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Binding of prostaglandins to human PPARγ: tool assessment and new natural ligands

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Abstract

The peroxisome proliferator-activated receptors (PPAR) form a family of nuclear receptors with a wide variety of biological roles from adipogenesis to carcinogenesis. More ligands (agonist and antagonist) are needed to explore the multiple functions of PPAR, particularly PPARγ. In order to complete such ligand screening, a binding test should be assessed versus the classical transactivation reporter gene assay. In the present work, the full-length human PPARγ protein as well as its ligand binding domain portion were expressed in *Escherichia coli*. Bacterial membrane preparations expressing those constructs were characterized using a classical binding competition assay [^{3}H]rosiglitazone as the radioligand. When the receptor preparations were soluble, binding had to be measured with a new alternative method. The systems were assessed using a series of reference PPAR (α, β and γ) ligands. The full-length human PPARγ fused to glutathione-S-transferase, expressed in *E. coli* and tested as a bacterial membrane-bound protein led to the most accurate results when compared to the literature. Furthermore, in an attempt to complete the panel of natural PPARγ ligands, 29 commercially available prostaglandins were screened in the binding assay. Prostaglandins H_1 and H_2 were found to be modest ligands, however as potent as $15\Delta^{12-14}$ prostaglandin J_2. These results were confirmed in the classical transactivation assay. The fact that these three prostaglandins were equally potent, suggests new pathways of PPARγ-linked gene activation.

Keywords: PPARγ; Prostaglandin H_1; Prostaglandin H_2; Glutathione-S-transferase fusion protein; Transactivation; PPARγ ligand, naturally occurring

1. Introduction

The peroxisome proliferator-activated receptor-γ (PPARγ) is a ligand-dependent nuclear receptor and a member of the steroid receptor superfamily (Issemann and Green, 1990; Schoonjans et al., 1997). PPARγ binds to its cognate DNA-response elements as an heterodimer with at least one other member of the steroid receptor superfamily, the retinoid acid receptor (RXRα) (Kliewer et al., 1992; Schulman et al., 1998). Activation of PPARγ has been linked to adipocyte differentiation (Tontonoz et al., 1994a), regulation of glucose homeostasis in rodents and humans (Spiegelmann, 1998) and enhancement of the insulin sensitivity in diabetic animals (Mukherjee et al., 1997). PPARγ is expressed at high levels in adipocyte tissue (Tontonoz et al., 1994b; Chawla et al., 1994).

Recent evidences also showed that ligands of PPARγ inhibit macrophage and monocyte activation (Ricote et al., 1998; Jiang et al., 1998), suppress tumor cell growth (Elstner et al., 1998; Sarraf et al., 1998; Suh et al., 1999), inhibit angiogenesis in vitro and in vivo (Xin et al., 1999) and induce terminal differentiation of human liposarcoma cells (Tontonoz et al., 1997).

PPARγ ligands range into two categories: (1) fatty acid-derived natural compounds, such as eicosanoid derivatives including prostaglandins, particularly 15-deoxy-$\Delta^{12,14}$ – prostaglandin J_2 (Yu et al., 1995; Kliewer et al., 1995; Forman et al., 1995), hydroxyeicosatetraenoic acid (Forman et al., 1997), fatty acids (Vanden Heuvel, 1999; Krey et al., 1997; Xu et al., 1999), all presenting affinities to PPARγ in the micromolar range and (2) synthetic compounds such as thiazolidinediones which are potent agonists with K_d in the nanomolar range, such as

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pioglitazone (Lehmann et al., 1995), the new ligand GW7845 [3-[3-[1-(*N*-Isopropyl-*N*-phenyl-carbamoylmethyl)-2,4-dioxo-5-phenyl-2,3,4,5-tetrahydro-1*H*-1,5-benzodiazepin-3-yl]ureido] benzoic acid] (Cobb et al., 1998) or poorer ligands such as NSAIDS (Lehmann et al., 1997).

Classical competition radioreceptor assays were successfully used to identify PPARγ ligands (Berger et al., 1996; Brown et al., 1997; Mais et al., 1997;Young et al., 1998; Cobb et al., 1998; Mukherjee et al., 2000). In addition, several new techniques were developed for the same purpose, such as: (1) a protease protection assay relying on a ligand-dependent reduced sensitivity of PPARγ to enzymatic proteolytic cleavage (Dowell et al., 1997; Leid, 1994); (2) a co-activator-dependent receptor ligand assay (CARLA) based on the hypothesis that ligand binding to PPARγ induces interactions of the receptor with transcriptional activators (Krey et al., 1997) and (3) a scintillation proximity assay (Nichols et al., 1998) which classically takes advantage of the fluorescence of the streptavidin-modified scintillation proximity beads on which the biotin-labeled ligand binding domain was immobilized and a fluorescence induced upon binding of [^{3}H]rosiglitazone to the ligand binding domain attached to beads.

Surprisingly, a complete and systematic measurement of prostaglandin binding on PPARγ is still lacking. Indeed, the initial description of Kliewer et al. (1995) of the agonistic effect of a prostaglandin J_2 metabolite on PPARγ was based on previous observations (Göttlicher et al., 1992; Keller et al., 1993; Banner et al., 1993) that arachidonate products could be natural ligands of PPARγ, by analogy to the arachidonic acid-related compound, 5,8,11,14-eicosatetraynoic acid. Systematic screening of other prostaglandins binding on PPARγ has been poorly reported, while reporter gene assays have been widely used for this purpose (Lehmann et al., 1997; Yu et al., 1995; Krey et al., 1997). These binding studies have been performed using different cloned PPARγ preparations (fused to glutathione-S-transferase and/or purified) and/or using the full-length PPARγ or only its ligand binding domain. They were also carried out with a wide variety of radioactive ligands. The variety of these assays makes accurate comparison between published data practically impossible.

In the present report, we tested several constructs expressing in bacteria various portions of the PPARγ. When the whole PPAR protein or the ligand binding domain is soluble, a classical filtration binding assay cannot be used. We, therefore, assessed a new technique for binding measurement using an original gel-sizing approach on a 96-well plate format. Using these tools, a complete binding survey of classical PPAR ligands was performed as well as a systematic study of prostaglandin binding. Prostaglandins H_1 and H_2 were found active in addition to the already described natural ligand, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2. All these observations were confirmed using the cell-based transactivation assay.

2. Materials and methods

2.1. Compounds

All regular chemicals were obtained at the highest purity grade available from Sigma. Prostaglandins were obtained from Cayman Chemicals (SpiBio, France).

2.2. Antibody against ligand binding domain and full-length PPARγ

Rabbit polyclonal antisera were raised against either a peptide derived from the ligand binding domain (SDIDQLNPESADLRC) or a peptide derived from a non-ligand binding domain sequence (DSPIDPESDSFTD) of the human PPARγ sequence. The first sequence was checked for its uniqueness in the PPARγ protein using Blast (peptide sequence against nucleotide non-redundant database). Rabbit (×2) immunisation was performed using keyhole limpet hemocyanin-coupled peptides, once with 200 μg of antigen injected in Freund complete adjuvant followed by three injections (days 14, 28 and 42) of antigen (200 μg) in Freund incomplete adjuvant. Sera titers evaluated by ELISA with plastic coated peptide were 1/5000 and 1/10,000, respectively. Those antibodies are referred to in the following text as anti-ligand binding domain and anti-PPARγ antibodies.

2.3. Cloning, expression and production of glutathione-S-transferase-PPARγ ligand binding domain and glutathione-S-transferase-PPARγ

cDNA encoding human ligand binding domain (from amino acid 200 to 506) and full-length PPARγ2 were amplified by polymerase chain reaction using primers chosen according to the published sequence (Accession number U63415). The resulting PCR products were digested by *Bam*HI and *Sal*I for the human PPARγ ligand binding domain and by *Sal*I and *Not*I for the complete PPARγ. The cDNA were then inserted into the bacterial expression vector pGEX-5X1 (Pharmacia, Les Ulis, France) for expression as glutathione-S-transferase fusion protein. The *Escherichia coli* strain BL21(DE3)pLysS was transformed with resulting plasmids and grown overnight at 37°C in Luria Broth supplemented with ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml). The culture was diluted 1:25 in fresh medium and grown at 37°C, until it reached a $D.O._{595}$ of 0.7. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was then added to a final concentration of 0.2 mM and the culture maintained at 24°C for 22 h to reach a $D.O._{595}$ of 8. The cells were harvested by centrifugation (5000 × *g*, 4°C, 10 min), homogenized with phosphate buffer saline (2×) containing 10 mM dithiothreitol pH 7.4, frozen in dry ice and stored at −80°C until further use.

2.4. Purification and cleavage of glutathione-S-transferase-PPARγ-ligand binding domain and glutathione-S-transferase-PPARγ

All the procedures were performed at 0–4°C. Approximately 10 g of a frozen bacteria pellet expressing glutathione-S-transferase-ligand binding domain or glutathione-S-transferase-PPARγ was thawed in 40 ml of a 20 mM Tris, pH 8.2 buffer containing 500 mM KCl, 2 mM dithiothreitol, a cocktail of protease inhibitors (Complete, Boeringher Mannheim, 1 tablet/50 ml) and 2% (v/v) of Triton X-100. The thawed bacterial suspension was heated at 37°C then frozen down in liquid nitrogen three times. After the first of these three cycles, the preparation was incubated at 4°C for 30 min and centrifuged (50,000 × *g*, 30 min). For the second and third extractions, the pellets were suspended in 44 ml of a 10 mM Tris buffer, pH 8.2 containing 250 mM KCl, 1 mM dithiothreitol, 1% (v/v) Triton X-100 and the protease inhibitor cocktail. The final pellet was adjusted to 4 mg/ml of protein. This suspension was solubilized with 0.2% *N*-lauroylsarcosine for 1 h at 4°C and centrifuged (100,000 × *g*, 60 min). The supernatant was diluted four times and dialyzed against 10 l of 10 mM Tris, pH 8.2 buffer containing 50 mM KCl, and 1 mM dithiothreitol. The supernatant was passed (40 ml/h) through a glutathione column (Pharmacia, 10-ml-packed volume), equilibrated in 10 mM Tris, pH 8.2 containing 50 mM KCl and 10 mM dithiothreitol. The column was washed three times with 40 ml of the same buffer. The fusion proteins were eluted with 40 ml of 50 mM glutathione in the washing buffer.

The eluted fractions containing glutathione-S-transferase-ligand binding domain were dialyzed against 10 l of 50 mM Tris, pH 8.0 buffer containing 1 mM dithiothreitol, 150 mM NaCl and 1 mM $CaCl_2$, overnight, using Slide-A-Lyser (Pierce Chemicals, Interchim, Montluçon, France) to remove free glutathione. The dialyzed sample was then incubated (4°C, 8h) with factor Xa (Boehringer Mannheim, 10 U/ml final). The fractions were assayed for binding and those containing the [^{3}H]rosiglitazone binding capacity were pooled and stored at −80°C. The protein concentration was determined by the Bradford assay (Protassay, BioRad, Ivry s/Seine, France) with bovine serum albumin as standard. The extraction and cleavage of glutathione-S-transferase-PPARγ was the same as above.

2.5. Membrane-bound PPARγ binding assay

The association rate of [^{3}H]rosiglitazone (40 nM) binding was determined using parallel sets of tubes in which a

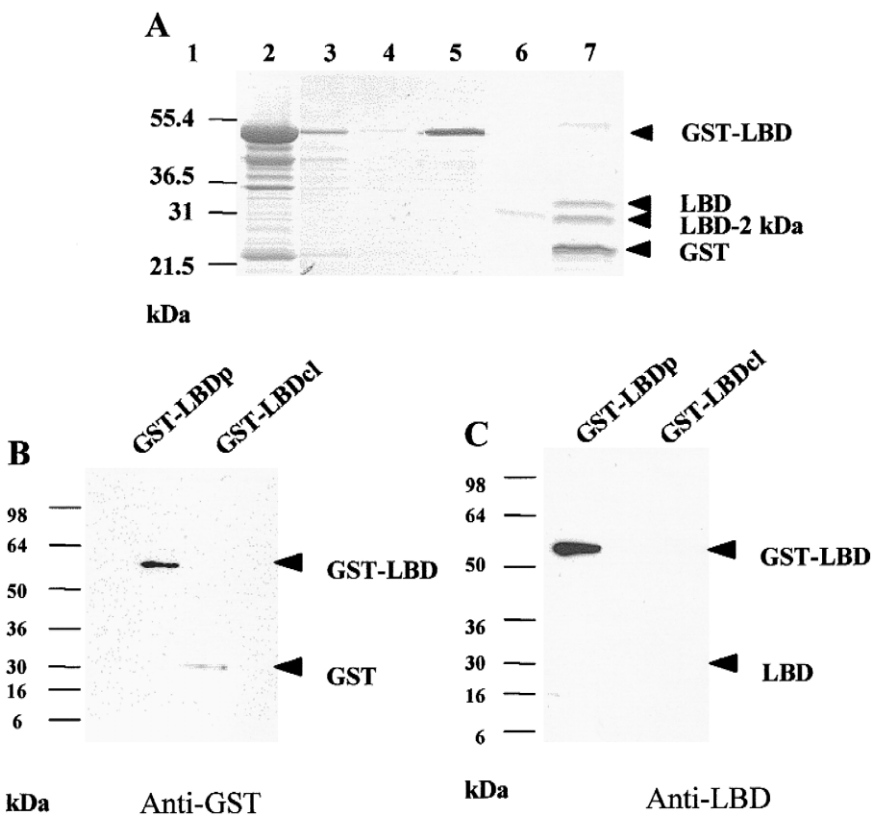

Fig. 1. Characterization of affinity purified human glutathione-S-transferase-ligand binding domain after *N*-laurylsarcosine (0.1%) extraction from *E. coli*. SDS-polyacrylamide gel electrophoresis was performed on samples from the various stages of the purification process. (A) The gel was stained by Coomassie Blue. (1) Molecular weight markers; (2) total bacteria homogenate; (3) five-fold diluted total homogenate; (4) GSH-agarose column flow-through; (5) elution by 50 mM GSH; (6) factor Xa cleavage; (7) cleaved glutathione-S-transferase-ligand binding domain. Western blot analyses with either anti-glutathione-S-transferase antibodies (B) and anti-ligand binding domain antibodies (C) were performed. Blots were developed using an enhanced chemiluminescence kit (Amersham).

concentration range of membrane preparations (9–800 μg) was incubated for 180 min at 4°C in the presence (non-specific binding) or absence (total binding) of an excess of unlabelled pioglitazone (100 μM) in the assay buffer. Specific binding was defined as the difference between total and non-specific binding at each time point. Incubation was terminated by the addition of ice cold 50 mM Tris/HCl buffer pH 8.0 followed by rapid filtration under reduced pressure through Whatman GF/C filters. The filters were washed twice rapidly with 1 ml of ice-cold buffer, dried and treated with the liquid scintillation cocktail for measurement of the radioactive content.

2.6. Solubilized or purified PPARγ gel filtration binding assay

Twenty microliters of assay buffer were mixed with 20 μl of 40 nM [^{3}H]rosiglitazone and 160 μl of 1.25 nM protein target and incubated in 96-well plates for 3 h at 4°C. One-hundred-microliter aliquots of each well was simultaneously loaded on an equilibrated 96-well gel filtration plate (Multiscreen, Millipore, St Quentin en Yveline, France) filled with Sephadex G25 fine (Pharmacia-Biotech) previously equilibrated in starting buffer. The separation plate assembled with a collector microtiter plate (Optiplate, Packard), was centrifuged at $1250 \times g$ for 5 min. Two hundred microliters of scintillation fluid was added to each recovery well, and the plates were sealed and allowed to equilibrate for 20 h before counting in a 96-well filter plate counter (Topcount-NXT, Packard). Non specific binding was determined in the presence of 100 μM unlabeled pioglitazone. Calibration and assessment are described in Section 3.

2.7. Saturation and displacement experiments

Saturation experiments (4–150 nM [^{3}H]rosiglitazone were performed in parallel sets of tubes (each set in triplicate) in which a fixed amount of membrane was incubated in the presence (non-specific binding) or absence (total binding) of 100 μM unlabeled pioglitazone. Displacement studies were performed by adding increasing concentrations of the displacing compound to a set of tubes (in triplicate) containing [^{3}H]rosiglitazone (40 nM) and a fixed amount of membrane preparation. Incubations for saturation and displacement studies were done in a final volume of 500 μl for membrane studies (classical filtration assay) and 200 μl for the purified protein preparation (gel-filtration assay), for 180 min at 4°C. The incubation was terminated as described above.

2.8. Analytical electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (1970) followed by Coomassie Blue staining. Immunoblotting was carried out using standard procedures and blots were developed using an enhanced chemiluminescence kit (Amersham) with a goat anti-glutathione-S-transferase polyclonal antibody as described by the manufacturer (Pharmacia-Biotech).

2.9. Transactivation assay

Full-length cDNAs for hPPARγ2 and hRXRα, (accession number X52773) were amplified by polymerase chain reaction and subcloned into an Okayama and Berg expression vector (pSR) to generate pSR/hPPARγ2 and pSR/hRXRα, respectively. The reporter plasmid, which comprises the peroxisome proliferator responsive element, the thymine kinase promoter and luciferase (PPRE-Tk-Luc) and the Tk-Luc control plasmid were kindly provided by Dr. Ronald M. Evans. CV1 cells seeded at 15.10^6 cells per 225-cm^2 flasks in Dulbecco's minimal essential medium (DMEM) were transfected with 1.5 μg per million cells of an equal mixture of the pSR/hPPARγ2, pSR/hRXR and either PPRE-Tk-Luc or Tk-Luc plasmids using lipofec-

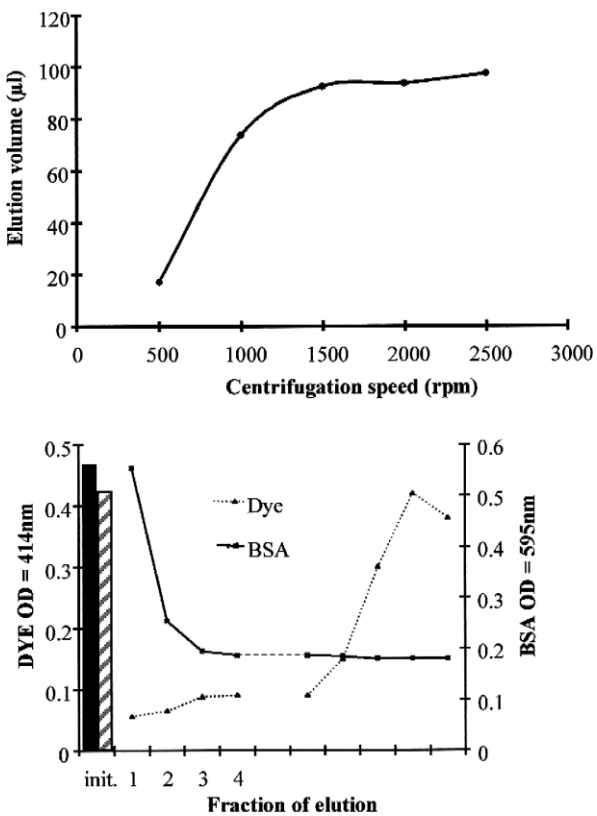

Fig. 2. Assessment of the original binding assay for soluble PPARγ or PPARγ-ligand binding domain ligands. The technique is based on gel filtration in 96-well-column plates filled with Sephadex G25 medium. The upper panel described the elution volume as a function of centrifugation speed. Optimal volume recovery was found for 1500 rpm. The lower panel describes the separation of a low molecular weight dye, methyl red, from bovine serum albumin (BSA). A minimal percentage of dye is eluted together with bovine serum albumin in the first fractions.

Table 1
Binding characterization of different ligand binding domain and full length PPARγ preparation

Products	GST-LBD	GST-LBD Purified	LBD	GST-PPARγ	PPARγ	Reference values
K_d (nM)						
Rosiglitazone	13 ± 2.4	105 ± 21	156 ± 12	20 ± 0.7	93 ± 7	3–40 (Lehmann et al., 1995; Kliewer et al., 1995; Young et al., 1998; Mais et al., 1997)
K_i (μM)						
Rosiglitazone	0.47 ± 0.03	0.06 ± 0.02	0.09 ± 0.01	0.043 ± 0.002	0.05 ± 0.005	0.04 (Nichols et al., 1998)
Pioglitazone	4.7 ± 1.7	3.1 ± 0.8	0.9 ± 0.0	0.86 ± 0.12	3.2 ± 0.5	4.8 (Young et al., 1998)
$15\Delta^{12,14}$Prostaglandin J2	13.03 ± 0.4	3.7 ± 1.4	11.6 ± 0.1	4.05 ± 0.55	12.3 ± 2.7	2.5 (Kliewer et al., 1995)
Bezafibrate	> 100	> 100	> 100	> 100	> 100	(PPARα ligand)
Linolenic acid	19.2 ± 1.1	3.7 ± 1.3	99.9 ± 6.8	> 100	53.7 ± 35.8	1.7–17 (Kliewer et al., 1997)
Indomethacin	53 ± 4.1	34 ± 1.9	42 ± 1.7	38 ± 3.2	37 ± 4.5	50 (Lehmann et al., 1997)

4–150 nM [^{3}H]rosiglitazone were performed in parallel sets of tube (each set in triplicate) in which a fixed amount of membrane or receptors was incubated in the presence (non-specific binding) or absence (total binding) of various concentrations of the unlabeled non specific ligand (pioglitazone). Incubation for saturation and displacement studies were done in a final volume of 500 μl for membrane studies and 200 μl for purified preparation, respectively, for a period of 180 min at 4°C.

tamine plus (Life Technologies) as described by the manufacturer. Twenty-four hours after the beginning of the transfection, the cells were detached using trypsin/EDTA, resuspended in DMEM phenol red free medium supplemented with 2% fetal calf serum and seeded as 50-μl aliquots into white 96-well tissue culture plates containing the drugs previously distributed at 50 μl/well in the same medium. After a 20-h incubation period at 37°C in a 5% CO_2 atmosphere, 100 μl of luclite buffer (Packard) were added to each well for 30 min in the dark at room

Table 2
Binding characterization of different ligand binding domain and full length PPARγ preparation

Products	GST-LBD	GST-LBD Purified	GST-PPARγ	Reference values
K_d (nM)				
Rosiglitazone	13 ± 2.4	105 ± 21	20 ± 0.7	14–40 (Lehmann et al., 1995; Kliewer et al., 1995; Young et al., 1998; Mais et al., 1997)
K_i (μM)				
Rosiglitazone	0.47 ± 0.031	0.060 ± 0.018	0.043 ± 0.002	0.04 (Nichols et al., 1998)
Pioglitazone	4.7 ± 1.7	3.1 ± 0.8	0.86 ± 0.12	4.8 (Young et al., 1998)
Ciglitazone	15 ± 3	12 ± 0.6	8 ± 0.3	?
WY 14643	47 ± 7	28.1 ± 8.7	25 ± 3.1	100 (Lehmann et al., 1997)
Prostaglandin J_2	30.9 ± 9.0	35.2 ± 3	37 ± 3.3	40 (Kliewer et al., 1995)
15ΔProstaglandin J_2	13.03 ± 0.4	3.7 ± 1.4	4.05 ± 0.55	2.5 (Kliewer et al., 1995)
8-[±]-HETE	32.6 ± 9.0	1.8 ± 0.4	1.9 ± 0.5	?
all trans-retinoic acid	52.0 ± 12.3	97.0 ± 0.0	> 100	> 100 (Kliewer et al., 1992)
Bezafibrate	> 100	> 100	> 100	?
Clofibrate	> 100	> 100	> 100	?
Linoleic acid	21.2 ± 1.7	4.9 ± 2.1	15.1 ± 2	1.7–17 (Kliewer et al., 1997)
Linolenic acid	19.2 ± 1.1	3.7 ± 1.3	> 100	1.7–17 (Kliewer et al., 1997)
Indomethacin	53 ± 4.1	34 ± 1.9	38 ± 3.2	50 (Lehmann et al., 1997)
ETYA	26.4 ± 7	3 ± 0.5	12 ± 0.5	2.4 (Young et al., 1998)

4–150 nM [^{3}H]rosiglitazone were performed in parallel sets of tube (each set in triplicate) in which a fixed amount of membrane or receptors was incubated in the presence (non-specific binding) or absence (total binding) of various concentrations of the unlabeled non specific ligand (pioglitazone). Incubation for saturation and displacement studies were done in a final volume of 500 μl for membrane studies and 200 μl for purified preparation, respectively, for a period of 180 min at 4°C.

temperature and the luciferase activity was read in a Packard Topcount.

3. Results

3.1. Production, purification and cleavage of glutathione-S-transferase-ligand binding domain

The expression of the glutathione-S-transferase-ligand binding domain encoding gene in *E. coli* resulted in the production of active, poorly soluble glutathione-S-transferase-ligand binding domain (> 10 mg/l of culture). As a first step towards purification, two types of extraction (sonication or heat shock) were tested. Heat shock was selected because less proteins were obtained (187 mg versus 477 mg) with higher specific binding (B_{max}: 50500 versus 30400 fmol/mg proteins using sonicated extracts) and similar K_D: 54 versus 57 nM. As a second step towards purification, the solubilization of glutathione-S-transferase-ligand binding domain was attempted with 14 different detergents (deoxycholate, *N*-laurylsarcosine, sodium dodecylsulfate, Triton X-114, Triton 770, Triton X-405, Tween-20 and -85, octyl-β-glucoside, Brij 35, Lubrol, Igepal, CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1 propane-sulfonate) and Nonidet P40) at two concentrations of 1% and 0.1%. The amount of proteins solubilized during those experiments was evaluated by electrophoresis on SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. Two anionic detergents (Triton 770 and *N*-laurylsarcosine) were selected for their capacity to solubilize glutathione-S-transferase-ligand binding domain. The binding of the resulting solubilized membranes to affinity (glutathione) chromatography was tested. Only the solubilization with *N*-laurylsarcosine led to a fused protein recognizing glutathione agarose in affinity chromatography after extensive dialysis and dilution.

The partial purification of the glutathione-S-transferase-ligand binding domain solubilized by *N*-laurylsarcosine was achieved with a glutathione affinity chromatography developed under standard conditions (50 mM glutathione for elution). A 58-kDa band was identified by SDS-polyacrylamide gel electrophoresis and Western blot (Fig. 1) as pure glutathione-S-transferase-ligand binding domain fused protein. This glutathione-S-transferase-ligand binding domain protein was cleaved by factor Xa to give both a double band (30 and 32 kDa) corresponding to the ligand binding domain and a 26-kDa band for glutathione-S-transferase (Fig. 1A). The light double band corresponding to the ligand binding domain was probably due to a minimal proteolytic process. Western blot analysis of pure and cleaved glutathione-S-transferase-ligand binding domain with anti-glutathione-S-transferase (Fig. 1B) and anti-PPARγ antibodies (Fig. 1C) confirmed those identities. Purity was superior to 90% as determined by densitometric scanning of the 10% SDS-polyacrylamide gel electrophoresis stained with Coomassie blue.

3.2. Binding assays for the different ligand binding domain preparations

Two analytical methods were tested: a classic filtration for glutathione-S-transferase-ligand binding domain embedded in bacterial membranes and an original 96 well plate method for solubilized or purified proteins. For the latter, a dry Sephadex G25 (Pharmacia) gel filtration

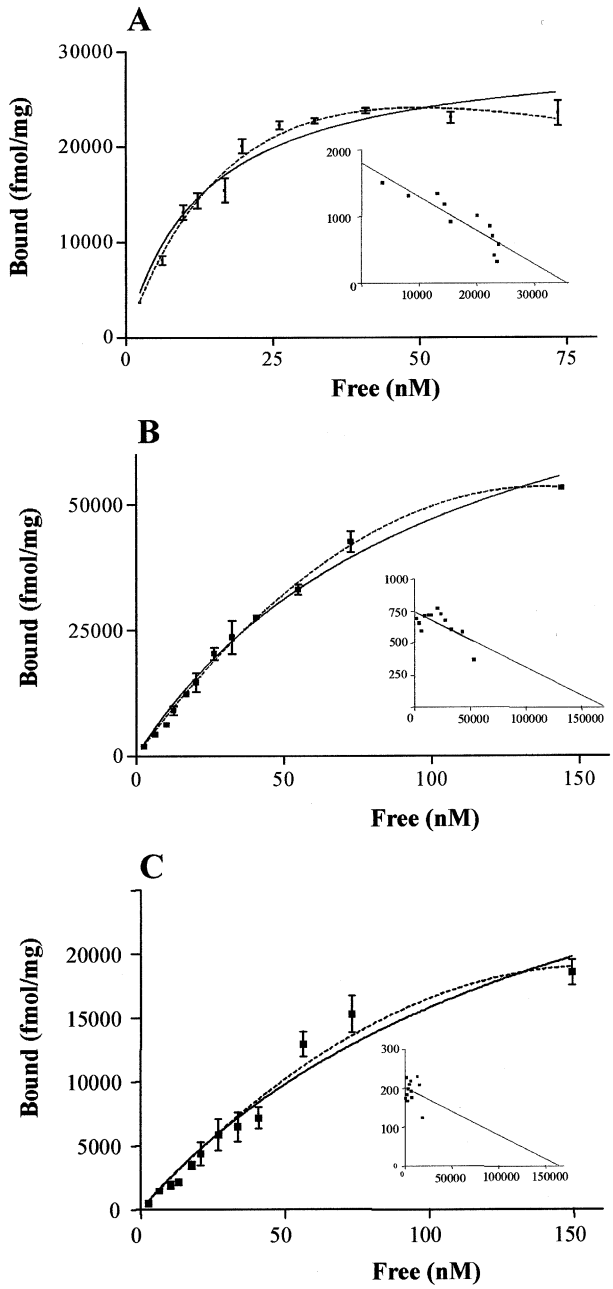

Fig. 3. Scatchard plot analysis of glutathione-S-transferase-ligand binding domain at various stages of the purification protocol using [^{3}H]rosiglitazone as radioligand. (A) bacterial membranes (K_d = 13 nM, B_{max} = 30,000 fmol/mg); (B) glutathione-S-transferase-ligand binding domain (K_d = 105 nM, B_{max} = 97,000 fmol/mg); (C) cleaved pure ligand binding domain (K_d = 156 nM, B_{max} = 40,000 fmol/mg). Inserts: *x*-axis: bound (fmol/mg); *y*-axis: Bound/free (fmol/mg/nM).

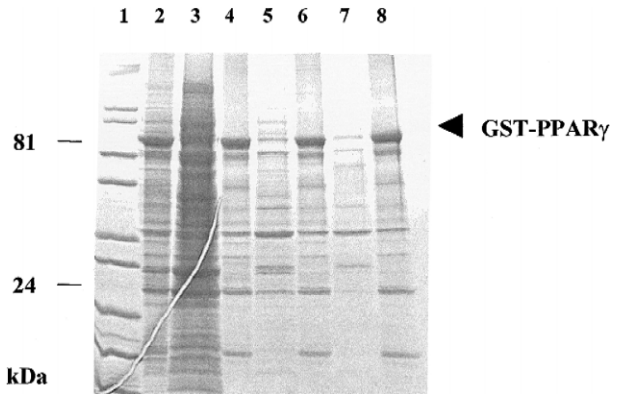

Fig. 4. Extraction procedure for the solubilization of glutathione-S-transferase-PPARγ expressed in *E. coli*. SDS-polyacrylamide gel electrophoresis was performed on samples from the various stages of the purification process. The gel was stained by Coomassie Blue. (1) molecular weight markers; (2) total homogenate of the bacteria pellet; (3) supernatant after 1st extraction with Triton X-100 (0.1%); (4) pellet after the first extraction with Triton X-100 (0.1%); (5) supernatant after the second extraction with Triton X-100 (0.1%); (6) Pellet after the second extraction with Triton X-100 (0.1%); (7) supernatant after the 3rd extraction with Triton X-100 (0.1%); (8) Pellet after the third extraction with Triton X-100 (0.1%).

medium was loaded, hydrated and equilibrated in a 96-well plate. To calibrate the centrifugation speed, a mixture of 100 μl of bovine serum albumin (67 kDa) and methyl red (0.3 kDa) was applied on the 100-μl column top. Following the application of 100 μl of the mixture onto the columns, a collecting plate was attached to the column block and centrifuged. A maximal recovery was observed for the ideal centrifugation speed of 1500 rpm (see left panel of Fig. 2). The experiment was repeated until all the dye applied initially on the column was recovered. The protein with a minor contaminant from the dye was in the first fraction (as shown on the right panel of Fig. 2) while the dye was totally eluted from the column between the 13th to 16th fractions. This method made possible the measurement of binding affinities using different preparations of receptor in solution (purified glutathione-S-transferase-ligand binding domain, pure ligand binding domain and pure PPARγ) while for the other membrane-associated preparations, a standard filtration assay was used. The methodology implies a single centrifugation during which the proteins labeled with the radioligand [^{3}H]rosiglitazone are excluded. As shown on Tables 1 and 2, the results

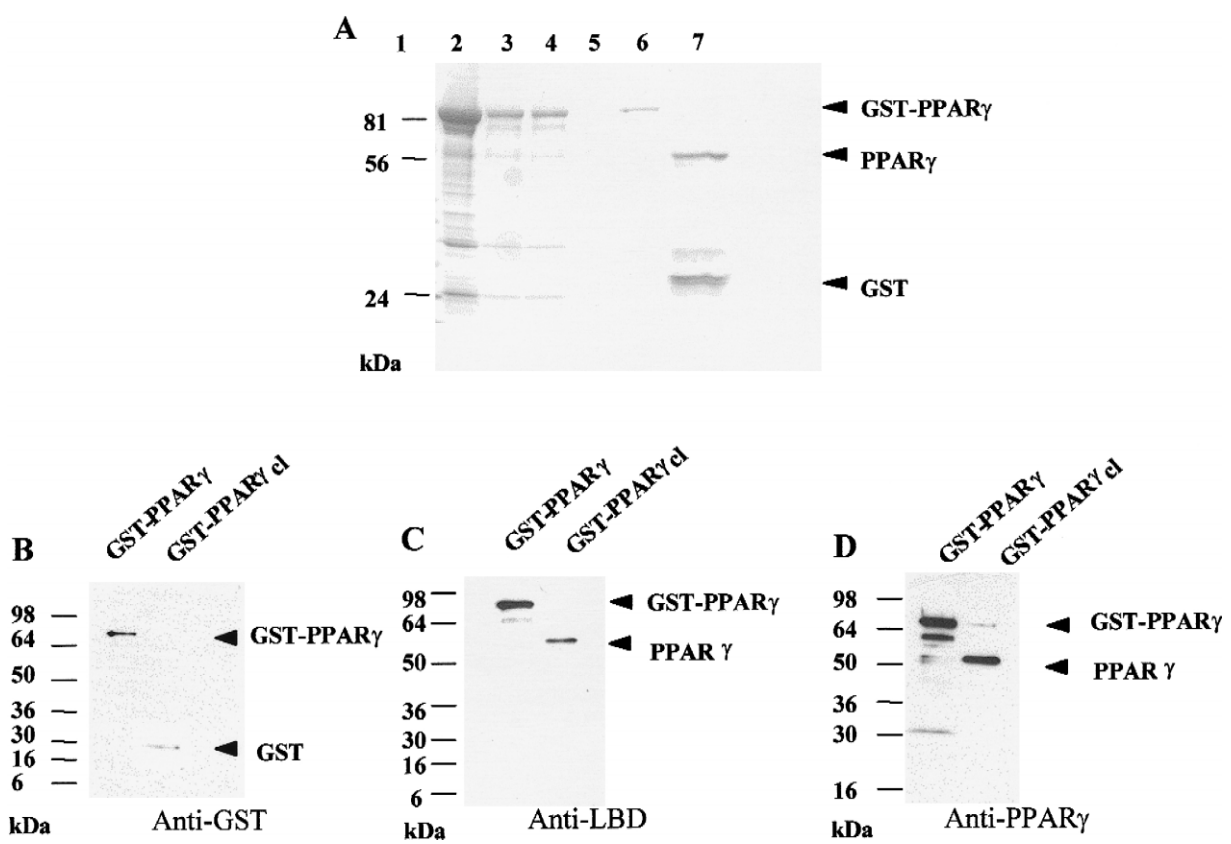

Fig. 5. Characterization of affinity purified human glutathione-S-transferase-PPARγ after *N*-laurylsarcosine pellet solubilisation (0.1%) from *E. coli*. (A) After separation by SDS-polyacrylamide gel electrophoresis , the gel was stained by Coomassie Blue. (1) molecular weight markers; (2) third extraction Triton X-100, solubilized by *N*-lauroylsarcosine (see Fig. 4); (3) five-fold diluted homogenate; (4) flow-through; (5) elution by GSH 50 mM; (6) factor Xa cleavage; (7) cleaved glutathione-S-transferase-ligand binding domain. Then, after transfer, Western blot analyses with either anti-glutathione-S-transferase antibodies (B), anti-ligand binding domain antibodies (C), anti-PPARγ (D) were performed. Blots were developed using an enhanced chemiluminescence kit (Amersham).

obtained with this method compared well with those of the classical binding assay and with the available literature, despite a 10-fold shift of K_i, which seems to be due to the use of the soluble forms of the receptors.

3.3. Ligand binding on the different forms of ligand binding domain

An optimal concentration of proteins was evaluated for the three preparations, membrane-bound glutathione-S-transferase-ligand binding domain, purified glutathione-S-transferase-ligand binding domain and purified ligand binding domain proteins: 70, 70, and 80 μg/ml, respectively. In all of these preparations, the detergent concentrations were far less than the critical micellar concentrations. Therefore, no interference with detergent micelles could be implicated in these measurements. Kinetic data revealed a plateau at 30 min which remained stable for 24 h (not shown). All the experiments were performed with 40 nM of [^{3}H]rosiglitazone for 1 h. The concentration of cold pioglitazone was 100 μM. The characterization of these preparations was by Scatchard plots revealed a 13 nM K_d and a B_{max} of 30 pmol/mg of proteins for the glutathione-S-transferase-ligand binding domain in bacterial membranes (Fig. 3A), a 105 nM K_d and a B_{max} of 97 pmol/mg protein for the purified glutathione-S-transferase-ligand binding domain (Fig. 3B) and a 156 nM K_d and a B_{max} of 40 pmol/mg proteins for the ligand binding domain (Fig. 3C). As a control, pure glutathione S-transferase (Pharmacia) was used in the [^{3}H]rosiglitazone-binding assay. No binding could be recorded using the pure enzyme, ruling out a possible interference in the [^{3}H]rosiglitazone binding measurement.

3.4. Extraction and purification of the full-length glutathione-S-transferase-PPARγ

E. coli transfected with glutathione-S-transferase-PPARγ produced a protein that was extracted from the bacterial homogenate after 3 heat shocks in the presence of 2% (first extraction) and 1% (second extraction) of Triton X-100. This solubilization step was very powerful in terms of protein extraction. Indeed, as can be seen from Fig. 4, the pellet obtained after solubilization contained mainly the fused protein. Glutathione-S-transferase-PPARγ solubilization from this pellet was obtained in the presence of 0.2% of *N*-lauroylsarcosine. Nevertheless, after dilution and dialysis, part of the protein precipitated while the remaining solubilized proteins were not retained on glutathione affinity chromatography probably due to the anionic nature of this detergent (data not shown). Dilution and dialysis after solubilization permitted to decrease the detergent concentration but proteins then precipitated making purification impossible (data not shown). Surprisingly, though, the diluted N-laurylsarcosine solubilization supernatant of these bacteria showed a clean preparation of glutathione-S-transferase-PPARγ (~90% pure, Fig. 5A, lane 7). Glutathione-S-transferase-PPARγ was then cleaved by factor Xa to give the 58 kDa PPARγ and 26 kDa glutathione-S-transferase bands (Fig. 5A). Western blot analyses of soluble and cleaved glutathione-S-transferase-PPARγ with anti-glutathione-S-transferase, anti-ligand binding domain and anti-PPARγ antibodies (Fig. 5B, C and D) revealed a band at 89 kDa (glutathione-S-transferase-PPARγ) and another at 26 kDa (glutathione-S-transferase) for the first antibodies, ligand binding domain and PPARγ bands (89 and 58 kDa, respectively) for the second and third antibodies with minor contaminants revealed by the anti-PPARγ antibody.

3.5. Binding assay for membrane-bound glutathione-S-transferase-PPARγ and purified PPARγ

An optimal concentration of protein was evaluated for membrane-bound glutathione-S-transferase-PPARγ or cleaved PPARγ, and the concentration chosen were 100 and 300 μg/ml, respectively. The kinetic behavior was similar to that obtained with the ligand binding domain

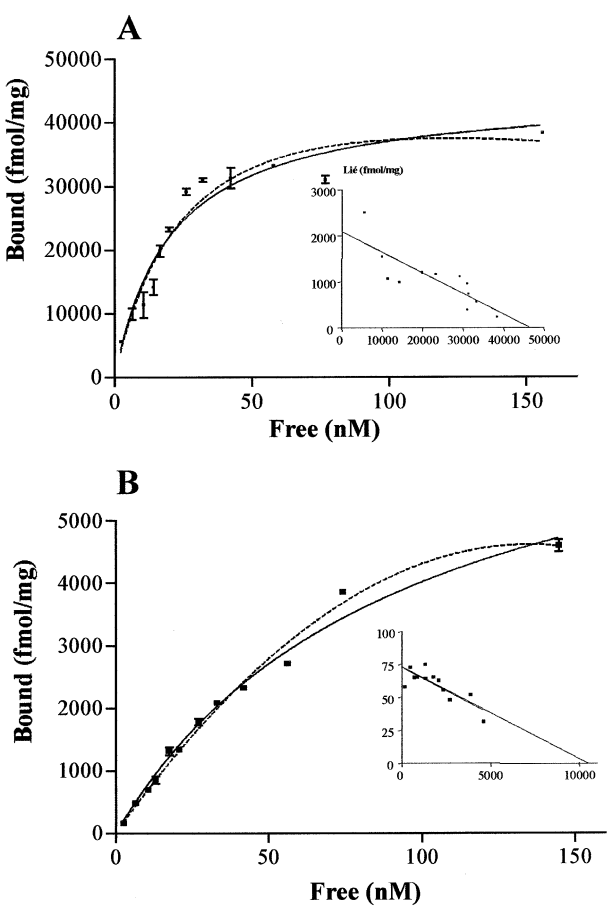

Fig. 6. Scatchard plot analysis of glutathione-S-transferase-PPARγ at various stages of the purification protocol using [^{3}H]rosiglitazone as radioligand. (A) bacterial membranes (K_d = 20 nM, B_{max} = 44500 fmol/mg); (B) cleaved PPARγ (K_d = 93 nM, B_{max} = 7800 fmol/mg). Inserts: *x*-axis: bound (fmol/mg); *y*-axis: Bound/free (fmol/mg/nM).

Table 3
Displacement experiments with prostaglandin derivatives for glutathione-S-transferase-PPARγ embedded in bacterial membrane preparations
Experimental conditions are described in the legend of Table 2.
Experiments were conducted at least three times for each compounds.

Ligands	K_i (μM) ± S.E.M.
Pioglitazone	0.86 ± 0.12
Rosiglitazone	0.043 ± 0.002
Prostaglandin A_1	> 1000
Prostaglandin A_2	> 1000
Prostaglandin A_3	187.83 ± 3.1
Prostaglandin B_1	26.28 ± 8.7
Prostaglandin B_2	77.00 ± 37.7
Prostaglandin B_3	> 1000
Prostaglandin D_1	57.50 ± 9.2
Prostaglandin D_2	51.83 ± 3.2
15-deoxy-Δ^{12-14}- Prostaglandin D_2	26.50 ± 3.7
Prostaglandin D_3	> 1000
Prostaglandin E_1	36.30 ± 1.3
Prostaglandin E_2	81.20 ± 36
Prostaglandin E_3	> 1000
Prostaglandin $F_{1\alpha}$	> 1000
Prostaglandin $F_{2\alpha}$	> 1000
Prostaglandin $F_{2\beta}$	36.20 ± 0.5
Prostaglandin $F_{3\alpha}$	> 1000
Prostaglandin G_2	15.19 ± 0.9
Prostaglandin H_1	4.54 ± 0.1
Prostaglandin H_2	3.43 ± 0.04
6β-Prostaglandin I_1	> 1000
Prostaglandin I_2	39.50 ± 1.8
Prostaglandin I_3	> 1000
Prostaglandin J_2	37.00 ± 3.3
15-deoxy-$\Delta^{12,14}$ – Prostaglandin J_2	4.05 ± 0.4
Prostaglandin K_1	> 1000
Prostaglandin K_2	> 1000
Misoprostol	> 1000
U46619	> 1000

preparations. All the experiments were performed as previously with 40 nM of [^{3}H]rosiglitazone and 100 μM of cold rosiglitazone. Scatchard plots revealed a K_d of 20 nM and a B_{max} of 44.5 pmol/mg of protein for glutathione-S-transferase-PPARγ in bacterial membrane (Fig. 6A), a 93 nM K_d and a B_{max} of 7.8 pmol/mg for the cleaved PPARγ (Fig. 6B). As stated above, glutathione-S-transferase is not able to bind [^{3}H]rosiglitazone, therefore allowing the use of the fused protein as the source of biological material.

3.6. Displacement experiments with reference ligands

A selected series of compounds was tested on the various forms of PPARγ produced during this study. The results are gathered in Table 1. K_d for [^{3}H]rosiglitazone ranges from 13 to 156 nM while the literature gives figures between 14 and 40 nM. No clear explanation was found for some of the discrepancies. The K_i obtained with rosiglitazone clearly showed that all the biological materials were equivalent. None of the compounds presented a dramatically different affinity for one among all the nuclear receptor forms. The thiazolidinediones were clearly the best ligands of PPARγ in any biological form, purified or not, in agreement with the literature. The preparation was not contaminated by any PPARα-related binding sites, since clofibrate was not a ligand in this system. Linolenic acid also showed affinity for this binding site of ~100 μM. The prostaglandin data also fitted the available reports although affinities in the 1–10 μM range seem modest for natural, short-life ligands such as those compounds. Furthermore, a more complete description of the molecular pharmacology of three out of the five 'constructs' of PPARγ is presented in Table 2. There are some major discrepancies between the data recorded with these three preparations (glutathione-S-transferase-ligand binding domain or glutathione-S-transferase-PPARγ associated with the membranes or purified glutathione-S-transferase-ligand binding domain). Fourteen compounds described in the literature as ligands of PPAR isoforms (α, β/δ or γ) were checked in this system showing in most cases, a fair agreement of the binding data with the literature.

3.7. Prostaglandins as ligands of the PPARγ

A series of commercially available prostaglandins was tested in the binding assay, using [^{3}H]rosiglitazone as a radioligand and the crude bacterial membrane preparation as source of PPARγ. The results are presented in Table 3. While most of these prostaglandins showed a moderate affinity for the receptor, and the standard so-called 'natural

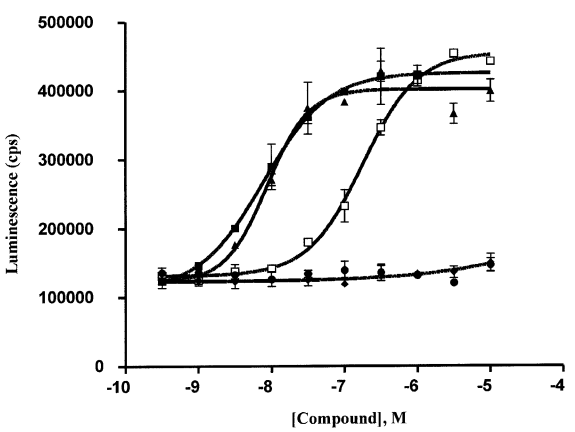

Fig. 7. Transactivation assay with the standard ligands for PPAR. The HEK293-based cellular system is described in Materials and Methods. The various compounds were added to cells at various concentrations, and the luciferase assay was run 24 h later. Rosiglitazone, a PPARγ specific ligand (close squares) led to an EC_{50} of 7.8 nM; GW1929, another PPARγ-specific ligand (close triangles) presents an EC_{50} of 8.78 nM; bezafibrate, a PPARβ-specific ligand (close diamonds) and WY 14643, a PPARα-specific ligand (close circles) are not active in this system, and pioglitazone, a third PPARγ-specific ligand (open squares) has an EC_{50} of 0.2 μM. Results are expressed in luminescence units, counts per second.

Table 4
EC_{50} of some prostaglandins in a PPARγ-related transactivation assay. Comparison with pioglitazone
Experiments were conducted as described in Materials and Methods. Results are the mean ± S.E.M. of at least three independent determinations, conducted in triplicate.

Products	EC_{50} (μM ± S.E.M.)
Pioglitazone	1 ± 0.055
Prostaglandin D_2	27 ± 10
Prostaglandin D_3	> 100
Prostaglandin H_1	5.5 ± 2.5
Prostaglandin H_2	9.9 ± 1.5
15-deoxy-Δ^{12-14}-Prostaglandin J_2	9.1 ± 5.4

ligand' of PPARγ was a relatively good ligand, two 'new' prostaglandins (never reported so far) showed significant affinity at this receptor: prostaglandin H_1 and H_2. Their affinities were close to the affinity of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, in the 1–10 μM range. The stable analog of endoperoxide prostaglandin, U-46619 [(15*S*)-Hydroxy-11α,9α-(epoxymethano)prosta-5*Z*,13*E*-dienoic acid], showed no affinity for this binding site.

3.8. Transactivation assay

The widely described transactivation assay was standardized in our laboratory and compared with published data using a series of known ligands of PPARγ, as shown in Fig. 7, including rosiglitazone, WY 14643 ([[4-chloro-6-[(2,3-dimethylphenyl)amino]-2-pyrimidinyl]thio]-acetic acid), bezafibrate and GW1929 [*N*-(2-benzoyphenyl)-*O*-[2-[*N*-methyl-*N*-(2-pyridinyl) amino]ethyl]-L-tyrosine]. Part of this standardization comprised control transfections with luciferase alone. No transactivation could be observed under those conditions. The compounds behaved as reported in the literature, namely: the bezafibrate and WY14643 were not active on this PPARγ-mediated assay and rosiglitazone, GW1929 and pioglitazone were as expected powerful agonists, with $EC_{50} \sim 0.2$ μM, as reported in the literature. Furthermore, we found prostaglandin J_2 and its derivative to behave as activators as previously shown (Kliewer et al., 1995). Two other prostaglandins, namely, H_1 and H_2 were found in the present work to be as potent as the prostaglandin J_2 derivative in the μM range (Table 4). Some other prostaglandin derivatives were also active in this transactivation assay, but with potencies beyond the 10 μM range.

4. Discussion

A number of publications have pointed out the importance of the nuclear receptor PPARγ in several types of pathological situations (see reviews by Spiegelmann, 1998; Sorensen et al., 1998; Rocchi and Auwerx, 1999; Lowell, 1999; Desvergne and Wahli, 1999; Giguère, 1999; Vamecq and Latruffe, 1999; Willson et al., 2000). Recent evidences have demonstrated the central role of PPARs, particularly of PPARγ, in obesity and related diseases (Kubota et al., 1999; Barak et al., 1999; Rosen et al., 1999; Mukherjee et al., 2000) and, preliminarily, in some colon cancers models (Lefebvre et al., 1998; Saez et al., 1998; Sarraf et al., 1998). PPARγ has been used in screening tests throughout the literature either as fused PPARγ (Kliewer et al., 1997), fused ligand binding domain (Krey et al., 1997), pure PPARγ (Mukherjee et al., 1997) or as pure ligand binding domain (Kliewer et al., 1995). We engineered these various constructions and overproduced them in bacteria. In order to compare the numerous data obtained from fundamentally different model systems, we measured the K_i of a series of reference compounds on these biological materials. We also compared the different possible techniques to obtain a biological material suitable for biological testing. It is clear that a robust system can be obtained by transfecting bacteria (*E. coli*) with a construct encoding the full-length PPARγ protein under the control of appropriate regulation sequences. A rapid preparation of the plasma membrane by standard centrifugation led to an efficient and robust binding system. This system was compared with glutathione-S-transferase-ligand binding domain obtained under the same conditions, and with glutathione-S-transferase-PPARγ prepared from bacterial expression, followed by solubilization, purification and cleavage. This latter system led to results similar to those obtained with the simpler procedure but it required intensive biochemical work.

Since most of the knowledge on PPARg ligands has been built upon data obtained with transactivation assays, it seemed important to develop the molecular tools necessary for binding studies. Indeed, direct binding and transactivation studies are both equally important tools for mechanistic studies and complete each other. The binding profiles were compared using the various biological materials either in a standard filtration assay or in a newly developed 96-well-plate-based gel filtration assay for soluble receptors with [^{3}H]rosiglitazone as ligand. In all cases, the data were compared with the standard transactivation assay, which makes use of the luciferase under the control of PPAR-responsive elements (Mukherjee et al., 1997). The data obtained were comparable with the two assays, the latter taking into account the cellular penetration (and possible metabolism) of the compounds before their action at the nuclear receptor level. Our results also compared well with those published in the literature as shown in Table 1 where reference values are reported (far right column). This methodology can be easily applied as primary screening (binding) in the search for new compounds, with a medium rate throughput. In this prospect, the gel-filtration-based assay also compared well with other assays developed for PPARγ ligand screening, such as CARLA (Krey et al., 1997) or scintillation proximity

assay (Mais et al., 1997; Nichols et al., 1998). The direct binding technique has also the advantage over the transactivation assay to be a 'pure' PPARγ assay, while the cellular-based transactivation assay involved (1) the PPARγ/RXRα heterodimers in which RXRα ligands are active as well as (2) the cellular penetration capacity of the tested compounds. This method is also more straightforward than the hydroxyapatite column separation used by others (Mais et al., 1997; Mukherjee et al., 2000).

Furthermore, we investigated a series of commercially available prostaglandins as possible binders of the PPARγ. Surprisingly, beside the naturally occurring, already described, ligand of PPARγ, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, we found two other PPAR ligands: prostaglandin H_1 and prostaglandin H_2, with an affinity close to that of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (~ 5 μM). This result was confirmed in the transactivation assay where the three prostaglandins (including the 'reference' 15-deoxy-Δ12,14-prostaglandin J_2) behaved in a similar manner (with an EC_{50} in the μM range). Such 'high' concentrations (i.e., μM range) are often reported in the literature whenever prostaglandins are concerned, for instance, for angiogenesis inhibition in which concentrations of 1, 10 and 100 μM were used with prostaglandins of the A, B and J types (Xin et al., 1999), but also in various circulatory models for the prostaglandin H_1 and analogues (Morita et al., 1979), in apoptosis induction in endothelial cells (Ikai et al., 1998) or for anti-inflammatory properties (Rossi et al., 2000). The acellular (binding) and cellular (transactivation) experiments led to similar results for the two endoperoxide cyclopentane prostaglandins H_1 and H_2, as well as for the prostaglandin J_2 derivative, strongly suggesting that those compounds are also 'natural' ligands of PPARγ. The biosynthesis pathway of prostaglandin J_2 from arachidonic acid involves the synthesis of prostaglandin G_2 catalyzed by cyclooxygenases. Prostaglandin G_2 then leads to prostaglandin H_2, which in turn is transformed into prostaglandin D_2 (Taketo, 1998), to give both in vitro or in vivo the prostaglandins of the J series (Fukushima, 1992). All these steps are catalyzed by different enzymes. Prostaglandin D_2 proved to be only marginally active (Tables 3 and 4) in both PPARγ systems (acellular and cellular), suggesting that the effects observed with its parent compound(s) [prostaglandins H_1 and H_2] are not due to metabolism leading via prostaglandin D_2 to prostaglandin J_2 in our assays. Furthermore, recent evidence has shown that cyclooxygenase-2, one of the core enzyme in the prostaglandin synthesis, is regulated by a negative feedback loop mediated by PPARγ (Inoue et al., 2000). Prostaglandins H or G were neither tested in the few binding experiments reported by Kliewer et al. (1995), nor in the transactivation assay reported by Yu et al. (1995). Due to the importance of the finding that 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 was a natural ligand of PPARγ, and the subsequent experiments that strengthened this observation (Chinery et al., 1999; Keelan et al., 1999; Bishop-Bailey and Hla, 1999; Marvin et al., 2000), we believe that the finding of new natural ligands of PPARγ may suggest new investigation routes in the understanding of PPAR pharmacology.

In summary, a novel 96-well plate binding assay with the human ligand binding domain fused with glutathione-S-transferase was developed in order to discover new ligands to the nuclear receptor PPARγ. Using this assay on glutathione-S-transferase-PPARγ, we found the affinity of reference PPARγ ligands to be similar to those reported in the literature although these figures varied according to the nature of the biological material used. The partially automated set-up described in the present work, has throughput capacity and may serve for the screening of large numbers of potential PPARγ ligands. Furthermore, we pointed out that a series of structurally related (endoperoxides) prostaglandins H_1 and H_2 are in vivo ligands and may also be 'natural ligands' of the PPARγ nuclear receptor, in addition to the prostaglandin J_2 and its metabolites.

References

Banner, C.D., Göttlicher, M., Widmark, E., Sjovall, J., Rafter, J.J., Gustafsson, J.A., 1993. A systematic analytical/cell assay approach to isolate activators of orphan nuclear receptors from biological extracts: characterization of peroxisome proliferator-activated receptor activators in plasma. J. Lipid Res. 34, 1583–1891.

Barak, Y., Nelson, M.C., Ong, E.S., Jones, Y.Z., Ruiz-Lozano, P., Chien, K.R., Koder, A., Evans, R.M., 1999. PPARγ is required for placental, cardiac and adipose tissue development. Mol. Cell 4, 585–595.

Berger, J., Bailey, P., Biswas, C., Cullinan, C.A., Doebber, T.W., Hayes, N.S., Saperstein, R., Smith, R.G., Leibowitz, M.D., 1996. Thiazolidinediones produce a conformational change in peroxisomal proliferator-activated receptor-γ: binding and activation correlate with antidiabetic actions in *db/db* mice. Endocrinology 137, 4189–4195.

Bishop-Bailey, D., Hla, T., 1999. Endothelial cell apoptosis induced by the peroxisome proliferator-activated receptor (PPAR) ligand 15-deoxy-Δ12,14-prostaglandin J_2. J. Biol. Chem. 274, 17042–17048.

Brown, P.W., Smith-Oliver, T.A., Charifson, P.S., Tomkinson, T.C.O., Fivush, A.M., Sternbach, D.D., Wade, L.E., Orband-Miller, L., Parks, D.J., Blanchard, S.G., Kliewer, S.A., Lehman, J.M., Willson, T.M., 1997. Identification of peroxisome proliferator-activated receptor ligands from a biased chemical library. Chem. Biol. 4, 909–918.

Chawla, A., Schwarz, E.J., Dimaculangan, D.D., Lazar, M.A., 1994. Peroxisome proliferator-activated receptor (PPAR) gamma: adipose-predominant expression and induction early in adipocyte differentiation. Endocrinology 35, 798–800.

Chinery, R., Coffey, R.J., Graves-Deal, R., Kirkland, S.C., Sanchez, S.C., Zackert, W.E., Oates, J.A., Morrow, J.D., 1999. Prostaglandin J_2 and 15-deoxy-Δ12,14-prostaglandin J2 induce proliferation of cyclooxygenase-depleted colorectal cancer cells. Cancer Res. 59, 2739–2746.

Cobb, J.E., Blanchard, S.G., Boswell, E.G., Brown, K.K., Charifson, P.S., Cooper, J.P., Collins, J.L., Dezube, M., Henke, B.R., Hull-Ryde, E.A., Lake, D.H., Lenhard, J.M., Oliver, W., Oplinger, J., Pentti, M., Parks, D.J., Plunket, K.D., Tong, W.Q., 1998. *N*-(2-benzoylphenyl)-L-tyrosine PPARγ: 3. Structure–activity relationship and optimization of the *N*-aryl substituent. J. Med. Chem. 41, 5055–5069.

Desvergne, B., Wahli, W., 1999. Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr. Rev. 20, 649–688.

Dowell, P., Peterson, V.J., Zabriskie, T.M., Leid, M., 1997. Ligand

induced peroxisome proliferator-activated receptor α conformational change. J. Biol. Chem. 272, 2013–2020.

Elstner, E., Muller, C., Koshizuka, K., Williamson, E.A., Park, D., Asou, H., Shintaku, P., Said, J.W., Heber, D., Koeffler, H.P., 1998. Ligands for peroxisome proliferator-activated receptor γ and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells in vitro and in BNX mice. Proc. Natl. Acad. Sci. U. S. A. 95, 8806–8811.

Forman, B.M., Tontonoz, P., Chen, J., Brun, R.P., Spiegelman, B.M., Evans, R.M., 1995. 15-deoxy-Δ12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPARγ. Cell 83, 803–812.

Forman, B.M., Chen, J., Evans, R.M., 1997. Hypolipidemic drugs, polyunsaturated fatty acids and eicosamoids are ligands for peroxisome proliferator-activated receptors α and δ. Proc. Natl. Acad. Sci. U. S. A. 94, 4312–4317.

Fukushima, M., 1992. Biological activities and mechanisms of action of prostaglandin J2 and related compounds: an update. Prostaglandins Leukotrienes Essent. Fatty Acids 47, 1–12.

Giguère, V., 1999. Orphan nuclear receptors: from gene to function. Endocrinol. Rev. 20, 689–725.

Göttlicher, M., Widmark, E., Li, Q., Gustafsson, J.A., 1992. Fatty acids activate a chimera of the clofibric acid-activated receptor and glucocorticoid receptor. Proc. Natl. Acad. Sci. U. S. A. 89, 4653–4657.

Ikai, K., Kudo, H., Toda, K., Fukushima, M., 1998. Induction of apoptosis, p53 and heme oxygenase-1 by cytotoxic prostaglandin Δ^{12}-prostaglandin J_2 in transformed endothelial cells. Prostaglandins Leukotiene Essent. Fatty Acids 58, 295–300.

Inoue, H., Tanbe, T., Umesono, K., 2000. Feedback control of cyclooxygenase-2 expression through PPARγ. J. Biol. Chem. 275, 28028–28032.

Issemann, I., Green, S., 1990. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature 347, 645–650.

Jiang, C., Ting, A.T., Seed, B., 1998. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature 391, 82–86.

Keelan, J.A., Sato, T.A., Marvin, K.W., Lander, J., Gilmour, R.S., Mitchell, M.D., 1999. 15-deoxy-Δ12,14-prostaglandin J_2, a ligand for peroxisome proliferator-activated receptor-γ, induces apoptosis in JEG3 choriocarcinoma cells. Biochem. Biophys. Res. Commun. 262, 579–585.

Keller, H., Dreyer, C., Medin, J., Mahfoudi, A., Ozato, K., Wahli, W., 1993. Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. Proc. Natl. Acad. Sci. U. S. A. 90, 2160–2164.

Kliewer, S.A., Umesomo, K., Noonan, D.J., Heyman, R.A., Evans, R.M., 1992. Convergence of 9-*cis* retinoic acid and peroxisome proliferator signaling pathways through heterodimer formation of their receptors. Nature 358, 771–774.

Kliewer, S.A., Lenhard, J.M., Wilson, T.M., Patel, I., Morris, D.C., Lehman, J.M., 1995. A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation. Cell 83, 813–819.

Kliewer, S.A., Sundseth, S.S., Jones, S.A., Brown, P.J., Wisely, G.B., Koble, C.S., Devchand, P., Wahli, W., Willson, T.M., Lenhard, J.M., Lehmann, J.M., 1997. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. Proc. Natl. Acad. Sci. U. S. A. 94, 4318–4323.

Krey, G., Braissant, O., L'Horset, F., Kalkhoven, E., Perroud, M., Parker, M.G., Wahli, W., 1997. Fatty acids, eicosanoids and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by co-activator-dependent receptor ligand assay. Mol. Endocrinol. 11, 779–791.

Kubota, N., Terauchi, Y., Miki, H., Tamemoto, H., Yamauchi, T., Komeda, K., Satoh, S., Nakano, R., Ishii, C., Sugiyama, T., Eto, K., Tsubamoto, Y., Okuno, A., Murakami, K., Sekihara, H., Hasegawa, G., Naito, M., Toyoshima, T., Tanaka, S., Shiota, K., Kitamura, T., Fujita, T., Ezaki, O., Aizawa, S., Nagai, R., Tobe, K., Kimura, S., Kadowaki, T., 1999. PPARγ mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. Mol. Cell 4, 597–609.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lefebvre, A.M., Chen, I., Desreumaux, P., Najib, J., Fruchart, J.C., Geboes, K., Briggs, M., Heyman, R., Auwerx, J., 1998. Activation of the peroxisome proliferator-activated receptor γ promotes the development of colon tumors in C57BL/6J-APC^{min}/+ mice. Nat. Med. 4, 1053–1057.

Lehmann, J.M., Moore, L.B., Smith-Oliver, T.A., Wilkison, W.O., Willson, T.M., Kliewer, S.A., 1995. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPAR γ). J. Biol. Chem. 270, 12953–12956.

Lehmann, J.M., Lenhard, J.M., Oliver, B.B., Ringold, G.M., Kliewer, S.A., 1997. Peroxisome proliferator-activated receptors α and γ are activated by indomethacin and other non-steroidal anti-inflammatory drugs. J. Biol. Chem. 272, 3406–3410.

Leid, M., 1994. Ligand-induced alteration of the protease sensitivity of retinoid X receptor α. J. Biol. Chem. 269, 14175–14180.

Lowell, B.B., 1999. PPARγ: an essential regulator of adipogenesis and modulator of fat cell function. Cell 99, 239–242.

Mais, D.E., Hamann, L.G., Klausing, K.U., Paterniti, J.R., Mukherjee, R., 1997. Characterization of synthetic tritium-labeled peroxisome proliferator-activated receptor (hPPAR-γ) ligand. Med. Chem. Res. 7, 325–334.

Marvin, K.W., Eykholt, R.L., Keelan, J.A., Sato, T.A., Mitchell, M.D., 2000. The 15-deoxy-Δ12,14-prostaglandin J2 receptor, peroxisome proliferator activated-receptor-γ (PPARγ) is expressed in human gestational tissues and is functionally active in JEG3 choriocarcinoma cells. Placenta 20, 436–440.

Morita, A., Nishino, H., Mori, M., Hasegawa, K., Sakai, K., Kobayashi, S., 1979. Biological activites of prostaglandin H1 analogs. Prostaglandins 18, 529–540.

Mukherjee, R., Davies, P.J., Crombie, D.L., Bischoff, E.D., Cesario, R.M., Jow, L., Hamann, L.G., Boehm, M.F., Mondon, C.E., Nadzan, A.M., Paterniti, J.R., Heyman, R.A., 1997. Sensitization of diabetic and obese mice to insulin by retinoid X receptor agonists. Nature 386, 407–410.

Mukherjee, R., Hoener, P.A., Jow, L., Bilakovics, J., Klausing, K., Mais, D.E., Faulkner, A., Croston, G.E., Paterniti, J.R., 2000. A selective peroxisome-activated receptor-γ (PPARγ) modulator blocks adipocyte differentiation but stimulates glucose uptake inb 3T3-L1 adipocytes. Endocrinology 14, 1425–1433.

Nichols, J.S., Parks, D.J., Consler, T.G., Blanchard, S.G., 1998. Development of a scintillation proximity assay for peroxisome proliferator-activated receptor γ ligand binding domain. Anal. Biochem. 257, 112–119.

Ricote, M., Li, A.C., Willson, T.M., Kelly, C.J., Glass, C.K., 1998. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. Nature 391, 79–82.

Rocchi, S., Auwerx, J., 1999. Peroxisome proliferator-activated receptor-γ: a versatile metabolic regulator. Ann. Med. 31, 342–351.

Rosen, E.D., Sarraf, P., Troy, A.E., Bradwin, G., Moore, K., Milstone, D.S., Spiegelman, B.M., Mortensen, R.M., 1999. PPARγ is required for the differentiation of adipose tissue in vivo and in vitro. Mol. Cell 4, 611–617.

Rossi, A., Kapahi, P., Natoli, G., Takahashi, T., Chen, Y., Karin, M., Santoro, M.G., 2000. Anti-inflammatory cyclopentenione prostaglandins are direct inhibitors of IκB kinase. Nature 403, 103–108.

Saez, E., Tontonoz, P., Nelson, M.C., Alvarez, J.G.A., U, T.M., Baird, S.M., Thomazy, V.A., Evans, R.M., 1998. Activators of the nuclear receptor PPARγ enhance colon polyp formation. Nat. Med. 4, 1058–1061.

Sarraf, P., Mueller, E., Jones, D., King, F.J., DeAngelo, D.J., Partridge,

J.B., Holden, S.A., Chen, L.B., Singer, S., Fletcher, C., Spiegelman, B.M., 1998. Differentiation and reversal of malignant changes in colon cancer through PPARγ. Nat. Med. 9, 1046–1052.

Schoonjans, K., Martin, G., Staels, B., Auwerx, J., 1997. Peroxisome proliferator-activated receptors, orphans with ligands and functions. Curr. Opin. Lipidol. 8, 159–166.

Schulman, I.G., Shao, G., Heyman, R.A., 1998. Transactivation by retinoid X receptor-peroxisome proliferator-activated receptor γ (PPARγ) heterodimers: intermolecular synergy requires only the PPARγ hormone dependent activation function. Mol. Cell Biol. 18, 3483–3494.

Sorensen, H.N., Treuter, E., Gustafsson, J.A., 1998. Regulation of peroxisome proliferator-activated receptors. Vitam. Horm. 54, 121–166.

Spiegelmann, B.M., 1998. PPARγ: adipogenic regulator and thiazolidinedione receptor. Diabetes 47, 507–514.

Suh, N., Wang, Y., Williams, C.R., Risingsong, R., Gilmer, T., Willson, T.M., Sporn, M.B., 1999. A new ligand for the peroxisome proliferator-activated receptor-γ (PPAR-γ), GW7845, inhibits rat mammary carcinogenesis. Cancer Res. 59, 5671–5673.

Taketo, M.M., 1998. Cyclooxygenase-2 inhibitors in tumorigenesis. J. Natl. Cancer Inst. 90, 1529–1536.

Tontonoz, P., Hu, E., Graves, R.A., Budavari, A.I., Spiegelman, B.M., 1994a. mPPAR γ 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev 8, 1224–1234.

Tontonoz, P., Hu, E., Spiegelman, B.M., 1994b. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell 79, 1147–1156.

Tontonoz, P., Singer, S., Forman, B.M., Sarraf, P., Fletcher, J.A., Fletcher, C.D., Brun, R.P., Mueller, E., Altiok, S., Oppenheim, H., Evans, R.M., Spiegelman, B.M., 1997. Terminal differentiation of human liposarcoma cells induced by ligands for peroxisome proliferator-activated receptor gamma and the retinoid X receptor. Proc. Natl. Acad. Sci. U. S. A. 94, 237–241.

Vamecq, J., Latruffe, N., 1999. Medical significance of peroxisome proliferator-activated receptors. Lancet 354, 141–148.

Vanden Heuvel, J.P., 1999. Peroxisome proliferator-activated receptors: a critical link among fatty acids, gene expression and carcinogenesis. J. Nutr. 129, 575S–580S.

Willson, T.M., Brown, P.J., Sternbach, D.D., Henke, B.R., 2000. The PPARs: from orphan receptors to drug discovery. J. Med. Chem. 43, 527–550.

Xin, X., Yang, S., Kowalski, J., Gerritsen, M.E., 1999. Peroxisome proliferator-activated receptor γ ligands are potent inhibitors of angiogenesis in vitro and in vivo. J. Biol. Chem. 274, 9116–9121.

Xu, H.E., Lambert, M.H., Montana, V.G., Parks, D.J., Blanchard, S.G., Brown, P.J., Sternbach, D.D., Lehman, J.M., Wisely, G.B., Willson, T.M., Kliewer, S.A., Milburn, M.V., 1999. Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. Mol. Cell 3, 397–403.

Young, P.W., Buckle, D.R., Cantello, B.C.C., Chapman, H., Clapham, J.C., Coyle, P.J., Haigh, D., Hindley, R.M., Holder, J.C., Kallender, H., Latter, A.J., Lawrie, K.W.M., Mossakowska, D., Murphy, G.J., Cox, L.R., Smith, S.A., 1998. Identification of high-affinity binding sites for the insulin sensitizer rosiglitazone in rodent and human adipocytes using a radioionated ligand for peroxisome proliferator-activated receptor γ. J. Pharm. Exp. Ther. 284, 751–759.

Yu, K., Bayona, W., Kallen, C.B., Harding, H.P., Ravera, C.P., McMahon, G., Brown, M., Lazar, M.A., 1995. Differential activation of peroxisome proliferator-activated receptors by eicosanoids. J. Biol. Chem. 270, 23975–23983.