A Novel Method for Analysis of Nuclear Receptor Function at Natural Promoters: Peroxisome Proliferator-Activated Receptor γ Agonist Actions on aP2 Gene Expression Detected Using Branched DNA Messenger RNA Quantitation

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Peroxisome proliferator-activated receptor-γ (PPARγ), a member of the nuclear hormone receptor superfamily, plays an essential role in the mediation of the actions of antidiabetic drugs known as thiazolidinediones (TZDs). PPARγ activates many target genes involved in lipid anabolism including the adipocyte fatty acid binding protein (aP2). In this study, induction of aP2 gene expression by PPARγ agonists was examined in both cultured cells and diabetic mice using branched DNA (bDNA)-mediated mRNA quantitation. bDNA technology allows for the direct measurement of a particular mRNA directly within cellular lysate using a 96-well plate format in a time frame comparable to a reporter gene assay. In cultured human subcutaneous preadipocytes, the TZDs, troglitazone and BRL-49653, both rapidly induced aP2 mRNA as detected with the bDNA method. In these cells, the effect of BRL-49653 on aP2 mRNA levels was detectable as early as 30 min after treatment (47% increase) and was maximal after 24 h of treatment (12-fold increase). The effects of troglitazone on aP2 mRNA induction were similar to those of BRL-49653 except that the maximal level of induction was consistently lower (*e.g.* 24 h treatment = 4-fold increase). Dose-response relationships for both of the TZDs were also determined using the 24-h treatment time point. EC_{50}s for both BRL-49653 and troglitazone were estimated to be 80 nM and 690 nM, respectively. A natural PPARγ ligand, 15-deoxy-$\Delta^{12,14}$-PGJ_2, was also active in this assay with a maximal induction of aP2 mRNA of approximately 5-fold when tested at 1 μM. Since the PPARγ:retinoid X receptor (RXR) heterodimer has been characterized as a permissive heterodimer with respect to RXR ligands, the ability of 9-*cis*-retinoic acid (9-*cis*-RA) to induce aP2 mRNA was examined. Although 9-*cis*-RA had very low efficacy (2-fold induction), the maximal effect was reached at 100 nM. No synergism or additivity in aP2 mRNA induction was detected when 9-*cis*-RA was included with either of the TZDs used in this study. Significant induction of aP2 mRNA in bone marrow of *db*/*db* mice treated with either troglitazone or BRL-49653 was also detected, indicating that the bDNA assay may be a simple method to monitor nuclear receptor target gene induction *in vivo*. (Molecular Endocrinology 13: 410–417, 1999)

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INTRODUCTION

Peroxisome-proliferator activated receptor-γ (PPARγ) is a member of the nuclear hormone receptor superfamily that has been demonstrated to play an essential role in the mediation of the actions of antidiabetic drugs known as thiazolidinediones (TZDs) (1–3). TZDs have been demonstrated to bind directly to PPARγ with high affinity, causing activation of target genes (1–3). These compounds were first demonstrated to be specific PPARγ ligands by Lehmann *et al.* with affinities [dissociation constants (K_ds)] ranging from the relatively weak ciglitazone (>1 μM) to the potent BRL-49653 (40 nM) (4). It was later shown that there

was a direct correlation between the affinity of the various TZDs for PPARγ and their relative antihyperglycemic effect *in vivo* (5).

Endogenous PPARγ ligands have also been identified such as 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) (6, 7) and 9- and 13-hydroxyoctadecadienoic acid (HODE) (8). Although the physiological role of 15d-PGJ_2 as a PPARγ agonist is unclear, 15d-PGJ_2 has been characterized as efficacious in both transcriptional reporter and adipocyte differentiation assays, although the potency is relatively low ($K_d > 1$ μM) (6, 7). 9- and 13-HODE are also relatively low potency ligands for PPARγ ($K_d > 1$ μM) and are active in reporter assays (8). It has been suggested that 9- and 13-HODE, components of oxidized low-density lipoprotein, may play a role in the PPARγ-mediated differentiation of macrophages to foam cells (8, 9).

Activation of PPARγ leads to increased expression of many target genes involved in lipid anabolism and energy balance such as the adipocyte fatty acid binding protein (aP2), acyl-CoA oxidase, lipoprotein lipase, and acyl-CoA synthase (1–3, 10). Recently, with the description of a role of PPARγ in the monocyte, a new class of responsive genes has been identified, *e.g*. CD14, CD36, and SR-A, which function in the differentiation of these cells to foam cells (8, 9) and inducible nitric oxide synthase, gelatinase B, and tumor necrosis factor-α, which function in the regulation of the inflammatory response (11, 12).

Analysis of the response of various target genes, such as those described above, in response to ligands for nuclear receptors has been limited by the techniques with which the induction of the gene of interest is detected. Typically, Northern analysis, RT-PCR, or ribonuclease (RNAse) protection is used to detect changes in expression of nuclear receptor target genes. However, if the effects of multiple ligands and doses of these ligands are to be analyzed, these classic molecular biology methods are cumbersome. The transcriptional reporter assay has solved the problem concerning the level of throughput required for these types of studies, but in many cases it may be advantageous or more appropriate to examine the expression of an authentic target gene with its entire natural regulatory sequences instead of a reporter in a heterologous system. To address this, we have developed a method to quantitate a nuclear receptor target in a 96-well format. This method utilizes branched DNA (bDNA) amplification technology that was originally developed for clinical detection of viral DNA and RNA (13–16). The method is as simple to perform as a transcriptional reporter assay and is completed in the same time frame. In this study, we used the bDNA assay to monitor the level of induction of the PPARγ target gene, aP2, after treatment of various cell types with a variety of agonists. We also demonstrate that this assay provides a simple method for detecting the actions of nuclear receptor ligands on target genes *in vivo*.

RESULTS

bDNA mRNA Detection Methodology and aP2 Probe Design

An overview of the bDNA mRNA detection methodology is illustrated in Fig. 1. After treatment of hormone-sensitive cells, cultured in a 96-well plate, with ligand for various amounts of time, the cells are lysed in the presence of target oligonucleotides (probes). Multiple short probes are used during the hybridization to span the mRNA of interest. Three types of hybrid target probes are used and include capture probes, target probes, and spacer probes. Capture probes are designed so that a portion hybridizes with an oligonucleotide that is fixed to the well surface of a 96-well plate and another portion that hybridizes to the mRNA of interest. Label probes are designed so that a portion hybridizes to the mRNA of interest and the other portion hybridizes to a branched DNA molecule that is essential for the amplification of the hybridization signal. The probes are designed to completely span the mRNA of interest; the stability of the hybrid is increased by leaving no gaps that would increase the susceptibility of the hybrid to single-stranded nucleases. An aliquot of the lysate from each well is transferred to a capture plate that consists of wells coated with an oligonucleotide complementary to a portion of the capture probe. Hybridization is carried out overnight at 53 C. A solution of bDNA is then added followed by a short hybridization step (30 min at 53 C). The bDNA hybridizes to a portion of the label probe that has previously complexed with the mRNA of interest. A covalently modified oligonucleotide (coupled to alkaline phosphatase) complementary to the branches of the bDNA is added followed by another short hybridization step (15 min at 53 C). A luminescent alkaline phosphatase substrate is then added, followed by quantitation in a luminometer.

To validate this method for quantitation of nuclear receptor target gene induction, we selected the PPARγ target gene aP2. ProbeDesigner (Chiron Diagnostics, Emeryville, CA) software was used to design the probe set for detection of the human aP2 mRNA. Figure 2 is a schematic illustrating the location of the capture, label, and spacer probes within the aP2 mRNA seqeunce. A total of 18 oligonucletides were required to span the aP2 mRNA sequence.

aP2 mRNA Induction by PPARγ Agonists Measured with bDNA Technology

To validate the bDNA method for measurement of aP2 in human subcutaneous preadipocytes, we treated the cells with 1 μM BRL-49653 for 3 and 24 h and compared the level of expression of aP2 mRNA as detected by both quantitative PCR (QPCR) and the bDNA method (17–19). At both the 3- and 24-h timepoints, the two methods are in agreement with respect to the level of induction of aP2 mRNA. After 3 h of treatment,

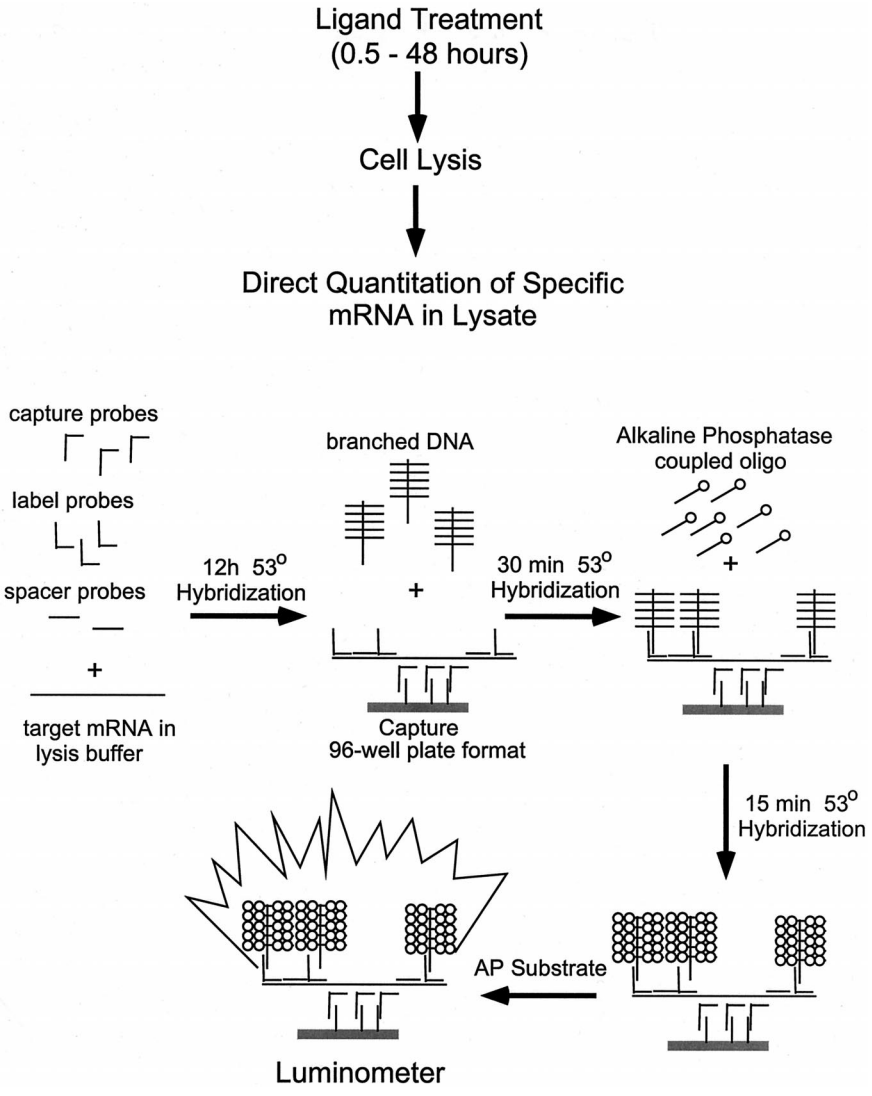

Fig. 1. Summary of the Assay Procedure and Mechanism of the bDNA-Mediated mRNA Detection Assay

Cells are treated with ligands for various amounts of time followed by cell lysis in the presence of oligonucleotides. Multiple oligonucleotides are used during the hybridization to span the mRNA of interest. Three types of hybrid target probes are used and include capture probes, target probes, and spacer probes. Capture probes are designed so that a portion hybridizes with an oligonucleotide that is fixed to the well surface of a 96-well plate and another portion that hybridizes to the mRNA of interest. Label probes are designed so that a portion hybridizes to the mRNA of interest and the other portion hybridizes to a branched DNA molecule that is essential for the amplification of the hybridization signal. The probes are designed to completely span the mRNA of interest; the stability of the hybrid is increased by leaving no gaps so as to decrease the susceptibility of the hybrid to single stranded nucleases. An aliquot of the lysate from each well is transferred to a capture plate that consists of wells coated with an oligonucleotide complementary to a portion of the capture probe. Hybridization is carried out overnight at 53°C. A solution of bDNA is then added followed by a short hybridization step (30 min at 53°C). The bDNA hybridizes to a portion of the label probe that has previously complexed with the mRNA of interest. A covalently modified oligonucleotide (coupled to alkaline phosphatase) complementary to the branches of the bDNA is added followed by another short hybridization step (15 min at 53 C). A luminescent alkaline phosphatase substrate is then added followed by quantitation in a luminometer.

QPCR detected a 3.0-fold increase in aP2 mRNA expression while the bDNA method detected a 3.1 ± 0.30-fold increase (Table 1). After 24 h of treatment with BRL-49653, QPCR detected a 12.7-fold increase in expression, whereas the bDNA method detected a 9.9 ± 0.74-fold increase (Table 1).

To assess the kinetics of induction of aP2 mRNA by BRL-49653, human subcutaneous preadipocytes were cultured in 96-well culture plates and challenged with 1 μM BRL-49653, for various amounts of time ranging from 0.5 h to 5 h followed by assessment of aP2 gene expression using the bDNA assay. As shown in Fig. 3A, aP2 expression increased approximately 50% 0.5 h after treatment with BRL-49653 and continued to increase to a level 3-fold higher than controls after 5 h of treatment. Figure 3B illustrates a longer

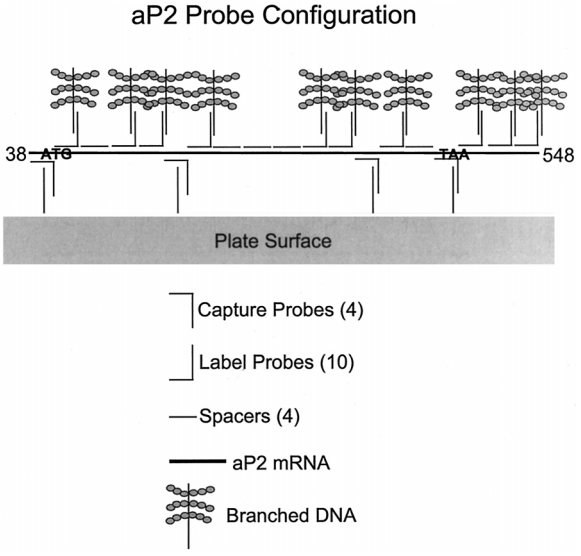

Fig. 2. Schematic Illustrating the Localization of Oligonucleotides Annealing to the aP2 mRNA and Also Functioning in the bDNA mRNA Detection System

Table 1. Induction of aP2 mRNA Expression Induced by 1 μM BRL-49653 as Detected by QPCR and bDNA Methods

Duration of Treatment	Quantitative PCR		bDNA Fold Increase
	aP2 cDNA (attomoles/μg total RNA)	Fold Increase	
0 h	6	1	1.0 ± 0.38
3 h	18	3.0	3.1 ± 0.30
24 h	76	12.7	9.9 ± 0.74

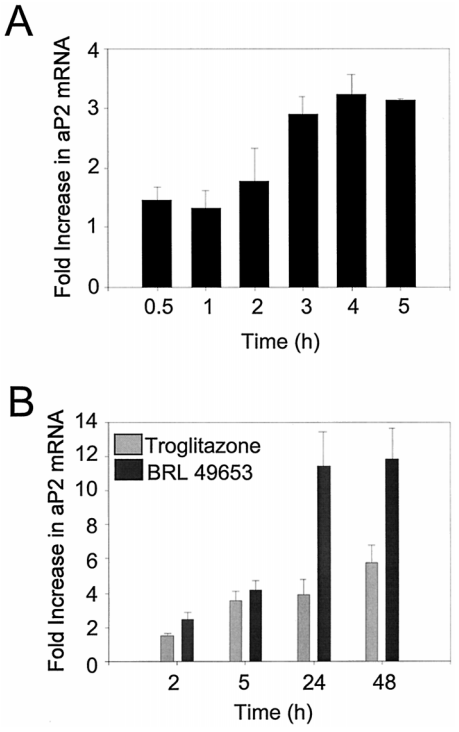

Fig. 3. The Effect of the PPARγ Agonists, Troglitazone and BRL 49653, on aP2 mRNA Levels in Preadipocytes after Various Incubation Times

aP2 mRNA levels were assessed with the bDNA assay. A, BRL 49653 (1 μM) increases aP2 mRNA after very short incubation times. An approximate 50% increase in aP2 mRNA levels is detectable in as little as 30 min after addition of the drug. B, The effects of BRL49653 (1 μM and troglitazone (1 μM) on aP2 mRNA levels peak after approximately 24 h. BRL 49653 has a larger effect on the induction of aP2 mRNA than troglitazone. Values are expressed as a mean of the values from three individual wells ± SEM.

time course experiment in which the cells were treated with either BRL-49654 or troglitazone (1 μM) for times ranging from 2–48 h. BRL-49653-treated cells reached a maximal 12-fold increase in the level of aP2 gene induction after 24 h treatment. Interestingly, troglitazone did not induce this great an increase in aP2 gene expression, with a maximal level of only approximately 6-fold over control levels (48 h). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were monitored in parallel wells, and no significant changes were noted upon addition of PPARγ agonists (data not shown).

As detected by the bDNA assay, both troglitazone and BRL-49653 dose dependently increased aP2 expression as shown on Fig. 4. Troglitazone was approximately 10-fold less potent than BRL-49653 in this assay (EC_{50} 690 nM *vs.* 80 nM). 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) was quite active with efficacy similar to the thiazolidinediones (Fig. 5). The retinoic acid receptor (RAR)/retinoid X receptor (RXR) agonist, 9-*cis*-retinoic acid, showed minimal efficacy, increasing aP2 expression only 2-fold (Fig. 5). The effects of 9-*cis*-retinoic acid treatment are attributable to its actions at RXR since a selective RAR agonist, TTNPB, was not active in this assay (data not shown). We also examined the effect of 9-*cis*-retinoic acid treatment concurrent with either troglitazone or BRL-49653. No additive or synergistic effects were noted (Table 2). The effects of the various PPARγ agonists on aP2 gene expression in human omental preadipocytes were also examined. As shown in Fig. 6, consistent with previous findings (20), no effect of these compounds were noted.

Detection of PPARγ Action *in Vivo* with the bDNA Assay

Since the bDNA assay measures the induction of an actual nuclear receptor target gene, we believed that this method may be useful to monitor the actions of nuclear receptor ligands *in vivo*. To examine this, we treated diabetic mice (*db*/*db*) with 30 mg/kg of either BRL-49653 or troglitazone by oral gavage once a day for five consecutive days. After this treatment, the relative expression of aP2 was examined in bone marrow collected from the femurs of the animals. Bone

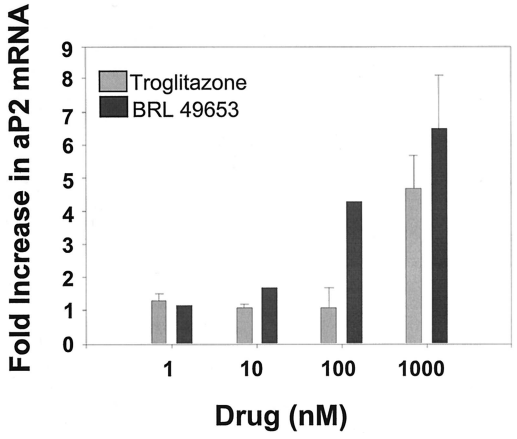

Fig. 4. The PPARγ Agonists, Troglitazone and BRL 49653, Increase aP2 mRNA Levels in a Dose-Dependent Manner

Various concentrations of the agonists were incubated with the preadipocytes, and the experiment was terminated after 24 h. aP2 mRNA levels were assessed with the bDNA assay. BRL 49653 was significantly more potent (EC_{50} estimated at 80 nM) than troglitazone (EC_{50} estimated at 690 nM). Values are expressed as a mean of the values from three individual wells ± SEM.

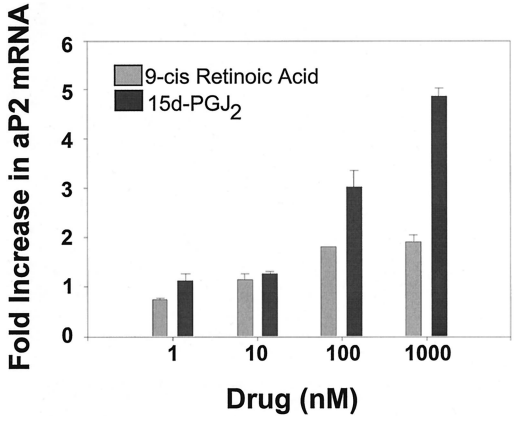

Fig. 5. 9-*cis*-Retinoic Acid and PGJ2 Increase aP2 mRNA Levels in a Dose-Dependent Manner

Various concentrations of the compounds were incubated with the preadipocytes, and the experiment was terminated after 24 h. aP2 mRNA levels were assessed with the bDNA assay. 9-*cis*-Retinoic acid had only limited efficacy with a maximal induction of aP2 mRNA levels of approximately 2-fold. 15d-PGJ2 had efficacy comparable to troglitazone. Values are expressed as a mean of the values from three individual wells ± SEM.

marrow, which has been previously characterized as a target tissue for PPARγ agonist actions (21), was selected as the tissue to examine because of the low basal levels of aP2 expression and the ease of sample collection. Relative levels of aP2 expression were normalized to the expression of GAPDH to control for variability during the preparation of the tissue. As shown in Fig. 7, BRL-49653 and troglitazone significantly increased aP2 gene expression in bone marrow 3.8- and 3.2-fold, respectively.

Table 2. Lack of Additivity or Synergy of 9-*cis*-Retinoic Acid with Thiazolidinediones

	Fold Activation of aP2 mRNA	
	−9-*cis* RA	+9-*cis* RA (100 nM)
No drug	1.00 ± 0.12	1.82 ± 0.02
Troglitazone		
100 nM	1.09 ± 0.59	1.29 ± 0.10
1000 nM	4.69 ± 1.0	6.25 ± 0.33
BRL 49653		
100 nM	4.29 ± 0.01	2.83 ± 0.55
1000 nM	6.50 ± 1.6	6.82 ± 0.08

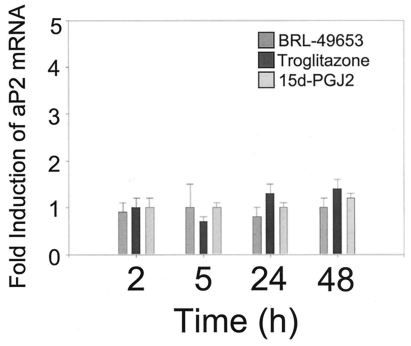

Fig. 6. PPARγ Ligands Have no Effect on aP2 mRNA Expression in Omental Preadipocytes

BRL-49653, troglitazone, and 15d-PGJ2 were applied to the cells at a concentration of 1 μM for 2, 5, 24, and 48 h. aP2 mRNA levels were assessed with the bDNA assay. Values are expressed as a mean of the values from three individual wells ± SEM.

DISCUSSION

Using PPARγ and aP2 as a model nuclear receptor and target gene, respectively, we demonstrated that the bDNA assay can be effectively used to monitor nuclear receptor-mediated alterations in gene expression. The bDNA assay is simple to perform and is completed along the same time frame as a transcriptional reporter assay. The assay is easily adapted to different cell types including tissue directly from an animal source. With the availability of software to aid in the design of the oligonucleotide probes, the assay is also adaptable to virtually any gene of interest.

The results of the bDNA-mediated aP2 induction assay were consistent with other assays that examine the activity of PPARγ. BRL-49653 and troglitazone had EC_{50}s for induction of aP2 that correlated well with published values for their affinities for PPARγ and EC_{50}s in the transcriptional reporter assay (4, 6, 7). The time frame of induction of aP2 was also similar to

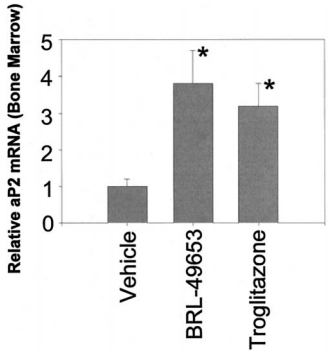

Fig. 7. Thiazolidinediones, BRL-49653 and Troglitazone, Increase aP2 Expression *in Vivo* as Detected by the bDNA Assay

Female *db*/*db* mice, 8–10 weeks of age, were dosed once per day for five consecutive days with 30 mg/kg of either BRL-49653, troglitazone, or vehicle. On the fifth day, bone marrow collected from the femur and tibia was collected and assayed for aP2 mRNA using the bDNA assay. Animals treated with either of the two TZDs showed significant ($P < 0.01$) increases in aP2 expression.

previous values obtained by Northern analysis (21, 22). Our studies with the RAR/RXR agonist, 9-*cis*-retinoic acid, confirm that the PPARγ/RXR heterodimer is permissive (23); however, our data indicate that, at least in terms of aP2 gene induction, activation of RXR does not yield the same degree of efficacy as activation of PPARγ, and the RXR ligand does not function additively or synergistically with a PPARγ ligand. Previously, it was demonstrated that RXR ligands may act additively (24) or synergistically (25) with PPARγ ligands, although this does not appear to be the case for induction of aP2 expression in preadipocytes. Mukherjee *et al*. (25) found that the RXR ligand, LGD 100268, is as efficacious in the activation of the PPARγ/RXR heterodimer as a PPARγ ligand in reporter assays. These investigators also showed that PPARγ and RXR ligands synergistically activated reporter gene transcription. Similar results were obtained in diabetic rodent models using plasma glucose and glucose tolerance tests as endpoints (25). The reason for this discrepancy is not clear, but may arise from the fact that we are monitoring the expression of a single PPARγ target gene under the regulation of its natural promoter; thus, complex regulation within the aP2 promoter may preclude RXR ligands from functioning, as previously described at the DR1 element (25). The fact that RXR ligands have a much greater effect on insulin sensitivity *in vivo* than on aP2 gene expression *in vitro* may be ascribed to the notion that activation of many PPARγ target genes is required to increase insulin sensitivity by RXR ligands, and aP2 is not one of these. Alternatively, the differences in the ligands used (9-*cis*-retinoic acid *vs.* LG 100268) in these studies may account for the discrepancies.

In this study, using the bDNA assay, we also examined the ability of the TZDs to induce aP2 gene expression *in vivo*. Previously, it was demonstrated that TZDs induce adipocyte differentiation in bone marrow (21). Since bone marrow is a relatively accessible source of tissue and has low basal levels of aP2 expression, we examined the effects of troglitazone and BRL-49653 on the expression of aP2 in this tissue. We found that treatment of *db*/*db* mice with either of these TZDs induced significant (3.2- to 3.8-fold induction) aP2 expression. This assay is extremely efficient at detecting induction of genes *in vivo*, as we could evaluate the results of the assay within 16 h after harvesting tissue with no RNA purification required.

In summary, we have adapted a novel assay, originally developed for detection of viral nucleic acids, for use to detect activation of nuclear receptor target genes. This method is advantageous in that one can quickly and simply assay the relative effects of a ligand on target genes within target tissues. We believe this assay will complement the transcriptional reporter assay. Whereas the reporter assay has its strengths in the area of investigation of the mechanistics and specificity of nuclear receptor function, the bDNA assay focuses on the physiological relevance of receptor activation in both cell culture and animals.

MATERIALS AND METHODS

Cell Culture

Human preadipocytes obtained from cosmetic liposuction procedures were purchased from Zen-Bio (Research Triangle Park, NC). Confluent cells were shipped in 96-well plates and used on the day of receipt. Cells were plated in preadipocyte medium [DMEM/Ham's F-10 nutrient broth (1:1) supplemented with HEPES (pH 7.4, 15 mM), biotin (33 μM), pantothenate (17 μM), insulin (100 nM), dexamethasone (1 μM), FBS (10%), and antibiotics (streptomycin, penicillin, and fungizone). Cells were maintained at 37 C and 5% CO_2 throughout the study. Cells were challenged with various PPARγ agonists on the day the cells were received from Zen-Bio. DMEM/F-12 medium containing the agonists was added to the cells after removal of the preadipocyte media. The media remained on the cells for various amounts of time (0.5 h–48 h) depending on the experiment. At the termination of the challenge, medium was removed and 100 μl of lysis buffer were added as described below.

Quantitative RT-PCR

Human subcutaneous preadipocytes were induced with differentiation medium (see *Cell Culture*), and the cells were harvested for RNA purification after 3 and 24 h. Total RNA was prepared using the protocol of guanidinium/acid-phenol extraction (17). The method for RT and QPCR has been described previously (17–19). Briefly, total RNA was mixed with nuclease-free water to a final volume of 7 μl, heated at 65 C for 5 min, and chilled on ice. Aliquoted RT mixture was added into individual tubes bringing the final volume to 20 μl. Each RT reaction contained 1.5 μM oligodT_{12-18}, 0.5 mM deoxynucleoside triphosphate), 1 U of RNase inhibitor, 10 μM dithiothreitol, 1 $\times$ RT buffer (GIBCO BRL, Gaithersburg, MD) and 100 U of M-MLV (GIBCO BRL). The reaction was incubated at 37 C for 1 h, heated at 85 C for 4 min, and chilled on ice. For QPCR, a 1:3 dilution of internal control for aP2 was added into a series of PCR reaction tubes containing equal

volumes of RT reaction mixture (1 μl/50 ng total RNA). The PCR reaction contained 0.4 μM of corresponding primers specific for aP2 cDNA (5′-TGAAA GAAGT AGGAG TGGGC, 5′-CTTCA GTCCA GGTCA ACGTC), 50 μM deoxynucleoside triphosphate, 1 × PCR buffer and 2.5 U of *Taq* polymerase (Perkin-Elmer, Norwalk, CT) in a final volume of 50 μl. The amplification conditions for PCR were detailed previously (23). The internal standard of aP2 was the corresponding aP2 cDNA fragment with 90-bp deletion (from 134–213 bp of the coding sequence) engineered by PCR method. The authenticity of the internal control DNA was confirmed by DNA sequencing. Fifteen microliters of PCR product were loaded onto a 6% Tris-borate-EDTA acrylamide gel and subjected to electrophoresis. After staining with ethidium bromide, the gel was photographed, and the intensity of the DNA bands was quantified by a computerized camera and computer analysis system (Bio-Rad Gel Docu 1000; Bio-Rad Laboratories, Richmond, CA). The ratio of the integrated DNA bands was plotted out on a log-log scale against the amount of internal standard present in the PCR reactions. The amount of aP2 cDNA before the PCR was determined from the plot (17).

bDNA Assay

The bDNA assay was performed according to the manufacturers protocol (Chiron Diagnostics; Emeryville, CA). Briefly, after the challenge of the preadipocytes, cells were lysed with lysis buffer (provided by Chiron Diagnostics) containing the aP2 oligonucleotides (described below). After a 15-min incubation, 80 μl of the lysis buffer from each well were added to a corresponding capture well (preincubated with 100 μl of blocking buffer (provided by Chiron Diagnostics). The capture plate was incubated overnight at 53 C in a plate incubator (Chiron Diagnostics). After this incubation, the bDNA and label probes were annealed as directed by the manufacturer. After a 30-min incubation with the luminescent alkaline phosphatase substrate, dioxitane, the luminescence was quantitated in a Dynex MLX microtiter plate luminometer. A summary of the procedure is provided in Fig. 1

aP2 Probe Design

Oligonucleotide probes were designed to anneal to the aP2 mRNA and function in the bDNA mRNA detection system were designed with ProbeDesigner software (Chiron Diagnostics). This software package analyzes a target sequence of interest with a series of algorithms to determine which regions of the sequence can perform as locations for capture, label, or spacer probe annealing. The sequence of the oligonucleotides is summarized below:

ap2001.CE CATTTTGTGAGTTTTCTAGGATTATTCTTTTCTCTTGGAAAGAAAGT

ap2002.CE
ATGTTAGGTTTGGCCATGCCTTTCTCTTGGAAAGAAAGT

ap2003.CE
CCTCTCGTTTTCTCTTTATGGTTTTCTCTTGGAAAGAAAGT

ap2004.CE GCTTATGCTCTCTCATAAACTCTCGTGGTTTCTCTTGGAAAGAAAGT

ap2005.LE CCAGGTACCTACAAAAGCATCACATTTAGGCATAGGACCCGTGTCT

ap2006.LE GCCCACTCCTACTTCTTTCATATAATCATTTAGGCATAGGACCCGTGTCT

ap2007.LE AGCCACTTTCCTGGTGGCAAATTTAGGCATAGGACCCGTGTCT

ap2008.LE CATCCCCATTCACACTGATGATCTTTAGGCATAGGACCCGTGTCT

ap2009.LE GTACCAGGACACCCCCATCTAAGGTTTTTAGGCATAGGACCCGTGTCT

ap2010.LE GGTTGATTTTCCATCCCATTTCTGCACATTTTAGGCATAGGACCCGTGTCT

ap2011.LE GCATTCCACCACCAGTTTATCATTTTAGGCATAGGACCCGTGTCT

ap2012.LE GCGAACTTCAGTCCAGGTCAACGTCCCTTGTTTAGGCATAGGACCCGTGTCT

ap2013.LE TCCCACAGAATGTTGTAGAGTTCAATTTTAGGCATAGGACCCGTGTCT

ap2014.LE AAAACAACAATATCTTTTTGAACAATATATTTAGGCATAGGACCCGTGTCT

ap2015.BL TCAAAGTTTTCACTGGAGACAAGTTT

ap2016.BL AAAGGTACTTTCAGATTTAATGGTGATCA

ap2017.BL
CTGGCCCAGTATGAAGGAAATCTCAGTATTTTT

ap2018.BL TCTGCAGTGACTTCGTCAAATTC

ap2019.BL ATGGTGCTCTTGACTTTCCTGTCA

ap2020.BL AAGTGACGCCTTTCATGAC

A schematic illustrating the design of the aP2 oligonucleotide probes is shown in Fig. 2. The GAPDH probes designed and validated by Chiron Diagnositics are shown below:

H.GAPDH.118.26.L AATTTGCCATGGGTGGAATCATATTGQTTTQAGGCATAGGACCCGTGTCT

H.GAPDH.144.20.L CAGCCTTGACGGTGCCATGGQTTTQAGGCATAGGACCCGTGTCT

H.GAPDH.164.22.L TTGATGACAAGCTTCCCGTTCTQTTTQAGGCATAGGACCCGTGTCT

H.GAPDH.186.21.L AAGATGGTGATGGGTTTACCAQTTTQAGGCATAGGACCCGTGTCT

H.GAPDH.229.20.L CCAGCATCGCCCCACTTGATQTTTQAGGCATAGGACCCGTGTCT

H.GAPDH.249.25.L AGTGGACTCCACGACGTACTCAGCGQTTTQAGGCATAGGACCCGTGTCT

RH.GAPDH.274.22.L TCTCCATGGTGGTGAAGACGCCQTTTQAGGCATAGGACCCGTGTCT

H.GAPDH.375.23.C CATACTTCTCATGGTTCACACCCQTTTQCTCTTGGAAAGAAAGT

H.GAPDH.398.26.C ATTGCTGATGATCTTGAGGCTGTTGTQTTTQCTCTTGGAAAGAAAGT

H.GAPDH.71.21.C CAATGAAGGGGTCATTGATGGQTTTQCTCTTGGAAAGAAAGT

H.GAPDH.92.26.C GAACATGTAAACCATGTAGTTGAGGTQTTTQCTCTTGGAAAGAAAGT

H.GAPDH.207.22.B TTTGGAGGGATCTCGCTCCTGG

Treatment of *db*/*db* Mice

For determination of aP2 expression in bone marrow, *db*/*db* mice (8- to 10-week-old females; C57Bl/KFJ; Jackson Laboratories, Bar Harbor ME) were dosed by oral gavage once a day for five consecutive days with either vehicle (0.5% carboxymethylcellulose) or 30 mg/kg BRL-49653. The bone marrow was aspirated from the femur and tibia of one leg of each mouse and collected in RPMI 1640 media. The cells were diluted to 0.4 ml with media and evenly distributed into 4 wells of a 96-well plate. After allowing the cells to adhere for 2 h, the wells were washed twice with medium, and adherent cells were lysed as described above. aP2 was assessed using the bDNA assay using GAPDH levels to control for variance in tissue preparation.

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