Insulin resistance-inducing cytokines differentially regulate SOCS mRNA expression via growth factor- and Jak/Stat-signaling pathways in 3T3-L1 adipocytes

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Abstract

Various cytokines, including tumor necrosis factor (TNF) α, growth hormone (GH) and interleukin (IL)-6, induce insulin resistance. Recently, it was demonstrated that induction of suppressor of cytokine signaling (SOCS)-3 by TNFα and GH is an important mechanism by which these cytokines impair insulin sensitivity. The current study investigated in 3T3-L1 adipocytes whether TNFα and GH also upregulate SOCS-1 and SOCS-6, which have both been shown to inhibit insulin signaling potently, and whether IL-6 might alter synthesis of SOCS-1, -3 and -6. Interestingly, 10 ng/ml TNFα, 500 ng/ml GH and 30 ng/ml IL-6 induced SOCS-1 mRNA time-dependently with maximal stimulation detectable after 8 h of TNFα and 1 h of GH and IL-6 addition respectively. Furthermore, TNFα and GH caused sustained upregulation of SOCS-1 for up to 24 h, whereas stimulation by IL-6 was only transient, with SOCS-1 mRNA returning to basal levels 2 h after effector addition. Induction of SOCS-1 was dose-dependent, and significant stimulation was detectable at concentrations as low as 3 ng/ml TNFα, 50 ng/ml GH and 10 ng/ml IL-6. Furthermore, stimulation experiments and studies using pharmacologic inhibitors suggested that the positive effect of TNFα, GH and IL-6 on SOCS-1 mRNA is, at least in part, mediated by Janus kinase (Jak) 2. Finally, SOCS-3 expression was dose- and time-dependently induced by IL-6, at least in part via Jak2, but none of the cytokines affected SOCS-6 expression. Taken together, our results show a differential regulation of SOCS mRNA by insulin resistance-inducing hormones, and suggest that SOCS-1, as well as SOCS-3, may be an important intracellular mediator of insulin resistance in fat cells and a potential pharmacologic target for the treatment of impaired insulin sensitivity.

Introduction

Insulin resistance, a diminished biologic response of tissues to insulin, is frequently associated with obesity and is a major risk factor for the development of type 2 diabetes, hypertension and coronary artery disease. Defects of various insulin signaling molecules are observed in states of impaired insulin sensitivity and are regarded as a major cause of this pathologic condition (Matthaei et al. 2000). Thus, various studies have shown that impaired activation of insulin receptor (IR), insulin receptor substrate (IRS) proteins and phosphatidylinositol (PI) 3-kinase in insulin resistance leads to impaired stimulation of insulin-induced metabolic endpoints, such as glucose uptake (Kahn & Flier 2000). In recent years, it has been shown that various cytokines, including tumor necrosis factor (TNF) α (Hotamisligil 1999), growth hormone (GH) (Takano et al. 2001) and interleukin (IL)-6 (Senn et al. 2002), are dysregulated in insulin resistance and obesity, and impair insulin signaling profoundly. However, the intracellular proteins mediating this insulin resistance-inducing effect are mostly unknown.

Suppressor of cytokine signaling (SOCS) proteins have been shown to inhibit cytokine-activated Janus kinase (JAK)/signal transducer and activator of transcription (Stat) pathways by binding to either Jak or the cytoplasmic tail of cytokine receptors (Endo et al. 1997, Naka et al. 1997, Starr et al. 1997). Recently, we and others have demonstrated that GH, TNFα, insulin and β-adrenergic agonists stimulate SOCS-3 mRNA expression and that SOCS-3 inhibits insulin signaling in 3T3-L1 adipocytes (Emanuelli et al. 2000, 2001, Fasshauer et al. 2002). Furthermore, it has been shown that SOCS-1 and -6 are also potent inhibitors of insulin signaling (Mooney et al. 2001, Rui...
et al. 2002), and it appears plausible that cytokines such as TNFα, GH, and IL-6 might further decrease insulin sensitivity in fat cells by upregulating either of these proteins.

Therefore, in the current study, we examined the effect of TNFα, GH and IL-6 on SOCS-1 and -6 gene expression in 3T3-L1 adipocytes in vitro. We demonstrate for the first time that the three cytokines potently induce SOCS-1, but not SOCS-6, mRNA in insulin-sensitive fat cells. In addition, we show that IL-6, like TNFα and GH, induces SOCS-3 expression in 3T3-L1 adipocytes. Furthermore, we present evidence that the stimulatory effect of TNFα, GH and IL-6 is mediated, at least in part, via Jak2.

Materials and Methods

Materials

Dexamethasone, GH, IL-6, insulin, isobutylmethylxanthine and TNFα were obtained from Sigma AG490, LY294002 and PD98059 were obtained from Calbiochem (Bad Soden, Germany). Cell culture reagents were from Life Technologies (Grand Island, NY, USA), and oligonucleotides were from MWG-Biotech (Ebersberg, Germany). Phospho-specific p44/42 MAP kinase and Akt antibodies were from Cell Signaling Technology (Beverly, MA, USA), Jak2 and p85 PI 3-kinase antibodies were from Upstate Biotechnology (Lake Placid, NY, USA) and phosphotyrosine antibody PY20 was purchased from BD Biosciences (San Jose, CA, USA).

Culture and differentiation of 3T3-L1 cells

3T3-L1 preadipocytes (American Type Culture Collection, Rockville, MD, USA) were cultured and differentiated as described recently (Fasshauer et al. 2001b,c). Briefly, confluent cells were maintained for 3 days in DMEM containing 25 mM glucose, 10% fetal bovine serum, and antibiotics (culture medium) further supplemented with 1 µM insulin, 0.5 mM isobutylmethylxanthine and 0.1 µM dexamethasone; and for 3 days in culture medium with 1 µM insulin. After an additional 3–6 days in culture medium, more than 90% of the cells had accumulated fat droplets.

Quantification of SOCS-1, -3 and -6 gene expression

SOCS-1 and -6 gene expression was measured by quantitative real-time RT-PCR in a fluorescent temperature cycler (Taqman, Applied Biosystems, Darmstadt, Germany). Total RNA was isolated from 3T3-L1 adipocytes using TRIzol (Life Technologies), and 1 µg RNA was reverse transcribed with standard reagents (Life Technologies). A volume of 2 µl of each RT reaction was amplified in a 26 µl PCR with 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, followed by 40 PCR cycles, 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: SOCS-1 (accession no. AF180302) TGGTTGT AGCAGCTTGTGTCTT (sense) and ACCAAACTG GCTGTAAAAAT (antisense); SOCS-6 (accession no. AF121907) GTGCCGCTTTGTPATCCGTCAGTA (sense) and GAAACAGGGCTCCAAATCTCTT (antisense); and 36B4 (accession no. NM007475) AAGCG CGTCCCTGGCA5TGCT (sense) and CGCGAG GGCAGCAGTTG (antisense). SYBR Green I fluorescence emissions were monitored after each cycle, and mRNA levels of SOCS-1, SOCS-6 and 36B4 were quantified by using 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. SOCS-1 and -6 expression was calculated relative to 36B4, which was used as an internal control due to its resistance to hormonal regulation (Lin et al. 2002). To confirm amplification of specific transcripts, melting curve profiles (cooling the sample to 68 °C and heating slowly to 95 °C with measurement of fluorescence) were produced at the end of each PCR. The specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis. SOCS-3 gene expression was measured by using LightCycler technology (Roche Molecular Biochemicals, Mannheim, Germany), as previously described (Fasshauer et al. 2002).

Immunoprecipitation and Western blotting

Immunoprecipitation and Western blotting were performed essentially as described previously (Klein et al. 1999). Briefly, after the stimulation period, cells were harvested in lysis buffer (50 mM HEPES, 137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM Na2P2O7, 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 (100 and 500 µg respectively) were either solubilized directly in Laemmli sample buffer or immunoprecipitated for 2 h at 4 °C with the indicated antibodies. Immunocomplexes were collected by adding 50 µl of protein A-Sepharose for 2 h at 4 °C, washed in lysis buffer and solubilized in Laemmli sample buffer. Proteins were resolved by SDS–PAGE, transferred to nitrocellulose membranes, blocked for 1 h and immunoblotted with the appropriate antibodies for 2 h. Specifically bound primary antibodies were

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detected with peroxidase-coupled secondary antibody and enhanced chemiluminescence.

Statistical analysis

Results are expressed as mean ± s.e. Comparisons between groups were carried out by unpaired Student’s t-test and, in case of multiple time points and treatments, by one-way ANOVA. P values of < 0.05 were considered significant; those of < 0.01, highly significant.

Results

Quantification of SOCS-1 and -6 mRNA levels in 3T3-L1 adipocytes

First, the reliability of quantitative real-time RT-PCR was tested. For this purpose, increasing amounts of total cellular RNA from differentiated 3T3-L1 cells were reverse-transcribed and analyzed by using specific primer pairs for SOCS-1 and -6 respectively (Fig. 1A and B). Linearity between total RNA used per reaction and amount of mRNA measured by the Taqman software was obtained between 2 and 200 ng of total RNA for both mRNA products (Fig. 1A and B).

SOCS-1, but not SOCS-6, mRNA expression is time-dependently induced by TNFα, GH and IL-6

As SOCS-1 and -6 have recently been suggested as potent negative regulators of insulin signaling, we tested whether insulin resistance-inducing cytokines might influence mRNA synthesis of these genes in 3T3-L1 adipocytes in vitro. Interestingly, 10 ng/ml TNFα significantly induced SOCS-1 expression as early as 4 h after effector addition, with maximal 8.6-fold stimulation being seen at 8 h of treatment and stimulation persisting for up to 24 h (P < 0.05) (Fig. 2A). GH more rapidly stimulated SOCS-1 expression, with maximal 5.6-fold induction being seen after 1 h of effector addition (P < 0.05) (Fig. 2A). Again, significant stimulation was seen as long as 24 h after GH was added (P < 0.05) (Fig. 2A). In contrast, IL-6 only transiently induced SOCS-1 mRNA, with maximal 4.1-fold stimulation being detectable after 1 h of treatment, and SOCS-1 synthesis returning to basal values after 2 h (P < 0.01) (Fig. 2A). In contrast, SOCS-6 was not significantly regulated by TNFα, GH and IL-6 (Fig. 2B).

SOCS-1 mRNA expression is dose-dependently induced by TNFα, GH and IL-6

The effect of various concentrations of TNFα, GH and IL-6 on SOCS-1 gene expression in 3T3-L1 adipocytes was determined. TNFα treatment for 16 h induced SOCS-1 mRNA dose-dependently, with significant stimulation at concentrations as low as 3 ng/ml, and maximal 9.1-fold induction at 100 ng/ml effector (P < 0.01) (Fig. 3A). Moreover, GH addition for 16 h significantly increased SOCS-1 mRNA expression by up to 2.6-fold, an effect already seen at 50 ng/ml of the cytokine (P < 0.01) (Fig. 3B). IL-6 treatment for 1 h also induced SOCS-1 synthesis in a dose-dependent manner, with significant, 1.5-fold stimulation first being detected at 10 ng/ml and maximal 3.2-fold stimulation at 100 ng/ml effector (P < 0.01) (Fig. 3C).

Signaling molecules mediating the positive effects of TNFα, GH and IL-6 on SOCS-1 expression

We further tested which molecules implicated in TNFα-, GH- and IL-6-signaling might mediate the positive effect of these cytokines on SOCS-1 expression. To this end,
3T3-L1 adipocytes were pretreated with specific pharmacologic inhibitors of Jak2 (AG490, 10 µM), p44/42 MAP kinase (PD98059, 50 µM) or PI 3-kinase (LY294002, 10 µM) for 1 h before TNFα (10 ng/ml, 16 h), GH (500 ng/ml, 16 h) or IL-6 (30 ng/ml, 1 h) was added. Treatment of 3T3-L1 adipocytes with AG490 for 17 h significantly suppressed basal SOCS-1 expression to 62% of control levels (P<0.05), whereas PD98059 and LY294002 did not significantly influence SOCS-1 mRNA synthesis (Fig. 4A and B). Again, SOCS-1 expression was increased by more than 10-fold after 16 h of TNFα treatment (P<0.01) (Fig. 4A). This induction was significantly blunted to almost control levels in cells pretreated with the Jak2 inhibitor AG490 (P<0.01) (Fig. 4A). Furthermore, TNFα-induced SOCS-1 mRNA induction was partially reversed by pharmacologic inhibition of PI 3-kinase with LY294002 (P<0.05) (Fig. 4A). In contrast, PD98059 treatment did not significantly affect SOCS-1 stimulation (Fig. 4A). Furthermore, SOCS-1 was significantly induced 3·2-fold after 16 h of GH treatment,
and this stimulation was reduced by about 50% in AG490-pretreated adipocytes ($P<0.05$) (Fig. 4B). In contrast, PD98059 and LY294002 preincubation did not reduce, but instead augmented, GH-induced SOCS-1 expression (Fig. 4B). Treatment of 3T3-L1 adipocytes with AG490 alone for 2 h did not influence SOCS-1 gene expression, but PD98059 and LY294002 significantly stimulated SOCS-1 mRNA by up to threefold ($P<0.05$) (Fig. 4C). Again, IL-6 treatment for 1 h significantly induced SOCS-1 expression by almost sixfold ($P<0.01$) (Fig. 4C). This induction was significantly decreased by 50% in cells pretreated with the Jak2 inhibitor AG490 ($P<0.05$) (Fig. 4C). In contrast, PD98059 and LY294002 did not significantly influence IL-6-induced SOCS-1 expression (Fig. 4C).

**IL-6 induces SOCS-3 gene expression**

We and others have recently established that SOCS-3 is a potential mediator of TNFα- and GH-induced insulin resistance in fat cells. However, it has not been determined so far whether IL-6, another major factor implicated in the induction of insulin resistance, may also mediate its insulin resistance-inducing effects via SOCS-3 upregulation in adipocytes. Therefore, the influence of this cytokine on SOCS-3 gene expression was studied. As shown in Fig. 5A, treatment of 3T3-L1 cells with IL-6 for 16 h dose-dependently induced SOCS-3 gene expression, with significant twofold stimulation being seen at 1 ng/ml effector and the maximal, more than ninefold induction at 100 ng/ml IL-6 ($P<0.01$). Furthermore, 30 ng/ml IL-6 significantly induced SOCS-3 expression as early as 30 min after effector addition, with the maximal, 46-fold induction being seen at 1 h of IL-6 treatment and stimulation persisting for up to 24 h ($P<0.01$) (Fig. 5B). Treatment of 3T3-L1 cells with AG490, PD98059 or LY294002 alone for 17 h did not significantly influence SOCS-3 mRNA (Fig. 5C). SOCS-3 expression was again increased by more than ninefold after 16 h of IL-6 treatment ($P<0.01$) (Fig. 5C). This induction was significantly blunted by more than 50% in cells pretreated with the Jak2 inhibitor AG490 ($P<0.01$) (Fig. 5C). Similarly, pretreatment of 3T3-L1 cells with AG490 for 1 h decreased IL-6-induced SOCS-3 expression by about 80% when adipocytes were treated with IL-6 for 1 h (data not shown). In contrast, PD98059 or LY294002 did not have any effect on IL-6-stimulated SOCS-3 mRNA expression (Fig. 5C).

**Signaling molecules acutely activated by TNFα, GH and IL-6**

Finally, intracellular signaling molecules acutely activated by the three different cytokines and the effectiveness of the inhibitors used were tested. Therefore, after serum starvation, 3T3-L1 adipocytes were treated with 10 ng/ml TNFα, 500 ng/ml GH and 30 ng/ml IL-6 for up to...
Interestingly, TNFα and GH potently induced phosphotyrosine-associated Jak2 in a time-dependent manner, with maximal effects being seen after 2 and 15 min of TNFα and GH addition respectively (Fig. 6A). In contrast, IL-6 did not significantly induce Jak2 activity (Fig. 6A). Furthermore, all three cytokines stimulated p44/42 MAP kinase phosphorylation time-dependently, with maximal effects being seen 15 min after effector addition (Fig. 6B). Moreover, a consistent upregulation of phosphotyrosine-associated p85 PI 3-kinase by TNFα and GH was found, maximal stimulation being observed after 1 and 5 min respectively, whereas IL-6 inhibited this signaling intermediate (Fig. 6C). The efficiency of the inhibitors used was tested by pretreatment of 3T3-L1 adipocytes with 10 µM AG490, 50 µM PD98059 or 10 µM LY294002 for 16 h before stimulating Jak2 with GH (500 ng/ml, 15 min), p44/42 MAP kinase with IL-6 (30 ng/ml, 15 min) and Akt as a downstream target of PI 3-kinase with insulin (100 nM, 5 min). As shown in Fig. 6D, AG490, PD98059 and LY294002 effectively blocked activation of Jak2, p44/42 MAP kinase and Akt.

Discussion

Insulin’s metabolic effects are mediated through the IR, and it has been demonstrated that insulin resistance is accompanied by decreased activation of several insulin signaling molecules, such as IRS proteins, which are essential for insulin action (Fasshauer et al. 2000, 2001a, Matthaei et al. 2000). Furthermore, it has been shown that various cytokines, including adipocyte-secreted proteins such as IL-6 and TNFα, are dysregulated in insulin resistance and impair glucose homeostasis profoundly.

IL-6 has been considered an important proinflammatory cytokine. IL-6 plasma concentrations correlate with the development of type 2 diabetes mellitus (Tsigos et al. 1997, Pradhan et al. 2001). In vivo administration of recombinant IL-6 in rodent models and man induces hepatic gluconeogenesis, which, in turn, leads to hyperglycemia and compensatory hyperinsulinemia (Stith & Luo 1994, Tsigos et al. 1997). On a cellular level, Senn et al. (2002) reported impaired insulin signaling and insulin-induced glycogen synthesis in hepatocytes after IL-6 pretreatment. In the current study, we show for the first time that IL-6 induces SOCS-1 and -3 mRNA expression in adipocytes. Both SOCS family members negatively regulate insulin signaling (Emanuelli et al. 2000, 2001, Mooney et al. 2001, Rui et al. 2002). Thus, Emanuelli et al. (2000, 2001) recently demonstrated convincingly that SOCS-3 competitively binds to phosphotyrosine 960 of the IR, impairing insulin-induced activation of IRS-1 and Stat 5b, and thereby inducing insulin resistance. Similarly, IR-directed phosphorylation of IRS-1 is impaired by SOCS-1 in vitro (Mooney et al. 2001). Furthermore, SOCS-1 and -3 can block insulin signaling by ubiquitin-mediated degradation of IRS-1 and IRS-2 (Rui et al. 2002). Interestingly, decreased activation and expression of IRS-1 after IL-6 treatment have been observed in adipocytes; however, the mediators of this negative effect were not elucidated.
In view of these studies, it appears likely that induction of SOCS-1 and -3 is an important mechanism by which IL-6 induces insulin resistance in adipocytes. In accordance with this view, Senn et al. (2003) recently suggested that SOCS-3 is a primary mediator of the negative effect of IL-6 on insulin signaling.

**Figure 6** Signaling molecules acutely activated by cytokines. 3T3-L1 cells were serum-starved before (A–C) TNFα (10 ng/ml), GH (500 ng/ml), IL-6 (30 ng/ml) or insulin (Ins, 100 nM) was added for the indicated periods of time, or (D) AG490 (AG, 10 μM), PD98059 (PD, 50 μM) or LY294002 (LY, 10 μM) was added for 16 h, after which GH (500 ng/ml, 15 min, upper panel), IL-6 (30 ng/ml, 15 min, middle panel) and insulin (100 nM, 5 min, lower panel) were added. Immunoprecipitation and Western blotting were performed as described in Materials and Methods. Representative blots from at least two independent experiments are shown.
in liver cells. However, SOCS-1 expression was not studied in this system. IL-6 induces gp130 homodimerization at the plasma membrane, and associated kinases such as Jak2 and Tyk2 become activated upon binding and phosphorylate the cytoplasmic tail of gp130 (Murakami et al. 1993, Heinrich et al. 1998). In the current study, we demonstrate that pharmacologic inhibition of Jak2 by AG490 significantly reverses the positive effect of IL-6 on SOCS-1 and -3 mRNA expression, thus implicating Jak2 in IL-6-induced upregulation of either protein. However, it has to be emphasized that, in contrast to TNFα and GH, we cannot demonstrate significant activation of phosphorytrosine-associated Jak2 by IL-6 in the current study. From these findings, it appears possible that other signaling molecules inhibited by AG490, apart from Jak2, might mediate the positive effect of IL-6 on SOCS-1 and -3 mRNA expression. Thus, it has been suggested that AG490 might also inhibit the autokinase activity of Jak3 (Nielson et al. 1997). Alternatively, a small activation of Jak2 by IL-6, which is not readily detectable by immunoprecipitation and Western blotting, might already be sufficient to stimulate SOCS-1 and -3 mRNA synthesis. Stat 1 and 3, as well as SH2-domain-containing tyrosine phosphatase (SHP) 2, bind to the tyrosine-phosphorylated IL-6 receptor and stimulate downstream signaling proteins such as p44/42 MAP kinase and PI 3-kinase (Heinrich et al. 1998). Since pharmacologic inhibition of these signaling proteins does not significantly reverse SOCS-1 and -3 induction, our study does not support a role of these molecules in SOCS mRNA regulation by IL-6.

TNFα is a cytokine primarily produced by macrophages, as well as adipocytes. It potently induces insulin resistance (Hotamisligil et al. 1993, Spiegelman & Flier 2001). Furthermore, various, but not all, studies have demonstrated increased TNFα levels in obesity (Bluher et al. 2001, Spiegelman & Flier 2001). Recently, it has been shown that TNFα induces insulin resistance, at least in part via induction of SOCS-3 (Emanuelli et al. 2001, Fasshauer et al. 2002). The current study, for the first time that this cytokine also potently stimulates SOCS-1 expression, a fact that makes safe conclusions more difficult to draw. In accordance with previous findings (Ridderstrale et al. 1995, Love et al. 1998), p44/42 MAP kinase phosphorylation and the association of p85 PI 3-kinase with phosphotyrosines are stimulated by GH. However, both signaling intermediates do not appear to be involved in regulation of SOCS-1, since pharmacologic inhibition does not alter GH-induced expression.

In summary, we demonstrate for the first time that major insulin resistance-inducing cytokines, such as TNFα, IL-6 and GH, differentially regulate SOCS genes, which are important candidate molecules for the pathophysiological process of impaired insulin sensitivity. Of note, this study focuses on the effect of these cytokines on SOCS mRNA expression and the signaling molecules used. Although other studies have shown alterations on the mRNA level to be representative of changes on the SOCS protein level, these changes may follow divergent time
courses (Bjorbaek et al. 1999, Wang et al. 2000, Senn et al. 2003). TNFα, IL-6 and GH all stimulate SOCS-1 gene expression, whereas SOCS-6 expression remains unaffected, and SOCS-3 is strongly induced by IL-6. These data indicate that upregulation of SOCS-1 and SOCS-3 is a selectively regulated mechanism that might constitute an important element in the pathogenesis of insulin resistance and the insulin resistance syndrome.

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