Cross-talk between Orphan Nuclear Hormone Receptor RZRα and Peroxisome Proliferator-activated Receptor α in Regulation of the Peroxisomal Hydratase-Dehydrogenase Gene*

(Received for publication, June 5, 1998, and in revised form, August 18, 1998)

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The genes encoding the peroxisomal β-oxidation enzymes enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD) and fatty acyl-CoA oxidase (AOx) are coordinately regulated by peroxisome proliferator-activated receptor α (PPARα)/9-cis-retinoic acid receptor (RXRα) heterodimers that transactivate these genes in a ligand-dependent manner via upstream peroxisome proliferator response elements (PPRE). Here we demonstrate that the monomeric orphan nuclear hormone receptor, RZRα,§ modulates PPARα/RXRα-dependent transactivation in a response-element dependent manner. Electrophoretic mobility shift analysis showed that RZRα bound specifically as a monomer to the HD-PPRE but not the AOx-PPRE. Determinants in the HD-PPRE for binding of RZRα were distinct from those required for interaction with PPARα/RXRα heterodimers. In transient transfections, RZRα stimulated ligand-mediated transactivation by PPARα from an HD-PPRE luciferase reporter in the absence of exogenously added RZRα, but did not affect PPARα-dependent transactivation of an AOx-PPRE reporter gene. These data illustrate cross-talk between the RZRα and PPARα signaling pathways at the level of the HD-PPRE in the regulation of the HD gene and characterize additional factors governing the regulation of peroxisomal β-oxidation.

Nuclear hormone receptors are a diverse group of structurally related ligand-activated transcription factors that direct the expression of target genes in response to physiological and environmental stimuli (1, 2). Peroxisome proliferator-activated receptors (PPAR)¶ are members of the steroid hormone receptor superfamily that act to regulate a large number of genes involved in differentiation and lipid metabolism (3–9) in response to a variety of compounds collectively called peroxisome proliferators. Peroxisome proliferators include the fibrate family of hypolipidemic drugs, phthalate ester plasticizers, herbicides, pesticides, antidiabetic thiazolidinediones, as well as natural and synthetic fatty acids (10–15). Transactivation of target genes by PPARs is mediated through binding to cis-acting regulatory sequences called peroxisome proliferator response elements (PPRE) that consist of direct repeats of the hexameric TGAACCT/C core motif. PPARs heterodimerize with the 9-cis-retinoic acid receptor, RXRα, and bind with preference to response elements with spacing of one nucleotide between hexameric repeats (DR1) (16–19). PPREs have been identified in the regulatory regions of a number of genes, including those encoding the first two enzymes of the peroxisomal β-oxidation pathway, fatty acyl-CoA oxidase (AOx) (5, 20) and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD) (6, 21).

In addition to PPARs, several other nuclear hormone receptors have been shown to bind to PPREs and differentially modulate PPAR function. These include chicken ovalbumin upstream promoter transcription factor (COUP-TF) (22), hepatocyte nuclear factor-4 (HNF-4) (23), and thyroid hormone receptor (TR) (24). Transcriptional regulation via PPREs is thus a net aggregate response manifested in part by the availability of PPARs and other factors that bind to PPREs, the complexity of response elements, and the interplay of PPARs with other nuclear hormone receptors and cofactors. Overlying this network is a series of corepressors and coactivators that serve to mediate receptor signaling. The overall intricacy of the system enables the integration of information from multiple signaling pathways to ensure appropriate transcriptional responses of target genes to various stimuli.

The retinoid Z receptor (RZR) family, also known as the retinoid orphan receptor (ROR) family, is a recently described group of nuclear hormone receptors shown to be involved in regulating genes responsible for cellular differentiation, the inflammatory response, and lipid metabolism (25–28). RZR/ROR target response elements have been identified in the promoters of genes for chicken γ-F-crystallin, human and rat bone sialoprotein, human 5-lipoxygenase, N-myc proto-oncogene, and apolipoprotein A-I (28–32). Three RZR/ROR isoforms have been characterized (α, β, γ) that show differential tissue localization. The α isoform includes four splice variants (ROα1, ROα2, ROα3, and RZRα) and is widely expressed. RZRβ is found primarily in brain tissues, and RORγ is localized to skeletal muscle (26, 27, 33, 34). Although nuclear hormone receptors typically bind response elements as hetero- or homo-

* This work was supported in part by a grant from the Heart and Stroke Foundation of Canada (to J. P. C. and R. A. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.
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The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; AOx, fatty acyl-CoA oxidase; DR, direct repeat; EMSA, electrophoretic mobility shift analysis; HD, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase; PPRE, peroxisome proliferator response
modimers, RZR/ROR family members are distinct in that they are able to bind to, and transactivate from, target response elements as monomers (25, 26). RZR/ROR receptors recognize a TGACCT/C consensus half-site and have a demonstrated preference for sites flanked by a 6-base A/T-rich region (25, 26, 30, 35). This consensus half-site is embedded in the HD-PPRE, and recently RZRα was shown to bind to this element, suggesting that RZRα might be a candidate regulator of peroxisomal β-oxidation (30). To explore this possibility, we undertook to determine how RZRα might interact with PPARα and RXRα in regulating the transcription of genes encoding the first two enzymes of the peroxisomal β-oxidation pathway, AOx and HD. The results presented here indicate that RZRα binds to the HD-PPRE but not the AOx-PPRE, and selectively potentiates transactivation from the HD-PPRE in a manner dependent on the relative availability of RXRα.

EXPERIMENTAL PROCEDURES

**Plasmids**—The in vitro and in vivo expression plasmids for rat PPARα and human RXRα, and the luciferase reporter constructs pCPSluc containing the minimal promoter for the gene encoding carbamoyl-phosphate synthetase, pHD(X3)luc containing two copies of the minimal HD-PPRE, and pAOx(X2)luc containing two copies of the minimal AOx-PPRE, have been described previously (6, 17, 36). The plasmid pM2(X3)luc, constructed by cloning the mutant HD-PPRE synthetic oligonucleotide M2 (5'-GATCCTCTCCTTTAAAATATTGAACTATTACCTACATTTGA) and its complement into the BamHI site of pCPSluc, contains three direct tandem copies of the M2 element. The expression plasmid for human RZRα (RZRα/SG5) (30) was a kind gift of Carsten Carlberg (Hôpital Cantonal Universitaire de Genève, Geneva, Switzerland).

**In Vitro Transcription/Translation**—Transcription/translation of cDNAs encoding PPARα, RXRα, and RZRα (1 µl each) were incubated alone or in combination with radiolabeled HD-PPRE (A) or AOx-PPRE (B) probe, as indicated. Lysate volumes were kept constant by addition of unprogrammed lysate. EMSA was performed as described under "Experimental Procedures." The positions of the heterodimeric PPARα/RXRα and monomeric RZRα complexes are indicated by arrows.

**Fig. 1.** Comparison of the sequence of the RZRα consensus binding site (30) to sequences within the AOx- and HD-PPREs. Arrows and Roman numerals indicate the locations and directions of TGACCT-like motifs.

**Fig. 2.** RZRα binds as a monomer to the HD-PPRE but not the AOx-PPRE. In vitro synthesized PPARα, RXRα, and RZRα (1 µl each) were incubated alone or in combination with radiolabeled HD-PPRE (A) or AOx-PPRE (B) probe, as indicated. Lysate volumes were kept constant by addition of unprogrammed lysate. EMSA was performed as described under "Experimental Procedures." The positions of the heterodimeric PPARα/RXRα and monomeric RZRα complexes are indicated by arrows.

**Fig. 3.** RZRα monomer binds to the HD-PPRE in a sequence-specific manner. EMSA was performed with in vitro synthesized receptors (1 µl each) and radiolabeled HD-PPRE probe, as shown. Lysate volumes were kept constant by addition of unprogrammed lysate. Where indicated, unlabeled competitor DNA (5'-GATCCGGTTGAGCTAAATGATATTCT and its complement) (Nonspecific) or unlabeled HD-PPRE (Specific) was present in 10-, 50-, or 100-fold molar excess. The positions of the heterodimeric PPARα/RXRα and monomeric RZRα complexes are indicated by arrows.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The in vitro and in vivo expression plasmids for rat PPARα and human RXRα, and the luciferase reporter constructs pCPSluc containing the minimal promoter for the gene encoding carbamoyl-phosphate synthetase, pHD(X3)luc containing two copies of the minimal HD-PPRE, and pAOx(X2)luc containing two copies of the minimal AOx-PPRE, have been described previously (6, 17, 36). The plasmid pM2(X3)luc, constructed by cloning the mutant HD-PPRE synthetic oligonucleotide M2 (5'-GATCCTCTCCTTTAAAATATTGAACTATTACCTACATTTGA) and its complement into the BamHI site of pCPSluc, contains three direct tandem copies of the M2 element. The expression plasmid for human RZRα (RZRα/SG5) (30) was a kind gift of Carsten Carlberg (Hôpital Cantonal Universitaire de Genève, Geneva, Switzerland).

**In Vitro Transcription/Translation**—Transcription/translation of cDNAs encoding PPARα, RXRα, and RZRα was performed using the TNT T7 coupled rabbit reticulocyte lysate system according to the manufacturer’s protocol (Promega). Translation products labeled with L-[35S]methionine were analyzed on 15% SDS-polyacrylamide gels. Synthesis of proteins for use in electrophoretic mobility shift analysis (EMSA) was carried out in parallel with unlabeled methionine.

**Electrophoretic Mobility Shift Analysis**—EMSA was carried out as described previously (22, 36) with oligonucleotide probes corresponding to the HD-PPRE (5'-GATCCTCTCTTGTACCTACATTTGAAAAT) and its complementary strand (Nonspecific) or unlabeled HD-PPRE (Specific) was present in 10-, 50-, or 100-fold molar excess. The positions of the heterodimeric PPARα/RXRα and monomeric RZRα complexes are indicated by arrows.
0.4 mM MgCl$_2$, 0.1 mM EDTA, 7% (v/v) glycerol, 4 mg of bovine serum albumin, 4 mg of nonspecific competitor DNA (poly(dI-dC) and sonicated salmon sperm DNA, 1:1 weight ratio), 150 mM phenylmethylsulfonyl fluoride, and 0.2 mM dithiothreitol. The total amount of reticulocyte lysate in each reaction was kept constant by addition of unprogrammed lysate. Binding reactions were analyzed by electrophoresis at 4 °C on prerun 3.5% polyacrylamide gels (30:1 acrylamide/N,N$_9$-methylenebisacrylamide weight ratio) with 22 mM Tris base, 22 mM boric acid, 1 mM EDTA as running buffer, followed by autoradiography.

**RESULTS**

**RZRα Binds as a Monomer to the HD-PPRE but Not the AOx-PPRE**—Several potential RZRα consensus binding sites exist within the HD- and AOx-PPREs (Fig. 1), and interaction of RZRα with the HD-PPRE has recently been observed (30). To explore how RZRα might cooperate with PPARα and RXRα to regulate transactivation from PPREs, we first examined the
DNA-binding characteristics of these nuclear hormone receptors to the HD- and AOx-PPREs. EMSA was performed with radiolabeled HD- and AOx-PPREs and in vitro translated receptors. PPARa and RXRa formed a characteristic heterodimer that bound strongly to the HD-PPRE (Fig. 2A). RZRa also bound to the HD-PPRE, apparently as a monomer based on a comparison of the mobility of the RZRa complex with that of the heterodimeric PPARa/RXRa complex. Coincubation of RZRa with either PPARa or RXRa alone did not yield heterodimeric complexes. When all three receptors were present in the binding reaction, only heterodimeric PPARa/RXRa and monomeric RZRa complexes were observed, suggesting that these receptors do not co-occupy the HD-PPRE under these conditions. Therefore, RZRa binds as a monomer to the HD-PPRE and does not form complexes with PPARa or RXRa on the HD-PPRE in vitro. PPARa/RXRa heterodimers readily formed a complex on the AOx-PPRE, as expected; however, RZRa was unable to bind to this element either alone or in combination with PPARa or RXRa (Fig. 2B).

Competition analysis demonstrated that the interaction of RZRa with the HD-PPRE was specific. As shown in Fig. 3, both the RZRa and the PPARa/RXRa complexes were refractory to competition by nonspecific unlabeled oligonucleotide (compare lanes d-f to lanes a-c and g), whereas increasing amounts of unlabeled HD-PPRE oligonucleotide effectively competed for binding of both RZRa and PPARa/RXRa (compare lanes h-j to lanes a-c and g). These results show that binding of RZRa is specific for the HD-PPRE.

We next examined whether PPARa might modulate binding of RZRa to the HD-PPRE. EMSA was performed with radiolabeled HD-PPRE probe, constant amounts of RXRa and RZRa, and decreasing amounts of PPARa. Dilution of PPARa resulted in progressively decreased amounts of the PPARa/RXRa complex, as expected; however, there was no effect on formation of the RZRa monomer complex (Fig. 4A), suggesting that RZRa binding to the HD-PPRE in vitro is not influenced by PPARa. Similarly, increasing amounts of RZRa resulted in correspondingly greater amounts of the RZRa monomeric complex, but no change in the PPARa/RXRa complex (Fig. 4B). Additional analyses carried out using different amounts of radiolabeled HD-PPRE probe and titration of either RZRa (Fig. 5A) or PPARa/RXRa (Fig. 5B) showed that PPARa/RXRa and RZRa did not affect each other’s binding to the HD-PPRE. Therefore, RZRa and PPARa/RXRa bind independently to the HD-PPRE.

RZRa and PPARa/RXRa Bind to Different Regions of the HD-PPRE in Vitro—The above results suggest that RZRa recognizes distinct determinants on the HD-PPRE compared with the requirements for PPARa/RXRa interaction. The HD-PPRE
is a complex response element that consists of 4 consensus TGACCT hexameric half-sites (sites I–IV; Fig. 1). To determine which elements are responsible for RZRα binding specificity, oligonucleotide probes containing mutations in each of the four hexameric half-sites were used in binding studies (Fig. 6). EMSA analysis showed that mutations in sites I, III, or IV did not adversely affect binding of RZRα, whereas disruption of site II eliminated binding (Fig. 6). This result is consistent with the fact that site II most closely matches the RZRα consensus sequence (TGACCT/C/A/T). By comparison, the integrity of sites III and IV were essential for PPARα/RXRα interaction, whereas sites I and II were dispensable (Fig. 6). This latter result is consistent with previous observations that the downstream DR1 element is necessary and sufficient for PPARα/RXRα binding specificity, which is abrogated in the reporter construct pM2(X3)luc (Fig. 7B), in which site II of the HD-PPRE required for binding of RZRα (see below). Importantly, functional cooperativity between PPARα and RZRα in transactivation from the HD-PPRE is apparently required to achieve transcriptional stimulation by PPARα.

**Fig. 8. Melatonin does not stimulate transactivation by PPARα/RZRα from the HD-PPRE.** BSC40 cells were transfected with 5 μg of the luciferase reporter pHDX3luc and the expression plasmids for PPARα (2 μg) and RZRα (4 μg), with addition of vehicle (dimethyl sulfoxide) (open bars), 0.1 mM Wy-14,643 (hatched bars), or 1 μM melatonin (dashed bars). Plasmid dosage was normalized by the addition of the appropriate empty vectors, where required. Cells were harvested 48 h after transfection, and luciferase activity was quantitated. Values are normalized to the appropriate control, with the values obtained for cells transfected with reporter plasmid alone (Fig. 7A). Treatment of PPARα-expressing cells with the peroxisome proliferator and PPARα ligand, Wy-14,643, increased luciferase activity an additional 4-fold. Transfection of increasing amounts of RZRα expression plasmid in the presence of fixed amounts of PPARα expression plasmid led to a dose-dependent increase in the level of luciferase activity in cells treated with Wy-14,643, but did not affect the ligand-independent response. At the maximal level of RZRα plasmid used (4 μg), ligand-dependent PPARα activity was 23 times the basal level of activity of control cells transfected with reporter plasmid alone (and compared with the 8-fold PPARα, ligand-dependent induction observed in the absence of RZRα expression plasmid). RZRα expression plasmid alone showed at most a 1.5-fold increase in luciferase activity over basal levels of the HD-PPRE luciferase reporter (data not shown), in agreement with previous studies that demonstrated that RZRα only weakly activates transcription via this element (30). The modest increase observed likely reflects cooperation of RZRα with endogenous PPARα, as RZRα had no effect on basal expression levels of the parental pCPSluc reporter gene, which lacks a PPRE (see below). Importantly, functional cooperativity between PPARα and RZRα in transactivation from the HD-PPRE is abrogated in the reporter construct pM2(X3)luc (Fig. 7B), in which site II of the HD-PPRE required for binding of RZRα has been mutated. These results show that binding of RZRα to the HD-PPRE is apparently required to achieve transcriptional stimulation by PPARα.
Cross-talk between RZRα and PPARα Receptor Signaling Pathways

The above experiments demonstrate that RZRα cooperates with PPARα to potentiate transactivation from the HD-PPRE in response to treatment with a peroxisome proliferator. In contrast, RZRα did not significantly affect PPARα-dependent transactivation from the AOx-PPRE, in either the absence or presence of ligand (Fig. 7C). These data are in keeping with the in vitro binding data presented above, and together the results demonstrate that RZRα binds specifically to the HD-PPRE and modulates transactivation by PPARα from this response element.

We next investigated whether melatonin, which has been suggested to be a putative ligand for RZRα (31, 37), could influence the functional cooperation between PPARα and RZRα in stimulating transcription from the HD-PPRE. Under our experimental conditions, we were unable to observe any effect of melatonin on the functional cooperativity of these two receptors in stimulating transcription from the HD-PPRE (Fig. 8).

Exogenous RXRα Abrogates the Potentiation Effect of RZRα on Transactivation by PPARα from the HD-PPRE—RXRα is an obligate heterodimerization partner for PPARα, and the PPARα-dependent activation presented above presumably arises from cooperation with low levels of endogenous RXRα present in BSC40 cells. We therefore examined the effects of RZRα under conditions where RXRα expression plasmid was included in the transfections. Coexpression of exogenous RXRα and PPARα increased luciferase activity 14- and 10-fold over basal levels for the HD-PPRE (Fig. 9A) and AOx-PPRE (Fig. 9B) luciferase reporter plasmids, respectively. In the presence of Wy-14,643, activity was increased 60-fold for the HD-PPRE reporter (Fig. 9A) and 20-fold (Fig. 9B) for the AOx-PPRE reporter. The robust activity obtained with coexpressed RXRα is consistent with the fact that the endogenous level of this receptor is limiting for PPARα-mediated transactivation. Interestingly, under these conditions, transactivation from the HD- and AOx-PPRE reporter plasmids was not significantly affected by addition of RZRα (Fig. 9, A and B, respectively). Control transfections with the parental construct pCPSluc, which lacks a PPRE, showed that the presence of PPARα, RXRα, and RZRα did not influence the levels of relative luciferase activity observed (Fig. 9C), indicating that these receptors did not alter the basal activity of the parental reporter construct. Together, these data suggest that the stimulatory effect observed with RZRα on transactivation by PPARα is attenuated by increasing the level of RXRα.

DISCUSSION

Several observations have suggested a degree of interplay between the RZRα/ROR and PPAR nuclear hormone receptor families in the regulation of genes, particularly those encoding proteins involved in lipid metabolism. For instance, both PPARα and RZRα regulate transcription of the apolipoprotein A-I gene (28, 38). PPARα has been shown to be a critical regulator of the adipogenic program, and RORα and RORγ mRNA transcripts are induced early in adipogenesis (4, 39). Finally, the antidiabetic thiazolidinediones, which are potent activators of PPARγ, have recently been shown to also be specific ligands for RZRα (15, 34).

Both the HD and AOx genes are regulated by a number of nuclear hormone receptors from several different signaling networks that converge on the respective PPREs or that directly modulate PPARα activity (22–24, 40). Consistent with these observations, Schrader et al. (30) reported that RZRα weakly interacted with the HD-PPRE and was able to minimally activate transcription via this element (30). Our results extend this finding to show that RZRα can strongly potentiate transactivation by PPARα from the HD-PPRE when RXRα levels are limiting. We found that the monomeric binding of RZRα to the HD-PPRE was specific and required for potentiation of transactivation by PPARα from the HD-PPRE, and that RZRα did not bind to, or stimulate PPARα-dependent transactivation from, the AOx-PPRE.

The mechanism by which RZRα stimulates PPARα activity on the HD-PPRE is not known. EMSA analysis did not show any obvious antagonizing or stabilizing effects between RZRα monomers and PPARα/RXRα heterodimers on the HD-PPRE in vitro. This result agrees with our findings that the RZRα monomer and PPARα/RXRα heterodimer target distinct and non-overlapping hexameric determinants for binding to the HD-PPRE. Moreover, we did not observe a higher order ternary complex containing PPARα/RXRα/RZRα on the HD-PPRE. However, this does not preclude the possibility of such a com-
plex forming in vivo, which may be dependent on cooperativity or interaction with auxiliary cofactors. The involvement of auxiliary factors such as SRC-1, p300, and N-COR in transcriptional regulation by PPARs and other nuclear hormone receptors is well established (41–43). Additionally, PPARα, RXRα, and/or RXRα may require phosphorylation or other modification not provided in the in vitro transcription/translation system. We are currently investigating whether auxiliary factors and receptor post-translational modification affect cooperative transcriptional regulation by PPARα, RXRα and RXRα.

The HD-PPRE is a complex response element and among a select few that contain four hexameric direct repeats. In the HD-PPRE, these hexameric half-sites are organized in two tandem DR1 arrays that are separated by 2 nucleotides, an arrangement that is thought to facilitate diverse receptor interactions and thereby permit multiple levels of control. A model has been proposed in which either one or two PPARα/RXRα heterodimers bind to the HD-PPRE to determine the state of transcriptional activation (16). As we have shown previously, the 3’ DR1 (sites III/IV) array is essential and sufficient for PPARα/RXRα binding and activity (36); however, the arrangement that has been suggested to yield the highest level of transactivation has PPARα/RXRα heterodimers bound to both DR1 sites. Under certain conditions, a single PPARα/RXRα heterodimer may also bind to the DR2 element (sites II/III), but this complex is in a transcriptionally inactive form. Since RXRα occupies site II within the HD-PPRE, this may preclude binding of PPARα/RXRα to the DR2 element and thereby favor binding of the heterodimer to the transcriptionally competent 3’ DR1 array. RXRα may also contribute directly to transcriptional responses by stabilizing the PPARα/RXRα complex in vivo or facilitating interactions with an auxiliary factor(s). This pathway of stimulation may be operative when RXRα is present in limiting amounts, for example in transfections carried out with PPARα alone, as RXRα had no effect when transfections were carried out in the presence of excess exogenous RXRα. Cotransfection of RXRα significantly increased the overall level of transactivation by PPARα, as one would expect if endogenous RXRα is limiting. It is possible that this level of activity is beyond a threshold level at which RXRα might be expected to have a stimulatory effect. Moreover, when RXRα is present in excess in vivo, PPARα/RXRα heterodimers may occupy both DR1s, thereby resulting in a maximal transcriptional response and preventing RXRα from accessing site II. This model suggests ligand-mediated regulation of the HD-PPRE is dependent on input and dynamic interplay among the PPARα, RXRα, and RXRα signaling pathways.

In summary, our findings demonstrate that the orphan receptor RXRα can work in cooperation with PPARα to regulate expression of the gene encoding HD, the second enzyme of the peroxisomal β-oxidation pathway. Transcriptional regulation of peroxisomal β-oxidation has proven to be a dynamic process, integrating cues from a host of signaling pathways and rapidly responding to variations in levels of key components. An understanding of the principal regulators of peroxisomal β-oxidation, including transcription factors controlling and modulating the expression of the genes encoding the enzymes of this pathway, may provide for the development of pharmacologic agents that specifically target the peroxisomal β-oxidation system as a means to influence overall lipid metabolism and homeostasis.