

Effects of Pioglitazone on Suppressor of Cytokine Signaling 3 Expression

Potential Mechanisms for Its Effects on Insulin Sensitivity and Adiponectin Expression

Yukiko Kanatani,¹ Isao Usui,¹ Ken Ishizuka,¹ Agussalim Bukhari,¹ Shiho Fujisaka,¹ Masaharu Urakaze,¹ Tetsuro Haruta,¹ Tadimitsu Kishimoto,² Tetsuji Naka,³ and Masashi Kobayashi¹

Pioglitazone is widely used for the treatment of diabetic patients with insulin resistance. The mechanism of pioglitazone to improve insulin sensitivity is not fully understood. Recent studies have shown that the induction of suppressor of cytokine signaling 3 (SOCS3) is related to the development of insulin resistance. Here, we examined whether the insulin-sensitizing effect of pioglitazone affects the SOCS induction. In *db/db* mice and high-fat-fed mice, expression of SOCS3 mRNA in fat tissue was increased compared with that in lean control mice, and pioglitazone suppressed SOCS3 levels. In 3T3-L1 adipocytes, mediators of insulin resistance such as tumor necrosis factor- α (TNF- α), interleukin-6, growth hormone, and insulin increased SOCS3 expression, which was partially inhibited by pioglitazone. The ability of pioglitazone to suppress SOCS3 induction by TNF- α was greatly augmented by peroxisome proliferator-activated receptor γ overexpression. SOCS3 overexpression and tyrphostin AG490, a Janus kinase 2 inhibitor, or dominant-negative STAT3 expression partially inhibited adiponectin secretion and was accompanied by decreased STAT3 phosphorylation. Conversely, pioglitazone increased adiponectin secretion and STAT3 phosphorylation in fat tissue of *db/db* mice and in 3T3-L1 adipocytes. These results suggest that pioglitazone exerts its effect to improve whole-body insulin sensitivity in part through the suppression of SOCS3, which is associated with the increase in STAT3 phosphorylation and adiponectin production in fat tissue. *Diabetes* 56:795–803, 2007

From ¹The First Department of Internal Medicine, Faculty of Medicine, University of Toyama, Toyama, Japan; the ²Osaka University Graduate School of Frontier Bioscience, Osaka, Japan; and the ³Laboratory for Immune Signal, National Institute of Biomedical Innovation, Osaka, Japan.

Address correspondence and reprint requests to Isao Usui, MD, PhD, The First Department of Internal Medicine, University of Toyama, 2630 Sugitani Toyama 930-0194, Japan. E-mail: isaousui-tym@umin.ac.jp.

Received for publication 26 July 2006 and accepted in revised form 26 November 2006.

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IRS, insulin receptor substrate; JAK, Janus kinase; MAP, mitogen-activated protein; PPAR γ , peroxisome proliferator-activated receptor γ ; SOCS, suppressor of cytokine signaling; TNF- α , tumor necrosis factor- α ; TZD, thiazolidinedione.

DOI: 10.2337/db06-1039

© 2007 by the American Diabetes Association.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Insulin resistance is an important and cardinal factor for type 2 diabetes, metabolic syndrome, and obesity (1–3). Although the precise mechanisms underlying insulin resistance remain uncertain, a large body of evidence has accumulated in the past decade implicating activators of inflammatory signaling cascades as potential mediators of insulin resistance. Pro-inflammatory cytokines, fatty acids, amino acids, cellular stress, angiotensin II, and hyperinsulinemia, which are known to cause insulin resistance, activate serine/threonine (Ser/Thr) kinases, such as I κ B kinase, jun NH₂-terminal kinase, and mTOR/S6K1 (4–7). The Ser-phosphorylated insulin receptor substrate (IRS) has been reported to become a poor substrate for insulin receptor tyrosine kinase or to become a good substrate for a ubiquitin-proteasome degradation system, thereby attenuating downstream insulin signaling.

In addition, the suppressor of cytokine signaling (SOCS) proteins plays a pivotal role in the pathogenesis of metabolic disorders (8–10). SOCSs were originally cloned as inducible proteins that participate in a negative feedback loop in cytokine signaling (11–13). The SOCS protein family consists of eight members that have characteristic SH2 domains and COOH-terminal SOCS boxes. SOCS suppresses insulin signaling and cytokine signaling (8,9,14–16) through the direct association with insulin receptor (9), IRSs (8), and Janus kinase (JAK) (10). Furthermore, SOCS enhances ubiquitination of IRS, resulting in the enhanced ubiquitin-proteasomal degradation of IRS (8). This mechanism is active in vivo as well as overexpression of SOCS3 in mouse fat tissues elicits local insulin resistance (17). In contrast, tyrosine phosphorylation of IRS-1 is increased in SOCS1 knockout mice (18,19). The depletion of SOCS1/3 expression in the liver using a SOCS1/3 antisense oligonucleotide leads to not only increased insulin signaling in the liver but also improved insulin sensitivity as measured by insulin tolerance testing (10).

Pioglitazone, a thiazolidinedione, is widely used for the diabetic patients with insulin resistance. It is well known that pioglitazone ameliorates insulin resistance via the activation of peroxisome proliferator-activated receptor γ (PPAR γ). Because PPAR γ is predominantly expressed in fat tissue, pioglitazone may act mainly in fat tissue. How-

ever, glucose uptake by fat tissue is only a small part of the total-body glucose disposal, and several hypotheses have been raised as the mechanisms for the insulin-sensitizing effects of pioglitazone. First, pioglitazone increases fatty acid uptake and storage in fat tissues and decreases fatty acid in skeletal muscle and liver. As a result, insulin resistance due to the fat accumulation in skeletal muscle and liver is ameliorated (20,21). Second, pioglitazone increases the number of small adipocytes and induces apoptosis of large adipocytes. Secretion of adiponectin mainly from small adipocyte increases, whereas pro-inflammatory cytokines or fatty acids in particular from large adipocytes decline. Through these changes in secretion of these adipocytokines, pioglitazone modulates whole-body insulin sensitivity not only locally in fat tissue (22,23). Third, because a small number of PPAR γ are expressed in muscle or liver, pioglitazone may directly improve insulin sensitivity in the organs except for the fat tissue. Therefore, to understand the mechanisms of increased insulin sensitivity by pioglitazone, one must consider both direct effects in insulin target tissues and indirect effects through altered adipokine production from fat tissues.

The effect of pioglitazone treatment on SOCS-induced insulin resistance has not been extensively studied. In the current study, we examined the effects of pioglitazone on SOCS3 expression, STAT3 phosphorylation, adiponectin production, and insulin sensitivity.

RESEARCH DESIGN AND METHODS

Monoclonal anti-phosphotyrosine antibodies (PY 20) were purchased from Transduction Laboratories (Lexington, KY). Anti-*myc* antibody was from Upstate Biotechnology (Lake Placid, NY). Anti-phospho-Akt, anti-STAT3, anti-phospho-STAT3, and anti-actin antibodies were from Cell Signaling Technology (Beverly, MA). Anti-SOCS3 antibody was from Immuno-Biological Laboratories (Gunma, Japan). Interleukin (IL)-6 and growth hormone (GH) were from Sigma (St. Louis, MO). AG490 was from Sigma. Mouse Adiponectin ELISA kit was from R&D Systems (Minneapolis, MN). IL-1 α and TNF- α were provided by Dainippon Pharmaceutical (Osaka, Japan). Pioglitazone was a gift of Takeda Pharmaceutical (Osaka, Japan).

Cell culture. Murine 3T3-L1 cells were obtained from American Type Culture Collection (Manassas, VA). Briefly, after confluence, cells were left for 2 more days in Dulbecco's modified Eagle's medium/high glucose supplemented with 100 units/ml streptomycin and 10% fetal bovine serum in a 10% CO $_2$ environment. Differentiation was induced by changing the culture medium to the same one containing 0.5 mmol/l 3-isobutyl-1-methylxanthine, 1 μ mol/l dexamethasone, and 1 μ mol/l insulin for 3 days, followed by the culture in the medium containing 0.8 μ mol/l insulin for another 3 days. The medium was changed every 3 days. The cells were used for experiments 14–16 days after the induction of differentiation.

Adenovirus vectors. Adenovirus vectors containing cDNAs encoding wild-type PPAR γ and PPAR γ S112A, in which Ser112 is replaced with Ala, were constructed as previously described (24). A virus vector encoding β -galactosidase (LacZ) was used as a control (24). Adenovirus vectors encoding *myc*-tagged SOCS1 and SOCS3 were provided by Dr. Naka (Osaka University, Osaka, Japan). Adenovirus vectors encoding dominant-negative STAT3 were provided by Dr. Kunisada (Osaka University, Osaka, Japan) (25). Differentiated 3T3-L1 adipocytes were infected with adenovirus 48 h before the experiments.

Quantitative RT-PCR. Total RNA was extracted from 3T3-L1 adipocytes or mouse tissues using an ISOGEN kit or a RNeasy kit, respectively. Quantitative RT-PCR was conducted according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Briefly, we synthesized cDNA using oligo (dT) primers with the TaqMan Reverse Transcription Reagents. Reverse-transcribed cDNA was mixed with PCR Master Mix and gene-specific Assays-on-Demand Gene Expression Products and amplified on an ABI PRISM 7700 (Applied Biosystems). Results were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression.

Animals. Six-week-old C57BLKS/J *db/db* mice, C57BLS/J *db/+m* mice, and C57BL/6J mice were purchased from Clea Japan (Tokyo, Japan). All animals were housed on a 12-h light-dark cycle. In the experiments, on high-fat-fed

mice, C57BL/6J mice were fed a standard chow or a high-fat diet (Quick Fat; Clea Japan) for 12 weeks. Pioglitazone (10 mg \cdot kg $^{-1}$ \cdot day $^{-1}$) was mixed with the food and orally administered to *db/db* mice or high-fat-fed mice for 2 weeks. All protocols for animal use and euthanasia were approved by Guide for Animal Experiments of University of Toyama.

Western blotting and immunoprecipitation analysis. For animal experiments, human insulin (5 units/kg) was injected intraperitoneally. Epididymal fat pad, liver, and quadriceps muscles were removed at 10 min after injection. These tissues were homogenized for 1 min at 4°C in lysis buffer containing 25 mmol/l Tris-HCl (pH 7.4), 10 mmol/l Na $_3$ VO $_4$, 100 mmol/l NaF, 50 mmol/l Na $_4$ P $_2$ O $_7$, 10 mmol/l EGTA, 10 mmol/l EDTA, 5 mg leupeptin/ml, 5 mg aprotinin/ml, 2 mmol/l phenylmethylsulfonyl fluoride, and 1% Nonidet-P 40. 3T3-L1 adipocytes were lysed in a cell-solubilizing buffer containing 30 mmol/l Tris, pH 7.4, 150 mmol/l NaCl, 10 mmol/l EDTA, 1% Nonidet-P40, 1 mmol/l phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 1 μ mol/l leupeptin, 1 mmol/l Na $_3$ VO $_4$, and 50 mmol/l NaF. For immunoprecipitation, whole-cell lysates were centrifuged at 4°C for 20 min to remove the insoluble materials. Western blot and immunoprecipitation were performed as described previously (7).

Quantification of adiponectin secretion. The amounts of adiponectin in culture medium for 3T3-L1 adipocytes or in mouse serum were measured using Quantikine Mouse Adiponectin/Acrp30 Immunoassay kit according to the manufacturer's instruction (R&D Systems). The absorbance was measured by Labsystem iEMS Reader MF (Labsystems, Franklin, MA).

Statistical analysis. All data are presented as means \pm SE. The statistical comparison between the groups was carried out using ANOVA or Student's *t* test. *P* values <0.05 were considered statistically significant.

RESULTS

Pioglitazone decreases SOCS3 expression in epididymal fat of *db/db* mice and high-fat-fed mice. We first examined the expression levels of SOCS3 mRNA in insulin target tissues of *db/db* mice. As recently reported in *db/db* mice and high-fat-fed mice (9,14,16), SOCS3 mRNA level in epididymal fat tissues of 8-week-old *db/db* mice was increased to 2.7-fold of that in control C57BL/6J mice. After the oral administration of pioglitazone for 2 weeks, SOCS3 mRNA was decreased by ~50% of that in epididymal fat tissues of *db/db* mice without pioglitazone (Fig. 1A). In the liver, whereas baseline SOCS3 expression of *db/db* mice was not increased compared with control lean mice, it was decreased by pioglitazone (Fig. 1B). In quadriceps muscle, no changes were observed between pioglitazone-treated and nontreated animals (Fig. 1C). The expression of SOCS3 in high-fat-fed mice was also changed by pioglitazone, in a manner similar to the changes seen in the *db/db* mice. SOCS3 expression in epididymal fat of high-fat-fed mice was increased compared with that in control mice fed standard diet and was decreased by pioglitazone administration (Fig. 1D). No changes of SOCS3 expression were observed in either liver or skeletal muscle of high-fat-fed mice, and subsequent pioglitazone treatment did not alter SOCS3 mRNA in these tissues (data not shown). We confirmed the previous reports that the same treatment with pioglitazone for 2 weeks improved the glucose profile during intraperitoneal glucose tolerance test and intraperitoneal insulin tolerance test in *db/db* mice (data not shown). Pioglitazone treatment also restored Akt phosphorylation in fat, liver, and skeletal muscle of *db/db* mice (data not shown), as reported recently (26,27).

SOCS3 is induced by various causes of insulin resistance in 3T3-L1 adipocytes. Small molecule mediators of insulin resistance may have as a common mechanism the ability to increase the expression of SOCS proteins in insulin target tissues. Thus, we examined the effects of the representative mediators for insulin resistance on the induction of SOCSs in cultured 3T3-L1 adipocytes. All the stimuli, including IL-6, GH, TNF- α , and insulin, enhanced

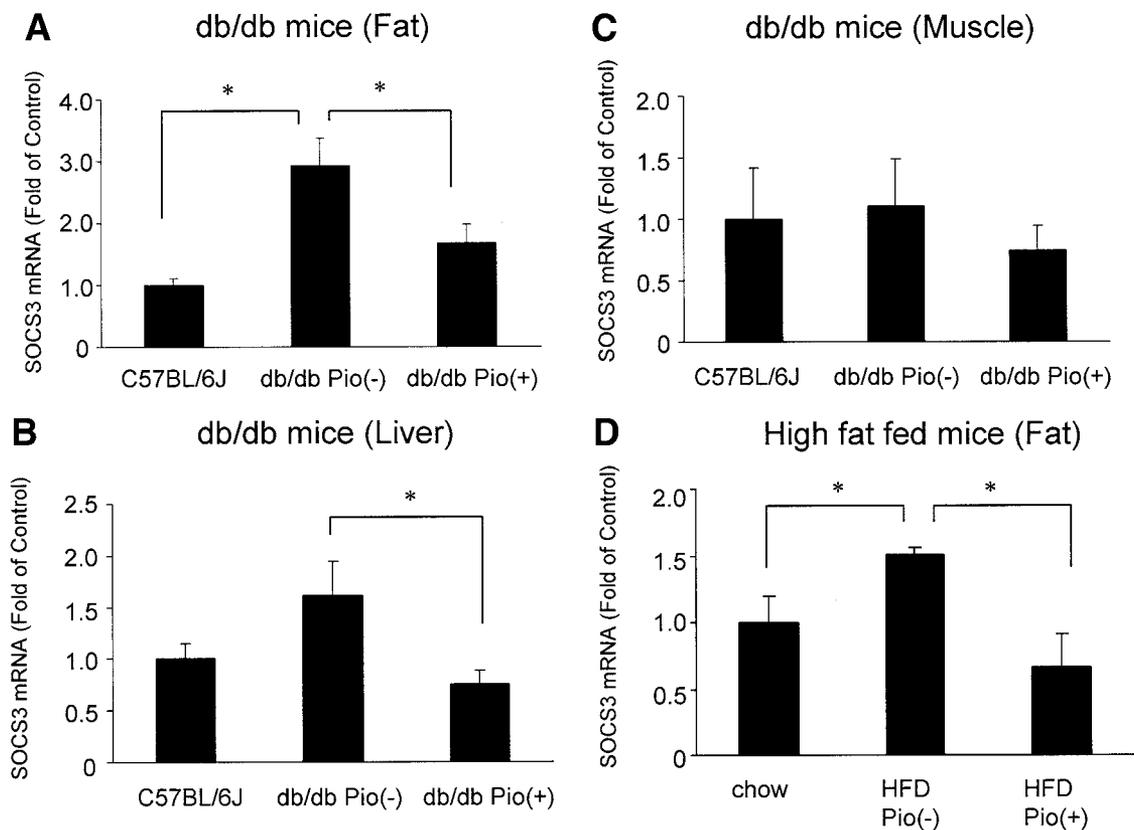


FIG. 1. Effects of pioglitazone on SOCS3 expression in insulin target tissues of *db/db* mice and high-fat-fed mice. SOCS3 mRNA in epididymal fat of *db/db* mice (A), liver of *db/db* mice (B), quadriceps muscles of *db/db* mice (C), and epididymal fat of high-fat-fed mice (D). Results are shown as means \pm SE from eight independent experiments. * $P < 0.05$ by ANOVA.

the expression of mRNAs for SOCS1 and SOCS3. Interestingly, the induction patterns of SOCS3 were varied among the mediators, e.g., the maximal induction was observed at the different time points (data not shown).

Pioglitazone inhibits SOCS3 induction in a PPAR γ -dependent manner. So far, it has not been clarified whether thiazolidinedione can prevent increases in SOCS expression by insulin-desensitizing agents. To examine this issue, we evaluated the effect of pioglitazone on SOCS3 and SOCS1 induction in 3T3-L1 adipocytes. SOCS3 mRNA induced by the stimulations with 1-h IL-6, 1-h GH, 8-h TNF- α , and 2-h insulin were suppressed by the pre-treatment with 10 μ mol/l pioglitazone by 35, 49, 53, and 61%, respectively (Fig. 2A). In contrast, pioglitazone did not change the SOCS1 mRNA level induced by the same stimuli (Fig. 2B). Figure 2C shows a dose-dependent effect of pioglitazone on the suppression of SOCS3 mRNA level induced by TNF- α . Although the suppression by pioglitazone was dose dependent, the maximal suppressive effect was partial. Because of this partial response, we wondered whether the level of PPAR γ protein present in the target tissue influenced that the response of the tissue to pioglitazone treatment. Pioglitazone is reported to exert its various functions mainly through the PPAR γ -dependent mechanisms (23,28). Therefore, we next examined the involvement of PPAR γ in reducing SOCS3 expression by pioglitazone by overexpressing exogenous PPAR γ proteins. Expression of wild-type PPAR γ alone did not affect TNF- α -induced SOCS3 expression, but wild-type PPAR γ clearly enhanced the ability of pioglitazone to suppress SOCS3 induction at an almost complete level (90% of suppression) (Fig. 2D). Mitogen-activated protein (MAP

kinase is known to phosphorylate Ser112 on PPAR γ to decrease PPAR γ transcriptional activity, when it is activated by TNF- α stimulation. Thus, we used an adenovirus vector encoding a mutant PPAR γ , in which Ser112 is replaced by Ala, which cannot be phosphorylated by MAP kinase (S112A-PPAR γ). In contrast to wild-type PPAR γ , S112A-PPAR γ expression inhibited TNF- α -induced SOCS3 expression by itself, which was further suppressed by pioglitazone. This result suggests that the reason for the failure of wild-type PPAR γ to suppress SOCS3 expression may be the phosphorylation on Ser112 by TNF- α . Taken together, these results further suggest the involvement of active PPAR γ in the inhibition of SOCS3 induction by pioglitazone.

SOCS3 partially inhibits adiponectin production by inhibiting STAT3 pathway. Adiponectin is an adipokine that is secreted from fat tissue and antagonizes pro-inflammatory signals. To test the hypothesis that SOCS3 expression in fat cells may affect insulin sensitivity in the other tissues, we examined whether altered expression of SOCS3 affects adiponectin production in 3T3-L1 adipocytes. The concentration of adiponectin in the original culture media with 10% FCS was negligible, which became detectable by our enzyme-linked immunosorbent assay detection kit after culturing with fully differentiated 3T3-L1 adipocytes for >24 h. Overexpression of SOCS3 by adenovirus vectors significantly lowered the concentration of adiponectin in the media cultured with the cells for 72 h to \sim 70% of the control without SOCS3 overexpression (Fig. 3A). In contrast, adenovirus-mediated SOCS1 overexpression, which was much more than the maximal induction of endogenous SOCS1 by TNF- α (data not shown), did not

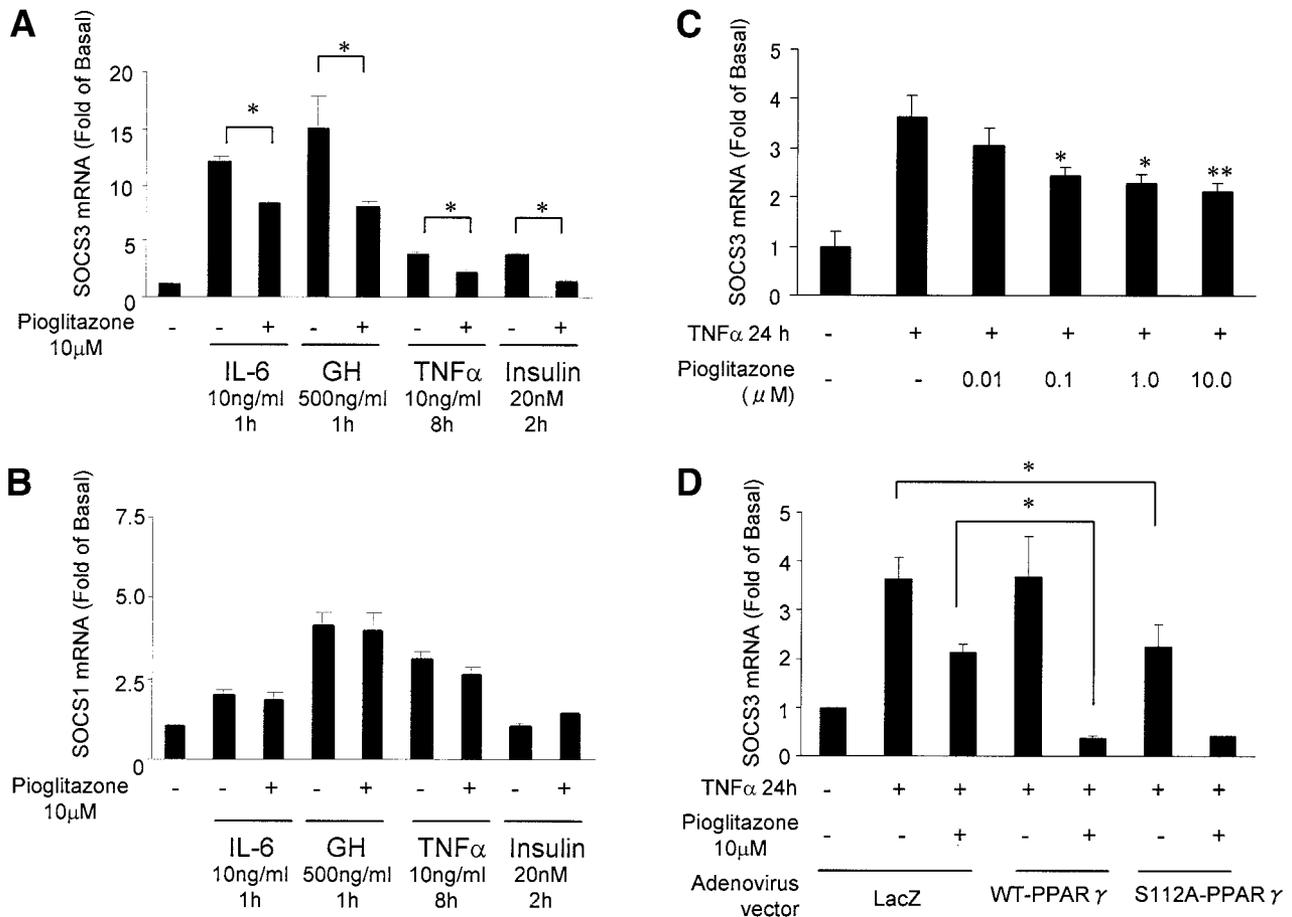


FIG. 2. Effects of pioglitazone or PPAR γ overexpression on SOCS3 induction in 3T3-L1 adipocytes. **A** and **B**: Effect of pioglitazone on SOCS3 mRNA (**A**) and SOCS1 mRNA (**B**) induced by IL-6, GH, TNF- α , and insulin. **C**: Dose-dependent effect of pioglitazone on SOCS3 mRNA induced by TNF- α . **D**: Effect of PPAR γ and/or pioglitazone on SOCS3 mRNA induced by TNF- α . Differentiated 3T3-L1 adipocytes were infected with adenovirus vectors encoding β -galactosidase (LacZ), wild-type PPAR γ , or S112A-PPAR γ for 2 days. Data were normalized to GAPDH and shown relative to untreated cells. Results are shown as means \pm SE from three independent experiments. * P < 0.05; ** P < 0.01.

affect the concentration of adiponectin (Fig. 3A). Quantitative RT-PCR analysis confirmed that SOCS3 overexpression suppressed adiponectin expression at mRNA level (Fig. 3B). However, overexpression of SOCS3 did not affect STAT3 protein level, but it completely inhibited STAT3 phosphorylation (Fig. 3C).

Because SOCS3 is an endogenous inhibitor of JAK2-STAT3 pathway, these findings suggested that inactivation of JAK2-STAT3 pathway may be involved in the partial inhibition of adiponectin production by SOCS3 overexpression. To test this possibility, we next examined the effect of tyrphostin AG490, a specific inhibitor of JAK2, on adiponectin level. As expected, AG490 completely inhibited phosphorylation of JAK2 and STAT3 (Fig. 4C). AG490 also suppressed the expression of adiponectin at the protein level (Fig. 4A) and at the mRNA level (Fig. 4B) in a dose-dependent manner. To directly investigate the roles of STAT3, the effects of adenovirus-mediated overexpression of a dominant-negative STAT3 on adiponectin production was examined. Secreted adiponectin and adiponectin mRNA were reduced by dominant-negative STAT3 overexpression in a multiplicity of infection-dependent manner (Fig. 4D and E). Expression of dominant-negative STAT3 almost completely inhibited STAT3 phosphorylation (Fig. 4F), but the inhibitory effects of dominant-negative STAT3 on adiponectin production were less potent than those of

SOCS3 overexpression or JAK2 inhibitor on adiponectin production (Figs. 3 and 4A-C).

Pioglitazone increases tyrosine phosphorylation of STAT3 in parallel with increased adiponectin levels, both in 3T3-L1 adipocytes and in *db/db* mice. Finally, we examined the effects of pioglitazone on adiponectin production and SOCS3 expression in vitro and in vivo. In contrast to the effect of JAK2 inhibitor or expression of dominant-negative STAT3, pioglitazone increased adiponectin expression at the protein level (Fig. 5A) and at the mRNA level (Fig. 5B) in a dose-dependent manner, which was paralleled with the increased STAT3 phosphorylation (Fig. 5C) in 3T3-L1 adipocytes. Furthermore, oral administration of pioglitazone for 2 weeks increased serum adiponectin concentration (Fig. 5D) and mRNA of adiponectin in epididymal fat (Fig. 5E) of *db/db* mice with increased tyrosine phosphorylation of STAT3 in the fat tissue (Fig. 5F).

DISCUSSION

Recent studies have shown that SOCS proteins may impair insulin signaling and glucose metabolism in experiments with cultured cells (8,9) or mouse models (10) overexpressing SOCS proteins. In addition, when the expression of SOCS is suppressed in knockout mice or in mouse

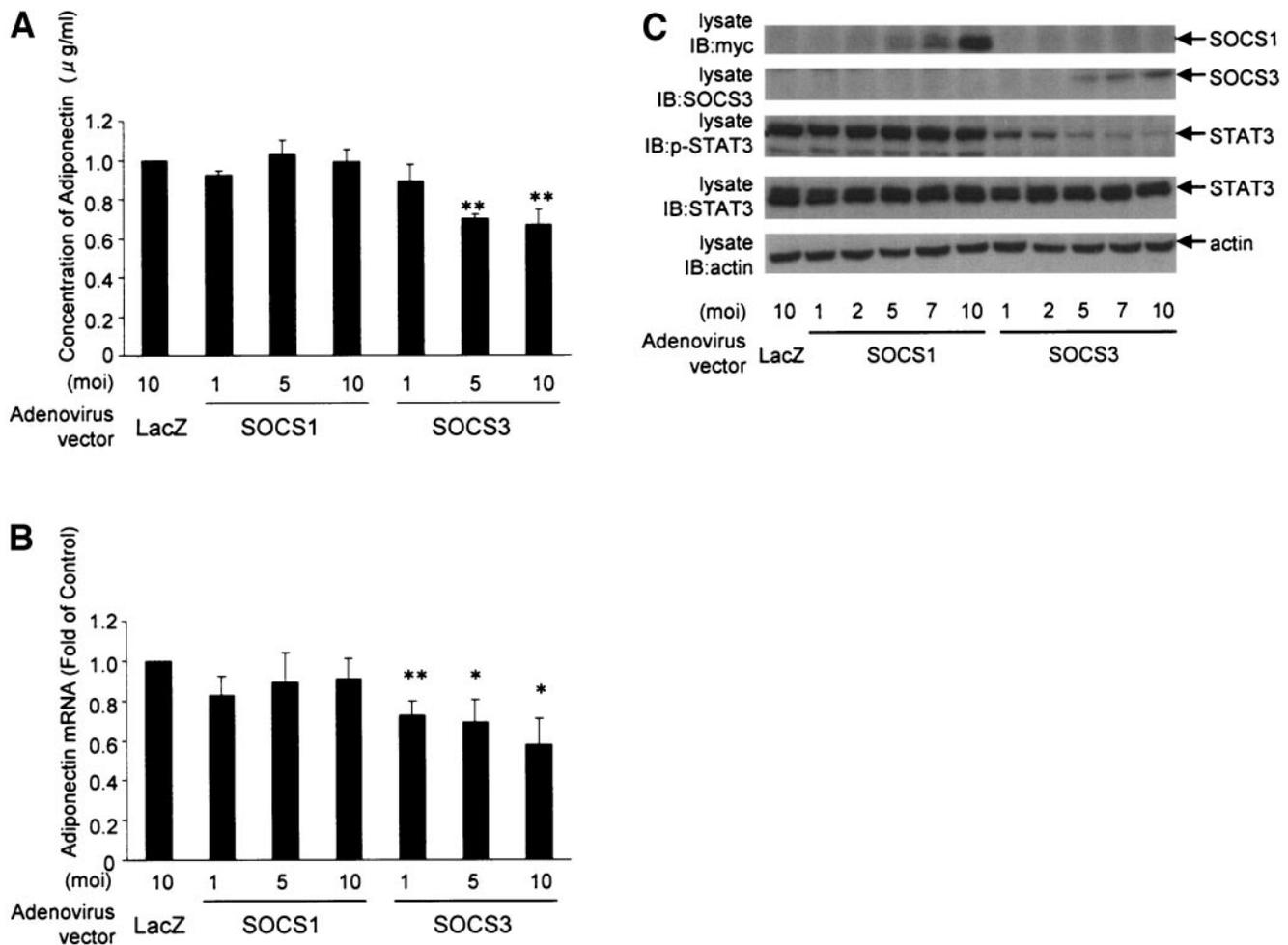


FIG. 3. Effects of SOCS3 overexpression on adiponectin production in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were infected with adenovirus vectors encoding β -galactosidase (LacZ), *myc*-tagged SOCS1, or SOCS3 for 4 days. **A:** Adiponectin concentration in the medium. **B:** Adiponectin mRNA. Data were normalized to GAPDH and were shown relative to LacZ-infected cells. **C:** STAT3 phosphorylation. Results are shown as means \pm SE from three independent experiments. * $P < 0.05$; ** $P < 0.01$ compared with LacZ-infected cells.

models treated with an antisense oligonucleotide for SOCS3 gene, insulin sensitivity is improved (10,19). In this study, we now show that the expression of endogenous SOCS3 is increased in fat tissues of insulin-resistant mice (Fig. 1) and in 3T3-L1 adipocytes treated with cytokines or hormones known to induce insulin resistance (Fig. 2) as previously reported (9,14,16,29). The synthetic insulin sensitizer, pioglitazone, suppresses the expression of SOCS3 and improves insulin sensitivity in these in vivo and in vitro insulin-resistant models via PPAR γ -dependent mechanisms. These results indicate that altered expression levels of endogenous SOCS3 in fat cells may modulate insulin sensitivity in conditions associated with insulin resistance and that SOCS3 is a target gene, whose expression is modified by pioglitazone.

In the second half of this study, we have shown that SOCS3 in fat cells inhibits adiponectin production via JAK2-STAT3-dependent mechanisms. These results are very important when we understand how SOCS3 in the fat tissue regulates whole-body insulin sensitivity, i.e., STAT3 phosphorylation was inhibited in experiments in which SOCS3 overexpression decreased adiponectin production (Fig. 3). Additionally, a chemical inhibitor of JAK2 and expression of dominant-negative STAT3 also inhibited adiponectin production (Fig. 4). These results suggest that the physiological activation of JAK2 and, at least in part,

STAT3 may be necessary for the production of adiponectin in fat cells and that increased expression of SOCS3 may decrease adiponectin production by inhibiting this pathway. The mechanism of how the activation of JAK2-STAT3 is related to adiponectin production is currently unclear. The consensus sequence of the STAT3 binding site has not yet been found in the promoter region of adiponectin gene, whereas those of several transcription factors, such as PPAR γ , SREBP1-c, and C/EBP, have been reported (30–32). One of the possibilities is that activation of STAT3 may increase adiponectin mRNA at the step other than the transcription level, e.g., increasing the stability of mRNA. Alternatively, STAT3 may indirectly enhance adiponectin production via enhancing the activity of other transcription factors. It should also be noted that the inhibitory effects of SOCS3, the JAK2 inhibitor, and dominant-negative STAT3 on adiponectin production were all partial, as shown in Figs. 3 and 4. This result suggests the involvement of the transcription factors other than STAT3, such as PPAR γ , in the regulation of gene expression of adiponectin. SOCS3 may not inhibit the activation of all these transcription factors. If so, this not only helps explain the partial inhibition of adiponectin production by SOCS3, dominant-negative STAT3, or JAK2 inhibitor (Figs. 3 and 4), but also may account for the small increase in adiponectin production by pioglitazone, which decreased

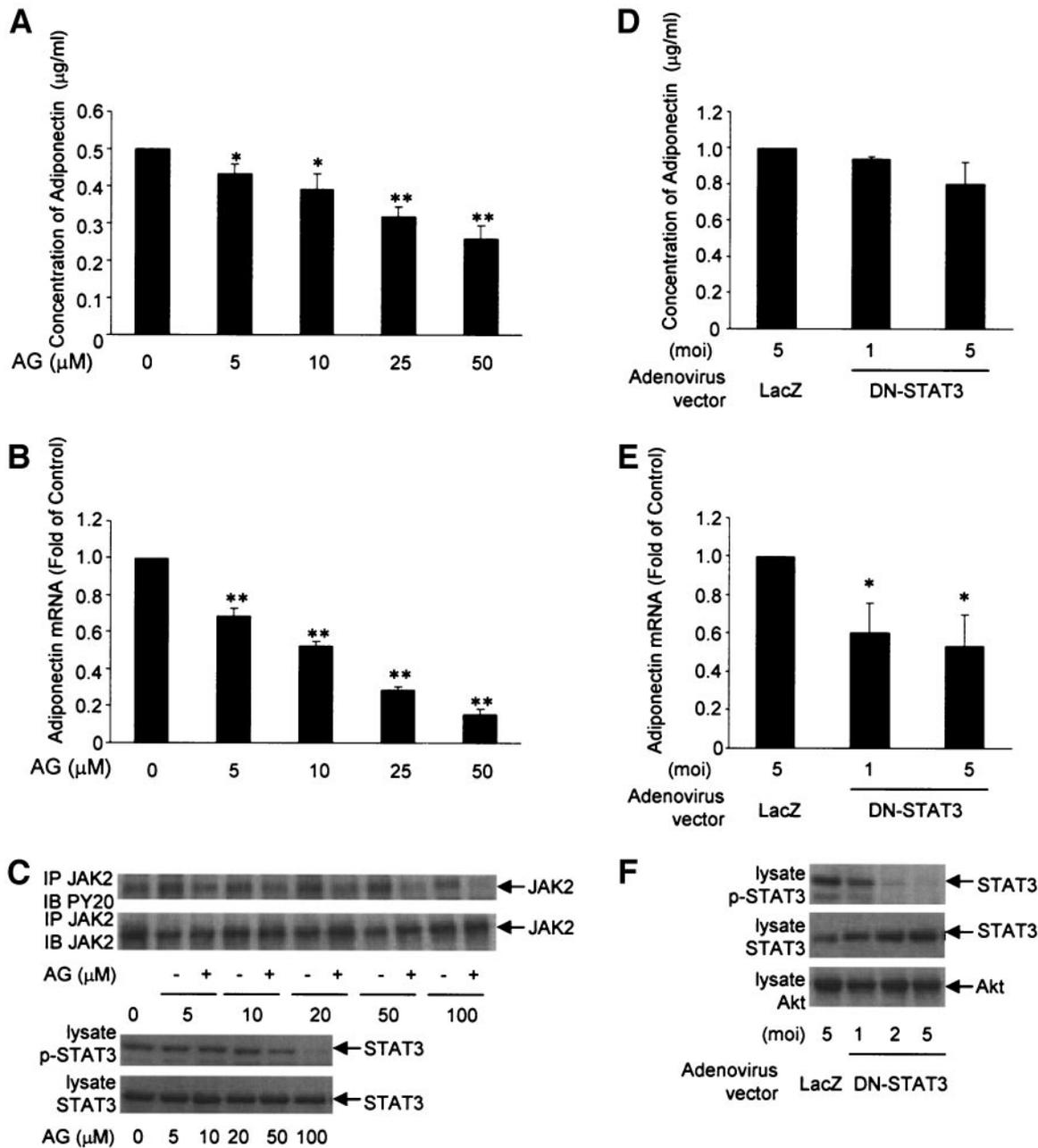


FIG. 4. Effects of JAK2 inhibitor or dominant-negative STAT3 on adiponectin production in 3T3-L1 adipocytes. *A–C:* Differentiated 3T3-L1 adipocytes were treated with the indicated concentrations of tyrphostin AG490 for 24 h. *A:* Adiponectin concentration in the medium. *B:* Adiponectin mRNA. *C:* Tyrosine phosphorylation of JAK2 and STAT3. *D–F:* Differentiated 3T3-L1 adipocytes were infected with adenovirus vectors containing LacZ or dominant-negative STAT3 (DN-STAT3) for 4 days. *D:* Adiponectin concentration in the medium. *E:* Adiponectin mRNA. Data were normalized to GAPDH and were shown relative to LacZ-infected cells. *F:* STAT3 phosphorylation. Data were normalized to GAPDH and were shown relative to control cells. Results are shown as means ± SE from three or four independent experiments. **P* < 0.05; ***P* < 0.01 compared with control cells.

SOCS3 expression and enhanced STAT3 phosphorylation (Fig. 5D). The precise mechanisms by which active JAK2-STAT3 pathway enhances adiponectin production should be more thoroughly investigated.

We have proposed that increased adiponectin production, after pioglitazone-mediated suppression of SOCS3 expression, plays an important role in improved insulin sensitivity. But in general, SOCS3 regulates signal transduction of many other factors related to insulin sensitivity. Therefore, improved insulin sensitivity after reduced SOCS3 expression may not be explained only by increased adiponectin production. For example, insulin signaling is

the most important target directly affected by SOCS3 expression among such factors. As reported in several previous papers, SOCS3 directly inhibits insulin signaling via the association to insulin receptor or the degradation of IRS proteins (8,9,14–16). Thus, decreased expression of SOCS3 by pioglitazone directly leads to the enhancement of insulin signaling in insulin-target tissues. Furthermore, some adipokines secreted from adipocytes should also be considered. We examined the effects of forced expression of SOCS3 on the production of adiponectin, TNF-α, IL-1β, and IL-6. Only the adiponectin production was significantly changed by the forced expression of SOCS3, but the

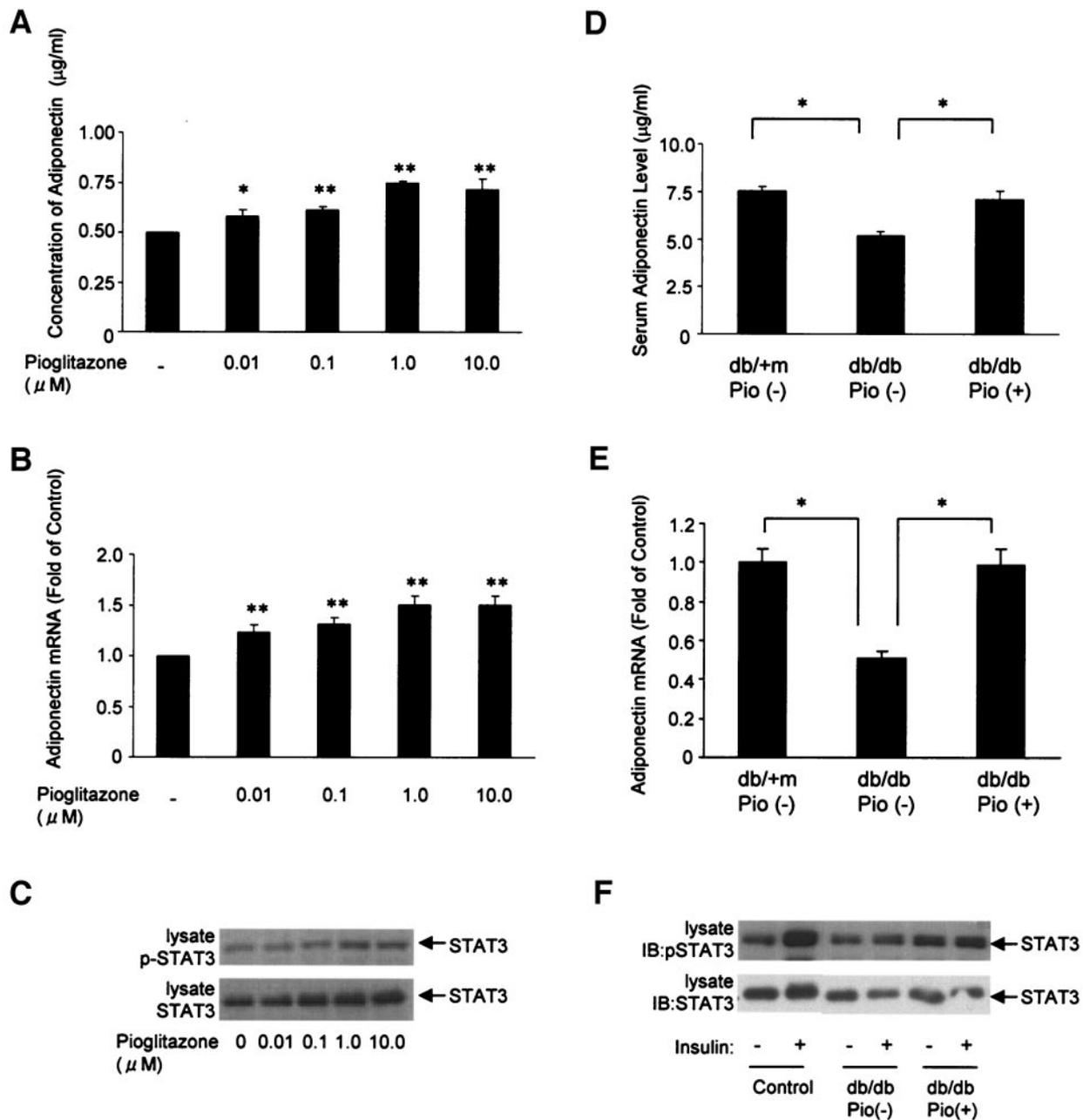


FIG. 5. Effects of pioglitazone on STAT3 phosphorylation and adiponectin production in 3T3-L1 adipocytes and in *db/db* mice. *A–C*: Differentiated 3T3-L1 adipocytes were treated with the indicated concentrations of pioglitazone for 24 h. *A*: Adiponectin concentration in the medium. *B*: Adiponectin mRNA. *C*: Tyrosine phosphorylation of STAT3. Data were normalized to GAPDH and shown relative to untreated cells. Results are shown as means \pm SE from three independent experiments. * P < 0.05; ** P < 0.01. *D–F*: *db/+m* control mice or *db/db* mice (8-weeks old) were fed standard chow with or without pioglitazone (Pio) for 2 weeks. *D*: Serum adiponectin levels. *E*: Adiponectin mRNA of epididymal fat. *F*: STAT3 phosphorylation. Results are shown as means \pm SE from four independent experiments. * P < 0.05; ** P < 0.01.

others were not (data not shown). Finally, it is highly possible that SOCS3 negatively regulated leptin signaling in our system of *db/db* mice as already reported (33,34). Unfortunately, we could not test the effects of SOCS3 on leptin signaling in the current study. It should be examined in the near future. Moreover, the improvement of insulin sensitivity by pioglitazone is not fully regulated by the expression level of SOCS3, but some other possible mechanisms are suggested, such as decreases of adipocyte size, free fatty acid, resistin, and TNF- α (35,36). An increase of adiponectin observed in our *db/db* mouse model (Fig. 5D) is a part of such complex mechanisms by which pioglitazone ameliorates insulin sensitivity.

Adiponectin exists in plasma creating three major oligomeric forms: low molecular weight, middle molecular weight, and a high molecular weight. Thiazolidinediones (TZDs) are reported to upregulate high-molecular weight adiponectin predominantly (27). Unfortunately, we could not examine the effects of pioglitazone on each form of adiponectin. But it is highly possible that pioglitazone may increase high-molecular weight adiponectin predominantly in our experimental system as in the previous reports. At least two possible mechanisms are recently reported by which TZDs increase high-molecular weight adiponectin, i.e., pioglitazone directly facilitates the generation of high-molecular weight adiponectin in addition

to the activation of adiponectin gene transcription (35). We have demonstrated that pioglitazone increases adiponectin production accompanied by the enhancement of STAT3 activation via the reduced expression of SOCS3. It is not clear whether the present data on STAT3 and SOCS3 are related to the facilitated generation of high-molecular weight adiponectin. Further studies are necessary to address this issue.

A recent study investigated the role of SOCS3 in fat tissue by creating fat-specific SOCS3 transgenic mice (17). In these mice, overexpression of SOCS3 in adipose tissue inhibited local insulin action but improved systemic glucose metabolism with high-fat diet conditions. Adiponectin production was increased, which was accompanied by the decreased amount of fat tissue. Their data seem quite different from our current results, in which suppression of SOCS3 by pioglitazone was observed together with improved glucose metabolism and increased adiponectin production. The improvement of systemic glucose metabolism and the increment of adiponectin observed in these transgenic mice may be associated with decreased adipocyte cell size as the authors suggested. In their transgenic mice, insulin sensitivity and adiponectin production may be regulated by the factors associated with altered adipocyte differentiation. Because the systems are very different, their results are not necessarily inconsistent with our current results.

In conclusion, our results indicate that SOCS3 levels are increased in the pathological conditions of insulin resistance and that pioglitazone suppresses SOCS3 expression through the activation of PPAR γ . Some parts of the insulin-sensitizing effect of pioglitazone involve its effect on SOCS3 expression in fat tissue, which is associated with the enhanced JAK2/STAT3 activity and increased production of adiponectin, leading to improved whole-body insulin sensitivity.

ACKNOWLEDGMENTS

M.K. has received a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture, Japan (17590918).

REFERENCES

1. Kahn BB, Flier JS: Obesity and insulin resistance. *J Clin Invest* 106:473–481, 2000
2. Mokdad AH, Ford ES, Bowman BA, Dietz WH, Vinicor F, Bales VS, Marks JS: Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. *JAMA* 289:76–79, 2003
3. Spiegelman BM, Flier JS: Obesity and the regulation of energy balance. *Cell* 104:531–543, 2001
4. Aguirre V, Uchida T, Yenush L, Davis R, White MF: The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *J Biol Chem* 275:9047–9054, 2000
5. Gao Z, Hwang D, Bataille F, Lefevre M, York D, Quon MJ, Ye J: Serine phosphorylation of insulin receptor substrate 1 by inhibitor kappa B kinase complex. *J Biol Chem* 277:48115–48121, 2002
6. Gao Z, Zuberi A, Quon MJ, Dong Z, Ye J: Aspirin inhibits serine phosphorylation of insulin receptor substrate 1 in tumor necrosis factor-treated cells through targeting multiple serine kinases. *J Biol Chem* 278:24944–24950, 2003
7. Haruta T, Uno T, Kawahara J, Takano A, Egawa K, Sharma PM, Olefsky JM, Kobayashi M: A rapamycin-sensitive pathway down-regulates insulin signaling via phosphorylation and proteasomal degradation of insulin receptor substrate-1. *Mol Endocrinol* 14:783–794, 2000
8. Rui L, Yuan M, Frantz D, Shoelson S, White MF: SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. *J Biol Chem* 277:42394–42398, 2002
9. Ueki K, Kondo T, Kahn CR: Suppressor of cytokine signaling 1 (SOCS-1)

- and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms. *Mol Cell Biol* 24:5434–5446, 2004
10. Ueki K, Kondo T, Tseng YH, Kahn CR: Central role of suppressors of cytokine signaling proteins in hepatic steatosis, insulin resistance, and the metabolic syndrome in the mouse. *Proc Natl Acad Sci U S A* 101:10422–10427, 2004
11. Endo TA, Masuhara M, Yokouchi M, Suzuki R, Sakamoto H, Mitsui K, Matsumoto A, Tanimura S, Ohtsubo M, Misawa H, Miyazaki T, Leonor N, Taniguchi T, Fujita T, Kanakura Y, Komiyama S, Yoshimura A: A new protein containing an SH2 domain that inhibits JAK kinases. *Nature* 387:921–924, 1997
12. Naka T, Narazaki M, Hirata M, Matsumoto T, Minamoto S, Aono A, Nishimoto N, Kajita T, Taga T, Yoshizaki K, Akira S, Kishimoto T: Structure and function of a new STAT-induced STAT inhibitor. *Nature* 387:924–929, 1997
13. Starr R, Willson TA, Viney EM, Murray LJ, Rayner JR, Jenkins BJ, Gonda TJ, Alexander WS, Metcalf D, Nicola NA, Hilton DJ: A family of cytokine-inducible inhibitors of signalling. *Nature* 387:917–921, 1997
14. Emanuelli B, Peraldi P, Filloux C, Chavey C, Freidinger K, Hilton DJ, Hotamisligil GS, Van Obberghen E: SOCS-3 inhibits insulin signaling and is up-regulated in response to tumor necrosis factor-alpha in the adipose tissue of obese mice. *J Biol Chem* 276:47944–47949, 2001
15. Emanuelli B, Peraldi P, Filloux C, Sawka-Verhelle D, Hilton D, Van Obberghen E: SOCS-3 is an insulin-induced negative regulator of insulin signaling. *J Biol Chem* 275:15985–15991, 2000
16. Shi H, Tzamelis I, Bjorbaek C, Flier JS: Suppressor of cytokine signaling 3 is a physiological regulator of adipocyte insulin signaling. *J Biol Chem* 279:34733–34740, 2004
17. Shi H, Cave B, Inouye K, Bjorbaek C, Flier JS: Overexpression of suppressor of cytokine signaling 3 in adipose tissue causes local but not systemic insulin resistance. *Diabetes* 55:699–707, 2006
18. Jamieson E, Chong MM, Steinberg GR, Jovanovska V, Fam BC, Bullen DV, Chen Y, Kemp BE, Proietto J, Kay TW, Andrikopoulos S: Socs1 deficiency enhances hepatic insulin signaling. *J Biol Chem* 280:31516–31521, 2005
19. Kawazoe Y, Naka T, Fujimoto M, Kohzaki H, Morita Y, Narazaki M, Okumura K, Saitoh H, Nakagawa R, Uchiyama Y, Akira S, Kishimoto T: Signal transducer and activator of transcription (STAT)-induced STAT inhibitor 1 (SSI-1)/suppressor of cytokine signaling 1 (SOCS1) inhibits insulin signal transduction pathway through modulating insulin receptor substrate 1 (IRS-1) phosphorylation. *J Exp Med* 193:263–269, 2001
20. Mayerson AB, Hundal RS, Dufour S, Lebon V, Befroy D, Cline GW, Enocksson S, Inzucchi SE, Shulman GI, Petersen KF: The effects of rosiglitazone on insulin sensitivity, lipolysis, and hepatic and skeletal muscle triglyceride content in patients with type 2 diabetes. *Diabetes* 51:797–802, 2002
21. Yki-Jarvinen H: Thiazolidinediones. *N Engl J Med* 351:1106–1118, 2004
22. Maeda N, Takahashi M, Funahashi T, Kihara S, Nishizawa H, Kishida K, Nagaretani H, Matsuda M, Komuro R, Ouchi N, Kuriyama H, Hotta K, Nakamura T, Shimomura I, Matsuzawa Y: PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. *Diabetes* 50:2094–2099, 2001
23. Okuno A, Tamemoto H, Tobe K, Ueki K, Mori Y, Iwamoto K, Umehara K, Akanuma Y, Fujiwara T, Horikoshi H, Yazaki Y, Kadowaki T: Troglitazone increases the number of small adipocytes without the change of white adipose tissue mass in obese Zucker rats. *J Clin Invest* 101:1354–1361, 1998
24. Iwata M, Haruta T, Usui I, Takata Y, Takano A, Uno T, Kawahara J, Ueno E, Sasaoka T, Ishibashi O, Kobayashi M: Pioglitazone ameliorates tumor necrosis factor-alpha-induced insulin resistance by a mechanism independent of adipogenic activity of peroxisome proliferator-activated receptor-gamma. *Diabetes* 50:1083–1092, 2001
25. Kunisada K, Tone E, Fujio Y, Matsui H, Yamauchi-Takahara K, Kishimoto T: Activation of gp130 transduces hypertrophic signals via STAT3 in cardiac myocytes. *Circulation* 98:346–352, 1998
26. Chick WL, Lavine RL, Like AA: Studies in the diabetic mutant mouse. V. Glucose tolerance in mice homozygous and heterozygous for the diabetes (db) gene. *Diabetologia* 6:257–262, 1970
27. Pajvani UB, Hawkins M, Combs TP, Rajala MW, Doebber T, Berger JP, Wagner JA, Wu M, Knopps A, Xiang AH, Utzschneider KM, Kahn SE, Olefsky JM, Buchanan TA, Scherer PE: Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione-mediated improvement in insulin sensitivity. *J Biol Chem* 279:12152–12162, 2004
28. Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA: An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J Biol Chem* 270:12953–12956, 1995
29. Fasshauer M, Kralisch S, Klier M, Lossner U, Bluher M, Klein J, Paschke R:

- Insulin resistance-inducing cytokines differentially regulate SOCS mRNA expression via growth factor- and Jak/Stat-signaling pathways in 3T3-L1 adipocytes. *J Endocrinol* 181:129–138, 2004
30. Iwaki M, Matsuda M, Maeda N, Funahashi T, Matsuzawa Y, Makishima M, Shimomura I: Induction of adiponectin, a fat-derived antidiabetic and antiatherogenic factor, by nuclear receptors. *Diabetes* 52:1655–1663, 2003
 31. Park SK, Oh SY, Lee MY, Yoon S, Kim KS, Kim JW: CCAAT/enhancer binding protein and nuclear factor- κ B regulate adiponectin gene expression in adipose tissue. *Diabetes* 53:2757–2766, 2004
 32. Seo JB, Moon HM, Noh MJ, Lee YS, Jeong HW, Yoo EJ, Kim WS, Park J, Youn BS, Kim JW, Park SD, Kim JB: Adipocyte determination- and differentiation-dependent factor 1/sterol regulatory element-binding protein 1c regulates mouse adiponectin expression. *J Biol Chem* 279:22108–22117, 2004
 33. Howard JK, Cave BJ, Oksanen LJ, Tzamelis I, Bjorbaek C, Flier JS: Enhanced leptin sensitivity and attenuation of diet-induced obesity in mice with haploinsufficiency of Socs3. *Nat Med* 10:734–738, 2004
 34. Mori H, Hanada R, Hanada T, Aki D, Mashima R, Nishinakamura H, Torisu T, Chien KR, Yasukawa H, Yoshimura A: Socs3 deficiency in the brain elevates leptin sensitivity and confers resistance to diet-induced obesity. *Nat Med* 10:739–743, 2004
 35. Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K, Tobe K: Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *J Clin Invest* 116:1784–1792, 2006
 36. Kubota N, Terauchi Y, Kubota T, Kumagai H, Itoh S, Satoh H, Yano W, Ogata H, Tokuyama K, Takamoto I, Mineyama T, Ishikawa M, Moroi M, Sugi K, Yamauchi T, Ueki K, Tobe K, Noda T, Nagai R, Kadowaki T: Pioglitazone ameliorates insulin resistance and diabetes by both adiponectin-dependent and -independent pathways. *J Biol Chem* 281:8748–8755, 2006