The peroxisome proliferator-activated receptor:retinoid X receptor heterodimer is activated by fatty acids and fibrate hypolipidaemic drugs

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ABSTRACT

The peroxisome proliferator-activated receptor (PPAR) is a member of the steroid hormone receptor superfamily and is activated by a variety of fibrate hypolipidaemic drugs and non-genotoxic rodent hepatocarcinogens that are collectively termed peroxisome proliferators. A key marker of peroxisome proliferator action is the peroxisomal enzyme acyl CoA oxidase, which is elevated about tenfold in the livers of treated rodents. We have previously shown that a peroxisome proliferator response element (PPRE) is located 570 bp upstream of the rat peroxisomal acyl CoA oxidase gene and that PPAR binds to it. We show here that the retinoid X receptor (RXR) is required for PPAR to bind to the PPRE, and that the RXR ligand, 9-*cis* retinoic acid, enhances PPAR action. Retinoids may therefore modulate the action of peroxisome proliferators and PPAR may interfere with retinoid action, perhaps providing one mechanism to explain the toxicity of peroxisome proliferators. We have also shown that a variety of hypolipidaemic drugs and fatty acids can activate PPAR. This supports the suggestion that the physiological role of PPAR is to regulate fatty acid homeostasis, and provides further evidence that PPAR is the target of the fibrate class of hypolipidaemic drugs. Finally, we have demonstrated that a metabolically stabilized fatty acid is a potent PPAR activator, suggesting that fatty acids, or their acyl CoA derivatives, may be the natural ligands of PPAR.

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INTRODUCTION

Fibrate hypolipidaemic drugs were developed to lower cholesterol and triglyceride levels as a treatment for coronary heart disease (Thorp & Waring, 1962; Sirtori *et al.* 1977). Clinical trials using fibrate drugs, such as gemfibrozil, indicate that they are well tolerated and effective hypolipidaemic drugs in patients with severe hypertriglyceridaemia; however, their mechanism of action is unknown (Leaf *et al.* 1989). A disturbing feature of these drugs is that they induce liver tumours when chronically administered to rats and mice (Reddy & Lalwani, 1983). These drugs form part of a larger class of rodent hepatocarcinogens, known as peroxisome proliferators, that increase both the size and the number of hepatic peroxisomes (Reddy & Lalwani, 1983; Green, 1992). The hypolipidaemic drugs (e.g. Wy-14,643) are some of the most potent peroxisome proliferators, whereas plasticizers, such as di-(2-ethylhexyl) phthalate (DEHP), are much weaker (Marsman *et al.* 1988). The levels of several peroxisomal enzymes (Lazarow & de Duve, 1976), as well as members of the cytochrome P-450 IV gene family (Orton & Parker, 1982), are elevated some 10- to 20-fold in response to peroxisome proliferators.

Changes in the transcription rates of the genes encoding these enzymes appear to account for the increases in enzyme levels (Reddy *et al.* 1986; Hardwick *et al.* 1987). The induced peroxisomal enzymes, acyl CoA oxidase, bifunctional enzyme and thiolase, are all part of the peroxisomal β-oxidation pathway that is important for the metabolism of long chain fatty acids (Osmundsen *et al.* 1991). The P-450 IV enzymes have ω-hydroxylase activity using a variety of substrates including fatty acids (e.g. lauric acid) and prostaglandins E_1 and $F_{2\alpha}$ (Orton & Parker, 1982; Aoyama *et al.* 1990). One suggestion to explain the hepatocarcinogenic effects of peroxisome proliferators argues that the increase in acyl CoA oxidase activity produces an elevation in hydrogen peroxide, which is a biproduct of the reaction catalysed by this enzyme (Reddy & Lalwani, 1983). This could result in the formation of DNA reactive

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oxygen radicals, and hence lead to mutation, or could activate transcription factors such as nuclear factor-κB (Schreck & Baeuerle, 1991), that may in turn regulate genes that are important in the carcinogenic process. Alternatively, peroxisome proliferators may act as tumour promoters and in some way influence the growth of selected hepatocytes (Marsman *et al.* 1988).

We have shown that peroxisome proliferators activate a murine member of the steroid hormone receptor superfamily, the murine peroxisome proliferator-activated receptor-α (mPPARα) (Issemann & Green, 1990). More recently, a rat PPARα (Göttlicher *et al.* 1992), three *Xenopus* PPARs (α, β and γ; Dreyer *et al.* 1992) and a human homologue (hNUC1; Schmidt *et al.* 1992) have been identified which can also be activated by the hypolipidaemic drug Wy-14,643. PPARα binds to a specific response element (PPRE) located upstream of the gene encoding peroxisomal acyl CoA oxidase and is important in regulating its transcription (Tugwood *et al.* 1992). Furthermore, similar PPREs have been identified in other peroxisome proliferator-regulated genes, including the peroxisomal bifunctional enzyme (Zhang *et al.* 1992*a*) and the rabbit P-450 IVA6 gene (Muerhoff *et al.* 1992). There is, therefore, good evidence to suggest that PPAR mediates the biological action of peroxisome proliferators and hypolipidaemic drugs. Interestingly, there is evidence to suggest that the physiological role of PPAR is to regulate lipid homeostasis and that fatty acids may be the natural PPAR ligands. For example, a high fat diet can produce peroxisome proliferation in rodents, and many of the enzymes induced by peroxisome proliferators have some role in fatty acid metabolism (Osmundsen *et al.* 1991).

We have examined the interaction between PPARα and its response element. We show that the retinoid X receptor (RXR) (Mangelsdorf *et al.* 1990), which binds the ligand 9-*cis* retinoic acid (Heyman *et al.* 1992; Levin *et al.* 1992), enhances the binding of PPAR specifically to the PPRE, and that the RXR ligand contributes to the activation of transcription. Furthermore, we show that a wide variety of hypolipidaemic drugs and fatty acids can activate PPAR.

MATERIALS AND METHODS

Chemicals

The following fibrate hypolipidaemic drugs were used in this study: Wy-14,643 (Chemsyn Science Laboratories, Lenexa, KS, U.S.A.), ciprofibrate (Sterling Winthrop Pharmaceuticals, Rensselaer, NY, U.S.A.), gemfibrozil (Warner Lambert, Ann Arbor, MI, U.S.A.), bezafibrate (Boehringer Mannheim, Mannheim, Germany), nafenopin (Ciba-Giegy, Basel, Switzerland) and methyl clofenapate (ZENECA Pharmaceuticals, Macclesfield, Cheshire, U.K.). Mono-(2-ethylhexyl) phthalate (MEHP) was obtained from ICI Chemicals and Polymers, Runcorn, Cheshire, U.K. 9-*cis* retinoic acid was a kind gift of Hoffmann-La Roche, Nutley, NJ, U.S.A. Tetradecylthioacetic acid (TTA) was a kind gift of Prof. K. Gautvik, Institute of Medical Biochemistry, Oslo, Norway. Clofibric acid, fatty acids, dehydroepiandrosterone (DHEA), DHEA sulphate and trichloroacetic acid (TCA) were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

Construction and analysis of reporter plasmids

Oligonucleotides containing the PPRE sequences were ligated between the HindIII and BamHI restriction enzyme sites of the polylinker in the pG.CAT vector. This vector contains the rabbit β-globin (G) promoter (−109 to +10) placed upstream of the chloramphenicol acetyltransferase (CAT) coding sequence (Issemann & Green, 1990; Tugwood *et al.* 1992). The acyl CoA oxidase (ACO) reporter plasmids were constructed from ACO(−1273/+20)CAT (Tugwood *et al.* 1992). Different regions of the acyl CoA oxidase promoter were amplified using ten cycles of the polymerase chain reaction and subcloned between the HindIII and BamHI restriction enzyme sites of the pG.CAT vector. Reporter plasmids (1 μg) were transfected individually into the mouse hepatoma cell line Hepa1c1c7 (in 9 cm Petri dishes), either in the absence or presence of PPAR expression vector pSG5-mPPARα (300 ng) and the potent peroxisome proliferator Wy-14,643 (10 μM) (Tugwood *et al.* 1992). In addition, 3 μg of the β-galactosidase internal control plasmid pCH110 (Pharmacia, Milton Keynes, Bucks, U.K.) were included and the final DNA concentration adjusted to 10 μg with pBluescribe plasmid DNA (Stratagene, Cambridge, Cambs, U.K.). Note that when pSG5-mPPARα was not used it was replaced with the parent expression vector pSG5 (Stratagene; Green *et al.* 1988). After 48 h, cytosol extracts were prepared by three rounds of freeze-thawing, and the β-galactosidase and CAT assays were performed as described previously (Tugwood *et al.* 1992).

DNA binding assays

COS1 cells were transfected with 10 μg of the appropriate expression plasmid using N-[1-(2, 3-dioleyloxy)propyl]-N,N,N-trimethylammonium

chloride (DOTMA) (Tugwood *et al.* 1992). After 48 h, whole cell extracts were prepared by lysing the cells in a buffer containing 0·6 M KCl with three rounds of freeze-thawing, and the extracts were cleared by centrifugation for 15 min at 10 000 r.p.m. at 4 °C in a microfuge (Tugwood *et al.* 1992). The pSG5-based expression plasmids (mPPARα and mRXRα) were linearized with XbaI and SmaI respectively and mRNA was transcribed using T7 polymerase. This was translated *in vitro* using rabbit reticulocyte lysates (Amersham International plc, Aylesbury, Bucks, U.K.) as directed by the manufacturer. DNA binding assays were performed using 1 μl (5 μg) COS cell extract or 0·5–1 μl programmed lysate (approximately 75 fmol receptor) as described previously (Tugwood *et al.* 1992), except that the binding assay buffer used for the in-vitro translated proteins was 10 mM Hepes, pH 7·9, 50 mM KCl, 1 mM dithiothreitol and 2·5 mM $MgCl_2$, 20% (v/v) glycerol and 5 μg poly(d(I)d(C)). The competition experiments were performed using equimolar amounts of in-vitro translated mPPARα and mRXRα. These were incubated with a mixture of the RDR-1 (reverse direct repeat-1; see Fig. 1) oligonucleotide probe (50 fmol, approximately 80 000 c.p.m.) and a 100-fold excess of the unlabelled competitor oligonucleotide. The DNA–protein complexes were resolved by polyacrylamide gel electrophoresis (Tugwood *et al.* 1992).

Testing PPAR activators

Hepa1c1c7 cells in 9 cm Petri dishes were transfected with the ACO(−1273/−471)G.CAT reporter plasmid (1 μg; Tugwood *et al.* 1992), pSG5-mPPARα expression vector (1 μg), pCH110 (3 μg) and pBluescribe (5 μg) using DOTMA. Potential ligands were added in dimethyl sulphoxide (DMSO; 0·1% final concentration) 5 h later with the addition of serum, and were refreshed with the next change of medium 20 h later. After a further 24 h, the cells were harvested and extracts analysed as above.

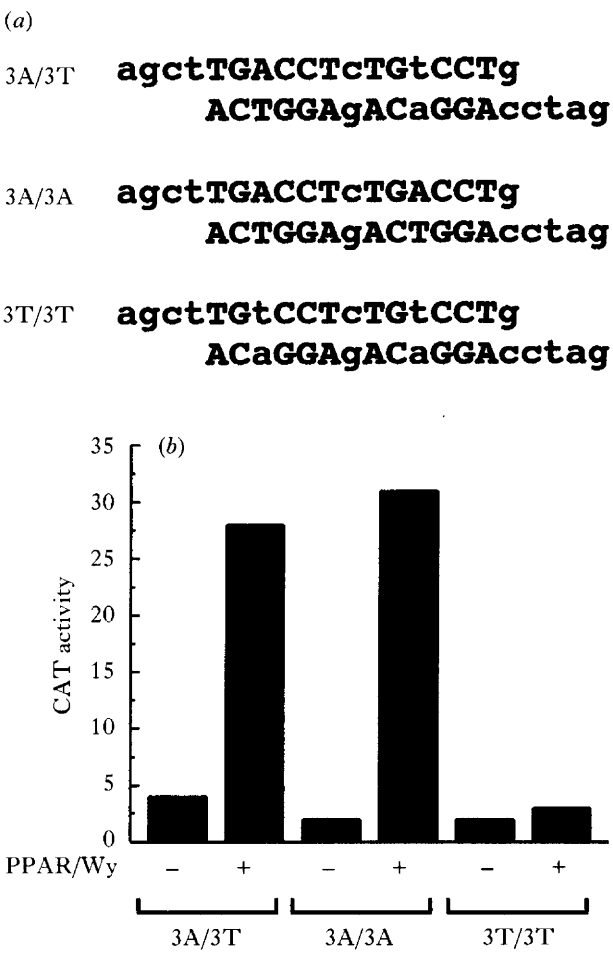

FIGURE 1. Sequence specificity of the peroxisome proliferator response element (PPRE) half-site. (*a*) Sequence of the synthetic PPREs. The half-sites contain either an adenine (TGACCT) or thymine (TGtCCT) in the third position. The consensus half-site (TGACCT) is denoted in capitals. Note that the perfect direct repeat (sequence 3A/3A) is identical to RDR-1 (reverse direct repeat-1) in Fig. 2. (*b*) Activities of reporter plasmids constructed using the synthetic PPREs transfected into Hepa1 cells in the absence (−) or presence (+) of both the peroxisome proliferator-activated receptor expression plasmid and Wy-14,643 (PPAR/Wy). Chloramphenicol acetyltransferase (CAT) activity is expressed as the percentage of acetylated chloramphenicol. The averages of triplicate results are shown.

RESULTS

The PPRE identified in the 5′ flanking sequence of the rat peroxisomal acyl CoA oxidase gene is an almost perfect direct repeat with the sequence 5′-TGACCTTTGTCCT-3′ (Tugwood *et al.* 1992). Nuclear hormone receptors for retinoic acid (RAR), vitamin D_3 (VDR), thyroid hormones (TR) and oestrogens (ER) all recognize an AGGTCA half-site that is complimentary to the TGACCT PPRE sequence (Mader *et al.* 1989; Umesono *et al.* 1991; Green, 1993). We investigated whether a direct repeat of the TGACCT or TGTCCT half-site constitutes a better PPRE than the natural acyl CoA oxidase response element. Reporter plasmids were constructed using synthetic oligonucleotide response elements placed upstream of the rabbit β-globin promoter (−109/+10) and the CAT

coding sequence (Fig. 1). The response elements contained either the natural sequence of the rat acyl CoA oxidase promoter, with an adenine in the third position of the first half-site and a thymine in the third position of the second (3A/3T; Fig. 1*a*), or a direct repeat of either the TGACCT (3A/3A; Fig. 1*a*) or TGTCCT (3T/3T; Fig. 1*a*) half-sites. These reporter plasmids were transfected into the mouse hepatoma cell line, Hepa1, either in the presence or in the absence of the PPARα expression plasmid pSG5-mPPARα and the potent peroxisome proliferator Wy-14,643 (Fig. 1*b*). In the presence of Wy-14,643, PPARα stimulated the transcription of both the 3A/3T and 3A/3A reporter plasmids equally, but had no effect on 3T/3T, indicating that a response element containing TGACCT (AGGT CA) half-sites is preferred.

It is of particular interest that a direct repeat of the AGGTCA half-site separated by 1 bp is also a response element for RXR (Mangelsdorf *et al.* 1991). The relative spacing of the half-sites is an important feature that determines the specificity of response elements for receptors that recognize a direct repeat of the AGGTCA half-site (Umesono *et al.* 1991). We constructed a series of reporter plasmids containing a direct repeat of the AGGTCA half-site spaced by 0–5 bp, with the orientation of the sequence the same as the natural acyl CoA oxidase PPRE (RDR-0 to RDR-5; Fig. 2*a*). None of these reporter plasmids were activated when transfected into Hepa1 cells in the absence of PPARα (Fig. 2*b*, upper panel). However, when cotransfected with PPAR expression plasmid and Wy-14,643, transcription of the RDR-1 reporter plasmid was stimulated 15-fold whilst that of RDR-0 and RDR-2 was only stimulated weakly (Fig. 2*b*, lower panel). Therefore, the RXR response element (RDR-1) is also the preferred PPRE.

RXR forms heterodimers with, and enhances the DNA-binding of, other nuclear receptors that bind to direct repeats of the TGACCT half-site (Yu *et al.* 1991; Bugge *et al.* 1992; Kliewer *et al.* 1992*a*; Leid *et al.* 1992; Marks *et al.* 1992; Zhang *et al.* 1992*b*). We therefore examined whether RXR could also stimulate the binding of PPAR to the acyl CoA oxidase PPRE. PPARα and RXRα were transiently expressed in COS-1 cells and aliquots of the whole cell extracts used in a gel retardation assay to assess DNA binding. Only a weak retarded band was observed when using extracts that contained either PPARα (Fig. 3*a*, lane 2) or RXRα (lane 3) alone. However, a much stronger retarded band was seen when the PPARα and RXRα extracts were mixed (lane 4), indicating that RXRα and PPARα co-operatively bind to the acyl CoA oxidase PPRE. Similar experiments were also performed using

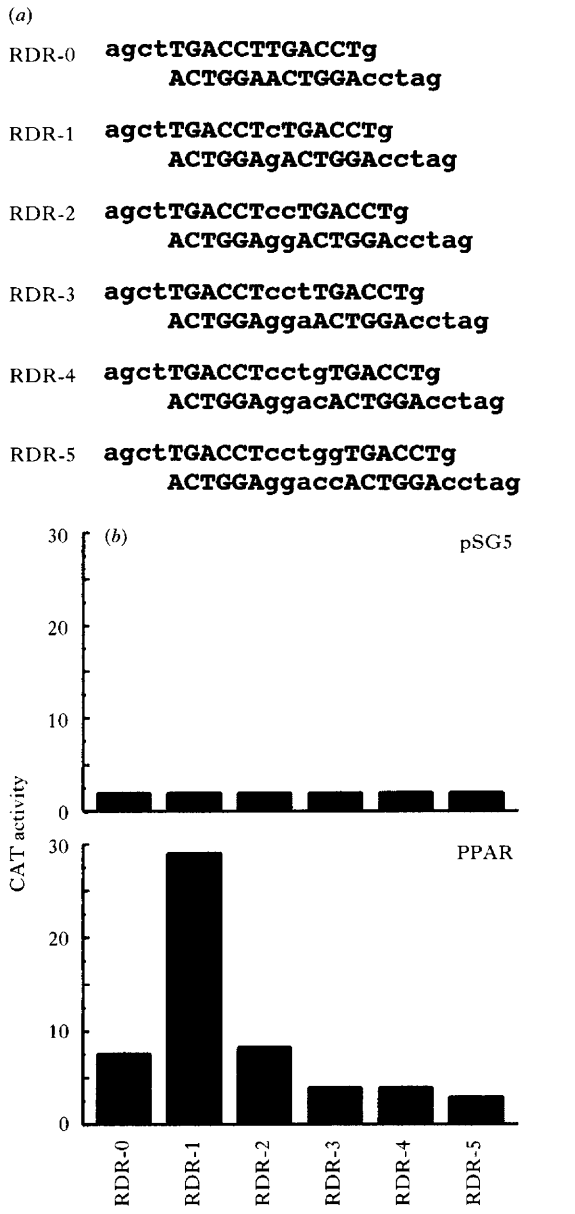

FIGURE 2. Specificity of peroxisome proliferator response element half-site spacing and activation by the peroxisome proliferator-activated receptor (PPAR). (*a*) Sequences of the spaced half-site elements. The response elements contain a direct repeat of the TGACCT half-site separated by 0–5 bp (reverse direct repeat sequences RDR-0 to RDR-5). (*b*) Activities of reporter plasmids constructed using the synthetic spaced half-sites. The reporter plasmids (1 μg) were cotransfected with either pSG5 (300 ng) in the absence of activator (upper panel) or the pSG5-mPPARα expression plasmid (300 ng) in the presence of 10 μM Wy-14,643 (lower panel). Chloramphenicol acetyltransferase (CAT) activity is expressed as the percentage of acetylated chloramphenicol. The averages of triplicate results are shown.

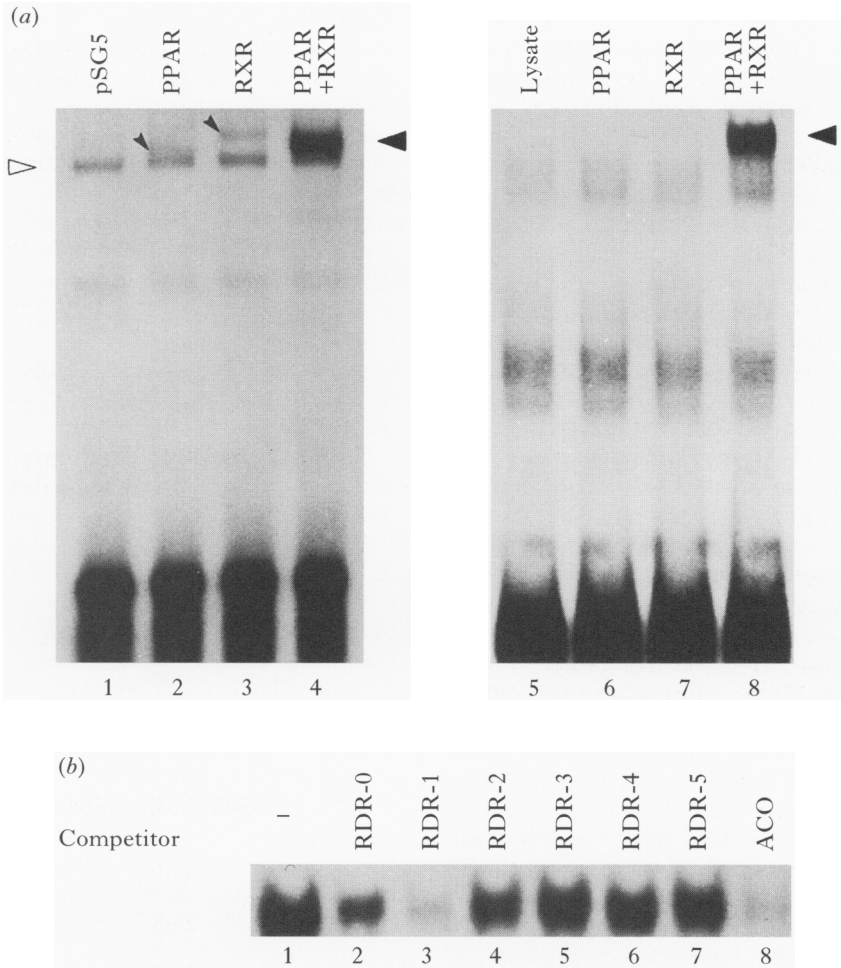

FIGURE 3. The peroxisome proliferator-activated receptor (PPAR) and the retinoid X receptor (RXR) bind co-operatively and specifically to the peroxisome proliferator response element (PPRE). (*a*) Co-operative binding of PPAR and RXR to the acyl CoA oxidase PPRE. Cellular extracts were prepared from COS cells transfected with pSG5 (lane 1), pSG5-mPPARα (lane 2) or pSG5-mRXRα (lane 3). These were used either alone (lanes 1–3) or in combination (lane 4) in an in-vitro DNA binding assay with a radiolabelled 43 bp oligonucleotide probe containing the acyl CoA oxidase PPRE. The open arrowhead indicates a non-specific protein–DNA complex and the solid arrowhead the specific one. Similar experiments were performed using proteins translated *in vitro* (lanes 5–8). (*b*) Specificity of PPAR/RXR binding to the RDR-1 (reverse direct repeat-1) response element. DNA binding was performed using the RDR-1 probe either in the absence (lane 1) or presence (lanes 2–8) of a 100-fold excess of competitor oligonucleotide. The competitors used were the spaced half-site elements (RDR-0 to RDR-5) and the acyl CoA oxidase PPRE (ACO).

in-vitro translated PPARα and RXRα (lanes 6–8). In these cases no DNA binding was observed with the individual receptors (lanes 6 and 7) but extensive binding was observed when the receptors were mixed (lane 8). Notably, DNA binding did not require the addition of either 9-*cis* retinoic acid or Wy-14,643. The DNA binding specificity of the PPARα–RXRα interaction was examined using the

acyl CoA oxidase response element or the spaced response element oligonucleotides (RDR-0 to RDR-5) as competitors in a gel retardation experiment. As anticipated, both the acyl CoA oxidase and RDR-1 response elements competed well with the RDR-1 probe for binding to the PPARα–RXRα complex (Fig. 3*b*, lanes 3 and 8). Some competition was also observed using the RDR-0 (lane 2) and RDR-2 (lane 4) oligonucleotides, but not with RDR-3 (lane 5), RDR-4 (lane 6) or RDR-5 (lane 7). These results agree well with the transactivation results observed with PPARα using the reporter plasmids that contain these response elements (Fig. 2*b*).

We next determined the effect of RXRα on the response of the acyl CoA oxidase promoter to PPARα. We have demonstrated previously that the PPRE contained within the 5′ flanking sequence of the rat acyl CoA oxidase gene is located 570 bp upstream from the transcription start site (Tugwood *et al.* 1992). We therefore created a reporter plasmid, ACO(−581/+20)CAT, that contains the 5′ flanking sequence of the rat acyl CoA oxidase gene from −581 bp to +20 bp, placed upstream of the CAT coding sequence (Fig. 4*a*). This was transfected into Hepa1 cells either in the absence or in the presence of the PPARα and RXRα expression plasmids and their activators (Fig. 4*a*). A 4-fold response to PPAR plus Wy-14,643 was observed (Fig. 4*a*, group B) and a 5·4-fold response to RXRα plus 9-*cis* retinoic acid (Fig. 4*a*, group C). Notably, the addition of both receptors and activators resulted in a 9·4-fold response, indicating an additive effect (Fig. 4*a*, group D). We next tested a reporter plasmid, ACO(−549/+20)CAT, where the PPRE was deleted (Fig. 4*a*). As expected, this reporter plasmid was unresponsive to PPARα but, interestingly, could still be stimulated 2·2-fold by RXRα. Furthermore, PPARα had no effect upon the RXRα response. A third reporter plasmid, ACO(−124/+20)CAT, that only contains 124 bp of 5′ flanking sequence, was unresponsive to either PPARα or RXRα (Fig. 4*a*).

To determine the effect of activators on the response to PPARα and RXRα, transfections were performed using the ACO(−581/+20)CAT reporter plasmid in the absence or presence of PPARα, RXRα, Wy-14,643 and 9-*cis* retinoic acid (Fig. 4*b*). In the absence of either receptor expression plasmid, the addition of Wy-14,643 had no effect (lane 2) and the addition of 9-*cis* retinoic acid had only a weak effect (lane 3), suggesting the presence of a small amount of endogenous RXR in Hepa1 cells. When cotransfected with pSG5-mPPARα, a 4-fold induction was observed with Wy-14,643 (lane 6) but no additional effect was observed with 9-*cis* retinoic acid (lane 8). Cotransfections with pSG5-mRXRα produced a 5·4-fold effect with 9-*cis* retinoic acid (lane 11) but little additional effect with Wy-14,643 (lane 12). Notably, 9-*cis* retinoic acid had no effect on PPARα (lane 7) and Wy-14,643 had no effect upon RXRα (lane 10). In transfections using both receptor expression plasmids, Wy-14,643 (lane 14) and 9-*cis* retinoic acid (lane 15) had similar effects to those observed when using either receptor alone (compare lane 6 with lane 14 and lane 11 with lane 15). Importantly, however, a greater response was obtained in the presence of both activators (lane 16), indicating that both PPARα and RXRα contribute to activate transcription.

We next examined the ability of several hypolipidaemic drugs to activate PPAR. Hepa1 cells were transfected with the mPPARα expression vector and the ACO(−1273/−471)G.CAT reporter plasmid in the presence of 100 μM, 10 μM or 1 μM of a potential ligand. Transfection of PPARα alone produced a small increase in CAT activity when compared with cells not transfected with PPARα (Table 1, DMSO). This may be due to the presence of endogenous fatty acids in the Hepa1 cells (see below). A comparison of the hypolipidaemic drugs indicated that they all activated PPAR, with Wy-14,643 being the most potent followed by nafenopin, ciprofibrate, methyl clofenapate, bezafibrate, gemfibrozil and clofibric acid (Table 1). The activities of the peroxisome proliferators MEHP, TCA, DHEA and DHEA sulphate were also examined. MEHP, which is an active metabolite of DEHP, was as potent as methyl clofenapate when tested at 10 μM, yet far less active than Wy-14,643 when tested at 1 μM. TCA, which is a weak peroxisome proliferator, activated PPAR only very weakly at 100 μM. Interestingly, neither the steroid DHEA nor its sulphate activated PPAR. This finding supports other data indicating that DHEA causes peroxisome proliferation *in vivo* but not in primary hepatocyte cultures, suggesting that DHEA acts through some indirect mechanism (Hertz *et al.* 1991).

A high fat diet produces peroxisome proliferation in rats (Osmundsen *et al.* 1991), and a high concentration of some fatty acids has been shown to activate a chimera between the glucocorticoid receptor and the rat PPARα in transfection assays (Göttlicher *et al.* 1992). We wished to examine whether there was any specificity amongst a variety of fatty acids to activate the wild-type mPPARα using a natural PPRE (ACO(−1273/−471) G.CAT). A number of saturated and polyunsaturated fatty acids were tested at 100 μM for their ability to activate PPARα (Fig. 5). A wide range of

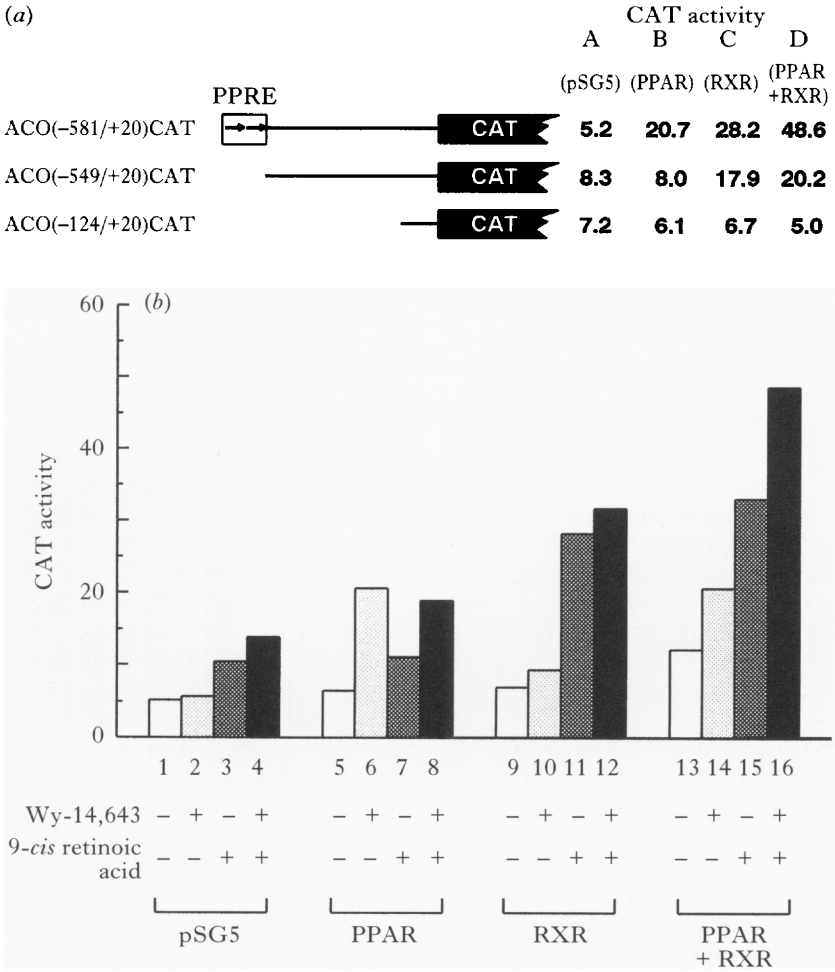

FIGURE 4. The peroxisome proliferator-activated receptor (PPAR) and the retinoid X receptor (RXR) co-operatively activate transcription of the acyl CoA oxidase (ACO) reporter plasmid. (*a*) Transcriptional activation of the acyl CoA oxidase reporter plasmid by PPAR and RXR. Reporter plasmids (5 μg) containing different regions of the acyl CoA oxidase promoter region were cotransfected into Hepa1 cells with (A) pSG5 (600 ng) and no ligands; (B) pSG5-mPPARα (300 ng), pSG5 (300 ng) and 10 μM Wy-14,643; (C) pSG5-mRXRα (300 ng), pSG5 (300 ng) and 1 μM 9-*cis* retinoic acid or (D) both receptor expression vectors (300 ng each) and both ligands. (*b*) Effect of ligand on PPAR/RXR activation of the acyl CoA oxidase reporter plasmid. The ACO(−581/+20)CAT reporter plasmid (5 μg) was transfected into Hepa1 cells with (bars 1–4) pSG5 (600 ng), (bars 5–8) pSG5 (300 ng) and pSG5-mPPARα (300 ng), (bars 9–12) pSG5 (300 ng) and pSG5-mRXRα (300 ng) or (bars 13–16) both receptor expression vectors (300 ng each), in either the absence or presence (−/+) of Wy-14,643 (10 μM) and 9-*cis* retinoic acid (1 μM). Chloramphenicol acetyltransferase (CAT) activity is expressed as the percentage of acetylated chloramphenicol. The averages of triplicate results are shown.

fatty acids activated PPARα to a level of between 33 and 50% of that observed when using 10 μM Wy-14,643 (Fig. 5). A minimum chain length of 10 carbon atoms was required for good activity (capric acid), with all fatty acids up to the large polyunsaturated fatty acid eicosapentaenic acid (C20:5) having similar activities (Fig. 5). Fatty acids that are shorter than C10 were either less active

TABLE 1. Hypolipidaemic drugs and peroxisome proliferators activate the peroxisome proliferator-activated receptor (PPAR). Hepal cells were transfected with the PPAR expression vector (pSG5-mPPARα), the reporter plasmid pACO(−1273/−471)G.CAT and the β-galactosidase internal control plasmid pCH110. The hypolipidaemic drugs Wy-14,643, nafenopin, ciprofibrate, bezafibrate, gemfibrozil, methyl clofenapate and clofibric acid and peroxisome proliferators mono-(2-ethylhexyl) phthalate (MEHP), trichloroacetic acid (TCA), dehydroepiandrosterone (DHEA) and DHEA sulphate were added at final concentrations of 1, 10 and 100 μM in dimethyl sulphoxide (DMSO; 0·1% final concentration) for 44 h. Chloramphenicol acetyltransferase (CAT) activities were determined and normalized using the β-galactosidase internal control. Each compound was tested in triplicate and the averages of two experiments are shown

	CAT activity (arbitrary units)		
	100 μM	10 μM	1 μM
Addition			
DMSO	7·0	7·0	7·0
Wy-14,643	85·0	100*	63·6
Nafenopin	104·7	77·6	12·7
Ciprofibrate	81·8	44·8	14·3
Methyl clofenapate	toxic	32·0	7·1
Bezafibrate	67·1	25·2	7·7
Gemfibrozil	81·4	18·4	6·1
Clofibric acid	71·4	12·4	5·5
MEHP	toxic	32·1	9·0
TCA	10·8	—	—
DHEA	toxic	5·6	—
DHEA sulphate	4·1	—	—

*Reference value (100 arbitrary units=35% acetylated chloramphenicol).

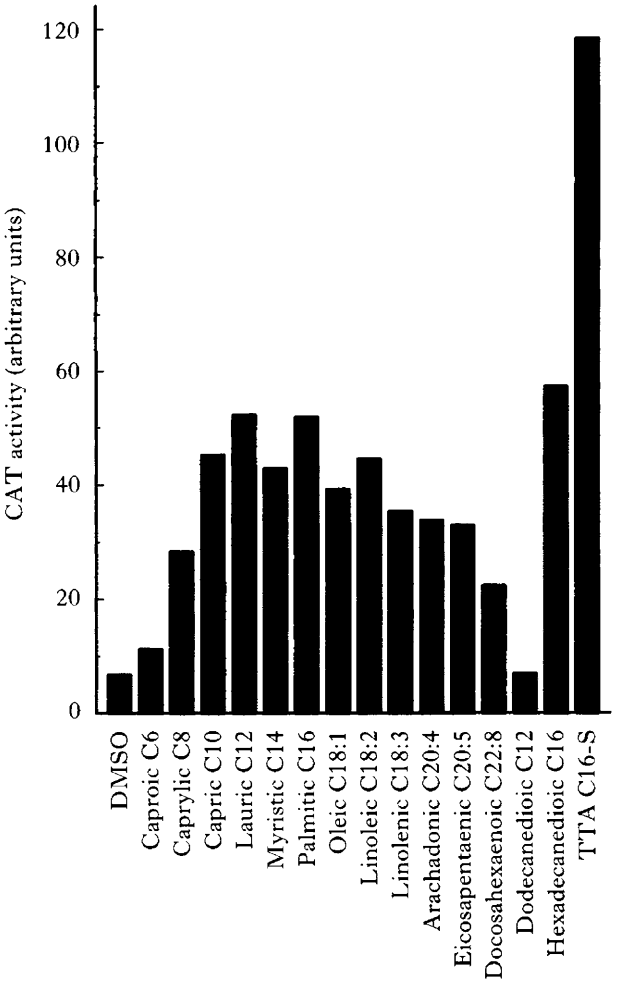

FIGURE 5. A wide range of fatty acids activate the peroxisome proliferator-activated receptor (PPAR). Hepal cells were transfected with the PPARα expression vector and the ACO(−1273/−471)G.CAT reporter plasmid in the presence of 100 μM fatty acids (in 0·1% dimethyl sulphoxide (DMSO), final concentration). Chloramphenicol acetyltransferase (CAT) activities were determined 44 h later and the averages of triplicate experiments are shown with each experiment repeated three times. Arbitrary CAT activity units are defined using the value obtained with 10 μM Wy-14,643 as a reference. TTA, tetradecylthioacetic acid.

(caprylic, C8) or weakly active (caproic, C6). Neither chain length (above C10) nor the number or position of unsaturated double bonds had any major influence upon activity, suggesting that no one fatty acid is an exclusive natural PPAR ligand. The cytochrome P-450 IVA1 induced by peroxisome proliferators has lauric acid ω-hydroxylase activity that is partly responsible for the formation of dicarboxylic acids (Orton & Parker, 1982; Aoyama *et al.* 1990). Since these are candidates for the natural PPAR ligand, we tested dodecanedioic acid (C12) and hexadecanedioic acid (C16) as PPAR activators. Surprisingly, dodecanedioic acid was inactive whereas hexadecanedioic acid was one of the most potent fatty acids tested. One possible explanation of these findings is that dodecanedioic acid may be rapidly metabolized to a chain length which is shorter than C10. Rapid metabolism may also explain why high concentrations of fatty acids are required to activate PPAR. To test this, we examined a thiol-substituted fatty acid, TTA, that is unable to be metabolized by β-oxidation and is therefore more stable than the related fatty acid palmitic acid. Interestingly, TTA was almost as active as Wy-14,643 at 10 μM but was less active at 1 μM (Fig. 6). As expected, TTA was far more active than palmitic acid, supporting the suggestion that the weak activity of fatty acids is due to their rapid metabolism within the cell.

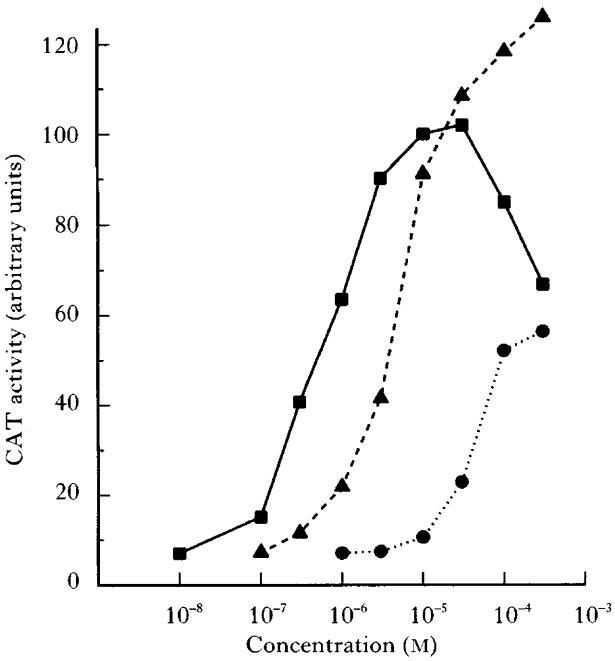

FIGURE 6. The thiol-substituted fatty acid, tetradecylthioacetic acid (TTA), is a potent peroxisome proliferator-activated receptor activator. Hepa1 cells were transfected with the pSG5-mPPARα expression vector (1 μg) and the ACO(−1273/−471)G.CAT reporter plasmid in the presence of Wy-14,643 (■), TTA (▲) or palmitic acid (●). Chloramphenicol acetyltransferase (CAT) activities were determined after 44 h and the averages of triplicate experiments are shown. Arbitrary CAT activity units are defined using the value obtained with 10 μM Wy-14,643 as a reference.

DISCUSSION

In common with RAR, TR, and VDR, the ability of PPARα to bind to DNA is dramatically enhanced by the addition of RXR and is independent of ligand. Our results therefore support similar recent findings (Kliewer *et al.* 1992*b*). Furthermore, our results indicate that a direct repeat of the TGACCT half-site with a single base pair spacing is the preferred binding site for the PPARα–RXRα heterodimer, and is the optimal PPARα response element. Of particular interest is the finding that the functional interaction between PPARα and RXRα is dependent upon both Wy-14,643 and 9-*cis* retinoic acid. This is in contrast to experiments with RAR where cotransfection with RXR is sufficient to enhance the activity of RAR and the addition of 9-*cis* retinoic acid has no further effect (Zhang *et al.* 1992*c*). Our results therefore demonstrate that RXRα contributes to the stimulation of transcription and is not simply required to enhance DNA binding.

Fibrate hypolipidaemic drugs have been used for many years to lower the level of plasma triglyceride and, to some extent, cholesterol, as a treatment for coronary heart disease (Sirtori *et al.* 1977; Leaf *et al.* 1989). The mechanism of action of this important class of drugs is unknown but the finding that a number of them are potent activators of PPAR (Table 1) suggests that this receptor mediates at least part of their activity. It remains to be established, however, whether PPAR mediates the therapeutic, or merely the toxicological, action of fibrate drugs. Nevertheless, there is some support for a physiological role for PPAR in regulating triglyceride levels. For example, a high fat diet produces peroxisome proliferation in rats and elevates peroxisomal acyl CoA oxidase (Osmundsen *et al.* 1991) and we show here that a wide variety of fatty acids can activate PPAR. To examine this question further it will be important to determine the expression and function of PPARs in humans, looking in particular at tissues that are important for fatty acid metabolism and utilization. Interestingly, since fatty acids activate PPAR, one could speculate that fatty acids and 9-*cis* retinoic acid may co-operate to regulate genes containing upstream direct repeat-1 motifs, such as those that are important for gluconeogenesis (e.g. phosphoenolpyruvate carboxykinase; Lucas *et al.* 1991) or lipid homeostasis (e.g. apolipoprotein; Ladias *et al.* 1992; Mietus-Snyder *et al.* 1992).

The finding that the metabolically stabilized fatty acid TTA activates PPAR almost as well as the potent hypolipidaemic drug Wy-14,643 suggests that fatty acids, or perhaps their CoA derivatives, are natural ligands for PPAR. It is somewhat surprising, however, that such a large variety of fatty acids can activate PPAR (Fig. 5). One possibility is that all of the fatty acids could be metabolized to a common ligand or could induce the formation of the PPAR ligand. Alternatively, the fatty acids could bind to PPAR (perhaps as CoA esters) with PPAR recognizing determinants on the carboxylic acid or CoA group, and extra stability provided by hydrophobic interactions with the fatty acid backbone. Related to this is whether fibrate hypolipidaemic drugs and peroxisome proliferators activate PPAR by binding directly to it. Interestingly, some peroxisome proliferators interact with liver fatty acid-binding protein (FABP), resulting in the displacement of fatty acids, such as oleic acid, and may therefore activate PPAR by increasing intracellular fatty acid levels (Cannon & Eacho, 1991). Note, however, that since both peroxisome proliferators and fatty acids bind to FABP it would not be unreasonable for them to compete for a binding site on PPAR. Furthermore, there are

interesting parallels with retinoic acid action. Retinoic acid binds to both a cellular binding protein (CRABP) and a nuclear hormone receptor (RAR). CRABP is related to FABP and is thought to regulate the free retinoic acid concentration, whereas RAR, which is related to PPAR, mediates the biological action of retinoic acid. A further possibility is that there are two classes of peroxisome proliferator, one that binds to PPAR directly and a second that acts indirectly by elevating fatty acid levels.

Finally, a surprisingly large number of nuclear receptors recognize the single spaced direct repeat either as hetero- or homodimers, and act either negatively (chicken ovalbumin upstream promoter transcription factor, apolipoprotein AI regulatory protein-1) or positively (RXR, PPAR, hepatocyte nuclear factor-4) to regulate gene expression (Sladek *et al.* 1990; Ladias & Karathanasis, 1991; Mangelsdorf *et al.* 1991; Cooney *et al.* 1992; Ladias *et al.* 1992; Mietus-Snyder *et al.* 1992). Learning more about how PPAR influences these interactions should lead to a better understanding of why peroxisome proliferators are rodent hepatocarcinogens and how they act as hypolipidaemic drugs in man.

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