

Pioglitazone Ameliorates Tumor Necrosis Factor- α -Induced Insulin Resistance by a Mechanism Independent of Adipogenic Activity of Peroxisome Proliferator-Activated Receptor- γ

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Tumor necrosis factor (TNF)- α is one of the candidate mediators of insulin resistance associated with obesity, a major risk factor for the development of type 2 diabetes. The insulin resistance induced by TNF- α is antagonized by thiazolidinediones (TZDs), a new class of insulin-sensitizing drugs. The aim of the current study was to dissect the mechanism whereby pioglitazone, one of the TZDs, ameliorates TNF- α -induced insulin resistance in 3T3-L1 adipocytes. Pioglitazone restored insulin-stimulated 2-deoxyglucose (DOG) uptake, which was reduced by TNF- α , with concomitant restorations in tyrosine phosphorylation and protein levels of insulin receptor (IR) and insulin receptor substrate (IRS)-1, as well as association of the p85 regulatory subunit of phosphatidylinositol (PI) 3-kinase with IRS-1 and PI 3-kinase activity. Adenovirus-mediated gene transfer of either wild-type human peroxisome proliferator-activated receptor (PPAR)- γ 2 or a mutant carrying a replacement at the consensus mitogen-activated protein kinase phosphorylation site (hPPAR- γ 2-S112A) promoted adipogenesis of 3T3-L1 fibroblasts and restored TNF- α -induced decrease of triglyceride in adipocytes as effectively as pioglitazone. Overexpression of the PPAR- γ proteins in TNF- α -treated adipocytes restored protein levels of IR/IRS-1, but did not improve insulin-stimulated tyrosine phosphorylation of IR/IRS-1 or insulin-stimulated 2-DOG uptake. These results indicate that the ability of pioglitazone to restore insulin-stimulated tyrosine phosphorylation of IR/IRS-1, which is necessary for amelioration of TNF- α -induced insulin resistance, may be independent of the adipogenic activity of PPAR- γ that regulates protein levels of IR/IRS-1. *Diabetes* 50: 1083–1092, 2001

Tumor necrosis factor (TNF)- α is an inflammatory cytokine that elicits pleiotropic biological effects, including inflammation, tumor necrosis, cell proliferation, differentiation, and apoptosis (1,2), and it has been suggested to play a role in the development of insulin resistance associated with obesity, a major risk factor for the development of type 2 diabetes. TNF- α is secreted from adipocytes, and it blocks insulin action in cultured cells and whole animals (3–8). However, the mechanism of TNF- α -induced cellular insulin resistance is still controversial. For example, TNF- α was shown to impair tyrosine kinase activity of insulin receptor (IR), thereby decreasing tyrosine phosphorylation of insulin receptor substrate (IRS)-1, a major endogenous substrate for the activated IR (7,8). In contrast, another report indicated that TNF- α decreases expression of IR, IRS-1, and GLUT4 without affecting tyrosine kinase activity of IR (9).

Thiazolidinediones (TZDs) are a new class of insulin-sensitizing drugs used for the treatment of insulin resistance associated with diabetes (10). TZD is a high-affinity ligand for peroxisome proliferator-activated receptor (PPAR)- γ , a member of the nuclear receptor superfamily of ligand-activated transcription factors, a family that includes the steroid, retinoid, and thyroid hormone receptors (11,12). PPAR- γ is induced early during the course of adipocyte differentiation, binds to its cognate DNA-response elements as a heterodimer with the retinoid X receptor, and plays a key role in adipocyte differentiation, in concert with other adipogenic factors such as CAAT/enhancer-binding proteins (C/EBPs) and adipocyte determination and differentiation factor-1/sterol response element binding protein-1 (13). Two forms of PPAR- γ (γ 1 and γ 2) are produced because of alternative promoter usage and splicing (13). PPAR- γ 2 is expressed selectively in fat tissue, whereas γ 1 is found at low levels in many tissues (13), although the functional difference of these variants is not yet clear. Since the rank order of potency of TZDs correlates well between PPAR- γ binding and antidiabetic effects in vivo (14,15), PPAR- γ is assumed to be the major target of antidiabetic activity of TZDs. TZDs have been reported to antagonize TNF- α -induced insulin resistance both in vitro and in vivo. (16–23). However, the precise nature of the insulin-sensitizing activity of TZDs and the

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ATCC, American Type Culture Collection; C/EBP, CAAT/enhancer-binding protein; DMEM, Dulbecco's modified Eagle's medium; DOG, deoxyglucose; FCS, fetal calf serum; HRP, horseradish peroxidase; IR, insulin receptor; IRS, insulin receptor substrate; LacZ, β -galactosidase; MAP, mitogen-activated protein; PBS, phosphate-buffered saline; PI, phosphatidylinositol; PMSF, phenylmethylsulfonyl fluoride; PPAR, peroxisome proliferator-activated receptor; PVDF, polyvinylidene difluoride; TG, triglyceride; TNF, tumor necrosis factor; TZD, thiazolidinedione.

role of PPAR- γ in the insulin-sensitizing mechanism of TZDs are not completely understood.

In this study, to obtain further insights into the insulin-sensitizing mechanism of TZDs, we examined the effects of pioglitazone, one of the TZDs, and overexpression of wild-type or mutated human PPAR- γ 2 proteins on the insulin-signaling cascade in TNF- α -treated 3T3-L1 adipocytes. We provide evidence for an ability of pioglitazone that is independent of the adipogenic activity of PPAR- γ and is required for amelioration of TNF- α -induced insulin resistance.

RESEARCH DESIGN AND METHODS

Materials. Recombinant human TNF- α was from Dainippon Pharmaceutical (Osaka, Japan). Pioglitazone was from Takeda Chemical Industries (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from GIBCO BRL (Life Technologies, Rockville, MD). [γ - 32 P]ATP was acquired from Amersham (Arlington Heights, IL). [1,2- 3 H]2-deoxyglucose (DOG) was from DuPont New England Nuclear (Boston, MA). Porcine insulin was provided by the Lilly Research Laboratories (Indianapolis, IN). Monoclonal anti-phosphotyrosine antibodies (PY20 and PY20H) were purchased from Transduction Laboratories (Lexington, KY). Anti-IRS-1 and anti-p85 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-insulin receptor β -subunit, anti-PPAR- γ , and horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protein G-Sepharose was purchased from Pharmacia Biotech (Uppsala, Sweden). Electrophoresis reagents were from Bio-Rad (Hercules, CA). Determiner TG-S555 was purchased from Kyowa Medex (Tokyo, Japan). Adenovirus Expression Vector Kit was purchased from Takara Biomedicals (Tokyo, Japan). All other reagents were from standard suppliers.

Cell culture and treatment. Murine 3T3-L1 cells obtained from American Type Culture Collection (ATCC); (Rockville, MD) were cultured, maintained, and differentiated essentially as previously described (24). Briefly, cells were plated and grown for 2 days postconfluence in DMEM/high glucose supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% FCS in a 10% CO₂ environment. Differentiation was then induced by changing to the same medium containing 0.5 mmol/l 3-isobutyl-1-methylxanthine, 1 μ mol/l dexamethasone, and 1 μ mol/l insulin for 3 days, and the medium containing 0.8 μ mol/l insulin for another 3 days. The medium was then changed every 3 days until the cells were used for experimentation, i.e., 14–16 days after the induction of differentiation, when >95% of the cells had the morphologic and biochemical properties of adipocytes. TNF- α dissolved in phosphate-buffered saline (PBS) and pioglitazone in DMSO were added to the cell culture medium for 96 and 48 h, respectively, unless otherwise indicated. During the treatment period, fresh TNF- α or pioglitazone was added every 24 h.

Human embryonic kidney 293 cells obtained from ATCC were cultured in DMEM/high glucose containing 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% FCS in 5% CO₂ atmosphere.

2-DOG uptake. The 3T3-L1 adipocytes were deprived of serum for 3 h and then were stimulated with 20 nmol/l insulin for 15 min in a buffer containing Krebs-Ringer phosphate and HEPES (10 mmol/l HEPES, pH 7.4, 131.2 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l MgSO₄, 2.5 mmol/l CaCl₂, 2.5 mmol/l NaH₂PO₄) with 1% bovine serum albumin at 37°C. Unlabeled 2-DOG and [3 H]2-DOG (0.1 mmol/l, 0.74 kBq/well) were added and the cells were incubated for 4 min. Reaction was stopped by adding 10 μ mol/l cytochalasin B and washing cells with ice-cold PBS three times. The cells were solubilized in 1 ml of 0.2% SDS and 0.2 N NaOH. The radioactivity was quantitated in a liquid scintillation counter. The results were corrected for nonspecific uptake and absorption determined by [3 H]2-DOG uptake in the presence of 10 μ mol/l cytochalasin B. Nonspecific uptake and absorption were always <10% of total uptake.

Immunoprecipitation and immunoblotting. The 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 (PMSF), 10 μ g/ml aprotinin, 1 μ mol/l leupeptin, 1 mmol/l Na₃VO₄, and 50 mmol/l NaF. For immunoprecipitation, the whole-cell lysates were centrifuged at 4°C for 20 min to remove the insoluble materials and the supernatants were incubated with the indicated antibody for 16 h at 4°C, followed by incubation with protein G-Sepharose for 2 h and washing the beads with the lysis buffer three times. The cell lysates or immunoprecipitates were boiled with Laemmli sample buffer for 5 min, resolved by 7.5 or 12% SDS-PAGE, and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore,

Bedford, MA) in the Trans-Blot cell apparatus (Bio-Rad). The membranes were blocked and incubated with the indicated antibodies, followed by incubation with HRP-conjugated secondary antibodies. The proteins were visualized with chemiluminescence reagents according to the manufacturer's protocol (Amersham). In some experiments, the intensities of blots were quantitated using a scanning densitometer.

Phosphatidylinositol 3-kinase assay. Serum-starved 3T3-L1 adipocytes in 6-cm dishes were stimulated with 20 nmol/l insulin for 10 min and solubilized in a buffer containing 20 mmol/l Tris, pH 7.6, 100 mmol/l NaCl, 0.8 mmol/l MgCl₂, 0.8 mmol/l CaCl₂, 0.1 mmol/l Na₃VO₄, 1% Nonidet-P40, 10% glycerol, 1 mmol/l PMSF, and 100 μ g/ml aprotinin. The cell lysates were centrifuged at 10,000g for 20 min at 4°C to remove the insoluble materials. The supernatant was incubated with anti-phosphotyrosine antibody for 2 h at 4°C, and then the immune complexes were collected by incubation with protein G-Sepharose for 1 h at 4°C. The beads were washed and suspended in 20 μ l of solution containing 0.5 mg/ml phosphatidylinositol (PI), 50 mmol/l HEPES, pH 7.6, 1 mmol/l NaH₂PO₄, and 1 mmol/l EGTA. The phosphorylation reaction was carried out by adding 10 μ l of the reaction mixture containing 250 μ mol/l [γ - 32 P]ATP (0.37 MBq/tube), 100 mmol/l HEPES, and 50 mmol/l MgCl₂ for 5 min at 25°C. The reaction was terminated by the addition of 15 μ l of 4 mol/l HCl. The products were extracted by adding 130 μ l chloroform/methanol (1:1) and centrifugation. The lower organic phase was removed and spotted on Silica Gel 60 plate. The plates were developed in CHCl₃:CH₃OH:H₂O:NH₄OH (60:47:11.3:2) and dried. The phosphorylated inositol was visualized by autoradiography, and phosphate incorporated into inositol was determined by BAS 2000 (Fuji Film, Tokyo, Japan).

Generation and infection of recombinant adenovirus. The S112A point mutation was introduced into the full-length human PPAR- γ 2 cDNA using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Adenovirus vectors were generated using Adenovirus Expression Vector Kit (Takara) essentially according to the manufacturer's instructions. Briefly, the full-length human PPAR- γ 2 cDNA (either wild-type or S112A mutant) was excised by digestion with *Sma*I and was inserted into the unique *Swa*I site of the full-length adenovirus genome cloned in a cassette cosmid, pAxCawt. Obtained recombinant cosmid or control cosmid pAxCaILacZ containing a cDNA encoding β -galactosidase (LacZ) was cotransfected into human embryonic kidney 293 cells together with the adenovirus DNA-terminal protein complex digested at several sites by the calcium phosphate method using CellPfect Transfection Kit (Pharmacia). The recombinant adenoviruses produced by homologous recombination were amplified in 293 cells, and viral stock solutions with the viral titer >10⁸ plaque-forming units/ml were prepared.

For adenovirus-mediated gene transfer, 3T3-L1 cells were infected with the vectors by incubating with indicated multiplicity of infection of viral stock solution in DMEM containing 2% heat-inactivated FCS. The medium was replaced with DMEM containing 10% FCS, and the cells were used 48 h after infection. When the control recombinant adenovirus containing β -galactosidase cDNA was infected, >95% of fully differentiated 3T3-L1 adipocytes had successful gene transfer, as determined by histochemical staining for β -galactosidase (data not shown).

Oil Red O staining. Cells were washed three times with PBS, fixed by 10% formalin in PBS for 1 h at room temperature, washed once again with PBS, then stained with 60% filtered Oil Red O stock solution (0.5 g of Oil Red O [Sigma] in 100 ml isopropanol) for 15 min, washed four times with water, and analyzed under a microscope.

Measurement of triglyceride content. Cells were washed with PBS three times, and triglyceride (TG) in the cells was extracted with isopropanol for 20 min. TG concentrations were measured using the Determiner TG-S 555 according to the manufacturer's instructions (Kyowa Medex) and were corrected for cellular proteins.

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

RESULTS

Pioglitazone improves 2-DOG uptake in TNF- α -treated 3T3-L1 adipocytes. We first examined the effect of pioglitazone on the basal and insulin-stimulated 2-DOG uptake in 3T3-L1 adipocytes treated with or without TNF- α . Incubation of the cells with TNF- α inhibited insulin-stimulated 2-DOG uptake in a dose- and time-dependent manner (data not shown), and 96 h of treatment with 10 ng/ml TNF- α decreased the basal and insulin-stimulated 2-DOG uptake by 58 \pm 19 and 42 \pm 11%, respectively (Fig.

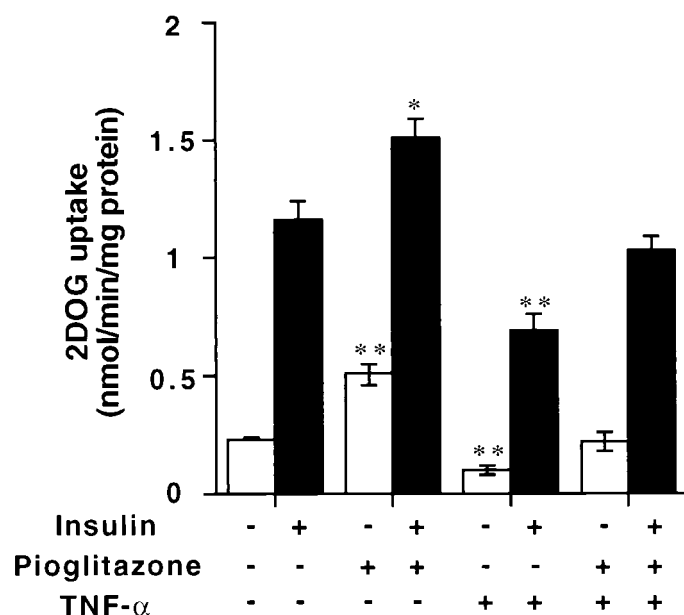


FIG. 1. Effects of TNF- α and pioglitazone on 2-DOG uptake. Fully differentiated 3T3-L1 adipocytes were treated with TNF- α (10 ng/ml) for 96 h, pioglitazone (10 μ M) for 48 h, or both (TNF- α for 96 h and pioglitazone for the last 48 h of the TNF- α -treatment period), followed by stimulation with insulin (20 nmol/l) for 15 min. [3 H]2-DOG uptake was measured as described under RESEARCH DESIGN AND METHODS. Means \pm SE for three independent experiments are shown (* P < 0.05; ** P < 0.01 as compared with the values in control cells). In cells treated with both TNF- α and pioglitazone, the values for the basal (P < 0.05) and insulin-stimulated (P < 0.05) 2-DOG uptake were significantly greater as compared with the values in cells treated only with TNF- α .

1). Addition of pioglitazone antagonized the effect of TNF- α in a dose- and time-dependent manner (data not shown) and completely restored the TNF- α -induced decrease in 2-DOG uptake when 10 μ M pioglitazone was added for 48 h (Fig. 1). This concentration of pioglitazone was within the therapeutic range when administered to rats or humans (21). Pioglitazone alone increased the basal and insulin-stimulated 2-DOG uptake by 117 ± 8 and $30 \pm 6\%$, respectively (Fig. 1). These results are consistent with previous reports (6,9,18–20,22,23) and indicate that TNF- α causes insulin resistance and pioglitazone antagonizes the effects of TNF- α under these conditions.

Pioglitazone restores tyrosine phosphorylation and protein levels of IR/IRS-1 in TNF- α -treated 3T3-L1 adipocytes. We then determined which components of the insulin-signaling cascade are affected by TNF- α and pioglitazone. First, to examine the effect of these agents on the insulin-stimulated tyrosine phosphorylation and protein levels of IR and IRS-1, the cell lysates were immunoprecipitated with antibody against either IR or IRS-1, resolved by SDS-PAGE, and immunoblotted with anti-phosphotyrosine antibody or antibody against each protein (Fig. 2). Immunoblotting with anti-phosphotyrosine antibody showed that insulin-stimulated tyrosine phosphorylation of IR was decreased in the cells treated with TNF- α , and addition of pioglitazone restored it to the control level (Fig. 2). Similarly, TNF- α decreased tyrosine phosphorylation of IRS-1, and pioglitazone restored it to the control level (Fig. 2). Immunoblotting the same samples with antibody against either IR or IRS-1 revealed that TNF- α decreased the protein levels of IR and IRS-1, and

pioglitazone restored them to the levels comparable with those found in control cells (Fig. 2). Pioglitazone alone increased the insulin-stimulated tyrosine phosphorylation and protein levels of IR and IRS-1 (Fig. 2). These results indicated that tyrosine phosphorylation and the protein levels of IR and IRS-1 apparently correlated with the effects of TNF- α and pioglitazone on 2-DOG uptake.

Pioglitazone restores association of p85 with IRS-1 and PI 3-kinase activity in TNF- α -treated 3T3-L1 adipocytes. PI 3-kinase plays a key role in insulin-stimulated glucose transport and GLUT4 translocation in insulin-responsive tissues (24–27). Therefore, we next determined the effect of pioglitazone on the basal and insulin-stimulated association of p85 with IRS-1 and PI 3-kinase activity in the cells treated with or without TNF- α . First, cell lysates were immunoprecipitated with anti-IRS-1 antibody and the immune complexes were resolved by SDS-PAGE and immunoblotted with anti-p85 antibody (Fig. 3). Insulin-stimulated association of p85 with IRS-1 was reduced in the cells treated with TNF- α and was restored by pioglitazone to the level comparable with that in control cells (Fig. 3). Pioglitazone alone increased the basal and insulin-stimulated association of p85 with IRS-1 (Fig. 3).

Similarly, insulin-stimulated PI 3-kinase activity in the immunoprecipitates with anti-phosphotyrosine antibody was reduced by TNF- α by $35 \pm 12\%$, which was restored by pioglitazone to the control level (Fig. 4). Pioglitazone alone increased the basal and insulin-stimulated PI 3-kinase activity by 70 ± 19 and $45 \pm 9\%$, respectively (Fig. 4). Taken together, these results indicated that insulin-stimulated association of p85 with IRS-1 and PI 3-kinase activity was affected by TNF- α and pioglitazone, apparently in parallel with the effects of these agents on the protein levels and tyrosine phosphorylation of IR/IRS-1, and also with the effects on insulin-stimulated 2-DOG uptake.

Adenovirus-mediated gene transfer of hPPAR- γ 2 promotes adipogenesis of 3T3-L1 cells. Previous reports indicated that TNF- α decreases the amount of PPAR- γ (29,29). TNF- α was also suggested to reduce transcriptional activity of PPAR- γ through phosphorylation of serine 112 in its amino-terminal A/B domain by activation of mitogen-activated protein (MAP) kinases such as p44/42 MAP kinase (30–32) and c-Jun NH₂-terminal kinase (30,33). On the other hand, most of the insulin-sensitizing effects of TZDs are assumed to be induced through activation of PPAR- γ . Therefore, to evaluate the role of PPAR- γ and its adipogenic activity in the insulin-sensitizing effects of TZDs against TNF- α -induced insulin resistance, we examined the effects of overexpression of wild-type hPPAR- γ 2 as well as hPPAR- γ 2-S112A, which carries a replacement of the serine to alanine at the consensus MAP kinase phosphorylation site and is assumed to be resistant to phosphorylation by MAP kinases.

First, to test adipogenic activities of the wild-type and mutant hPPAR- γ 2 in undifferentiated 3T3-L1 cells, we infected confluent 3T3-L1 fibroblasts with adenoviruses containing these cDNA constructs and evaluated adipose conversion of the infected cells by Oil Red O staining and the amount of TG in the cells after 10 days of infection (Fig. 5). Only a small percentage ($\sim 2\%$) of the cells that were infected with control adenovirus (LacZ) differentiated into adipocytes (Fig. 5A). Infection of wild-type

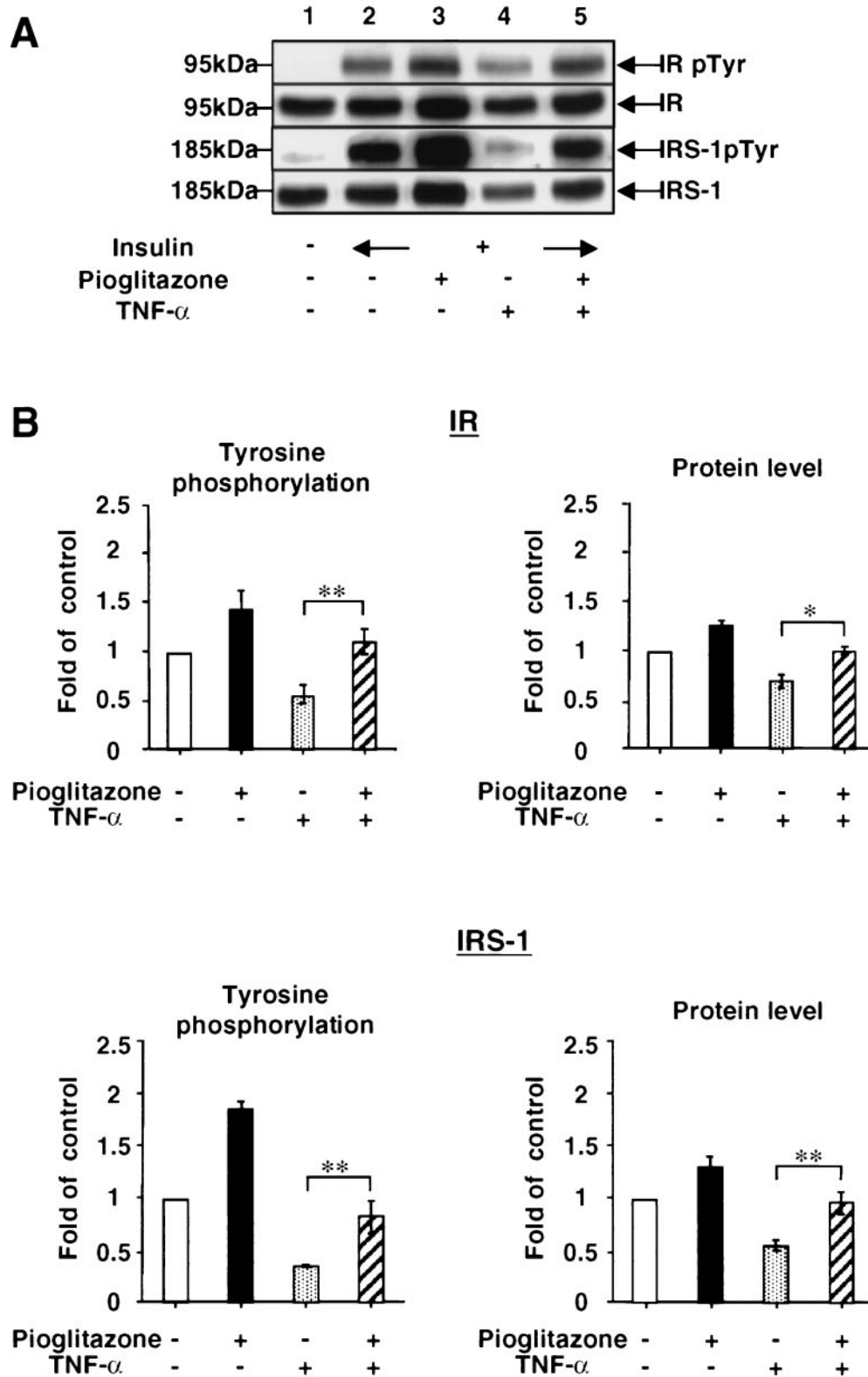


FIG. 2. Effects of TNF- α and pioglitazone on tyrosine phosphorylation (pTyr) and protein levels of IR and IRS-1. The 3T3-L1 adipocytes were treated as in Fig. 1, followed by stimulation with insulin (20 nmol/l) for 5 min. Proteins in the cell lysates were immunoprecipitated with either anti-IR or anti-IRS-1 antibody, separated by SDS-PAGE, and transferred to PVDF membranes. The membranes were probed with anti-phosphotyrosine (PY20H), anti-IR, or anti-IRS-1 antibody. **A:** The immunoblots representative of five independent experiments are shown. **B:** The immunoblots were scanned and the intensities of the bands were quantitated by a densitometer. Results for insulin-stimulated tyrosine phosphorylation and protein levels of IR and IRS-1 are normalized for the values in control cells. Error bars represent SE for five independent experiments (* $P < 0.05$; ** $P < 0.01$).

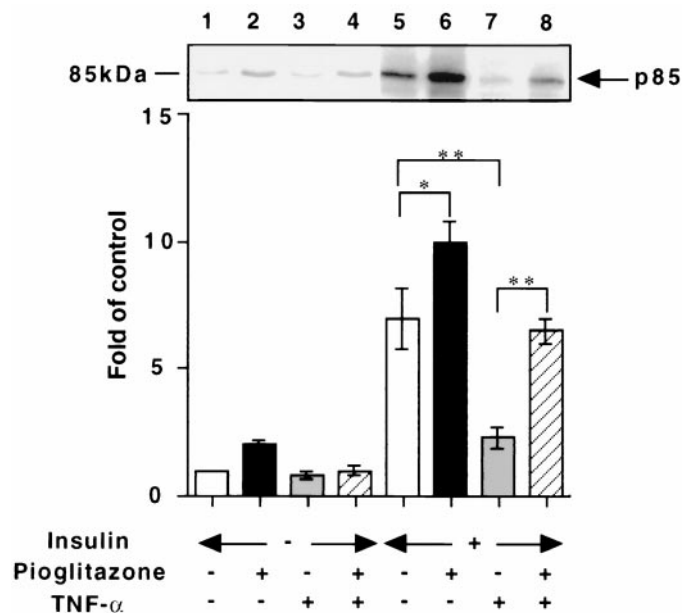


FIG. 3. Effects of TNF- α and pioglitazone on association of p85 with IRS-1. The 3T3-L1 adipocytes were treated as in Fig. 1, followed by stimulation with insulin (20 nmol/l) for 5 min. Proteins in the cell lysates were immunoprecipitated with anti-IRS-1 antibody, separated by SDS-PAGE, and transferred to PVDF membrane, which was probed with an antibody against the p85 subunit of PI 3-kinase. *Upper panel*: The immunoblot representative of three independent experiments is shown. *Lower panel*: The immunoblots were scanned and the intensities of the bands were quantitated by a densitometer. Results are normalized for the values in control cells. Error bars represent SE for three independent experiments (* $P < 0.05$; ** $P < 0.01$).

hPPAR- γ 2 adenovirus stimulated adipose conversion in ~40% of the cells (Fig. 5A), with a modest increase in TG content in the cells (Fig. 5B). Overexpression of S112A mutant hPPAR- γ 2 converted more cells (~60%) into adipocytes (Fig. 5A) and accumulated more TG in the cells

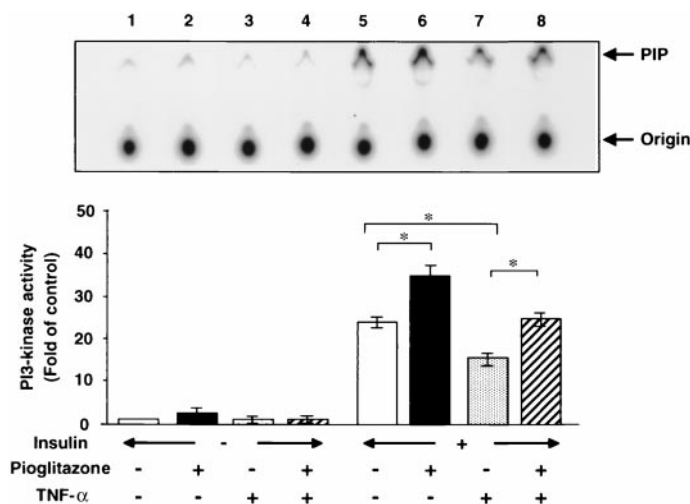


FIG. 4. Effects of TNF- α and pioglitazone on PI 3-kinase activity. The 3T3-L1 adipocytes were treated as in Fig. 1, followed by stimulation with insulin (20 nmol/l) for 10 min. PI 3-kinase activity in the anti-phosphotyrosine antibody (PY20) immunoprecipitates was measured as described under RESEARCH DESIGN AND METHODS. *Upper panel*: A representative autoradiogram for three independent experiments is shown. The arrows indicate the positions of the origin of sampling (Origin) and PI 3-phosphate (PIP). *Lower panel*: The labeled lipid products were quantitated by BAS 2000. Results are normalized for the values in control cells. Error bars represent SE for three independent experiments (* $P < 0.05$).

(Fig. 5B). Treatment of the cells with pioglitazone alone converted ~20% of the cells into adipocytes (Fig. 5A), with a small increase in TG content (Fig. 5B). Addition of pioglitazone to the cells infected with either wild-type or mutant hPPAR- γ 2 adenovirus resulted in adipose conversion of almost all the cells (Fig. 5A) and accumulation of large amounts of TG in the cells (Fig. 5B). These results are similar to those described for 3T3-L1 cells stably expressing mPPAR- γ 2 or mPPAR- γ 2-S112A using a retroviral expression strategy (30), and they indicate that the adenovirus constructs produce PPAR- γ molecules with expected activities to induce adipocyte differentiation.

Next, to evaluate adipogenic activities of the PPAR- γ proteins in differentiated adipocytes, we examined the effects of overexpression of the PPAR- γ proteins on TG content in the adipocytes treated with TNF- α (Fig. 6). TNF- α decreased the TG content in the adipocytes, and pioglitazone partially restored it (Fig. 6). Overexpression of either wild-type hPPAR- γ 2 or hPPAR- γ 2-S112A also increased the TG content in the TNF- α -treated adipocytes as effectively as pioglitazone (Fig. 6).

Overexpression of hPPAR- γ 2 restores protein levels but not tyrosine phosphorylation of IR/IRS-1 or 2-DOG uptake in TNF- α -treated 3T3-L1 adipocytes.

We then determined the effect of overexpression of the PPAR- γ proteins on the protein levels and insulin-stimulated tyrosine phosphorylation of IR/IRS-1 in TNF- α -treated adipocytes (Fig. 7). As reported previously (28,29), TNF- α decreased the amount of endogenous PPAR- γ by ~70% (Fig. 7A, lane 5). Infection of the cells with wild-type or S112A mutant hPPAR- γ 2 adenovirus vector resulted in increases in PPAR- γ levels approximately threefold of the control levels (Fig. 7A). As shown in Fig. 2, TNF- α decreased protein levels and tyrosine phosphorylation of IR and IRS-1 (Fig. 7). Overexpression of either wild-type or S112A mutant hPPAR- γ 2 restored the amounts of IR and IRS-1 protein to the levels comparable with those in control cells (Fig. 7). In contrast, the decreases in insulin-stimulated tyrosine phosphorylation of IR and IRS-1 in TNF- α -treated cells were not restored by overexpression of wild-type hPPAR- γ 2 or hPPAR- γ 2-S112A (Fig. 7). Overexpression of the hPPAR- γ proteins in untreated cells did not significantly increase the protein levels of IR and IRS-1 (Fig. 7), which may be due to the high endogenous PPAR- γ levels in untreated cells.

Finally, we examined the effect of overexpression of the hPPAR- γ 2 proteins on 2-DOG uptake (Fig. 8). In the cells infected with control adenovirus, TNF- α decreased insulin-stimulated 2-DOG uptake by ~50% (Fig. 8), as shown in Fig. 1. Overexpression of either wild-type or mutant hPPAR- γ 2 did not restore 2-DOG uptake in TNF- α -treated cells, nor did it affect 2-DOG uptake in untreated cells (Fig. 8).

DISCUSSION

We demonstrated in this study that pioglitazone restores both protein levels and insulin-stimulated tyrosine phosphorylation of IR/IRS-1, thereby ameliorating TNF- α -induced insulin resistance. Insulin-stimulated association of p85 with IRS-1, PI 3-kinase activity, and 2-DOG uptake were also restored by pioglitazone, apparently in parallel with the protein levels and tyrosine phosphorylation of

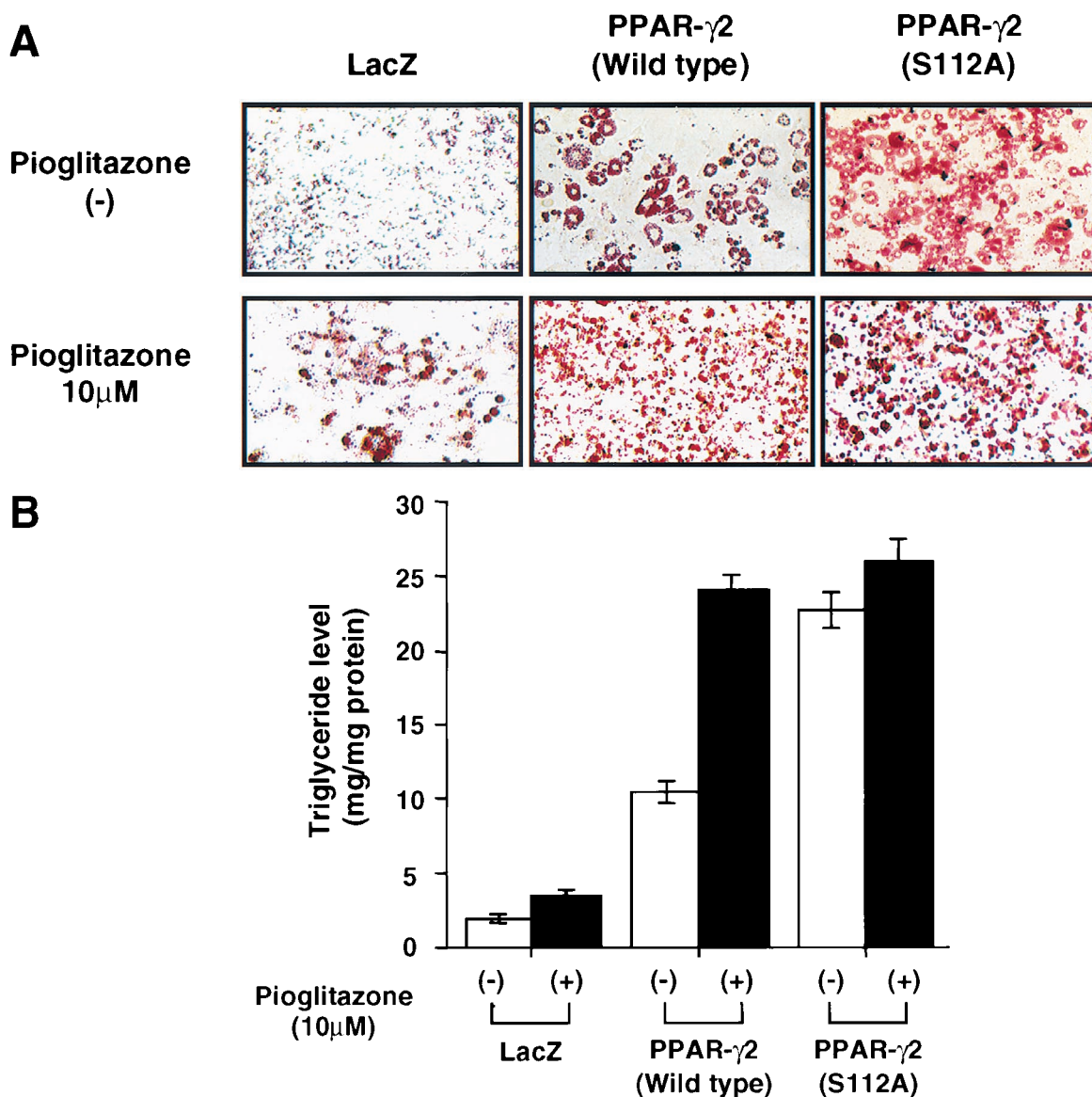


FIG. 5. Effect of PPAR- γ 2 overexpression on differentiation of 3T3-L1 cells. Confluent 3T3-L1 fibroblasts were infected with adenovirus vectors containing β -galactosidase (LacZ), hPPAR- γ 2, or hPPAR- γ 2-S112A cDNA at a multiplicity of infection of 50. The cells were then treated with 10 μ M pioglitazone or vehicle (0.1% DMSO) for 10 days. The adipose conversion of the cells were evaluated by Oil Red O staining (A) and measurement of triglyceride in the cells (B) as described under RESEARCH DESIGN AND METHODS. Error bars represent SE for three independent experiments.

IR/IRS-1 in TNF- α -treated cells, indicating that the effect of pioglitazone on IR/IRS-1 is the major mechanism for antagonizing TNF- α -induced insulin resistance. Our results clearly indicate that insulin-stimulated tyrosine phosphorylation of IR/IRS-1, in addition to IR/IRS-1 protein levels, was decreased by TNF- α , because tyrosine phosphorylation of IR/IRS-1 remained suppressed even when the amounts of these proteins were restored by overexpression of PPAR- γ in TNF- α -treated cells (Fig. 7). In addition, the protein levels of p85 and GLUT4 were not significantly affected by either TNF- α or pioglitazone (data not shown), supporting the notion that the major targets of these agents are IR and IRS-1 in determining insulin sensitivity. The enhancement of the basal and insulin-stimulated 2-DOG uptake by pioglitazone alone in the absence of TNF- α treatment also seems to be due at least in part to the increased protein levels and tyrosine phos-

phorylation of IR/IRS-1, because the basal and insulin-stimulated association of p85 with IRS-1 and PI 3-kinase activity were increased by pioglitazone alone. Previous studies in 3T3-L1 adipocytes indicated that TNF- α attenuates insulin signaling by decreasing IR tyrosine kinase activity (7,8), whereas another study showed that TNF- α decreases expression of IR/IRS-1 and GLUT4 without affecting IR tyrosine kinase activity (9). The discrepancies in the previous studies and the current study may be due to differences in experimental conditions. In 32D cells, pure inhibition of tyrosine phosphorylation was demonstrated using exogenously expressed IR/IRS-1 (7,8). Therefore, inhibition of IR/IRS-1 tyrosine phosphorylation by TNF- α seems to occur even in cell types other than adipocytes. In contrast, suppression of IR/IRS-1 protein levels may be observed only under conditions that favor dedifferentiation of adipocytes by TNF- α .

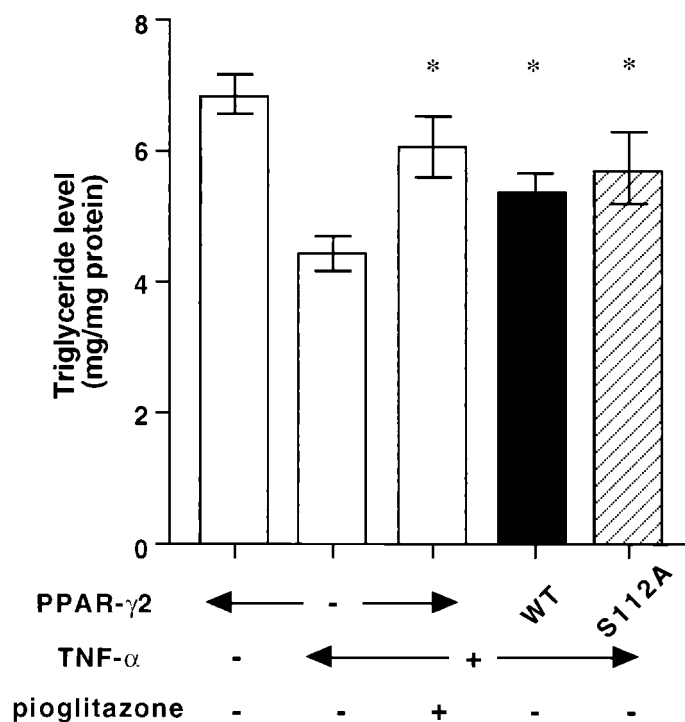


FIG. 6. Effect of PPAR- γ 2 overexpression on TG content in TNF- α -treated 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were treated with TNF- α (10 ng/ml) for 96 h, or TNF- α and pioglitazone (10 μ M) for 96 h and pioglitazone for the last 48 h of the TNF- α -treatment period). The cells were infected with adenoviruses containing wild-type (WT) hPPAR- γ 2, hPPAR- γ 2-S112A, or control β -galactosidase (LacZ) cDNA at a multiplicity of infection of 50, 48 h before the measurement of TG content in the adipocytes as described under RESEARCH DESIGN AND METHODS. Error bars represent SE for three independent experiments (* P < 0.05 as compared with the values in the cells treated with TNF- α and infected with the control adenovirus).

Expression of IR and IRS-1 increases during the course of adipocyte differentiation (34 and data not shown), and TNF- α blocks and reverses the differentiation process of adipocytes (18,28). On the other hand, PPAR- γ is an important positive regulator of adipocyte differentiation (35,36). Therefore, the effects of TNF- α and pioglitazone on the protein levels of IR/IRS-1 are likely due to modulation of differentiation process of adipocytes mediated by PPAR- γ . In support of this notion, TNF- α decreased the protein levels of IR/IRS-1 to levels similar to those in the less-differentiated state, when the levels of p85 and GLUT4 were not significantly different (data not shown), and TG content in the cells measured as a marker for adipocyte differentiation was reduced by TNF- α and was restored by pioglitazone. As previously reported (28,29), TNF- α markedly reduced the amount of PPAR- γ , which is likely to account at least in part for the TNF- α -induced dedifferentiation of adipocyte and concomitant decreases in IR/IRS-1 protein levels. Furthermore, overexpression of the hPPAR- γ 2 proteins, which possess potent adipogenic activities, restored both the protein levels of IR/IRS-1 and TG content in TNF- α -treated adipocytes as effectively as pioglitazone, supporting the role for the adipogenic activity of PPAR- γ in increasing IR/IRS-1 protein levels. Since PPAR- γ has been reported to cross-regulate C/EBP α , and the regulatory regions in IR and IRS-1 genes were reported to contain C/EBP-binding sequences (37), the decrease in PPAR- γ level and/or its transcriptional activity by TNF- α and the

activation of PPAR- γ by pioglitazone may affect expression of C/EBP α , which in turn regulates expression of IR/IRS-1.

The current study indicated that pioglitazone improves tyrosine phosphorylation of IR/IRS-1 in addition to the amounts of these proteins, thereby ameliorating TNF- α -induced insulin resistance. Adenovirus-mediated overexpression of hPPAR- γ 2 mimicked only the ability of pioglitazone to restore protein levels of IR/IRS-1, which was not sufficient to improve the TNF- α -induced decrease in insulin-stimulated 2-DOG uptake. Insulin-stimulated association of p85 with IRS-1 and PI 3-kinase activity were also not improved by overexpression of hPPAR- γ 2 (data not shown). TNF- α may decrease transcriptional activity of the overexpressed hPPAR- γ 2 by serine phosphorylation through activation of MAP kinases (30–33,38). However, such a mechanism is unlikely to account for the failure to restore tyrosine phosphorylation of IR/IRS-1, because overexpression of hPPAR- γ 2-S112A, which is assumed to be resistant to serine phosphorylation and has more potent adipogenic activity than wild-type hPPAR- γ 2, also did not restore tyrosine phosphorylation of IR/IRS-1 and 2-DOG uptake. These findings, therefore, suggest that the ability of pioglitazone to improve insulin-stimulated tyrosine phosphorylation of IR/IRS-1 may be independent of the adipogenic activity of PPAR- γ , which regulates the expression of IR/IRS-1. One of the possible explanations for the inability of overexpressed PPAR- γ to mimic all of the effects of pioglitazone is that the natural ligand produced in the adipocyte and the synthetic ligands such as pioglitazone may be different in activating endogenous and ectopic PPAR- γ . Thus, it is likely that activation of endogenous PPAR- γ by pioglitazone leads to expression of the genes that are involved in amelioration of both protein levels and tyrosine phosphorylation of IR/IRS-1, whereas activation of overexpressed PPAR- γ by endogenous ligand stimulates expression of only the genes that regulate IR/IRS-1 protein levels through promoting adipocyte differentiation. In fact, recent studies have suggested that different ligands can have differential effects on PPAR- γ activity, which results in expression of different sets of genes (39–41). Alternatively, the possibility remains that a PPAR- γ -independent mechanism is involved in the ability of pioglitazone to restore tyrosine phosphorylation of IR/IRS-1.

Heterozygous PPAR- γ knockout mice have been reported to be more insulin-sensitive than wild-type mice (42), and to be protected from high-fat diet-induced insulin resistance (43). A lack of adipocyte hypertrophy, together with increased leptin secretion, was suggested to play a role in the mechanism (43). These observations imply that PPAR- γ -mediated adipogenesis may even deteriorate insulin resistance in certain circumstances, and support the idea that stimulation of adipogenesis may not be the major mechanism of insulin-sensitizing effects of TZDs. On the other hand, recent identification of dominant-negative PPAR- γ gene mutations in human subjects with severe insulin resistance (44) highlights the critical role of PPAR- γ in maintaining normal insulin sensitivity. The observation that these patients had no evidence of abnormal fat distribution (44) is consistent with the notion that the activity of PPAR- γ that is independent of its adipogenic activity regulates insulin sensitivity.

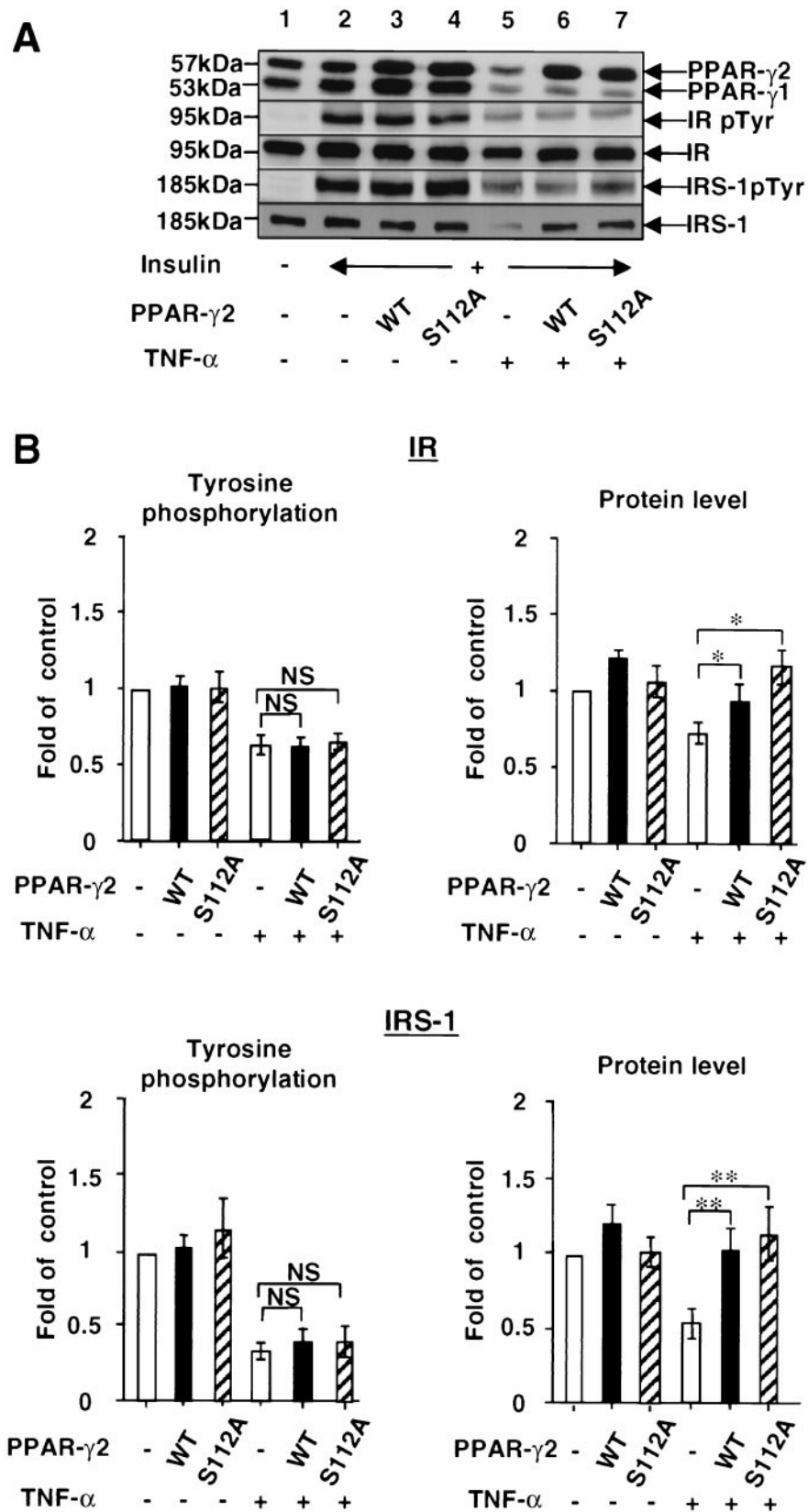


FIG. 7. Effect of PPAR- γ 2 overexpression on the protein levels and tyrosine phosphorylation (pTyr) of IR and IRS-1. Fully differentiated 3T3-L1 adipocytes were treated with TNF- α (10 ng/ml) for 96 h. The cells were infected with adenoviruses containing wild-type (WT) hPPAR- γ 2, hPPAR- γ 2-S112A, or control β -galactosidase (LacZ) cDNA at a multiplicity of infection of 50, 48 h before the stimulation with insulin (20 nmol/l) for 5 min. The cell lysates or immunoprecipitates with either anti-IR or anti-IRS-1 antibody were subjected to immunoblot analysis with anti-PPAR- γ , anti-phosphotyrosine (PY20H), anti-IR, or anti-IRS-1 antibody. **A:** The immunoblots representative of three independent experiments are shown. **B:** The immunoblots were scanned and quantitated by a densitometer. Results for insulin-stimulated tyrosine phosphorylation and protein levels of IR and IRS-1 are normalized for the values in control cells. Error bars represent SE for three independent experiments (* P < 0.05; ** P < 0.01; NS, no significant difference).

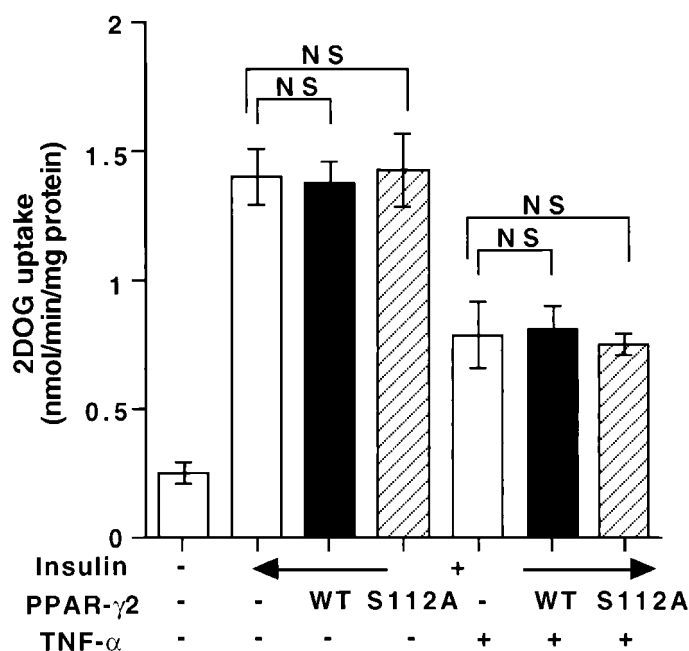


FIG. 8. Effect of PPAR- γ 2 overexpression on insulin-stimulated 2-DOG uptake. Fully differentiated 3T3-L1 adipocytes were treated with TNF- α (10 ng/ml) for 96 h. The cells were infected with adenoviruses containing wild-type (WT) hPPAR- γ 2, hPPAR- γ 2-S112A, or control β -galactosidase (LacZ) cDNA at a multiplicity of infection of 50, 48 h before the stimulation with insulin (20 nmol/l) for 15 min. [3 H]2-DOG uptake was then measured as described under RESEARCH DESIGN AND METHODS. Means \pm SE for three independent experiments are shown (NS, no significant difference).

In conclusion, the current results indicate that pioglitazone ameliorates TNF- α -induced insulin resistance by restoring both protein levels and insulin-stimulated tyrosine phosphorylation of IR/IRS-1, and that the ability of pioglitazone to improve insulin-stimulated tyrosine phosphorylation of IR/IRS-1 may be independent of the adipogenic activity of PPAR- γ . The dissection of the distinct activities of TZD/PPAR- γ should lead to the development of better therapeutic agents for diabetes.

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