

# Troglitazone, an Antidiabetic Agent, Inhibits Cholesterol Biosynthesis Through a Mechanism Independent of Peroxisome Proliferator-Activated Receptor- $\gamma$

Minghan Wang, Scott C. Wise, Todd Leff, and Ti-Zhi Su

Troglitazone is an antidiabetic agent of the thiazolidinedione family. It is generally believed that thiazolidinediones exert their insulin-sensitizing activity through activation of peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ), a member of the steroid nuclear receptor superfamily. In the present study, we examined the effect of troglitazone on cholesterol biosynthesis in cultured Chinese hamster ovary (CHO) cells. Troglitazone inhibited biosynthesis of cholesterol, but not that of total sterols, in a dose-dependent manner, with a half-maximal concentration ( $IC_{50}$ ) value of 8  $\mu\text{mol/l}$ . At 20  $\mu\text{mol/l}$ , troglitazone inhibited cholesterol biosynthesis by more than 80%, resulting in the accumulation of lanosterol and several other sterol products. This inhibitory effect observed in CHO cells was also reproduced in HepG2, L6, and 3T3-L1 cells, suggesting that there is a common pathway for this troglitazone action. One hour after removal of troglitazone from the culture medium, disappearance of the accumulated sterols was accompanied by restored cholesterol synthesis, indicating that those accumulated sterols are precursors of cholesterol. PPAR- $\gamma$  reporter assays showed that PPAR- $\gamma$  activation by troglitazone was completely blocked by actinomycin D and cycloheximide. In contrast, the inhibition of cholesterol synthesis by troglitazone remained unchanged in the presence of the above compounds, suggesting that this inhibition is mechanistically distinct from the transcriptional regulation by PPAR- $\gamma$ . Like troglitazone, two other thiazolidinediones, ciglitazone and englitazone, exhibited similar inhibitory effect on cholesterol synthesis; however, other known PPAR- $\gamma$  ligands such as BRL49653, pioglitazone, and 15-deoxy- $^{12,14}$ -prostaglandin  $J_2$  showed only weak or no inhibition. The dissociation of PPAR- $\gamma$  binding ability

from the potency for inhibition of cholesterol synthesis further supports the conclusion that inhibition of cholesterol biosynthesis by troglitazone is unlikely to be mediated by PPAR- $\gamma$ . *Diabetes* 48:254–260, 1999

**T**roglitazone is a novel oral hypotriglyceridemic agent of the thiazolidinedione (TZD) family. TZDs are a class of insulin-sensitizing compounds that enhance insulin actions by increasing insulin sensitivity in target tissues such as liver, muscle, and fat (1). These compounds markedly decrease plasma glucose, insulin, and triglyceride levels in both genetic and nongenetic models of insulin resistance (1). Accumulating evidence supports the concept that these actions are mediated by peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ), a member of the PPAR nuclear receptor superfamily (1–4). This family of nuclear receptors, including subtypes of  $\alpha$ ,  $\gamma$ , and  $\delta$  ( $\beta$ , NUC-1, FAAR), heterodimerize with retinoid receptor X- $\alpha$  (RXR $\alpha$ ), bind to specific response elements called peroxisome proliferator response elements (PPREs), and control the expression of genes involved in lipid and carbohydrate metabolism in target tissues (3–7). PPAR- $\gamma$  binding studies have demonstrated that TZDs and a natural product, 15-deoxy- $^{12,14}$ -prostaglandin  $J_2$ , are ligands of PPAR- $\gamma$  (2,5). The binding of TZDs to PPAR- $\gamma$  correlates well with many of their *in vivo* activities, including stimulation of adipogenesis (8–10), induction of terminal differentiation of human liposarcoma cells (11), repression of leptin gene expression (12–14), activation of lipoprotein lipase (15), and attenuation of hyperglycemia (16,17).

The PPAR- $\gamma$ -mediated transcriptional response to TZDs requires synthesis of new RNAs and proteins. Usually, cells need to be treated with these compounds for as long as 24 h before functional responses can be observed (18,19). In addition to its ability to decrease circulating triglyceride (20,21) and nonesterified fatty acids (22), troglitazone also exhibits other favorable effects on lipid metabolism in several *in vivo* models. For example, treatment with troglitazone results in significant decreases in serum total cholesterol level in humans with NIDDM (23,24) and in obese Zucker rats (25). Similar decreases in cholesterol levels have also been observed in *ob/ob* mice and nondiabetic rats treated with englitazone (26,27) and dogs treated with ciglitazone (28). It has been reported that plasma cholesterol concentrations decrease synergistically when cholesterol-fed rats are treated with a combination of insulin and pioglitazone (29).

From the Departments of Molecular Biology (M.W., T.-Z.S.) and Cellular Biology (S.C.W., T.L.), Parke-Davis Pharmaceutical Research Division of Warner-Lambert Company, Ann Arbor, Michigan.

Address correspondence and reprint requests to Ti-Zhi Su, Department of Molecular Biology, Parke-Davis Pharmaceutical Research Division of Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, MI 48105. E-mail: sut@aa.wl.com.

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CMV, cytomegalovirus; DMEM/F12, Dulbecco's modified Eagle's medium and Ham's F12 medium; ER, endoplasmic reticulum; FBS, fetal bovine serum; FCS, fetal calf serum;  $IC_{50}$ , half-maximal concentration; LBD, ligand binding domain; LPDS, lipoprotein-deficient serum; luc, luciferase; MEM, minimum essential medium; PBS, phosphate-buffered saline; PPAR- $\gamma$ , peroxisome proliferator-activated receptor- $\gamma$ ; PPRE, PPAR response element; RXR $\alpha$ , retinoid receptor X- $\alpha$ ; TLC, thin layer chromatography; tet, tetracyclin; TZD, thiazolidinedione.

Moreover, an increase in HDL cholesterol has been observed in troglitazone-treated NIDDM patients (22,24). The mechanisms underlying the effects of TZDs on cholesterol synthesis are yet to be addressed. It is unclear whether these effects are related to the activation of PPAR- $\gamma$  or regulation of cholesterol biosynthesis or both. To test if cholesterol biosynthesis is affected by troglitazone and other TZDs, we measured cholesterol production in Chinese hamster ovary (CHO) cells and other cells. Our findings suggest a novel mechanism for the metabolic effect of troglitazone on lipid metabolism independent of PPAR- $\gamma$ .

## RESEARCH DESIGN AND METHODS

**Materials.** [ $^{14}\text{C}$ ]acetate (55 mCi/mmol) and [ $1\alpha$ ,  $2\alpha$ - $^3\text{H}$ ]cholesterol (47 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). TLC silica gel plates were from EM Sciences (Gibbstown, NJ). Petroleum ether, ethyl ether, and heptane were from VWR (Chicago, IL). All other chemicals were from Sigma (St. Louis, MO). Cell culture media and fetal bovine serum (FBS) were from Gibco BRL (Grand Island, NY). Lipoprotein-deficient fetal bovine serum was from Cocalico Biological (Reamstown, PA). Troglitazone, BRL49653, pioglitazone, englitazone, and ciglitazone were synthesized in the Department of Chemistry, Parke-Davis Research Division/Warner-Lambert Company (Ann Arbor, MI). Prostaglandin analog 15-deoxy- $^{12,14}$ -prostaglandin  $\text{J}_2$  was purchased from Cayman (Ann Arbor, MI).

**Cell culture and treatment.** All cells were cultured at  $37^\circ\text{C}$  in an atmosphere of 5%  $\text{CO}_2$ . CHO cells were maintained in minimum essential medium (MEM)- $\alpha$  containing 10% FBS, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. HepG $_2$  cells were cultured in MEM plus 10% FBS. L $_6$  muscle cells and 3T3L1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% FBS and 10% calf bovine serum, respectively. All the above media contained penicillin and streptomycin. At least 2 days before the labeling experiments, cells were switched to growth in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12) containing 5% lipoprotein-deficient serum (LPDS), penicillin, and streptomycin. Troglitazone and other thiazolidinedione compounds were dissolved in DMSO and added to the culture medium without serum. The final DMSO concentration in the medium did not exceed 0.2% (vol/vol). For transcription and protein synthesis dependency assays, actinomycin D and cycloheximide were added to a final concentration of 1  $\mu\text{g}/\text{ml}$  1 h before troglitazone treatment.

**Sterol assays.** Cells were seeded on 24-well plates at  $5 \times 10^4$  cells per well 3 days before the labeling experiments. On day 3, cells were treated with troglitazone or other thiazolidinedione compounds in a 1:1 mixture of DMEM/F12 without serum. Two hours later, [ $^{14}\text{C}$ ]acetate was added to a final concentration of 0.5 mmol/l. After 4-h incubation at  $37^\circ\text{C}$ , the cells were washed with phosphate-buffered saline (PBS) and lysed with 100  $\mu\text{l}$  of 0.1 N NaOH per well. The samples were saponified, and the radiolabeled sterols were extracted as described (30), with modifications (31). The sterols were resolved by thin-layer chromatography (TLC) on silica gel plates in petroleum ether:diethyl ether:acetic acid (60:40:1, vol/vol/vol), and the plates were subjected to autoradiography on x-ray films for 3–7 days at  $-70^\circ\text{C}$  (20). The x-ray films after exposure were scanned for densitometry of specific or total sterols.

**Pulse-chase labeling of cellular sterol products.** Cells on 24-well plates were treated with 20  $\mu\text{mol}/\text{l}$  troglitazone for 2 h in a 1:1 mixture of DMEM/F12 without serum. [ $^{14}\text{C}$ ]acetate was added to a final concentration of 0.5 mmol/l. The cells were labeled for 1 h and washed with cold PBS. One milliliter DMEM/F12 containing 5% LPDS and 2 mmol/l sodium acetate was added to each well. The cells were harvested with 0.1 N NaOH at different time points.

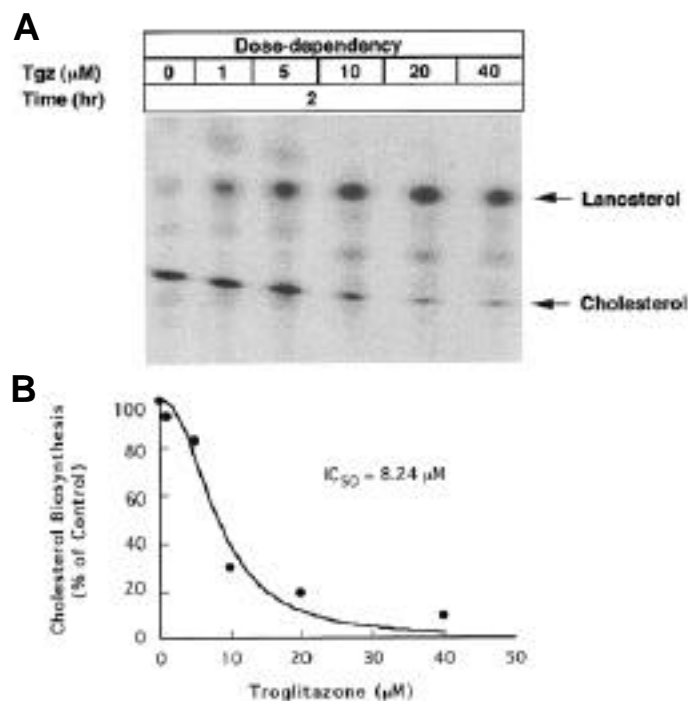
**PPAR- $\gamma$  reporter assay.** PPAR- $\gamma$  reporter assay with troglitazone was carried out using a transient transfection system. In this system, we used a chimeric form of mouse PPAR- $\gamma$  containing amino acids 205–506 of mouse PPAR- $\gamma$ 2 and amino acids 1–214 of *Escherichia coli* tetracyclin repressor (PPAR- $\gamma$  LBD-tet) and a reporter (pTET-luc) containing TET repressor binding site, a minimum cytomegalovirus (CMV) promoter, and the luciferase gene.  $\beta$ -Galactosidase was used as an internal control. After electroporation, cells were plated in a 96-well plate and allowed to attach. The cells were then pretreated with 1  $\mu\text{g}/\text{ml}$  cycloheximide, actinomycin D, or both for 1 h. After treatment, troglitazone was added at a final concentration of 20  $\mu\text{mol}/\text{l}$  and the cells were incubated for 24 h. Samples were then lysed in 50  $\mu\text{l}$  cell lysis buffer (Promega, Madison, WI). Luciferase and  $\beta$ -galactosidase activities were determined using the dual light kit (Tropix, Bedford, MA) on an E&G Berthold Microlumet 96P luminometer.

Transcription experiments with other PPAR- $\gamma$  ligands were carried out in a CHO-based stable cell line containing a Gal4-PPAR- $\gamma$ 1 chimeric receptor (amino

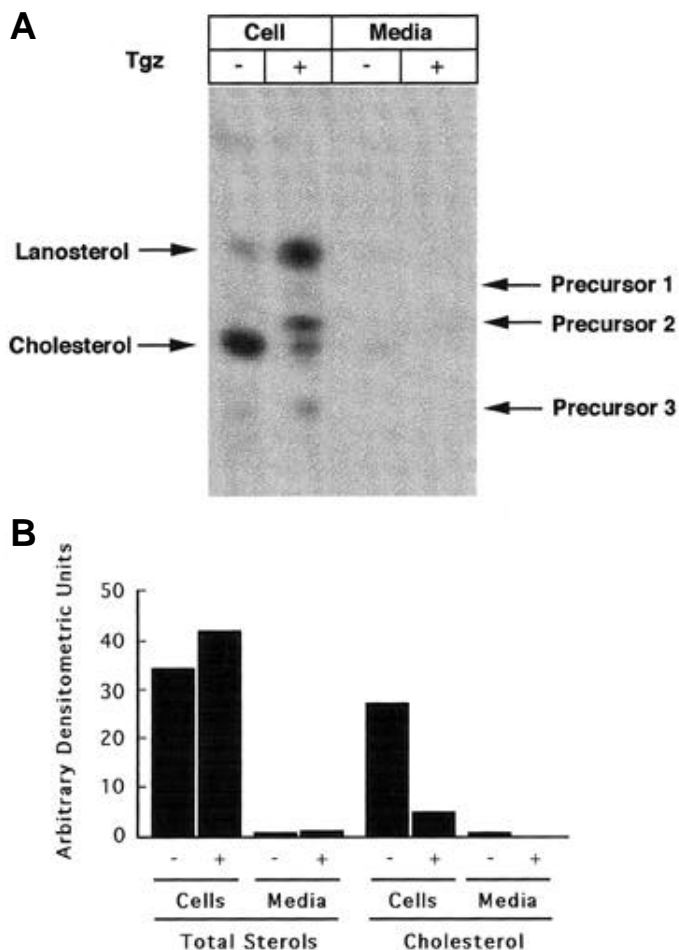
acids 1–147 of Gal4 fused with amino acids 205–505 of mouse PPAR- $\gamma$ 1) and a reporter construct containing seven copies of the Gal4 UAS fused to a minimal TATA promoter driving the expression of  $\beta$ -lactamase (constructed at Aurora Biosciences Corporation, San Diego, CA). The CHO cell line was cultured in phenol red-free DMEM/F12 (Gibco BRL) containing 10% charcoal/dextran-treated FCS (Hyclone, Logan, UT) and 1% penicillin/streptomycin/glutamine (Gibco BRL). Cells ( $2 \times 10^4$ ) were plated in each well of a 96-well microtiter plate (Costar, Corning, NY) in phenol red-free DMEM/F12, 1% charcoal/dextran-treated FCS, 1% penicillin/streptomycin/glutamine and allowed to attach for 4 h. The various compounds were then added as indicated, and the cells were incubated for an additional 16 h. The cells were loaded with the fluorescent  $\beta$ -lactamase substrate CCF2/AM (Aurora Biosciences Corporation, San Diego, CA) (32) at a final concentration of 2  $\mu\text{mol}/\text{l}$  for 1 h at room temperature.  $\beta$ -Lactamase activity was determined using a Cytofluor (Perspective Biosystems, Framingham, MA) plate reader as described (32), and fold activation was calculated relative to vehicle control.

## RESULTS

**Troglitazone inhibits cholesterol biosynthesis in CHO cells.** The effect of troglitazone on cholesterol biosynthesis was examined in cultured CHO cells. Cholesterol synthesis was measured by monitoring the incorporation of [ $^{14}\text{C}$ ]acetate into cellular cholesterol after 2- to 4-h incubation at  $37^\circ\text{C}$ . All samples were saponified before extraction so that the total cellular cholesterol could be measured. Cells were treated with different concentrations of troglitazone for 2 h, and the total cholesterol synthesis was resolved by TLC. As shown in Fig. 1, troglitazone inhibited cholesterol biosynthesis in a dose-dependent manner. The half-maximal concentration

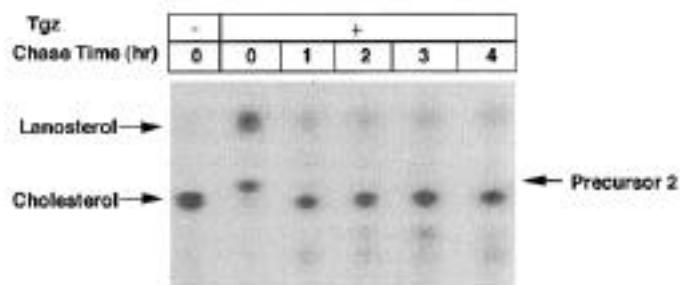


**FIG. 1.** Dose-dependent inhibition of cholesterol biosynthesis by troglitazone. **A:** CHO cells were seeded at  $5 \times 10^4$  cells per well on 24-well plates, as described in METHODS, and treated with different concentrations of troglitazone for 2 h. The cells were then labeled with 0.5 mmol/l [ $^{14}\text{C}$ ]acetate in DMEM/F12 at 0.5 ml per well for 4 h in the presence of troglitazone. Cells were harvested with 0.1 N NaOH, and sterols were extracted and resolved by TLC on silica gel plates. **B:** The cholesterol bands in Fig. 1A were scanned for density, and values were obtained by normalizing the densities by total protein. Cholesterol biosynthesis is expressed as percent of the values obtained for the untreated cells.



**FIG. 2.** Incorporation of [ $^{14}\text{C}$ ]acetate into cellular and media sterols. **A:** CHO cells were untreated or treated with 20  $\mu\text{mol/l}$  troglitazone for 2 h and labeled with 0.5 mmol/l [ $^{14}\text{C}$ ]acetate for 4 h as described in Fig. 1. Cells and media were harvested, and sterols were extracted and resolved by TLC as described in METHODS. **B:** The autoradiogram in **A** was scanned on a densitometer for cholesterol and total sterol values. The values were normalized by total protein. The contents of cholesterol and total sterols are shown in arbitrary units.

( $\text{IC}_{50}$ ) for inhibition was 8  $\mu\text{mol/l}$ . The inhibition of cholesterol biosynthesis was accompanied by the accumulation of several sterol products including lanosterol (Fig. 1A and Fig. 2A). The accumulation of lanosterol was apparent at 1  $\mu\text{mol/l}$  troglitazone. At 40  $\mu\text{mol/l}$ , troglitazone inhibited cholesterol production by more than 90% (Fig. 1B). In contrast with the longer period of time (24 and 72 h) required for troglitazone to trigger a variety of other metabolic responses (18,19), prominent inhibition of cholesterol production was observed after only 2 h of treatment with troglitazone (Fig. 1A). After troglitazone treatment, lanosterol and at least three other products accumulated in the cells (Fig. 2A). To determine whether inhibition of cholesterol synthesis results from a decrease in total sterol synthesis, the total sterols from both the cells and the media were analyzed (Fig. 2B). After troglitazone treatment, the total sterols did not change significantly. In contrast, the intracellular cholesterol level decreased by at least fourfold. Because there was no detectable cholesterol in the medium after treating the cells with troglitazone, the decrease in cellular cholesterol level

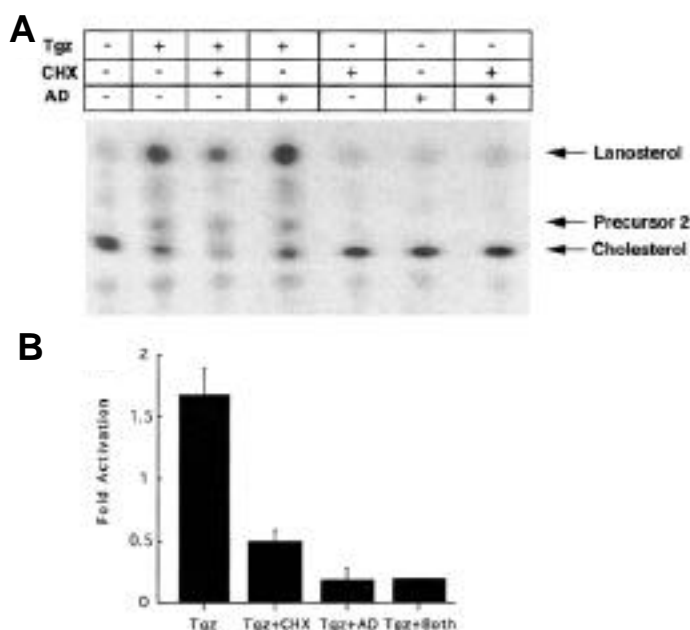


**FIG. 3.** Pulse-chase labeling of cellular sterols in cells treated or untreated with troglitazone. CHO cells were seeded on 24-well plates at  $5 \times 10^4$  cells per plate. On day 3, cells were treated with 20  $\mu\text{mol/l}$  troglitazone in DMEM/F12 for 2 h and labeled for 1 h in the presence of troglitazone. The cells were washed with cold PBS several times and refed 1 ml DMEM/F12 containing 5% LPDS and 2 mmol/l sodium acetate. The cells were harvested at the indicated time after chase and sterols were extracted. A sample from untreated but labeled cells was used as a control.

was very unlikely because of the enhancement of cholesterol exodus (Fig. 2B). These data suggest that the decrease in cholesterol is caused by metabolic inhibition.

The metabolites formed following inhibition by troglitazone were similar to those accumulated by progesterone treatment (31). To determine whether these metabolites are cholesterol precursors as in the case of progesterone treatment, pulse-chase labeling was used to monitor the relationship between cholesterol and the accumulated sterol products. The troglitazone-treated cells were labeled with [ $^{14}\text{C}$ ]acetate for 1 h and then chased with medium containing cold acetate for various times. At time point 0, the majority of the labels were lanosterol and precursor 2 (Fig. 3). One hour after the chase, lanosterol and precursor 2 disappeared and cholesterol reappeared, suggesting that these accumulated sterols are cholesterol precursors. Moreover, these data also suggest that troglitazone reversibly blocks one or more intermediate steps downstream of the biochemical reactions producing the accumulated sterols but upstream of that for cholesterol production.

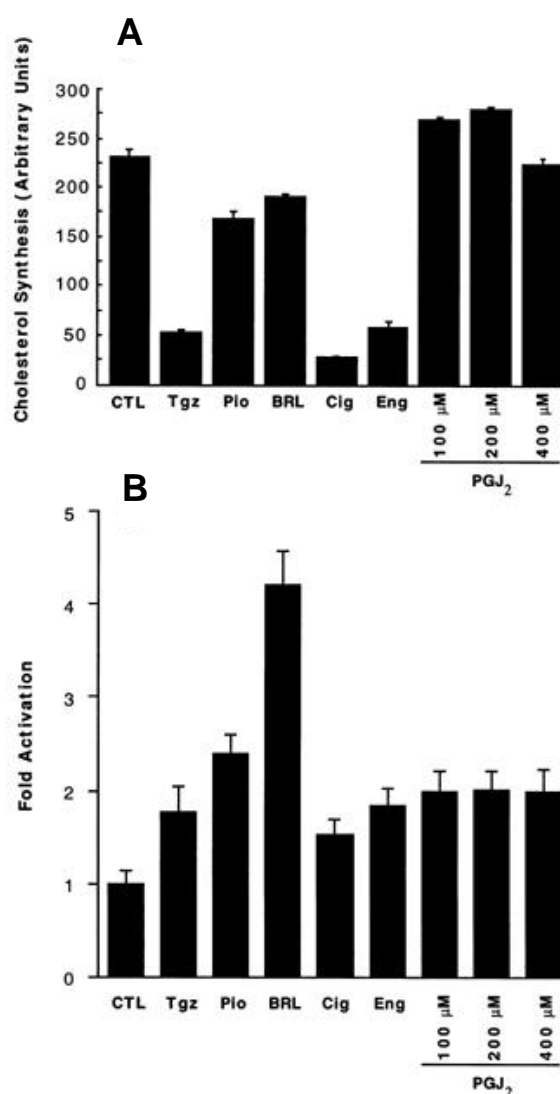
**Inhibition of cholesterol biosynthesis by troglitazone is independent of de novo RNA and protein synthesis.** The inhibition of cholesterol synthesis was reversed within 1 h after the cells were switched to medium without troglitazone, in clear contrast with several known transcription-dependent metabolic effects by this and other TZDs (19,33). To test whether transcriptional regulation is involved in the inhibition of cholesterol biosynthesis by troglitazone, actinomycin D and cycloheximide were used to block RNA and protein synthesis, respectively. As shown in Fig. 4A, these two agents had no effect on the inhibition of cholesterol synthesis by troglitazone. These data suggest that in contrast with the events mediated by PPAR- $\gamma$ , inhibition of cholesterol production by troglitazone does not require synthesis of new proteins. To ensure that the cycloheximide and actinomycin D used in our experiments were effective in blocking PPAR- $\gamma$ -mediated responses, we used a reporter system to monitor PPAR- $\gamma$  activity. We used a transient transfection system with a chimeric form of mouse PPAR- $\gamma$  containing amino acids 205–506 of mouse PPAR- $\gamma$ 2, amino acids 1–214 of *E. coli* tetracyclin repressor (PPAR- $\gamma$  LBD-tet), and a reporter (pTET-luc) containing TET repressor binding site, a minimum CMV promoter, and the luciferase gene. The chimeric PPAR- $\gamma$  and the



**FIG. 4. A:** Effects of actinomycin D and cycloheximide on the inhibitory activity of troglitazone on cholesterol biosynthesis. CHO cells seeded on 24-well plates were pretreated (+) or untreated (-) with cycloheximide (CHX) or actinomycin D (AD) for 1 h at 1  $\mu\text{g}/\text{ml}$ . Troglitazone was then added to a final concentration of 20  $\mu\text{mol}/\text{l}$ . The incubation lasted 2 h, and the cells were labeled with [ $^{14}\text{C}$ ]acetate for 4 h. Sterols were extracted and resolved by TLC as described in METHODS. **B:** Inhibition of reporter activity by cycloheximide or actinomycin D in CHO cells. CHO cells were electroporated with PPAR- $\gamma$  LBD-tet, reporter pTET-luc, and a  $\beta$ -galactosidase expression vector. The cells were plated in a 96-well plate and allowed to attach. The cells were then pretreated with 1  $\mu\text{g}/\text{ml}$  cycloheximide, actinomycin D, or both as indicated for 1 h. After this treatment, troglitazone was added at a final concentration of 20  $\mu\text{mol}/\text{l}$  and the cells were incubated for 24 h. The luciferase activity was normalized with  $\beta$ -galactosidase activity.

reporter DNAs were cotransfected into CHO cells, and the cells were treated as indicated (Fig. 4B). Figure 4B shows that addition of troglitazone increased luciferase activity by about twofold. As expected, both cycloheximide and actinomycin D blocked troglitazone-induced luciferase activity. Under the same conditions, however, neither cycloheximide nor actinomycin D altered the inhibition of cholesterol synthesis, confirming the above result that the inhibitory effect of troglitazone is independent of de novo RNA and protein synthesis.

**Differential inhibition of cholesterol synthesis by other PPAR- $\gamma$  activators.** Given the fact that inhibition of cholesterol synthesis by troglitazone is not a result of activation of PPAR- $\gamma$ , the ability of TZDs to bind PPAR- $\gamma$  may not be a reflection of their potency for inhibition of cholesterol synthesis. We monitored cholesterol synthesis in the presence of various PPAR- $\gamma$  activators including ciglitazone, englitazone, BRL49653, pioglitazone, and 15-deoxy- $^{12,14}$ -prostaglandin  $\text{J}_2$ . Ciglitazone and englitazone exhibited similar strength of inhibition on cholesterol synthesis to that of troglitazone (Fig. 5A). Despite the fact that BRL49653 and pioglitazone are potent ligands of PPAR- $\gamma$  (2,10), they exhibited only slight inhibitory activity on cholesterol synthesis. The PPAR- $\gamma$  natural ligand 15-deoxy- $^{12,14}$ -prostaglandin  $\text{J}_2$  was essentially ineffective even at 400  $\mu\text{mol}/\text{l}$ , a concentration much higher than that required for activation of PPAR- $\gamma$  (5,6). The rank order of potency for inhibition of cholesterol synthesis was ciglita-



**FIG. 5. A:** Effects of other thiazolidinediones or PPAR- $\gamma$  ligands on cholesterol biosynthesis. CHO cells seeded on 24-well plates were treated with troglitazone (Tgz), pioglitazone (Pio), BRL49653 (BRL), ciglitazone (Cig), englitazone (Eng), and 15-deoxy- $^{12,14}$ -prostaglandin  $\text{J}_2$  (PGJ<sub>2</sub>). The final concentration of the thiazolidinediones was 20  $\mu\text{mol}/\text{l}$ , and PGJ<sub>2</sub> was used at the indicated concentrations. The cells were labeled with [ $^{14}\text{C}$ ]acetate for 4 h and harvested for analysis of labeled sterols. The cholesterol bands were scanned and the obtained values were normalized by total protein. The final values are in arbitrary units. **B:** Activation of PPAR- $\gamma$  by the same series of PPAR- $\gamma$  ligands as in A. A CHO cell line stably transfected with a Gal4-PPAR- $\gamma$ 1 chimeric receptor and a reporter construct containing seven copies of the Gal4 UAS fused to a minimal TATA promoter driving the expression of  $\beta$ -lactamase (METHODS) was used in the reporter assay. Each ligand (final 20  $\mu\text{mol}/\text{l}$  except PGJ<sub>2</sub> at indicated concentrations) was added to the cells, and the incubation lasted for 16 h. The cells were loaded with the fluorescent  $\beta$ -lactamase substrate CCF2/AM (32),  $\beta$ -lactamase activity was determined as described (METHODS), and fold activation was calculated relative to vehicle control. The value for each ligand represents the average of eight assays.

zone > englitazone > troglitazone > pioglitazone > BRL49653, which is inconsistent with their affinities for PPAR- $\gamma$  (2,5,6,10). The activation of PPAR- $\gamma$ -mediated transcription by these compounds was analyzed in Fig. 5B. The rank order of PPAR- $\gamma$  activation by these compounds does not match the rank order of their inhibitory activity on cholesterol synthesis.

TABLE 1  
Effect of troglitazone on cholesterol biosynthesis in different cell lines

Cell line	Troglitazone ( $\mu\text{mol/l}$ )	Treatment time (h)	Cholesterol synthesis (%)
CHO	20	2	23.29 $\pm$ 0.77
HepG2	20	2	21.76 $\pm$ 1.24
L6	20	2	4 $\pm$ 0.15
3T3L1	20	2	36.07 $\pm$ 6.95

Cells were treated with 20  $\mu\text{mol/l}$  troglitazone, and cholesterol synthesis was assayed as in Fig. 1. The cholesterol bands were scanned for densitometric values and normalized by total protein. Cholesterol synthesis is expressed as percent of the values obtained for the untreated cells.

**Troglitazone inhibits cholesterol synthesis in other cell types.** To determine whether inhibition of cholesterol synthesis by troglitazone is unique to CHO cells, we selected cell lines of different origin, including HepG2, 3T3L1, and L6, for a further test. The cells were treated with troglitazone for 4 h, and cholesterol synthesis was determined. As shown in Table 1, 20  $\mu\text{mol/l}$  troglitazone inhibited cholesterol synthesis in all cell types tested. The inhibition in HepG2 cells was similar to that in CHO cells (~80%). Under the same conditions, there was less inhibition in 3T3-L1 cells (64%) but more inhibition in L6 cells (96%).

## DISCUSSION

The present study demonstrates that troglitazone, ciglitazone, and englitazone are potent inhibitors of cholesterol biosynthesis in CHO cells. Our results are consistent with the cholesterol-lowering ability of these three drugs *in vivo* (23–28). In addition, the weak inhibition of cholesterol synthesis by pioglitazone is in agreement with its ineffectiveness in lowering plasma cholesterol level in a rodent model of type 2 diabetes (34). It should be noted that when rats are fed with hypercholesterolemic diet, pioglitazone may effectively decrease plasma cholesterol, but this effect results from decreased cholesterol absorption rather than inhibition of cholesterol synthesis (29). The dose-dependent inhibition of cholesterol synthesis by troglitazone produced an  $\text{IC}_{50}$  of 8  $\mu\text{mol/l}$ . This  $\text{IC}_{50}$  value is close to its clinical concentrations (35) and is in accordance with the low micromolar range of  $\text{EC}_{50}$  values for many other TZD-mediated metabolic events, including glucose transport (36–38), fructose-2,6-bisphosphate production (39), adipocyte lipid-binding protein gene expression (40), and glycogen synthase generation (41).

The present study also demonstrates that the cholesterol-lowering activity by troglitazone is reproduced in cell types of different origin such as HepG2, 3T3-L1, and L6 cells, suggesting that the inhibitory effect by troglitazone is mediated by a common mechanism. It is generally true that the insulin-sensitizing effects of TZDs are direct or indirect consequences of activation of PPAR- $\gamma$  (1); however, this is not without exception. For example, troglitazone can inhibit LDL and HDL oxidation *in vitro* (42). In contrast with troglitazone, pioglitazone, another PPAR- $\gamma$  activator, does not show any antioxidant activity. Therefore, the scavenging effect of troglitazone is thought to be related to its structural similarity to the antioxidant  $\alpha$ -tocopherol rather than its ability to

bind PPAR- $\gamma$  (43). Furthermore, it has been shown that troglitazone inhibits long-chain acyl-CoA synthetase activity through its sulfoconjugate derivative, and this inhibition can be observed in a cell-free system (20). These observations suggest that troglitazone may exert some of its effects via mechanisms independent of PPAR- $\gamma$ . Indeed, our results indicate that inhibition of cholesterol synthesis by troglitazone is not mediated by PPAR- $\gamma$ . This conclusion is primarily based on the following two properties of the inhibition. First, the inhibition is not dependent on *de novo* synthesis of RNA and protein, a condition necessary for transcriptional regulation by PPAR- $\gamma$ . We have confirmed the expression of PPAR- $\gamma$  in CHO cells, which is consistent with the report by Zhang et al. (44). Second, the potencies for inhibition of cholesterol synthesis by different PPAR- $\gamma$  activators do not correlate with their affinities for PPAR- $\gamma$ . For example, englitazone is a relatively poor ligand for both mouse and human PPAR- $\gamma$  molecules compared with pioglitazone (2,10), but its inhibitory activity on cholesterol biosynthesis is much stronger than that of pioglitazone. In addition, BRL49653 has been known as the most potent ligand for PPAR- $\gamma$  among the TZDs (Fig. 5B) (2), but BRL49653 has essentially no effect on cholesterol biosynthesis. The transcription-independence and dissociation between the ability to bind PPAR- $\gamma$  and the inhibitory activity on cholesterol synthesis strongly suggest that inhibition of cholesterol synthesis by troglitazone is independent of PPAR- $\gamma$ .

The fold activations by PPAR- $\gamma$  ligands we observed in our system (Fig. 5B) differ from those reported elsewhere (5). This discrepancy could be attributed to the different cell lines and reporter constructs used. For example, we used CHO cells, and Forman et al. (5) used CV-1 cells. These different conditions might give different backgrounds and therefore affect fold activation values. We have experienced high background and low activation problems when using too much DNA in transient transfection experiments. Our system uses a stable cell line highly expressing both the reporter and the chimeric receptor. This could significantly increase basal luciferase activity and therefore affect activation values. Finally, our reporter plasmid contains different promoter elements from those reported by Forman et al. (5). This could also result in additional "leakiness" of the reporter.

The mechanism underlying the inhibitory effects of troglitazone on cholesterol biosynthesis remains to be explored. Troglitazone may inhibit a common pathway required for the biochemical reactions producing the accumulated sterols. One primary candidate for such inhibition is the transport of intracellular cholesterol precursors and cholesterol, the majority of which are at the plasma membrane, to the endoplasmic reticulum (ER), where enzymes involved in cholesterol homeostasis reside (31,45). The translocation of the sterols including cholesterol is rate-limiting for cholesterol synthesis and esterification and is sensitive to a large group of structurally unrelated amphophiles including progesterone (31,46,47). The striking similarity in the accumulated cholesterol precursors produced by progesterone (31) (M.W., S.C.W., T.L., T.-Z.S., unpublished observations) and troglitazone suggests that troglitazone may inhibit cholesterol synthesis by blocking the intracellular trafficking of sterols from the plasma membrane to the ER and therefore limit substrate availability to the cholesterol synthesis enzymes at the ER. Because most of the amphophiles inhibiting sterol traf-

ficking also interact with the P-glycoproteins and inhibit their activities, it was proposed that the progesterone-sensitive movement of sterols from the plasma membrane to the ER may be mediated by the P-glycoproteins (48). Their role in the intracellular sterol trafficking is not clear, however. P-glycoproteins are generally considered to be plasma membrane transporters responsible for pumping out a large number of structurally unrelated compounds from cells (49). Despite the similar inhibition patterns exhibited by troglitazone and progesterone on cholesterol synthesis, troglitazone does not inhibit drug transport activity of the P-glycoproteins as progesterone does. In wild-type CHO cells and a P-glycoprotein-overproducing CHO cell line, CH<sup>R</sup>C5, troglitazone did not cause a significant change in the accumulation of [<sup>3</sup>H]vinblastine, a typical substrate for the P-glycoproteins (data not shown). The dissociation between the inhibitions of P-glycoprotein activity and cholesterol synthesis is consistent with a previous finding that cholesterol traffic from the plasma membrane to the ER can still be blocked by a number of P-glycoprotein inhibitors in a cell line where P-glycoprotein activity has never been detected (45,46). This evidence raises the possibility that an amphiphile-sensitive protein other than the P-glycoproteins might mediate sterol transport from the plasma membrane to the ER. Another interpretation for the inhibitory effects of troglitazone on cholesterol synthesis is that troglitazone might partition into the plasma membrane, affect its sterol "sensor," and reduce the delivery of sterols to the cytoplasmic membrane (47,50). Moreover, it cannot be ruled out that troglitazone might interact with the enzymes involved in the synthesis of late cholesterol precursors such as zymosterol. Further investigation of the above possibilities may lead to the identification of new biological targets for cholesterol-lowering drugs.

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