Serum amyloid A (SAA) protein enhances formation of cyclooxygenase metabolites of activated human monocytes

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Abstract As serum amyloid A (SAA), an apolipoprotein associated with HDL during the acute-phase reaction may induce Ca^{2+} mobilization in human monocytes we raised the question whether SAA_1 the predominant isoform of human acute-phase SAA is able to alter eicosanoid formation. In resting monocytes SAA_1 was without effect on the secretion of cyclooxygenase metabolites while in calcium ionophore A_{23187} (0.5 and 2.5 μM) stimulated cells SAA_1 led to a pronounced dose-dependent increase of TXA_2, PGE_2, and $PGF_{2\alpha}$. In addition a time-dependent increase of cyclooxygenase metabolites in between 1.5- and 3-fold in the presence of SAA_1 was observed; apo A-I, the main HDL-apolipoprotein under non-acute-phase conditions, had no effect. Using sequence-specific anti-human SAA_1 peptide (40–63) $F(ab)_2$ fragments we could show that the proposed Ca^{2+}-binding tetrapeptide Gly^{48}-Pro^{49}-Gly^{50}-Gly^{51} of SAA_1 is not responsible for enhanced biosynthesis of cyclooxygenase metabolites. Finally, we could demonstrate that human SAA_1 is unable to bind Ca^{2+}-ions, suggesting that SAA_1 does not directly enhance eicosanoid biosynthesis via Ca^{2+} mobilization leading to enhanced phospholipase A_2 activity.

Key words: Acute-phase reaction; Atherosclerosis; Calcium-binding property; Cyclooxygenase; Eicosanoid; Gas chromatography/mass spectrometry

1. Introduction

Mammalian liver responds to various systemic injuries, such as acute inflammation, infection, or burns with profound changes in plasma concentrations of a group of proteins, termed acute-phase proteins [1]. In humans serum amyloid A (SAA) protein is expressed during the acute-phase reaction in response to cytokines released by activated monocytes/macrophages. SAA may reach plasma levels up to 1000-fold greater than that found in the non-inflammatory state (2–5 μg/ml), thus representing an ideal marker for clinical use [2,3]. Serum levels of SAA are increased during bacterial and viral infections and a number of chronic inflammatory and neoplastic diseases which may predispose to amyloidosis [3]. In amyloidosis, SAA (104 amino acids, 12 kDa) is usually processed by cleavage between residues 76 and 77, and the N-terminal residue, the amyloid A protein, is incorporated into the bulk of fibrils in secondary reactive amyloidosis [4].

Upon release into the circulation SAA is mainly complexed with lipoproteins of the high density range [5] by displacing apolipoprotein A-I (apo A-I) the major protein component of native high density lipoproteins (HDL) [6]. While remodeling its physiological carrier during the acute-phase reaction, SAA may significantly impede the protective function of HDL in reverse cholesterol transport in humans [7]. Displacement of HDL-associated apo A-I by SAA was demonstrated to reduce the binding properties of SAA-enriched HDL to hepatocytes which was accompanied with a 3- to 4-fold higher binding affinity to macrophages resulting in a significant shift in HDL-cholesterol carrying capacity towards the macrophage [8].

Besides its principal role as acute-phase protein, amyloidogenic protein, and apolipoprotein, some specific functions have been proposed for SAA. Among these are anti-tumor growth properties [9], platelet activation [10], inhibition of lymphocyte antibody response [11], induction of T-lymphocyte migration and adhesion [12], directional migration of monocytes and polymorphonuclear leukocytes as well as expression of adhesion proteins and adhesion molecules [13]. Recent results suggested that recombinant SAA (rSAA) may be effective in chemoattracting monocytes resulting in transient elevation of cytoplasmic Ca^{2+} concentrations [14] and that SAA might even enhance the activity of secretory non-pancreatic phospholipase A_2 (PLA_2) [15].

After activation the PLA_2 hydrolyses the glyceryl-fatty-acid ester bond at the *sn*-2 position of phospholipid molecules where arachidonic acid (AA) is most frequently found. AA is the precursor to a broad array of structurally diverse and potent bioactive lipids that include prostaglandins, thromboxanes, leukotrienes, lipoxins, as well as other oxygenated fatty acids [16,17]; collectively, these molecules are termed eicosanoids. In human tissues, prostaglandins and thromboxanes, both cyclooxygenase metabolites, are potent mediators of inflammation involved in the regulation of platelet-vessel wall interactions and in the regulation of blood vessel tone and hemostasis [18]. The fact that SAA contains a highly conserved tetrapeptide Gly^{48}-Pro^{49}-Gly^{50}-Gly^{51} a sequence homologous to Gly^{30}-Xaa^{31}-Gly^{32}-Gly^{33}, the main Ca^{2+}- and

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Abbreviations: AA, arachidonic acid; apo A-I, apolipoprotein A-I; HDL, high density lipoproteins; NICI-GC/MS, negative ion chemical ionization-gas chromatography/mass spectrometry; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PLA_2, phospholipase A_2; PKC, protein kinase C; rSAA, recombinant SAA; SAA, serum amyloid A protein; TXB_2, thromboxane B_2

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lipid-binding site in PLA_2 molecules [19,20], suggested that properties of altered eicosanoid balance in resting or activated cells might be due to the proposed Ca^{2+}-sensitive epitope present in SAA.

As SAA contributes to enhanced migration of monocytes and polymorphonuclear leukocytes to inflamed tissues [13] and the fact that both SAA and secretory PLA_2 are rapidly induced during inflammatory events [15], the present study was aimed at investigating whether SAA might alter the secretion of biologically active AA-metabolites, i.e. TXA_2, PGE_2, and $PGF_{2\alpha}$. As in humans the predominant SAA isoform is SAA_1 (>95% of total SAA); all experiments were performed with this protein.

2. Materials and methods

2.1. Isolation and purification of apolipoproteins

Crude SAA_1 was prepared directly from human plasma (obtained from patients with rheumatoid arthritis or patients undergoing plasmapheresis for therapeutic purposes) by hydrophobic interaction chromatography on Octylsepharose CL 4B followed by gel permeation-fast protein liquid chromatography on Superdex 75 prep grade as described [21]. Subsequently SAA_1 was purified from remaining SAA isoforms and other apolipoproteins (apo A-I, apo A-II, and apo Cms) by preparative IEF using the Rotofor system (Bio-Rad) and buffers containing 6 M urea, 10% (w/v) glycerol and 6.4% (w/v) carrier ampholytes [21]. Non-acute-phase HDL subclass 3 (d=1.125–1.21 g/ml), isolated by density gradient ultracentrifugation, was incubated with 6 M guanidine/HCl for 3 h at 37°C. After recentrifugation at a density of 1.21 g/ml, apo A-I was isolated from the lipid-free bottom fraction by gel permeation chromatography as described above. Aliquots of SAA_1 and apo A-I tested were negative for endotoxin in *Limulus* amebocyte lysate (BioWhittaker, sensitivity 0.06 EU/ml).

2.2. Preparation of sequence-specific F(ab)$_2$ fragments

Sequence-specific antibodies to humans SAA_1 were raised in rabbits using Freud's complete adjuvant (Sigma). The immunogen was a synthetic peptide (corresponding to amino acid residues 40–63 of human SAA_1) coupled via its C-terminal residue to *N*-maleimido-butyryl-*N*-hydroxysuccinimide ester-activated keyhole-limpet haemocyanin [22]. To obtain $F(ab)_2$ fragments cleavage of both, the sequence-specific anti-SAA_1 peptide IgGs and a non-immune rabbit IgG fraction was performed by enzymatic digestion with pepsin [23]. The $F(ab)_2$ fragments were then purified by gel permeations chromatography to ensure the absence of residual IgG or Fc fragments. Both, intact anti-SAA_1 peptide IgGs [22] and corresponding $F(ab)_2$ fragments did immunochemically react with either SAA_1 or the corresponding peptide (40–63).

2.3. Preparation of human monocytes and culture conditions

The mononuclear cell fraction of buffy coats from healthy blood donors was obtained by Ficoll-Hypaque separation as described [24]. The mononuclear cell fraction was washed in phosphate buffered saline (pH 7.4, without Ca^{2+} and Mg^{2+}), separated from platelets by overnight settlement at 4°C, and resuspended in RPMI 1640 medium supplemented with 5% heat-inactivated (56°C, 30 min) AB serum, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), pyruvate (2 mM), and HEPES buffer (50 mM). Mononuclear cells were seeded into 24-well culture plates (Falcon, Germany), and after 2 h of incubation at 37°C in a humidified 5% CO_2, 95% air atmosphere, the non-adherent leukocytes were removed be several extensive washing procedures. Platelet-depleted monocyte cultures (5×10^5/ml RPMI medium plus supplements without the addition of serum) were then incubated with different concentrations of SAA_1 or apo A-I (3.2 to 200 μg/ml) up to 12 h. Stimulation of cells was performed with calcium ionophore A_{23187} (final concentration: 0.5 and 2.5 μM) up to 4 h in the presence/absence of the corresponding apolipoprotein or in the presence of a 10- to 20-fold molar excess of sequence-specific anti-SAA_1 peptide $F(ab)_2$ or non-immune rabbit $F(ab)_2$ fragments. At the end of the incubation the culture supernatants were removed, rendered cell-free by centrifugation (3500 rpm, 4°C), and stored at −70°C until eicosanoid analysis.

2.4. Quantification of cyclooxygenase metabolites

After acidification to pH 3.2 and addition of $^{18}O_2$-labelled TXB_2 (prepared as described [25]), D_4-$PGF_{2\alpha}$, and D_4-PGE_2, respectively, eicosanoids were extracted from cell supernatants with diethyl ether and separated by silicic acid column chromatography [26]. TXB_2 (the stable hydrolysis product of TXA_2), PGE_2, and $PGF_{2\alpha}$ were analyzed as trimethyl silyl ether pentafluorobenzyl ester methoxime derivative by negative ion chemical ionization-gas chromatography/mass spectrometry chromatography (NICI-GC/MS) as described [27]. A Finnigan gas chromatograph 9619 coupled to a Finnigan 4500 mass spectrometer and an INCOS data system was used. Values for detection limits (5 to 20 pg of AA-metabolites measured by NICI-GC/MS) are based on a signal to noise ratio of 4:1. Interassay and intraassay coefficients of variations were 2.0 to 2.4 and 1.1 to 2.2%, respectively [26].

2.5. Electrophoresis, transfer to nitrocellulose and Ca^{2+}-binding properties

SDS-PAGE under non-reducing or reducing conditions was performed on 3.5–15% gradient slab gels at 180 V for 60 min using minigels (Bio-Rad) [23]. Analytical IEF was carried out as described previously [21]. Proteins were electrophoretically transferred to nitrocellulose membrane in 20 mM Tris-HCl (0.15 M glycine buffer, pH 8.4) for 90 min at 200 mA. Detection of Ca^{2+}-binding proteins/peptides on nitrocellulose membranes after SDS-PAGE and IEF or dot blot experiments were performed by washing membranes in imidazole buffer (10 mM imidazole/HCl, 60 mM KCl, 5 mM $MgCl_2$, pH 6.8) exactly as described [28]. Membranes were incubated in the same buffer containing 15–20 μCi $^{45}Ca^{2+}$ (Du Pont NEN)/ml prior to autoradiography.

3. Results

As rSAA might induce calcium mobilization in resting human monocytes [14] we initially analyzed whether the addition of SAA_1 to resting and stimulated monocytes affects eicosanoid production. Coincubation of resting cells with SAA_1 (3.15 to 200 μg/ml, 2–12 h) was without effects on secretion of TXB_2, PGE_2, and $PGF_{2\alpha}$. We therefore studied a possible involvement of SAA_1 during eicosanoid biosynthesis by stimulated cells. The major cyclooxygenase metabolite released from calcium ionophore-stimulated, platelet-depleted monocyte cultures after 4 h was TXB_2 (66.3 ± 28.2 ng/5×10^5 cells, n=12). Analysis of prostaglandins secreted into the cell medium revealed 0.83 ± 0.5 ng/5×10^5 cells and

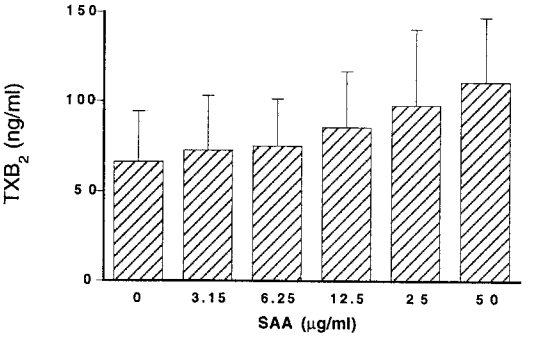

Fig. 1. Effects of SAA_1 on TXB_2 production by A_{23187}-stimulated human monocytes. Adherent human monocytes (5×10^5 cells) were incubated with increasing SAA_1 concentrations and stimulated with 2.5 μM calcium ionophore A_{23187} for 4 h in RPMI medium at 37°C. The cell supernatant was then harvested on ice, centrifuged at 3500 rpm and acidified to pH 3.2. After addition of stable isotope-labelled internal standards (50 ng/ml) eicosanoids were extracted, separated by silicic acid chromatography and TXB_2 was analyzed by NICI-GC/MS. Values represent mean ± S.D. of three experiments performed in triplicate determinations.

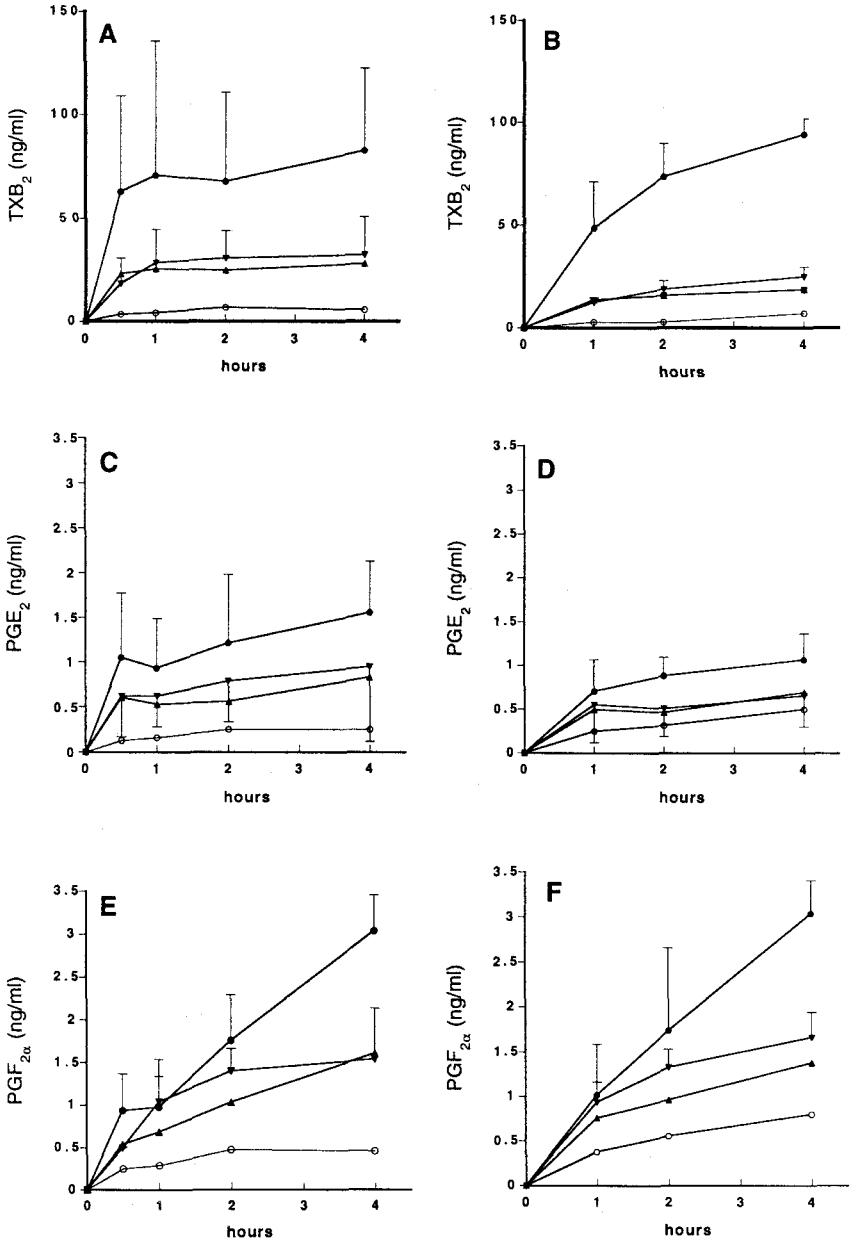

Fig. 2. Formation of cyclooxygenase metabolites by A_{23187}-stimulated monocytes in the presence of apolipoproteins. Adherent monocytes (5×10^5/ml) were stimulated with either 2.5 μM (A, C, E) or 0.5 μM (B, D, F) calcium ionophore A_{23187} (▲) in the presence of 25 μg SAA_1 (●) or apo A-I (▼) at 37°C for the indicated time intervals. Quantification of TXB_2 (A, B), PGE_2 (C, D), and $PGF_{2\alpha}$ (E, F) was performed by NICI-GC/MS. Values represent mean ± S.D. of four experiments performed in triplicate determinations. (○) refers to blank values, i.e. without stimulation by A_{23187}. For more clarity S.D. values are shown only for eicosanoid secretion in the presence of SAA_1 and apo A-I.

1.6 ± 0.3 ng/5×10^5 cells for PGE_2 and $PGF_{2\alpha}$, respectively. When monocytes were stimulated by 2.5 μM A_{23187} in the presence of SAA_1 (3.15 to 50 μg/ml) a dose-dependent formation of TXB_2 reaching levels from 60 ± 29 (3.15 μg/ml) to 110 ± 36 ng (50 μg/ml) after 4 h was estimated (Fig. 1). Similar effects were observed at SAA_1 concentrations up to 200 μg/ml and when lower concentrations of A_{23187} (0.5 μM) were used under the same assay conditions. In addition to TXB_2, also PGE_2 and $PGF_{2\alpha}$ concentrations were increased to a similar extent in A_{23187}-stimulated monocytes in the presence of SAA_1 (data not shown). SAA_1 concentrations higher than 200 μg/ml did not lead to a further increase of cyclooxygenase metabolites released.

In the next set of experiments the time-dependent formation of cyclooxygenase metabolites in the presence of either SAA_1 or apo A-I was investigated. From Fig. 2A it is evident that the formation of TXB_2 in activated cells reached a plateau value 1 h after activation (25.5 ± 9.6 ng/5×10^5 cells, 2.5 μM A_{23187}). Incubation of stimulated cells with SAA_1 led to a pronounced increase in TXB_2 release in between 2- and 3-fold (82.6 ± 39.9 ng/5×10^5 cells) after 4 h. To verify that the effect is specific for SAA, the same experiments were performed with purified apo A-I (a negative acute-phase reactant [29]) present almost exclusively on native HDL. From Fig. 2A it is evident that apo A-I was without any effect on the formation of TXB_2 under these experimental conditions. Stimu-

lation of monocytes with 0.5 μM A_{23187} led to almost identical results as observed with 2.5 μM A_{23187} (Fig. 2B).

In addition to TXB_2, we have also analyzed the time-dependent formation of PGE_2 (Fig. 2C and D) and $PGF_{2\alpha}$ (Fig. 2E and F) in A_{23187}- (2.5 and 0.5 μM) stimulated monocytes incubated in the presence of SAA_1 or apo A-I. Again, apo A-I was without effect on eicosanoid release, similar to data presented in Fig. 2A and B. PGE_2 release was, however, stimulated by SAA_1 (almost 3-fold, 0.6 vs. 1.6 ng/5×10^5 monocytes) (Fig. 2C and D). Finally, also $PGF_{2\alpha}$ secretion was enhanced in the presence of SAA_1. While we have observed $PGF_{2\alpha}$ concentrations of about 1.5 ng/5×10^5 in cells stimulated with A_{23187} or A_{23187} in the presence of apo A-I (Fig. 2E and F) $PGF_{2\alpha}$ concentrations secreted in the presence of SAA_1 were 3.0 ± 0.4 ng/5×10^5 cells. It is noteworthy that the secretion of cyclooxygenase metabolites was very similar during experiments when cells were stimulated with either 0.5 or 2.5 μM A_{23187} (compare Fig. 2A–F).

We further addressed the question whether the tetrapeptide Gly^{48}-Pro^{49}-Gly^{50}-Gly^{51} of human SAA_1 [19] might be the reason for enhanced formation of cyclooxygenase metabolites by activated monocytes. Therefore, A_{23187}-activated cells were coincubated with SAA_1 in the presence of $F(ab)_2$ fragments (raised against position 40–63 of human SAA_1) to block the proposed Ca^{2+} site in the molecule. Irrespective whether A_{23187}- (0.5 or 2.5 μM) activated cells were incubated in the presence of SAA_1 and $F(ab)_2$ fragments from either anti-SAA_1 peptide IgGs or non-immune IgGs (as a negative control) TXB_2 formation was nearly identical (Fig. 3). These data strongly suggest that the proposed Ca^{2+}-binding region is either not exposed on the protein surface or not responsible for enhanced formation of cyclooxygenase metabolites in the presence of SAA_1.

To clarify whether isolated SAA_1 is able to bind Ca^{2+} (either via the proposed Ca^{2+}-binding domain or another non-identified epitope) increasing SAA_1 concentrations were dotted on nitrocellulose and incubated with $^{45}Ca^{2+}$. It is evident from the autoradiograph (Fig. 4) that neither SAA_1 nor the corresponding peptide carrying the putative Ca^{2+}-binding domain did bind $^{45}Ca^{2+}$ even at highest protein/peptide concentrations used. As expected PLA_2 effectively bound $^{45}Ca^{2+}$ in a dose-dependent manner. The same results were obtained when purified SAA_1 or PLA_2 (1 to 20 μg) were subjected to SDS-PAGE (under reducing or non-reducing conditions) or IEF and then electrophoretically transferred to nitrocellulose prior to incubating membranes with $^{45}Ca^{2+}$.

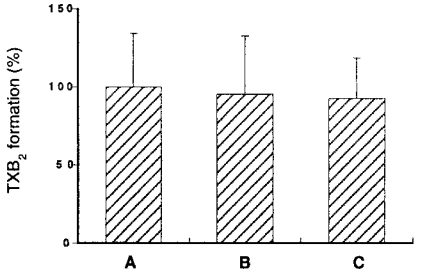

Fig. 3. Synthesis of TXB_2 by A_{23187}-stimulated monocytes in the presence of $F(ab)_2$ fragments. 5×10^5 monocytes/ml were stimulated with 2.5 μM calcium ionophore A_{23187} in the presence of 50 μg SAA_1 (A). SAA was coincubated with either a 10-fold molar excess of rabbit anti-SAA_1 peptide (40–63) $F(ab)_2$ (B) or non-immune rabbit $F(ab)_2$ fragments (C). Values represent mean ± S.D. of three experiments performed in duplicate determinations. TXB_2 concentrations in the presence of SAA_1 and A_{23187} are given as 100%.

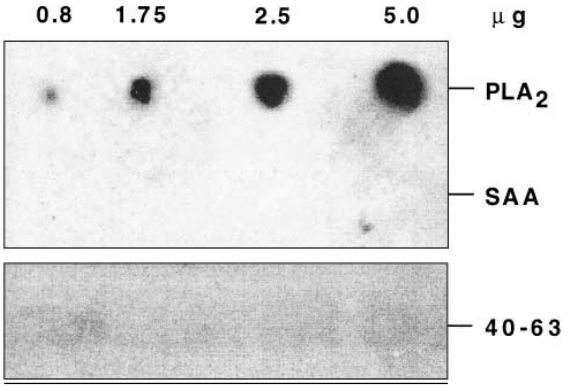

Fig. 4. $^{45}Ca^{2+}$-binding properties by dotted proteins/peptide. PLA_2, human SAA_1, and synthetic peptide (corresponding to amino acid residues 40–63 of human SAA_1) were dotted to nitrocellulose membranes at the indicated concentrations and incubated with radioactive Ca^{2+} as described in Section 2. Membrane bound ligands were visualized by autoradiography at −70°C.

4. Discussion

The presence of a-SAA mRNA in cells, e.g. endothelial cells, smooth muscle cells, and monocyte-derived macrophages [30,31], ultimately involved in the development of atherosclerosis, suggest a proinflammatory role of SAA in tissues. The major findings of the present study are that SAA_1 did not spontaneously induce eicosanoid secretion in resting monocytes. However, SAA_1 very efficiently enhanced biosynthesis of cyclooxygenase metabolites in stimulated monocytes up to 3-fold. Although a proposed Ca^{2+}-binding epitope (Gly^{48}-Pro^{49}-Gly^{50}-Gly^{51}) is present on all SAA isoforms in mammals [2], we presented two lines of evidence that SAA_1, the main representative of human acute-phase SAA, is not able to bind and/or transport Ca^{2+}-ions. First, the use of sequence-specific antibodies raised against the proposed Ca^{2+}-binding domain of SAA_1 failed to impair formation of cyclooxygenase metabolites; secondly, as demonstrated by autoradiography, human SAA_1 is not able to bind Ca^{2+}-ions.

Previous investigations by Badolato and coworkers [14] reported the rSAA (a protein homologous to the primary structure of human SAA_1 except for the addition of a methionine at the N-terminus) might induce Ca^{2+} mobilization in human monocytes. rSAA (at concentrations between 0.4 and 4 μM) caused an increase in cytoplasmic calcium concentrations in a dose-dependent manner similar to that when cells were activated by either FMLP or MCP-1, both chemoattractants. Findings that rSAA apparently does not induce Ca^{2+} mobilization from intracellular stores but more likely stimulates influx of extracellular Ca^{2+} present in the medium have prompted us to investigate the combined effects of A_{23187} and SAA_1 on eicosanoid biosynthesis by monocytes. During the present study we have observed enhanced formation of cyclooxygenase metabolites by activated monocytes in the presence of SAA_1 concentrations similar to those mentioned in [14] and corresponding to in vivo concentrations about 50- to 100-fold higher than baseline levels; levels reflecting acute-

phase conditions in various inflammatory disorders which are observed particularly early after the onset of the inflammatory event.

When occurring in vivo, enhanced TXA_2 biosynthesis by activated monocytes must be considered a potent mediator of inflammation involved in the regulation of platelet-vessel wall interactions and in the regulation of blood vessel tone and hemostasis [18,32]. Observations that apo A-I does not exert proinflammatory properties under the same conditions described for SAA_1 (Fig. 2) could be seen in context of apo A-I as an antiatherogenic apolipoprotein [33]. The inhibitory effect of HDL, recently reported to block the chemotactic effect of rSAA on monocytes in a dose-dependent manner [13], is currently being examined.

The mechanism of how SAA_1 may alter intracellular Ca^{2+} concentrations [14] and eicosanoid biosynthesis in further consequence (Figs. 1 and 2) is presently not clear. It is important to note that the acute-phase response and activation of protein kinase C (PKC) in neutrophils and lymphocytes, cells involved in the inflammatory response, are concurrent events. In a previous paper [34] it was reported that SAA_1 but not apo A-I could serve as a substrate for PKC in vitro; maximum phosphorylation required the presence of Ca^{2+}-ions. In addition, the inhibition of rSAA-induced chemotaxis of monocytes by staurosporine and H-7 seems to strengthen PKC-dependent mechanisms delivering chemotactic signals [14]. SAA_1 apparently induces calcium mobilization by activating a pertussis toxin-sensitive signalling pathway suggesting that SAA_1 could bind to transmembrane G protein-coupled cell surface receptors on monocytes. These receptors, however, appear to belong to a different class of chemotactic receptors known to bind RANTES, MIP-1α, or MCP-1 [14]. If SAA_1 activates a receptor that impairs adenylate cyclase and prevents the rise in cAMP via an inhibitory G protein [14], one could speculate that the low cAMP concentrations in resting/activated cells would not induce monocyte function per se but more likely enhance signal generation via PLC or PLA_2. From our studies we may hypothesize that SAA has an additive effect on calcium ionophore A_{23187}, which can activate also membrane bound PKC [35]. However, identification of a possible SAA-receptor on monocytes will clarify the underlying mechanisms of enhanced eicosanoid biosynthesis. As demonstrated by Walker and coworkers [36] the binding of intact HDL to a non-identified receptor on human fibroblasts very efficiently stimulated the activities of PLC and PLD, both enzymes involved in the intracellular signalling cascade.

In conclusion we have shown that SAA_1, a proinflammatory apolipoprotein present on acute-phase HDL in vivo, is able to enhance the formation and secretion of TXA_2, PGE_2, and $PGF_{2\alpha}$ from activated monocytes. However, enhanced biosynthesis and secretion of cyclooxygenase metabolites mediating cell-cell interaction in vivo is not mediated via Ca^{2+}-binding properties of SAA_1 but more likely the result of intracellular signalling pathways and requires further elucidation.

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