

Activation of Peroxisome Proliferator–Activated Receptor- β/δ (PPAR- β/δ) Ameliorates Insulin Signaling and Reduces SOCS3 Levels by Inhibiting STAT3 in Interleukin-6–Stimulated Adipocytes

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OBJECTIVE—It has been suggested that interleukin (IL)-6 is one of the mediators linking obesity-derived chronic inflammation with insulin resistance through activation of STAT3, with subsequent upregulation of suppressor of cytokine signaling 3 (SOCS3). We evaluated whether peroxisome proliferator-activated receptor (PPAR)- β/δ prevented activation of the IL-6-STAT3-SOCS3 pathway and insulin resistance in adipocytes.

RESEARCH DESIGN AND METHODS—Adipocytes and white adipose tissue from wild-type and PPAR- β/δ -null mice were used to evaluate the effect of PPAR- β/δ on the IL-6-STAT3-SOCS3 pathway.

RESULTS—First, we observed that the PPAR- β/δ agonist GW501516 prevented both IL-6-dependent reduction in insulin-stimulated Akt phosphorylation and glucose uptake in adipocytes. In addition, this drug treatment abolished IL-6-induced SOCS3 expression in differentiated 3T3-L1 adipocytes. This effect was associated with the capacity of the drug to prevent IL-6-induced STAT3 phosphorylation on Tyr⁷⁰⁵ and Ser⁷²⁷ residues in vitro and in vivo. Moreover, GW501516 prevented IL-6-dependent induction of extracellular signal-related kinase (ERK)1/2, a serine-threonine-protein kinase involved in serine STAT3 phosphorylation. Furthermore, in white adipose tissue from PPAR- β/δ -null mice, STAT3 phosphorylation (Tyr⁷⁰⁵ and Ser⁷²⁷), STAT3 DNA-binding activity, and SOCS3 protein levels were higher than in wild-type mice. Several steps in STAT3 activation require its association with heat shock protein 90 (Hsp90), which was prevented by GW501516 as revealed in immunoprecipitation studies. Consistent with this finding, the STAT3-Hsp90 association was enhanced in white adipose tissue from PPAR- β/δ -null mice compared with wild-type mice.

CONCLUSIONS—Collectively, our findings indicate that PPAR- β/δ activation prevents IL-6-induced STAT3 activation by inhibiting ERK1/2 and preventing the STAT3-Hsp90 association, an effect that may contribute to the prevention of cytokine-induced insulin resistance in adipocytes. *Diabetes* 60:1990–1999, 2011

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Accumulating evidence suggests that type 2 diabetes is associated with a cytokine-related acute-phase reaction, as part of an overall inflammatory state. Indeed, insulin resistance correlates with increased acute-phase response marker levels, including tumor necrosis factor- α (TNF- α) (1), interleukin (IL)-1 β (2), and IL-6 (3–5). Of these cytokines, IL-6 shows a strong association with obesity in both human and rodent models. Thus elevated levels of IL-6 in humans positively correlate with obesity and insulin resistance and predict the development of type 2 diabetes (5–7), whereas depletion of IL-6 ameliorates insulin signaling in obese mice (8).

IL-6 signals through a transmembrane receptor complex containing the common signal transducing receptor glycoprotein gp130, which activates Janus tyrosine kinases (Jak1, Jak2, Tyk2), with subsequent Tyr⁷⁰⁵ phosphorylation of STAT3 (9–11). Phosphorylated STAT3 dimerizes and translocates to the nucleus, where it regulates the transcription of target genes through binding to specific DNA-responsive elements (12). In addition to activation by Tyr⁷⁰⁵ phosphorylation, STAT3 also requires phosphorylation on Ser⁷²⁷ to achieve maximal transcriptional activity (13,14). Protein kinases involved in STAT3 serine phosphorylation include protein kinase C, Jun NH₂-terminal kinase, extracellular signal-regulated kinase (ERK), the mitogen-activated protein kinase p38, and mammalian target of rapamycin (mTOR) (15). Interestingly, interaction of STAT3 with the chaperone heat shock protein 90 (Hsp90) contributes to many steps in STAT3 activation (16).

Suppressor of cytokine signaling (SOCS) is a family of target genes that are upregulated through IL-6-mediated activation of STAT3. These SOCS proteins were originally described as cytokine-induced molecules involved in a negative feedback loop of cytokine (17) and insulin signaling (18). Several studies have reported that SOCS3 can inhibit insulin signaling (18–20) by direct interaction with the insulin receptor and by preventing the coupling of insulin receptor substrate (IRS)-1 with the insulin receptor, thereby inhibiting IRS-1 tyrosine phosphorylation and downstream insulin signaling (18,19). In addition, SOCS3 inhibits insulin signaling by proteasomal-mediated degradation of IRS-1 (20). Thus overexpression of SOCS3 in adipocytes inhibits insulin signal transduction (19,21), whereas SOCS3 deficiency in adipocytes increases insulin-stimulated IRS-1 phosphorylation and glucose uptake (22).

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily of ligand-inducible transcription factors that form heterodimers with retinoid X receptors (RXRs) and bind to consensus DNA sites (23). In addition, PPARs may suppress inflammation through diverse mechanisms, such as reduced release of inflammatory factors or stabilization of repressive complexes at inflammatory gene promoters (24–27). Of the three PPAR isotypes found in mammals, PPAR- α (NR1C1) (28) and PPAR- γ (NR1C3) are the targets for hypolipidemic (fibrates) and antidiabetic (thiazolidinediones) drugs, respectively. Finally, activation of the third isotype, PPAR- β/δ (NR1C2, called PPAR- δ below), enhances fatty acid catabolism in adipose tissue and skeletal muscle; therefore, it has been proposed as a potential treatment for insulin resistance (29). Recently, it was reported that agonist-activated PPAR- δ interferes with IL-6-mediated acute phase reaction in the liver by inhibiting the transcriptional activity of STAT3 (30), although the exact molecular mechanism involved remains unknown. Given the prominent role of the STAT3-SOCS3 pathway in IL-6-mediated insulin resistance in adipocytes, we explored whether PPAR- δ activation by GW501516 prevented IL-6-mediated insulin resistance in adipocytes and the mechanisms involved. PPAR- δ activation by GW501516 prevented the reduction in insulin-stimulated Akt phosphorylation and glucose uptake, indicating that this drug prevents IL-6-induced insulin resistance. In addition, we found that this drug prevented IL-6-mediated induction of SOCS3 mRNA levels and STAT3 phosphorylation in 3T3-L1 adipocytes. Consistent with the role of PPAR- δ in blocking IL-6-induced STAT3 activity, STAT3-DNA binding activity and STAT3 phosphorylation was higher in white adipose tissue from PPAR- δ -null mice than in wild-type mice. Our findings also show that PPAR- δ activation elicited STAT3 dissociation from Hsp90 in adipocytes, whereas the association of these two proteins was greatly enhanced in white adipose tissue in PPAR- δ -null mice compared with wild-type mice. Overall, on the basis of our findings, we suggest that PPAR- δ activation can ameliorate insulin resistance in adipose tissue by preventing activation of the STAT3-SOCS3 pathway by cytokines.

RESEARCH DESIGN AND METHODS

Materials. The PPAR- δ ligand GW501516 was obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Other chemicals were from Sigma (St. Louis, MO).

Cell culture. 3T3-L1 preadipocytes [American Type Culture Collection (ATCC)] were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum. Two days after confluence (day 0), differentiation of the 3T3-L1 cells was induced in DMEM containing 10% FBS, methylisobutylxanthine (500 $\mu\text{mol/L}$), dexamethasone (0.25 $\mu\text{mol/L}$), and insulin (5 $\mu\text{g/mL}$) for 48 h. The cells were then incubated in 10% FBS/DMEM with insulin for 3 days and this was then replaced with FBS/DMEM. Medium was changed every 2 days. Fat droplets were observed in more than 90% of cells after day 10. Adipocytes were then incubated with 10 $\mu\text{mol/L}$ GW501516 and IL-6 (10 or 100 ng/mL) for the times indicated. After incubation, RNA and total and nuclear protein extracts were extracted from adipocytes as described below. Inhibitors were added 30 min before incubation with IL-6.

Animals. Obese male ZDF rats (ZDF/Gmi, *fa/fa*) and their lean littermates (*fa/+* or *+/+*) were used. Both strains were maintained under standard light-dark cycle (12-h light/dark cycle) and temperature ($21 \pm 1^\circ\text{C}$) conditions and fed with Purina 5008 chow. Male ZDF and lean rats were killed at 12 weeks of age. CD-1 male mice (12 weeks old) were treated for 48 h with vehicle (100 μL PBS-0.1% BSA and 0.5% w/v carboxymethylcellulose medium viscosity), IL-6 (0.8 $\mu\text{g/g}$ body wt i.p.), or IL-6 plus GW501516 (one daily oral gavage of 3 mg/kg/day GW501516 dissolved in carboxymethylcellulose). Epididymal white adipose

tissue of rats and mice was rapidly removed, frozen in liquid nitrogen, and stored at -80°C . All procedures were conducted in accordance with the principles and guidelines established by the University of Barcelona Bioethics Committee, as stated in Law 5/1995, 21 July, passed by the Generalitat de Catalunya.

The generation of PPAR- δ -null mice was described previously (31). Eight male PPAR- δ -null mice and eight of their control male PPAR- δ wild-type mice were used (5 to 6 months of age). In agreement with the guidelines specified by the veterinary office of Lausanne (Switzerland), the mice were housed under standard light-dark cycle (12-h light/dark cycle) and temperature ($21 \pm 1^\circ\text{C}$) conditions and fed with Provimi Kliba 3436 chow. Epididymal white adipose tissue was rapidly removed, frozen in liquid nitrogen, and stored at -80°C .

2-Deoxy-D-[^{14}C]glucose uptake experiment. Determination of 2-Deoxy-D-[^{14}C]glucose (2-DG) uptake was performed as reported elsewhere (32).

Measurements of mRNA. Levels of mRNA were assessed by RT-PCR as previously described (33). Total RNA was isolated using the Ultraspec reagent (Biotecx, Houston, TX). The total RNA isolated by this method is nondegraded and free of protein and DNA contamination. The sequences of the sense and antisense primers used for amplification were: *Socs3* (suppressor of cytokine signaling 3) 5'-TTTTCGCTGCAGAGTGACCCC-3' and 5'-TGGAGGAGAGAGATCGGCTCA-3'; early growth response (*Egr-1*), 5'-CTTCTCTGCTCCCA-GAGCC-3' and 5'-TGGGAACCTGGAACACCCT-3', and *Aprt* (adenosyl phosphoribosyl transferase), 5'-GCCTCTTGCCAGTCACCTGA-3' and 5'-CCA-GGCTCACACTCCACCA-3'. Amplification of each gene yielded a single band of the expected size (*Socs3*: 250 bp, *Egr-1*: 210 bp, and *Aprt*: 329 bp). Preliminary experiments were carried out with various amounts of cDNA to determine nonsaturating conditions of PCR amplification for all the genes studied. Under these conditions, relative quantification of mRNA was then assessed by the RT-PCR method used in this study (34). Radioactive bands were quantified by video-densitometric scanning (Vilbert Lourmat Imaging). The results for the expression of specific mRNAs are always presented relative to the expression of the control gene (*Aprt*).

Isolation of nuclear extracts. Nuclear extracts were isolated as previously described (35). Cells were scraped into 1.5 mL of cold PBS, pelleted for 10 s, and resuspended in 400 μL cold Buffer A (10 mM HEPES [pH 7.9 at 4°C], 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, and 5 $\mu\text{g/mL}$ aprotinin) by flicking the tube. Cells were allowed to swell on ice for 10 min and then vortexed for 10 s. Samples were then centrifuged for 10 s, and the supernatant fraction was discarded. Pellets were resuspended in 50 μL of cold Buffer C (20 mM HEPES-KOH [pH 7.9 at 4°C], 25% glycerol, 420 mM NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 5 $\mu\text{g/mL}$ aprotinin, and 2 $\mu\text{g/mL}$ leupeptin) and incubated on ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation for 2 min at 4°C , and the supernatant fraction (containing DNA-binding proteins) was stored at -80°C . Nuclear extract concentration was determined by the Bradford method.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was performed using double-stranded oligonucleotides (Santa Cruz) for the consensus binding site of the STAT3 nucleotide (5'-GATCCTCTGGGAA-TTCCTAGATC-3'). Oligonucleotides were labeled in the following reaction: 2 μL of oligonucleotide (1.75 pmol/ μL), 2 μL $5\times$ kinase buffer, 1 μL T4 polynucleotide kinase (10 units/ μL), and 2.5 μL [$\gamma\text{-}^{32}\text{P}$]ATP (3,000 Ci/mmol at 10 mCi/mL) incubated at 37°C for 2 h. The reaction was stopped by adding 90 μL of TE buffer (10 mmol/L Tris-HCl [pH 7.4] and 1 mmol/L EDTA). To separate the labeled probe from the unbound ATP, the reaction mixture was eluted in a Nick column (Amersham) following the manufacturer's instructions. Crude nuclear protein (μg) was incubated for 10 min on ice in binding buffer (10 mmol/L Tris-HCl [pH 8.0], 25 mmol/L KCl, 0.5 mmol/L DTT, 0.1 mmol/L EDTA [pH 8.0], 5% glycerol, 5 mg/mL BSA, and 50 $\mu\text{g/mL}$ poly[di-dC]), in a final volume of 15 μL . Labeled probe ($\sim 75,000$ cpm) was added, and the reaction was incubated for 30 min at 4°C . Where indicated, specific competitor oligonucleotide was added before the labeled probe and incubated for 20 min on ice. STAT3 antibody was added 15 min before incubation with the labeled probe at 4°C . Protein-DNA complexes were resolved by electrophoresis at 4°C on a 5% acrylamide gel and subjected to autoradiography.

Antibodies, immunoprecipitation, and immunoblotting. Antibodies against total and phospho-ERK1/2 and phospho-STAT3 (Tyr⁷⁰⁵ and Ser⁷²⁷) were purchased from Cell Signaling. Antibodies against total STAT3 and Hsp90 were purchased from Santa Cruz.

To obtain total protein, cells and adipose tissue were homogenized in radioimmunoprecipitation assay (RIPA) buffer (Sigma) with phosphatase inhibitors (0.2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 5.4 $\mu\text{g/mL}$ aprotinin). The homogenate was centrifuged at 16,700g for 30 min at 4°C . Protein concentration was measured by the Bradford method.

Whole-cell lysates and nuclear extracts were mixed with various antibodies (as specified under RESULTS) and protein A coupled to agarose beads. Proteins from whole-cell lysates, nuclear extracts, and immunoprecipitates were separated by SDS-PAGE and then transferred to immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA) and blotted with various antibodies (as specified in RESULTS). Detection was achieved using the EZ-ECL chemiluminescence kit (Amersham). Size of detected proteins was estimated using protein molecular-mass standards (Invitrogen, Barcelona, Spain).

Statistical analyses. Data are presented as mean \pm SD of five separate experiments. Significant differences were established by one-way ANOVA, using the GraphPad InStat program (GraphPad Software V2.03, GraphPad Software, San Diego, CA). When significant variations were found, the Tukey-Kramer multiple comparisons test was applied. Differences were considered significant at $P < 0.05$.

RESULTS

PPAR- δ activation restores IL-6 defects in Akt activation and glucose uptake in response to insulin. We first examined the effects of the PPAR- δ agonist GW501516 on IL-6-induced insulin resistance, which was assessed as the inhibition of insulin-stimulated Akt phosphorylation and glucose uptake. Differentiated 3T3-L1 adipocytes were stimulated with IL-6 in the absence or in the presence of GW501516, a selective ligand for PPAR- δ with a 1,000-fold higher affinity toward PPAR- δ than PPAR- α and PPAR- γ (36). Exposure of adipocytes to IL-6 for 24 h caused

a reduction in insulin-stimulated Akt phosphorylation (Fig. 1A). In contrast, when cells preincubated with 10 $\mu\text{mol/L}$ GW501516 were exposed to IL-6 the inhibitory effect of this cytokine on insulin-stimulated Akt phosphorylation was prevented. The effect of GW501516 on insulin-stimulated Akt phosphorylation was also observed at lower concentrations and the effect attained at 10 $\mu\text{mol/L}$ was dependent on PPAR- δ since it was abolished by coincubation with the PPAR- δ antagonist GSK0660 (Fig. 1B). Similarly, GW501516 significantly reversed the reduction observed in glucose uptake in IL-6-stimulated cells (Fig. 1C). Drug treatment in the absence of insulin did not affect the phosphorylation status of Akt (data not shown). Thus GW501516 treatment offered protection against a reduction in insulin responsiveness by IL-6.

PPAR- δ activation inhibits IL-6-induced SOCS3 expression in 3T3-L1 adipocytes by preventing STAT3 activation. Because IL-6-induced insulin resistance in 3T3-L1 adipocytes has been attributed to SOCS3 (22), we then examined the effect of PPAR- δ activation on the mRNA levels of the STAT3-target gene *SOCS3*. Differentiated 3T3-L1 adipocytes were stimulated with 10 ng/mL of IL-6 for 1 h in the absence or in the presence of GW501516. Under these conditions, the increase in SOCS3

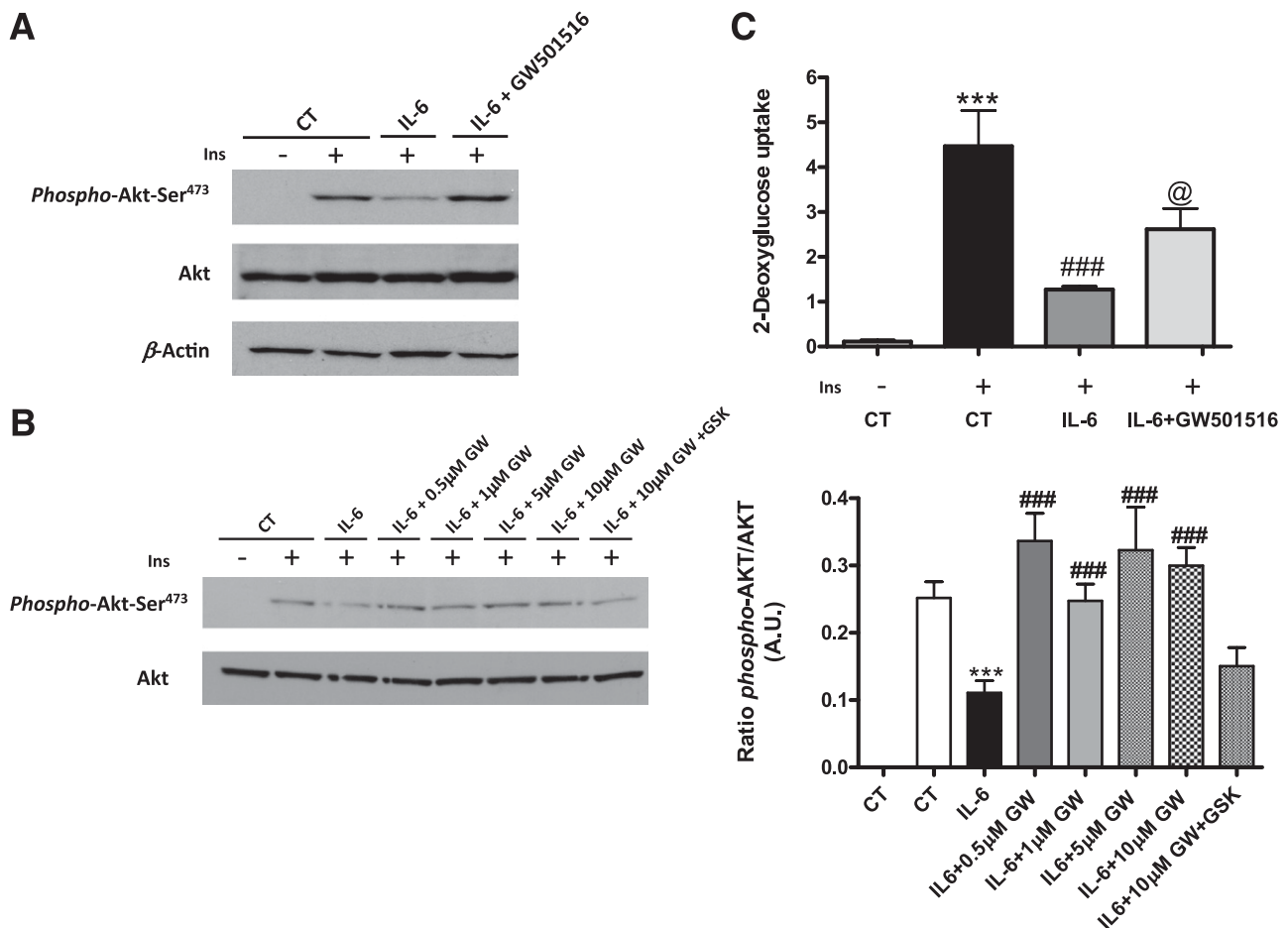


FIG. 1. PPAR- δ activation antagonizes IL-6 action by restoring insulin responsiveness. Differentiated adipocytes were stimulated with 100 nmol/L insulin for 30 min, with or without pretreatment with either 10 $\mu\text{mol/L}$ GW501516 or 100 ng/mL IL-6 for 24 h. **A:** Cell lysates were subjected to Western blot analysis for phospho-Akt(Ser⁴⁷³) and total Akt and β -actin. **B:** Different concentrations of GW501516 were assayed on insulin-stimulated Akt-phosphorylation in cells exposed to IL-6. Indicated cells were pretreated for 30 min with 10 $\mu\text{mol/L}$ GSK0660 before treatment with GW501516. *** $P < 0.001$ vs. control cells stimulated with insulin; ### $P < 0.001$ vs. IL-6-stimulated cells in the presence of insulin. **C:** 2-Deoxyglucose uptake was assessed without or with insulin. Values are means \pm SD of six independent experiments. *** $P < 0.001$ vs. control cells without insulin stimulation; ### $P < 0.001$ vs. control cells stimulated with insulin; @ $P < 0.05$ vs. IL-6-exposed cells.

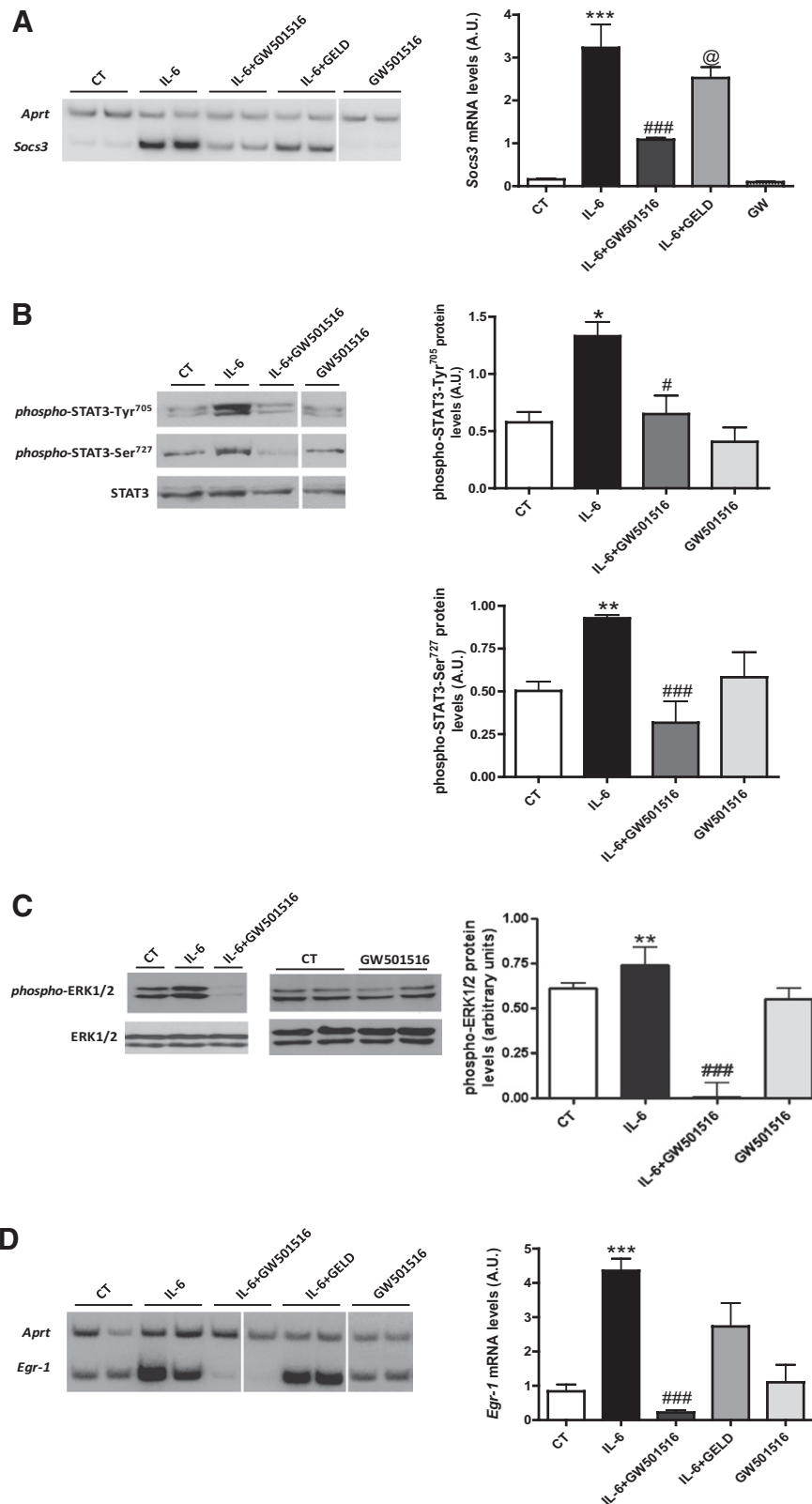


FIG. 2. The PPAR- δ agonist GW501516 prevents IL-6-induced SOCS3 expression and STAT3 phosphorylation in 3T3-L1 adipocytes. **A:** Analysis of the mRNA levels of *Socs3* in serum-starved differentiated adipocytes untreated or treated with 10 $\mu\text{mol/L}$ GW501516 for 24 h or 2 $\mu\text{mol/L}$ geldanamycin for 30 min before stimulation with 10 ng/mL IL-6 for 1 h. Total RNA was isolated and analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the *Appt* mRNA levels are shown. Data are the means \pm SD of five independent experiments. 3T3-L1 adipocytes were treated with 10 $\mu\text{mol/L}$ GW501516 for 24 h before stimulation with 10 ng/mL IL-6 for 24 h. Nuclear (**B**) or total cell extracts (**C**) were subjected to Western blot analysis with phospho-STAT3 (Tyr⁷⁰⁵ and Ser⁷²⁷) or STAT3 antibodies (**B**) or phospho-ERK1/2 and ERK1/2 (**C**) antibodies. **D:** Analysis of the mRNA levels of *Egr-1* in 3T3-L1 serum-starved differentiated adipocytes untreated or treated with 10 $\mu\text{mol/L}$ GW501516 for 24 h before stimulation with 10 ng/mL IL-6 for 1 h. Total RNA was isolated and analyzed by RT-PCR. Bars are the means \pm SD of five independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. control; @ $P < 0.05$ vs. IL-6+GW501516-exposed cells; # $P < 0.05$ and ### $P < 0.001$ vs. IL-6-stimulated cells. Dividing lines indicate grouping of images from different parts of the same gel.

mRNA levels caused by IL-6 exposure (23-fold induction; $P < 0.001$) was reduced in cells coincubated with IL-6 plus GW501516 (sevenfold induction; $P < 0.001$ vs. IL-6-stimulated cells) (Fig. 2A). Furthermore, because of recent reports showing suppression of IL-6 signaling through inhibition of STAT3-Hsp90 interaction by the selective Hsp90 inhibitor geldanamycin (16,37,38), we also evaluated the effects of the latter. Geldanamycin treatment significantly reduced the increase in SOCS3 mRNA levels caused by IL-6 ($P < 0.05$ vs. IL-6-stimulated cells). Overall, these findings suggest that PPAR- δ activation inhibits STAT3. Dimerization, nuclear translocation, and increase in transcriptional activity of STAT3 require its phosphorylation on tyrosine residue 705. In addition, STAT3 phosphorylation on Ser⁷²⁷ is required for its maximal activation (13,14). When we analyzed the phosphorylation status of STAT3 we observed that IL-6 exposure increased both Tyr⁷⁰⁵ and Ser⁷²⁷ phosphorylation, whereas in the presence of GW501516 these changes were prevented (Fig. 2B). Because IL-6 activates ERK1/2 (10), which has been reported to be a kinase for STAT3 phosphorylation on Ser⁷²⁷ (15), and we have previously reported that GW501516 prevents LPS-induced ERK1/2 activation in adipocytes (36), we evaluated the effect of the PPAR- δ agonist on the activation of this kinase. IL-6 exposure caused a slight increase in ERK1/2 phosphorylation, whereas GW501516 strongly suppressed ERK1/2 protein phosphorylation (Fig. 2C). Consistent with these changes,

the increase in Egr-1 mRNA levels, which has been attributed to IL-6-mediated activation of ERK1/2 (10), was abolished by GW501516 (Fig. 2D). To demonstrate the involvement of the ERK1/2 activation in IL-6-induced insulin resistance in adipocytes, we took advantage of U0126, a potent and specific ERK1/2 inhibitor, which binds to mitogen-activated protein kinase (MAPK)-ERK1/2 (MEK1/2), thereby inhibiting its catalytic activity as well as phosphorylation of ERK1/2. Similarly to GW501516, U0126 prevented the reduction in insulin-stimulated Akt phosphorylation (Fig. 3A) and glucose uptake (Fig. 3B) and prevented the increase in STAT3 phosphorylation on Ser⁷²⁷ (Fig. 3C) caused by IL-6. U0126 treatment alone did not affect the phosphorylation status of Akt (data not shown). **GW501516 prevents the increase in SOCS3 protein levels and STAT3 activity induced by IL-6 in vivo.** We have previously reported that PPAR- δ expression and activity is reduced, whereas ERK1/2 phosphorylation is increased in white adipose tissue from an animal model of obesity and insulin resistance, the ZDF (*fa/fa*) rat (36). In the current study we found that STAT3 phosphorylation at Ser⁷²⁷ and SOCS3 protein levels were increased in white adipose tissue of ZDF rats compared with lean animals (Fig. 4A and B). Hence, we hypothesized that reduced PPAR- δ activity and enhanced ERK1/2 activation may contribute to the increase in STAT3 activity in the white adipose tissue of the ZDF rat. We then evaluated whether GW501516 might prevent the increase

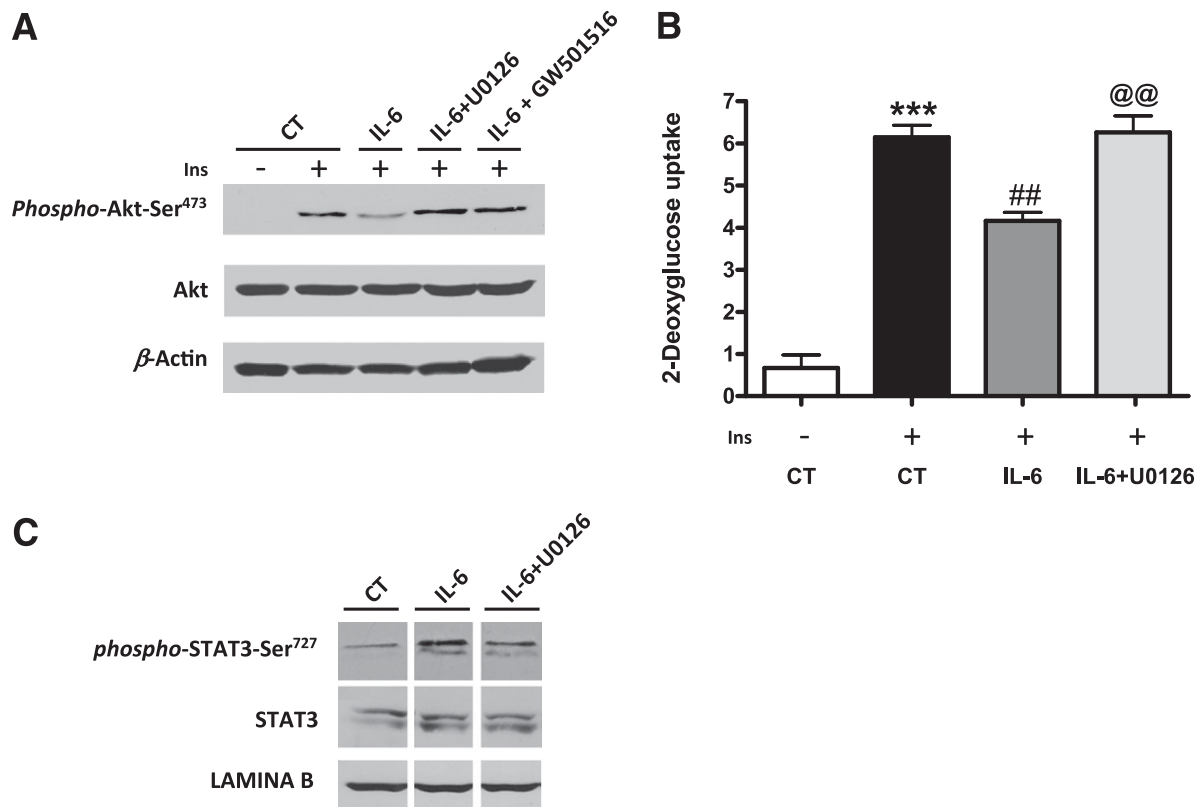


FIG. 3. ERK1/2 inhibition prevents IL-6-induced insulin resistance and STAT3 phosphorylation on Ser⁷²⁷. Differentiated adipocytes were stimulated with 100 nmol/L insulin for 30 min, with or without pretreatment with either 10 μ mol/L U0126, 10 μ mol/L GW501516, or 100 ng/mL IL-6 for 24 h. **A:** Cell lysates were subjected to Western blot analysis for phospho-Akt(Ser⁴⁷³) and total Akt and β -actin. **B:** 2-Deoxyglucose uptake was assessed without or with insulin. Values are means \pm SD of six independent experiments. **C:** Nuclear cell extracts were subjected to Western blot analysis with phospho-STAT3 (Ser⁷²⁷), STAT3, or Lamin B antibodies. *** $P < 0.001$ vs. control cells without insulin stimulation; ## $P < 0.01$ vs. control cells stimulated with insulin; @@ $P < 0.01$ vs. IL-6-exposed cells. Dividing lines indicate grouping of images from different parts of the same gel.

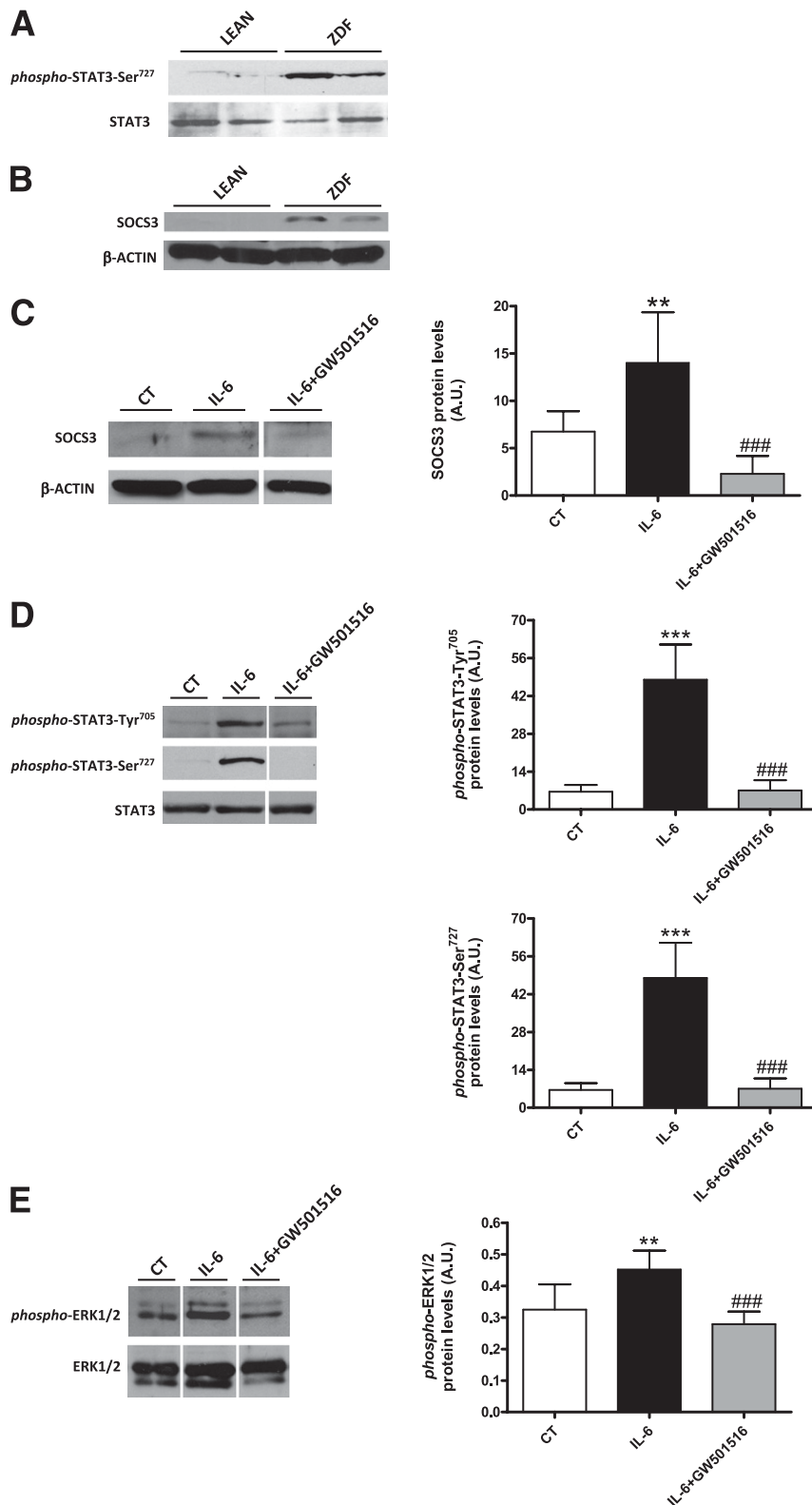


FIG. 4. The PPAR- δ agonist GW501516 prevents IL-6-induced SOCS3 expression and STAT3 phosphorylation in white adipose tissue. Phospho-STAT3 (Ser⁷²⁷) and SOCS3 protein levels are increased in white adipose tissue of ZDF rats. **A:** Analysis of phospho-STAT3 (Ser⁷²⁷) and total STAT3 by immunoblotting of nuclear or total protein extracts from white adipose tissue of lean and ZDF rats. **B:** Total cell extracts from white adipose tissue of lean and ZDF rats were subjected to Western blot analysis with SOCS3 and β -actin antibodies. Mice were treated for 48 h with vehicle, IL-6, or IL-6 plus GW501516. SOCS3 (**C**), phospho-STAT3 (Tyr⁷⁰⁵ and Ser⁷²⁷) (**D**), and phospho-ERK1/2 (**E**) protein levels. Nuclear (phospho-STAT3-Ser⁷²⁷) or total cell extracts were subjected to Western blot analysis with phospho-STAT3 (Tyr⁷⁰⁵) or STAT3 antibodies or phospho-ERK1/2 and ERK1/2 antibodies. Bars are the means \pm SD of four independent experiments. *** P < 0.001; ** P < 0.01 vs. control; ### P < 0.001 vs. IL-6-treated mice. Dividing lines indicate grouping of images from different parts of the same gel.

in STAT3-SOCS3 pathway in white adipose tissue after IL-6 stimulation *in vivo* in a similar fashion as observed *in vitro*. When we examined the SOCS3 protein levels in mice exposed to IL-6 (Fig. 4C) we observed that the increase caused by IL-6 treatment was prevented in those mice treated with the PPAR- δ agonist. Similarly, drug treatment prevented the increase in the phosphorylation status of STAT3 in both Tyr⁷⁰⁵ and Ser⁷²⁷ (Fig. 4D). In agreement with data obtained *in vitro*, GW501516 inhibited the increase in phospho-ERK1/2 levels induced by IL-6 treatment (Fig. 4E).

Increased STAT3 activity in the white adipose tissue of the PPAR- δ -null mouse. To clearly demonstrate the involvement of PPAR- δ in the regulation of STAT3 activity in white adipose tissue we took advantage of the PPAR- δ -null mouse. In the absence of PPAR- δ , Tyr⁷⁰⁵ and Ser⁷²⁷ phosphorylation of STAT3 and SOCS3 protein levels were increased compared with wild-type mice (Fig. 5A). Consistent with these changes, the DNA-binding activity of STAT3 was strongly increased in nuclear extracts of white adipose tissue of PPAR- δ -null mice compared with wild-type animals (Fig. 5B).

PPAR- δ elicits STAT3 dissociation from Hsp90. Hsp90 is thought to contribute to many steps in STAT3 activation, such as binding to its docking sites on gp130 and subsequent phosphorylation by associated JAKs, as well as enhanced trafficking of the activated cytosolic STAT3 to the

nucleus (16,37). Thus we examined whether PPAR- δ suppressed IL-6 signaling through inhibition of STAT3-Hsp90 interaction. This interaction was studied by using nuclear extracts of 3T3-L1 adipocytes stimulated with IL-6 in the presence or in the absence of GW501516 and the Hsp90 inhibitor geldanamycin, which were immunoprecipitated with anti-Hsp90, and analyzed by Western blot (Fig. 6A). IL-6 stimulation caused an increase in the interaction of Hsp90 with STAT3, whereas in the presence of GW501516 or geldanamycin the IL-6-induced recruitment of Hsp90 to STAT3 was blocked. Consistent with this, the association of STAT3 with Hsp90 was very faint in white adipose tissue of wild-type mice but strongly increased in PPAR- δ -null mice (Fig. 6B). Overall, these findings indicate that PPAR- δ may inhibit IL-6-induced STAT3 activation by promoting STAT3 dissociation from Hsp90.

DISCUSSION

Insulin resistance and type 2 diabetes are closely associated with low-grade chronic inflammation characterized by abnormal proinflammatory cytokine production (39), such as TNF- α (1) and IL-6 (4,5,21). Of these cytokines, IL-6 plasma levels correlate more strongly with the severity of insulin resistance in insulin-resistant patients than with TNF- α (3,6). Adipocytes play an important role in IL-6-induced insulin resistance since adipose tissue is an

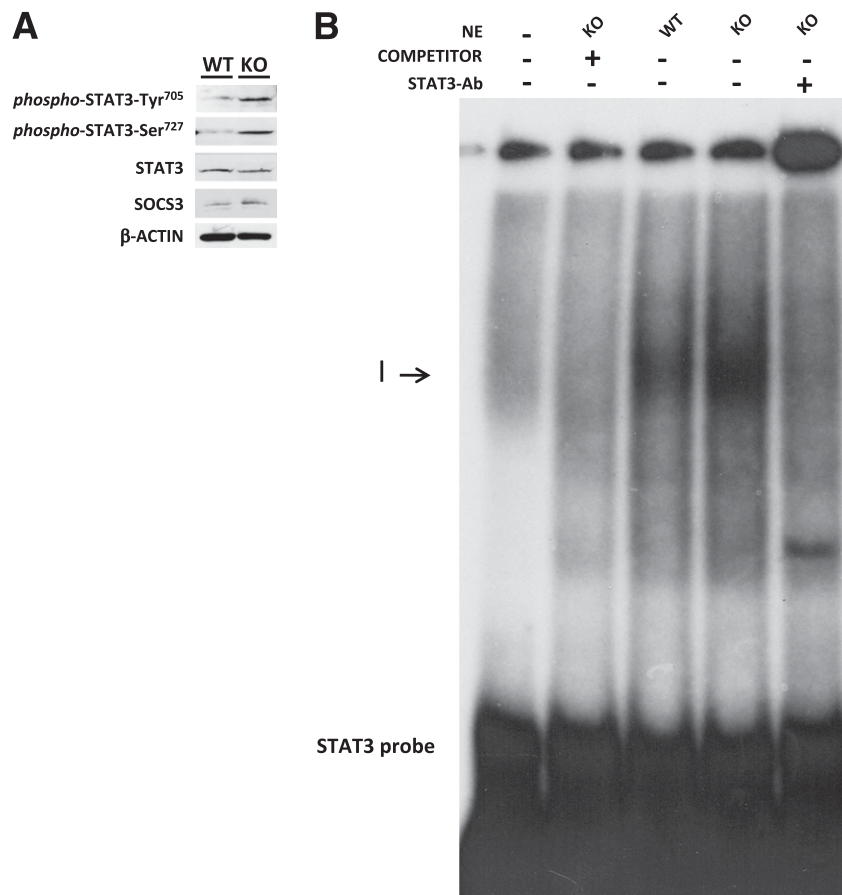


FIG. 5. The PPAR- δ -null mouse shows STAT3 activation and enhanced SOCS3 protein levels in white adipose tissue. **A:** Cellular extracts from wild-type (WT) or PPAR- δ -null (knockout [KO]) mouse white adipose tissue were analyzed by Western blot with phospho-STAT3 (Tyr⁷⁰⁵ and Ser⁷²⁷), STAT3, SOCS3, and β -actin antibodies as indicated. **B:** Autoradiograph of EMSA performed with a ³²P-labeled STAT3 nucleotide and nuclear protein extracts (NE). One specific complex (I), based on competition with a molar excess of unlabeled probe, is shown. An analysis performed by incubating NE with an antibody directed against STAT3 is also shown.

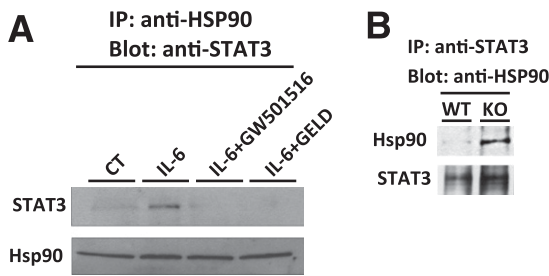


FIG. 6. PPAR- δ activation dissociates the complex formed between Hsp90 and STAT3. **A:** Differentiated adipocytes untreated or treated with 10 μ mol/L GW501516 for 24 h or 2 μ mol/L geldanamycin for 30 min before stimulation with 10 ng/mL IL-6 for 24 h. Nuclear extracts were immunoprecipitated (IP) with anti-Hsp90 followed by Western blot analysis using antibodies directed against STAT3. **B:** Cellular extracts from wild-type (WT) or PPAR- δ -null (KO) mouse white adipose tissue were immunoprecipitated (IP) with anti-STAT3 followed by Western blot analysis using antibodies directed against Hsp90 or STAT3.

important source (40) and a major target of this cytokine. IL-6 acts primarily by activating STAT3 and upregulating the transcription of its target gene *SOCS3*, which causes insulin resistance by interfering with insulin receptors and/or IRS-1 (19,41–43). Our findings demonstrate that PPAR- δ activation by GW501516 prevents IL-6-induced expression of *SOCS3* in adipocytes, showing a similar effect to that reported for the PPAR- γ activators rosiglitazone (21) and pioglitazone (44). These data suggest that PPAR- δ prevents STAT3 activation in adipocytes, which is in agreement with a previous report showing that GW501516 prevented IL-6-induced STAT3 activation in hepatocytes (30), although in this latter study the molecular mechanism involved was not elucidated. The activity of STAT3 is dependent on its phosphorylation status. Thus STAT3 phosphorylation on Tyr⁷⁰⁵ leads to dimerization and translocation to the nucleus, where it regulates the transcription of its target genes (12), whereas phosphorylation of Ser⁷²⁷ is required to achieve maximal activity (13,14). Our findings show that the PPAR- δ activator GW501516 inhibits IL-6-induced STAT3 phosphorylation on both residues. The increase in STAT3 phosphorylation, STAT3 DNA-binding activity, and *SOCS3* protein levels in white adipose tissue from PPAR- δ -null mice compared with wild-type animals clearly indicates that PPAR- δ inhibits STAT3 activation in adipocytes. Interestingly, white adipose tissue from ZDF rats showed increased STAT3 serine phosphorylation and *SOCS3* protein levels. These changes could be related to the reduction in PPAR- δ expression observed in white adipose tissue of ZDF rats (36). In addition, because overexpression of *SOCS3* in adipose tissue causes local but not systemic insulin resistance (45), these findings suggest that the increase in *SOCS3* levels in white adipose tissue from ZDF rats might, at least, exacerbate insulin resistance in this tissue, thereby contributing to the metabolic alterations observed in this animal model of type 2 diabetes.

The prevention of IL-6-induced STAT3 activation after PPAR- δ activation might be the result of several mechanisms of action. First, protein kinases responsible for STAT3 serine phosphorylation include, among others, ERK1/2 (46), and we have previously reported that GW501516 inhibits ERK1/2 phosphorylation in adipocytes (36). GW501516 abolished both ERK1/2 phosphorylation and the expression of *Egr-1*, which is upregulated after ERK1/2 activation (10), suggesting that inhibition of this protein kinase by PPAR- δ activation might be involved in the suppression of STAT3 Ser⁷²⁷ phosphorylation in IL-6-exposed cells. These

data are consistent with previous studies reporting that the MEK/ERK inhibitor PD98059 suppresses STAT3 serine phosphorylation, whereas STAT3 tyrosine phosphorylation was blunted by the JAK2 inhibitor AG490 (47). However, our findings also showed a reduction of STAT3 Tyr⁷⁰⁵ phosphorylation after GW501516 treatment, suggesting that additional mechanisms were involved. This second mechanism might involve a reduction in the interaction of STAT3 with Hsp90. In fact, activation of STAT3 requires its association with Hsp90 in many steps, including binding to its docking sites on gp130 and subsequent phosphorylation by associated JAKs and its translocation to the nucleus (16). Thus it has been reported that both geldanamycin, a selective Hsp90 inhibitor, and pyrrolidine dithiocarbamate (PDTC) inhibit STAT3 tyrosine and serine phosphorylation and its translocation to the nucleus by reducing the association of STAT3 with Hsp90 (16). Our immunoprecipitation studies in adipocytes exposed to IL-6 indicate that PPAR- δ activation by GW501516 dissociates Hsp90 from STAT3, a mechanism that may prevent STAT3 activation by phosphorylation. In agreement with this, white adipose tissue from PPAR- δ knockout mice showed enhanced STAT3 phosphorylation and DNA-binding activity. Our data do not explain how PPAR- δ activation reduces the physical interaction between STAT3 and Hsp90. However, because it has been reported that PPAR- δ interacts with Hsp90 (48), it is likely that competition between PPAR- δ and STAT3 for binding to Hsp90 may reduce the availability of this heat shock protein to interact with STAT3.

The mechanisms of action responsible for STAT3 inhibition by PPAR- δ agonists reported here are different to that previously reported for PPAR- γ activators (49,50). Thus ligand binding to PPAR- γ promotes its dissociation from the corepressor silencing mediator for retinoid and thyroid hormone receptors (SMRT), which, in turn, interacts with STAT3 and inhibits its transcriptional activity. It remains a matter of further study whether PPAR- γ ligands inhibit STAT3 by promoting its dissociation from Hsp90. However, this possibility seems unlikely since it has been reported that, in contrast with PPAR- δ and PPAR- α , PPAR- γ does not interact with Hsp90 (48). Further studies are necessary to evaluate whether PPAR- δ -mediated inhibition of STAT3 involves enhanced interaction between SMRT and STAT3.

It has been suggested that the proinflammatory cytokine IL-6 is one of the mediators linking obesity-derived chronic inflammation with insulin resistance. In addition, it has previously been reported that STAT3 activation is required for IL-6 inhibition of insulin signaling in hepatocytes (51) and that the negative effect of IL-6 on insulin signaling in adipocytes is linked to the upregulation of *SOCS3* (21). Thus we wanted to explore whether PPAR- δ activation prevented IL-6-induced insulin resistance in adipocytes. In agreement with the reported inhibition of the STAT3-*SOCS3* by GW501516, PPAR- δ activation by this drug prevented the reduction in insulin-stimulated Akt phosphorylation and glucose uptake caused by IL-6 exposure. These findings suggest that the inhibition of STAT3 and the subsequent reduction in *SOCS3* levels after PPAR- δ activation in IL-6-stimulated adipocytes might contribute toward preventing IL-6-induced insulin resistance. Given that impairment of insulin resistance in adipocytes by exposure to TNF- α (52) and IL-1 α (53) has been largely associated with IL-6 production and *SOCS3* induction, it is likely that the effects of PPAR- δ on the improvement in insulin sensitivity can also be extended to these cytokines.

In summary, on the basis of our findings in adipocytes, we suggest that PPAR- δ activation prevents IL-6-induced STAT3 activation and SOCS3 upregulation, thereby contributing toward preventing the cytokine-mediated development of insulin resistance.

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L.S.-M., R.R.-C., and I.E.K. performed experiments. X.P. performed experiments and contributed to discussion. L.M. and W.W. developed the PPAR- β - δ -null mice and contributed to discussion. M.V.-C. wrote the manuscript and performed experiments.

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