Thiazolidinedione- and Tumor Necrosis Factor Alpha–Induced Downregulation of Peroxisome Proliferator–Activated Receptor Gamma mRNA in Differentiated 3T3-L1 Adipocytes

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Thiazolidinediones (TZDs) are antidiabetic insulin-sensitizing agents that bind to peroxisome proliferator–activated receptor gamma (PPARγ) and have potent adipogenic effects on 3T3-L1 preadipocytes. In fully differentiated 3T3-L1 adipocytes, TZDs markedly decreased PPARγ mRNA levels without reducing the expression of genes that are positively regulated by PPARγ, such as adipocyte lipid-binding protein 2 (aP2) or lipoprotein lipase-(LPL). PPARγ mRNA levels were also downregulated by tumor necrosis factor alpha (TNFα), an antiadipogenic cytokine. We propose that the downregulation of PPARγ is not the common denominator of the metabolic effects of TZDs and TNFα on mature adipocytes.

ADIPOCYTES are terminally differentiated cells that play an essential role in energy homeostasis. Their functional dysregulation leads to various disorders such as obesity, glucose intolerance, hyperlipidemia, and hypertension, which collectively contribute to an increased risk of ischemic heart disease (reviewed in Schwartz and Brunzell[1]). Several transcription factors that act cooperatively and sequentially to trigger the terminal differentiation program of adipocytes have been identified. These include members of the CAAT/enhancer binding protein (C/EBP) and peroxisome proliferator–activated receptor (PPAR) families of transcription factors. In particular, PPARγ is most specifically expressed in adipocytes.[2,3] Thus far, several factors have been reported to be positive or negative regulators of PPARγ mRNA expression. Adipocytes differentiation,[4] obesity in humans,[5] and feeding with a high-fat diet in rodents[6] are associated with increased expression of PPARγ mRNA. On the other hand, tumor necrosis factor alpha (TNFα) suppresses PPARγ mRNA levels in adipocytes in culture,[7] and weight loss is associated with a reduction in PPARγ mRNA levels in humans.[5] Furthermore, the forced expression of PPARγ in fibroblasts converts their phenotype to adipocytes, underscoring its critical role in adipogenesis.[4,8] PPARs regulate gene expression by binding to DNA sequence elements termed PPAR response elements (PPREs) as a heterodimer with retinoid X receptor (RXR).

Recently, antidiabetic insulin-sensitizing compounds, the thiazolidinediones (TZDs), have been found to be high-affinity ligands for PPARγ.[9] Most of the pharmacologic actions of TZDs are thought to be mediated by activation of PPARγ.[2] In vitro studies have shown that TZDs have a strong adipogenic effect on preadipocytes and mesenchymal stem cells.[10,11] Besides upregulating the expression of genes that are characteristic of mature adipocytes, TZDs antagonize the effects of TNFα on adipocytes.[12] With regard to their in vivo effects, Pearson et al[13] reported that the TZD BRL49653 increased PPARγ mRNA levels in adipose tissue in high-fat–fed rats but not in carbohydrate-fed rats. Burant et al[14] reported that treatment with troglitazone normalized glucose intolerance and abnormalities of plasma lipoprotein metabolism in adipocyte lipid-binding protein 2 (aP2)/diphtheria toxin A (DTA) mice whose adipose tissues were genetically ablated. They showed that troglitazone did not alter PPARγ mRNA levels in either wild-type or aP2/DTA mice. With regard to in vitro effects, Brun et al[8] showed that pioglitazone induced the expression of endogenous PPARγ mRNA in NIH-3T3 cells ectopically expressing PPARγ. Finally, BRL49653 induced PPARγ mRNA expression in C3H10T1/2 cells after 7 days' exposure.[9]

In the current study, we investigated the effects of TZDs, as well as 15-deoxy-$\Delta^{12,14}$ prostaglandin J2 (15-$\Delta^{12,14}$PGJ2), a putative natural ligand of PPARγ,[15,16] on PPARγ mRNA levels in 3T3-L1 cells, a widely used adipocyte cell line that undergoes differentiation from a fibroblastic adipoblast to a mature adipocyte capable of insulin-regulated lipogenesis.[17] TZDs induced the expression of PPARγ mRNA when added to undifferentiated 3T3-L1 preadipocytes as previously reported,[9] whereas, TZDs suppressed the expression of PPARγ mRNA when added to 3T3-L1 mature adipocytes that were fully differentiated by insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX). Despite the reduced mRNA levels of PPARγ, TZD-treated cells retained the phenotypes of mature adipocytes.

MATERIALS AND METHODS

Materials

Troglitazone and pioglitazone were generous gifts from Sankyo Pharmaceutical (Tokyo, Japan) and Takeda Pharmaceutical (Osaka, Japan), respectively. The compounds were solubilized in dimethylsulfoxide (DMSO), and an equivalent concentration of DMSO was used as a control; the final concentration of DMSO was kept at 0.1% (vol/vol). Recombinant mouse TNFα was purchased from Genzyme Diagnostics (Boston, MA), 15-$\Delta^{12,14}$PGJ2 was obtained from Cayman Chemical (Ann Arbor, MI). Dulbecco's modified Eagle's medium (DMEM) was

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from GIBCO-BRL (Gaithersburg, MD). Calcium pantothenate, biotin, and actinomycin D were from Sigma (St Louis, MO). cDNA polymerase chain reaction fragments were used as probes for PPARγ, lipoprotein lipase (LPL), aP2, CD36, hormone-sensitive lipase (HSL), acyl-coenzyme A synthase (ACS), and sterol regulatory element binding protein-1 (SREBP-1).

3T3-L1 Cell Culture and Differentiation

The mouse fibroblast 3T3-L1 cell line was a generous gift from Dr M. Kawakami (Jichi Medical School, Omiya, Japan). 3T3-L1 preadipocytes were grown in DMEM supplemented with 10% (vol/vol) calf serum, 4,500 mg/L D-glucose, 8 mg/L biotin, and 4 mg/L calcium pantothenate. The 3T3-L1 cells were differentiated as previously described.[18] Briefly, 2 days after the cells were grown to confluence, differentiation was initiated by incubation in a fresh medium containing 10 μg/mL insulin, 0.39 μg/mL dexamethasone, and 115 μg/mL IBMX. Forty-eight hours later, insulin was added to the medium at a final concentration of 10 μg/mL and the medium was replaced every 2 days.

Northern Blot Analysis

Total RNA was isolated from the cells by Trizol (GIBCO-BRL) according to the manufacturer's instructions. For Northern blot analysis, 10 μg total RNA was subjected to electrophoresis in 1% (wt/vol) agarose gel containing formamide and transferred to a nylon filter (Hybond N^-, Amersham Pharmacia, Uppsala, Sweden). cDNA probes were radiolabeled with [α-^{32}P]deoxy-cytidine triphosphate. After prehybridization for 30 minutes, the blots were hybridized in Rapid-hyb buffer (Amersham Pharmacia) for 1 hour at 65°C with the probes. The relative signal intensity was calculated by a phosphoimager (BAS 2000; Fuji Film, Tokyo, Japan).

Cellular Triglyceride Content

After extensive washing with phosphate-buffered saline, the cellular lipids were extracted by hexane-isopropanol (2:1). The organic solvents were evaporated, and triglycerides were determined by a kit (Determiner TG 555; Kyowa Medex, Tokyo, Japan). Cellular proteins were dissolved in 0.1N NaOH and determined by protein assay kit (BCA, Pierce, Rockford, IL).

Glycerol and Free Fatty Acid Release From the Cells

Media conditioned with the cells for 24 hours were collected, and the glycerol concentration was determined by a kit (Determiner TG555; Kyowa Medex) without the use of a reagent containing lipase. The free fatty acid concentration in the medium was determined by a kit (Determiner NEFA; Kyowa Medex).

RESULTS

Five days after the start of differentiation, mature 3T3-L1 cells were treated with increasing concentrations of either troglitazone or pioglitazone for 24 hours and the expression level of PPARγ mRNA was compared by Northern blot analysis (Fig 1). Both compounds reduced the PPARγ mRNA level in a dose-dependent manner, with a 50% inhibitory concentration (IC_{50}) of about 4 and 0.4 μmol/L for troglitazone and pioglitazone, respectively, indicating that pioglitazone is more potent than troglitazone. These differences in potency appear to parallel differences in the insulin-sensitizing action and affinity for the PPARγ protein. The synthetic activator 15-$\Delta^{12,14}$PGJ2 reduced PPARγ mRNA levels also (Fig 1). To determine the time course of the PPARγ-suppressing effects of TZD, mature 3T3-L1 cells were incubated with 10 μmol/L troglitazone for different periods (Fig 2). The suppression of PPARγ mRNA was apparent 2 hours after the addition of troglitazone, and reached a maximum reduction after 4 hours. The maximal suppression (70%) was sustained for at least 24 hours after the addition of troglitazone.

To determine the mechanisms by which TZDs decrease PPARγ mRNA levels, we examined the effects of actinomycin D, an inhibitor of mRNA synthesis, on the degradation of mRNA (Fig 3). After the addition of 5 μg/mL actinomycin D to the cells treated with troglitazone for 4 hours, the PPARγ mRNA level declined. The disappearance rates were comparable between troglitazone-treated and untreated cells, with a 50% reduction 3 to 4 hours after the addition of actinomycin D, indicating that the suppression of mRNA levels was primarily mediated by the reduced transcriptional activity, not the increased degradation rate.

To determine whether the suppression of PPARγ mRNA is associated with any change in the transcriptional activity of genes that are specifically expressed in white adipose tissue, we examined the effects of troglitazone on the mRNA level of LPL, aP2, CD36, HSL, ACS, and SREBP-1. LPL, aP2, and ACS contain PPREs in their promoter sequences. Two different modes of exposure to troglitazone were used, (1) incubation with troglitazone from day 0 to day 6 (chronic effects) and (2) incubation with troglitazone on day 5 for 24 hours (acute effects, Fig 4). There were no significant differences in the mRNA level of LPL, CD36,

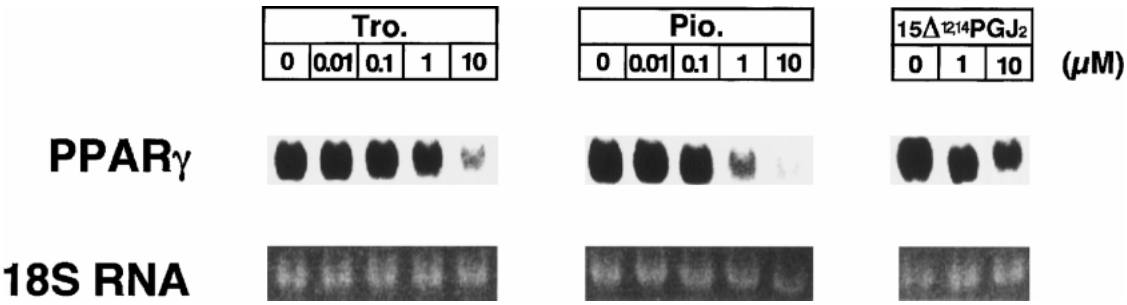

Fig 1. Dose-dependent effects of TZDs and 15-deoxy-$\Delta^{12,14}$PGJ2 on the PPARγ mRNA level in 3T3-L1 mature adipocytes. 3T3-L1 preadipocytes were differentiated by incubation with media containing insulin, dexamethasone, and IBMX for 2 days. Cells were further cultured with media containing only insulin for 3 days before the addition of troglitazone (Tro), pioglitazone (Pio), or 15-deoxy-$\Delta^{12,14}$PGJ2 (15-$\Delta^{12,14}$PGJ2) at the indicated concentration for 24 hours. Northern blot analysis was performed for PPARγ.

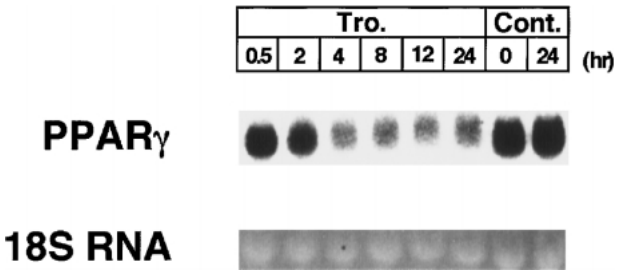

Fig 2. Time course of the effects of troglitazone on the PPARγ mRNA level in 3T3-L1 mature adipocytes. 3T3-L1 preadipocytes were differentiated by incubation with media containing insulin, dexamethasone, and IBMX for 2 days, and were further cultured with media containing only insulin for 3 days before the addition of the compounds. Cells were incubated with (Tro) or without (Cont) 10 μmol/L troglitazone for the indicated time. Northern blot analysis was performed for PPARγ.

HSL, ACS, and SREBP-1, while the aP2 mRNA level increased 1.7-fold when the cells were treated long-term with troglitazone. Morphologic examination showed that long-term exposure to troglitazone significantly increased the efficiency of adipocyte differentiation. As for the acute effects, there were no significant differences in the mRNA level of LPL and CD36; however, the aP2 mRNA level increased 1.6-fold, while HSL mRNA decreased 2-fold. Therefore, only the HSL expression level changed in a manner comparable to the PPARγ expression level in response to exposure to TZDs.

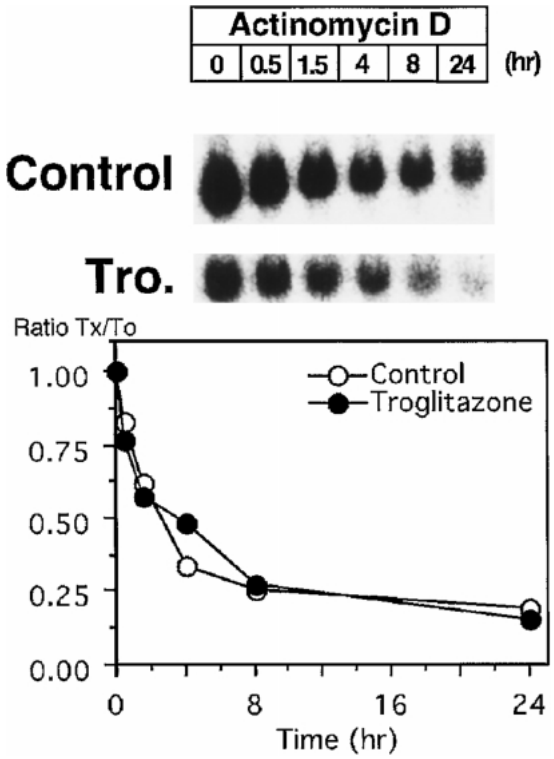

Fig 3. Effects of troglitazone on PPARγ mRNA stability. 3T3-L1 preadipocytes were differentiated by incubation with media containing insulin, dexamethasone, and IBMX for 2 days, and were further cultured with media containing only insulin for 3 days. The cells were incubated with the previous media with (Tro) or without (Cont) 10 μmol/L troglitazone added to the media for 4 hours (Tmax). Thereafter, 5 mg/mL actinomycin D was added to the media and incubated for the indicated time before harvest. Northern blot analysis was performed for PPARγ. The band intensity was measured by a phosphoimager, and the ratio to the value for time 0 is plotted in the inset.

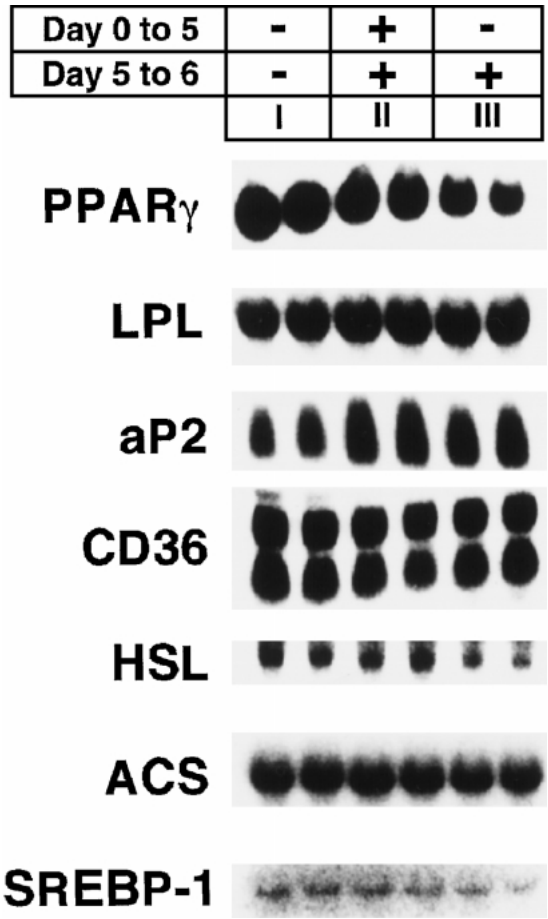

Fig 4. Chronic and acute effects of troglitazone on the mRNA level of various genes essential for adipocyte functions. The cells were incubated with the differentiation media with or without 10 μmol/L troglitazone for an additional 24 hours. Three different conditions were used: without troglitazone for 6 days (I), with troglitazone for 6 days (II, chronic), and with troglitazone only in the last 24 hours (III, acute). Northern blot analysis was performed using 7 different probes: PPARγ, LPL, aP2, CD36, HSL, ACS, and SREBP-1. Samples were loaded in duplicate. The fold-change in the mRNA level of each gene is as follows: 0.69, 1.23, 1.71, 0.64, 1.21, 1.10, and 0.95, respectively, for II; and 0.35, 1.15, 1.60, 0.71, 0.49, 1.06, and 0.87, respectively, for III.

Furthermore, we compared the triglyceride content in cells treated with troglitazone (Table 1). In mature 3T3-L1 cells, troglitazone did not change the net triglyceride content. On the other hand, troglitazone increased the amount of glycerol, but decreased the amount of free fatty acid in the medium.

Finally, we examined the effects of TNFα on lipolysis and the PPARγ mRNA level. TNFα stimulated the release of both free fatty acids and glycerol (Table 1). The simultaneous addition of both agents increased the release of glycerol without

Table 1. Effect of Troglitazone and TNFα on Triglyceride Content and Lipolysis in 3T3-L1 Adipocyte

Condition	Cell	Medium	
	Triglycerides (μg/mg)	Glycerol (μg/mg)	Free Fatty Acids (nEq/mg)
Control	144 ± 8	136 ± 5	101 ± 14
Troglitazone	154 ± 2	169 ± 16*	57 ± 7*
TNFα	—	185 ± 17*	170 ± 36*
Troglitazone + TNFα	—	171 ± 20*	108 ± 15

NOTE. 3T3-L1 preadipocytes were differentiated by incubation with media containing insulin, dexamethasone, and IBMX for 2 days. Thereafter, the cells were incubated with the differentiation media containing insulin with or without 10 μmol/L troglitazone or 3 nmol/L TNFα. Cellular triglyceride at day 6 and the release of glycerol and free fatty acids in the medium during the last 24 hours were determined in triplicate wells. Values are expressed as the mean ± SD.

* $P < .05$ *v* control by Student's *t* test.

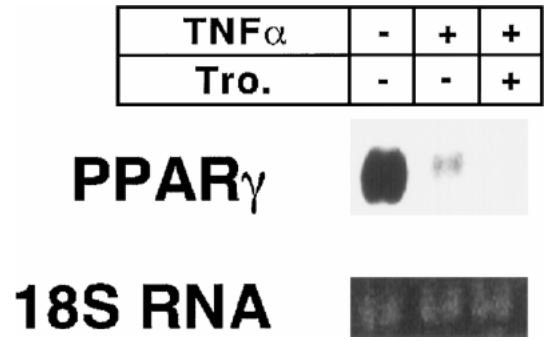

Fig 5. Effects of TNFα on the PPARγ mRNA level of 3T3-L1 adipocytes. 3T3-L1 preadipocytes were differentiated by incubation with media containing insulin, dexamethasone, and IBMX for 2 days, and were further cultured with media containing only insulin for 3 days. After incubation with 10 μmol/L troglitazone or 3 nmol/L TNFα for 24 hours, the cells were harvested for RNA extraction. Northern blot analysis was performed for PPARγ, and 18S RNA was stained with ethidium bromide as the loading control.

changing the level of free fatty acids in the medium. TNFα markedly reduced PPARγ mRNA levels in fully differentiated 3T3-L1 adipocytes. In contrast to their effects on lipolysis, the simultaneous addition of both troglitazone and TNFα decreased the expression of PPARγ (Fig 5).

DISCUSSION

In the present study, we show that short-term administration of TZDs (troglitazone and pioglitazone) and 15-$\Delta^{12,14}$PGJ2 acutely downregulated the expression of PPARγ in 3T3-L1 mature adipocytes at the transcriptional level (Figs 1 to 3). In agreement with this, Camp et al[19] have recently reported that troglitazone reduced the PPARγ protein level and the DNA binding activity of PPARγ in fully differentiated 3T3-L1 adipocytes. BRL49653 reportedly has similar suppressive effects on PPARγ mRNA levels.[20] In this context, it is noteworthy that other nuclear factors such as glucocorticoid receptor and the thyroid receptor are downregulated by their ligands, dexamethasone and thyroxine, respectively.[21,22] Although the precise mechanisms underlying the downregulation of nuclear receptors are yet to be determined, this phenomenon may protect the receptors and their heterodimerizing partner, RXR, from excessive consumption. In the present study, we did not separately measure the transcript and/or protein levels of two isoforms of PPARγ, PPARγ1 and PPARγ2. This may be one of the reasons that the effects of TZDs on PPARγ mRNA levels were not complete.

However, paradoxically, the TZD-induced downregulation of the PPARγ mRNA level was not associated with comparable changes in the mRNA level of other genes such as LPL, aP2, and ACS, which are assumed to be under the control of PPARγ (Fig 4). These results suggest that the transcriptional activities of these genes are not simply determined by the PPARγ mRNA level. Instead, other factors such as the phosphorylation of PPARγ may play a more important role.[2]

TNFα and TZDs have almost opposite effects on lipid and glucose metabolism, as well as adipocyte differentiation.[23] TZDs even antagonize the inhibitory effects of TNFα on differentiation, insulin-stimulated glucose uptake, and gene expression in 3T3-L1 cells.[12] Supporting this, free fatty acid release was stimulated by TNFα but suppressed by TZDs in 3T3-L1 adipocytes, and their combined effects were neutral (Table 1). Despite these contrasting biologic effects, both TNFα and TZDs induced downregulation of PPARγ mRNA in 3T3-L1 mature adipocytes, in agreement with previous reports.[7,20] Their combination further decreased the PPARγ mRNA level (Fig 5). Therefore, it is unlikely that PPARγ serves as a common denominator of the actions of TNFα and TZDs. Rather, it may be that TNF stimulates the release of fatty acids, which in turn downregulate PPARγ expression.

In conclusion, TZDs potently downregulate the expression of their cognate receptor, PPARγ, without reducing the expression of genes that are positively regulated by PPARγ such as aP2 or LPL. TNFα a cytokine with opposing effects versus TZDs, similarly reduces PPARγ mRNA levels. Therefore, the PPARγ mRNA level is not the common denominator of adipocyte function. Further studies are needed to clarify the mechanisms underlying the dissociation.

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