PRODUCTION OF SERUM AMYLOID A IN RESPONSE TO INFLAMMATORY CYTOKINES BY HUMAN ADIPOCYTES

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Serum amyloid A (SAA) is a major acute-phase protein in humans, and elevated plasma levels represent a risk factor for cardiovascular diseases. SAA was thought to be produced by hepatocytes only in response to inflammatory stimuli; moreover, recent studies have shown that adipose tissue can secrete several proinflammatory factors. Therefore, we investigated whether cells in adipose tissue can synthesize SAA in response to inflammatory stimuli. Adipocytes and preadipocytes isolated from abdominal adipose tissue were incubated with IL-1, IL-6, TNF-α, LPS, or resistin at different concentrations. After 48 hours, the supernatants were analyzed by ELISAs for human SAA. Preadipocytes did not show any production in SAA. In contrast, in adipocytes, incubation with TNF-α led to a significant increase in SAA production, peaking after LPS or resistin (~3 times greater *vs* unstimulated adipocytes). The greatest increase in SAA occurred with all stimuli combined (~5 times greater *vs* control cells). Subsequently, we investigated whether treatment with some drugs could modulate SAA production in adipocytes, and observed that fluvastatin led to a significant inhibition of SAA release, whereas a larger modulation of SAA release was observed after treatment with troglitazone or aspirin. These results show for the first time that human adipocytes, and not preadipocytes, can produce SAA in response to inflammatory cytokines and that this process can be modulated.

Obesity, the most common nutritional disorder in industrial countries, is associated with increased cardiovascular mortality and morbidity (1-3). Adipose tissue secretes various bioactive substances, generally referred to as adipocytokines, including interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), leptin, C-reactive protein (CRP), adiponectin, and resistin, that may contribute to obesity-linked metabolic and vascular diseases (4-5).

Serum amyloid A (SAA) is a family of closely related proteins discovered in the 1970s as the circulating precursor of AA amyloid fibrils (6). It is an important acute-phase protein that may increase in levels up to 1000-fold in response to inflammatory stimulus (7).

At the beginning, SAA was thought to be synthesized in human hepatocytes in response to, cytokines in particular, such as interleukin-1β (IL-1β), IL-6, TNF-α, but also to microbial infection, tissue injury, autoimmune disorders, stress, and other inflammatory stimuli (8). Subsequently, the extra-hepatic synthesis of SAA was found to occur under similar proinflammatory conditions (9-10). Moreover, SAA has also been found in human

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atherosclerotic plaques, suggesting a potential role for this protein in atherosclerosis (11). Elevated levels of SAA have been found in subjects at risk for developing future coronary heart disease, as well as in patients with coronary and peripheral vascular disease (12).

Recent studies have shown that SAA is expressed by subcutaneous white adipose tissue, and its production at this site is regulated by nutritional status or by an anti-inflammatory drug (13-14). However, it is not clear which are the stimuli able to induce SAA in adipose tissue and how this expression can be modulated.

Accordingly, in the present study, we investigated whether SAA is produced by cells present in adipose tissue, such as preadipocytes and mature adipocytes, in response to inflammatory stimuli and whether some drugs with anti-inflammatory actions are able to inhibit this synthesis.

MATERIALS AND METHODS

Cell isolation and culture

Primary cultures of human adipocytes and preadipocytes were performed as described previously (15).

Adipose tissue samples were obtained from different patients (n=20), who gave informed consent for the study, undergoing a mastectomy and subsequent autologous breast reconstruction with a transverse rectus abdominis myocutaneous (TRAM) flap, which uses lower abdominal skin and fat. This procedure produces redundant abdominal skin and fat (~10- to 50-gram), which would normally be discarded. Each experiment was conducted using cells from a different donor. Adipose tissue was removed with sterile instruments and preadipocytes and adipocytes were obtained as previously described (15).

In the case of the adipocytes, 1 ml of the suspension of freshly obtained adipose cells was placed in each tube containing specific adipocyte medium (Zen-Bio, Inc., Research Triangle Park, NC), and the cells were incubated under the conditions required for each particular experiment.

The preadipocytes were plated and incubated for 24 h to allow the attachment and multiplication of the cells and after 24 h the medium was removed and replaced with the specific preadipocyte medium (Zen-Bio, Inc., Research Triangle Park, NC). In order to obtain a pure preadipocyte population by negative selection, the preadipocytes were subcultured. Preadipocytes were used at passages 2 to 3 and incubated under the conditions required for each particular experiment.

Serum amyloid A protein assay

The SAA levels in the cell supernatants were measured by using a commercial ELISA kit specific for human SAA (Anogen Laboratories, Mississauga, Ontario, Canada) according to the manufacturer's directions. The minimum concentration detected by the assay was 0.6 ng/mL. All experiments were performed in duplicate. Adipocytes were cultured in tubes, and cells from a different donor were used for each experiment. Preadipocytes were cultured in 6-well plates until they reached 80%-90% confluency. The cells were then incubated for 48 h with recombinant human IL-1β (25 ng/mL), recombinant human IL-6 (10 ng/mL), recombinant human TNF-α (50 ng/mL), lipopolysaccharide (LPS) derived from *Escherichia coli* O113:H10 (1000 EU/mL), or human recombinant resistin (100 ng/ml).

In order to mimic a more pathophysiological state when more than a single stimulus is present, we also performed experiments using several combinations of the different cytokines to reach concentrations similar to that found in human circulation.

For the modulation experiments, at the same time of stimulation, cells were incubated with vehicle (DMSO), or several anti-inflammatory agents separately such as: troglitazone, a member of the drug class of the thiazolidinediones, agonist of proliferator-activated receptors (PPARs), a group of transcription factors regulating the expression of genes involved in lipid, carbohydrate and protein metabolism (10 μM); aspirin or salicylic acid that modulates signaling through NF-kB, a transcription factor that plays a central role in inflammation (5 μM); fluvastatin, a member of a class of medication called HMG-CoA reductase inhibitors that not only works by reducing the production of cholesterol by the liver, but also has pleotropic anti-inflammatory effects (5 μM); fenofibrate, an agonist of PPAR type α (10 μM); or 15-deoxy-d12,14-prostaglandin J2 (15d-PGJ2), a natural ligand of PPAR type γ (10 μM).

Doses were chosen on the basis of findings from previous experiments (15). After 48 hours, the culture supernatants were concentrated (about 10-fold) by using centrifugal filter units and assayed to determine the SAA levels. The overall inter-assay and intra-assay coefficients of variation of the ELISA kit used were 3.0% and 6.0% respectively.

Statistical analysis

Data are presented as the mean ± SD. The data was analyzed using one-way ANOVA followed by the Scheffe test for multiple comparisons. Statistical significance was indicated at the level $P<0.05$.

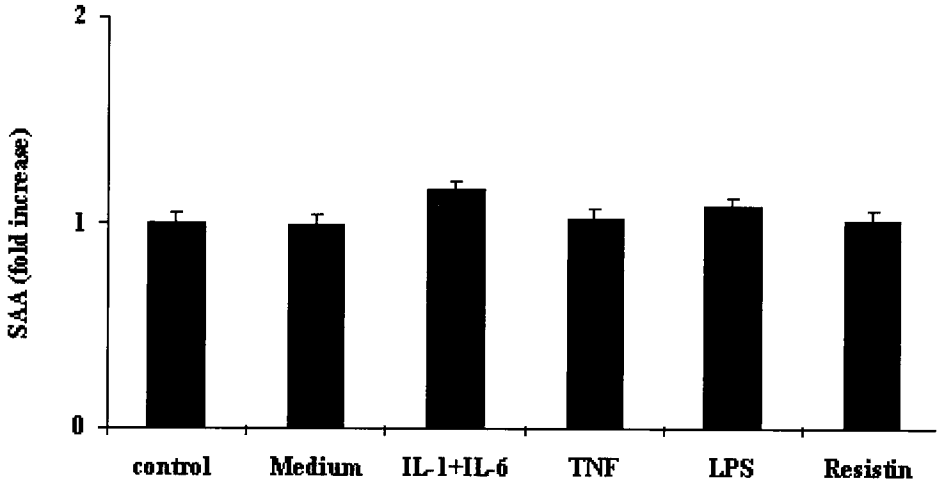

Fig. 1. *SAA production in human preadipocytes. Effect of cytokines, LPS, or resistin on SAA protein production in human preadipocytes. Neither incubation with TNF-α, LPS, or resistin alone, nor the combination of IL-1β and IL-6 led to a statistically significant increase in SAA production compared with unstimulated cells (control).*

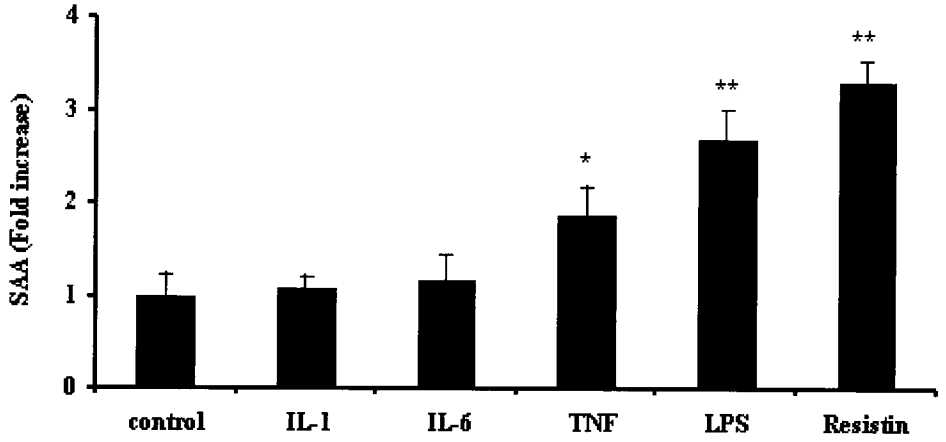

Fig. 2. *Effect of cytokines, LPS, or resistin on SAA protein production in human adipocytes. Shown are the results of two experiments in which human adipocytes were incubated with different stimuli for 48 hours and the supernatants were then concentrated and analyzed for SAA. Values are expressed as the fold increase in the level compared with the level in untreated cells, and each bar represents the mean±SD of duplicate determinations. *P<0.05 vs. untreated cells; **P<0.01 vs. untreated cells.*

RESULTS

SAA production by preadipocytes

We studied SAA production by human preadipocytes following treatment with inflammatory cytokines from two patients. As shown in Fig. 1, neither incubation with TNF-α, LPS, or resistin alone, nor the combination of IL-1β and IL-6 led to a statistically significant increase in SAA production compared with unstimulated cells.

SAA production by adipocytes

We next studied the production of SAA under similar circumstances in human adipocytes, and these results are shown in Figs. 2 and 3. As shown in Fig. 2, incubation of adipocytes with IL-1β and IL-6 alone led to a small but not statistically significant increase in SAA production compared with unstimulated cells.

In contrast, incubation with TNF-α led to a significant increase in SAA production (~2 times

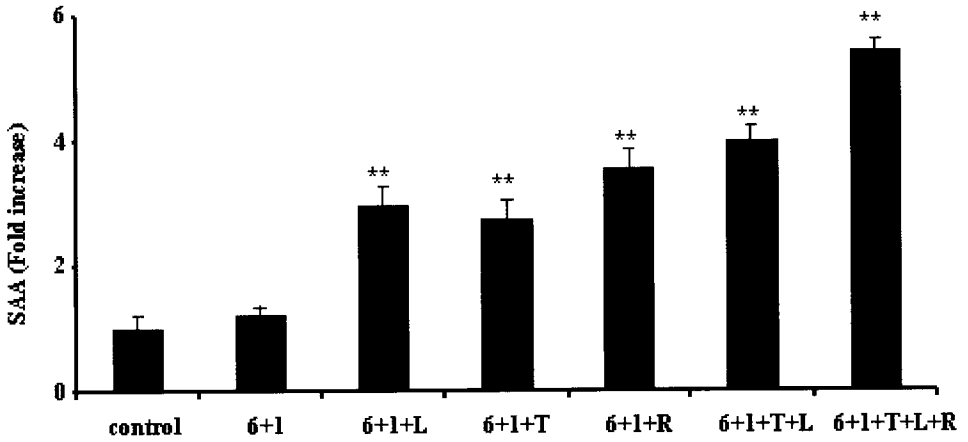

Fig. 3. *Effect of the combination of cytokines, LPS, or resistin on SAA protein production in human adipocytes. Effect of the combination of IL-6 (6), IL-1β (1), LPS (L), TNF-α (T), and resistin (R) on SAA protein production in human adipocytes. Shown are the results of two experiments in which human adipocytes were incubated with several combinations of the different cytokines for 48 hours and the supernatants were then concentrated and analyzed for SAA. Values are expressed as the fold increase in the level compared with the level in untreated cells, and each bar represents the mean±SD of duplicate determinations. *P<0.05 vs. untreated cells; **P<0.01 vs. untreated cells.*

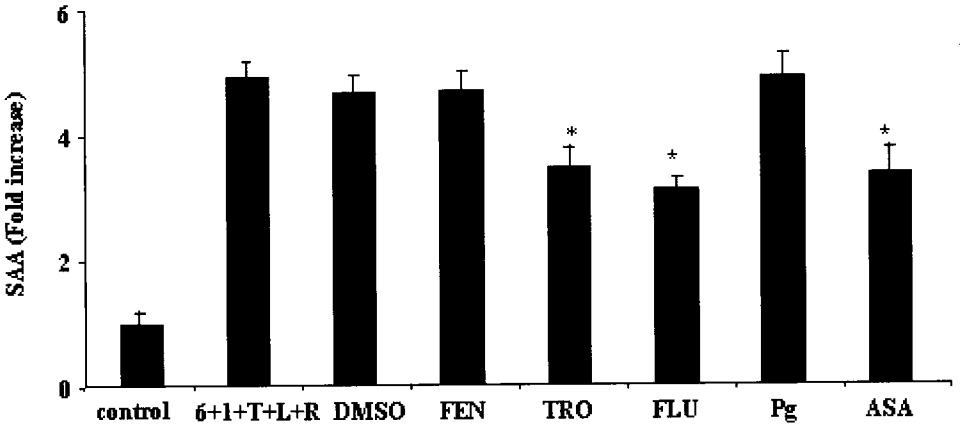

Fig. 4. *Modulation of SAA production in human adipocytes. Modulation of SAA synthesis in human adipocytes treated with medium control DMSO (control), fenofibrate (FEN), troglitazone (TRO), fluvastatin (FLU), 15d-PGJ2 (Pg), or aspirin (ASA). Shown are two experiments in which human adipocytes were treated with various drugs and incubated with combinations of IL-6 (6), IL-1β (1), LPS (L), TNF-α (T), and resistin (R) for 48 hours. The supernatants were then concentrated and analyzed for SAA. Values are expressed as the fold increase in the level compared with the level in untreated cells, and each bar represents the mean±SD of duplicate determinations. *P<0.05 vs. combination of cytokines.*

greater than that in control adipocytes, $P<0.05$). Finally, a maximal increase in SAA production was observed after incubation with either LPS or resistin (~3 times greater than that in control adipocytes, $P<0.01$). We then analyzed the effect of different combinations of the cytokines, the results of which are shown in Fig. 3. The combination of IL-1β and IL-6 still did not induce significant SAA production; moreover, the response to these two cytokines in combination with LPS, TNF-α, or resistin did not differ from the response to the three alone.

A small increase in SAA production was observed

when LPS and TNF-α together were added to the combination of the two cytokines (~3 times greater than that in unstimulated adipocytes, $P<0.01$). In contrast, the biggest increase in SAA released by the human adipocytes occurred in cells exposed to all stimuli combined (~5 times greater than that in control cells, $P<0.01$).

Modulation of SAA production by adipocytes

Previous studies have shown that weight loss and rosiglitazone treatment decrease adipose SAA expression and secretion as well as circulating SAA levels (13-14, 16-18).

However, little is known about the effects of other cardiovascular drugs on SAA production. We therefore investigated whether treatment with some of the cardiovascular drugs could modulate SAA production by human adipocytes in response to inflammatory cytokines.

Fig. 4 shows the effect of treatment for 48 h with 15d-PGJ2 and troglitazone, two different PPAR-γ agonists, fluvastatin, fenofibrate, and aspirin on the production of SAA in human adipocytes (n=2).

Treatment with 15d-PGJ2 and fenofibrate did not affect SAA production compared with the production in the stimulated cells not treated with drugs. In contrast, treatment with troglitazone or aspirin led to a significant, but not complete, inhibition of SAA release from adipocytes ($P<0.05$).

Moreover, larger, but still un-complete, modulation of SAA release from adipocytes was observed after treatment with fluvastatin.

DISCUSSION

In the present study, we showed for the first time the production of SAA by human adipocytes isolated from adipose tissue in response to inflammatory cytokines, and that this phenomenon may be modulated by several anti-inflammatory drugs, thereby confirming a new link between SAA, obesity and vascular inflammation in humans.

In contrast, preadipocytes did not show any SAA production in response to inflammatory stimuli, probably due to different maturation status of the cells.

Until recently, the adipocyte was largely thought to be an inert storage cell. It is now clear that adipocytes have a more complex role in the organism, producing a large number of hormones, peptides, and smaller molecules that affect metabolism and cardiovascular function (5). Of these, the most important substances are leptin (19), adiponectin and resistin (20-21).

In addition, obese individuals have high circulating levels of a range of inflammatory markers produced by adipose tissue, including TNF-α, IL-1, and IL-6 (22-23). Interestingly, these inflammatory molecules are the ones responsible for both the hepatic and extrahepatic production of SAA (8-10).

In addition, *in vivo* and *in vitro* studies performed in rodents have showed that proinflammatory cytokines, as IL-1β, TNF-α and LPS, can induce SAA expression in adipose tissue (24). More recently, Poitou et al. found the overexpression of SAA mRNA isoforms in human subcutaneous white adipose tissue by healthy, morbidly obese subjects. They next observed that circulating SAA levels significantly reduced after a short period of very-low-calorie diet, showing a regulation of SAA production by the nutritional status of the individual (13). However, nothing is known about which are, in humans, the stimuli able to induce an overexpression of SAA in adipose tissue. Here we found that mature adipocytes isolated from human adipose tissue produced SAA due to inflammatory cytokines such as TNF-α, LPS, or resistin but not to IL-1β, IL-6, or these two cytokines together. Our experiments showed that the various stimuli alone, in various combinations, or all together, incrementally induced SAA production.

In 2006, Yang et al. confirmed the predominant SAA expression in human adipose tissue, more specifically in adipocytes, and the reduction of serum SAA levels, after weight loss with a hypocaloric diet program. In addition, they treated obese non-diabetic individuals with rosiglitazone, a drug with an anti-inflammatory action, and they observed a reduction in adipose SAA secretion and in serum SAA levels (14).

This interesting paper has some important differences with the present: first of all, they did not test the effects of rosiglitazone at cellular level, therefore they could not define if the effects of rosiglitazone were direct or mediated by different molecules. In the second place, they excluded other classes of drugs with well known anti-inflammatory

properties.

In a previous work we showed the production of CRP by human adipocytes in response to similar inflammatory stimuli, and the reduction of CRP levels after incubation with anti-inflammatory drugs, such as aspirin, troglitazone and fluvastatin (15). These represent a group of drugs that, due to their pleiotropic effects, are indispensable for the prevention and the treatment of cardiometabolic diseases.

In a similar way, in this report, we show that cellular incubation with anti-inflammatory drugs, leads to reduction, but not complete inhibition of SAA release from adipocytes. This might explain in part the beneficial cardiovascular effects of these drugs.

In conclusion, our study demonstrates that human adipocytes can produce SAA under the stimulation of several proinflammatory cytokines; moreover, SAA production may be modulated by selected pharmacological intervention.

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