Regulation of the Activity and Fatty Acid Specificity of Lecithin-Cholesterol Acyltransferase by Sphingomyelin and Its Metabolites, Ceramide and Ceramide Phosphate†

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ABSTRACT: Sphingomyelin (SM), the second most abundant phospholipid in plasma lipoproteins, was previously shown to be a physiological inhibitor of the lecithin-cholesterol acyltransferase (LCAT) reaction. In this study, we investigated the effects of its metabolites, ceramide and ceramide phosphate, on the activity and fatty acid specificity of LCAT in vitro. Treatment of SM-containing substrate with SMase C, which hydrolyzes SM to ceramide, abolished the inhibitory effect of SM, whereas treatment with SMase D, which hydrolyzes it to ceramide phosphate, increased the level of inhibition. Although incorporation of ceramide into the substrate in the absence of SM activated the LCAT reaction only modestly, its co-incorporation with SM neutralized the inhibitory effect of SM. Ceramide phosphate, on the other hand, inhibited the LCAT reaction more strongly than SM. The effects of the sphingolipids on the phospholipase A and cholesterol esterification reactions of the enzyme were similar, indicating that they regulate the binding of phosphatidylcholine (PC) to the active site, rather than the esterification step. Incorporation of ceramide into the substrate stimulated the synthesis of unsaturated cholesteryl esters at the expense of saturated esters. However, these effects on fatty acid specificity disappeared when the PC substrates were incorporated into an inert diether PC matrix, suggesting that ceramide increases the availability of polyunsaturated PCs to the enzyme by altering the macromolecular structure of the substrate particle. Since the plasma ceramide levels are increased during inflammation, these results indicate that the activity and fatty acid specificity of LCAT may be altered during the inflammatory response.

Sphingomyelin (SM)[1] is one of the most abundant phospholipids in cell membranes and lipoproteins, constituting up to 30% of certain lipoprotein fractions (*1*, *2*). Although its role as a structural component of membranes and as a precursor of signaling molecules has been well recognized, its function in plasma lipoproteins has received less attention. Recent studies show that plasma SM may be an independent risk factor for atherosclerosis (*3*) and that a reduction in SM levels by myriocin treatment reduces the severity of atherosclerosis in apo E deficient mice (*4*, *5*). While the exact mechanism of the proatherogenic effect of SM is unknown, one proposed mechanism involves the generation of ceramide in LDL by the action of arterial SMase, which causes enhanced retention, aggregation, and oxidation of LDL, with subsequent uptake by the scavenger receptors of the macrophage (*3*). The formation of large amounts of ceramide in the plasma compartment is unlikely because of the absence of an active SMase in normal plasma. However, under inflammatory conditions, significant amounts of SMase may be released into circulation and cause the hydrolysis of lipoprotein SM to ceramide (*6*). Furthermore, de novo synthesized ceramide is secreted with the newly assembled lipoproteins by the liver during inflammatory reactions (*7*, *8*), and the presence of an active secretory SMase is not necessary for this (*8*). Holopainen et al. (*9*) also reported the presence of a LDL-associated SMase that may be derived from apoprotein B of LDL, and which could therefore hydrolyze LDL SM in the circulation. More recently, Lee et al. (*10*) reported that cultured endothelial cells and fibroblasts secrete SMase in association with nascent lipoprotein particles, supporting the formation of ceramides in the circulation. The concentration of ceramides is increased in sepsis patients, and the ceramide:SM ratio correlates positively with mortality in the patients (*11*).

Although the cellular functions of ceramide have been investigated well, its possible effects in the plasma compartment are unknown. Studies from several laboratories, including ours, have shown that ceramide is an activator of secretory and cytosolic phospholipases in vitro (*12*−*16*). Because of its tendency to form inverted hexagonal phase structures (H_{II}), it disrupts the membrane bilayer (*12*, *17*), and this may be the basis for its activation of phospholipase activities. Furthermore, ceramide was reported to specifically stimulate the release of unsaturated fatty acids from phospholipids by $sPLA_2$ IIa (*13*), suggesting that it facilitates the

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[1] Abbreviations: apo, apolipoprotein; CE, cholesteryl ester; FC, free (unesterified) cholesterol; HDL, high-density lipoproteins; LCAT, lecithin-cholesterol acyltransferase; LDL, low-density lipoproteins; PC, phosphatidylcholine; PLA, phospholipase A; SM, sphingomyelin; SMase, sphingomyelinase.

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interaction of lipolytic enzymes with specific phospholipid species. The possible effect of ceramide on the activity and specificity of LCAT, an enzyme that is essentially a modified PLA_2, has not been investigated, although SM, its precursor, has been shown to inhibit LCAT activity (*18*−*21*). In this study, we addressed the effect of SM and its metabolites, ceramide and ceramide phosphate, on the activity and fatty acid specificity of human LCAT. The results show that SM and ceramide have opposing effects on the enzyme activity and that ceramide phosphate is a more potent inhibitor of the enzyme than SM. Furthermore, ceramide stimulated the transfer of polyunsaturated fatty acids to cholesterol, at the expense of saturated fatty acids. This function of ceramide may be important in the regulation of LCAT specificity and could possibly influence the atherogenic properties of the lipoproteins.

MATERIALS AND METHODS

Materials. Egg PC, human serum albumin, egg ceramide, and unlabeled cholesterol and SMase C (*Staphylococcus aureus*, 174 units/mg of protein) were obtained from Sigma-Aldrich. Labeled cholesterol (4-^{14}C, 53 mCi/mmol) and labeled PC (1-palimtoyl-2-[1-^{14}C]linoleoyl-PC, 52 mCi/mmol) were purchased from Amersham Biosciences. Dioctadecenyl-PC (18:1 diether PC) and synthetic PCs (16:0−16:0, 16:0−18:1, 16:0−18:2, and 16:0−20:4) were purchased from Avanti Polar Lipids. Recombinant SMase D from *Corynebacterium pseudotuberculosis* was purified from the transfected *Escherichia coli*, as described previously (*22*). This enzyme was stored at −30 °C in 50% glycerol at a protein concentration of 100 μg/mL. The specific activity of this preparation was 4.6 units/mL, using the Amplex Red SMase assay kit from Molecular Probes, Inc. (1 unit hydrolyzes 1.0 μmol of SM per minute at 37 °C). It has no activity toward PC or other diacyl phosphoglycerides, although it exhibited some activity against lyso PC. Ceramide phosphate was prepared by treating egg SM liposomes containing 10 mol % egg phosphatidylethanolamine (PE) with recombinant SMase D and purified by silica gel TLC in a chloroform/methanol/water solvent system (65:25:4, v/v). The presence of PE was necessary for the optimal hydrolysis of PC, although PE itself was not hydrolyzed by the enzyme.

LCAT and Apo AI Preparation. LCAT was purified, as described previously (*23*), from normal human plasma obtained from the local blood bank. For most experiments, the phenyl-Sepharose eluate was used instead of the more purified enzyme, because of the instability of the pure enzyme. The major contaminant in the phenyl-Sepharose eluate was albumin, which is a normal constituent of the reaction mixture. The amounts of PC, as measured by lipid phosphorus (*24*) and cholesterol (measured with an enzymatic kit from Waco) in the enzyme preparation, were negligible in the sample (the detection limits were 50 ng and 1 μg for lipid phosphorus and cholesterol, respectively). Apoprotein AI was purified from normal human HDL, as described previously (*25*).

Substrate Preparation. Proteoliposome substrates were prepared by a modification of the method of Chen and Albers (*23*). Briefly, 375 nmol of PC, 18.75 nmol of unlabeled cholesterol, 0.3 μCi of [^{14}C]cholesterol, and varying amounts of sphingolipids, where indicated, were added to a conical bottom screw-cap glass tube, and the solvent was evaporated under nitrogen. The lipids were redissolved in 120 μL of ethanol and dried again under nitrogen. The sample was then dispersed in 12 μL of 750 mM sodium cholate in standard buffer [0.15 M NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.05% sodium azide]. To the resulting dispersion was added 30.0 μL of standard buffer, and the sample was incubated at 60 °C for 10 min and then at 37 °C for 10 min. To the cooled sample was added 30.0 μg of apo A1 in standard buffer, and the final volume was adjusted to 150 μL with standard buffer. Samples were vortexed for 1 min and incubated for 30 min at 37 °C. Sodium cholate was removed by extensive dialysis against 3 × 1 L of standard buffer (overnight at 4 °C, 7−8 h at room temperature, and finally 2 days at 4 °C).

Diether PC-containing proteoliposomes were prepared as described above, except that 80% of the PC was replaced with dioctadecenyl (di-18:1) ether PC. The amount of cholesterol was decreased accordingly to keep the (diester) PC:cholesterol molar ratio at 20:1. The standard reaction mixture contained 75 nmol of PC, 3.75 nmol of cholesterol, and 300 nmol of di-18:1 PC ether in 150 μL. Proteoliposomes for the determination of the phospholipase A activity of LCAT in the ether matrix were prepared as described above, except that 0.5 μCi of 16:0−[1-^{14}C]18:2 PC was used as the label, and cholesterol was omitted.

Assay of the LCAT Activity. LCAT activity was measured in a total volume of 200 μL containing 2.5 mg/mL human serum albumin, 10 mM β-mercaptoethanol, 20 μL of proteoliposome preparation (containing 50 nmol of PC and 2.5 nmol of labeled cholesterol unless indicated otherwise), and 10−30 μL (5−15 μg of protein) of the enzyme preparation (phenyl-Sepharose eluate). The amount of enzyme was adjusted to give a 15−25% esterification of labeled cholesterol in 30 min. The reaction was stopped by adding 800 μL of ethanol, and the lipids were extracted with 2 × 2 mL of hexane, containing 25 μg/mL cholesterol and 25 μg/mL cholesteryl oleate as carriers. The lipids were separated on a silica gel TLC plate using petroleum ether and ethyl acetate (85:15, v/v) as the mobile phase. Cholesterol and cholesterol ester bands were scraped from the plate, and their radioactivity was measured in a liquid scintillation counter.

Assay of Phospholipase Activity. The reaction conditions for phospholipase activity of LCAT were similar to those for the cholesterol esterification assay, except that the substrate contained labeled PC (16:0−[1-^{14}C]18:2 PC) and did not contain cholesterol, unless otherwise indicated. The reaction was stopped by the addition of 1 mL of methanol containing 100 μg of cholesteryl oleate and 100 μg of free oleic acid, and the samples were extracted by the method of Bligh and Dyer (*26*). The lipids were separated on a silica gel TLC plate using a hexane/ethyl acetate/acetic acid mobile phase (80:20:1, v/v). PC, cholesteryl ester, and free fatty acid bands were scraped from the plate, and their radioactivity was determined in a liquid scintillation counter.

SMase Treatments. When the SM in the substrate was hydrolyzed before reaction with LCAT, the proteoliposome substrate was first incubated (for 30−60 min) with the indicated concentration of SMase C or SMase D at pH 7.4 in standard buffer in the presence of 0.2 mM Mg^{2+} and 0.05 mM Mn^{2+}, in a final volume of 0.2 mL. The reaction was stopped by the addition of 4 mM EDTA in 20 mM phosphate

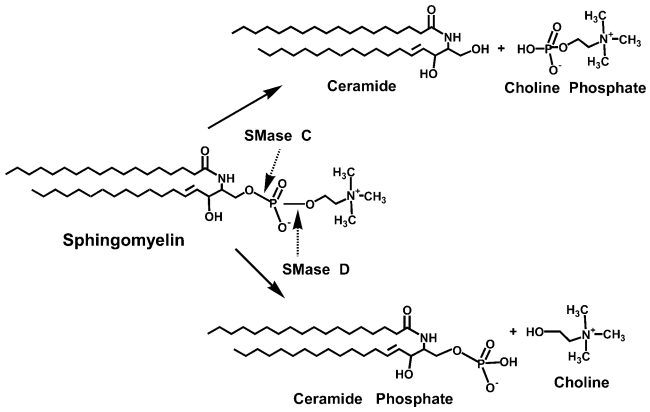
buffer (pH 7.5), after which human serum albumin, mercaptoethanol, and LCAT were added as described for the cholesterol esterification assay, and the incubation was continued for an additional 30 min in a final volume of 0.4 mL. The LCAT reaction was stopped by the addition of 1 mL of methanol, and the lipids were extracted by the procedure of Bligh and Dyer (*26*) and processed as described above.

Determination of the Fatty Acid Composition. For the determination of the composition of free fatty acids released by LCAT, the reaction mixture was scaled up by using 120 μL of proteoliposome substrate (containing 300 nmol of total PC, but no cholesterol). The composition of the reaction mixture was identical to that used for the LCAT activity measurement, except that the reaction volume was increased to 400 μL, the amount of enzyme was increased 10 times, and the incubation time was extended to 60 min. In some experiments, the substrate was completely hydrolyzed with snake venom phospholipase A to determine the *sn*-2 fatty acid composition of the PC substrate that was used. This was performed in a reaction mixture identical to LCAT but supplemented with 10 mM Ca^{2+}, 1.2 units of *Naja mozambique* phospholipase A_2, and 12 μL of the standard proteoliposome preparation (containing 30 nmol of total PC).

The reactions were stopped by the addition of 1.6 mL of ethanol, and the lipids were extracted with 2 × 4 mL of hexane after addition of 2 μg of heptadecanoic acid (17:0) as a carrier and internal standard. The solvent was evaporated under nitrogen, and the lipids were separated on a silica gel plate using a hexane/diethyl ether/acetic acid solvent system (70:30:1, v/v) or a hexane/ethyl acetate/acetic acid solvent system (80:20:1, v/v). The free fatty acid spot was identified by exposing only the lane containing the oleic acid standard (100 μg) to iodine vapors. The area corresponding to the free fatty acid was scraped from each sample lane and transferred to a screw-cap glass tube. The fatty acids were methylated with an instant methanolic HCl kit (acetyl chloride in methanol, Alltech Associates), according to the manufacturer's protocol. Water (1 mL) was added, and the fatty acid methyl esters were extracted twice with 2 mL of hexane. The solvent was evaporated and the sample redissolved in 20 μL of hexane, and 3 μL of it was injected into a Shimadzu GC-17A instrument, equipped with a flame ionization detector. The fatty acid methyl esters were analyzed under the conditions described previously (*27*). The concentrations of individual fatty acids were calculated with the help of the internal standard (17:0).

Determination of the Fatty Acid Specificity of LCAT. For the analysis of CE species formed by LCAT, the reaction mixture was scaled up to contain 50 μL of the proteoliposome preparation (125 nmol of PC and 6.25 nmol of cholesterol), and the lipids were extracted as described for the standard LCAT assay, after which 10% of the lipid extract was used for the determination of the total enzyme activity. The remainder was concentrated under nitrogen and loaded onto a C-18 reverse phase HPLC column. CE species were separated from each other and from free cholesterol using an acetonitrile/tetrahydrofuran/water solvent system (65:35:1.9, v/v), and the radioactivity of the peaks was determined online with a radioactivity detector, as described previously (*28*).

Scheme 1: Sites of Action of SMase C and SMase D



RESULTS

Effect of SMase C and SMase D on LCAT Activity. Although previous studies from our laboratory and others showed that SM is a physiological inhibitor of the LCAT reaction (*18*−*21*), the structural requirements for this inhibition were not investigated. As a first step in understanding the molecular requirements for LCAT inhibition, we treated the SM-containing proteoliposome substrate with either SMase C, which hydrolyzes SM to ceramide, or SMase D, which hydrolyzes it to ceramide phosphate (see Scheme 1), and then determined the effect on LCAT activity. As shown in Figure 1, the presence of SM at 50 mol % PC resulted in ~65% inhibition of the activity, and this inhibition was abolished by pretreatment of the substrate with SMase C, in agreement with our previous studies (*18*). Compared to the untreated control, SMase C treatment resulted in a 3-fold stimulation of the enzyme activity. In contrast, when the substrate was treated with SMase D before incubation with LCAT, there was a further inhibition of LCAT activity, compared to the untreated sample. Approximately 80% of SM was hydrolyzed under these conditions by both SMases. When the substrate was first treated with SMase C and then with SMase D, there was no inhibition of LCAT activity, showing that SMase D has no direct effect on LCAT activity. Since most of the SM was converted to ceramide before the addition of SMase D, these results also show that the formation of ceramide phosphate was necessary for the improved inhibition of LCAT by SMase D. When the substrate was first treated with SMase D and then with SMase C, the LCAT reaction was inhibited, showing that the depletion of SM is not enough, but the formation of ceramide was necessary, for the activation to occur. Treatment of SM-free liposomes with SMase D did not affect the LCAT activity (results not shown), supporting the conclusion that the formation of ceramide phosphate was necessary for the inhibition.

Correlation of LCAT Activity with Degradation of SM. To further establish that the differential effects of the two SMases are due to the different products formed rather than due to different levels of SM hydrolysis, we compared the amount of SM remaining after treatment with increasing concentrations of SMase C or SMase D, with the subsequent effect on LCAT activity. For studying the SMase C effect, we used a substrate that contained 50 mol % SM (relative to PC), whereas for the SMase D effect, we used a substrate that contained 35 mol % SM. We used different initial

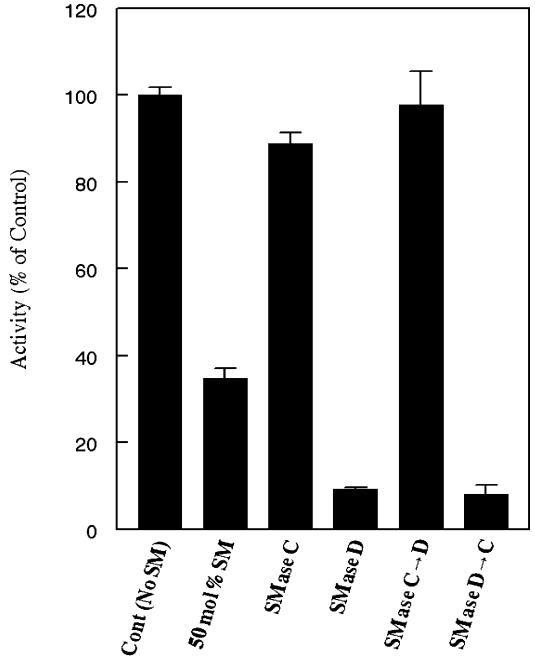


FIGURE 1: Effect of pretreatment of a SM-containing substrate with SMase C or D on LCAT activity. Egg PC/[^{14}C]cholesterol proteoliposomes containing 50 mol % SM (with respect to PC) were first treated for 30 min with either SMase C (100 milliunits) or SMase D (100 milliunits), or a sequential combination of the two, in the presence of 50 μM Mn^{2+} and 200 μM Mg^{2+}, as described in Materials and Methods. The SMase reaction was stopped by the addition of 4 mM EDTA, and the substrate was then incubated with human LCAT (phenyl-Sepharose eluate) for an additional 30 min. The LCAT reaction was stopped by the addition of ethanol containing unlabeled FC and CE, and the percent of labeled FC esterified was determined as described in Materials and Methods. All activities are expressed as the percent of activity obtained with SM-free substrate. The values shown are means ± the standard error of the mean of four to six experiments.

concentrations of SM because we expected the LCAT activity to be stimulated by SMase C but inhibited further by SMase D. It is therefore necessary to start with weaker initial inhibition of LCAT activity in the case of SMase D, to show further inhibition by this enzyme. As shown in Figure 2, the LCAT reaction was activated in proportion to the SM hydrolyzed by SMase C, whereas it was inhibited in parallel with the SM hydrolysis by SMase D. It is noteworthy that the full enzyme activity (i.e., the activity observed in the absence of SM) was restored after only a partial hydrolysis of SM by SMase C, suggesting that ceramide may either independently stimulate LCAT reaction or neutralize the effect of the remaining SM. The data in Figure 2 show that the full activity was restored after treatment with 120 milliunits of SMase C, although the SM remaining in the reaction mixture was ~32 mol % PC. This amount of SM by itself (in the absence of SMase C reaction) normally inhibits LCAT activity by ~40% (see the control value for SMase D studies at the bottom). Treatment with larger amounts of SMase C did not further stimulate LCAT activity and, in fact, inhibited the reaction by ~15%, although the SM content was reduced to less than 10 mol % PC (result not shown).

Effect of Ceramide and Ceramide Phosphate. The results described above strongly suggest that the enzymatic generation of ceramide activates the LCAT reaction, whereas the generation of ceramide phosphate inhibits it. To investigate

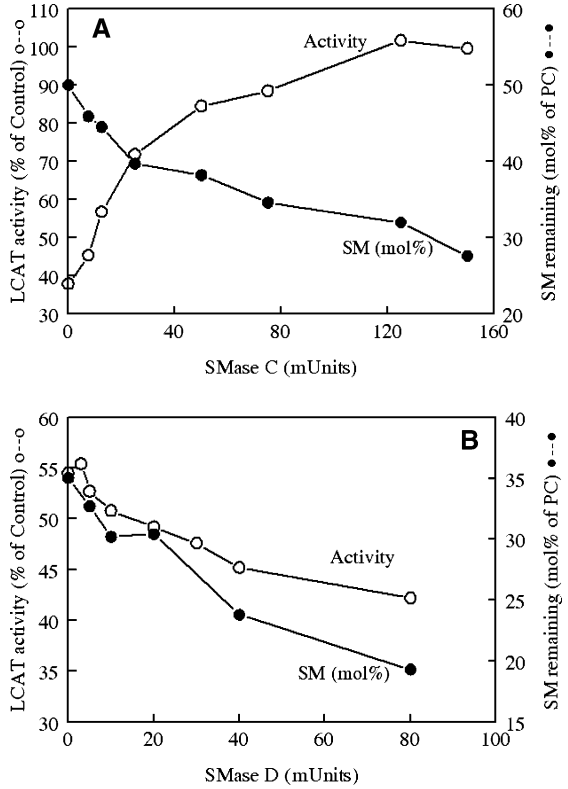


FIGURE 2: Correlation of LCAT activity with the extent of SM hydrolysis by SMase C or SMase D. (A) For SMase C, egg PC/[^{14}C]FC proteoliposomes containing 50 mol % SM (with respect to PC) were treated with varying amounts of SMase C for 30 min in the presence of 50 μM Mn^{2+} and 200 μM Mg^{2+}. The SMase reaction was stopped by the addition of 4 mM EDTA, and the sample was then incubated with LCAT for 30 min. LCAT activity was determined as described in Materials and Methods. The extent of SM hydrolysis was determined by estimating the amount of SM remaining in separate aliquots of the reaction mixture treated identically with SMase. For this purpose, the lipids were extracted by the procedure of Bligh and Dyer (*26*), the lipid extract was separated on silica gel TLC plates with a chloroform/methanol/water solvent system (65:25:4, v/v), the spot corresponding to authentic SM was scraped, and the amount of the lipid phosphorus was determined by the modified Bartlett procedure (*24*). The LCAT activity is expressed as a percent of the activity obtained with the SM-free substrate. The values shown are averages of duplicate samples. (B) For SMase D, egg PC/[^{14}C]cholesterol liposomes containing 35 mol % SM (with respect to PC) were treated with varying concentrations of recombinant SMase D for 30 min, and the LCAT activity and SM levels were determined as described above for SMase C. All values are means of two separate experiments.

the effects of ceramide and ceramide phosphate directly, we incorporated varying amounts of these lipids into the proteoliposomes in the absence of SM and determined the effect on LCAT activity. As shown in Figure 3, low concentrations of ceramide (<0.2 mol % PC) stimulated the LCAT activity by ~15%, but higher concentrations either had no effect or inhibited the enzyme. Ceramide phosphate, on the other hand, had no stimulatory effect at any concentration but consistently inhibited the reaction above 1 mol % PC. At a ceramide phosphate concentration of 25 mol % PC, the enzyme activity was inhibited by 80%. In comparison, SM at 25 mol % inhibited the reaction by only ~25−35% (see Figures 4 and 5). Thus ceramide phosphate is a stronger inhibitor of LCAT than SM at an equivalent concentration, and this accounts for the inhibitory effect of SMase D on SM-containing substrate.

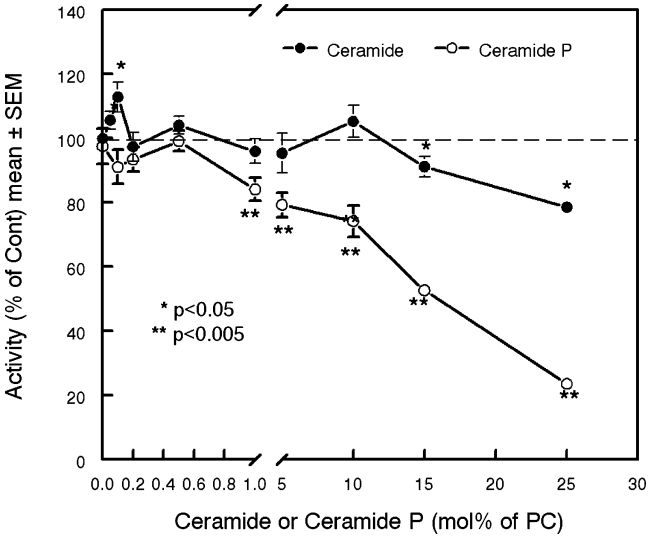


FIGURE 3: Effect of ceramide or ceramide phosphate in SM-free proteoliposomes. Substrates containing the indicated mole percent of ceramide (●) or ceramide phosphate (○) were prepared as described in Materials and Methods. The esterification of labeled cholesterol by LCAT was assessed as described in the text. Values shown are means ± the standard error of the mean of at least three experiments. In comparison with sphingolipid-free substrate, one asterisk indicates $p < 0.05$ and two asterisks indicate $p < 0.005$.

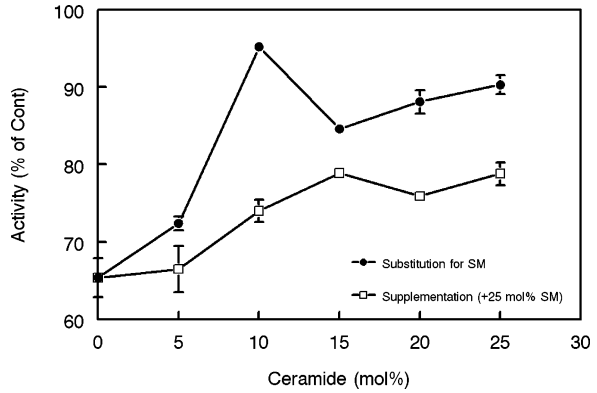


FIGURE 4: Effect of incorporation of ceramide into the SM-containing substrate. Empty squares indicate the substrates in which the indicated mole percent of ceramide was incorporated in addition to the 25 mol % SM (supplementation). Filled circles indicate substrates in which the indicated mol percent of ceramide replaced equimolar amounts of SM, keeping the total sphingolipid concentration at 25 mol % PC (substitution). All enzyme activities are expressed as the percent of activity measured in the absence of any sphingolipid (means ± the standard error of the mean of three experiments).

Effect of Ceramide in the Presence of SM. Results in Figure 2 show that partial hydrolysis of SM restored the LCAT activity to that of the SM-free substrate, whereas the results in Figure 3 show that ceramide is not a strong activator of LCAT in the absence of SM. This raises the possibility that ceramide counteracts the effect of SM, rather than directly activating the enzyme. To test this possibility, we incorporated increasing amounts of ceramide into the proteoliposome substrate containing 25 mol % SM, either as a supplement or as a substitute for SM. The latter condition simulates the results of the SMase C reaction and keeps the total sphingolipid concentration at 25 mol % PC. As shown in Figure 4, the enzyme activity was inhibited by ~35% in the presence of 25 mol % SM alone. When ceramide was added as a supplement (i.e., without decreasing the SM concentration), there was a partial restoration of the activity (up to 80% of the SM-free control) at 15 mol % ceramide (with 25 mol % SM, bringing the total sphingolipid concentration to 40 mol %). When ceramide replaced SM, however, almost complete restoration of the activity was observed with only partial replacement (10 mol % ceramide and 15 mol % SM). Replacing a further amount of SM with ceramide showed no further stimulation. It may be noted that 15 mol % SM, in the absence of ceramide, inhibits the enzyme activity by 20−30% (see Figure 5). Since this inhibition is abolished by the presence of 10 mol % ceramide, these results show that ceramide and SM have opposing effects on LCAT activity.

Effect of Sphingolipids on the Phospholipase A Activity of LCAT. LCAT reaction takes place in two distinct steps, namely, the formation of an acyl−enzyme intermediate with the acyl group of PC and the active site serine followed by the transfer of the acyl group to cholesterol. In the absence of a sufficient amount of cholesterol or another acyl acceptor, the acyl group is released as free fatty acid (phospholipase A reaction) (*29*). The first step of the reaction can, therefore, be studied as the phospholipase A activity by measuring the rate of release of labeled free fatty acid from labeled PC. Previous studies showed that ceramide is an activator of various phospholipase reactions due to its bilayer-disrupting properties (*12*, *13*, *15*, *16*). Recent studies also show that ceramide influences the distribution of cholesterol in the membrane bilayer, by specifically displacing it from the raft domains (*30*, *31*). It is, therefore, possible that ceramide has independent effects on the two steps of the LCAT reaction. To test this possibility, we prepared proteoliposome substrates with 16:0−[1-^{14}C]18:2-PC in the presence of 5 mol % cholesterol. Under these conditions, both phospholipase A and cholesterol esterification can be assessed (*25*). The effect of incorporation of ceramide, ceramide phosphate, or SM into this substrate on the two reactions of LCAT was investigated. As shown in Figure 5, ceramide at 5 and 15 mol % activated the phospholipase A activity by 25−30% but had a much weaker effect on cholesterol esterification. In fact, the higher concentration of ceramide slightly inhibited cholesterol esterification. Ceramide phosphate inhibited both reactions to the same extent at low concentrations but inhibited phospholipase activity more at higher concentrations. SM, at 15 mol %, inhibited both reactions to the same extent. It should be pointed out since cholesterol esterification is the sum of both reactions, the inhibition of the second step by ceramide may be greater than is apparent here, since the first step is actually stimulated by it.

Effect of Ceramide on the Fatty Acid Specificity of LCAT. Studies by Koumanov et al. (*13*) showed that ceramide influences the fatty acid specificity of group IIa $sPLA_2$, selectively stimulating the release of polyunsaturated fatty acids from phosphatidylethanolamine/phosphatidylserine (PE/PS) mixtures. To investigate whether ceramide also influences the CE species formed by LCAT, we prepared proteoliposomes with defined PC composition and determined the CE species formed after reaction with LCAT. The substrate contained an equimolar mixture of four common PC species of human plasma (16:0−18:2, 16:0−18:1, 16.0−20:4, and 16:0−16:0), [^{14}C]cholesterol, apo AI, and 0, 5, or 15 mol % ceramide (relative to PC). After incubation with LCAT, the labeled CE species that formed were separated

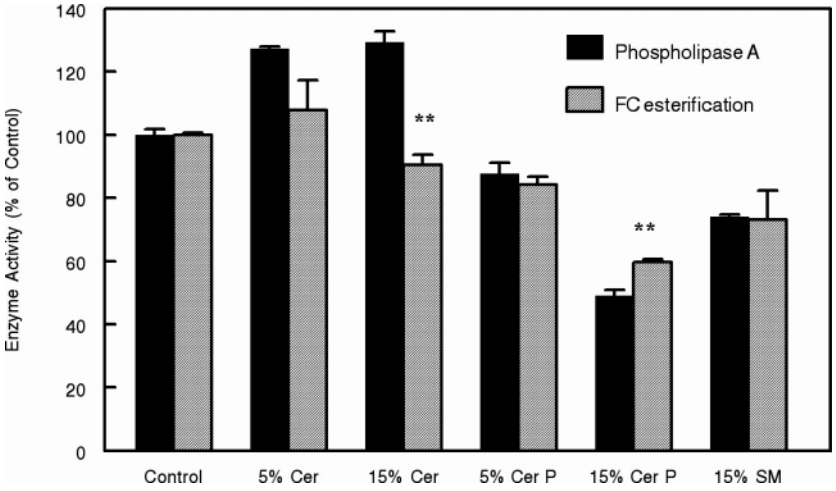


FIGURE 5: Comparative effects of sphingolipids on the two steps of the LCAT reaction. Proteoliposome substrates containing 16:0−[1-^{14}C]18:2 PC, unlabeled cholesterol (at a PC:cholesterol molar ratio of 20:1), and the indicated mole percent of sphingolipid were prepared as described in the text. After incubation with purified LCAT for 30 min, the lipids were extracted with ethanol and hexane, and the radioactivity in free fatty acids and CE was determined as described in Materials and Methods. All activities are expressed as the percent of the activity obtained in the absence of added sphingolipid. Values are means ± the standard error of the mean of at least three experiments. Where indicated with asterisks ($p < 0.005$), the effect on phospholipase A activity was significantly different from the effect on FC esterification activity.

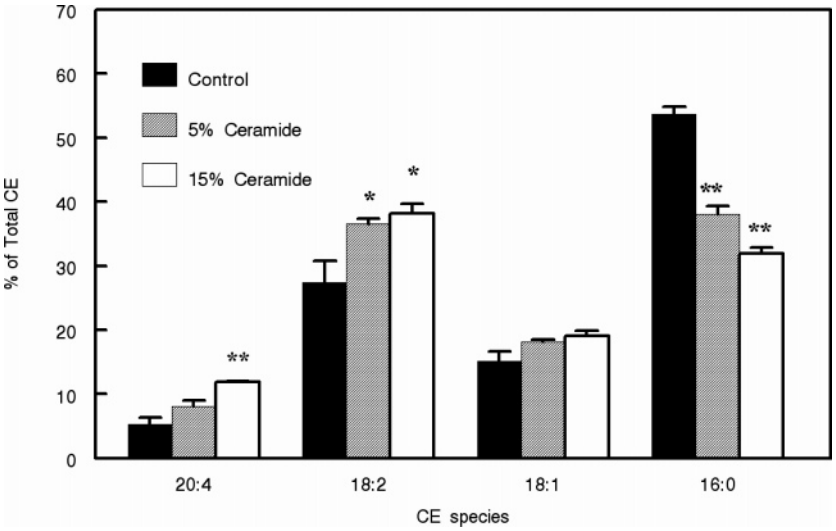


FIGURE 6: Effect of ceramide on the fatty acid specificity of the LCAT reaction. Proteoliposomes containing an equimolar mixture of 16:0−16:0 PC, 16:0−18:1 PC, 16:0−18:2 PC, and 16:0−20:4 PC, labeled cholesterol (at a total PC:FC ratio of 20:1), and 0, 5, or 15 mol % ceramide (with respect to total PC) were prepared as described in Materials and Methods. The reaction mixture was scaled up to contain 125 nmol of PC and 6.25 nmol of labeled cholesterol per reaction. After lipid extraction, 10% of the lipid extract was taken for the determination of total enzyme activity by TLC, and the rest was used for the separation of CE species by reverse phase HPLC as described in Materials and Methods. The activity of the enzyme (picomoles of CE formed in 30 min) in this experiment was 412 ± 32 for control, 553 ± 47 for 5 mol % ceramide, and 606 ± 56 for 15 mol % ceramide. Values are means ± the standard error of the mean of three experiments. Statistical significance of the difference between control and ceramide-containing substrates was determined by a paired *t* test: $p < 0.05$ (one asterisk) and $p < 0.005$ (two asterisks).

by HPLC, and their radioactivity was determined as described in Materials and Methods. As shown in Figure 6, the presence of ceramide significantly stimulated the synthesis of 18:2 CE and 20:4 CE, while it had no influence on 18:1 CE. The synthesis of 16:0 CE was significantly inhibited by the presence of ceramide. These results are similar to the effect of ceramide on sPLA$_2$ IIa hydrolysis of a PE/PS mixture, where the release of 20:4 and 18:2 was stimulated more than the release of 18:1 (*13*). It may be pointed out that in the study presented here, the level of formation of 16:0 CE was greater than that expected from the percent of 16:0−16:0 PC in the mixture (25%), although LCAT is not known to be specific for this PC species (*32*, *33*). This disproportionate synthesis of 16:0 CE is due to the presence of *sn*-2-16:0 PC isomers as impurities in the synthetic PCs used. Thus, 16:0−18:2 PC, 16:0−18:1 PC, and 16:0−20:4 PC each had 7−15% of their *sn*-2 position occupied by 16:0, as determined by the snake venom PLA$_2$ treatment. Consequently, the total 16:0 present at the *sn*-2 position of the PC mixture was ~33%, instead of 25%. In addition, we have previously shown that human LCAT transfers some 16:0 from the *sn*-1 position of 16:0−20:4 PC (*25*, *34*). While these factors resulted in the formation of a higher-than-expected percent of 16:0 CE, the presence of ceramide significantly decreased the extent of 16:0 CE synthesis, suggesting a specific inhibitory effect of ceramide on the synthesis of saturated CE. However, since the total activity of the enzyme was stimulated by ~30% in the presence of ceramide in these

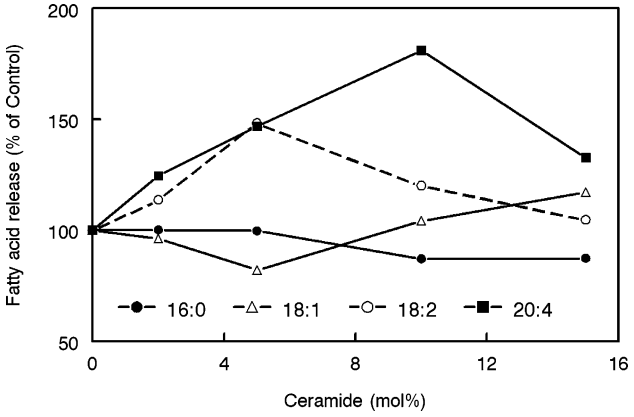


FIGURE 7: Effect of ceramide on the fatty acid specificity of the phospholipase A reaction of LCAT. Varying amounts of ceramide were incorporated into proteoliposomes containing an equimolar mixture of 16:0−16:0 PC, 16:0−18:1 PC, 16:0−18:2 PC, and 16:0−20:4 PC, in the absence of cholesterol. After reaction with LCAT, the released free fatty acids were analyzed by gas−liquid chromatography, using 17:0 free fatty acid as an internal standard. The concentration of individual fatty acids released was calculated as the percent of the amount released in the absence of ceramide. Values are averages of two experiments.

experiments, the net effect is the stimulation of synthesis of 20:4 CE and 18:2 CE, rather than an inhibition of 16:0 CE synthesis.

Effect of Ceramide on Fatty Acid Specificity of the Phospholipase Reaction of LCAT. To determine whether ceramide influenced the fatty acid specificity at the formation of the acyl−enzyme intermediate or at the cholesterol esterification step, the effect of incorporation of ceramide on the composition of fatty acids released from a defined mixture of PC species (in the absence of cholesterol) was tested. Varying amounts of egg ceramide were incorporated into a proteoliposome substrate that contained an equimolar mixture of 16:0−16:0 PC, 16:0−18:1 PC, 16:0−18:2 PC, and 16:0−20:4 PC, and after reaction with LCAT, the composition of the fatty acids released was determined by gas−liquid chromatography as described in Materials and Methods. The percent of individual fatty acids released in the presence of ceramide was calculated relative to its percentage in the absence of ceramide. As shown in Figure 7, the release of both 20:4 and 18:2 was stimulated by the presence of ceramide. Since the percent stimulation is comparable to that observed for cholesterol esterification (see Figure 6), the specificity effect of ceramide appears to be exerted at the first (acyl−enzyme intermediate formation) step of the LCAT reaction. The release of 16:0 was inhibited by the presence of ceramide, but this effect was weaker than that observed for cholesterol esterification.

Influence of the Matrix on the Ceramide Effect. The individual PCs used in the experiment described above differ significantly in their physical properties, and therefore, it is possible that the effect of ceramide on the fatty acid specificity of LCAT is due to its macromolecular effects on the proteoliposome rather than on the interaction of the individual PC molecules with the active site. To minimize the physical differences in the PCs, we incorporated the four PCs at equimolar concentrations into the di-18:1 ether PC matrix, which provides an inert milieu for the substrate PCs (*35*). The diether PC made up 80% of the total phospholipid, while the four diester PCs constituted 5% each of the total. When ceramide was incorporated into this substrate at either 5 or 15 mol % of the total PC and the CE species formed by LCAT were analyzed, the differential effect of ceramide on the various CE species disappeared (Figure 8). These results indicate that the major effect of ceramide is on the macromolecular structure of the substrate and that when this effect is minimized by the use of the PC ether matrix, the ceramide effect on fatty acid specificity is also eliminated.

DISCUSSION

LCAT reaction is responsible for the synthesis of most of the cholesteryl esters present in human plasma and is a critical component of the reverse cholesterol transport pathway (*29*, *36*). The plasma levels of HDL are positively correlated with this enzyme activity, and therefore, the regulation of its activity is of great clinical interest. Furthermore, since different CE species appear to have different atherogenic properties (*37*, *38*), the regulation of its fatty acid specificity is also of importance. The physiological factors involved in the regulation of LCAT activity and fatty acid specificity are poorly understood. Previous studies from our laboratory (*18*) as well as others (*19*−*21*) showed that SM, a key phospholipid constituent of all lipoproteins, is a physiological inhibitor of the LCAT reaction. Depletion of SM by bacterial SMase C stimulates the reaction significantly in both native lipoproteins and synthetic substrates (*18*), supporting the inhibitory role of SM in the lipoproteins. A recent report by Lee et al. (*10*) showed that HDL from patients with Niemann-Pick disease, which contains a high concentration of SM, also exhibits impaired cholesterol esterification by LCAT and that this is mostly attributable to the lack of secretory SMase in these patients. However, studies with various phospholipases showed that ceramide, the product of SMase C reaction, is an independent activator of the phospholipases (*12*−*16*). Since LCAT is essentially a specialized phospholipase A that utilizes cholesterol as an acyl acceptor in place of water, this raised the possibility that the stimulation of LCAT reaction by SMase C treatment of lipoproteins is due to the formation of an activator (ceramide), rather than depletion of the inhibitor (SM). The availability of recombinant SMase D, which degrades SM without the generation of ceramide, provides a new tool for addressing this question (*22*) (see Scheme 1). Our previous studies showed, for instance, differential effects of SMase D and SMase C in cultured cells on the metabolic fate of the plasma membrane cholesterol, suggesting an independent effect of ceramide (*22*). The results presented here show that the degradation of SM by the two SMases yields diametrically opposite effects on the LCAT reaction. While degradation by SMase C activated the reaction by up to 3-fold and completely reversed the inhibitory effect of SM, degradation by SMase D inhibited it further, indicating that the depletion of SM alone does not account for the effects of SMase C. The physiological significance of these findings is not clear because while the presence of ceramide in the lipoproteins has been established (*6*−*8*), the presence of ceramide phosphate has not been demonstrated. Nevertheless, these findings offer a clue about the mechanism of action of sphingolipids on LCAT. First, they show that the presence of choline is not necessary for the SM inhibition of LCAT. Second, the presence of a phosphate moiety is critical because while ceramide is an activator of the reaction,

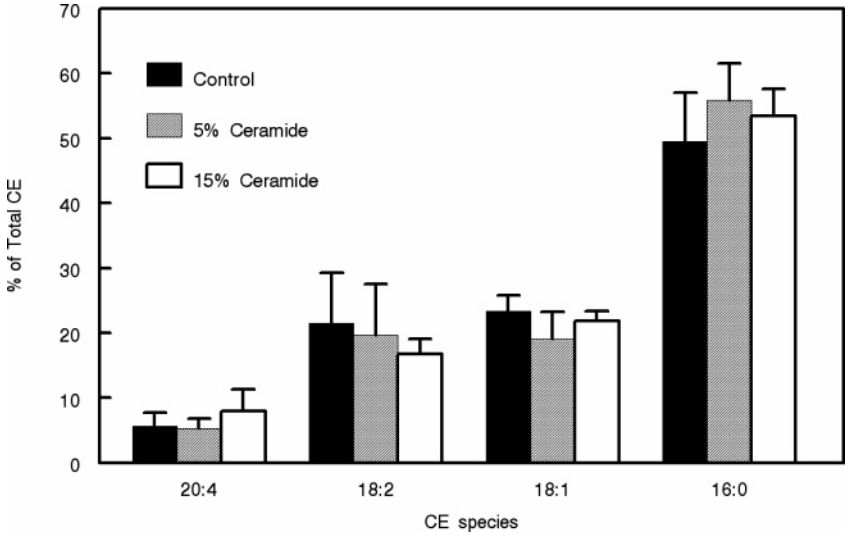


FIGURE 8: Effect of ceramide on LCAT fatty acid specificity in the presence of the diether PC matrix. The substrate preparation was similar to that described in the legend of Figure 6, except that 80% of the PC was replaced with dioleyl ether PC, which provides an inert matrix for the substrate. The diacyl PC:labeled FC ratio was maintained at 20:1, and the mole percent of ceramide was calculated with respect to diacyl PC only. The analysis of CE species composition by HPLC was as described in the text. Values are means ± the standard error of the mean of three separate experiments. The total activity of the enzyme (in picomoles of CE formed in 30 min) was 156 ± 4 (control), 198 ± 7 (5 mol % ceramide), and 142 ± 5 (15 mol % ceramide). None of the CE species exhibited a significant difference (by a Student's *t* test) between control and ceramide-containing substrates.

ceramide phosphate is a more potent inhibitor than SM. Since ceramide phosphate is a phospholipid, it is likely to be integrated into the membrane bilayer, unlike ceramide which is known to disrupt the bilayer structure (*12*, *17*). These results are in sharp contrast to the findings of Pettus et al. (*39*), who reported that ceramide phosphate activates cytosolic PLA_2 by direct interaction with the CaLB/C2 domain of the enzyme, as well as by translocation of the enzyme to the Golgi apparatus. This interaction of ceramide phosphate with PLA_2, however, requires Ca^{2+}. Since the LCAT reaction is carried out in the absence of Ca^{2+}, such an interaction with LCAT is unlikely. Moreover, since the amount of ceramide phosphate required to inhibit LCAT is rather large (>1 mol % PC), it is more likely to be acting through its effects on the substrate.

An interesting finding in our studies is that while ceramide by itself is only a weak activator of LCAT, it effectively neutralizes the inhibitory effect of SM. Since both ceramide and SM are present in the same lipoprotein particle (*7*, *8*), this observation could be physiologically significant in the regulation of LCAT activity and substrate specificity. The inhibitory effect of SM on LCAT is due to its competition with PC substrate (*18*), as well as due to its physical effects on the lipoprotein substrate (*19*). How exactly ceramide counteracts the effect of SM is not yet clear, but several possibilities could be considered. First, it is known that SM helps retain cholesterol in the ordered domains of the membrane (*40*, *41*), while ceramide specifically displaces cholesterol from the membrane rafts (*30*, *31*). It is therefore possible that ceramide offsets the inhibitory effect of SM by "liberating" the SM-sequestered cholesterol to accept the acyl group in the LCAT reaction. Our results, however, show that the major stimulatory effect of ceramide is on the first step of the reaction (acyl−enzyme intermediate formation) rather than on the acyl transfer step. Furthermore, the inhibitory effect of SM is also predominantly on the first step, because the phospholipase A reaction of the enzyme is as sensitive as the cholesterol esterification reaction (Figure 5) (*18*), even though SM is known to specifically interact with the cholesterol molecule (*40*−*42*). Thus, it appears unlikely that the ceramide effect is due to the displacement of cholesterol from the ordered domains of the membrane. A second possibility is that ceramide counteracts the effects of SM by disordering the membrane structure and thereby allowing an increased penetration of the enzyme into the bilayer. Since the membrane is less ordered in the absence of SM, this effect of ceramide would not be noticeable in the absence of SM. Third, the long chain ceramides are known to induce lateral phase separation of the PC bilayer into gel and liquid crystalline domains (*12*). Such phase separation could lead to the formation of long-lived boundary regions in which the individual PC molecules are less tightly bound (*43*) and therefore can dissociate from the bulk phase and interact with the enzyme's active site. Studies by Holopainen et al. (*44*) also show that while SM inhibits the lateral rates of diffusion of lipids in the bilayer, the presence of even a small percent of ceramide offsets this effect. It is therefore possible that the neutralizing effect of low concentrations of ceramide on the SM inhibition of LCAT is due to its ability to counteract the inhibitory effect of SM on the lateral diffusion rates. Higher concentrations of ceramide, on the other hand, increase the level of molecular packing of the PC molecules, in addition to increasing the lateral diffusion rates (*44*). Since the higher packing density of PC is inhibitory to LCAT reaction (*19*), this could explain the inhibitory effect of higher ceramide levels both in the presence and in the absence of SM. Ceramide is also believed to activate some phospholipases by direct interaction with the enzyme (*45*), but this is unlikely in the case of LCAT because the addition of ceramide separately to the reaction mixture did not stimulate the reaction (results not shown). Furthermore, as shown in Figure 3, incorporation of ceramide into the substrate (in the absence of SM) activated the reaction by less than 20%, in contrast to the 2−3-fold activation of $sPLA_2$ IIa (*13*). It also appears unlikely that ceramide acts on apo AI (the protein activator of LCAT),

because our earlier studies (*18*) showed that the inhibition by SM is not due to its effect on apo AI. On the basis of these considerations, we propose that the stimulation of LCAT activity by SMase C treatment is due to membrane reorganization by ceramide rather than due to a simple depletion of SM.

Our studies also show that ceramide stimulates the synthesis of polyunsaturated CE and inhibits the synthesis of saturated CE. These effects are similar to those reported for $sPLA_2$ activity with a mixture of PE and PS as a substrate (*13*). However, unlike the 15-fold increase in the rate of release of polyunsaturated fatty acids reported for $sPLA_2$, the synthesis of 20:4 CE by LCAT was stimulated only ~2-fold by ceramide. Although the presence of cholesterol has been shown to weaken the effect of ceramide on PLA_2 specificity (*13*), it is unlikely to be a factor in case of LCAT, because even in the absence of cholesterol, the release of 20:4 as free fatty acid (phospholipase A activity) was only stimulated ~2-fold (Figure 7). Interestingly, the specificity effect is abolished when the PC substrates were incorporated into an inert PC diether matrix, which minimizes the differences in the physical properties of the various PC substrates. This strongly suggests that ceramide affects the distribution of PC molecular species on the surface of the substrate particle, possibly through the microdomain formation. On the basis of small-angle X-ray refraction studies, Koumanov et al. (*13*) suggested that the long chain polyunsaturated PCs are concentrated in the intermediate structures between the hexagonal and lamellar phases created by the ceramide-induced phase separation and that these structures are more susceptible to the enzymatic attack. It is conceivable that the enrichment of lipoproteins with ceramide, either through SMase C action or through direct secretion from the tissues (*7*, *8*, *10*), could facilitate interaction of more polyunsaturated PCs with LCAT. Our previous studies also showed that the action of LCAT on 16:0−20:4 PC results in the formation of 20:4 lyso PC, due to an alteration in its positional specificity (*25*). Therefore, an increase in the level of ceramide during the inflammatory reactions could theoretically increase the rate of formation of 20:4 lyso PC, which is known to be a preferred source of the pro-inflammatory 20:4 for certain tissues (*46*).

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