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Inhibition of lecithin cholesterol acyltransferase by phosphatidylcholine hydroperoxides

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Abstract To gain insight into the nature of the lecithin-cholesterol acyltransferase inhibitory factor(s), we separated and collected the oxidation products from oxidized lipoproteins after lipoxygenase treatment. Isolated fractions identified by chemiluminescence, as hydroperoxides of phosphatidylcholine, were found to produce a significant reduction of lecithin-cholesterol acyltransferase activity. The reaction kinetics of lecithin-cholesterol acyltransferase with reconstitued high density lipoproteins were studied in the presence of 0.6 and 1.2 μM hydroperoxides of phosphatidylcholine. No significant changes in the apparent V_{max} were observed but a concentration-dependent increase in slope of the reciprocal plots and in the apparent K_m values was observed with increasing hydroperoxide concentrations. These results show that the active site of lecithin-cholesterol acyltransferase is not affected by the presence of phosphatidylcholine hydroperoxides. Nevertheless, hydroperoxides of phosphatidylcholine altered the reactivity of lecithin-cholesterol acyltransferase for reconstitued high density lipoproteins suggesting either an alteration of the binding of lecithin-cholesterol acyltransferase to the reconstitued high density lipoproteins or a competitive inhibition mechanism.

Key words: Lecithin-cholesterol acyltransferase; Hydroperoxide; Phosphatidylcholine; Enzyme inhibition

1. Introduction

It is now generally believed that the oxidative modifications of plasma lipoproteins enhance their atherogenicity [1]. Oxidized low density lipoproteins (LDL) are taken up by the scavenger receptor mechanism, resulting in cholesterol accumulation and subsequent foam cell formation. High density lipoproteins (HDL) serve a protective role in inhibiting the progression of atherosclerosis. First, HDL play a crucial role in the removal of cholesterol from peripheral tissues and its transfer to the liver [2]. Secondly, HDL protect LDL from oxidative damage, possibly through the exchange of oxidized phospholipids (PL) from LDL to HDL or through competitive oxidation between HDL and LDL phospholipids [3].

However, lipid peroxidation products carried on HDL could impair the ability of HDL to promote cholesterol efflux [4] and lecithin-cholesterol acyl transferase (LCAT) activation [5], two key processes in the reverse cholesterol transport.

Recently, Bielicki et al. [6] have observed that minimally oxidized LDL were potent inhibitors of the LCAT activity and they supposed that the inhibition was due to a lipophilic oxidation product. Moreover, Subbaiah and Liu [7] showed that LCAT activity was inactivated by polar phospholipids containing short chain acyl groups at the *sn-2* position (short chain polar phosphatidylcholine), which were produced during phospholipid peroxidation by chain fragmentation of phosphatidylcholine hydroperoxide derivatives.

We used a high performance liquid chromatography (HPLC) procedure to separate and collect the oxidation products from oxidized lipoproteins. To clarify the role of these products in the inhibition of LCAT, we prepared reconstitued HDL (rHDL). This substrate consists of the mixture of egg-phosphatidylcholine (egg-PC), cholesterol and purified apolipoprotein A-I (apoA-I). We studied the reaction kinetics of LCAT with the rHDL in the presence of isolated fractions of oxidized PL.

2. Materials and methods

HPLC grade solvents and analytical grade chemicals were from Carlo Erba (Milano, Italy) or from Merck (Darmstadt, Germany). Microperoxidase, isoluminol (6-amino-2,3-dihydro-1,4-phthalazinedione) and soybean lipoxygenase (type IB, EC 1 13 11 12), egg-PC, cholesterol and defatted bovine serum albumin were from Sigma (St. Louis, MO, USA). Nordihydroguaiaretic acid (NDGA) was from TEBU-Biomol (USA).

2.1. Chromatographic equipment

The HPLC equipment (Thermo Separation Products, Les Ulis, France) included an automatic injector with a 200 μl sample loop, a 250×4.6 mm C18 and a 150×4.6 mm C8 Kromasil 5 μm (Touzart et Matignon, Les Ulis, France) analytical columns and a UV-visible light detector (Thermo Separation Products, Les Ulis, France). The fluorometer (Spectroflow 980 fluorescence detector, Applied Biosystems, Ramsey, NJ, USA) was equipped with a 5 μl flow cell.

2.2. Isolation of LDL from human plasma

Blood was collected from healthy individuals into tubes containing EDTA (Becton Dickinson, Rutherford, NJ, USA) and samples were chilled to 4°C. Plasma was obtained after low speed centrifugation (1000×*g*) to remove blood cells. Low density lipoproteins (LDL, d 1.019–1.063 g/ml) were isolated by preparative, sequential ultracentrifugation [8] and dialyzed (at 4°C) against 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl.

2.3. LDL oxidation

Oxidation of LDL (1.6 g/l LDL phospholipids) was performed with soybean lipoxygenase (type IB, EC 1 13 11 12, Sigma, St. Louis, MO, USA), dissolved in 0.1 M borate buffer, pH 9.2, (400 mg/l). The mixture was incubated with gentle shaking at 37°C for 16 h. The oxidation was stopped by addition of NDGA (60 μM).

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2.4. Isolation of oxidized phospholipid products

The major products of lipid oxidation were isolated for their subsequent analysis as potential substrates for LCAT inhibition. For this purpose, the lipids were extracted from oxidized LDL. Hydroperoxides from free fatty acids, PC and cholesterol ester (CE), short chain polar phosphatidylcholine and non-oxidized molecular species from PC and CE were extracted with methanol and hexane (2:10, v/v). The mixture was partitioned between the hexane layer (upper phase containing CE) and the methanol/water layer (lower phase containing PL and free fatty acids). These layers were separated by centrifugation at 1500×*g* for 5 min. The two layers were collected and evaporated to dryness under a nitrogen stream. The dried lipid residues were then dissolved in 200 μl methanol and injected into the HPLC system. Phospholipid separation was done using two serial analytical columns, a C8 (150×4.6 mm) and a C18 (250×4.6 mm) Kromasil 5 μm, maintained at 40°C. The mobile phase was 6% 10 mM ammonium acetate, pH 5 and 94% methanol (flow rate 1.5 ml/min) as previously described by Laureaux et al. [9]. Cholesteryl ester separation was performed with a 250×4.6 mm C18 Kromasil column, the mobile phase was 100% methanol. Molecular species of PL and CE were detected at 205 nm and the eluate was then mixed with the chemiluminescence reagent prepared as described by Yamamoto et al. [10] with slight modifications (55 mg/l isoluminol dissolved in 0.1 M borate buffer, pH 9.2 and 10 mg/l microperoxidase) to specifically quantify hydroperoxides from PC, CE and free fatty acids (flow rate 1 ml/min). The solution was passed through a fluorometer used as a photon detector with the excitation source turned off. Identification of molecular species of PC and CE and their corresponding hydroperoxides was realized as previously described by Therond et al. [11]. From each extract of lipoxygenase-oxidized LDL, three fractions of oxidized lipids were obtained after the HPLC separation and collected in glass vials. The solvent was evaporated under a gentle stream of nitrogen. Each fraction from PC and CE was redissolved in 250 μl methanol and kept frozen at −75°C until use.

2.5. Purification of apoA-I and LCAT

Human apoA-I used in the preparation of the complexes was purified from plasma of healthy donors as previously described by us [12]. LCAT was purified as previously described from 1 l of human serum [13].

2.6. Preparation of discoidal apoA-I-egg-PC-cholesterol complexes used as a substrate for the LCAT activity measurement

The activity of LCAT was assessed using a synthetic substrate that consisted of recombinant complexes of apoA-I with egg-PC and cholesterol that were formed using the sodium cholate dialysis procedure of Matz and Jonas [14]. ApoA-I:egg-PC:cholesterol molar ratios of 1:120:6 (initial ratio) were used assuming a molecular weight of 28 000, 780 and 387 for apoA-I, egg-PC and cholesterol, respectively. Radiolabelled [^{3}H]cholesterol (Amersham, Les Ulis, France) was incorporated into the particle preparation at a ratio of 2 μCi/μM of cholesterol. The diameters of rHDL were estimated by non-denaturing 8–25% polyacrylamide gradient gel electrophoresis (Pharmacia PHAST gradient gel electrophoresis).

2.7. LCAT reaction kinetics

Initial velocity (V_o) determinations of enzymatic activity were performed in capped glass tubes. The incubation mixtures contained 1.5–36 nmol of cholesterol in complexes with apoA-I and egg-PC, 2 mg of defatted bovine serum albumin, 4 mM mercaptoethanol, 1.25 U of LCAT activity (1 unit=1 nmol of cholesterol ester (CE) formed/h) in a final volume of 0.5 ml of 1 mM EDTA, 1 mM NaN_3, 10 mM Tris-HCl buffer, pH 7.4. 5 or 10 μl of each isolated fraction containing oxidized lipids dissolved in methanol, were mixed with the reaction mixture and incubated at 37°C in a metabolic shaker during 1 h to assay the LCAT activity. The reactions were stopped and the lipids were extracted with chloroform/methanol (2/1, v/v) according to the procedure of Folch et al. [15]. Carrier cholesterol and CE (∼100 μg each) were added to the organic solution of the lipids prior to drying. CE was separated from unesterified cholesterol using thin layer silicic acid chromatography and was quantified by scintillation counting. Results were expressed as nmol CE formed per h. Under our conditions, less than 20% of the cholesterol was esterified, ensuring that initial velocity values were being measured. Determination of the apparent maximal initial velocity (app V_{max}) and apparent Michaelis-Menten constant (app K_m) was performed using a Lineweaver-Burk plot of the kinetic data.

3. Results

To identify the nature of the inhibitor for the LCAT reaction produced during lipid peroxidation, we extracted lipids from oxidized LDL and we separated the oxidized phospholipids and sterol fractions on a reverse phase HPLC. When incubated with the reaction mixture to assay the LCAT activity, none of the sterol fractions recovered in the hexane layer, produced a reduction in the LCAT activity after 1 h of incubation (data not shown).

Fig. 1 shows the HPLC patterns from phospholipids extract monitored by UV absorption at 205/234 nm before and after lipoxygenase treatment (respectively Fig. 1A and B). Fig. 1C shows the specific detection (after mixing with the chemiluminescence reagent) of hydroperoxides corresponding to the peaks absorbing at 234 nm. Three major fractions were collected. These fractions were characterized by their retention times, their UV light absorption and their chemiluminescence detection. Fraction I, eluted between 0 and 4 min, presented a strong absorption at 234 nm and was detected by the chemiluminescence system. This fraction was identified as free fatty acid hydroperoxides coming from linoleic acid or arachidonic acid (13 (S) HPODE and 15 (S) HPETE, respectively). Fraction II (elution time between 4 and 9 min), contained peaks which absorbed at 234 nm but were not detectable by the chemiluminescence reaction. We could speculate that this fraction contained short chain polar PC (e.g. esterified conjugated enols at the *sn-2* position) resulting from the chain fragmentation of PC hydroperoxides. Fraction III, eluted between 9 and 22 min, contained peaks which strongly absorbed at 234 nm and were detectable by the chemiluminescence reaction. These peaks were identified as the hydroperoxides coming from 16:0-18:2, 16:0-20:4, 16:0-22:6 PC and 18:0-18:2, 18:0-20:4, 18:0-22:6 PC (PCOOH).

When incubated with the reaction mixture to assay LCAT activity, only the fraction III, corresponding to PCOOH, led to a 40% decrease in the LCAT activity after 1 h of incubation.

For the study of the activity of LCAT in the presence of PCOOH, we used a synthetic substrate (rHDL). The size of the rHDL was showed to be monodispersed and centered around 98 Å. The molar composition of these complexes was determined and the final composition of apoA-I:egg-PC:cholesterol was ∼1:99:4.

Fig. 2 shows the Lineweaver-Burk plots for the LCAT reaction with rHDL in the absence or in the presence of PCOOH. The maximal initial velocity (V_{max}) was not affected by the presence of 0.6 μmol/l of PCOOH but we observed an increase of the app K_m from 1.5×10^{-6} M to 2.4×10^{-6} M. In the presence of 1.2 μM PCOOH, the app K_m increased to 4.2×10^{-6} M and furthermore, we observed a small decrease of the V_{max} from 1 nmol CE/h to 0.93 nmol CE/h. We also determined an apparent inhibition constant K_i ($K_i = 5\times10^{-7}$ M) using the following equation app K'_m = app $K_m\times(1+[I]/K_i)$ (see the insert in Fig. 2). In this expression, K_m is the apparent Michaelis constant in the absence of the inhibitor, K'_m is the apparent Michaelis constant in the presence of different inhibitor concentrations, [I] is the inhibitor concentration, K_i is the apparent dissociation constant of the enzyme-inhibitor complex.

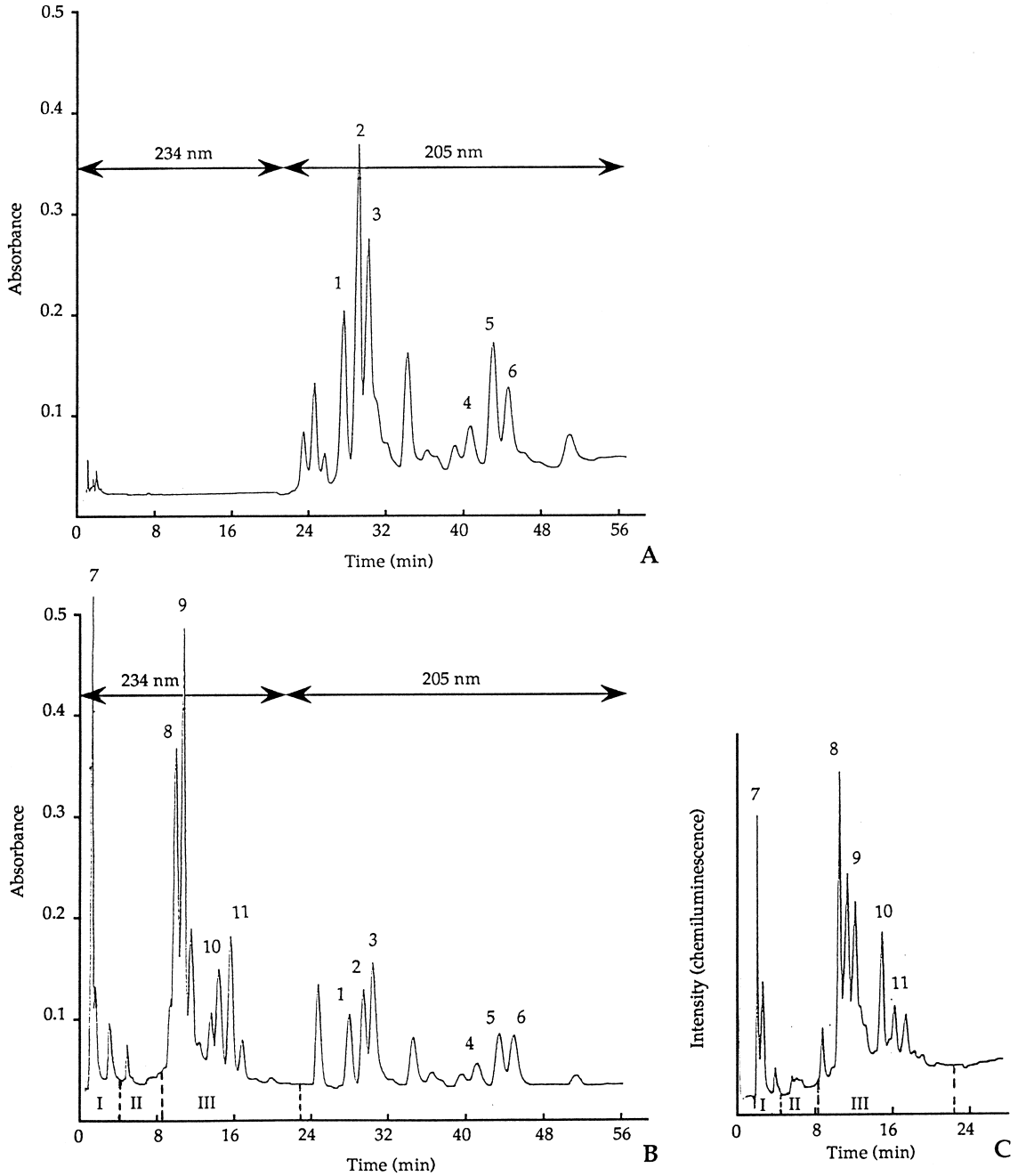

Fig. 1. HPLC elution profile of phospholipid molecular species and their corresponding hydroperoxides in LDL before lipoxygenase treatment (A) and after lipoxygenase treatment (B) using two serial Kromasil C8 and C18 analytical columns at 40°C. The mobile phase was 6% 10 mM ammonium acetate, pH 5 and 94% methanol (flow rate 1.5 ml/min). Eluates were monitored by UV absorbance at 234 nm until 22 min to detect conjugated dienes and then at 205 nm to detect the fatty acid molecular species. Hydroperoxides were identified with the chemiluminescence reagent (C). The numbered peaks represent (1) PC 16:0/22:6, (2) PC 16:0/20:4, (3) PC 16:0/18:2, (4) PC 18:0/22:6, (5) PC 18:0/20:4, (6) PC 18:0/18:2. Peak 7 containing free fatty acid hydroperoxides (mainly linoleic acid hydroperoxide) was fraction I. Fraction II contained short chain polar PC as tentatively identified by Subbaiah et al. [7], based on their chromatographic behavior. Peak 8 contained PCOOH 16:0/18:2, peak 9 contained two PCOOH compounds identified as PCOOH 16:0/20:4 and PCOOH 16:0/22:6, peak 10 contained PCOOH 18:0/18:2 and peak 11 contained PCOOH 18:0/20:4+18:0/22:6, they were collected together as fraction III. 16:0/18:2 PC, 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine; 16:0/20:4 PC, 1-pamitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine; 16:0/22:6 PC, 1-pamitoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine; 18:0/18:2 PC, 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphocholine; 18:0/20:4 PC, 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine; 18:0/22:6 PC, 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phospho-choline.

4. Discussion

Two recent studies have reported the inhibitory effect of lipoperoxidation products on the LCAT activity. Bielicki et al. [6] have observed that a lipophilic oxidation product, contained in minimally oxidized LDL, inhibited the LCAT activity. Subbaiah et Liu [7] have reported that the short chain polar PC, produced during lipoperoxidation, could inhibit the cholesterol esterification by modifying the interfacial binding domain of LCAT. In our study, we have extracted phos-

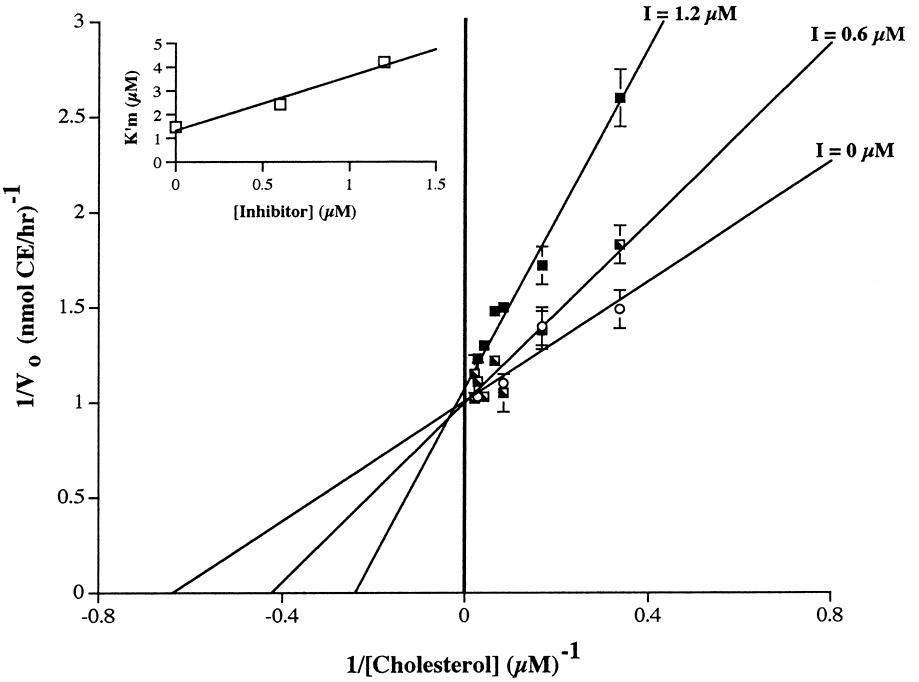

Fig. 2. Lineweaver-Burk plots of LCAT reaction kinetics with rHDL in the presence of increasing concentrations of the inhibitor in the medium. PCOOH were incubated at a final concentration of 0.6 μM or 1.2 μM, for 1 h, with the reaction mixture to assay the LCAT activity (37°C). V_o is the initial reaction velocity measured at 37°C. All data are expressed as mean ± S.D. of triplicate samples. When not shown, the S.D. is smaller than the size of the symbols. The insert shows the variation of apparent K'_m with PCOOH concentrations in order to obtain the inhibition constant, K_i.

pholipids from oxidized LDL and we have isolated among the polar products of oxidized phospholipids those presenting free fatty acids with a hydroperoxide function, short chain polar PC and PC with a hydroperoxide function on the poly-unsaturated fatty acid located at the *sn-2* position of PC. With the lipoxygenase treatment, PCOOH were mainly produced while the other more polar products such as free fatty acid hydroperoxides and short chain polar PC were poorly produced. Moreover, among the fractions isolated from oxidized lipids, only the fraction with PCOOH produced a significant inhibition in the LCAT activity.

We examined the reactivity of rHDL particles with LCAT to investigate the effects of the PCOOH on LCAT reaction kinetics.

The reaction of LCAT with rHDL involves multiple steps: the association of LCAT with lipid interface, followed by activation by apolipoproteins, binding of lipid substrate(s) to the active site and subsequent catalytic events. LCAT utilizes phospholipids and unesterified cholesterol for cholesterol ester formation on HDL. The active site of LCAT contains a catalytic triad of aspartic acid, histidine and serine residues [16,17]. Although the three dimensional structure of LCAT has not been determined, the presence of separate catalytic and interfacial domains may be inferred for this enzyme [16,18,19]. The possible roles of PCOOH are reflected in the kinetic parameters of the LCAT reaction.

As the V_{max} was weakly changed by the presence of increased PCOOH concentrations, we could conclude that PCOOH did not modify amino acids in the active site of the enzyme. On the other hand, we observed changes in the slopes of the Lineweaver-Burk plots (Fig. 2) and app K_m with increasing PCOOH concentrations. Many factors, size, morphology, phospholipid composition and apolipoprotein composition, have been shown to modulate the LCAT activity when substrate analogs such as proteoliposomes or rHDL are used [16]. The close interrelation of all these factors makes it difficult to examine one of these parameters. In the present work, by using rHDL of a defined composition and by the introduction of a very low amount of PCOOH during the incubation, we avoided major changes in the particle size and total phospholipid content and therefore in the conformation of apoA-I. We observed changes in the slopes of the Lineweaver-Burk plots (Fig. 2) and app K_m with increasing PCOOH concentrations suggesting that PCOOH compete with PC for the active site or alter the binding of LCAT to the rHDL surface. Indeed, due to the hydrophilic nature of the hydroperoxides, PCOOH may have direct access to the active site of LCAT and act as a competitor with PC molecules. The variations in app K'_m values could also reflect, as previously reported by Bolin and Jonas [20,21], the affinity of LCAT for rHDL. However, whatever the substrate concentration used in this work, the inhibitor concentration in the interface represents less than 1% molar ratio PCOOH/PC. Thus, the addition of PCOOH does not change the interfacial lipid composition of the rHDL and cannot affect significantly the binding of the LCAT to the lipid interface. Nevertheless, we have also to take into account that PCOOH could react with a domain of LCAT that is involved in the interfacial recognition or with a domain of apoA-I that is implicated in the interfacial binding with the enzyme. Indeed, on the one hand, PCOOH could modify the free cysteine residues of LCAT [22] or could react with the free amino group of proteins as previously reported by Fruebis et al. [23] and lead to the formation of Schiff base or cross-linking of proteins. On the other hand, Garner et al. have recently reported [24] the role for methionine residues of apoA-I and apoA-II in

reducing PCOOH to PC hydroxides. As the Met residues are located within the known lipid binding domains of these apolipoproteins, the oxidation of Met residues of apoA-I could affect their ability to interact with lipids, which is crucial for activation of LCAT. Thus, the inhibition of LCAT obtained with PCOOH could be linked to oxidative modifications of apoA-I and this possibility could also explain the results of Bielicki et al. [6] which reported cross-linking of apoA-I molecules induced by copper-oxidized LDL associated with a reduced LCAT activity.

Our study confirms that LCAT activity is inhibited by oxidized phospholipids that we have further characterized as hydroperoxides of PC. Thus, the accumulation of PCOOH in oxidized lipoproteins could contribute to impair the reverse cholesterol transport and pre-dispose to the development of atherosclerosis.

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