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PPARγ activators down-regulate the expression of PPARγ in 3T3-L1 adipocytes

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Abstract Transcriptional activation of PPARγ by the anti-diabetic compound troglitazone enhances the rate of 3T3-L1 adipocyte differentiation. In this study, we examined the effects of troglitazone, a specific PPARγ ligand, on the expression of PPARγ during and after 3T3-L1 adipocyte differentiation. Troglitazone treatment caused a significant decrease in PPARγ proteins and DNA binding activity. This reduction was associated with a similar decrease in transcription of PPARγ mRNA. These data suggest that in 3T3-L1 cells, the expression of PPARγ is auto-regulated.

Key words: PPAR; Desensitization; Troglitazone; Anti-diabetic

1. Introduction

Treatment of confluent 3T3-L1 preadipocytes with fetal bovine serum (FBS), insulin, dexamethasone (DEX) and methylisobutylxanthine (IBMX) induces the differentiation of 3T3-L1 cells into adipocytes within 7 to 8 days [1]. Acquisition of the adipocyte phenotype requires the coordinate expression of specific transcription factors, and the subsequent induction of adipocyte-enriched genes including aP2 (intracellular lipid binding protein), glucose transporter 4 (Glut4) and stearoyl-CoA desaturase 1 (SCD1) [2,3]. Initial work showed that expression of the CCAAT enhancer binding protein alpha (C/EBPα) was necessary and sufficient for expression of the adipocyte phenotype [4]. However, the late expression of C/EBPα during differentiation led researchers to discover an adipocyte-enriched new transcription factor, PPARγ.

In mouse, several PPAR subtypes, PPARα, PPARγ and PPARδ (NUC1/FAAR), have been identified [5–7]. PPARγ is expressed predominantly in mouse white and brown fat, with lower levels in liver, whereas PPARα is present in heart, kidney and liver [7,8]. PPARδ expression is ubiquitous [6,9]. Heterodimerization with a second steroid receptor family member, the retinoic X receptor (RXR), is required for DNA binding of PPAR to its direct repeat DNA consensus site, the PPRE. Transcriptional activity is established by the binding of the isoform specific ligands to the C-terminal ligand binding domain of the PPAR [10,11].

Several pieces of evidence establish PPARs as important regulators of fat cell differentiation. First, in several preadipocyte cell lines, PPAR activators such as 5,8,11,14-eicosatetraynoic acid (ETYA) or thiazolidinediones enhance adipocyte conversion [8,12,13,21]. Second, the induction of PPARγ and PPARδ occurs very rapidly during adipocyte differentiation. Third, the ectopic expression of either PPARγ or PPARα can induce fat cell differentiation in the presence of PPAR activators in NIH 3T3 cells [14,15].

Therefore, the ability of thiazolidinediones to decrease plasma glucose, insulin and triglyceride levels in non-insulin dependent diabetic patients [16] may result from PPARγ dependent regulation of gene transcription. Because the biologic effect of PPAR ligands must depend on the cellular concentration of its functional receptors, we sought to examine the regulation of PPARγ by troglitazone in the 3T3-L1 adipocyte system. We show that PPARγ undergoes ligand dependent down-regulation in 3T3-L1 adipocytes.

2. Materials and methods

2.1. Materials

DEX, IBMX and insulin were purchased from Sigma. 2-Bromopalmitate was obtained from Aldrich. Troglitazone was synthesized at Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Co. Fetal bovine serum (FBS) was purchased from Summit Biotechnology. Dulbecco's modified Eagle's medium (DMEM) was from Gibco Bethesda Research Laboratories (BRL).

2.2. Cell culture

3T3-L1 preadipocytes were cultured and induced to differentiate as reported previously [1]. Briefly, 2 days post-confluent preadipocytes were treated with 0.25 μM DEX, 0.5 mM IBMX and 1 μg/ml insulin for 2 days. Cells were then switched to 10% FBS/DMEM media containing only insulin (1 μg/ml) for 2 days, and finally to 10% FBS/DMEM. For PPAR activator treatment, a final concentration of 5 μM troglitazone in DMSO, or 30 μM 2-bromopalmitate in ethanol, was added to the media. For the differentiation experiments, the compounds were added to the media at the initiation of differentiation and replenished with every media change.

2.3. Preparation of nuclear extracts

Nuclei were isolated from 3T3-L1 cells as published previously [17]. Briefly, cells were rinsed twice with PBS and incubated with 50 mM EDTA for 10 min to allow cells to detach from the plates. Cells were pelleted at 3000 rpm for 10 min, incubated in hypotonic buffer for 5 min, and homogenized using a Dounce homogenizer fitted with a tight pestle. Nuclear extracts were prepared as published previously [18]. Protein concentrations were determined using the BCA protein Reagent (Pierce Chemical Co.).

2.4. Antibody preparation and immunodetection procedures

A full-length PPARγ protein was expressed in *E. coli* as a fusion protein linked to a maltose binding protein using the pMAL-C2 cloning vector (New England Biolabs). The rabbit polyclonal antibody was prepared using this fusion protein. Standard Western blotting and immunoprecipitation protocols were used to identify or immunodeplete PPARγ from nuclear extracts [19]. The Western blot was quantitated with the BioImage system (Imaging Systems, Millipore Corp.).

2.5. Gel shift analysis

A double stranded oligonucleotide corresponding to the ARE7 site (5′ AATTCAAGGCAGAAAGTGAACTCTGATCCAGTAAGAAG

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3′) of the aP2 enhancer region or a double stranded NFκB oligonucleotide (5′ GATCCGAGGGGACTTTCCGCTGGGGACTTTCCAGG 3′) (Oncogene Science) was end-labeled using [γ-^{32}P]ATP. The labeled probe was then gel purified to remove single stranded products. Approximately 20 fmoles of the labeled probe were incubated with 5 μg of nuclear extracts in 1×mobility shift assay buffer (15 mM HEPES pH 7.9, 80 mM KCl, 10% glycerol, 1 μg poly dI/dC, 0.2 mM EDTA and 0.4 mM DTT) at room temperature for 20 min. Protein-DNA complexes were analyzed in 5% 1×TBE polyacrylamide gels.

2.6. Northern blot analysis

Total RNA was isolated from 3T3-L1 cells treated with the appropriate ligands in the presence or absence of actinomycin D (5 μg/ml) using the Ultraspec RNA isolation system (Biotecx). Approximately 10–15 μg of total RNA was resolved on 1.2% agarose-formaldehyde gels and transferred to Hybond-nylon membranes (Amersham Corp.) in 10×SSC. Northern blots were prehybridized in a solution consisting of 10% PEG, 1.5×SSPE, 7% SDS, and 100 μg/ml of sonicated salmon sperm DNA at 65°C for 1 h. The PPARδ (a gift from P.A. Grimaldi, Centre de Biochimie, France), mouse C/EBPα (a gift from P.F. Johnson, Frederick Cancer Research and Development Center, Frederick, MD, USA), mouse aP2 (a gift from C. Burrant, University of Chicago) and rat β-actin (pCRACTF) (a gift from R. Hart, Rutgers University, New Brunswick, NJ, USA) cDNAs were labeled with [α-^{32}P]dCTP (3000 Ci/mmol, Amersham) using random primed labeling (BRL). Denatured, labeled probe was then added and incubation continued at 65°C overnight. High stringency wash conditions were employed by soaking filters twice in 2×SSC, 0.1% SDS (w/v) at room temperature for 30 min and twice in 0.1×SSC/0.1% SDS (w/v) at 65°C for 60 min. Filters were removed and subjected to autoradiography at −70°C, or exposed to a PhosphorImager screen (Molecular Dynamics) for quantitation.

2.7. RNAse protection assay (RPA)

A set of primers (nucleotide positions 481–502 and 785–806) was designed from the published PPARγ cDNA sequences [20] and used to amplify a 325 base pair (bp) PPARγ specific fragment by polymerase chain reaction (PCR). The PCR fragment was inserted into the pCRII vector (Invitrogen) and the nucleotide sequence was verified using Sequenase chemistry according to the manufacturer's recommendation (UBI). An antisense riboprobe was synthesized using SP6 RNA polymerase (BRL). RPA was carried out following the instructions for the RPA II protocol (Ambion). For normalization, a 340 bp mouse β-actin riboprobe was synthesized from pTRI-β-mouse DNA template (Ambion) using SP6 RNA polymerase and hybridized in the same reactions with the PPARγ probe. The size of the protected PPARγ and actin riboprobes was 300 and 250 base pairs, respectively.

3. Results

3.1. Effects of troglitazone on PPARγ protein levels in differentiating 3T3-L1 cells

Because ligands often auto-regulate the expression of their own receptors, it was of interest to assess the effects of troglitazone and other PPAR activators on PPARγ protein levels during 3T3-L1 cell differentiation. Fig. 1 shows the level of PPARγ1 and PPARγ2 protein present in nuclear extracts at various time points after the initiation of differentiation with or without troglitazone. In preadipocytes, both isoforms of PPARγ proteins were detected at low levels. The expression of both proteins increased dramatically within 2 days of differentiation in control cells. This correlates well with the published reports on the early induction of PPARγ mRNA during 3T3-L1 cell differentiation [15]. However, within 2 days of differentiation, the presence of troglitazone caused a 72% decrease in PPARγ1 and a 50% decrease in PPARγ2 protein levels relative to the control. The PPARγ protein levels continue to decline throughout the differentiation in both treated and the control cells. However, PPARγ protein levels remained lower in treated cells at all times.

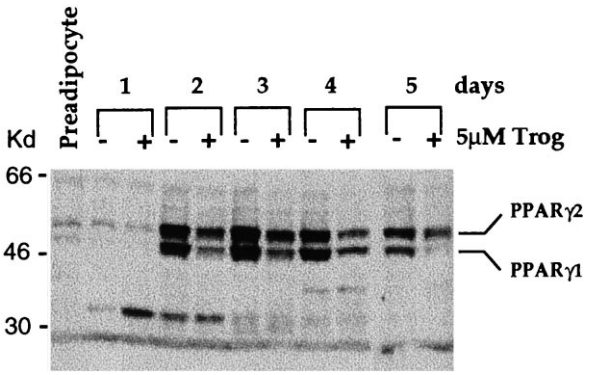

Fig. 1. The effects of troglitazone on the expression of PPARγl and g2 proteins. Nuclear extracts were prepared from 3T3-L1 cells during differentiation treated with either DMSO or 5 μM troglitazone in the presence of standard adipogenic inducers for the indicated times. Equal amounts of nuclear extract (30 μg of protein) as determined by BCA protein reagent (Pierce Chemical Co.) were subjected to gel electrophoresis, and immunoblotted using the anti-PPARγ antibody. Shown is a representative of two separate experiments.

3.2. Effects of troglitazone on the DNA binding activity of PPARγ

Gel shift analysis was performed to determine if the troglitazone-induced changes in the PPARγ protein levels affected changes in PPARγ DNA binding activity. Nuclear extracts isolated from differentiating cells with or without troglitazone were subjected to gel shift analysis. As the ARE7 PPRE has previously been shown to specifically bind to PPARγ and RXR heterodimer, a double stranded oligonucleotide containing the ARE7 PPRE was used as a probe [14]. In control cell extracts, the PPARγ DNA binding activity reached maximal levels after 3–4 days of differentiation and decreased slightly thereafter (Fig. 2A). In comparison, nuclear extracts prepared from cells treated with troglitazone showed a significant decrease in PPARγ DNA binding activity (30–45%) (days 2–5). Addition of specific or non-specific competitor oligonucleotides indicated that the binding to ARE7 was specific. Moreover, to verify that the protein complexes formed in ARE7 PPRE contain PPARγ, immunodepletion experiments were performed using the anti-PPARγ antibody (Fig. 2C). 3T3-L1 adipocyte extracts were immunoprecipitated with no antibody, pre-immune sera or anti-PPARγ antibody prior to gel shift analysis. Immunoprecipitation of the nuclear extract with the anti-PPARγ antibody specifically prevented retardation of the PPRE in the mobility shift assay, indicating the presence of PPARγ in the shifted complex (Fig. 2A and C).

To show that the troglitazone dependent decrease was specific for PPARs, an identical amount of nuclear extract was used to shift a double stranded ^{32}P-labeled NFκB oligonucleotide. No significant change in the binding activity of NFκB was found in troglitazone treated cells compared to that in untreated control cells (Fig. 2B). These results demonstrate that troglitazone specifically decreases the amount of PPARγ DNA binding activity without generally affecting the levels of other transcription factors.

3.3. Troglitazone decreases PPARγ steady state mRNA levels in differentiating 3T3-L1 cells

To further examine if this receptor down-regulation also occurs at the level of transcription, the effect of troglitazone compared to that of other PPARγ activators such as the non-metabolizable fatty acid 2-bromopalmitate on the expression

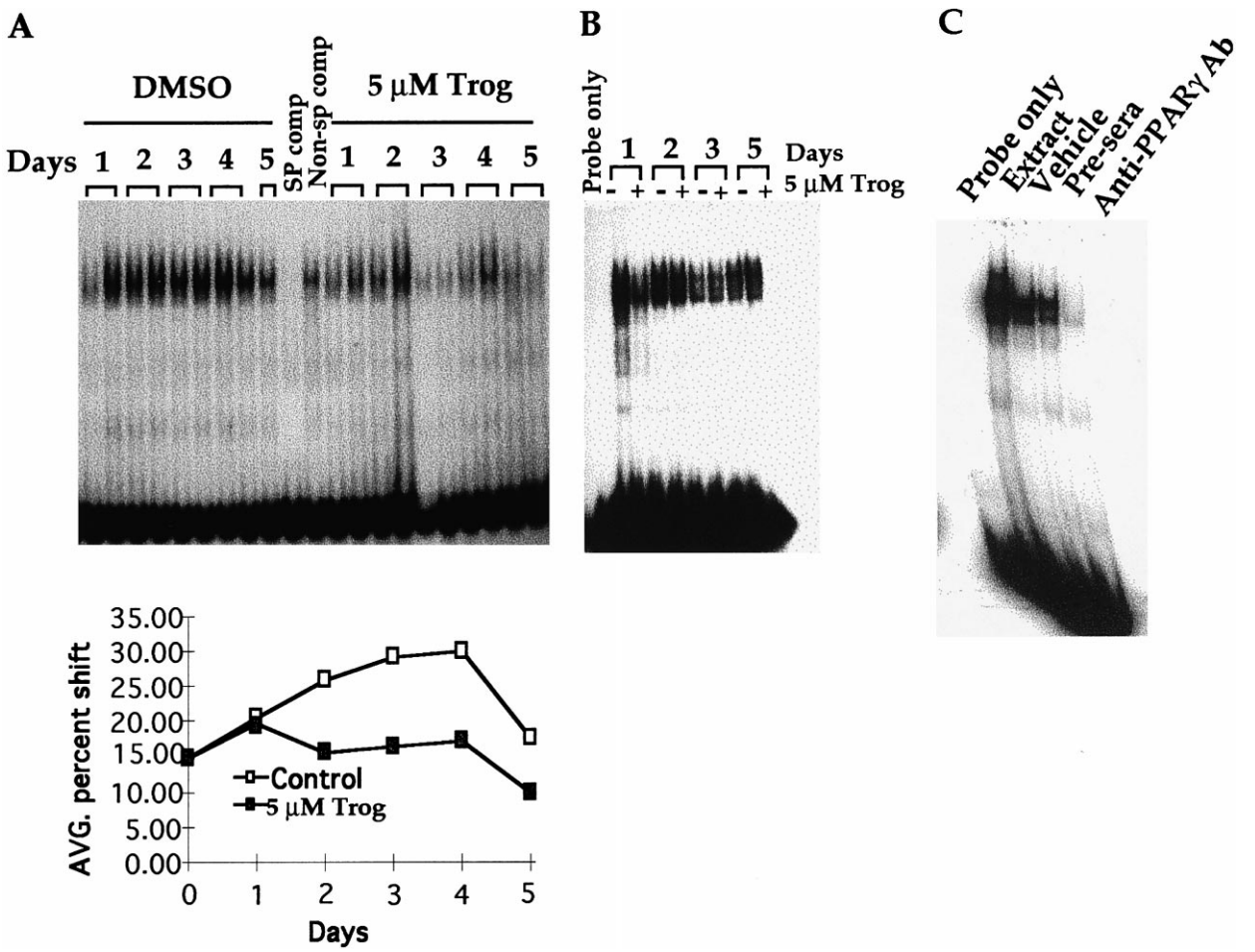

Fig. 2. DNA binding activity of PPARγ in troglitazone treated differentiating 3T3-L1 cells. Gel shift assay was performed using approximately 20 fmoles of the ^{32}P-labeled oligonucleotide corresponding to the PPARγ binding site (ARE7) (A) or a double stranded ^{32}P-labeled NFκB oligonucleotide (B), and 5 μg of nuclear extracts prepared as described in the legend of Fig. 1. To demonstrate specificity of PPARγ binding to the probe, a 100-fold excess of either cold specific (ARE7) or non-specific oligonucleotides were added during the incubation of nuclear extracts with the labeled ARE7 probe. Each shift was performed in duplicate and the percent of total probe shifted, i.e. the amount of PPARγ binding, in each sample was quantitated using the PhosphorImager. The average value for each time point is graphed beneath. The data shown are representatives of six separate experiments. C: Nuclear extracts were immunoprecipitated with either no antibody, pre-sera or anti-PPARγ antibody (see Section 2) and used in mobility shift assays.

of PPARγ was determined. 3T3-L1 cells were chronically treated with either 2-bromopalmitate or troglitazone for 5 days. Treatment with troglitazone or 2-bromopalmitate caused approximately 30 to 60% reduction of PPARγ mRNA levels, respectively (Fig. 3A). This effect was also seen with BRL-49653 suggesting that the decrease in PPARγ gene expression occurs with all PPARγ activators, not just troglitazone (data not shown). Because PPARδ was shown to be stimulated by fatty acids in transient reporter assays [9,23], PPARδ mRNA levels in cells treated with troglitazone or 2-bromopalmitate were also examined. Although PPARδ mRNA levels increased approximately 8-fold as differentiation progressed, the chronic treatment of cells with PPAR activators did not cause notable changes in PPARδ mRNA levels (Fig. 3B).

3.4. *Troglitazone down-regulates PPARγ mRNA levels in fully differentiated adipocytes*

Because the troglitazone-mediated PPARγ down-regulation was much more pronounced in the later stages of differentiation, the effects of PPAR activators on PPARγ expression in fully differentiated adipocytes were examined. Eight days post-differentiated cultures were stimulated with troglitazone for 1–12 h, and analyzed for the expression of PPARγ. A 60% decrease in PPARγ transcripts was observed within 2 h of troglitazone treatment and that level was maintained for 12 h (Fig. 4A). Again, nuclear extracts were prepared from adipocytes treated with either troglitazone or 2-bromopalmitate for 3 or 6 h to see if the reduction of PPARγ mRNA levels correlated with PPARγ DNA binding activity. A significant reduction of PPARγ DNA binding activity was observed in cell extracts treated with either troglitazone or 2-bromopalmitate for 3 or 6 h (Fig. 4B). Therefore, this decrease in PPARγ expression upon PPAR ligand treatment occurs at both mRNA and DNA binding activity levels. To further investigate troglitazone-mediated receptor down-regulation, we examined the effect of RNA synthesis inhibition by stimulating cells with troglitazone in the presence of actinomycin D. As shown above, troglitazone treatment caused a 50% reduction of PPARγ mRNA levels. However, the rate of PPARγ mRNA

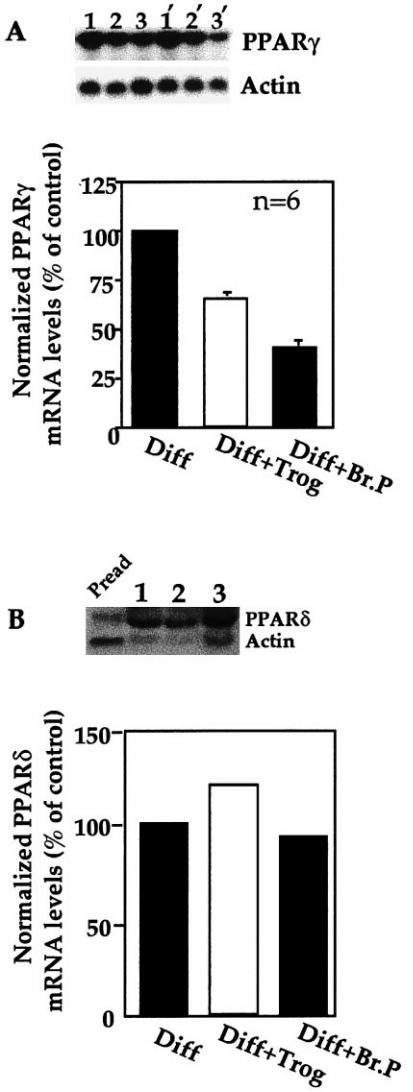

Fig. 3. Effect of PPAR activator on PPARγ and PPARδ mRNA levels during differentiation of 3T3-L1 cells. Confluent 3T3-L1 preadipocytes were treated with either DMSO (lane 1), 5 μM troglitazone (lane 2) or 30 μM 2-bromopalmitate (lane 3) in the presence of adipogenic hormones for 5 days. Total RNA was isolated and analyzed for PPARγ mRNA and β-actin levels using RPA (A), or PPARδ and β-actin mRNA levels using Northern blot analysis (B). The histogram in A represents the average of six separate experiments, whereas that in B represents two experiments. Duplicate samples were analyzed and designated 1′, 2′ and 3′. Histograms compared the actin normalized RNA levels for PPARγ or PPARδ as quantitated by a Molecular Dynamics PhophorImager.

degradation was unaffected by the drug (Fig. 5). Thus, the effects of PPARγ ligand treatment on PPARγ mRNA levels must occur at the level of transcription.

4. Discussion

Through the use of ligand binding assays, Kliewer and coworkers demonstrated that the anti-diabetic thiazolidinediones including troglitazone, are specific ligands for PPARγ [11]. In this report, we show that during the late stages of adipocyte differentiation and in adipocytes, ligand treatment results in a down-regulation of PPARγ mRNA, protein, and DNA binding activity. Based on several observations, we suggest that this receptor down-regulation occurs via a ligand-mediated negative feedback mechanism which is dependent upon the differentiation state of the 3T3-L1 cell.

We have noticed that the effects of ligand on PPARγ expression are dependent on the overall competency of preadipocytes to become adipocytes (i.e. cell passage number and plating density). In a well differentiating line, the receptor down-regulation occurs as early as 24 to 48 h after the addition of troglitazone in the presence of a mixture of adipogenic

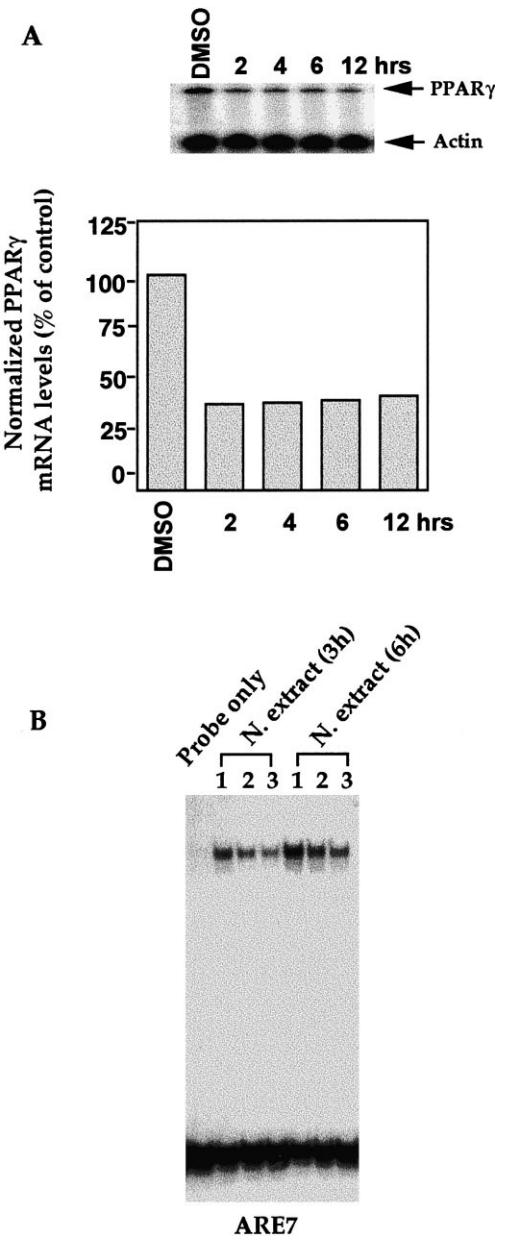

Fig. 4. Effects of troglitazone and 2-bromopalmitate on PPARγ expression in fully differentiated 3T3-L1 adipocytes. A: Total RNA was isolated from adipocytes treated with 5 μM troglitazone or 30 μM 2-bromopalmitate for indicated times and analyzed for PPARγ expression by RPA. PPARγ levels were normalized to β-actin and quantitated values are presented in histogram. B: Mobililty shift on ARE7 PPRE using duplicate nuclear extracts isolated from (1) DMSO, (2) 5 μM troglitazone or (3) 30 μM 2-bromopalmitate treated cells for 3 or 6 h. The data shown represent two separate experiments.

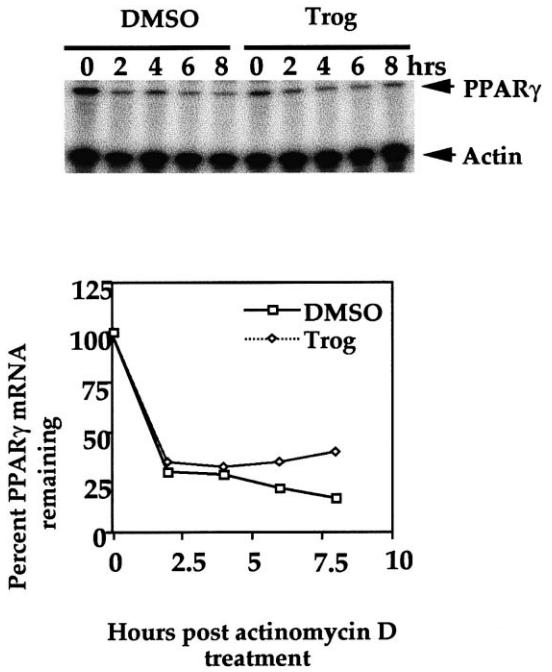

Fig. 5. Troglitazone does not alter the stability of PPARγ mRNA. 3T3-L1 adipocytes were pre-treated with either DMSO or 5 μM troglitazone for 12 hours and followed by actinomycin D (5 μg/ml). RNase protection assay of PPARγ message was performed on RNA isolated at different time points after the actinomycin D treatment.

hormones. The continuous presence of troglitazone causes a decrease in PPARγ gene expression and DNA binding activity during the later stages of differentiation. We speculate that during the course of differentiation there is a threshold point for which a specific level of PPARγ activity is required for adipogenesis to proceed and PPAR activators serve to enhance the rate at which this threshold is achieved. Upon attainment of the required level of PPAR activity, the continued presence of ligands causes the receptor down-regulation similar to that seen with other nuclear receptors.

Auto-regulation of receptor concentration by the ligands is well documented for numerous receptor subtypes including the nuclear receptors [22–25]. In most studied systems, both dexamethasone and L-triiodothyronine have been shown to down-regulate the levels of the glucocorticoid and thyroid receptors, respectively [23,24]. In rat hepatoma cells, a ligand-mediated transient decrease in glucocorticoid receptor was observed [23], similar to that seen with PPARγ in the 3T3-L1 adipocytes. The mechanisms for the ligand-induced down-regulation of these other nuclear receptors include both the reduced transcription and increased turnover of the receptor mRNA and protein levels [23–25]. Based on actinomycin D experiments, we have found no significant changes in the stability of PPARγ mRNA by troglitazone treatment. Therefore, we suggest that a decrease in mRNA synthesis accounts for the reduction in PPARγ message. As protein levels also decrease dramatically after 2 h, protein degradation must also affect PPARγ expression.

How the auto-regulation of PPARγ expression manifests itself in vivo is unclear. The effects of BRL-49653 treatment on PPARγ expression in adipose tissue have been described and are dependent upon diet and the nutritional state of the animals [26]. Growth factor dependent activation of MAP kinase and phosphorylation of Ser-84 (Ser-112 in PPARγ2) also regulate PPARγ1 activity [27]. Thus, many factors contribute to the expression and regulation of PPARγ activity. The additional knowledge that PPARγ expression is subject to ligand dependent down-regulation may help us to understand the anti-diabetic effects of thiazolidinediones.

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