Modulation of the activity and arachidonic acid selectivity of group X secretory phospholipase A_2 by sphingolipids

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Abstract **To investigate the role of sphingomyelin (SM) in the regulation of inflammatory reactions, we studied its effect on the activity and fatty acid specificity of group X secretory phospholipase A_2 ($sPLA_2X$). Compared with other phospholipases, recombinant $sPLA_2X$ released more arachidonate from HDL. Pretreatment of HDL with sphingomyelinase (SMase) C activated the $sPLA_2X$ activity, but the release of arachidonate was stimulated less than that of linoleate. In liposomes containing synthetic phosphatidylcholines (PCs), $sPLA_2X$ showed no clear selectivity among the various *sn*-2 unsaturated fatty acids. However, when SM was incorporated into liposomes at 30 mol%, the enzyme exhibited strong preference for arachidonate, although its overall activity was inhibited. Degradation of liposomal SM by SMase C resulted in $sPLA_2X$ activation and loss of its arachidonate preference. Incorporation of ceramide into HDL or PC liposomes activated the enzyme activity, the release of arachidonate being stimulated more than that of linoleate. SM-deficient cells released more arachidonate than normal cells in response to exogenous $sPLA_2X$. SMase pretreatment of normal cells stimulated the release of arachidonate by the exogenous $sPLA_2X$. These results show that SM not only inhibits $sPLA_2X$ activity but also contributes to its selectivity for arachidonate, whereas ceramide stimulates the hydrolysis of arachidonate-containing PCs.—** Singh, D. K., and P. V. Subbaiah. **Modulation of the activity and arachidonic acid selectivity of group X secretory phospholipase A_2 by sphingolipids. *J. Lipid Res.* 2007.** 48: **683–692.**

Supplementary key words sphingomyelin • ceramide • fatty acid specificity • inflammation

Sphingomyelin (SM) is the most abundant phospholipid, next to phosphatidylcholine (PC), in the plasma lipoproteins and is a major constituent of the plasma membrane of all mammalian cells. It is also a critical component of membrane rafts, which serve as platforms for several cellular functions, including receptor-ligand interactions and signal transduction (1, 2). The major metabolite of SM is ceramide, which is formed by the action of neutral or acid sphingomyelinases (SMases) and may be converted back to SM by the SM synthases. This SM-ceramide cycle is stimulated by various hormones, cytokines, and growth factors (3, 4) and plays an important role in the inflammatory response. Although the role of ceramide as a signaling molecule and as a promoter of apoptosis is well established (5–7), the possible direct role of SM in the membranes, other than as a structural component, has not received much attention. Previous studies from our laboratory and others showed that SM is a physiological inhibitor of several lipolytic enzymes, including LCAT (8–11), secretory phospholipase A_2 IIa ($sPLA_2$IIa) (12, 13), $sPLA_2$V (13), cytosolic PLA_2 (14), and lipoprotein lipase (15, 16), all of which use PC as a substrate. We proposed that, because of its structural similarity to PC, SM may act as a competitive inhibitor of the lipolytic enzymes by competing with PC in binding to the active site of the enzyme (8, 17).

SM may also affect the interfacial properties of the substrate and inhibit the binding or penetration of the enzyme into the bilayer (9). The bulk of cellular SM is present in the outermost monolayer of the cells (18), and this property may be important in the protection of membrane phospholipids against unregulated hydrolysis by exogenous phospholipases. There are several known $sPLA_2$s that are secreted by a variety of cells and that can potentially hydrolyze membrane phospholipids and cause cell damage. Approximately 10 different $sPLA_2$s that differ in their structure, calcium requirements, and substrate specificity have been identified (19). Although the physiological roles of individual $sPLA_2$s are not known, several studies indicate that they may be important in the inflammatory reactions (19, 20). Because the PLA_2 reactions can generate not only arachidonic acid, the precursor of proinflammatory lipid mediators such as prostaglandins and leukotrienes, but also lyso PC, a proinflammatory and

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Abbreviations: PC, phosphatidylcholine; SM, sphingomyelin; SMase, sphingomyelinase; $sPLA_2$, secretory phospholipase A_2.

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proatherogenic lipid (21), the regulation of these activities is physiologically important. By far the most important $sPLA_2$ in the generation of arachidonate from the cell membranes is the group X $sPLA_2$ ($sPLA_2X$), which is secreted by several inflammatory cells, including macrophages and endothelial cells (19). Hanasaki et al. (22) reported that $sPLA_2X$ releases arachidonate from PC and monocytes more efficiently than $sPLA_2IB$, $sPLA_2IIa$, or $sPLA_2V$. Bezzine et al. (23) similarly showed that exogenous $sPLA_2X$ efficiently releases arachidonate from the adherent mammalian cells, whereas $sPLA_2$ groups IB, IIa, and V were not able to do so. Furthermore, the enzyme appears to release the arachidonate from the cells by direct hydrolysis of membrane phospholipids, unlike $sPLA_2V$, which works through the activation of cytosolic PLA_2 (24, 25). Recent studies by Pruzanski et al. (26) with lipoprotein substrates also showed a preferential hydrolysis of arachidonate-containing PCs by $sPLA_2X$.

Although we reported previously the regulation of $sPLA_2IIa$ and $sPLA_2V$ by SM and ceramide (13), the possible modulation of $sPLA_2X$ by the sphingolipids has not been investigated. Because of the enzyme's importance in the preferential release of the proinflammatory arachidonate from the cells, the regulation of its activity would be of greater clinical interest. Therefore, we studied the effect of SM and ceramide on the activity and fatty acid specificity of recombinant human $sPLA_2X$ acting on plasma lipoproteins, synthetic liposomes, and cultured cells. The results presented here show that the activity of $sPLA_2X$ is inhibited by SM but activated by ceramide. Furthermore, the enzyme shows greater specificity for arachidonate in the presence of the SM, apparently because SM preferentially inhibits the release of 18:2. On the other hand, ceramide appears to directly stimulate the release of 20:4. These results suggest that SM and ceramide may play important roles in the inflammatory response by cells.

MATERIALS AND METHODS

Materials

Recombinant $sPLA_2X$ was purified from *Escherichia coli* expressing the gene for human $sPLA_2X$ (23). The plasmid encoding the gene was kindly supplied by Dr. Michael Gelb (University of Washington). Egg SM, brain SM, egg ceramide, synthetic ceramides of various *n*-acyl chain lengths, snake venom PLA_2, and SMase C from *Staphylococcus aureus* (120 U/mg) were all purchased from Sigma Chemical Co. (St. Louis, MO). SMase D from *Corynbacterium pseudotuberculosis* was purified from transfected *E. coli* as described previously (27). The SMase C and D activities were assayed using the Amplex Red SMase kit from Invitrogen. Synthetic PCs were purchased from Avanti Polar Lipids (Alabaster, AL) and were used without further purification. The positional purity of the PCs was determined from the composition of fatty acids released by the action of excess snake venom PLA_2, which hydrolyzed the *sn*-2 acyl esters completely (28). The contamination of each PC with the corresponding positional isomer was as follows: 16:0-18:1 PC, 5%; 16:0-18:2 PC, 1.5%; and 16:0-20:4 PC, 12%. Labeled PCs with [^{14}C]fatty acid at the *sn*-2 position (16:0-18:2 and 16:0-20:4) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Both preparations contained at least 97% of the label in the *sn*-2 position, as determined by snake venom PLA_2. CHO cells deficient in serine palmitoyl transferase activity (Ly-B cells) and the corresponding control cells (Ly-B/cLCB1) (29) were kindly supplied by Dr. K. Hanada (National Institutes of Infectious Diseases, Tokyo, Japan). Mouse macrophage cell line RAW 274.7 was obtained from the American Type Culture Collection.

HDL preparation

HDL was isolated from normal human plasma (obtained from a local blood bank, LifeSource) by sequential ultracentrifugation in KBr between the densities of 1.063 and 1.21 g/ml. The preparation was dialyzed against 10 mM Tris/Cl buffer, pH 7.4, containing 0.15 M NaCl and 1 mM EDTA and stored at 4°C. It was used for the enzyme assays within 3 weeks of preparation. In experiments in which HDL was first treated with SMases, the indicated amounts of SMase C (*S. aureus*) or SMase D (*C. pseudotuberculosis*) were added to 100 μg (cholesterol) of HDL in the presence of 0.1% BSA, 10 mM $MnCl_2$, and 10 mM $MgCl_2$. After incubation at 37°C for 60 min, 5 μg of $sPLA_2X$ and 10 mM $CaCl_2$ were added, and the activity was determined as described above. When HDL was enriched with ceramide, varying amounts of egg ceramide were added to HDL as an ethanol solution and incubated for 16 h at 37°C, before reacting with recombinant $sPLA_2X$.

Liposome substrate preparation

Except where indicated, the PCs were incorporated into liposomes by sonication. Briefly, the chloroform solution of PC (and ceramide or SM, where indicated) was dried under nitrogen, dissolved in 1 ml of ethanol, and again dried under nitrogen. The sample was then hydrated with 1 ml of Tris/Cl buffer (100 mM, pH 8.0) at 50°C under nitrogen for 1 h and sonicated for 2 min (15 s pulses) in a Sonics Vibra Cell sonicator at 4°C at 28% of maximum energy. The substrate was used within 7 days of preparation.

Enzyme assay

The reaction mixture for the assay of $sPLA_2X$ activity contained 100 μM PC (labeled or unlabeled), the indicated mol% ceramide or SM, 100 mM Tris/Cl, pH 8.0, 0.1% BSA, and 10 mM $CaCl_2$ in a final volume of 0.2 ml. The reaction was carried out for 30 min at 37°C and stopped by the addition of 0.5 ml of methanol. After adding 3 μg of 17:0 free fatty acid as an internal standard, the lipids were extracted by the Bligh and Dyer procedure (30) and separated on a silica gel TLC plate with the solvent system of chloroform-methanol-water (65:25:4, v/v). A mixture of egg PC, egg lyso PC, and free oleic acid was run on a separate lane for the purpose of identification. After chromatography, the sample lanes were covered with a glass plate, and the lane containing the standards was exposed to iodine vapors. The region of each sample lane corresponding to the free fatty acid standard was immediately scraped and was either analyzed by GC, as described below, or counted for radioactivity (when a labeled PC substrate was used). The enzyme activity was calculated as micrograms of fatty acid released and was corrected for the value of the control sample, in which the substrate was incubated in the absence of $sPLA_2X$.

Fatty acid specificity

The fatty acid specificity of the enzyme in the presence of HDL as a substrate was determined from the composition of free fatty acids released after the enzyme reaction. Normal HDL, ceramide-enriched HDL, or SMase-treated HDL (100 μg of cho-

lesterol) was incubated with $sPLA_2X$ (5 μg), 0.1% BSA, and 10 mM $CaCl_2$ at 37°C for 1 h. The total lipids were extracted by the Bligh and Dyer procedure (30) after adding 3 μg of 17:0 free fatty acid as an internal standard, and the lipids were separated on a TLC plate as described above. The spot corresponding to the free fatty acid standard was scraped from the plate, and methyl esters were prepared using the instant methanolic HCl kit (Alltech). The methyl esters were extracted with 2 ml of hexane (twice), concentrated, and analyzed by capillary GC as described previously (31).

The fatty acid specificity was also determined in the presence of a mixture of synthetic PCs. In this case, the reaction mixture contained an equimolar mixture of four synthetic PCs: 16:0-16:0, 16:0-18:1, 16:0-18:2, and 16:0-20:4 PCs, SM, or ceramide as indicated, 5 μg of $sPLA_2X$, 100 mM Tris/Cl, pH 8.0, 0.1% BSA, and 10 mM $CaCl_2$ in a final volume of 0.2 ml. The reaction was stopped by the addition of methanol, the lipids were extracted after adding 17:0 as an internal standard, and the composition of the free fatty acids was determined by GC as described above.

Correction for the positional impurity of synthetic PCs

As mentioned above, the synthetic PCs used here contained varying amounts of positional isomers as contamination, which results in overestimation of 16:0 released when the mixtures of four synthetic PCs was used as a substrate. Therefore, a correction factor was applied using the total hydrolysis of the four PC substrate with snake venom PLA_2, which is absolutely specific for the *sn*-2 position. The liposome preparations containing the equimolar PC mixture were hydrolyzed completely with excess snake venom PLA_2, and the deviation of each *sn*-2 fatty acid from the expected value (25%) was used as the correction factor for the experimental values obtained with $sPLA_2X$. Thus, for example, if the percentage of 18:1 released by snake venom PLA_2 in the mixture is 21% instead of the expected 25%, a correction factor of 1.19 (25 ÷ 21) was applied for the 18:1 value obtained with the same substrate and $sPLA_2X$, by multiplying it by 1.19.

RESULTS

Fatty acid specificity of $sPLA_2X$

To investigate the fatty acid specificity of $sPLA_2X$ in the presence of physiological substrates, we incubated normal human HDL with recombinant human $sPLA_2X$ and determined the composition of free fatty acids released. For comparison, the fatty acids released by $sPLA_2V$ under the same conditions were also determined. In addition, we analyzed the fatty acid composition of the *sn*-2 position of the HDL glycerophospholipids by treating HDL with excess snake venom PLA_2, which is not known to exhibit fatty acid specificity and which hydrolyzed the HDL phosphoglycerides completely under the conditions used. As shown in **Fig. 1**, the major fatty acids released by $sPLA_2X$ were 18:2 > 20:4 > 18:1 > 22:6, in agreement with the reports of Hanasaki et al. (32) and Pruzanski et al. (26). Minor fatty acids released (<1% of total) are not shown. As reported previously by us (13) and recently by Pruzanski et al. (26), the group V $sPLA_2$ released a lower percentage of 20:4 than expected from the *sn*-2 acyl composition. In contrast, $sPLA_2X$ released a significantly higher percentage of 20:4 than expected from the *sn*-2 acyl composition. It also released more 22:6 and less 18:1 than expected, although the former was not statistically significant. These results confirm the relative preference of $sPLA_2X$ compared with $sPLA_2V$ for the release of 20:4 from physiological substrates.

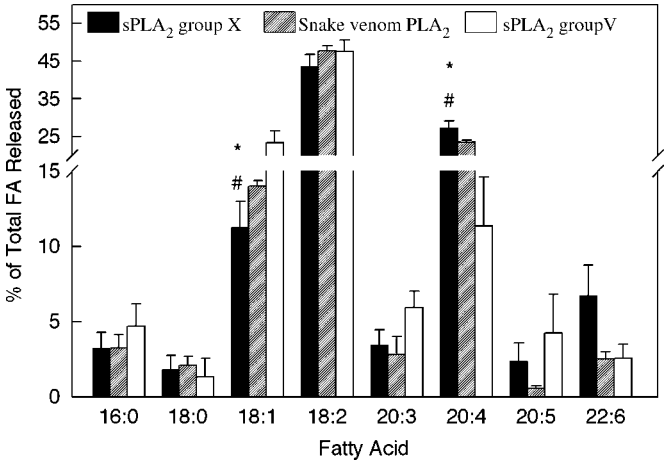

Fig. 1. Fatty acid specificity of secretory phospholipase A_2 X ($sPLA_2X$) with HDL as a substrate. One hundred micrograms of normal human HDL (cholesterol) was incubated with 5 μg of $sPLA_2X$, 2.5 μg of $sPLA_2V$, or 2.5 μg of snake venom PLA_2 for 60 min at 37°C in the presence of 10 mM Ca^{2+}, 0.1% BSA, and 100 mM Tris/Cl, pH 8.0, in a final volume of 200 μl. The reactions were stopped by the addition of 0.5 ml of methanol, and the total lipids were extracted (30) after adding 3 μg of 17:0 free fatty acid as an internal standard. The total lipids were separated by TLC on silica gel using the solvent system of chloroform-methanol-water (65:25:4, v/v). The spot corresponding to free fatty acid was scraped, methylated, and analyzed by GC, as described in Materials and Methods. The values shown are means ± SEM of six separate experiments for $sPLA_2X$ and nine experiments each for snake venom PLA_2 and $sPLA_2V$. * $P < 0.05$, comparison between $sPLA_2X$ and snake venom PLA_2; # $P < 0.05$, comparison between $sPLA_2X$ and $sPLA_2V$ (two-tailed *t*-test).

Effect of SM on $sPLA_2X$ activity

We next studied the effect of SM on the hydrolysis of 16:0-20:4 PC, which appears to be the preferred substrate of the enzyme, based on the results presented above as well as on the published data of Pruzanski et al. (26). For this purpose, we incorporated varying amounts of egg SM into liposomes containing 16:0-[^{14}C]20:4 PC and determined the release of labeled fatty acid after incubation with recombinant $sPLA_2X$. In addition, we pretreated the SM-containing liposomes with either SMase C or SMase D before incubation with $sPLA_2X$ to study the reversibility of the SM effect. SMase C hydrolyzes SM to ceramide and choline phosphate, whereas SMase D hydrolyzes it to ceramide phosphate and choline (27). As shown in **Fig. 2**, SM inhibited the hydrolysis of 16:0-20:4 PC by ~35% at 42 mol% concentration. This inhibition is lower than that found with $sPLA_2V$ (13) but was completely reversible by treatment with either SMase C or SMase D. Moreover, as observed with $sPLA_2V$ (13), treatment with SMase C stimulated the $sPLA_2X$ reaction above the SM-free control, showing that the ceramide generated by SMase stimulated

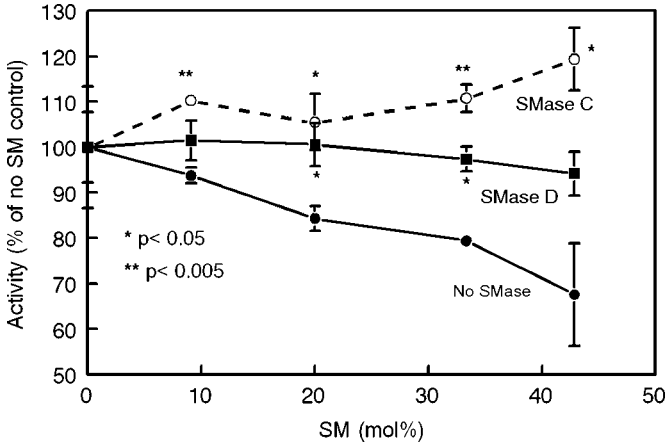

Fig. 2. Inhibition of $sPLA_2X$ by sphingomyelin (SM) and reversal of inhibition by sphingomyelinase (SMase) treatment. 16:0-[^{14}C]20:4 phosphatidylcholine (PC) liposomes containing varying amounts of egg SM were prepared by sonication. The exact concentration of SM in the liposomes (mol% PC) were 0, 9.1, 20.0, 33.3, and 42.9, as determined by lipid phosphorus. Aliquots of the liposomes containing SM were treated with either SMase C (0.05 units) or SMase D (0.125 units) for 60 min in the presence of 10 mM $MgCl_2$ and 10 mM $MnCl_2$. Then, $sPLA_2X$ (5 μg) was added, and the incubation continued for another 60 min in the presence of 10 mM $CaCl_2$. More than 75% of SM was hydrolyzed by both enzymes under these conditions (results not shown). The reaction was stopped with methanol, and after extraction of the lipids and TLC separation, the radioactivity in the free fatty acid spot was determined by scintillation counting. The enzyme activity in the absence of SM was taken as 100%, and all other values are expressed as percentages of this value. The values shown are means ± SEM of three experiments. * $P < 0.05$, ** $P < 0.01$, compared with the corresponding control without SMase treatment.

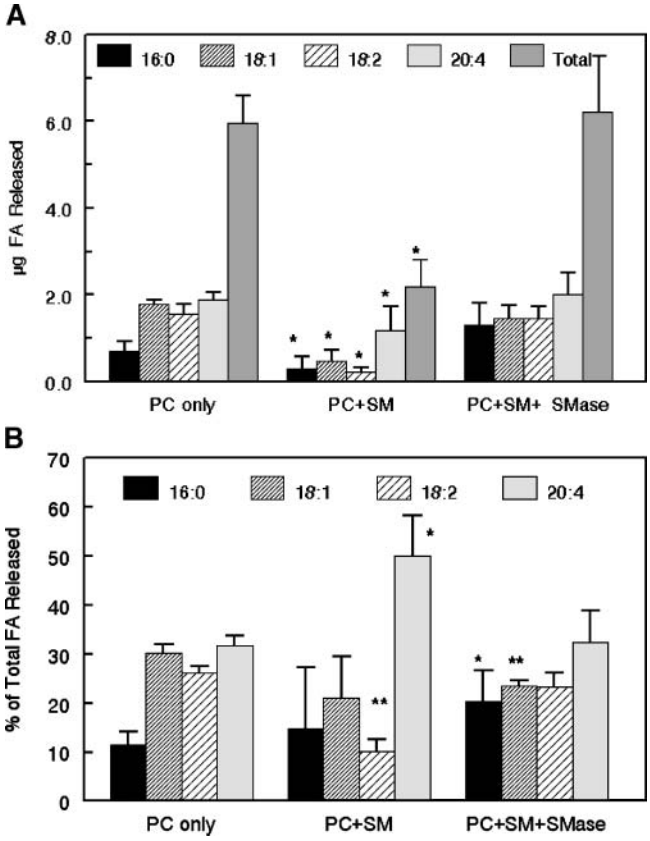

Fig. 3. Fatty acid specificity of $sPLA_2X$ in the presence of a synthetic PC mixture. Liposomes containing an equimolar mixture of 16:0-16:0 PC, 16:0-18:1 PC, 16:0-18:2 PC, and 16:0-20:4 PC were prepared by sonication. Where indicated, the liposomes contained egg SM at 30 mol% total PC. An aliquot of the SM-containing PC was treated with 0.05 units of SMase C before incubation with $sPLA_2X$. More than 80% of SM in the substrate was hydrolyzed under these conditions (results not shown). After reaction with $sPLA_2X$, 3 μg of 17:0 was added, and all samples were extracted (30) and separated on a TLC plate, and the free fatty acids were analyzed by GC, as described in Materials and Methods. The value of each fatty acid released after correction for the blank, as shown in A and the percentage of each fatty acid in the total fatty acids released, as shown in B. A correction was applied for the presence of positional isomers of the PC used based on the values obtained with excess snake venom PLA_2, as described in Materials and Methods. * $P < 0.05$, ** $P < 0.005$, compared with the PC-only control.

the PC hydrolysis by $sPLA_2X$. Treatment with SMase D restored the activity but did not stimulate it further. These results show that $sPLA_2X$ is similar to other phospholipases in being inhibited by SM and activated by ceramide.

SM and fatty acid specificity of $sPLA_2X$

To determine the possible effect of SM on the fatty acid specificity of the enzyme, we first studied the effect of egg SM in liposomes containing an equimolar mixture of four synthetic PCs that differ only in the *sn*-2 acyl group: 16:0-16:0, 16:0-18:1, 16:0-18:2, and 16:0-20:4. The SM was incorporated at 30 mol% total PC, and after the reaction with recombinant $sPLA_2X$, we determined the fatty acids released by GC. In addition, the effect of SMase C treatment of the SM-containing substrate was determined. Because the synthetic PCs used here contained varying amounts of positional isomers with 16:0 in the *sn*-2 position, we also applied a correction factor for the values, using the results of snake venom PLA_2 treatment of the liposomes, as described in Methods. As shown in **Fig. 3**, the release of all unsaturated fatty acids was greater than 16:0 in the absence of SM, but no clear selectivity for 20:4 was seen. However, in the presence of 30 mol% SM, the enzyme showed a clear preference for the release of 20:4, although the overall activity was inhibited by 60%. When expressed as a percentage of the total fatty acids released (Fig. 3, bottom panel), 20:4 constituted 32% of the total in the absence of SM but 50% of the total in the presence of SM. Moreover, SM exerted a preferential inhibition of the release of 18:2, which decreased from 26% of the total in the absence of SM to 10% of the total in the presence of SM. The ratio of 20:4 to 18:2 in the released fatty acids increased from 1.2 to 4.9 by the presence of SM. Treatment of the SM-containing substrate with SMase C resulted in a 2.8-fold activation of the total activity of $sPLA_2X$, but the activation of the individual fatty acids was unequal. Thus, the activation was highest for the release of 18:2 (6-fold) and lowest for the release of 20:4 (1.7-fold).

Therefore, these results show that SM, in addition to inhibiting the overall activity of $sPLA_2X$ in the native lipoproteins and cell membranes, markedly influences the fatty acid specificity of the enzyme.

We next studied the effect of SM depletion of HDL on the composition of fatty acids released by $sPLA_2X$ to determine the effect of SM in the physiological substrates. Normal HDL was first treated with varying amounts of SMase C before reacting with $sPLA_2X$ and the determination of the fatty acids released. As shown in **Fig. 4**, the release of all fatty acids was stimulated by treatment with SMase C, resulting in up to 4-fold stimulation of the overall activity. Quantitatively, 18:2 and 20:4 were the major fatty acids released in the control, but SMase C treatment stimulated the release of 18:2 more than that of 20:4 (Fig. 4, inset), similar to the results obtained with the synthetic substrates (Fig. 3). We also observed an unexpected stimulation in the release of saturated fatty acids (16:0 and 18:0) in these studies. Expressed as a percentage of the total fatty acids released, the release of the two saturated fatty acids increased by 6- to 20-fold after treatment with SMase C, although no increase was seen after treatment with SMase D (**Fig. 5**). On the other hand, the percentages of 20:4 and 18:2 decreased after SMase C treatment but not after SMase D treatment.

The increase in saturated fatty acids mentioned above is unlikely to be attributable to the action of $sPLA_2X$ itself but is probably the result of SMase C, for the following reasons. First, as mentioned above, the depletion of SM by SMase D did not have the same effect (Fig. 5). Second, the $sPLA_2X$ does not have any PLA_1 or lysophospholipase activity. Third, whereas SMase C alone did not show any phospholipase activity when tested with labeled PC substrates (results not shown), the saturated fatty acids were released in higher amounts in the combined presence of SMase C and $sPLA_2X$. These results suggest that the 16:0 and 18:0 were derived from the hydrolysis of lysophospholipids that were generated by the action of $sPLA_2X$. This is also supported by the fact that the amounts of 16:0 and 18:0 released in the combined presence of SMase C and $sPLA_2X$ exceeded the amount of saturated fatty acids in the s*n*-2 position of the HDL phospholipids (results not shown). Further studies with labeled lyso PC are needed to investigate the apparent lysophospholipase activity of the commercial SMase C, because this enzyme is used widely for the specific depletion of SM from the cells (33–35), and the hydrolysis of lyso PC by the enzyme may have unintended cellular effects.

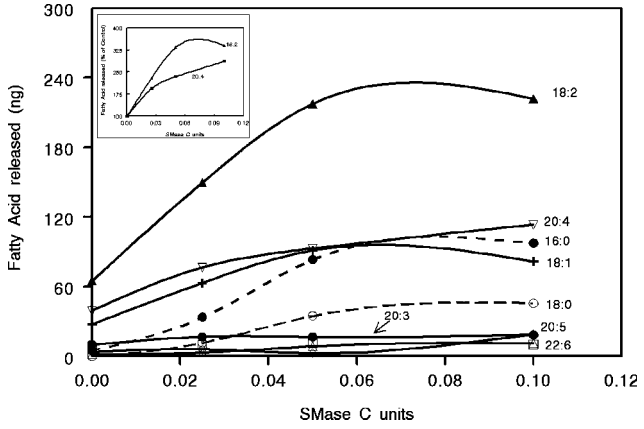

Fig. 4. Effect of SM depletion of HDL on the fatty acid specificity of $sPLA_2X$. Normal human HDL (100 μg of cholesterol) was treated with the indicated amount of SMase C for 60 min in 100 mM Tris/Cl, pH 8.0, containing 10 mM each $MnCl_2$ and $MgCl_2$. $sPLA_2X$ was then added along with 10 mM $CaCl_2$, and the incubation continued for 60 min. The lipids were extracted after spiking the samples with 3 μg of 17:0 free fatty acid, and the fatty acids released were determined by GC as described in Materials and Methods. The values shown are averages of at least two separate experiments. The inset shows the percentage stimulation of the release of 18:2 and 20:4 by SMase C treatment.

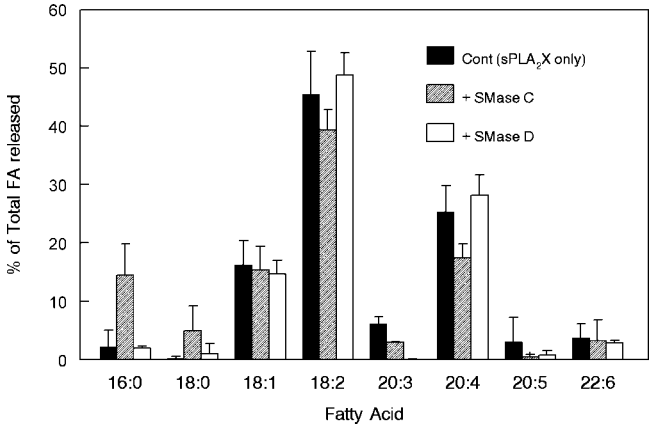

Fig. 5. Effect of SMase C or SMase D treatment of HDL on fatty acid specificity. Normal human HDL (100 μg of cholesterol) was treated with either 0.05 units of SMase C or 0.125 units of SMase D in the presence of 0.1% BSA and 10 mM each $MnCl_2$ and $MgCl_2$. The sample was then incubated with 5 μg of $sPLA_2X$ and 10 mM $CaCl_2$, and the composition of fatty acids released was determined by GC, as described in the text. The values shown are means ± SEM of three experiments.

Effect of ceramide on $sPLA_2X$

Previous studies showed that ceramide, the product of the SMase C reaction, independently stimulates several phospholipases (10, 13, 36–39) and influences the fatty acid specificity of $sPLA_2IIa$ (39) as well as LCAT (10). To determine the possible effect of ceramide on $sPLA_2X$, we incorporated varying amounts of egg ceramide into normal HDL before treatment with the recombinant $sPLA_2X$. The total activity of the enzyme was increased by 2.5-fold at a ceramide concentration of 20 mol% HDL PC. The composition of the released fatty acids showed that, similar to the effect of SMase C treatment, the ceramide incorporation stimulated the release of all fatty acids with the exception of saturated fatty acids (**Fig. 6**). The stimulation of 20:4 release was higher than that of 18:2, although this was not statistically significant (Fig. 6, inset). At high concentrations, ceramide stimulated the release of 18:1 more than other major fatty acids. However, there was no signifi-

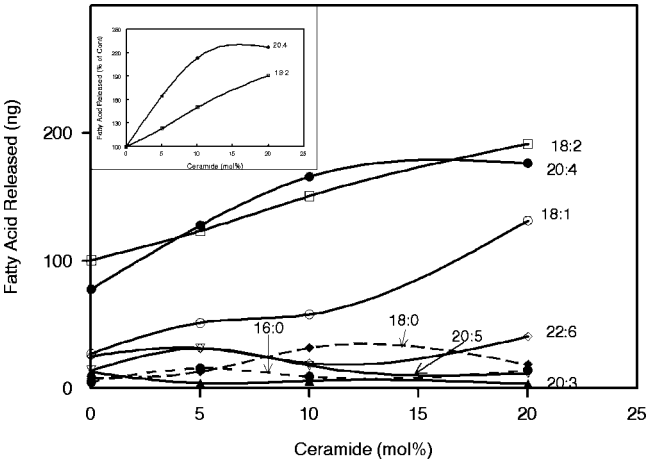

Fig. 6. Effect of ceramide incorporation into HDL on the fatty acid specificity of $sPLA_2X$. Egg ceramide was incorporated into normal HDL by incubation for 16 h as an ethanol solution of the indicated amount. The mol% ceramide was calculated on the basis of HDL PC. The ceramide-enriched HDL was treated with 5 μg of $sPLA_2X$, and the released fatty acids were analyzed by GC as described in the text. The inset shows the percentage stimulation of the release of 18:2 and 20:4 compared with the PC-only control.

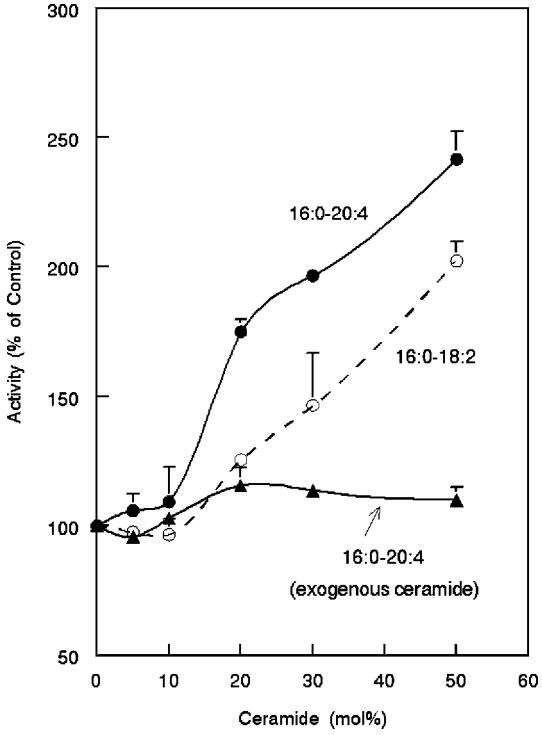

Fig. 7. Effect of ceramide incorporation into liposomes containing single PCs. The indicated mol% egg ceramide was incorporated by sonication into liposomes containing either 16:0-[^{14}C]18:2 PC or 16:0-[^{14}C]20:4 PC at 20 mol%. After reaction with $sPLA_2X$, the released fatty acids were separated from the substrate by TLC, and their radioactivity was determined by scintillation counting. The exogenous ceramide was added as separate liposomes to the substrate liposomes containing only 16:0-[^{14}C]20:4 PC. The values shown are expressed as percentages of control containing no ceramide. All values are means ± SEM of three estimations.

cant increase in the release of the saturated fatty acids by ceramide, unlike the results with SMase C treatment. These results further support the conclusion that the increase of these fatty acids in the presence of SMase C is attributable to the hydrolysis of lyso PC by SMase C, rather than to the direct action of $sPLA_2X$. Ceramide phosphate, up to 20 mol% PC, did not affect the $sPLA_2X$ activity, but it inhibited the enzyme above this concentration (results not shown). This is in contrast to the effect of ceramide phosphate on cytosolic PLA_2, which was shown to be activated (40).

To investigate the effect of ceramide under more defined conditions, we incorporated ceramide (at 20 mol%) into liposomes containing equimolar amounts of four synthetic PC species (16:0-16:0, 16:0-18:1, 16:0-18:2, and 16:0-20:4) and determined the fatty acids released by $sPLA_2X$. The overall activity of the enzyme was stimulated by 50–100% in the presence of 20 mol% ceramide (results not shown). Similar to the studies with HDL, ceramide stimulated the release of 20:4 more than that of the other fatty acids and inhibited the release of 16:0 (results not shown).

The effect of ceramide concentration was also tested on the hydrolysis of individual PCs using s*n*-2 acyl-labeled substrates. **Figure 7** shows the effect of the incorporation of increasing amounts of ceramide on liposomes containing either 16:0-[^{14}C]20:4 PC or 16:0-[^{14}C]18:2 PC. The results clearly show that ceramide stimulates the hydrolysis of 16:0-20:4 PC more than that of 16:0-18:2 PC. Furthermore, the ceramide must be incorporated into the same liposome as the PC substrate, because when the ceramide was added as separate liposomes to the reaction mixture containing 16:0-20:4 PC liposomes (exogenous ceramide), there was very little stimulation of enzyme activity. These results show that ceramide influences the properties of PC substrates rather than acts on the enzyme directly.

The structural requirements for ceramide activation were determined by incorporating ceramides of various *n*-acyl chain lengths into 16:0-20:4 PC liposomes at 20 mol% concentration. As shown in **Fig. 8**, the short-chain ceramides (C2–C8) were inhibitory, whereas the long-chain ceramides (C10–C20) all stimulated the activity. Maximum stimulation was obtained with C14–C17 ceramides, although the differences between the various long-chain ceramides were not statistically significant. Interestingly, the brain ceramide, which contains predominantly 18:0 and 24:0 acyl groups, was less active than the egg ceramide, which contains predominantly the C16:0 acyl group, suggesting that there is an optimum *n*-acyl chain length (C14–C17) for the enzyme stimulation.

Effect of SM on the release of arachidonate from cultured cells

$sPLA_2X$ has been shown to be the most effective $sPLA_2$ in releasing arachidonate and in stimulating eicosanoid synthesis in inflammatory cells (22, 23). Because SM is concentrated in the outermost monolayer of the cells, it may inhibit the activity of the externally added $sPLA_2X$. To

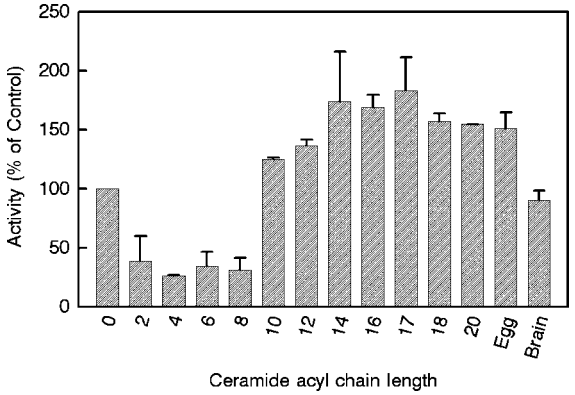

Fig. 8. Effect of ceramide *n*-acyl chain length. Synthetic ceramide of various *n*-acyl chain lengths or ceramides from egg or bovine brain were incorporated into liposomes containing 16:0-[^{14}C]20:4 PC at 20 mol% PC. After incubation with sPLA$_2$X for 60 min, the radioactivity in the liberated fatty acid was measured by scintillation counting. The values shown are relative to the control that contained no ceramide. The values shown are means ± SEM of three separate experiments.

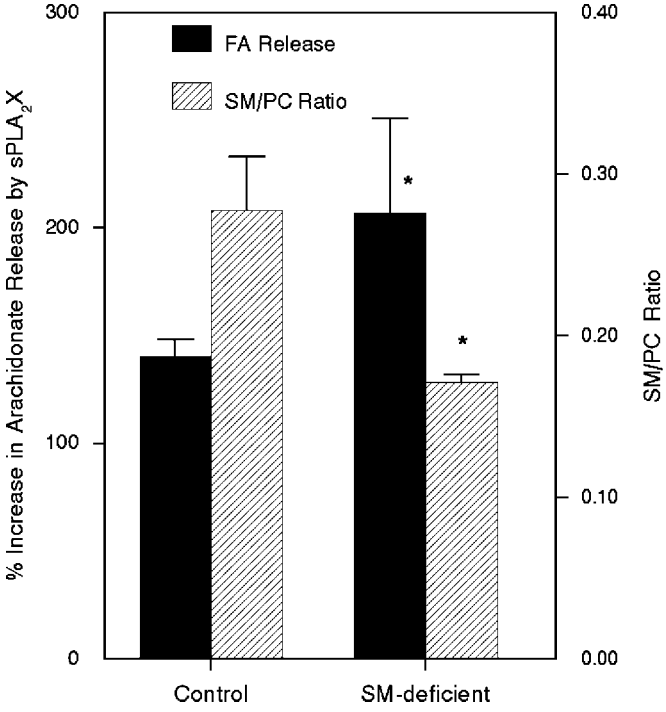

Fig. 9. Release of labeled arachidonate from CHO cells in response to exogenous sPLA$_2$X. Ly-B cells were grown in sphingolipid-deficient medium for 2 days. Ly-B/cLCB1 cells, in which the missing enzyme subunit was replaced (42), were used as control cells. The cells were prelabeled with [^{14}C]arachidonate by incubation in serum-free medium for 18 h. After washing the cells of adherent radioactivity, they were treated with 5 μg of sPLA$_2$X for 1 h. Radioactivity in the medium and the cells was determined after extraction of lipids by scintillation counting. The percentage of total radioactivity (cells + medium) appearing in the medium was calculated, and all values are expressed as percentages of radioactivity above the control (cells incubated without sPLA$_2$X). The values shown are means ± SEM of three experiments. * $P <$ 0.05, compared with control (*t*-test).

test this possibility, we first used mutant CHO cells that are deficient in serine palmitoyltransferase activity because of a mutation in the LCB1 subunit of the enzyme (Ly-B cells; obtained from Dr. K. Hanada) (29). These cells have reduced levels of SM when grown in defined medium that is deficient in sphingolipids. We also used the corresponding control cells (also obtained from Dr. K. Hanada), in which the missing subunit of SPT is replaced (Ly-B/cLCB1) and that have normal levels of SM under the same conditions. If the SM in the plasma membrane is inhibitory to exogenous sPLA$_2$X, we should find an increased release of arachidonate from Ly-B cells compared with the Ly-B/cLCB1 cells after exposure to the enzyme. The cells were first labeled with [^{14}C]arachidonate by incubation with the labeled fatty acid for 18 h, and after washing with PBS to remove the excess label, they were incubated with the recombinant sPLA$_2$X for 60 min and the labeled fatty acid in the medium and the cells was determined. As shown in **Fig. 9**, there was significantly more arachidonate released from the Ly-B cells in response to sPLA$_2$X compared with the control cells. As expected, the SM/PC ratio was significantly lower in the Ly-B cells. These results support the hypotheses that the membrane SM is a physiological inhibitor of exogenous sPLA$_2$X and that the membranes deficient in SM release more arachidonate in response to the extracellular phospholipase A.

In another study, mouse leukemia-derived macrophages (RAW 264.7 cells) were prelabeled overnight with [^{14}C] arachidonate and then treated with bacterial SMase C (0.05 units) or SMase D (0.125 units) for 60 min before incubation with the recombinant human sPLA$_2$X for an additional 60 min. The amount of labeled arachidonate released into the medium was measured by scintillation counting and expressed as percentage increase over the release from untreated cells. As shown in **Fig. 10**, neither SMase C nor SMase D by itself released much arachidonate from the cells, whereas sPLA$_2$X alone released significant amounts. Treatment with either SMase followed by incubation with sPLA$_2$X released significantly more arachidonate than a simple additive effect. These results further support the role of membrane SM in the inhibition of exogenous sPLA$_2$X. Additional studies are needed to determine whether the eicosanoid synthesis is also stimulated in these cells after treatment with SMase C.

DISCUSSION

Although several secretory PLA$_2$s have been identified in mammalian systems, the biological functions of these enzymes and their physiological regulation are poorly understood. Although all sPLA$_2$s hydrolyze the glycerophospholipids with varying efficiencies, sPLA$_2$X appears to be unique in its relative specificity for the release of arachidonate from the liposomes, lipoproteins, and cell membranes (22, 23, 26, 32). The regulation of arachidonate generation is of obvious clinical and physiological signifi-

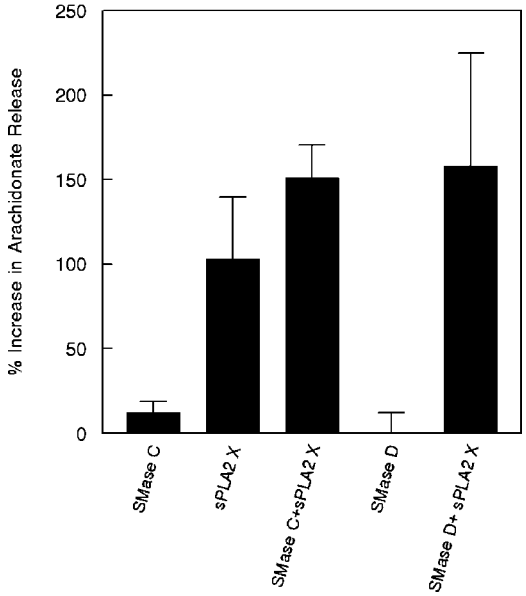

Fig. 10. Effect of SMase treatment on the release of labeled arachidonate from macrophages. RAW cells were grown in DMEM to near confluence and labeled with [^{14}C]arachidonate by overnight incubation with ethanolic solution of the labeled fatty acid (final concentration of ethanol was 0.2%). After washing the cells (two times with PBS), they were incubated with 0.05 units of SMase C for 60 min, and sPLA$_2$X (5 μg) was then added and the incubation continued for another 60 min. The labeled arachidonate released into the medium and remaining in the cells was counted in a scintillation counter. Results are expressed as percentage increase in counts in the medium over the control cells, which were incubated in the absence of any added enzyme. The values shown are means ± SEM of three experiments.

cance because the release of this fatty acid is the rate-limiting step in the synthesis of the proinflammatory eicosanoids, including prostaglandins and leukotrienes. Unlike sPLA$_2$IIa and sPLA$_2$V, which also can generate some arachidonate through stimulation of the cytosolic PLA$_2$ (24, 25), sPLA$_2$X acts directly on the cell membrane to hydrolyze the arachidonate-containing phosphoglycerides (41). Another important distinction between sPLA$_2$X and other secretory phospholipases is that it is constitutively expressed in most tissues examined; therefore, its synthesis is not controlled by inflammatory cytokines or hormones (19). Consequently, the regulation of its function should occur by modulation of its activity. One possible mechanism to regulate the enzyme activity is through the presence of physiological inhibitors and activators, which may in turn be independently regulated. The results presented here suggest that SM, a major phospholipid of membranes and lipoproteins, and its metabolite ceramide may fulfill this function.

The physiological role of SM in the regulation of various lipolytic enzymes has been reported by several laboratories. The list of enzymes inhibited by SM includes LCAT (8–11), triglyceride lipase (15, 16), hepatic lipase (P. V. Subbaiah, unpublished data), sPLA$_2$IIa (12, 13), cytosolic PLA$_2$ (14), and sPLA$_2$V (13). Because it is concentrated in the cell surface that is exposed to the environment, the cellular SM is positioned ideally as a potential inhibitor of exogenous phospholipases. It is degraded primarily to ceramide by neutral or acidic SMases, which are in turn regulated by external stimuli such as cytokines, growth factors, and hormones (3, 4). Furthermore, the SM that is degraded by the SMases is rapidly regenerated by the SM synthases, ensuring the integrity of the plasma membrane and the restoration of the PLA$_2$-inhibitory environment after the external stimulus is removed. It is significant that ceramide, the product of the SMase C reaction, is an independent activator of the sPLA$_2$ reactions (13, 36–39) and therefore provides another point of regulation of sPLA$_2$ activity. The formation of ceramide by de novo synthesis or by SM degradation is known to be regulated by cytokines and stress conditions (5, 42, 43). Previous studies showed that ceramide disturbs the membrane bilayer and allows greater penetration of the membrane by PLA$_2$ (36). Our results showing that only the long-chain ceramide activates the enzyme reaction (Fig. 9), and that the ceramide has to be incorporated into the PC liposomes to be effective (Fig. 8), support this mechanism.

An important observation in this study is that SM not only affects the enzyme activity but also its fatty acid specificity. The presence of SM in the substrate particle enhanced the specificity of the enzyme for 20:4 release by a preferential inhibition of the hydrolysis of 18:2, the major unsaturated fatty acid in lipoproteins and cell membranes. Previous studies by Singer et al. (44) showed the sPLA$_2$X does not exhibit a clear preference in defined liposomes, whereas it releases 20:4 preferentially when added to highly organized structures such as cell membranes. Based on the results presented here, the presence of SM in the membrane may be one factor responsible for the apparent specificity of the enzyme for the arachidonate in the organized structures. In contrast to SM, which increases the specificity for 20:4 release by preferential inhibition of 18:2 release, ceramide appears to directly stimulate the release of 20:4 in preference to 18:2. Koumanov et al. (39) previously reported that the incorporation of ceramide into phosphatidylethanolamine-phosphatidylserine substrate preferentially stimulated the hydrolysis of unsaturated phospholipids by sPLA$_2$IIa.

The mechanism by which SM and ceramide affect the fatty acid specificity of the enzyme is not clear. Our results show that although the enzyme releases more 20:4 than 18:2 from native HDL (relative to their concentrations in the s*n*-2 position), the SMase C treatment of HDL results in greater stimulation of the release of 18:2 compared with that of 20:4, indicating that the presence of SM in the native HDL suppresses the hydrolysis of 18:2 more than that of 20:4. This is also supported by our data with the liposomes containing an equimolar mixture of four synthetic PCs, in which the release of 18:2 is inhibited much more (−85%) than that of 20:4 (−38%) (Fig. 3). Because the 18:2-containing phospholipids are more abundant than the 20:4-containing phospholipids in HDL (45), this may reflect either the association of SM with the major PC species or a specific interaction between SM and the 18:2-

containing phosphoglycerides. On the other hand, the inclusion of 20 mol% ceramide in the four PC mixture resulted in the stimulation of hydrolysis of all unsaturated PC species but the inhibition of hydrolysis of a disaturated PC. Furthermore, the hydrolysis of 16:0-20:4 PC was stimulated more than that of 16:0-18:2 PC. These results are in agreement with those of Koumanov et al. (39) with $sPLA_2$IIa, who suggested that ceramide facilitates the phase separation of susceptible PC species.

SM and its metabolites have long been implicated in inflammatory reactions (46). The synthesis and secretion of SMases is increased significantly during the acute phase reaction (3). Similarly, the hepatic synthesis of sphingolipids is increased markedly by endotoxin administration, which results in the enrichment of lipoproteins with ceramide and SM (47). The cytokines, such as tumor necrosis factor-α and γ-interferon, which trigger the inflammatory response in macrophages and other cells, also specifically stimulate the synthesis and secretion of SMase C as well as the increased conversion of cellular SM to ceramide (3, 48). In fact, a 10-fold increase in lipoprotein-ceramide has been reported during the acute phase response (49). It is well established that the ceramide thus generated plays a critical role in apoptosis, cellular differentiation, cellular senescence, etc. Stimulation of eicosanoid synthesis through the specific activation of arachidonate release may be added to the list of pathways influenced by ceramide.

$sPLA_2X$ has been shown to be present in atherosclerotic lesions, and modification of LDL by it results in the increased uptake of LDL by macrophages, leading to foam cell formation (32). Interestingly, SMase C treatment of LDL also leads to increased uptake by the macrophages, albeit through a different mechanism that involves LDL aggregation (50, 51). It has also been reported that LDL-apolipoprotein B contains SMase C activity (52), which could also stimulate $sPLA_2$ activity in the lesions. It is possible that the increased $sPLA_2X$ activity in the lesions could not only cause LDL modification but also increase arachidonate release from the hydrolysis of LDL and cell membrane phospholipids, leading to inflammatory conditions prevalent in the lesions.

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