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Thiazolidinediones Downregulate PPARγ Expression via Induction of aP2 During Mouse 3T3-L1 Preadipocyte Differentiation

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Thiazolidinediones, such as troglitazone and rosiglitazone, are anti-diabetic insulin-sensitizing agents that bind to the peroxisome proliferator-activated receptor γ (PPARγ) and have potent adipogenic effects on 3T3-L1 preadipocytes. During 3T3-L1 preadipocyte differentiation, which was induced by isobutyl methylxanthine, dexamethasone, and insulin, troglitazone treatment increased lipid content and decreased PPARγ protein levels compared with DMSO-treated control cells. However, the level of CCAAT/enhancer binding protein α (C/EBPα) and C/EBPβ proteins did not decrease in troglitazone-treated cells compared with DMSO-treated cells. Real-time PCR analysis showed that PPARγ mRNA but not C/EBPα mRNA was downregulated in troglitazone-treated adipocytes, suggesting that PPARγ protein reduction occurred due to the decrease in its transcription level. Rosiglitazone treatment also increased lipid content but decreased PPARγ expression during 3T3-L1 preadipocyte differentiation. Both thiazolidinediones significantly increased the levels of adipokines such as adipocyte protein 2 (aP2) and adiponectin in 3T3-L1 adipocytes compared with that in DMSO-treated cells. We propose that thiazolidinediones are involved in adipogenic homeostasis rather than act as agonists of PPARγ during 3T3-L1 adipocyte differentiation.

Key words peroxisome proliferator-activated receptor γ, 3T3-L1 cells, thiazolidinediones, adipogenesis

INTRODUCTION

Adipocyte differentiation is controlled by molecular and cellular mechanisms that include transcription factors and genetic and environmental factors. Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor superfamily of ligand-inducible transcription factors that regulate adipocyte differentiation, thereby controlling the gene networks involved in lipid metabolism and glucose homeostasis.[1)] Previous research has shown that PPARγ is associated with the differentiation and maintenance of adipocyte phenotypes, which have roles in metabolic disorders such as type 2 diabetes and obesity.[2)] The differentiation of preadipocytes into mature adipocytes is accompanied by the sequential expression and activation of adipogenic transcription factors, including PPARγ and members of the CCAAT/enhancer binding protein (C/EBP) transcription factor family.[3,4)] These proteins regulate the downstream target genes involved in adipogenesis.[5)] PPARγ antagonists that inhibit PPARγ expression have been suggested as candidate anti-obesity agents.[6,7)] Hyperactivation of PPARγ has been shown to induce adipose tissue expansion, weight gain, and development of fatty liver in human and animal studies and adipogenesis *in vitro*.[8)] However, normal activation of PPARγ has been shown to provide beneficial effects, such as improving insulin sensitivity, which play a role in glucose homeostasis.

Anti-diabetic insulin-sensitizing compounds—thiazolidinediones (TZDs)—have been reported to be high-affinity ligands for PPARγ. Most pharmacological actions of TZDs are thought to be mediated by the activation of PPARγ. *In vitro* studies have shown that TZDs have a strong adipogenic effect on preadipocytes.[9)] Previous research using 3T3-L1 preadipocytes or diabetic animal models has demonstrated the effectiveness of many natural compounds, with fewer side-effects, compared with PPARγ agonists, such as TZDs.[10–18)] Many previous studies reported that in 3T3-L1 preadipocyte differentiation, induction of lipid accumulation by natural compounds was associated with increased PPARγ expression. To investigate the effects of natural compounds on insulin sensitivity, TZDs have been used as positive control agents due to increased PPARγ protein expression in post-confluent 3T3-L1 cells.[19,20)]

In this study, we observed that TZD treatment reduced PPARγ gene and protein expression during differentiation of methylxanthine, dexamethasone, and insulin (MDI)-treated 3T3-L1 preadipocytes to adipocytes, despite enhancing MDI-induced adipocyte differentiation. Lipid accumulation by TZD treatment may not be a direct result of PPARγ expression. TZDs may not be positive control agents to evaluate natural compounds for activating of PPARγ using 3T3-L1 preadipocyte differentiation system. Therefore, we evaluated the increased insulin sensitivity induced by TZDs and studied the mechanism associated with the reduction of PPARγ expression during 3T3-L1 adipocyte differentiation. Our results will help identify a novel mechanism of anti-diabetic agents.

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MTERIALS AND METHODS

Chemicals Troglitazone and rosiglitazone were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and dissolved in dimethyl sulfoxide (DMSO; Wako) to a final concentration of 10 and 20 mM, respectively. Both chemicals were diluted in the culture medium to a final concentration of 0.05% DMSO for cell treatments.

Cell Culture 3T3-L1 cells were obtained from the JCRB Cell Bank (Osaka, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM; Wako) with 10% bovine calf serum (HyClone, South Logan, UT) and 100 U/mL penicillin and 0.1 mg/mL streptomycin (Nacalai Tesque, Kyoto, Japan) at 37°C in 5% CO_2. At post-confluence, cell differentiation was induced by the Cayman Induction Medium containing 1 × MDI (Cayman Chemical, Ann Arbor, MI) for 3 d. The culture medium was replaced every 2 d thereafter, with DMEM containing 10% fetal bovine serum and insulin (Cayman Chemical).

Oil Red O Staining 3T3-L1 adipocytes (day 9), seeded in 96-well cell culture plates, were rinsed with phosphate-buffered saline (PBS) and fixed in formalin for 15 min at room temperature. Thereafter, formalin was removed and the cells were rinsed twice with wash solution (60% isopropanol). Oil red O solution (Cayman Chemical) was added to the dried wells and incubated with the cells for 20 min. The cells were rinsed three times with water, rinsed twice with 60% isopropanol, and dried. Lipid droplet accumulation was observed under a microscope. The stained lipid droplets were dissolved in isopropanol and the absorbance of the extracted solution was measured at 492 nm.

Immunoblot Analysis For immunoblot analysis, 3T3-L1 cells were cultured in 24-well plates. The cells were rinsed twice with PBS; thereafter, 200 µl of cell lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, and 1% protease inhibitor cocktail; Sigma-Aldrich, St. Louis, MO) was added to each well, and the lysates were collected in Eppendorf tubes. The protein content of the cell lysate samples was measured using the Quick Start Protein Assay (Bio-Rad, Hercules, CA) kit. The cell lysate samples were mixed with 4× sample buffer (Sigma-Aldrich) and the samples (4–8 µg protein) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were electro-transferred onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Bedford, MA). The membranes were blocked for 1 h at room temperature in 5% skim milk (in PBS) with 0.05% Tween-20 and incubated with the primary antibody overnight at 4°C. Thereafter, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary donkey anti-rabbit IgG (Cell Signaling Technology, Beverly, MA) or HRP-conjugated secondary rabbit anti-mouse IgG (Cell Signaling Technology). Anti-C/EBPβ, anti-PPARγ, and anti-β-actin antibodies were purchased from Cell Signaling Technology. Anti-C/EBPα antibody was from Invitrogen. HRP substrate (Luminata Forte Western HRP Substrate; Millipore) was added to the membrane and chemiluminescence was detected using the LAS 3000 imaging system (Fujifilm, Tokyo, Japan). The blots were stripped and re-probed with other primary antibodies and subjected to chemiluminescence detection. Target protein band intensity was quantified using the ImageJ software (National Institutes of Health, Bethesda, MD).

Quantitative RT-PCR The cells were washed twice with PBS and suspended in RNAiso Plus (Takara Bio, Shiga, Japan). Reverse transcription was performed on 2 µg total RNA using MMLV reverse transcriptase (Invitrogen, Carlsbad, CA). RT-PCR was carried out using StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA). The conditions used for the PCR reactions were: 95°C for 7 min, followed by 40 cycles at 95°C for 30 s and 59°C for 30 s. Data analysis was performed using the ΔΔCT method. mRNA levels were normalized relative to β-actin expression levels and compared with that of untreated control cells. The primer sequences used for real-time PCR are as follows: β-actin: 5′-CAGCCTTCCTTCTTGGGTATGG-3′, 5′-CTGTGTTGGCATAGAGGTCTTTACG-3′; C/EBPα: 5′-TTACAACAGGCCAGGTTTCC-3′, 5′-GGCTGGCGACATACAGTACA-3′; PPARγ: 5′-GGAGCCTAAGTTTGAGTTTGCTGTG-3′, 5′-TGCAGCAGGTTGTCTTGGATG-3′; adiponectin: 5′-AGCCTGGAGAAGCCGCTTAT-3′, 5′-TTGCAGTAGAACTTGCCAGTGC-3′; and aP2: 5′-CATGGCCAAGCCCAACAT-3′, 5′-CGCCCACTTTGAAGGAAATC-3′. PPARγ primers recognize all PPARγ isoforms (PPARγ1, PPARγ2 and PPARγ3). It is confirmed that the efficiency of each PCR reactions is approximately equal (Fig. S1).

Statistical Analysis The results are presented as the means of at least triplicate determinations ± standard deviation. Significance was determined by the Tukey-Kramer test or Dunnett's test. For all statistical comparisons, a p-value < 0.05 was considered statistically significant.

RESULTS

Effect of Troglitazone on Lipid Accumulation and PPARγ Expression 3T3-L1 preadipocytes were induced to differentiate into adipocytes. At day 9 after differentiation, oil red O staining showed significant lipid accumulation in troglitazone and MDI-treated cells compared with cells treated with MDI alone (Fig. 1A).

PPARγ protein levels were analyzed at day 6 after 3T3-L1 cells were induced to differentiate into adipocytes. We observed three isoforms of PPARγ, and especially PPARγ1 and PPARγ2 increased in MDI-induced 3T3-L1 adipocytes. As shown in Fig. 1B, induction with MDI and DMSO resulted in a marked increase in PPARγ expression; however, addition of troglitazone resulted in an approximately 70% reduction in MDI-induced PPARγ expression (Fig. 1B). Time course experiment showed that total PPARγ protein levels were much lower in troglitazone-treated cells than in DMSO-treated cells at day 4 after differentiation and the lower levels continued to persist on day 8 (Fig. 2A).

The conversion of preadipocytes into adipocytes requires the activation of key transcription factors such as PPARγ, C/EBPα, and C/EBPβ. During the differentiation process, increased levels of C/EBPβ induce the transcription of C/EBPα and PPARγ. Therefore, we investigated C/EBPα and C/EBPβ expression during 3T3-L1 preadipocyte differentiation. Western blot analysis showed that C/EBPα protein levels were slightly increased or unchanged in troglitazone-stimulated cells compared with DMSO-treated cells (Fig. 2A). C/EBPβ protein levels were slightly increased at days 4 and 6 in troglitazone-stimulated cells compared with DMSO-treated cells (Fig. 2B).

Effects of Troglitazone on PPARγ and C/EBPα Gene Expression To investigate whether the reduction of PPARγ

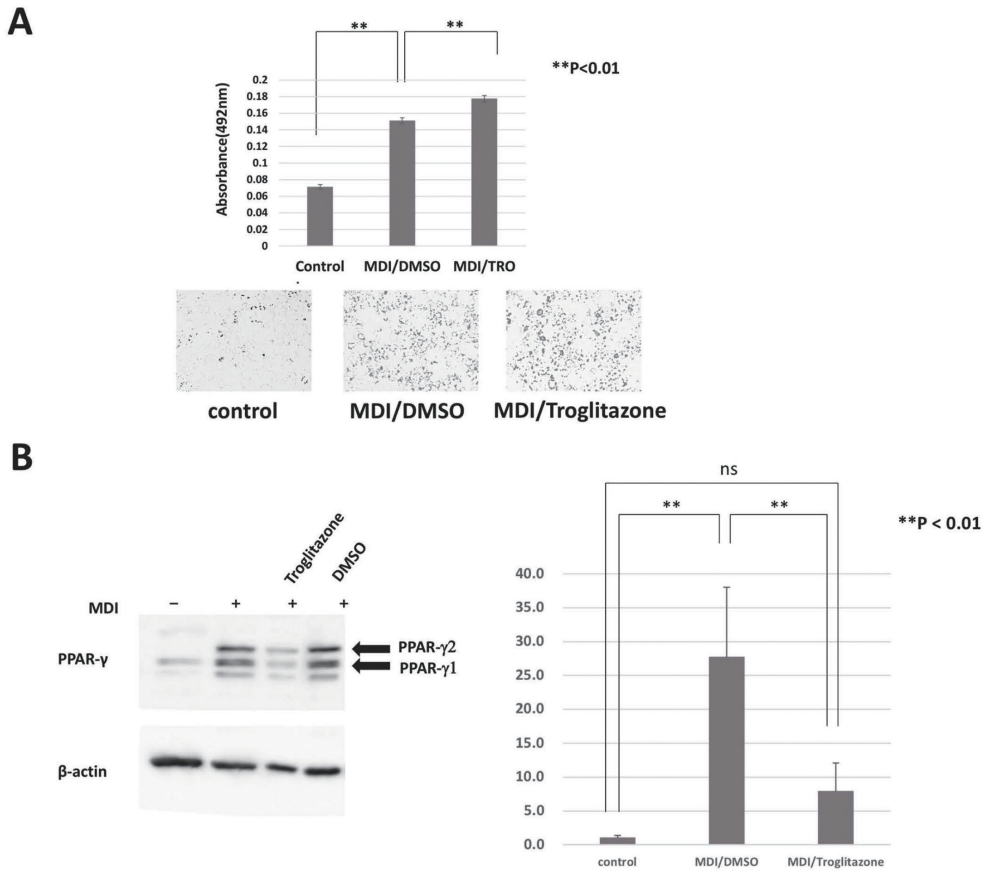

Fig. 1. Effect of Troglitazone on Lipid Accumulation and Peroxisome Proliferator-Activated Receptor γ (PPARγ) Expression in Methylxanthine, Dexamethasone, and Insulin (MDI)-Induced 3T3-L1 Preadipocytes

Post-confluent 3T3-L1 cells were differentiated in the presence of troglitazone (5 μM) or dimethyl sulfoxide (DMSO) up to day 9. (A) The cells were fixed and stained with oil red O and the eluted fractions were analyzed at 492 nm (upper panel). Oil red-stained 3T3-L1 adipocytes were observed under a microscope (lower panel). (B) The cell lysate was prepared on day 6 and PPARγ protein expression was analyzed by western blotting (left panel). Anti-PPARγ antibody recognized three PPARγ isoforms (PPARγ1, PPARγ2 and PPARγ3). Relative PPARγ protein levels normalized to β-actin protein levels were calculated (right panel). n = 3; *p < 0.05, **p < 0.01 (Tukey-Kramer multiple comparisons test).

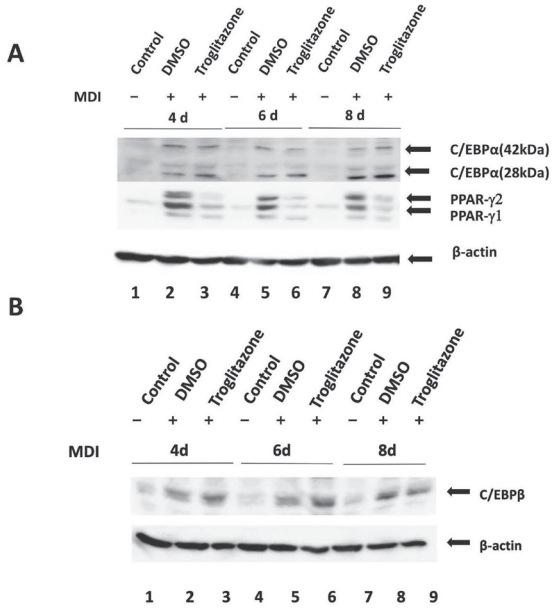

Fig. 2. Time Course Effect of Troglitazone on the Expression of Peroxisome Proliferator-Activated Receptor γ (PPARγ), CCAAT/Enhancer Binding Protein α (C/EBPα), and C/EBPβ During 3T3-L1 Preadipocyte Differentiation

Post-confluent 3T3-L1 cells were differentiated in the absence or presence of troglitazone (5 μM) and the cell lysate was isolated on day 4 (lanes 1–3), 6 (lanes 4–6), and 8 (lanes 7–9). C/EBPα and PPARγ protein expression (A) and C/EBPβ protein expression (B) were analyzed by western blotting.

protein expression was accompanied by changes at the gene level, real-time PCR analysis was performed at days 4, 6, and 8 after troglitazone treatment. As shown in Fig. 3, troglitazone treatment downregulated PPARγ mRNA expression, but not C/EBPα mRNA expression, at days 4, 6, and 8 after the induction of differentiation in 3T3-L1 cells. These results indicated that troglitazone treatment reduced PPARγ gene expression, but not C/EBPα expression, in MDI-stimulated 3T3-L1 adipocytes.

Effect of Rosiglitazone on PPARγ Expression During 3T3-L1 Preadipocyte Differentiation To examine the effects of other TZDs on PPARγ expression during 3T3-L1 preadipocyte differentiation, 3T3-L1 cells were treated with 10 μM rosiglitazone during MDI-induced differentiation. Cell lysates were prepared at days 2, 4, 6, and 8 after MDI treatment, and the cells were stained with oil red O at day 9. As shown in Fig. 4A, rosiglitazone significantly increased the accumulation of triglycerides. Rosiglitazone treatment downregulated PPARγ expression at days 2, 4, 6, and 8 after stimulation, but not C/EBPα protein levels, similar to the effect of troglitazone (Fig. 4B, and 4C).

Real-time PCR analysis indicated that rosiglitazone treatment significantly downregulated PPARγ mRNA expression, but not C/EBPα mRNA expression, at days 4, 6, and 8 after MDI-induction (Fig. 4D).

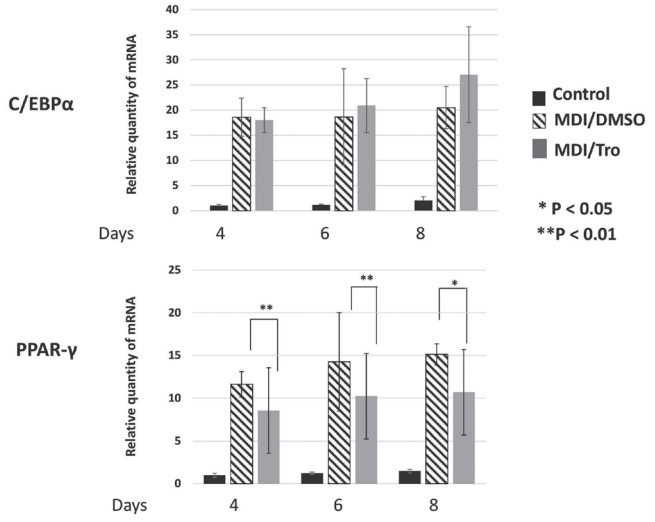

Fig. 3. Real-Time PCR Analysis in 3T3-L1 Adipocytes

Post-confluent 3T3-L1 cells were differentiated in the absence or presence of troglitazone (5 μM). Total RNA was extracted on days 4, 6, and 8 and mRNA levels of peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα) were evaluated by real-time PCR. All expression levels were normalized to that of β-actin. Values are mean ± S.D. (n = 3). *p < 0.05, **p < 0.01.

Induction of Adipokines in TZD-Treated 3T3-L1 Adipocytes TZD-treated 3T3-L1 adipocytes showed increased lipid content and reduced PPARγ expression compared with MDI-treated adipocytes. To investigate the expression of adipokines, which is regulated by PPARγ expression, real-time PCR analysis of adiponectin and aP2 expression was performed. Adiponectin expression increased on day 8 but not on day 4 after induction by both TZDs in 3T3-L1 cells (Fig. 5A). Both TZDs increased aP2 expression on days 4 and 8 after differentiation induction (Fig. 5B).

PPARγ Expression Is Downregulated at the Early Stages of Preadipocyte Differentiation by TZDs We investigated the effect of TZDs on PPARγ expression at the early stages of differentiation. Post-confluent 3T3-L1 preadipocytes were stimulated with MDI and used to prepare cell lysates 6–48 h after stimulation. PPARγ protein expression was increased for up to 48 h in MDI-treated cells, but the addition of TZDs increased PPARγ expression at a lower level compared with MDI stimulation in 3T3-L1 preadipocytes (Fig. 6).

Real-time PCR analysis indicated that TZDs did not enhance MDI-induced PPARγ expression at the early stage (Fig. 7A). The induction of C/EBPα expression was not changed or enhanced by the addition of troglitazone or rosiglitazone (Fig. 7B). Interestingly, aP2 expression increased sig

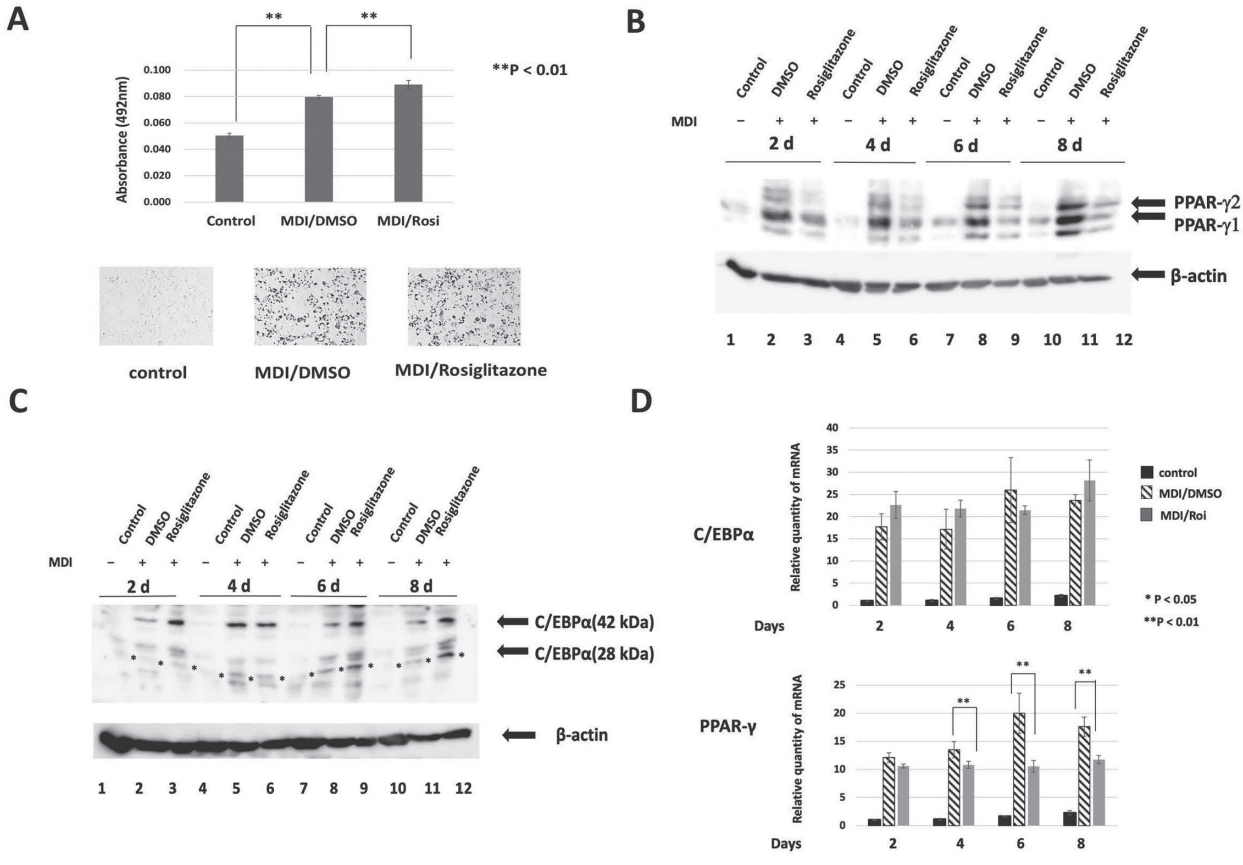

Fig. 4. Effect of Rosiglitazone on Lipid Accumulation in 3T3-L1 Adipocytes

(A) Post-confluent 3T3-L1 cells were differentiated in the presence of rosiglitazone (10 μM) or dimethyl sulfoxide (DMSO) up to day 9. The cells were fixed and stained with oil red O and the eluted fractions were analyzed at 492 nm (upper panel). Stained cells were observed under the microscope (lower panel). Post-confluent 3T3-L1 cells were differentiated in the presence of rosiglitazone or DMSO and lysed on days 2 (lanes 1–3), 4 (lanes 4–6), 6 (lanes 7–9), and 8 (lanes 10–12) and subjected to western blot analysis with peroxisome proliferator-activated receptor γ (PPARγ) (B) and CCAAT/enhancer binding protein α (C/EBPα) (C) antibodies. * indicates C/EBPα band (28 kDa). (D) Total RNA was extracted on days 2, 4, 6, and 8 after treatment and mRNA levels of PPARγ and C/EBPα were evaluated by real-time PCR. All expression levels were normalized to that of β-actin. Values are mean ± S.D. (n = 3). *p < 0.05, **p < 0.01.

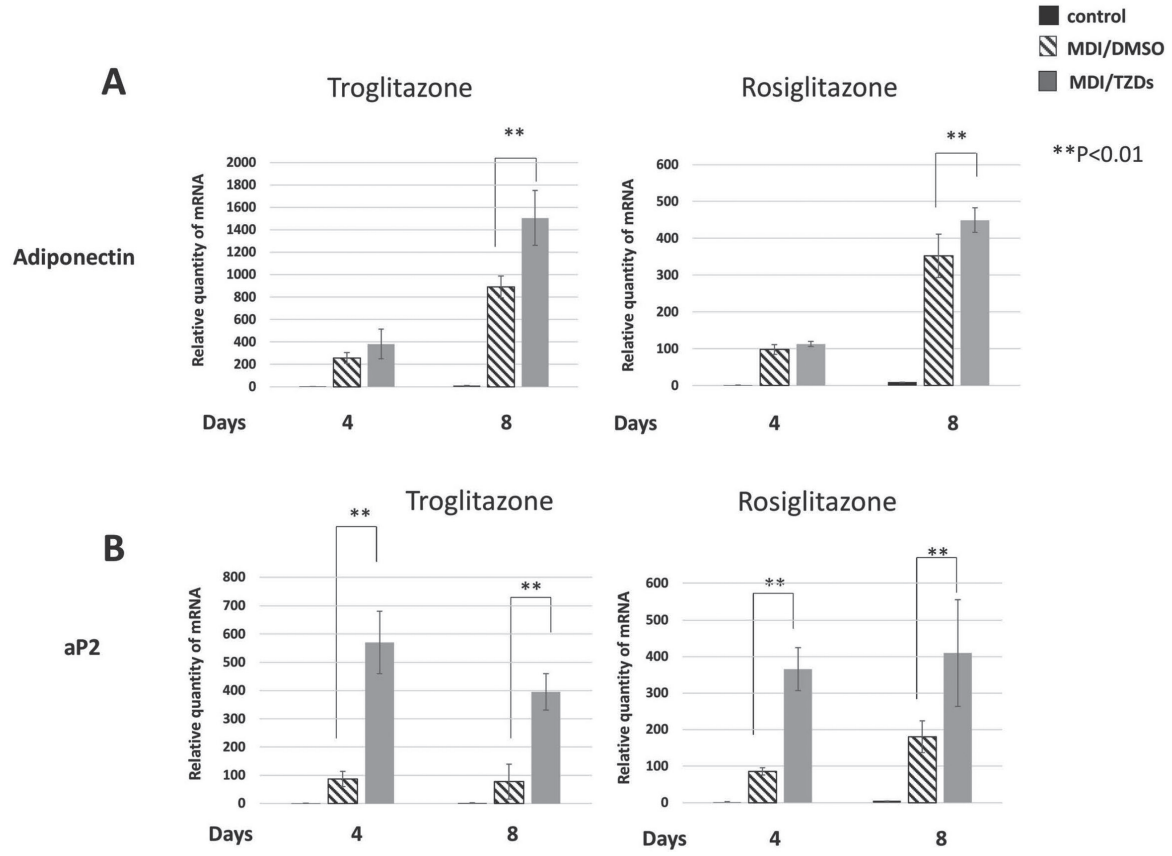

Fig. 5. Real-Time PCR Analysis in 3T3-L1 Adipocytes

Post-confluent 3T3-L1 cells were differentiated in the absence or presence of troglitazone (5 μM) or rosiglitazone (10 μM). Total RNA was extracted on days 4 and 8; thereafter, mRNA levels of adiponectin (A) and aP2 (B) were evaluated by real-time PCR. All expression levels were normalized to that of β-actin. Values are mean ± S.D. (n = 3). *p < 0.05, **p < 0.01.

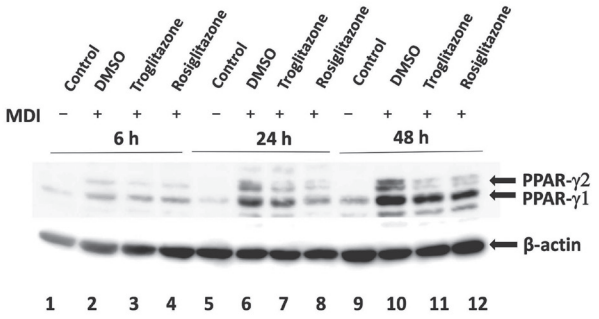

Fig. 6. Effect of Thiazolidinediones on Peroxisome Proliferator-Activated Receptor γ (PPARγ) Expression in Early Stages of 3T3-L1 Preadipocyte Differentiation

Post-confluent 3T3-L1 cells were differentiated in the presence of troglitazone, rosiglitazone, or dimethyl sulfoxide (DMSO). Cells were lysed at 6 h (lanes 1-4), 24 h (lanes 5-8) and 48 h (lanes 9-12) and subjected to western blot analysis with anti-PPARγ and anti-β-actin antibodies.

nificantly, starting at 24 h after treatment with either of the TZDs, and continued to increase for up to 48 h, compared with that after MDI control treatment (Fig. 7C).

DISCUSSION

PPARγ is a nuclear hormone receptor that forms obligate heterodimers with the retinoid X receptor and binds to PPRE motifs in the promoter regions of target genes.[21–23] PPARγ is an important factor in the development and homeostasis of adipogenesis.[2,24]

Anti-diabetic TZDs, including troglitazone and rosiglitazone, have been developed as specific ligands and activators for PPARγ.[9,20,25,26] In this report, we have shown that during adipocyte differentiation, TZD treatment with MDI resulted in the downregulation of PPARγ protein expression in 3T3-L1 adipocytes. Continuous exposure to troglitazone with MDI induced lipid accumulation but caused a decrease in PPARγ gene expression at late stages of differentiation. However, adipogenic-specific genes, such as adiponectin and aP2, were induced more in 3T3-L1 cells treated with both TZD and MDI than in cells treated with MDI alone. Based on several observations, we suggest that receptor downregulation occurs via a ligand-mediated negative feedback mechanism that is dependent upon the differentiation state of 3T3-L1 cells.

Previous reports have indicated that TZDs downregulate PPARγ in differentiated 3T3-L1 adipocytes.[27,28] Camp *et al*. have shown that PPARγ undergoes ligand-dependent downregulation in 3T3-L1 adipocytes.[27] They have suggested a threshold point for adipogenesis during differentiation; the continued presence of ligands downregulates PPARγ levels, similar to that seen with other nuclear receptors.[27,28] Perrey *et al*. have indicated that in fully differentiated 3T3-L1 adipocytes, TZDs markedly decrease PPARγ mRNA levels without reducing the expression of genes that are positively regulated

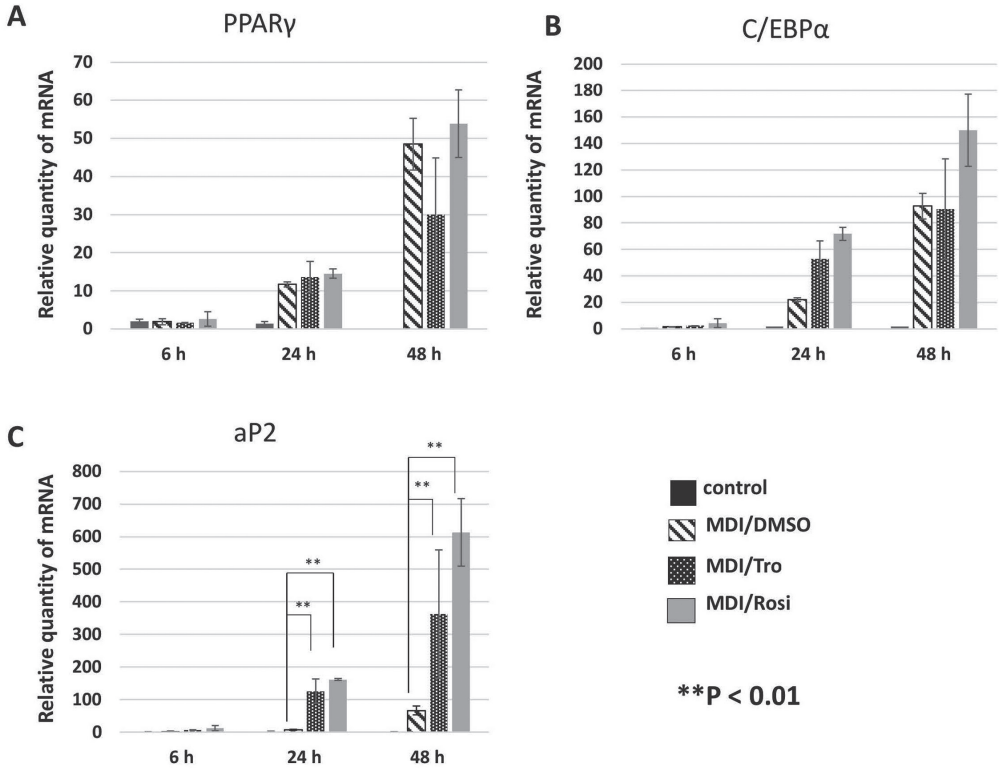

Fig. 7. Real-Time PCR Analysis in the Early Stages of 3T3-L1 Adipocyte Differentiation

Post-confluent 3T3-L1 cells were differentiated in the absence or presence of troglitazone (5 μM) or rosiglitazone (10 μM). Total RNA was extracted 6, 24, and 48 h after treatment and mRNA levels of peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer binding protein α (C/EBPα) (B), and aP2 (C) were evaluated by real-time PCR. All expression levels were normalized to that of β-actin. Values are mean ± S.D. (n = 3). *p < 0.05, **p < 0.01.

		MDI	MDI/TZDs
24-48 h	C/EBPα	+	+
	PPARγ	+	+
	aP2	±	++
day 2-8	C/EBPα	++	++
	PPARγ	++	±
	aP2	+	+++
	adipogenesis	++	+++

Negative feedback

Fig. 8. Relationship Between the Expression and Action of Various Adipogenic Factors During Differentiation of 3T3-L1 Cells at the Early and Late Stage

±: low induction, +: medium induction, ++: high induction, +++: very high induction

by PPARγ and propose that PPARγ mRNA level is not a common denominator of adipocyte function.[28] These reports demonstrate that TZDs downregulate PPARγ genes and proteins in 3T3-L1 adipocytes without affecting any cellular function. In contrast, our study indicates that aP2 expression is hyper-induced at early stages of MDI and TZD-induced 3T3-L1 adipocyte differentiation. MDI treatment induced aP2 expression at day 2 after treatment; MDI and TZD treatment caused a considerable increase in aP2 expression within 24 h compared with that caused by MDI treatment alone. The aP2 expression level is reported to be regulated by the C/EBP family and PPARγ in 3T3-L1 cells.[5] However, aP2 expression increased before enhancing PPARγ expression and continued to increase up to day 8 in the presence of TZDs (Fig. 7). Thus, significant induction of aP2 expression by TZDs negatively regulates PPARγ expression at the early stages of differentiation, as shown in Fig. 8. Garin-Shkolnik *et al* demonstrated that FABP4 (aP2) attenuates both PPARγ expression and adipogenesis in adipocytes.[29] In our study, however, aP2 induction by TZD treatment attenuated PPARγ expression but induced lipid accumulation in 3T3-L1 adipocytes (Fig. 8). We speculate that aP2 activation but not PPARγ enhances lipid accumulation during TZD/MDI-induced 3T3-L1 adipocyte differentiation. Thus, TZDs seem to be a PPARγ regulator for adipogenic homeostasis, which maintains a normal adipogenesis state by regulating the normal PPARγ expression level. Further experimentation regarding the induction mechanism of aP2 expression by TZDs is necessary. We are convinced that these novel findings contribute to the development and evaluation of anti-obesity or diabetes reagents with few side-effects.

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Conflict of interest The authors declare no conflict of interest.

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