Articles

A Novel Lecithin-Cholesterol Acyltransferase Antioxidant Activity Prevents the Formation of Oxidized Lipids during Lipoprotein Oxidation†

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ABSTRACT: Recent investigations suggest that high-density lipoprotein (HDL) may play an anti-atherogenic role as an antioxidant and inhibit the oxidative modification of low-density lipoprotein (LDL). The antioxidant activity of HDL has been proposed to be associated with several HDL-bound proteins. We have purified one HDL-associated protein, lecithin:cholesterol acyltransferase (LCAT), to apparent homogeneity and have found that LCAT is not only capable of esterifying cholesterol in the plasma, but can also prevent the accumulation of oxidized lipids in LDL. Addition of pure human LCAT to LDL or palmitoyl-linoleoyl phosphatidylcholine/sodium cholate (PLPC) micelles inhibits the oxidation-dependent accumulation of both conjugated dienes and lipid hydroperoxides. LCAT also inhibits the increase of net negative charge that occurs during oxidation of LDL. LCAT has the ability to prevent spontaneous oxidation and Cu^{2+} and soybean lipoxygenase-catalyzed oxidation of lipids. The antioxidant activity of LCAT appears to be enzymatic, since the enzyme is active for up to 10 h in the presence of mild free-radical generators. The catalytic serine, residue 181, may mediate this activity and act as a reusable proton donor. Chemical modification of the active serine residue with diisopropylfluorophosphate completely inhibits the ability of LCAT to prevent lipid oxidation. Thus, in addition to its well-characterized phospholipase and acyltransferase activities, LCAT can also act as an antioxidant and prevent the accumulation of oxidized lipid in plasma lipoproteins.

Epidemiological studies have demonstrated an inverse relationship between plasma high-density lipoprotein (HDL)[1] cholesterol levels and coronary artery disease (CAD) (*1*−*3*). HDL may protect against CAD by its ability to promote the clearance of plasma cholesterol (*4*−*6*) and by preventing oxidative modifications of low-density lipoprotein (LDL) in the artery wall (*7*−*9*). A number of HDL-bound enzymes such as paraoxonase (*8*, *9*), platelet activating factor-acetylhydrolase (PAF-AH) (*10*), and lecithin-cholesterol acyltransferase (LCAT) (*7*) may confer antioxidant properties on HDL. It has also recently been reported that apolipoprotein (apo) AI and AII, the two major proteins of HDL, play a role in the detoxification of potentially atherogenic lipid hydroperoxides (L-OOH) (*11*, *12*).

LCAT is a 67 kDa plasma enzyme that catalyzes the transfer and esterification of *sn*-2 fatty acids from phosphatidylcholine to the 3-hydroxyl group of cholesterol and is then responsible for the synthesis of most of the cholesteryl esters (CE) in human plasma (review in ref *13*). LCAT is also able to hydrolyze water-soluble esters (*14*) and platelet-activating factor (*15*) as well as oxidized polar phosphatidylcholine (PC) generated during lipoprotein oxidation (*16*). LCAT may play an important role in preventing the oxidation of LDL (*7*). Klimov et al. (*7*) showed that the addition of partially purified (∼650-fold) LCAT to LDL, during incubations with Fe^{2+}, completely inhibited both the formation of malonic dialdehyde and LDL aggregates. Since this inhibition may have been due to some antioxidant contaminant in the partially purified LCAT preparation (e.g., paraoxonase), we have performed experiments with highly purified (>15 000-fold) LCAT and determined whether this enzyme indeed possesses an antioxidant activity and can prevent the oxidative modification of LDL. We show that LCAT can prevent the oxidation of both phospholipids and CE via a cofactor

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[1] Abbreviations: AAPH, 2.2′-azo-bis-(2-amidinopropane) dihydrochloride; ABSF, aminoethyl benzenesulfonyl fluoride; apoA-I, apolipoprotein A-I; CAD, coronary artery disease; CE, cholesteryl ester; CE-OOH, cholesteryl ester-hydroperoxides; DFP, diisopropylfluorophosphate; FC, free cholesterol; HDL, high-density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; LDL, low-density lipoproteins; L-OOH, lipid hydroperoxides; PAF-AH, platelet-activating factor acetylhydrolase; PC, phosphatidylcholine; PLPC, palmitoyl-linoleoyl phosphatidylcholine; PLPC-OOH, PLPC-hydroperoxides; PMSF, phenylmethylsulfonyl fluoride; POPC, palmitoyl-oleoyl phosphatidylcholine.

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and metal ion independent mechanism that may utilize the active serine residue in the enzyme.

EXPERIMENTAL PROCEDURES

Materials

Cholesterol linoleate, phenylmethanesulfonyl fluoride (PMSF), diisopropylfluorophosphate (DFP), choline chloride, soybean lipoxygenase (EC.1.13.11.12), and aminoethyl benzenesulfonyl fluoride (ABSF) were purchased from Sigma Chemical Co. (St. Louis, MO). 1-Palmitoyl 2-oleoyl phosphatidylcholine (POPC) and 1-palmitoyl 2-linoleoyl phosphatidylcholine (PLPC) were obtained from Avanti Polar Lipids (Birmingham, AL). Acetonitrile, 2-propanol, chloroform, hexane, methanol, and H_2O were HPLC grade. All aqueous solutions were prepared using the highest grade chemicals and double distilled, deionized water.

Methods

Preparation and Oxidation of Lipoproteins and PLPC Micelles. LDLs (density 1.019−1.063 g/mL) were isolated from fresh plasma of normolipidemic donors by sequential ultracentrifugation. LDLs were used immediately after dialysis against an EDTA-free phosphate buffered saline (PBS, 50 mM phosphate buffer, and 150 mM NaCl, pH 7.2). Optically clear PLPC micelles were prepared freshly for each experiment by incubation of PLPC and sodium cholate (PLPC:cholate = 0.74, mol:mol) at 37 °C for 0.5 h, punctuated by vortexing every 10 min. Oxidation of LDL or PLPC micelles, in the presence and absence of LCAT, was carried by incubation at 37 °C with 5−20 μM $CuSO_4$ or soybean lipoxygenase in EDTA-free PBS (*8*, *17*).

Measurement of Oxidation. L-OOH formation in the lipoprotein samples was determined over 0−8 h incubations by the method of El-Saadani et al. (*18*), as modified by Gebicki et al. (*19*). After reacting the lipoprotein with a potassium iodide (KI) reagent, the absorbance of I_3^- produced from the reaction of L-OOH and KI was measured at 365 nm, and the concentration of L-OOH was calculated using a molar extinction coefficient of 2.46×10^4 M^{-1} cm^{-1} (*8*). Conjugated diene production was measured by monitoring the increase in absorbance at 234 nm. Surface potential was calculated from the electrophoretic mobility on precast 0.5% agarose gels (Beckman, Paragon Lipo Kit) according to the previously described methods (*20*).

HPLC Detection of L-OOH in LDL and PLPC Micelles. The formation of PLPC-OOH under various oxidative conditions was assessed using the HPLC protocol of Bao and Williamson (*17*) and a Supelco TSKgel ODS-120T (25 cm × 4.6 mm) column. PLPC micelles were oxidized with 5−20 μM $CuSO_4$, in the presence or absence of LCAT, and were extracted with twice the volume of chloroform-methanol (2:1). After vortexing, the mixtures were centrifuged for 10 min at 3000 rpm. The lower phase was dried under N_2 and then dissolved in methanol and analyzed immediately**.** Aliquots of these mixtures were chromatographed under the conditions recommended by Bao and Williamson (*17*) using a mobile phase of acetonitrile-methanol-H_2O 60/39.5/0.5 (v/v/v) containing 10 mM choline chloride. Each sample was analyzed simultaneously for unmodified PLPC and PLPC-OOH using wavelengths of 205 and 234 nm, respectively. CE hydroperoxides (CE-OOH) were measured in Cu^{2+} oxidized LDL using the method of Sattler and collaborators (*21*) and the reversed-phase column described above. Briefly, oxidized LDLs were extracted with 2 mL of methanol and 10 mL of hexane. The hexane phase was dried down and dissolved in the mobile phase acetonitrile/2-propanol/H_2O (22/27/1, v/v/v). The lipids were chromatographed at flow rate of 1 mL/min and CE-OOH chromatograms were obtained at 234 nm.

Purification and Characterization of LCAT. LCAT was purified to homogeneity (greater than 15000-fold and shown to exhibit one band on a silver stained SDS PAGE gel) from normolipidemic human plasma as previously described (*22*). After ultracentrifugation of fresh human plasma at 1.21 g/mL, the $d < 1.21$ lipoproteins were removed and the upper 50% of the $d > 1$ .21 infranate (albumin poor) was chromatographed on a phenyl-Sepharose CL-4B column and subsequently on DE52 cellulose and hydroxylapatite columns. LCAT specific activity was 20.7 nmol of cholesterol esterified/μg of protein/h and was determined using a stock substrate [POPC:free cholesterol (FC):apoA-I ratio of 160: 20:2, mol/mol/mol] (*23*). A unit of enzyme is defined as 1 nmol of cholesterol esterified/h. The effect of LCAT on the oxidation of LDL was monitored after a 3 h incubation with the addition of 0−50 units of LCAT. In some experiments, purified LCAT was inhibited with 1.0−5.0 mM PMSF or 0.1−0.5 mM DFP (*24*, *25*). In some studies, LDL was preincubated with 1 mM PMSF or 0.1 mM aminoethyl benzenesulfonyl fluoride (ABSF) to inactivate any surface bound phospholipase (PAF-AH) activity (*26*). Paraoxonase activity was measured spectroscopically (A_{412nm}) by monitoring the conversion of paraoxon to *p*-nitrophenol (*27*). The purified LCAT preparations contained no detectable paraoxonase activity.

RESULTS

Characterization of the Effect of Purified LCAT on the Formation/Accumulation of L-OOH in LDL. Co-incubation of pure LCAT and LDL shows that this enzyme is able to prevent the formation of L-OOH in LDL during oxidation with Cu^{2+} or soybean lipoxygenase. Figure 1 shows a dose-dependent relationship between LCAT concentration and the inhibition of L-OOH formation. Preincubation of LCAT with a serine esterase inhibitor, DFP, completely inhibits the phospholipase activity of LCAT and also inhibits its ability to prevent L-OOH accumulation in LDL (see inset, Figure 1). The same results were obtained when LCAT was preincubated with PMSF, another serine esterase inhibitor (data not shown). LCAT prevents the loss of color and aggregation of LDL during oxidation with Cu^{2+} or lipoxygenase (data not shown). Table 1 shows that LCAT prevent changes in LDL surface charge induced by oxidation for 2−4 h and reduces the increase in net negative charge on oxidized LDL between 4 and 6 h. As shown in Figure 2A, the addition of LCAT to a mixture of LDL and Cu^{2+} prevents L-OOH formation in LDL for up to 7 h (Figure 2A). To determine if LCAT could specifically prevent oxidation of CE in LDL, we also measured the formation of CE-OOH in LDL by HPLC. Consistent with the iodometric assay (Figure 2A), the formation of CE-OOH during a 3 h oxidation of LDL with Cu^{2+} was completely inhibited by the addition of LCAT

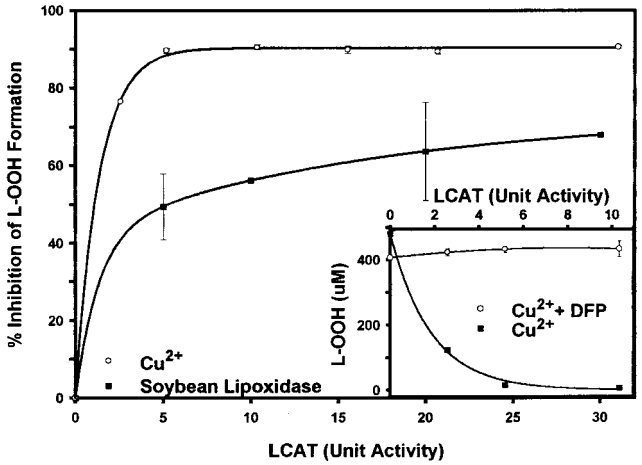

FIGURE 1: Effect of LCAT on the oxidation of LDL. LDL (density 1.019−1.063 g/mL) was isolated from fresh plasma of normolipidemic donors by sequential ultracentrifugation. LDL (final concentration 0.75 μg of protein/μL) was incubated at 37 °C with 2.5 μM Cu^{2+} or 0.02 mg/mL soybean lipoxygenase and 0−35 units of purified LCAT (1 unit = 1 nmol of cholesterol esterified/h) for 3 h. The concentration of L-OOH in the mixture was determined by the KI spectroscopic method (*19*) and expressed as the percent inhibition of L-OOH formation. Values are the mean ± SD of triplicate determinations and are representative of three different experiments. (Inset) LCAT was preincubated with 0.5 mM DFP and then incubated with LDL and 2.5 μM Cu^{2+} as described above. The concentration of L-OOH was determined by the KI spectroscopic method.

Table 1: Effect of LCAT and Oxidation on the Surface Charge of LDL

	LDL surface potential[a]			
time (h)	control LDL	Ox-LDL	Ox-LDL + LCAT	Ox-LDL + LCAT + PMSF
2	−4.4	−5.1	−4.4	−5.3
4	−4.9	−7.1	−5.6	−7.1
6	−4.6	−9.3	−8.3	−9.5

[a] LDL was isolated from a normolipidemic donor and was incubated at 37 °C with 5.0 μM Cu^{2+} for 3 h in the presence or absence of 10 units of LCAT and 0.1 mM PMSF. Aliquots underwent electrophoresis on 0.5% agarose gels and LDL surface potentials were determined by electrokinetic analysis (*20*). Data are from one experiment (±0.2 mV, SD) and are representative of two different experiments.

(Figure 2B). Chromatograms in Figure 2B also show no evidence of fatty acid hydroperoxides, in incubations with LCAT.

Characterization of the Effect of Purified LCAT on the Formation/Accumulation of PLPC−OOH. To eliminate any potential influence of LDL constituents on the antioxidative activity of LCAT and to characterize the ability of LCAT to specifically prevent oxidation of phospholipids, we produced a reconstituted phospholipid micellar substrate (PLPC micelles) that could be readily oxidized. The effect of LCAT on the formation of PLPC-OOH in PLPC:cholate micelles is shown in Figure 3. The micelle preparation was oxidized with Cu^{2+} and the formation of PLPC-OOH was followed over time. Inclusion of LCAT in the Cu^{2+}/PLPC mixture significantly inhibited the formation of PLPC-OOH for up to 7 h. To determine whether LCAT prevents the formation of other oxidized lipid aducts, the effect of LCAT on conjugated diene formation was also measured (Figure 4). PLPC micelles were oxidized spontaneously (in deionized solutions) by incubation at 37 °C or with Cu^{2+} and the

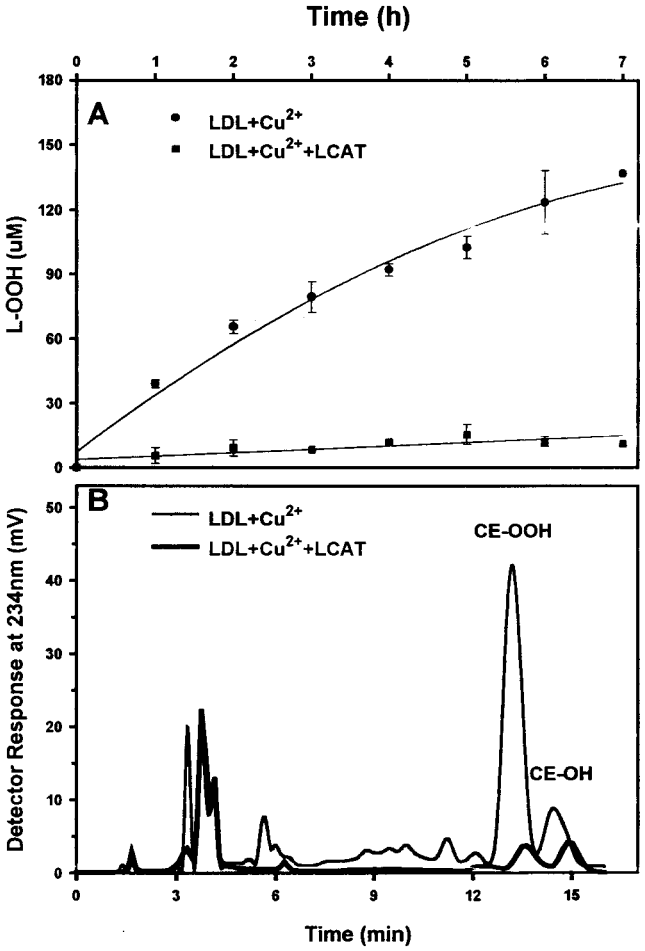

FIGURE 2: The effect of LCAT on the accumulation of L-OOH in LDL. LDL (0.75 μg of protein/μL) was incubated at 37 °C with 2.5 (panel A) or 10 (panel B) μM Cu^{2+} in the presence and absence of 10 units of LCAT for various times. After the incubations, L-OOH formation was measured using two different methods. (A) The concentration of L-OOH in the mixture was determined by the KI spectroscopic method. Values are the mean ± SD of triplicate determinations and are representative of three different experiments. (B) Oxidized LDL was prepared as above. After a 3 h incubation, oxidized LDL was extracted and submitted to HPLC analysis as described in the Experimental Procedures. The chromatograms show the resolution of CE-OOH formed in LDL. Data are from one experiment and are representative of three different experiments.

formation of conjugated dienes was followed over time. Figure 4 shows that LCAT has the ability to prevent the formation of conjugated dienes in PLPC during spontaneous oxidation or Cu^{2+}-catalyzed oxidation. Experiments with Cu^{2+} and LDL show that LCAT can also prevent conjugated diene formation in LDL (data not shown). HPLC analysis of extracted lipids shows that LCAT can specifically prevent the formation of PLPC-OOH in the micelles after an oxidative challenge. As shown in Figure 5, LCAT can completely inhibit the formation of PLPC-OOH during oxidation with Cu^{2+} for up to 9 h. The inset of Figure 5 shows that LCAT also inhibits the spontaneous oxidation of PLPC micelles and prevents the formation of PLPC-OOH during an incubation of 10−24 h (identical chromatograms, not shown) at 37 °C.

DISCUSSION

Several years ago, Klimov et al. (*7*) showed that partially pure human LCAT (650-fold) was able to completely inhibit

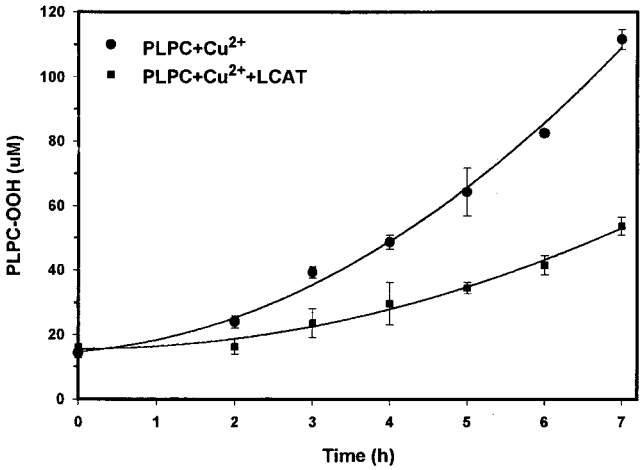

FIGURE 3: Effect of LCAT on the oxidation of PLPC/cholate micelles. PLPC/cholate micelles (4.3 mg of PLPC) were prepared as described in the text (PLPC/cholate = 0.74) and then incubated at 37 °C with 10 μM Cu^{2+} and 10 units of purified LCAT. The concentration of L-OOH in the mixture was determined over time by the KI spectroscopic method. Values are the mean ±SD of triplicate determinations and are representative of two different experiments.

LDL oxidation and LDL aggregate formation during incubations with Fe^{2+}. Various studies over the past few years, however, have raised the question of whether this activity was specifically due to LCAT or due to some other contaminant in the partially purified preparation (*8*, *10*). In the present study, we show that highly purified LCAT (>15000-fold) can prevent L-OOH formation in LDL and PLPC micelles under a variety of oxidative conditions (Cu^{2+}, soybean lipoxygenase and aerobic). This activity of LCAT could not be due to a contaminant oxidoreductase since the activity is unaffected by the classic inhibitors sodium azide and aminotriazole. While we observe antioxidative activity with pure PLPC micellar substrate, the ability of LCAT to prevent lipid oxidation appears much greater with LDL. This suggests that other endogenous lipophilic or enzymatic antioxidants in the lipoprotein may also be active. However, since LCAT is active with the PLPC substrate, this indicates that no endogenous constituents of LDL are critical to LCAT's ability to inhibit oxidation. This is consistent with data from an experiment where any endogenous lipase activity in LDL was inhibited by incubation with 0.1 mM ABSF or 1 mM PMSF (results not shown). While chemical modification of LCAT with PMSF inhibits its antioxidant activity, chemical modification of any endogenous LDL proteins had no effect on the ability of LCAT to inhibit oxidized lipid formation in LDL. This indicates that LCAT acts independently of any other LDL-associated enzyme activity in preventing the formation of oxidized lipids.

LCAT can also completely prevent the formation of conjugated dienes in LDL and in PLPC micelles. While this appears contrary to the reported inability of HDL to prevent diene formation in LDL (*8*), it should be noted that when we tested the ability of LCAT to inhibit conjugated diene formation using the conditions described by Mackness et al. (*8*, *9*), LCAT also could not inhibit diene formation. However, when supraoxygenated buffer was omitted from the reaction mixture, LCAT could completely inhibit diene formation (Figure 4). This suggests that the oxygenated buffer may modify LCAT and neutralize its ability to inhibit oxidation. The data are consistent with what we observed in the present study with other potent oxidation initiators; AAPH (data not shown) and soybean lipoxygenase. Incubations of LCAT and high concentrations of either initiator completely inactivate the antioxidant ability of LCAT. LCAT is, however, active as an antioxidant at low concentrations of these compounds, but appears to be the most efficient in incubations with Cu^{2+} (see Figure 1). The data suggest that excessive free-radical bombardment of LCAT may modify the protein and neutralize its antioxidant activity.

To explore the involvement of the lipolytic capability of LCAT in its antioxidant function, the enzyme was treated with well-characterized phospholipase A_2 inhibitors and then characterized. Preincubation of LCAT with the serine modifying agents, PMSF or DFP, completely inactivates the phospholipase activity of LCAT and also destroys its ability to prevent oxidized lipid formation in LDL. These results appear in agreement with the observation of Klimov et al. (*7*) that heat inactivation of LCAT also abolished the antioxidant activity of the enzyme. Their observations prompted Klimov to suggest that LCAT may utilize its cholesterol esterifying activity to repair oxidized lipids in an LDL particle. However, more recent studies have shown that the ability of LCAT to transfer a fatty acid from PC to cholesterol is completely inhibited under oxidative conditions (*28*−*30*). LCAT may retain its phospholipase activity in the presence of oxidants, as Goyal et al. (*16*) have recently shown that LCAT is capable of hydrolyzing oxidized polar PC in lipoprotein particles. However, in our experiments, LCAT was not directly hydrolyzing oxidized lipids, since when we determined the extent of fatty acid liberation we observed no increase in free fatty acids after a 3 h incubation. Similarly, HPLC analyses of lipid extracts from LDL or PLPC micelles failed to show any evidence of newly liberated fatty acids (at 205 nm) or fatty acid hydroperoxides (at 234 nm). It is possible that LCAT may be acting as a hydroperoxidase to directly repair oxidized lipids, but this also appears unlikely as we have observed no evidence of the formation of lipid-OH in co-incubation experiments (Figure 2B) or after incubations of LCAT with preoxidized LDL or PLPC micelles (Vohl, M. C., and Sparks, D. L., unpublished data). These results suggest that LCAT does not directly eliminate oxidized lipids by repairing or hydrolyzing them, but instead probably acts early in the oxidative cascade by scavenging free radicals.

Our data suggests that LCAT may prevent lipid oxidation much like other chain-breaking antioxidants. Treatment of LCAT with serine-modifying reagents suggests that it is not the sulfhydryl-containing residues that are functioning to neutralize free radicals, but instead it is probably the -OH-containing serine residues. The mechanism by which chain breaking antioxidants such as ascorbic acid function also involves the deprotonation of −OH groups through two one-electron transfer steps, which produce a dehydroascorbate (*31*). While these molecules are an effective first line of oxidative defense, they are short lived and rapidly consumed. In contrast, LCAT is not rapidly inactivated and under some circumstances; its antioxidant activity can be very long-lived (Figures 2−5). This suggests that the antioxidant activity of LCAT is enzymatic. While it is possible that LCAT may utilize other -OH-containing residues as proton donors, our chemical inactivation studies suggest that the active serine

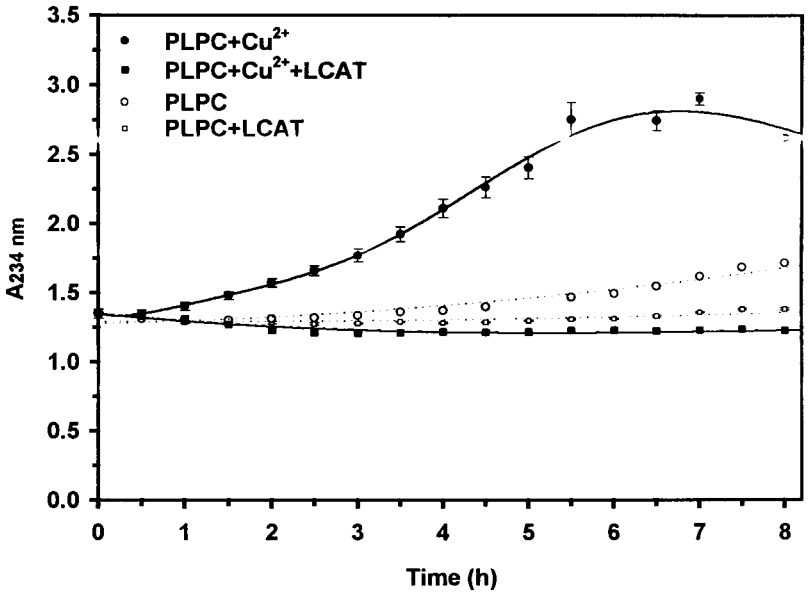

FIGURE 4: Effect of LCAT on conjugated diene formation in PLPC micelles. PLPC/cholate micelles (4.3 mg of PLPC) were prepared as described in the text (PLPC/cholate = 0.74) and then incubated at 37 °C with/without 5 μM Cu^{2+} and with/without 10 units of purified LCAT for various times and the concentration of conjugated dienes was determined as an increase in optical absorbance at 234 nm. Values are the mean ± SD of triplicate determinations and are representative of 2 different experiments.

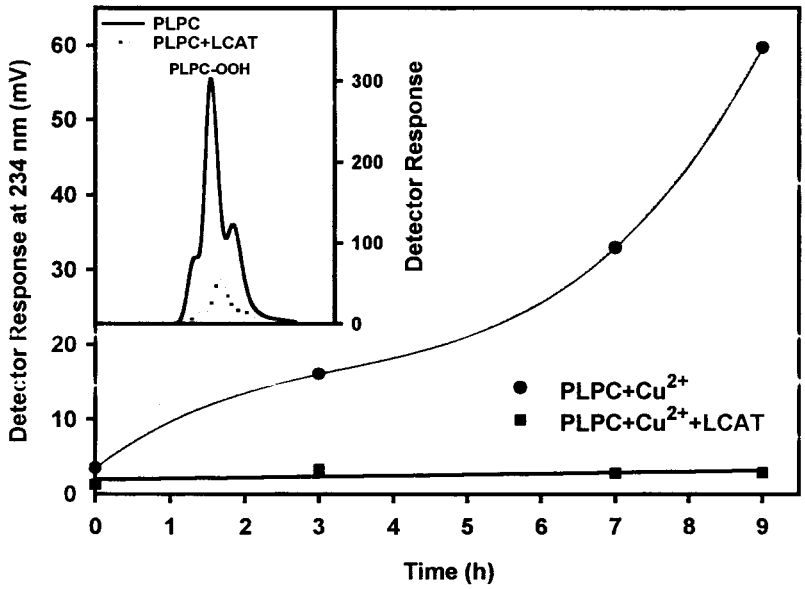

FIGURE 5: Effect of LCAT on the formation of lipid hydroperoxides in PLPC/cholate micelles. PLPC/cholate micelles (4.3 mg of PLPC) were prepared as described in the text (PLPC/cholate = 0.74) and then incubated at 37 °C with 20 μM Cu^{2+} and with/without 10 units of purified LCAT for various times. After incubation, oxidized PLPC was extracted and submitted to HPLC analysis as described in the Experimental Procedures. The detector response at 234 nm (mV) is plotted as a function of the duration of the oxidation. Data are from a single experiment and are representative of 3. (Inset) PLPC/cholate micelles (8.6 mg of PLPC) were prepared and incubated as above, but in the absence of Cu^{2+}. The chromatograms show the resolution of PLPC-OOH (PLPC-OOH, retention time ≈ 22.5 min) formed in PLPC micelles ± LCAT after a 10 h incubation. The two unresolved peaks represent subspecies of PLPC-OOH (PLPC-OH retention time ≈ 24.5 min). The data are the results of a single experiment and are representative of three different experiments.

is involved. Inhibition studies with proteases (*32*) and with LCAT (*24*, *25*) show that DFP modifies the active serine in these enzymes. DFP has been shown to primarily modify the serine residue 181 in LCAT (*24*, *25*) and to a lesser extent, serine 216 (*25*). The data suggest that LCAT may require a serine for its antioxidant activity and that the enzyme may have the ability to regenerate its active serine residue via proton transfer. It therefore appears that the redox mechanism that LCAT utilizes may be distinct and not sacrificial to the enzyme. This may explain why LCAT can remain active for up to 10 h (Figure 5) in the presence of relatively mild oxidation initiators. This mechanism may be related to the classic proton relay mechanism described for proteases (*32*, *33*) and lipases (*34*, *35*), wherein active serine residues participate with proximal histidine and aspartic acid residues in a proton relay prior to the nucleophilic attack of the serine oxygen. The active serine of LCAT may act as a reusable proton donor to neutralize free radicals or lipoxygenase-generated carbon-centered radicals. LCAT may restore the hydrogen atom on the serine -OH group by direct extraction from medium or from the imidazole group of a proximal histidine. This integrated antioxidant/esterase reac-

tion may partially explain why injection of DFP into rats increases lipid peroxidation in vivo (*36*). In this study, treatment of rats with DFP caused a dose-dependent increase in thiobarbituric acid−malondialdehyde complex and F2-isoprostanes, a response that could be potentiated by buthionine sulfoximine and inhibited by an antioxidant (U-78517f).

In summary, LCAT appears to be able to utilize its catalytically active serine residue in two distinct but chemically related functions (1) for a nucleophilic attack as a phospholipase and (2) as a proton donating free radical scavenger. This latter function confers on LCAT a potent enzymatic antioxidant activity that can effectively prevent lipid oxidation in lipoprotein particles. While the physiologic importance of this novel activity remains unclear, clinical studies suggest that a deficiency of this enzyme can result in oxidative tissue damage (*37*). Patients with complete LCAT deficiency have premature CAD and severe renal disease that is associated with the accumulation of oxidized lipid-enriched foam cells in glomerular tufts (*37*, *38*). LCAT may therefore play an important role in maintaining a normal metabolic redox equilibrium.

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