In vivo stabilization of nuclear retinoid X receptor α in the presence of peroxisome proliferator-activated receptor α

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Abstract Retinoid X receptor α (RXRα) can reveal diverse functions through forming a heterodimer with peroxisome proliferator-activated receptor α (PPARα). However, the mechanism of regulation of the cellular RXRα level is unclear. Thus, quantitative change of RXRα was investigated in mouse liver. Nuclear RXRα level was constitutively lower in PPARα-null mice than in wild-type mice. The level was also increased by clofibrate treatment in wild-type mice without a concomitant increase of RXRα mRNA, but not in PPARα-null mice. Pulse chase experiments demonstrated that the presence of PPARα and its activation by ligands significantly affected the stability of nuclear RXRα. These findings suggest a novel regulatory mechanism of nuclear RXRα in vivo.

Key words: Retinoid X receptor α; Peroxisome proliferator-activated receptor α; Heterodimer; Stabilization

1. Introduction

The peroxisome proliferator-activated receptor (PPAR) family is a member of the steroid/thyroid hormone receptor superfamily, and consists of distinct genes designated PPARα, β or δ, and γ [1,2]. PPARα is highly expressed in hepatocytes, epithelial cells of the digestive tract and renal proximal tubular cells of both rodents and humans [3,4], and plays diverse roles including the maintenance of lipid and glucose homeostasis [5,6], regulation of cell proliferation [7,8] and modulation of inflammatory responses [9,10].

PPAR forms a heterodimer with retinoid X receptor (RXR) to generate a complex that binds to the peroxisome proliferator response element in the promoter region of target gene and activates its transcription [1,2,11–13]. The extent of the transcriptional activation is known to be influenced by various factors, including its ligands, coactivators, transcription factors and other nuclear receptors [1,2].

Of the three isoforms of RXR, RXRα is the most abundant in the liver [14]. Recently, Wan et al. established a mouse line lacking RXRα specifically in the hepatocytes, demonstrating that RXRα plays an important role in the action of hepatic PPARα [15,16]. Moreover, the mRNA level of hepatic PPARα is constitutively upregulated in these mice [15], suggesting that RXRα can affect the quantity of its heterodimeric partner PPARα. Since heterodimer formation between PPAR and RXRα might be important for quantitative regulation of these nuclear receptors, we examined it in detail. PPARα-null mice were used, because they have relatively normal phenotypes [17] and are thus suitable for serial in vivo analyses [18,19].

2. Materials and methods

2.1. Animals and clofibrate treatment

PPARα-null mice on an Sv/129 genetic background were generated as described elsewhere [17]. Twelve-week-old wild-type Sv/129 male mice ($n = 12$) and age- and sex-matched PPARα-null mice ($n = 12$) were used. The mice were fed either a control diet or one containing 0.75% (w/w) clofibrate (Wako Chemicals, Osaka, Japan) for 2 weeks. After treatment, the mice were killed and their livers excised. The feeding period and clofibrate content in the diet respectively were changed in the time course and dose–response experiment (see figure legends).

2.2. Immunoblot analysis

Nuclear extracts prepared from the mouse liver homogenates were used for the immunoblot analysis. Protein of the hepatocyte nuclear extracts (100 μg) was subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes. The membranes were incubated first with the primary antibody, and then with alkaline phosphatase-conjugated anti-rabbit or anti-goat IgG. The primary antibodies used (RXRα, D-20; PPARα, H-98; PPARβ, H-74; PPARγ, H-100) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RXRα (D-20) reacts specifically with RXRα of mouse and human origin and is non-cross-reactive with RXRβ and RXRγ. PPARα (H-98), PPARβ (H-74) and PPARγ (H-100) react specifically with PPARα, PPARβ and PPARγ of mouse and human origin, respectively. In the preliminary immunoblot experiments, the band positions of these four proteins were confirmed. Briefly, the band positions of nuclear RXRα and PPARα were determined by co-electrophoresis with cDNA-expressed RXRα [20] and PPARα [21], respectively. The band positions of nuclear PPARβ and PPARγ were estimated by co-electrophoresis with cDNA-expressed PPARα and double immunostaining, respectively, because the molecular weight of PPARα (52 kDa) is closely similar to that of PPARβ or PPARγ [22].

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Abbreviations: CRBPII, cellular retinol-binding protein II; DMSO, dimethyl sulfoxide; PPAR, peroxisome proliferator-activated receptor; PT, peroxisomal thiolase; RXR, retinoid X receptor; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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2.3. mRNA analysis

Northern blotting was used for mRNA analysis. Total liver RNA was extracted with an RNeasy Mini Kit™ (Qiagen, Valencia, CA, USA), electrophoresed on 1.1 M formaldehyde-containing 1% agarose gels and transferred to nylon membranes. The membranes were incubated with ^{32}P-labelled cDNA probes and analyzed on a Fuji system analyzer (Fuji Photo Film, Tokyo, Japan). The cDNAs for PPARs, RXRα and cellular retinol-binding protein II (CRBPII), used as probes, were generated by reverse transcription-polymerase chain reaction. Total RNA extracted from the liver of the clofibrate-treated mice was used as a template. The primer pairs were designed on the basis of the following published cDNA sequences: 5′-TGACGTTTGTGGCTGGTCAA-3′ and 5′-CAGAGATTTGAGGTCTGCAG-3′ for PPARα [21]; 5′-AGAACACACGCTTCCTTCCA-3′ and 5′-ATTGAGGAAGAGGCTGCTGA-3′ for PPARβ [22]; 5′-ACCAACTTCGGAATCAGCTC-3′ and 5′-GGGAAGGACTTTATGTATGA-3′ for PPARγ [23]; 5′-GTTGGGCGACTTTTGCAACA-3′ and 5′-TTTGCGTACTGTCCTCTTGA-3′ for RXRα [20], and 5′-GGTGGAGTTTGACGAACACAC-3′ and 5′-TTTGAACACTTGTCGGCACAC-3′ for CRBPII [24]. The amplified cDNA fragments for PPARα, β, γ, RXRα, and CRBPII were 705, 730, 644, 589, and 192 bp in size, respectively. The authenticity of these fragments was confirmed by bidirectional sequencing using a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit® and an ABI Prism 310 genetic analyzer (both from Applied Biosystems, Foster City, CA, USA). The origin of mouse cDNAs for peroxisomal thiolase (PT) and β-actin is described elsewhere [5,17].

2.4. Hepatocyte isolation, culture and clofibrate treatment

Parenchymal hepatocytes were isolated from the control or clofibrate-treated mice with the modified in situ perfusion method [25]. After perfusion with 0.05% collagenase solution (Wako Chemicals), the isolated hepatocytes were washed three times by means of differential centrifugation and the dead cells were removed by density gradient centrifugation on Percoll (Amersham Pharmacia Biotech, Buckinghamshire, UK). The live parenchymal hepatocytes were then washed and suspended in CL15 medium containing 8.3% fetal bovine serum. The viability of the isolated hepatocytes was $>90\%$ as determined by the trypan blue test. Primary monolayer cultures of hepatocytes and monolayer harvesting were performed as described previously [26]. Clofibrate was dissolved in dimethyl sulfoxide (DMSO) and added at each change of the medium for a final clofibrate concentration of 0.3 mM to maintain the effects. Control cells were cultured in medium containing the same final concentration of DMSO (0.15%, v/v). The hepatocytes were cultured for 5 days.

2.5. Pulse label and pulse chase experiment

Cultured hepatocytes were washed with methionine-free medium containing 5% (v/v) dialyzed fetal bovine serum and then incubated for 2 h in the same medium at 37°C. The medium was replaced with the same medium containing 300 μCi/ml of [^{35}S]methionine (Amersham Pharmacia Biotech). After a 2-h incubation, the labelling medium was changed to the standard medium, and the preparation was chased for 3, 6, or 12 h. The labelled cells were then washed twice with phosphate-buffered saline, homogenized and centrifuged in preparation for nuclear fraction. The radioactivity in the homogenates was measured and showed values almost identical to those of the pulse-labelled preparations, suggesting that the [^{35}S]methionine uptake capacity is similar for these hepatocyte groups. The nuclear fraction was lysed in RIPA buffer (10 mM Tris–HCl, pH 7.5, 0.2% sodium deoxycholate, 0.2% Nonidet P-40, 0.1% SDS, 0.25 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin). The lysate, in a volume of 350 μl, was reacted for 3 h at 4°C with purified anti-RXRα antibody. The immune complexes were precipitated with *Staphylococcus aureus* protein A bound to agarose beads. After the precipitates had been washed three times in RIPA buffer, the labelled proteins were resolved by means of 10% SDS–PAGE and visualized by autoradiography with the aid of a Fuji system analyzer (Fuji Photo Film). The nuclear fractions of the pulse-labelled preparations were also used for immunoblot analysis of RXRα.

2.6. Hepatocyte culture and 9-cis-retinoic acid treatment

Parenchymal hepatocytes were isolated from wild-type or PPARα-null mice and cultured. 9-*cis*-Retinoic acid (ICN Biomedicals, Costa Mesa, CA, USA) was dissolved in DMSO and added to the medium to make a final concentration of 1 μM. Control cells were cultured in medium containing the same final concentration of DMSO (0.1%, v/v). The hepatocytes were incubated for 24 h, harvested and subjected to mRNA analysis.

2.7. Statistical analysis

Results are expressed as the means ± S.D. Group means were compared using analysis of variance followed by Student's *t*-test.

3. Results

3.1. Analysis of RXRα and PPARs in mouse livers

To examine the expression of hepatic RXRα and PPARs, immunoblot analysis using nuclear fractions was performed. The nuclear RXRα level in the wild-type mice was constitutively higher than that in the PPARα-null mice (Fig. 1A,B). The level was significantly increased by clofibrate treatment, which is known to induce hepatic PPARα and stimulate the transcription of its target genes such as fatty acid-metabolizing enzymes [5,17,27]. The PPARα levels in the nuclear fractions from the wild-type mouse livers increased by clofibrate

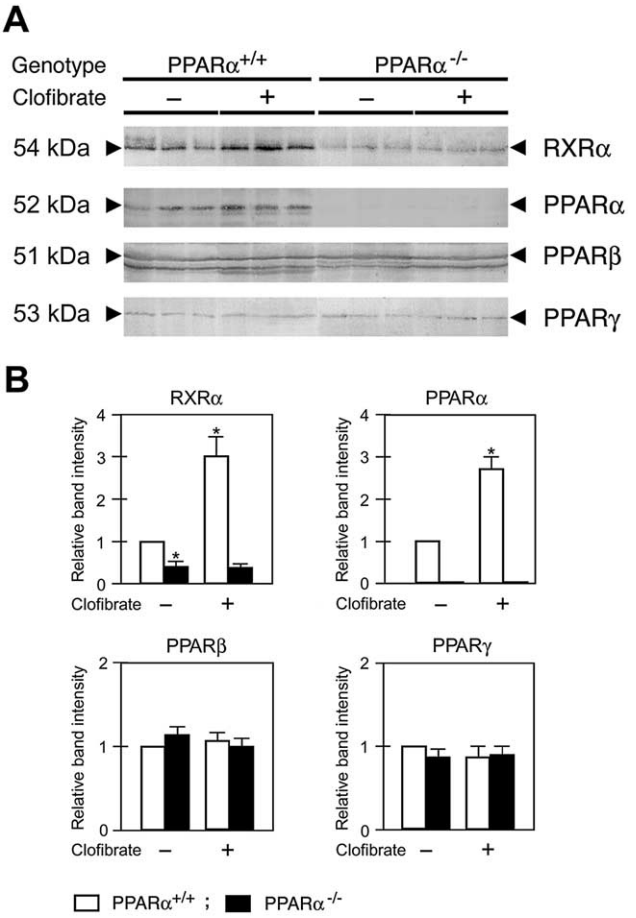

Fig. 1. Immunoblot analysis of RXRα and PPARs. A: Each lane was loaded with 100 μg of protein of nuclear extracts prepared from the individual mouse livers. After electrophoresis, the blots were stained with antibodies against RXRα, PPARα, β and γ. The arrowheads represent the positions of the proteins. Genotype and diet are indicated. PPARα$^{+/+}$, wild-type Sv/129 mice; PPARα$^{-/-}$, PPARα-null mice. B: The band intensity of nuclear RXRα and PPARs was quantified densitometrically and expressed as the fold change relative to the control wild-type mice. Values are expressed as the means ± S.D. ($n=6$). *$P<0.01$ compared with the control wild-type mice. PPARα$^{+/+}$ (open bar), wild-type Sv/129 mice; PPARα$^{-/-}$ (closed bar), PPARα-null mice.

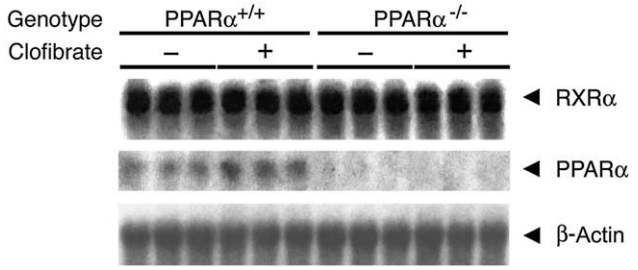

Fig. 2. mRNA analysis of RXRα and PPARα. Total RNA extracted from three individual mouse livers was electrophoresed (10 μg), blotted and hybridized with radiolabelled cDNA probes for RXRα, PPARα and β-actin, respectively. The abbreviations used are shown in the legend of Fig. 1

treatment, as expected. The levels of PPARβ and PPARγ remained relatively constant in all cases (Fig. 1A,B). These results demonstrate that the protein level of RXRα is affected by cellular level of PPARα and its ligand.

3.2. mRNA analysis of RXRα and PPARα

To determine whether the correlative changes in RXRα and PPARα at the protein level correspond to those at the transcriptional level, Northern blot analysis was performed. Interestingly, the levels of RXRα mRNA remained virtually constant in all cases. In contrast, the level of PPARα mRNA in the wild-type mice was increased by clofibrate treatment, as expected (Fig. 2). The levels of PPARβ and PPARγ mRNA remained unchanged in all the samples (data not shown). These results suggest that the changes in RXRα at the protein level are due to post-transcriptional events.

3.3. Correlative changes in nuclear RXRα and PPARα

To further characterize the correlative changes in nuclear RXRα and PPARα, time course and dose–response experiments concerning clofibrate treatment were performed. The levels of nuclear RXRα and PPARα in the wild-type mice increased rapidly until 4 and 2 days after starting clofibrate

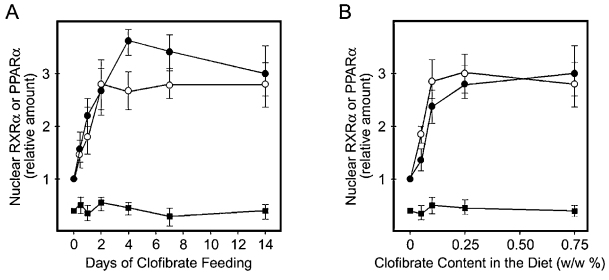

Fig. 3. Changes in nuclear RXRα and PPARα. A: Time course experiment concerning clofibrate treatment. The wild-type and PPARα-null mice were fed with a control diet or one containing 0.75% (w/w) clofibrate for 0.5, 1, 2, 4, 7, or 14 days. Protein of nuclear extracts (100 μg) prepared from the individual mouse livers was subjected to immunoblot analysis. The band intensity of nuclear RXRα and PPARα was quantified densitometrically, expressed as the fold change relative to the control wild-type mice and plotted. Values are expressed as the means ± S.D. ($n = 4$). ●, RXRα level in the wild-type mice; ○, PPARα level in the wild-type mice; ■, RXRα level in the PPARα-null mice. B: Dose–response experiment concerning clofibrate treatment. The wild-type and PPARα-null mice were fed with a control diet or one containing 0.05, 0.10, 0.25, or 0.75% (w/w) clofibrate for 2 weeks. Nuclear RXRα and PPARα levels were plotted as described in A. Values are expressed as the means ± S.D. ($n = 4$). The symbols are as described in A.

treatment, respectively, and these levels were nearly constant until 14 days. The level of nuclear RXRα in the PPARα-null mice was practically unchanged. The changes of RXRα level in the wild-type mice were similar to those of PPARα level (Fig. 3A). In the dose–response experiment, the levels of nuclear RXRα and PPARα in the wild-type mice were increased to 0.1% (w/w) clofibrate concentration, and these levels were practically constant at the higher concentrations. The dose–response changes of RXRα in the wild-type mice were similar to those of PPARα (Fig. 3B). These results confirm the synchronous changes in nuclear RXRα and PPARα.

3.4. Stabilization of nuclear RXRα by PPARα

To investigate the stability of nuclear RXRα, pulse chase experiments were performed using hepatocytes isolated from the mouse livers. There was little difference in the band intensity of pulse labelling (P in Fig. 4A) among the four isolated hepatocyte groups, demonstrating that transfer of RXRα into the nucleus does not differ between the hepatocyte groups. The half-life of nuclear RXRα in the hepatocytes from the control wild-type mice was approximately 7.5 h. This increased to approximately 12 h by clofibrate treatment (Fig. 4A,B). On the other hand, the half-life in the hepatocytes from the control PPARα-null mice was approximately 4.5 h, and was not changed by clofibrate treatment (Fig. 4A,B). The levels of RXRα in the nuclear extracts used in the pulse chase experiments (Fig. 4C) showed a similar tendency to those in vivo (Fig. 1A). To summarize, the stability of nuclear RXRα in the control wild-type mice was higher than that in the control PPARα-null mice and considerably increased by clofibrate treatment, which was probably reflected in the levels of RXRα in the hepatocyte samples. The stability and the resultant protein level of nuclear RXRα seem to be regulated by nuclear PPARα, since RXRα can heterodimerize with PPARα [1,2,11,13]. Indeed, the degree of its stability roughly corre-

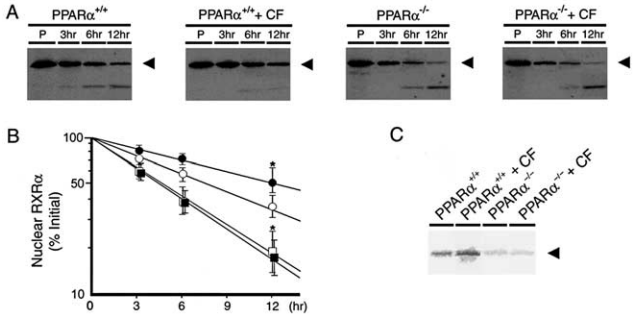

Fig. 4. Pulse label and pulse chase experiment using isolated hepatocytes. A: Labelled RXRα bands on X-ray film. Pulse chase and pulse label experiment was performed as described in Section 2. The arrowheads indicate the position of RXRα. P, pulse label; 3, 6, 12 h, pulse chase for 3, 6, 12 h; PPARα$^{+/+}$, wild-type Sv/129 mice; PPARα$^{-/-}$, PPARα-null mice; +CF, clofibrate treatment. B: Intensity plot of RXRα band in five individual experiments. The band intensity of labelled RXRα was quantified densitometrically, expressed as a percentage of pulse-labelled band intensity and plotted. Values are expressed as the means ± S.D. *$P < 0.01$ compared with isolated hepatocyte group from the control wild-type mice; ○, control wild-type mice; ●, clofibrate-treated wild-type mice; □, control PPARα-null mice; ■, clofibrate-treated PPARα-null mice. C: Immunoblot analysis of isolated hepatocyte nuclear fractions. The same samples as those used in A were electrophoresed and blotted. The blot was stained with the antibody against RXRα. The arrowhead indicates the position of RXRα.

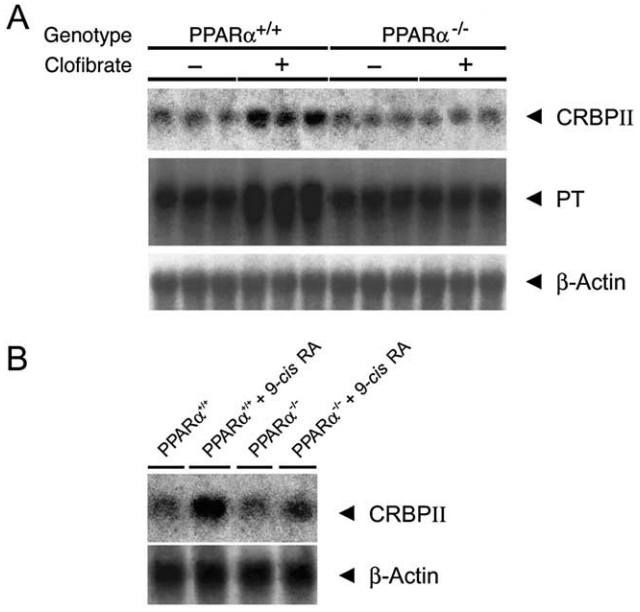

Fig. 5. mRNA analysis of CRBPII and PT. A: The effect of clofibrate on the transcription of CRBPII and PT genes in mouse livers. The same samples used in Fig. 2 were adopted and hybridized with radiolabelled cDNA probes for CRBPII, PT and β-actin. B: The effect of 9-*cis*-retinoic acid on the transcription of CRBPII gene in isolated hepatocytes. Primary cultured hepatocytes were incubated with or without 1 μM 9-*cis*-retinoic acid for 24 h. After the cells were harvested, total RNA was extracted from the respective groups, electrophoresed (40 μg), blotted and hybridized with cDNA probes used in A. PPARα$^{+/+}$, wild-type Sv/129 mice; PPARα$^{-/-}$, PPARα-null mice; +9-*cis* RA, 9-*cis*-retinoic acid treatment.

lates with the protein level of nuclear PPARα (Figs. 1, 3 and 4).

3.5. Influence of RXRα stabilization on its function

The CRBPII gene possesses a retinoid X response element in the promoter region and its transcription is enhanced by the binding of RXRα homodimer [28]. The transcription of the PT gene is also activated by the PPARα/RXRα heterodimer, similar to other PPARα target genes such as peroxisomal acyl-CoA oxidase gene and mitochondrial medium chain acyl-CoA dehydrogenase gene [2,5]. When analyzing the mRNA levels of CRBPII and PT, the former level increased and the latter increased markedly by clofibrate treatment (Fig. 5A). Additionally, the increase of functional activation of RXRα was further examined in the experiment using primary hepatocytes and 9-*cis*-retinoic acid, a RXRα-specific agonist. The mRNA level of CRBPII significantly increased in the wild-type hepatocytes treated with 9-*cis*-retinoic acid and increased to some degree in the treated PPARα-null hepatocytes (Fig. 5B). These results demonstrate that the nuclear RXRα stabilization and the resultant protein increase can enhance its activity in the transcription of target genes.

4. Discussion

Nuclear RXRα was increased in clofibrate-treated wild-type mice through its stabilization. A similar stabilization and increase of nuclear RXRα was found in the control wild-type mice, in comparison with the control PPARα-null mice. These findings indicate the presence of a novel mechanism for the regulation of the nuclear RXRα level through the interaction with PPARα. Since PPARα/RXRα heterodimer formation is essential for its functional activation and the resultant transcriptional activation or suppression of its target genes [1,2,11,13], these findings may well be important for maintaining a balanced simultaneous increase of RXRα and PPARα in order to enhance their functional association. Moreover, the significant RXRα stabilization accompanied an enhancement of target gene expression regulated by the RXRα homodimer. This suggests that the activation of PPARα may affect the functional activation of various RXRα-associated metabolic pathways.

Hirotani et al. showed that, as a result of coexpression of RXRα in HeLa cells, ectopically expressed PPARα was stabilized through heterodimerization with RXRα [29]. In the present study, the levels of nuclear RXRα and PPAR were examined in mouse livers, leading to a more accurate reflection of in vivo phenomena.

In the absence of PPARα, RXRα forms a homodimer or various heterodimer species with other nuclear receptors such as retinoic acid receptor, farnesoid X receptor and the other PPAR isoforms [1,13,30,31]. Since RXRα is less stable and its nuclear level is lower in PPARα-null mice, it is supposed that the absence of PPARα influences many signalling pathways regulated by the RXRα homodimer and several other heterodimers in the liver [28,31]. In human livers, the level of PPARα is much lower than that in rodent livers [32]. Therefore, PPARα-null mice may be a useful model animal to estimate the role of RXRα-mediated signalling pathways in humans.

The precise mechanism of stabilization of RXRα by PPARα ligand and by exposure to PPARα is not yet understood. Perhaps, binding of RXRα to its partner protects it against proteolytic degradation. In the absence of PPARα, RXRα would be expected to bind many other heterodimeric partners such as the thyroid hormone receptor, vitamin D receptor, liver X receptor, or others [1,13,33,34]. Although the precise cellular levels of these nuclear receptors in the liver are not known in the mouse, binding of RXRα to these nuclear receptors seems to be unable to compensate the role of PPARα.

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