Role of Group IIa and Group V Secretory Phospholipases A_2 in the Metabolism of Lipoproteins. Substrate Specificities of the Enzymes and the Regulation of Their Activities by Sphingomyelin†

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ABSTRACT: Although many isoforms of secretory phospholipases A_2 ($sPLA_2$) are known to be secreted by various inflammatory cells, and are present in plasma, their role in lipoprotein metabolism is unknown. We studied the in vitro hydrolysis of lipoprotein phospholipids by group IIa and group V $sPLA_2$, two structurally related enzymes with differing phospholipid specificities. The group V $sPLA_2$ was about 30 times more efficient than the group IIa enzyme in the hydrolysis of lipoprotein phosphatidylcholine (PC), and both enzymes were more active on high density liporotein (HDL) than on low density lipoprotein (LDL). The lower activity on LDL appears to be due to the higher sphingomyelin (SPH) concentration in this lipoprotein. PC hydrolysis in lipoproteins was stimulated significantly by enzymatic depletion of their SPH. The hydrolysis of PC in liposomes was inhibited by the incorporation of SPH, and this inhibition was reversed by treatment with sphingomyelinase. The incorporation of ceramide, on the other hand, stimulated the $sPLA_2$ activity significantly. Unlike most $sPLA_2$, which show no fatty acid preference, group V $sPLA_2$ released disproportionately more linoleate, and less arachidonate from lipoproteins. These studies show that group V $sPLA_2$ is physiologically more important than group IIa enzyme in lipoprotein metabolism, that the $sPLA_2$ activities are regulated by sphingomyelin and ceramide, and that the pathological effects of $sPLA_2$ may not be mediated through stimulation of eicosanoid synthesis.

Several types of low molecular weight (~14 kDa) phospholipases A_2 ($sPLA_2$)[1] are known to be secreted by mammalian tissues (*1*, *2*). These secretory PLA_2 ($sPLA_2$) have, in common, a large number of disulfide linkages, a requirement for millimolar concentrations of Ca^{2+}, and an apparent nonselectivity for the acyl group at the *sn*-2 of the phosphoglycerides. Although human plasma normally contains very little PLA_2 activity, significant amounts of the enzyme(s) are secreted into plasma in response to infection, inflammation, or trauma (*1*, *3*). Previous studies reported that the plasma concentration of group IIa PLA_2 can increase by 100-fold during the acute phase (*4*), although the effects of this enzyme on plasma lipoproteins are not well understood. The studies of Pruzanski et al (*5*) showed that the acute phase high density lipoprotein (HDL) is a better substrate than normal HDL for the group IIa enzyme, suggesting a possible role for the enzyme in the inflammatory response and in the metabolism of acute phase lipoproteins. Furthermore, transgenic mice overexpressing the group IIa $sPLA_2$ show a dramatic increase in atherosclerosis (*6*, *7*). This $sPLA_2$ is also present in the atherosclerotic lesions, apparently on the surface of the macrophages (*8*). The mechanisms for the increased atherosclerosis in these animals are not clear, although the possibilities include a decrease in HDL levels (*9*), an increase in the oxidation of low density liporoteins (LDL) (*7*), and a change in the surface properties of LDL leading to their increased retention in artery (*10*). One confounding property of the group IIa $sPLA_2$, however, which conflicts with the above results, is its apparent inability to act on phosphatidylcholine (PC), the major phospholipid of cell membranes and lipoproteins. The steric and electrostatic properties of the active site of this enzyme and its interfacial properties appear to be unfavorable for the hydrolysis of PC (*11*). The more recently discovered group V $sPLA_2$, which is structurally similar to group IIa enzyme, however, is more efficient in binding and hydrolyzing PC in the membranes (*12*). Since potentially both these enzymes can be secreted into circulation by the inflammatory cells, and be present in arterial lesions, the relative roles of these two enzymes in the metabolism of lipoproteins and in atherosclerosis need to be reassessed. This is especially important because many of the antibodies previously employed to detect or inhibit the group IIa $sPLA_2$ have later been shown to cross react with group V $sPLA_2$ (*1*, *13*). In this study, we investigated the comparative effects of

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[1] Abbreviations: FFA, free fatty acids; HDL, high-density lipoproteins; LCAT, lecithin-cholesterol acyltransferase; LDL, low-density lipoproteins; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phophatidylglycerol; SPH, sphingomyelin; SPHase, sphingomyelinase; $sPLA_2$, secretory phospholipase A_2

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recombinant group IIa and group V $sPLA_2$ on isolated plasma lipoproteins. The results presented here show that the group V $sPLA_2$ is up to 30 times more efficient than the group IIa enzyme in the hydrolysis of lipoprotein PC. Since this enzyme is known to be secreted by macrophages (*13*), it may play an important role in the metabolism of lipoproteins in arterial tissues, and in the progression of atherosclerotic lesions.

Sphingomyelin (SPH) is the most abundant phospholipid in plasma lipoproteins, next to PC. Previous studies from our laboratory showed that SPH is a physiological inhibitor of lecithin-cholesterol acyltransferase (LCAT) activity in the lipoproteins, possibly because of competition with PC in binding to the active site (*14*). It also appears to inhibit the interfacial binding of the enzyme to the lipoproteins (*15*). A similar inhibition of group IIa PLA_2 in erythrocyte membranes (*16*), as well as lipoprotein lipase in emulsions (*17*), was later reported by others. SPH has also been shown to inhibit phospholipase C-δ1, possibly by inhibiting the penetration of the enzyme into the lipid interface (*18*). On the basis of these observations, and its structural similarity to PC, we postulated that SPH may be a physiological inhibitor of phospholipase A activities, especially those involving PC (*14*, *19*). Since the $sPLA_2$ activities are believed to be involved in the inflammatory responses, it would be of interest to know whether SPH plays an antiinflammatory role by inhibiting these phospholipase activities in the lipoproteins. In this study, we provide evidence for the inhibitory effect of lipoprotein SPH on group V $sPLA_2$, which appears to be more relevant in lipoprotein metabolism than the group IIa enzyme. The SPH content of the lipoprotein was found to be negatively correlated with the hydrolysis of PC by $sPLA_2$, and the incorporation of SPH into liposomes reversibly inhibited the hydrolysis of PC. Although previous studies showed that there is no acyl group specificity for most $sPLA_2$s (*20*, *21*), we found that the release of linoleate (18:2) is favored over the release of arachidonate (20:4) from the lipoprotein PC by the group V $sPLA_2$.

MATERIAL AND METHODS

Materials. Egg SPH, 16:0−18:2 PC, 16:0−20:4 PC, 14:0−14:0 phosphatidylglycerol (PG), brain ceramide, and C2 and C6 ceramides were all obtained from Avanti Polar Lipids (Alabaster, AL). 17:0 FA, SPHase C from *Staphylococcus aureus* (297 units/mg), *Naja mocambique mocambique* venom PLA_2 (1540 units/mg), and bovine serum albumin were obtained from Sigma-Aldrich Inc (St. Louis, MO). Pyrenoyl PG substrate (1-hexadecanoyl-2(1-pyrnedecanoyl)-*sn*-glycero-3-phosphoglycerol) was purchased from Molecular Probes (Eugene, OR). All solvents (HPLC grade) were purchased from Allegiance (McGaw Park, IL). 1−16:0−2 [1-^{14}C] 18:2 PC was obtained from American Radiolabeled Chemicals. BF_3/methanol was purchased from Alltech (Deerfield, IL). Recombinant human $sPLA_2$ group IIa (*22*), and group V (*23*) $sPLA_2$ were prepared as described previously. The enzyme activities were assayed using anionic polymerized mixed liposome substrates (*24*). The specific activities of the enzymes were respectively 4.4 and 4.5 μmol of PE hydrolyzed min^{-1} mg^{-1} for group IIa and group V $sPLA_2$. The enzymes were also assayed using a common fluorescent substrate, 2-pyrene decanoyl PG, in micellar form. With this substrate, the specific activity of the group IIa $sPLA_2$ was about 40 times higher than that of the group V enzyme (0.66 vs 0.015 pmol s^{-1} μg^{-1}, respectively, for group IIa and group V).

Preparation of Plasma LDL and HDL. Fresh plasma, prepared from nonfasting normal volunteers, was purchased from a commercial source (LifeSource, Chicago). LDL and HDL were isolated by sequential ultracentrifugation between the densities of 1.019 and 1.063 g/mL for LDL, and between 1.063 and 1.21 g/mL for HDL. The lipoproteins were dialyzed against Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, filtered through a 0.2 μm filter, and stored in the dark at 4 °C. Protein concentrations were determined by the modified method of Lowry et al (*25*).

Preparation of Liposomes. Liposome substrates were prepared by extrusion through a 100-nm polycarbonate membrane (Mini-Extruder, Avanti Polar Lipids) according to the method of MacDonald et al (*26*), at 42 °C for liposomes containing PC plus SPH or ceramide, and at 37 °C for liposomes containing only PC. SPH-containing liposomes were also prepared using 16:0-[^{14}C]18:2 PC (60 000 dpm and 1000 nmol) and 0−1000 nmol of egg SPH in 1 mL of Tris-HCl buffer, pH 7.4. The labeled liposomes containing ceramide were similarly prepared by substituting 0−200 nmol of brain ceramide for SPH.

For the preparation of liposomes with lipoprotein PC, the total lipids of LDL and HDL lipids (10 mg of protein) were first extracted by the Bligh and Dyer procedure (*27*), and separated by TLC, using the solvent system of chloroform/methanol/water (65:25:4, by vol). The spot corresponding to PC was scraped and extracted by chloroform/methanol/water (1:1: 0.5, by vol), and the PC concentration was determined by estimation of lipid phosphorus (*28*) in an aliquot. The PC was incorporated into liposomes by extrusion through a 100-nm membrane after mixing with 10 mol % 14:0−14:0 phosphatidylglycerol (PG). Liposomes containing synthetic 16:0−20:4 PC and 16:0−18:2 PC in different ratios were similarly prepared by lipid extrusion at 37 °C, after mixing with 10 mol % (final concentration) of 14:0−14:0 PG.

PLA_2 Activity on Isolated Lipoproteins. LDL or HDL (0.25 mg of protein) were incubated with group IIa or V $sPLA_2$ (65 μg) in a total volume of 1 mL of Tris-HCl buffer, pH 7.4, containing 10 mM $CaCl_2$ and 0.01% BSA, for 1 h at 37 °C. The reactions with liposome substrates were carried out under the same conditions excepting that 160 μg of group V $sPLA_2$ was used for liposomes containing 300 nmol PC, and the incubation time was 2 h. The reaction was stopped by addition of 2.5 mL of methanol, and the total lipids were extracted by the Bligh and Dyer procedure (*27*). The lipid extracts were separated on silica gel TLC with a two-step procedure to separate both neutral and polar lipids. First, the plate was developed halfway (10 cm) in chloroform/methanol/water (65:25:4, v/v) to separate the phospholipids. The plate was then briefly dried in air and developed in the solvent system of hexane/diethyl ether/acetic acid (70:30:2, v/v) to separate the neutral lipids. The spots corresponding to lyso PC, PC, PE, SPH, and free fatty acids (FFA) were detected by brief exposure to iodine vapor and scraped into glass tubes. The phospholipids were then estimated by lipid phosphorus estimation using the modified Bartlett procedure (*28*). When radioactive PC (2-acyl labeled) was used as

Table 1: Hydrolysis of Lipoprotein Phospholipids by $sPLA_2$[a]

	LDL				HDL			
	PE		PC		PE		PC	
	molar rate[b]	fractional rate[c]	molar rate[b]	fractional rate[c]	molar rate[b]	fractional rate[c]	molar rate[b]	fractional rate[c]
$sPLA_2$ IIa ($n=3$)	4.2 ± 1.7	4.0 ± 1.8	31.6 ± 12.1	1.5 ± 0.7	3.4 ± 2.4	4.7 ± 3.5	45.0 ± 18.4	0.9 ± 0.4
$sPLA_2$ V ($n=7$)	17.9 ± 6.5*	25.5 ± 11.3*	1017 ± 242**	41.4 ± 8.6**	21.2 ± 15.2	43.3 ± 32.7	1102 ± 447**	51.6 ± 16.8**

[a] Recombinant group IIa and group V sPLA2 (65 μg) were incubated with human plasma LDL or HDL (0.25 mg of protein) for 1 h in the presence of 10 mM Ca^{2+}, and the decrease in PC and PE was determined by TLC separation and lipid phosphorus determination. The values shown are mean ± SD of three experiments. *$p < 0.05$; **$p < 0.01$ compared to $sPLA_2$ IIa. [b] nmol of phospholipid hydrolyzed h^{-1} (mg of protein)$^{-1}$. [c] % of the phospholipid hydrolyzed/h.

substrate, the radioactivity in the FFA and PC spots was determined in a liquid scintillation counter. The enzyme activity was expressed as the percentage of PC or PE hydrolyzed (fractional rate) or nmol of PC or PE hydrolyzed per hour (molar rate).

Sphingomyelinase C (SPHase C) Treatment. Where indicated, SPHase C (0.5 U/mL) was added at the same time as PLA_2 to the lipoprotein or liposome preparations in a total volume of 1 mL of Tris-HCl buffer, pH 7.4, containing 10 mM $CaCl_2$, 0.01% BSA, and 0.8 mM $MnCl_2$, for 1 h, at 37 °C. The SPHase was found to hydrolyze only SPH in the lipoproteins as well as liposomes, as reported earlier (*14*). Nevertheless, a control containing only SPHase (without PLA_2) was included in all experiments to confirm the lack of phosphoglyceride hydrolysis by the SPHase.

Specificity toward Molecular Species of PC. LDL or HDL (0.5 mg of protein) was incubated at 37 °C for 2 h with either group V PLA_2 (160 μg) or *Naja m. mocambique* venom PLA_2 (0.2 U/mL) in a total volume of 1 mL of Tris-HCl buffer, pH 7.4, containing 10 mM $CaCl_2$, 0.01% BSA. Total lipids were extracted, and applied to silica gel TLC plates. The lipids were separated with the two-step development procedure as described above. Spots corresponding to PC were scraped, and extracted with chloroform/methanol/water (*27*), and an aliquot was used for the determination of lipid phosphorus (*28*). The PC was then incorporated into liposomes, and incubated with the phospholipase A_2 as described above. The molecular species of PC were separated by reverse phase HPLC, using a C-18 column with the solvent system of methanol/acetonitrile/water (60: 39: 1, v/v) at a flow rate of 0.8 mL/min. The temperature of the column was maintained at 30 °C with the help of a column heater. The PC species were detected with a light scattering detector (ELSD, Mark III, Alltech Associates), and the percentages of individual species were determined with EZChrom program (Scientific Software, Inc, San Ramon, CA).

The decrease in total PC after hydrolysis with either $sPLA_2$ or *Naja* PLA_2 was determined from the decrease in lipid phosphorus. The decrease in the concentration of individual PC species was calculated from the HPLC data, and was expressed as percent of the hydrolyzed PC. The specificity of $sPLA_2$ for the individual molecular species of PC was then calculated as:

$$\frac{\%\text{ of the given PC species in total PC hydrolyzed}}{\%\text{ of its initial concentration}}$$

To correct for the possible differences in the accessibility of individual PC species in native lipoproteins, we normalized the specificity values with those obtained with *Naja* PLA_2, which is known to be nonselective for the PC species. The relative specificity of $sPLA_2$ was calculated as:

$$(\text{specificity of } sPLA_2 \div \text{specificity of } \textit{Naja}\ PLA_2) - 1$$

PC species with positive values are preferred substrates for the $sPLA_2$ in native lipoproteins, while those with negative values are relatively poor substrates for the enzyme.

Fatty Acid Specificity. LDL, HDL (0.5 mg of protein), or PC liposomes were incubated, as described above, at 37 °C with group V PLA_2 (160 μg) or *Naja m. mocambique* venom PLA_2 (0.2 U/mL) in a total volume of 1 mL of Tris-HCl buffer, pH 7.4, containing 10 mM $CaCl_2$, 0.01% BSA, for 2 h. Total lipids were extracted by Bligh and Dyer procedure (*27*), and separated on silica gel TLC plates with the two-step solvent system as described above. Spots corresponding to FFA were scraped and transmethylated with BF_3/methanol, and analyzed by capillary gas liquid chromatography as described previously (*29*). The concentration of the individual FA released was determined with the help of an internal standard (17:0). Since the *Naja* PLA_2 is known to be nonselective for the acyl groups at the *sn*-2 position of PC, the specificity of group V enzyme was determined by comparing the fatty acids released by the two PLA_2 under similar conditions.

Statistics. Results are expressed as mean ± standard deviation (SD). Differences between group means were compared by using the paired or unpaired Student's *t*-test (VassarStats).

RESULTS

Hydrolysis of LDL and HDL Phosphoglycerides by Group IIa and V $sPLA_2$. We first determined the activity of group IIa and V $sPLA_2$ to hydrolyze the two major phosphoglycerides (PC and PE) of the native lipoproteins. LDL and HDL, isolated from normal human plasma, were incubated with either recombinant group IIa or group V $sPLA_2$ for 1 h at 37 °C and pH 7.4 in the presence of 10 mM Ca^{2+}. The total lipids were extracted (*27*), and the decrease in PC and PE was determined by the estimation of lipid phosphorus after TLC separation, as described in the Methods. At equal protein concentrations (65 μg), the group V enzyme was found to be up to 30 times more active than the group IIa enzyme in the hydrolysis of lipoprotein PC (molar rate)

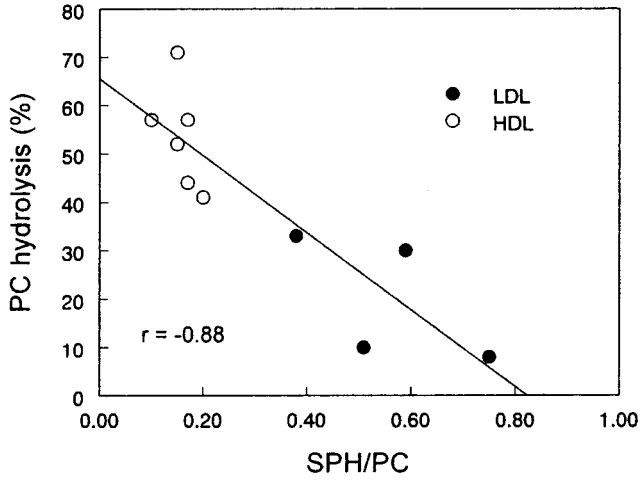

FIGURE 1: Negative correlation of PC hydrolysis in lipoproteins with their SPH/PC ratio. The percentage of PC hydrolyzed in various LDL and HDL preparations by group V $sPLA_2$ (1 h, 65 μg of enzyme) was plotted against the SPH/PC ratio of the lipoprotein.

(Table 1). In terms of the fractional rate (% of the lipoprotein phospholipid hydrolyzed) the group IIa enzyme was 2−5 times more active on PE than on PC in both LDL and HDL, whereas the group V enzyme hydrolyzed PC more efficiently than PE. These results are in variance with the recent study of Pruzanski et al (*5*) who reported that lipoprotein PC is a good substrate for the group IIa enzyme, but are consistent with the known specificities of the two enzymes in membranes (*2*, *12*). It may be pointed out that the specific activities of the two phospholipases are similar when assayed with anionic polymerized mixed liposomes. In fact, with the micellar PG, the group IIa enzyme was 40 times more active than the group V enzyme.

Our results also indicate that HDL is a better substrate than LDL for group V enzyme, in accordance with the results of Pruzanski et al (*5*) for the group IIa enzyme. Although the results in Table 1 do not show this clearly because of the variations in activities from different batches of lipoproteins, LDL from a given plasma was consistently a poorer substrate than the HDL from the same plasma. Since SPH has been shown to be inhibitory to several lipolytic enzymes (*14*, *16*, *17*, *30*), and since the ratio of SPH/PC is higher in LDL than in HDL, we next examined the relationship between the SPH content of the lipoprotein substrate and the percent of PC hydrolyzed by the group V $sPLA_2$. As shown in Figure 1, there was an inverse correlation ($r = -0.88$) between the SPH content of the lipoprotein and the percentage of PC hydrolyzed by this enzyme. Therefore, the lower activity toward LDL may be, at least partly, due to the higher concentration of SPH in this lipoprotein.

Effect of SPHase Treatment of the Lipoproteins on PLA_2 Activities. To determine if the group V $sPLA_2$ activity is affected by the SPH content of the lipoproteins, we included in the reaction mixture, bacterial SPHase C, which specifically hydrolyzes SPH to ceramide and phosphorylcholine. As shown in Figure 2, this treatment decreased the SPH/PC ratio by about 95% in LDL and HDL. There was no effect on other phospholipids or the neutral lipids of the lipoproteins, as we reported in previous studies (*14*). The density of the lipoproteins was also not affected by the SPHase treatment because the lipoproteins could be isolated at their

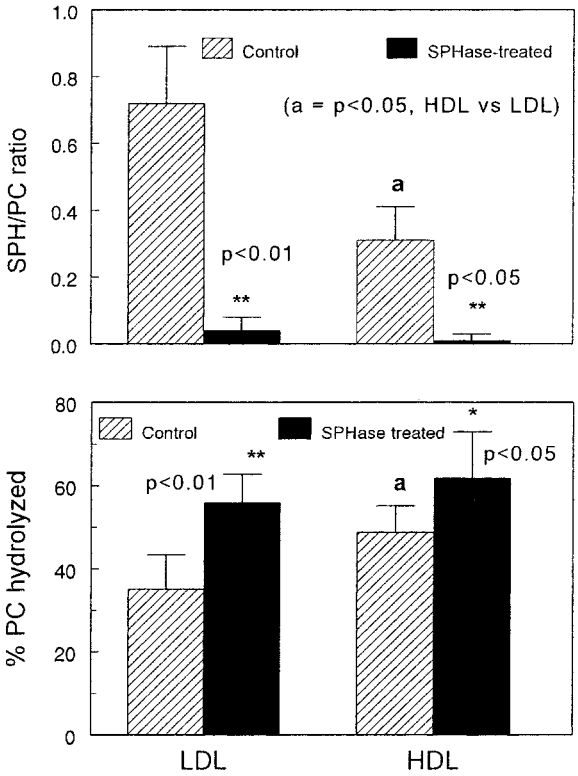

FIGURE 2: Effect of SPHase treatment of lipoproteins on group V $sPLA_2$ activity. Group V $sPLA_2$ was incubated with LDL or HDL (0.25 mg of protein) in the absence or presence of SPHase C from *S. aureus* (0.5 U/mL) for 1 h at 37 °C. The SPH/PC ratio and the percentage of PC hydrolyzed were determined by lipid phosphorus estimations, following TLC separation, as described in text. Results shown are mean ± SD of five separate analyses. $*p < 0.05$, $**p < 0.01$ (control vs SPHase-treated); *a* $p < 0.05$ (HDL vs LDL).

original flotation densities after the enzyme reaction. SPH depletion led to the activation of the PLA_2 activity by 60% ($p < 0.01$) in LDL and by 20% ($p < 0.05$) in HDL. Furthermore, the differences in PC hydrolysis between LDL and HDL disappeared in the presence of SPHase. The activity of group IIa enzyme was also stimulated by the treatment of lipoproteins with SPHase C. Since the activity of this enzyme is very low in the presence of the lipoprotein substrates, we estimated the activity by incorporating labeled PC (16:0-[1-^{14}C]-18:2 PC) into the lipoprotein, and determining the release of labeled fatty acid following incubation with $sPLA_2$. As shown in Figure 3, inclusion of SPHase C resulted in a 5-fold stimulation of group IIa enzyme activity against LDL, and a 65% stimulation against HDL substrate. Thus, the depletion of SPH had a more profound effect on LDL than on HDL with both phospholipase activities. These results suggest that the difference in the hydrolysis of PC of the two lipoproteins is related to the amount of SPH originally present in them.

The effect of treatment of LDL with varying amounts of SPHase on group V enzyme activity is shown in Figure 4. We found that the progressive decrease of the SPH content of LDL is accompanied by a reciprocal increase in the hydrolysis of both PC and PE by group V PLA_2.

Effect of SPH in Liposomes. To determine the inhibitory effect of SPH directly, we studied the effect of incorporation of SPH into PC liposomes on the hydrolysis of PC by group V PLA_2. Liposomes containing 16:0-[1-^{14}C] 18:2 PC and

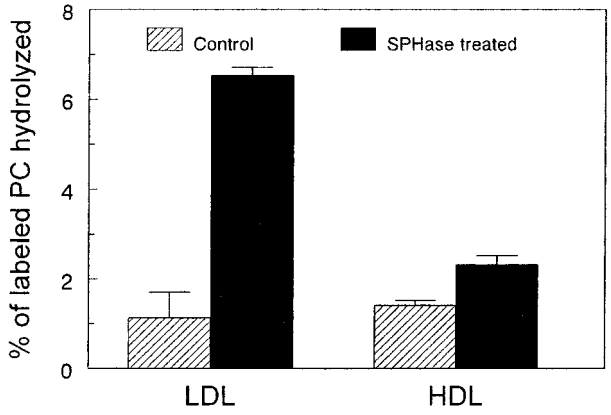

FIGURE 3: Effect of SPHase treatment of lipoproteins on group IIa $sPLA_2$ activity. LDL or HDL preparations were first labeled with 16:0-[1-^{14}C]-18:2 PC by incubating labeled PC (in ethanol, 1% total volume) at 37 °C for 30 min. Recombinant group IIa $sPLA_2$ (65 μg) was then added and incubated in the absence or presence of *S. aureus* SPHase (0.5 U/mL) for 2 h. The percentage of labeled PC hydrolyzed was determined from the labeled fatty acid released. The results shown are mean ± SD of two experiments each performed in triplicate.

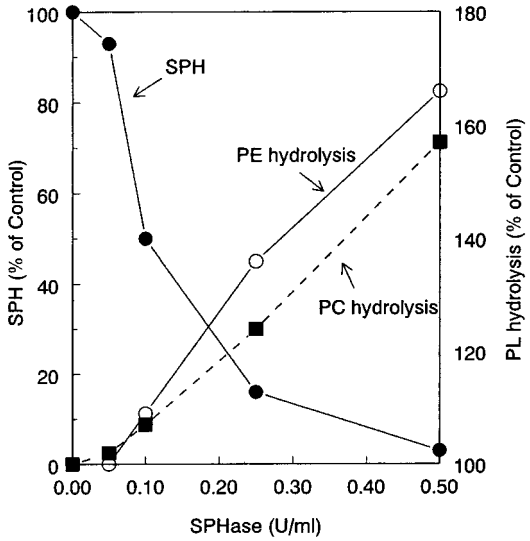

FIGURE 4: Effect of increasing concentration of SPHase C on the hydrolysis of LDL phospholipids by group V $sPLA_2$. LDL (0.5 mg of protein) was incubated with 65 μg of recombinant group V $sPLA_2$, and the indicated concentration of SPHase C from *S. aureus* for 1 h, and the hydrolysis of PE and PC was determined from the decrease in lipid phosphorus, as described in the text. The enzyme is expressed as percent of activity in the absence of added SPHase C (control).

varying amounts of egg SPH were prepared by the membrane extrusion method (*31*), and reacted with group V PLA_2 as described in Methods. The results in Figure 5 show that SPH inhibited PC hydrolysis, in a concentration-dependent manner. At the molar ratio of SPH/PC of 1.0, the hydrolysis of PC was inhibited by over 60%. In these experiments, the amount of labeled PC was kept constant, and only the amount of SPH was altered, and therefore, the lower activity was not due to a limitation of the substrate PC. We then studied whether the enzymatic degradation of SPH would reverse the inhibitory effect of SPH. As shown in Figure 5, the SPHase C treatment not only reversed the inhibition of SPH, but actually stimulated the activity above the control (without SPH), especially at higher initial concentration of SPH. There

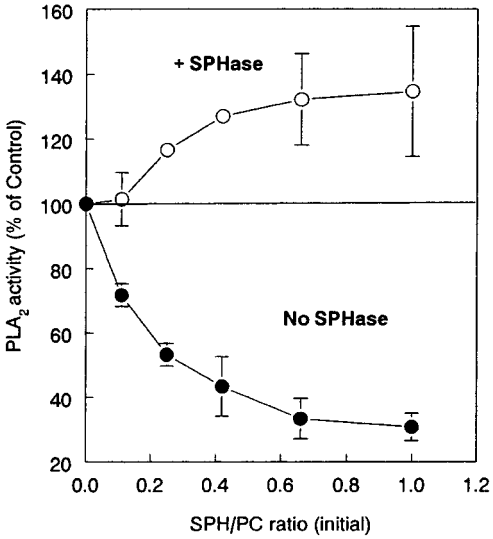

FIGURE 5: Effect of SPH on PC hydrolysis in liposomes by group V $sPLA_2$. Liposomes containing 16:0-[1-^{14}C]-18:2 PC (1 μmol/mL) and increasing amounts of egg SPH were prepared by membrane extrusion, as described in the text. The samples were then incubated with $sPLA_2$ alone or with $sPLA_2$ and *S. aureus* SPHase C (0.5 U/mL) for 2 h at 37 °C. The amount of labeled fatty acid liberated was determined after TLC separation, and was expressed as percentage of the control value (no SPH). The closed circles show the PLA_2 activity in the presence of SPHase C and the open circles the activity in the absence of SPHase C. The SPH/PC ratios shown on *x* axis are the initial ratios in the samples (before SPHase treatment). There was about a 40% decrease in SPH content in all samples containing the SPHase C, compared to those without SPHase C.

was no change in the size of liposomes after SPHase treatment, as reported earlier (*14*). It should be pointed out that under these conditions only about 40% of the SPH was hydrolyzed in all the samples. Thus, in the sample containing the maximum amount of SPH, the SPHase treatment decreased the ratio from 1.0 to 0.57. However, this sample showed a 3-fold higher activity than the untreated sample containing a comparable amount of SPH. These results suggest that the increase in PLA_2 activity is not only due to a depletion of SPH but also due to the formation of a stimulatory product, probably ceramide, which has previously been reported to activate some phospholipases (*32*, *33*).

Effect of Ceramide on PLA_2 Activity. To investigate whether the stimulation of $sPLA_2$ activity is due to the independent effects of the ceramide generated by the SPHase treatment, we determined the activity of PLA_2 in liposomes containing 16:0- [1-^{14}C]-18:2 PC and varying amounts of brain ceramide. The results in Figure 6 show that the incorporation of brain ceramide into PC liposomes increased the PC hydrolysis, in a dose-dependent manner. When 20 mol % of ceramide was present in the liposomes, there was a 78% stimulation of PLA_2 activity. Higher amounts of ceramide were not tested because the liposomes appeared to undergo phase transition, similar to that reported for diacylglycerol (*34*), as indicated by the cloudiness of the solution, at >20 mol % of brain ceramide concentration. Interestingly, the stimulation obtained with 20 mol % ceramide was higher than that found when the liposomes containing SPH/PC ratio of 1.0 were treated with SPHase (Figure 5). We calculate that these liposomes should also contain 20 mol % ceramide, in addition to the 30 mol % of

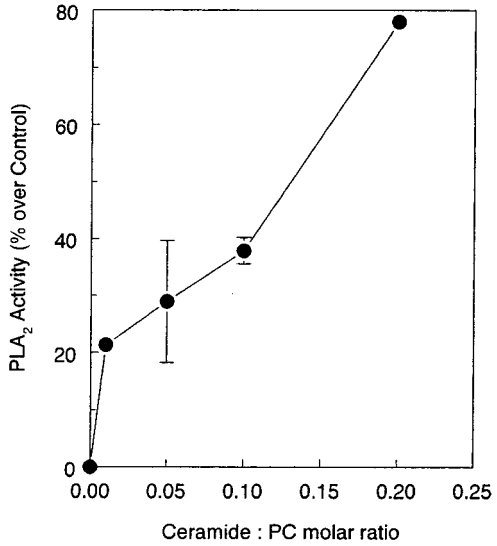

FIGURE 6: Effect of long chain ceramide on group V $sPLA_2$ activity. Liposomes containing 16:0-[1-^{14}C]-18:2 PC (1 μmol/mL) and varying amounts of brain ceramide were incubated for 2 h with group V $sPLA_2$ (160 μg) and the enzyme activity was determined from the release of labeled free fatty acids. The percentage increase in activity over the control (no ceramide) was plotted against the mol % of brain ceramide in the liposomes. The values shown are mean ± SD of three separate experiments, each performed in duplicate.

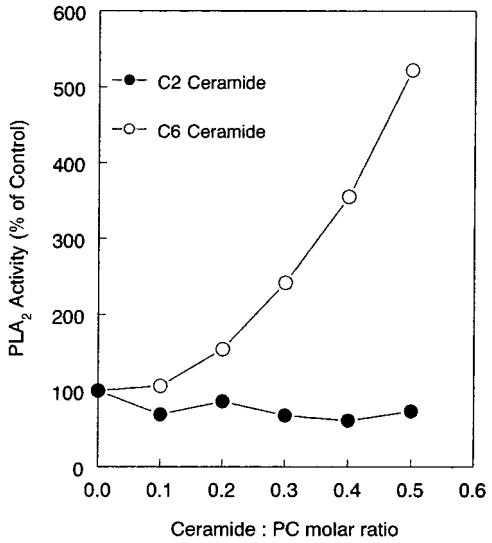

FIGURE 7: Effect of short chain ceramides on group V $sPLA_2$ activity. 16:0-[1-^{14}C]-18:2 PC liposomes were prepared as described in the text, and incubated with group V $sPLA_2$ in the presence of various amounts of C-2 or C-6 ceramides which were added in DMSO (1% of total volume). The enzyme activities are expressed as percentage of control activity (no ceramide).

unhydrolyzed SPH. Since the activity is higher in the presence of ceramide alone, when compared to ceramide + SPH, these results therefore suggest that SPH and ceramide have opposing effects on the activity of $sPLA_2$.

The effect of addition of short chain ceramides to the reaction mixture containing PC liposomes is shown in Figure 7. The exogenous C-6 ceramide stimulated the enzyme activity by up to 5-fold, while the C-2 ceramide showed a slight inhibition at similar concentrations. We found that dioleoyl glycerol, at a concentration of 20 mol % also stimulated the $sPLA_2$ activity by 100% (results not shown).

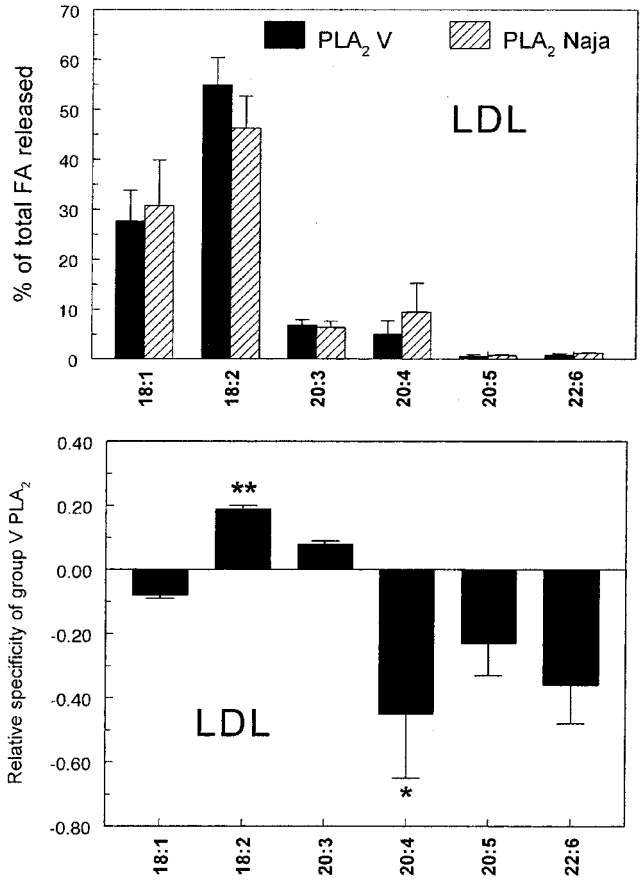

FIGURE 8: Fatty acid specificity of group V $sPLA_2$ in LDL. Group V $sPLA_2$ (160 μg) or *Naja m. mocambique* PLA_2 (0.2 U) was incubated with human plasma LDL (0.5 mg of protein) for 2 h, and the released fatty acids were analyzed by gas chromatography, as described in the text. In the upper panel, the percentage composition of the released fatty acids by the two enzymes is shown. In the lower panel, the relative specificity of the group V $sPLA_2$ is shown. The relative specificity of the enzyme for each fatty acid was calculated as (% of the fatty acid released in the presence of group V $sPLA_2$ ÷ of the fatty acid released in the presence of *Naja* PLA_2) − 1. A value above zero shows the preference of group V $sPLA_2$ for the given fatty acid, whereas a value below zero shows that the enzyme discriminates against it. Results shown are mean ± SD of four separate experiments. *p < 0.05, **p < 0.005.

These results suggest that the perturbation of the PC bilayer may be involved in the activation of the $sPLA_2$ V, as suggested for the intracellular phospholipases (*30*, *33*).

Fatty Acid Specificity of Group V PLA₂. Although the $sPLA_2$ isoforms have been shown to be generally nonspecific toward the fatty acid at the *sn*-2 position of PC in synthetic liposomes (*20*, *21*), the studies of Chen and Dennis (*35*) on synthetic substrates suggested that the group V PLA_2 may have some selectivity for 18:2. However, the specificity of the enzyme has not been investigated using natural substrates. Therefore, we studied the composition of fatty acids released from the lipoprotein phospholipids by the group V enzyme and compared it to the fatty acids released by *Naja m. mocambique* PLA_2. Since the snake venom PLA_2 is known to be nonselective for the acyl group at the *sn*-2 position of PC, the fatty acids released by it represent the composition at *sn*-2 position of plasma PC. As shown in Figure 8 (LDL) and Figure 9 (HDL), group V PLA_2 released more 18:2 and

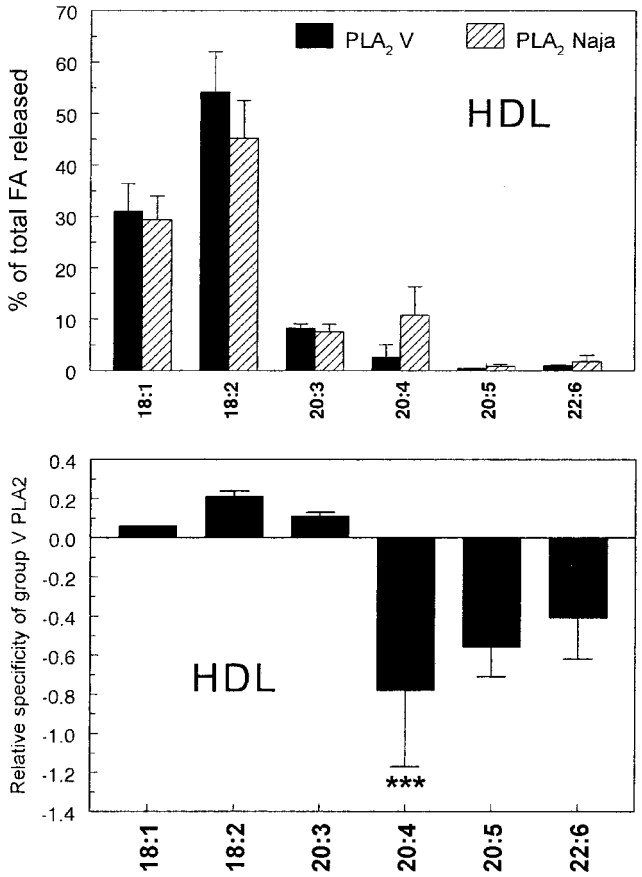

FIGURE 9: Fatty acid specificity of group V $sPLA_2$ in HDL. Group V $sPLA_2$ or *Naja* PLA_2 was incubated with human HDL (0.5 mg) for 2 h at 37 °C, and the released fatty acids were analyzed by gas chromatography as described in the text. The upper panel shows the percentage composition of the fatty acids released in the presence of the two enzymes. The lower panel shows the relative specificity of group V $sPLA_2$, which was calculated as described under Figure 8 and in the text. Results shown are mean ± SD of four experiments. ***$p < 0.001$ (paired t test).

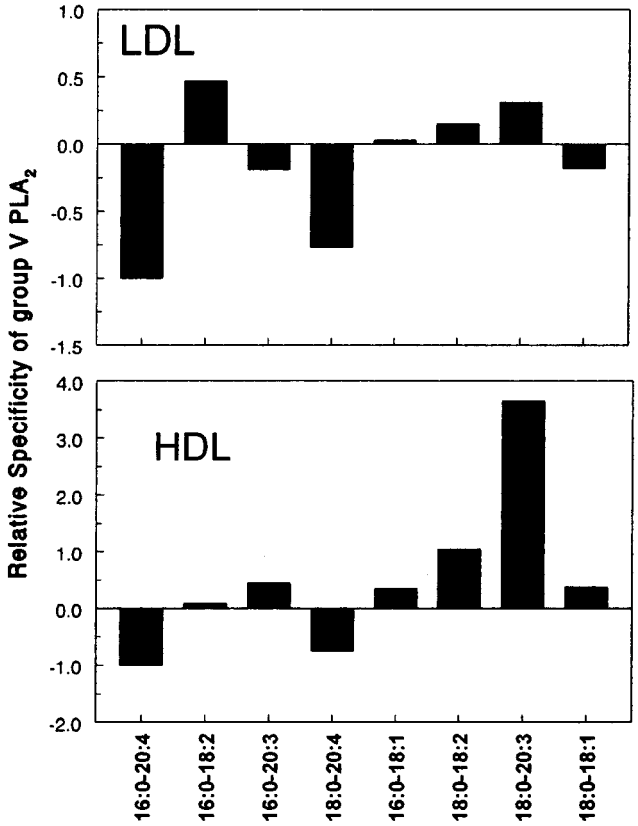

FIGURE 10: Specificity of group V $sPLA_2$ for molecular species of PC. After incubation of the lipoproteins with either group V $sPLA_2$ or *Naja* PLA_2, as described under Figure 8 and Figure 9, the total lipids were extracted, and the molecular species composition of PC was analyzed by HPLC. The decrease in total PC during incubation was determined from the change in lipid phosphorus, while the decrease in individual PCs was calculated from their percent composition and the total lipid phosphorus before and after the reaction. The specificity of group V enzyme was calculated relative to that of *Naja* PLA_2, as described in the text. Positive values show the preference of the group V $sPLA_2$ for the given PC species whereas negatives values show that the PC species is a poor substrate for group V $sPLA_2$, relative to *Naja* PLA_2. The values shown are averages of two separate experiments.

20:3 FA compared to *Naja* PLA_2 from both lipoproteins. Furthermore, the percentages of 20:4, 20:5, and 22:6 in the released FA were significantly lower in the presence of $sPLA_2$ than in the presence of the snake venom enzyme. These results suggest that the role of group V $sPLA_2$ might not be to provide arachidonate from the lipoproteins for eicosanoid biosynthesis.

The molecular species of lipoprotein PC hydrolyzed by the two phospholipases were next analyzed by HPLC, to confirm the apparent specificity of group V enzyme toward 18:2 containing PC species. As shown in Figure 10, group V s PLA_2 was less reactive with 16:0−20:4 PC and 18:0−20:4 PC in both LDL and HDL. Moreover, it appeared to be more specific for PCs containing 18:1, 18:2, and 20:3 in HDL, whereas only the specificity for 16:0−18:2, 18:0−18:2, and 18:0−20:3 PC was higher in LDL.

To investigate whether the apparent lack of specificity for arachidonate is due to a lower accessibility of the group V $sPLA_2$ for the arachidonate-containing PC, we determined the activity of PLA_2 on liposomes reconstituted from the PC extracted from LDL and HDL. As shown in Figure 11, we found that even in the absence of apoproteins, the group V PLA_2 preferentially hydrolyzed 18:2, and discriminated against 20:4, in comparison to *Naja m. mocambique* venom PLA_2. Therefore, the PCs containing arachidonate appear to be hydrolyzed at a lower rate than other PCs because of the inherent property of the enzyme. The $sPLA_2$ showed a decreased preference for 20:3 in liposomes, although in intact lipoproteins this fatty acid was released more efficiently (Figures 8−10). Further evidence for the specificity was obtained by using liposomes prepared with synthetic 16:0−20:4 PC and 16:0−18:2 PC at varying ratios. We found that the percent of arachidonate released by the group V enzyme was significantly lower than by that released by the snake venom enzyme at all ratios of the two PCs (Figure 12). At equal concentrations of 16:0−18:2 and 16:0−20:4 PCs, only 13% of the released fatty acid was 20:4, the rest being 18:2. On the other hand, the percentage of 20:4 in the free fatty acids released by the snake venom enzyme was 40%. These results thus show that 16:0−20:4 PC is not a preferred substrate for the group V enzyme, at least in comparison to the major PC species of plasma.

DISCUSSION

Although several isoforms of phospholipases A have been identified in mammalian tissues, their potential role in the

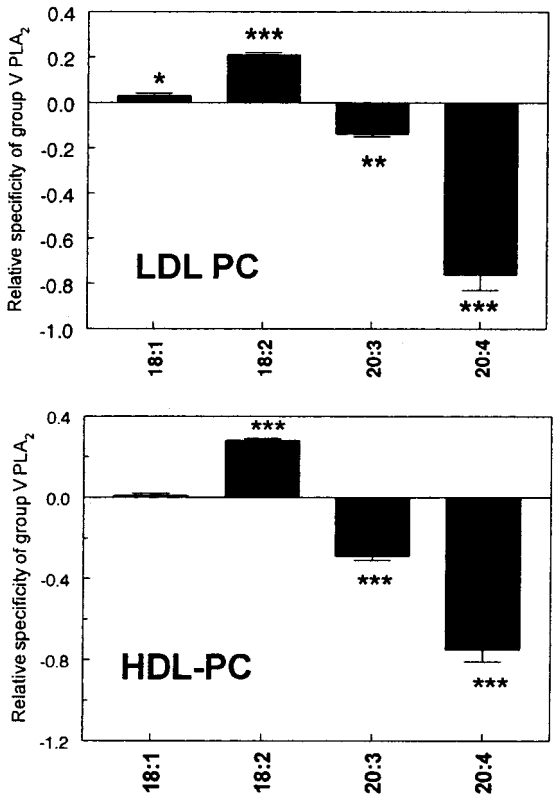

FIGURE 11: Fatty acid specificity of group V $sPLA_2$ in liposomes prepared from lipoprotein PC. Total PC was prepared from LDL or HDL, and incorporated into liposomes (with 10 mol % di-14:0 PG) by lipid extrusion. The liposomes were then incubated with either group V $sPLA_2$ or *Naja* PLA_2, and the relative specificity of the group V enzyme was calculated as described under Figure 8. Results shown are mean ± SD of four experiments. $*p < 0.05$, $**p < 005$, $***p < 0.001$, (paired t test).

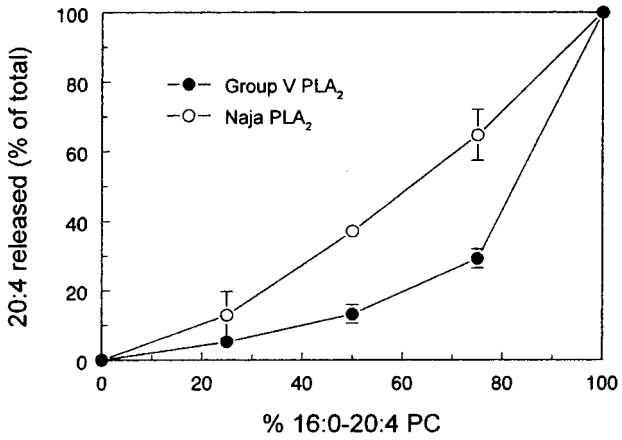

FIGURE 12: Fatty acid specificity of group V and Naja PLA_2 in synthetic PC mixture. Liposomes containing 16:0−18:2 PC and 16:0−20:4 PC in various ratios (and 10 mol % di-14:0 PG) were prepared by lipid extrusion, and reacted with the two PLA_2. The released free fatty acids were analyzed by gas chromatography, and the percent of 20:4 released was plotted against the percent of 16:0−20:4 PC in the substrate. A lower value from the linear straight line shows the preference of 16:0−18:2 PC over 16:0−20:4 PC by the enzyme. Results shown are mean ± SD of two separate experiments, each performed in triplicate.

metabolism of plasma lipoproteins has not received much attention. There is indirect evidence that the hydrolysis of lipoprotein phospholipids by the PLA_2 has profound effects on the metabolism of the lipoproteins in vivo. Thus, the degradation of LDL phosphoglycerides by snake venom PLA_2 results in an accelerated disappearance of the lipoprotein from the plasma compartment (*36*). Since this is considered to be an anti-atherogenic effect, the extra corporal degradation of LDL phospholipids by PLA_2 has been proposed as a therapeutic approach for the treatment of hypercholesterolemia (*36*). On the other hand, the hydrolysis of LDL phospholipids in the arterial tissue may be pro-atherogenic, because the PLA_2-modified LDL binds strongly to proteoglycans (*37*), and is taken up avidly by the macrophages, leading to the formation of foam cells (*38*, *39*). Therefore, the characterization of the phospholipases which can potentially hydrolyze the lipoprotein phospholipids in the plasma compartment as well as in arterial tissue is of great interest. The PLA_2 that has attracted most of the attention in this regard is the group IIa $sPLA_2$, which is secreted by a variety of cells, including platelets, hepatocytes, and smooth muscle cells, especially in response to stimulation by inflammatory cytokines (*1*, *2*). Its concentration has been reported to increase over 100-fold in acute phase (*4*), and its overexpression in transgenic mice results in a dramatic increase in atherosclerotic lesions (*6*). However, several in vitro studies show that while this enzyme can hydrolyze the negatively charged phospholipids such as PG, it is ineffective in the hydrolysis of the major phospholipid of the lipoproteins, namely, PC (*2*, *12*). Recent studies by Pruzanski et al (*5*) on the other hand, show that the lipoproteins, especially the acute phase HDL, are good substrates for the group IIa $sPLA_2$, and that their PC is hydrolyzed efficiently by it. The results presented here, in contrast, show that the PC of human plasma LDL and HDL is a poor substrate for the group IIa $sPLA_2$, whereas it is hydrolyzed effectively by the group V enzyme. The reason for the discrepancy between the two studies is not clear. As mentioned before, the specific activity of group IIa enzyme is much higher than that of group V enzyme, when assayed with a micellar PG substrate. It is also more stable than the group V enzyme during storage. Therefore, the lack of activity against lipoproteins is not due to lower specific activity. It is not known how much of the increase in the $sPLA_2$ activity during inflammatory reactions is due to group V enzyme. Because of the close structural similarities between the group IIa and group V enzymes, and the cross-reactivity of the antibodies generally used for their determination, it is necessary to reexamine the relative concentrations of the two isoforms present in acute phase plasma, as well as in arterial lesions. Since the study of Pruzanski et al (*5*) did not include the group V enzyme, it is not possible to compare the relative activities of the two enzymes. Since PC is hydrolyzed efficiently by the group V enzyme, but not by the group IIa enzyme, the activity of the former may be more relevant for the metabolism of lipoproteins both in the plasma compartment and in arteries. One recent study reported that only group IIa enzyme is found in the sera of acute chest syndrome patients and certain trauma victims (*40*). It should, however, be pointed out that the macrophages express predominantly the group V $sPLA_2$, and this enzyme has been shown to stimulate the release of arachidonic acid, as well as the production of eicosanoids in these cells (*13*). In contrast, the group IIa $sPLA_2$ does not elicit eicosanoid production in peritoneal macrophages stimulated with LPS (*41*), suggesting that it may not be involved in the metabolism of macrophages in vivo. Another

recently discovered $sPLA_2$, the group X enzyme, has also been shown to be specific for PC (*42*), and to release arachidonate from the adherent cells (*43*), although its presence in plasma or in atherosclerotic lesions has not been reported.

An important finding of the present study is the inhibition of group V $sPLA_2$ by SPH in the lipoproteins. Although SPH is the most abundant phospholipid in the lipoproteins next to PC, its physiological function in the lipoprotein metabolism has not been established. While it does contribute to the structural integrity of the lipoproteins, it is not essential for the lipoprotein structure. However, the protection of the surface PC from hydrolysis by the various phospholipase activities may be an important physiological function of this sphingophospholipid. In addition to the inhibition of group V and group IIa $sPLA_2$, SPH has previously been shown to inhibit the activities of LCAT (*15*), lipoprotein lipase (*17*), and hepatic lipase (P. V. Subbaiah, and J. Goyal, unpublished results), all of which can hydrolyze the phosphoglycerides of the lipoproteins, and destabilize their structure. Although the mechanism of inhibition of $sPLA_2$ by SPH has not been investigated, previous studies from our laboratory and others (*14*, *15*) suggested that it may act as a competitive inhibitor of LCAT, because of its structural similarity to PC, and its lack of the easily hydrolyzable acyl ester linkage. In addition, SPH may inhibit the binding of the phospholipases to the lipoprotein substrates, and their penetration into the PC monolayer, because of its condensing effects on monolayer structure (*15*). Previous studies also showed that SPHase treatment enhances the activation of $sPLA_2$ and other other phospholipases in cell membranes and monolayers (*44*, *45*). It is important to note that the bulk of the cellular SPH is located in the outer monolayer of the plasma membrane (*46*), indicating the potential physiological significance of SPH as a protective agent against hydrolysis by the phospholipases which may be present in the surrounding medium. We previously showed that SPH inhibits lipid peroxidation in lipoproteins and PC bilayers, possibly by retarding the propagation of the lipid peroxy radicals (*47*). The combined effects of PLA_2 inhibition and the inhibition of lipid peroxidation would make SPH an unique regulatory lipid that maintains the integrity of cell membranes and lipoproteins (*19*).

In addition to the inhibitory effect of SPH, our studies show a stimulation of group V $sPLA_2$ by the ceramides. When the SPH-containing liposomes were treated with bacterial SPHase C, there was not only a reversal of the SPH inhibition, but also a stimulation of the enzyme activity over and above the control (which contained only PC). Furthermore, the inclusion of natural ceramide in the PC liposomes significantly stimulated the enzyme activity, suggesting that the ceramide may decrease the packing density of the liposomes, enabling the PLA_2 to penetrate the monolayer better. Studies by Huang et al (*32*) showed that cobra venom PLA_2 activity was inhibited by C6 and C8 ceramides, but was activated by a long chain (C16) ceramide, which induces a lateral phase separation of the bilayer into gel and crystalline domains. Hashizume et al (*33*), on the other hand, reported that C6 ceramide also stimulated the cytosolic PLA_2 (group IV PLA_2) in platelets, probably though modification of the membrane structure. We found that C6 ceramide, but not C2 ceramide, activated the group V enzyme when added to the PC liposomes, suggesting that the major effect of ceramides is to modify the bilayer structure, and facilitate penetration of the enzyme. We also found that 1,2-dioleoyl glycerol, which is structurally similar to long chain ceramide, stimulated the group V $sPLA_2$ activity significantly when incorporated into PC liposomes (results not shown). The stimulation of other phospholipases by diacylglycerol has been reported earlier (*30*, *48*, *49*), although its effect on secretory PLA_2 has not been reported. It is of interest to note that the diacylglycerol has been shown to be most predominant glyceride in HDL, and is present on the surface (*50*), suggesting that it may stimulate the $sPLA_2$ activity against HDL in vivo.

Both group IIa and group V $sPLA_2$ have been implicated in the release of arachidonate and the formation of eicosanoids by various cells (*13*, *51*). However, unlike the cytosolic PLA_2, these enzymes do not cause a preferential release of arachidonate, and therefore their function in eicosanoid synthesis during the inflammatory response may be incidental. Most of the previous studies showed that the $sPLA_2$ in general do not exhibit fatty acid specificity at the *sn*-2 position of the phosphoglycerides (*20*, *21*), and therefore the composition of the released fatty acids should reflect the acyl group composition at the *sn*-2 position of plasma PC. The results presented here show that the group V enzyme, however, liberates less archidonate, and more linoleate than expected from the acyl group composition of lipoprotein PC. It is possible that this apparent specificity of the enzyme for the linoleate is the function of the PC composition in the lipoproteins, the effect of the apoproteins, or the topography of the lipoprotein structure, where certain molecular species of PC are more accessible to the enzyme. However, the studies with synthetic PCs showed that 16:0−18:2 PC is preferred over 16:0−20:4 PC by about 5−7-fold, when presented as equimolar mixture in the same liposome in the absence of apoproteins, suggesting a true preference of $sPLA_2$ for this PC species. Using synthetic PC liposomes, Chen and Dennis (*35*) have previously reported the fatty acid preference of the enzyme to be 18:2 > 16:0 > 20:4. The apparent bias against arachidonate release indicates that the pathological effects of the enzyme may be less likely due to the direct stimulation of eicosanoid synthesis, but more due to the generation of lyso PC, which is known to have several cytotoxic and chemotactic effects on aortic cells (*52*−*54*), as well as independently stimulate the prostaglandin synthesis (*55*). In addition, the FFA released by the enzyme may promote LDL uptake by the macrophages by altering the surface charge of the lipoprotein (*10*).

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