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 dexamethasone 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.


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 secretory on whole-body glucose homeostasis and endothelial function has been well established. Thus, adipocytes secrete various proteins, including tumor necrosis factor (TNF) α, 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), α1-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 TNFα 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 TNFα 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 (Fasshauer et al. 2004a, M Fasshauer, U Lossner & R. Paschke, unpublished observations). Dexamethasone, growth hormone (GH), IL-6, insulin, isobutylmethylxanthine, isoproterenol and TNFα 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 μM insulin, 0.5 mM isobutylmethylxanthine and 0.1 μM dexamethasone and an additional 3 days in culture medium with 1 μM 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 μg RNA was reverse-transcribed 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’-GTTCACGGGACATGAGCGAGAGGA-3’ (sense) and 5’-GCAGGCCCAGAGGTCCGAAGTG-3’ (antisense); 36B4 (NM007475) 5’-AAGCAGCTCTGGCATTGCT-3’ (sense) and 5’-CCGCAGGGCCAGCAGTGGT-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 ± 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).
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 dexamethasone 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 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, TNFα 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 TNFα (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 µM), p44/42 mitogen-activated protein kinase (MAP kinase; PD98059; 50 µM), p38 MAP kinase (SB203580; 20 µM) or phosphoinositide 3-kinase (PI 3-kinase; LY294002; 10 µM) 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
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 TNFα, IL-6, monocyte chemoattractant protein 1 and...
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 TNFα 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, TNFα 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 TNFα 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 TNFα levels such as obesity. In contrast, we do not find an effect of insulin, GH or the β-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 TNFα 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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