

# Differential Activation of Peroxisome Proliferator-Activated Receptor- $\gamma$ by Troglitazone and Rosiglitazone

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The antidiabetic thiazolidinediones, which include troglitazone and rosiglitazone, are ligands for the nuclear receptor peroxisome proliferator-activated receptor (PPAR)- $\gamma$  and exert their antihyperglycemic effects by regulation of PPAR- $\gamma$ -responsive genes. We report here that PPAR- $\gamma$  activation by troglitazone depends on the experimental setting. Troglitazone acts as a partial agonist for PPAR- $\gamma$  in transfected muscle (C2C12) and kidney (HEK 293T) cells, producing a sub-maximal transcriptional response (1.8- to 2.5-fold activation) compared with rosiglitazone (7.4- to 13-fold activation). Additionally, troglitazone antagonizes rosiglitazone-stimulated PPAR- $\gamma$  transcriptional activity. Limited protease digestion of PPAR- $\gamma$  suggests conformational differences in the receptor bound to troglitazone versus rosiglitazone. Consistent with this finding, an *in vitro* coactivator association assay demonstrated that troglitazone-bound PPAR- $\gamma$  recruited the transcriptional coactivators p300 and steroid receptor coactivator 1 less efficiently than rosiglitazone-bound receptor. In contrast to these observations, troglitazone behaves as a full agonist of PPAR- $\gamma$  in 3T3L1 adipocytes. Two-dimensional protein gel electrophoresis demonstrated that troglitazone and rosiglitazone regulated distinct but overlapping sets of genes in several cell types. Thus, troglitazone may behave as a partial agonist under certain physiological circumstances and as a full agonist in others. These differences could be caused by variations in the amount of specific cofactors, differences in PPAR response elements, or the presence of different isoforms of PPAR- $\gamma$ . *Diabetes* 49:539–547, 2000

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2d, two-dimensional; CAP, c-Cbl-associated protein; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EC<sub>50</sub>, concentration at half-maximal stimulation; FBS, fetal bovine serum; GST, glutathione S-transferase; IC<sub>50</sub>, concentration at half-maximal inhibition; LBD, ligand binding domain; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response element; SRC-1, steroid receptor coactivator 1; TK, thymidine kinase; TZD, thiazolidinedione.

Type 2 diabetes is characterized by decreased insulin sensitivity of peripheral tissues. Glucose homeostasis is maintained under these circumstances by increased insulin secretion from pancreatic  $\beta$ -cells. In some cases, the  $\beta$ -cell is unable to maintain increased output. The antidiabetic thiazolidinediones (TZDs), such as troglitazone, improve peripheral insulin sensitivity, leading to reduced blood glucose and insulin levels and the preservation of pancreatic function (1–4). Improvement of insulin sensitivity by TZDs is most likely due to the activation of the peroxisome proliferator-activated receptor (PPAR)- $\gamma$  (5). The TZDs are high-affinity ligands for PPAR- $\gamma$  *in vitro*, and the rank order of receptor affinity correlates with their *in vivo* hypoglycemic activity (6), with one reported exception (7). Although many of the molecular details are not clearly understood, a model has emerged in which activated PPAR- $\gamma$  modulates the transcriptional activity of a set of genes encoding proteins that are important in glucose and lipid metabolism. However, the identity of these genes and the precise pathways leading to the normalization of insulin sensitivity remain largely unknown.

Recently, the X-ray crystal structure of the ligand-binding domain of PPAR- $\gamma$  has been elucidated (3,8), revealing that ligand binding causes a conformational change within PPAR- $\gamma$  such that the receptor is converted to an "activated mode" that promotes the recruitment of coactivators such as steroid receptor coactivator 1 (SRC-1) and p300. It has been postulated that these coactivators act as bridges to transmit the nuclear receptor regulatory signals to the cellular transcriptional machinery. Studies on estrogen and progesterone receptors have suggested that a range of distinct receptor conformations can be induced by the binding of different ligands (9–13). Although less is known about PPAR- $\gamma$  in this regard, it is possible that different PPAR- $\gamma$  ligands induce unique conformational changes in the receptor, eliciting distinct downstream biological effects. We have compared troglitazone to other TZDs in terms of its ability to activate PPAR- $\gamma$ 1 in several different experimental paradigms, including transcriptional activation, coactivator binding, and receptor conformation. We report here that in certain situations, troglitazone behaves as a partial agonist of the receptor.

## RESEARCH DESIGN AND METHODS

Chemicals. Troglitazone and BRL49653 (rosiglitazone) were synthesized at Parke-Davis Pharmaceutical Research Division of Warner-Lambert (Ann Arbor,

MI). Trypsin, insulin, dexamethasone, and isobutylmethylxanthin were purchased from Sigma Chemical (St. Louis, MO).

Transient reporter assays. The chimeric receptor plasmid pTET-mPPAR- $\gamma$ -LBD was constructed by fusing the ligand binding domain of mouse (m) PPAR- $\gamma$ 1 (amino acids 175–476) to the DNA binding domain of the *Escherichia coli* tetracycline (tet) repressor (amino acids 1–214). The luciferase reporter plasmid pLTetP is composed of a HindIII-XhoI fragment from the plasmid pTET-tTAK (Life Technologies, Gaithersburg, MD) (which contains the tet repressor binding site embedded in a minimal cytomegalovirus [CMV] promoter), inserted upstream of the luciferase coding region in the plasmid pGL3-basic (Promega, Madison, WI). Transfection of the human kidney epithelial cell line 293T was carried out by electroporation using the Cell Porator system from Life Technologies according to the manufacturer's instructions. After transfection, cells were plated onto 96-well plates and treated immediately with ligand as indicated.

The reporter construct used in the native PPAR response element (PPRE) reporter assay (ARE6.3XTKpGL3) contained three copies of the PPRE site (ARE6) from the aP2 enhancer (14) inserted upstream of the minimal thymidine kinase (TK) promoter in the pGL3 luciferase vector (Promega). C2C12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), plated into 24-well plates at 50% confluence, and transfected with expression plasmids encoding mouse PPAR- $\gamma$ 1 (200 ng), mouse RXR- $\alpha$  (50 ng), the ARE6.3XTKpGL3 (200 ng), and the internal reference plasmid pCMV  $\beta$ -galactosidase (50 ng) using lipofectamine (Life Technologies). After transfection, cells were treated for 48 h with appropriate ligands and then harvested. Luciferase and  $\beta$ -galactosidase activities were determined using the Dual-Light luciferase and  $\beta$ -galactosidase reporter gene assay system (Tropix, Bedford, MA). For 3T3-L1 adipocytes, cells were first differentiated into adipocytes as described (15). Approximately  $1 \times 10^7$  cells were transfected with the reporter plasmid ARE6.3XTKpGL3 (100  $\mu$ g) and the pCMV  $\beta$ -galactosidase (50  $\mu$ g) using the Gene Pulser II system (Bio-Rad Laboratories, Hercules, CA) at 960  $\mu$ F and 160 V. Transfected cells were replated into 24-well plates and treated with appropriate ligands for 36 h and then harvested. In all transfection assays, the expression of pCMV  $\beta$ -galactosidase plasmid was used as an internal control for the normalization of transfection efficiency.

In vitro coactivator binding assay. The COOH-terminal portion of human PPAR- $\gamma$ 1 (amino acids 175–476) containing the ligand binding domain (LBD) was fused to the glutathione S-transferase (GST) coding region in the plasmid pGEX-5X (Amersham Pharmacia Biotech, Uppsala, Sweden). The resulting GST-PPAR- $\gamma$  LBD fusion protein was expressed in *E. coli*, isolated, and partially purified using glutathione agarose. Full-length human SRC-1, or the amino-terminal portion of p300 (amino acids 1–443), was labeled with [<sup>35</sup>S]methionine in a coupled in vitro transcription and translation system (Promega). The coactivator binding assay was carried out essentially as described by Cheng et al. (16). Briefly, 3–5  $\mu$ l [<sup>35</sup>S]methionine-labeled p300 or SRC-1 was incubated with the immobilized GST-PPAR- $\gamma$  LBD in GST binding buffer (50 mmol/l KCl, 20 mmol/l HEPES, pH 7.9, 2 mmol/l EDTA, 0.1% Nonidet P-40, 10% glycerol, 0.5% nonfat dry milk, 5 mmol/l dithiothreitol [DTT], and protease inhibitors) together with ligands as indicated. The mixture was incubated for 2 h at 4°C with gentle rocking. The beads were washed four times with 1 ml GST binding buffer containing 150 mmol/l NaCl. The bound proteins were eluted in 20  $\mu$ l of 2 $\times$  SDS loading buffer, run on a 10% SDS-polyacrylamide gel, and visualized by autoradiography. The amount of [<sup>35</sup>S]methionine-labeled p300 or SRC-1 was quantified by phosphor-image analysis using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Limited protease sensitivity assay. Approximately 5  $\mu$ l of in vitro synthesized [<sup>35</sup>S]methionine-labeled, full-length mouse PPAR- $\gamma$ 1 was preincubated with 1 mmol/l rosiglitazone or 1 mmol/l troglitazone in 25 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.0, and 10 mmol/l DTT for 15 min. Trypsin was added to a final concentration of 10  $\mu$ g/ml and incubated for 15, 30, 60, 120, and 150 min. The reaction was terminated by adding 2 $\times$  SDS sample loading buffer and resolved in 12% SDS-polyacrylamide gel. Labeled PPAR- $\gamma$  was visualized by phosphor-image analysis.

Analysis of RNA. Total cellular RNA was isolated from 3T3-L1 adipocytes using the Ultraspec RNA isolation system (Biotech Laboratories, Houston, TX). Northern blot analysis was performed on ~15  $\mu$ g of total RNA with purified mouse aP2 and rat  $\beta$ -actin probes as described previously (17). RNase protection assays were performed using the RPA II ribonuclease protection assay kit (Ambion, Austin, TX). The antisense [<sup>32</sup>P]UTP-labeled single-stranded RNA probe for c-Cbl-associated protein (CAP) mRNA has been described previously (18).

Two-dimensional gel electrophoresis. HUVEC (human umbilical vein) cells were purchased from BioWhittaker (Walkersville, MD) and maintained as recommended by the manufacturer. HepG2 (human hepatic) cells were cultured in minimal essential medium containing 10% FBS. CV-1 (African green monkey kidney) cells, Caco2 (human colonic) cells, and 3T3-L1 (mouse) adipocytes were maintained in DMEM containing 10% FBS. U937 (human) monocytes were cultured in RPMI containing 10% FBS. Cells grown in 60-mm plates were treated with DMSO, 20  $\mu$ mol/l rosiglitazone, or 20  $\mu$ mol/l troglitazone for 18 h at a cell density

of ~60–80% confluence. Medium was replaced with methionine-free medium containing the same concentrations of the drugs and 500  $\mu$ Ci/ml of Trans <sup>35</sup>S-label (*E. coli* hydrolysate labeling reagent containing 70% L-methionine) (ICN Pharmaceuticals, Irvine, CA) and incubated for an additional 12 h. Whole cell extracts required for the two-dimensional (2d) gel electrophoresis system were prepared as recommended in the Millipore Investigator 2d gel electrophoresis manual (Millipore, Bedford, MA). 2d Gels were run on the Investigator system (Genomic Solution, Ann Arbor, MI) with pH 3–10/2D ampholines in the first dimension and 10% acrylamide in the second dimension.

## RESULTS

Troglitazone is a partial agonist of PPAR- $\gamma$  in a chimeric receptor transcription system. Although troglitazone and rosiglitazone are identical in their efficacy regarding the lowering of plasma glucose levels in animal models of diabetes (J.H. Johnson, personal communication), other physiological differences have been noted (19). To determine if these ligands display differential activities at the molecular level, we compared the ability of troglitazone and rosiglitazone to activate PPAR- $\gamma$  in different cell types and in different promoter contexts. The activation of PPAR- $\gamma$ -dependent transcription by troglitazone and rosiglitazone was examined using a chimeric receptor assay. We constructed a PPAR- $\gamma$  receptor composed of the ligand binding domain of mouse PPAR- $\gamma$ 1 fused to the bacterial tetracycline repressor DNA binding domain. A plasmid producing this receptor (pTET-mPPAR- $\gamma$  LBD) was cotransfected into 293T cells with a reporter containing the tet repressor binding site driving expression of the luciferase gene. Transfected cells were then treated with increasing concentrations of either troglitazone or rosiglitazone. As shown in Fig. 1A, both rosiglitazone and troglitazone caused an increase in PPAR- $\gamma$ -dependent transcriptional activity in a dose-dependent manner, with concentration at half-maximal stimulation (EC<sub>50</sub>) values of 6.5 and 11.0  $\mu$ mol/l, respectively. However, troglitazone induced a submaximal response compared with rosiglitazone (2.5- vs. 13-fold). This finding suggested that troglitazone may be acting as a partial agonist of PPAR- $\gamma$  in this experimental setting.

A partial agonist is defined as a ligand that induces a submaximal response even at full receptor occupancy. In addition, a partial agonist can competitively occupy the same drug-binding site as the agonist and consequently decrease the agonist-stimulated cellular response. To determine whether troglitazone can compete with rosiglitazone and inhibit its transcriptional activity, transfected 293T cells were cotreated with 8  $\mu$ mol/l rosiglitazone and increasing concentrations of troglitazone. Troglitazone caused a dose-dependent decrease in rosiglitazone-stimulated PPAR- $\gamma$  transcriptional activity, with a concentration at half-maximal inhibition (IC<sub>50</sub>) of ~25  $\mu$ mol/l (Fig. 1B). These results demonstrate that in this system, troglitazone can competitively reduce the transcriptional activity of a full agonist. To further explore this activity, transfected 293T cells were treated with increasing concentrations of rosiglitazone in the presence or absence of 50  $\mu$ mol/l troglitazone. As shown in Fig. 1C, the rosiglitazone dose-response curve was shifted to the right in the presence of troglitazone, again indicating that troglitazone competes with rosiglitazone and reduces its transcriptional activity. These results suggest that in this particular experimental setting, troglitazone acts as a partial agonist.

It has been reported that the order of the PPAR- $\gamma$  binding potencies of known TZDs are rosiglitazone > pioglitazone >

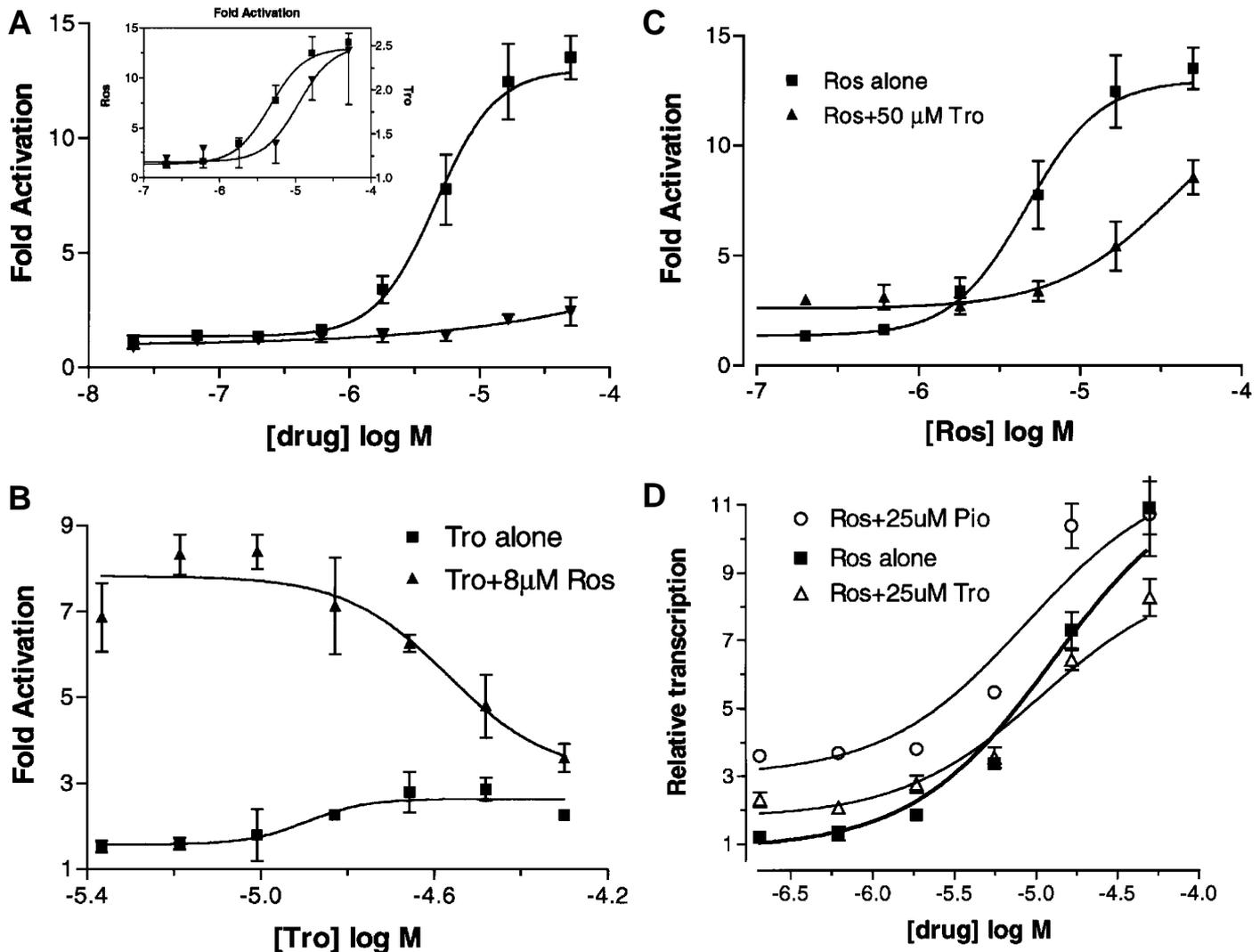


FIG. 1. Troglitazone acts as a partial agonist of PPAR- $\gamma$  in a chimeric receptor transcription assay. 293T cells were transfected with 15  $\mu$ g of the reporter plasmid pLTetP and 1  $\mu$ g pTET-mPPAR- $\gamma$  LBD for each 96-well plate and treated with the indicated concentration of TZDs for 48 h. Luciferase activity was normalized to  $\beta$ -galactosidase activity from a cotransfected reference plasmid. Data are plotted as fold activation relative to the vehicle control (0.5% DMSO). Error bars represent standard deviation ( $n = 3$ ). A: Dose response of activation of pTET-mPPAR- $\gamma$  LBD by troglitazone ( $\blacktriangledown$ ) and rosiglitazone ( $\blacksquare$ ). The inset shows the same data plotted with separate y-axes for troglitazone and rosiglitazone. B: Repression of rosiglitazone (Ros)-mediated pTET-PPAR- $\gamma$  activity by increasing concentrations of troglitazone (Tro). C: Rightward shift of the rosiglitazone dose response in the presence of 50  $\mu$ mol/l troglitazone. D: Pioglitazone (Pio) does not cause a rightward shift of the rosiglitazone dose-response curve.

troglitazone = ciglitazone (6). To determine if any of the TZDs with potencies closer to that of troglitazone behave as partial agonists, transfected cells were treated with increasing concentrations of rosiglitazone in the presence of these TZDs. In contrast to troglitazone, the inclusion of 25  $\mu$ mol/l pioglitazone did not cause a rightward shift in the rosiglitazone dose-response curve (Fig. 1D). Very similar results were observed with ciglitazone (data not shown). These findings indicate that pioglitazone and ciglitazone are full agonists of PPAR- $\gamma$  in this system and, further, that troglitazone is unique among this set of TZDs.

Troglitazone behaves as a partial agonist in a native receptor transcription system. To determine if troglitazone also acts as a partial agonist of the native (full-length) receptor, an experiment was carried out with vectors expressing full-length PPAR- $\gamma$ 1, RXR $\alpha$ , and a reporter construct containing a triple copy of a PPARE from the mouse aP2 gene

enhancer (ARE6.3XTKpGL3). C2C12 myoblasts were transfected with these plasmids and treated with TZDs. Troglitazone and rosiglitazone caused increases in PPAR- $\gamma$  transcriptional activity, with EC<sub>50</sub> values of 7.6 and 3.0  $\mu$ mol/l, respectively (Fig. 2A). However, troglitazone induced a submaximal transcriptional response compared with rosiglitazone (1.7- vs. 7.5-fold) (Fig. 2A). Similar results were obtained in HeLa cells as well as with a reporter containing the entire aP2 enhancer region or PPAREs from PEPCK and malic enzyme (data not shown).

To determine if the native receptor system behaves like the chimeric receptor system with regard to competition by troglitazone, transfected C2C12 cells were treated with a constant amount of rosiglitazone (8  $\mu$ mol/l) and increasing concentrations of troglitazone. Similar to the results observed in the chimeric receptor system, troglitazone caused a dose-dependent decrease in rosiglitazone-stimu-

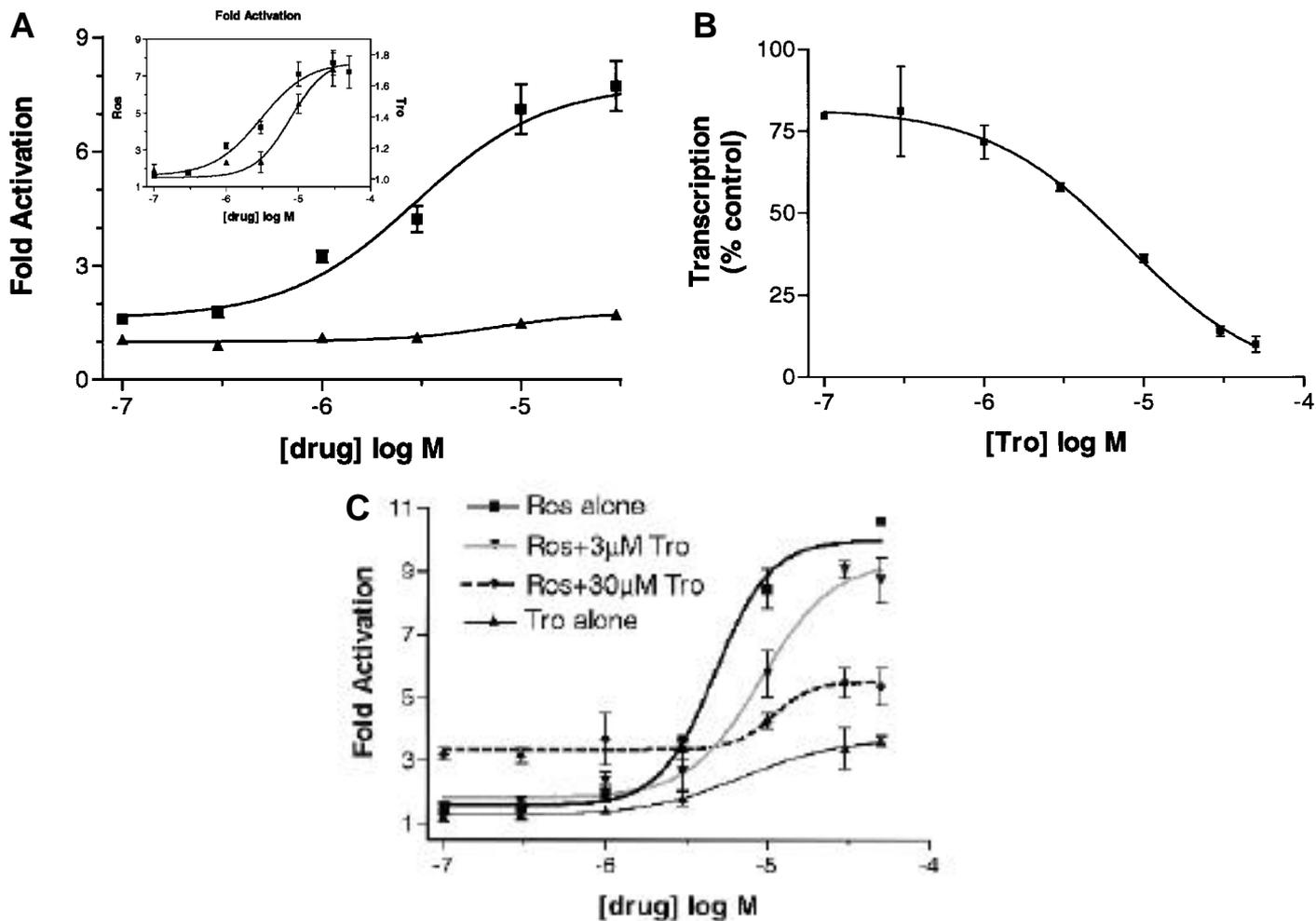


FIG. 2. Effects of rosiglitazone and troglitazone on PPAR- $\gamma$  transcriptional activity in a native PPAR- $\gamma$  reporter assay. C2C12 myoblasts were transfected with the reporter construct (ARE6.3XTKpGL3) and the full-length mouse PPAR- $\gamma$ 1 and RXR $\alpha$  followed by stimulation with the TZDs. Error bars represent standard deviation ( $n = 3$ ). A: Dose response of PPAR- $\gamma$  activation by rosiglitazone (■) and troglitazone (▲). The inset shows the same data plotted with separate y-axes for troglitazone and rosiglitazone. B: Repression of rosiglitazone-mediated PPAR- $\gamma$  activity by increasing concentrations of troglitazone (Tro). The concentration of rosiglitazone was constant at 8  $\mu$ mol/l. C: Rightward shift of the rosiglitazone (Ros) dose-response curve on PPAR- $\gamma$  activation in the presence of constant amounts of troglitazone (3 and 30  $\mu$ mol/l). Data are plotted relative to the vehicle control (0.1% DMSO alone).

lated PPAR- $\gamma$  transcriptional activity, with an  $IC_{50}$  of 8  $\mu$ mol/l (Fig. 2B). Likewise, the effect of troglitazone (3 or 30  $\mu$ mol/l) on the rosiglitazone dose-response curve was similar to that observed in the chimeric receptor system. The addition of 3  $\mu$ mol/l troglitazone caused a rightward shift of the rosiglitazone dose-response curve, while 30  $\mu$ mol/l troglitazone flattened the curve almost completely (Fig. 2C). At lower concentrations of rosiglitazone, the presence of 30  $\mu$ mol/l troglitazone increased PPAR- $\gamma$  transcriptional activity to the level expected for that amount of troglitazone (2.5-fold). In two different experimental settings, troglitazone acts as a partial agonist of PPAR- $\gamma$  with respect to the induction of PPAR- $\gamma$ 1 transcriptional activity.

Troglitazone and rosiglitazone induce a differential sensitivity to limited trypsin digestion of PPAR- $\gamma$ . The differential transcriptional responses displayed by troglitazone and rosiglitazone could be due to differences in the receptor conformation induced by the binding of these two ligands. To determine if there are conformational differences in PPAR- $\gamma$  bound to these two drugs, a limited trypsin digestion on a full-

length in vitro translated [ $^{35}$ S]methionine-labeled mPPAR- $\gamma$ 1 was carried out in the presence or absence of saturating concentrations of rosiglitazone or troglitazone. Compared with unliganded or troglitazone-bound receptor, rosiglitazone induced a stronger protection of the 34- and 35-kDa fragments (Fig. 3). Troglitazone binding yielded several protected fragments (>35 kDa) that were not present in the DMSO control samples and only weakly present in rosiglitazone-treated samples. In addition, a strong 29-kDa fragment detected in the troglitazone-bound receptor was only faintly represented in the DMSO- and rosiglitazone-treated samples. These results indicate that troglitazone-bound PPAR- $\gamma$  has a distinct trypsin digestion pattern compared with the rosiglitazone-bound receptor, presumably due to differences in receptor conformation.

Troglitazone behaves as a partial agonist in an in vitro coactivator recruitment assay. The change in receptor conformation induced by ligand binding causes the recruitment of transcriptional coactivators (20,21). PPAR- $\gamma$  has been shown to interact with the coactivators SRC-1 and p300/CBP

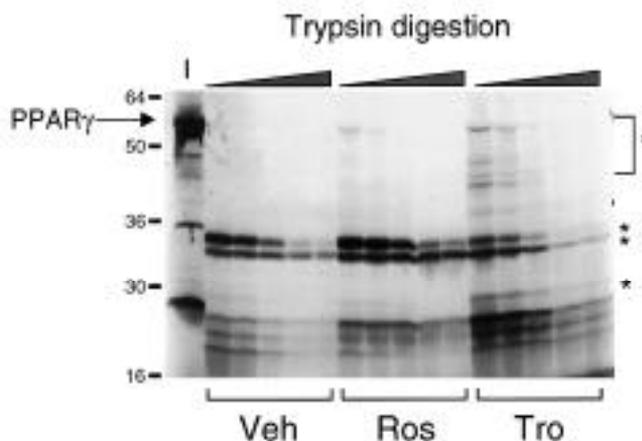


FIG. 3. Differential protease sensitivity of rosiglitazone- and troglitazone-bound PPAR- $\gamma$ . Autoradiogram of an SDS-polyacrylamide gel showing digested [ $^{35}$ S]methionine-labeled full-length PPAR- $\gamma$ 1 digested with trypsin (10  $\mu$ g/ml) for increasing lengths of time (15, 30, 60, 120, and 150 min, indicated with wedge shapes at the top of the figure). The receptor was preincubated with DMSO (Veh), rosiglitazone (Ros), or troglitazone (Tro). This experiment was repeated three times with similar results. The lane on the far left (I) shows the input amount of labeled PPAR- $\gamma$  that was not subjected to protease treatment. Migration of protein size marker is indicated on the left in kilodaltons. \*Trypsin-resistant protein fragments.

(22–24). To determine if the conformational differences in troglitazone- versus rosiglitazone-bound receptor lead to differential interactions with coactivators, an *in vitro* GST-fusion protein pull-down assay was performed with SRC-1 and p300. Bacteria-expressed GST-PPAR- $\gamma$  LBD fusion protein was incubated with *in vitro* translated [ $^{35}$ S]methionine-labeled NH $_2$ -terminal region of p300, or full-length SRC-1, in the presence of increasing concentrations of ligands (Fig. 4). Both ligands induced cofactor recruitment to PPAR- $\gamma$  in a dose-dependent manner. However, the level of cofactor recruitment at maximal ligand concentrations was much lower for troglitazone (20% of rosiglitazone-mediated binding). These results support the hypothesis that troglitazone and rosiglitazone induce different receptor conformations and suggest that the differential transcriptional activity of the two ligands is due to differential recruitment of coactivators.

Troglitazone behaves as a full agonist of PPAR- $\gamma$  in 3T3-L1 adipocytes. 3T3-L1 preadipocytes can be differentiated into fat droplet-containing adipocytes with appropriate hormonal treatment (25). PPAR- $\gamma$  appears to play a role in this system, since treatment of preadipocytes with PPAR- $\gamma$  ligands can induce differentiation, presumably by activating endogenous PPAR- $\gamma$  (5). In this cell line, we have found that troglitazone and rosiglitazone induce a similar level of adipocyte conversion at maximal concentrations, although with different EC $_{50}$  values (data not shown). To examine the behavior of these two ligands on endogenous PPAR- $\gamma$ , the transcriptional activity of a transfected PPAR- $\gamma$ -responsive reporter was measured in 3T3-L1 adipocytes treated with troglitazone and rosiglitazone. Preadipocytes were converted into adipocytes by treatment with an adipogenic hormonal cocktail (15). Fully differentiated adipocytes were then electroporated with the reporter plasmid ARE6.3XTKpGL3 and treated with increasing concentrations of ligands. Both drugs caused a dose-dependent increase in

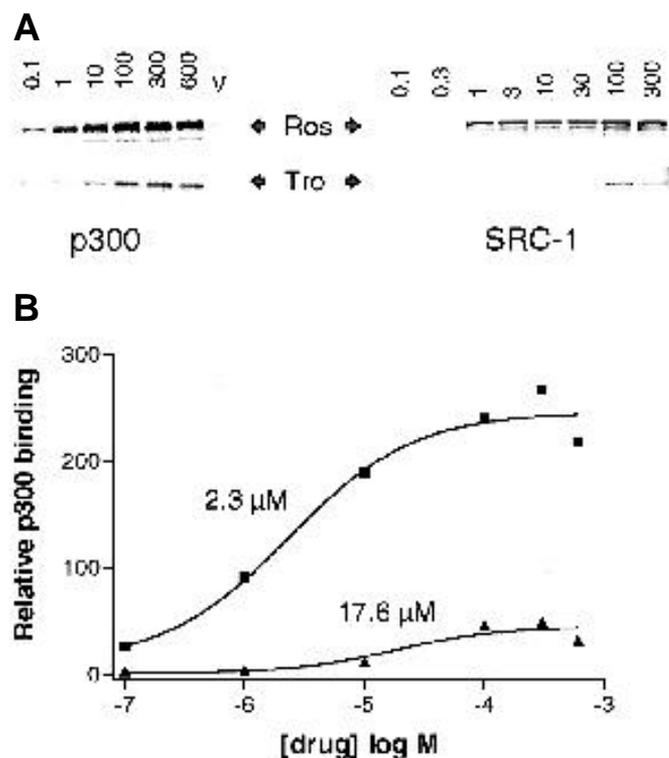


FIG. 4. Comparison of troglitazone- and rosiglitazone-induced recruitment of p300 and SRC-1 to PPAR- $\gamma$ . The GST-PPAR- $\gamma$  LBD fusion protein bound to glutathione-Sepharose beads was incubated with increasing doses of rosiglitazone or troglitazone and either [ $^{35}$ S]p300 or [ $^{35}$ S]SRC-1. A: Autoradiograms of SDS-polyacrylamide gels showing the amount of coactivator bound to PPAR- $\gamma$  LBD in the presence of either troglitazone (Tro) or rosiglitazone (Ros). The amount of p300 bound to PPAR- $\gamma$  LBD in the presence of the vehicle (2% DMSO) is indicated (V). The concentration of drug in micromoles per liter is indicated at the top of each lane. B: Results of the quantification of the p300 gel shown in A by phosphor-imager analysis. The calculated EC $_{50}$  values for troglitazone ( $\blacktriangle$ ) and rosiglitazone ( $\blacksquare$ ) dose-response curves are indicated. This experiment was repeated three times with similar results.

PPAR- $\gamma$  activity, with lower EC $_{50}$  values than observed with transfected receptors (150 nmol/l for rosiglitazone, 1  $\mu$ mol/l for troglitazone) (Fig. 5A). In contrast to the experiments described above, at maximal concentrations, troglitazone activated transcription to the same degree as rosiglitazone.

There are several PPAR- $\gamma$  target genes expressed in 3T3-L1 adipocytes. The aP2 gene encodes an adipose-specific intracellular lipid binding protein and contains two functional PPRES in its enhancer region (ARE6 and ARE7) (14). Recently, it was shown that CAP is a PPAR- $\gamma$  target gene in adipocytes (18). To examine if these PPAR- $\gamma$  target genes are differentially induced in response to troglitazone and rosiglitazone, 3T3-L1 adipocytes were treated with maximal concentrations of rosiglitazone or troglitazone for 13 or 42 h, followed by measurement of mRNA levels. As shown in Fig. 5B, treatment with either drug caused approximately equivalent increases in aP2 mRNA expression at both time points. A similar increase of CAP mRNA was also produced by both compounds. Taken together, these results indicate that in this insulin-responsive cell line, troglitazone behaves as a full agonist of PPAR- $\gamma$ , activating PPAR- $\gamma$  target gene expression to the same degree as rosiglitazone.

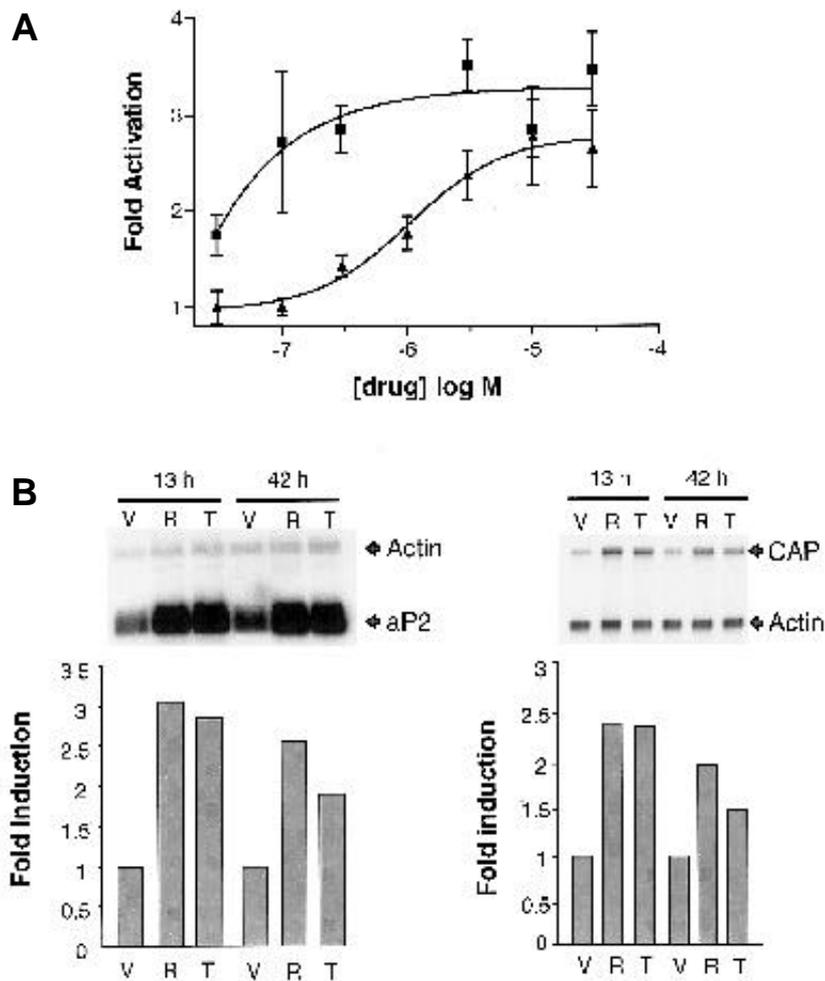


FIG. 5. Troglitazone behaves as a full agonist of PPAR- $\gamma$  in 3T3-L1 adipocytes. A: 3T3-L1 cells were differentiated into adipocytes and transfected with the ARE6.3XTKpGL3 reporter plasmid and the internal reference plasmid pCMV- $\beta$ -galactosidase as described in RESEARCH DESIGN AND METHODS. Transfected cells were treated with increasing concentrations of rosiglitazone (■) or troglitazone (▲) for 48 h and harvested, and luciferase and  $\beta$ -galactosidase levels were determined. Data are plotted relative to the vehicle control (0.1% DMSO). Error bars represent standard error of the mean ( $n = 4$ ). B: Total RNA was harvested from 3T3-L1 adipocytes treated with vehicle (0.1% DMSO) (V), 20  $\mu$ M rosiglitazone (R), or 20  $\mu$ M troglitazone (T) for 13 and 42 h. Northern blot analysis was used to determine the level of aP2 mRNA (left panel). RNase protection analysis was used to determine the level of CAP mRNA (right panel).

Troglitazone and rosiglitazone regulate distinct but overlapping sets of genes in several cell lines. Based on the above observations, we speculated that troglitazone behaves as a partial agonist of PPAR- $\gamma$  in some cellular circumstances and as a full agonist in others. Consequently, it seems possible that troglitazone and rosiglitazone may have distinct downstream biological effects, perhaps by regulating different sets of genes. As an initial step in testing this hypothesis, using 2d protein gel electrophoresis, we indirectly examined gene expression patterns in cells treated with ligands. Changes in gene transcription will result in altered rates of protein synthesis, which can be determined by incorporation of labeled amino acids into specific proteins. Metabolic labeling and 2d protein gel electrophoresis were carried out using CV1, HepG2, HUVEC, 3T3-L1, Caco2, and U937 cells, all of which are known to express some level of endogenous PPAR- $\gamma$ . As presented in Table 1, the expression level of a number of proteins was changed by treatment with PPAR- $\gamma$  ligands. Although this method does not directly measure transcriptional activity, troglitazone and rosiglitazone appeared to activate or repress distinct but overlapping sets of genes in most of these cell lines. Several examples of changes induced by troglitazone and rosiglitazone are shown in Fig. 6. Two examples of coregulated genes can be seen in gels of proteins from 3T3-L1 adipocytes, where both drugs induced protein 46 but decreased the level of protein 66 (Fig. 6A). An example of a troglitazone-specific change from

HUVEC cells is presented in Fig. 6B, where the expression of protein 13 is increased by troglitazone but not rosiglitazone. A rosiglitazone-specific change in CV-1 cells is presented in Fig. 6C, where protein 10 is increased by rosiglitazone but not by troglitazone. These results suggest that different PPAR- $\gamma$  ligands may have distinct downstream effects, possibly in a cell-specific or tissue-specific manner.

#### DISCUSSION

Numerous studies with steroid hormone receptors suggest that individual ligands of single receptors can have multiple downstream biological effects depending on tissue type, cellular environment, and promoter architecture (9–13). A good example of this phenomenon is seen with the anti-estrogen tamoxifen, which functions as an antagonist in some estrogen-mediated responses and as a partial agonist in others (26,27). In the current study, we provide evidence that the antidiabetic drug troglitazone can behave as a partial agonist for PPAR- $\gamma$  in certain cellular settings. In two different transfected cell systems, troglitazone induced a submaximal PPAR- $\gamma$  transcriptional response compared with the more potent ligand rosiglitazone (Figs. 1 and 2). In addition, cotreatment of transfected cells with these ligands demonstrated that troglitazone antagonized rosiglitazone-induced PPAR- $\gamma$  transcriptional activity. Two other TZDs, pioglitazone and ciglitazone, did not show partial agonist activity in the same experimental conditions (Fig. 1D). Results from in

TABLE 1

Number of protein spots on autoradiograms of 2d gels from metabolically labeled cells that were increased or decreased by drug treatment

	Common		Troglitazone-specific		Rosiglitazone-specific	
	Up	Down	Up	Down	Up	Down
CV1 (kidney)	3	4	4	0	4	6
HepG2 (liver)	3	3	2	3	3	0
HUVEC (vascular endothelium)	14	5	11	5	0	0
3T3L1 (adipose)	2	10	0	5	0	1
Caco2 (intestinal epithelium)	8	0	7	0	3	0
U937 (macrophage)	10	2	3	0	0	0

The number of protein spots scored as "up" or "down" was based on approximately twofold changes compared with vehicle control. Common, proteins whose expression was changed to the same degree by both drugs; troglitazone- and rosiglitazone-specific, proteins whose expression was changed by one drug and unchanged by the other.

in vitro coactivator binding experiments suggest that this reduced transcriptional activity may be due to a decreased ability of the troglitazone-bound receptor to recruit transcriptional coactivators. These findings suggest that troglitazone induces a conformation of the receptor distinct from that of rosiglitazone. This interpretation was supported by structural studies demonstrating that the two ligands induced distinct protease digestion patterns upon receptor occupations (Fig. 3).

In contrast to its behavior in transcription systems using exogenous (transfected) PPAR- $\gamma$ , troglitazone appeared to

behave as a full agonist when transcription was analyzed in cells containing endogenous receptors (3T3L1 adipocytes). In these cells, the transcription from either a transfected PPRE driven reporter or endogenous PPAR- $\gamma$ -responsive genes was activated to a similar degree by both troglitazone and rosiglitazone (Fig. 5). These results indicate that troglitazone may differentially activate PPAR- $\gamma$  in a manner dependent on the cellular environment. The full agonist activity of troglitazone in 3T3L1 adipocytes may be analogous to its activity as an antidiabetic agent in vivo, where it appears to be as efficacious as rosiglitazone.

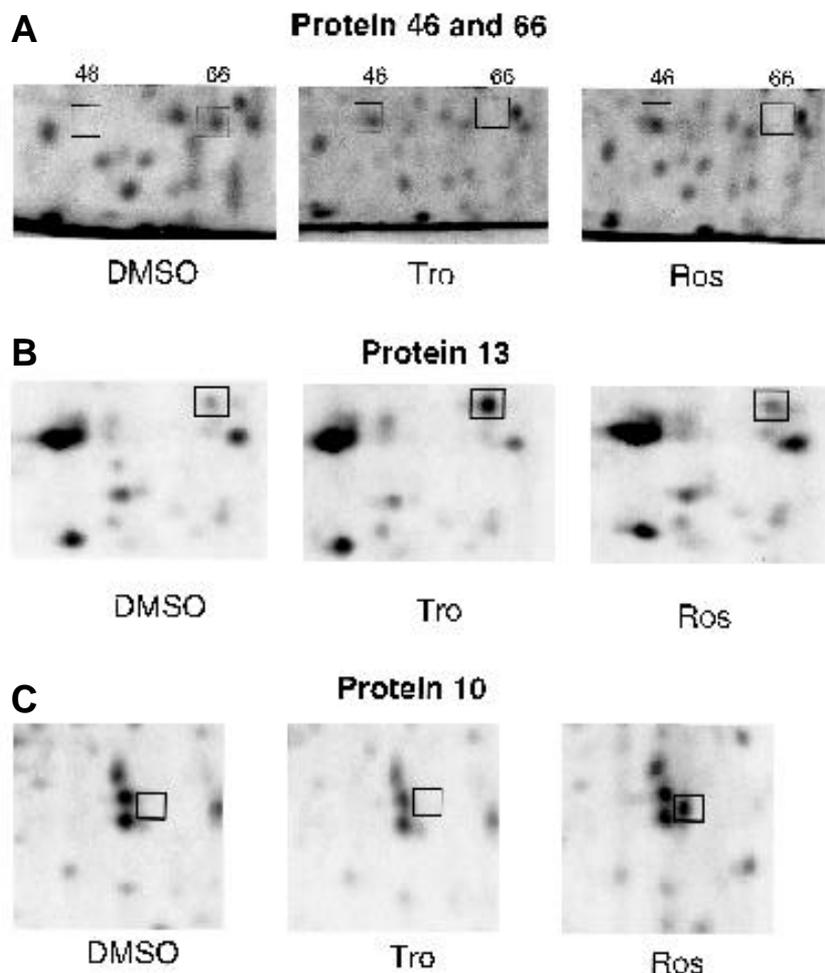


FIG. 6. Analysis of the effect of troglitazone and rosiglitazone on gene expression by 2d protein gel electrophoresis. Representative autoradiograms of 2d protein gels of whole cell extracts from [ $^{35}$ S]methionine-labeled cells treated with vehicle (DMSO), troglitazone (Tro), or rosiglitazone (Ros). A: Section of a 2d gel derived from 3T3L1 adipocytes showing two proteins that are coordinately regulated by troglitazone (Tro) and rosiglitazone (Ros). Both drugs caused a significant and approximately equal increase in the expression level of protein 46 and a decrease in the level of protein 66. B: A section of a 2d gel from HUVEC cell extracts showing differential regulation by troglitazone. Protein 13 is strongly increased by troglitazone but not rosiglitazone. C: A section of a 2d gel from CV1 cell extracts showing differential regulation by troglitazone. Protein 10 is strongly increased by rosiglitazone but not troglitazone.

What is the molecular basis for the different transcriptional activities of troglitazone and rosiglitazone? One possibility is that the two ligands cause differential cofactor recruitment. The immediate consequence of ligand binding is a conformational change in the receptor that increases its affinity for transcriptional coactivator proteins (20,21). Many of these coactivators are expressed ubiquitously, although a cell type-specific coactivator has been described (28). We have shown that two coactivators, p300 and SRC-1, appear to have a lower affinity for PPAR- $\gamma$  bound to troglitazone compared with rosiglitazone. These coactivators interact with a domain of the receptor that undergoes a conformational change upon ligand binding (3). Our findings suggest that the conformation of this domain must be somewhat different in troglitazone- versus rosiglitazone-bound receptors. The observation that troglitazone and rosiglitazone behave similarly in 3T3L1 cells suggests that there may be additional coactivators in this cell type that interact with a different domain of the receptor than that recognized by p300 or SRC-1. This second domain might undergo the same conformational change upon either troglitazone or rosiglitazone binding and would therefore interact equally well with receptors bound to either ligand. A model can be constructed in which troglitazone behaves as a partial agonist in cell types that contain primarily coactivators such as p300 or SRC-1, and as a full agonist in cell types that contain "nondiscriminating" coactivators.

Another potential factor that could contribute to the differential activity of troglitazone in different cell types is the PPAR- $\gamma$ 2 isoform. Mouse PPAR- $\gamma$ 2 is derived by alternative splicing from the same gene as PPAR- $\gamma$ 1 and contains an additional 30 amino acids at the NH<sub>2</sub>-terminus (29,30). PPAR- $\gamma$ 2 expression is limited to adipose tissue (31), whereas PPAR- $\gamma$ 1 is expressed in adipose tissue, liver, muscle, macrophages, and colon (32–34). The relative contribution of the two isoforms to the antidiabetic activity of the TZDs is unknown. It is possible that the additional NH<sub>2</sub>-terminal sequence affects the action of some ligands. It has been reported that the additional sequences of PPAR- $\gamma$ 2 contribute a ligand-independent transactivation function (35). In addition, it has been reported that modification of the NH<sub>2</sub>-terminal portion of PPAR- $\gamma$ 2 (by phosphorylation) can influence ligand-receptor interactions (36). One possible explanation of our results is that troglitazone behaves differently on these two isoforms of PPAR- $\gamma$ .

Finally, it is also possible that the different transcriptional effects of troglitazone and rosiglitazone are caused by special circumstances that occur in cells transiently transfected with PPAR- $\gamma$ . It is likely that in these systems, the cellular concentration of exogenously introduced receptor exceeds the normal level of endogenous PPAR- $\gamma$  in cells such as 3T3L1 adipocytes. This may contribute to the higher EC<sub>50</sub> values we have observed in transiently transfected cells. The excess amount of receptor could change characteristics of the transcriptional apparatus in unpredictable ways that might influence the ability of a ligand to activate the receptor. This possibility seems less likely, since not all TZDs behave as partial agonists in the same experimental setting. In addition, our results from the coactivator binding assay and protease sensitivity assay clearly showed differential effects of troglitazone and rosiglitazone in a noncellular in vitro setting.

The findings reported here have potential general implications with regard to the physiological actions of PPAR

ligands. The observation that ligands can have distinct effects on the receptor raises the possibility that different PPAR- $\gamma$  ligands induce different sets of genes in different tissues that in turn have distinct downstream biological effects. In this respect, PPAR- $\gamma$  may be analogous to the steroid hormone receptors. Numerous studies on estrogen and progesterone receptors have indicated that a range of distinct receptor conformations can be induced by the binding of different ligands. There is a strong correlation between receptor conformations elicited by different ligands and downstream biological activity (9–13). The hypothesis that different PPAR- $\gamma$  ligands have distinct downstream effects is supported by our protein profiling experiment that demonstrated differential regulation of gene expression by troglitazone and rosiglitazone in several cell types.

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