SOCS-3 Inhibits Insulin Signaling and Is Up-regulated in Response to Tumor Necrosis Factor-α in the Adipose Tissue of Obese Mice*

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SOCS (suppressor of cytokine signaling) proteins are inhibitors of cytokine signaling involved in negative feedback loops. When expressed, SOCS-3 binds to phosphorylated Tyr960 of the insulin receptor and prevents Stat5B activation by insulin. Here we show that in COS-7 cells SOCS-3 decreases insulin-induced insulin receptor substrate 1 (IRS-1) tyrosine phosphorylation and its association with p85, a regulatory subunit of phosphatidylinositol-3-kinase. This mechanism points to a function of SOCS-3 in insulin resistance. Interestingly, SOCS-3 expression was found to be increased in the adipose tissue of obese mice, but not in the liver and muscle of these animals. Two polypeptides known to be elevated during obesity, insulin and tumor necrosis factor-α (TNF-α), induce SOCS-3 mRNA expression in mice. Insulin induces a transient expression of SOCS-3 in the liver, muscle, and the white adipose tissue (WAT). Strikingly, TNF-α induced a sustained SOCS-3 expression, essentially in the WAT. Moreover, transgenic ob/ob mice lacking both TNF receptors have a pronounced decrease in SOCS-3 expression in the WAT compared with ob/ob mice, providing genetic evidence for a function of this cytokine in obesity-induced SOCS-3 expression. As SOCS-3 appears as a TNF-α target gene that is elevated during obesity, and as SOCS-3 antagonizes insulin-induced IRS-1 tyrosine phosphorylation, we suggest that it is a player in the development of insulin resistance.

Based on population studies, type 2 diabetes is now considered as an epidemic disease affecting almost 150 million people worldwide. It results from a diminished biological response of tissues to insulin, termed insulin resistance, and the failure of increased insulin secretion to compensate for this resistance. Its progression parallels that of obesity. Indeed, association between insulin resistance and obesity has been recognized for decades (1). However, the molecular basis linking these two syndromes is not yet clearly defined. In humans the concentration of several circulating molecules, such as glucose, free fatty acids, and insulin, as well as the levels of TNF-α in animal models, are increased during obesity, and these molecules have been proposed to play a role in the development of insulin resistance (2, 3). At the cellular level, a general decrease in insulin signaling is observed and defects occur via several mechanisms (4). First, the insulin receptor tyrosine kinase activity is diminished (2). Moreover, the coupling of the insulin receptor substrates with the insulin receptor is impaired (2). As a consequence, all the signaling events downstream of the insulin receptor are defective, leading to decreased cellular responses. Several hypotheses, which are not mutually exclusive, have been proposed to explain these defects (4). Among them the following have been reported: (i) a decrease in the expression of key molecules such as the insulin receptor and GLUT 4 (5), (ii) an increased expression of the phosphotyrosine phosphatases LAR and PTP-1B, potentially involved in the dephosphorylation of the insulin receptor and IRS-1 (6), and (iii) serine phosphorylation of the insulin receptor by certain protein kinase C isoforms (7, 8) and of IRS-1 by several serine kinases such as MAPK (mitogen-activated protein kinase), TOR (target of rapamycin), PI3K,1 and Jnk (Jun N-terminal kinase) (9–12). The serine phosphorylation of IRS-1 results in diminished insulin receptor tyrosine kinase activity and coupling of IRS-1 with the insulin receptor. However, due to the complexity of the insulin resistance syndrome, it is anticipated that other mechanisms are involved, which are likely to act in concert.

Recently, a new class of inhibitory molecules, the SOCS (suppressor of cytokine signaling) proteins, has been identified (13–15). The SOCS protein family consists presently of eight members that all contain a central SH2 domain and a conserved C-terminal domain, the SOCS box (16). SOCS proteins are induced by several cytokines, in various tissues, in a cytokine- and tissue-specific manner. Once induced, they are involved in negative feedback loops leading to decreased cytokine signaling. Different mechanisms allow the SOCS to modulate the cytokine-activated Jak/Stat pathway (17). They can either bind to Jak and inhibit their tyrosine kinase activity (14) or bind to the cytoplasmic tail of the receptors, thus preventing Stat binding and activation (18). By their ability to be rapidly induced and to efficiently inhibit Jak/Stat pathway, SOCS proteins are potent modulators of cytokine physiology in vivo. This

1 The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; SOCS, suppressor of cytokine signaling; IRS, insulin receptor substrate; Shc, Src homology collagen; Stat, signal transducer and activator of transcription; TNF-α, tumor necrosis factor-α; WAT, white adipose tissue.
is illustrated by the marked phenotype of the knock-out mice. Indeed, the SOCS-3 knock-out is embryonic lethal for reasons still under debate (19, 20), and SOCS-1-invalidated mice die rapidly after birth (21–23), while SOCS-2-deficient mice have an increased body size (24).

Although the SOCS were originally described in cytokine signaling pathways, we have shown recently that they also belong to the insulin signaling circuitry (25). Indeed, insulin increases SOCS-3 mRNA expression in 3T3-L1 adipocytes. When expressed, SOCS-3 is located in the cytoplasm under basal conditions. Following insulin treatment, we found that it translocates to the plasma membrane, where it colocalizes with the insulin receptor. SOCS-3 probably binds directly to phosphotyrosine 960 of the insulin receptor, since the hormone-induced translocation and colocalization are not observed in cells expressing an insulin receptor mutated on this residue. Furthermore, yeast two-hybrid experiments show that SOCS-3 binds through its SH2 domain to this receptor residue. This association results in a competition with Stat 5B to bind to the insulin receptor, thus preventing the activation of the translocation factor by insulin. Interestingly, this site also belongs to the unique binding motif for most of the insulin receptor substrates.

Here we studied whether SOCS-3 could be considered as a more general inhibitor of insulin signaling pathways, and we investigated its potential involvement in insulin resistance. We show that SOCS-3 expression decreases insulin-induced tyrosine phosphorylation of IRS-1 and its subsequent association with p85, a regulatory subunit of PI 3-kinase. Moreover, SOCS-3 expression was found to be increased in the white adipose tissue (WAT) of obese animals. Interestingly, TNF-α, which has been proposed to play a role in the insulin resistant state observed in obesity, induces SOCS-3 expression mainly in the WAT. Finally, compared with ob/ob mice, a pronounced decrease in SOCS-3 expression was observed in the WAT of transgenic ob/ob mice devoid of TNF-α signaling. This study shows that TNF-α, a molecule known to be involved in the insulin resistant state of obese rodents, is responsible, at least in part, for an increase of SOCS-3 expression in the WAT of obese rodents and that SOCS-3 is an inhibitor of insulin signaling.

MATERIALS AND METHODS

Constructions, Cell Culture, and Transfection—The human insulin receptor cDNA (obtained from A. Ulrich) and the human insulin receptor substrate 1 cDNA (obtained from M. White) were subcloned in pcDNA3.

COS-7 fibroblasts were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum. Cells were transfected by the DEAE-dextran method as described in Ref. 26 and were depleted in Dulbecco’s modified Eagle’s medium, 0.2% (w/v) bovine serum albumin 18 h before the experiments.

Antibodies, Immunoprecipitation, and Immunoblotting—Monoclonal anti-FLAG antibodies (M2) were purchased from Sigma. Rabbit polyclonal serum against IRS-1, and monoclonal antibody against insulin receptor (used for immunoprecipitation) were produced in our laboratory. Monoclonal antibody against insulin receptor (for Western blotting) and rabbit polyclonal anti p85α were purchased from Santa Cruz (Santa Cruz, CA) and Upstate Biotechnology (Lake Placid, NY), respectively.

Cells were starved overnight and then stimulated or not with 100 nm insulin for 5 min. Cells were washed in phosphate-buffered saline and lysed in ice-cold lysis buffer. Whole-cell lysates were mixed with various antibodies (as specified under “Results”) and protein A- or G-coupled to agarose beads. Proteins from whole-cell lysates and immunoprecipitates were separated by SDS-PAGE, then transferred to Immobilon-P membrane (Millipore, Bedford, MA) and blotted with various antibodies (as specified under “Results”). Finally, membranes were incubated with radioactive iodinated protein A, and proteins were revealed by autoradiography or by a Storm 840 apparatus, Molecular Dynamics (Amer sham Biosciences, Uppsala, Sweden) and quantified using NIH Image or Imagequant.

Animal Treatment—All mouse strains were housed and raised on open formula rations. Water was given ad libitum with a 12-h light-dark cycle.

Male OF1 mice (IFFA CREDO, L’Arbresle, France) (about 40 g) were starved overnight and then injected intraperitoneally with a saline solution (9% NaCl (w/v)) or insulin (1 millinunit/g). Mice were euthanized 1, 2, 3, or 4 h after injection. In a second set of experiments, male OF1 mice were injected intraperitoneally with recombinant human TNF-α (TEBU, Le Perray-en-Yvelines, France), 4 µg per mouse, or with saline solution. Mice were euthanized 1, 2, 4, or 8 h after injection.

Male C57BL6/0Ah-Led-Pep +/+ (ob/ob) mice, male C57BL6/Ko0Ah-Led-Pep +/+ (db/db) mice, and lean controls were obtained from Harlan (Gannat, France). They were euthanized at 12 or 14 weeks of age, respectively, when the lean controls weighed 30 g and the obese mice 60 g.

In each experiment, skeletal muscle, liver, and peri-epididymal fat were quickly removed for RNA isolation.

ob/ob p55 α/α mice were obtained and characterized as described previously (27, 28). These mice and their ob/ob p55 α/β and p55 α/− littermates were euthanized at 20 weeks of age for RNA isolation.

Northern Blot Analysis—Total RNA from mouse tissues was isolated with the Trizol reagent (Life Technologies, Inc.) according to the manufacturer’s instructions. 20 µg of denatured total RNA was separated by electrophoresis on formaldehyde-containing 1.2% (w/v) agarose gels and transferred to positively charged nylon membranes (Ambion, Austin, TX). Full-length SOCS-3 cDNA was used as probe. The probe was labeled by random priming with [α-32P]dCTP using the Rediprime kit (Amersham Biosciences, Buckinghamshire, UK) and purified with the Probequant kit (Amersham Biosciences). A radiolabeled oligonucleotide corresponding to a 18 S RNA specific sequence was used as an internal control for RNA loading and integrity. Hybridizations were performed at 42 °C in hybridization buffer (Ambion, Austin, TX). Membranes were washed three times in 0.5× SSC, 0.5% (w/v) SDS at 42 °C and revealed by autoradiography or by a Storm 840 apparatus, Molecular Dynamics and quantified using NIH Image or Imagequant.

RESULTS

SOCS-3 Expression Reduces Insulin-induced IRS-1 Tyrosine Phosphorylation—We have previously shown that SOCS-3 inhibits the insulin-induced stimulation of Stat 5B (25). We hypothesized that this inhibition was the result of a competition between SOCS-3 and Stat 5B to bind to the phosphorylated Tyr1168 of the insulin receptor. As established substrates of the insulin receptor, such as IRS-1, bind to this residue to undergo phosphorylation by the insulin receptor, we investigated whether SOCS-3 could interfere with IRS-1 tyrosine phosphorylation in response to insulin. COS-7 cells were transfected with plasmids encoding the insulin receptor or IRS-1 and with pEF-SOCS-3. Cells were stimulated or not with insulin for 5 min and lysed. The insulin receptor and IRS-1 were immunoprecipitated and analyzed by an antiphosphotyrosine Western blot. Expression levels of IR, IRS-1, and SOCS-3 were measured in total cell lysates. As shown in Fig. 1, expression of SOCS-3 decreased the insulin-induced tyrosine phosphorylation of IRS-1 by 80%. Under these conditions, the decrease occurred without modification of IRS-1 or of insulin receptor expression. However, higher expression of pEF-SOCS-3 lead to a decrease in both insulin receptor and IRS-1 proteins (data not shown). As described previously, SOCS-3 expression did not modify the insulin receptor tyrosine kinase activity (25, 29).

SOCS-3 Expression Decreases Insulin-induced Association of p85α with IRS-1—Once phosphorylated on tyrosine residues by the insulin receptor, IRS-1 becomes a docking molecule for SH2-domains containing proteins, such as the p85 regulatory subunit of PI 3-kinase. As SOCS-3 expression decreases insulin-induced tyrosine phosphorylation of IRS-1, we investigated whether it could impair the association between IRS-1 and p85α. COS-7 cells were transfected with plasmids encoding the insulin receptor or IRS-1 and with increasing amounts of pEF-SOCS-3. Cells were stimulated with insulin for 5 min and thereafter lysed. IRS-1 was immunoprecipitated, and the obtained immunocomplexes were analyzed by an anti-phosphoty-
Insulin Induces SOCS-3 mRNA Expression in Mice—We have reported previously that in 3T3L1 adipocytes insulin increases SOCS-3 mRNA expression. Here we studied SOCS-3 mRNA expression in insulin target tissues of animals injected with the hormone. OF1 mice were injected intraperitoneally either with a saline solution or with insulin. Mice were euthanized 1, 2, 3, or 4 h after injection and the liver, hindleg muscles, and WAT were removed. Total RNAs were isolated and analyzed by Northern blot using SOCS-3 cDNA as a probe. As shown in Fig. 5, SOCS-3 mRNA expression was increased 4-fold and 5.5-fold in the WAT of ob/ob and db/db mice, respectively, compared with their lean counterparts. By contrast, SOCS-3 mRNA was not detected in the muscle and the liver of either the obese or the lean animals. These data indicate that SOCS-3 is up-regulated preferentially in the WAT of obese animals. Obesity is usually associated with hyperinsulinemia and with an up-regulation of TNF-α mRNA and an anti-p85 Western blot (Fig. 2). Expression levels for IRS-1 and SOCS-3 were measured on total cell lysates. As observed, the decrease in the insulin-induced tyrosine phosphorylation of IRS-1 upon SOCS-3 expression is associated with a loss of association between endogenous p85<sup>PI3K</sup> and IRS-1.

**Fig. 1.** SOCS-3 expression results in decreased insulin-induced IRS-1 tyrosine phosphorylation. COS-7 cells were transfected with pCDNA3-IR (2 μg), pCDNA3-IRS-1 (2 μg), and pEF-SOCS3 (0.5 μg). Cells were starved overnight and treated or not with insulin (10<sup>-8</sup> m) for 5 min. Immunoprecipitated IR and IRS-1 were analyzed by Western blot with an antibody to phosphotyrosine. Cell lysates were analyzed by Western blot using anti-IR, anti-IRS-1, or anti-FLAG antibodies to monitor the expression levels of IR, IRS-1, and SOCS-3, respectively. This experiment was performed five times, and a representative experiment out of five is shown.

**DISCUSSION**

The insulin receptor mediates its biological effects through tyrosine phosphorylation of several substrates, among them the IRS, Shc, and Stat 5B. This phosphorylation is dependent on a functional coupling between the insulin receptor and its substrates. The coupling can be direct, for instance through the interaction between phosphorylated tyrosine 960 (pTyr<sup>960</sup>) of the insulin receptor and the phosphotyrosine binding domains of IRS and Shc (30, 31) or with the SH2 domain of Stat 5B (32, 33). The coupling insulin receptor/substrate can also be indirect through the PH domain of IRS (34). These pleckstrin homology domains may bind to acidic peptide motifs in membrane proteins or to phospholipids that link substrates to activated cell surface insulin receptors (35). As a consequence, any molecular event impairing the coupling between the insulin receptor and
its substrates should lead to a decrease in the tyrosine phosphorylation of the latter molecules. Recently, we have revealed that SOCS-3 could be considered as such a molecule, which is able to hamper the interaction between the insulin receptor and one of its substrates, Stat 5B (25). Indeed, SOCS-3 expression reduces insulin-induced DNA binding activity of Stat 5B, probably through a competition between Stat 5B and SOCS-3 for binding to the insulin receptor pTyr960.

We have evaluated the impact of SOCS-3 on insulin signaling by investigating whether SOCS-3 interferes with the tyrosine phosphorylation of the key insulin receptor substrate, IRS-1. Indeed, although Stat 5B may participate in the transcriptional control of some insulin target genes (36), IRS-1, through its engagement with PI3K, Grb2 and SHP2, is thought to control several major cellular processes regulated by insulin such as glucose uptake, protein and glycogen synthesis, and gene expression (37). Since, however, Stat 5B and IRS-1 interact with the insulin receptor through distinct mechanisms, the effect of SOCS-3 on IRS-1 tyrosine phosphorylation could differ. Indeed, Stat 5B and SOCS-3 bind to the insulin receptor through their SH2 domain, while IRS-1 uses its PTB domain. Even if the affinity of PTB and SH2 domains for the association with pTyr960 are likely to be in the same order of magnitude (38), they do not recognize the same binding motif. A SH2 domain is expected to interact with pTyr960 and amino acids located in the N terminus of the motif containing pTyr960, whereas a PTB domain is anticipated to bind pTyr960 but is more dependent upon residues located in the C terminus of the motif containing pTyr960. Moreover, IRS-1 possesses a PH do-
main, which has been proposed to play a crucial role in insulin-induced IRS-1 phosphorylation in intact cells (39).

Here we show that SOCS-3 expression reduces insulin-induced tyrosine phosphorylation of IRS-1 and its subsequent association with p85<sub>110k</sub>. From these results and previous studies, it would appear that the binding of SOCS-3 to Tyr<sup>360</sup> prevents the coupling of IRS-1 with the insulin receptor both through its PTB and PH domains. Therefore, we suggest that SOCS-3 is a potent inhibitor of insulin signaling. This is of particular interest, since we find that SOCS-3 expression is increased in several situations associated with insulin resistance.

At least two recent reports have pointed toward a function of the SOCS in insulin resistance. Using transfectant cells, Mooney et al. (40) showed that SOCS-1 and SOCS-6 inhibit insulin-induced activation of MAP kinase and of PKB. Based on in vitro reconstitution experiments with partially purified insulin receptor and recombinant IRS-1, the authors suggest that these SOCS inhibit insulin receptor tyrosine kinase activity. However, using intact cells, they did not observe a modification of insulin receptor tyrosine phosphorylation, and furthermore IRS-1 phosphorylation was not studied. A role for SOCS-1 in the control of glucose homeostasis has been proposed by Kawazoe et al. (29). They reported that insulin sensitivity was increased in SOCS-1-deficient mice. In transfected cells, they show that SOCS-1, and SOCS-3 to a lesser extent, inhibit insulin-induced tyrosine phosphorylation of IRS-1. They suggest that this inhibition is linked to a decrease in Jak activity. This work could have important physiological significance, since it provides evidence for a role of SOCS-1 in the regulation of insulin sensitivity. However, one can argue that although the Jaks phosphorylate IRS, it is generally assumed that most of the insulin-induced tyrosine phosphorylation of IRS-1 is due to the insulin receptor itself. Therefore, our hypothesis of a competition occurring between SOCS-3 and IRS-1 to bind to the insulin receptor provides us with a more plausible explanation for the observed decrease in IRS-1 tyrosine phosphorylation. Second, SOCS-1 is nearly undetectable in insulin-sensitive tissues, and when induced, its expression is transient (data not shown). Therefore, the situations in which SOCS-1 triggers insulin resistance remain uncertain.

Here we show that SOCS-3 expression is increased in the WAT of obese mice. No modification was observed in skeletal muscle and, in agreement with a previous study, in the liver (41). It is tempting to propose that this SOCS-3 expression may participate in the development of insulin resistance in the WAT, since we show that sustained high SOCS-3 expression is hampering insulin signaling. Interestingly, an obesity-induced up-regulation of SOCS-3 mRNA expression has also been reported to occur in the hypothalamus of obese mice, and it has been proposed that this up-regulation is involved in leptin resistance (42). In diet-induced obese rats, an up-regulation of SOCS-1 and SOCS-3 mRNA in the adipose tissue has also been observed (43).

Among other factors, insulin resistance has been known to be elevated during obesity. We have reported previously that insulin leads to SOCS-3 mRNA expression in 3T3-L1 adipocytes, and we have proposed that this induction was linked to Stat 5B activation (25). Recently, our results were confirmed in C2C12 myotubes by Sadowski et al. (44). We find here that SOCS-3 is an insulin-target gene in major classical insulin-sensitive tissues. However, insulin-induced SOCS-3 expression is not limited to one tissue, as it is observed in the muscle, liver, and WAT. Moreover, the increase in SOCS-3 expression was transient. This suggests that hyperinsulinemia is not the leading cause of the WAT-specific increased expression of SOCS-3 during obesity.

Obesity has also been associated with an increased expression of TNF-α in the WAT (45). Several reports have illustrated the role of TNF-α in obesity-associated insulin resistance in rodents, while its precise contribution to insulin resistance in humans is debated (3, 46). We show in Fig. 5 that in mice intraperitoneally injected with TNF-α SOCS-3 mRNA expression is induced in insulin-sensitive tissues. This induction is more pronounced and sustained in the WAT compared with liver and skeletal muscle. So far, the molecular mechanisms leading to SOCS-3 expression in response to TNF-α have not been identified. Usually, SOCS transcription is induced by cytokines through activation of transcription factors of the Stat family (17). Although TNF-α has been shown to activate certain Stat to some extent (47), it is likely that the effect of TNF-α on SOCS-3 expression is indirect. Indeed, two reports indicate that TNF-α induces SOCS-3 expression in the liver (48, 49), but not in primary hepatocytes and in HepG2 cells. Therefore, it was suggested that other cytokines, induced by TNF-α, were responsible for SOCS-3 expression. Conflicting observations were obtained with transgenic mice lacking interleukin-6 on the effect of this interleukin on TNF-α induction of SOCS-3 in the liver (48, 49).

At this point we hypothesized that a paracrine mechanism involving TNF-α could explain the increased SOCS-3 expression observed in WAT of obese mice. To test this we studied SOCS-3 expression in ob/ob mice and in ob/ob transgenic mice deficient in both p55TNFFR and p75TNFFR (ob/ob p55<sup>−/−</sup>/p75<sup>−/−</sup>), the two known receptors for TNF. It has previously been reported that these transgenic mice have a higher insulin sensitivity compared with their controls (27, 28). We found a pronounced decrease in SOCS-3 expression in obese mice lacking TNF-α signaling. These data provide genetic evidence supporting the idea that the elevated levels of SOCS-3 expression found in obesity is linked to increased TNF-α expression. Several molecular mechanisms have been proposed to explain TNF-α-induced insulin resistance. Although some divergence exists among the different reports,
they all point toward an important role of IRS-1 phosphorylation on serine residues, thought to be responsible for the decrease in insulin signaling (3, 46). TNF-α-induced SOCS-3 expression could represent a newly identified means for this cytokine to diminish the cellular responses to insulin.

Altogether our results show that SOCS-3 negatively regulates insulin signaling. Moreover, our evidence of increased SOCS-3 expression in situations associated with insulin resistance leads us to propose that SOCS-3 participates in the development of this syndrome. Such deleterious actions of SOCS-3 could be operational not only at the periphery but also in the central nervous system. Jointly, these SOCS-3 effects would result in insulin resistance and disturbed energy expenditure. To conclude, SOCS-3 may constitute a newly recognized molecular target for the prevention and/or treatment of insulin resistance.

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