mediating HDL maturation is revealed in cases of familial LCAT deficiencies (FLD) where there are marked reductions in plasma HDL-cholesterol concentrations (4, 5). Cholesterol esterification catalyzed by LCAT also reduces the amount of unesterified cholesterol in plasma; thus, a concentration gradient is established that favors net efflux of cellular unesterified cholesterol to HDL (6, 7). Indeed, patients with FLD frequently exhibit a build-up of unesterified cholesterol in peripheral tissues and red blood cells; renal dysfunction, hypertension, and atherosclerosis of renal and coronary arteries are frequently associated with FLD (4).

Because of the critical role that LCAT plays in the maturation of HDL particles and in the maintenance of cellular cholesterol homeostasis, it stands to reason that factors which reduce the amount of functional LCAT enzyme may predispose to an HDL-deficiency state and accelerate the atherosclerotic process. We recently found that inactivation of LCAT activity was, potentially, one the earliest pro-atherogenic responses induced by the minimal oxidation of LDL, suggesting that HDL-cholesterol transport may be compromised during early atherogenesis (8). Subfractions of LDL more susceptible to oxidation, as well as oxidatively modified forms of LDL, have been identified in human plasma and in atherosclerotic lesions (9–14). A number of recently published studies indicate that oxidized LDL is atherogenic as it can promote the recruitment/retention of monocyte/macrophages in the artery wall and stimulate the production of cytokines and growth factors which exacerbate proinflammatory reactions (15–18).

Our previous observation that minimally oxidized LDL is a potent inhibitor of LCAT activity suggests that expo-

Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; L-OOH, lipid hydroperoxides; PL-OOH, phospholipid hydroperoxides; DTNB, 5, 5′-dithiol-bis-nitrobenzoic acid; TBARS, thiobarbituric acid reactive substances.

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sure of HDL and LCAT to oxidized lipids may represent a key metabolic event in the progression of atherosclerosis. Cell culture model systems of LDL oxidation indicate that HDL may protect LDL from oxidation and thereby lessen the biological acitivity of minimally oxidized LDL (19–21). This protection, presumably, involves the transfer of oxidized lipids from minimally oxidized LDL to HDL and the degradation of the oxidation products by specific anti-inflammatory enzymes associated with HDL. Because of the central role of LCAT in reducing cholesterol accumulation and foam cell development, we set out to define boundaries wherein HDL may accept and degrade peroxidized lipids without adversely affecting LCAT activity. As a first step in attaining this goal, we sought to identify the chemical nature of the LCAT inhibitory compounds associated with the minimal oxidation of LDL. We found that the earliest of LDL lipid peroxidation products, namely the lipid hydroperoxides, were potent inhibitors of LCAT activity.

MATERIALS AND METHODS

Isolation of LDL and the d > 1.063 g/ml fraction from human plasma

Blood was collected from normolipidemic individuals into tubes containing EDTA (2.7 mm). Plasma was obtained by pelleting blood cells by centrifugation (1000 *g* for 25 min at 4°C). Plasma lipid and lipoprotein concentrations were 174 ± 35, 96 ± 28, 59 ± 17, and 92 ± 46 for total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides, respectively (n = 12 human donors). Low density lipoproteins (d 1.019–1.063 g/ml) were isolated by sequential, preparative ultracentrifugation using NaBr to adjust densities (22). LDL was dialyzed into phosphate (10 mm)-buffered saline (PBS, pH 7.4) for copper oxidation studies, and against PBS (pH = 7.4) containing EDTA (2.7 mm) for lipoxygenase treatment (see below). After dialysis, gentamicin sulfate (50 μg/ml) was added to LDL, and samples were stored (4°C) under nitrogen in the dark prior to use usually within 2 days from isolation. The d > 1.063 g/ml fraction which contains HDL, LCAT, and plasma proteins was dialyzed into PBS-EDTA; after dialysis, BHT (20 μm) and gentamicin were added.

LDL oxidation

Minimally oxidized LDL were prepared with copper ions (20 nmol/mg LDL protein/ml) at 37°C for 0.5 to 3 h as previously described (8). Reactions were quenched by adding EDTA (2.7 mm), BHT (20 μm), and by cooling samples to 4°C. The degree of LDL lipid peroxidation was quantified by measuring the thiobarbituric reactive substances (TBARS) as described by Kosugi, Kojina, and Kikugawa (23). The phrase "minimally oxidized LDL" is used throughout this manuscript to describe copper-oxidized LDL possessing <7.0 TBARS. The concentration of lipid hydroperoxides (L-OOH) associated with these minimally oxidized LDL averaged 44 ± 16 nmol total L-OOH/mg LDL protein as measured by an iodide to iodine conversion assay (24).

To "seed" LDL with small amounts of L-OOH via a mechanism that was relatively more specific than copper oxidation, an enzymatic method developed by Sparrow, Parthasarathy, and Steinberg (25) was used. Samples of LDL (3 mg protein/ml) in PBS-EDTA (pH = 7.4) were incubated (37°C) with 3 U/μl of soybean lipoxygenase (Sigma Chemicals, St. Louis, MI). Detailed time-course studies revealed that modest increases in conjugated dienes (234 nm absorbance) reached maximal levels (24 ± 5% above controls, n = 4) by 3 h. The lipid hydroperoxide content (20 ± 4 nmol of total lipid hydroperoxide/mg LDL protein) of lipoxygenase-treated LDL was calculated using the molar absorptivity coefficient (ε = 29,500 $Lcm^{-1}m^{-1}$) of conjugated dienes. This method yields concentrations of L-OOH that are within the range of clinical values for oxidized LDL in vivo (12–14). Lipoxygenase-treated LDL were added (1 mg protein/ml) to the d > 1.063 g/ml fraction of human plasma for the times shown in Fig. 3. Thereafter, plasma LCAT activity was assessed as described below. Control experiments revealed that small amounts of the lipoxygenase enzyme carried over with the LDL into the recombined plasma mixtures did not appreciably inhibit plasma LCAT activity, and that reductions in LCAT activity were contingent upon prior pretreatment of LDL with lipoxygenase (data not shown).

Preparations of phospholipid hydroperoxides and additions to human plasma

Phospholipid hydroperoxides (PL-OOH) were prepared by standard methodologies using soybean lipoxygenase and 1-palmitoyl, 2-linoleoyl (18:2) phosphatidycholine (26, 27). The phospholipid (0.3 mm) was dispersed in 0.2 m borate buffer (pH = 9.0) containing 3 mm sodium deoxycholate, BHT (20 μm) and EDTA (2.7 mm). Reactions were initiated by adding soybean lipoxygenase (3 U/μl) and increases in conjugated dienes were monitored at 234 nm. Because of the presence of EDTA and BHT, the PL-OOH generated are relatively stable and are not subject to spontaneous breakdown. The PL-OOH were extracted from reaction mixtures by the method of Bligh and Dyer (28). The mass of PL-OOH was calculated from the molar absorptivity coefficient (ε = 29,500 $Lcm^{-1}m^{-1}$) of conjugated dienes. Defined concentrations of PL-OOH were added to human plasma was in a small amount of ethanol (0.5% final concentration) and plasma incubated (37°C) for the times shown in the figures. Control phospholipid was similarly treated except the lipoxygenase enzyme was omitted from incubations. Because the values of PL-OOH were quantified by measuring conjugated dienes, the concentrations used may overestimate the actual plasma concentrations of PL-OOH if, during handling and incubations, the PL-OOH are reduced to their alcohol form (PC-OH) which also exhibits diene conjugation.

DTNB "capping" of LCAT's free cysteines and catalytic residues

Two different studies were performed with the thiol "capping" reagent 5,5′ dithiobisnitrobenzoic acid (DTNB). In the first set of experiments, we asked whether DTNB "capping" of LCAT's free cysteine and/or active site residues protected the enzyme from copper-oxidized LDL. Oxidized LDL that varied in the degree of lipid peroxidation were prepared with copper ions as described above. The d > 1.063 g/ml fraction of human plasma (containing HDL and LCAT) was treated with the reversible sulfhydryl compound, DTNB (1.7 mm), for 0.5 h at 37°C. After this incubation, the d > 1.063 g/ml fraction was dialyzed against PBS at 4°C to remove unbound DTNB. This DTNB treatment completely inhibited (>99%) plasma LCAT activity, indicating that the enzyme was effectively blocked (data not shown). The minimally oxidized LDL preparations were recombined (1 mg protein/ml) with control (no DTNB) and DTNB-treated d > 1.063 g/ml fractions and incubated at 37°C. At specified times, samples were taken for measurement of LCAT activity. The LCAT assay was performed in the presence of β-mercaptoethanol (see below) to reverse the DTNB "cap" and "unmask" protected LCAT activity.

In the second set of experiments, whole plasma was used to determine whether DTNB "capping" protected LCAT activity from PL-OOH. Plasma was treated with DTNB (1.7 mm) exactly

as descibed above. Control and DTNB-treated plasma were exposed (2 h) to relatively high concentrations (2 mole %) of PL-OOH previously determined to produce maximal reductions in LCAT activity.

Quantification of plasma LCAT activity

LCAT activity was measured using an exogenous proteoliposome substrate containing [^{14}C]cholesterol as described by Chen and Albers (29). This procedure quantifies initial rates of plasma LCAT activity that are independent of endogenous cofactors and lipoprotein profiles. The ^{14}C-labeled proteoliposome substrate was composed of human apoA-I: egg-yolk phosphatidylcholine: unesterified cholesterol (0.8:250:12.5 mole ratios). In addition to the proteoliposome substrate, reaction mixtures (0.25 ml final volume) contained 20 mm Tris-HCl (pH = 8.0), 0.15 m NaCl, 0.27 mm EDTA, 0.5% human serum albumin, and 5 mm β-mercaptoethanol (BME). Aliquots (7.5 μl) of plasma were added to start reactions, and samples were incubated at 37°C for 0.5 h. Reactions were stopped by cooling samples to 4°C and by adding ethanol (50% final concentration). Lipids were extracted with hexane, and [^{14}C]cholesterol and [^{14}C]cholesteryl esters were separated by instant thin-layer chromatography using fiber glass sheets coated with silica (Gelman Sciences, MI) and a toluene mobile phase. Bands corresponding to unesterified cholesterol and cholesteryl ester were cut, and the radioactivity associated with each was quantified by liquid scintillation counting. Results were expressed as a percentage of the initial [^{14}C]cholesterol that was converted to [^{14}C]cholesteryl ester in 0.5 h.

Other methods

The protein content of isolated lipoproteins was determined by the method of Markwell et al. (30). Phospholipid was quantified by measuring lipid phosphorus as described by Chen, Torihara, and Warner (31), and cholesterol was measured enzymatically by the method of Sale et al. (32).

Statistical analyses

Student's unpaired *t*-test was performed and $P < 0.05$ was used as the criterion for significance.

RESULTS

DTNB "capping" of LCAT's free cysteine and active site residues protects the enzyme from minimally oxidized LDL

The capacity of minimally oxidized LDL to inhibit plasma LCAT activity is shown in **Fig. 1**. When copper-oxidized LDL, possessing as little as 4.1 ± 0.1 TBARS, was recombined (1 mg protein/ml) with the d > 1.063 g/ml fraction of human plasma, 52 ± 8% reductions in LCAT activity were observed by 1 h.

It is well known that DTNB forms adducts with LCAT's free cysteine residues and sterically blocks the active site of the enzyme (33), but this modification is fully reversible under the reducing conditions of our proteoliposome assay of LCAT activity. Thus, we asked whether the presence of DTNB, temporarily bound to the LCAT enzyme, could protect plasma LCAT activity from minimally oxidized LDL. At low levels of LDL lipid peroxidation (4.1 ± 0.1 TBARS), LCAT activity was completely protected, indicating that products of LDL lipid peroxidation inhibited LCAT activity by modifying the enzyme's free cysteine and/or active site residues (Fig. 1). However, as the degree of LDL oxidation was increased (approx. 15 TBARS), DTNB no longer afforded such protection (**Fig. 2**) suggesting that products associated with more extensively oxidized LDL inhibited LCAT activity via mechanism(s) unrelated to active site modifications.

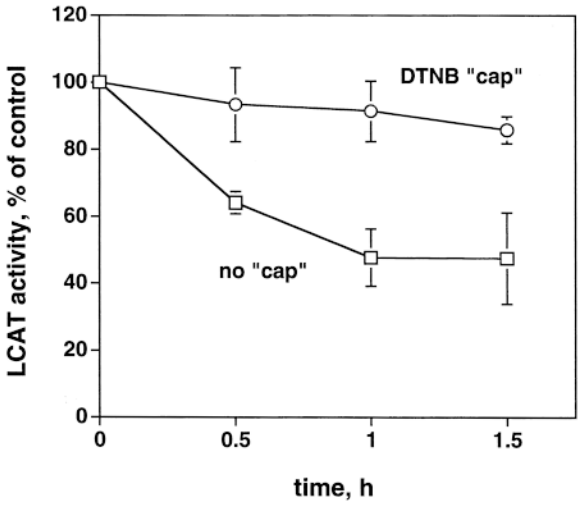

Fig. 1. DTNB "capping" of LCAT's free cysteine and active site residues protects LCAT activity from minimally oxidized LDL. The d > 1.063 g/ml fraction of human plasma (containing HDL and LCAT) was treated with DTNB (1.7 mm) at 37°C for 30 min followed by dialysis (PBS-EDTA, pH = 7.4) to remove unbound DTNB. Control and DTNB-treated d > 1.063 g/ml fractions were exposed to minimally oxidized LDL (1 mg protein/ml) possessing 4.1 ± 0.1 TBARS. LCAT activity was measured by the exogenous substrate method in the presence of β-mercaptoethanol (BME, 5.0 mm) to reverse the DTNB adduct and "unmask" protected LCAT activity. Results are expressed as a percentage of control LCAT activity at t = 0 (8.0 ± 1.3% esterification of [^{14}C]cholesterol/0.5 h). Values are means ± SD, n = 3.

Lipoxygenase-treated LDL inhibits plasma LCAT activity

Previously, we found that most of the LCAT inhibitory capacity of minimally oxidized LDL occurred during the lag-phase of LDL oxidation. This is the time when lipid hydroperoxides (L-OOH) accumulate. For this reason, we hypothesized that L-OOH might have been responsible for the observed reductions in LCAT activity. Treatment of LDL with soybean lipoxygenase has been found by Sparrow et al. (25) to mimic cell induced oxidation and "seed" LDL with L-OOH. Indeed, we have found that lipoxygenase treatment of LDL produced a small increase in L-OOH (20 ± 4 nmol L-OOH/mg LDL protein). Despite this small increase in L-OOH, lipoxygenase-treated LDL produced 25 ± 4% and 65 ± 16% reductions in plasma LCAT activity by 1 and 3 h, respectively (**Fig. 3**).

The lipoxygenase enzyme can utilize either free fatty acid (FFA) or phospholipid as substrates for L-OOH formation, but the FFA content of isolated lipoproteins is low compared to the phospholipid content of the particles. Thus, very little FFA hydroperoxides (FFA-OOH) were likely generated upon lipoxygenase treatment of LDL. Therefore, we tested whether addition of excess FFA to plasma may facilitate the lipoxygenase reaction and pro-

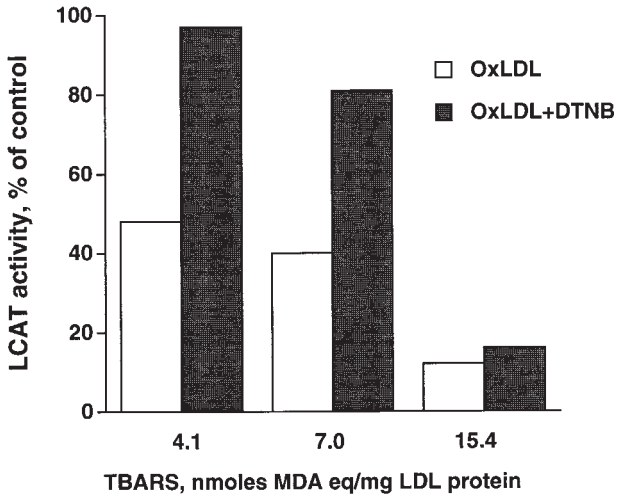

Fig. 2. DTNB fails to protect LCAT activity as the levels of LDL lipid peroxidation increases. The d > 1.063 g/ml fraction of human plasma was treated with DTNB as described in Fig. 1. Minimally oxidized LDLs that varied in the degree of lipid peroxidation (indicated as nmol of MDA equivalents) were recombined (1 mg LDL protein/ml) with control (open bars) and DTNB-treated (shaded bars) d > 1.063 g/ml fractions and incubated (37°C) for 1 h. LCAT activity was measured by the exogenous substrate method and in the presence of BME (5 mm). Results are expressed as a percentage of control LCAT activity at t = 0 (8.2 ± 0.5% esterification of [^{14}C]cholesterol/0.5 h). A representative experiment using three different preparations of minimally oxidized LDLs is shown. LCAT activity was measured in duplicate.

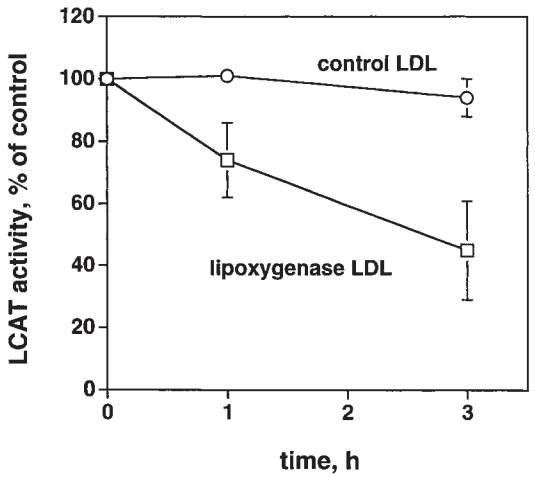

Fig. 3. Lipoxygenase-treated LDL inhibits plasma LCAT activity. Low density lipoproteins (d = 1.019–1.063 g/ml) were treated (3 h at 37°C) with soybean lipoxygenase (3 U/μl) in PBS-EDTA (pH = 7.4) to yield maximal increases (24 ± 5%, n = 4) in conjugated dienes. The concentration of lipid hydroperoxides was 20 ± 4 nmol L-OOH/mg LDL protein as determined from the molar absorptivity coefficient (29,500 $Lcm^{-1}M^{-1}$) of conjugated dienes. Control and lipoxygenase-treated LDL were added (1 mg protein/ml) to the d > 1.063 g/ml fraction of human plasma. At the times indicated, LCAT activity was measured using the exogenous substrate method. Results were expressed as a percentage of control activity at t = 0 (7.2 ± 0.7% esterification of [^{14}C]cholesterol/0.5 h). Values are means ± SD, n = 4.

duce an inhibition of plasma LCAT activity. In these experiments, 1 mm linolenate (18:3 fatty acid substrate) was added to plasma in the presence and absence of 3 U/μl of soybean lipoxygenase. In the absence of lipoxygenase, no reductions in plasma LCAT activity were observed during the time-course of these experiments (data not shown). However, when plasma was supplemented with FFA plus lipoxygenase, LCAT activity rapidly decreased as FFA-OOH started to accumulate in plasma (**Fig. 4**).

Phospholipid hydroperoxides inhibit LCAT activity by modifying the enzymes' free cysteines and/or active site residues

As discussed above, the phospholipid on the surface of LDL is the most abundant substrate for the lipoxygenase enzyme. As a result, it is likely that PL-OOH were responsible for the inhibition of LCAT activity induced by lipoxygenase-treated LDL. To directly test this hypothesis, PL-OOH were added to plasma at defined concentrations. As shown in **Fig. 5**, as little as 0.23±0.06 mole% additions of PL-OOH to plasma (i.e. 1 molecule of PL-OOH /500 molecules of total phospholipid) produced 20% reductions in LCAT activity by 1 h. More dramatic, dose-dependent reductions in LCAT activity were observed over a 10-fold concentration range where nearly all (>80%) of the plasma LCAT activity was inhibited at the upper limit of 2.0 mole % PL-OOH in plasma (Fig. 5).

As phospholipids are used as substrates for the LCAT reaction, it is likely that PL-OOH may inhibit LCAT activ-

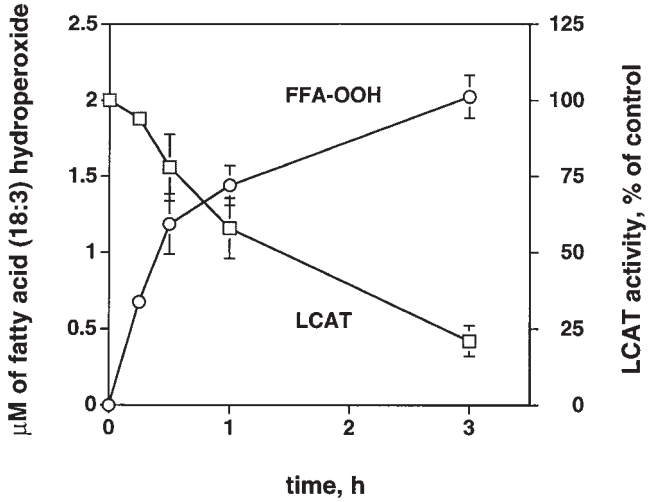

Fig. 4. Free fatty acid hydroperoxides (FFA-OOH) inhibit plasma LCAT activity. Human plasma was supplemented with 1 mm linolenate (18:3 lipoxygenase substrate) and 3 U/μl of soybean lipoxygenase. At the times indicated, samples of plasma were removed for measurements of conjugated dienes (234 nm absorbance) and plasma LCAT activity. The concentration of FFA-OOH was calculated from the molar absorptivity coefficient of conjugated dienes as described in Fig. 3. LCAT activity was quantified using the exogenous substrate method. Results are expressed as a percentage of control activity in plasma treated with 1 mm linolenate (no lipoxygenase). The control LCAT activity was 7.6 ± 0.8% esterification of [^{14}C]cholesterol/0.5 h and it did not differ from LCAT activity in fresh human plasma at t = 0 prior to addition of linolenate.

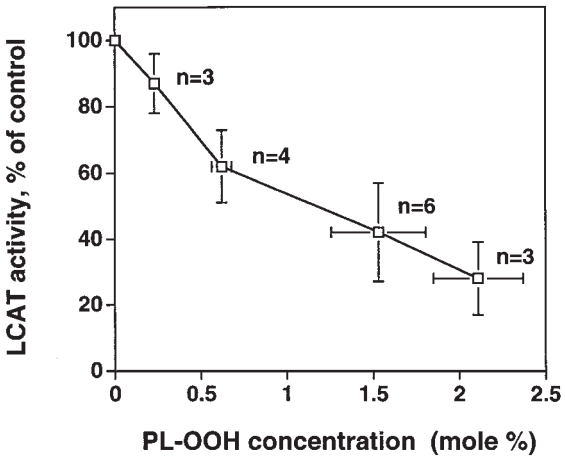

Fig. 5. Dependence of LCAT inhibition on the concentration of phospholipid hydroperoxides (PL-OOH) added to human plasma. PL-OOH were prepared enzymatically with soybean lipoxygenase (3 U/μl) and 1-palmitoyl, 2-linoleoyl phosphatidylcholine as described in Methods. Human plasma was supplemented with increasing concentrations of PL-OOH added in ethanol (0.5% final concentration). Plasma samples were incubated for 2 h, and LCAT activity was assessed using the exogenous substrate method. Results are expressed as a percentage of control activity in plasma incubated with unoxidized PL (no lipoxygenase). The LCAT activity of this control was 8.9 ± 1.6% esterfication of [^{14}C]cholesterol/0.5 h and it did not differ from fresh human plasma at t = 0 prior to the addition of phospholipid.

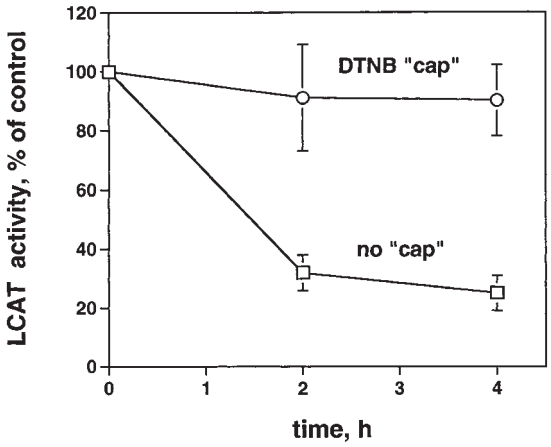

Fig. 6. DTNB "capping" protects LCAT from PL-OOH. Human plasma was treated with DTNB as described in Methods. PL-OOH (2.0 mole %) were added to control and DTNB-treated plasma and incubated (37°C) as described in Fig. 5. At the times indicated, samples of plasma were taken for measurements of plasma LCAT activity utilizing the exogenous substrate method. Results are expressed as percentages of control activity in dialyzed plasma at t = 0 prior to the addition of PL-OOH. Values are means ± SD, n = 3.

ity by modifying residues in or around the catalytic center. As indicated in Fig. 1, DTNB "capping" of the enzyme's free cysteine residues completely protected LCAT from minimally oxidized LDL. To determine whether PL-OOH behave in a similar fashion with regards to the underlying mechanism of LCAT impairment, we asked whether DTNB "capping" of LCAT's active site protected the enzyme from relatively high concentrations of PL-OOH. **Figure 6** shows that when LCAT's free cysteines were "capped" with DTNB during exposures to PL-OOH (2 mole %), enzymatic activity was fully retained. These observations indicate that PL-OOH, in a manner analogous to minimally oxidized LDL, inhibited LCAT activity by modyfing the enzyme's free cysteine and/or catalytic residues.

DISCUSSION

A number of previously published findings indirectly support our hypothesis that PL-OOH were the principal molecular species involved in the inactivation of LCAT activity induced by minimally oxidized LDL (8). First, LCAT activity was found to be rapidly inhibited during the the lag-phase of LDL oxidation. This is the time when lipid hydroperoxides accumulate. Second, polar lipids isolated from minimally oxidized LDL inhibited plasma LCAT activity thus ruling out the possibility that water-soluble, aldehydic decomposition end-products of LDL lipid peroxidation were involved. It is well known that LCAT utilizes phospholipid and cholesterol derived from LDL as substrates for the cholesterol esterification reaction, and recently published studies suggest that lipid peroxides can transfer from minimally oxidized LDL to HDL (19–21). In our previous study (8), we found that the LCAT inhibitory compound(s) was transferable and could associate with HDL. These experimental findings occurring in a plasma background, which is expected to have a high capacity to buffer oxidation events, suggested a specificity regarding the underlying mechanism of LCAT inactivation. We therefore hypothesized that this specificity was conferred via a targeting of the LCAT enzyme via a peroxidized phospholipid substrate.

In the present study, we now provide direct evidence that PL-OOH inhibit LCAT activity. Treatment of LDL with soybean lipoxygenase, while resulting in only modest increases in total L-OOH (20 nmol L-OOH/mg LDL protein), produced a dramatic inhibition (65%) of plasma LCAT activity. Although the lipoxygenase enzyme can utilize either FFA or phospholipids as substrates for L-OOH formation, the phospholipid on the surface of LDL particles is probably the most abundant substrate for the lipoxygenase enzyme. Thus, PL-OOH were most likely responsible for the inactivation of LCAT activity by lipoxygenase-treated LDL. To establish that PL-OOH do, indeed, inhibit LCAT activity, PL-OOH were added directly to plasma and LCAT activity was quantified. We found that as little as 0.23 ± 0.01 mole % additions of PL-OOH to plasma (i.e., 1 molecule of PL-OOH/500 molecules total phospholipid) produced significant reductions (20%) in LCAT activity. Recently, subfractions of small, dense LDL particles with enhanced electronegativity have been isolated from human plasma (12–14). These particles represented approximately 10–30% of the total plasma LDL pool and were found to be enriched in L-OOH (40–50 nmol/mg LDL protein). Our findings that lipoxygenase-

treated LDL, as well as PL-OOH, have such a profound inhibitory effect on LCAT activity suggest that even if a small percentage of plasma LDL is oxidized, the concentrations of L-OOH can reach levels found in this study to inhibit LCAT activity.

Bowry, Stanley, and Stocker (34) recently found, however, that HDL, rather than LDL, was the major carrier of L-OOH in human plasma, but the principal molecular species of oxidized lipids was found to be cholesteryl ester hydroperoxides (CE-OOH). The mechanism by which CE-OOH accumulate in HDL is unknown, but it has been suggested that cholesteryl ester transfer protein (CETP) may be involved (35). Our observation that PL-OOH have such a profound inhibitory effect on LCAT activity suggests that it is unlikely that PL-OOH are used as substrates for cholesteryl ester formation, thus ruling out the possibility that CE-OOH arise from the oxidized phospholipid pool. Indeed, it has been shown that the reduced forms of PL-OOH, namely PL-OH, are esterified by LCAT, but PL-OOH themselves are poor substrates for LCAT (26). As discussed by Bowry et al. (34), the antioxidant content of HDL is low compared to LDL suggesting, that HDL may be more susceptible to oxidation in vivo. Our findings that PL-OOH inhibit LCAT activity even in the presence of a plasma background support the notion that HDL and HDL components are highly susceptible to oxidative damage.

In an attempt to rationalize the extreme susceptiblity that LCAT exhibits towards inactivation in a plasma background, we hypothesized that PL-OOH may target amino acid residue in, or near, the enzyme's active site to produce an inhibition of enzymatic activity. It is well known that DTNB forms adducts with LCAT's free cysteine residues thereby blocking the active site via steric hindrance. Utilizing DTNB to block the enzyme's free cysteine residues, we found that LCAT was completely protected from both minimally oxidized LDL (<7.0 TBARS) and relatively high concentrations of PL-OOH. These observations suggest that PL-OOH inhibited plasma LCAT activity by modifying either the enzyme's free cysteines or catalytic residues. Moreover, minimally oxidized LDL behaved similarly suggesting that PL-OOH were likely to be the principal molecular species involved in the inhibition of LCAT activity. At this time, however, we cannot exclude the possibility that several LCAT inhibitory compounds are generated as a result of LDL oxidation. As shown in Fig. 2, as the levels of LDL oxidation are increased (>7 TBARS), DTNB "capping" of the enzymes free cysteine residues no longer affords LCAT protection, suggesting that as the levels of LDL oxidation are increased additional LCAT inhibitors are generated which have the potential of inhibiting LCAT activity by mechanisms unrelated to active site modifications.

To account for LCAT's exquisite susceptibility to inactivation upon exposure to minimally oxidized LDL (<7 TBARS), we propose that the PL-OOHs delivered to HDL target the enzyme's active site and/or substrate binding domain. Once bound to the enzyme, it is likely that PL-OOHs break down, liberating low molecular weight carbon-centered radicals and/or aldehydes which have the potential to covalently modify residues at the active site. Adducts forming between reactive aldehydic breakdown products and LCAT's free cysteine residues (Cys31 and Cys184) could conceivably inhibit LCAT activity by sterically blocking the active site of the enzyme. Aldehydic functional groups are extremely reactive toward thiols, forming stable thiol–ether linkages. We have previously found that unsaturated aldehydes, such as acrolein and 4-hydroxynonenal, inhibit plasma LCAT activity by modifying the enzymes free cysteine residues (36). Such modification required relatively high concentrations (0.16 mm) of aldehydes added to plasma. Aldehydes are short lived and react nonspecifically with plasma proteins, thus explaining why such high concentrations are needed to inhibit LCAT activity. Our model predicts that these same breakdown products may very well be generated as peroxidized phospholipids break down within the substrate binding domain of the LCAT enzyme, and that modification of the enzyme's free cysteine residues renders LCAT catalytically inactive. Experiments are now in progress to determine the nature of the specific amino acid adduct directly involved in LCAT inactivation.

The results presented in this study clearly demonstrate that small quantities of PL-OOH added to plasma can inhibit LCAT activity; however, plasma is not the only compartment wherein HDL and LCAT may be exposed to PL-OOH. Phospholipid hydroperoxides may also accumulate in the subendothelial space of the artery wall (37). During the initial stages of atherogenesis, LDL is thought to be retained in the subendothelial spaces (38). Respiratory burst released from inflammatory cells in developing atherosclerotic lesions promote LDL lipid peroxidation and the formation of PL-OOH (39). The observation that these early lipid peroxidation products can directly inhibit LCAT activity suggest that during the initial stages of atherogenesis, HDL-cholesterol transport may be impaired. Cholesterol esterification catalyzed by LCAT has been shown to facilitate the net efflux of cellular unesterified cholesterol to HDL (6). Impairment of LCAT activity could facilitate the atherogenic process by limiting the cholesterol efflux abilities of HDL thus allowing cholesterol to accumulate in macrophage foam cells.

The inhibition of LCAT activity by small quantities of PL-OOH generated in the artery wall or in plasma may also predispose to an HDL deficiency state. The esterification of cholesterol on HDL results in the accumulation of cholesteryl esters in the core of HDL particles. As a result, the core of HDL expands causing particle growth and maturation. Although speculative, the inhibition of LCAT activity by PL-OOH may limit HDL particle expansion, favoring the accumulation of small dense HDL which are rapidly catabolized. Accelerated clearance of HDL from the circulation, if occurring over time, could lead to reductions in HDL-cholesterol concentrations. Thus, the inhibition of plasma LCAT activity by PL-OOH not only interferes with the cholesterol transport functions of HDL but may also predispose to an HDL deficiency state thereby increasing the risk of atherosclerosis.

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