

# Serum amyloid A3 expression is stimulated by dexamethasone and interleukin-6 in 3T3-L1 adipocytes

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## Abstract

A chronic increase in systemic levels of acute-phase reactants contributes to the development of insulin resistance and associated disorders such as cardiovascular disease. Recently, serum amyloid A3 (SAA3) has been characterized as an adipocyte-secreted acute-phase reactant, expression of which is dramatically increased in insulin resistance and obesity. To further clarify expression and regulation of this adipocytokine in fat, SAA3 mRNA was measured by quantitative real-time reverse transcriptase PCR during differentiation of 3T3-L1 adipocytes and after treatment with various hormones known to induce insulin resistance and contribute to atherosclerosis. SAA3 mRNA was dramatically induced up to 77-fold during differentiation of 3T3-L1 preadipocytes. Furthermore, 100 nM dexa-

methasone and 30 ng/ml interleukin (IL)-6 induced SAA3 mRNA by up to 11- and 4.8-fold, respectively, in a time-dependent fashion with significant stimulation observed at concentrations as low as 10 nM dexamethasone and 1 ng/ml IL-6. In contrast, insulin, isoproterenol and growth hormone did not influence SAA3 synthesis. Inhibitor studies suggested that the positive effect of IL-6 on SAA3 expression is at least in part mediated by Janus kinase 2. Taken together, our results show a differential regulation of SAA3 by glucocorticoids and IL-6 supporting an integrative role of this acute-phase reactant in the pathogenesis of insulin resistance and its link to obesity and cardiovascular disease.

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## Introduction

Insulin resistance and type 2 diabetes are associated with obesity and cardiovascular disease. During the last couple of years a better understanding of the connection between increased adiposity on the one hand and impaired insulin sensitivity and cardiovascular disease on the other hand has been obtained (Fasshauer & Paschke 2003). In particular, the central role of fat cell metabolism and secretion on whole-body glucose homeostasis and endothelial function has been well established. Thus, adipocytes secrete various proteins, including tumor necrosis factor (TNF)  $\alpha$ , interleukin (IL)-6 and resistin, which are upregulated in insulin resistance and profoundly impair insulin sensitivity (Fasshauer & Paschke 2003). Furthermore, the adipocytokine adiponectin has been established as both an endogenous insulin sensitizer and a protector of endothelial function diminishing the development of atherosclerosis (Kubota *et al.* 2002). Moreover, various acute-phase reactants produced in fat cells and implicated in cardiovascular disease such as serum amyloid A3 (SAA3),  $\alpha$ 1-acid glycoprotein, plasminogen activator inhibitor-1

and fibrinogen are chronically increased in insulin resistance (Pickup *et al.* 1997). Recently, a report by Lin and co-workers (Lin *et al.* 2001) demonstrated that SAA3 is strongly upregulated in adipose tissue of obese mice as compared with lean controls and that white adipose tissue is a major source of this adipocyte-secreted protein. Furthermore, the authors demonstrated that SAA3 is stimulated by TNF $\alpha$  which has been shown to induce insulin resistance *in vivo* and *in vitro* (Lin *et al.* 2001). Thus, the data accumulated so far suggest that SAA3 is not only a well-known acute-phase reactant but also an adipocyte-secreted factor which might provide a molecular link between insulin resistance, obesity and cardiovascular disease. Therefore, we tested in the current study whether other hormones besides TNF $\alpha$  known to induce insulin resistance might alter SAA3 expression in 3T3-L1 adipocytes *in vitro*. We demonstrate for the first time that the glucocorticoid dexamethasone and the cytokine IL-6 potently induce SAA3 mRNA. Furthermore, we present evidence that the stimulatory effect of IL-6 is mediated via Janus kinase (Jak) 2.

## Materials and Methods

### Materials

Cell-culture reagents were from Life Technologies (Grand Island, NY, USA), oligonucleotides were obtained from MWG-Biotech (Ebersberg, Germany) and AG490, LY294002, PD98059 and SB203580 were from Calbiochem (Bad Soden, Germany). All pharmacological inhibitors were used at concentrations that have been shown to potently inhibit the respective signaling molecule in 3T3-L1 adipocytes (Fasshauer *et al.* 2004a, M Fasshauer, U Lossner & R Paschke, unpublished observations). Dexamethasone, growth hormone (GH), IL-6, insulin, isobutylmethylxanthine, isoproterenol and TNF $\alpha$  were obtained from Sigma Chemical Co. (St Louis, MO, USA).

### Culture and differentiation of 3T3-L1 cells

3T3-L1 cells (American Type Culture Collection, Rockville, MD, USA) were differentiated as described previously (Fasshauer *et al.* 2001). In brief, confluent preadipocytes were cultured for 3 days in Dulbecco's modified Eagles medium containing 25 mM glucose (DMEM-H), 10% fetal bovine serum and antibiotics (culture medium) further supplemented with 1  $\mu$ M insulin, 0.5 mM isobutylmethylxanthine and 0.1  $\mu$ M dexamethasone and an additional 3 days in culture medium with 1  $\mu$ M insulin. After another 3–6 days in culture medium about 95% of the cells showed accumulation of fat droplets. All treatments were carried out after serum starvation as further detailed in the figure legends.

### Analysis of SAA3 mRNA synthesis

Expression of SAA3 mRNA was determined by quantitative real-time reverse transcriptase (RT) PCR in a Taqman fluorescence temperature cycler (Applied Biosystems, Darmstadt, Germany) as described previously (Fasshauer *et al.* 2004b). Briefly, after total RNA was isolated from 3T3-L1 adipocytes with TRIzol (Life Technologies), 1  $\mu$ g RNA was reverse-transcribed using standard reagents (Life Technologies). Ten per cent of each RT reaction was amplified in a 26  $\mu$ l PCR using the Brilliant SYBR Green QPCR Core Reagent Kit from Stratagene (La Jolla, CA, USA) according to the manufacturer's instructions. Samples were incubated in the Taqman for an initial denaturation at 95 °C for 10 min after which 40 PCR cycles were performed, each cycle consisting of 95 °C for 15 s, 60 °C for 1 min and 72 °C for 1 min. The following primers were used: SAA3 (accession no. NM\_011315), 5'-GTTACACGGGACATGGAGCAGAGGA-3' (sense) and 5'-GCAGGCCAGCAGGT CGGAAGTG-3' (antisense); 36B4 (NM007475) 5'-AAGCGGTCCTGGCATTGTCT-3' (sense) and 5'-CCGCAGGGGCAGCAGTGGT-3' (antisense). SYBR

Green I fluorescence emissions were monitored after each cycle and expression of SAA3 and 36B4 mRNA was quantified by the second derivative maximum method of the Taqman software (Applied Biosystems). This method determines the crossing points of individual samples by an algorithm identifying the first turning point of the fluorescence curve. SAA3 expression was calculated relative to 36B4 which was used as an internal control due to its resistance to hormonal regulation (Laborda 1991). Amplification of specific transcripts was confirmed by melting curve profiles (cooling the sample to 68 °C and heating slowly to 95 °C with measurement of fluorescence) at the end of each PCR.

The specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis and restriction analysis (data not shown).

### Statistical analysis

Results are shown as means  $\pm$  S.E. Differences between various treatments were analysed by unpaired Student's *t*-tests.  $P < 0.01$  was considered highly significant and  $P < 0.05$  significant. The number of independent single experiments performed is indicated in the figure legends.

## Results

### Quantification of SAA3 mRNA levels in 3T3-L1 fat cells

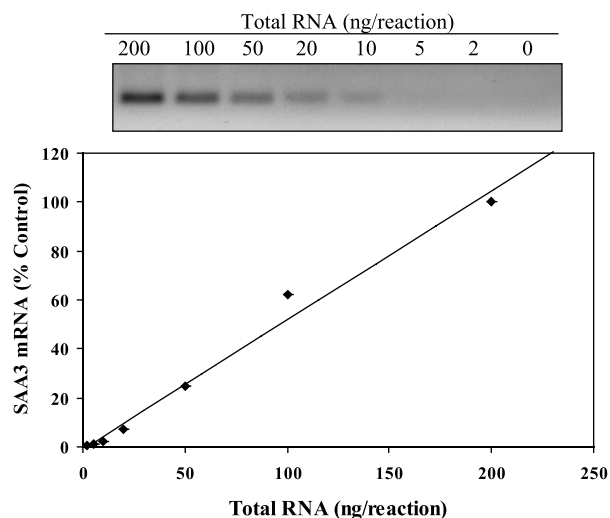
First, the reliability of the quantitative real-time RT-PCR method was tested. For this purpose, increasing amounts of total cellular RNA were reverse-transcribed and quantified using specific primer pairs for SAA3. As shown in Fig. 1, linearity between total RNA used per reaction and amount of mRNA measured by the Taqman software was obtained between 2 and 200 ng total RNA.

### SAA3 mRNA is induced during differentiation

Expression of SAA3 mRNA during differentiation of 3T3-L1 cells was determined. The acute-phase reactant was strongly induced 77-fold on day 3, 63-fold on day 6 and 57-fold on day 9 of differentiation as compared with undifferentiated preadipocytes (day 0;  $P < 0.01$ ; Fig. 2).

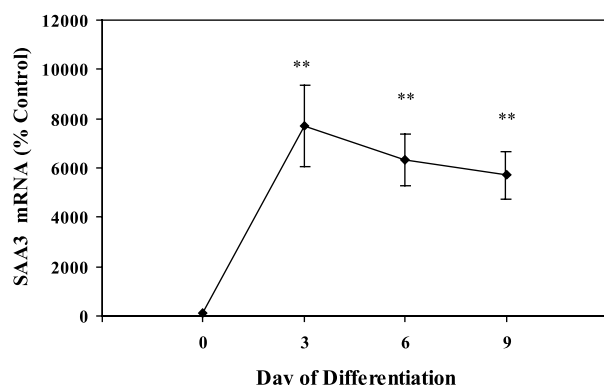
### Dexamethasone and IL-6 are novel stimulators of SAA3 synthesis

Various hormones inducing insulin resistance *in vivo* and *in vitro* were tested concerning regulation of SAA3. Treatment of 3T3-L1 adipocytes with 100 nM dexamethasone for 16 h significantly induced SAA3 expression 11-fold ( $P < 0.01$ ; Fig. 3). Furthermore, 30 ng/ml IL-6 significantly induced SAA3 mRNA synthesis 2.7-fold ( $P < 0.01$ ; Fig. 3). In contrast, insulin, isoproterenol and GH did not significantly influence expression of SAA3 (Fig. 3).

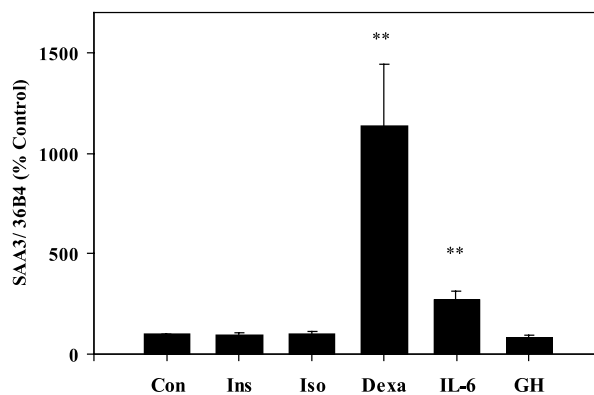


**Figure 1** Quantification of SAA3 mRNA in 3T3-L1 fat cells. Increasing amounts of total RNA from differentiated 3T3-L1 adipocytes were subjected to quantitative real-time RT-PCR with primers specific for SAA3 as described in the Materials and Methods section. Data are shown relative to mRNA levels measured with 200 ng RNA (=100%). Results are the means from three independent experiments. Top panel, agarose gel electrophoresis of the PCR product at cycle 31.

Dexamethasone induced SAA3 time-dependently with maximal 9.1-fold upregulation detectable after 8 h of treatment and significant stimulation persisting for up to 24 h ( $P<0.05$ ; Fig. 4A). Moreover, stimulation of SAA3 was dose-dependent with significant 3.3-fold induction first seen at 10 nM effector and a maximal 9.8-fold increase at 1000 nM dexamethasone ( $P<0.05$ ; Fig. 4D). IL-6 (30 ng/ml) significantly activated SAA3 expression in



**Figure 2** SAA3 expression during differentiation. Confluent preadipocytes (day 0) were induced to differentiate and, at the days indicated, total RNA was subjected to quantitative real-time RT-PCR. SAA3 mRNA expression is presented relative to preadipocytes on day 0 (=100%). Results are the means  $\pm$  s.e. from four independent experiments.  $**P<0.01$ , comparing confluent cells (day 0) with differentiating adipocytes (days 3, 6 and 9).

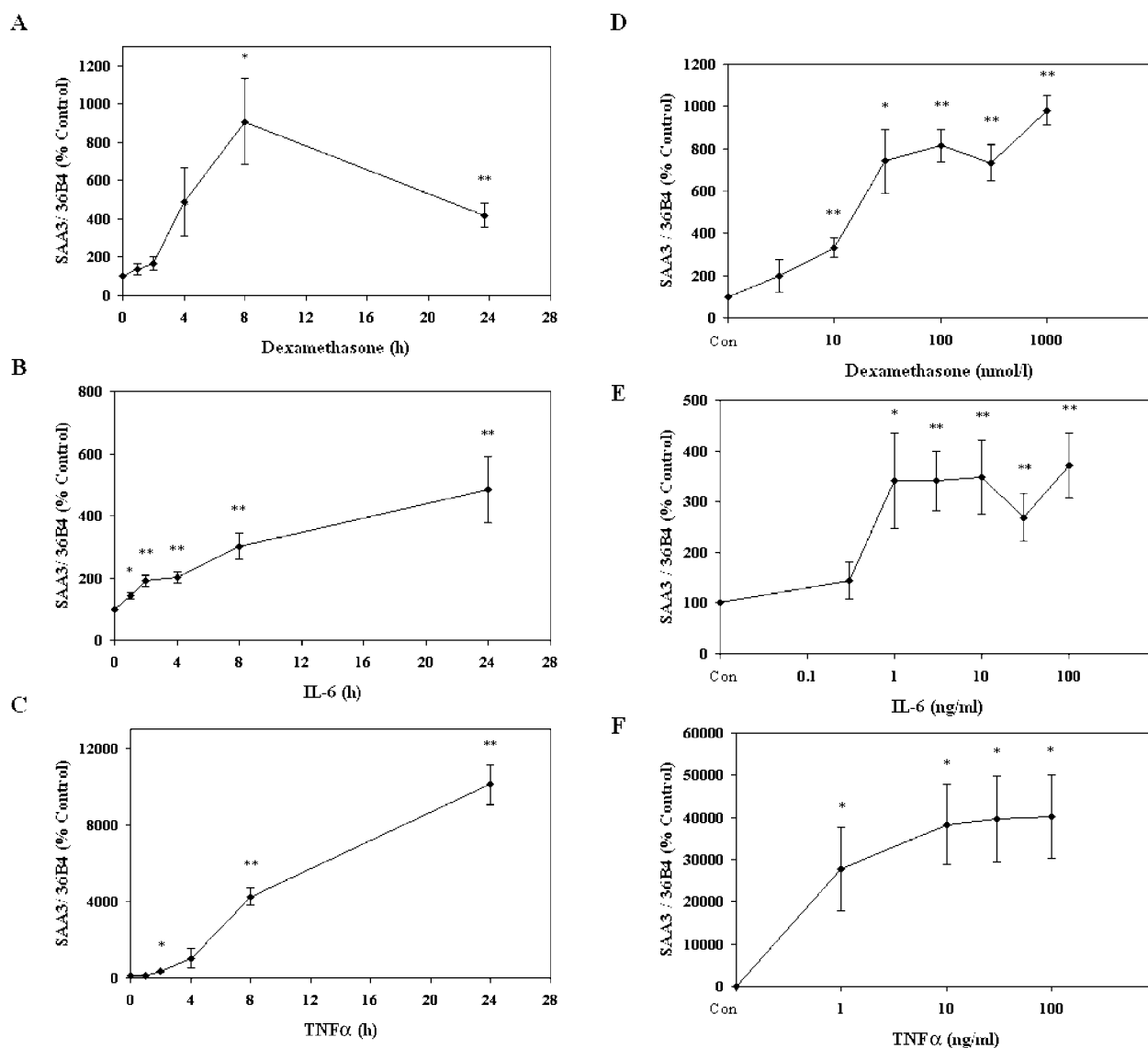


**Figure 3** SAA3 mRNA synthesis is stimulated by dexamethasone and IL-6. Fully differentiated 3T3-L1 cells were serum deprived for 16 h before 100 nM insulin (Ins), 10  $\mu$ M isoproterenol (Iso), 100 nM dexamethasone (Dexa), 30 ng/ml IL-6 and 500 ng/ml GH were added for 16 h. After total RNA isolation, quantitative real-time RT-PCR was performed determining SAA3 mRNA levels normalized to 36B4 relative to untreated control (Con) cells (=100%) as described in the Materials and Methods section. Results are the means  $\pm$  s.e. from at least three independent experiments.  $**$ Highly significant ( $P<0.01$ ) differences comparing hormone-treated with non-treated cells.

a time-dependent manner with 1.4-fold stimulation observed as early as 1 h after IL-6 addition and maximal 4.8-fold induction after 24 h of treatment ( $P<0.05$ ; Fig. 4B). Furthermore, IL-6 induced SAA3 mRNA more than 3-fold at concentrations as low as 1 ng/ml ( $P<0.05$ ; Fig. 4E). Moreover, TNF $\alpha$  stimulated SAA3 expression in a time- and dose-dependent fashion with significant induction seen as early as 2 h after effector addition ( $P<0.05$ ; Fig. 4C) and at concentrations as low as 1 ng/ml TNF $\alpha$  ( $P<0.05$ ; Fig. 4F).

#### *The stimulatory effect of IL-6 on SAA3 synthesis is mediated via Jak2*

Finally, we tested which molecules implicated in IL-6-signaling potentially mediate induction of SAA3 synthesis. To this end, 3T3-L1 fat cells were pretreated with specific pharmacological inhibitors of Jak2 (AG490; 10  $\mu$ M), p44/42 mitogen-activated protein kinase (MAP kinase; PD98059; 50  $\mu$ M), p38 MAP kinase (SB203580; 20  $\mu$ M) or phosphoinositide 3-kinase (PI 3-kinase; LY294002; 10  $\mu$ M) for 1 h before 30 ng/ml IL-6 was added for 16 h. Treatment of 3T3-L1 adipocytes with PD98059 and LY294002 alone significantly increased basal SAA3 expression ( $P<0.01$ ) whereas AG490 and SB203580 did not have any effect (Fig. 5). Again, SAA3 synthesis significantly increased 4.2-fold after IL-6 addition ( $P<0.01$ ; Fig. 5). This induction was significantly blunted in cells pretreated with the Jak2 inhibitor AG490 to 167% of control levels ( $P<0.05$ ; Fig. 5). In contrast, addition of the p44/42 MAP kinase inhibitor PD98059 and the PI



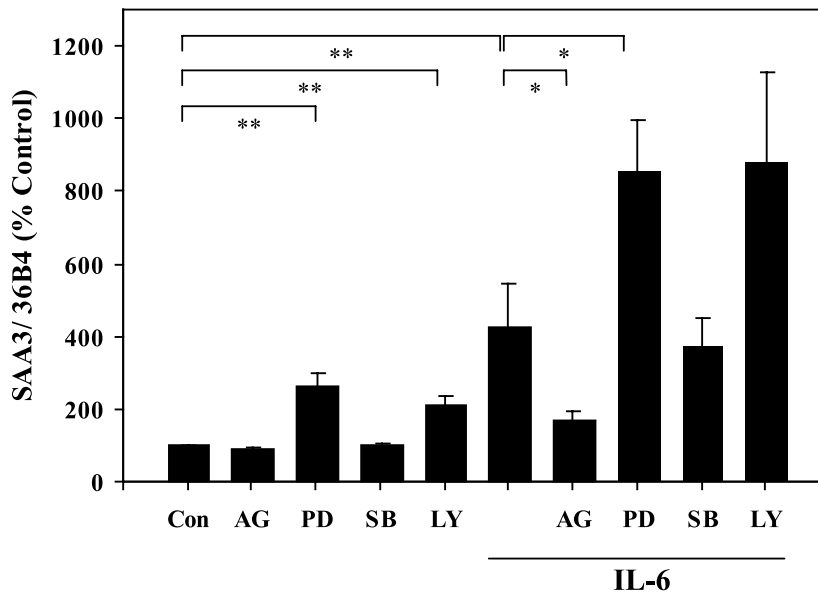
**Figure 4** Dose- and time-dependent upregulation of SAA3 expression by dexamethasone, IL-6 and TNF $\alpha$ . 3T3-L1 cells were serum starved for 16 h (A–C) or 6 h (D–F) before (A) 100 nM dexamethasone, (B) 30 ng/ml IL-6 and (C) 20 ng/ml TNF $\alpha$  were added for the indicated periods of time or the indicated concentrations of (D) dexamethasone, (E) IL-6 and (F) TNF $\alpha$  were added for 16 h. Total RNA was extracted and subjected to quantitative real-time RT-PCR determining SAA3 mRNA levels normalized to 36B4 as described in the Materials and Methods section. Data are expressed relative to untreated control (Con) cells (=100%). Results are the means  $\pm$  s.e. from at least three independent experiments. \*\*Highly significant ( $P < 0.01$ ), \*significant ( $P < 0.05$ ) differences comparing hormone-treated with non-treated cells.

3-kinase inhibitor LY294002 augmented IL-6-induced SAA3 expression; however, only in the case of PD98059 was statistical significance reached ( $P < 0.05$ ; Fig. 5). SB203580-preincubation did not significantly influence IL-6-induced SAA3 expression (Fig. 5).

## Discussion

During the last couple of years, it has been suggested that an ongoing cytokine-mediated acute-phase response

initiated by the body's innate immune system might be an important contributor to insulin resistance and its link to other accompanying disease states including obesity and atherosclerosis (Pickup & Crook 1998). Although the liver has been conventionally considered as the major site of many acute-phase reactants, cytokine production by fat tissue has become a focus of current research since fat cells themselves and macrophages infiltrating adipose tissue produce various proinflammatory cytokines including TNF $\alpha$ , IL-6, monocyte chemoattractant protein 1 and



**Figure 5** Signaling molecules mediating induction of SAA3 by IL-6. After serum starvation for 5 h, 3T3-L1 adipocytes were cultured in the presence or absence of AG490 (AG; 10  $\mu$ M), PD98059 (PD; 50  $\mu$ M), SB203580 (SB; 20  $\mu$ M) or LY294002 (LY; 10  $\mu$ M) for 1 h before 30 ng/ml IL-6 was added for 16 h. After RNA extraction and reverse transcription, quantitative real-time RT-PCR was performed to determine SAA3 normalized to 36B4 as described in the Materials and Methods section. Data are expressed relative to non-treated control (Con) cells (=100%) and the results are the means  $\pm$  s.e. from three independent experiments. \*\* $P$ <0.01, \* $P$ <0.05 comparing effector-treated with non-treated and inhibitor-pretreated adipocytes.

SAA3 (Weisberg *et al.* 2003, Wellen & Hotamisligil 2003, Xu *et al.* 2003). Among these, SAA3 is a member of a family of at least four highly homologous high-density lipoprotein (HDL) apoproteins actively transcribed in mice. In contrast to SAA1, SAA2 and SAA5, SAA3 mRNA is expressed in many tissues including fat, liver and macrophages (Meek *et al.* 1992). All SAA family members share an amphipathic helical structure which appears essential for their lipid-associating character. The function of adipocyte-derived SAA3 and other SAA family members has become more evident in recent years. Thus they displace apolipoproteins A1 and E from HDL, thereby increasing HDL binding to macrophages and impairing HDL clearance in the liver (Meek *et al.* 1992). Therefore, SAA proteins may contribute to dyslipidemia associated with insulin resistance and promote cardiovascular disease. In agreement with this notion, increased SAA levels at baseline predict an increased risk of patients suffering from future coronary artery occlusion (Ridker 1999). In a recent study, SAA3 was shown to be upregulated in obesity *in vivo* and by TNF $\alpha$  and lipopolysaccharide in adipocytes *in vitro* (Lin *et al.* 2001).

Glucocorticoids have been demonstrated to cause insulin resistance *in vivo* (Andrews & Walker 1999). In the current study, we show for the first time that dexamethasone potently induces SAA3 expression in 3T3-L1

adipocytes *in vitro*. These findings indicate that upregulation of SAA3 may be one mechanism by which impaired insulin sensitivity in hypercortisolism is linked to obesity and increased cardiovascular risk. However, glucocorticoid-induced downregulation of the endogenous insulin sensitizer and vascular protector adiponectin in fat cells probably also contributes (Fasshauer *et al.* 2002). *In vivo* data from patients with Cushing's syndrome will be helpful to further define the role of SAA3 and adiponectin in glucocorticoid-induced insulin resistance, adiposity and endothelial dysfunction. Furthermore, our data indicate that despite their profound anti-inflammatory effects used in clinical medicine, glucocorticoids are able to differentially regulate acute-phase mediators. Thus, SAA3 expression is induced by dexamethasone whereas the major proinflammatory cytokine IL-6 is, in fact, downregulated (Fasshauer *et al.* 2003a).

IL-6 has been implicated as an important proinflammatory cytokine, plasma concentrations of which correlate with the development of insulin resistance and cardiovascular disease (Tsigos *et al.* 1997, Pradhan *et al.* 2001). Administration of recombinant IL-6 in rodent models and humans *in vivo* induces hepatic gluconeogenesis which, in turn, leads to hyperglycemia and compensatory hyperinsulinemia (Stith & Luo 1994, Tsigos *et al.* 1997). In the current study, we demonstrate for the first time that SAA3

is upregulated by IL-6 *in vitro* in 3T3-L1 fat cells. Therefore, our data suggest that increased levels of this acute-phase reactant may contribute to IL-6-induced insulin resistance and vascular dysfunction besides other well-studied mechanisms including induction of IL-6 itself (Fasshauer *et al.* 2003a) and suppression of adiponectin (Fasshauer *et al.* 2003b). IL-6 induces homodimerization of its receptor gp130 at the plasma membrane, thereby activating associated kinases such as Jaks and Tyk2 which phosphorylate the cytoplasmic tail of gp130 (Heinrich *et al.* 1998). In the current study, we show that pharmacological inhibition of Jak2 by AG490 significantly reverses stimulation of SAA3 mRNA by IL-6 without affecting basal SAA3 synthesis. Therefore, these data support Jak2 as a principal positive mediator of IL-6-induced SAA3 gene expression. Stat-1 and -3, and SH2-domain-containing tyrosine phosphatase (SHP) 2 bind to tyrosine-phosphorylated gp130 and stimulate downstream signaling proteins including p44/42 MAP kinase, p38 MAP kinase and PI 3-kinase (Heinrich *et al.* 1998). Since pharmacological inhibition of p44/42 MAP kinase and PI 3-kinase by PD98059 and LY294002, respectively, increases basal and IL-6-induced SAA3 synthesis, both signaling proteins might be negative mediators of SAA3 mRNA expression. In contrast, p38 MAP kinase is probably not involved in basal and IL-6-induced SAA3 synthesis.

Furthermore, TNF $\alpha$  is closely linked to obesity-associated insulin resistance and cardiovascular disease (Hotamisligil *et al.* 1993). Consistent with previous data (Lin *et al.* 2001) we find strong stimulation of SAA3 by TNF $\alpha$  in the current study further supporting the notion that SAA3 might be an important mediator of cardiovascular disease and insulin resistance in states of increased TNF $\alpha$  levels such as obesity. In contrast, we do not find an effect of insulin, GH or the  $\beta$ -adrenergic agonist isoproterenol on SAA3 expression in 3T3-L1 adipocytes *in vitro*, pointing to the fact that this acute-phase reactant is not involved in insulin resistance and endothelial dysfunction induced by these hormones (Reaven *et al.* 1996, Gerich 1998, Frank 2001).

Taken together, we demonstrate for the first time that glucocorticoids and IL-6, beside TNF $\alpha$  and lipopolysaccharide, are potent stimulators of SAA3 expression in 3T3-L1 adipocytes *in vitro*. Furthermore, we present evidence that the positive effect of IL-6 is mediated via Jak2. These data indicate that stimulation of SAA3 expression in fat is a selectively regulated mechanism that might constitute an important element in the pathogenesis of insulin resistance, obesity and associated cardiovascular disease.

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