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The Adipokine SAA3 is Induced by Interleukin-1β in Mouse Adipocytes

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Abstract Serum amyloid A (SAA) 3 has been characterized as an inflammatory adipocyte-secreted acute-phase reactant. In the current study, regulation of SAA3 by the proinflammatory and insulin resistance-inducing cytokine interleukin (IL)-1β was determined in 3T3-L1 and brown adipocytes. Interestingly, SAA3 mRNA and protein synthesis were dramatically increased by IL-1β in a time-dependent fashion with maximal induction after 24 h. Furthermore, IL-1β significantly induced SAA3 mRNA expression dose-dependently with maximal 36.4-fold upregulation seen at 2 ng/ml effector. Moreover, IL-1β-induced SAA3 expression was mediated by nuclear factor-κB and janus kinase 2. Taken together, our data show a potent upregulation of SAA3 by IL-1β. J. Cell. Biochem. 104: 2241–2247, 2008.

Key words: 3T3-L1 adipocyte; adipokine; interleukin-1β; serum amyloid A3

Adipocyte-derived factors, the so-called adipokines, have been proposed to link insulin resistance, obesity, and cardiovascular disease (CVD). Among those, adiponectin is an insulin-sensitizing and anti-atherogenic fat-secreted factor [Yamauchi et al., 2001; Ouchi et al., 2003]. Furthermore, tumor necrosis factor (TNF) α and interleukin (IL)-6 have been suggested to induce insulin resistance [Hotamisligil et al., 1993; Fasshauer and Paschke, 2003]. Recently, the acute-phase reactant protein serum amyloid A (SAA) 3 has been introduced as another proinflammatory adipokine. SAA3 belongs to a family of several serum amyloid proteins of which the members SAA1, SAA2, SAA3 [Yamamoto and Migita, 1985; Yamamoto et al., 1986; Lowell et al., 1986a,b], and SAA5 [de Beer et al., 1991] are actively transcribed in mice. All SAA proteins function as high-density lipoprotein (HDL) apoproteins that displace the apolipoproteins A1 and E from HDL. This exchange, in turn, leads to increased HDL binding to macrophages and altered HDL clearance in the liver [Meek et al., 1992]. SAA3 production is found in various cell types including adipocytes in rodents whereas the other SAA proteins are synthesized primarily by the liver [Yamamoto and Migita, 1985; Yamamoto et al., 1986; Lowell et al., 1986a,b]. In contrast, SAA3 expression is not found in humans [Kluve-Beckerman et al., 1991]. It has been shown convincingly that SAA3 is strongly upregulated in adipose tissue of obese mice as compared to lean controls [Lin et al., 2001]. The same group demonstrated that white adipose tissue is a major source of this secreted protein [Lin et al., 2001]. Moreover, SAA3 in complex with hyaluronan showed monocyte chemotactic activity independent of monocyte chemoattractant protein (MCP)-1 in mice, thereby, leading to local inflammation in adipose tissue [Han et al., 2007]. Similarly, circulating levels of acute-phase serum amyloid A (A-SAA) are elevated in obese humans as compared to lean

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subjects and expression of A-SAA is correlated with BMI and fat cell size [Yang et al., 2006]. Furthermore, it has been shown that A-SAA is not only a marker of inflammation but also directly induces the production of different proinflammatory cytokines including IL-6, IL-8, TNFα, and MCP-1 in vascular endothelial cells and monocytes [Yang et al., 2006]. Moreover, an important role of SAA proteins in the development of atherosclerosis has been proposed. Thus, high concentrations of these acute-phase proteins are found in various cell types in atherosclerotic plaques including smooth muscle cells, foam cells, and macrophages [Meek et al., 1994; Urieli-Shoval et al., 1994]. In accordance with these findings, SAA was an independent predictor of cardiovascular disease in women [Johnson et al., 2004].

We have demonstrated that SAA3 mRNA expression is induced by the proinflammatory and insulin resistance-inducing cytokines TNFα and IL-6 in adipocytes in vitro [Fasshauer et al., 2004a]. Recently, IL-1β has been introduced as another cytokine besides TNFα and IL-6 which impairs insulin sensitivity and induces proinflammatory gene expression in various tissues [Lagathu et al., 2006; Jager et al., 2007]. However, the effect of this cytokine on expression of SAA3 in adipocytes has not been elucidated so far. Therefore, we determined regulation of SAA3 by IL-1β in the current study in fat cells.

MATERIALS AND METHODS

Materials

Cell culture reagents were obtained from Life Technologies, Inc. (Grand Island, NY) and PAA (Pasching, Austria), oligonucleotides from MWG-Biotech (Ebersberg, Germany). Dexamethasone, IL-1β, insulin, and isobutylmethylxanthine were purchased from Sigma Chemical Co. (St. Louis, MO). AG490, parthenolide, PD98059, and LY294002 were from Calbiochem (Bad Soden, Germany). SAA3-polyclonal antibody was generated as described [Lin et al., 2001].

Culture and Differentiation of 3T3-L1 and Brown Adipocytes (BAT)

Immortalized brown preadipocytes and 3T3-L1 cells (American Type Culture Collection, Rockville, MD) were cultured as previously described [Fasshauer et al., 2000, 2004a]. In brief, preadipocytes were grown to confluence in DMEM containing 25 mM glucose (DMEM-HG), 10% fetal bovine serum, and antibiotics (culture medium). After this period, cells were induced for 3 days in culture medium further supplemented with 1 μM insulin, 0.5 mM isobutylmethylxanthine, and 0.1 μM dexamethasone. Subsequently, they were grown for 3 days in culture medium with 1 μM insulin and for additional 3–6 more days in culture medium. Various effectors were added to cells starved in DMEM-HG only for the indicated periods of time. At the time of the stimulation experiments at least 95% of the cells had accumulated fat droplets.

Western Blotting

Detection of SAA3 protein synthesis was performed essentially as described previously [Fasshauer et al., 2001]. Briefly, after indicated stimulation periods, cells were harvested in lysis buffer (50 mM HEPES, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM $Na_4P_2O_7$, 10 mM NaF, 2 mM EDTA, 10% glycerol, 1% Igepal CA-630, 2 mM vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 2 mM phenylmethylsulfonyl fluoride, pH 7.4). Lysates were clarified, and equal amounts of protein were solubilized directly in Laemmli sample buffer. Proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose membranes, blocked for 1 h and immunoblotted with a SAA3 antibody [Lin et al., 2001] overnight. Specifically bound primary antibody was detected with peroxidase-coupled secondary antibody and enhanced chemiluminescence.

Analysis of SAA3 mRNA

SAA3 mRNA was quantified by relative real-time polymerase chain reaction (RT-PCR) in a fluorescent temperature cycler (ABI Prism 7000, Applied Biosystems, Darmstadt, Germany) as described previously [Fasshauer et al., 2004a]. Briefly, total RNA was isolated from BAT and 3T3-L1 adipocytes with TRIzol reagent (Invitrogen, Life Technologies, Inc., Carlsbad, CA) and 1 μg RNA was reverse transcribed using standard reagents (Invitrogen, Life Technologies, Inc.). Two microliters of each RT reaction was amplified in a PCR with a total volume of 26 μl. After initial denaturation at 95°C for 10 min, 40 PCR cycles were performed using the following conditions: 95°C

for 15 s, 60°C for 1 min, and 72°C for 1 min. The following primer pairs were used: SAA3 (accession no. NM_011315), 5′-gttcacgggacatggagcagagga-3′ (sense) and 5′-gcaggccagcaggtcggaagtg-3′ (antisense); 36B4 (accession no. NM_007475) 5′-aagcgcgtc ctggcattgtct-3′ (sense) and 5′-ccgcaggggcagcagtggt-3′ (antisense). SYBR Green I fluorescence emissions were monitored after each cycle and synthesis of SAA3 and 36B4 mRNA was quantified using the second derivative maximum method of the ABI Prism 7000 software (Applied Biosystems, Darmstadt, Germany). In brief, crossing points of individual samples were determined by an algorithm identifying the first turning point of the fluorescence curve. 36B4 was used as internal control due to its resistance to hormonal regulation [Laborda, 1991], and SAA3 expression was calculated relative to 36B4. Specific transcripts were 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 and the specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis.

Statistical Analysis

Results are shown as mean ± SE. Differences between various treatments were analyzed by unpaired Student's *t*-tests with *P*-values < 0.01 considered highly significant and <0.05 considered significant.

RESULTS

SAA3 Protein Expression Is Induced by IL-1β

SAA3 protein expression was quantified in differentiated 3T3-L1 cells in the presence or absence of 20 ng/ml IL-1β. Interestingly, IL-1β induced SAA3 protein synthesis time-dependently with significant induction seen after 4, 8, and 24 h of treatment as compared to control conditions (Fig. 1).

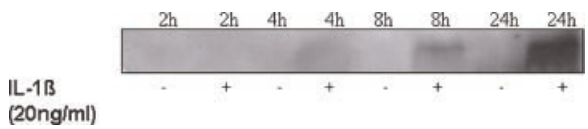

Fig. 1. Time-dependent stimulation of SAA3 protein synthesis by IL-1β. Fully differentiated 3T3-L1 adipocytes were serum-deprived overnight before IL-1β (20 ng/ml) was added for the indicated periods of time. Total protein was isolated and immunoblotted as described in Materials and Methods Section. A representative blot of two independent experiments is shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

SAA3 mRNA Expression Is Induced Time- and Dose-Dependently by IL-1β in Murine Adipocytes

Treatment with 20 ng/ml IL-1β rapidly increased SAA3 mRNA expression in a time-dependent manner in both 3T3-L1 adipocytes and BAT (Fig. 2A,B). Thus, in 3T3-L1 cells significant 2.9-fold upregulation of SAA3 mRNA synthesis was seen after 1 h ($P < 0.05$) and maximal 10.4-fold induction after 24 h ($P < 0.01$) of IL-1β stimulation (Fig. 2A). In BAT, significant 14-fold ($P < 0.01$) upregulation was first seen after 1 h and maximal nearly 500-fold ($P < 0.01$) induction was observed after 24 h of treatment (Fig. 2B).

Furthermore, IL-1β-induced SAA3 synthesis in a dose-dependent manner in 3T3-L1 after 16 h of treatment (Fig. 3). Here, significant 22.7-fold stimulation was observed at IL-1β concentrations as low as 0.02 ng/ml ($P < 0.05$), and maximal 36.4-fold ($P < 0.01$) upregulation was seen at 2 ng/ml effector (Fig. 3).

Jak2 and NFκB Mediate the Effect of IL-1β on SAA3 Expression

We elucidated which signaling molecules implicated in IL-1β signaling might mediate the positive effect of IL-1β on SAA3 expression. To this end, 3T3-L1 cells were treated with specific pharmacological inhibitors of janus kinase (Jak) 2 (AG490, 10 μM), nuclear factor-κB (NFκB) (parthenolide, 50 μM), p44/42 mitogen-activated protein (MAP) kinase (PD98059, 50 μM), and phosphatidylinositol (PI) 3-kinase (LY294002, 10 μM) before IL-1β (20 ng/ml) was added. Treatment of 3T3-L1 adipocytes with AG490 for 17 h significantly induced basal SAA3 expression to 150% of control levels ($P < 0.05$) (Fig. 4A). In contrast, parthenolide, PD98059, and LY294002 treatment for 17 h suppressed basal SAA3 mRNA synthesis to 35%, 66%, and 64% of controls, respectively (Fig. 4A). Again, SAA3 expression was increased 31.6-fold after 16 h of IL-1β treatment ($P < 0.01$) (Fig. 4B). This induction was partially reversed from 3160% to 891% of control levels in cells pretreated with the Jak2 inhibitor AG490 ($P < 0.05$) (Fig. 4B). Furthermore, IL-1β-induced SAA3 mRNA induction was completely blocked to 84% of untreated controls by pharmacological inhibition of

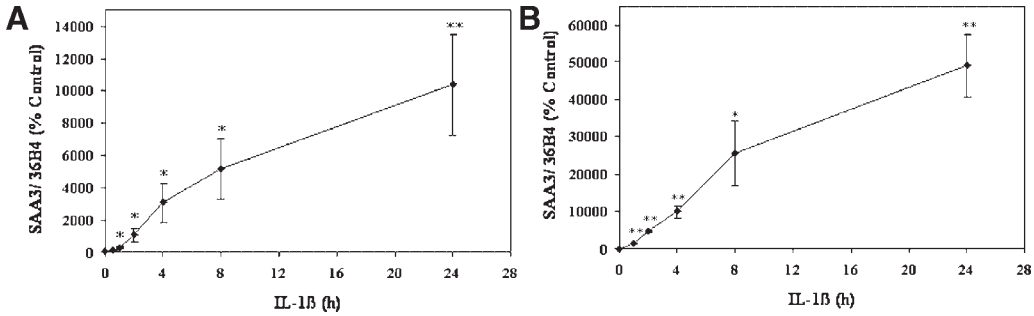

Fig. 2. Time-dependent stimulation of SAA3 gene expression by IL-1β. Fully differentiated 3T3-L1 adipocytes (**A**) and BAT (**B**) were serum-deprived overnight before IL-1β (20 ng/ml) was added for the indicated periods of time. Total RNA was extracted and subjected to relative real-time RT-PCR determining SAA3 mRNA levels normalized to 36B4 expression relative to untreated control cells (=100%) as described in Materials and Methods Section. Results are the means ± SE of at least three independent experiments. **indicates $P < 0.01$, *$P < 0.05$ comparing IL-1β-treated with non-treated cells.

NFκB with parthenolide ($P < 0.01$) (Fig. 4B). In contrast, PD98059 and LY294002 treatment did not significantly influence IL-1β-induced SAA3 expression (Fig. 4B). PD98059 and LY294002 treatment for 3 h induced basal SAA3 mRNA expression to 182% and 174% ($P < 0.01$) of control levels, respectively (Fig. 4C). Again, treatment of fully differentiated 3T3-L1 cells with IL-1β for 2 h upregulated SAA3 expression 135-fold ($P < 0.01$) (Fig. 4D). This induction was reversed by more than 90% in cells pretreated for 1 h with the Jak2 inhibitor AG490 ($P < 0.05$) (Fig. 4D). Furthermore, IL-1β-induced SAA3 mRNA induction was almost completely blocked to 180% of untreated controls by pharmacological inhibition of NFκB with parthenolide ($P < 0.05$) (Fig. 4D). Again, PD98059 and LY294002 treatment did not significantly influence IL-1β-induced SAA3 expression (Fig. 4D).

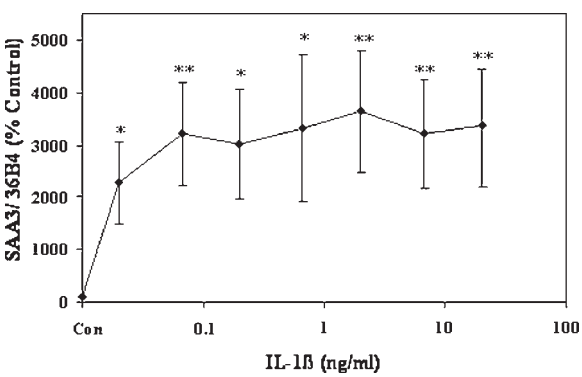

Fig. 3. Dose-dependent stimulation of SAA3 mRNA expression by IL-1β. 3T3-L1 cells were serum-starved for 6 h before various concentrations of IL-1β were added for 16 h. Total RNA was extracted and subjected to relative real-time RT-PCR to determine SAA3 mRNA levels normalized to 36B4 expression as described in Materials and Methods Section. Data are expressed relative to untreated control (Con) cells (=100%). Results are the means ± SE of at least seven independent experiments. **denotes $P < 0.01$ and *denotes $P < 0.05$ comparing IL-1β-treated with non-treated cells.

DISCUSSION

It has recently been shown in a clinical setting that blockade of IL-1 by anakinra improves glycemic control and reduces markers of systemic inflammation [Larsen et al., 2007]. In the current study, we show for the first time that IL-1β significantly stimulates both SAA3 protein and mRNA expression in 3T3-L1 adipocytes. Two recent studies demonstrate convincingly that IL-1β potently induces insulin resistance in human and rodent adipocytes by interacting with insulin signaling molecules [Lagathu et al., 2006; Jager et al., 2007]. Here, IL-1β inhibits insulin-induced phosphorylation of the insulin receptor, insulin receptor substrate 1, protein kinase B, and p44/42 MAP kinase [Lagathu et al., 2006]. Furthermore, in differentiating 3T3-F442A cells, fully differentiated 3T3-L1 cells, as well as human adipocytes, IL-1β severely reduces expression and secretion of insulin-sensitizing adiponectin [Lihn et al., 2004; Lagathu et al., 2006].

We further elucidate in the current study by which signaling molecules IL-1β induces SAA3 mRNA production in fat cells. Binding of IL-1β to the extracellular domain of the IL-1 receptor leads to activation and phosphorylation of IL-1 receptor-associated kinase (IRAK) which, in turn, stimulates NFκB [Cao et al., 1996; Cooke et al., 2001]. Activated NFκB translocates in

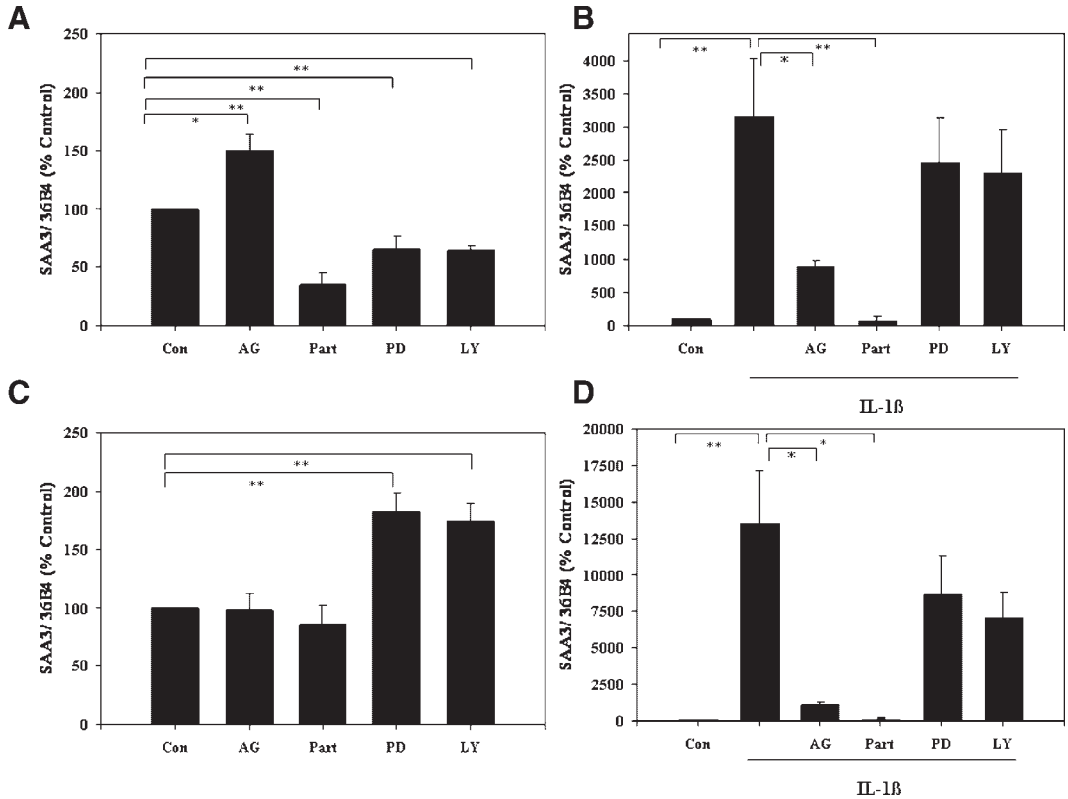

Fig. 4. SAA3 mRNA induction by IL-1β is mediated via Jak2 and NFκB. After serum-starvation for 5 h, 3T3-L1 adipocytes were cultured in the presence or absence of AG490 (AG, 10 μM), parthenolide (Part, 50 μM), PD98059 (PD, 50 μM), or LY294002 (LY, 10 μM) for 1 h before IL-1β (20 ng/ml) was added for 16 h (**A**,**B**) and 2 h (**C**,**D**). Total RNA was extracted and subjected to relative real-time RT-PCR to determine SAA3 normalized to 36B4 expression as described in Materials and Methods Section. Data are expressed relative to non-treated control (Con) cells (=100%). Results are the means ± SE of at least four independent experiments. ** denotes $P < 0.01$, *denotes $P < 0.05$ comparing untreated with inhibitor-pretreated (A,C) or IL-1β-treated cells (B,D), as well as comparing IL-1β-treated with inhibitor-pretreated adipocytes (B,D).

the nucleus and acts as a transcription factor for target genes involved in inflammatory processes. In the current study, we demonstrate that IL-1β-mediated upregulation of SAA3 mRNA expression is significantly and completely reversed by the NFκB inhibitor parthenolide. This finding suggests that IL-1β-induced SAA3 mRNA synthesis is mediated via NFκB in adipocytes. Jak2, p44/42 MAP kinase, and PI 3-kinase have been implicated in IL-1β signaling besides NFκB [Reddy et al., 1997; Stylianou and Saklatvala, 1998; Doi et al., 2002]. Here, inhibition of Jak2 by AG490 reverses IL-1β-induced SAA3 mRNA expression. Interestingly, our group has recently shown that Jak2 also plays an important role in IL-6-stimulated SAA3 mRNA synthesis [Fasshauer et al., 2004a] suggesting that Jak2 is a principal positive regulator of SAA3. In contrast, pharmacological inhibition of p44/42 MAP kinase and PI 3-kinase does not influence IL-1β-induced SAA3 expression indicating that both signaling molecules are probably not involved in regulation of SAA3. We have recently demonstrated that both 50 μM PD98059 and 10 μM LY294002, in fact, effectively block activation of p44/42 MAP kinase and the PI 3-kinase downstream target Akt, respectively [Fasshauer et al., 2004b]. It has to be pointed out that in another set of experiments PD98059 and LY294002 increased basal SAA3 expression whereas AG490 did not have any effect on SAA3 synthesis [Fasshauer et al., 2004b]. The discrepancies between the effects of the pharmacological inhibitors on basal SAA3 gene expression in the two studies remain unclear at present but most probably arise from experiment-to-experiment differences.

Interestingly, IL-1β treatment also significantly induces mRNA expression and protein secretion of the adipokines MCP-1 and IL-6 in 3T3-L1 adipocytes whereas adiponectin

mRNA synthesis is significantly inhibited by the cytokine (data not shown). These data indicate that IL-1β is a multifunctional cytokine regulating the expression and secretion of several adipokines in fat cells.

Taken together, the current study shows for the first time that the proinflammatory and insulin resistance-inducing cytokine IL-1β potently stimulates SAA3 expression in murine adipocytes.

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