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Central role of suppressors of cytokine signaling proteins in hepatic steatosis, insulin resistance, and the metabolic syndrome in the mouse

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Insulin resistance, obesity, diabetes, dyslipidemia, and nonalcoholic fatty liver are components of the metabolic syndrome, a disease complex that is increasing at epidemic rates in westernized countries. Although proinflammatory cytokines have been suggested to contribute to the development of these disorders, the molecular mechanism is poorly understood. Here we show that overexpression of suppressors of cytokine signaling (SOCS)-1 and SOCS-3 in liver causes insulin resistance and an increase in the key regulator of fatty acid synthesis in liver, sterol regulatory element-binding protein (SREBP)-1c. Conversely, inhibition of SOCS-1 and -3 in obese diabetic mice improves insulin sensitivity, normalizes the increased expression of SREBP-1c, and dramatically ameliorates hepatic steatosis and hypertriglyceridemia. In obese animals, increased SOCS proteins enhance SREBP-1c expression by antagonizing STAT3-mediated inhibition of SREBP-1c promoter activity. Thus, SOCS proteins play an important role in pathogenesis of the metabolic syndrome by concordantly modulating insulin signaling and cytokine signaling.

RNA Isolation from Mice. Mice were starved overnight, then killed under anesthesia with the tissues removed. Total RNA was isolated from mouse tissues by using an RNaseasy kit (Qiagen, Valencia, CA).

Semi-quantitative RT-PCR and Northern Blot Analysis. Five hundred nanograms of total RNA was applied to RT-PCR reaction by using the One-Step RT-PCR system (Invitrogen). The primer pairs were: 5′-TCGGATTACCGGCATCACG-3′ and 5′-CTCCAGCAGTCGAAAGGCA-3′ for SOCS-1; 5′-CAGCAAAATGTCCGCCGCGGCC-3′ and 5′-GTGCCACAGCTTGAATGATCACA-3′ for SOCS-3; and 5′-ACCCATGGAGAAGGCGCGG-3′ and 5′-CTCAGTGAGGCCAAGATGC-3′ for GAPDH. The PCR reaction profile was as follows: one cycle at 94°C for 5 min followed by 38 cycles at 94°C for 1 min; 60°C for 30 s and 94°C for 30 s and 72°C for 1 min; and finally one cycle at 72°C for 10 min. For Northern blot analysis, we applied 20 µg of the total RNA and used phosphonolpyruvate carboxykinase, peroxisome proliferator-activated receptor γ-coactivator-1α (PGC-1α), SREBP-1, or fatty acid synthase cDNA fragment as a probe, as described (19, 20).

Generation of Recombinant Adenoviruses. The cDNAs of SOCS-1 and -3 were subcloned between BamHI and EcoRI sites of pCMV-Tag2 vector, respectively, and amplified the full-length SOCS-1 and -3 cDNAs with an N-terminal FLAG tag by using the primer pairs: 5′-GCCGCCACCAGTTAAGGATAGATCACA-3′ and 5′-TCAGATCTGGGAGGGAAGCTGAC-3′ for SOCS-1.
and 5'-GCGCACCATTAGGTTACAGGAT-3' and 5'-CTAAAGTGGAGCATCAGTAC-3' for SOCS-3. After confirming the sequences, we treated the amplified fragments with Klenow enzyme and subcloned them into the SwaI site of the pAdexiC1Awt cosmid cassette. The recombinant adenoviruses, Adex1CASOCs-1-FLAG and Adex1CASOCs-3-FLAG, were constructed by homologous recombination between the expression cosmid cassette and parental virus genome, as described (21).

Adenovirus-Mediated Gene Transfer. Eight-week-old male C57BL/6 mice were injected with the adenoviruses at a concentration of 5 x 10^8 plaque-forming units per gram of body weight in a suspension of 200 μl of PBS through the tail vein, as described (21). Blood samples were obtained on the day before adenoviral injection (day 0) and 5 days after injection (day 5) for measurement of glucose and insulin. Insulin tolerance tests were performed at day 6 and insulin signaling after i.v. insulin injection at day 8.

Antisense Treatment. Two oligonucleotides, designated as AS1 and AS3, were synthesized for antisense treatment against SOCS-1 and -3, respectively. AS1 was designed as a 26-bp single-strand oligonucleotide covering the −5 to +21 region of murine SOCS-1 mRNA: 5'-CACCTGTTGCTGCTAGCACCTCCTAC-3', whereas AS3 was designed covering the −5 to +21 region of the murine SOCS-3 mRNA: 5'-AAACTTGTGGTTGACCATGGCGC-3' (22). Two oligonucleotides, designated as C1 (5'-CAGCTCGTAGGAGCAACCATCGTAC-3'), a six-base mismatch to AS1, and C3 (5'-AATCTAGCTCTGGTGACATGGCGGC-3', a six-base mismatch to AS3), were also synthesized for controls. All oligonucleotides were synthesized as uniform phosphorothioate chimeric oligonucleotides, with 2'-O-methoxymethyl groups on bases 1-5 and 22-26. To inhibit expression of SOCS-1 and -3 in vivo, db/db mice were treated by i.p. injection with 25 mg/kg of AS1, AS3, C1, C3, or PBS once per week for 2 weeks as described (23).

Metabolic Studies. Blood glucose values were determined by using a Glucometer Elite XL (Bayer, Elkhart, IN), and plasma insulin concentrations were measured by ELISA with mouse insulin as a standard (Crystal Chem, Downers Grove, IL). For the insulin tolerance test, blood samples were obtained by tail bleeding at 0, 15, 30, and 60 min after i.p. injection of 0.75 units/kg insulin (Lilly Research Laboratories, Indianapolis). Plasma triglyceride (Lilly Research Laboratories, Indianapolis), 0.5 μg of a promoterless Renilla luciferase construct (pRL-null), and 1 μg of pCDA3.1 vector encoding STAT3, STAS5 (kindly provided by H. Shimano, Tsukuba University, Tsukuba, Japan), 0.5 μg of a promotorless Renilla luciferase construct (pRL-null), and 1 μg of pCDA3.1 vector encoding STAT3, STAS5 (kindly provided by H. Shimano, Tsukuba University, Tsukuba, Japan) were cotransfected with pGL2 mouse SREBP-1c promoter luciferase vector (25) (kindly provided by H. Shimano, Tsukuba University, Tsukuba, Japan) by using the Fugene 6 transfection kit (Roche Applied Science). Cells were lysed with passive lysis buffer (Promega) and examined with a fluorescent microscope.

**In Vitro Insulin Stimulation and Analysis of Insulin Signaling.** Mice were starved overnight, anesthetized with pentobarbital, and injected with 5 units of regular human insulin (Lilly) into the inferior vena cava. Five minutes after injection, the liver was removed and frozen in liquid nitrogen. Immunoprecipitation and immunoblot analysis of insulin signaling molecules were performed by using tissue homogenates extracted with buffer A containing 25 mM Tris-HCl, pH 7.4; 10 mM NaNO3; 100 mM NaF; 50 mM Na2PO4; 10 mM EGTA; 10 mM EDTA; 5 μg/ml leupeptin; 5 μg/ml aprotinin; 2 mM PMSF; and 1% Nonidet-P 40, as described (21).

**Immunostaining for SOCS Proteins.** After a wash with PBS, 10-μm frozen sections from liver were incubated with 3% normal goat serum in 2.5% Triton X-100/PBS for 2 h for blocking, followed by incubation with anti-SOCS-1 or anti-SOCS-3 antibody in blocking solution at 1:100 dilution for overnight. After six washes with PBS, slides were incubated with Alexa Fluor 488 (Molecular Probes) for SOCS-1 and Alexa Fluor 546 (Molecular Probes) for SOCS-3 for 20 min at 21°C. After rinsing with PBS, slides were mounted with Slow Fade kit (Molecular Probes) and examined with a fluorescent microscope.

**Luciferase Assay.** Fao cells were plated at 1 x 10^5/well in 12-well plates 24 h before transfection. Cells were transfected with 0.5 μg of pGL2 mouse SREBP-1c promoter luciferase vector (25) (kindly provided by H. Shimano, Tsukuba University, Tsukuba, Japan), 0.5 μg of a promoterless Renilla luciferase construct (pRL-null), and 1 μg of pCDA3.1 vector encoding STAT3, STAS5 (kindly provided by T. Tanaka, Harvard School of Public Health, Boston), pEF-Boss vector encoding SOCS-1 or -3 (kindly provided by T. Naka, Osaka University, Osaka), or pME18S encoding a mutant STAT3 lacking the C-terminal region [dominant negative (dn)STAT3, kindly provided by A. Miyajima, Institute of Cellular and Molecular Biosciences, Tokyo] by using the Fugene 6 transfection kit (Roche Applied Science). Cells were lysed with passive lysis buffer (Promega) 24 h after transfection. Luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instructions. Relative light units were determined by quantitation of luminescent signal from the Firefly luciferase normalized with cotransfected Renilla luciferase activity in the same sample.

**Results and Discussion**

**Increased SOCS Proteins in Liver Cause Systemic Insulin Resistance.** Several lines of evidence have suggested that insulin resistance in obesity is a component mediated by chronic inflammation associated with elevated cytokines (4, 26, 27), which may induce SOCS proteins in insulin-sensitive tissues. Indeed, in livers of mice on a high-fat diet (Fig. 6, which is published as supporting information on the PDAS web site), the levels of SOCS-1 and -3 mRNA were increased 2- to 3-fold (Fig. 1a and b). Similar up-regulation of SOCS mRNAs was also observed in other insulin-resistant models, such as db/db mice and mice on a high-fat diet (Fig. 6, which is published as supporting information on the PDAS web site). Thus, SOCS proteins may be a candidate linking between the metabolic syndrome and proinflammatory cytokine signaling.

To directly assess the role of increased SOCS proteins in insulin resistance and the metabolic syndrome, mice were injected with adenoviruses encoding either SOCS-1, SOCS-3, or LacZ, respectively. Under these conditions, the levels of expression of SOCS-1 and -3 were within the pathophysiological range and only slightly higher than those in livers of db/db mice (Fig. 1a and b). β-Galactosidase staining of liver infected with adenovirus encoding LacZ revealed that, using our system, >90% of hepatocytes were infected (data not shown).

Overexpression of either SOCS-1 or -3 in liver by adenoviral
infection resulted in significantly increased plasma insulin concentrations (Fig. 1c), with only slight increases in blood glucose levels (Fig. 1c), suggesting insulin resistance in these mice. This was confirmed by a marked impairment in glucose lowering during the insulin tolerance test in mice overexpressing SOCS-1 or -3 (Fig. 1d). This is consistent with findings produced by others and ourselves that SOCS proteins can attenuate insulin signaling by binding to the insulin receptor and reducing their ability to phosphorylate IRS proteins (14, 28, 29). SOCS proteins have also been suggested to interfere with insulin signaling by promoting ubiquitin-mediated degradation of IRS proteins (30).

Consistent with the impaired insulin sensitivity in mice overexpressing SOCS-1 or -3, there was an up-regulation of the mRNA of phosphoenolpyruvate carboxykinase, a key enzyme of gluconeogenesis (Fig. 1e). This occurred without a change in the expression level of peroxisome proliferator-activated receptor γ-coactivator-1α (PGC-1α), one of the key regulators of expression of gluconeogenic enzymes (20). On the other hand, expression of the SREBP-1c, a key regulator of fatty acid synthesis in liver (31), was increased 1.83 ± 0.17 fold (n = 8) in livers overexpressing SOCS-1 and 5.21 ± 0.31 fold (n = 8) in those overexpressing SOCS-3 (Fig. 1e), leading to an increase in levels of SREBP-1 protein in the nuclei of hepatocytes overexpressing SOCS-1 or -3 (Fig. 1f). This increase in SREBP-1c after increased SOCS expression could act as a trigger in the regulation of fatty acid synthesis in liver.

Amelioration of Insulin Resistance by Suppressing SOCS-1 and -3 in the db/db Mouse. To directly address whether SOCS-1 and -3 contribute to insulin resistance, hepatic steatosis, and other metabolic derangement in obese diabetic animals, we treated db/db mice with antisense oligonucleotides against SOCS-1 and -3 (AS1 and AS3). After 2 weeks of treatment with specific antisense oligonucleotides, there was a marked and specific down-regulation of the elevated levels of the expression of the targeted SOCS mRNA. Combination therapy (AS1+AS3) reduced the expression of both isoforms in liver, whereas the control oligonucleotides (C1 and C3) and PBS treatment did not affect expression of either isoform (Fig. 2a Left). Immunostaining using the specific antibody against SOCS-1 or -3 revealed that each antisense treatment specifically reduced the targeted SOCS protein to the level in wild-type mice, whereas combination therapy down-regulated both (Fig. 2a Right). These effects were specific to liver (23); expression of SOCS-1 and -3 in muscle and fat was not significantly altered (data not shown). Because there were no differences in the three control groups (PBS, C1, and C3), in subsequent analyses, these were combined.

In concordance with the reduction of the SOCS protein in liver, antisense treatment, especially AS3 and the combination of AS1 and AS3, partially restored phosphorylation of both IRS-1 and -2 (Fig. 2b). This is consistent with the greater ability of SOCS-3 to inhibit IRS-1 and -2 phosphorylation (29). By contrast, the impaired phosphorylation of the insulin receptor in db/db mice was unaffected by the reduction of SOCS protein, consistent with the inhibitory mechanisms of SOCS proteins to specifically block IRS phosphorylation (14, 29) and the pre-existing down-regulation of the insulin receptor in liver of these obese diabetic mice (32). As a result of the enhancement of phosphorylation of IRS proteins, mice treated with antisense oligonucleotides to each of the SOCS proteins showed modest improvement in phosphoinositide 3-kinase and Akt activity, with combined therapy increasing these to up to 45% of the level of wild-type (Fig. 2c and d). The residual defects reflect the residual insulin resistance due to reduced insulin receptor signaling and protein in obese animals (32).

As a consequence of reducing SOCS expression after antisense treatment, there was a decrease in the elevated circulating insulin levels in the db/db mice consistent with improved insulin sensitivity (Fig. 2e). This was confirmed by insulin tolerance testing, which showed a significantly improved glucose-lowering effect after suppression of SOCS-3 and a tendency to improvement after reduction of SOCS-1 (Fig. 7, which is published as supporting information on the PNAS web site). Furthermore, the high levels of expression of PGC-1α in the livers of db/db mice that contribute to the increased gluconeogenesis in obese diabetic animals (20) were almost normalized by either single antisense treatment or combined therapy (Fig. 3e). These changes, however, were not sufficient to lower blood glucose concentrations in these severely diabetic mice, in part because the effect of antisense treatment was limited in liver and in part because, even in liver, the insulin receptor signaling was still impaired through other mechanisms, including down-regulation of the insulin receptor itself (32) and SOCS-independent cytokine-mediated pathways (6, 33).
These changes in hepatic and plasma triglycerides paralleled the triglyceride concentrations. Antisense treatment resulted in reductions of the elevated plasma toward normal, as evidenced by measurement of tissue triglyceride reduced the elevated lipid content in livers 70% of the way by the specific antisense oligonucleotide treatment (AS1 for SOCS-1, AS3 for SOCS-3 and their specific controls, C1 and C3; AS1 + 3 indicates combination therapy of AS1 and AS3). (Left) Representative results of RT-PCR using RNA from the liver of mouse treated with the indicated material for 2 weeks. (Right) Representative results of immunostaining using liver sections. (b) Tyrosine phosphorylation of IR, IRS-1, and IRS-2 in liver. The immunoprecipitates with antinsiulin receptor (αIR), anti-IRS-1 (αIRS-1), or anti-IRS-2 (αIRS-2) antibodies from liver lysates of the indicated mouse with or without insulin stimulation were immunoblotted with antiphosphotyrosine antibody (αPY). (c) Phosphoinositide 3-kinase activity associated with phosphotyrosine complexes in liver. (Upper) Representative result. (Lower) Each bar represents the mean SE (n = 3). (*, P < 0.05 PBS vs. AS1 or AS3.) (d) Insulin-induced Akt activity in liver. Each bar represents the mean SE (n = 3). (*, P < 0.05 PBS vs. AS1 or AS3.) (e) Decreased insulin concentrations caused by suppression of SOCS-1, SOCS-3, or both in liver. Each bar represents the mean ± SE (n = 6–18) of the plasma insulin concentrations in random fed state at day 14 after the first injection of antisense oligonucleotides. (*, P < 0.05 control vs. AS1 or AS3; **, P < 0.05 AS1 or AS3 vs. AS1 + 3.) AS1 + 3 indicates the combination therapy of AS1 and AS3, and control (Cont) includes C1, C3, and PBS groups.

**Improvement of Hepatic Steatosis and Reduction of Plasma Triglyceride Concentrations by Suppressing SOCS-1 and -3.** The most striking effects of SOCS antisense treatment in the db/db mice were on two other aspects of the metabolic syndrome, hepatic steatosis, and hyperlipidemia (34), which aggravate hyperinsulinemia and insulin resistance (35). Thus, control db/db mice exhibited severe hepatic steatosis with a 5.8-fold increase in triglyceride content in liver and more than a doubling of fasting plasma triglyceride levels compared to wild-type mice (Fig. 3 a and b). Antisense treatment, especially with AS3 and AS1 + AS3, reduced the elevated lipid content in livers 70% of the way toward normal, as evidenced by measurement of tissue triglyceride content and oil red O staining (Fig. 3a). Likewise, the antisense treatment resulted in reductions of the elevated plasma triglyceride concentrations 45–75% toward normal (Fig. 3b). These changes in hepatic and plasma triglycerides paralleled the

**changes in the expression of SREBP-1c (Fig. 3c).** SREBP-1c was prominently up-regulated in control db/db mouse by suppression of SOCS-1 and -3. In the graph, each bar represents the mean ± SE (n = 6–18; *, P < 0.05 control vs. AS1 + 3). (Right) Representative results of oil red O staining of liver with the indicated treatment. (b) Reduced concentrations of plasma triglyceride by suppression of SOCS-1 and -3 in db/db mice. Each bar represents the mean ± SE (n = 6–18; *, P < 0.05 control vs. AS3 or AS1 + 3). (c) Decreased expression of SREBP-1 in liver of db/db mouse by suppression of SOCS proteins. Shown are representative results of Northern blot analysis for SREBP-1, fatty acid synthase, or PGC-1α. (d) Decreased levels of nuclear SREBP-1 protein in liver of db/db mouse by suppression of SOCS proteins. Shown are representative results of immunoblot analysis with αSREBP-1 using nuclear fractions. AS1 + 3 indicates the combination therapy of AS1 and AS3, and control (Cont) includes C1, C3, and PBS groups.
SOCS proteins inhibit insulin signaling, suggesting that SOCS proteins modulate SREBP-1c expression by an insulin-independent mechanism. Indeed, obese animals show abnormal up-regulation of SREBP-1c expression in the fasting state (34), although persistent hyperinsulinemia caused by insulin resistance can also contribute to the up-regulation of SREBP-1c expression by elevating basal insulin signaling.

SOCS proteins have been identified as negative regulators of STAT proteins, and the promoter regions of the mouse and rat SREBP-1c genes have at least two potential STAT-binding motifs [TT(N)5AA] at -616 and -540 in the murine sequence. This raises the possibility that STAT proteins could regulate SREBP-1c promoter activity directly or indirectly by modulating expression of other regulatory factors. Indeed, several STAT proteins tested in transfection experiments, STAT3 and -5b showed inhibitory effects on SREBP-1c promoter activity (Fig. 4b; also see Fig. 8a, which is published as supporting information on the PNAS web site). The STAT3-mediated inhibition was antagonized by coexpression of SOCS-1 or -3, whereas STAT5b-mediated inhibition was little affected (Fig. 4b). Similarly, STAT3-mediated inhibition of SREBP-1c transcription and SOCS-mediated antagonism were also observed in HepG2 human hepatoma cells (Fig. 8b). By contrast, expression of a dominant negative form of STAT3 (dnSTAT3), which inhibits activation of endogenous STAT3, significantly increased SREBP-1c promoter activity. The effect of dnSTAT3 was slightly less than that produced by SOCS-3. Furthermore, the combination of dnSTAT3 and SOCS-3 was not additive, suggesting that SOCS proteins increase SREBP-1c expression, at least in part, through STAT3-mediated inhibition. Indeed, STAT3 inhibited SREBP-1c promoter activity in a dose-dependent manner, and SOCS-3 appeared to competitively attenuate this inhibition (Fig. 4d). Consistent with this, STAT3 phosphorylation was decreased by 50% in liver of the control db/db mouse and was restored by suppression of SOCS proteins (Fig. 4e), whereas there was no alteration in STAT5 phosphorylation among all groups (Fig. 8c).

Recently, Kasuga and coworkers (37) have shown that liver-specific STAT3 knockout causes marked increases in SREBP-1 expression and hepatic triglyceride content, and that adenoviral-mediated gene transfer of a constitutively active form of STAT3 in liver of db/db mouse ameliorates hepatic steatosis with down-regulation of SREBP-1 expression. Together with our data, this indicates the importance of SOCS proteins and STAT3 in the regulation of lipid synthesis in liver and a paradigm for integrating several components of the metabolic syndrome, insulin resistance, dyslipidemia, and hepatic steatosis (nonalcoholic fatty liver) in a common regulatory pathway (Fig. 5).

**Conclusion**

Obese subjects with persistently elevated cytokine levels may have down-regulated STAT3-mediated signaling and insulin signaling by increased SOCS proteins in liver. Because liver is the primary site for insulin clearance (19, 38), insulin resistance caused by increased SOCS proteins in liver may lead to persistent hyperinsulinemia that further exacerbates insulin resistance (19, 38, 39). Thus, increased SOCS proteins correspondantly increase fatty acid synthesis by up-regulation of SREBP-1c expression presumably through suppression of STAT3 phosphorylation and persistent hyperinsulinemia even in the fasting state. Overproduction of fatty acid and lipotoxicity results in further insulin resistance (35), creating a vicious cycle leading to the metabolic syndrome (Fig. 5). The present study indicates that the overexpression of SOCS proteins may be a critical step in this vicious cycle, and that reducing expression of SOCS proteins in liver presents a previously undescribed approach to treatment/prevention of hepatic steatosis and several components of the metabolic syndrome associated with diabetes and obesity.
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