Phosphatidylcholine fluidity and structure affect lecithin:cholesterol acyltransferase activity

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Abstract **The purpose of this study was to test the hypothesis that lipid fluidity regulates lecithin:cholesterol acyltransferase (LCAT) activity. Phosphatidylcholine (PC) species were synthesized that varied in fluidity by changing the number, type (*cis* vs. *trans*), or position of the double bonds in 18 or 20 carbon *sn*-2 fatty acyl chains and recombined with [³H]cholesterol and apolipoprotein A-I to form recombinant high density lipoprotein (rHDL) substrate particles. The activity of purified human plasma LCAT decreased with PC *sn*-2 fatty acyl chains containing *trans* versus *cis* double bonds and as double bonds were moved towards the methyl terminus of the *sn*-2 fatty acyl chain. The decrease in LCAT activity was significantly correlated with a decrease in rHDL fluidity (measured by diphenylhexatriene fluorescence polarization) for PC species containing 18 carbon ($r^2 = 0.61$, n = 18) and 20 carbon ($r^2 = 0.93$, n = 5) *sn*-2 fatty acyl chains. rHDL were also made containing 10% of the 18 carbon *sn*-2 fatty acyl chain PC species and 90% of an inert PC ether matrix (*sn*-1 18:1, *sn*-2 16:0 PC ether) to normalize rHDL fluidity. Even though fluidity was similar among the PC ether-containing rHDL, the order of PC reactivity with LCAT was significantly correlated ($r^2 = 0.71$) with that of 100% PC rHDL containing the same 18 carbon *sn*-2 fatty acyl chain species, suggesting that PC structure in the active site of LCAT determines reactivity in the absence of measurable differences in bilayer fluidity.** ▪ **We conclude that PC fluidity and structure are major regulators of LCAT activity when fatty acyl chain length is constant.**—Parks, J. S., K. W. Huggins, A. K. Gebre, and E. R. Burleson. **Phosphatidylcholine fluidity and structure affect lecithin:cholesterol acyltransferase activity.** *J. Lipid Res.* 2000. **41:** 546–553.

Supplementary key words cholesteryl ester • high density lipoproteins • *trans* fatty acids • n–3 fatty acids

Lecithin:cholesterol acyltransferase (LCAT; EC 2.3.1.43) is a glycoprotein enzyme that is responsible for the synthesis of most cholesteryl esters (CE) in human plasma (1, 2). The LCAT reaction consists of two steps: a phospholipase A₂ (PLA₂) step, in which the *sn*-2 fatty acyl group is hydrolyzed from phosphatidylcholine (PC) to form an acyl-enzyme intermediate and lysoPC, and a transacylase step, in which the fatty acyl group is transferred to the hydroxyl group of cholesterol to form CE (3). The reaction is activated by apolipoprotein A-I (apoA-I), the major apolipoprotein of HDL (4). LCAT plays a major role in the maturation of nascent high density lipoproteins (HDL) secreted by the liver and intestine, and in determining HDL subfraction distribution. LCAT also has a pivotal role in reverse cholesterol transport, a process that facilitates the net movement of excess cholesterol from peripheral tissues back to the liver for excretion (5). LCAT activity is important in determining the concentration of plasma HDL, as LCAT deficiency states lead to reduced HDL concentrations (6–8), whereas transgenic or adenovirus mediated over-expression of LCAT results in increased HDL concentrations in plasma (9–11).

The fatty acyl composition of substrate PC molecules appears to be the primary determinant of LCAT activity. Jonas et al. (12) have shown a 100-fold variation in initial reaction velocity of human plasma LCAT among recombinant HDL (rHDL) with PC species consisting of fatty acyl groups that differed in chain length and degree of unsaturation. The molecular explanation for this remarkable variation in enzyme activity is not well understood, but is thought to be related to molecular effects at the active site of the enzyme as well as lipid packing in the substrate particle. One study has suggested that rHDL fluidity or PC molecular surface area is important in controlling LCAT activity, with a more fluid interface resulting in a higher

reactivity with LCAT (13). However, the phospholipid species used in the study varied in the length of the *sn*-1 and *sn*-2 fatty acyl chains and in the head group, making it difficult to determine whether LCAT activity was controlled primarily by fluidity or fatty acyl chain length. Indeed, another study by Pownall, Pao, and Massey (14) demonstrated that increasing *sn*-1 and *sn*-2 fatty acyl chain length decreased LCAT activity, whereas increasing the number of double bonds in 18 carbon diacyl PC substrates increased LCAT activity. A study by Jonas and Matz (15) has shown that the gel to liquid-crystalline transition of PC had little impact on the activity of LCAT. However, none of these studies has systematically investigated the effect of lipid fluidity on LCAT reactivity when PC fatty acyl chain length is controlled.

The purpose of this study was to determine whether lipid fluidity was a major determinant of LCAT activity when fatty acyl chain length was held constant. We hypothesized that LCAT activity would be directly correlated with PC fluidity and tested our hypothesis using substrate PC that varied in the number, type (*cis* vs. *trans*), and position of the *sn*-2 fatty acyl double bonds to change PC fluidity. Substrate rHDL used for the study were similar in size, had two apoA-I molecules per particle, and contained PC species with *sn*-1 16:0 and *sn*-2 18 or 20 carbon fatty acyl chains. We also investigated the *sn*-2 18 carbon fatty acyl PC species in an unreactive PC ether matrix that normalized rHDL bilayer fluidity. Our results indicate that PC fluidity and structure are major regulators of LCAT activity when fatty acyl chain length is constant.

EXPERIMENTAL PROCEDURES

Materials

The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO): *cis*-5,8,11,14,17-eicosapentaenoic acid (20:5 n–3), *cis*-5,8,11,14-eicosatetraenoic acid (20:4), *trans*-9-octadecenoic acid (18:1 *trans*), *trans*-9-*trans*-12-octadecadienoic acid (18:2 *trans*), *cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid (alpha-18:3 n–3), *cis*-6,*cis*-9,*cis*-12-octadecatrienoic acid (gamma-18:3), *cis*-8, *cis*-11,*cis*-14 eicostrienoic acid, *cis*-11,*cis*-14,*cis*-17 eicostrienoic acid, 1-palmitoyl-*sn*-glycero-3-phosphocholine (lysoPC), 4-dimethylaminopyridine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (P18:1PC), 1-palmitoyl-2-linoleoyl-*sn*-3-phosphocholine (P18:2PC), ultrapure guanidine-HCl and sodium cholate. *cis*-5,*cis*-8,*cis*-11 Eicostrienoic acid was purchased from Oxford Biomedical. OPPC ether was purchased from Serdary Research Labs (London, Ontario, Canada). Butylated hydroxytoluene, BHT (2,6-di-tert-butyl-4-methylphenol) was purchased from Aldrich Fine Chemicals (Milwaukee, WI). Radiolabeled cholesterol ([7-^{3}H]cholesterol; 21.8 Ci/mmol) was obtained from DuPont/New England Nuclear (Boston, MA). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Molecular Probes, Inc. (Eugene, OR). Cholesterol was obtained from NuChek Prep, Inc. (Elysian, MN). All other reagents, chemicals, and solvents were purchased from Fisher Scientific Co. (Pittsburgh, PA).

Recombinant HDL (rHDL) synthesis and characterization

rHDL were used as substrate particles for measurement of CE formation by LCAT as described previously (16). rHDL were made with purified human plasma apoA-I, [^{3}H]cholesterol (50,000 dpm/μg), and PC in a starting molar ratio of 1:5:80. ApoA-I was purified from human plasma (17). In the first experiment, six PC species were used for rHDL synthesis as summarized in Table 1. P18:1PC and P18:2PC were purchased from Sigma Chemical Co.; the remainder were synthesized from the corresponding fatty acid and lysoPC as described previously (18). In the second experiment, rHDL were made that contained PC species with 20 carbon *sn*-2 fatty acyl chains and *sn*-1 16:0. The PC species with 20 carbon fatty acyl chains were synthesized (20:5 n–3, and three isomers of 20:3), except for P20:4PC, which was purchased. All PC species used for the studies were characterized as described (18). In a third experiment, rHDL were made that contained 10% of the PC species containing 18 carbon *sn*-2 fatty acyl chains (from Study 1) and 90% OPPC ether as described previously (16). Compositional analysis of the rHDL, chemical crosslinking of apoA-I, and gradient gel electrophoresis were performed as described previously (16, 19, 20).

LCAT assays

LCAT incubations were performed in duplicate or triplicate as described previously (16). Assays of initial reaction velocity were performed in 0.5 ml buffer (10 mm Tris, 140 mm NaCl, 0.01% EDTA, 0.01% NaN$_3$, pH 7.4) containing: rHDL (0.2 μg cholesterol), 0.6% bovine serum albumin (fatty acid-free; Sigma Chemical Co.), 2 mm β-mercaptoethanol, and 25–50 ng of purified human plasma LCAT, isolated as described previously (21).

Kinetic analysis of LCAT activity was performed on a subset of rHDL using substrate cholesterol concentrations ranging from 0.52 to 26 μm and 50 ng of purified human plasma LCAT (21). Assays were performed in duplicate and analyzed using a nonlinear least squares program (Graph Pad Prism, San Diego, CA) to determine apparent V_{max} and apparent K_m as described previously (16).

Activation energy for the initial reaction velocity of LCAT was determined from Arrhenius plots on a subset of rHDL over a temperature range of 25–37°C (16).

Fluorescence studies

Fluorescence measurements were made using an ISS K2 multifrequency phase fluorometer (ISS, Inc., Champaign, IL). rHDL lipid fluidity was measured by fluorescence polarization using diphenylhexatriene (DPH). The DPH probe was dissolved in tetrahydrofuran at 10^{-4} m concentration and added to rHDL solutions containing 25 μg rHDL PC in a total volume of 1 ml. The molar ratio of PC:probe was 400:1. The probe was allowed to equilibrate for at least 15 min at 37°C before measurements were taken. Samples were routinely kept in the dark under an argon atmosphere during all incubations. Polarization measurements were taken in the L-format (22) with blank subtraction using the following conditions: excitation wavelength = 366 nm, 2 mm slit widths, and KV418 emission filters. The temperature was regulated to ±0.1°C with a circulating water bath. The average intra-assay coefficient of variation for the polarization values, determined from triplicate measurements of each rHDL, was 4.5% (n = 10 rHDL).

Frequency domain fluorometry was used to measure the fluorescence lifetimes of DPH in rHDL. Sample preparation and other settings were the same as that for the polarization measurements. The phase and modulation data were obtained over the frequency range of 2–150 MHz. Data analysis was performed by minimizing the reduced chi-square with the ISSL Decay Acquisition Software provided by ISS. The data were fit to a two-lifetime component with an error of 0.004 and 0.2 for the modulation and phase data, respectively. Values reported are the major lifetime component of DPH. A scattering solution of glycogen was

used as a lifetime reference. The temperature was regulated to ±0.1°C with a circulating water bath.

rHDL apoA-I denaturation studies

The stability of rHDL apoA-I was determined by measuring the ellipticity at 222 nm of rHDL in the presence of increasing concentrations of GndHCl as previously described (16). Briefly, 10 μg of rHDL apoA-I was incubated with 0–5 m GndHCl (200 μl final volume) for 72 h at room temperature under an argon atmosphere and then scanned from 223 to 221 nm using a 1 mm quartz cell in a Jasco 720 spectropolarimeter (Jasco, Inc., Easton, MD). The concentration of GndHCl at which denaturation of apoA-I was 50% completed ($D_{1/2}$) was calculated as previously described (16).

Data analysis

Results are presented as mean ± standard error of the mean. Regression analysis was performed using Statview software.

RESULTS

Three separate experiments were performed to test the experimental hypothesis that PC fluidity affects LCAT activity, when fatty acyl chain length is constant. In the first experiment, a series of biologically relevant PC molecules were selected to make six rHDL with a wide range of fluidity values. The PC *sn*-1 fatty acyl group was palmitate in all cases and the *sn*-2 position contained 18 carbon fatty acyl chains that varied in double bond number, position, and

isomerization (*cis* vs. *trans*) to change fluidity. In the second experiment, PC species containing *sn*-1 palmitate and 20 carbon *sn*-2 fatty acyl chains were used to determine whether similar results were obtained when the *sn*-2 fatty acyl chain contained 20 carbons. Three of the PC species used in the second experiment contained isomers of eicosatrienoic acid ($\Delta^{5,8,11}$, $\Delta^{8,11,14}$, and $\Delta^{11,14,17}$) in the *sn*-2 position to determine the effect of double bond position on LCAT activity and PC fluidity. In the third experiment, rHDL were made containing 10% PC species with 18 carbon *sn*-2 fatty acyl chains and 90% OPPC ether, which is unreactive with LCAT. These rHDL were used to determine whether LCAT reactivity for the 18 carbon *sn*-2 fatty acyl PC substrates was similar when rHDL fluidity was normalized by the 90% OPPC ether matrix.

Characteristics of rHDL containing 18 and 20 carbon *sn*-2 fatty acyl chain PC are shown in **Table 1**. In experiment one, the compositions of rHDL were similar except those containing P18:1tPC, which in all cases had a higher PC to apoA-I molar ratio. This difference was consistent with the larger sized rHDL observed with non-denaturing gradient gels (**Fig. 1**). All rHDL had one major band ranging in size from 8.2 to 10 nm diameter, except P18:1tPC (lane c), which contained larger average size particles compared to the other rHDL. The LCAT activity of the rHDL containing the 18 carbon *sn*-2 fatty acyl species ranged 7-fold. The LCAT activities for rHDL containing P18:1PC, P18:2PC, and P18:3$^{\Delta6,9,12}$PC were similar; how-

TABLE 1. Characteristics of rHDL containing different 18 and 20 carbon *sn*-2 fatty acyl chains

rHDL PC[a]	rHDL Composition[b] (PC:chol.:apoA-I)	LCAT Activity[c]	DPH Polarization (37°C)	$D_{1/2}$[d]	LCAT Activation Energy[e]	DPH Fluorescence Lifetime[f] (37°C)
	molar ratio	*nmol/h/ml/LCAT*		*M*	*kcal/mol*	*ns*
Experiment 1						
P18:1PC	68 ± 6:4.3 ± 0.4:1	1166 ± 255	0.192 ± 0.006	2.9 ± 0.07	13.7	8.4
P18:2PC	70 ± 2:4.0 ± 0.2:1	1227 ± 114	0.171 ± 0.002	1.5 ± 0.16	16.0	8.2
P18:1tPC	90 ± 4:4.1 ± 0.4:1	175 ± 12	0.243 ± 0.009	3.3 ± 0.12	23.1	9.3
P18:2tPC	69 ± 8:3.2 ± 0.4:1	361 ± 79	0.193 ± 0.006	2.4 ± 0.03	12.8	8.4
P18:3$^{\Delta6,9,12}$PC	58 ± 11:2.8 ± 0.4:1	1272 ± 248	0.181 ± 0.009	1.9 ± 0.28	12.0	6.9
P18:3$^{\Delta9,12,15}$PC	60 ± 14:2.9 ± 0.2:1	741 ± 111	0.177 ± 0.011	1.7 ± 0.10	9.4	7.0
Experiment 2						
P20:4PC	71:3.9:1	342	0.146	ND	ND	ND
P20:5PC	74:4.6:1	165	0.163	ND	ND	ND
P20:3$^{\Delta5,8,11}$PC	69:4.3:1	260	0.168	ND	ND	ND
P20:3$^{\Delta8,11,14}$PC	77:4.3:1	196	0.171	ND	ND	ND
P20:3$^{\Delta11,14,17}$PC	76:4.4:1	13	0.229	ND	ND	ND

Values are the mean of 2–3 separate rHDL preparations made over a period of 5 months for the 18 carbon *sn*-2 fatty acyl chain PC species and a single rHDL preparation for the 20 carbon species. The standard error of the mean is reported for measurements made on three separate rHDL preparations; ND, not determined.
[a] Phosphatidylcholine (PC) species all contained palmitate (P) in the *sn*-1 position and the indicated fatty acyl group in the *sn*-2 position; *t, trans*.
[b] rHDL were made using cholate dialysis procedure as described in Experimental Procedures. The final composition of the rHDL was determined by assays for phosphorus, cholesterol, and protein and the values were normalized to the moles of apoA-I protein (20); chol., free cholesterol.
[c] Initial reaction velocity of purified human plasma LCAT. Average reproducibility of duplicate assay values was 6% for the 20 carbon *sn*-2 fatty acyl PC species.
[d] $D_{1/2}$, concentration of guanidine HCl needed to denature one-half of apoA-I on rHDL particles.
[e] LCAT activation energy was determined from initial reaction velocities of rHDL assayed over the temperature range of 25–37°C as described in Experimental Procedures (n = 2 rHDL).
[f] DPH fluorescence lifetime represents the major lifetime component of DPH after a two-component fit of the phase and modulation data obtained over the frequency range of 2–150 MHz (20) (n = 2 rHDL).
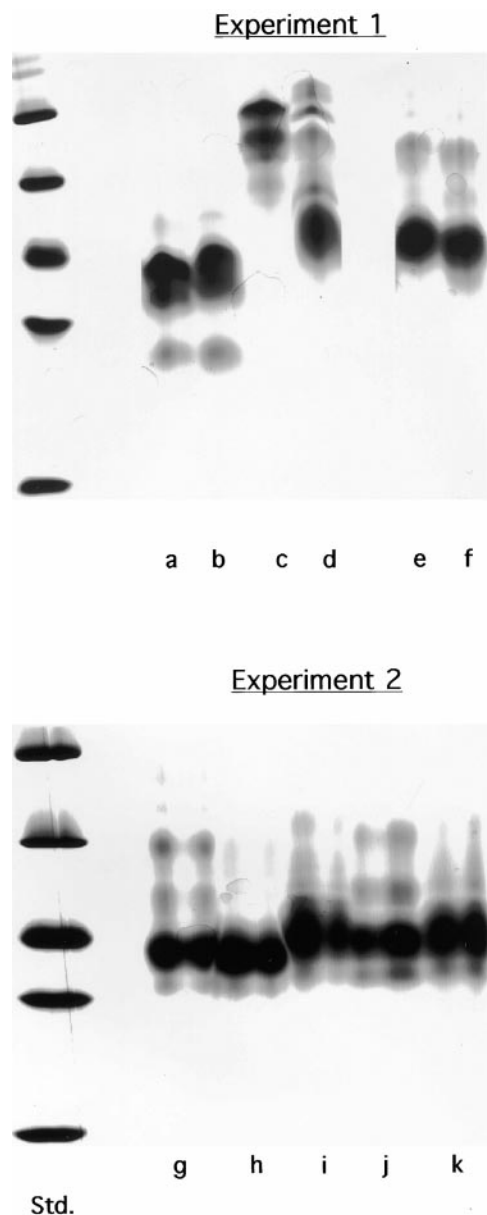
Experiment 1

a b c d e f

Experiment 2

g h i j k

Std.

Fig. 1. Size distribution of representative rHDL from two experiments analyzed by nondenaturing 4–30% polyacrylamide gel electrophoresis. After sample application, the gels were electrophoresed at 1400 Volt-h, stained with Coomassie blue, and destained. Standard (Std) lane contains the following protein standards (hydrated Stokes' diameter): thyroglobulin (17 nm), apoferritin (12.2 nm), catalase (10.4 nm), lactate dehydrogenase (8.2 nm), and bovine serum albumin (7.1 nm). a = P18:2PC, b = P18:1PC, c = P18:1tPC, d = P18:2tPC, e = P18:3$^{\Delta6,9,12}$PC, f = P18:3$^{\Delta9,12,15}$PC, g = P20:4PC, h = 20:5PC, i = P20:3$^{\Delta5,8,11}$PC, j = P20:3$^{\Delta8,11,14}$PC, k = P20:3$^{\Delta11,14,17}$PC.

ever, LCAT reactivity of the 18:1 *trans sn*-2 fatty acyl chain was 15% that of 18:1 *cis* and 18:2 *trans* was 29% that of 18:2 *cis* (P18:2PC). The position of double bonds along the *sn*-2 fatty acyl chain also affected LCAT activity, with the activity of P18:3$^{\Delta9,12,15}$PC being 58% that of P18:3$^{\Delta6,9,12}$PC. Thus, the positioning of the *sn*-2 fatty acyl double bonds towards the methyl terminus ($\Delta^{9,12,15}$) resulted in decreased LCAT reactivity compared to positioning to-

wards the carbonyl region of the fatty acyl chain ($\Delta^{6,9,12}$). This result could not be explained by differences in the chemical composition or size distribution of rHDL containing P18:3$^{\Delta6,9,12}$PC and P18:3$^{\Delta9,12,15}$PC (Fig. 1, lanes e and f and Table 1). Further characterization of the rHDL containing 18 carbon *sn*-2 fatty acyl chains revealed that, in general, the rHDL containing polyunsaturated *sn*-2 fatty acyl chains (i.e., 18:2, 18:3$^{\Delta6,9,12}$, and 18:3$^{\Delta9,12,15}$) had the lowest values for DPH polarization (more fluid), apoA-I stability (i.e., $D_{1/2}$), LCAT activation energy, and average DPH florescence lifetime. On the other hand, rHDL containing P18:1tPC had the highest values for all of those measurements.

To determine whether the variation in LCAT activity among the various PC species containing 18 carbon *sn*-2 fatty acyl chains was related to the fluidity of the rHDL particle, we plotted DPH polarization measured at 37°C versus LCAT activity for 18 individual rHDL (three separate studies of six rHDL particles) studied over a 5-month period. The results are shown in **Fig. 2**. There was a nonlinear relationship between fluidity measured by DPH polarization and LCAT activity, such that, an increase in fluidity (i.e., decreased DPH polarization) was associated with an increase in LCAT activity. The data were fit to an exponential function with which 61% of the variability in the LCAT activity could be explained by the DPH polarization measurement. rHDL DPH polarization also was significantly correlated ($r^2 = 0.66$; $P < 0.001$; n = 12) with the stability of apoA-I, such that apoA-I was more easily denatured on rHDL that contained more fluid PC species. Consistent with these relationships, there was also a significant negative correlation between LCAT activity and rHDL apoA-I stability ($r^2 = -0.41$; $P = 0.03$; n = 12). Fi-
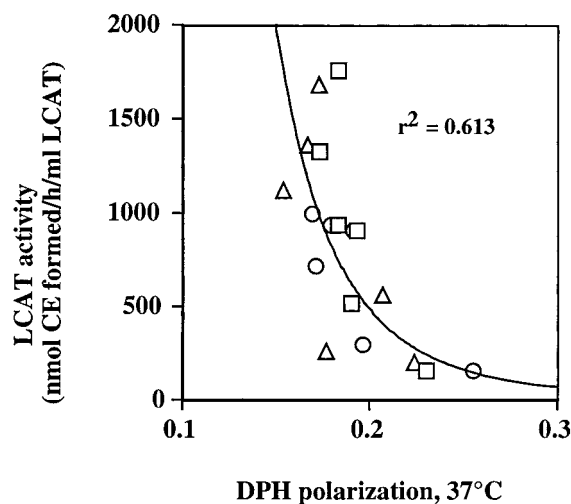

Fig. 2. Relationship between initial LCAT reaction velocity and DPH fluorescence polarization for rHDL-containing PC species with 18 carbon *sn*-2 fatty acyl chains. Values were measured with 18 rHDL preparations made over a 5-month period (three separate studies of six rHDL; particles within a study are represented with like symbols). Each rHDL contained PC species with *sn*-1 16:0 and an 18 carbon *sn*-2 fatty acyl chain (see Table 1). The line of best fit ($y = 0.183x^{-4.9}$) was determined using an exponential function and is shown with the individual data points.

nally, there was a significant positive relationship between DPH polarization and LCAT activation energy ($r^2 = 0.37$; $P = 0.035$; n = 12), such that the rHDL containing the more fluid PC species had lower activation energy for the LCAT reaction.

To determine whether the relationship between LCAT activity and rHDL fluidity was also apparent for PC molecules containing a 20 carbon *sn*-2 fatty acyl chain, we performed a second experiment using five rHDL preparations. All five rHDL were similar in size (Fig. 1) and chemical composition (Table 1). Table 1 also shows the LCAT activities for these rHDL. LCAT activity was less with *sn*-2 20:5 n–3 compared to 20:4 and there was a step-wise decrease in reactivity for the P20:3PC species as the double bond of the *sn*-2 fatty acyl chain moved from the carbonyl end of the chain to the methyl terminal end. These results suggest that the position of the *sn*-2 double bonds has a profound impact on LCAT reactivity and agree with those found for the P18:3PC isomers (Table 1). Thus, the presence of a double bond within three carbons of the methyl terminus of the *sn*-2 fatty acyl chain reduces LCAT reactivity regardless of whether the fatty acyl chain is 18 or 20 carbons in length.

Kinetic analysis of the rHDL used for experiment 2 was performed to determine whether the low reactivity of P20:3$^{\Delta11,14,17}$PC was a function of apparent V_{max} or apparent K_m (**Table 2**). Kinetic values derived for P20:4PC and P20:5PC were similar to those published in another study (16). For the rHDL containing the P20:3PC isomers, there was a trend towards a lower appV_{max} and higher appK_m as the position of the double bonds moved closer to the methyl terminus of the fatty acyl chain.

A plot of DPH polarization versus LCAT activity was constructed for the *sn*-2 20 carbon fatty acyl chain-containing PC species (**Fig. 3**) to determine whether a relationship existed between LCAT activity and rHDL lipid fluidity as observed in Fig. 2 for the 18 carbon PC species. The values for LCAT activity and DPH polarization for the individual rHDL are given in Table 1. There was a strong positive relationship between lipid fluidity, measured as DPH polarization, and LCAT activity, so that LCAT activity increased as lipid fluidity increased (decreased DPH polarization). Ninety-three percent of the variability in LCAT

Fig. 3. Relationship between initial LCAT reaction velocity and DPH fluorescence polarization for rHDL containing PC species with 20 carbon *sn*-2 fatty acyl chains. Values were measured with five rHDL preparations containing *sn*-1 16:0 and a 20 carbon *sn*-2 fatty acyl chain (see Table 1). The line of best fit ($y = 0.00022x^{-7.6}$) was determined using an exponential function and is shown with the individual data points.

activity could be explained by the measurement of rHDL fluidity, similar to the results found for 18 carbon *sn*-2 fatty acyl chain PC substrate.

In a third experiment, rHDL were made containing 10% of the 18 carbon *sn*-2 fatty acyl chain PC and 90% of an inert PC ether matrix, OPPC ether, to normalize bilayer fluidity. The composition was similar among the rHDL with PC molar ratios ranging from 89 to 94 and cholesterol molar ratios ranging from 4.2 to 4.5 relative to apoA-I. The size distribution was also similar for the rHDL (data not shown). A plot of DPH polarization versus LCAT activity is shown in **Fig. 4**. Note that the range of LCAT activities is approximately 10% that of the 100% PC rHDL,

Fig. 4. Relationship between initial LCAT reaction velocity and DPH fluorescence polarization for rHDL containing 10% PC–90% OPPC ether. Values were measured with 10 rHDL preparations containing 10% PC with *sn*-1 16:0 and a 18 carbon *sn*-2 fatty acyl chain and 90% OPPC ether.

TABLE 2. LCAT kinetic constants for rHDL-containing PC species with 20 carbon *sn*-2 fatty acyl chains

PC Type	appK_m	appV_{max}
	μM *cholesterol*	*nmolCE/h/ml LCAT*
P20:4PC	2.0	1038
P20:5PC	3.9	1469
P20:3$^{\Delta5,8,11}$PC	1.1	623
P20:3$^{\Delta8,11,14}$PC	1.6	584
P20:3$^{\Delta11,14,17}$PC	3.9	270

Assays were performed in duplicate using rHDL cholesterol concentrations ranging from 0.5 to 26 μm, and 50 ng of purified human plasma LCAT. Nonlinear least squares regression analysis was used to determine the best fit line of the data to the Michaelis-Menten equation and to calculate the kinetic constants. The average variation among duplicate assay values was 6.2% (n = 40).
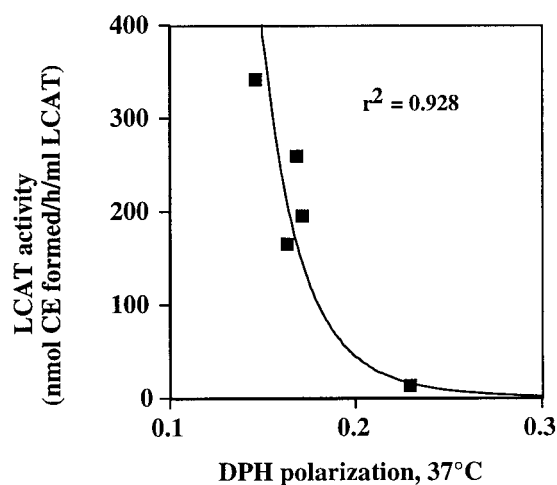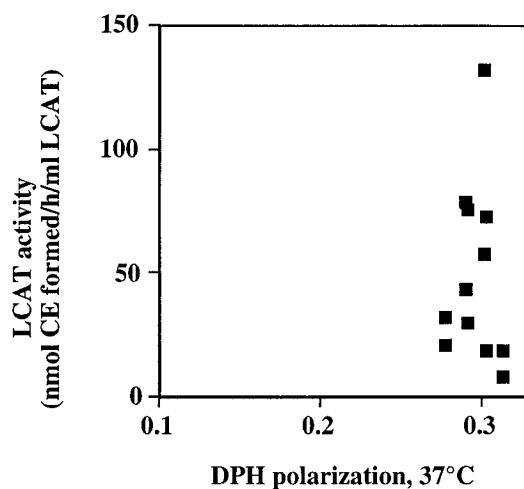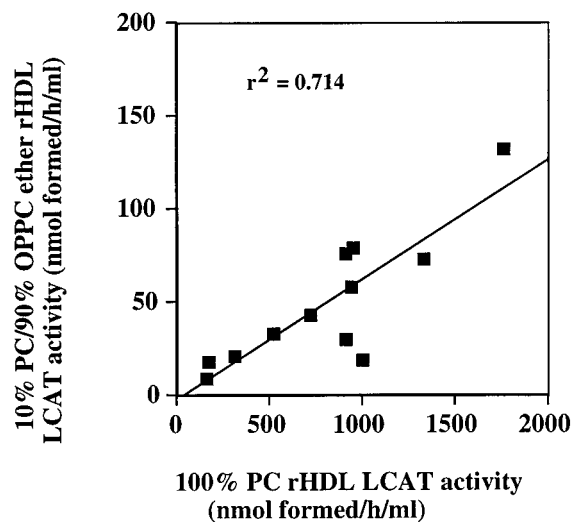
Fig. 5. Relationship of initial LCAT activity for rHDL containing 100% PC vs. 10% PC–90% OPPC ether. rHDL were made with similar overall composition that consisted of 100% of the 18 carbon sn-2 PC species listed in Table 1 or 10% of the same PC species and 90% OPPC ether. The OPPC ether is unreactive with LCAT. The line of best fit was determined by linear regression analysis.

consistent with the replacement of 90% of the rHDL PC matrix with an inert PC ether. The substitution of PC ether for PC essentially normalized the interfacial fluidity of the rHDL as seen in Fig. 4 compared to the same 18 carbon PC species in a 100% PC matrix (Fig. 2). However, despite the normalization of fluidity, LCAT activities varied 15-fold. To determine whether there was a correlation between LCAT activity of the 100% PC rHDL compared to that of the 10% PC/90% OPPC ether rHDL, a plot of LCAT activity was made (**Fig. 5**). There was a highly significant linear relationship between the reactivity of the 100% versus 10% PC rHDL ($r^2 = 0.71$), suggesting the LCAT activity differences among individual PC substrates were maintained even when rHDL fluidity was normalized.

DISCUSSION

The purpose of this study was to test the hypothesis that lipid fluidity affects LCAT activity when PC sn-1 and sn-2 fatty acyl chain length is constant. Previous studies have shown that the initial reaction velocity of LCAT can vary nearly 100-fold as a function of PC substrate, suggesting that LCAT activity is primarily controlled by PC substrate fatty acyl composition (12). However, from previous studies it was unclear whether this remarkable difference in LCAT activity was due to differences in lipid fluidity, sn-1 and sn-2 fatty acyl composition and/or sn-2 fatty acyl chain length of the substrate PC. In the present study, we varied PC fluidity by changing the number, type, and position of double bonds, while keeping the length of the sn-1 and sn--2 fatty acyl chains constant. The results demonstrate that there was a strong non-linear positive relationship between rHDL PC fluidity and LCAT activity for both 18 and 20 carbon sn-2 fatty acyl chains. The results could not be

explained by differences in rHDL composition, size, or heterogeneity. Normalization of rHDL bilayer fluidity with 90% OPPC ether/10% PC maintained the same order of reactivity for the 18 carbon sn-2 fatty acyl chains as with the 100% PC rHDL, suggesting that monomolecular PC structure at the active site of the enzyme is an important determinant of LCAT reactivity and that bulk PC fluidity is a surrogate measurement that reflects PC structure. To our knowledge, this is the first study to systematically examine the role of PC fluidity on LCAT activity when fatty acyl chain length is controlled and shows that LCAT activity is strongly influenced by rHDL PC fluidity and structure.

Several studies have reported the effects of PC fluidity and PC fatty acyl composition on LCAT activity. Jonas and Matz (15) concluded that LCAT reaction rates were not dependent on the phase state of rHDL lipid using P18:1PC, P16:0PC, and sn-1 14:0,sn-2 14:0 PC as the test PC species. In another study using a larger number of PC substrates, Jonas et al. (12) speculated, based on empirical considerations, that lipid fluidity did not directly affect the reactivity of LCAT. However, the PC substrates used in these two studies varied in the length of the sn-1 and sn-2 fatty acyl chains, which have independent effects on LCAT activity (13). For instance, there may be additional constraints on LCAT activity dictated by fatty acyl chain length, such as ability to fit into the active site of LCAT, that supersede the effect of PC fluidity on enzyme reactivity. To sort out interdependencies of PC fluidity and fatty acyl chain length, Pownall et al. (13) performed a seminal study on the influence of rHDL PC composition on LCAT activity using different PC species. rHDL were made that contained 90% of a diether analogue of PC and the other 10% of the rHDL phospholipid consisted of reactive diacyl PC substrate that differed in sn-1 and sn-2 fatty acyl chain length and in the degree of fatty acyl chain saturation. Their results clearly showed a dependence of sn-1 and sn-2 fatty acyl chain length on LCAT activity. In nearly all cases, 16:0 in the sn-2 position was more reactive than 18:1. For symmetrical diacyl PC species, LCAT activity decreased as fatty acyl chain length progressed from 14 to 18 carbons in length. To determine the effect of rHDL bilayer fluidity on LCAT reactivity of different PC species, Pownall et al. (13) investigated the LCAT reactivity of several diacyl PC species in three different phospholipid matrices that were unreactive with LCAT: POPC ether, DMPC ether, and sphingomyelin. Bilayer lipid fluidity, measured as DPH fluorescence polarization, was rank-ordered in the same direction as LCAT activity, with the POPC ether interface having the greatest fluidity and LCAT activities and sphingomyelin having the least. Based on these results, the authors suggested that interfacial fluidity or unsaturation was an important factor in determining LCAT activity. However, the three interfaces tested had differences other than fluidity that might have influenced LCAT activity, including differences in fatty acyl chain length, head group, and degree of unsaturation. The results of our study support and extend those previously provided by Pownall and coworkers (13) by investigating more unsaturated sn-2 fatty acyl species without changing fatty acyl chain length.

How can PC fluidity be related to LCAT activity yet there be no obvious effect of PC gel to liquid crystalline transition on LCAT activity? We speculate that this occurs because LCAT does not use bulk PC in rHDL for catalysis but rather PC that is activated by its cofactor apoA-I. We envision this activation as a "melting" of the bulk PC by apoA-I, resulting in monomeric PC that is used as substrate for the reaction. Thus, the bulk PC physical state would have little direct effect on the activity of LCAT, but the structure of the monomeric PC would. This was shown previously by Pownall et al. (13) and in our study (Fig. 5) as the rank order of individual PC species was maintained when they were in an inert PC ether matrix. If this explanation is correct, fluidity is a surrogate measurement of PC structure at the active site of the enzyme. Thus, although monomeric PC substrate molecules no longer have bulk lipid properties, they do maintain the structural elements that determine their bulk lipid fluidity and phase transition properties. It is these structural elements, we speculate, that influence PC interaction at the active site of LCAT and overall enzymatic activity.

The PC structural elements that contribute to bulk fluidity and phase behavior are likely to be the van der Waals interaction between sn-1 and sn-2 fatty acyl chains of the PC molecule. Several studies have systematically investigated the gel to liquid-crystalline transition of phosphatidylethanolamine (PE) and PC molecules containing one to five sn-2 chain double bonds and the influence of double bond position on the phase transition temperature (23, 24). These studies have shown that increasing the number of double bonds from one to two reduces the phase transition of the PC, but increasing from two to three or more actually increases the transition temperature. This observation was independent of fatty acyl chain length as it occurred for PC species containing 18, 20, and 22 carbon sn-2 fatty acyl chains. The increase in transition temperature with three or more double bonds in the sn-2 position has been hypothesized to occur because of decreased van der Waal interactions between intramolecular sn-1 and sn-2 fatty acyl chains resulting in a greater interaction of the saturated intermolecular sn-1 fatty acyl chains (24). An alternative explanation is that the position of the double bonds along the fatty acyl chain influences the intramolecular interaction of the sn-1 and sn-2 chains resulting in a U-shaped dependence of phase transition temperature and double bond position (23). When double bonds occupy the middle part of the sn-2 fatty acyl chain, the transition temperature is lowest because the longest effective length of interaction for the sn-2 chain with the sn-1 chain is minimized. However, as double bonds move towards the carboxy or methyl terminus of the chain, there is a longer region of the sn-2 chain that is not interrupted with double bonds that can interact with the sn-1 chain, leading to a higher transition temperature. Our fluorescence polarization studies are in general agreement with the calorimetric studies in that P20:3$^{\Delta 8,11,14}$PC was more fluid (lower polarization value; Table 1) than P20:3$^{\Delta 11,14,17}$PC. However, the polarization value for P20:3$^{\Delta 5,8,11}$PC was similar to that of P20:3$^{\Delta 8,11,14}$PC (0.168 vs. 0.171, respectively)

which does not follow the trend found for the calorimetric data using PE species. Because the calorimeter measures an actual phase transition whereas our fluorescence polarization measurement indirectly monitors fluidity of liquid crystalline PC, the calorimetric measurement may be more sensitive at detecting differences among these PC species. However, our fluorescence polarization data for PC containing 18:3 fatty acyl species do agree well with a previously published study of PC vesicles (25) and show that the placement of the double bond on 18 carbon chains has minor effects on PC fluidity, unlike the data for the sn-2 20 carbon chains.

To our knowledge this is the first report that LCAT activity decreases as double bonds are positioned closer to the methyl terminus of PC sn-2 fatty acyl chains. Our previous studies have shown that n–3 fatty acyl chains in the sn-2 position of PC were less reactive with LCAT than n–6 or n–9 species, but in those studies the fatty acyl chain length and number of double bonds was not controlled (16, 20). The effect of sn-2 fatty acyl double bond position on LCAT activity cannot be explained by bulk PC fluidity alone for several reasons. First, the order of reactivity of these PC species was maintained in 90% OPPC ether rHDL, where there was no obvious difference in PC fluidity among rHDL (Figs. 4 and 5). Second, although we observed an increase in LCAT activity that paralleled an increase in fluidity for the rHDL containing the three P20:3PC isomeric species, this was not the case for rHDL containing P18:3PC species (Table 1). As discussed above, we speculate that the positioning of the double bonds towards the methyl terminus of the sn-2 fatty acyl chain of PC results in a steric hindrance of monomeric PC binding at the active site of LCAT. These results taken together support the hypothesis that LCAT activity is influenced by rHDL fluidity, but individual PC structural elements override PC fluidity and dictate reactivity at the active site of LCAT.

It is also likely that PC fluidity affects surface binding of LCAT on rHDL as well as binding of individual PC molecules to the active site. As discussed earlier, Pownall et al. (13) suggested that bilayer fluidity affected LCAT reactivity based on studies using three PC ether species that varied in fatty acyl chain length and degree of unsaturation. The work of Bolin and Jonas (26) proposed that differences in appK_m of the LCAT reaction likely reflect differences in LCAT interfacial binding, whereas changes in appV_{max} occur through monomeric PC binding to the active site of LCAT. In experiment 2, using 20:3 isomers in the sn-2 position of PC, we found that appK_m increased and appV_{max} decreased as the three double bonds moved closer to the methyl terminus of the fatty acyl chain. The difference in appK_m was 4-fold between P20:3$^{\Delta 5,8,11}$PC and P20:3$^{\Delta 11,14,17}$PC, whereas the difference in appV_{max} was 2-fold. These results suggest that the position of the sn-2 fatty acyl double bonds influenced LCAT interfacial binding to a greater extent than PC binding to the active site, but both contributed to the reduced activity of LCAT. Mechanistically, a more fluid rHDL interface may allow easier penetration of LCAT into the PC interface; mono-

layer studies have demonstrated that lateral surface pressure can influence interfacial binding of LCAT (27). More fluid PC species may present a preferred orientation of the molecule in the active site of LCAT, resulting in increased substrate hydrolysis. Regardless of the mechanism, our results demonstrate that PC fluidity markedly affects LCAT activity when *sn*-2 fatty acyl chain length is controlled.▮

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