Apolipoprotein serum amyloid A down-regulates smooth-muscle cell lipid biosynthesis

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The addition of acute-phase apolipoprotein serum amyloid A (SAA) to cultured aortic smooth-muscle cells caused a decrease in the incorporation of [¹⁴C]acetate into lipids. Optimal inhibition of lipid biosynthesis was achieved with 2 μ M SAA, and the effect was maintained for up to 1 week when SAA was included in the culture medium. Lipid extracts were subjected to TLC and it was determined that the SAA-induced decrease in [¹⁴C]acetate incorporation into lipids was attributable to decreases in cholesterol, phospholipid and triglyceride levels. The accumulated mass of cholesterol and phospholipid in SAA-treated cultures was significantly less than that of controls, with no change in the accumulated protein. Moreover, SAA had no effect on either

protein synthesis or DNA synthesis, suggesting that SAA specifically alters lipid synthesis. By using a peptide corresponding to the cholesterol-binding domain of acute-phase SAA (amino acids 1–18), it was shown that this region of the molecule was as effective as the full-length protein in decreasing lipid synthesis and the accumulation of cholesterol and phospholipid. The implications of these findings for atherosclerosis and Alzheimer's disease are discussed.

Key words: atherosclerosis, cholesterol, fatty acids, inflammation, phospholipids.

INTRODUCTION

The serum amyloid A (SAA) proteins are a family of apolipoproteins that are up-regulated by inflammatory cytokines, including interleukin (IL)-1 and IL-6, which are major regulators of biochemical changes in the body when homeostasis has been disrupted [1,2]. The family of six known human isoforms is encoded by three genes (SAA₁, SAA₂ and SAA₃). During inflammation there are marked changes in the concentration of the acute-phase isoforms SAA₁ and SAA₂, but not of the constitutively expressed isoform, SAA₃ [3]. In addition, SAA₃, expressed in rabbit, hamster and mouse, is an acute-phase isoform but appears to be a pseudogene in human. The acute-phase isoforms are synthesized by the liver, in which maximal levels of SAA are achieved by a combination of IL-1 and IL-6 [4–6]. The extrahepatic synthesis of SAA₃ by synovial fibroblasts, macrophages and adipocytes [7–9] has also been documented. More recently, work in our laboratory demonstrated that, in response to IL-1 α alone, cultured aortic smooth muscle cells synthesize and secrete acute-phase SAA [10].

An acute-phase response including elevated plasma SAA levels in connection with myocardial infarction has been documented [11–19]. Evidence suggests that SAA is associated with the atherosclerotic plaque in mice [20–24] and humans [8,25,26]. Moreover, it has been shown that SAA accumulates in the brains of those afflicted with Alzheimer's disease, a condition in which chronic inflammation in the brain is thought to occur [27]. Although the regulation of the synthesis of SAA in response to inflammatory mediators has been the subject of many investigations, the function of this family of proteins remains speculative. It has been suggested that the association of SAA with high-density lipoprotein (HDL) might alter the metabolism of

the lipoprotein particle, thereby compromising its protective effect against atherosclerosis [28–32]. SAA was shown to decrease lecithin:cholesterol acyltransferase activity [32] and to enhance the activity of phospholipase A₂ [33]. Moreover, SAA might contribute to the decreased half-life of apolipoprotein A-I [34]. Mitchell et al. [7] reported that SAA is produced by rabbit synovial fibroblasts as an autocrine factor that stimulates collagenase and stromelysin. A variety of additional effects have been noted, including the inhibition of the oxidative burst by neutrophils [35] and others as reviewed by Husby et al. [3]. Our group has shown a potential role for SAA in intracellular lipid metabolism by studies in which acute-phase SAA bound and enhanced the uptake of cholesterol by HepG2 cells [36] and rabbit neonatal aortic smooth muscle cells [37].

Here we show that SAA down-regulates lipid biosynthesis in smooth muscle cells. Moreover, a synthetic peptide corresponding to the cholesterol-binding region of acute-phase SAA is as effective as the full-length molecule in decreasing the ability of the cells to synthesize lipids.

EXPERIMENTAL

Reagents

Recombinant SAA (SAA_p), purchased from PeproTech (Rocky Hill, NJ, U.S.A.), corresponds to acute-phase human SAA, as described previously [37]. Freeze-dried SAA_p was dissolved in water at 1 mg/ml and stored at –20 °C. The purity was confirmed by electrospray ionization MS (Boston University School of Medicine Mass Spectrometry Resource, Boston, MA, U.S.A.). Synthetic peptides corresponding to residues 1–18 of human SAA₁ (SAA₁ 1–18), the reverse peptide corresponding to residues

Abbreviations used: DMEM, supplemented Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HDL, high-density lipoprotein; IL, interleukin; LDL, low-density lipoprotein; LDS, lipoprotein-deficient serum; SAA, apolipoprotein serum amyloid A; SAA_p, recombinant human SAA; TCA, trichloroacetic acid.

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18–1 of human SAA₁ (SAA₁ 18–1) and residues 1–18 of human SAA₄ (SAA₄ 1–18) were synthesized and purified as described previously [37]. Freeze-dried peptides were dissolved in DMSO at 10 mg/ml and stored at –20 °C until use. A stock solution of cholesterol (10 mg/ml) was prepared in ethanol.

Isolation and culture of neonatal rabbit aortic smooth muscle cells

Neonatal rabbit aortic smooth muscle cells were isolated aseptically from the aortae of 3-day-old New Zealand White rabbits (Pine Acres Rabbitry, Brattleboro, VT, U.S.A.) as described previously [38]. The primary cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) (cat. no. 56-469-111; J.R.H. Biosciences, Lenexa, KS, U.S.A.) supplemented with 3.7 g/l NaHCO₃, 100 i.u./ml penicillin, 100 µg/ml streptomycin, 0.1 mM MEM non-essential amino acids (Gibco Laboratories, Grand Island, NY, U.S.A.), 1 mM MEM sodium pyruvate solution (Gibco Laboratories) and 20% (v/v) fetal bovine serum (FBS; Sigma Chemical Co) for 1 week, after which they were treated with 0.5% trypsin/0.02% EDTA (Gibco Laboratories) and plated in DMEM containing 10% (v/v) FBS at a density of 2.0×10^4 /cm². Studies were performed with cells in their second or third passage. Spent medium was replaced twice weekly.

Treatment of cells with free and SAA_p-bound cholesterol

Cholesterol bound to SAA_p was prepared by diluting free cholesterol and/or SAA_p in medium containing 10% (v/v) lipoprotein-deficient serum (LDS; prepared as described previously [39]) at 37 °C for 2 h (vehicle controls included the appropriate volumes of ethanol and/or water). Before the addition of the reagents, the media were removed and the cells were washed twice with Puck's saline [140 mM NaCl/5.4 mM KCl/1.1 mM KH₂PO₄/1.1 mM Na₂HPO₄/6.1 mM glucose (pH 7.4)]. The media containing the reagents were then added and incubated for 7 days. The media and reagents were replaced twice weekly. The cell cultures were radiolabelled with [¹⁴C]acetate to determine the levels of lipid biosynthesis as described below.

Treatment of cells with SAA_p and synthetic peptides

Before the addition of reagents to the cells, the media were removed and the cells were washed twice with Puck's saline. Media containing 10% (v/v) LDS were added to the cells and then SAA_p, synthetic peptides, BSA or vehicle control (water or DMSO) was added. In cultures treated for more than 3 days, the media were aspirated and the cells re-fed with fresh media plus reagents twice weekly, including the day before harvest in each case. After the indicated incubation, the biosynthesis of lipids, the accumulation of cholesterol, phospholipid or protein, the synthesis of proteins and the synthesis of DNA were measured as described below.

Incorporation of [¹⁴C]acetate into lipid

[¹⁴C]Acetate incorporation into lipid was determined in cells plated into multi-well tissue-culture trays (3.8 cm² per well). The cells were treated with reagents including SAA_p, synthetic peptides, BSA or vehicle control, as described above, for the indicated durations. Before harvest, sodium [1,2-¹⁴C]acetate (58.2 mCi/mmol; NEC-553, Dupont NEN Research Products, Boston, MA, U.S.A.), 0.5 µCi/ml of medium, was added to each well as indicated. At the time of harvest, the media were removed and the cells were washed with cold PBS containing 0.4% BSA followed by three washes with cold PBS. Lipids were extracted

with hexane/propan-2-ol (3:2, v/v) as described previously [38] and total lipid synthesis was determined by measuring an aliquot of the extract by liquid-scintillation spectrometry. Individual lipid classes were evaluated by drying the extracts under a stream of nitrogen, redissolving them in toluene and subjecting them to TLC. The plates were developed in hexane/diethyl ether/ acetic acid (70:30:1, by vol.). Staining with iodine was used to reveal the migration of standards, which allowed cutting and counting of the lipid classes including phospholipid (origin), cholesterol and triglyceride. In addition, cell layer protein was solubilized with 0.2 M NaOH and quantified by the procedure of Lowry et al. [40], with BSA as a standard. Results are expressed as total c.p.m. of [¹⁴C]acetate/mg of protein or as c.p.m. of [¹⁴C]acetate in phospholipid, triglyceride or cholesterol/mg of protein (means ± S.D.).

Measurement of total cholesterol accumulation

Total cholesterol levels were determined in cells plated on multi-well tissue-culture trays (3.8 cm² per well). The cells were treated with reagents including SAA_p, synthetic peptides, BSA or vehicle control, as described above, for the indicated durations. On the day of harvest, the cells were washed and extracted with hexane/propan-2-ol, as described above. Total cholesterol levels were measured with a fluorometric technique as previously described [39]. The hexane/propan-2-ol in the lipid extracts was removed by evaporation under a stream of N₂. The lipids were resolubilized in propan-2-ol; total cholesterol was determined by comparison with standard curves run with each assay. In addition, cell layer protein was solubilized with 0.2 M NaOH and quantified by the procedure of Lowry et al. [40], with BSA as a standard. Results are expressed as µg of cholesterol/mg of protein (means ± S.D.).

Measurement of total phospholipid accumulation

Total phospholipid levels were determined in cells plated into multi-well tissue-culture trays (3.8 cm² per well). The cells were treated with reagents including SAA_p, synthetic peptides, BSA or vehicle control as described above, for the indicated duration. On the day of harvest, the cells were washed and extracted with hexane/propan-2-ol as described above. An aliquot to be analysed was dried under a stream of N₂ and water/chloroform/methanol (1:2:1, by vol.) was added. After vortex-mixing, samples were incubated on ice for 15 min, after which they were subjected to centrifugation on a Microfuge for 10 min on high speed. The organic phase was transferred to Pyrex test tubes and the solution was dried under a stream of nitrogen. Total phospholipid levels were determined by measuring phosphorus with the technique of Svanborg and Svennerholm [41]. Essentially, total phosphorus was determined by comparison with standard curves by using KH₂PO₄ run with each assay. To each sample was added 0.5 ml of 4.5% HClO₄/27% H₂SO₄ and tubes were heated at 180 °C for 3 h. After cooling to room temperature, 5 ml of the colour reagent (a 10:1 dilution of solutions containing 2.5 mg/ml ammonium molybdate, 8.2 mg/ml sodium acetate and 100 mg/ml ascorbic acid respectively) was added and the tubes were incubated for 2 h at 37 °C. The absorbance was measured spectrophotometrically at 820 nm. The mass of phospholipid was estimated by multiplying the phosphorus content by 25, as described by Svanborg and Svennerholm [41]. In addition, cell layer protein was solubilized with 0.2 M NaOH and quantified by the procedure of Lowry et al. [40], with BSA as a standard. Results are expressed as µg of phospholipid/mg of protein (means ± S.D.).

Measurement of protein synthesis

Protein synthesis was determined in cells plated into multi-well tissue-culture trays (3.8 cm² per well). The cells were treated with reagents including SAA_p, BSA or vehicle control as described above, for the indicated duration. At 4 h before harvest, L-[3,4,5-³H]leucine (180 Ci/mmol; NET-460, Dupont NEN Research Products), 25 μ Ci/ml of media, was added. At the time of harvest, media were removed and PMSF and *p*-chloromercuribenzoate were added to final concentrations of 50 and 300 μ M respectively. The cell layers were washed three times with Puck's saline, water was added (0.5 ml per well) and the cells were scraped with a Teflon cell scraper. The cells were transferred to a tube and the wells were rewashed with a further 0.5 ml of water. The cell layers were then homogenized with a glass/glass homogenizer, followed by freeze-drying.

Trichloroacetic acid (TCA) was added to an aliquot of radiolabelled medium (0.5 ml) to achieve a final concentration of 10% (w/v); the reaction mixture was incubated on ice for 15 min, after which it was subjected to centrifugation in a Microfuge for 10 min on high speed. The supernatant was discarded and the pellet was washed twice with 10% (w/v) TCA diluted in PBS. The final pellet was dissolved in 0.5 ml of 0.1 M NaOH and the TCA-insoluble radioactivity was determined by liquid-scintillation spectrometry. The cell layers were reconstituted in 0.5 ml of PBS containing 3 mg/ml BSA; TCA was added to achieve a final concentration of 10% (w/v). The cell layers were then processed as described above for the culture medium. Results are expressed as c.p.m. in [³H]leucine in each well (means $\pm$ S.D.).

Analysis of incorporation of [³H]thymidine into DNA

Cells were plated into 96-well flat-bottomed tissue-culture plates in DMEM containing 10% (v/v) FBS. The cells were treated with reagents including SAA_p, BSA or vehicle control, as described above, for the indicated durations. On the day of harvest, [³H]thymidine (5 Ci/mmol; NET-027; Dupont NEN

Figure 1 SAA inhibits lipid synthesis in smooth muscle cells

Neonatal rabbit aortic smooth muscle cells were incubated in the presence or absence (control) of SAA_p [SAA; 2 μ M (24 μ g/ml)], cholesterol (Chol; 2 μ M), or cholesterol bound to SAA_p (SAA + Chol), as indicated in the Experimental section. After 7 days of incubation, the cells were radiolabelled with [¹⁴C]acetate and both lipids and proteins were extracted as described in the Experimental section. The amount of [¹⁴C]acetate incorporated into lipids was determined by liquid-scintillation counting; expressed as c.p.m./mg of protein (means $\pm$ S.D.). Statistical analysis of the data demonstrated that [¹⁴C]acetate incorporation in control cultures was significantly different from that of cultures treated with SAA_p, cholesterol and SAA_p plus cholesterol.

Figure 2 Effect of time and dose of SAA on the inhibition of lipid synthesis

Neonatal rabbit aortic smooth muscle cells were incubated in the presence or absence (0) of increasing concentrations of SAA_p as indicated [SAA; 1–4 μ M (12–48 μ g/ml)]. After the indicated duration of incubation, the cells were radiolabelled with [¹⁴C]acetate and both lipids and proteins were extracted as described in the Experimental section: (A) the amount of [¹⁴C]acetate incorporated into lipids was determined by liquid-scintillation counting, and expressed as c.p.m./mg of protein (means $\pm$ S.D.); (B) the amount of total protein per well was determined and expressed as mg (means $\pm$ S.D.). Statistical analysis of the data demonstrated that [¹⁴C]acetate incorporation in control cultures was significantly different from that of cultures treated with SAA_p (2 and 4 μ M) on days 4 and 7 (A) and that protein accumulation was not significantly different in control cultures and in SAA_p-treated cultures (B).

Research Products), 20 μ Ci/ml of medium, was added and the cells were radiolabelled for 4 h. Cells were harvested and the incorporation of [³H]thymidine into DNA was assessed as described previously [38]. Results are expressed as c.p.m. in [³H]thymidine in each well (means $\pm$ S.D.).

Statistical analysis

Experimental groups were compared by one and two-factor analysis of variance. Statistically significant differences of relevant comparisons are reported when $P < 0.05$ by post-hoc analysis with Fisher protected least-squares difference.

RESULTS

SAA decreases lipid biosynthesis in smooth muscle cells

Our observations that acute-phase SAA binds and transports cholesterol into aortic smooth muscle and HepG2 cells [36,37] suggest that SAA might have a role in cholesterol homeostasis during chronic inflammation in diseases including atherosclerosis

Table 1 SAA inhibits the synthesis of phospholipids, triglycerides and cholesterol

Neonatal rabbit aortic smooth-muscle cells were incubated in the presence or absence (control) of SAA_p [SAA; 2 μ M (24 μ g/ml)] and cells were radiolabelled with [¹⁴C]acetate for 24 h, after which both lipids and proteins were extracted as described in the Experimental section. The lipid extracts were subjected to TLC and the amounts of [¹⁴C]acetate incorporated into cholesterol, phospholipids and triglycerides were determined by liquid-scintillation counting; they are expressed as means $\pm$ S.D. A statistical analysis of the data demonstrated that [¹⁴C]acetate incorporation into cholesterol, phospholipids and triglycerides in control cultures was significantly different from that in SAA_p-treated cultures.

Sample	10 ⁻³ $\times$ [¹⁴ C]Acetate incorporated (c.p.m./mg of protein)		
	Cholesterol	Phospholipid	Triglyceride
Control	131.9 $\pm$ 33.9	346.9 $\pm$ 106.7	11.2 $\pm$ 4.7
SAA	79.2 $\pm$ 6.0	115.9 $\pm$ 22.5	3.4 $\pm$ 0.8

and Alzheimer's disease. This led us to consider the possibility that cholesterol transported into the cell via SAA might have the ability to down-regulate cholesterol biosynthesis, as does lipoprotein-bound cholesterol entering the cell via the low-density lipoprotein (LDL; apolipoprotein B/E) receptor [42]. To test this hypothesis, neonatal rabbit aortic smooth muscle cells were treated with cholesterol, either in its free form or bound to SAA_p. The cells were incubated in the presence of these reagents (or the appropriate vehicle control) for 7 days in medium containing 10% (v/v) LDS, after which the cells were radiolabelled with sodium [¹⁴C]acetate to measure lipid biosynthesis. Surprisingly, the data in Figure 1 show that the total radioactivity incorporated into lipids decreased in the presence of SAA_p whether or not it had been added in the presence of cholesterol, i.e. SAA_p alone was capable of down-regulating lipid synthesis.

In a study designed to determine the optimal dose of SAA_p, and the time course of SAA_p-induced down-regulation of lipid biosynthesis, smooth muscle cells were incubated with 1, 2 or 4 μ M SAA_p or under control conditions for up to 7 days. On the day of harvest, the cells were radiolabelled with [¹⁴C]acetate, the cell layers were harvested and lipid biosynthesis was measured by evaluating the amount of radioactivity incorporated into lipid. The data in Figure 2(A) show that treatment with 2 μ M SAA_p resulted in a significant decrease in [¹⁴C]acetate incorporation. Lipid synthesis in SAA_p-treated cultures was 39% and 49% of that in control cultures on days 4 and 7 respectively. The effect of the higher dose (4 μ M SAA_p) was approximately equivalent to that of 2 μ M SAA_p. Figure 2(B) shows that, despite the decrease in lipid synthesis, the total protein remaining in the cultures treated with SAA_p was equal to that in the control cultures, and that the expected accumulation of protein by the cultured cells during the duration of the experiment was not inhibited by SAA_p.

Synthesis of cholesterol as well as that of triglycerides and phospholipids is affected by SAA

To ascertain the contribution of various lipid classes to the SAA_p-induced effect, extracts of cells incubated in the presence or absence of SAA_p were subjected to TLC. Interestingly, the results in Table 1 show that the down-regulation of [¹⁴C]acetate incorporation into lipids was attributable to a decrease in triglyceride and phospholipid synthesis in addition to decreased synthesis of cholesterol.

Figure 3 SAA decreases accumulation of cholesterol and phospholipid

Neonatal rabbit aortic smooth muscle cells were incubated in the presence or absence (control) of SAA_p [SAA; 2 μ M (24 μ g/ml)] for 6 days, after which both lipids and proteins were extracted as described in the Experimental section; the amount of total cholesterol was determined and is expressed as μ g/mg of protein (means $\pm$ S.D.; hatched columns) and the amount of total phospholipid was determined and is expressed as mean $\times 10^{-1} \times (\mu$ g/mg of protein) (means $\pm$ S.D.; grey columns). A statistical analysis of the data demonstrated that both total cholesterol and total phospholipid accumulation in control cultures were significantly different from those in SAA_p-treated cultures.

Figure 4 SAA has no effect on protein synthesis

Neonatal rabbit aortic smooth muscle cells were incubated in the presence or absence (control) of SAA_p [SAA; 2 μ M (24 μ g/ml)] or BSA (24 μ g/ml) for 8 days, after which the cell cultures were radiolabelled with [³H]leucine as described in the Experimental section. The amounts of [³H]leucine incorporated into protein in the medium (hatched columns) and cell-layer (grey columns) fractions were determined and are expressed as c.p.m. per well (means $\pm$ S.D.). A statistical analysis of the data demonstrated that there were no significant differences between control and treated cultures.

To determine whether the SAA_p-induced decrease in lipid biosynthesis resulted in a decrease in cellular lipid mass, the cells were exposed to SAA_p for 6 days. In considering the results of these experiments, it is important to note that the confluent cultures accumulate a substantial amount of cellular cholesterol and phospholipid, so comparisons are always against the relatively large mass of lipid in control cultures. The data in Figure 3 demonstrate that despite this, the total in SAA_p-treated cultures was significantly lower than that in control cultures. The same cultures were also evaluated for total phospholipid: SAA_p-treated cells had significantly less phospholipid than the control cultures.

Figure 5 SAA has no effect on DNA synthesis

Neonatal rabbit aortic smooth muscle cells were incubated in the presence or absence (control) of SAA_p [SAA; 2 μ M (24 μ g/ml)] or BSA (24 μ g/ml) for 4 days (hatched columns) or 8 days (grey columns) as indicated, after which the cell cultures were radiolabelled with [³H]thymidine as described in the Experimental section. The amount of [³H]thymidine incorporated into DNA at each time point was determined and is expressed as c.p.m. per well (means $\pm$ S.D.). A statistical analysis of the data demonstrated that there were no significant differences between control and treated cultures.

SAA has no effect on synthesis of protein or DNA

To establish that acute-phase SAA specifically altered lipid biosynthesis, protein synthesis and DNA synthesis were measured after incubation with the reagents. The effect of SAA_p on protein synthesis was ascertained by evaluating the incorporation of [³H]leucine into TCA-precipitable radioactivity in the media and cell-layer fractions of cultures that had been incubated with the reagents for 8 days and then radiolabelled with [³H]leucine for 4 h before harvest. When compared with control (vehicle-treated) or BSA-treated cultures, the data in Figure 4 show that protein synthesis remained unchanged in cells that had been treated with SAA_p.

To assess the effect of SAA_p on the rate of cell proliferation, the cells incubated in its presence were radiolabelled with [³H]thymidine and its incorporation into DNA was analysed as described in the Experimental section. The data in Figure 5 demonstrate that exposure to SAA_p for either 4 or 8 days had no effect on this parameter in that [³H]thymidine incorporation into DNA was unchanged in comparison with control or BSA-treated cultures.

The cholesterol-binding region of acute-phase SAA is responsible for the decrease in lipid biosynthesis

In previous studies we reported that SAA_p binds and enhances cholesterol uptake into HepG2 and aortic smooth muscle cells via the N-terminal region corresponding to the first 18 amino acid residues of the protein [36,37]. The ability to bind cholesterol was unique to the acute-phase isoforms of SAA (SAA₁ and SAA₂); in other words, the constitutive isoform (SAA₄) did not display the ability to transport cholesterol into cells. To determine whether the lipid-lowering effect of SAA_p was dependent on its ability to bind cholesterol, the effect of a synthetic peptide corresponding to residues 1–18 of acute-phase SAA was evaluated for its effect on lipid biosynthesis. Two additional peptides were tested, including one in which residues 1–18 of acute-phase SAA were placed in the reverse order (i.e. 18–1) and a peptide corresponding to residues 1–18 of SAA₄. The peptides were introduced in DMSO (as opposed to H₂O for the full-length

Figure 6 Residues 1–18 of acute-phase SAA inhibit lipid synthesis

Neonatal rabbit aortic smooth muscle cells were incubated in the presence or absence (water or DMSO as vehicle controls) of SAA_p [SAA; 2 μ M (24 μ g/ml)], BSA (24 μ g/ml), the synthetic peptide corresponding to residues 1–18 of human SAA₁ [SAA₁ 1–18; 2 μ M (4 μ g/ml)], the peptide synthesized in reverse, i.e. residues 18–1 of human SAA₁ [SAA₁ 18–1; 2 μ M (4 μ g/ml)] or the synthetic peptide corresponding to residues 1–18 of human SAA₄ [SAA₄ 1–18; 2 μ M (4 μ g/ml)] for 8 days, after which the cells were radiolabelled with [¹⁴C]acetate and both lipids and proteins were extracted as described in the Experimental section. Upper panel: the amount of [¹⁴C]acetate incorporated into lipids was determined by liquid-scintillation counting and is expressed as c.p.m./mg of protein (means $\pm$ S.D.). Lower panel: the amount of total protein per well was determined and is expressed as mg (means $\pm$ S.D.). A statistical analysis of the data demonstrated that [¹⁴C]acetate incorporation in control (water-treated) cultures was significantly different from that of SAA_p-treated cultures and [¹⁴C]acetate incorporation in control (DMSO-treated) cultures was significantly different from that of SAA₁ 1–18-treated cultures (upper panel), and that protein accumulation was not significantly different in control cultures and in treated cultures (lower panel).

SAA_p), so an additional vehicle control was therefore included. Neonatal aortic smooth muscle cells were incubated with the reagents for 8 days, after which the cultures were radiolabelled for 4 h with [¹⁴C]acetate and its incorporation into total lipids was measured. The data in Figure 6 (upper panel) confirm that the radioactivity in [¹⁴C]acetate incorporated/mg of protein in cultures incubated with full-length SAA_p was significantly less than in cultures incubated with either vehicle alone or BSA. The data also show that when peptides corresponding to residues 1–18 of acute-phase SAA (SAA₁ 1–18) were used, lipid biosynthesis was similarly down-regulated; however, neither the reverse peptide (SAA₁ 18–1) nor the peptide corresponding to residues 1–18 of constitutive SAA (SAA₄ 1–18) was active. Once again, comparisons of the mass of total protein in all treatment groups showed no statistically significant differences (Figure 6, lower panel).

Both total cholesterol and total phospholipid were also analysed; the data in Figure 7 show that the decrease in lipid

Figure 7 Residues 1–18 of acute-phase SAA decrease the accumulation of cholesterol and phospholipid

Neonatal rabbit aortic smooth muscle cells were incubated in the presence or absence (water or DMSO as vehicle controls) of SAA_p [SAA; 2 μ M (24 μ g/ml)], BSA (24 μ g/ml), the synthetic peptide corresponding to residues 1–18 of human SAA₁ [SAA₁ 1–18; 2 μ M (4 μ g/ml)], the peptide synthesized in reverse, i.e. residues 18–1 of human SAA₁ [SAA₁ 18–1; 2 μ M (4 μ g/ml)] or the synthetic peptide corresponding to residues 1–18 of human SAA₄ [SAA₄ 1–18; 2 μ M (4 μ g/ml)] for 8 days, after which both lipids and proteins were extracted as described in the Experimental section. Upper panel: the amount of total cholesterol was determined and is expressed as μ g/mg of protein (means $\pm$ S.D.). Lower panel: the amount of total phospholipid was determined and is expressed as μ g/mg of protein (means $\pm$ S.D.). A statistical analysis of the data demonstrated that the accumulation of both total cholesterol (upper panel) and total phospholipid (lower panel) in control (water-treated) cultures was significantly different from that in SAA_p-treated cultures, and that control (DMSO-treated) cultures were significantly different from SAA₁ 1–18-treated cultures.

biosynthesis resulted in an equivalent and significant diminution in the mass of both cholesterol (Figure 7, upper panel) and phospholipid (Figure 7, lower panel) in cultures treated with SAA_p and SAA₁ 1–18.

DISCUSSION

The data reported here demonstrate that SAA inhibits lipid synthesis in aortic smooth muscle cells and that these metabolic changes might be of consequence during the acute-phase response and/or situations in which chronic inflammation persists. During an acute-phase response, plasma levels of SAA rise markedly and can reach more than 1000 μ g/ml. The levels attained in the atherosclerotic plaque have not been determined but localized concentrations in chronically affected areas are likely to be substantially lower than those achieved during an acute-phase response. The demonstration that as little as 2 μ M SAA (24 μ g/ml) effectively down-regulates lipid synthesis suggests that this effect is potentially physiologically relevant.

The alteration in lipid biosynthesis results in a significant decrease in the mass of both cholesterol and phospholipid in the cell cultures. Frank toxicity was not evident from measurements of DNA synthesis as well as protein synthesis and accumulated total protein. As previous work demonstrated that the proliferative rate of the cells diminishes as the cultures become confluent and form multilayers [39], the rate of DNA synthesis was low in the confluent cultures examined in these studies. It would be of interest to determine whether factors related to the proliferation and/or differentiation of smooth muscle cells have any effect on the SAA-induced decrease in lipid biosynthesis. Such studies could shed additional light on the potential for SAA-induced changes in atherosclerosis, a disease associated with the migration of smooth muscle cells from the medial layer of the blood vessel to its intima, a process accompanied by phenotypic changes in the cell [43–47]. Several reports have suggested that SAA is associated with the atherosclerotic plaque [9,20–26]. It has been speculated that SAA might appear at the site of lesion development in atherosclerosis via plasma-derived HDL or LDL [25,26]. Although SAA is most often found associated with HDL in plasma, there is evidence to suggest that it can be dissociated from HDL in tissues [48]. Several important functional studies of SAA have addressed the role of non-lipid-bound SAA on the chemotaxis of monocytes, neutrophils and T-lymphocytes [49–52]; these authors discuss the possibility that local increases in SAA at the site of inflammation could form a gradient of the free protein. Our previously reported results demonstrating that IL-1 α induces SAA₃ synthesis and secretion by cultured aortic smooth muscle cells [10] suggest (1) that an inflammatory response to injury in the vasculature can lead to the local synthesis of SAA, which might be at least partly responsible for its accumulation at the site of lesion development, and (2) that as a result of local synthesis, SAA is potentially available in the smooth muscle cell microenvironment in a free, non-lipoprotein-bound form. It is also worthy of note that cultured neonatal aortic smooth muscle cells have been shown to assemble an apolipoprotein-E-containing HDL particle in the culture medium [53]. It is therefore likely that at least some of the exogenously added SAA was associated with lipoproteins in the medium and was therefore lipid-bound during the time course of the experiments described here. Comparisons of lipid biosynthesis in the presence of free SAA and of SAA bound to HDL will address the question of whether conformational changes might alter the ability of SAA to down-regulate lipid biosynthesis in smooth muscle cells and whether circulating and/or locally available SAA in the vessel wall can modify lipid homeostasis.

Of particular interest is consideration of the finding that the peptide corresponding to residues 1–18 of the acute-phase isoforms of SAA was capable of down-regulating lipid biosynthesis as effectively as the full-length protein. Previous comparisons showed that differences in amino acid composition between SAA₁ and SAA₄ in this region account for the ability of the acute-phase isoform to bind cholesterol, whereas the constitutive isoform cannot [37]. As it is this portion of SAA that is responsible for binding and enhancing the uptake of cholesterol into smooth muscle cells [36,37], these results show clearly that the ability to bind cholesterol is critical to the processes involved in regulating lipid synthesis. Because the addition of exogenous cholesterol is not a requirement for the SAA-induced decrease in lipid biosynthesis (see Figure 1), the process must be dependent on the trafficking of endogenously synthesized cholesterol. Cholesterol homeostasis in cells is critical in that excess accumulation can cause cell death and deposition in arteries leading to atherosclerosis; however, many cellular functions are dependent on it. Studies of the regulation of lipid metabolism in other cell

systems led to the identification of a novel family of transcription factors known as the sterol-response-element-binding proteins (SREBPs) whose function is dependent on the cholesterol status of cells [54]. Target genes containing sterol response elements (SREs) to which SREBPs bind and activate transcription include those associated with cholesterol biosynthesis, cholesterol uptake via lipoproteins, and fatty acid synthesis [54–56]. The ability of SAA to contribute to changes in SREBP-mediated smooth muscle cell gene transcription via alterations in its ability to transport cholesterol into the cells is the subject of current investigation.

Finally, recent novel findings have shown that patients afflicted with Alzheimer's disease accumulate acute-phase SAA isoforms in the brain [27]. The decrease in lipid synthesis by SAA suggests a link between the documentation of an inflammatory response associated with the illness and the demonstration that the brains of patients with Alzheimer's disease have decreased levels of cholesterol and phospholipid [57,58].

In summary, the results in this paper demonstrate that acute-phase SAA down-regulates lipid biosynthesis in cultured aortic smooth muscle cells in a time- and dose-dependent manner. Moreover, the mass of both cholesterol and phospholipid decreases in SAA-treated cells. Finally, it appears that the ability of SAA to bind and transport cholesterol is critical to its capability of inducing changes in lipid homeostasis.

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