Serum amyloid A protein enhances the activity of secretory non-pancreatic phospholipase A₂

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The acute-phase proteins serum amyloid A protein (SAA) and secretory phospholipase A₂ (sPLA₂) are simultaneously expressed during inflammatory conditions. SAA associates with high-density lipoprotein (HDL) altering its physicochemical composition. We found that purified acute-phase SAA, but not the constitutive form, markedly enhances the lipolytic activity of sPLA₂ in a dose-related manner with phosphatidylcholine/lysophosphatidylcholine or phosphatidylethanolamine/lysophosphatidylethanolamine liposomal substrates. Normal HDL

was found to reduce activity of sPLA₂ in a dose-dependent manner, but when acute-phase HDL containing 27% SAA was tested, it enhanced sPLA₂ activity. Immunopurified monospecific antibodies against SAA completely abolished the enhancing activity of SAA and acute-phase HDL. Given the central role of HDL in lipoprotein metabolism, the interaction between HDL, SAA and sPLA₂ may account for changes detected in lipoprotein metabolism during the acute phase.

INTRODUCTION

The serum amyloid A proteins (SAAs) comprise a family of molecules that are apolipoproteins of high-density lipoprotein (HDL) [1–4]. They can be divided into two groups. The first contains the well-characterized acute-phase SAAs which increase dramatically during inflammatory events [1–3]. They displace apolipoprotein AI with resultant remodelling of the HDL particle yielding larger particles with a higher hydrated density, and even becoming the major apolipoprotein component of acute-phase HDL [1–4]. The second group comprises the recently discovered mouse SAA₅ [5] and human SAA₄ [6] group which are constitutively expressed as minor apolipoproteins associated with HDL during homeostasis. They are structurally and probably functionally distinct from the acute-phase SAA group. The effect of acute-phase SAA on HDL is still not known, but it seems likely that it equips the HDL particle for an altered role to promote host defence.

In defining the function of acute-phase SAA, one needs to recognize that, as SAA increases in the circulation, there is concomitant cytokine-driven synthesis and extracellular release of non-pancreatic (group-II) secretory phospholipase A₂ (sPLA₂) [7,8]. Both SAA and sPLA₂ are rapidly induced during inflammatory events [7–10] with mRNA for both being detectable at 2 h and the resultant proteins similarly produced with respect to time [5,11]. Whereas acute-phase SAAs are, in practice, only produced in the liver [5,6], sPLA₂ is produced by a variety of mesenchymal cells that would be present at sites of inflammation [7,8]. The concentration of sPLA₂ can increase hundreds of fold in inflammatory fluids [7,8,12] and in the circulation [13,14].

The influence of phospholipase activity on HDL is important in the understanding of the function of this particle [15,16]. In the healthy state, the effect of phospholipase activity on normal HDL has been shown with respect to hepatic lipase [15–17]. Hydrolysis of surface phospholipid molecules is associated with redistribution of cholesterol from the core to the surface of the HDL particle and increased polar group segmental motion of

surface phospholipids [15]. The result is a shift in the equilibrium of free cholesterol between HDL and the plasma membrane with an increased net delivery of free cholesterol to the cell by a surface transfer process [15]. Given that the phospholipase activity of hepatic lipase during homeostasis would be fixed on the surface of hepatocytes, the net result has been postulated to be cholesterol delivery to the liver for excretion, an important part of reverse cholesterol transport [15–17].

During the acute phase the situation is dramatically changed. SAA now dominates on the HDL particle and a new phospholipase activity, that of sPLA₂, is highly induced by cytokines at inflammatory sites. The interaction between SAA-bearing acute-phase HDL and sPLA₂ is thus important in the altered HDL function during inflammation. We here report that SAA can promote the activity of sPLA₂. Although a number of agents have been described that can block the activity of this enzyme [7,8], SAA is the first protein identified that can promote its activity. These data have the potential to advance our understanding of the major changes in lipoprotein metabolism that occur during inflammation.

MATERIALS AND METHODS

1,2-Dipalmitoylphosphatidylcholine (dipalmitoylPC) was obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.). L- α -[2-palmitoyl-1-¹⁴C]dipalmitoylPC (55.5 mCi/mmol) and [1-¹⁴C]oleic acid (40–60 mCi/mmol) were purchased from DuPont–NEN products. 1-Linoleoyl-[2-¹⁴C]palmitoyl-3-phosphatidylethanolamine (PE) (50–60 mCi/mmol) was obtained from Amersham. Recombinant human PLA₂ was generously given by Dr. Jeffrey Browning, Biogen Corporation (Cambridge, MA, U.S.A.); it was produced from a stable transfected Chinese hamster ovary cell line and purified. Bio-Rad protein assay was purchased from Bio-Rad (Richmond, CA, U.S.A.). Palmitoyl-L- α -lysoPC β -linoleoyl- γ -palmitoyl-L- α -phosphatidylethanolamine, palmitoyl-L- α -lysoPE, BSA and silica gel were purchased

Abbreviations used: SAA, serum amyloid A protein; HDL, high-density lipoprotein; sPLA₂, secretory phospholipase A₂; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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from Sigma (St. Louis, MO, U.S.A.). All other reagents were analytical grade or better.

Preparation of acute-phase and normal HDL

The respective HDLs were isolated by sequential ultracentrifugation from the blood of healthy donors or from patients experiencing an acute-phase response after surgery. HDL was further subfractionated according to plasma density (ρ) [HDL₂ (ρ = 1.063–1.13 g/ml), HDL_{3A} (ρ = 1.13–1.155 g/ml), HDL_{3B} (ρ = 1.155–1.18 g/ml)] by re-centrifugation of the total HDL fraction into a linear KBr gradient as described previously [18]. Only HDL_{3A} fractions were used in these studies. Protein concentrations were determined and individual apolipoprotein content was established by pyridine extraction of Coomassie Blue-stained bands from SDS/PAGE as described [4]. Normal HDL_{3A} contained 71% apolipoprotein AI and 29% apolipoprotein AII. Acute-phase HDL_{3A} contained 56% apolipoprotein AI, 17% apolipoprotein AII and 27% acute-phase SAA.

Purification of constitutive SAA₄

SAA₄ was isolated from normal human HDL [6] by Rotofor (Bio-Rad) preparative isoelectric focusing according to the manufacturer's instructions. Normal HDL (200 mg) was delipidated at -20°C with ethanol/ether (3:2, v/v), and the protein pellets were suspended in 15 ml of 8 M urea/1% (w/v) decyl sodium sulphate (Eastman Kodak Co., Rochester, NY, U.S.A.)/5% (v/v) 2-mercaptoethanol. The Rotofor running buffer consisted of 8 M urea, 1.2% Ampholines pH 4–6.5, 1.2% Ampholines pH 7–9 and 0.6% Ampholines pH 3–10. The anionic and cationic chambers contained 0.1 M phosphoric acid and 0.1 M NaOH respectively. The sample was electrofocused at constant power (12 W) until equilibrium was reached. Twenty fractions were harvested and analysed by SDS/PAGE. The SAA₄-containing fractions were dialysed against 15 mM NaCl/2 mM Tris/HCl, pH 8.4, and lyophilized. The lyophilized pellet was suspended in 2 ml of 7 M urea/150 mM NaCl/20 mM Tris/HCl, pH 8.4, and subjected to molecular-sieve chromatography on a Sephacryl S200 column (1 cm $\times$ 120 cm) according to the manufacturer's instructions (Pharmacia-LKB Biotechnology, Piscataway, NJ, U.S.A.).

Preparation of acute-phase SAA and antibodies to SAA

Acute-phase HDL₃ was delipidated and the SAA separated by molecular-sieve chromatography as described previously [6,18]. Monospecific rabbit anti-(human SAA) antibodies were raised [18]. Immunopurified antibodies were prepared by coupling purified SAA to CNBr-activated Sepharose 4B (Pharmacia-LKB) according to the manufacturer's instructions. Antiserum was passed over the column, washed in PBS, uncoupled with 0.2 M glycine/HCl and dialysed into PBS.

Liposome assays

Aliquots of dipalmitoylPC, [^{14}C]dipalmitoylPC, with or without lysoPC [or phosphatidylethanolamine (PE) with or without lysoPE] were prepared in chloroform and evaporated to dryness. Multilamellar liposomes were prepared by dispersing the resulting lipid mixture in 100 mM Tris/HCl buffer, pH 8.0, followed by heating for 2 min at 41°C and vortexing for 2 min before use. Only freshly prepared liposomes were used. Assays were carried out in a total volume of 0.2 ml of 100 mM Tris/HCl, pH 8.0, containing 2.5 or 10 mM CaCl_2 , 0.1% BSA and

5–30 nmol of PC vesicles (containing 2–16 nCi of [^{14}C]dipalmitoylPC per assay). The optimal concentration was found to be 20 nmol/assay and this was used in all experiments. If acute-phase SAAs or other agents were included, they were preincubated with 20 nmol of liposomes for 1 h at 41°C before the assay. The reaction was then started by addition of 20 μl of recombinant human sPLA₂ stock solution with final sPLA₂ concentrations ranging between 10 and 200 ng/200 μl of assay volume unless otherwise stated. The reaction mixture was incubated for 30 min at 41°C . The reaction was stopped by the addition of 1.32 ml of propan-2-ol/heptane/0.5 M H_2SO_4 (40:10:1, by vol.). The mixture was heated for 1 min at 60°C before addition of 0.66 ml of water and 0.8 ml of heptane. The two phases were allowed to separate and after centrifugation for 10 min at 500 g (1500 rev./min), 0.8 ml of the upper phase was added to 1.0 ml of heptane containing 100 mg of silica gel. The mixture was spun again for 10 min at 500 g and 1.0 ml of the supernatant was used for scintillation counting of ^{14}C -labelled non-esterified palmitic acid. All assays were performed in triplicate [19].

Inhibition studies

SAA (20 μl ; 250 $\mu\text{g}/\text{ml}$) was mixed with 30 μl of appropriately diluted immunopurified monospecific rabbit anti-(human SAA) antibody and the mixture incubated at room temperature for 60 min. Final concentrations of antibody were 25, 50 and 300 $\mu\text{g}/\text{ml}$. Then 50 μl of 0.1 M Tris/HCl buffer, pH 8.0, 20 μl of 100 mM Ca^{2+} , 20 μl of BSA (concentration 10 mg/ml) and 40 μg of substrate were added and the final mixture was further incubated for 30 min at 41°C . PLA₂ activity is expressed as nmol of substrate hydrolysed/30 min. The controls included antibody alone, β_2 -microglobulin (25 $\mu\text{g}/\text{ml}$) alone and β_2 -microglobulin-anti- β_2 -microglobulin complex. Anti-(human β_2 -microglobulin) antibody (mouse IgG1) (clone BM-63) was obtained from Sigma. The working dilution was at least 1:1000 per μg of antigen in an indirect ELISA. In assays employing anti-SAA antibodies, the antigens were preincubated with appropriately diluted antibodies for 60 min at room temperature. Liposomal substrate was added and the mixture was further incubated for 60 min at 41°C . The sPLA₂ was finally added and the reaction was allowed to proceed for 30 min at 41°C .

Statistical analysis was carried out using standard tests such as Student's t test and correlation coefficients.

RESULTS

Effect of normal and acute-phase HDL on sPLA₂ activity

Normal HDL was found to inhibit the activity of sPLA₂ in a dose-dependent manner with an IC_{50} of 10 $\mu\text{g}/\text{ml}$. With PC/lysoPC liposomes as substrate (Figure 1), inhibitory activity was already observed at an HDL concentration of 1 $\mu\text{g}/\text{ml}$, and a concentration of 100 $\mu\text{g}/\text{ml}$ inhibited sPLA₂ activity by 80% ($P < 0.001$). when PE/lysoPE liposomes were used as substrate, the inhibition was very similar to that obtained using PC/lysoPC liposomes (52% at 10 $\mu\text{g}/\text{ml}$, 58% at 50 $\mu\text{g}/\text{ml}$ and 70% at 100 $\mu\text{g}/\text{ml}$). Preincubation with phosphorylcholine (10 μM) did not abolish the inhibitory activity of normal HDL.

The inhibitory activity of normal HDL was related to the amount of sPLA₂ used in the assays. Keeping the HDL concentration constant at 10 $\mu\text{g}/\text{ml}$, the inhibition of hydrolytic activity by 10 ng of sPLA₂ was 59%, by 100 ng 34% and by 200 ng 11%.

As, during the acute phase, newly synthesized and released extracellular acute-phase SAA combines with HDL to form

Figure 1 Dose-dependence of inhibition of lipolytic activity of sPLA₂ by normal HDL

PC/lysoPC (2:1, v/v) liposomes were used as substrate. Each point represents the mean $\pm$ S.D. of three determinations. —, Inhibition of sPLA₂; ----, sPLA₂ activity.

Figure 2 Enhancement of lipolytic activity of sPLA₂ by acute-phase SAA

●, PC/lysoPC (2:1, v/v) liposomal substrate; ▲, PE/lysoPE (2:1, v/v) liposomal substrate. Each point represents the mean $\pm$ S.D. of three determinations.

acute-phase HDL, we compared the effects of physiological normal and acute-phase HDL on sPLA₂ activity. The former (at a concentration of 15 μ g/ml) produced 62% inhibition of sPLA₂ activity ($P < 0.001$) whereas the latter (also at 15 μ g/ml) enhanced sPLA₂ activity by 188% ($P < 0.001$).

Effect of purified SAA on sPLA₂ activity

Purified inflammatory SAA enhanced the activity of sPLA₂ in a dose-dependent manner (Figure 2). With PC/lysoPC liposomes (2:1, v/v) as substrate, a 135% increase was seen at an SAA concentration of 3 μ g/ml, and a concentration of 10 μ g/ml doubled (195%) sPLA₂ activity ($P < 0.001$). When PC alone was used as substrate, the enhancement was less pronounced with 10 μ g/ml acute-phase SAA increasing sPLA₂ activity to 130%, 20 μ g/ml to 151% ($P < 0.01$) and 50 μ g/ml to 185% ($P < 0.001$) (results not shown). Liposomes composed of PE/lysoPE (2:1, v/v) were also hydrolysed but less actively than

Table 1 Inhibition of enhancement of sPLA₂ activity by SAA using monospecific anti-SAA antibody

Results are means $\pm$ S.D. of three determinations. * $P < 0.005$, ** $P < 0.001$ compared with activity in the presence of SAA alone.

SAA (μ g/ml)	Anti-SAA antibody (μ g/ml)	sPLA ₂ (nmol/30 min)	Percentage of control
0	0	1.97 $\pm$ 0.06	100
25	0	3.39 $\pm$ 0.13	172
25	25	2.82 $\pm$ 0.10*	143
25	50	2.66 $\pm$ 0.09**	135
25	300	1.58 $\pm$ 0.03**	80

PC/lysoPC; 10 μ g/ml of acute-phase SAA increased sPLA₂ activity to 129% and 50 μ g/ml to 167% ($P < 0.001$). Constitutive SAA in the concentration range 10–50 μ g/ml did not significantly enhance sPLA₂ activity (9–34%) ($P > 0.05$), in either the PC/lysoPC or the PE/lysoPE system (results not shown).

Inhibition of acute-phase-SAA-mediated sPLA₂ increase by anti-SAA antibody

Acute-phase SAA preincubated with increasing concentrations of anti-SAA antibody gradually lost its sPLA₂-enhancing activity (Table 1). Neither antibody alone nor an irrelevant β_2 -microglobulin-anti- β_2 -microglobulin complex of similar molecular mass had any effect on sPLA₂ activity.

To support the notion that the enhancing activity of acute-phase HDL on sPLA₂ is caused by the inflammatory SAA associated with HDL, acute-phase HDL_{3A} (25 μ g/ml) was incubated with immunopurified monospecific anti-SAA antibody. When no antibody was added, the increase in sPLA₂ activity by acute-phase HDL_{3A} was 165% ($P < 0.001$). Incubation of the HDL_{3A} with 100 μ g/ml anti-SAA antibody reduced the enhancement of sPLA₂ activity to 12%; incubation with 200 μ g/ml antibody abolished the increase completely and actually resulted in inhibition of sPLA₂ activity by 29%. The antibody itself had no effect on sPLA₂ activity.

DISCUSSION

During the acute phase, the concentration of the acute-phase protein SAA can increase up to a thousandfold in body fluids [1]. The presence of SAA on HDL remodels the latter [1] and alters its binding characteristics, thereby augmenting its association with macrophages [20] and neutrophils [21] and reducing its affinity for hepatocytes [20]. SAA thus has the potential to dramatically alter HDL function.

Concomitant with the stimulation of SAA production, sPLA₂ activity increases markedly under conditions that induce the acute-phase reaction [7,8]. Circulating sPLA₂ activity may increase more than 3000-fold during septic shock [13]. Extremely high sPLA₂ activity has been found in patients with malaria [14], multi-organ dysfunction caused by salicylate poisoning [22] and inflammatory processes such as peritonitis [23] or inflammatory arthritis [24].

There is a paucity of specific information about the possible link between sPLA₂ activity and lipoprotein metabolism, even though a substantial body of evidence exists that this group of lipases alter HDL-mediated lipid flow [15–17]. It has been reported that hydrolysis of HDL phospholipids by hepatic lipase,

an enzyme that possesses PLA₂-like activity, results in the appearance of HDL with enhanced ability to deliver cholesterol to cells [15,16]. This process was found to be associated with relocation of cholesterol molecules from the core of HDL to the surface, inducing a shift in equilibrium of free cholesterol between HDL and the plasma membrane [15]. It has also been reported that stimulation of the release of non-esterified fatty acids from PC-labelled human platelets by HDL is probably mediated via activation of membrane-associated PLA₂ [25].

At inflammatory sites there is an enormous increase in fluid-phase sPLA₂ activity in addition to an alteration of HDL containing SAA (acute-phase HDL). As acute-phase HDL enhances the lipolytic activity of sPLA₂, one can envisage a mechanism whereby lipid, particularly cholesterol, would flow from HDL towards cells at such sites. Such lipid delivery might in the short term be of potential benefit during an acute inflammatory event, providing materials for repair of damaged cell membranes.

Given the central role of HDL in lipoprotein metabolism [26], the interaction of sPLA₂ with SAA and HDL could account for some of the changes detected in lipoprotein metabolism during the acute phase. This is of particular relevance for patients with chronic inflammatory diseases such as rheumatoid arthritis, who have prolonged periods of high sPLA₂ activity and SAA induction [27,28]. In these patients a high incidence of atherosclerosis and high mortality from cardiovascular diseases have been reported [29].

In addition, our findings may be important for events leading to the development of atherosclerotic lesions in the vessel wall. The presence of acute-phase SAA molecules in atherosclerotic lesions in mouse and man has been reported [30,31]. *In situ* hybridization studies have indicated SAA production in endothelial cells, macrophage-derived 'foam' cells and vascular smooth-muscle cells in lesions [31], presumably induced by oxidized lipids present in the milieu [32]. If one considers that vascular smooth-muscle cells are probably a major site of sPLA₂ synthesis [11], the enhancement of sPLA₂ activity by SAA at such sites could lead to aberrant lipid delivery and conceivably promote 'foam' cell formation, a key event in atherogenesis.

The finding that constitutive SAA₄ had no significant effect on sPLA₂ activity suggests that the very conserved region of acute-phase SAA between amino acids 35 and 48 could be the active site for promotion of this activity, as constitutive SAAs are the only members of the SAA family with differences in this region [6]. The mechanism by which SAA promotes sPLA₂ activity is not known. sPLA₂ hydrolyses phospholipids only when they are in a specific spatial orientation. Unperturbed cell-membrane phospholipids for instance resist this activity. It is conceivable that the amphiphilic SAA, when associated with cell membranes, alters their fluidity, making phospholipids accessible to sPLA₂-induced hydrolysis. Another possibility is that the SAA region between amino acids 48 and 51, which is similar to the Gly³⁰-X-Gly³²-Gly³³ phospholipid- and Ca²⁺-binding site of PLA₂ molecules, is involved. This possibility is lessened by the fact that constitutive SAA [6] has an identical sequence in the above region but does not activate sPLA₂.

In conclusion, the present data indicate the existence of a potential mechanism involving SAA-sPLA₂ interaction that may significantly alter lipid flow during inflammation.

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