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Role of sphingomyelin and ceramide in the regulation of the activity and fatty acid specificity of group V secretory phospholipase A₂

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Abstract

We previously showed that group V secretory phospholipase A₂ (sPLA₂V) is inhibited by sphingomyelin (SM), but activated by ceramide. Here we investigated the effect of sphingolipid structure on the activity and acyl specificity of sPLA₂V. Degradation of HDL SM to ceramide, but not to ceramide phosphate, stimulated the activity by 6-fold, with the release of all unsaturated fatty acids being affected equally. Ceramide-enrichment of HDL similarly stimulated the release of unsaturated fatty acids. Incorporation of SM into phosphatidylcholine (PC) liposomes preferentially inhibited the hydrolysis of 16:0–20:4 PC. Conversely, SMase C treatment or ceramide incorporation resulted in preferential stimulation of hydrolysis of 16:0–20:4 PC. The presence of a long chain acyl group in ceramide was essential for the activation, and long chain diacylglycerols were also effective. However, ceramide phosphate was inhibitory. These studies show that SM and ceramide in the membranes and lipoproteins not only regulate the activity of phospholipases, but also the release of arachidonate, the precursor of eicosanoids.

Keywords

Group V sPLA₂; Sphingomyelin; Ceramide; Ceramide phosphate; Fatty acid specificity; Diacylglycerol; Arachidonate; Sphingomyelinase

Introduction

The secretory phospholipases A₂ (sPLA₂) are low molecular weight, Ca²⁺-requiring enzymes which are expressed by several cells in mammals, and which have been implicated in many biological processes including inflammation, host-defense and atherosclerosis [1–3]. At least 10 types of sPLA₂ have been described in the mammalian systems, differing in calcium requirements, catalytic site architecture, and substrate specificities [1]. Although these enzymes have been fairly well characterized with respect to their structure, specificity, and biochemical effects, their physiological roles and regulation are not well understood. For example, one of the best characterized mammalian sPLA₂, the group IIa sPLA₂, is known to be elevated up to 100- fold in plasma during the acute phase reaction [4], but it acts poorly on plasma lipoproteins and cell membranes [5], and therefore its role in inflammatory response

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is not clear. Transgenic expression of sPLA₂ IIa in mice greatly accelerates the development of atherosclerosis [6]. Recent studies by Tietge *et al* [7] suggest that the accelerated atherogenesis in these mice may be due to overexpression of the enzyme in macrophages, leading to increased LDL oxidation and foam cell formation. The group V and group X sPLA₂ which act more readily on lipoproteins and cell membranes [8–10], are present in much lower levels in plasma even under inflammatory conditions. However, these enzymes may be more potent in the mobilization of arachidonic acid and in stimulation of prostaglandin synthesis in macrophages [8;9], and therefore may play important role in inflammation and atherogenesis. The group V enzyme (sPLA₂V) is expressed in heart, lung, placenta, spleen and macrophages [8;10]. This enzyme can also induce eicosanoid synthesis in various inflammatory cells including neutrophils and eosinophils [10], and acts in concert with the cytosolic PLA₂ in releasing the arachidonate [11]. The physiological factors involved in the regulation of this enzyme activity are not known. Our previous studies show that sphingomyelin (SM), a major phospholipid in lipoproteins and cell membranes, is an inhibitor of sPLA₂V [12], whereas its degradation product, ceramide, is an activator. Our results also showed that the enzyme exhibits specificity towards release of 18:2, and discriminates against 20:4 (relative to snake venom PLA₂), when acting on plasma HDL. However, it is not known whether the fatty acid specificity of the enzyme is affected by the presence of SM or ceramide. Previous studies by Koumanov *et al* on sPLA₂ group IIa showed that ceramide specifically stimulated the release of polyunsaturated fatty acids from a substrate of PE/PS mixture [13]. However, as mentioned above, since this enzyme does not readily hydrolyze the lipoprotein or membrane phospholipids [5], the physiological relevance of this finding is unclear. In the present study, we investigated the effect of SM and its metabolite ceramide on the fatty acid specificity of sPLA₂V, which does act on the lipoproteins. The results obtained with synthetic PC substrates show that the release of arachidonate is specifically inhibited by SM, whereas the degradation of SM to ceramide, or incorporation of exogenous ceramide into the substrate specifically stimulated the release of arachidonate. These studies show that the sphingolipids of lipoproteins and cell membranes not only regulate the overall activity of sPLA₂, but also its fatty acid specificity, and thus may play a role in the regulation of the inflammatory responses.

Materials and Methods

Materials

All synthetic PCs were purchased from Avanti Polar Lipids (Alabaster, AL), and were used without further purification. The positional purity of the PCs was ascertained from the composition of fatty acids released by the action of snake venom PLA₂ [14]. 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%; and 16:0–20:4 PC, 15%. Labeled PCs with ¹⁴C fatty acid at *sn*-2 position (16:0–16:0, 16:0–18:2, and 16:0–20:4) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All ceramides and SMase C (*S. aureus*, 174 units/mg) were purchased from Sigma Chemical Co (St. Louis, MO). SMase D (*Corynebacterium pseudotuberculosis*) was purified from transfected *E. Coli* as described previously [15]. The SMase activities were assayed using the Amplex Red SMase Kit from Invitrogen Inc. Ceramide phosphate was prepared by the action of SMase D on egg SM and PE, as described before [16].

Substrates

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 ethanol, and again dried under nitrogen. The sample was then hydrated with 1 ml Tris/Cl buffer (100 mM, pH 8.0) at 50 °C under nitrogen for 1h and sonicated for 2 min (15 sec pulses) in a Sonics Vibra Cell sonicator at 4 °C at 28% of maximum energy. The substrate was used within 7 days of the preparation.

Assay of enzyme activity

Recombinant sPLA₂V was kindly supplied by Dr. Wonhwa Cho, Department of Chemistry, University of Illinois at Chicago. The enzyme was prepared and purified as described before [17], and was stored at -20 °C in 0.1 M Tris/Cl buffer, pH 8.0. The specific activity of the enzyme, as assayed with the polymerized mixed substrate [17] was 4.0 μmol PE hydrolyzed min⁻¹ mg⁻¹.

The reaction mixture for the assay of the enzyme activity contained 100 μM PC (either labeled or unlabeled), indicated amount of ceramide or SM, 100 mM Tris/Cl, pH 8.0, 0.1% BSA, and 10 mM CaCl₂, in a final volume of 0.2 ml. The reaction was carried out for 1 h at 37 °C, and stopped by the addition of 0.5 ml methanol. The lipids were extracted by Bligh and Dyer procedure [18], and separated on a silica gel TLC plate with the solvent system of chloroform: methanol: water (65: 25: 4 v/v). A mixture of standard PC, lyso PC, and free oleic acid was run on a separate lane for the identification purpose. After chromatography, the sample lanes were covered with a glass plate, and the standard lane was exposed to iodine vapors. The region corresponding to the free fatty acid standard was scraped from each lane, and was either counted for radioactivity (when a labeled substrate was used) or was analyzed by GC as described below (when unlabeled substrate was used). The enzyme activity was calculated as % of PC hydrolyzed, and was corrected for the control sample which was incubated in the absence of the enzyme.

HDL preparations

Normal human plasma, prepared in acid citrate dextrose, was purchased from a local blood bank (LifeSource, Chicago). HDL was isolated by sequential centrifugation 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 where HDL was treated with SMases, 0.025 or 0.05 units of SMase C from *S. aureus*, or 0.125 or 0.25 units of SMase D from *C. pseudotuberculosis* was added to 100 μg of HDL protein in presence of 10 mM MnCl₂, and 10 mM MgCl₂. After incubation at 37 °C for 60 min, 2.5 μg sPLA₂V, and 10 mM CaCl₂ were added, and the activity determined as described above. When HDL was enriched with ceramide, varying amounts of egg ceramide were added to HDL as ethanol solution, and incubated for 16 h at 37 °C, before reacting with sPLA₂V.

Fatty acid specificity

The fatty acid specificity of sPLA₂V with HDL as substrate was determined from the composition of free fatty acids released after the enzyme reaction. HDL (100 μg cholesterol) was incubated with sPLA₂V (2.5 μg), 0.1% BSA, and 10 mM CaCl₂ at 37 °C for 2 h. The total lipids were extracted by Bligh and Dyer procedure [18] after adding 3 μg 17:0 free fatty acid as internal standard, and the lipids were separated on TLC plate as described above. The spot corresponding to the free fatty acid standard was scraped from the plate, and methyl esters prepared using the instant methanolic HCl kit (Alltech). The methyl esters were extracted with 2 ml hexane (twice), concentrated, and analyzed by capillary GC, as described before [19]. When the substrate was composed of mixture of synthetic PCs, the reaction mixture contained equimolar mixture of 16:0–18:1, 16:0–18:2, and 16:0–20:4 PCs with or without SM or ceramide, 2.5 μg of sPLA₂V, 10 mM Tris/Cl pH 8.0, 0.1% BSA, and 10 mM CaCl₂, in a final volume of 0.2 ml. The reaction was stopped by the addition of methanol, and the lipids were extracted and the composition of the free fatty acids determined by GC as described above.

Results

1. Effect of lipoprotein SM on the fatty acid specificity of sPLA₂V

Our previous studies showed that treatment of plasma lipoproteins with recombinant sPLA₂V releases relatively higher percent of 18:2 and lower percent of 20:4 than expected from the fatty acid composition at the *sn*-2 position of plasma phospholipids [12]. Although we showed that SM inhibits the overall activity, the effect of SM on the fatty acid specificity of the enzyme was not investigated. For this purpose, we first determined the effect of SM degradation by SMase C on the composition of fatty acids released by the action of sPLA₂V on native HDL. Normal human HDL was first incubated with varying amounts of SMase C from *S. aureus* for 1h, followed by further incubation in the presence of recombinant sPLA₂V. In agreement with the previous results, sPLA₂V showed relative specificity towards 18:2, the major fatty acid in HDL phospholipids (Fig. 1, top). About 50% of the total fatty acids released was 18:2, whereas 18:1 and 20:4 constituted about 20% each, and 16:0 about 7%. Minor amounts of 20:5, 22:6 and 20:3 fatty acids were also released (results not shown). SMase C treatment of HDL resulted in up to 4-fold stimulation of the total activity of sPLA₂V. The stimulation was comparable for the three major unsaturated fatty acids (18:1, 18:2, and 20:4) (Fig. 1, bottom). An unexpected finding in these studies, however, was that the treatment with SMase C resulted in up to a 10-fold stimulation of the release of 16:0, a minor constituent at the *sn*-2 position of plasma phospholipids [20;21]. Since the amount of 16:0 released was much greater than that expected from the fatty acid composition at the *sn*-2 position (about 3% in human plasma lipoproteins [19]), it most probably originated from the *sn*-1 position of the phospholipids, in the presence of SMase C. However, when the SMase C preparation used here was tested with 16:0–18:2 PC liposomes, it did not show any phospholipase A activity (results not shown). The release of 16:0 occurred only in the presence of both SMase C and sPLA₂V. These results suggest that the excess 16:0 came from the hydrolysis of lyso PC generated by the sPLA₂ activity. Further studies are required to characterize the putative lysophospholipase activity of the commercial preparations of SMase C, because this enzyme is extensively used as a probe to specifically deplete SM from cell membranes [22–24], and to induce LDL aggregation [25]. It is, however, unlikely that the overall activity or specificity of sPLA₂V was substantially influenced by the lysophospholipase activity of SMase C because quantitatively, the amount of 16:0 released is much lower than that of the unsaturated fatty acids (Fig. 1 top).

In contrast to the effect of SMase C, treatment of HDL with SMase D (which hydrolyzes SM to ceramide phosphate rather than to ceramide) had only a modest effect on the activity and fatty acid specificity of sPLA₂V. The overall activity of sPLA₂V was stimulated by less than 20% after SMase D treatment, compared to a 6-fold stimulation after the SMase C treatment, although the amount of SM degraded by the two SMases was similar. There was also no effect on the specificity of sPLA₂V (results not shown). This suggests that the formation of ceramide, rather than the simple depletion of SM was responsible for most of the stimulation observed after SMase C treatment.

2. Effect of ceramide incorporation into HDL

To test the possibility that the generation of ceramide by SMase C was responsible for the sPLA₂ activation and altered specificity, we incorporated varying amounts of ceramide into native HDL by incubation for 16 h at 37°C, and then treated the HDL with sPLA₂V. Since the SM in the lipoprotein is not degraded, any effect should be due to the exogenous ceramide alone. As shown in Fig. 2, the total activity of the enzyme was activated by up to 2.5-fold by the presence of ceramide in HDL. The stimulation was uniform for all the unsaturated fatty acids, but was significantly less for 16:0. These studies show that ceramide activates the release of unsaturated fatty acids by sPLA₂V, similar to its previously reported effect on sPLA₂ IIa acting on PE/PS substrate [13]. These results also support our conclusion that the increased

release of 16:0 in the presence of SMase C and sPLA₂V is not due to the direct action of the PLA₂ on PC or ceramide.

3. Effect of ceramide on the hydrolysis of synthetic PCs

To study the effect of ceramide on sPLA₂ specificity in a more defined system, we incorporated varying concentrations of egg ceramide into liposomes containing a single labeled PC species, and compared the activity of sPLA₂V on three different PCs. As shown in Fig. 3, the presence of ceramide dose-dependently stimulated the hydrolysis of all PC species, although to different degrees. The order of activation was 16:0–20:4 PC > 16:0–16:0 PC > 16:0–18:2 PC. Addition of ceramide separately to the reaction mixture (extrinsic) did not stimulate the enzyme activity. This shows that the ceramide and PC have to be incorporated into the same liposome for the activation to occur. In contrast to ceramide, the incorporation of ceramide phosphate into the liposome inhibited the hydrolysis of 16:0–20:4 PC (Fig. 4). We have previously reported a similar inhibition of LCAT activity by ceramide phosphate, whereas ceramide activated this enzyme activity also [16].

The above results with individual PCs are somewhat in variance with the effect of ceramide in native lipoproteins, where the release of 16:0 was stimulated less than that of unsaturated fatty acids (Fig. 2). One possible explanation for this discrepancy is that the effect of ceramide depends upon the overall composition of PC species in the substrate. Since several PC species occur together in HDL at varying and unequal concentrations, ceramide may interact preferentially with certain PC species in the mixture, or get incorporated into specific domains of the lipoprotein. To investigate this, we tested the effect of ceramide incorporation into a defined mixture of synthetic PCs containing the three most abundant fatty acids in *sn*-2 position of plasma phospholipids, namely 18:1, 18:2, and 20:4 [19–21]. For these studies, equimolar amounts of 16:0–18:1 PC, 16:0–18:2 PC, and 16:0–20:4 PC were incorporated into liposomes in the absence or presence of SM or ceramide. Where indicated, the liposomes contained either 16 mol % SM or 20 mol % ceramide. This amount of SM was chosen to keep the SM concentration in the physiological range. The SM-containing liposomes were also treated with SMase C, to produce ceramide *in situ*. As shown in Fig. 5 (top panel), the hydrolysis of all PCs was inhibited in the presence of physiological concentration of SM. The percent inhibition was greater for the release of 20:4 compared to 18:2 or 18:1 (Fig. 5, bottom panel). When these liposomes were first treated with SMase C before incubation with PLA₂, the stimulation by SMase C was also the greatest for the release of 20:4. When compared with the untreated sample, there was a 14-fold increase in the release of 20:4, and about 4 to 5-fold increase in the release of 18:1 and 18:2 after SMase C treatment. Incorporation of ceramide in the absence of SM or SMase reaction also resulted in the preferential activation of 20:4 release (4.5-fold) and 18:1 (4.3-fold) compared to 18:2 (2.3-fold). The net effect of ceramide incorporation or *in situ* generation of ceramide was therefore an increase in the ratio of 20:4/18:2 in the free fatty acids generated by sPLA₂V. It should be pointed out that the effect of ceramide in liposomes is different from its observed effect in HDL, where the release of all unsaturated fatty acids was stimulated to a similar extent (Fig. 2). This suggests that other components of HDL may modulate the effect of ceramide.

4. Effect of ceramide chain length

To determine the specificity of ceramide effect on the sPLA₂ activation, we tested the effect of incorporation of ceramides of various *n*-acyl chain lengths on the release of labeled fatty acid from 16:0-[¹⁴C] 16:0 PC). All ceramides were incorporated into the liposomes at 20 mol % of PC. As shown in Fig. 6, the short chain ceramides (up to C8) were ineffective or inhibitory for the hydrolysis of disaturated PC, whereas the long chain ceramides (> C10) were all stimulatory to a comparable extent. Although our previous studies [12] showed a stimulation of the enzyme activity by high concentration of C6 ceramide (20–50 mol%, added in DMSO)

with 16:0-[¹⁴C] 18:2 PC as substrate, the present studies employing 16:0-[¹⁴C] 16:0 PC and 20 mol % of ceramide show an inhibition by this compound. These results also suggest that the effect of ceramide is also dependent upon the fatty acid composition of the PC substrate.

5. Effect of diacylglycerol

Since diacylglycerol has been shown to be an activator of phospholipases [26], and it has structural similarities to ceramide, we have also tested the effect of incorporation of diacylglycerols of various chain lengths on the hydrolysis of 16:0-[¹⁴C]16:0 PC by sPLA₂ V. As shown in Fig. 7, the long chain DAG (>10 carbon) activated sPLA₂ V by about 3-fold, whereas the 8:0 DAG inhibited it. Interestingly, the unsaturated DAG (1,2 18:1 and 1,3 18:1) did not show any activation of the enzyme. We also found that the activation of sPLA₂V by DAG was dependent upon the type of PC substrate used. While the hydrolysis of diunsaturated PC was stimulated up to 3 fold by long chain ceramide, there was virtually no stimulation of the hydrolysis of 16:0–18:2 PC (results not shown). Since DAG is a major neutral lipid in some lipoprotein fractions [27], its stimulation of sPLA₂ activity may be physiologically relevant, especially in the type of fatty acids released.

Discussion

The results presented here show that both the activity, as well as the substrate specificity of sPLA₂V are regulated by SM, and its major metabolite, ceramide. Although sPLA₂V is expressed in several tissues, neither its physiological role, nor its *in vivo* regulation are clearly established. Structurally, sPLA₂V is closely related to sPLA₂Ia, from which it may have originated through gene duplication [10]. Unlike sPLA₂Ia, however, sPLA₂V binds efficiently to the major phospholipid (PC) in membranes and lipoproteins [10], and therefore may be physiologically more relevant in phospholipid turnover and eicosanoid synthesis. Balboa *et al* [8] showed that the arachidonate release and prostaglandin production in mouse macrophage cell line P388D was dependent upon the presence of sPLA₂V. Similarly, Murakami *et al* [11] reported that in fibroblasts and CHO cells, sPLA₂V acts in concert with the cytosolic PLA₂ in the release of arachidonate. sPLA₂V also has strong affinity to proteoglycans on the cell surface, and this property appears to be critical for its function [11]. The enzyme has been reported to be present in atherosclerotic lesions [28], and may be involved in the conversion of LDL to a more atherogenic particle [29]. Therefore, the regulation of its activity is physiologically important. Previous results from our laboratory showed that a physiologically relevant modulator of sPLA₂V activity is SM, which is the most abundant phospholipid in plasma, next to PC [12], and which accumulates in atherosclerotic lesions [30]. It is noteworthy that the structure of sPLA₂V is uniquely suited for binding the zwitterionic phospholipids, PC and SM [10]; the former being its substrate and the latter its inhibitor. Since SM is concentrated in the outermost monolayer of all mammalian cells, it is possible that its role in the inhibition of PLA₂ activity is physiologically relevant in preventing the unregulated hydrolysis of membrane phospholipids by exogenous phospholipases. SM itself is, however, susceptible to the action of SMase C, which is secreted by several cells, and is increased significantly during inflammatory reactions [31]. Our results show that the hydrolysis of SM by SMase C not only reverses the inhibition of sPLA₂, but also activates the phospholipase reaction further through the generation of an activator, the ceramide. Therefore, the balance between SM and ceramide may play an important role in the modulation of phospholipase activities as well as the inflammatory reactions mediated by its products, lyso PC and arachidonic acid. Further studies with intact cells are required to determine whether SM protects the membrane PC against exogenous phospholipases.

In general, the various Ca²⁺-dependent phospholipases do not exhibit high selectivity with respect to the fatty acids released from the phospholipids [32;33], presumably because the

active sites of the enzymes interact with less than 10 carbon atoms of the acyl chain [10]. However, our previous studies showed that sPLA₂V releases more 18:2 than 20:4 from the lipoproteins [12]. Moreover, the release of 20:4 is much lower than expected from the *sn*-2 acyl composition of plasma phospholipids. The preference of 18:2 over 20:4 is maintained in presence of liposome substrates containing equimolar mixtures of 16:0–18:1, 16:0–18:2, and 16:0–20:4 PCs, and is augmented by the presence of SM (Fig. 5). However, when SM is degraded to ceramide, or when exogenous ceramide is incorporated into the substrate, the difference between the release of 18:2 and 20:4 is nearly eliminated (Fig. 5). These results suggest that SM inhibits, whereas ceramide activates, specifically the release of 20:4 by sPLA₂V. Interestingly, the effect of SM is significantly different on sPLA₂X, where it inhibits specifically the hydrolysis of 16:0–18:2 PC, not 16:0–20:4 PC (Singh and Subbaiah, unpublished data). The preferential activation of 20:4 release by ceramide is also observed when individual PC substrates were used (Fig. 6). Because of its role as precursor of prostaglandins and leukotrienes, the regulation of arachidonate release is of obvious importance in the regulation of inflammatory processes.

The role of ceramide as an activator of other phospholipase reactions has been reported by several laboratories. Huang *et al* [34] reported that the activity of snake venom PLA₂ was stimulated up to 3-fold by the long chain ceramides, but was inhibited by the short chain ceramides, similar to the results found here for sPLA₂V (Fig. 6). The stimulation of activity by the long chain ceramides was attributed to the lateral phase segregation of the bilayer into gel and liquid crystalline domains, which facilitates the penetration of the enzyme into the bilayer, as well as the binding of substrate PC to the active site. Koumanov *et al* [13] reported that the long chain ceramide not only simulated sPLA₂Ia, but also promoted the release of polyunsaturated fatty acid from PE/PS substrate. They proposed that the polyunsaturated phospholipids are specifically excluded from the ceramide-rich lamellar phase, and this results in their increased susceptibility to PLA₂ attack. Recent studies from our laboratory showed that LCAT, a specialized phospholipase A that esterifies cholesterol in plasma, is also activated by ceramide, and that the synthesis of unsaturated cholesteryl esters by the enzyme is specifically stimulated [16]. In addition, we found that group X sPLA₂, an enzyme that is more active in the release of arachidonate from inflammatory cells [9], is inhibited by SM, and activated by ceramide, and alters its the fatty acid specificity in presence of ceramide (Singh and Subbaiah, unpublished data). The influence of ceramide on the fatty acid specificity of LCAT was minimized when the fluidity differences among the PC substrates was eliminated by incorporating the PCs into an inert common matrix [16], suggesting that ceramide affects the physical presentation of the substrate PC to the enzyme, rather than directly interacting with the enzyme. The results from the present study also show that the preferential activation of 20:4 release by ceramide was not apparent when the ceramide was incorporated into normal HDL (Fig. 2). This indicates either that the other components of HDL such as apoproteins and cholesterol modulate the action of ceramide, or that the site of incorporation of exogenous ceramide in HDL is different from that in PC liposomes. The results of Kumanov *et al* [13] indeed showed that cholesterol suppresses the effect of ceramide on sPLA₂Ia. Nevertheless, the effect of ceramide on the fatty acid specificity of sPLA₂V is consistently observed in defined PC liposomes. It is of interest to note that ceramide phosphate, which has been reported to directly interact with, and activate cytosolic PLA₂ [35], is actually inhibitory to sPLA₂V (Fig. 7) as well as to LCAT [16]. The cytosolic PLA₂, however differs significantly from the secretory enzymes both structurally and in its calcium requirements, and therefore the differential effect of ceramide phosphate is not surprising. A further regulation of PLA₂ activities could theoretically be achieved in the cells by the phosphorylation of ceramide by ceramide kinase. Although the presence of ceramide phosphate in plasma has not yet been reported, significant amounts of ceramide are present in association with lipoproteins, especially during inflammatory response [36;37], and therefore the role of ceramide in the generation of arachidonate and eicosanoids needs further investigation.

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Abbreviations

DAG	diacylglycerol
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PS	phosphatidylserine
SM	sphingomyelin
SMase	sphingomyelinase
sPLA₂V	secretory phospholipase A ₂ , group V

Figure 1. Effect of SMase C treatment of HDL on the fatty acids released by sPLA₂V

Normal human HDL (100 µg protein) was incubated with 0, 0.025 or 0.05 units of SMase C in the presence of 10 mM MgCl₂ and 10 mM MnCl₂ for 60 min, and then sPLA₂V (2.5 µg) was added. The incubation (in a final volume of 0.2 ml) was continued for another 60 min after adding 10 mM CaCl₂, and the reaction was stopped by the addition of 0.5 ml of methanol. The total lipids were extracted by Bligh and Dyer procedure [18], and separated on TLC as described in the text. The spot corresponding to free fatty acids was scraped, methylated and analyzed by GC as described in the text. Only the major fatty acids (>2% of total) are shown. The results shown are mean ± S.D. of 3 separate experiments. Top panel shows the absolute amounts of fatty acids, while in the bottom panel, the results are calculated as percent of the control sample (0 units of SMase C). As shown previously with LDL [12], about 80% of HDL SM was hydrolyzed in presence of 0.05 units of SMase C, and about 50% of SM

Figure 2. Effect of ceramide incorporation into HDL on the fatty acids released by sPLA₂V
 The indicated amount of ceramide (dissolved in ethanol) was incubated with 100 µg of HDL (protein) for 16 h at 37 °C, and then 2.5 µg of sPLA₂V was added and the incubation continued for another 1 h in presence of 10 mM CaCl₂. The composition of the fatty acids released was determined as described in the text. In the upper panel, the absolute amounts of individual fatty acids are shown, whereas in the lower panel, the values are expressed as percent of the value obtained with control HDL (no ceramide). The results shown are averages of two separate experiments which differed from each other by < 15%.

Figure 3. Effect of ceramide on the hydrolysis of individual PC species

PCs labeled at the *sn*-2 acyl group were incorporated into liposomes by sonication in the presence of indicated mol% of egg ceramide. The liposomes were then incubated with 2.5 μ g of sPLA₂V, and the amount labeled fatty acid released was determined by TLC and scintillation counting, as described in Methods. In the sample labeled “extrinsic”, the ceramide was added as aqueous dispersion to the liposome that contained only 16:0–20:4 PC (i.e., the ceramide and PC were in different particles). There was no activation of sPLA₂V in this case.

Figure 4. Comparison of the effect of ceramide and ceramide phosphate on the hydrolysis of 16:0–20:4 PC

Liposomes containing 16:0-[^{14}C] 20:4 PC and the indicated mol% of egg ceramide or egg ceramide phosphate were prepared by sonication. After reaction with sPLA₂V, the labeled fatty acid released was determined as described in the text. The values are expressed as % of the values obtained in the absence of either ceramide or ceramide phosphate (Mean $\pm$ SD, n = 3).

Figure 5. Effect of SM and ceramide on the specificity of sPLA₂V in presence of synthetic PC mixture

Equimolar mixture of 16:0–18:1 PC, 16:0–18:2 PC, and 16:0–20:4 PC was incorporated into the liposomes, alone or in combination with the 20 mol% of egg ceramide or 16 mol% of egg SM. Aliquots of the SM-containing liposomes were also treated with SMase C, to generate ceramide in situ. All liposomes were incubated with sPLA₂V for 60 min, and the released fatty acids were analyzed by GC, as described in the text. Upper panel: absolute amount of individual and total fatty acids released in the presence of each substrate. Lower panel: percent composition of the fatty acids released. Small amounts of 16:0 coming from the positional

isomers in the substrate are not included in the calculation. The values shown are mean $\pm$ SD of 3 determinations.

Figure 6. Effect of ceramide chain length on the activation of sPLA₂V

Synthetic ceramides of various n-acyl chain lengths were incorporated into liposomes containing 16:0-[¹⁴C] 16:0 PC at 20 mol% of PC. The amount of labeled free fatty acid released after reaction with sPLA₂V was determined by TLC separation and scintillation counting, as described in the text. The values are expressed as the percent of the labeled fatty acid released in the absence of added ceramide.

Figure 7. Effect of diacylglycerol (DAG) fatty acid composition on sPLA₂V activity

1,2 DAG containing the indicated fatty acid at both positions, or 1,3 DAG with 18:1 at both positions, was incorporated into 16:0-[¹⁴C] 16:0 PC liposomes at 20 mol% of PC. The liposomes were reacted with sPLA₂V for 60 min, and the label in the released fatty acid was determined as described in the text. Values are expressed as percent of the control (no DAG), and are mean $\pm$ S.D of 3 experiments.