Inhibition of lecithin:cholesterol acyltransferase activity by synthetic phosphatidylcholine species containing eicosapentaenoic acid or docosahexaenoic acid in the *sn*-2 position

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Abstract Phospholipids isolated from the plasma of monkeys fed a diet enriched in fish oil were poor substrates for cholesteryl ester (CE) synthesis by the lecithin:cholesterol acyltransferase (LCAT) reaction relative to those from animals fed a lard containing diet when the phospholipids were used for the preparation of recombinant particles by cholate dialysis (Parks, J. S., B. C. Bullock, and L. L. Rudel. 1989. *J. Biol. Chem.* **264:** 2545–2551). The purpose of the present study was to directly test the influence of eicosapentaenoic acid (20:5 n-3) and docosahexaenoic acid (22:6 n-3) in the *sn*-2 position of phosphatidylcholine (PC) on the activity of LCAT. PC species containing 1-palmitoyl-2-oleoyl PC (POPC), 1-palmitoyl-2-linoleoyl PC (PLPC), 1-palmitoyl-2-arachidonoyl PC (PAPC), 1-palmitoyl-2-eicosapentaenoyl PC (PEPC), or 1-palmitoyl-2-docosahexaenoyl PC (PDPC) were purchased or synthesized and made into recombinant particles of uniform size and composition with [^{14}C]cholesterol and apoA-I using the cholate dialysis procedure. The recombinant particles (PC:cholesterol:apoA-I molar ratio = 42:1.9:1) exhibited the following order of reactivity towards purified human LCAT in vitro: POPC > PLPC > PEPC = PAPC > PDPC. The apparent V_{max}/K_m for recombinant particles containing PEPC and PDPC was 17% and 7% that of particles containing POPC, respectively. There was a linear decrease in CE formation when the percentage of PEPC or PDPC was increased from 0 to 100% relative to POPC in recombinant particles with a constant PC:cholesterol:apoA-I molar ratio, suggesting that the PEPC and PDPC were competitive inhibitors of the LCAT reaction. The phospholipase activity of LCAT was measured on monolayers of POPC, PEPC, and PDPC at a constant surface pressure of 25 mN/m using a zero order reaction trough. The hydrolysis rates for POPC, PEPC, and PDPC by LCAT were 2.5, 1.1, and 0 nmol/ml LCAT preparation per min (n = 2), respectively, and were inversely related to the mean molecular area at the argon–water interface of the PC species (72, 79, and 86 Å^2 per molecule, respectively). We conclude from these studies that PEPC and PDPC were less reactive with LCAT than POPC or PLPC. The decreased reactivity of PEPC and PDPC with LCAT appears related to the increased molecular surface area and/or conformation of these molecules. The data also suggest that PEPC and PDPC are competitive inhibitors of the phospholipase A_2 half of the LCAT reaction. The studies suggest that enrichment of plasma phospholipids with n-3 fatty acids may result in decreased plasma CE synthesis by LCAT.—**Parks, J. S., T. Y. Thuren, and J. D. Schmitt.** Inhibition of lecithin:cholesterol acyltransferase activity by synthetic phosphatidylcholine species containing eicosapentaenoic acid or docosahexaenoic acid in the *sn*-2 position. *J. Lipid Res.* 1992. **33:** 879–887.

Supplementary key words recombinant particles • cholesterol • apoA-I • n-3 fatty acids • monolayers

Lecithin:cholesterol acyltransferase (LCAT) is a 416 amino acid glycoprotein that transesterifies the *sn*-2 fatty acid of a phospholipid molecule to the 3-β-hydroxyl group of cholesterol resulting in two products, lysophosphatidylcholine (lysoPC) and cholesteryl ester (CE) (1, 2). The enzyme functions at the lipid–water interface of lipoprotein particles and can be found associated with lipoprotein particles including plasma HDL, LDL, nascent discoidal HDL (secreted by the liver and intestine or generated from redundant surface of chylomicron particles during lipolysis) and recombinant HDL, which mimic the composition and size of nascent HDL (3). LCAT activity is essential for the maturation of nascent HDL particles to spherical plasma HDL and for the maintenance of normal lipoprotein particle structure. ApoA-I, the major

Abbreviations: HPLC, high performance liquid chromatography; LCAT, lecithin:cholesterol acyltransferase; PC, phosphatidylcholine; CE, cholesteryl ester; apo, apolipoprotein; HDL, high density lipoprotein(s); LDL, low density lipoprotein(s); PL, phospholipid; TLC, thin-layer chromatography; BHT, butylated hydroxytoluene; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PLPC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; PEPC, 1-palmitoyl-2-eicosapentaenoyl-*sn*-glycero-3-phosphocholine; PDPC, 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine.

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apolipoprotein of HDL, serves as the preferred cofactor for the LCAT reaction (3).

There are ample data to show that LCAT activity is influenced by the type of substrate with which it interacts. The substrate effects can be divided into those involving the macromolecular particle (i.e., lipoprotein particle) and those involving monomeric substrates (i.e., phospholipid and cholesterol). With regard to lipoprotein particle effects, discoidal particles are more reactive than spherical HDL particles and smaller discoidal and spherical particles are more reactive than larger ones (3). In addition, Pownall, Pao, and Massey (4) and Jonas et al. (5) have shown that the bulk matrix lipid of the substrate particles can affect activity as well as the chain length and degree of saturation of PL fatty acids and the PL head group. Thus, numerous factors have been shown to influence LCAT activity although, in most cases, the exact nature of the substrate effects on activity are not well understood.

In a previous study we found that phospholipids isolated from the plasma of African green monkeys fed a diet enriched in fish oil were poor substrates for the LCAT reaction compared to those from animals fed saturated fat when the phospholipids were used for the preparation of recombinant particles by the cholate dialysis procedure (6). The results of the study suggested that n-3 fatty acids were poorly utilized by LCAT for the generation of CE. However, the explanation for the overall low reactivity of recombinant particles made from plasma phospholipids of animals fed fish oil versus saturated fat was not readily apparent. Possible explanations included dilution of the preferred fatty acid substrate of LCAT by the n-3 fatty acids or a direct inhibition of LCAT by n-3 fatty acids. Our more recent studies demonstrate that dietary fish oil alters not only the fatty acid composition of plasma PL but also the distribution of plasma PL species of nonhuman primates; animals fed the fish oil diet had a greater proportion of sphingomyelin and lysoPC and less PC relative to those animals fed saturated fat (7). The extent to which diet-induced plasma PL compositional changes could have influenced the results of our previous study is unknown. Results of our most recent studies demonstrate that the effect of n-3 fatty acids on LCAT activity warrants further investigation.

The purpose of the present study was to directly test the influence of *sn*-2 n-3 fatty acids of PC on the reactivity of LCAT. Based on our previous results we hypothesized that eicosapentaenoic acid and docosahexaenoic acid relative to oleic acid and linoleic acid would be poor substrates for the LCAT reaction. To accomplish our experimental goal we synthesized 1-palmitoyl-2-eicosapentaenoyl PC (PEPC) and 1-palmitoyl-2-docosahexaenoyl PC (PDPC) and compared then to 1-palmitoyl-2-oleoyl PC (POPC), 1-palmitoyl-2-linoleoyl PC (PLPC), and 1-palmitoyl-2-arachidonoyl PC (PAPC) with regard to LCAT activity using recombinant particles and a monolayer trough. Specifically, we tested the LCAT reactivity of recombinant complexes containing PEPC and PDPC against recombinant complexes of similar size and composition containing POPC, PLPC, and PAPC to determine the effect of the *sn*-2 fatty acid on CE formation. This experimental design eliminated potentially confounding variables that were in our previous study (6) such as heterogeneous fatty acid and phospholipid distribution among isolated plasma PL and allowed us to specifically address the effect of the type of fatty acid in the *sn*-2 position of PC on LCAT reactivity.

MATERIALS AND METHODS

Materials

The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO): *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA); *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA); 1-palmitoyl-*sn*-glycero-3-phosphocholine (LysoPC); 4-dimethylaminopyridine; *cis*-5,8,11,14-eicosatetraenoic acid; 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC); 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine, and sodium cholate. N,N'-dicyclohexylcarbodiimide and 2,6-di-tert-butyl-4-methylphenol (butylated hydroxytoluene, BHT) were purchased from Aldrich Fine Chemicals (Milwaukee, WI). [4-^{14}C]Cholesterol (57.5 mCi/mmol) was obtained from Dupont/New England Nuclear (Boston, MA). All other reagents, chemicals, and solvents were purchased from Fisher Scientific Co. (Pittsburgh, PA).

Phospholipid synthesis

1,2-Diacyl-*sn*-glycerol-3-phosphocholine (PC) species were synthesized from 1-palmitoyl lysoPC and the appropriate fatty acid (8, 9). Briefly, all reagents were vacuum-desiccated over P_2O_5 for 12–18 h at room temperature in the dark. Then 50 nmol of 1-palmitoyl lysoPC, 80 nmol fatty acid, 120 nmol N,N'-dicyclohexyl-carbodiimide, and 200 nmol 4-dimethylaminopyridine were combined in a small, brown, screw-cap tube with ~1 ml of CH_2Cl_2 that had been distilled over P_2O_5 and stored over 5 Å molecular sieves (Fisher Scientific Co.). All transfers were made rapidly with flamed Pasteur pipettes or spatulas to exclude traces of water. The tube was gassed with Ar and placed in a sonic water bath for 2 h and then stirred overnight at room temperature.

Synthesized PC species were purified by silicic acid chromatography. Two g of heat-activated silicic acid (silica gel 60; EM Science, Gibbstown, NJ) was suspended in $CHCl_3$ and poured into a disposable 1.5 × 15 cm column (Isolabs, Akron, OH). The column was washed with 50 ml of $CHCl_3$ and the crude PC preparation was loaded onto the column in $CHCl_3$. The column was then washed with 100 ml $CHCl_3$, 100 ml 25% methanol in $CHCl_3$, and

the PC fraction was eluted with 100 ml of 40% methanol in $CHCl_3$. The solvent was removed by rotary evaporation and 5 ml of $CHCl_3$ was added to solubilize the PC. The PC samples were filtered through a 0.2-μm Prep-Disc Membrane (Bio-Rad, Richmond, CA) to remove any residual silicic acid and brought to a final volume of 10 ml with $CHCl_3$. Aliquots were taken for phosphorus analysis and thin-layer chromatography (TLC) as described below. Immediately after the phosphorus assay, BHT was added to a final concentration of 0.02 wt%. The PC samples were flushed with Ar and stored at −20°C. At all steps during the isolation procedure, where feasible, the PC preparations were under an Ar atmosphere.

Characterization of PC preparations

The purified PC preparations were analyzed by TLC in neutral (hexane–diethyl ether–acetic acid 70:30:1, v/v) and polar ($CHCl_3$–CHl_3OH–acetic acid–water 65:45:12:6, v/v) solvent systems using silica gel H TLC plates (Analtech, Newark, DE). Detection of bands on TLC plates was accomplished with I_2 vapor, molybdenum spray (10), and primulin spray. A stock solution of 0.1% Primulin (Sigma Chemical Co.) in water was diluted 1:100 with acetone–water 4:1 and sprayed on the TLC plate. The lipid bands were then visualized with UV light. Molecular species of the PC preparations were determined after hydrolysis with phospholipase C, benzoylation of the diacylglycerides, and HPLC separation of the benzoylated derivatives as described by Blank et al. (11). The position of the fatty acids in the PC species was also investigated by phospholipase A_2 digestion and subsequent fatty acid analysis as described previously (6). The thiobarbituric acid-reactive substance assay was used to determine whether lipid peroxidation of the PC species had occurred during the studies (12).

Apolipoprotein A-I and LCAT isolation

ApoA-I was isolated from HDL of fresh human plasma by gel filtration chromatography (13) and its purity was checked by SDS-polyacrylamide gel electrophoresis as described previously (14). The isolated apoA-I was stored at −20°C in 0.01% EDTA, 0.01% NaN_3, pH 7.4, at a concentration of 500 μg/ml, as determined by the published extinction coefficient (ϵ = 1.21 ml/mg; ref. 15) and protein assay (16).

LCAT was isolated from fresh human plasma by the procedure of Chen and Albers (17). Briefly, plasma lipoproteins were precipitated with dextran sulfate–Mn^{2+} and LCAT was isolated from lipoprotein-free plasma using, sequentially, phenyl Sepharose, DEAE Sepharose, and Affigel blue chromatography. This procedure resulted in an LCAT preparation with a specific activity of 4800 nmol CE formed/h per mg protein using recombinant particles containing egg yolk lecithin, [^{14}C]cholesterol, and apoA-I (80:5:1 molar ratio) and represented a 4340-fold purification of the enzyme from plasma. The enzyme preparation was stored at 4°C in 20 mM phosphate buffer, pH 7.0, containing 0.01% NaN_3.

Recombinant complex formation

Recombinant complexes were formed using the sodium cholate dialysis procedure (18). Aliquots of PC species in $CHCl_3$ and [^{14}C]cholesterol (3.28×10^5 dpm/μg) in ethanol were transferred to glass screw-cap tubes and the solvent was evaporated under N_2 atmosphere at room temperature. Then the sample was flushed with argon and placed on a lyophilizer for 30 min to remove residual solvent. Buffer (10 mM Tris, 140 mM NaCl, 0.01% EDTA, 0.01% NaN_3, pH 7.4) and the appropriate amount of human apoA-I were added to the tube to give a molar ratio of PC-[^{14}C]cholesterol–apoA-I of 55:2.1:1 and a final volume of 2 ml. Sufficient sodium cholate (25 μmol; 35 μl of a 312 mg/ml solution) was added to render the solution isotropic after incubation of the tube at room temperature for 30 min under Ar atmosphere. The recombinant complexes were then transferred to dialysis bags and dialyzed against 6 × 1 liter of buffer that had previously been bubbled with Ar, at 4°C over a period of 3 days in a closed container under Ar atmosphere.

In some recombinant particle preparations the mole of fraction of PDPC or PEPC was varied from 0 to 1.0 relative to POPC while maintaining a constant PC-[^{14}C]cholesterol–apoA-I ratio of 55:2.5:1. All other aspects of the procedure were the same as described above.

Characterization of recombinant complexes

The PC, cholesterol, and apoA-I composition of recombinant complexes was determined by phosphorus assay (19), liquid scintillation spectrometry, and protein assay (16), respectively. The size distribution of each preparation of recombinant complexes was monitored by gradient gel electrophoresis (14). Lipid extraction and TLC analysis (above) were repeated on the recombinant complexes to monitor potential lysoPC formation during the experimental procedures.

LCAT incubations

LCAT incubations were conducted in 0.5 ml buffer containing: recombinant complexes (0.25–1.4 nmol cholesterol; 0.5–2.8 μM), 2% bovine serum albumin (fatty acid-free), 10 mM β-mercaptoethanol, and enough LCAT to esterify <10% of the substrate free cholesterol during incubations, which were routinely conducted for 1 h at 37°C. The reaction was stopped by the addition of 0.5 ml of redistilled 95% ethanol. Free and esterified cholesterol were extracted, separated by TLC, and quantitated by liquid scintillation spectrometry as described previously (6). Percentage CE formed was converted to nmole CE formed/h per ml of LCAT preparation.

Monolayer studies

The surface barostat method was used to determine the rate of hydrolysis of lipid monolayers by LCAT phospholipase A_2 action at a constant surface pressure of 25 mN/m using a KSV LB5000 monolayer apparatus (KSV-Instruments USA, Inc., Stamford, CT). Enzyme reaction was followed in a key-type Verger-deHaas zero-order trough (20) as described previously for hepatic lipase (21). The trough consisted of a magnetically stirred reaction compartment (total volume 35 ml and total surface 23.2 cm^2), a compartment for measuring surface pressure, and a reservoir (20 × 55 mm). A Wilhelmy platinum plate was used for surface pressure measurements, a personal computer for data collection, and the LB5000 software for data analysis.

PC species (POPC, PEPC, and PDPC) were spread onto a clean argon-water interface from chloroform containing no BHT. The subphase consisted of 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0, and the subphase in the reaction compartment contained 0.2 mg/ml fatty acid-free bovine serum albumin (0.02%). ApoA-I (1.2 μg) was injected into the subphase in the reaction compartment through an inlet port without disturbing the lipid film 5 min prior to the injection of LCAT enzyme (5 μg) which started the reaction. The monolayer apparatus was maintained under an argon atmosphere during the entire study. Phospholipase A_2 activity of LCAT was expressed as nmole of PC hydrolyzed/min per ml LCAT solution after monitoring phospholipid hydrolysis for 25–45 min.

Lateral compression isotherms of POPC, PEPC and PDPC were measured in a rectangular Teflon trough (50 × 20 cm) under an argon atmosphere. PC species were spread from $CHCl_3$ containing no BHT at a clean argon-water surface (subphase buffer was the same as in hydrolysis experiments) and were compressed laterally at a rate of 5 mm/min while surface pressure was continuously monitored.

RESULTS

All synthetic and purchased PC species used in these studies were characterized by HPLC, phospholipase A_2 digestion, TLC, and thiobarbituric acid assay. All PC species migrated with authentic egg yolk lecithin in both neutral and polar TLC solvent systems and contained no detectable contaminating species when 300 μg of PC was loaded on the plate. HPLC analysis of each PC benzoate derivative gave only one peak and the retention times for the peaks were similar to those reported by Blank et al. (11). Results from the phospholipase A_2 digestion demonstrated that 80–90% of the fatty acid in the *sn*-2 position was the expected fatty acid with 10–20% contamination from 16:0, which was likely the result of acyl migration from the *sn*-1 position during the phospholipase A_2 digestion. The amount of thiobarbituric acid-reactive substance was < 0.25 mole % with respect to phospholipid for all recombinant particles tested.

The composition of representative recombinant complexes used in these studies is shown in **Table 1.** All recombinant complexes had compositions that were similar within each experiment. We chose to work with particles with PC-cholesterol-apoA-I molar ratios of approximately 40:2:1 because the size and composition of these particles mimic those of HDL particles isolated from recirculating nonhuman primate liver perfusate (22).

Densitometric scans of recombinant particles separated by size on 4–30% nondenaturing gradient gels are shown in **Fig. 1** for recombinant particles made for experiment 1. The recombinant particles were heterogeneous in size, containing several major subfractions of particles between 7.0 and 10.0 nm diameter. However, the distribution and size of subfractions were quantitatively similar among the recombinant particles made from the various PC species, which agreed with the similarity in particle compositions noted in Table 1.

Fig. 2 shows representative data for CE formation versus substrate cholesterol concentration for the recombinant particles used in experiment 1 (Table 1). CE formation increased with substrate cholesterol concentration and was highest for the recombinant particles containing POPC and PLPC. The amount of CE formed during the incubation was much less for the particles containing PEPC, compared to POPC and PLPC, and was equivalent to that for particles containing PAPC. The recom-

TABLE 1. Recombinant particle chemical composition

Recombinant Particle #	PC Composition	Molar Ratio PC-Chol-ApoA-I
Experiment 1		
67	POPC	41:1.9:1
68	PLPC	39:1.9:1
72	PAPC	44:1.7:1
70	PEPC	46:1.8:1
71	PDPC	38:2.0:1
Experiment 2		
62	100% POPC/0% PEPC	36:2.4:1
63	75% POPC/25% PEPC	35:2.3:1
64	50% POPC/50% PEPC	36:2.3:1
65	25% POPC/75% PEPC	32:2.3:1
66	0% POPC/100% PEPC	35:2.3:1
Experiment 3		
74	100% POPC/0% PDPC	42:2.2:1
75	78% POPC/22% PDPC	42:2.2:1
76	55% POPC/45% PDPC	38:2.1:1
77	29% POPC/71% PDPC	40:2.2:1
78	0% POPC/100% PDPC	37:2.1:1

Recombinant particles containing different species of PC or combinations of two PC species with [^{14}C]cholesterol and human apoA-I were made by the cholate dialysis procedure. The PC, cholesterol, and apoA-I composition of the recombinant complexes was determined by phosphorus assay, liquid scintillation spectrometry, and Lowry protein assay, respectively, as described in the Methods section.

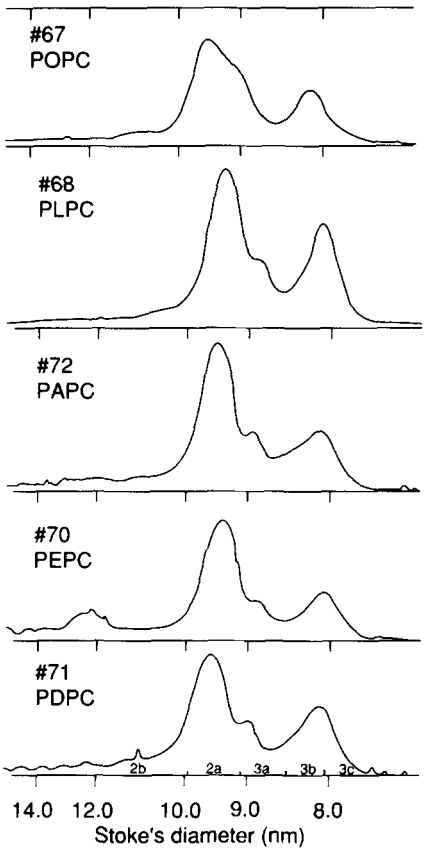

Fig. 1. Densitometric scans of recombinant particles on 4–30% nondenaturing gradient gels. Gradient gels were run as described previously, stained with Coomassie Blue, destained, and scanned using a laser densitometer (14). Standard proteins were used to construct a curve relating Stoke's diameter to migration distance. Recombinant complex number and PC species of the complex are given in the upper left corner of each scan. The size range of human plasma HDL subfractions is also shown on the bottom axis.

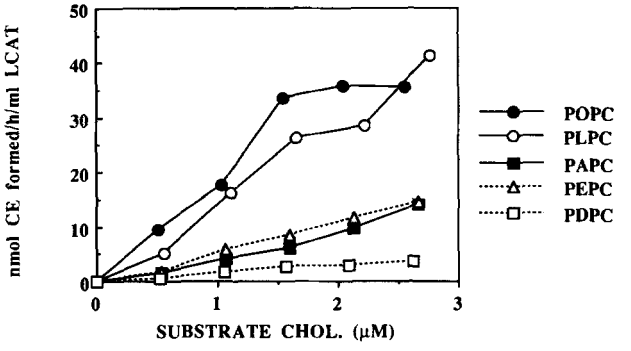

Fig. 2. Cholesteryl ester formation by LCAT using recombinant complexes containing the different PC species in experiment 1 (Table 1). Details of the incubation are given in the Methods section. The chemical composition and gradient gel profiles of the recombinant complexes are shown in Table 1 and Fig. 1, respectively.

binant particles containing PDPC had the lowest rate of CE formation of any of the preparations studied. These results were found to be reproducible in several separate experiments using recombinant particles made from at least two separate synthetic preparations of PEPC, PAPC, and PDPC.

The average value for apparent V_{max}/K_m for the recombinant particles made for five different species of PC are shown in **Table 2.** These data were obtained from linear regression analysis of the double reciprocal plot of CE formation versus substrate cholesterol concentration. The apparent V_{max}/K_m for recombinant particles containing PEPC and PAPC was only 15% of that of particles containing POPC. The particles containing PDPC had even lower values that were 7% of those containing POPC.

Since recombinant particles containing only PC species with eicosapentaenoic acid and docosahexaenoic acid had lower apparent activity with LCAT, we decided to test the hypothesis that these PC species were acting as competitive inhibitors of the LCAT reaction. Recombinant particles were made in which the PC-cholesterol-apoA-I ratio was held constant and the mole percentage of PEPC or PDPC relative to POPC was varied from 0 to 100. The composition of these particles is given in Table 1 and the data show that the PC-cholesterol-apoA-I molar ratio was approximately 35:2.3:1 for the recombinant particles containing POPC and PEPC and was approximately 40:2.2:1 for those particles containing POPC and PDPC. The results of LCAT incubation with an increasing concentration of these particles is shown in **Fig. 3.** It is apparent from the data in Fig. 3 that CE formation decreased as the percentage of PEPC or PDPC was increased relative to POPC. The double reciprocal plots of these data demonstrated lines that were not parallel and intersected near the ordinate of the plots (data not shown) suggesting that the PEPC and PDPC were acting as competitive inhibitors of the LCAT reaction.

According to the kinetic model of Verger and deHaas (23), a competitive inhibitor of an interfacial lipolytic enzyme such as LCAT is characterized by a linear plot of activity versus increasing percentage of the inhibitor relative

TABLE 2. Apparent V_{max}/K_m for recombinant complexes containing different PC species

PC Species	Apparent V_{max}/K_m
	nmol/h · ml/μM
POPC	13.4 ± 2.4 (n = 4)
PLPC	5.8 (n = 2)
PAPC	2.0 (n = 2)
PEPC	2.3 (n = 2)
PDPC	0.9 ± 0.2 (n = 4)

Data are the reciprocal slope of the double reciprocal plot of CE formation versus substrate cholesterol concentration after linear regression analysis was performed to find the line of best fit for the data points. Either two or four separate recombinant particles for each species of PC were analyzed and the mean ± SE of the mean is shown when n = 4.

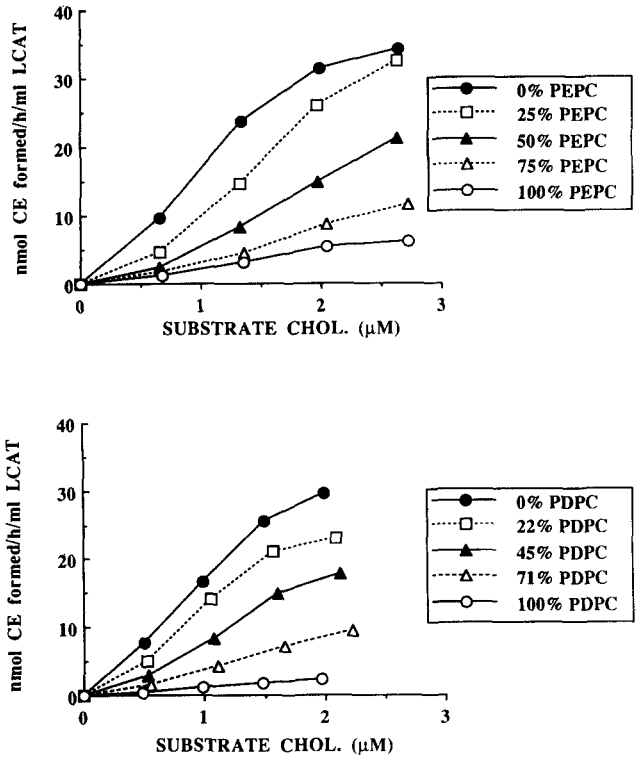

Fig. 3. Effect of increasing percentage of PEPC (top) or PDPC (bottom) relative to POPC at different substrate cholesterol concentrations on CE formation by LCAT. The PC-[^{14}C]cholesterol-apoA-I molar ratio was approximately 35:2.3:1 for the PEPC/POPC recombinant particles and approximately 40:2.2:1 for the PDPC/POPC recombinant particles. The composition for each recombinant particle used is given in Table 1.

to the substrate when $K_m = K_i$. In other words, when a competitive inhibitor has an affinity for the enzyme that is equal to that of the substrate, there will be a linear decrease in enzyme activity with an increasing concentration of inhibitor relative to substrate as the total amount of substrate plus inhibitor is held constant. **Fig. 4** shows a plot of LCAT activity versus an increasing percentage of PEPC or PDPC relative to POPC at several concentrations of substrate cholesterol. The experimental data appear to best fit a straight line determined by regression analysis and suggest the PEPC was acting as a competitive inhibitor of CE formation by LCAT with a K_i value that was similar to the K_m of the substrate POPC. Similar results were also obtained for the recombinant particles containing an increasing percentage of PDPC relative to POPC.

In order to determine whether the apparent inhibition of PEPC and PDPC on CE formation was affecting the phospholipase A_2 half of the LCAT reaction, monolayer hydrolysis studies were performed using POPC, PEPC, and PDPC. Each phospholipid was spread at an argon-water interface and maintained at a surface pressure of 25 mN/m using a zero order reaction trough, so that substrate concentration remained constant in the reaction compartment by laterally transferring substrate from a reservoir throughout the hydrolysis experiments. **Fig. 5** shows the results of the study. LCAT activity, measured as nmole of PC hydrolyzed/ml of LCAT preparation per min, is shown in the top panel and the surface area per molecule measured at a surface pressure of 25 mN/m is shown in the bottom panel. Note that POPC had the highest hydrolysis rate at 2.5 nmol/ml per min (n = 2). The hydrolysis rate for PEPC was 45% that of POPC and averaged 1.12 nmol/ml per min (n = 2). However, there was no detectable hydrolysis of PDPC under the same experimental conditions (n = 2). By comparing the PC hydrolysis rates (top panel) with the molecular area of the PC species (bottom panel), an apparent inverse relationship between LCAT hydrolysis rate and molecular area was observed. These data suggested that the molecular size and/or conformation of the PC molecule may influence its reactivity with LCAT.

DISCUSSION

The purpose of the present study was to determine the effect of *sn*-2 eicosapentaenoic acid and docosahexaenoic acid in PC compared to oleic acid, linoleic acid, and arachidonic acid on the reactivity of LCAT. PC species

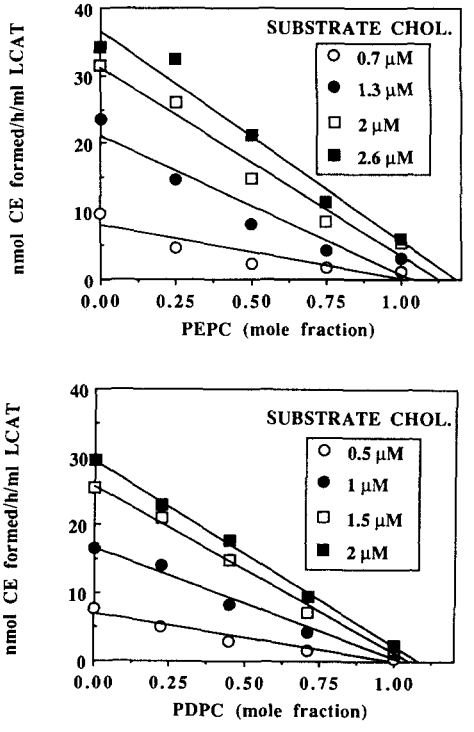

Fig. 4. Effect of increasing mole fraction of PEPC (top) or PDPC (bottom) relative to POPC at different substrate cholesterol concentrations on CE formation by LCAT. The PC-[^{14}C]cholesterol-apoA-I molar ratio of the recombinant complexes averaged 35:2.3:1 for the PEPC/POPC recombinant particles and 40:2.2:1 for the PDPC/POPC recombinant particles. The line of best fit, determined by linear regression analysis, is shown for recombinant particles at each substrate concentration.

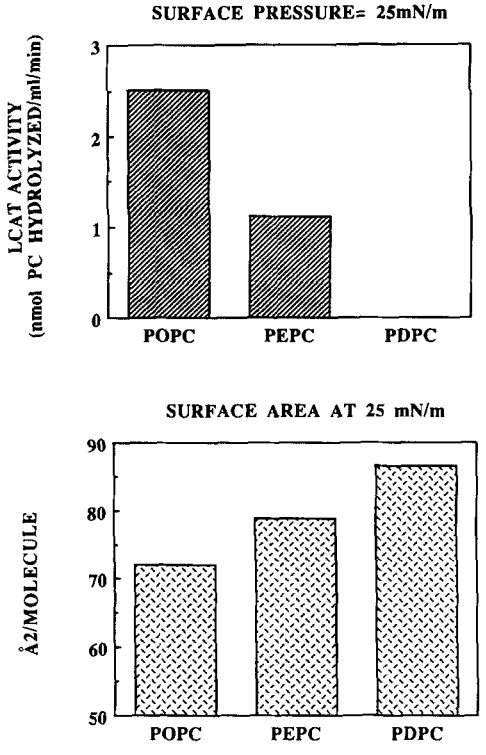

Fig. 5. LCAT-mediated hydrolysis of POPC, PEPC, and PDPC at the argon-water interface of a monolayer trough (top panel) and the molecular area of POPC, PEPC, and PDPC at a surface pressure of 25 mN/m. Details of the monolayer experiments are given in the Methods section. Data represent the mean of two separate experiments and the duplicate values varied not more than 7% from the mean.

were synthesized and made into uniform macromolecule particles (i.e., recombinant particles) by the cholate dialysis procedure. The recombinant particles containing PC species that varied only in the *sn*-2 fatty acid were then analyzed in vitro for reactivity with semi-purified human LCAT. In addition, the phospholipase A_2 half of the LCAT reaction was studied using POPC, PEPC, and PDPC on a monolayer apparatus. The results demonstrated that pure PEPC and PDPC were each less reactive with LCAT relative to POPC and PLPC (Fig. 2). This was also true when POPC/PDPC or POPC/PEPC mixtures were made into recombinant particles (Fig. 3). Kinetic analysis of the data suggested that PEPC and PDPC acted as competitive inhibitors of the LCAT reaction relative to POPC and the monolayer studies suggested that the phospholipase A_2 half of the reaction was probably the inhibited reaction step. Furthermore, the monolayer studies suggested that the inhibition of the LCAT phospholipase A_2 activity was related to the increased molecular area and perhaps the conformation of PEPC and PDPC relative to POPC. These results taken together confirm and extend our previous studies in which plasma phospholipids obtained from animals consuming saturated fat or fish oil-enriched diets were used to make recombinant particles to study LCAT reactivity (6). Both studies show that LCAT reactivity to n-3 fatty acids is low relative to oleic acid and linoleic acid. Thus, enrichment of plasma phospholipid with n-3 fatty acids may result in decreased CE synthesis by LCAT and may result in the CE-depleted LDL (7, 24) and HDL (25), as observed in nonhuman primates fed diets containing fish oil.

The data suggest that PEPC and PDPC act as competitive inhibitors of the LCAT reaction relative to POPC. Verger and de Haas (23) have described a general model for the interaction of lipolytic enzymes with macromolecular substrate particles. The first step involves the binding of the enzyme to the interface of the particle followed by the binding of the substrate(s) to the active site of the enzyme; the overall catalysis rate is a function of both steps. According to this model, if the K_m for the substrate is equal to the K_i^* for the inhibitor the enzymatic velocity equation becomes:

$$V = \frac{K_{cat} \cdot Eo \cdot S}{K_m^* \left(1 + \frac{i+S}{K_i^*}\right)}$$

where K_{cat} = catalytic rate constant; Eo = total enzyme concentration; K_m^* = interfacial Michaelis-Menten constant; i = inhibitor concentration; S = substrate concentration, and K_i^* = interfacial inhibition constant. Thus, a plot of the mole fraction of inhibitor relative to substrate, where i + S is constant, versus enzyme velocity should yield a straight line with a negative slope. The results in Fig. 4 show that this was the case as the LCAT activity decreased in a linear manner when the mole fraction of PDPC or PEPC relative to POPC in recombinant particles was varied from 0 to 1.0. Based on these kinetic results we conclude that PEPC and PDPC act as competitive inhibitors of LCAT relative to POPC and have a K_i^* value that is similar to the K_m^* of POPC for LCAT in these recombinant particles. If PEPC and PDPC were merely diluting the surface concentration of POPC in the recombinant particles with no affinity for the enzyme, the plots in Fig. 4 would have been curvilinear (23). These data also indirectly suggest that PEPC and PDPC do not affect the binding of LCAT to the interface but rather the subsequent phospholipase A_2 hydrolysis step. This assumes that nearly all of the available LCAT was bound to recombinant particles so that the dissociation rate of LCAT from the recombinant particle surface could be neglected in the kinetic analysis. This is likely the case in our experiments since there was a linear decrease in LCAT activity with an increased mole fraction of PEPC or PDPC at saturating concentrations of substrate cholesterol (i.e., 2-2.6 μM cholesterol; Fig. 4). However, direct binding studies of LCAT to the surface of these PC species or studies of LCAT reactivity with a standard inert lipid

matrix (i.e., PC ether) containing a small amount of the test PC (4, 5) will be necessary to test this hypothesis. These studies are currently underway in our laboratory.

The inhibition of LCAT by PEPC and PDPC may be due to the increased molecular area of these PC molecules relative to POPC. At a surface pressure of 25 mN/m POPC, PEPC, and PDPC had molecular areas of 72, 79, and 86 Å²/molecule, respectively. Pownall et al. (26) have shown that diphytanoyl PC (di-3,7,11,15-tetramethylpalmitoyl PC) incorporated into recombinant particles with POPC or POPC ether also resulted in LCAT inhibition. Monolayer studies indicated that diphytanoyl PC had molecular areas greater (16.4 Å² on average) than those of POPC at all surface pressures and at a surface pressure of 25 mN/m the area of diphytanoyl PC was >80 Å²/molecule. They suggested that PC molecules with molecular areas greater than ~70 Å²/molecule could not be handled by LCAT in a catalytically productive manner. Our results, in general, agree with those of Pownall et al. (26), although PEPC, which had an area of 79 Å²/molecule, was still reactive with LCAT albeit at approximately half the rate of POPC. Nonetheless, it appears that the size of PC molecules may be an important factor in determining LCAT activity. Alternatively, a difference in the conformation of the PEPC and PDPC relative to that of POPC may result in decreased reactivity with LCAT. For instance, if PEPC had a conformation that was distinct from that of PDPC it may explain why PEPC, which has a molecular surface area similar to that of diphytanoyl PC, was still relatively reactive towards LCAT despite its similarity in size to diphytanoyl PC. Taken together, our data suggest that the size and/or conformation but not the presence of n-3 fatty acids in PC molecules, per se, is responsible for the observed decrease in LCAT activity. This concept is supported by our studies with PAPC (Fig. 2) which had reaction rates that were similar to those of PEPC but contained no n-3 fatty acids as well as past studies that have shown that long chain fatty acids are poor substrates for the LCAT reaction (4, 5).

In light of our monolayer results it is interesting that Applegate and Glomset (27–29), using computer-based modeling of the conformation and packing of docosahexaenoic acid, predicted that under certain circumstances docosahexaenoic acid could promote tight, regular acyl chain packing arrays in membrane systems. This was clearly not the case in our experimental results where PDPC had a greater molecular area than POPC. However, the modeling studies were based on the packing of diacylglycerol molecules that were constrained to contain an *sn*-1 stearic acid chain and to have conformations that resembled those of the diacylglyceryl moiety in crystalline dilauryl phosphatidylethanolamine oriented normal to the monolayer surface. Thus, the extent to which the phosphocholine group alters the modeled packing of the fatty acyl chains was not ascertained. Additional studies will be necessary to understand the differences between the predicted versus empirically determined molecular packing of docosahexaenoic acid in PC. Our isotherms of POPC, PEPC, and PDPC with and without cholesterol will be the subject of a separate publication (J. S. Parks and T. Y. Thuren, unpublished results).

There may be several trivial explanations for our experimental observations that PEPC and PDPC inhibit LCAT activity relative to POPC; these include differences in macromolecular substrate composition and the presence of oxidized lipids in our PC preparations containing n-3 fatty acids. The data in Table 1 and Fig. 1 suggest that the recombinant particles within each experiment were comparable in composition and size and that any small differences in these parameters were unlikely to explain the large differences in LCAT reactivity. In addition, we could detect no more than 0.25 mol % thiobarbituric acid-reactive substance in the PC preparations. We also routinely checked the recombinant particles at the time of LCAT incubation for lysoPC resulting from PC breakdown and excluded any data from those recombinant particles that demonstrated detectable lysoPC accumulation. Thus, we feel our results cannot be nullified by any of these trivial explanations.

In summary, our studies demonstrate that eicosapentaenoic acid and docosahexaenoic acid in the *sn*-2 position of PC are poor substrates for LCAT in the generation of cholesteryl esters. Our kinetic analysis and monolayer studies suggest that PEPC and PDPC are competitive inhibitors of the enzyme; however, the possibility that LCAT binds less avidly to recombinant substrate particles containing PEPC or PDPC cannot be excluded based on our results. We speculate that the decreased reactivity of LCAT towards PEPC and PDPC is related to the molecular area and/or conformation of the PC molecules. However, the observed results are not uniquely a property of PC species containing n-3 fatty acids as similar results were obtained for PAPC. These data suggest that enrichment of n-3 fatty acids in plasma PL may decrease LCAT reactivity enough to contribute to the relative depletion of the plasma CE pool in nonhuman primates fed diets enriched in n-3 fatty acids (7, 24, 25).

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