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Metabolism of Oxidized Phosphatidylcholines Formed in Oxidized Low Density Lipoprotein by Lecithin-Cholesterol Acyltransferase[1]

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The possible involvement of lecithin-cholesterol acyltransferase (LCAT) in the metabolism of oxidized phosphatidylcholine (PC) in plasma was investigated. A variety of oxidized products are formed from PC following oxidation of low density lipoproteins (LDL). A significant increase in LDL oxidation levels in patients with familial LCAT deficiency (FLD) has been previously demonstrated by a sensitive sandwich ELISA for oxidized LDL using the monoclonal antibody DLH3 which recognizes oxidized products of PC. In the present study, we found that LCAT produces various metabolites from oxidized PC and that oxidized PC molecules in LDL particles serve as substrates. When the neutral lipid fraction was separated by TLC after the incubation of oxidized 1-palmitoyl-2-[1-^{14}C]linoleoyl PC with human plasma, a number of radioactive bands were formed in addition to cholesteryl ester. These products were not formed from native 1-palmitoyl-2-[1-^{14}C]linoleoyl PC. Plasma from FLD patients also failed to form the additional products from oxidized PC. The addition of dithio-bis(nitrobenzoate) (DTNB), an LCAT inhibitor, or the inactivation of LCAT activity by treating the plasma at 56°C for 30 min abolished the generation of these products from oxidized PC. The activity was recovered in the high density lipoprotein (HDL) fraction but not in the LDL fraction separated from normal plasma. When 1-palmitoyl-2-[1-^{14}C](9-oxononanoyl) PC and 1-stearoyl-2-[1-^{14}C](5-oxovaleroyl)PC, PC oxidation products that contain short chain aldehydes, were incubated with human plasma, radioactive products in the neutral lipid fraction were observed on TLC. LDL containing oxidized PC was measured by sandwich ELISA using an anti-apolipoprotein B antibody and DLH3. The reconstituted oxidized PC-LDL particles were found to have lost their ability to bind DLH3 upon incubation with HDL, while the reactivity of the reconstituted oxidized PC-LDL remained unchanged in the presence of DTNB. These results suggest that LCAT is capable of metabolizing a variety of oxidized products of PC and preventing the accumulation of oxidized PC in circulating LDL particles.

Key words: familial LCAT deficiency, LCAT, monoclonal antibody, oxidized LDL, oxidized PC.

Lecithin-cholesterol acyltransferase (LCAT) is an enzyme that cleaves an acyl chain of phosphatidylcholine (PC) and subsequently transfers it to cholesterol to form cholesteryl ester (CE) (*1*). The physiological role of this enzyme is thought to be reverse cholesterol transport, in which high density lipoprotein (HDL) particles retrieve cholesterol molecules from peripheral tissues, stabilize them as esters, and finally transfer them back to the liver and adrenal gland (*2, 3*). The transfer of cholesterol between the lipoproteins and cellular membranes is performed by LCAT together with the CE transfer protein, both of which regulate the amount and subclasses of HDL particles. LCAT, which is synthesized and secreted from the liver, is found mainly on HDL particles where apolipoprotein A-I, an activator of LCAT, is present (*1*).

Familial LCAT deficiency (FLD) is a rare autosomal recessive disease in which patients show decreased plasma HDL levels and a reduced ratio of CE to free cholesterol. They are also prone to hemolytic anemia, cataracts, renal dysfunction, and early atherosclerosis (*4*). "Fish eye" disease is also the result of hereditary partial LCAT malfunc-

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Abbreviations: LCAT, lecithin-cholesterol acyltransferase; FLD, familial LCAT deficiency; PC, phosphatidylcholine; 9-CHO PC, 1-palmitoyl-2-(9-oxononanoyl)PC; 5-CHO PC, 1-stearoyl-2-(5-oxovaleroyl) PC; PCOOH, hydroperoxides prepared from PC; CE, cholesteryl ester; 2-ME, 2-mercaptoethanol; DG, diacylglycerol; DTNB, dithio-bis(nitrobenzoate); PAF-AH, platelet-activating factor acetylhydrolase; ELISA, enzyme-linked immunosorbent assay.

tion, although the symptoms are less severe than in FLD. Many mutations of the human LCAT gene have been cloned and have recently been classified into five types (*5*). Haemolytic anaemia and cataracts, two of the major symptoms of FLD are thought to be caused by an accumulation of cholesterol in erythrocytes and lens cells (*4*). Transgenic rabbits that overexpress LCAT show resistance to diet-induced atherosclerosis, suggesting a possible antiatherogenic role of LCAT (*6*). However, the mechanisms of renal failure and early atherosclerosis observed in FLD have not yet been clarified.

LCAT exhibits a fairly broad substrate specificity. Although LCAT prefers unsaturated fatty acyl chains on the *sn*-2 position of PC, saturated *sn*-1 acyl chains are also utilized (*7, 8*). It has been reported that very short acyl chains, such as the acetyl group at the *sn*-2 position of platelet-activating factor (PAF), are hydrolyzed and transferred to another lysoPC molecule by purified LCAT (*9*). The acyl acceptor is not restricted to cholesterol since various alcohols including oxysterols and diacylglycerol (DG) have also been shown to undergo transesterification by LCAT (*10, 11*). The enzyme transfers acyl groups to water molecules, demonstrating phospholipase A activity in the absence of particular acyl acceptors (*12*). However, the acyl moiety of acylCoA is not transferred to cholesterol (*13*). LCAT is also active in the absence of HDL, although the enzyme tends to exhibit phospholipase A and lysolecithin acyltransferase (LAT) activities under these conditions (*14*).

In our previous study a sensitive method for measuring oxidatively modified low density lipoprotein (LDL) in human plasma was developed using a monoclonal antibody that recognizes oxidized LDL (*15*). This antibody has been shown to recognize oxidized PC molecules including PC hydroperoxide (PCOOH) and 1-palmitoyl-2-(9-oxononanoyl)PC, (9-CHO PC), a PC containing an aldehyde group (*16*). We found a greater than threefold increase in the level of plasma-oxidized LDL in FLD patients as compared with normal subjects (*17*). Additionally, immunohistochemical analysis showed the presence of oxidized PC-related deposits in the glomeruli of FLD patients (*17*). Based on these observations, it seems possible that plasma levels of oxidized LDL are increased in FLD because of decreased metabolism. Recent reports have suggested that oxidatively modified acyl groups in oxidized PC are transferred to cholesterol or lysoPC by LCAT (*18, 19*). In the present study we have shown that a variety of oxidized PC products are metabolized by LCAT with oxidized PC molecules in LDL particles serving as substrates.

MATERIALS AND METHODS

Materials—1-Palmitoyl-2-linoleoyl PC, 1-palmitoyl-2-oleoyl PC, and 1-stearoyl-2-arachidonoyl PC were purchased from Avanti (Perham, AL, USA). 1-Palmitoyl-2-[1-^{14}C]linoleoyl PC, 1-palmitoyl-2[1-^{14}C]-oleoyl PC, 1-stearoyl-2-[1-^{14}C]arachidonoyl PC, and [4-^{14}C]cholesterol were purchased from New England Nuclear. The monoclonal antibody DLH3 (murine IgM) was established as described previously (*20*). The antibody used in this study was IgM fractions partially purified from murine ascites by ammonium sulfate precipitation followed by Toyopearl HW-65^{R} (Tosoh, Tokyo) gel filtration column chromatography. PCOOH prepared from egg PC by photooxidation was a kind gift from Mr. Yoshio Nakano of Nippon Oil and Fat. 1-Palmitoyl-2-[^{14}C]oleoyl DG and 1-palmitoyl-2-[1-^{14}C]-linoleoyl DG were prepared from corresponding PCs by treatment with phospholipase C (from *C. perfringens*, Sigma). Osmium tetroxide (2% solution) and sodium periodate were purchased from Wako Pure Chemical.

Preparation of LDL and HDL—Human LDL and HDL were prepared from human plasma by sequential centrifugation in the presence of 0.25 mM EDTA. After centrifugation, fractions with densities of 1.019-1.063 g/cm^3 and 1.063-1.21 g/cm^3, were recovered as LDL and HDL, respectively (*15, 21*). All of the LDL, HDL, and plasma samples contained EDTA except for oxidized LDL preparations. Prior to the oxidation of LDL, an aliquot of LDL was passed through a 10DG^{R} desalting column (Bio-Rad) to remove EDTA. LDL (0.2 mg/ml) in 10 mM phosphate-buffered saline, pH 7.4 (PBS), was incubated with 5 μM $CuSO_4$ at 37°C for 3 h (*15*).

Preparation of Oxidized PC—Oxidized PC was prepared as described previously (*16*). Briefly, after drying under argon gas, 1-palmitoyl-2-linoleoyl PC (2 mM) was suspended in PBS. Ascorbic acid and $FeSO_4$ were dissolved in distilled water just before use. Reaction mixtures containing 0.4 mM PC, 0.4 mM ascorbic acid, and 40 μM $FeSO_4$ in PBS were incubated at 37°C for 3 h under air with mild agitation. The reaction products were extracted with chloroform and methanol by the method of Bligh and Dyer (*22*). 9-CHO PC and 5-CHO PC were prepared from 1-palmitoyl-2-oleoyl PC and 1-stearoyl-2-arachidonoyl PC by reductive cleavage using osmium tetroxide based on the method of Kamido *et al.* with a slight modification (*23*). After PC (4 μmol) dissolved in diethylether was incubated with 0.6 μmol of osmium tetroxide at room temperature for 1 h, 25 μmol of sodium periodate was added and the mixture was further incubated for 4 h. 9-CHO PC and 5-CHO PC were purified by TLC followed by reversed-phase HPLC. They showed single spots on TLC, and were positively stained with Schiff's reagent, indicating they contain an aldehyde group.

Measurement of Oxidized PC by Mixing with LDL—Nanogram quantities of copper-induced oxidized LDL were measured using the monoclonal antibody DLH3 and an anti-apolipoprotein B antibody (*15*). Although native LDL is inactive in this sandwich ELISA, it becomes reactive in the presence of a small amount of oxidized PC, 9-CHO PC or egg PCOOH. Utilizing this property, it was possible to measure oxidized PC using the sandwich ELISA technique with a slight modification. Microtiter wells precoated with the monoclonal antibody DLH3 (3 μg/ml in PBS, 100 μl/well) were blocked with 1% bovine serum albumin (BSA) in 50 mM Tris-buffered saline, pH 8.0 (TBS). One hundred microliters of LDL (0.2 μg) mixed with oxidized PC (1 nmol) were added the wells and the mixtures were left overnight at 4°C. The remaining oxidized LDL, after washing with TBS containing 0.05% Tween 20, was detected by the addition of 100 μl of sheep anti-human apolipoprotein B antibody (Binding Site, Birmingham, England) and 100 μl of alkaline phosphatase-conjugated donkey anti-sheep IgG antibody (Chemicon, CA, USA). The reactivity of alkaline phosphatase was measured by incubating 1 mg/ml of *p*-nitrophenylphosphate at 37°C for appropriate time intervals. Each measurement was run with 1-10 ng/well of

copper-induced oxidized LDL as a standard.

Oxidized PC Metabolism—Reaction mixtures containing 30 nmol of radioactive PC or oxidized PC, 1 mM of 2-mercaptoethanol (2-ME), 15 μl of plasma and PBS were incubated at 37°C for 18 h. The reaction products were extracted by the method of Bligh and Dyer (*22*) and separated by TLC with two solvent systems developed in the same direction (1st solvent: chloroform/methanol/acetic acid = 98:2:1 up to 10 cm from the origin; 2nd solvent: hexane/diethyl ether/acetic acid = 80:20:1 up to 2 cm from the top). The TLC was then visualized with a Fuji BAS-1500 bioimaging analyzer. To observe the acyl transfer reaction to cholesterol and DG, [4-^{14}C]cholesterol or 1-palmitoyl-2-[1-^{14}C]-oleoyl DG (4 μCi/200 μl of 4% BSA in PBS) was incubated with normal plasma at 37°C for 4 h in the presence of 1 mM dithio-bis(nitrobenzoate) (DTNB) (*24*). After this, the pre-labelled plasma was incubated with various cold oxidized PCs (30 nmol) at 37°C for 18 h with or without the addition of 2-ME.

PAF Acetylhydrolase and Paraoxonase Activities—Platelet-activating factor acetylhydrolase (PAF-AH) activity was measured as described previously (*25*). Briefly, plasma (5 or 10 μl) was incubated with [^{3}H]acetyl-PAF (250 dpm/nmol) in 50 mM Hepes-NaOH buffer, pH 7.2, at 37°C for 5 min. The reaction was stopped by the addition of acetic acid. After buffering by the addition of sodium acetate, the reaction mixture was passed through a C_{18}-sep pack column. The radioactivity in the eluate was counted by a liquid scintillation counter. Paraoxonase activity was measured using phenylacetate as a substrate according to the method described in Ref. *26*.

Other Methods—Protein concentration was determined by the bicinchoninic acid (BCA) method (*27*). The level of phosphorus from PC was determined by the method of Zhou and Arthur (*28*).

RESULTS

The ability of LCAT to metabolize oxidized PC was investigated by incubating radioactive oxidized PC with human plasma. Incubation of 1-palmitoyl-2-[1-^{14}C]linoleoyl PC with plasma in the presence of 2-ME produced radioactive CE (Fig. 1A, lane 3). When 1-palmitoyl-2-[1-^{14}C]linoleoyl PC oxidized with ferrous ion and ascorbic acid was incubated with plasma under the same conditions, various radioactive products, in addition to CE, were detected by TLC (Fig. 1A, lane 4). The oxidized PC prepared under these conditions contained approximately 60% intact PC (*16*), thus explaining the production of CE. These bands were divided into two groups, "A" and "B." Authentic free fatty acid and free cholesterol migrated about a quarter of the way from the bottom of the chromatograph, between products "A" and "B". This TLC was run under analytical conditions optimised for neutral lipids, so polar lipids such as PC and lysoPC remained at the origin. CE and products A and B were produced in time- and dose-dependent manners (data not shown). Since the reaction was not saturated even after 18 h of incubation, all experiments were carried out with an incubation period of 18 h to allow for the accumulation of the products.

When plasma obtained from an FLD patient was incubated with PC no CE was formed, indicating that there was no LCAT activity in the patient's plasma. Under the same conditions, FLD plasma failed to form product "A" and CE from oxidized PC (Fig. 1A, lanes 5 and 6). The experiments were repeated using plasmas from five different FLD patients (three homozygotes and two heterozygotes; patient backgounds are described precisely in Ref. *17*). As shown in Fig. 1B, none of the five plasma samples produced CE from unoxidized PC and they produced little "A" products, confirming that FLD plasma is unable to produce "A" products from oxidized PC (Fig. 1B).

LCAT activity was inactivated by preincubating the plasma at 56°C for 30 min, resulting in no formation of CE from native PC. This preheated plasma also failed to form "A" products from oxidized PC (Fig. 2B). LCAT is active in the presence of reducing agents such as 2-ME and is strongly inhibited by the SH-reagent DTNB (*13*). The formation of "A" products, as well as CE, was slightly higher in the presence of 2-ME than in the absence of reducing agent, and was abolished by the addition of DTNB (Fig. 2A).

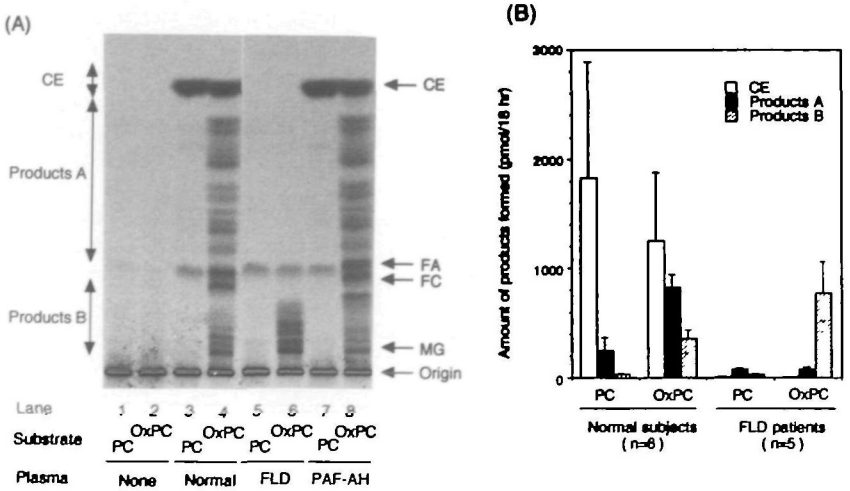

Fig. 1. **Nonpolar products from oxidized PC are formed by normal plasma but not by FLD plasma.** (A) TLC profile of the radioactive products formed from oxidized 1-palmitoyl-2-[1-^{14}C]linoleoyl PC. 1-Palmitoyl-2-[1-^{14}C]linoleoyl PC (0.4 mM) was oxidized with ferrous sulfate (40 μM) and ascorbate (0.4 mM) at 37°C for 3 h. Untreated PC or oxidized PC suspended in PBS (30 nmol) was incubated with (lanes 3 to 8) or without (lanes 1 and 2) 25 μl of human plasma in the presence of 2 mM 2-ME at 37°C for 18 h. The human plasma was obtained from a normal subject (lanes 3 and 4), an FLD patient (lanes 5 and 6), or a partial PAF-AH deficient subject (lanes 7 and 8). The reaction products were extracted with chloroform and methanol and analyzed by TLC using two solvent systems developed in the same direction (1st solvent: chloroform/methanol/acetic acid = 98:2:1 up to 10 cm from the origin; 2nd solvent: hexane/diethyl ether/acetic acid = 80:20:1 up to 2 cm from the top). (B) The amounts of products formed from PC or oxidized PC using plasma from normal subjects (n = 5) and FLD patients (n = 6) were determined using a BAS-1500 bioimaging analyzer. Values are indicated as means ± standard deviations.

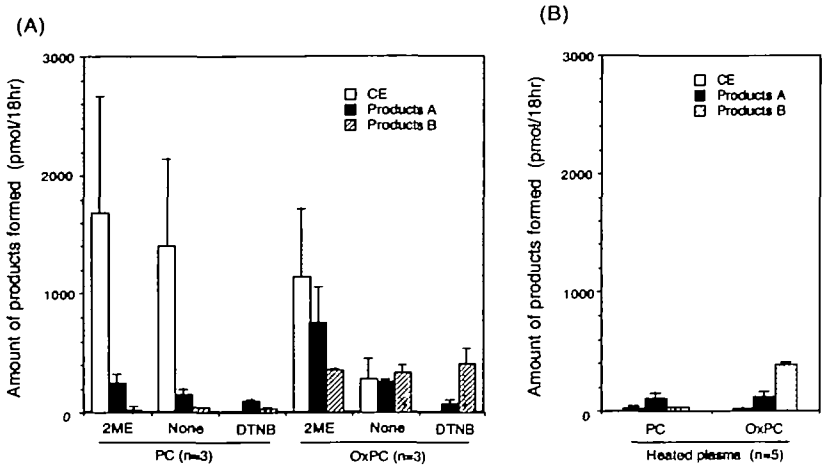

Fig. 2. **Oxidized PC-derived products "A" are not formed by normal plasma when LCAT activity is inactivated.** (A) Untreated PC or oxidized PC suspended in PBS (30 nmol) was incubated with 25 μl of human normal plasma in the presence of either 2-ME (10 mM) or DTNB (1 mM), or without the addition of these reagents at 37°C for 18 h. (B) Normal plasma was preincubated at 56°C for 30 min to inactivate LCAT activity. The amounts of products formed from PC or oxidized PC by preheated plasma ($n=5$) were determined. Values are indicated as means±standard deviations.

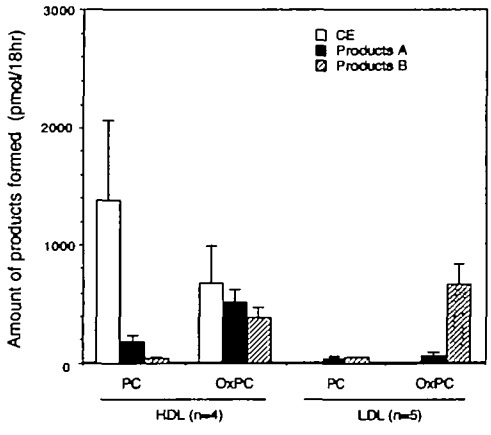

Fig. 3. **Metabolism of PC and oxidized PC by LDL and HDL fractions.** LDL and HDL fractions were separated from the plasma of three healthy subjects by sequential ultracentrifugation as described in "MATERIALS AND METHODS." Untreated PC or oxidized PC suspended in PBS (30 nmol) was incubated with either the LDL or HDL fractions (80 μg protein) in the presence of 2 mM 2-ME at 37°C for 18 h. Values are indicated as means±standard deviations ($n=3$).

LDL and HDL fractions were separated from normal plasma by sequential ultracentrifugation. When radioactive oxidized PC was incubated with these lipoproteins (80 μg each) instead of whole plasma, the HDL produced radioactive CE and "A" products, while the LDL produced only "B" products (Fig. 3). It is well-known that LCAT is mainly distributed on HDL particles (*1*), and these results suggest that the formation of "A" products is due to LCAT.

Conversely, platelet-activating factor acetylhydrolase (PAF-AH), another plasma enzyme that hydrolyzes short chain PC analogues, is mainly found on LDL particles (*29, 30*). PAF-AH activity in the FLD plasma was slightly higher than that found in normal plasma (Table I). A plasma sample was obtained from a subject with partial PAF-AH deficiency, and shown to contain approximately 30% of the normal level of PAF-AH activity. Products "A" were formed from radioactive OxPC upon incubation with the partially PAF-AH-deficient plasma, and the TLC pattern was almost the same as that from normal plasma (Fig. 1A, lanes 7 and 8 *versus* lanes 3 and 4). These results

TABLE I. **PAF-AH activity in plasma obtained from normal subjects and FLD patients.** PAF-AH activity of the plasma samples used in the Fig. 1B was determined as described in "MATERIALS AND METHODS."

Plasma	PAF-AH activity (nmol/5 min/μl plasma)
Normal ($n=3$)	0.97±0.16
FLD ($n=4$)	1.20±0.50

confirm that PAF-AH is not responsible for the formation of products "A".

Paraoxonase, a plasma enzyme that hydrolyzes organic ester compounds, has been reported to metabolize oxidized PC (*31*). This enzyme is mainly distributed in the HDL fraction, however, its enzyme activity is dependent on calcium ions (*26*). Under our experimental conditions, all plasma and lipoprotein preparations contained 0.25 mM EDTA throughout the procedure. When paraoxonase activity was measured using phenyl acetate as a substrate, the plasma paraoxonase activity in the presence of 0.25 mM EDTA was less than 1% of that found in the presence of 2 mM $CaCl_2$. In addition, virtually no activity was detected in our HDL preparations (data not shown). Therefore, neither paraoxonase nor PAF-AH appear to be responsible for the formation of "A" products from oxidized PC under the conditions of this study. However, it is possible that some other enzyme activity that metabolizes oxidized PC may be responsible for the formation of products "B", since they were formed from oxidized PC by FLD plasma and by normal plasma even when LCAT was inactivated.

A variety of oxidized PC products is formed from PC treated with metal ions. PCOOH, a PC containing a long chain polyunsaturated fatty acid hydroperoxide, is an intermediate product of PC oxidation. PCOOH can be cleaved by radical-mediated reactions to form various short-chain PCs including 9-CHO PC. To determine whether LCAT is active against oxidized PC products with short chain aldehydes, components found in oxidized LDL (*16, 20, 32*) that are biologically active in blood vessel wall cells (*32, 33*), radioactive 9-CHO PC and 5-CHO PC, were prepared. Incubation of ^{14}C-labeled 9-CHO PC with normal plasma in the presence of 2-ME resulted in new radioactive bands in the area corresponding to products "A", but these were not formed in the presence of DTNB (Fig. 4A).

Similar results were obtained using ^{14}C-labeled 5-CHO PC (Fig. 4B). Using the HDL fraction instead of whole plasma, the DTNB-sensitive formation of radioactive bands from ^{14}C-labeled 9-CHO PC was observed (data not shown).

We next examined whether products "A" were formed by transacylation of oxidized fatty acyl moieties to cholesterol. Normal plasma was labeled with radioactive cholesterol by incubation with [4-^{14}C]cholesterol-BSA complex prior to carrying out the LCAT assay with PC analogues (Fig. 5). During the preincubation step, 1 mM DTNB was added to the plasma to prevent the conversion of radiolabeled cholesterol to CE. The pre-labeled plasma was then incubated with PC analogues with or without the addition of 10 mM 2-ME, which re-activates LCAT. Radioactive CE was formed even without the addition of PC, indicating that endogenous PC was utilized as an acyl donor (Fig. 5, lane 1). The addition of oxidized PC to the reaction mixture did not increase the formation of either CE or "A" products. Two synthetic oxidized PC products, 9-CHO PC, a PC analogue containing a 9-carbon aldehyde group, and PCOOH, were added to the reaction mixture. Two new radioactive bands were formed in the presence of PCOOH near product "A", while the addition of 9-CHO PC to the reaction mixture did not increase the formation of CE or products "A" as in the case of oxidized PC (Fig. 5, lanes 3 to 5). These radioactive bands were not produced when LCAT was not re-activated (Fig. 5, lanes 6 to 9). The same results were observed using isolated HDL fractions instead of whole plasma (data not shown).

It is known that various alcoholic compounds including DG can be used as acyl acceptors in the LCAT reaction (*11*). We prepared radioactive DG from 1-palmitoyl-2[1-^{14}C]-oleoyl PC by phospholipase C digestion to determine whether acyl groups are transferred to DG from oxidized

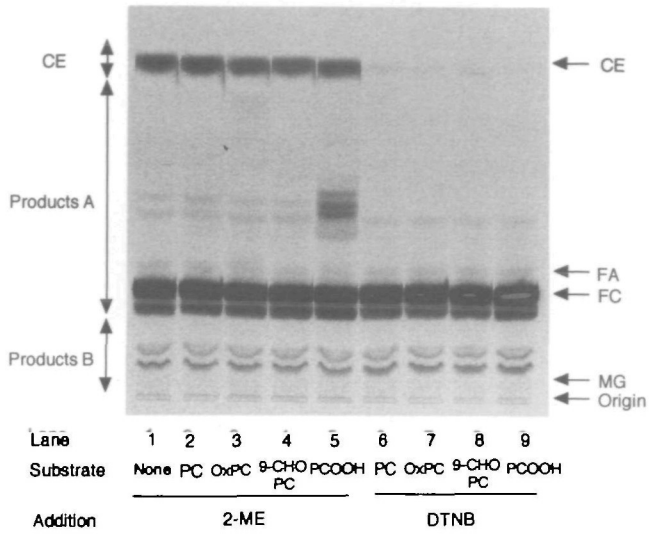

Fig. 5. **TLC profile of the radioactive products formed from ^{14}C-labeled cholesterol preincubated with normal plasma.** [4-^{14}C]Cholesterol mixed with BSA (4 μCi/200 μl of 4% BSA in PBS) was incubated with normal plasma at 37°C for 4 h in the presence of DTNB (1 mM). The plasma (25 μl) was then further incubated with 30 nmol of oxidized PC, egg PCOOH, or 9-CHO PC at 37°C for 18 h with or without the addition of 2-ME (10 mM). The reaction products were extracted with chloroform and methanol and analyzed by TLC.

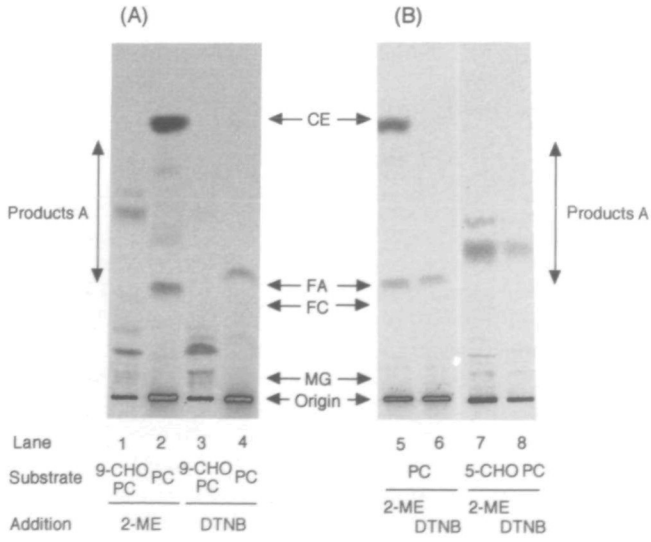

Fig. 4. **TLC profile of the radioactive products formed from ^{14}C-labeled 9CHO-PC and 5CHO-PC.** Short chain aldehyde-containing PCs were prepared from radioactive PC by treatment with OsO_4 and $NaIO_4$ (see "MATERIALS AND METHODS"). (A) 1-Palmitoyl-2-[1-^{14}C]linoleoyl PC or 1-palmitoyl-2-([1-^{14}C] 9-oxononanoyl)PC (30 nmol) was incubated with normal plasma (25 μl) at 37°C for 18 h in the presence of either 2-ME (10 mM) or DTNB (1 mM). (B) 1-Stearoyl-2-[1-^{14}C] arachidonoyl PC or 1-stearoyl-2-([^{14}C] 5-oxovaleroyl) PC (30 nmol) was incubated with normal plasma (25 μl) under the same conditions as in (A). The reaction products were extracted with chloroform and methanol and analyzed by TLC as described in the legend to Fig. 1.

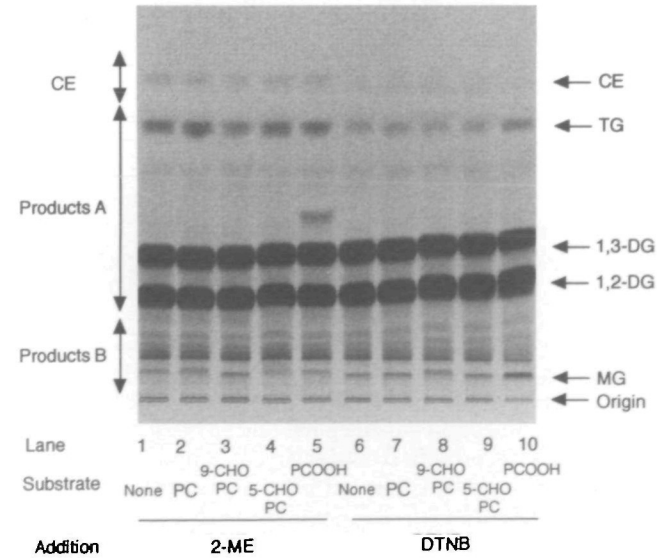

Fig. 6. **TLC profile of the radioactive products formed from 1-palmitoyl-2-[1-^{14}C]oleoyl DG preincubated with normal plasma.** 1-Palmitoyl-2-[1-^{14}C]linoleoyl DG was prepared from the corresponding PC by treatment with phospholipase C. The radioactive DG mixed with BSA (4 μCi/200 μl of 4% BSA in PBS) was incubated with normal plasma at 37°C for 4 h in the presence of DTNB (1 mM). The plasma (25 μl) was then further incubated with 30 nmol of 9-CHO PC, 5-CHO PC, or egg PCOOH at 37°C for 18 h with or without the addition of 2-ME (10 mM). The reaction products were extracted with chloroform and methanol and analyzed by TLC.

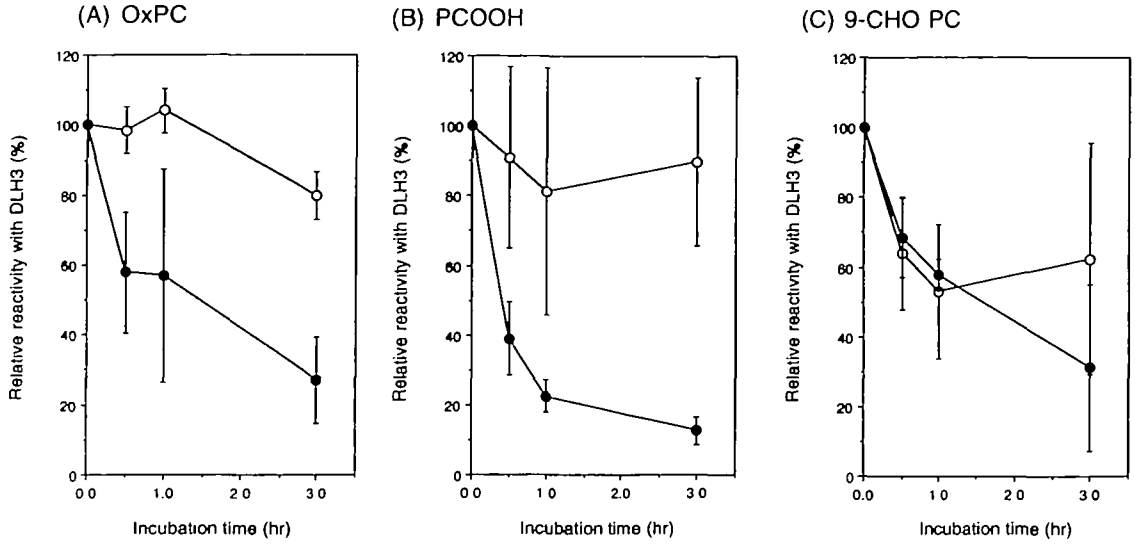

Fig. 7. **Effect of LCAT on the binding of oxidized PC-LDL mixtures to anti-oxidized PC antibody DLH3.** LDL (100 ng/20 μl) was incubated with oxidized PC (2.5 nmol), egg PCOOH (1 nmol), or 9-CHO PC (1 nmol) at 37°C for 15 min. These oxidized PC analogue-LDL complexes were then further incubated with 80 μg of HDL in the presence of either DTNB (1 mM) or 2 ME (10 mM) at 37°C for up to 3 h. After incubation, the immunological reactivities of the oxidized PC-LDL complexes as oxidized LDL were determined by sandwich ELISA using anti-oxidized PC monoclonal antibody (DLH3) and an anti-apolipoprotein B polyclonal antibody as described in "MATERIALS AND METHODS." Values are indicated as means ± standard deviations of four independent experiments.

PCs. Normal plasma pre-labeled with radioactive DG was incubated with cold oxidized PCs (Fig. 6). Radioactive triacylglycerol and CE were formed without the addition of PC, suggesting that endogenous PC was utilized as an acyl donor in this case as well, and that the radioactive linoleate in DG may be transferred to cholesterol by LCAT. This result is very similar to that shown in Fig. 5. The LCAT-dependent formation of new bands was observed when PCOOH was added to the reaction mixture, while the addition of oxidized PC, 9-CHO PC or 5-CHO PC, failed to produce new radioactive products. These results suggest that although LCAT can transfer the fatty acid hydroperoxide of PCOOH to cholesterol and to DG, short chain acyl groups may not be transferred to these alcohol compounds under the same conditions. It is noteworthy that 9-CHO PC and 5-CHO PC, but not PCOOH, inhibited LCAT activity, since the radioactive bands of TG and CE were significantly reduced. This suggests that short chain aldehyde PCs have inhibitory activity against LCAT.

In previous studies, an increased level of LDL plasma oxidation was observed in FLD patients (*17*). The LDL oxidation level was determined by a sandwich ELISA using the anti-oxidized PC monoclonal antibody DLH3 and anti-human apolipoprotein B antibody (*15*). The antibody DLH3 reacts with several oxidized PC products, including 9-CHO PC and PCOOH (*16*). LDL mixed with oxidized PC and copper-induced oxidized LDL are good DLH3 antigens and are easily detected using the sandwich ELISA. In order to investigate whether LCAT reacts with oxidized PC molecules on LDL particles, a mixture of LDL and oxidized PC was incubated with HDL as the LCAT source in the presence of either DTNB or 2-ME. The reactivity of the LDL mixed with oxidized PC in the sandwich ELISA decreased following incubation with HDL in the presence of 2-ME, but remained unchanged in the presence of DTNB (Fig. 7A). The "A" products produced from OxPC did not inhibit OxLDL binding to DLH3 (data not shown), indicating the products were no longer antigenic. Incubation of LDL mixed with egg PCOOH with HDL decreased the reactivity to DLH3 in a similar manner. However, the reactivity of LDL mixed with 9-CHO PC decreased by 40% in the presence of DTNB. It is likely that a spontaneous transfer of hydrophilic phospholipids such as 9-CHO PC from LDL particles to HDL may occur under the experimental conditions of the study.

DISCUSSION

In the present study we have shown that LCAT is capable of metabolizing oxidized PC to form a variety of apolar products. These products (products "A") migrate between CE and free fatty acids on TLC. It is unlikely that products "A" are oxidatively modified fatty acyl groups liberated from the *sn*-2 position of oxidized PC, since oxidized fatty acids would not migrate above authentic free fatty acids on the chromatogram. The involvement of LCAT in the formation of "A" products is suggested by four pieces of evidence; firstly, plasma from FLD patients did not produce the "A" products; secondly, inhibition of LCAT by heat treatment diminished the production of these bands; thirdly, the addition of DTNB, an LCAT inhibitor, abolished the formation of "A" products; and, finally, HDL, separated from plasma, but not LDL, showed the activity together with CE formation. In all cases, "A" products were not formed in the absence of CE formation from PC. We then tested whether LCAT is active against OxPC molecules in modified LDL particles. The reduction in antigenicity of oxidized PC-LDL mixtures following incubation with HDL suggests that oxidized PC distributed on LDL particles is also metabolized by LCAT. These results, together with our previous observation that the plasma concentration of oxidized LDL in FLD patients is signifi-

cantly higher than that of normal controls, suggest that LCAT is a circulating scavenger of oxidized PC.

Oxidized LDL has been thought to be involved in the early development of atherosclerosis (*34*). Although the precise modified structures and behavior of oxidized LDL formed *in vivo* have not yet been clarified, several studies have reported the presence of oxidized LDL in circulating plasma (*15, 17, 35-37*). We have developed a sandwich ELISA method capable of measuring oxidatively modified LDL in human plasma sensitively using a monoclonal antibody that recognizes oxidized PC molecules including 9-CHO PC (*15*). Using this method, we observed a small but detectable amount of oxidized LDL in normal human plasma. Significantly higher levels of LDL oxidation were detected in patients receiving hemodialysis (*15*) and in patients with cardiovascular disease (*37*). Furthermore, LDL oxidation in FLD patients is at least three-fold higher than in normal subjects (*17*). It is likely that LDL oxidation levels are affected by a variety of factors including local oxidative stress in damaged tissues or inflamed regions, the concentrations of pro-oxidants and antioxidants in circulation, and the capability of metabolizing and clearing oxidized LDL. The increased oxidation levels of plasma LDL in FLD patients may be explained at least in part by the loss of metabolic activity of LCAT against oxidized PC on modified LDL.

Recently LCAT has been reported to be inhibited by oxidation of lipoproteins or oxidized phospholipids (*19, 38, 39*). These studies demonstrated that the radioactive products formed from ^{14}C-labeled cholesterol are reduced either by the addition of copper-induced oxidized LDL in exogenous substrate assays, or by oxidation of whole plasma in endogenous substrate assays. These observations are in agreement with our finding that the formation of radioactive CE from radioactive PC is reduced when PC is oxidized with ferrous ion prior to incubation with plasma. Since the amount of unoxidized PC remaining in our oxidized PC preparations was approximately 60% of the total, the observed reduction in the formation of radioactive CE (Fig. 1B) may be explained by the decreased amount of radioactive PC available. A specific inhibitory effect of oxidized PC on LCAT cannot, however, be ruled out, because earlier reports achieved nearly complete inhibition of LCAT activity by the addition of oxidized LDL or oxidation of whole plasma (*38, 39*). It is noteworthy that the intensities of the radioactive bands of TG and CE formed from ^{14}C-labeled DG were significantly reduced by incubation with 9-CHO PC and 5-CHO PC, but not with PCOOH (Fig. 6). It is possible that short chain aldehyde PCs have inhibitory properties against LCAT.

When the reaction products formed from oxidized 1-palmitoyl-2[1-^{14}C]linoleoyl PC were hydrolyzed with NaOH, no bands corresponding to the "A" products other than free fatty acid were observed. Furthermore, treatment of the reaction products formed from 1-^{14}C-labeled 9-CHO PC with NaOH diminished the two "A" products bands and part of the radioactivity was recovered in the water phase (data not shown). The "A" products should contain the first carbon atom of the linoleoyl group at the *sn*-2 position, since these products are radioactive. They migrate between CE and free fatty acid on TLC under these analytical conditions, in which phospholipids should stay at the origin of the TLC plate. No radioactive bands, however, were produced from ^{14}C-labeled cholesterol and DG upon incubation with cold oxidized PC. These results suggest that the "A" products are likely to be esters containing oxidized analogues of short chain fatty acids and certain alcohol compounds other than cholesterol and DG. When ^{14}C-labeled 9-CHO PC was incubated with plasma, two radioactive products were formed, suggesting that there may be at least two different acceptors for short chain fatty aldehydes. Alternatively, it is possibile that "A" products may be formed by more than two processes. For example transfer of fatty acid from normal PC to "B" products may result in the formation of "A" products. More work is needed to identify the components comprising "A" products.

The "B" products, formed in the presence of DTNB or by FLD plasma, migrate between free fatty acid and the origin under the TLC conditions employed. Judging from the TLC pattern, it is possible that the "B" products include oxidized fatty acids and/or diacylglycerols containing oxidized acyl chains.

Hydrolysis of oxidized PC was not confirmed by measuring the formation of lysoPC in this study, since it is hard to distinguish lysoPC derived from oxidized PC from the remaining unoxidized PC. Moreover, re-esterification of lysoPC by the lecithin-lysolecithin acyltransferase activity of LCAT would affect the amount of lysoPC.

Radioactive bands were formed when ^{14}C-labeled cholesterol was incubated with egg PCOOH, while no band was observed in the case of ferrous ion-induced oxidized PC and 9-CHO PC (Fig. 5). Nagase *et al.* reported that cholesteryl linoleate hydroperoxide and its alcohol are formed when PCOOH is incubated with human plasma (*18*). LCAT may hydrolyze a variety of oxidized PC products including short chain PC while it may only transfer long-chain acyl groups. Several reports have suggested that HDL is a major carrier of CE hydroperoxides (*40, 41*). The accumulation of CE hydroperoxides on HDL particles may be achieved by the action of LCAT.

Oxidized PC has been reported to be hydrolyzed by several enzymes including plasma PAF-AH (*29*), paraoxonase (*31*), and cytosolic PAF-AH type II (*42*). Plasma PAF-AH is mainly distributed on LDL particles and is able to hydrolyze short chain PC analogues. However, PAF-AH activity is even higher in FLD patients than in normal subjects, and partially PAF-AH deficient plasma shows no differences from normal plasma (Table I, Fig. 1A). Paraoxonase is another plasma enzyme that hydrolyzes a variety of ester compounds. It has been proposed that this enzyme can hydrolyze oxidized PC products containing two hydroperoxide groups (*31*). Since all of our experiments were performed in the presence of EDTA, which inhibits this enzyme, it is unlikely that the formation of products "A" and "B" was due to paraoxonase. Actually, no paraoxonase activity was observed in the HDL fraction in which "A" products were formed most potently from oxidized PC (Fig. 3 and text). Although, cytosolic PAF-AH type II possesses some characteristics similar to plasma PAF-AH, it is unlikely that the cytosolic enzyme is present in the plasma or HDL fraction. Therefore, we conclude that LCAT is resposible for at least the formation of "A" products. However, it is possible that the "B" products may be formed by PAF-AH, since isolated LDL produced these bands. It appears that many enzymes work together to

regulate the *in vivo* concentration of oxidized PC and thereby play a role in protecting tissues from oxidative damage. Our results suggest that LCAT is also a member of this family of oxidized PC metabolizing enzymes.

The transfer of oxidized fatty acids from PC to less polar lipids by LCAT does not reduce the oxidized lipid mass. We assume, however, that such metabolism would be beneficial for two possible reasons: Firstly, products "A" would be distributed in the hydrophobic core of LDL particles. Conversion of oxidized PC to "A" products may reduce the amount of oxidized lipids expressed on the surface of LDL particles. Secondly, oxidized PC has been shown to have various stimulatory activities on vessel wall cells. For example, 5CHO-PC, found in mildly oxidized LDL, induces the surface expression of monocyte chemoattractant protein-1 (MCP-1) on endothelial cells (*31, 32*). Hörkkö *et al.* recently found that oxidized PC is directly involved in macrophage recognition of oxidized LDL (*43*). Therefore, reducing oxidized PC in LDL particles may be a protective factor againt the development of atherosclerosis.

Oxidized PC-related deposits were found in the glomeruli of FLD patients by immunohistochemical analysis using the anti-oxidized PC monoclonal antibody (*17*). It is well-known that renal dysfunction is one of the major symptoms of FLD, although the mechanism of renal damage is not understood (*5*). The observation that LDL oxidation levels in patients receiving haemodialysis is higher than in normal subjects suggests that oxidized LDL and renal damage may be related to each other. Oxidized PC induces a variety of responses in many types of cells including chemotaxis, expression of signal molecules, and cytotoxicity (*44*). It is possible that increased levels of oxidized PC in circulation may be toxic to glomeruli.

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