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Identification and regulation of novel PPAR- γ splice variants in human THP-1 macrophages

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Abstract

We have previously identified four novel isoforms of PPAR- γ transcripts in monkey macrophages (J. Zhou, K.M. Wilson, J.D. Medh, Genetic analysis of four novel peroxisome proliferator receptor- γ splice variants in monkey macrophages. *Biochem. Biophys. Res. Commun.*, 293 (2002) 274–283). The purpose of this study was to ascertain that these isoforms are also present in humans. Specific primers were designed to amplify individual isoform transcripts. The presence of PPAR- γ 4, PPAR- γ 5, and PPAR- γ 7 transcripts in human THP-1 macrophages was confirmed by RT-PCR and sequencing. A transcript corresponding to PPAR- γ 6 was not detected. The presence of novel full-length transcripts and protein was also ascertained by Northern and Western blot analysis. Treatment of THP-1 cells with 15-deoxy- Δ 12,14-prostaglandin J₂ (15d-PGJ₂) resulted in more than 20% induction in the expression of PPAR- γ 5 and PPAR- γ 7 transcripts by both Northern blot analysis and RT-PCR. Another PPAR- γ ligand, troglitazone, induced expression of only PPAR- γ 5. Both ligands inhibited the expression of PPAR- γ 1 and PPAR- γ 2. Additionally, 15d-PGJ₂ and troglitazone increased the level of apolipoprotein E transcript by 60% but decreased lipoprotein lipase expression by 15% in THP-1 cells. The differential regulation of PPAR- γ transcripts suggests that each transcript isoform may contribute to macrophage function.

Keywords

PPAR- γ isoforms; THP-1 macrophages; 15-deoxy- Δ 12,14-prostaglandin J₂; Troglitazone; Lipoprotein lipase; Apolipoprotein E

1. Introduction

Peroxisome proliferators activated receptors (PPAR) are members of the nuclear receptor superfamily. Based on an analysis of their highly conserved DNA- and ligand-binding domains, PPAR have been assigned to a subfamily of nuclear receptors that includes the retinoic acid receptors (RAR), the thyroid hormone receptors (TR) and other steroid hormone nuclear receptors [2]. PPAR are divided into three distinct subtypes, PPAR- α , PPAR- β , and PPAR- γ . Generally, PPAR- α and PPAR- β display a ubiquitous pattern of expression, while the expression of PPAR- γ is more restricted and mostly prevalent in adipose tissue, adrenal gland and large colon [3].

All PPAR subtypes form a heterodimer with the 9-cis-retinoic acid receptor (RXR) [3,4]. Upon activation by ligand-binding, the complex binds to a specific DNA sequence called peroxisome proliferator regulatory element (PPRE) in the promoter region of specific genes. As a result,

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this complex transduces the ligand's signal to the transcriptional machinery and regulates the expression of various genes with a wide range of physiological functions.

PPAR- γ is the most extensively studied of the three PPAR subtypes as it is thought to induce genes that participate in several pathophysiological processes [5–7]. PPAR- γ is a key regulator of adipocyte differentiation and carbohydrate and lipid metabolism [7–10]. Endogenously, it is activated by natural long chain polyunsaturated fatty acids, lipid hydroperoxides, and various eicosanoids, especially prostaglandin J₂ (PGJ₂) metabolites [11,12]. Synthetic ligands for PPAR- γ also include thiazolidinediones (TZDs), a class of agents that improve insulin sensitivity and are used for the management of Type II diabetes [13,14]. Of the TZDs, troglitazone is most commonly used to study activation of PPAR- γ in macrophages [15–17]. In certain cell types, troglitazone has been shown to be a partial agonist of PPAR- γ , but in macrophages, troglitazone appears to regulate more genes than other TZDs such as rosiglitazone [18]. Activation of PPAR- γ results in growth inhibition, changes in cell morphology and lipid accumulation [19]. PPAR- γ 's anti-mitogenic function may be exploited to overcome a differentiation block of transformed cells, such as liposarcoma cells and human colon cancer cells. Recent studies show that PPAR- γ is also implicated in dyslipidemia, inflammation, and atherosclerosis [6,20].

PPAR- γ was found to be abundantly expressed in macrophage foam cells in atherosclerotic lesions while normal vascular tissue expressed low levels of PPAR- γ [21,22]. Oxidized LDL (oxLDL) induced PPAR- γ expression [22,23] and, conversely, ligand activation of PPAR- γ led to the up-regulation of CD36, a scavenger receptor responsible for the uptake of oxLDL [23]. Other gene targets of PPAR- γ include several macrophage genes that have contradictory roles in atherogenesis. PPAR- γ induces scavenger receptor CD36, but represses scavenger receptor type A [20]. The anti-inflammatory activity of PPAR- γ is atheroprotective, but its pro-apoptotic activity is thought to promote plaque maturation [24]. PPAR- γ activation also regulates other atherosclerosis-relevant genes including lipoprotein lipase (LPL), apolipoprotein E (apoE), ABCA1 (a cholesterol transporter), gelatinase B, and intracellular nitric oxide synthase (iNOS) [21,25–28]. Such multiple and opposing functions suggest the existence of multiple PPAR- γ isoforms, each with unique ligand specificity and gene targets. For example, it has been shown that PPAR- γ 2 is expressed specifically in adipose tissue and plays a key role in regulating adipocyte differentiation [29,30]. The overall biological effect of PPAR- γ activation may be determined by abundance of individual transcript isoforms, their translational efficiencies, and the sum total of their regulation of various genes.

Structural studies of the PPAR- γ gene and mRNA transcript support the existence of multiple PPAR- γ isoforms. The open reading frame (ORF) of the PPAR- γ gene consists of exons 1 to 6 [31–34]. Exons 2 and 3 encode the DNA binding domain, while exons 5 and 6 encode the ligand-binding domain. The 5'-terminal region of the transcript is the most variable and is the determinant of the PPAR- γ isoform. Until recently, three exons had been identified in the 5'-terminal region in many species including rhesus monkey and human. They are referred to as exon A1, exon A2 and exon B. They are alternatively spliced with exons 1–6 of the ORF to generate three well established isoforms of PPAR- γ as shown in Fig. 1A [33,34]. PPAR- γ 1 consists of exon A1 and A2 spliced together with exons 1–6. The mRNA for PPAR- γ 2 consists of exon B and exons 1–6. A third isoform, PPAR- γ 3, identified in humans, consists of only exon A2 in its 5'-terminal region and exons 1–6.

Recently, we identified two novel exons in PPAR- γ cDNA from monkey macrophages, which we have called exon C and exon D [1]. Both of these exons combine with either exons A1–A2 or with exon B to form four novel PPAR- γ isoforms which we have called PPAR- γ 4, - γ 5, - γ 6 and - γ 7 (Fig. 1A). Thus, in monkey macrophages, the PPAR- γ gene encodes for at least seven unique transcript isoforms resulting from alternative splicing of the five exons at the 5'-terminal

region. The ORF region encoded by exons 1–6 remains identical in all isoforms. There is a translation initiating ATG in exon B; however, in isoforms of PPAR- γ 4 and PPAR- γ 5, this site is inactivated by stop codons present in both exons C and D. Thus, exon B contributes 30 NH₂-terminal amino acids in monkey PPAR- γ 2 but not in PPAR- γ 4 and PPAR- γ 5. A translation initiating ATG is also present in exon C resulting in an additional eight amino acids at the NH₂-terminus of PPAR- γ 4 and PPAR- γ 6. Thus, the seven transcripts encode for a total of 3 different PPAR- γ protein isoforms (Table 1).

Earlier studies have shown that the PPAR- γ sequence is highly conserved between species [31,32]. The cDNA sequences of all monkey PPAR- γ exons (including novel exons C and D) were aligned with sequences in the human genome database (Fig. 1B). We found PPAR- γ sequences encoded by a single gene located in region 10498K to 10384K on human chromosome 3 and divided into 11 exons and 10 introns, with 93–100% sequence identity to the monkey sequence. Based on the high homology of human and monkey PPAR- γ 1 to - γ 3, and alignment of new exons C and D with the human genome database, we hypothesized that human macrophages may also express novel PPAR- γ isoforms - γ 4 to - γ 7. In this study we report the identification of PPAR- γ 4, - γ 5 and - γ 7 in human THP-1 macrophages. Our results indicate that the expression of isoform-specific transcripts may be regulated by PPAR- γ ligands. Future studies aimed at identifying specific inducers as well as targets of each isoform should enable us to modulate abundance of individual isoform transcripts for optimum physiological benefits.

2. Materials and methods

2.1. THP-1 cells

THP-1 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). They are human monocytes derived from the peripheral blood of a 1-year old male with acute monocytic leukemia. They were maintained in THP-1 growth media, consisting of RPMI 1640 (Cellgro) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 4.5 g/L glucose, 20 mM HEPES, 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol, 2 mM glutamine, and 100 units/mL each of penicillin and streptomycin. The THP-1 cells were seeded at 1.5×10^6 cells/mL on a 6-well plate in THP-1 differentiation media, which was the same as growth media except that the 10% FBS was replaced with 1 mg/mL BSA. One day after plating, the monocytes were differentiated into macrophages by treatment of 200 nM of phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich). After 2 days of differentiation, THP-1 macrophages were incubated without or with different ligands (all ligands were from Cayman Chemical) at the indicated concentrations for 1 to 3 days.

2.2. RNA isolation and RT-PCR

THP-1 macrophages were harvested in TRI REAGENT (Sigma-Aldrich), mixed and stored at -80°C until use. Total RNA was isolated by extraction with chloroform and precipitation with isopropanol. The concentration of RNA and any protein contamination was determined by measuring the absorbance at 260 nm and 280 nm respectively using a SPECTRONIC GENESYSTM 5 Spectrophotometer. Two μ g of total RNA isolated from untreated and ligand-treated THP-1 macrophages was used as a template for synthesis of the first strand of cDNA using random hexamers or oligodT primers (Promega). The reaction was catalyzed by Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega) at 42°C for 50 min. Three μ L of cDNA was used in a reaction mixture consisting of 1.5 mM MgCl₂ and 400 μ M dNTP mix for second strand synthesis by *Taq* DNA polymerase (Promega). Amplification of isoform-specific PPAR- γ transcripts was accomplished by polymerase chain reaction (PCR) using 1 μ M of primer sets shown in Table 2. The 3-step PCR reaction was performed at 94°C for 10 min followed by 40 cycles of 94°C for 1 min, $48\text{--}60^\circ\text{C}$ for 90 s, 72°C for 1 min and

a final incubation at 72 °C for 10 min. The specific primer sets were designed based on the differences in the exon-splicing pattern of each monkey isoform as shown in Fig. 2A. A 227-bp fragment of apolipoprotein E (apoE) was amplified using the following primers: Sense: 5' GGCACGGCTGTCCAAGGAGCT3' (bases 384–404 of hu apoE cDNA) and antisense: 5' GCCCGGCCTGGTACACT3' (bases 594–611 of hu apoE cDNA). A 742 bp fragment of human lipoprotein lipase (LPL) was obtained using the following primers: sense: 5' GGTATTATCAACTGGATGGAGGAGG3' (bases 584–607) and antisense (bases 1302–1326): 5' GGAAACTTCAGGCAGAGT-GAATGG3'. PCR products were analyzed by electrophoresis on a 2% agarose gel in 1× TAE buffer (40 mM Tris–Acetate and 1 mM EDTA) alongside a 100-bp DNA Ladder (Promega).

2.3. Purification and cloning of isoform-specific RT-PCR products

The entire 5' region of each PPAR- γ transcript was amplified from THP-1 total RNA by RT-PCR using primer sets shown in Table 2. Primer pairs A+I and E+I efficiently synthesized unique regions of PPAR- γ 7 and - γ 5, respectively. Primer set CS-B and CX-AS was used for amplification of the 5'-region of PPAR- γ 4. The desired PCR products, identified by size, were purified using the GENECLON II Kit from BIO101 Inc. Briefly, bands of interest were excised from the agarose gel, weighed and melted in three volumes of 6 M NaI at 55 °C. The DNA was bound to GLASSMILK and precipitated. After washing unbound contaminants with a solution containing 10 mM Tris–HCl, 0.5 mM EDTA, 50 mM NaCl and 50% ethanol, the DNA was eluted with 20 μ L of water. The gel-purified PCR products were inserted into a plasmid vector using the TOPO TA Cloning Kit for sequencing (Invitrogen). The plasmids were used to transform TOP10 One-shot chemically competent *Escherichia coli* cells (Invitrogen) and spread on LB agar-plates (1.0% Tryptone, 0.5% Yeast Extract, and 1.0% NaCl, 15 g/L bacto-agar, pH 7.0) containing 50 μ g/mL ampicillin. The plates were incubated at 37 °C overnight and three to five visible colonies were picked for plasmid isolation using the plasmid miniprep kit (Qiagen).

2.4. Sequence analysis

The TOPO-PCR Plasmids were subjected to sequencing at the DNA Sequencing Facility of California State University, Northridge by using a ABI Prism™ 377 DNA Sequencer platform with BigDye™ v.3.1 chemistry. Standard M13 primers, which are contained on the TOPO vector, were used for sequencing. The homology between the sequence of PCR fragments from human THP-1 PPAR- γ isoform transcripts and monkey PPAR- γ isoform transcripts were analyzed using the BLAST alignment software available at the NCBI website (<http://www.ncbi.nlm.nih.gov/>).

2.5. Northern blot analysis

Total RNA from control or ligand-treated macrophages was resolved on 1% agarose gels containing 0.6 mol/L formaldehyde and transferred to Nytran membrane by capillary action. Hybridization was done with radiolabeled DNA probes for 3 h at 65 °C. DNA probes specific for either exon C or exon D were prepared by PCR using primer pairs CS and CX-AS (exon C) and DS and D (exon D) shown in Table 2. Plasmids containing full-length PPAR- γ 4 and PPAR- γ 5 were used as templates respectively, for amplification of exon C and exon D. The gel-purified PCR products were radiolabeled with α -[³²P]-dCTP (Amersham, 50 μ Ci per 25 ng DNA per reaction). To ascertain equal loading of RNA, membranes were also evaluated for the relative amounts of β -actin.

2.6. Western blot analysis

Ligand-treated or untreated THP-1 cells were solubilized in buffer containing 50 mM Tris–HCl, pH 7.4, 2 mM CaCl₂, 0.15 M NaCl, 0.1% Triton-X-100 and 10 μ g/mL each of aprotinin,

benzamidine and phenyl methyl sulphonyl fluoride. Protein concentration in cell lysates was determined by the method of Lowry. Equal amounts of protein mixed with Laemmli's sample buffer were separated by 7.5% SDS-PAGE and electrophoretically transferred to Immobilon membrane. Western blot detection was accomplished using the SuperSignal West Pico Chemiluminiscent system (Pierce). A rabbit polyclonal antibody capable of detecting all PPAR- γ protein isoforms was obtained from Santa Cruz Biotechnology (SC-7196, directed against amino acids 5–106 of PPAR- γ 1 protein) and used as the primary antibody. A goat anti-rabbit IgG was used as the secondary antibody.

3. Results

3.1. Detection of PPAR- γ isoforms in differentiated but untreated THP-1 macrophages

Based on the high homology of human and monkey PPAR- γ 1 to - γ 3, and an analysis of the human genome database, we predicted that novel PPAR- γ isoforms may also exist in humans. We examined the human monocytic leukemia cell line THP-1 for the presence of PPAR- γ 4 to - γ 7 mRNA by RT-PCR. Total RNA isolated from THP-1 cells served as a template for the synthesis of cDNA in the presence of reverse transcriptase and random hexamers or oligodT primers. The cDNA was subjected to PCR using primer pairs that amplified segments unique to specific PPAR- γ transcript isoforms (Table 2). Thus, a sense primer from exon A1 and an antisense primer from exon 1 should amplify only isoforms - γ 6, - γ 7 and - γ 1 (Fig. 2A). Similarly, a sense primer from exon B and an antisense primer from exon 1 should amplify only isoforms - γ 4, - γ 5 and - γ 2. The size of the PCR product helped identify the specific isoform (Table 2). Using this technique, we found that none of the PPAR- γ isoforms were expressed in THP-1 monocytes (not shown). The cells were allowed to differentiate into macrophages by treatment with 200 nM Phorbol 12-myristate 13-acetate (PMA) for 2 days prior to RNA isolation and the RT-PCR procedure was repeated. Instead of three expected bands for each primer pair (Table 2), only a single band was obtained for the reaction with primers E and I or F and I (Fig. 2C). The size of these PCR products was consistent with the expected size of PPAR- γ 2 fragment. Primer set A and I or B and I gave the expected size of PPAR- γ 1 and some non-specific bands (Fig. 2B). However, there was no amplification of PPAR- γ 6 or - γ 7. Therefore, prolonged differentiation for 48 h induced the expression of PPAR- γ 1 and PPAR- γ 2, however none of the novel isoforms were detected in non-ligand treated human THP-1 macrophages by RT-PCR.

3.2. Detection of PPAR- γ transcript isoforms in ligand-treated THP-1 macrophages

It has been reported previously that differentiation of adipocytes leads to an induction of PPAR- γ expression [7]. Also, PPAR- γ activation is known to promote differentiation [30]. We investigated if the expression of the novel isoforms PPAR- γ 4 to PPAR- γ 7 was regulated by PPAR- γ activation.

Differentiated THP-1 macrophages were treated with a potent, endogenous PPAR- γ -specific ligand, 15-deoxy- Δ 12,14-prostaglandin J₂ (15d-PGJ₂) at a concentration of 5–10 μ M. A control experiment without ligand treatment was performed at the same time. Total RNA was isolated and used as a template to perform RT-PCR. The primer pairs E and I and F and I were used in two separate reactions to amplify segments for PPAR- γ 2, PPAR- γ 4 and PPAR- γ 5. As shown in Fig. 3A, in addition to the expected band of PPAR- γ 2, we were able to amplify bands with sizes corresponding to PPAR- γ 5 fragments from the total RNA of 15d-PGJ₂-treated THP-1 macrophages. Once again, only PCR fragments of PPAR- γ 2 were obtained from total RNA of control THP-1 macrophages. Unexpectedly, a band corresponding to PPAR- γ 4 was not obtained in these PCR reactions.

Primer pairs A and I and B and I were used to amplify segments of PPAR- γ 1, PPAR- γ 6 and PPAR- γ 7 by RT-PCR using total RNA from both 10 μ M 15d-PGJ₂-treated and untreated (control) THP-1 macrophages as templates. As shown in Fig. 3B, only one band with expected size corresponding to PPAR- γ 1 was obtained in RNA samples from untreated cells when primer set A and I was used. In addition to a PPAR- γ 1 band, a non-specific band with size of more than 300 bp was present when primer set B and I was used. When total RNA from THP-1 cells treated with 10 μ M 15d-PGJ₂ was used as a template for RT-PCR, we were able to obtain a band with expected size of PPAR- γ 7. The expected band for PPAR- γ 6 was not obtained in these PCR reactions.

Detection of PPAR- γ 4 and PPAR- γ 6 (both of which contain novel exon C) proved to be difficult with the primer pairs described above. Hence, we designed a new sense primer, CS, located at the 5' end of exon C (Fig. 4A). We paired it with a new antisense primer, CX-AS, at the 3' end of exon C. Together, this primer pair from within exon C amplified (by RT-PCR) a single fragment of exon C with an expected length of 251 bp from RNA of untreated THP-1 macrophages (Fig. 4B). Thus, we were able to confirm the presence of a transcript containing exon C. Next, additional primers were designed to ascertain that exon C was spliced adjacent to exon A2 or exon B as seen, respectively, in PPAR- γ 6 and - γ 4. Primer CSA2 is a sense primer at the 3'-end of exon A2. Primer CSB is a sense primer on the 3'-end of exon B (Fig. 4A). They were paired with the anti-sense primer, CX-AS, on exon C. Total RNA of untreated THP-1 macrophages was used as template to perform RT-PCR with these newly designed primers. We also tried different Mg⁺² concentrations to optimize amplification. At 2.5 mM of [Mg²⁺], a very strong band with expected size (297 bp) for PPAR- γ 4 was obtained as the PCR product when primers CSB and CX-AS were used (Fig. 4B). However, PCR using primer sets for PPAR- γ 6 did not show any band at all.

All of the isoform-specific PPAR- γ fragments were amplified even when oligodT primers were used for the reverse transcription reaction. Thus, our results strongly suggest the presence of full-length functional transcripts for PPAR- γ 4, - γ 5 and - γ 7 in human THP-1 macrophages. We next proceeded to confirm the identity of the PCR products by DNA sequencing.

3.2.1. Confirmation of PPAR- γ 4, PPAR- γ 5 and PPAR- γ 7 sequences—The *Taq* polymerase-amplified DNA fragments for PPAR- γ 5, - γ 7 and - γ 4 were purified by agarose gel electrophoresis and cloned into the pCR4-TOPO cloning vector (Invitrogen) for sequencing. Sequencing was carried out using standard M13 forward and M13 reverse primers. The sequences obtained were aligned with corresponding monkey PPAR- γ isoform transcripts using the BLAST software available at the NCBI website (<http://www.ncbi.nlm.nih.gov/>). We found that all of the sequences obtained from THP-1 macrophages matched the ones published for isoforms from monkey macrophages. Human PPAR- γ 5 had 100% identity to the corresponding monkey sequence. The PPAR- γ 4 and PPAR- γ 7 sequences were 99% identical to monkey sequences. The sole difference was a one-base mismatch (PPAR- γ 4) or gap in alignment (PPAR- γ 7). Since this variation is in the untranslated region of the transcript, it does not change the reading frame or the amino acid sequence of the protein product.

3.2.2. Expression of full-length PPAR- γ transcript and protein isoforms—To further demonstrate the presence of full-length novel PPAR- γ transcripts in THP-1 cells, we performed Northern blot analysis. Total RNA isolated from control, 15d-PGJ₂- or troglitazone-treated THP-1 macrophage was resolved on a 1% agarose gel and transferred to Nytran membrane. Hybridization was done using [³²P]-labeled exon D or exon C prepared as described in Materials and methods. With both probes, we were able to detect a band of ~1.8 kb, just above the 18S rRNA band (Fig. 5A). These bands correspond to full-length transcripts of PPAR- γ isoforms - γ 5 and - γ 7 (for exon D) and - γ 4 (for exon C). This confirmed the presence of functional novel transcripts in THP-1 macrophages. A probe for β -actin was also hybridized

as a control for RNA loading. The abundance of PPAR- γ transcripts appeared to be significantly lower than that of β -actin, but this could be a function of the low specific activity of exon D and exon C probes. We used the ImageJ software (NIH) to quantitate any alteration in the level of PPAR- γ 5/ γ 7 and - γ 4 as a result of ligand treatment. Treatment with 10 μ M 15d-PGJ₂ resulted in a 22% induction of PPAR- γ 5/- γ 7 transcript and a 24% repression in PPAR- γ 4 transcript. Troglitazone was effective at repressing PPAR- γ 4 levels by 23% but it did not significantly induce PPAR- γ 5/ γ 7 transcript. Regulation of transcription of PPAR- γ isoforms by PPAR- γ ligands was confirmed by RT-PCR experiments (Figs. 6 and 7).

Of the 3 novel PPAR- γ transcripts identified in THP-1 macrophages, only PPAR- γ 4 encodes for a novel protein (Table 1). We investigated the presence of a PPAR- γ 4 protein in THP-1 macrophage cell lysates. Equal mass of total protein in lysates of control and 15d-PGJ₂-treated macrophages were resolved by 7.5% SDS-PAGE. Western blot analysis was performed using a polyclonal antibody against PPAR- γ (SantaCruz Biotechnology). This antibody recognizes all protein isoforms of PPAR- γ . We clearly detected a polypeptide, corresponding to the size (55.3 kDa) of PPAR- γ 4, just above the PPAR- γ 1 band in control cell lysates. This band was absent in 15d-PGJ₂-treated cells. The 15d-PGJ₂-mediated repression of PPAR- γ 4 protein is consistent with its negative regulation of PPAR- γ 4 transcript (Figs. 5A, 6). We also detected protein bands corresponding to PPAR- γ 1 (54.5 kDa) and - γ 2 (57.6 kDa) in both control and 15d-PGJ₂-treated macrophages. The levels of both PPAR- γ 1 and - γ 2 protein isoforms decreased by almost 50% after treatment with 15d-PGJ₂ (Fig. 5B). Due to the identical polypeptides for PPAR- γ 1, - γ 3, - γ 5, and - γ 7 transcripts, it was not possible to determine the relative contribution of each transcript to the expression of PPAR- γ 1 protein. However, our results suggest that, at the level of protein expression, 15d-PGJ₂-mediated induction of PPAR- γ 5 and - γ 7 transcripts did not significantly compensate for repression of PPAR- γ 1 transcript.

3.2.3. Effect of PPAR- γ ligands on regulation of PPAR- γ isoform transcription—

As described above, expression of different PPAR- γ isoforms was regulated not only by differentiation, but also by PPAR- γ ligand activation. We saw a differential induction of the different isoforms, suggesting a role for these novel transcripts in macrophage function. When differentiated cells were stimulated for 24 h with the endogenous PPAR- γ ligand, 15d-PGJ₂, expression of both PPAR- γ 5 and PPAR- γ 7 was significantly induced (Fig. 6). Quantitative analysis using ImageJ software revealed a 21% increase in PPAR- γ 5 and a 24% increase in PPAR- γ 7 transcripts (Fig. 6 table). While PPAR- γ 5 expression was induced at 15d-PGJ₂ concentrations from 3 μ M to 10 μ M (not shown), induction of PPAR- γ 7 was dependent on the dose of ligand used for stimulation. It was induced at a concentration of 10 μ M 15d-PGJ₂, but not at 5 μ M concentration (data not shown). The expression of β -actin transcript, measured as a control, did not change upon treatment with 15d-PGJ₂. Interestingly, 15d-PGJ₂ treatment significantly inhibited the expression of PPAR- γ 1 and PPAR- γ 2 (Fig. 6). As shown in the table alongside Fig. 6, 15d-PGJ₂ treatment repressed the expression of both PPAR- γ 1 and - γ 2 by more than 30%. Similarly, the level of PPAR- γ 4 transcript was reduced by 32% lower after treatment of THP-1 macrophages with 15d-PGJ₂ (Fig. 6). Since the amplification was done in the presence of excess dNTPs and primers and equal amounts of starting total RNA, it is unlikely that this decrease is an artifact of the experimental conditions.

We also examined the effect of other PPAR- γ ligands on expression of novel PPAR- γ isoforms. Treatment with 10 μ M troglitazone for 24 h induced PPAR- γ 5 by 22% expression over control levels (Fig. 7A). There was no clear expression of PPAR- γ 7 in control or treated samples, though there were several non-specific bands of similar size fragments (Fig. 7A). Troglitazone induced PPAR- γ 7 expression only after 72 h of treatment (Fig. 7B), but such prolonged incubation of cells in the presence of differentiation agent PMA alone (control) also resulted in PPAR- γ 7 induction (Fig. 7B). Troglitazone treatment had no effect of PPAR- γ 4 expression but it significantly reduced the expression of PPAR- γ 1 and PPAR- γ 2 to 83% and 71% of control

levels after 24-h treatment. Ciglitazone, another TZD, give identical results as troglitazone (not shown). We also examined the effect of various ligands and growth factors on the expression different PPAR- γ isoforms. Wy14643, is primarily a PPAR- α ligand but at micromolar concentrations it activates PPAR- γ as well [35]. As shown in Fig. 7A, Wy14643 induced the expression of PPAR- γ 5 by 22% but cells treated with 10 μ M Wy14643 did not show any detectable expression of PPAR- γ 7 after 24 h. It repressed PPAR- γ 1 expression by 16% (Fig. 7A) but did not alter PPAR- γ 4 expression. Similarly, Prostaglandin D₂, which is a major cyclooxygenase product and potential precursor of 15d-PGJ₂, induced the expression of PPAR- γ 5 but not PPAR- γ 7 at 10 mM concentration. Another selective and potent PPAR- α activator, 8S-hydroxy-eicosatetraenoic acid (8(S)-HETE) did not regulate the expression of any of the novel PPAR- γ isoforms (not shown). Insulin and dexamethasone also did not alter the level of any PPAR- γ transcript isoforms (not shown).

Based on these studies, we hypothesize that PPAR- γ 5 is a differentiation-dependent isoform that is further induced upon ligand activation with various PPAR- γ ligands including 15d-PGJ₂ and its precursor, TZDs and Wy14643. PPAR- γ 7 expression is more stringently regulated and is induced only by high concentrations of 15d-PGJ₂. Thus, treatment with PPAR- γ ligands appears to favor the formation of transcripts containing exon D. On the other hand, transcripts containing exon C are repressed by 15d-PGJ₂ treatment. PPAR- γ 1 and - γ 2 transcripts also appear to be inhibited by 15d-PGJ₂ as well as TZDs.

3.2.4. Regulation of PPAR- γ target genes—Both LPL and apoE have a PPRE in their promoter regions suggesting a role for PPAR- γ in regulation of their transcription [25,26]. However, there is no information about a specific isoform being responsible. We investigated the correlation between expression of PPAR- γ isoforms, and of LPL and apoE in THP-1 cells by PCR analysis. The relative abundance of amplified bands was quantified using the Image J software. We found that treatment with both 15d-PGJ₂ and troglitazone induced the level of apoE transcript by 60% (Fig. 8). On the other hand, both ligands marginally reduced the expression of LPL (Fig. 8) to ~85% of the level in control cells. The amplification of β -actin was uniform in all samples.

4. Discussion

Our results demonstrate the presence of novel transcripts for PPAR- γ 4, - γ 5 and - γ 7 in human THP-1 macrophages. We also confirmed the expression of previously known isoforms PPAR- γ 1, - γ 2 and - γ 3 in these cells. As shown in Table 1, the seven different transcripts encode for only three different proteins. The existing literature on PPAR- γ does not address the physiological basis for multiple protein isoforms or the possibility of unique functions for each of these isoforms. Current information about isoform-specific functions of PPAR- γ s is limited to the role of PPAR- γ 2 in adipocyte differentiation and carbohydrate metabolism [30]. The differences in PPAR- γ protein isoforms are restricted to the NH₂-terminal region of the protein [1,32], but they may alter protein folding and thereby ligand specificity and affinity. Each isoform may also regulate unique target genes.

The significance of multiple PPAR- γ transcripts that encode for identical proteins is also not clear. Most of the variability between isoforms is located in the 5' untranslated region. This region may bind regulatory proteins that dictate mRNA splicing and translation efficiency. In most eukaryotic cells, post-transcriptional regulation of translation is controlled at the initiation step by structural elements of mRNA (such as 5' UTR) that may modify binding of the ribosome initiation complex to the mRNA. The 5' UTR may contain sequences with a potential to form stable secondary structures. Other mechanisms may include alteration of stability of mRNA and transport of mRNA into the cytoplasm. There are several instances where the 5' UTR exerts post-transcriptional regulation on translation. The 5' UTR of apolipoprotein B was shown to

increase the efficiency of translation in luciferase reporter gene assays and by in vitro translation assays [36]. The expression of serum amyloid A2 apolipoprotein is also post-transcriptionally regulated by both its 5' and 3' UTRs [37]. The translation of glutamate receptor 2 is inhibited by a polymorphic repeat sequence in its 5' UTR [38]. Similarly, variability in the 5' UTR of PPAR- γ transcript isoforms may contribute to their level of translation.

Sequencing data show that human PPAR- γ 4, - γ 5 and - γ 7 transcripts are identical to that of the corresponding monkey isoform. A one-base gap had to be introduced in the human PPAR- γ 7 sequence for optimum alignment. A one-base mismatch was also found in the PPAR- γ 4 sequence. However, being in the untranslated 5'-region, these do not alter the sequence of the protein product. The identity of the sequences is consistent with the belief that PPAR- γ gene is highly conserved across species.

15d-PGJ₂ and troglitazone are, respectively, the most widely used natural and synthetic ligands for PPAR- γ [12,13]. Both are highly selective and equally potent activators of PPAR- γ mediated transcriptional regulation. Various in vitro assays showed that 15d-PGJ₂-bound PPAR- γ interacts with known nuclear receptor coactivators including SRC-1, TIF2 and AIB-1 [39]. Troglitazone binds to the ligand-binding domain of PPAR- γ but it fails to induce receptor interaction with known transcriptional coactivators. Therefore, troglitazone is described as a partial agonist of PPAR- γ . A comparison of PPAR- γ activation by troglitazone in various cell types revealed that while troglitazone does act as a partial agonist in PPAR- γ -? transfected muscle (C2C12) and kidney (HEK 293T) cells, it is a full PPAR- γ agonist in 3T3L1 adipocytes and U937 macrophages [18]. In fact, in macrophages, troglitazone regulated the expression of more genes than another TZD, rosiglitazone, which is considered to be a full PPAR- γ agonist [18]. Thus, while troglitazone may not always be the ideal TZD, it continues to be used extensively to study PPAR- γ -dependent functions in macrophages [15–17].

It is interesting that PPAR- γ -specific ligands regulated the expression of PPAR- γ isoforms. Such self-regulation was unexpected since the PPAR- γ gene is not known to have a PPRE. It is likely that regulation of PPAR- γ transcripts by both 15d-PGJ₂ and troglitazone are independent of PPAR- γ activation. Several reports indicate that both ligands are capable of PPAR- γ independent functions in macrophages and other cell types [20,40–44]. Hinz et al. used the PPAR- γ antagonist, bisphenol A diglycidyl ether, to demonstrate that the anti-inflammatory effect of 15d-PGJ₂ in lipopolysaccharide-stimulated monocytes was PPAR- γ independent [41]. Similarly, troglitazone inhibited cell proliferation and cyclin D1 expression in keratinocytes in a PPAR- γ independent manner [43]. Troglitazone also inhibited cholesterol biosynthesis in various cell lines independently of PPAR- γ activation [44]. Thus, in the absence of a PPRE on the PPAR- γ gene, it is probable that the 15d-PGJ₂- and troglitazone-induced regulation of PPAR- γ transcript expression is by a PPAR- γ -independent mechanism. Alternatively, PPAR- γ -dependent alteration of macrophage environment may indirectly lead to a shift in expression of specific PPAR- γ transcript isoforms expressed. For example, it has been shown previously that activation of PPAR- γ regulates the expression of different macrophage genes [23,45]. Induction of CD36, a scavenger receptor leads to an accumulation of lipid droplets and morphological changes characteristic of foam cell formation [20,23]. Lipid-loading of macrophages has been shown to induce PPAR- γ expression [22]. Thus, ligand activation of PPAR- γ may modify macrophage function and cause the indirect induction of specific PPAR- γ isoform expression. Also, activation of PPAR- γ promotes cellular differentiation and PPAR- γ expression is differentiation-dependent. Tontonoz et al. showed that differentiation of monocytes to macrophages is accompanied by transcriptional induction of PPAR- γ [23].

In our studies, the effect of 15d-PGJ₂ was consistent between Northern blot analysis and RT-PCR experiments. Troglitazone induced PPAR- γ 5 but not - γ 7 in RT-PCR studies. The lower induction of the PPAR- γ 5/- γ 7 band in the Northern blot is probably reflective of the fact that the band represents both PPAR- γ 5 and - γ 7 together. The only discrepancy is in the increased troglitazone-mediated repression of PPAR- γ 4 in Northern blot analysis. This could be due to the different sensitivities of the two methods and the low level of PPAR- γ 4 mRNA abundance.

Our results demonstrate parallel regulation of both isoforms containing the same novel exon (C or D). Both PPAR- γ 5 and - γ 7 contain exon D and are induced by treatment with 15d-PGJ₂. Transcripts containing exon C were inhibited after 24 h treatment with 15d-PGJ₂. This suggests that the exons may contribute to regulation of expression of individual isoforms, possibly by binding to specific regulatory elements. The regulatory elements may differ depending on the specific ligand or stimulant. Our studies also demonstrate that PPAR- γ 7 is not expressed as immediately as PPAR- γ 5. While PPAR- γ 5 is expressed after an acute treatment with 15d-PGJ₂ and Troglitazone, PPAR- γ 7 requires a prolonged differentiation with PMA.

Both LPL and apoE have a PPRE in their promoter regions suggesting that their regulation by PPAR- γ ligands may be mediated by PPAR- γ activation [25,26]. In our studies, PPAR- γ ligands induced apoE expression but repressed LPL transcription. There is contradictory evidence in the literature about the direction of regulation of LPL by PPAR- γ ? [46–48]. Treatment of human monocyte derived macrophages with PPAR- γ ligands resulted in an increase in LPL mRNA and extracellular LPL immunoreactive mass and activity [46]. In another study, PPAR- γ ligands increased LPL mRNA but decreased LPL protein mass and activity in human macrophages [47]. Ranganathan et al. showed that thiazolidinediones inhibit LPL activity in adipocytes [48]. Various fatty acid products of LPL's hydrolytic activity were shown to be ligands for PPARs [49,50]. Of these, arachidonic acid was found to activate PPAR- γ and repress LPL transcription [49].

In macrophages, the expression of apoE mRNA correlates with that of PPAR- γ . Similar to PPAR- γ expression, apoE mRNA levels increased both during differentiation and as a result of lipid-loading [51,52]. Our result is consistent with a previous study which reported that the PPAR- γ -specific ligand, ciglitazone (a thiazolidinedione), doubled the levels of apoE mRNA levels in THP-1 cells [26]. A specific inhibitor of PPAR- γ , bisphenolA diglycidyl ether, resulted in significant reduction of apoE mRNA levels [26]. In macrophages isolated from genetically engineered mice with a conditional disruption of the PPAR- γ gene, the levels of apoE mRNA were significantly reduced compared to macrophages from control mice [53].

These studies suggest a role for PPAR- γ in troglitazone and 15d-PGJ₂-mediated regulation of LPL and apoE expression. It is important to determine the contribution of each PPAR- γ isoform to this regulation. Future studies with over-expression of individual PPAR- γ isoforms should divulge this information.

Evidence for PPAR- γ 's involvement in atherogenesis and diabetes/obesity is compelling [6, 8,14,19]. Current literature indicates contradictory roles for PPAR- γ in atherosclerosis. We suggest that the multiple isoforms may be responsible for the opposing functions. We have clearly demonstrated that PPAR- γ 5 and - γ 7 expression is induced whereas PPAR- γ ? and - γ 2 expression is repressed by PPAR- γ ligands. Thus, it is likely that these isoforms may have contradictory functions. An investigation of the ligand specificity and gene targets of each isoform is underway with the use of isoform-specific Lentivirus expression vectors

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References

1. Zhou J, Wilson KM, Medh JD. Genetic analysis of four novel peroxisome proliferator receptor- γ splice variants in monkey macrophages. *Biochem Biophys Res Commun* 2002;293:274–283. [PubMed: 12054596]
2. Olefsky JM. Nuclear receptors: mini review series. *J Biol Chem* 2001;276:36863–36864. [PubMed: 11459855]
3. Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr Rev* 1999;20:649–688. [PubMed: 10529898]
4. Willson TM, Brown PJ, Sternbach DD, Henke BR. The PPARs: from orphan receptors to drug discovery. *J Med Chem* 2000;43:527–550. [PubMed: 10691680]
5. Medh JD. PPARs in atherosclerosis. *Curr Opin Lipidol* 1999;10:69–71. [PubMed: 10095993]
6. Glass CK. Potential roles of the peroxisome proliferator-activated receptor- γ in macrophage biology and atherosclerosis. *J Endocrinol* 2001;169:461–464. [PubMed: 11375116]
7. Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM. Transcriptional regulation of adipogenesis. *Genes Dev* 2000;14:1293–1307. [PubMed: 10837022]
8. Lee CH, Olson P, Evans RM. Minireview: lipid metabolism, metabolic diseases and peroxisome proliferator-activated receptors. *Endocrinology* 2003;114:2201–2207. [PubMed: 12746275]
9. Walczak R, Tontonoz P. PPARadigms and PPARadoxes: expanding roles for PPAR- γ in the control of lipid metabolism. *J Lipid Res* 2002;43:177–186. [PubMed: 11861659]
10. Ricote M, Glass CK. New roles for PPARs in cholesterol homeostasis. *Trends Pharmacol Sc* 2001;22:441–443.
11. Corton JC, Anderson SP, Stauber A. Central role of peroxisome proliferators-activated receptors in the actions of peroxisome proliferators. *Annu Rev Pharmacol Toxicol* 2000;40:491–518. [PubMed: 10836145]
12. Foreman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM. 15-deoxy- $\Delta^{12,14}$ -prostaglandin J2 is a ligand for the adipocyte determination factor PPAR γ . *Cell* 1995;83:803–812. [PubMed: 8521497]
13. Lehmann JM, Moore LB, Smith-Oliver TA, Wilkinson WO, Willson TM, Kliewer SA. An antidiabetic thiazolidinedione is a high-affinity ligand for peroxisome proliferators-activated receptor gamma (PPAR γ). *J Biol Chem* 1995;270:12953–12956. [PubMed: 7768881]
14. Olefsky JM. Treatment of insulin resistance with peroxisome proliferators-activated receptor γ agonists. *J Clin Invest* 2000;106:467–472. [PubMed: 10953021]
15. Tanaka T, Fukunaga Y, Itoh H, Doi K, Yamashita J, Chun TH, Inoue M, Masatsugu K, Saito T, Sawada N, Sakaguchi S, Arai H, Nakao K. Therapeutic potential of thiazolidinediones in activation of peroxisome proliferator-activated receptor gamma for monocyte recruitment and endothelial regeneration. *Eur J Pharmacol* 2005;508:255–265. [PubMed: 15680279]
16. Sundararajan S, Gamboa JL, Victor NA, Wanderi EW, Lust WD, Landreth GE. Peroxisome proliferator-activated receptor- γ ligands reduce inflammation and infarction size in transient focal ischemia. *Neuroscience* 2005;130:685–696. [PubMed: 15590152]
17. Minge CE, Ryan NK, Van Der Hoek KH, Robker RL. Troglitazone regulates peroxisome proliferator-activated receptors and inducible nitric oxide synthase in murine ovarian macrophages. *Biol Reprod*. 2005 Sep 28;(Electronic publication ahead of print)
18. Camp HS, Li O, Wise SC, Hong YH, Frankowski CL, Shen X, Vanbogelen R, Leff T. Differential activation of peroxisome proliferators-activated receptor- γ by troglitazone and rosiglitazone. *Diabetes* 2000;49:539–547. [PubMed: 10871190]
19. Rosen ED, Spiegelman BM. PPAR γ : a nuclear regulator of metabolism, differentiation and cell growth. *J Biol Chem* 2001;276:37731–37734. [PubMed: 11459852]

20. Chawla A, Barak Y, Nagy L, Liao D, Tontonoz P, Evans RM. PPAR- γ dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. *Nat Med* 2001;7:48–52. [PubMed: 11135615]
21. Marx N, Sukhova G, Murphy C, Libby P, Plutzky J. Macrophages in human atheroma contain PPAR γ . Differentiation-dependent peroxisomal proliferator-activated receptor γ (PPAR γ) expression and reduction of MMP-9 activity through PPAR γ activation in mononuclear phagocytes in vitro. *Am J Pathol* 1998;153:17–23. [PubMed: 9665460]
22. Ricote M, Huang J, Fajas L, Li A, Welch J, Najib J, Witztum JL, Auwerx J, Palinski W, Glass WCK. Expression of the peroxisome proliferator-activated receptor gamma in human atherosclerosis and regulation in macrophages by colony stimulating factors and oxidized low density lipoprotein. *Proc Natl Acad Sci U S A* 1998;95:7614–7619. [PubMed: 9636198]
23. Tontonoz P, Nagy L, Alvarez JGA, Thomazy VA, Evans RM. PPAR- γ promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell* 1998;93:241–252. [PubMed: 9568716]
24. Chinetti G, Griglio S, Antonucci M, Torra IP, Delerive P, Majd Z, Fruchart JC, Chapman J, Najib J, Staels B. Activation of proliferator-activated receptors α and γ induces apoptosis of human monocyte-derived macrophages. *J Biol Chem* 1998;273:25573–25580. [PubMed: 9748221]
25. Schoonjans K, Peinado-Onsurbe J, Lefebvre AM, Heyman RA, Briggs M, Deeb S, Staels B, Auwerx J. PPAR α and PPAR γ activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *EMBO J* 1996;15:5336–5348. [PubMed: 8895578]
26. Galetto R, Albajar M, Polanco JI, Zakin MM, Rodriguez-Rey JC. Identification of a peroxisome-proliferator-activated-receptor response element in the apolipoprotein E gene control region. *Biochem J* 2001;357:521–527. [PubMed: 11439103]
27. Chinetti G, Lestavel S, Bocher V, Remaley AT, Neve B, Torra IP, Teissier E, Minnich A, Jaye M, Duverger N, Brewer HB, Fruchart J, Clavey V, Staels B. PPAR- α and PPAR- γ activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. *Nat Med* 2001;7:53–58. [PubMed: 11135616]
28. Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK. The PPAR- γ is a negative regulator of macrophage activation. *Nature* 1998;391:79–82. [PubMed: 9422508]
29. Hotta K, Gustafson TA, Yoshioka Y, Ortmeyer HK, Bodkin NL, Hansen BC. Relationships of PPAR γ and PPAR γ 2 mRNA levels to obesity, diabetes and hyperinsulinaemia in rhesus monkeys. *Int J Obes* 1998;22:1000–1010.
30. Tontonoz P, Hu E, Spiegelman BM. Stimulation of adipogenesis in fibroblasts by PPAR γ 2, a lipid-activated transcription factor. *Cell* 1994;79:1147–1156. [PubMed: 8001151]
31. Zhu Y, Qi C, Korenberg JR, Chen X, Noya D, Rao MS, Reddy JK. 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* 1995;92:7921–7925. [PubMed: 7644514]
32. Fajas L, Auboeuf D, Raspe E, Schoonjans K, Lefebvre A, Saladin R, Najib J, Laville M, Fruchart J, Deeb S, Vidal-Puig A, Flier J, Briggs MR, Staels B, Vidal H, Auwerx J. The organization, promoter analysis, and expression of the human PPAR γ gene. *J Biol Chem* 1997;272:18779–18789. [PubMed: 9228052]
33. Elbrecht A, Chen Y, Cullinan CA, Hayes N, Leibowitz MD, Moller DE, Berger J. Molecular cloning, expression and characterization of human peroxisome proliferator-activated receptors γ 1 and γ 2. *Biochem Biophys Res Commun* 1996;224:431–437. [PubMed: 8702406]
34. Fajas L, Fruchart J, Auwerx J. PPAR γ 3 mRNA: a distinct PPAR γ mRNA subtype transcribed from an independent promoter. *FEBS* 1998;438:55–60.13.
35. Lehmann JM, Lenhard JM, Oliver BB. Peroxisome proliferator-activated receptors α and γ are activated by indomethacin and other non-steroidal anti-inflammatory drugs. *J Biol Chem* 1997;272:3406–3410. [PubMed: 9013583]
36. Pontrelli L, Sidiropoulos KS, Adeli K. Translational control of apolipoprotein B mRNA: regulation via cis elements in the 5' and 3' untranslated regions. *Biochemistry* 2004;43:6734–6744. [PubMed: 15157107]
37. Longley DB, Steel DM, Whitehead AS. Posttranscriptional regulation of acute phase serum amyloid A2 expression by the 5'- and 3'-untranslated regions of its mRNA. *J Immunol* 1999;163:4537–4545. [PubMed: 10510397]

38. Myers SJ, Huang Y, Genetta T, Dingledine R. Inhibition of glutamate receptor 2 translation by a polymorphic repeat sequence in the 5'-untranslated leaders. *J Neurosci* 2004;24:3489–3499. [PubMed: 15071096]
39. Kodera Y, Takeyama K, Murayama A. Ligand type-specific interactions of peroxisome proliferator-activated receptor γ with transcriptional coactivators. *J Biol Chem* 2000;275:33201–33204. [PubMed: 10944516]
40. Narce M, Poisson JP. Novel PPAR- γ -dependent and independent effects of thiazolidinediones. *Curr Opin Lipidol* 2003;14:651–652. [PubMed: 14624145]
41. Hinz B, Brune K, Pahl A. 15-deoxy- $\Delta^{12,14}$ -prostaglandin J2 inhibits the expression of proinflammatory genes in human blood monocytes via a PPAR- γ -independent mechanism. *Biochem Biophys Res Commun* 2003;302:415–420. [PubMed: 12604364]
42. Nencioni A, Lauber K, Grunebach F, Brugger FW, Denzlinger C, Wesselborg S, Brossart P. Cyclopentenone prostaglandins induce caspase activation and apoptosis in dendritic cells by a PPAR- γ -independent mechanism: regulation by inflammatory and T cell-derived stimuli. *Exp Hematol* 2002;30:1020–1028. [PubMed: 12225793]
43. He G, Thuiller P, Fischer SM. Troglitazone inhibits cyclin D1 expression and cell cycling independently of PPAR γ in normal mouse skin keratinocytes. *J Invest Dermatol* 2004;123:1110–1119.
44. Wang M, Wise SC, Leff T, Su TZ. Troglitazone, an antidiabetic agent, inhibits cholesterol biosynthesis through a mechanism independent of peroxisome proliferator-activated receptor γ . *Diabetes* 1999;48:254–260. [PubMed: 10334298]
45. Moore KJ, Rosen ED, Fitzgerald ML, Randow F, Andersson LP, Altshuler D, Milstone DS, Mortensen RM, Spiegelman BM, Freeman MW. The role of PPAR- γ in macrophage differentiation and cholesterol uptake. *Nat Med* 2001;7:41–47. [PubMed: 11135614]
46. Li L, Beauchamp MC, Renier G. Peroxisome proliferator-activated receptor α and γ agonists upregulate human macrophage lipoprotein lipase expression. *Atherosclerosis* 2002;165:101–110. [PubMed: 12208475]
47. Gbaguidi FG, Chinetti G, Milosavljevic D, Teissier E, Chapman J, Olivecrona G, Fruchart JC, Griglio S, Fruchart-Najib J, Staels B. Peroxisome proliferator-activated receptor (PPAR) agonists decrease lipoprotein lipase secretion and glycated LDL uptake by human macrophages. *FEBS Lett* 2002;512:85–90. [PubMed: 11852057]
48. Ranganathan S, Kern PA. Thiazolidinediones inhibit lipoprotein lipase activity in adipocytes. *J Biol Chem* 1998;273:26117–26122. [PubMed: 9748292]
49. Michaud SE, Renier G. Direct regulatory effect of fatty acids on macrophage lipoprotein lipase: potential role of PPARs. *Diabetes* 2001;50:660–666. [PubMed: 11246888]
50. Ziouzenkova O, Perrey S, Asatryan L, Hwang J, MacNaul KL, Moller DE, Rader DJ, Sevanian A, Zechner R, Hoefler G, Plutzky J. Lipolysis of triglyceride-rich lipoproteins generates PPAR ligands: evidence for an anti-inflammatory role for lipoprotein lipase. *Proc Natl Acad Sci* 2003;100:2730–2735. [PubMed: 12606719]
51. Auwerx JH, Deeb S, Brunzell JD, Peng R, Chait A. Transcriptional activation of the lipoprotein lipase and apolipoprotein genes accompanies differentiation in some human macrophage-like cell lines. *Biochemistry* 1988;27:2651–2655. [PubMed: 3401441]
52. Mazzone T, Basheeruddin K, Poulos C. Regulation of human apolipoprotein E gene expression by cholesterol. *J Lipid Res* 1989;30:1055–1064. [PubMed: 2794789]
53. Akiyama TE, Sakai S, Lambert G, Nicol CJ, Matsusue K, Pimprale S, Lee YH, Ricote M, Glass CK, Brewer HB Jr, Gonzalez FJ. Conditional disruption of the peroxisome proliferator-activated receptor γ gene in mice results in lowered expression of ABCA1, ABCG1, and apoE in macrophages and reduced cholesterol efflux. *Mol Cell Biol* 2002;22:2607–2619. [PubMed: 11909955]

Fig. 1.
Structure of Monkey PPAR- γ isoforms and alignment of exons to human chromosome 3. (A) cDNA structures of seven PPAR- γ isoforms identified in monkey macrophages are shown. The ATG codon indicates the translation initiation site for each isoform. (B) Partial structure of human chromosome 3 with a blast analysis showing alignment of all monkey PPAR- γ exon sequences.

Fig. 2.

RT-PCR amplification of PPAR- γ isoforms using RNA from untreated macrophages. Total RNA isolated from THP-1 macrophages 48 h after differentiation in 200 nM phorbol 12-myristate 13-acetate (PMA) was used to synthesize cDNA. PCR amplification of isoform-specific DNA fragments was accomplished using specific primer pairs. (A) Location of primers used for PCR amplification of isoform specific transcripts is shown. Primers A, B, E and F are sense primers whereas primers C, D and I are antisense primers. These primer pairs will also amplify fragments of PPAR- γ 1, - γ 2 and - γ 3 that are not shown here. Expected sizes of DNA fragments are shown in Table 2. (B) Amplification of PPAR- γ 1, - γ 6 and - γ 7 using primer pairs A and I or B and I. Only PPAR- γ 1 fragment was amplified. (C) Amplification of PPAR- γ 2, - γ 4 and - γ 5 using primer pairs E and I or F and I. Only PPAR- γ 2 fragment was amplified. Marker lanes contain a 100-bp DNA ladder with the most intense band being that of 500 bp.

Fig. 3.

Identification of PPAR-γ5 and -γ7 transcripts in human THP-1 macrophages. Total RNA was isolated from untreated (control) or 10 μM 15d-PGJ₂-treated THP-1 macrophages. RT-PCR was performed as described using the indicated primer pairs. (A) Primer pairs E and I or F and I show the expression of PPAR-γ5 transcript after treatment with 15d-PGJ₂. (B) Primer pairs A and I or B and I show the expression of PPAR-γ7 transcript after treatment with 15d-PGJ₂. A 100-bp ladder was resolved in the marker lanes.

Fig. 4. Identification of transcripts for PPAR- γ 4 and exon C in human THP-1 macrophages. (A) Primers were designed as indicated in the schematic. Antisense primer CX-AS was used with sense primers CSA2, CSB, or CS to amplify PPAR- γ 4, PPAR- γ 6 or exon C, respectively. Expected sizes are listed in the table. (B) Total RNA was isolated from differentiated but untreated THP-1 macrophages and subjected to RT-PCR using the described primer pairs. The gel shows amplification of transcripts for PPAR- γ 4 and exon C but not of a transcript corresponding to PPAR- γ 6. A 100-bp ladder (marker) was used to determine the size of amplified fragments.

Fig. 5. Identification of full-length PPAR- γ transcript and protein isoforms in THP-1 macrophages. After differentiation in 200 nM PMA for 2 days, THP-1 macrophages were treated for 24 h with 10 μ M 15d-PGJ₂ or 10 μ M troglitazone. (A) Total RNA was isolated and processed for Northern blot analysis as described in Materials and methods. [³²P]-labeled exon D- or exon C-DNA probes were used to detect, respectively, full-length PPAR- γ 5/- γ 7 or PPAR- γ 4. A probe for β -actin was used to control for RNA loading. Quantitative image analysis was done using Image J (NIH) and is shown in the table alongside. The percent change in each transcript after treatment is calculated. (B) Total cell lysates were prepared and equal protein mass was subjected to Western blot analysis as described in Materials and methods. The anti-PPAR- γ antibody recognizes all three protein isoforms. A prestained molecular weight marker was run in an adjacent lane and the position of 50 kDa size marker is labeled. The expected molecular size for PPAR- γ 1, - γ 4 and - γ 2 are respectively, 54.5 kDa, 55.3 kDa and 57.6 kDa (Table 1).

Fig. 6. Differential regulation of PPAR- γ isoforms by 15d-PGJ₂ activation. Differentiated THP-1 macrophages were incubated for 24 h in the absence (control) or presence of the 10 μ M 15d-PGJ₂. Total RNA was isolated and subjected to RT-PCR using primer pair E and I to amplify PPAR- γ 5 and - γ 2, primer pair A and I to amplify PPAR- γ 7 and - γ 1 and primer pair CS and CX-AS to amplify PPAR- γ 4. β -actin was amplified using primers obtained from Promega. Results of relative quantitative analysis and percent change in each isoform after treatment are shown in the table.

Fig. 7.

Differential regulation of PPAR- γ isoforms by ligand activation. Differentiated THP-1 macrophages were incubated for (A) 24 h or (B) the indicated duration in the absence (control) or presence of 10 μ M Troglitazone or 10 μ M Wy14643 (A only). Total RNA was isolated and subjected to RT-PCR using isoform-specific primers as above. Quantitative analysis was done for (A) and the results are shown in the attached table.

Image Analysis

	C	P	P/C (%)	C	T	T/C (%)
apoE	125	202	162	147	237	161
LPL	249	208	84	247	213	86
β-actin	238	239	100	236	240	102

Fig. 8.

Regulation of apoE and LPL by PPAR- γ activation. Differentiated THP-1 macrophages were incubated for 24 h in the absence of ligands (control) or in the presence of 10 μ M 15d-PGJ₂ or 10 μ M Troglitazone. Total RNA was isolated and subjected to RT-PCR using primers specific for human apoE, LPL or β -actin as described in Materials and methods. Results of densitometric analysis using the Image J software are shown in the table with the percent change in expression level after ligand treatment. C=control, P=15d-PGJ₂ and T=Troglitazone.

Table 1

Major features of PPAR γ isoforms

Transcript Isoform	PPAR- γ 1	PPAR- γ 2	PPAR- γ 3	PPAR- γ 4	PPAR- γ 5	PPAR- γ 6	PPAR- γ 7
Protein Isoform	PPAR- γ 1	PPAR- γ 2	PPAR- γ 1	PPAR- γ 4	PPAR- γ 1	PPAR- γ 4	PPAR- γ 1
Polypeptide (aa)	475	505	475	483	475	483	475
MW (kDa)	54.5	57.6	57.6	55.3	54.5	55.3	54.5
cDNA length(bp)	1800	1765	1713	2028	1849	2063	1884
ORF(bp)	1425	1515	1425	1449	1425	1449	1425
Accession no.	AY048694	AY048695	AY048696	AY048697	AY048698	AY048699	AY048700

Table 2
Primers used for isoform-specific PCR amplification

Sense primer	Antisense primer	Transcript amplified	Expected size of PCR product (bp)
A	I	PPAR- γ 1	190
cctttacctctgctggtgac	ccaaagttggtgggccagaatg	PPAR- γ 6	453
		PPAR- γ 7	274
B	I	PPAR- γ 1	185
acctctgctggtgacaagaaggc	ccaaagttggtgggccagaatg	PPAR- γ 6	448
		PPAR- γ 7	269
E	I	PPAR- γ 2	155
agcaaaccctattccatgct	ccaaagttggtgggccagaatg	PPAR- γ 4	418
		PPAR- γ 5	239
F	I	PPAR- γ 2	130
tgggtgaaactctgggagattctcc	ccaaagttggtgggccagaatg	PPAR- γ 4	393
		PPAR- γ 5	214
CS	CX-AS	Exon C	251
tcaggatgattctgtctctgg	gcctttcataggtcctttgtagtt		
CSA2	CX-AS	PPAR- γ 6	300
tacagcaagaagccatttctca	gcctttcataggtcctttgtagtt		
CSB	CX-AS	PPAR- γ 4	297
gcgattccttactgataactgt	gcctttcataggtcctttgtagtt		
DS	D	Exon D	82
tagagactgggttcacatggtg	taatcccagcactttgggaggccg		