

Novel Function of Lecithin-Cholesterol Acyltransferase

HYDROLYSIS OF OXIDIZED POLAR PHOSPHOLIPIDS GENERATED DURING LIPOPROTEIN OXIDATION*

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Although the major function of lecithin-cholesterol acyltransferase (LCAT) is cholesterol esterification, our previous studies showed that it can also hydrolyze platelet-activating factor (PAF). Because of the structural similarities between PAF and the truncated phosphatidylcholines (polar PCs) generated during lipoprotein oxidation, we investigated the possibility that LCAT may also hydrolyze polar PCs to lyso-PC during the oxidation of plasma. PAF acetylhydrolase (PAF-AH), which is known to hydrolyze polar PCs in human plasma, was completely inhibited by 0.2 mM *p*-aminoethyl benzenesulfonyl fluoride (Pefabloc), a new serine esterase inhibitor, which had no effect on LCAT at this concentration. On the other hand, 1 mM diisopropylfluorophosphate (DFP) completely inhibited LCAT but had no effect on PAF-AH. Polar PC accumulation during the oxidation of plasma increased by 44% in the presence of 0.2 mM Pefabloc and by 30% in the presence of 1 mM DFP. The formation of lyso-PC was concomitantly inhibited by both of the inhibitors. The combination of the two inhibitors resulted in the maximum accumulation of polar PCs, suggesting that both PAF-AH and LCAT are involved in their breakdown. Oxidation of chicken plasma, which has no PAF-AH activity, also resulted in the formation of lyso-PC from the hydrolysis of polar PC, which was inhibited by DFP. Polar PCs, either isolated from oxidized plasma or by oxidation of labeled synthetic PCs, were hydrolyzed by purified LCAT, which had no detectable PAF-AH activity. These results demonstrate a novel function for LCAT in the detoxification of polar PCs generated during lipoprotein oxidation, especially when the PAF-AH is absent or inactivated.

Lecithin-cholesterol acyltransferase (LCAT)¹ is a plasma enzyme that circulates mostly in association with the high density lipoproteins (HDL) and is responsible for the synthesis of

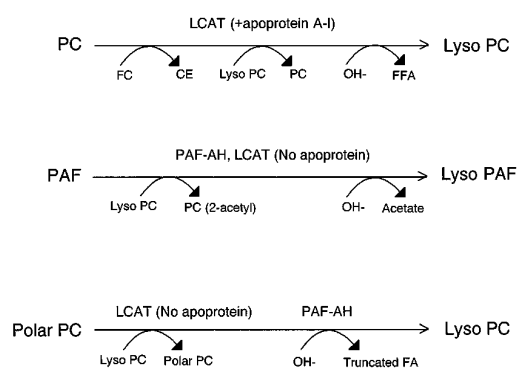
most of the cholesteryl esters present in human plasma (1, 2). Although it is generally described as a cholesterol-esterifying enzyme because of its well known physiological function, it is in fact a specialized phospholipase A, whose primary substrate is phosphatidylcholine (PC). Unlike the traditional phospholipases A, which transfer an acyl group from the PC to water, it normally transfers the acyl group to unesterified cholesterol, forming cholesteryl ester and lyso-PC. We previously demonstrated that LCAT can also transfer the acyl group from PC to lyso-PC instead of to cholesterol, thus catalyzing a futile cycle of PC-lyso-PC transesterification (3, 4). The enzyme does act as a phospholipase A in the absence of an acyl acceptor, releasing the acyl group as a free fatty acid (5, 6). All of the above reactions require the presence of an apoprotein activator, which is believed to facilitate the interaction of the enzyme with the lipid interface. They are also reversibly inhibited by 5,5'-dithiobis(nitrobenzoic acid) (DTNB), a thiol-blocking agent, which apparently inhibits the reactions by steric hindrance (7). In addition to the hydrolysis of the long chain acyl-ester groups of the diacyl PCs, LCAT has been shown to hydrolyze synthetic water-soluble esters (8) as well as the platelet-activating factor (PAF), a highly bioactive phosphoglyceride containing an acetyl group at the *sn*-2-position (9) (Scheme 1). However, unlike the long chain acyl transfer and hydrolytic reactions, the hydrolysis of the short chain esters by LCAT does not require an apoprotein activator and is not inhibited by DTNB (8, 9).

Oxidation of plasma lipoproteins is believed to be an important event in the formation of atherogenic particles, which are taken up by the vascular cells by unregulated pathways, leading to the formation of foam cells (10, 11). Some of the early products of the lipoprotein oxidation are polar derivatives of PC, which contain a short chain acyl group at the *sn*-2-position terminating in COOH, CHO, or CH₂OH and which are formed by the oxidative fragmentation of the di- and polyunsaturated fatty acids present at the *sn*-2-position of lipoprotein PC (12, 13). Many of these polar PC products have proinflammatory properties similar to PAF, because they can interact with the PAF receptor on various cells (14–17), and others have toxic effects on the cells (18). The biological activities of the oxidized PCs depend upon the presence of the *sn*-2 short chain acyl group, because treatment with phospholipase A abolishes their proinflammatory (14, 16) as well as cytotoxic (19) effects. The polar phospholipids containing an aldehyde function may also form Schiff-base complexes with the amino groups of the apoproteins and render the lipoproteins more atherogenic (20). Such complexes have in fact been demonstrated in the foam cells from human atherosclerotic lesions (21). The rapid hydrolysis of the oxidized PC products is therefore critical in preventing their proinflammatory and proatherogenic effects. It is generally believed that their hydrolysis is carried out by the PAF acetyl hydrolase (PAF-AH), an enzyme that is predominantly associated with LDL in plasma (16). However, this

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¹ The abbreviations used are: LCAT, lecithin-cholesterol acyltransferase; AAPH, 2,2'-azobis-(2-amidinopropane) dihydrochloride; DFP, diisopropylfluorophosphate; DTNB, 5,5'-dithio-bis-(nitrobenzoic acid); HDL, high density lipoprotein(s); LAT, lysolecithin acyltransferase; LDL, low density lipoprotein(s); PAF, platelet-activating factor; PAF-AH, platelet-activating factor acetylhydrolase; PC, phosphatidylcholine; Pefabloc, *p*-aminoethyl benzenesulfonyl fluoride.



SCHEME 1. Reactions catalyzed by LCAT and PAF-AH.

enzyme is not present in the plasma of certain species such as birds (22), and several cases of inherited or acquired deficiencies of the enzyme have been reported in the human population (23, 24). Furthermore, PAF-AH activity of human plasma is rapidly inactivated during the oxidation of LDL (25–27), and it is therefore possible that it cannot account for the hydrolysis of all the oxidized PCs generated during the oxidation of LDL. Our recent investigations on the effect of lipoprotein oxidation on LCAT showed that while its cholesterol esterification activity is destroyed rapidly, its ability to transfer the oxidized acyl group of PC to exogenous lyso-PC is retained or even stimulated (28). This reaction, which was named lysolecithin acyltransferase II (LAT II), is not dependent upon the presence of an apoprotein activator, and it was insensitive to sulfhydryl blocking agents. On the basis of these results we proposed that LCAT may also hydrolyze the oxidized PC to lyso-PC. The present study provides experimental evidence that LCAT can indeed hydrolyze the oxidized PCs generated during lipoprotein oxidation and that it may account for a significant percentage of the phospholipase A activity found in the plasma following lipoprotein oxidation.

EXPERIMENTAL PROCEDURES

Materials—Diisopropyl fluorophosphate (DFP), Pefabloc (4-(2-amino ethyl)benzenesulfonyl fluoride), DTNB, egg PC, cholesterol, and unlabeled *sn*-1-palmitoyl lyso-PC were purchased from Sigma. AAPH (2,2'-azobis-(2-amidinopropane) dihydrochloride) was obtained from Wako Chemicals (Richmond, VA). [4-¹⁴C]cholesterol (33 mCi/mmol), 1-[1-¹⁴C]palmitoyl lyso-PC (56.8 mCi/mmol), 2-[³H-acetyl]PAF, and *sn*-1-palmitoyl 2-linoleoyl [1-¹⁴C]PC (50 mCi/mmol) were from DuPont NEN. *Sn*-1-palmitoyl 2-linoleoyl PC, *sn*-1-palmitoyl 2-arachidonoyl PC, and PAF were obtained from Avanti Polar Lipids (Alabaster, AL), and *sn*-1-palmitoyl-2-arachidonoyl [1-¹⁴C]PC (55 mCi/mol) was from American Radiochemicals (St. Louis, MO).

Blood was drawn in EDTA (1.0 mg/ml) from normal healthy volunteers (ages 26–50) who had fasted overnight. Plasma was obtained by centrifugation at 2000 rpm for 15 min at 4 °C and was used immediately for oxidation studies. Pooled human plasma was also obtained from a local blood bank (United Blood Services) for the purification of LCAT and the preparation of HDL. Chicken blood, which was obtained from a local poultry supply company, was transported at 4 °C to the laboratory and was centrifuged immediately to prepare the plasma. Chicken serum (frozen) was purchased from Sigma for the purification of chicken LCAT.

Human LCAT and apolipoprotein A-1 were purified from plasma as described earlier (29). The final preparations gave single bands on SDS-polyacrylamide gel. In some cases, partially purified LCAT (after the phenyl-Sepharose step) was used, instead of the highly purified preparation. Chicken LCAT was purified by the same procedure as used for the human enzyme (29), except that the starting material was frozen serum.

Enzyme Assays—LCAT activity was determined by the esterification of labeled free cholesterol to cholesteryl ester using proteoliposome substrate (30), as described earlier (28, 29). LAT II activity was estimated by the esterification of labeled lyso-PC to polar PC, which moves with sphingomyelin on silica gel TLC (28). Briefly, 60 nmol of 1-[1-¹⁴C]palmitoyl lyso-PC was dispersed in Tris-NaCl-EDTA buffer (10 mM

Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl and 1 mM EDTA) and was added to 100 μ l of plasma, followed by the addition of 10 mM mercaptoethanol. The reaction mixture, in a final volume of 0.4 ml, was incubated at 37 °C for 2 h, the reaction was stopped by adding 1.0 ml of methanol, and the lipids were extracted by the Bligh and Dyer procedure (31). Extracted lipids were separated on silica gel TLC plates using the solvent system of chloroform/methanol/water (65:25:4, v/v). Spots corresponding to lyso-PC, PC, and sphingomyelin were scraped, and their radioactivity was determined in a liquid scintillation counter (Beckman LS 6500) after adding 0.5 ml of water and 5 ml of scintillation fluid (Cytosol, ICN Biomedical). Quench correction was made by the H number (Beckman Instruments). The radioactivity in the sphingomyelin region represents the LAT II activity (formation of labeled polar PC), whereas the radioactivity in the PC represents LAT I reaction (28). Control tubes were included in each assay where the reaction was stopped at 0 min.

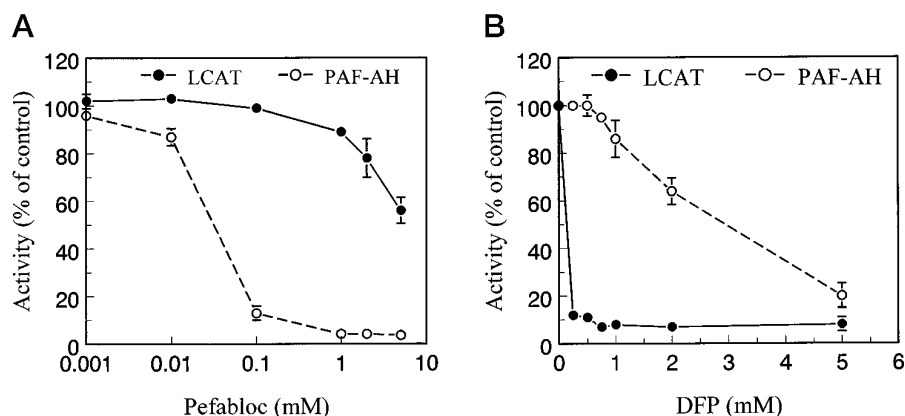
PAF-AH activity was determined by the release of labeled acetate from ³H-labeled PAF (9). The reaction mixture contained 32 nmol of ³H-acetate-labeled PAF in Tris-NaCl-EDTA buffer and the enzyme source in a final volume of 0.4 ml. After incubation at 37 °C for 5–30 min, the reaction mixture was extracted (31), and aliquots of the aqueous and chloroform layers were counted in a liquid scintillation counter to determine the radioactivity in free acetate and in the unhydrolyzed PAF, respectively.

Oxidation of Plasma—Oxidation of human or chicken plasma was carried out with varying concentrations of the free radical generator AAPH. For the assay of LCAT and LAT II reactions, 0.1 ml of plasma was diluted with Tris-NaCl-EDTA buffer and 2.0 mM DTNB. AAPH (0–60 mM final concentration) dissolved in Tris-HCl buffer, pH 7.4, was added, and oxidation was carried out at 37 °C for varying periods of time. Aliquots of the oxidized plasma were used for the enzyme assays. To determine the effects of inhibitors, 0.2 ml of plasma was oxidized with 50 mM AAPH in the presence of various combinations of 2.0 mM DTNB, 0.2 mM Pefabloc, and 1.0 mM DFP at 37 °C for 16 h. The reaction was stopped by adding 1.0 ml of methanol containing 0.01% butylated hydroxytoluene. Lipids were extracted (31) after acidification to pH 3.0 and separated on silica gel TLC using the solvent system of chloroform, methanol, 28% ammonium hydroxide (65:35:8, v/v/v). The spots corresponding to PC, sphingomyelin, lyso-PC, and origin were scraped into glass tubes, and the lipid phosphorus was estimated by the modified Bartlett procedure (32). When labeled PC was added in ethanol solution (1% of final volume).

Hydrolysis of Polar PC by Chicken LCAT—Human plasma was oxidized with 50 mM AAPH in the presence of 1.0 mM DFP and 0.2 mM Pefabloc, by 50 mM AAPH, for 16 h, at 37 °C. Lipids were extracted (31) after acidification to pH 3.0 with HCl and were separated on silica gel TLC using the solvent system of chloroform, methanol, 28% ammonium hydroxide (65:35:8, v/v/v). The area corresponding to sphingomyelin (and polar PC) was scraped into glass tubes, and the lipids were eluted (31). After evaporation of the solvent, the lipids were redissolved in Tris-NaCl-EDTA buffer and were reacted with purified chicken LCAT for 1 h at 37 °C. Lipids were extracted and separated on a silica gel TLC plate, and lipid phosphorus was measured in sphingomyelin (with polar PC) and lyso-PC regions (32). The percentage of hydrolysis was calculated as the percentage of total lipid phosphorus appearing in the lyso-PC spot.

Preparation and Hydrolysis of Labeled Polar PC—1 mg of 16:0–20:4 PC or 16:0–18:2 PC, labeled at the *sn*-2-acyl group (400,000 dpm), was solubilized in normal saline by adding 5 mM sodium deoxycholate. Oxidation was carried out with 50 mM AAPH for 2 h in a final volume of 0.8 ml. Lipids were extracted (31) and separated on TLC using a solvent system of chloroform, methanol, 28% ammonium hydroxide, (65:35:8, v/v/v). Spots corresponding to the origin, lyso-PC, sphingomyelin, and PC were scraped, and lipids were extracted again and redispersed in 1.0 ml of Tris-NaCl-EDTA buffer. After the determination of lipid phosphorus, 60 nmol of each product (containing 15,000 dpm) was treated with 50 μ l of purified chicken LCAT in a total volume of 400 μ l, at 37 °C for 1 h. After lipid extraction (31), 0.5 ml of water layer was used for counting radioactivity. The chloroform layer was evaporated to dryness under nitrogen and redissolved in 1.0 ml of methanol, from which 0.5 ml was used for counting radioactivity. The hydrolysis of polar PC by chicken LCAT was calculated as the percentage of the counts recovered in aqueous layer after subtraction of the blank (sample incubated in the absence of enzyme).

FIG. 1. Differential inhibition of LCAT and PAF-AH by Pefabloc (A) and DFP (B) in normal human plasma. Freshly drawn plasma (in EDTA) was preincubated for 30 min with the indicated concentration of the inhibitor. Aliquots (20 μ l) were then taken out for the determination of LCAT and PAF-AH activities, as described under "Experimental Procedures." The activities are expressed as the percentage of the control values (no inhibitor) and are mean $\pm$ S.E. of three separate experiments. The error bars are too small to be visible at some points.



RESULTS

Selective Inhibition of PAF-AH and LCAT Activities—To determine the relative roles of LCAT and PAF-AH in the hydrolysis of oxidized phospholipids we first sought to identify an inhibitor that can specifically inhibit PAF-AH, the enzyme known to carry out this hydrolysis in human plasma (16). Although both PAF-AH and LCAT are serine-dependent enzymes, we found that a recently described inhibitor of PAF-AH, namely Pefabloc (*p*-aminoethyl benzenesulfonyl fluoride) (33) has no effect on LCAT activity at low concentrations (Fig. 1). Freshly prepared plasma (in EDTA) was preincubated with increasing concentrations of either Pefabloc or DFP, and then the hydrolysis of PAF and the esterification of free cholesterol were studied using exogenous labeled substrates, as described under "Experimental Procedures." The PAF-AH activity of the plasma was almost completely inhibited by Pefabloc at a concentration of 0.1 mM, whereas the LCAT activity was not affected below 1.0 mM concentration (Fig. 1A). On the other hand, DFP, at a concentration of 0.25 mM was sufficient to inhibit most of the cholesterol esterification, while it had no effect on PAF-AH even at 1.0 mM concentration (Fig. 1B). A higher concentration of DFP (>1.0 mM) did inhibit PAF-AH, with the complete inhibition occurring at 10 mM, in accordance with published results (34–36). These results show that one can inhibit PAF-AH activity without affecting LCAT, and *vice versa*.

Effects of Specific Inhibitors on the Accumulation of Lyso-PC and Polar PC during the Oxidation of Plasma—Taking advantage of the differential inhibition of the two enzyme activities, we determined the relative contribution of each for the hydrolysis of polar PC to lyso-PC during lipoprotein oxidation. Freshly prepared human plasma was oxidized for 16 h at 37 °C with 50 mM AAPH in the presence of different inhibitors, and the concentrations of PC, polar PC (with sphingomyelin), and lyso-PC were determined by lipid phosphorus, following their TLC separation. Since lyso-PC is formed by the cholesterol esterification reaction also, we added DTNB (2 mM) to all samples to inhibit this reaction. Our previous results showed that while DTNB inhibits cholesterol esterification, it has no effect on the LAT II reaction (9) and therefore presumably has no effect on the hydrolysis of oxidized PC also. Our experiments also showed that when isolated plasma sphingomyelin was dispersed in 5 mM sodium deoxycholate and treated with 20 mM AAPH for 16 h at 37 °C, there was no change in its mobility on TLC plate or its phosphorus content, whereas under the same conditions plasma PC was extensively oxidized to polar PC (results not shown). These results demonstrate that the concentration of sphingomyelin is constant during the oxidation of plasma and that any increase in lipid phosphorus in this spot is due to an increase in polar PC. Therefore, we subtracted the

sphingomyelin value of control (unoxidized) sample from all of the experimental samples to calculate the increase in polar PC.

When the plasma was oxidized with AAPH in the presence of DTNB, there was an increase in the concentration of lyso-PC as well as polar PC with a concomitant decrease in PC concentration (Fig. 2). When the PAF-AH activity was inhibited by Pefabloc (0.2 mM) during oxidation, the increase in lyso-PC was inhibited by 62%, and the concentration of polar PC was further increased by 44%, while the decrease in PC was only marginally affected. When LCAT was specifically inhibited by DFP (1 mM), the increase in lyso-PC was inhibited by 67%, and the accumulation of polar PC was increased by 30%, when compared with the plasma that was oxidized in the presence of DTNB alone. As shown above, the PAF-AH activity is almost fully functional at this concentration of DFP. When both LCAT and PAF-AH were inhibited during the oxidation, there was a further increase in the accumulation of polar PC, compared with either inhibitor alone. However, the effect on lyso-PC formation was not additive. A possible reason for this is the presence of some polar PC in the lyso-PC region (see below). These results show that not all of the hydrolysis of polar PC can be attributed to PAF-AH in human plasma and that LCAT (or another DFP-sensitive enzyme) may also contribute to the hydrolysis.

Further evidence for the involvement of more than one enzyme in the hydrolysis of polar PC was obtained from the oxidation of labeled 16:0–20:4 PC after its equilibration with plasma PC. The labeled PC, containing [^{14} C]arachidonate at the *sn*-2-position, was first equilibrated with endogenous PC by incubation at 37 °C in the presence of 2 mM DTNB. The plasma was then oxidized with 20 mM AAPH for 16 h at 37 °C, in the presence of various inhibitors, and the amount of radioactivity was determined in the lipid and water-soluble products. After the separation of oxidized lipids on TLC plate, four zones, corresponding to PC, sphingomyelin (polar PC), lyso-PC, and origin were counted, as described by Tanaka *et al.* (12). Since the label was present in the *sn*-2-acyl group, the radioactivity in the lyso-PC region is also due to polar PC products and not lyso-PC itself. Based on the R_F values reported by Tanaka *et al.* (12), we tentatively identified the polar PCs in the sphingomyelin region as the *sn*-2-monocarboxylate and *sn*-2- ω -hydroxymonocarboxylate products and those in the lyso-PC region and origin as the *sn*-2-dicarboxylate products. The counts in the latter two were combined for the presentation (Fig. 3). The radioactivity in the aqueous layer represents the products of hydrolysis of polar PCs. The results show that the radioactivity in labeled polar PC was significantly increased in the presence of either Pefabloc or DFP, as expected, while that in water-soluble products is decreased. The decrease in aqueous products was greater in the presence of Pefabloc, compared with

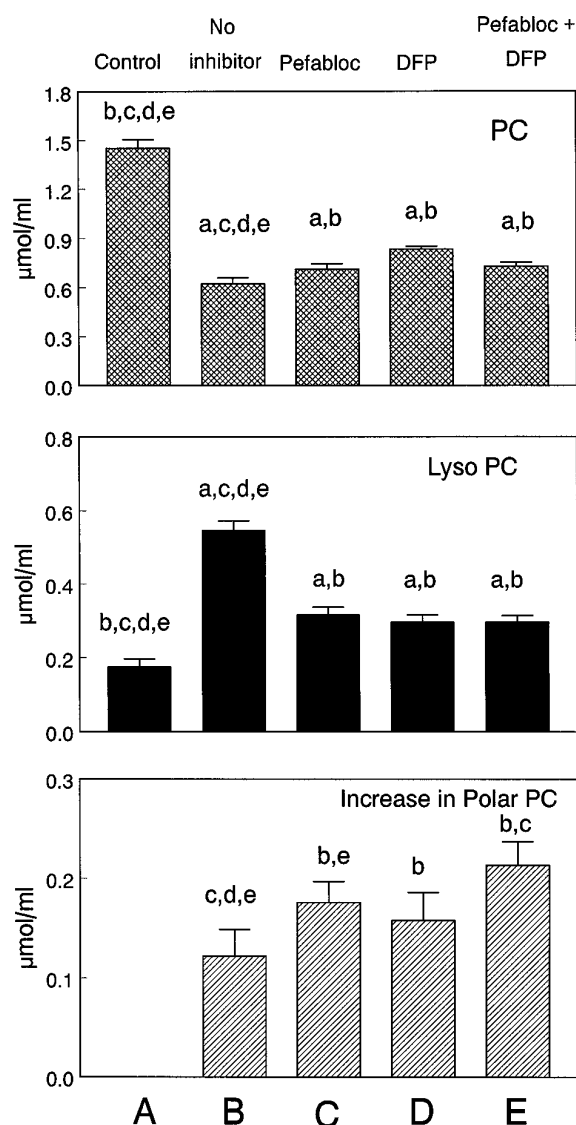


FIG. 2. Effect of selective inhibition of LCAT and PAF-AH on the accumulation of polar PC and lyso-PC. Normal human plasma was oxidized with 50 mM AAPH for 16 h in the presence of DTNB (2 mM), DFP (1 mM), and Pefabloc (0.2 mM) in various combinations as indicated. The oxidized lipids were separated on silica gel TLC and the spots corresponding to lyso-PC, sphingomyelin, and PC were quantitated by the estimation of lipid phosphorus. The sphingomyelin spot also contains the polar PC with the truncated acyl group. Since the amount of sphingomyelin itself does not change during oxidation, its value in unoxidized control was subtracted from the sphingomyelin value of the oxidized samples to calculate the increase in the polar PC concentration. All results shown are mean $\pm$ S.E. of six separate experiments. Statistical significance was calculated by a paired *t* test, comparing samples from the same experiment with each other. The lowercase letters above each bar show the presence of significant difference from the corresponding values at $p < 0.01$.

DFP. However, the combination of the two inhibitors did not decrease water-soluble radioactive products any further, although the increase in polar PC was additive. Since the combination of the two inhibitors resulted in significantly greater accumulation of polar PC than either inhibitor alone, these results show that both enzymes are involved in the hydrolysis of polar PCs. Similar results were obtained when the 2-acyl-labeled 16:0–18:2 was oxidized in the presence of whole plasma. However, the percentage of radioactivity appearing in the aqueous layer was much lower, and the percentage of label in the lyso-PC region was higher (results not shown).

The presence of label in the lyso-PC region indicates that

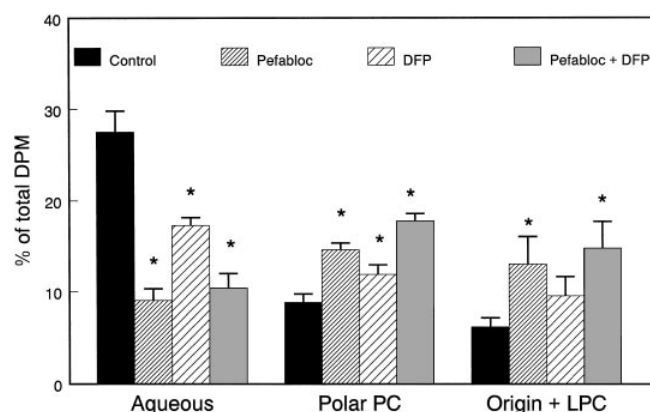


FIG. 3. Oxidation of 1-16:0-2-[1- ^{14} C]20:4 PC equilibrated with human plasma. Trace amounts of labeled 16:0–20:4 PC, containing [1- ^{14} C]arachidonate at the *sn*-2-position was added to normal plasma in ethanol (1% final concentration) and incubated at 37 °C for 1 h in the presence of 2 mM DTNB to equilibrate the label with endogenous PC. Then DFP (1 mM), Pefabloc (0.2 mM), or a combination of the two were added, followed by AAPH (20 mM, final concentration), and the samples were oxidized for 16 h at 37 °C. Control sample was oxidized in the presence of DTNB alone. The lipids were extracted (31) after acidification of the sample to pH 3.0 and separated on silica gel TLC. Spots corresponding to the origin, lyso-PC, sphingomyelin, and PC were scraped, and their radioactivity was determined in a liquid scintillation counter. The counts in the origin and lyso-PC spots were combined because of the incomplete separation of the two. Statistical significance between control and experimental samples ($n = 4$ each) was calculated by the paired *t* test (*, $p < 0.05$). Although not shown, the difference between Pefabloc alone and Pefabloc plus DFP was also significant for polar PC accumulation ($p < 0.05$). Differences between DFP alone and DFP plus Pefabloc were significant for aqueous, polar PC, and lyso-PC fractions ($p < 0.05$).

some of the lipid phosphorus estimated as lyso-PC in the previous experiment (Fig. 2) is actually polar PC, and we may have overestimated the lyso-PC in these experiments. This may account for the lack of complete inhibition of lyso-PC formation even in the presence of the two inhibitors, because the polar PC actually increases in the presence of inhibitors. This possibility is further supported by the recent findings of Tokumura *et al.* (37), who showed that the dicarboxylate derivatives of polar PC (which move with lyso-PC on TLC) accumulated only in the presence of DFP during oxidation of LDL.

Oxidation of Phospholipids in Plasma Deficient in PAF-AH—Cabot *et al.* (22) reported that PAF-AH activity is absent in the plasma of chicken and other avian species. However, the LCAT activity in these species is comparable with that in humans. The plasma from these species would therefore provide a convenient tool to study the possible role of LCAT in the metabolism of oxidized PC without the interference from the action of PAF-AH.

First, we established that labeled PAF is not hydrolyzed significantly by chicken plasma. As shown in Fig. 4, incubation of acetate-labeled PAF with chicken plasma did not result in appreciable hydrolysis of PAF. The PAF hydrolytic activity in chicken plasma was $<1\%$ of that found in human plasma. This residual activity may in fact be due to the LCAT itself, because we have shown that human LCAT can hydrolyze PAF, although at a slower rate than PAF-AH (9). This possibility is supported by the observation that unlike in human plasma, the PAF hydrolysis in chicken plasma was not inhibited by low concentration of Pefabloc (Fig. 5). The effect of Pefabloc on PAF hydrolysis resembled the effect on cholesterol esterification. The low PAF-AH activity in chicken plasma is not due to the presence of an inhibitor, because the addition of chicken plasma to human plasma did not result in an inhibition of the activity in the latter (results not shown).

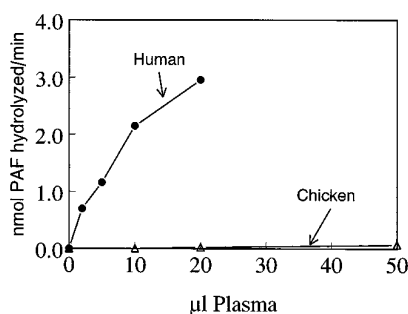


FIG. 4. **Assay of PAF-AH activity in human and chicken plasmas.** The indicated volume of fresh plasma was incubated with 32 nmol of [^{3} H]acetate-labeled PAF for 5 min (human) or 30 min (chicken) at 37 °C in a final volume of 0.4 ml. The extent of PAF hydrolysis was calculated from the radioactivity appearing in the water layer following the Bligh and Dyer extraction (9). Control tubes containing no enzyme were included to correct for any nonenzymatic hydrolysis. The results presented are averages of two separate experiments.

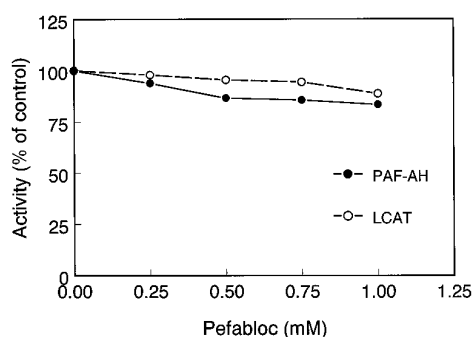


FIG. 5. **Effect of Pefabloc on PAF hydrolysis and cholesterol esterification in chicken plasma.** For the assay of PAF hydrolysis, 80 μ l of chicken plasma was used, and the incubation was carried out with 32 nmol of acetate-labeled PAF for 1 h at 37 °C. The activity in control (no inhibitor) was 12 nmol of PAF hydrolyzed/ml of plasma/h. For the assay of cholesterol esterification (LCAT), 50 μ l of plasma was incubated for 30 min with a proteoliposome substrate (30) and 2 mM mercaptoethanol. The LCAT activity in the control (no inhibitor) was 60 nmol of cholesterol esterified/ml of plasma/h. The results shown are averages of two separate experiments.

Next, we studied the effect of oxidation of chicken plasma on the various acyltransferase activities carried out by LCAT (Fig. 6). Oxidation with increasing concentrations of AAPH resulted in a progressive loss of LCAT activity (cholesterol esterification), as found in human plasma (28). The chicken enzyme appears to be relatively more resistant to oxidation than the human enzyme, possibly due to the absence of the sulfhydryl groups near the active site (38), because the modification of these SH groups by the products of oxidation is believed to be responsible for the loss of activity of human LCAT (39). There was also an activation of LAT II activity after oxidation, as seen in human plasma, indicating that this activity is not carried out by PAF-AH. These results show that the effects of oxidation on chicken plasma acyltransferase activities are similar to those on human plasma despite the absence of PAF-AH.

When the phospholipid composition of chicken plasma was analyzed after a 16-h oxidation by 50 mM AAPH in the presence of 2 mM DTNB, there was an increase in lyso-PC and polar PC, and a decrease in PC (Fig. 7). Although chicken LCAT lacks the two sulfhydryl groups present in the human enzyme (Cys³¹ and Cys¹⁸⁴) (38), 2 mM DTNB almost completely inhibited cholesterol esterification in chicken plasma, probably because of the presence of another sulfhydryl group (Cys²⁶) near the active site pocket that is derivatized by DTNB.² Since the oxidation

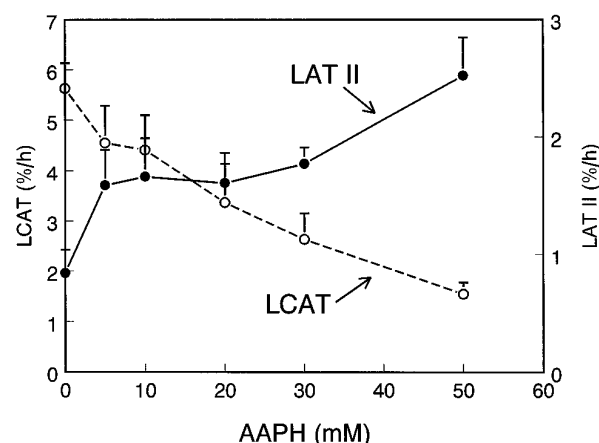


FIG. 6. **Effect of oxidation on LCAT and LAT II activities.** Human or chicken plasma was oxidized in the presence of 1 mM DTNB and varying concentrations of AAPH for 16 h at 37 °C. Aliquots of the oxidized plasma (20 μ l for LCAT and 100 μ l for LAT II) were then taken for the assay of enzyme activities as described under "Experimental Procedures." Values shown are mean $\pm$ S.E. of four experiments.

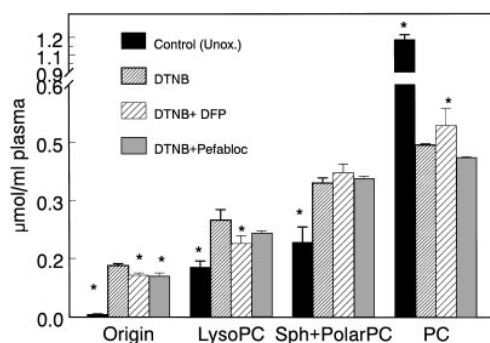


FIG. 7. **Effect of oxidation on chicken plasma phospholipid composition.** Chicken plasma was oxidized in the presence of 2 mM DTNB and 50 mM AAPH for 16 h. Where indicated, 1 mM DFP, and 0.2 mM Pefabloc were also included during oxidation. Total lipid extract was separated on TLC plates, and lipid phosphorus was determined in spots corresponding to lyso-PC, sphingomyelin (Sph) plus polar PC, and PC. The values shown are the mean $\pm$ S.E. of four separate experiments. Statistical significance of the difference between the DTNB-only sample and the rest was calculated by a paired *t* test (*, *p* < 0.05). The increase in lyso-PC and the decrease in PC were significantly affected by DFP but not by Pefabloc.

was performed in the presence of DTNB, the decrease in PC and increase in lyso-PC are not due to cholesterol esterification reaction but rather due to the hydrolysis of oxidized PC. The increase in lyso-PC was suppressed by DFP but not by Pefabloc. The apparent lack of complete inhibition of lyso-PC formation by DFP again may be due to the co-migration of some labeled polar PCs with lyso-PC on the TLC plate, as seen in human plasma. The accumulation of polar PC was increased by DFP and Pefabloc, although the increases were not statistically significant. The decrease in PC was significantly inhibited by DFP but not by Pefabloc. These results suggest that unlike in human plasma, PAF-AH contributes little to the hydrolysis of polar PCs in chicken plasma. Instead, it appears that LCAT (or another DFP-sensitive enzyme) carries out most of the hydrolysis.

Hydrolysis of Isolated Polar PC by Purified LCAT—While the above experiments showed that the hydrolysis of oxidized PC is not exclusively carried out by PAF-AH and that another enzyme that is more sensitive to DFP was also involved, they did not identify the putative enzyme as LCAT. To test the role of LCAT in this process directly, we first oxidized human plasma in the presence of DFP and Pefabloc and isolated the

² K. Wang and P. V. Subbaiah, unpublished data.

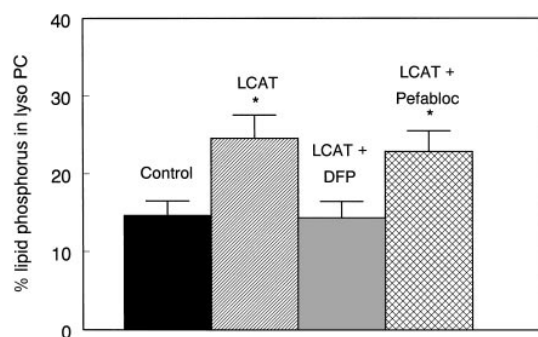


FIG. 8. **Hydrolysis of polar PC by purified LCAT.** Human plasma was first oxidized with 50 mM AAPH in the presence of 1 mM DFP and 0.2 mM Pefabloc for 16 h. The polar PC plus sphingomyelin spot from the oxidized plasma was eluted (31), and 250 nmol of the lipid was treated with purified chicken LCAT for 2 h at 37 °C in the presence or absence of inhibitors. The lipids were extracted and separated on TLC again, and lipid phosphorus was determined in lyso-PC and the polar PC regions. The percentage of total lipid phosphorus appearing in lyso-PC was calculated. Results presented are the mean $\pm$ S.E. of four experiments (*, $p < 0.05$ compared with control; paired t test).

polar PC formed by the elution of the sphingomyelin plus polar PC spot from the TLC plate. This mixture of sphingomyelin and polar PC (250 nmol of lipid phosphorus) was dispersed in Tris-HCl buffer, pH 7.4, and reacted with purified chicken LCAT for 2 h at 37 °C. Then the lipids were extracted and separated on a TLC plate, and the lipid phosphorus was determined in lyso-PC and polar PC spots. The percentage of total lipid phosphorus appearing in lyso-PC was calculated. As shown in Fig. 8, purified LCAT hydrolyzed the polar PC isolated from the oxidized plasma to lyso-PC, and this reaction was inhibited by DFP but not by Pefabloc. There was some lipid phosphorus in the lyso-PC region even in the absence of the enzyme (control), but it is not clear whether this was due to nonenzymatic breakdown of polar PC or to migration of some polar PC products in the lyso-PC region. Nevertheless, in all experiments, purified LCAT increased lyso-PC further at the expense of polar PC, and this increase was inhibited by DFP. When human apolipoprotein A-I was added at a concentration sufficient to activate the cholesterol esterification reaction (0.8 nmol/reaction), there was no additional hydrolysis of polar PC, indicating that this reaction does not require an apoprotein activator (results not shown). Since purified LCAT was obtained from chicken plasma rather than human plasma, the possibility of contamination with PAF-AH can be ruled out, and the absence of inhibition by Pefabloc further supports this conclusion.

Hydrolysis of Labeled Polar PCs Derived from the Oxidation of Synthetic PCs—As shown previously (28) (and Fig. 3), several polar PC molecules are formed by the oxidation of a single species of PC (16:0–20:4 PC). In the experiment described above, we used only the major product that co-migrates with sphingomyelin on the TLC plate. To determine whether LCAT can hydrolyze all of the polar PC products generated from the oxidation of various natural PCs, we isolated individual products of oxidation of synthetic labeled PCs in the absence of plasma and tested them as substrates for LCAT. *Sn*-2 acyl-labeled 16:0–20:4 PC, and 16:0–18:2 PC, the two major precursors of the short chain polar PCs formed during oxidation of the plasma, were separately dispersed in 5 mM sodium deoxycholate and oxidized with 50 mM AAPH for 2 h at 37 °C. The products formed were then separated on silica gel TLC plates with the solvent system of chloroform:methanol:water (65:25:4), and the spots corresponding to the origin (polar PC 1), lyso-PC (polar PC 2), and sphingomyelin (polar PC 3) were eluted (31). Each product (containing 15,000 dpm) was dispersed in Tris-NaCl-EDTA buffer at pH 7.4 and reacted with

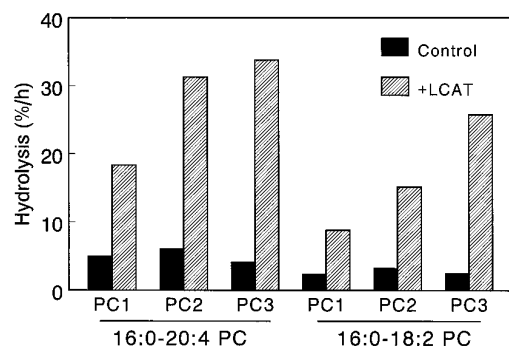


FIG. 9. **Hydrolysis of labeled polar PCs by purified LCAT.** 16:0–18:2 PC and 16:0–20:4 PC, labeled at the *sn*-2-acyl group, were first dispersed in 5 mM sodium deoxycholate and oxidized with 50 mM AAPH for 2 h at 37 °C. The total lipid extract was separated on silica gel TLC, and the spots corresponding to the origin (polar PC 1), lyso-PC (polar PC 2), and sphingomyelin (polar PC 3) were eluted. Each sample (containing 15,000 dpm and 60 nmol of lipid phosphorus) was then dispersed in Tris-NaCl-EDTA buffer and incubated with 50 μ l of purified chicken LCAT for 1 h at 37 °C (the activity of the enzyme was 9.0% cholesterol esterification in the standard proteoliposome assay). The lipids were extracted, and aliquots of the aqueous and chloroform layers were taken for radioactivity determination. The percentage of hydrolysis of the lipid was calculated from the percentage of total counts appearing in the aqueous layer. Control samples containing no enzyme were included for each polar PC. The results presented are averages of two experiments.

purified chicken LCAT for 1 h at 37 °C. The reaction mixture was extracted (31), and the radioactivity was determined in the aqueous and chloroform layers. The label in the aqueous layer represents the products of hydrolysis (short chain acyl group) of polar PC. As shown in Fig. 9, all of the polar PCs, derived either from the oxidation of 16:0–20:4 PC or 16:0–18:2 PC, were hydrolyzed by purified LCAT *in vitro*, although at varying rates. In both cases, the polar PC 3, corresponding to the spot co-migrating with sphingomyelin and containing products with a terminal methyl or hydroxyl function on the *sn*-2-acyl group (12), was hydrolyzed most rapidly. The small amount of “non-enzymatic hydrolysis” found in the control incubations (without the enzyme) may be due to the partition of some polar PC into the aqueous layer. These results provide further evidence that all of the polar PC products of lipoprotein oxidation are hydrolyzed by LCAT.

The effects of various inhibitors on the hydrolysis of labeled polar PC by purified chicken LCAT and human PAF-AH were tested to determine whether the differential inhibition of the two enzymes observed in whole plasma can be seen when a common substrate is used. For this experiment, the polar PC 3 fraction obtained from the oxidation of 16:0–18:2 PC was employed, but the same results were obtained with polar PC 3 obtained from the oxidation of 16:0–20:4 PC. As seen in Fig. 10, while both enzymes hydrolyzed the polar PC (control values were as follows: PAF-AH, 19.04% substrate hydrolyzed/h; LCAT, 13.95% substrate hydrolyzed/h), the reaction catalyzed by LCAT was strongly inhibited by 1 mM DFP but not by 1 mM Pefabloc, while that catalyzed by PAF-AH was only inhibited by Pefabloc but not by DFP. DTNB (2 mM), which inhibited the cholesterol esterification reaction of chicken LCAT by more than 90% (results not shown), inhibited the polar PC hydrolysis by the same enzyme by only 30%, suggesting that the hydrolysis of short chain acyl-ester is less susceptible to the steric hindrance caused by derivatization of the sulfhydryl groups by DTNB. Heating the enzyme preparations at 56 °C for 30 min destroyed the activity of LCAT but not of PAF-AH. These results confirm that both LCAT and PAF-AH are capable of hydrolysis of oxidized polar PCs and that the observed differ-

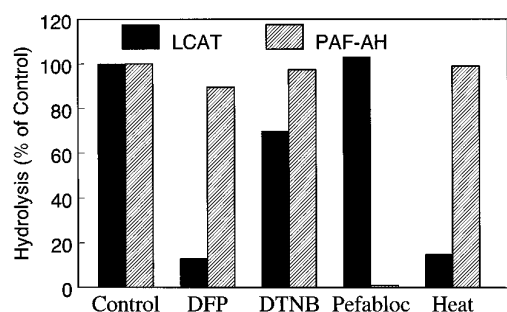


FIG. 10. **Effects of inhibitors on the hydrolysis of polar PCs by LCAT and PAF-AH.** Polar PC 3, obtained from the oxidation of labeled 16:0–18:2 PC was treated with partially purified LCAT (chicken) or PAF-AH (human) for 30 min in the presence of various inhibitors. The heat treatment of the enzymes was carried out at 56 °C for 30 min. All activities are expressed as percentage of the control values (without inhibitor). The control value for LCAT was 13.95% of substrate hydrolyzed/50 μ l of enzyme/h, while the control value for PAF-AH was 19.04% of substrate hydrolyzed/h/20 μ l of enzyme. All values are averages of two separate experiments.

ence in the inhibitor effects in whole plasma (Fig. 1) is not a function of the type of the substrate used for the assay.

DISCUSSION

The oxidation of LDL is believed to be a key early event in the development of atherosclerosis (10, 11). Several studies showed that a major end product of LDL oxidation is lyso-PC, which accumulates as a result of the hydrolysis of the oxidatively fragmented acyl group from the *sn*-2-position of PC (40, 41). Although lyso-PC itself has several biological effects, such as stimulation of monocyte chemotaxis (42), induction of adhesion molecules (43, 44), and impaired endothelium-dependent relaxation (45), its precursors, namely the oxidatively truncated PCs, have more varied and potent effects on vascular cells, probably because of their structural similarities to PAF (12, 14). Many of the biological effects of oxidized LDL, including the induction of monocyte chemotactic protein-1 (36), induction of adhesion molecules (46), and smooth muscle cell proliferation (17), have been attributed to these short chain polar PCs. Recent studies have also identified a submicromolar concentration of oxidatively fragmented PCs in normal human plasma (47). The hydrolysis of these potentially deleterious products to lyso-PC is therefore considered a defensive mechanism to protect the vascular cells (16). There is convincing evidence that the major enzyme responsible for this hydrolysis in human plasma is PAF-AH (16, 36). However, several lines of evidence suggest that not all of the hydrolysis of oxidized phospholipids in the plasma can be accounted for by the PAF-AH and that additional enzymatic mechanisms may exist: 1) PAF-AH is present on <1% of the LDL particles (16), and although it is proposed that it exchanges between various lipoprotein particles (16, 35), it is likely that not all of the LDL particles have access to the enzyme; 2) PAF-AH is inactivated rapidly during lipoprotein oxidation (25–27) and therefore may not be available for the hydrolysis of oxidized PC, when it is needed most; 3) some animal species, such as chicken, pigeon, and turkey, have been shown to have no PAF-AH activity in their plasma (22) and therefore should have other mechanisms to protect against the harmful effects of oxidized phospholipids and PAF; and 4) although a significant percentage of the Japanese population has been shown to completely lack PAF-AH in their plasma (23, 48), the clinical consequences of this deficiency appear to be rather mild, suggesting that back-up mechanisms exist for the detoxification of PAF and polar PCs in humans also. Several other enzymatic mechanisms have been proposed for the hydrolysis of oxidized polar PCs to lyso-PC. Thus, Parthasarathy and Barnett (49) reported the presence of

a phospholipase A intrinsic to LDL apoprotein B, but other studies suggested that this activity may be identical to PAF-AH (25, 34, 50). A calcium-dependent phospholipase A has been reported in human plasma, but it apparently does not hydrolyze lipoprotein PC (51, 52). This activity is probably due to an acute phase enzyme, rather than a constituent of normal plasma (53). Another potential source of hydrolytic activity against oxidized PC is paraoxonase, a phosphodiesterase associated with HDL (54, 55). However, unlike the reaction described here, this enzyme is not inhibited by DFP, and it is metal ion-dependent. Furthermore, it is primarily a phosphodiesterase, not an acyl-esterase, and its substrate does not appear to be the short chain PC (55). Therefore, it is unlikely that the observed formation of lyso-PC from polar PC in the present study is due to this enzyme.

Although our earlier studies showed that LCAT exhibited phospholipase A activity toward PAF, its activity was very low compared with that of PAF-AH (9). To determine the possible role of LCAT in the metabolism of PAF and oxidized PC species, it is necessary to block the activity of the more powerful PAF-AH. Until recently, all the inhibitors shown to inhibit the PAF-AH have also been known inhibitors of LCAT. For example, while DFP and phenylmethylsulfonyl fluoride inhibit PAF-AH activity, the concentrations required are very high (up to 10 mM) (34–36), and they completely inhibit LCAT activity at a much lower concentration (<1 mM). A recent report by Dentan *et al.* (33) showed that a new serine protease inhibitor, Pefabloc, inhibited PAF-AH at a much lower concentration. We therefore tested whether this inhibitor affects the LCAT activity and found that it has no effect below 1 mM concentration. This provided a valuable tool in determining the role of LCAT without interference from PAF-AH. The active sites of both of these enzymes have the Gly-X-Ser-X-Gly motif common to several mammalian and nonmammalian lipases (56, 57), but the differential effects of DFP and Pefabloc suggest that the environment around the active site serine is different for the two enzymes. Thus, it is possible that the active site serine of LCAT is surrounded by more hydrophobic residues, and DFP, being more hydrophobic than Pefabloc, can gain access to it more readily. On the other hand, the active site serine of PAF-AH is probably in a less hydrophobic environment; therefore, Pefabloc can react with it at a low concentration. It may be pointed out that the natural substrate of PAF-AH (PAF) is considerably less hydrophobic than the normal substrate for LCAT, namely the long chain PC.

We have suggested previously that the dramatic loss of cholesterol-esterifying activity of LCAT, but not its ability to transfer or hydrolyze short chain acyl groups, following the oxidation of plasma is due to a selective loss of the interfacial binding properties of the enzyme (28). The results presented here further support this hypothesis. First, the hydrolysis of polar PC by the enzyme is not activated by apoprotein A-I, and since the apoprotein is believed to increase the interaction of the enzyme with the interfacial substrate, but not with the monomeric substrate (57), it is unlikely that an interfacial binding is involved. Second, the lack of inhibition of polar PC hydrolysis by DTNB suggests that the derivatization of cysteine groups blocks only the hydrolysis of long chain ester linkages. It is likely that the cysteine SH groups are also derivatized by lipid oxidation (39), and this results in selective loss of long chain ester hydrolysis due to steric hindrance. It is well accepted that the sulfhydryl groups are not essential for the catalysis (7, 58); therefore, the short chain polar PC hydrolysis is not affected.

HDL is known to protect LDL against oxidative modification as well as minimize the effects of oxidized LDL on the cells (36, 59, 60). It has been suggested that most of this protection by

HDL is because of the presence of PAF-AH, which hydrolyzes the oxidized PC products, and abolishes the deleterious effects of oxidized LDL (36). However, in view of the present data, the possibility should be considered that part of the protection by HDL is due to the presence of LCAT, because most of the plasma LCAT is associated with HDL. The high concentration of DFP used in the previous studies to inhibit the PAF-AH activity of HDL (up to 10 mM) also would have completely inhibited the LCAT activity. Furthermore, since it has been reported that the PAF-AH associated with HDL is actually inactive under *in vivo* conditions (16), the observed beneficial effects of added HDL may have been due to LCAT instead of PAF-AH. It is necessary to explore this possibility by using Pefabloc in place of DFP to selectively inhibit PAF-AH. Klimov *et al.* (61) have also reported that the addition of large amounts (450 $\mu\text{g/ml}$) of purified LCAT to LDL prevented peroxidation of LDL by Fe^{2+} , although the mechanism of this protection is unknown. These authors speculated that LCAT stabilizes the LDL particle by transferring the unsaturated fatty acids from the surface phospholipids to the core cholesteryl esters. A recent preliminary report (62) suggested that LCAT exhibits a hydroperoxidase activity and thereby prevents lipid peroxide accumulation in the lipoproteins. However, this activity is unrelated to the hydrolytic activity of the enzyme reported here.

If the rapid hydrolysis of oxidized PCs in the plasma by the PAF-AH is essential for the protection against inflammatory and atherogenic effects of these compounds, then the complete absence of this activity, as reported in some Japanese subjects (23), should have resulted in more severe symptoms than observed. Many of the PAF-AH-deficient patients do not have any clinically apparent symptoms, suggesting that other enzymatic mechanisms such as LCAT (and paraoxonase) may be operative in addition to PAF-AH. While it is difficult to assess the quantitative contribution of LCAT to the overall hydrolysis of PAF and oxidized phospholipids *in vivo* in normal plasma, it is reasonable to assume that LCAT plays an important role in patients with PAF-AH deficiency.

The PAF-AH activity is also absent in the avian plasma, although the cDNA for the enzyme has been identified in chicken tissues (63). Furthermore, these species are also deficient in paraoxonase, another putative hydrolase of oxidized PC (64). It is not known, however, that they are more susceptible to inflammatory diseases compared with the species containing plasma PAF-AH activity, indicating that LCAT may be more important in detoxifying oxidized PCs in these species. The results presented here also show that the effects of oxidation on plasma phospholipid composition are similar in humans and chickens, including the formation of lyso-PC at the expense of PC and polar PC. Furthermore, the stimulation of LAT II activity, with a concomitant loss of cholesterol esterification, shows that the effects of oxidation on LCAT specificity are also the same in both species and that the LAT II reaction is due to LCAT, not PAF-AH.

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**Novel Function of Lecithin-Cholesterol Acyltransferase: HYDROLYSIS OF
OXIDIZED POLAR PHOSPHOLIPIDS GENERATED DURING LIPOPROTEIN
OXIDATION**

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