

Peroxisome Proliferator-Activated Receptor- γ Activation by Thiazolidinediones Induces Adipogenesis in Bone Marrow Stromal Cells

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SUMMARY

The thiazolidinediones improve insulin sensitivity in animal models and have promise as potent oral antidiabetic agents. Their clinical use has been limited because of the resulting anemia and cardiac hypertrophy. Some compounds of this class have been reported to induce bone marrow fat accumulation in animals, and this effect could account for the observed anemia. We examined the biological mechanism contributing to this phenomenon. The thiazolidinediones BRL49653 and pioglitazone induced adipocyte differentiation in the BMS2 bone marrow stromal cell line in a dose- and time-dependent manner. These actions were further enhanced by the presence of glucocorticoids and other adipogenic agonists. The thiazolidinediones

increased the mRNA levels of adipocyte-specific genes, including that of their receptor, the peroxisome proliferator-activated receptor- γ (PPAR γ). In contrast, mRNA levels of genes encoding other PPAR family members (PPAR α , PPAR δ , or NUC-1) were unchanged or decreased. Thiazolidinedione treatment of primary bone marrow stromal cells elicited a comparable dose-dependent response. Using a polyclonal antibody, PPAR γ was detected in protein lysates from adipose-rich bone marrow. Thus, thiazolidinedione directly regulates bone marrow stromal cell differentiation; induced PPAR γ expression may play a key regulatory role in this process.

The thiazolidinediones are potent oral antidiabetic agents. However, the clinical application of thiazolidinediones has been limited by their potential effects on blood cell production and cardiac hypertrophy. In both rodent and canine models, the administration of thiazolidinediones was accompanied by fat accumulation in the bone marrow cavity and impaired hematopoiesis, resulting in anemia (1, 2).

In vitro, thiazolidinediones have been found to induce adipocyte differentiation in preadipocyte cell lines derived from murine fetal tissue (3T3-L1) or ob/ob mice (Ob17) (3). Recently, the thiazolidinediones have been identified as ligands for PPAR γ (4). This protein was originally identified as an adipogenic transcription factor (5, 6). The PPAR γ gene is subject to alternative promoter use and splicing, giving rise

to the tissue-specific subtypes PPAR γ 1 (liver) and PPAR γ 2 (adipose) (5-9). Like other PPAR family members (e.g., PPAR α , PPAR δ), the PPAR γ isoforms are members of the steroid receptor family and are closely related to the retinoid, vitamin D₃, and thyroxine receptors (10). In its ligand-bound form, PPAR γ regulates transcription from adipocyte-specific genes and will induce adipocyte differentiation in fibroblasts (5, 6, 11, 12).

The mechanism underlying the bone marrow effects of the thiazolidinediones remains unknown. Normally, the bone marrow stroma consists of a heterogeneous population of hematopoietic supporting fibroblasts, adipocytes, and bone-forming osteoblasts (13, 14). It is hypothesized that this phenotypically diverse group of cells is derived from a multipotent stromal progenitor cell (15, 16). The murine BMS2 bone marrow stromal cell line provides a well-characterized *in vitro* model for this progenitor cell. The BMS2 cells support lymphohematopoiesis (17), display osteoblast-specific gene markers (18, 19), and undergo accelerated adipogenesis in

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ABBREVIATIONS: BRL, BRL49653 [5-(4-[(N-methyl-N(2-pyridyl)amino)ethoxy] benzyl)thiazolidine-2-4-dione]; FACS, fluorescence activated cell sorter; PIO, pioglitazone; MHI, Methylisobutylxanthine/hydrocortisone/indomethacin; PPAR, peroxisome proliferator-activated receptor; C/EBP α , CAAT/enhancer binding protein α ; LPL, lipoprotein lipase;

response to a cocktail of agonists (e.g., glucocorticoids, methylisobutylxanthine, indomethacin) (20). We determined that the thiazolidinediones PIO and BRL49653 induce BMS2 adipogenesis in a dose- and time-dependent manner. This correlates with increased levels of PPAR γ mRNA. Thiazolidinediones exert a similar effect on primary cultures of bone marrow stromal cells. Furthermore, the PPAR γ protein is detected in adipose-rich bone marrow. Together, these data indicate that bone marrow stromal cells are a direct target for thiazolidinedione actions *in vivo*.

Materials and Methods

Cell culture. Reagents were obtained from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless otherwise noted. The BMS2 cell line was originally cloned by limiting dilution from the adherent population of murine bone marrow stromal cells (17). The cells were selected based on their ability to support the proliferation of stromal-dependent B lineage lymphoid cell lines in culture (17). In these experiments, the BMS2 cells (17) were plated at a density of 4×10^4 cells/35-mm dish and cultured in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 1 mM Na pyruvate, 50 μ M 2-mercaptoethanol, 100 mg/ml streptomycin, and 100 units/ml penicillin; this is referred to as the standard medium. Confluence was achieved within 7 days. PIO and BRL were dissolved in DMSO and added at the indicated concentrations, with a constant final concentration of 0.5% DMSO. The DMSO carrier alone had no effect on cell differentiation. The adipogenic agonist cocktail MHI [500 μ M methylisobutylxanthine, 0.5 μ M hydrocortisone (Elkin Sinn, Cherry Hill, NJ), 60 μ M indomethacin] was used as a control (18). Confluent cultures were maintained in the presence of thiazolidinediones or MHI for 3 days. The medium was then removed and replaced with standard medium. Individual 35-mm plates were harvested after an additional 3 days in culture (day 6). Cell densities at this time averaged 4.7×10^5 cells/35-mm plate, independent of culture conditions (range, 4.2 – 5.2×10^5). Primary bone marrow stromal cells were harvested from the femurs and tibia of 6-week-old female Balb/c mice in standard medium. The mice were killed by CO₂ asphyxiation in accordance with an institutionally approved protocol, and the long bones of the lower extremities were removed under sterile conditions. The marrow cavity was flushed with standard medium with a 25-gauge needle, and 10^7 cells were cultured in a 25 cm² flask. After 2 hr in culture, the nonadherent (primarily hematopoietic) cells were removed, and the medium was replaced. This step enriched the adherent stromal population, which included fibroblasts, adipocytes, osteoblasts, and macrophages. At 2 weeks after the cultures were established, cells were treated with thiazolidinediones for 1 week, photographed using a Zeiss IM35 microscope (magnified 102 \times under phase contrast), and harvested for total RNA. Cell densities were not determined in these studies.

FACS. BMS2 were plated at a density of 10^4 cells/well in 24-well plates. After 7 days in culture, the cells were induced with agents for 3 days, and the medium was changed. After an additional 3 days of culture, the 24-well plates containing BMS2 cells were harvested by

treatment with 0.25% trypsin/1 mM EDTA, washed in phosphate-buffered saline, and fixed with the addition of a final concentration of 0.5% paraformaldehyde (18, 22). A stock solution of Nile red (1 mg/ml DMSO) was diluted 1:100 and added to the cells at a final concentration of 1 μ g/ml. Cells were analyzed on a FACScan (Becton-Dickinson, San Jose, CA) multiparameter flow cytometer. Gold fluorescence emission was detected between 564 and 604 nm with a band-pass filter of 585/42. Sample sizes of 7.5 – 10×10^3 cells were analyzed from each well.

Northern blot analysis. RNA was harvested from BMS2 cells cultured in 35-mm plates as described above and analyzed as described previously (21, 23). Northern blots were hybridized with the following probes: aP2 (provided by H. Green, Harvard University, Boston, MA) (24), actin and adipin (provided by W. Wilkison and B. M. Spiegelman, Dana Farber Cancer Center) (25), LPL (63117; American Type Culture Collection, Rockville, MD) (20), C/EBP α (provided by S. Enerback and K. Xanthopoulos, University Gotesborg), and PPAR γ 2 (5). The PPAR γ 2 probe was cloned by reverse transcription PCR using murine brown adipose total RNA and the following specific primers: amino-terminal primer 5'-TTTGAGCTC GCTGTTATGGGTGAAACTCTG-3' (base pairs 34–54) and carboxyl-terminal primer 5'-TTTGAGCTC CCTGCTAATACAAGTCTT-GTA-3' (base pairs 1540–1561) (5). The intensity of mRNA signals on Northern blots was quantified by using an Eagle Eye II Still Video System (Stratagene, La Jolla, CA).

Semiquantitative PCR. Reactions were performed according to previously reported methods (26). Aliquots containing 5 μ g of total RNA in a 12.5- μ l volume were heated for 5 min at 65°. The cDNA was reverse-transcribed in a 30- μ l volume of 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM dNTP, and 0.5 mM spermidine, containing RNasin (1.25 units), random hexamer and oligo(dT) primers (100 ng each), and avian myeloblastoma virus reverse transcription (2.5 units). The reaction was incubated for 60 min at 42°, 30 min at 52°, and 5 min at 95°. PCRs were conducted in 100- μ l volumes with oligonucleotide primers specific for PPAR α , PPAR δ , PPAR γ 1, PPAR γ 2, and β -actin (Table 1); a reaction cycle consisted of 45 sec at 94°, 45 sec at 62°, and 2 min at 72° using a Perkin-Elmer-Cetus DNA Thermal Cycler (Norwalk, CT). Aliquots (12 μ l) were removed at three-cycle intervals between cycles 17–35 and examined on 3% agarose gels stained with ethidium bromide.

Polyclonal antibody preparation. A multiple antigenic peptide (30) was synthesized based on the PPAR γ 2 amino acids 482–499 (5): HVIKKTETDMSLHPLLQE. Eight identical peptides were attached to a single polylysine-resin matrix core, providing a potent antigen (30). After the collection of preimmune serum, the goat was injected with 1 mg of the multiple antigenic peptide with incomplete Freund's adjuvant. Four weeks later, the animal was boosted with 1 mg of peptide alone. Immune serum was harvested weekly after the fifth week. Antibody was prepared by ammonium sulfate precipitation and affinity purified over a column prepared with the multiple antigenic peptide coupled to cyanogen bromide-activated Sepharose 4B.

Transfections. The PPAR γ 2 cDNA was subcloned into the pSG5 and the PPAR γ 1 cDNA (provided by Drs. F. Chen and B. O'Malley, Baylor College of Medicine, Houston, TX) (31) into the pEF-BOS eukaryotic expression vectors (provided by Dr. K. Oritani, Oklahoma Medical Research Foundation, Oklahoma City, OK) (32). The plasmids were transiently transfected into the human embryonic kidney

TABLE 1
Primers for semiquantitative PCR analyses

Gene	Ref.	5' Oligonucleotide	3' Oligonucleotide
PPAR α	27	CGACAAGTGTGATCGGAGCTGCAAG bp 574-598	GTTGAAGTCTTCAGGTAGGCTTC bp 800-777
PPAR δ	28	GGCCAACGGCAGTGGCTTCGTC bp 912-933	GGTCTCGGCTTAGTACATGTCCT bp 1390-1367
PPAR γ 1	7, 9	TTCTGACAGGACTGTGTGACAG bp 391-412	ATAAGGTGGAGATGCAGGTTTC bp 745-725
PPAR γ 2	5	GCTGTTATGGGTGAAACTCTG bp 34-54	ATAAGGTGGAGATGCAGGTTTC bp 384-364
β -Actin	29	CCTAAGGCCAACCCTGAAAAG bp 414-434	TCTTCATGGTCTAGGAGCCA bp 1059-1039

293T cell line through calcium phosphate precipitation. Cell lysates from the transiently transfected 293T cells provided an enriched source of the PPAR γ 2 protein. The nontransfected 293T cell lysates provided an appropriate negative control.

Western blot analysis. Cells or tissues were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM iodoacetamide, 0.1% NaN₃, 5% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 25 μ g/ml soybean trypsin inhibitor, 10 μ g/ml leupeptin). Equal protein aliquots were loaded onto each lane and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (BioRad, Richmond, CA), and blocked overnight in buffer (10 mM sodium phosphate, 150 mM NaCl, 0.5% gelatin, 0.05% Tween 20, 0.1% merthiolate) (33). Blots were sequentially incubated with goat primary antibody (21 μ g/ml) and an anti-goat horseradish peroxidase-coupled secondary antibody for 1 hr, followed by three washes (10 min) in phosphate-buffered saline/0.05% Tween 20, and visualized with the use of chemiluminescent reagents according to the manufacturer's instructions (Amersham, Arlington Heights, IL).

Results and Discussion

Dose-dependent effects of thiazolidinediones on the BMS2 bone marrow stromal cell line. The BMS2 cell line was used as an *in vitro* model to examine the response of bone marrow stromal cells to thiazolidinediones. Confluent cultures of BMS2 stromal cells were treated with varying concentrations of the thiazolidinediones BRL and PIO. Cellular accumulation of lipid vacuoles after 6 days in culture was quantified by staining with the lipophilic dye Nile red and FACS analysis (Fig. 1 and Table 2). In the absence of inducing factors, the cells did not contain lipid vacuoles after 6 days. As previously reported, a cocktail of adipogenic agonists (MHI) induced lipid droplets in >50% of the cells (18).

Both PIO and BRL induced adipocyte differentiation in a dose-dependent manner; the cell response was greater to BRL than to PIO at equal molar concentrations. At concentrations of 5 μ M BRL or 25 μ M PIO, \leq 40% of the cells contained lipid vacuoles. The actions of the thiazolidinediones were partially additive with those of the MHI cocktail (Table 3). Although 50% of the cells contained lipid vacuoles after treatment with the MHI cocktail alone, treatment with either thiazolidinedione in combination with the MHI cocktail induced adipogenesis in \leq 70% of the BMS2 cell population. Similar observations have been made in the ob/ob-derived preadipocyte cell line Ob1771, in which glucocorticoids further enhanced the actions of BRL on expression of adipocyte markers (34). This suggests that thiazolidinediones and glucocorticoids may activate distinct as well as common signaling pathways during adipogenesis. Evidence from previous studies supports this hypothesis. For example, dexamethasone up-regulated PPAR α mRNA levels in hepatic cells (35).

Thiazolidinedione induction of adipocyte gene markers in BMS2 cells. The temporal-dependent expression of adipocyte mRNAs was examined in thiazolidinedione-treated BMS2 cells. Confluent BMS2 cultures were treated with standard medium alone (control) or medium supplemented with either PIO (25 μ M) or BRL (5 μ M). Northern blot analyses were performed using total RNA harvested daily from the cells after treatment initiation (Fig. 2). The blots were hybridized with a β -actin probe to control for the relative RNA loading between lanes. The signal intensity of each mRNA on day 6 relative to β actin was quantified (Table 4). Two relatively late adipocyte differentiation marker genes, the fatty acid binding protein aP2 and adipsin, were not detected in control cells. The thiazolidinediones increased

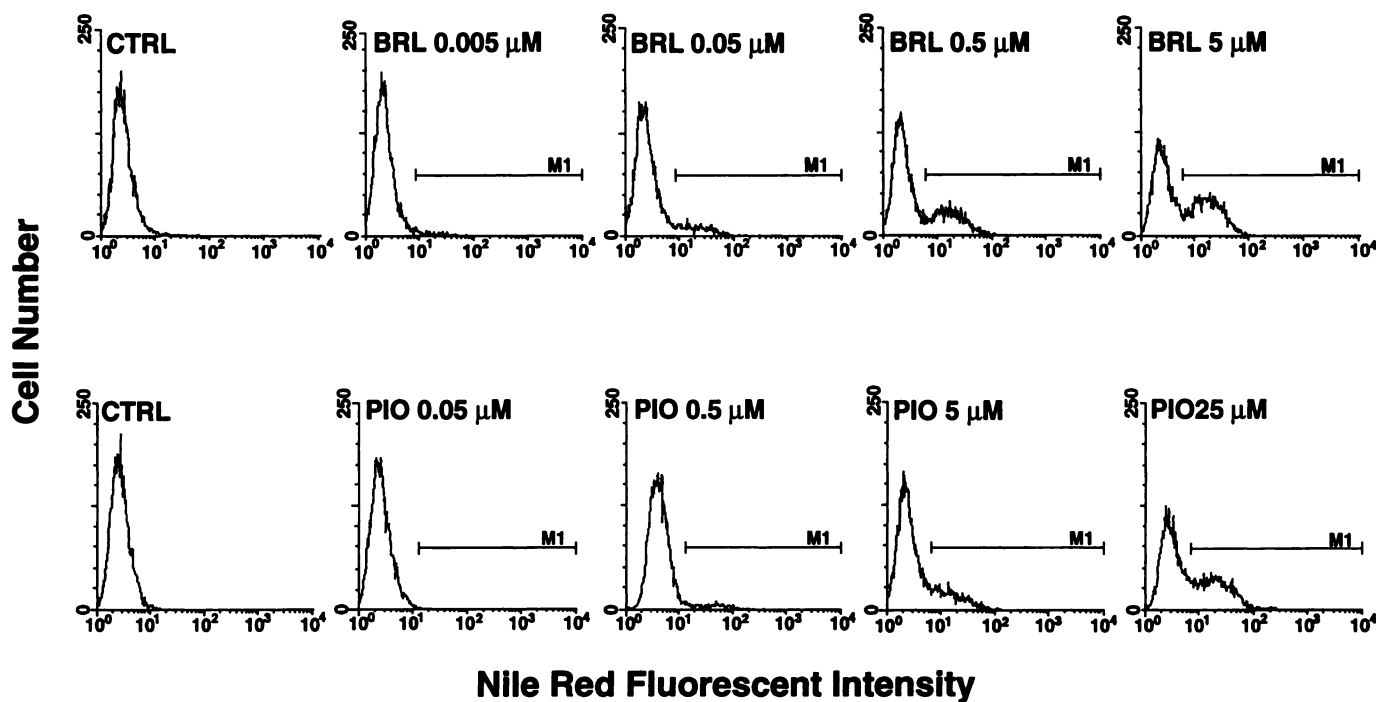


Fig. 1. FACS analysis of BMS2 stromal cell adipogenesis in response to thiazolidinedione compounds. Confluent, quiescent cultures of BMS2 stromal cells were untreated (CTRL) or induced with the thiazolidinedione compounds BRL or PIO at increasing concentrations for 3 days. The cells were returned to standard medium for an additional 3 days, at which time they were fixed, stained with the lipophilic fluorescent dye Nile red, and monitored by FACS for enhanced fluorescence in the gold wavelength (cells in the M1 region of the profile). This percentage value of the total cell population is reported in Table 2.

TABLE 2

Dose-dependent adipogenic response to thiazolidinediones

In all studies, values are reported as a percentage of all cells staining positive for Nile red based on fluorescence intensity. The *p* values are <0.0001 relative to the MHI point for all concentrations of thiazolidinediones. Results are mean \pm standard deviation from 10 data points collected in four experiments.

	0 μM	0.005 μM	0.05 μM	0.5 μM	5 μM	25 μM	MHI
PIO	0.00	N.D.	2.4 \pm 3.1	4.2 \pm 2.4	18.5 \pm 5.7	34.5 \pm 3.2	53.6 \pm 3.2
BRL	0.00	0.00	13.6 \pm 6.8	25.1 \pm 9.9	42.9 \pm 1.7	N.D.	53.4 \pm 4.1

N.D., not done.

TABLE 3

Additive effects of thiazolidinediones and MHI as adipogenic agonists

Values are expressed as a percentage of all cells positive for Nile red fluorescence. Results are mean \pm standard deviation from 14 data points collected in four experiments.

Control	0
MHI	50.5 \pm 12.2
MHI + BRL 5 μM	70.6 \pm 2.2 ^a
MHI + PIO 25 μM	68.9 \pm 5.9 ^a

^a *p* < 0.0001 relative to the MHI point.

both mRNAs, which reached maximal levels after 3–5 days. Although the relatively early adipocyte differentiation marker gene LPL was present in control cells, its level was further induced and sustained by treatment with the thiazolidinediones; near-maximal levels were reached after 3 days of treatment. These same genes exhibited a similar expression pattern in 3T3-derived and Ob1771 preadipocyte cell lines after induction with thiazolidinediones and other adipogenic agents (5, 28, 34, 36, 37).

The transcription factors C/EBP α and PPAR γ are both known to regulate adipocyte differentiation (3). Although control cells contained a detectable signal for each gene, thiazolidinediones accelerated the rate and extent of their accumulation by \leq 9-fold (Fig. 2 and Table 4). In 3T3-derived preadipocytes, adipogenic agents were found to induce PPAR γ expression in a similar time-dependent manner (5, 36, 37). Hybridization of the Northern blots with probes for PPAR α and PPAR δ detected a weak mRNA signal at best.

Specific induction of PPAR γ mRNA levels in BMS2 cells. Two distinct murine PPAR γ subtypes have been detected in adipose tissue (PPAR γ 2) and liver (PPAR γ 1), respectively. Compared with PPAR γ 1, the PPAR γ 2 isoform utilizes an alternative promoter and 5' noncoding region and contains an additional 30 amino acids (5, 7–9). To determine whether BMS2 cells expressed both PPAR γ isoforms and to more closely examine the PPAR α and PPAR δ genes, specific oligonucleotides (Table 1) were synthesized for semiquantitative PCRs (26). The β -actin gene was used as an internal standard to allow comparison between samples (Fig. 3). The signal intensity of each PCR product at 32 cycles under individual treatment conditions was quantified and normalized relative to control levels based on densitometry (Table 5); these values are intended only as an aid for comparative purposes and should not be viewed as quantitative. The control cells expressed detectable levels of mRNA for PPAR α , PPAR δ , and both PPAR γ isoforms (Fig. 3). After induction with either MHI or thiazolidinediones, the signal intensities of both PPAR α and PPAR δ were reduced relative to control levels. In contrast, after 6 days of exposure to adipogenic agonists, PPAR γ 1 and PPAR γ 2 levels were equal to or greater than control signals; the only exception was the

PPAR γ 1 level in response to PIO (Table 5). These findings document that BMS2 adipocytes express both the PPAR γ 1 and PPAR γ 2 isoforms.

Although the signals for PPAR α and PPAR δ are detected in BMS2 cells, adipogenesis reduces the levels of these rare mRNAs. This pattern of PPAR δ expression differs from that reported in 3T3-derived and Ob1771 preadipocytes (5, 28, 37). In 3T3-L1 cells, PPAR δ mRNA levels increased with adipocyte differentiation (37). Recently, Amri *et al.* (28) cloned the murine PPAR δ cDNA [also known as fatty acid-activated receptor (FAAR)]. Based on transfection experiments, they concluded that PPAR δ mediates the transcriptional effects of fatty acids on Ob1771 adipocyte differentiation. Because their previous work indicated that fatty acids and thiazolidinediones share a common mechanism of action, this indicates that PPAR δ mediates the effects of thiazolidinediones in epididymal-derived Ob1771 cells (34). The current results in bone marrow stromal cells are consistent with the original observations of Tontonoz and colleagues, indicating that the PPAR γ isoforms are induced during adipogenesis (5). This suggests that PPAR γ is partially responsible for the thiazolidinedione effects on bone marrow observed *in vivo*.

Thiazolidinedione induction of primary murine bone marrow stromal cells. To more closely approximate the *in vivo* bone marrow microenvironment, the response of primary murine stromal cells to thiazolidinediones was examined. Treatment of confluent primary stromal cultures for one week with BRL (0.005 μM to 5 μM) or PIO (0.025 μM to 25 μM) increased the number of adipocytes relative to control cultures (Fig. 4 A). Based on visual examination, up to 15% of the stromal cells contained lipid vacuoles in the presence of 5 μM BRL or 25 μM PIO. This was accompanied by increased mRNA levels for the adipocyte gene markers aP2, adipsin, and lipoprotein lipase, as well as the transcription factor, PPAR γ (Fig. 4 B, Table 6). Levels of induction were dose-dependent. Based on quantitation relative to the β actin control, maximum mRNA induction was achieved with concentrations of 0.5 μM BRL or 2.5 μM PIO (Table 6). Thus, these results demonstrate PPAR γ expression in primary stromal cells and are consistent with those obtained using the BMS2 stromal cell clone. However, the possibility cannot be excluded that macrophages in the heterogeneous primary cultures may account for some of the PPAR γ signal.

Antibody detection of PPAR protein in bone marrow. Additional experiments were undertaken to document the presence of the PPAR γ in the bone marrow *in vivo*. To directly detect the PPAR γ protein, a polyclonal antibody was prepared using a PPAR γ carboxyl-terminal peptide as antigen. Previously, a peptide from the comparable region of the retinoic acid receptor γ had been employed successfully for this same purpose (38). The final affinity-purified α -PPAR γ antibody was tested on Western

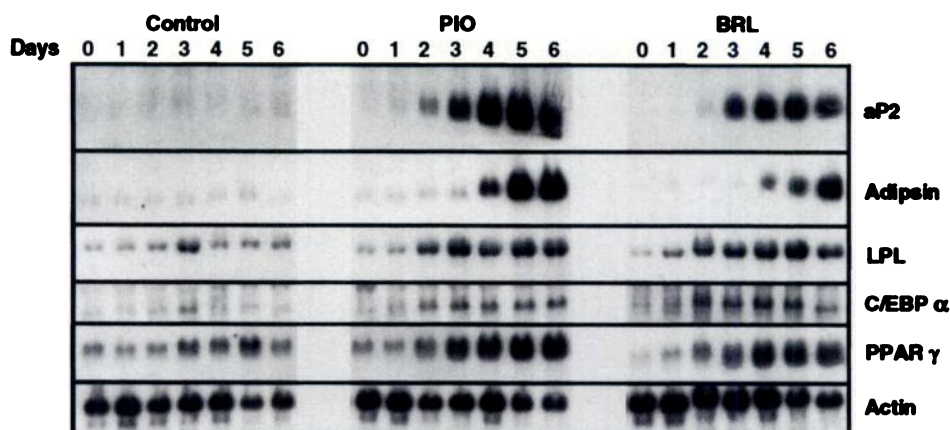


Fig. 2. Northern blot analysis of adipocyte-specific genes induced by thiazolidinediones. Stromal cells were cultured without inducing factors in "standard medium" (*Control*) or in the presence of either 25 μM PIO or 5 μM BRL for 3 days; at this time, all cultures were converted to "standard medium" alone. Individual cultures were harvested daily from day 0 to day 6. Northern blots prepared with total RNA from these cells were hybridized with the following cDNA probes and the autoradiographs exposed for the number of days indicated within parentheses: the fatty acid binding protein aP2 (1); adipsin (1); LPL (1); C/EBP α (9); PPAR γ (8); β -actin (1).

TABLE 4

Densitometric quantification of BMS2 mRNA levels on day 6 relative to actin, based on Northern blot analysis (Fig. 2)

Values are based on the signal intensity of the positive image from Northern blots measured as described in Materials and Methods. All signal intensities are normalized relative to actin at an equivalent day.

	Gene					
	aP2	Adipsin	LPL	C/EBP α	PPAR γ	Actin
Agent						
Control	0	0	0	0	0.2	1.0
PIO (25 μM)	2.6	2.5	0.9	0.2	1.9	1.0
BRL (5 μM)	0.8	1.3	0.5	0.1	0.9	1.0

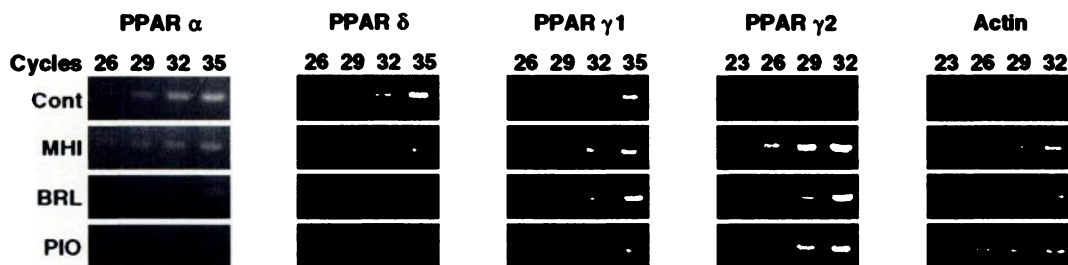


Fig. 3. Semi-quantitative PCR detection of peroxisome proliferator-activated receptor mRNA. Total RNA was prepared from BMS2 cells 6 days after treatment initiation. Cells were cultured in standard medium alone (*Cont*), with the classical adipogenic agonists MHI, with 5 μM BRL, or with 25 μM PIO. Equal aliquots of total RNA were reverse transcribed and amplified with oligonucleotide primers specific for PPAR α , PPAR δ , PPAR γ 1 and PPAR γ 2 (described in Table 1). The β -actin gene was used as a control. Aliquots were removed from each reaction volume at 3-cycle intervals and examined on 3% agarose gels to compare the relative signal intensity.

blots prepared with extracts from cells transfected with a PPAR γ 2 expression vector. The antibody specifically detected an approximately 63 kDa protein (Fig. 5). The protein was not detected in antisense oriented control expression constructs nor by the preimmune serum.

Control studies determined that the α -PPAR γ antibody detected an identically sized protein on Western blots of both murine and rat white adipose tissues (data not shown). Rats were used instead of mice to examine PPAR γ protein expression in bone marrow due to their greater size and the relative prominence of adipocytes in their marrow cavity. Of course, the marrow is heterogeneous, containing macrophages and other blood cell lineages in addition to the stromal cells themselves. It cannot be ruled out that proteins derived from the hematopoietic cell population might account for some component of the signal obtained from the bone marrow

specimens. On Western blots prepared with bone marrow, white, and brown adipose tissue specimens, the antibody detected a major protein of 63 kDa (Fig. 6 A). This was identical in size to the transfected PPAR γ 2 control vector and was not detected by preimmune antibody. Addition of the multi-antigenic peptide (MAP) antigen specifically competed away the protein signal in bone marrow (Fig. 6 B). Similar observations were made with white adipose tissue protein lysates (data not shown).

Conclusions. The current study has examined the mechanism underlying the effects of the thiazolidinediones on bone marrow. Both clonal and primary bone marrow stromal cell cultures underwent adipocyte differentiation in a time- and dose-dependent manner following thiazolidinedione treatment (Fig. 1, Tables 2 and 3). This induction was similar to that described in other murine preadipocyte cell lines (4,

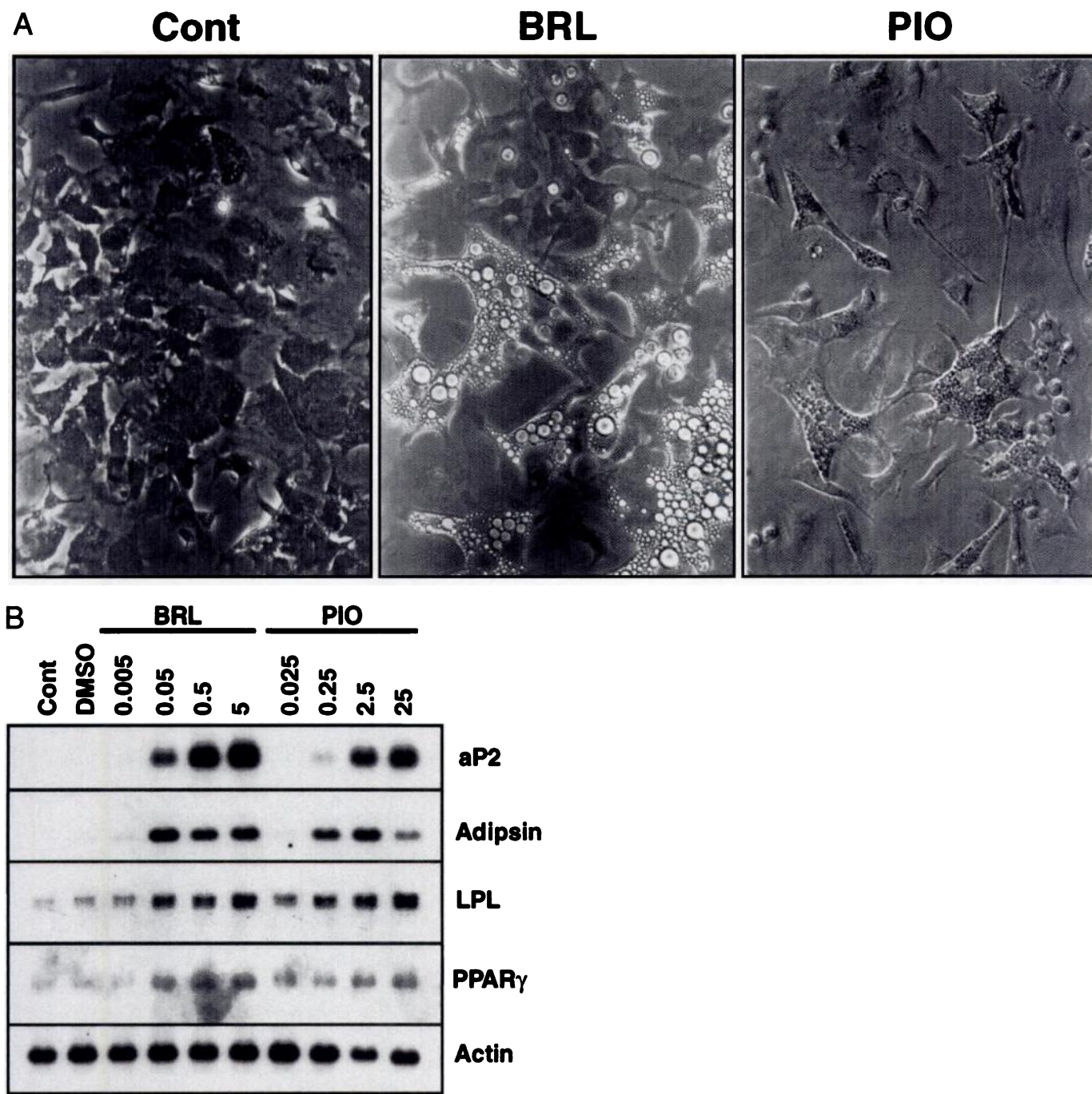


Fig. 4. Response of primary bone marrow stromal cells. **A**, Confluent cultures of primary murine bone marrow stromal cells were cultured in the absence (*cont*) or presence of thiazolidinediones [$0.5 \mu\text{M}$ BRL (*center*) or $2.5 \mu\text{M}$ PIO (*right*)] for 1 week. Cultures were photographed under phase contrast at $102\times$ magnification. **B**, Total RNA harvested from primary stromal cell cultures after 1 week in the presence of inducing agents (micromolar) was examined on Northern blots hybridized with the following cDNA probes, and the autoradiographs were exposed for the number of days indicated in parentheses: β -actin (1), aP2 (1), adipsin (1), LPL (1) or PPAR γ (5). Cells were cultured in standard medium (*Cont*), in the presence of vehicle alone (0.25% DMSO), in the presence of BRL 0.005 to $5 \mu\text{M}$, or in the presence of PIO (0.025 to $25 \mu\text{M}$).

39–41). In the BMS2 cells, adipogenesis correlated with a specific increase in the mRNA levels of the thiazolidinedione receptor, PPAR γ (4), while other PPAR mRNAs were unchanged or decreased. It is likely that the thiazolidinediones, acting as PPAR γ ligands, directly induce transcription of adipocyte-specific genes (Figs. 2 and 4). In transient transfection assays, expression of PPAR γ and its heterodimerization partner, RXR α , increases reporter gene expression under the regulation of the aP2 enhancer (5) or the LPL

promoter¹. Using an antibody reagent, the PPAR γ protein was detected in bone marrow tissue extracts (Fig. 6). Together, these findings suggest that thiazolidinedione interactions with PPAR γ as opposed to other receptor proteins underlies bone marrow stromal cell adipogenesis. In the future, it may be possible to decrease these bone marrow effects through the development of thiazolidinedione derivatives

¹ C. E. Robinson and J. M. Gimble, unpublished observations.

TABLE 5

Densitometric quantification of BMS2 mRNA levels on day 6 relative to actin, based on PCR analysis at 32 cycles (Fig. 3) and normalized relative to control

Values are based on the signal intensity of the negative image from PCR gels measured as described in Materials and Methods. All signal intensities are normalized relative to actin at a cycle number.

Agent	Gene				
	PPAR α	PPAR δ	PPAR γ 1	PPAR γ 2	Actin
Control	1.0	1.0	1.0	1.0	1.0
MHI ^a	0.6	0.7	1.2	1.6	1.0
BRL (5 μ M)	0.2	0.1	1.0	1.1	1.0
PIO (25 μ M)	0.2	0.2	0.5	1.0	1.0

^a 0.5 mM methylisobutylxanthine/60 μ M indomethacin/ 5×10^{-7} M hydrocortisone.

TABLE 6

Densitometric quantification of primary stromal cell mRNA levels normalized relative to actin mRNA signal intensity

Values are derived from data presented in Fig. 4 as described in Materials and Methods.

Gene	Agent		BRL					PIO			
	Control	DMSO vehicle alone	0.005	0.05	0.5	5	0.025	0.25	2.5	25	
aP2	0	0	0	0.3	1.1	1.3	0	0	1.0	1.1	
Adipsin	0	0	0	0.7	0.5	0.5	0	0.4	0.9	0.1	
LPL	0	0	0	0.4	0.3	0.6	0.1	0.3	0.9	0.9	
PPAR γ	0	0	0	0.1	0.6	0.3	0	0	0.1	0.2	
Actin	1	1	1	1	1	1	1	1	1	1	

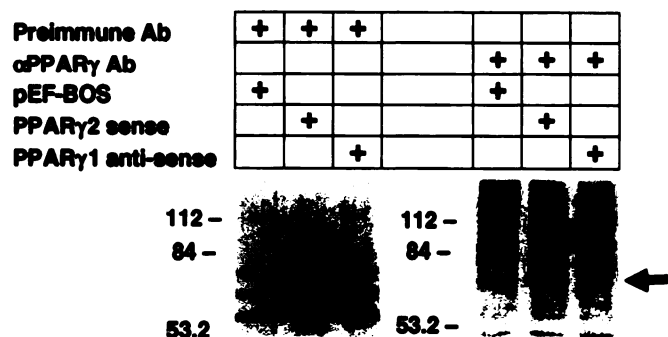


Fig. 5. Antibody detection of the recombinant PPAR γ protein. Equal aliquots of total cell lysates (51 μ g) from transiently transfected 293T kidney cells were examined on Western blots probed with either the goat preimmune antibody or the affinity-purified α -PPAR γ antibody. Eukaryotic expression vectors contained the murine PPAR γ 2 full-length cDNA in the sense orientation or the murine PPAR γ 1 full-length cDNA in the antisense orientation. The empty pEF-BOS vector served as an additional control. Antibody complexes were detected using chemiluminescent reagents.

that do not activate PPAR γ proteins in the marrow stromal cell lineages.

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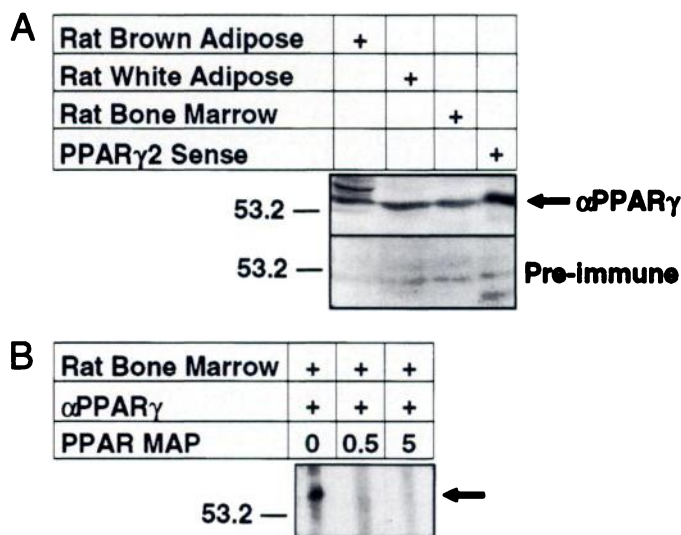


Fig. 6. Antibody detection of the native PPAR γ protein in bone marrow and adipose tissue. A, Total tissue lysates were prepared from rat bone marrow, brown adipose tissue, and white adipose tissue. Equal aliquots of tissue protein (124 μ g) were examined on Western blots with the α -PPAR γ antibody or preimmune antibody and complexes detected using chemiluminescent reagents. Lysates (25 μ g) from 293T cells transfected with the PPAR γ 2 expression construct served as a control. Arrows, PPAR γ specific protein of approximately 63 kDa. B, Western blots prepared with rat bone marrow protein lysates were examined with the anti-PPAR γ antibody in the absence or presence of increasing milligram concentrations of the multiple antigenic peptide antigen (MAP). Arrow, PPAR γ specific protein complex.

References

- Deldar, A., G. Williams, and C. Stevens. Pathogenesis of thiazolidinedione induced hematotoxicity in the dog. *Diabetes* 42 (suppl.):179 (1993).
- Williams, G. D., A. Deldar, W. H. Jordan, C. Gries, G. G. Long, and R. D. Dimarchi. Subchronic toxicity of the thiazolidinedione, Tanabe-174 (LY282449) in the rat and dog. *Diabetes* 42 (suppl.):186 (1993).

3. Smas, C. M., and H. S. Sul. Control of adipocyte differentiation. *Biochem. J.* **309**:697–710 (1995).
4. Lehmann, J. M., L. B. Moore, T. A. Smith-Oliver, W. O. Wilkison, T. M. Willson, and S. A. Kliewer. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPAR γ). *J. Biol. Chem.* **270**:12953–12956 (1995).
5. Tontonoz, P., E. Hu, R. A. Graves, A. I. Budavari, and B. M. Spiegelman. mPPAR γ 2: tissue-specific regulator of an adipocyte enhancer. *Genes Dev.* **8**:1224–1234 (1994a).
6. Tontonoz, P., E. Hu, and B. M. Spiegelman. Stimulation of adipogenesis in fibroblasts by PPAR γ 2, a lipid-activated transcription factor. *Cell* **79**:1147–1156 (1994b).
7. Zhu, Y., K. Alvares, Q. Huang, M. S. Rao, and J. K. Reddy. Cloning of a new member of the peroxisome proliferator-activated receptor gene family from mouse liver. *J. Biol. Chem.* **268**:26817–26820 (1993).
8. Zhu, Y., C. Qi, J. R. Korenberg, X.-N. Chen, D. Noya, M. S. Rao, and J. K. Reddy. Structural organization of mouse peroxisome proliferator-activated receptor γ (mPPAR γ) gene: alternative promoter use and different splicing yield two mPPAR γ isoforms. *Proc. Natl. Acad. Sci. USA* **92**:7921–7925 (1995).
9. Chen, F., S. W. Law, and B. W. O'Malley. Identification of two mPPAR related receptors and evidence for the existence of five subfamily members. *Biochem. Biophys. Res. Commun.* **196**:671–677 (1993).
10. Tsai, M. J., and B. W. O'Malley. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu. Rev. Biochem.* **63**:451–486 (1994).
11. Kletzien, R. F., L. A. Foellmi, P. K. W. Harris, B. M. Wyse, and S. D. Clarke. Adipocyte fatty acid-binding protein: regulation of gene expression *in vivo* and *in vitro* by an insulin-sensitizing agent. *Mol. Pharmacol.* **42**:558–562 (1992).
12. Harris, P. K. W., and R. F. Kletzien. Localization of a pioglitazone response element in the adipocyte fatty acid-binding protein gene. *Mol. Pharmacol.* **45**:439–445 (1994).
13. Kincade, P. W., G. Lee, C. E. Pietrangeli, S. I. Hayashi, and J. M. Gimble. Cells and molecules that regulate B lymphopoiesis in bone marrow. *Annu. Rev. Immunol.* **7**:111–143 (1989).
14. Gimble, J. M. The function of adipocytes in the bone marrow stroma. *New Biol.* **2**:304–312 (1990).
15. Beresford, J. N. Osteogenic stem cells and the stromal system of bone and marrow. *Clin. Orthop. Rel. Res.* **240**:270–280 (1989).
16. Owen, M. E. Marrow stromal stem cells. *J. Cell Sci. Suppl.* **10**:63–76 (1988).
17. Pietrangeli, C. E., S.-I. Hayashi, and P. W. Kincade. Stromal cell lines which support lymphocyte growth: characterization, sensitivity to radiation, responsiveness to growth factors. *Eur. J. Immunol.* **18**:863–872 (1988).
18. Dorheim, M. A., M. Sullivan, V. Dandapani, X. Wu, J. Hudson, P. R. Segarini, D. M. Rosen, A. L. Aulthouse, and J. M. Gimble. Osteoblastic gene expression during adipogenesis in hematopoietic supporting murine bone marrow stromal cells. *J. Cell. Physiol.* **154**:317–328 (1993).
19. Gimble, J. M., C. Morgan, K. Kelly, X. Wu, V. Dandapani, C. S. Wang, and V. Rosen. Bone morphogenetic proteins inhibit adipocyte differentiation by bone marrow stromal cells. *J. Cell. Biochem.* **58**:393–402 (1995).
20. Gimble, J. M., K. Youkhana, X. Hua, H. Baas, K. Medina, M. Sullivan, J. Greenberger, and C. S. Wang. Adipogenesis in a myeloid supporting bone marrow stromal cell line. *J. Cell. Biochem.* **50**:73–82 (1992).
21. Kelly, K., M. R. Hill, K. Youkhana, F. Wanker, and J. M. Gimble. Dimethyl sulfoxide modulates NF- κ B and cytokine activation in lipopolysaccharide-treated murine macrophages. *Infect. Immunol.* **62**:3122–3128 (1994).
22. Smyth, M. J., and W. Wharton. Differentiation of A31T6 preadipocytes to adipocytes: a flow cytometric analysis. *Exp. Cell Res.* **199**:29–38 (1992).
23. Chomczynski, P., and N. Sacchi. Single-step method of RNA isolation by acid guanidinium isothiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159 (1987).
24. Spiegelman, B. M., M. Frank, and H. Green. Molecular cloning of mRNA from 3T3 adipocytes: regulation of mRNA content for glycerophosphate dehydrogenase and other differentiation-dependent proteins during adipocyte development. *J. Biol. Chem.* **258**:10083–10089 (1983).
25. Wilkison, W. O., H. Y. Min, K. P. Claffey, B. L. Satterberg, and B. M. Spiegelman. Control of the adipin gene in adipocyte differentiation: identification of distinct nuclear factors binding to single- and double-stranded DNA. *J. Biol. Chem.* **265**:477–482 (1990).
26. Morgan, C. J., C. J. Hernandez, J. S. Ward, and C. G. Orosz. Detection of cytokine mRNA *in vivo* by polymerase chain reaction: problems and solutions. *Transplantation* **56**:437–443 (1993).
27. Issemann, I., and S. Green. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature (Lond.)* **347**:645–650 (1990).
28. Amri, E. Z., F. Bonino, G. Ailhaud, N. A. Abumrad, and P. A. Grimaldi. Cloning of a protein that mediates transcriptional effects of fatty acids in preadipocytes: homology to peroxisome proliferator-activated receptors. *J. Biol. Chem.* **270**:2367–2371 (1995).
29. Tokunaga, K., H. Taniguchi, K. Yoda, M. Shimuzi, and S. Sakiyama. Nucleotide sequence of a full-length cDNA for mouse cytoskeletal β -actin mRNA. *Nucleic Acids Res.* **14**:2829 (1986).
30. Posnett, D. N., H. McGrath, and J. P. Tan. A novel method for producing anti-peptide antibodies: production of site-specific antibodies to the T cell antigen receptor chain. *J. Biol. Chem.* **263**:1719–1725 (1988).
31. Chen, F., S. W. Law, and B. W. O'Malley. Identification of two mPPAR related receptors and evidence for the existence of five subfamily members. *Biochem. Biophys. Res. Commun.* **196**:671–677 (1993).
32. Mizushima, S., and S. Nagata. pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.* **18**:5322 (1990).
33. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature (Lond.)* **227**:680–685 (1970).
34. Ibrahim, A., L. Teboul, D. Gaillar, E. Z. Amri, G. Ailhaud, P. Young, M. A. Cawthorne, and P. A. Grimaldi. Evidence for a common mechanism of action for fatty acids and thiazolidinedione antidiabetic agents on gene expression in preadipose cells. *Mol. Pharmacol.* **46**:1070–1076 (1994).
35. Steineger, H. H., H. N. Sorensen, J. D. Tugwood, S. Skrede, O. Spydevold, and K. M. Gautvik. Dexamethasone and insulin demonstrate marked and opposite regulation of the steady-state mRNA level of the peroxisome proliferator-activated receptor (PPAR) in hepatic cells: hormonal modulation of fatty-acid-induced transcription. *Eur. J. Biochem.* **226**:967–974 (1994).
36. Chawla, A., and M. A. Lazar. Peroxisome proliferator and retinoid signaling pathways co-regulate preadipocyte phenotype and survival. *Proc. Natl. Acad. Sci. USA* **91**:1786–1790 (1994).
37. Chawla, A., E. J. Schwarz, D. D. Dimaculangan, and M. A. Lazar. Peroxisome proliferator-activated receptor (PPAR) γ : adipose predominant expression and induction early in adipocyte differentiation. *Endocrinology* **135**:798–800 (1994).
38. Rochette-Egly, C., Y. Lutz, I. Scheuer, M. P. Gaub, and P. Chambon. Retinoic acid receptor γ : specific immunodetection and phosphorylation. *J. Cell Biol.* **115**:535–545 (1991).
39. Hiragun, A., M. Sato, and M. Mitsui. Preadipocyte differentiation *in vitro*: identification of a highly active adipogenic agent. *J. Cell Physiol.* **134**:124–130 (1988).
40. Sparks, R. L., E. E. Straus, A. I. Zygmunt, and T. E. Phelan. Antidiabetic AD4743 enhances adipocyte differentiation of 3T3 T mesenchymal stem cells. *J. Cell. Physiol.* **146**:101–109 (1991).
41. Kletzien, R. F., S. D. Clarke, and R. G. Ulrich. Enhancement of adipocyte differentiation by an insulin-sensitizing agent. *Mol. Pharmacol.* **41**:393–398 (1991).

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