Lipoproteins are substrates for human secretory group IIA phospholipase A_2: preferential hydrolysis of acute phase HDL

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Abstract **Group IIA secretory phospholipase A_2 is an acute phase enzyme, co-expressed with serum amyloid A protein. Both are present in atherosclerotic lesions. We report that human normal and acute phase high density lipoproteins and low density lipoprotein are effective substrates for human group IIA phospholipase A_2. The enzyme hydrolyzed choline and ethanolamine glycerophospholipids at the *sn*-2 position resulting in an accumulation of the corresponding lysophospholipids, including the unhydrolyzed alkyl and alkenyl ether derivatives. The hydrolysis of acute phase high density lipoprotein was 2- to 3-fold more rapid and intensive than of normal high density lipoprotein. The hydrolysis of lipoproteins was noted at enzyme concentration as low as 0.05 μg/mg protein, which was within the range observed in the circulation in acute and chronic inflammatory diseases. The enzyme hydrolyzed the different molecular species of the residual glycerophospholipids in proportion to their mass, showing no preference for the release of arachidonic acid. Group IIA phospholipase A_2 preferentially attacked the hydroxy and hydroperoxy linoleates and possibly other oxygenated fatty acids, which were released from the glycerophospholipids at early times of incubation. There was no effect on the content or molecular species composition of the sphingomyelins or neutral lipids of the lipoproteins. In conclusion, human plasma lipoproteins are the first reported natural biological substrates for human group IIA phospholipase A_2. The enhanced hydrolysis of acute phase high density lipoproteins is probably due to its association with serum amyloid A protein, which enhances the activity of the enzyme and may promote its penetration to the lipid monolayer. As $sPLA_2$-induced hydrolysis of the lipoproteins leads to accumulation of lysophosphatidylcholine and potentially toxic oxygenated fatty acids, overexpression of this enzyme may be proatherogenic.—**Pruzanski, W., E. Stefanski, F. C. de Beer, M. C. de Beer, P. Vadas, A. Ravandi, and A. Kuksis. **Lipoproteins are substrates for human secretory group IIA phospholipase A_2: preferential hydrolysis of acute phase HDL.** ***J. Lipid. Res.*** 39: **2150–2160.**

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Human secretory non-pancreatic group IIA phospholipase A_2 ($sPLA_2$) is expressed as an acute phase protein and its concentration can increase hundreds-fold in the circulation and inflammatory fluids (1). $sPLA_2$ overactivity can persist for a long time in chronic inflammatory diseases such as rheumatoid arthritis (2) or systemic lupus erythematosus (3) and could contribute to altered lipoprotein levels in these diseases. Overexpression of human $sPLA_2$ in transgenic mice was found to be associated with marked reduction in high density lipoprotein levels (4). $sPLA_2$ expression has been identified in a variety of mammalian cells, including those that participate in atherogenesis, such as vascular smooth muscle cells (1, 5) and has also been detected in human atherosclerotic lesions (6–8). The possibility exists that $sPLA_2$ is concentrated in the vascular wall (6, 8) as it is bound to proteoglycans (7). Recent observation showing similarity of group V secretory PLA_2 to group IIA $sPLA_2$ (9) may necessitate reassessment of some of the above observations. In our studies we used human secretory PLA_2 proven to belong to group IIA.

High density lipoprotein (HDL) plays an important protective role in atherogenesis (10–12). It is conceivable that alteration of HDL by phospholipase A_2 activity could alter the function of this particle at inflammatory sites, such as developing atherosclerotic lesions. Hydrolysis of surface phospholipids of HDL is associated with redistribution of cholesteryl ester from the core to the surface of the particle resulting in increased net delivery of free cholesterol to cells by a surface transfer process (13). In the acute phase serum amyloid A protein (SAA) associates with HDL (APHDL) changing its apolipoprotein composition. Such HDL was shown to be proinflammatory and

Abbreviations: $sPLA_2$, secretory group IIA phospholipase A_2; SAA, serum amyloid A protein; LC, liquid chromatograph; MS, mass spectrometer; ESI, electrospray ionization.

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incapable of preventing LDL from oxidative modification in aortic wall cell culture (10). SAA can enhance the activity of $sPLA_2$ in hydrolyzing phospholipid vesicles (14). Thus, the interactive action of SAA and group IIA secretory PLA_2 on HDL functions merits consideration. Low density lipoprotein (LDL) is also a substrate for phospholipases A_2 (6, 15, 16). This could hold implications for LDL metabolism both with respect to clearance by scavenger receptors as well as enhancement of the susceptibility of LDL to lipid peroxidation (6).

Herein we report that human secretory group IIA $sPLA_2$ hydrolyses at the *sn*-2 position phospholipids from LDL, normal HDL (NHDL), and SAA-bearing acute phase HDL (APHDL) with APHDL being more susceptible to hydrolytic activity of $sPLA_2$. As lipoproteins are the first defined biological substrates for $sPLA_2$, the exact nature of the phospholipids hydrolyzed and free fatty acids liberated was analyzed. The effect of $sPLA_2$ on hydrolysis of lipoprotein phospholipid classes, phospholipid molecular species and neutral lipids was studied. Given the proinflammatory potential of many lipid molecules, the effect of $sPLA_2$ hydrolysis on the generation of lipid ester hydroperoxides and core aldehydes was also measured.

MATERIALS

Recombinant human group IIA secretory phospholipase A_2 ($sPLA_2$) was generously provided by Dr. J. Browning (Biogen Corporation, Cambridge, MA). Mixed R- and S-9- and 13-hydroperoxy and hydroxy linoleic acids along with the corresponding choline glycerophospholipids were prepared in the laboratory from oxidation of standards with methylene blue.

METHODS

Preparation of plasma HDL, APHDL, and LDL

Normal HDL (NHDL) was isolated by sequential ultracentrifugation from the plasma of healthy donors and acute phase HDL (APHDL) from patients experiencing an acute-phase response after cardiac surgery. APHDL and NHDL were exposed to identical stress during ultracentrifugation. Two types of preparations of HDL (17) were used in our studies: total HDL (plasma density [ρ]) 1.063–1.25 g/ml) and HDL_3 (ρ 1.13–1.18). As no differences in hydrolytic response of these preparations was noted, we labeled all preparations as normal HDL (NHDL). Protein concentrations were determined by the Lowry et al. method (18) and individual apolipoprotein content was established by pyridine extraction of Coomassie Blue-stained bands from SDS/PAGE as described (19). NHDL contained about 68% apolipoprotein A-I and 32% apolipoprotein A-II including other minor apolipoproteins. APHDL contained 56% apoA-I, 17% apoA-II, and 27% SAA. Low density lipoprotein (LDL, ρ 1.019–1.063 g/ml) was isolated from the plasma of normal blood donors by density gradient ultracentrifugation as described (17, 20). LDL isolation was accelerated by the use of 300,000 MW cut off Centricons for desalting and was completed in 48 h. LDL was used within 1–2 days of isolation. The concentration of lipoproteins in this study is expressed in terms of protein content.

Hydrolysis of lipoproteins with group IIA secretory phospholipase A_2 ($sPLA_2$)

Aliquots of NHDL, APHDL, and LDL were digested with $sPLA_2$. Digestion was carried out in a total volume of 1 ml Tris/HCl buffer, pH 7.5, containing $CaCl_2$ and 0.01% BSA. The reaction was started by adding 2 μl of recombinant human $sPLA_2$ stock solution with final $sPLA_2$ concentrations up to 1.25 μg $sPLA_2$ per 1 mg of NHDL, APHDL, or LDL. The reaction mixture was incubated at 37°C in a water bath for 0, 4, 8 and 24 hours. The total lipids were extracted with 5 ml of chloroform–methanol 2:1, (v/v). After adding 1 ml of 0.05 N KCl, the mixtures were centrifuged at 2000 *g* for 10 min and the organic phase was collected. The extracts were blown down under nitrogen and redissolved in chloroform–methanol 2:1 (v/v). In other experiments a fixed quantity of $sPLA_2$ of 2.5 μg/ml was added to various concentrations of lipoproteins (0.25–1.0 mg/ml), or $sPLA_2$ in concentrations of 0.1, 0.5, 1.0, and 2.5 μg/ml was added to fixed concentration of lipoproteins (1 mg/ml). The above concentrations of $sPLA_2$, when converted to units/ml, as determined by standard *E. coli* assay (21), corresponded to the range of 4000 U/ml to 100,000 U/ml (1 ng = 40 U), i.e., they were within the range observed during acute phase or in chronic inflammation in various diseases.[2] The incubation times were 1, 2, 4, 8, and 24 h. The experiments were repeated at least 3 times and done in triplicate. The reactions were stopped by adding the extracting solvents and completing a total lipid extraction. Total lipid profiles were determined by high temperature gas chromatography and calculated in relation to tridecanoin as internal standard.

Preparation of total lipid extracts

Total lipids of the NHDL, APHDL, and LDL preparations were obtained by chloroform–methanol 2:1 (v/v) extraction as described (22) without acidification in order to avoid plasmalogen decomposition. The chloroform phase was blown down to dryness under nitrogen and redissolved in 2 ml of chloroform–methanol 2:1, (v/v) and aliquots were taken for various analyses.

Isolation of free fatty acids

The total lipid extracts were resolved into the component lipid classes by thin layer chromatography in both neutral and polar solvent systems. The neutral lipids and free fatty acids (FFA) were separated using heptane–isopropyl ether–acetic acid 60:40:4 (v/v) which retained the polar phospholipids at the origin. The neutral lipid and FFA bands were detected by brief exposure to iodine vapor, and the neutral lipid fractions were recovered by extracting with chloroform the gel scrapings from appropriate areas of the TLC plate. The phospholipids were recovered from the origin of the plate with chloroform–methanol 2:1 (v/v) and were rechromatographed on another TLC plate using chloroform–methanol–ammonia–water 65:35:1:3 as previously described (23). The individual phospholipid classes were recovered by extracting with chloroform–methanol 2:1 (v/v). Aliquots of the various lipid extracts were reduced to dryness and redissolved in chloroform for LC/MS or were transmethylated for GLC with 6% sulfuric acid in methanol by heating at 80°C for 2 h. The methyl esters were recovered by repeat extraction with hexane after diluting the reaction mixture with distilled water. The solvents were blown down under nitrogen and the samples redissolved in hexane.

Analysis of free fatty acids

The fatty acids were analyzed on a polar capillary column (SP 2380, 15 m × 0.32 mm ID, Supelco, Mississauga, ON, Can-

[2] Maximal values of $sPLA_2$ protein (μg/ml) were: septic shock, 17.8 μg/ml; rheumatoid synovial fluid, 2.3 μg/ml; sera in juvenile arthritis, 1.97 μg/ml; sera in systemic lupus, 1.7 μg/ml; sera in adult rheumatoid arthritis, 0.075 μg/ml (W. Pruzanski, unpublished results).

ada) installed in a Hewlett-Packard (Palo Alto, CA) Model 5880 gas chromatograph equipped with a hydrogen flame ionization detector. Hydrogen was the carrier gas at 3 psi. Injections were made at 100°C and after 0.5 min the oven temperature was programmed at 20°C/min to either 130°C or 180°C and then to 240°C at 5°C/min (23). The fatty acid peaks were identified by comparing the relative retention times of the unknowns to those of standards run before and after analyzing the unknowns.

Determination of total lipid profiles

Aliquots of NHDL, APHDL, or LDL total lipid extracts (equivalent to 0.2 ml of plasma) were digested with phospholipase C (*Clostridium welchii*, Type I, Sigma Chemical Co.) as previously described (24). To the lipid was added 1 ml diethyl ether and 2 ml of a buffer solution (17.5 mm Tris buffer, pH 7.3) containing 8 mg $CaCl_2$ and 2 units of phospholipase C. The suspension was incubated with stirring for 2 h in a tightly closed screw-cap vial under nitrogen at 37°C. Reaction mixtures were extracted with chloroform–methanol 2:1 (v/v) containing 100 μg of tridecanoylglycerol as internal standard. The lower phase was passed through a Pasteur pipet containing anhydrous Na_2SO_4 and the solvent was evaporated to dryness under a stream of nitrogen. The lipids were then reacted for 30 min at 20°C with [N,0)-bis(trimethylsilyl] trifluoro-acetamide containing 1% trimethylchlorosilane (SYLON BFT) plus one part of dry pyridine. This reaction mixture was evaporated to dryness, diluted with hexane and used for GLC analysis by carbon number with a non-polar capillary column (quartz capillary, 8 m × 0.32 mm ID coated with permanently bonded SE-54 liquid phase, Hewlett-Packard) as previously described (25). The carrier gas was hydrogen and the column was temperature programmed as given in the legends to figures. The peaks were identified by comparing the relative retention times of the unknowns to those of standards run before and after analyzing the unknowns. The peak areas generated by the hydrogen flame ionization detector were quantified in relation to the peak area of the internal standard.

LC/ESI/MS analysis of glycerophospholipids and sphingomyelins

Normal phase HPLC separations of the total lipid extracts were performed on Spherisorb 3 micron columns (100 mm × 4.6 mm ID, Alltech Associates, Deerfield, IL) installed into a Hewlett-Packard Model 1060 Liquid Chromatograph (LC) connected to Hewlett-Packard Model 5988B quadruple mass spectrometer (MS) equipped with a nebulizer-assisted electrospray ionization (ESI) interface (HP 59987A) (26). Positive ESI spectra were taken in the mass range 350–1100. The capillary exit voltage was set at 150 volts, with the electron multiplier at 1795 volts. By injecting each sample two times, both positive and negative ion spectra were obtained. Selected ion chromatograms were retrieved from the total ion spectra by computer. The masses given in the tables and figures are actual masses of the $[M+1]^+$ (positive ion mode) or $[M-1]^-$ (negative ion mode) ions, rounded to the nearest integer. The nominal masses are one mass unit lower or higher, respectively.

The molecular species of the various phospholipids were identified on the basis of the molecular mass provided by ESI/MS, the knowledge of the fatty acid composition of the phospholipid classes and the relative HPLC retention time (longer chain species migrated ahead of the shorter chain species) of the glycerophospholipids and sphingomyelins.

Analysis of oxygenated fatty acids

The oxygenated acids among the fatty acids released by $sPLA_2$ were identified and quantified by LC/ESI/MS during total lipid profiling (see above). The oxygenated fatty acids were eluted as separate peaks following the elution of the non-oxygenated saturated and unsaturated fatty acids. The oxofatty acids were identified on the basis of the molecular weight and the HPLC retention time of appropriate standards. The fatty acids were quantified by single ion chromatography in the negative ion mode. The single ion chromatograms were retrieved from the total negative ion spectra by computer.

RESULTS

Effect of group IIA secretory phospholipase A_2 on lipoprotein phospholipid classes

The phospholipolysis was followed by changes in the relative proportions of phosphatidylcholine (PC) and lysophosphatidylcholine (LPC). As the PC content of the lipoproteins progressively fell, LPC content rose, while sphingomyelin concentration remained essentially constant. **Figure 1** shows typical pattern of the LC/ESI/MS total positive ion current profile of the NHDL, APHDL, and LDL phospholipids at 0 and 4 h of hydrolysis with $sPLA_2$. Approximately 30% of the LDL and NHDL PC was hydrolyzed in 4 h, whereas up to 80% of APHDL PC was hydrolyzed in the same time period. **Table 1** and **Fig. 2** summarize the quantitative results of 0–24 hrs hydrolysis as estimated by positive (PC, lyso PC) and negative (PE, lyso PE) LC/ESI/MS analysis of the phospholipid classes. Both NHDL and APHDL were nearly completely hydrolyzed in 8 h, while the LDL was only about 50% hydrolyzed at the same time. However, there was about three times more PC per gram of protein in LDL than in NHDL. Concentrations of the substrate of 0.25 to 1.0 mg/ml were equally well hydrolyzed by $sPLA_2$ 1.25 μg/1 mg protein of lipoproteins. Substantial hydrolysis was observed within 1 hour of incubation; 20%, 35%, and 17% of PC being converted into lyso PC in NHDL, APHDL, and LDL, respectively. Testing the lowest concentrations of $sPLA_2$ capable of inducing hydrolysis, it was found that as little as 0.05 μg/1 mg protein started to hydrolyze PC of APHDL and NHDL in less than 1 h of incubation (<10%). Hydrolysis of LDL was slightly slower, being significant in 2 h. The concentrations of $sPLA_2$ that hydrolyzed lipoproteins were well within the range observed in the circulation in various acute and chronic inflammatory diseases.[2] Lyso PC content increased whereas PC decreased, although not proportionately. During hydrolysis of NHDL, lyso PC increased in 24 h from 0.04 ± 0.02 (SD) μmol to 0.99 ± 0.2 μmol; in APHDL from 0.02 ± 0.01 μmol to 0.73 ± 0.5 μmol; and in LDL from 0.28 ± 0.1 μmol to 3.78 ± 0.2 μmol. The more rapid hydrolysis of the APHDL PC than that of NHDL by $sPLA_2$ was reproduced in nine experiments. Faster hydrolysis of APHDL was observed regardless of which concentration of $sPLA_2$ was used. Table 1, representative of four experiments, also indicates that ethanolamine-containing glycerophospholipids were hydrolyzed, and LC/ESI/MS in negative ion mode showed that both alkenyl and acyl lyso PE were formed. The content of phosphatidylinositol (PI) decreased in 24 h to about 50% of the initial val-

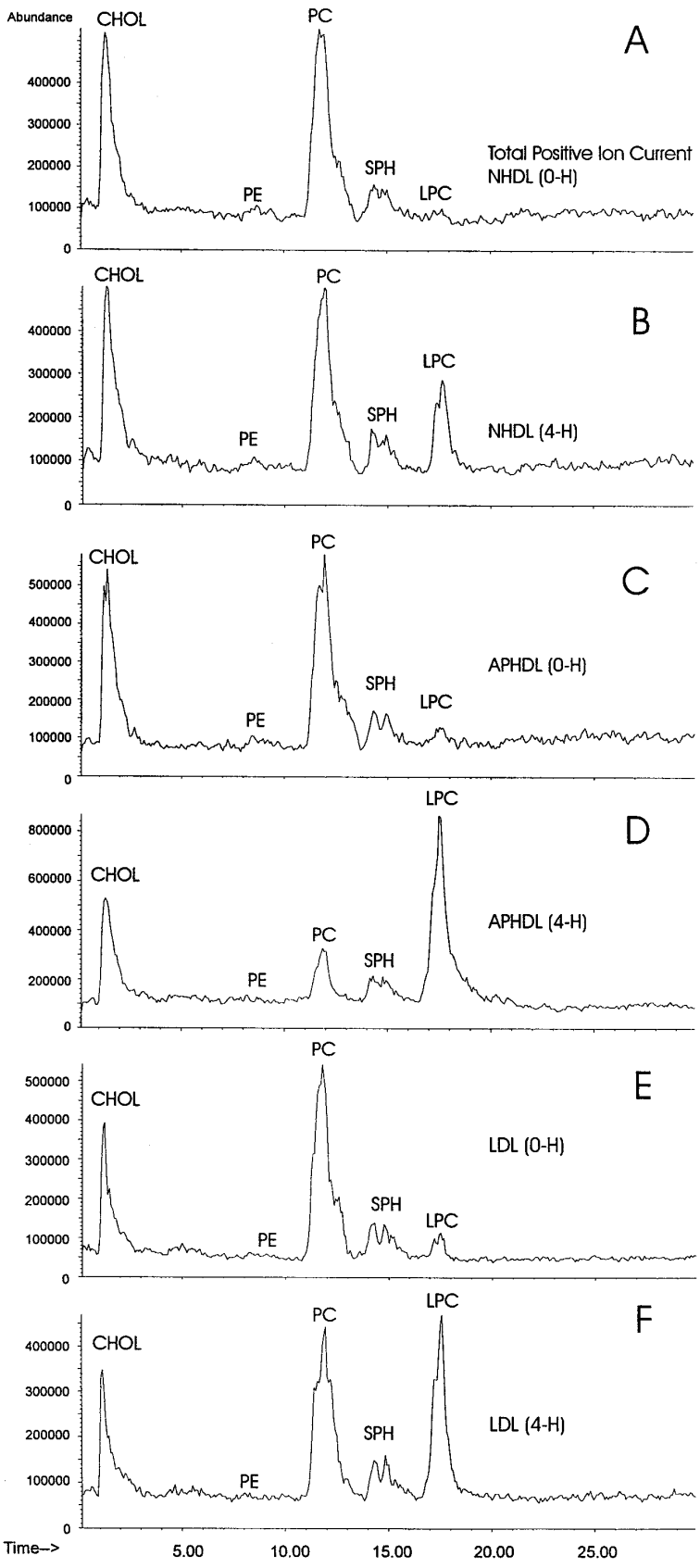

Fig. 1. Total positive ion current profiles of total lipid extracts of NHDL, APHDL, and LDL at selected stages of hydrolysis with secretory group IIA phospholipase A_2 obtained by normal phase liquid chromatography/mass spectrometry with electrospray ionization. A: Normal high density lipoprotein at 0 h (NHDL, O–H); B: at 4 h (NHDL, 4–H); C: acute phase HDL at 0 h (APHDL, O–H; D: at 4 h (APHDL, 4–H); E: low density lipoprotein at 0 h (LDL, O–H); F: at 4 h. (LDL, 4–H). Chol, free cholesterol along with other neutral lipids (cholesteryl esters and triacylglycerols); PE, phosphatidylethanolamine including its alkyl and alkenyl ether homologues and peroxidation products; PC, phosphatidylcholine including its alkyl ether homologues and peroxidation products; SPH, sphingomyelin; LPC, lysophosphatidylcholine including alkyl ether homologues. Column: Spherisorb 3μ (100 mm × 4.6 mm ID, Alltech Associates. Deerfield, IL); solvent: linear gradient of 100% solvent A to 100% solvent B in 14 min, then solvent B for 10 min; solvent A: chloroform–methanol–30% ammonium hydroxide 80:19.5:0.5 (by vol); solvent B: chloroform–methanol–water–30% ammonium hydroxide 60:34:5:0.5 (by vol); instrumentation: Hewlett-Packard Model 1060 Liquid Chromatograph interfaced with Hewlett-Packard Model 5988B quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray interface. Positive ion spectra were taken in the mass range 300–1100. The capillary exit voltage was set at 120 volts, with the electron multiplier at 1795 volts.

TABLE 1. Quantitative analysis of phospholipid classes during hydrolysis of lipoproteins by secretory group IIA phospholipase A_2

	NHDL				APHDL				LDL			
Time	PC	LPC	PE	LPE	PC	LPC	PE	LPE	PC	LPC	PE	LPE
h	*μmol/2 mg protein*				*μmol/2 mg protein*				*μmol/2 mg protein*			
0	1.05	0.004	0.05	0.00	1.06	0.002	0.05	0.00	3.29	0.004	0.03	0.00
4	0.78	0.26	0.02	0.03	0.20	0.45	0.02	0.03	2.28	0.287	0.02	0.02
8	0.38	0.67	0.01	0.03	0.07	0.49	0.01	0.03	1.82	0.735	0.01	0.02
24	0.06	0.99	0.00	0.04	0.07	0.50	0.00	0.03	0.45	1.083	0.00	0.03

Results are representative of one of four experiments; variations less than 5%. Substrate concentration, 1 mg/ml; $sPLA_2$ concentration, 1.25 μg/l mg protein of the substrate. NHDL, normal high density lipoprotein; APHDL, SAA-bearing acute phase high density lipoprotein; LDL, low density lipoprotein; PC, phosphatidylcholine; LPC, lyso-PC including alkyl and alkenyl species; PE, phosphatidylethanolamine; LPE, lyso-PE including alkyl and alkenyl species. Molecular weight of PC was calculated as distearoylglycerophosphocholine (MW 790); both PC and LPC were estimated by LC/ESI/MS as molar ratios to sphingomyelin. All values were calculated as μmol/2 mg protein.

ues of 0.02 μmol/2 mg protein which were similar in all three lipoproteins. There was no change in the content of sphingomyelin.

Effect of group IIA secretory phospholipase A_2 on phospholipid molecular species

The relative rates of hydrolysis of the PC species in the LDL, NHDL and APHDL PC were determined at each time of incubation. **Table 2** shows that the major PC species were hydrolyzed approximately in proportion to their molar concentration in the mixture. There was no preferential attack on the arachidonic acid containing PC. The major arachidonoyl PC species (16:0–20:4n–6) retained its relative proportion in relation to other species throughout the incubation period. **Figure 3** shows the relative proportions of the major oligoenoic diacyl species of PC along with the minor alkenylacyl species of PC in APHDL at 0 and 4 h after digestion with $sPLA_2$. In contrast to the random hydrolysis of the diacyl species, the alkenylacyl species tended to accumulate. Similar observations were made while testing LDL and NHDL. LC/ESI/MS analyses of the molecular species of ethanolamine glycerophospholipids of NHDL and APHDL indicated that representative proportions of the diacyl species were retained during incubation and yielded the corresponding lyso compounds, but that the alkenylacyl species again tended to accumulate in the digestion residue (data not shown).

Comparing the fatty acids release after 4 and 8 h of hydrolysis, it seems that the attack of $sPLA_2$ on the PC and PE of LDL, NHDL, and APHDL is largely random, however arachidonic and linoleic were predominant acids released (**Table 3**).

Effect of group IIA phospholipase A_2 on the neutral lipids

The neutral lipid classes of LDL, NHDL, and APHDL were not affected by incubation with $sPLA_2$ over a 24-h period. The concentrations of free cholesterol, cholesteryl

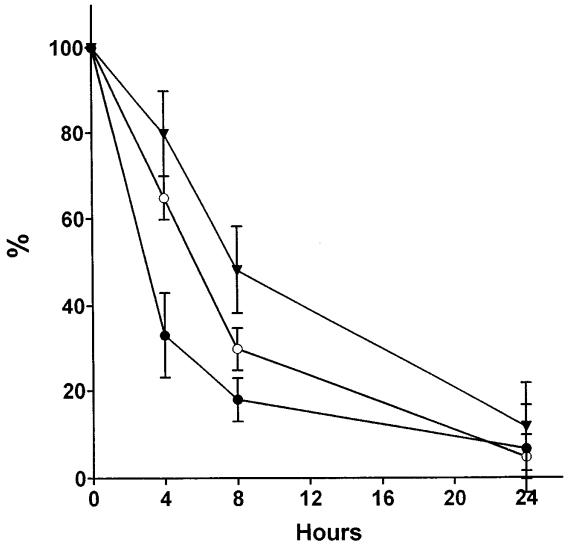

Fig. 2. Content of phosphatidylcholine in lipoproteins hydrolyzed by secretory group IIA phospholipase A_2. Closed circles, acute phase HDL; open circles, normal HDL; triangles, LDL. Concentration of lipoproteins, 1 mg/ml; concentration of $sPLA_2$, 1 μg/ml. Phosphatidylcholine estimated as percentage of non-hydrolyzed lipoproteins. At 4 h the difference between acute phase HDL and normal HDL, $P < 0.001$; at 8 h, $P < 0.01$ (n = 4). Bars = mean ± SD.

TABLE 2. Residual molecular species of phosphatidylcholine after hydrolysis with secretory group IIA phospholipase A_2

			NHDL		APHDL		LDL	
CN:DBN[a]	*m/z*	Molecular Species[b]	0 h	4 h	0 h	4 h	0 h	4 h
			mol %					
32:1	732	16:0–16:1	0.7	0.9	0.7	0.03	0.5	0.5
34:2	758	16:0–18:2	30.1	32.0	31.4	26.2	30.6	22.9
34:1	760	16:0–18:1	16.2	14.0	19.3	24.3	14.8	19.5
36:5	780	16:0–20:5	2.7	2.2	1.1	1.0	4.0	0.9
36:4	782	16:0–20:4	11.0	11.1	11.6	12.4	11.3	11.2
36:3	784	18:1–18:2 & 16:0–20:3	10.5	7.1	10.0	5.3	10.4	11.5
36:2	786	18:1–18:1 & 18:0–18:2	16.7	18.1	13.3	20.2	14.6	17.0
38:4	806	16:0–22:4	4.0	5.5	3.3	1.5	4.1	3.4
38:4	810	18:0–20:4	5.8	6.6	7.0	7.8	8.2	11.1
40:6	834	18:0–22:6	1.6	1.5	2.1	1.2	1.4	2.0
Total			100.3	99.0	99.8	99.9	99.9	99.7
PC/SPH, mol/mol			12.5	9.4	14.4	2.8	3.6	2.5

For peaks greater than 5%, the error may reach 3%; for peaks less than 5%, the error is up to 10% of the value. $sPLA_2$, 1.25 μg per 1 mg of protein of the substrate.

[a] CN:DBN, carbon number:double bond number.

[b] 16:0, palmitic; 16:1, palmitoloeic; 18:0, stearic; 18:1, oleic; 18:2, linoleic; 20:4, arachidonic; 20:5, eicosapentaenoic; 22.6, docosahexaenoic.

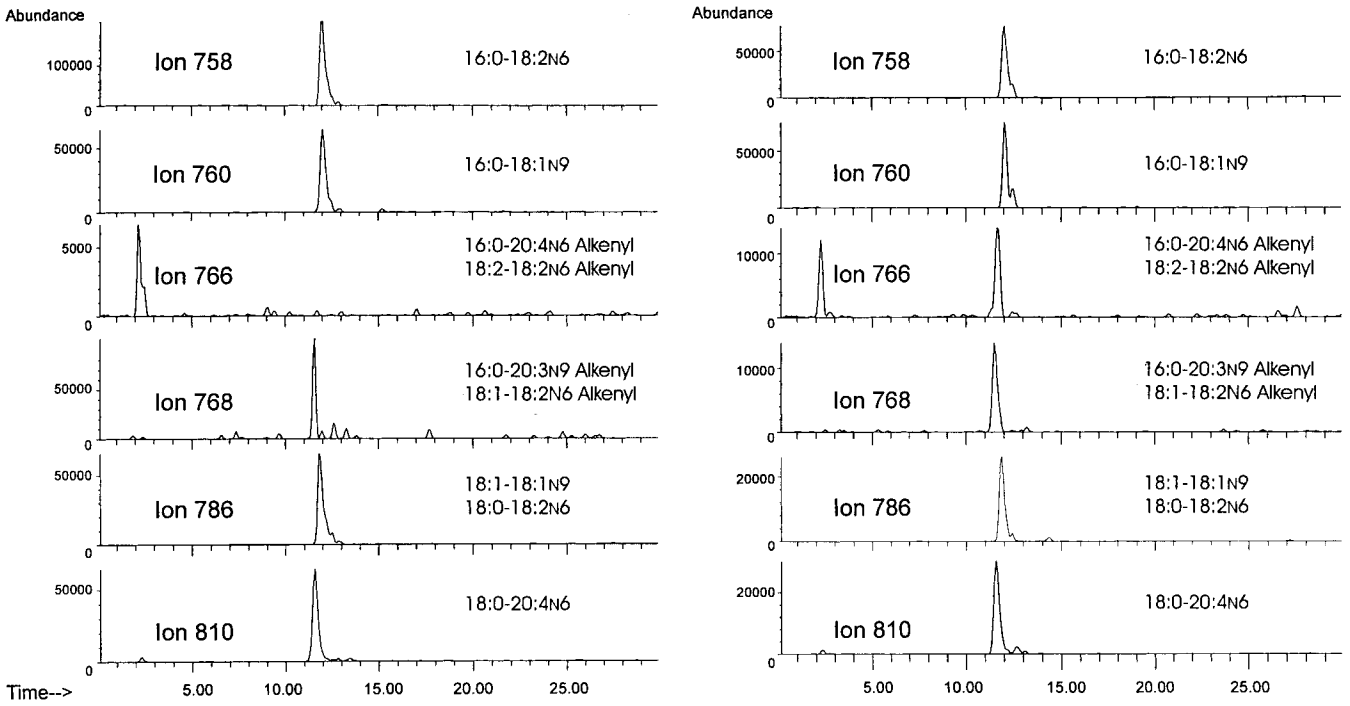

Fig. 3. Single ion chromatograms of molecular species of residual phosphatidylcholines of acute phase HDL (APHDL) hydrolyzed with secretory group IIA phospholipase A_2 at zero (left side panel) and 4 (right side panel) h of hydrolysis. Peak identification is as given in the figure. LC/ESI/MS conditions are as given in Fig. 1. Single ion chromatograms were retrieved from the total ion spectra by computer. The masses given in the figure are the actual masses of the $[M + H]^+$ ions. The nominal masses are one mass unit lower. Fatty acid abbreviations are as described in text. Alkenyl designates the vinyl ether species of phosphatidylcholine. Note absence of detectable amounts of *m/z* 766 (alkenyl species) from the PC species range in the original PC profile (left side panel).

esters, and triacylglycerols remained unchanged during the time course of the experiment. **Figure 4** shows the neutral lipid profiles recorded for LDL, NHDL, and APHDL after 4 h of hydrolysis. There was no detectable change in the peaks of free cholesterol, cholesteryl esters, and triacylglycerols, while those of the diacylglycerols, representing phospholipids, decreased steadily until only the ceramide profile remained by the end of 24 h incubation period (results not shown). Thus, $sPLA_2$ differs from hepatic and lipoprotein lipases, both of which also hydrolyze triacylglycerols.

Effect of group IIA phospholipase A_2 on lipid ester hydroperoxides and hydroxides

An examination of the total lipid profiles recorded by normal phase LC/ESI/MS for the total lipid extracts of the incubation mixtures revealed the presence of hydroperoxy and hydroxy derivatives of unsaturated fatty acids, which were released by the $sPLA_2$ from the glycerophospholipids present in the lipoproteins. **Figure 5** shows the total negative ion current profile along with selected single ion chromatograms for the mono- and dihydroperoxides and hydroxides of 18:2 and 20:4 acids as obtained for APHDL after 2 h of incubation with $sPLA_2$. The oxygenated fatty acids were eluted as separate peaks following the corresponding non-oxygenated fatty acids. There were overlaps among homologous acids and their oxides. The monohydroxides of the 18:2 (*m/z* 295) and 20:4 (*m/z* 319) predominated but the monohydroperoxide (*m/z* 311) and dihydroperoxide (*m/z* 343) of 18:2 as well as the monohydroperoxides of 20:4 (*m/z* 335) and 22:6 (*m/z* 359) could also be seen. The dihydroxyperoxides of the 20:4 and the 22:6 acids were de-

TABLE 3. Composition of free fatty acids released from *sn*-2 position of phosphatidylcholine and phosphatidylethanolamine by secretory group IIA phospholipase A_2

	NHDL			APHDL			LDL		
FAME	0 h	4 h	8 h	0 h	4 h	8 h	0 h	4 h	8 h
				mol %					
18:1n–9 (oleic)	14.5	12.2	23.1	19.9	26.8	19.9	15.6	14.1	14.0
18:2n–6 (linoleic)	2.3	7.0	23.8	3.5	26.9	25.8	8.6	36.7	31.7
20:4n–6 (arachidonic)	0.5	2.1	10.0	0.2	4.6	11.8	0.6	14.9	12.2
22:6n–3 (docosahexaenoic)	0.0	0.8	2.4	0.3	0.4	3.7	0.0	2.8	2.2
Others	82.7	78.7	40.6	66.1	41.7	38.8	75.2	31.5	42.3

Substrate concentration, 1 mg/ ml; $sPLA_2$ concentration, 1.25 μg/1 mg protein of the substrate.

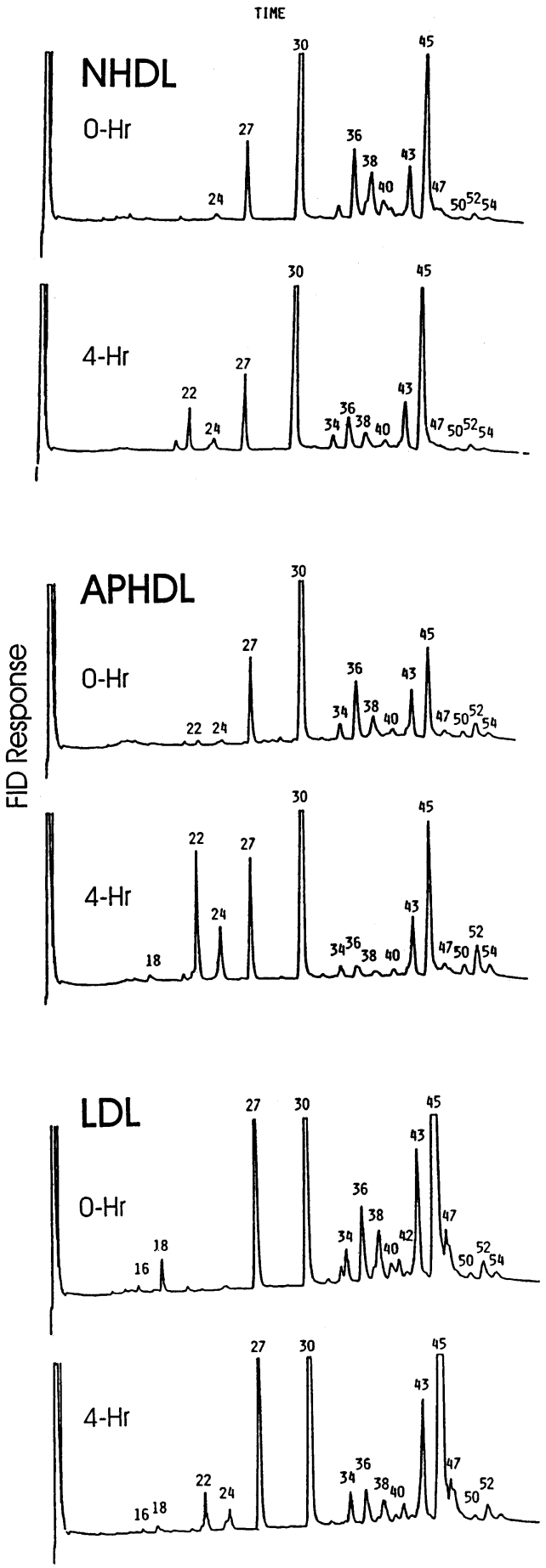

Fig. 4. Total lipid profiles of NHDL, APHDL and LDL at selected times of incubation with secretory group IIA phospholipase A_2; estimation by gas–liquid chromatography after dephosphorylation of the lipid extracts with phospholipase C and trimethylsilylation. Time 0 and 4 h; peak identification: 16 and 18, TMS esters of C_{16} and C_{18} fatty acids; 27, TMS ether of cholesterol; 30, tridecanoylglycerol (internal standard); 34, TMS ether of C_{34} ceramide (palmitoyl sphingosine); 36–40, TMS ethers of diacylglycerols with C_{34}–C_{38} acyl carbons; 43–47, cholesteryl esters with C_{16}–C_{20} fatty acids; 50–54, triacylglycerols with C_{50}–C_{54} acyl carbons. Instrument: Hewlett-Packard (Palo Alto, CA) Model 5880 gas chomatograph equipped with a flexible quartz capillary column (8 m × 0.32 mm id) coated with a nonpolar phenylmethylsilicone (DB-50, Hewlett-Packard) liquid phase; carrier gas, H_2; detector, flame ionization; temperature: programmed from 40° to 220°C ballistically; 220° to 320°C at 10°C/min; 320° to 360°C at 2°C/min.

tected only occasionally and in very small amounts. The oxygenated fatty acids were present in much greater proportions in the APHDL compared to LDL, with the NHDL values being found between these two extremes. **Table 4** compares the oxygenated fatty acid content in APHDL, NHDL, and LDL after 2 h of incubation with $sPLA_2$. There was little increase in the proportion of the oxygenated fatty acids with progressing exposure to $sPLA_2$. This suggests that all the oxygenated fatty acids were released from the parent glycerophospholipids at the beginning of the digestion period probably due to a preferential enzymatic attack. There may have been increases in the release of the oxygenated fatty acids with increasing time of incubation, which would suggest increased peroxidation during incubation. The possibility of increased peroxidation could have resulted from an exhaustion of the antioxidants present in the lipoproteins. Due to lower sensitivity of the electrospray ionization response in the positive ion mode, it was difficult to demonstrate the presence of the hydroperoxy fatty acid-containing species among the intact phosphatidylcholine molecules. Thus, an examination of the LC/ESI/MS profiles of the various incubation mixtures of LDL, NHDL, and APHDL failed to give significant ions for the mono (*m/z* 788) and dihydroperoxy (*m/z* 820) and monohydroxy (*m/z* 772) and the mixed function derivatives of the 1-palmitoyl-2-linoleoyl-*sn*-3-glycerophosphocholine. The presence of the oxofatty acids could be seen, however, among the molecular species of the phosphatidylethanolamine, which was analyzed in the negative ion mode (results not shown). Likewise, the monohydroperoxy and monohydroxy derivatives of 18:2 and 20:4 could be demonstrated (in the negative ion mode) to be released along with the steroid ring of cholesterol (*m/z* 369, positive ion mode) and 7-ketocholesterol (*m/z* 383, positive ion mode) during ESI fragmentation of the steryl ester fraction, which is eluted ahead of the other lipids during normal phase HPLC (data not shown).

DISCUSSION

The present study investigates the activity of human secretory group IIA phospholipase A_2 ($sPLA_2$) on human

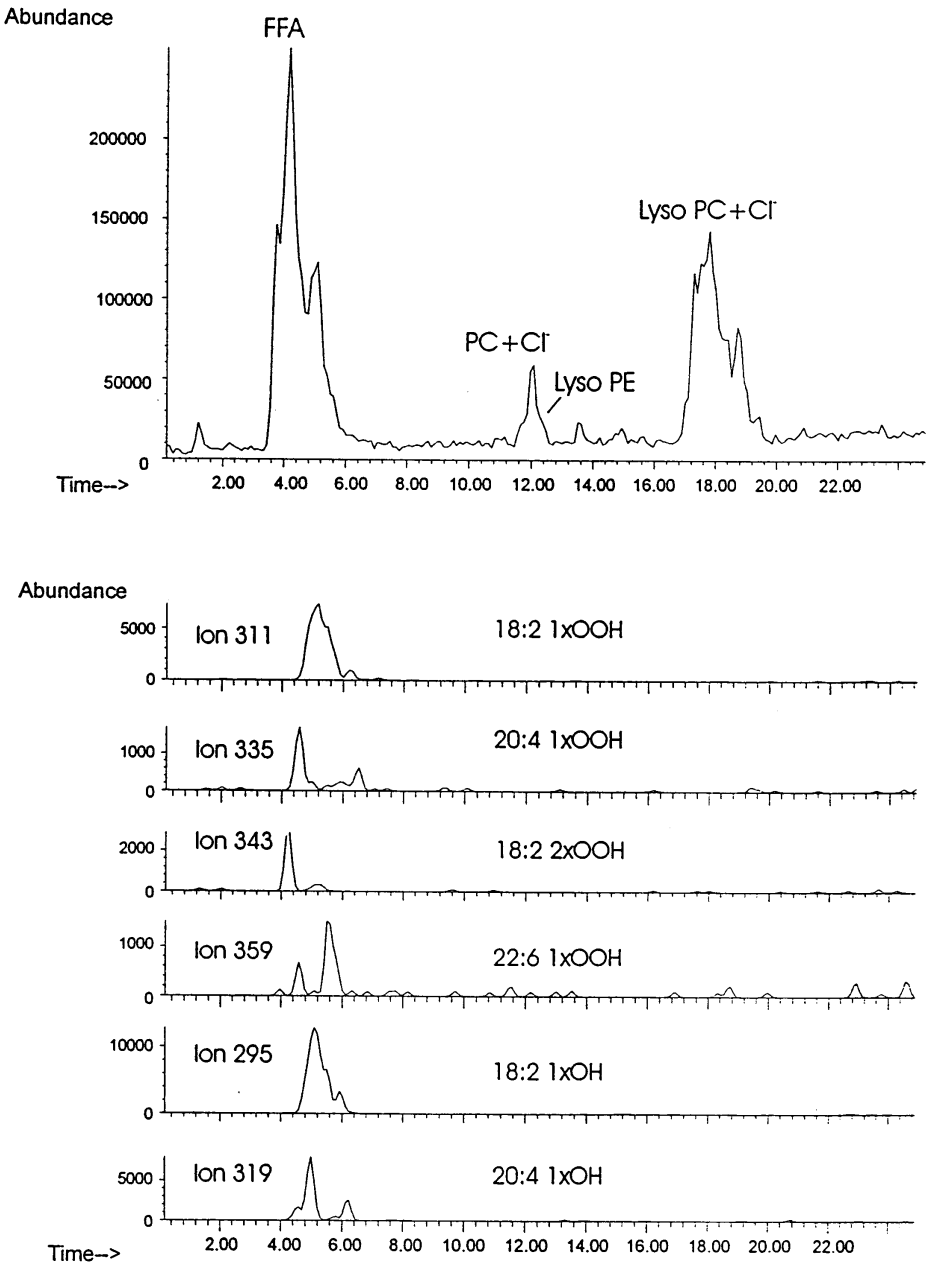

Fig. 5. Selected ion chromatograms of oxo-fatty acids released from APHDL during hydrolysis with secretory group IIA phospholipase A_2 obtained by normal phase liquid chromatography/mass spectrometry with electrospray ionization; 2-h hydrolysis. Upper panel, total negative ion current profile; lower panel, single negative ion chromatograms. Peak identification is given in the figures. Mono- and dihydroperoxy and hydroxy derivatives of the fatty acids are indicated by 1× or 2× the oxygen function. The peak doublets and multiplets are due to separation of positional and geometric isomers of the oxygenated fatty acids. Operating conditions as given in Fig. 1.

plasma lipoproteins in an autologous system. The concentration of $sPLA_2$ and the lipoproteins were selected to generally correspond to those commonly observed in acute phase and chronic inflammatory conditions.[2] Exposure of NHDL, APHDL, or LDL to $sPLA_2$ led to a rapid conversion of PC to lyso PC and PE to lyso PE. $sPLA_2$ did not attack triacylglycerols and in this respect differed from hepatic and lipoprotein lipases, which attack both glycerophospholipids and triacylglycerols. The $sPLA_2$ also did not affect the sphingomyelins present in the NHDL, APHDL, and LDL. The spingomyelins of LDL have been previously shown to be resistant to snake venom PLA_2 (27).

Our study showed that APHDL is hydrolyzed by $sPLA_2$ faster and more extensively than NHDL. Furthermore, addition of exogenous SAA to NHDL renders it more susceptible to hydrolysis (data not shown). Several reasons can be put forward to explain this fact. APHDL contains a large amount of SAA, which has been shown to enhance $sPLA_2$ activity (14), possibly by enhancing access to the *sn*-2 position of phospholipids. SAA association with NHDL

TABLE 4. Release of peroxidized fatty acids from lipoproteins by secretory group IIA phospholipase A_2

Substrate[a]	18:2	18:2 (OOH)	18:2 $(OOH)_2$	18:2 (OH)	20:4[b]	20:4 (OOH)	20:4 (OH)
NHDL	98.23	0.39	0.68	0.70	99.95	0	0.05
APHDL	80.04	2.60	1.35	6.75	89.40	0.97	8.90
LDL	99.33	0.28	0.38	trace	97.9	2.10	0.7

[a]Substrate concentration, 0.75 mg/ml; $sPLA_2$ concentration, 1.25 μg/1 mg of protein of the substrate; incubation time, 2 h. No further release was observed at later times of incubation.

[b]20:4 (OH) (*m/z* 319) corresponds to isomeric hydroxy-arachidonic acids.

changes NHDL, making the particle denser and larger (17) and increasing its cellular affinity (28). This acute phase HDL has been shown to be proinflammatory, most probably due to depletion of paraoxonase (10). As both $sPLA_2$ and SAA can be induced by the same group of cytokines and as they are co-overexpressed in acute phase and chronic inflammatory conditions and are present in the circulation (1, 29) and in atherosclerotic plaques (6–8, 30, 31), it is feasible that SAA serves as an enhancer/cofactor for $sPLA_2$ in altering lipoproteins. This would be of special importance in chronic inflammatory conditions in which prolonged overexpression of SAA and $sPLA_2$ takes place (31, 32) and in which an accelerated atherosclerotic process has been described (33).

Of interest is the fact that whereas $sPLA_2$ is active mainly against phosphatidylethanolamine (PE) in mammalian cell membranes (1), both PC and PE are attacked in lipoproteins. The activity of $sPLA_2$ was clearly non-specific with respect to the molecular species of diacyl GPC and the type of fatty acid located in the *sn*-2 position. There was no evidence of a preferential attack on the arachidonoyl species. In fact, there may have been an increase in the proportion of the arachidonoyl species of the plasmalogens, which appeared to be more resistant to hydrolysis by $sPLA_2$ than the diacyl species of GPC or GPE. A non-specific release of the fatty acids from the sn-2 position of the GPL by $sPLA_2$ had been previously observed using liposomal substrates (1). The enzyme also released the oxygenated fatty acids from both choline and ethanolamine GPL, which were identified as the hydroperoxides and hydroxides mainly of linoleic acid. It was not established whether the oxygenated fatty acids were racemic (non-enzymatic oxidation) or enantiomeric (enzymic oxidation).

The release was much more extensive from the APHDL particle, which is considerably richer in the oxygenated fatty acids than NHDL or LDL. This is in keeping with the observation that as opposed to NHDL, APHDL has proinflammatory properties with decrease in paraoxonase and PAF acetyl hydrolase (10).

Until now it was accepted that group IIA secretory PLA_2 acts on the outer surface of cells and is responsible for mostly extracellular sustained release of arachidonic acid (9, 34). It became obvious, however, that $sPLA_2$ cannot attack intact cellular membrane (1, 9, 35). Although experimentally induced injury of various cells and tissues by human $sPLA_2$ has been shown (36–38), until recently no extracellular physiological human substrates, that would be altered by interaction with human $sPLA_2$ were identified. The fact that lipoproteins such as NHDL, acute phase HDL, and LDL were hydrolyzed by $sPLA_2$ may provide insight into new biological and pathological roles which this enzyme could play in atherogenesis.

Exposure of NHDL, APHDL, or LDL to $sPLA_2$ leads to a rapid liberation of oxygenated fatty acids from the glycerophospholipids. Considering the fact that lyso PC causes membrane damage (39–41) and that oxygenated fatty acids are toxic (42, 43), $sPLA_2$-induced hydrolysis may initiate or enhance the inflammatory reaction in the atherosclerotic lesions. Altered HDL is probably less able to exert its protective action against detrimental activity of LDL (11, 44, 45) and altered LDL may become more prone to oxidation, promoting foam cell formation (45–48).

Recently the first NHDL receptor SR-BI has been cloned and studied in transfected cells (49, 50). This receptor mediates the selective transfer of lipids from NHDL into cells and is expressed most strongly in those tissues (liver and sterogenic tissues) that exhibit selective NHDL lipid uptake in vivo. SR-BI has a unique and broad specificity, being able to bind, in addition to NHDL, also acetylated LDL, native LDL, and autonomic phospholipids. How NHDL hydrolyzed by $sPLA_2$ would interact with the SR-BI receptor and influence lipid flow would be of obvious importance, particularly as this would likely be the type of HDL that exists in the atherosclerotic lesions.

LDL treated with non-human PLA_2s showed increase in lyso PC and loss of PC (15), decreased diacylphospholipid, and increased monoacylphospholipid content (14). Oxidative modification of LDL was accompanied not only by conversion of PC into lyso PC but also by liberation of oxidized fatty acids (51). It is feasible that these changes, especially those induced by autologous group IIA secretory PLA_2, could relate to promoting foam cell formation.

$sPLA_2$ has been detected in smooth muscle cells in the arterial wall of both normal arteries and in atherosclerotic lesions (1, 5, 6, 8, 30). In the latter, foam cells and fibroblast-like cells stained positively for $sPLA_2$ (6). $sPLA_2$ was also found in the extracellular lipid core of atheromatous plaques (6). $sPLA_2$ isolated from the vessel wall, hydrolyzed LDL phospholipids (6). It was suggested that in the atheromatous plaques in which $sPLA_2$ colocalizes with LDL, the former can modify the latter, enhancing atherosclerosis (6).

With the identification of lipoproteins as substrates for human group IIA $sPLA_2$, the implications that this holds for altered lipoprotein metabolism during systemic inflammation are important and need further study. Additionally, the role of group IIA secretory $sPLA_2$ in the inflammatory milieu of the developing atherosclerotic lesions must be further elucidated.

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