

PRO-INFLAMMATORY non-pancreatic phospholipase A₂ (sPLA₂) is markedly over-expressed in acute systemic and chronic local inflammatory processes. Since in acute phase reaction sPLA₂ is often over-expressed simultaneously with acute phase proteins (APP), it is important to determine whether APP interacts with sPLA₂. We tested ten APPs for interaction with sPLA₂ using as a substrate multilamellar liposomes composed either of PC:Lyso PC or PE:Lyso PE. Using PC:Lyso PC substrate, CRP, lactoferrin and SAP were found to inhibit sPLA₂ activity with an IC₅₀ of 25 µg/ml, 7.5 µg/ml and 50 µg/ml, respectively, corresponding to 0.21 µM, 0.1 µM and 0.21 µM respectively. Using PE:Lyso PE substrate only SAP was inhibitory, with an IC₅₀ of 10 µg/ml (0.04 µM). Phosphorylcholine abolished the inhibitory activity of CRP but not of SAP or lactoferrin. Addition of phosphorylethanolamine or of excess calcium had no effect on the inhibitory activity of APP. Limulin, lysozyme, transferrin, β₂-microglobulin, α₂-macroglobulin, human and bovine albumins had no effect on sPLA₂ activity. Therefore neither the structure of pentraxins, or iron-binding, bacteriostatic property or amyloidogenic property preclude whether APP modulates sPLA₂ activity. Inhibition of pro-inflammatory sPLA₂ by APP may be one of the protective mechanisms of the acute phase reaction.

Key words: Acute phase proteins, Inflammation, Phospholipase A₂

Inhibition of the activity of pro-inflammatory secretory phospholipase A₂ by acute phase proteins

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Introduction

Secretory non-pancreatic phospholipase A₂ (sPLA₂) belongs to the group of low-molecular-weight, calcium-dependent, lipolytic enzymes.¹ It plays an important physiological role in host defence participating in the destruction of Gram-negative microorganisms.^{2,3} sPLA was also found to exert modulatory activity on the cellular proliferation and tumour formation in the intestinal tract.^{4,5} Excessive activity of circulating sPLA₂ was discovered in several systemic inflammatory response syndromes (SIRS) such as clinical and experimental sepsis, multiorgan failure and salicylate intoxication,^{6–9} and in more localized processes such as peritonitis and inflammatory arthritis.^{10,11} A pathogenetic role of sPLA₂ was implicated by the observation that enzymatic activity and immunoreactivity of sPLA₂ in SIRS correlated with the severity and outcome of the disease,^{6–9} and by the fact that hypotension induced either by Gram-negative microorganisms⁷ or by infusion of sPLA₂⁶ could be attenuated by inhibition of the enzyme. Acute

inflammatory processes induced by sPLA₂ administered intracutaneously,¹² into subcutaneous air pouches¹³ or intraarticularly,^{14,15} could be blocked by inhibitors of PLA₂.^{12–15}

In general terms inhibition of sPLA₂ can be induced by three mechanisms: inhibition of synthesis, competition for substrate, or direct inhibition of sPLA₂ enzymatic activity. The best characterized inducers of its synthesis are cytokines, such as IL-1 and TNF.¹⁶ IL-6 and oncostatin M induce sPLA₂ in cells of hepatic origin such as Hep G₂^{17,18} and normal human liver cells (W. Pruzanski *et al.*, unpublished). Endotoxin was also found to be a strong inducer of sPLA₂.¹⁸ The above observations were consistent with the postulate that sPLA₂ plays an important pathogenetic role in inflammatory processes and led to an extensive search for exogenous inhibitors. However, very little is known about endogenous modulation of sPLA₂. Two endogenous inhibitors, glucocorticoid-inducible lipocortin¹⁹ and complement fragments²⁰ have been found, but neither has evolved into a useful therapeutic agent.

During the acute phase reaction the synthesis and release of sPLA₂ occurs simultaneously with that of a large number of acute phase proteins.^{21,22} Experimental studies have shown that endotoxin, IL-1, TNF and IL-6 are the main inducers of such release.^{21,22} Therefore the process of induction of acute phase proteins by liver cells seems to be similar to that of sPLA₂. Little is known about the possible interactions of acute phase proteins. We reported recently that CRP, one of the classical acute phase proteins is a strong inhibitor of sPLA₂, binding competitively to the phosphorylcholine-containing substrates²³ whereas SAA, another acute phase protein, enhances sPLA₂ activity.²⁴ Herein we report that two other acute phase proteins, serum amyloid P (SAP) and lactoferrin suppress sPLA₂ activity. These, previously unrecognized, interactions of acute phase proteins with proinflammatory sPLA₂, may add a new aspect to the understanding of the complex role of acute phase reaction.

Materials and Methods

1,2-Dipalmitoyl-phosphatidylcholine (dipalmitoyl PC) was obtained from Avanti Polar Lipids (Birmingham, AL). Phosphatidylcholine L- α -dipalmitoyl [2-palmitoyl-1-¹⁴C] (55.5 mCi/mmol) and oleic acid [1-¹⁴C] (40–60 mCi/mmol) were purchased from DuPont NEN Products. L-3-phosphatidylethanolamine 1-palmitoyl [2-¹⁴C linoleoyl] (50–60 mCi/mmol) was obtained from Amersham (Arlington Heights, IL). Recombinant human sPLA₂ (rh-sPLA₂) was a generous gift of Dr Jeffrey Browning, Biogen Corporation (Cambridge, MA). Bio-Rad protein assay reagent was purchased from Bio-Rad (Richmond, CA). L- α -lysophosphatidylcholine palmitoyl, L- α -phosphatidylethanolamine- β -linoleoyl- γ -palmitoyl, L- α -lysophosphatidylethanolamine palmitoyl, bovine serum albumin and silica gel were purchased from Sigma Chemical Corporation. All reagents were analytical grade or better.

Recombinant human lysozyme, bovine serum albumin (BSA), human serum albumin (HSA), lactoferrin purified from human milk, transferrin, β_2 -microglobulin purified from human urine, limulin from *Limulus polyphemus* and α_2 -macroglobulin were obtained from Sigma Chemical Company (St Louis, MO). CRP purified from human plasma was obtained from Helix Biotech Corporation, Richmond, BC and SAP purified from human serum, from Calbiochem, San Diego, CA.

Liposome assay: Aliquots of dipalmitoyl PC, [¹⁴C] dipalmitoyl PC, with or without LysoPC (2:1) or

phosphatidylethanolamine (PE) with or without LysoPE (2:1) were prepared in chloroform and evaporated to dryness. Multilamellar liposomes were made 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 CaCl₂, 0.1% bovine serum albumin and 5–30 nmoles of PC (or PE) vesicles (containing 2–16 nCi of [¹⁴C] dipalmitoyl PC per assay). The optimal concentration was found to be 20 nmoles/assay and this was used in all experiments. If acute phase proteins were included, they were preincubated with 20 nmoles of liposomes for 1 h at 41°C before the assay. The reaction was then started by addition of 20 μ l of recombinant human sPLA₂ stock solution with final sPLA₂ concentrations ranging between 10 and 200 ng/200 μ l assay volume unless otherwise stated. The reaction mixture was then incubated for 30 min at 41°C. The reaction was stopped by the addition of 1.32 ml isopropanol/heptane/0.5 M H₂SO₄ 40:10:1 (v/v/v). The mixture was heated for 1 min at 60°C before addition of 0.66 ml H₂O and 0.8 ml heptane. The two phases were allowed to separate and after centrifugation for 10 min at 1500 rpm, 0.8 ml of the upper phase was added to 1.0 ml heptane containing 100 mg silica gel. The mixture was spun again for 10 min at 1500 rpm and 1.0 ml of the supernatant was used for scintillation counting of ¹⁴C-labelled free palmitic acid. All assays were done in triplicate.

Immunologic assays: Anti-human (mouse IgG2) CRP/SAP antibody (clone CRP-20) C6552 was obtained from Sigma (St Louis, MO). It reacted with an epitope located on the 24 kDa subunit of denatured and reduced CRP and it recognized CRP independently of the Ca²⁺ binding site. This antibody did not recognize the calcium dependent phosphorylcholine binding site of CRP. It cross-reacted with human SAP, but not with CRP from *Limulus polyphemus*. Working dilutions were at least 1:4 000 per μ g of antigen in indirect ELISA assay. In antibody assays, the antigens were preincubated with appropriately diluted antibodies for 60 min at room temperature. Then the liposomal substrate was added and the mixture was further incubated for 60 min at 41°C. The PLA₂ was finally added and the incubation was carried on for an additional 30 min at 41°C.

Each experiment was repeated at least three times. The differences between the results and controls were assessed by Student's *t*-test.

Results

Ten acute phase proteins were tested for modulation of sPLA₂ activity, using as a substrate multilamellar liposomes composed either of PC:Lyso PC or PE:Lyso PE in the ratio of 2:1. Using the former substrate, lactoferrin (Fig. 1) CRP (Fig. 2) and SAP (Fig. 3) were inhibitory, with an IC₅₀ of 7.5 µg/ml, 25 µg/ml and 50 µg/ml respectively. These concentrations corresponded to 0.10 µM, 0.21 µM and 0.21 µM respectively (Fig. 4). Using the latter substrate, only SAP was inhibitory, with an IC₅₀ of 10 µg/ml (0.04 µM) (Fig. 3). Using mixed substrate (PE:Lyso PC), no inhibition of sPLA₂ activity by CRP was noted (Table 1).

Addition of phosphorylcholine in concentrations ranging from 0.5 to 10 µM abolished inhibitory activity of CRP. Fifty per cent attenuation was achieved by 2.0 µM and complete inhibition by 10 µM of phosphorylcholine.²³ Phosphorylcholine did not alter inhibitory activity of SAP or of lactoferrin. Phosphorylethanolamine, 10 µM,

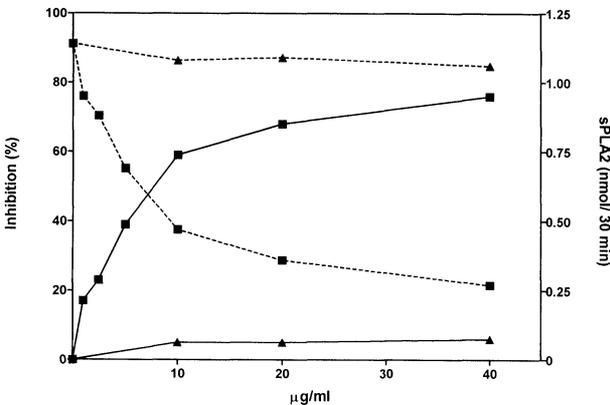


FIG. 1. Inhibition of sPLA₂ activity by lactoferrin. Closed squares, solid line—inhibition of sPLA₂ activity using PC:Lyso PC multilamellar liposomes as a substrate. Closed squares, interrupted line—as above; expression of sPLA₂ in nmol/30 min. Closed triangles, solid line—inhibition of sPLA₂ activity using PE:Lyso PE multilamellar liposomes as a substrate. Closed triangles, interrupted line—as above; expression of sPLA₂ in nmoles/30 min.

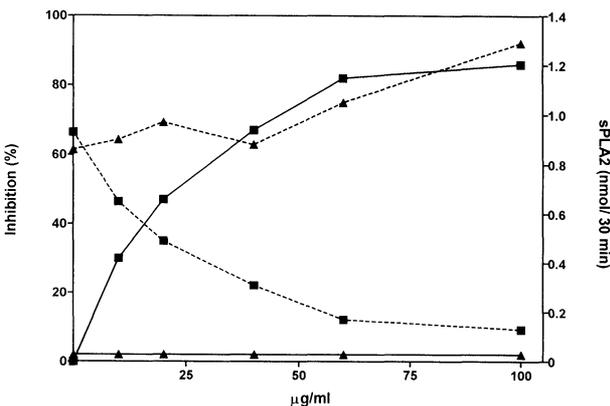


FIG. 2. Inhibition of sPLA₂ activity by CRP. Symbols as on Fig. 1.

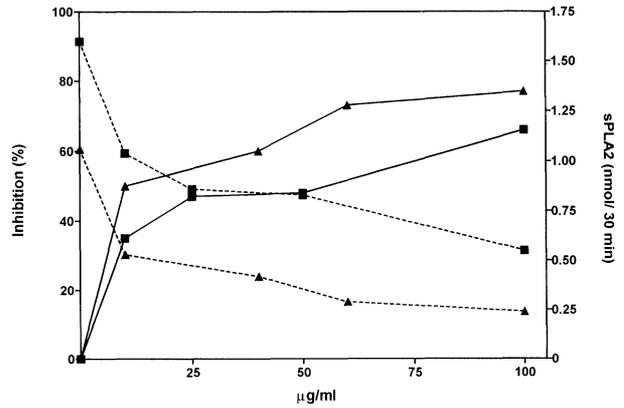


FIG. 3. Inhibition of sPLA₂ activity by SAP. Symbols as on Fig. 1.

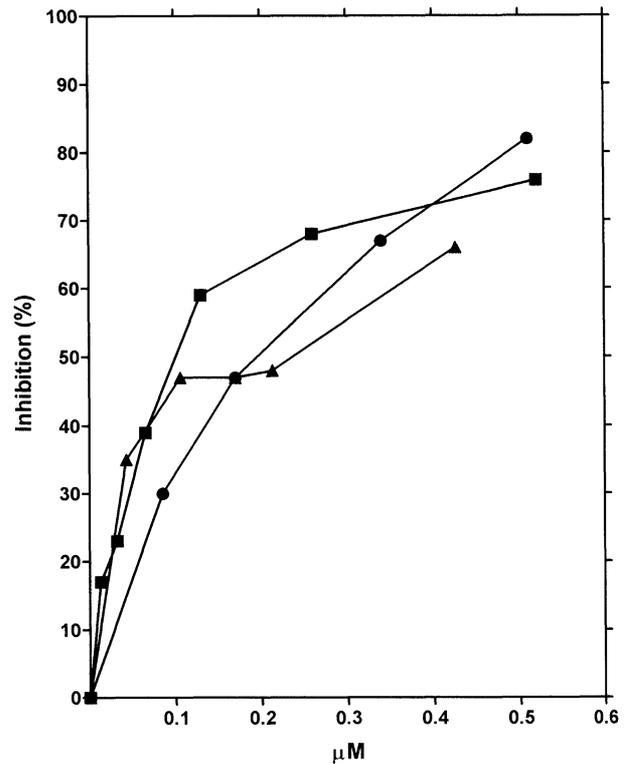


FIG. 4. Comparison of inhibitory activity of lactoferrin (squares), SAP (triangles) and CRP (circles) against sPLA₂, expressed in micromolar concentrations. PC:Lyso PC multilamellar liposomes used as a substrate.

had no effect on inhibitory activity of SAP, CRP or lactoferrin.

Anti CRP/SAP monoclonal antibody alone had no effect on sPLA₂ activity. This antibody pre-incubated with CRP or SAP did not alter their inhibitory activity in the range of concentrations used in the assays (CRP or SAP 40 µg/ml, sPLA₂ 100 ng).

To test whether chelation may have an effect on activity of sPLA₂, various concentrations of calcium were tested using either PC:Lyso PC or

Table 1. Impact of substrate on inhibitory activity of CRP

Substrate	sPLA ₂ (nmoles/30 ¹)
PE:Lyso PE	0.99
PE:Lyso PE + CRP	1.09
PC:Lyso PC	0.93
PC:Lyso PC + CRP	0.31
PE:Lyso PC	0.93
PE:Lyso PC + CRP	1.35

*sPLA₂ 100 ng, CRP 40 µg/ml.

PE:Lyso PE as a substrate. Maximum activity of sPLA₂ was observed at the level of 0.5–1.0 µM Ca²⁺. In some experiments with CRP, SAP, or lactoferrin, Ca²⁺ concentrations were increased up to 20 µM. Excess of Ca²⁺ had no effect on inhibitory activity of these proteins.

Human and bovine serum albumin, transferrin, lysozyme, β₂-microglobulin, α₂-macroglobulin and limulin had no effect on the activity of sPLA₂.

Discussion

An insult to an organism's homeostasis caused by injury, infection or inflammation, leads to a swift systemic response called the acute phase reaction (APR) (reviewed in References 21, 25–27). In the case of chronic or recurrent inflammation, APR may become quite prolonged.²⁵ An integral part of APR is a rapid synthesis and extracellular release of a large number of acute phase proteins (APP), and simultaneous decrease in some other proteins, the so called negative APP. Liver is the major source of APP synthesis, but other cells participate in the synthesis of APP as well.^{22,26,28} APR is orchestrated by a group of inflammatory mediators, including glucocorticoids, cytokines, anaphylotoxins and growth factors.^{21,25} The group of cytokines which induce APP synthesis includes, but is not limited to, IL-1, TNF, IL-6 and oncostatin.^{21,22,26,29,30}

The same group of cytokines was also found to induce the synthesis and release of the pro-inflammatory enzyme, secretory non-pancreatic phospholipase A₂ (sPLA₂).^{16,18} sPLA₂, first discovered in experimental peritonitis³¹ and in fluids draining inflammatory sites,³² was found to raise rapidly in the circulation in systemic inflammatory response syndromes such as septic shock,⁷ salicylate poisoning⁹ and malaria³³ and in the milieu of more localized inflammatory sites such as arthritis.¹¹ In the former group, circulating sPLA₂ correlated with both complications and the outcome of the disease,⁷ whereas in the latter it correlated with the disease activity.¹¹

Since the increase in circulatory sPLA₂ activity parallels temporally that of acute phase proteins, it was of interest to investigate whether there is an interaction between APPs and sPLA₂. Of ten APPs tested, three were found to inhibit sPLA₂ activity. These included CRP, SAP and lactoferrin. CRP is one of the best studied acute phase proteins, increasing rapidly up to 1000-fold in APR.³⁴ Both sPLA₂ and CRP bind to the phospholipids of perturbed membranes of living cells²⁷ and to PC:Lyso PC substrate.^{27,35} It was found that CRP is a strong inhibitor of sPLA₂ activity, acting most probably as a competitor for the substrate.²³ The substrates used in the former²³ and present study form vesicles of different phospholipid composition. It was found that binding of CRP to such vesicles is preferential when they are composed of PC:Lyso PC in the proportion of 2:1. Most probably it is related to optimally altered surface packing density of the substrate. PE:Lyso PE and PE:Lyso PC substrates were not susceptible to hydrolytic activity of sPLA₂.

Since CRP belongs to the family of pentraxins (PEP), two other pentraxins, SAP and limulin were also tested. The former was found to inhibit sPLA₂ whereas the latter did not. SAP is a major APP in mice²⁷ but a minor one in man,³⁶ increasing in APR only three-fold from the physiological level of 30–40 µg/ml to no more than 90 µg/ml.^{27,36} It shares 60% homology in amino acid sequence with CRP.²⁷ SAP circulates in the form of two pentameric molecules bound 'face to face' and, in contrast to CRP, is glycosylated.³⁷ The gene for SAP is located on the long arm of chromosome 1 (1q12–1q23), close to the gene coding for CRP. It was suggested that both are products of an ancestral duplication event.³⁸ Whereas CRP binds mainly to phosphorylcholine,^{36,39} SAP has high affinity to phosphorylethanolamine.³⁶ In our study, SAP inhibited sPLA₂ activity when either PE:Lyso PE or PC:Lyso PC were used as substrates; however, much lesser concentrations of SAP were needed to achieve IC₅₀ when PE:Lyso PE was employed. In human serum, SAP binds to C_{4b}-binding protein (C4BP) and to various types of phospholipid vesicles. These reactions were found to be calcium dependent and can be disrupted by phosphorylethanolamine. In our study phosphorylethanolamine did not block the inhibitory activity of SAP.

Limulin in *Limulus polyphemus* is analogous to CRP in humans. It shares only 25–30% amino acid homology with CRP^{40,41} and SAP⁴¹ and, similarly to CRP, binds avidly to phosphorylcholine.^{34,42,43} Limulin shares with human CRP two regions of preserved residues, 52–67, respon-

Table 2. Acute phase proteins as inhibitors of sPLA₂

Group	Inhibitors	Non-inhibitors
Pentraxins	CRP, SAP	Limulin
Iron binders	Lactoferrin	Transferrin
Bacteriostatic	Lactoferrin	Lysozyme
Amyloidogenic substances	SAP	SAA*, β_2 -m
Negative APP	HDL*	BSA, HSA
Proteinase inhibitors	N/D [†]	α_2 -Macroglobulin

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†N/D: not done.

sible for the binding site to phosphorylcholine and 139–153 that binds Ca²⁺.^{41,44} The fact that, in contrast to human CRP, limulin does not inhibit sPLA₂ activity, may mean that either the above two binding sites are not responsible for inhibition of sPLA₂ activity, or that the affinity of limulin to the substrate is much weaker than that of CRP. Therefore, pentraxin structure does not automatically confer anti-sPLA₂ activity, since in contrast to CRP and SAP, limulin was not inhibitory. Neither amyloidogenic property of SAP can be linked to the inhibition of sPLA₂, since SAA²⁴ and β_2 -microglobulin, both known participants in amyloidogenesis, did not inhibit sPLA₂ activity (Table 2).

Of three sPLA₂ inhibitors, lactoferrin was the most active. Lactoferrin is one of the iron-carrying proteins, produced mainly by polymorphonuclear cells.^{45,46} Its synthesis is induced by TNF^{45,46} and in sepsis it behaves like a classical acute phase protein^{46,47} with the potential to increase in the circulation ten or more fold from its physiological level of 0.2–2.8 mg/ml.^{46–48} Infusions of LPS to healthy volunteers⁴⁶ or to piglets,⁴⁸ or of *Escherichia coli* to piglets⁴⁷ lead to a marked increase in circulating lactoferrin. In turn, lactoferrin interacts with LPS, preventing iron-catalysed formation of hydroxyl radicals.⁴⁵ Lactoferrin protects mice against a lethal dose of *E. coli* *in vivo*,⁴⁷ acting as bacteriostatic glycoprotein and inducing damage to the outer membrane of Gram-negative bacteria.⁴⁹

It seems, therefore, that lactoferrin acts upon Gram-negative bacteria similarly to bacterial permeability increasing protein (BPI).² Since sPLA₂ hydrolyses membrane phospholipids of microorganisms killed by BPI,² the fact that lactoferrin inhibits sPLA₂ activity may mean that it acts as a limiting factor in hydrolytic activity of the latter. The property of iron chelation by lactoferrin was not related to inhibitory anti-sPLA₂ activity, since transferrin, another iron chelator did not inhibit sPLA₂.

The fact that during the acute phase there is simultaneous co-induction and over-expression of both inhibitors such as CRP, SAP and lacto-

ferrin, and enhancers such as SAA²⁴ of sPLA₂ activity, emphasizes the complexity of the organism's response to injury. Since, *in vivo*, various acute phase proteins are over-expressed to very different orders of magnitude, the end result regarding their impact on proinflammatory activity of sPLA₂ cannot be predicted.

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