Convergence of 9-*cis* retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors

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Abstract

Peroxisomes are cytoplasmic organelles which are important in mammals in modulation of lipid homeostasis, including the metabolism of long-chain fatty acids and conversion of cholesterol to bile salts (reviewed in refs 1 and 2). Amphipathic carboxylates such as clofibric acid have been used in man as hypolipidaemic agents and in rodents they stimulate the proliferation of peroxisomes. These agents, termed peroxisome proliferators, and all- *trans* retinoic acid activate genes involved in peroxisomal-mediated β -oxidation of fatty acids^{1–4}. Here we show that the receptor activated by peroxisome proliferators⁵ and the retinoid X-receptor α (ref. 6) form a heterodimer that activates acyl-CoA oxidase gene expression in response to either clofibric acid or the retinoid $\times$ receptor- α ligand, 9-*cis* retinoic acid, an all-*trans* retinoic acid metabolite^{7,8}; simultaneous exposure to both activators results in a synergistic induction of gene expression. These data demonstrate the coupling of the peroxisome proliferator and retinoid signalling pathways and provide evidence for a physiological role for 9-*cis* retinoic acid in modulating lipid metabolism.

The peroxisome proliferator responsive element (PPRE) located in the rat acyl-CoA oxidase (*AOX*) promoter is composed of two direct AGG(A/T)CA repeats separated by a single nucleotide (Fig. 1a)^{9–11} and thus conforms with previously described retinoid $\times$ response elements^{12,13}. Indeed, cotransfection of the retinoid $\times$ receptor- α (RXR α) expression plasmid in the presence of 9-*cis* retinoic acid (Fig. 1b, 9-*cis* RA, RXR) resulted in activation of a reporter construct containing the *AOX* promoter upstream of the luciferase gene (*AOX-LUC*). As previously shown⁹, the *AOX* promoter was also induced by the peroxisome proliferator-activated receptor (PPAR) in the presence of clofibric acid (Fig. 1b, clofibric acid, PPAR). Interestingly, cotransfection of expression plasmids for both PPAR and RXR α in the presence of clofibric acid and 9-*cis* retinoic acid resulted in a synergistic increase in the activity of the *AOX* promoter (Fig. 1b, clofibric acid + 9-*cis* RA, PPAR+RXR).

To investigate whether the cooperativity between PPAR and RXR α occurred through the PPRE, cotransfection experiments were performed with a reporter construct containing three copies of the PPRE upstream of the thymidine kinase promoter fused to the luciferase gene

(*PPRE₃-TK-LUC*). As expected, cotransfection of either PPAR or RXR α expression plasmids in the presence of clofibric acid or 9-*cis* retinoic acid, respectively, resulted in induction of reporter expression (Fig. 1c, clofibric acid or 9-*cis* RA). As in the case of the intact *AOX* promoter, coexpression of PPAR and RXR α in the presence of both activators resulted in a synergistic increase in *PPRE₃-TK-LUC* expression (Fig. 1c, clofibric acid + 9-*cis* RA). Thus, PPAR and RXR α cooperate in transactivating gene expression through the PPRE.

The synergism between PPAR and RXR α on the PPRE suggested the possibility of a heterodimeric interaction between the two receptors. To test this possibility, coimmunoprecipitation experiments were performed using bacterially expressed RXR α and *in vitro*-synthesized, [³⁵S]methionine-labelled PPAR. As shown in Fig. 2a, when mixed, polyclonal antiserum against RXR α efficiently coprecipitated PPAR, indicating the formation of a solution heterodimer. Using the PPRE as probe, gel mobility shift experiments were performed to examine the binding properties of the PPAR-RXR α complex. Neither PPAR nor RXR α alone bound efficiently to the radiolabelled PPRE (Fig. 2b, lanes 2 and 3). Mixing the PPAR and RXR α proteins, however, resulted in the appearance of a strong, shifted complex (Fig. 2b, lane 4) which was further shifted by inclusion of the polyclonal antiserum (Fig. 2b, lane 5). Thus, PPAR and RXR α are capable of forming a complex in solution that binds DNA in a cooperative fashion.

RXR also forms heterodimers with the receptors for vitamin D, thyroid hormone and retinoic acid (VDR, TR and RAR, respectively), which bind cooperatively to their cognate hormone response elements (HREs)¹⁴⁻¹⁷. *In vivo*, the selectivity in the hormonal response is achieved through these receptor complexes recognizing direct repeats spaced by 3,4 or 5 nucleotides, respectively (3-4-5 rule)¹⁸. The comparative binding specificity of the PPAR-RXR α complex was assessed by gel mobility shift experiments using as competitors a series of synthetic HREs composed of two AGGTCA direct repeats separated by spacers from zero to five nucleotides in length (DR 0-5; ref. 13). As predicted from *in vivo* studies¹⁸, heterodimers formed between RXR α and the VDR, TR or RAR interacted most strongly with DR-3, DR-4 and DR-5, respectively (Fig. 3a). In contrast, the PPAR-RXR α complex interacted with the DR-1 oligonucleotide only. The RAR-RXR α complex also bound to DR-1 (Fig. 3a) indicating that there is the potential for competitive interactions between these heterodimers.

Consistent with the observed DR-1 binding preference, the PPAR-RXR α complex interacted strongly with other natural DR-1-like HREs found in the 3-ketoacyl-CoA thiolase, chicken ovalbumin and cellular retinol-binding protein type II promoters as well as the PPRE (Fig. 3b, lanes 1-4). In contrast, the PPAR-RXR α complex bound only weakly to the retinoic acid response element of the RAR- β promoter and the thyroid hormone response element of the Moloney leukaemia virus long terminal repeat, and not at all to the osteopontin vitamin D response element (Fig. 3b, lanes 6-8). The observation that the PPAR-RXR α complex binds a sequence element found in the promoter of the 3-ketoacyl-CoA thiolase gene is particularly interesting, because this gene encodes another of the peroxisomal β -oxidation enzymes and is induced by peroxisomal proliferators¹⁹. The presence of DR-1-like HREs

upstream of the genes for both peroxisomal enzymes suggests that the PPAR- RXR α complex is involved in their coregulation.

The physiological significance of the PPAR-RXR α heterodimer is supported by the observations that both receptors are most highly expressed in the liver and kidney (data not shown, and refs 5 and 6), and that these tissues are the principal sites of action of peroxisome proliferators²⁰. Thus extracts prepared from these tissues should presumably contain factors that promote PPAR binding to the PPRE. As shown in Fig. 4, liver nuclear extracts have PPRE binding activity (lane 4), but addition of *in vitro*- synthesized PPAR results in a marked enhancement in DNA binding (Fig. 4; compare lanes 4 and 5), demonstrating a cooperative activity in the extract. Addition of RXR α -specific antiserum to the reaction resulted in a reduction in complex formation to roughly the level in nuclear extracts alone, and the concomitant formation of a new complex with reduced electrophoretic mobility (Fig. 4, lane 6). These results demonstrate that RXR α is the main protein in liver responsible for enhancing PPAR binding to the PPRE *in vitro*.

In summary, our results demonstrate that peroxisome proliferators and 9-*cis* retinoic acid regulate an overlapping set of target genes through the PPAR-RXR α complex. The observation that both retinoids and peroxisome proliferators stimulate transcription of the genes encoding the peroxisomal β -oxidation enzymes^{3,4} suggests that 9-*cis* retinoic acid may function as a hypolipidaemic agent through activation of the PPAR-RXR α heterodimer. Finally, our demonstration that 9-*cis* retinoic acid is present in the liver and kidney of mouse⁷ is consistent with this molecule serving as an endogenous signal in the modulation of peroxisomal-mediated metabolism of fatty acids and other lipids.

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FIG. 1.

PPAR and RXR α transactivate reporter expression cooperatively through the PPRE. *a*, The sequence of the PPRE located between nucleotides –558 and –570 of the *AOX* promoter is compared to that of the idealized DR-1 HRE. The positions of the AGGTCA direct repeats are indicated by arrows. X, nucleotide substitution in this motif, *b*, CV-1 cells were cotransfected with reporter construct *AOX-LUC* and either the control (CONT) Rous sarcoma-virus promotor-chloramphenicol acetyl transferase (RS-CAT) or expression plasmids RS-rat(r)PPAR (PPAR) and/or RS-human(h)RXR α (RXR) as indicated. Cells were subsequently treated with carrier dimethylsulphoxide (DMSO) (–) or hormones (+), including clofibric acid, 9-*cis* retinoic acid (RA), or clofibric acid and 9-*cis* RA as indicated. Luciferase activity is presented as per cent normalized response where induced PPAR activity in the presence of clofibric acid is arbitrarily set at 100%. *c*, CV-1 cells were cotransfected with reporter construct *PPRE₃-TK-LUC* and either the control RS-CAT or expression plasmids RS-rPPAR and/or RS-hRXR α as indicated.

METHODS. Expression plasmid RS-rPPAR was constructed by inserting the rat PPAR cDNA (D.J.N., submitted) into the *KpnI/BamHI* sites of pRS (ref. 6). The RS-human RXR α expression plasmid has been described¹². The reporter plasmid *AOX-LUC* was constructed by amplification from rat genomic DNA of the 5' flanking sequences (nucleotides -602 to +20) of the rat *AOX* promoter by 30 cycles of the polymerase chain reaction (PCR) and directional insertion into *BamHI/XhoI-cut*. pLUC (ref. 12). Oligonucleotides for PCR contained *BamHI* (5') and *XhoI* (3') restriction sites to facilitate subcloning. The reporter construct *PPRE₃-TK-LUC* was constructed by inserting 3 copies of an oligonucleotide encoding the PPRE (GTCGACAGGGGA-CCAGGACAAAGGTCACGTTCTGGGAGTCGAC)⁹⁻¹¹ in direct orientation into the unique *SaII* site of the basal reporter construct *TK-LUC* (ref. 12). The orientation of the PPREs was confirmed by sequencing. Transfection assays were performed using CV-1 cells as described^{12,18} and modified for automation in 96-well plates⁷. All transfections were performed on a Beckman Biomek Automated Workstation. Transfections contained 5.0 ng of receptor expression plasmid vector (or control RS-CAT), 50 ng of the reporter luciferase plasmid, 50 ng of pRS/3GAL (β -galactosidase) as an internal control, and 90 ng of carrier plasmid pGEM. Cells were transfected for 6 h, washed to remove DNA precipitates, and treated with hormones (1×10^{-3} M clofibric acid; 1×10^{-6} M 9-*cis* RA) for 36 h. Cell extracts were subsequently prepared and assayed for luciferase and β -galactosidase activities⁷. All experiments were done in triplicate in at least two independent experiments and were normalized for transfection efficiency by using β -galactosidase as the internal control.

FIG. 2.

Direct interactions between PPAR and RXR α in the absence or presence of DNA. *a*, PPAR and RXR α form a complex in solution. Immunoprecipitation was done using *in vitro*-synthesized [³⁵S]methionine-labelled PPAR in the presence of 150 ng of either bacterially expressed RXR α (lane 2) or control glutathione S-transferase (GST) (lane 1). Polyclonal antiserum prepared against RXR α was used. The position of immunoprecipitated PPAR is indicated by an arrow. Under identical conditions we failed to observe RXR α interactions with radiolabelled glucocorticoid receptor (data not shown, and ref. 14). *b*, PPAR and RXR α bind cooperatively to the PPPE. Gel mobility shift assays were done using *in vitro*-synthesized PPAR and/or RXR α as indicated, and ³²P-labelled PPPE oligonucleotide. Pre-immune (PI) or polyclonal antiserum prepared against RXR α (RXRab) were included in the reactions as indicated.

METHODS. PPAR and RXR α RNA was prepared and translated in rabbit reticulocyte lysates as directed by the supplier (Promega). Coimmunoprecipitation reactions (20 μ l) included 5 of *in vitro*-synthesized [³⁵S]methionine-labelled PPAR and 150 ng of either bacterially expressed GST-RXR fusion protein¹² or GST alone in 20 mM Tris, pH 8.0. Proteins were incubated for 20 min on ice before the addition of 5 μ l polyclonal antisera prepared against an RXR α peptide (amino acids 214–229). Antigen-antibody complexes were collected by the addition of protein A-Sepharose (Pharmacia) and the immunocomplexes washed three times with 400 μ l RIPA buffer (10 mM Tris, pH 8.0,

150mMNaCl, 1% Triton X-100, 1% sodium deoxycholate). Immunoprecipitated complexes were resolved by SDS-polyacrylamide gel electrophoresis on 10% gels which were then fixed in 30% methanol/10% acetic acid, dried, and subjected to autoradiography. Gel mobility shift assays (20 μ l) contained 10 mM Tris, pH 8.0, 40 mM KCl, 0.05% NP-40, 6% glycerol, 1 mM DTT, 0.2 μ g poly(dI-dC) and *in vitro*-synthesized PPAR (2.5 μ l) and RXR α (2.5 μ l) as indicated in the figure legends. The total amount of reticulocyte lysate was maintained constant in each reaction (5 μ l) through the addition of unprogrammed lysate. After a 10-min incubation on ice, 0.5–1 ng of 32 P-labelled oligonucleotide was added and the incubation continued for a further 10 min. DNA-protein complexes were resolved on a 4% polyacrylamide gel in 0.5 xTBE (1 xTBE is 90 mM Tris, 90 mM boric acid, 2mM EDTA, pH 8.0). Gels were dried and autoradiographed at -70° . The PPRE oligonucleotide was 5'-AGCTGGACCAGGACAAAGGTCACGT- TCAGCT-3'.

FIG. 3.

Binding specificity of the PPAR-RXR α complex on synthetic and natural HREs. *a*, The binding specificities of the PPAR-RXR α , VDR-RXR α , TR-RXR α and VDR-RXR α complexes were tested by gel mobility shift analysis in the absence (–) or presence of a 4- or 16-fold molar excess of synthetic HREs, DR-0 to DR-5. Radiolabelled oligonucleotides encoding the PPRE, osteopontin VDRE, Moloney leukaemia virus (MLV) LTR TRE, and β -RARE were used as radiolabelled probes in binding assays involving the PPAR-RXR α , VDR-RXR α , TR-RXR α and RAR-RXR α complexes, respectively. Only the regions of the autoradiographs displaying the bound complexes are shown. Asterisks indicate reactions in which maximal levels of competition were observed. *b*, The binding specificity of the PPAR-RXR α complex was tested using radiolabelled oligonucleotides encoding the PPRE, 3-ketoacyl-CoA thiolase (3KAT), cellular retinol binding protein type II (CRBP II), chicken ovalbumin (OVAL), DR-1, osteopontin (VDRE), MLV LTR (TRE), and β -RAR (β -RARE) HREs. The sequences of these HREs are shown on the right.

METHODS. PPAR, RXR α , TR β , VDR and RXR α RNAs were prepared and translated in rabbit reticulocyte lysates as directed by the supplier (Promega). Gel mobility shift assays were done as for Fig. 2. The 3-keioacyl-CoA thiolase oligonucleotide was 5'-AGC-TCTCAGAGACCTTTGAACCA-CTTC-3'. The CRBP II-RXRE, β -RARE, MLV TRE, osteopontin VDRE, chicken ovalbumin HRE, and synthetic HRE direct repeat series oligonucleotides have been described^{12,13,18}.

FIG. 4.

RXR α present in liver nuclear extracts enhances PPAR binding to the PPRE. Gel mobility shift assays were prepared using 32 P-labelled PPRE oligonucleotide and *in vitro*-synthesized PPAR and/or RXR α and 0.5 μ g of rat liver nuclear extract (NE) as indicated. Pre-immune (PI) or polyclonal antiserum prepared against RXR α (RXRab) was included in the reactions.

METHODS. Gel mobility shift assays were done as described for Fig. 2.