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The Peroxisomal 3-keto-acyl-CoA thiolase B Gene Expression Is under the Dual Control of PPARα and HNF4α in the Liver

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PPARα and HNF4α are nuclear receptors that control gene transcription by direct binding to specific nucleotide sequences. Using transgenic mice deficient for either PPARα or HNF4α, we show that the expression of the peroxisomal 3-keto-acyl-CoA thiolase B (Thb) is under the dependence of these two transcription factors. Transactivation and gel shift experiments identified a novel PPAR response element within intron 3 of the Thb gene, by which PPARα but not HNF4α transactivates. Intriguingly, we found that HNF4α enhanced PPARα/RXRα transactivation from TB PPRE3 in a DNA-binding independent manner. Coimmunoprecipitation assays supported the hypothesis that HNF4α was physically interacting with RXRα. RT-PCR performed with RNA from liver-specific HNF4α-null mice confirmed the involvement of HNF4α in the PPARα-regulated induction of Thb by Wy14,643. Overall, we conclude that HNF4α enhances the PPARα-mediated activation of Thb gene expression in part through interaction with the obligate PPARα partner, RXRα.

1. Introduction

Peroxisomes are essential organelles for various metabolic pathways including β-oxidation of very long-chain fatty acids (VLCFAs), prostaglandins, and leukotrienes as well as detoxification of xenobiotics and reactive oxygen species. Besides catabolic events, biosynthesis of cholesterol, bile acids, dolichol, and other lipids (plasmalogens) also occurs within the peroxisomal matrix. While both mitochondria and peroxisomes are the main sites for cellular fatty acid degradation by oxidation, β-oxidations by these two organelles differ substantially in the substrate specificity. Whereas mitochondria mainly oxidize short, medium, and most long-chain fatty acids, peroxisomes preferentially oxidize very long straight-chain fatty acids (VLCFAs) (C > 20) and branched-chain fatty acids (BCFAs) [1].

Peroxisomal β-oxidation of VLCFAs can be divided in four main steps: dehydrogenation, hydration, oxidation, and thiolytic cleavage. While different enzymes are involved in the different biochemical reactions, thiolytic cleavage is accomplished by three enzymes in rodents, namely, 3-ketoacyl-CoA thiolase A (ThA), 3-ketoacyl-CoA thiolase B (ThB, EC:2.3.1.16), and Sterol Carrier Protein X/Sterol Carrier Protein 2 thiolase (SCPx/SCP2).

In rodents, the Tha and Thb genes encode two distinct proteins that differ by 9 amino acids and display similar
substrate specificity in vitro [2]. However, the Tha and Thb genes do not exhibit overlapping expression patterns [3]. While Tha is ubiquitously expressed, Thb is mainly present in liver and kidney [4]. The difference in tissue distribution suggests that Tha and Thb may have different biological activities. While Thb is assumed to be important for VLCFA metabolism, the exact function of this protein in vivo remains to be established. Although Thb−/− animals are phenotypically indistinguishable from wild-type littermates under normal conditions, feeding the potent peroxisome proliferator activated receptor alpha (PPARα, NR1C1) agonist Wy14,643 (Wy) led to enrichment of the n-7 and n-9 medium chain unsaturated fatty acids (MUFA)s in the liver [5].

PPARs are ligand-activated nuclear hormone receptors involved in the regulation of numerous processes, including glucose, amino acid and lipid metabolism, inflammation and wound healing [6–8]. The three PPAR isotypes (α, β/δ, and γ) each regulate a distinct set of target genes by binding to DNA sequences consisting of two repeats of the consensus sequence AGGTCA separated by one nucleotide (Direct Repeat 1, DR1). DR1 sites specifically bound by PPARs and their dimerization partner retinoid x receptor (RXR) are also known as peroxisome proliferator response elements (PPREs) and are present either in the promoter region and/or intronic sequences of genes [9, 10]. The role of the nuclear receptor PPARα in hepatic fatty acid oxidation has been well documented [11]. PPARα serves as a nuclear receptor for fatty acids and is activated by the free fatty class of drugs, which are used in the treatment of dyslipidemia (low plasma HDL/high triglycerides). Transcriptional control of Tha and Thb by PPARs in the liver has been shown previously and involves a functional PPRE (TB PPRE2) in intron 3 of the Thb gene [12].

In addition to PPARα, the liver-enriched transcription factor hepatocyte nuclear factor-4α (HNF4α, NR2A1) also plays a pivotal role in glucose, amino acid, and lipid metabolism. Because HNF4α is also known to recognize DR1-binding sites, PPARα and HNF4α share common target genes, as previously shown for glycogen synthase-2, acyl-Coa thioesterase I, and ornithine transcarbamylase [7, 13, 14]. Another putative candidate for dual PPARα and HNF4α regulation is Thb. A DR1 sequence (TB PPRE1) found in the promoter of Thb was shown to be bound by both PPARα and HNF4α in vitro [15]. To confirm the in vivo relevance of this finding, the present study assesses the regulation of Thb by the nuclear receptors PPARα and HNF4α in vivo, using PPARα−/− and hepatic HNF4α-null mice, respectively [16–18]. These in vivo models in combination with cell culture tools provide evidence for the involvement of HNF4α in Wy induction of Thb by interacting with the PPARα dimerization partner retinoid x receptor alpha (RXRα) at a nonconventional PPRE located within intron 3 of the Thb gene.

2. Materials and Methods

2.1. Animal Experiments. Nine-week-old C57BL/6J PPARα-null male mice and age-matched WT mice were used.

Mice were kept in cages at 22°C, with equal proportions among males and females and free access to water and food containing 4.3% (w/v) lipids (U.A.R.A.-03, Epinay sur Orge, France). For the pharmacological intervention, wild-type (WT) and PPARα-null mice were fed by gavage for 8 days with Wy (30 mg kg−1·day−1 from Sigma). Control animals received the vehicle alone (3% arabic gum). Six-week-old liver-specifically HNF4α-disrupted (HNF4α ΔL) and control (HNF4α F/F) mice were fed a grain diet with or without (0.1% w/w) Wy for 5 days. Animals were sacrificed by cervical dislocation and tissues were rapidly snap-frozen in liquid nitrogen before storing at −80°C. Ethical considerations: in vivo studies were conducted under EU guidelines for the use and care of laboratory animals and were approved by an independent ethics committee.

2.2. Cell Culture. COS-7 cells were grown as previously described [12, 15]. Briefly, COS-7 cells, mouse hepatoma Hepa 1.6 and human HeLa cells were routinely grown in Dulbecco’s Modified Eagles Medium (DMEM) medium supplemented with 10% FCS and fetal calf serum (FCS) were from PAN Biotech GmbH. Rat hepatoma H4IEC3 cells were grown in DMEM/HAM’S F-12 (1/1) medium supplemented with 5% FCS. All cells were grown in absence of antibiotics in the culture medium. Regular testing for mycoplasma contamination was performed with a PCR-based test.

2.3. Isolation of Total RNA and Northern Blotting Experiments. Total RNA was extracted from 50 mg from liver using TRIzol reagent according to the method specified by the supplier (Invitrogen). Total RNA (15 µg) was resolved on 1% agarose gels containing 6% (v/v) formaldehyde and transfected to Hybond-N membranes (Amersham Biosciences). Filters were hybridized overnight with 32P-labeled cDNA probes. Thb and 36B4 probes were previously described [3]. The Acox-1 DNA probe was obtained by RT-PCR from mouse liver total RNA and verified by sequencing.

2.4. Isolation of Total RNA, Reverse-Transcription and Conventional PCR. Hnf4aloxP/loxP/Alfp.cre mutant mouse embryos [19] and Hnf4aloxP/loxP Alfp.cre adult mice [17] were previously reported elsewhere. Total RNA from 18.5-d.p.c and adult livers were extracted using Qiagen’s RNeasy mini kit following the manufacturer’s protocol. Genomic DNA was removed using 10 u RNase-free-DNase I/µg RNA. cDNA was synthesized using MMLV-RT (Invitrogen) with dNTP (0.5 mM) and random hexamer primers (5 µM). These DNA provided template using specific primers at the annealing temperature of 57°C in the presence of dNTP (0.1 mM), primers (0.5 µM), and Taq DNA polymerase (Roche).

2.5. Reverse Transcription and Real-Time Quantitative PCR (RT-qPCR). Total RNA from Hepa 1.6 cells was extracted and purified using Qiagen RNeasy columns (Qiagen). One µg of total RNA was used for reverse transcription with iScript Reverse Transcripase (BioRad). PCR reactions were performed using the qPCR MasterMix Plus for SYBR Green I with fluorescein (Eugenergent). All PCR reactions
were performed with MultiGuard Barrier Tips (Sorenson BioScience, Inc.) and an iCycler PCR machine (Bio-Rad Laboratories). Primers were designated to generate a PCR amplification product of 100–200 bp and were selected according to indication provided by the Primer 3 software (http://frodo.wi.mit.edu/primer3/). Sequences are available from S. Mandard on request. Specificity of the amplification was verified by melting curve analysis and evaluation of efficiency of PCR amplification. The “delta-delta Ct” quantification method was used and expression was related to the control gene 36B4, which did not change under any of the experimental conditions studied.

2.6. Transactivation Assays. COS-7 and HeLa cells were transfected with Exgen 500 (Euromedex) following manufacturer’s protocol. 5 × 10^4 cells/well were seeded in a 24-wells plate. Cells were transfected with a mixture of 1 µg of plasmid DNA containing 30 ng of the reporter vector pCMV β-galactosidase (Clontech) together with equivalent molar amount of Luciferase (Luc) vectors (about 250 ng). Different amounts of expression vectors encoding for different nuclear receptors such as pSG5-mPPARα, pSG5-mRXRα, and pCDNA3-hHNF4a were used. Corresponding empty vectors were used for control experiments. 4 h post transfection, the culture medium was replaced by 1 ml of complete medium with or without 10^{-5} M Wy (Alexis Biochemical). Luc and β-galactosidase activities were measured 48 h post transfection using the Promega Luc kit (Promega) and a standard assay. Chlorophenol red β-D-galactopyranoside was used as a substrate for β-galactosidase. For each condition, transfection assays were repeated four times. Reporter pGluC constructs was composed of a Luc expression vector containing the β-globin promoter upstream of the Luc coding sequence. TB PPRE3-pGluC was created by inserting synthetic double strand oligonucleotides between HindIII and BamHI of the pGlu vector (top strand 5’-TGCAGGTCAAGCTTTCCTAACTTTTTCTCTTCCTTTACTTCAGTTCATGACCTGACCTCTGCTGATACCTTTCTCTGACTTTTGAGCT-3’; lower strand 5’-CTTGAATGAGGAAAGTTACCTGCGAGAGAGGACTCTGAGGCTAACGTCAGTCAAGCT-3’). Oligonucleotides were end labelled with dCTP by using the Klenow fragment of DNA polymerase I. Nuclear extracts (0.5 µg for hHNF4α-enriched extracts) were incubated for 1 h on ice in 20 µl of the buffer (10 mM Hepes pH 7.9; 120 mM NaCl, 1 mM EDTA, 1 mM DTT, 7% (v/v) glycerol, 0.5 mM PMSE, 2 µg each of leupeptin, aprotonin, pepstatin), 1 µg of double-stranded poly (dIdC), and 35 fmol of radiolabelled rat PPRE3 oligonucleotide. Alternatively, a 1 to 100 fold molar excess of competitor oligonucleotides was added. The HNF4α antibody used for supershift was from Santa Cruz Biotechnology Inc. (HNF4α sc-8987x) and was added with the nuclear extract 30 minutes before adding the probe. Nucleoprotein complexes were resolved on a 6% (w/v) polyacrylamide gel in 1x TBE.

2.10. Coimmunoprecipitation (CoIP) Assays. For CoIP assays, nuclear extracts were adjusted to 25 mM HEPES (pH 7.9), 200 mM KCl, 1 mM EDTA, 0.5% NP-40, and 10% glycerol and incubated with 2 µg of antibody at 4°C for 12 h. 50 µl of protein G-Sepharose beads were washed twice with the same buffer before being incubated for 2 h with the upper mixture on a rotator. After a centrifugation step, pelleted beads were washed four times with the afore-mentioned buffer. After unbound proteins were washed away, bead pellets were finally resuspended in reducing loading buffer [25] and samples were boiled at 95–100°C for 3–5 minutes before being subjected to SDS-PAGE. The HNF4α (sc-6556), PPARα (sc-9000x), RXRα (sc-774), as well as the secondary (donkey anti-goat, sc-2020) antibodies used were all purchased from Santa–Cruz Biotechnology Inc.
coimmunoprecipitation assays were used except for the secondary antibody (goat antirabbit, sc-2004, Santa-Cruz Biotechnology Inc.).

2.12. Immunoblotting. Immunoblotting of the two peroxisomal 3-ketoacyl-CoA thiolases (PTL) was performed as previously described [27]. The rabbit polyclonal antibodies directed against both thiolase A and B proteins (PTL) was a gift from Dr. T. Hashimoto and Dr. N. Usuda (Shinshu University School of Medicine, Japan) and has been previously described [27]. Signals were detected with ECL-plus (Amersham Biosciences) according to the manufacturer's instructions.

2.13. Statistical Analyses. Unless indicated, the one-way ANOVA test was used to identify statistically significant differences. The cut off for statistical significance was set at a P-value of .05 or below.

3. Results

3.1. Thb mRNA Levels Are Robustly Induced by the PPARα Agonist Wy in Liver. Using a cDNA probe recognizing both Tha and Thb genes, it was previously shown that thiolase (A + B) mRNA levels were induced by PPARα agonists in a PPARα-dependent manner [18]. To evaluate whether Thb only was sensitive to PPARα agonists, northern blot analysis was performed using a specific nucleotide probe and hepatic RNA from WT and PPARα-null mice treated or not with Wy14,643 (Wy), a potent and specific agonist of PPARα (Figure 1(a)).

Under basal conditions, Thb mRNA levels were similar in wild-type (WT) and PPARα-deficient mice. The expression of the PPARα target gene Acox-I and of Thb were induced in a PPARα-dependent manner (Figure 1(a)). Of note, the augmentation of Thb mRNA levels upon Wy treatment was translated into higher peroxisomal 3-ketoacyl-CoA thiolases protein content (Figure 1(a)).

To further investigate whether the in vitro expression of Thb is also controlled by PPARα in other species, rat hepatoma H4IIEC3 cells were treated with Wy. Using RT-qPCR, it was shown that Thb mRNA levels were robustly induced by activated PPARα (Figure 1(b)). Given the dramatic activation of Thb expression by Wy in H4IIEC3 cells, more than a single PPRE in the rat Thb gene sequence may control its expression. Comparison with other established PPARα target genes such as Acox-I and Mfp-I indicated that the gene expression profile of Thb was indeed closer to that of Mfp-I (one of the most responsive gene to PPARα due to the presence of an atypical and composite PPRE in its gene sequence) than Acox-I (Figure 1(b)) [28, 29].

3.2. Identification of TB PPRE3 as a Novel Response Element for PPARα. To find out for more functional DR1 PPFEs, a comparative in silico analysis of the mouse and the rat Thb genes sequences from position −5600 bp upstream of the transcription start site to position +13000 bp downstream was performed with Nubiscan V2.0 software [30]. It revealed the presence of several conserved stretches of DNA sequences that harboured some previously characterized PPFEs as well as a novel PPRE that we named TB PPRE3 [12, 15] (Figure 1(c)). Gel mobility shift assays were used to test whether TB PPRE3 was bound in vitro by translated PPARα/RXRα proteins. In agreement with data previously reported by others, we found a retarded PPARα/RXRα complex on MFP-1 PPRE but also on TB PPRE3, further implying direct regulation of Thb by PPARα through TB PPRE3 [28] (Figure 1(d)).

To verify whether PPARα can transactivate the Thb gene through TB PPRE3, transactivation assays were performed in COS-7 cells with a reporter vector containing a single copy of TB PPRE3. Luc activity of the reporter vector containing TB PPRE3 responded significantly to PPARα overexpression in the absence of exogenous agonist (Figure 1(e)). Since the apparent large responsiveness of PPARα in absence of exogenous ligand may reflect the high constitutive activity mediated by the ligand-independent AF1 domain, we examined the behaviour of N-terminally deleted (AAF1) PPARα construct that lacks the AF1 in transactivation assays. It was found that ΔAF1 PPARα behaved similarly to WT PPARα, suggesting the presence of natural PPARα agonists in the culture medium (data not shown). As expected, Luc activity was significantly enhanced after Wy treatment. It is important to note that the MFP-1 PPRE is composed of two DR1 elements separated by two base pairs, thereby forming an internal DR2 element (Figure 1(c)) [28, 29]. With the exception of a single-base pair separating two putative DR1, the overall structure of TB PPRE3 was quite similar to that of MFP-1 PPRE. Yet, it is also worth noting that the nucleotide sequence composing the four different half-sites of TB PPRE3 significantly differs from that of MFP-1 PPRE. Of interest and compared to ACOX-I PPRE, the fold induction for TB PPRE3 and MFP-1 PPRE were quite similar as a probable consequence of their unusual and composite structure (Figures 1(c) and 1(e)). Overall, TB PPRE3 is a potent response element for PPARα and is likely critical for PPARα-dependent activation of Thb gene transcription.

3.3. The Nuclear Orphan Receptor HNF4α Controls Thb Gene Expression in Liver and in Hepatoma Hepa 1.6 Cells. The liver Hepatocyte Nuclear Factor-4α (HNF4α, NR2A1) plays an important role in the transcriptional regulation of genes involved in different metabolic pathways, including fatty acid, amino acid, glucose, and cholesterol metabolism. Although limited, there is some overlap in the identity of some of the HNF4α and PPARα regulated genes [6, 31, 32]. In view of this, HNF4α might be hypothesized to directly control Thb gene expression in liver. Because the complete deletion of Hnf4α is lethal in mice, we sought to check whether deletion of Hnf4α had any impact on liver Tha and Thb basal expression using samples from Hnf4α−/− 18.5 days old embryos (Figure 2(a)) [16, 19].

While mRNA levels of Tha were not affected, Thb mRNA levels were markedly decreased in liver of Hnf4α−/−loxP Alfp cre embryos supporting a critical role for HNF4α in regulating the basal expression of Thb during
Figure 1: Wy induces hepatic *Thb* gene expression in a PPARα-dependent manner through a novel PPRE. (a) Northern blot was performed with liver RNA from wild-type (*n* = 3) and PPARα −/− mice (*n* = 3) fed Wy (30 mg kg⁻¹·day⁻¹) for 8 days. Autoradiographs were quantified and average fold changes indicated below the different blots. The signal for WT mice NOT fed Wy was arbitrarily set to 1. 36B4 mRNA levels were evaluated and used as internal control of loading. Western blot experiment was conducted with liver protein samples of the same animals using an antibody that recognizes the two peroxisomal 3-ketoacyl-CoA thiolases (PTL) identified in rodents [35]. (b) Rat hepatoma H4IIEC3 cells were treated with Wy (10 or 50 µM, as indicated) for 48 h. Expression of Thiolase b (*Thb*), AcylCoA oxidase-1 (*Acox-1*), and Multifunctional protein-1 (*Mfp1*) was determined by quantitative RT-PCR analysis. Values are mean of three independent experiments ±SEM. Wy: Wy14,643. (c) The *Thb* gene contains a composite PPRE in intron 3. The nucleotide sequence of the different PPREs (TB PPRE3, ACOX-I PPRE, and MFP-1 PPRE) used in this study is shown. The nucleotide positions are given taking as +1 the transcription initiation site. The different arrows indicate half-site of a Direct Repeat (DR). (d) A double-strand oligonucleotide containing TB PPRE3 or MFP1-PPRE was incubated with increasing amounts of *in vitro* translated PPARα and RXRα proteins. Binding complexes were separated by electrophoresis. (e) Transactivation assays were performed in COS-7 cells with a Luc reporter vector containing either a single copy of TB PPRE3 or a copy of ACOX-I PPRE or MFP-1 PPRE. These constructs were transfected together with an expression vector for mouse PPARα (pSG5 mPPARα) in presence or absence of Wy (10 µM). DMSO was used as vehicle. Values are mean of four independent experiments ±SEM. **Effect statistically significant compared to control (no PPARα transfected, DMSO) with ***P < .001 with one-way Anova test. Errors bars represent SEM.
HNF4α is a master gene for the hepatic expression of Thb. (a) Expression of hepatic Thb in HNF4α null (Hnf4αloxP/loxP Alfp.cre; loxP/loxP) and control (Hnf4αloxP/+ Alfp.cre; loxP/) mouse embryos was determined by semiquantitative RT-PCR analysis (n = 3 per group). Hprt mRNA levels were evaluated and used as internal standard of loading. Tha: thiolase A; Thb: thiolase B; Hnf4α: Hepatocyte Nuclear Factor-4 alpha; Hprt: Hypoxanthine-guanine PhosphoRibosyl Transferase. (b) Mouse Hepa 1.6 hepatoma cells were transfected with expression vectors for WT HNF4α or dominant negative form of HNF4α (DN HNF4). Expression of Thb, Tha, and apolipoprotein AII (ApoA-II) was determined by quantitative RT-PCR. Crude results were standardized against 36B4 mRNA levels. Levels of gene expression in Hepa 1.6 cells transfected with empty pcDNA3.1 vector serve as reference point and are given the arbitrary value of 1.0. Values are mean of three independent experiments ± SEM. Apolipoprotein AII (ApoAII) gene expression was used as a positive control of experiment. Significantly different compared to control (transfection with empty pcDNA3.1 vector) with *P < .05 and **P < .01 by one-way ANOVA test. n.s.: no statistically different compared to control.

development of hepatocytes (Figure 2(a)). It may also be hypothesized that the decrease in Thb mRNA levels observed in liver of the Hnf4αloxP/loxP Alfp.cre embryos could be the consequence of metabolic perturbations due to the lack of HNF4α. To exclude this possibility, and since we have no way to pharmacologically activate HNF4α in vivo, we studied the expression of Thb in mouse Hepa 1.6 hepatocytes that were efficiently transfected (using The
Amoxa Nucleofector technology) with either a WT form or with the potent dominant-negative form of HNF4α (DN HNF4) previously characterized [22]. DN HNF4 is a selective dominant negative mutant that contains a defective DNA-binding domain. Hence, DN HNF4 forms defective heterodimers with WT HNF4α thereby preventing DNA binding and subsequent transcriptional activation by HNF4α. Compared to COS-7 and HeLa cells, mouse Hepa 1.6 and rat H4IIEC3 hepatoma cells express large amount of endogenous WT HNF4α (data not shown). Ectopic expression of WT HNF4α in Hepa 1.6 hepatocytes had a minor impact on Thb and Tha mRNA levels, as shown by RT-qPCR (Figure 2(b)). A similar picture was also observed for ApoAII, a previously characterized HNF4α target gene [33]. However, forced expression of DN HNF4 decreased Thb as well as ApoAII mRNA levels by 50% while Tha mRNA remained unchanged, confirming that HNF4α controls the basal Thb gene expression. Together, our data indicate that HNF4α is a novel regulator of Thb expression in cell lines of hepatic origin.

3.4. HNF4α Binds to TP PRE3. Given that HNF4α is critical for the basal expression of Thb and that HNF4α recognizes DR1 sequences, we examined whether HNF4α may bind to TP PRE3 [34]. Using TP PRE3 as a probe together with HNF4α-enriched nuclear extracts from HNF4α-transfected COS-7 cells, we performed electrophoresis mobility shift assays. A complex was seen when enriched HNF4α nuclear extracts were used (Figure 3(a), lane 2).

This complex mainly contained HNF4α since it was absent in untransfected COS-7 cells (lane 1) and it disappeared upon the addition of an excess amount of the unlabelled ACOX-I PRE consensus oligonucleotide (lane 3 to lane 5), previously shown to be efficiently bound by HNF4α. An excess amount of cold nonspecific Sp1 binding site oligonucleotide did not decrease HNF4α binding to PRE3 (lane 6 to lane 8). Addition of HNF4α specific antibody with the nuclear HNF4α enriched extracts supershifted the complex (Figure 3(b), lane 2). Together, our data demonstrate the in vitro binding of HNF4α to TP PRE3.

To assess whether TP PRE3 is able to mediate HNF4α-dependent transactivation, transient transfections were performed with HNF4α and reporter vectors containing a single copy of TP PRE3 sequence. While significant, HNF4α only modestly modulated transcription via TP PRE3 in COS-7 cells (Figure 3(c)) indicating that TP PRE3 behaves poorly as a HNF4α response element in classical transactivation assays and suggesting that binding of HNF4α to TP PRE3 is not necessarily translated into a massive transcriptional activation. Additional transactivation assays performed with 5.6kb of the mouse Thb gene promoter sequence also failed to demonstrate HNF4α-dependent promoter activation (data not shown). We concluded that the critical response element(s) for HNF4α, if any, was likely located elsewhere.

In light of this consideration, Bolotin et al. recently reported on an integrated approach for the identification of human HNF4α target genes using protein binding microarrays [35]. This strategy allowed the discovery of a DNA sequence bound by HNF4α in the human version of the peroxisomal 3-ketoacylCoA thiolase (also known as 3-acetyl-CoA acetyltransferase-1, ACAA1). Because we found a similar sequence in intron 5 (DR1int5) of the mouse Thb gene, transactivation assays were performed in COS-7 cells using a copy of DR1int5 cloned in front of the luciferase reporter gene (Figure 3(d)). Transfection with HNF4α enhanced reporter activity to about 3-fold, suggesting that DR1int5 is partly involved in the regulation of Thb by HNF4α.

3.5. HNF4α Enhances the PPARα-Mediated Activation of Transcription from TP PRE3. It is widely documented that HNF4α and PPARα share some similar binding motifs leading to competition between these two receptors. To check whether the same holds true for TP PRE3, both receptors were cotransfected either in nonhepatic (COS-7, HeLa) or hepatic cells (Hepa 1.6, H4IIEC3). Whatever the cell line used, PPARα/RXRα heterodimer increased reporter activity which was even further enhanced by cotransfection of HNF4α (Figures 4(a) and 4(b)).

As previously reported by others, cotransfection with HNF4α strongly suppressed PPARα/RXRα increased reporter activity when Luc activity was driven by the ACOX-I PRE motif (Figure 4(c)). Hence, we concluded that HNF4α promotes transactivation mediated by PPARα/RXRα from TP PRE3 through a molecular mechanism that remains to be determined.

3.6. Binding of HNF4α to TP PRE3 is Dispensable for the Cooperation with PPARα. Inasmuch HNF4α positively influenced the ability of PPARα to transactivate the TP PRE3, it may suggest a possible cooperation between both receptors. To test whether this synergistic effect by HNF4α depends on its capacity to bind to TP PRE3, transactivation assays were conducted in COS-7 cells using two different mutant forms of HNF4α for which DNA binding is either limited (−75%, as a consequence of a point mutation within its DNA binding domain, D126Y HNF4α2) or abolished (DN HNF4) while their stability remains unaffected (Figure 5(a)) [22, 23].

While the transactivation potential of D126Y HNF4α2 is reduced, dimersization of the receptor was not reported to be affected. Similar to WT HNF4α, over-expression of either D126Y HNF4α2 or DN HNF4 failed to transactivate the native TP PRE3 (Figure 5(b)). Noteworthy, both D126Y HNF4α2 and DN HNF4 potentiated further than WT HNF4α the transactivation by PPARα from TP PRE3. Together, we conclude that the cooperation between HNF4α and PPARα was not dependent on the physical binding of HNF4α to TP PRE3.

To finally check whether the cooperative effect between PPARα and HNF4α depends on the DNA nucleotide sequence itself, we performed transactivation assays using the DR-1 PRE of the Acyl-CoA Oxidase I (ACOX-I PRE) (Figure 5(c)). In contrast to TP PRE3, overexpression of
Figure 3: TB PPRE3 is a novel binding site for the nuclear receptor HNF4α. (a) Binding of HNF4α to native radiolabelled (32P) TB PPRE3 was determined by gel shift assay. A double strand oligonucleotide containing (32P) TB PPRE3 was incubated with lysates of transfected (by the expression vector HNF4α) COS-7 cells. Fold excess of specific (Sp.) cold probe (PPRE of the peroxisomal ACOX-I gene) was used for data shown lanes 3, 4 and 5. Nonspecific (Non Sp.) cold probe (Sp1) was used for data shown lanes 6, 7 and 8. Binding complexes were resolved on a 6% non-denaturing polyacrylamide gel. The arrow indicates the specific binding of HNF4α. The star indicates nonspecific binding. (b) Supershift assay (lane 2) was performed with an antibody directed against HNF4α. (c) COS-7 cells were transfected with increasing amounts (0 to 250 ng) of expression vectors encoding WT HNF4α with a Luc reporter vector containing TB PPRE3. (d) COS-7 cells were transfected with increasing amounts (0 to 250 ng) of expression vectors encoding wild-type HNF4α with a Luc reporter vector containing one copy of the DR1 localized in intron 5 of the mouse version of Thb (+4083 +4095 bp). Values are mean of three independent experiments ±SEM. Significantly different compared to control (transfection with empty pcDNA3.1 vector) with *P < .05 by one-way ANOVA test.
**Figure 4:** PPARα transactivation from TB PPRE3 is markedly enhanced by HNF4α. (a) Transactivation assays were performed in COS-7 and HeLa cells (b) or in Hepa 1.6 and H4IIEC3 cells with a Luc reporter vector containing isolated rTB PPRE3. These constructs were transfected together with expression vectors for both mouse PPARα (pSG5-mPPARα) and RXRα (pSG5-mRXRα) in absence (white bars) or presence (black bars) of Wy (10 µM). Cotransfection with pcDNA3.1 WT hHNF4α was performed as indicated. Note that the minimal promoter of the thiolase B gene was used instead of the globin gene promoter which was inactive in H4IIEC3 cells. (c) Transactivation assays were performed in COS-7 or Hepa 1.6 cells with a Luc reporter vector containing isolated ACOX-I PPRE. Normalized luciferase activity of each construct in the absence of PPARα and ligand was set at 1. Values are mean of four independent experiments ±SEM. DMSO was used as vehicle. Significantly different compared to control (transfection with a combination of empty pcDNA3.1 and pSG5 vectors) with ***P < .001 and **P < .01 by one-way ANOVA test. Significantly different between PPARα/RXRα and PPARα/RXRα + HNF4α with $$$P < .001 and $$$P < .001 by one-way ANOVA test.
WT HNF4α potently decreased Luc activity by the activated PPARα/RXRα heterodimer. Transfecting expression vectors encoding the two mutant forms of HNF4α led to a comparatively moderate decrease in Luc activity. Therefore, we hypothesized that the competition between PPARα and HNF4α was reduced. The two mutants were unable to drive Luc activity to a level higher than the PPARα/RXRα heterodimer alone, confirming the lack of cooperation with HNF4α. Overall, our data suggest that the cooperation between HNF4α and PPARα receptors may depend on both
the structure and the nucleotide sequence of the DNA response element.

3.7. HNF4α Does Not Favour the Binding of PPARα/RXRα to TB PPRE3. The molecular mechanisms by which the stimulatory effect of HNF4α on PPARα/RXRα-mediated activation of transcription via TB PPRE3 does not require the binding of HNF4α to DNA but at the present time, this mechanism remains unclear. We, therefore, questioned whether HNF4α could facilitate the binding of the PPARα/RXRα heterodimer to TB PPRE3, via a possible protein–protein interaction. We used DNA Affinity Precipitation Assay (DAPA) to assess complex formation on TB PPRE3 with PPARα, RXRα and HNF4α proteins coming from nuclear extracts of transfected COS-7 cells (Figure 6(a)).

Ectopic expression of PPARα in COS-7 cells led to the production of the PPARα protein (Figure 6(b), lane 1) and overexpressed HNF4α was not detected by the PPARα antibody (Figure 6(b), lane 4). Consistent with our previous findings (Figure 1(d)), we confirmed that PPARα was bound to TB PPRE3 (Figure 6(b), lane 2). When PPARα, RXRα, and HNF4α proteins were incubated altogether with TB PPRE3, the signal was similar indicating that HNF4α does not favor the binding of PPARα/RXRα to TB PPRE3.

We, therefore, concluded that the enhancement of the PPARα/RXRα mediated-activation of transcription from TB PPRE3 by HNF4α was likely not due to DNA binding stabilization of the PPARα/RXRα heterodimer.

To go a step further, immunorevelation of the complexes was also performed with a HNF4α antibody that recognized ectopic WT HNF4α (Figure 6(c), lane 1) but not PPARα or RXRα (data not shown). As previously shown in this study, HNF4α was either physically bound and/or was part of a complex bound to TB PPRE3 (Figure 6(c), lane 3). Intriguingly, when PPARα, RXRα and HNF4α proteins were incubated altogether (Figure 6(c), lane 2), the signal for HNF4α was stronger supporting the notion that PPARα/RXRα may favor the binding and/or the recruitment of HNF4α to TB PPRE3. In contrast to TB PPRE3 and in support of transactivation assays from others, competition between PPARα/RXRα and HNF4α for binding to ACOX-1 PPRE was found, validating our experimental system (Figure 6(c), compare lane 4 and lane 5) [36].

3.8. Physical Interaction between RXRα and HNF4α. The detection of increased HNF4α protein on the TB PPRE3 in the presence of PPARα and RXRα may result from physical interaction between PPARα/RXRα and HNF4α. This hypothesis merits further investigation especially because we found that DN HNF4α (deficient for DNA binding) was able to further enhance the PPARα/RXRα transactivation from TB PPRE3, possibly via protein–protein interactions (Figure 5(b)). Therefore, the interaction of PPARα, RXRα or PPARα/RXRα with HNF4α was assessed in solution in the absence of DNA using coimmunoprecipitation (CoIP) assays. Nuclear protein extracts from transfected COS-7 cells (see details, Figure 6(d)) were first immunoprecipitated with a polyclonal PPARα antibody that mapped the N-terminus part of the protein, before being analyzed by Western blotting with a polyclonal HNF4α antibody (Figure 6(d), lanes 2, 3, 5, and 7). In agreement with the lack of endogenous HNF4α in COS-7 cells, no signal was observed in absence of ectopic HNF4α (Figure 6(d), lane 7). Transient transfection of HNF4α led to the appearance of very faint bands (Figure 6(d), lanes 2 and 3) possibly indicating a weak interaction between PPARα and HNF4α. When PPARα, RXRα and HNF4α proteins were present altogether (Figure 6(d), lane 5), a little signal was still observed suggesting that PPARα either directly or via another protein partner such as RXRα, may interact with HNF4α. Alternatively, we cannot exclude the possibility that the interaction between HNF4α and PPARα/RXRα may partly mask the epitope recognized by the PPARα antibody.

To go a step further in our study, CoIP assays were also performed using a polyclonal RXRα (the obligate PPARα partner) for the immunoprecipitation step. Remarkably, it was found that RXRα massively interacts with HNF4α in solution (Figure 6(d), lane 6). It is worth noting that when the immunoprecipitation step was performed with the HNF4α antibody and the Western blotting with the RXRα antibody respectively, we and others failed to observe any signals [37] (data not shown). This might be the consequence of an epitope mapping at the C-terminus of HNF4α, a region critical for protein–protein interaction. Therefore, the epitope may be not accessible to the antibody used.

Because the endogenous PPARα expression is barely detected in COS-7 cells (data not shown) and since the signal observed in absence of ectopic PPARα was similar (Figure 6(d), compare lane 4 and 6), it can be concluded that HNF4α interacts with RXRα irrespective of the presence of PPARα. The signals observed in lanes 4 and 6 are in concordance with the apparent molecular weight obtained with the ectopic transfected WT HNF4α (Figure 6(d), lane 1).

Overall, our results point towards a physical interaction in solution between HNF4α and RXRα.

3.9. HNF4α is Involved in the PPARα-Regulated Induction of Thb in the Liver. In order to check whether our in vitro findings are translated in vivo and because a dichotomy may exist in the function of HNF4α in adult and fetal liver, we next explored the impact of Hnf4a deletion on basal and Wy-induced expression of Thb, Tha, and Scpx/Scp2 thiolase in adult mice with conditional hepatic disruption of HNF4α (ΔL HNF4α) [17]. Our experimental conditions reproduced the classical pattern of ApoAIV expression associated with the selective Hnf4a deficiency in the liver (Figure 7). When compared to WT mice, the expression of Thb and Scpx was significantly lower in ΔL HNF4a mice (P = .029 and P < .0001 for Thb and Scpx, resp.). Tha expression was not affected by hepatic Hnf4a deletion (P = .179). This piece of data supports our previous finding that Tha is not a HNF4α regulated gene. Furthermore, levels of mRNA for Thb, Tha, and Scpx were all significantly induced by Wy (P = .001, P < .0001 and P = .001 for Thb, Tha, and Scpx, resp.). Of
Nuclear extracts of transfected COS7 cells with a combination of expression vectors for PPARα, RXRα and HNF4α

Incubation with biotinylated dsDNA probe (TB PPRE3 or ACOX-I PPRE)

Precipitation of the biotinylated dsDNA probe (by means of streptavidin magnetic beads)

Washes, heat of the proteins bound to TB PPRE3 followed by SDS-PAGE

Immunoblot with anti-PPARα (Figure 6(b)) or anti-HNF4α (Figure 6(c))

Western blot anti-HNF4α

note, the effect of HNF4α was Wy-sensitive (Wy*genotype interaction, I: \( P = .05 \)) only for Thb. While the expression of Tha and Scpx tended to be lower in ΔL HNF4α mice fed Wy (\( P = .372 \) and .228 for Tha and Scpx, resp.), it was not significant. Together, our data support the involvement of HNF4α in the PPARα-regulated induction of Thb in vivo.

4. Discussion

This study contributes to the understanding of the regulation of the Thb gene by the nuclear receptors PPARα and HNF4α. Under basal conditions, hepatic Thb mRNA levels were not affected by PPARα deletion, similar to other direct
PPARα target genes such as the G0/G1 switch gene 2 or the gene encoding the soluble Interleukin-1 Receptor antagonist [38, 39]. This is not surprising since either a physiological stimulus such as fasting or a chronic stimulus like high fat diet is required for the activation of PPARα-dependent signalling system in liver [40, 41]. Disruption of PPARα completely abolished Wy-mediated induction of Thb mRNA levels. Because the induction of Thb gene expression by PPARα agonists was robust, we hypothesized the presence of more than a single functional PPRE within the promoter and/or Thb gene sequence. In line with this, we previously reported on the characterization of TB PPRE2 by which PPARα can transactivate [12]. This followup study now brings evidence that the molecular regulation of Thb by PPARα in liver is more complex than previously expected since it also depends on a novel cis-acting element that we named TB PPRE3, an atypical PPRE composed of two sequential DR1 sequences separated by one nucleotide thereby forming an internal DR1 element.

The presence of more than a single PPRE in close proximity (so-called PPRE clusters) has been proposed for the mouse and human version of the Fiaf/Angptl4 and Ucp3 genes as well as for the mouse catalase and rat Cyp4a1 genes [9, 42, 43]. Four adjacent PPREs are present in intron 3 of the highly PPAR sensitive target Fiaf/Angptl4 but only a single PPRE is functional [44]. It can be argued that in contrast to Thb, no overlapping was reported for these four PPREs rendering only speculative a potential comparison.

**Figure 7:** The PPARα-regulated induction of Thb is potentiated by HNF4α in the liver. Hepatic mRNA levels in the liver-specifically HNF4α-disrupted (HNF4α ΔL) and HNF4α F/F-fed control or Wy14,643-containing diet (0.1% w/w) for five days. Total RNA extracted from livers of these mice were subjected to real-time PCR analysis. The expression signals from the WT mice that did not receive Wy were arbitrarily set at 1. The results are shown as a relative expression to β-actin mRNA levels as normalization control. Error bars represent standard error (SE) and data are expressed as the mean ± S.E. (n = 4 for each condition). Significant effects were calculated using two-way ANOVA test for the genotype (G), Wy14,643 (Wy) and the interaction between both parameters (I). Results are indicated at the top of each figure. In bold, parameters that are under the cutoff for statistical significance (P value of .05 or below). Wy: Wy14,643.
between the two situations. While the presence of more than a single PPRE in a genomic sequence is not unique, overlapping of PPREs appears to be a rare occurrence. Wen et al. recently reported that the mouse Octn2 gene contains three overlapping PPREs in intron 1, yet only a single PPRE was predominant [34]. Similar to Thb, the presence of three PPREs in the genomic sequence of Octn2 is likely responsible for its massive transcriptional response to activated PPARα.

With respect to other peroxisomal genes, the SCPx/SCP2 thiolase gene was previously identified as a target of PPARα with two separated DR1 PPRE motifs localized in the promoter region [45]. It is also worth noting that Mfp1 displays a PPRE composed of four consensus hexameric TGACCT half-sites in an arrangement of two DR1 elements separated by two base pairs, thereby also forming an internal DR2 element [28, 29]. Similar to Mfp1, Thb appears to be the second peroxisomal oxidative gene that displays this particular feature.

Although the presence of multiple PPREs may be responsible for the large responsiveness of Thb mRNA levels to activated PPARα, alternative explanations are also considered. In this respect, previous data have shown that Thb mRNA was positively regulated by the liver X receptor alpha [46]. Given that transcription of the liver X receptor alpha gene has been proposed to be dependent on PPARα, it can be concluded that the PPARα signalling pathway modulates liver Thb expression through distinct but complementary mechanisms [47].

Furthermore, flanking the internal DR1 element of TB PPRE3 are two half-sites that have been shown to enhance and stabilize the formation of dimeric complexes made by type II nuclear receptors such as RXR and the thyroid hormone receptor [48]. Therefore, one can make the attractive hypothesis that such a mechanism also takes place as far as the PPAR/RXR heterodimer is concerned. It could then partly support the large response of the reporter vector to PPARα agonists in transactivation assays. Moreover, using electrophoretic mobility shift assays, others have previously classified the binding efficiency of PPARα to MFP-1 PPRE as high and transactivation assays further supported the functional relevance of this classification [49]. Given that the structure of TB PPRE3 is close to that of the MFP-1 PPRE, the presence of the TB PPRE3 in the genomic DNA sequence of Thb may be enough to explain the large responsiveness of Thb mRNA levels to PPARα agonists.

In addition to PPARα, the nuclear receptor HNF4α is abundantly expressed in liver and shares with PPARα some similar DNA-binding properties [50]. Because several DR1 motifs (potential candidates for HNF4α binding) have been identified in the sequence of Thb, regulation of Thb by HNF4α was studied using RNA from HNF4α null embryos and HNF4αL adult mice. It was found that hepatic Thb mRNA levels were dependent on HNF4α because it was either completely absent (embryo) or strongly reduced (adult) in liver upon HNF4α ablation. Yet, HNF4α transactivations from TB PPRE3 or DR1int5 were rather disappointing. At this point of investigation, our data of transactivation assays about HNF4α and TB PPRE3 or DR1int5 are at odds with the clear cut picture of Thb gene expression in the liver of HNF4α-deficient embryos/mice. A plausible explanation is that another critical HNF4α-response elements might be located elsewhere in the genomic DNA. Theoretically, it is also possible that taken individually, the DR1s tested are poor HNF4α-response elements while together in the genomic context of the Thb gene sequence, these distal elements simultaneously act to trigger a massive transcriptional response. Alternatively, we cannot rule out that a critical response element for HNF4α is located very far from the initiation start site of Thb in another part of the chromosome that would bend to specifically interact with the genomic sequence of Thb. Such a regulation has been recently described for the regulation of Ucp2 and Ucp3 by the nuclear receptor PPARγ [51].

Moreover, it is difficult to estimate to what extent PPARα might be involved in the lack of Thb observed in vivo in HNF4ΔL mice. Different studies have provided evidence that the steady-state levels of PPARα mRNA were decreased in the liver of HNF4ΔL adult mice [17, 52]. Ongoing investigations by DNA ChIP also showed that the PPARα promoter was physically bound by HNF4αa classifying PPARα as a novel and direct target of HNF4α [52]. Interestingly, binding of PPARα to the HNF4α promoter/enhancer was also reported [52]. Therefore, the authors concluded the existence of combinatorial regulation of the expression of Ppara and HNF4α, acting in a coordinated fashion on their downstream targets genes. Because basal Thb mRNA levels in the liver of PPARα null and WT mice was similar under regular conditions, a critical role for PPARα in Thb regulation is unlikely in HNF4ΔL mice.

Besides its classification as a strong constitutive transcriptional activator, it was previously demonstrated that HNF4α can form a stable affinity complex with other transcription factors such as SHP, SREBP-1c, SREBP-2, and HNF1α leading to the modulation of the expression of some of their respective target genes [53, 54]. These data raise an important conceptual question about the comparison between HNF4α as a conventional nuclear receptor/transcription factor and coactivator. Our data are consistent with the explanation that HNF4α interacts with RXRα in solution (Figure 6(d)) and in the presence of DNA (Figure 6(c)) without disrupting the binding of the liganded PPARα/RXRα heterodimer (Figure 6(b)).

While further studies are necessary to determine the stoichiometry of this interaction, it is worth underlining that HNF4α potentiated the transactivation from TB PPRE3 by liganded PPARα/RXRα in four different cell lines ruling out the hypothesis of an artefact due to the use of a particular cell line. Our data are reminiscent of those obtained by Winrow et al. who previously reported on the functional cooperation between PPARα and HNF4α in the induction of Luc activity from the MFP-1 PPRE [36]. As previously reported by others, HNF4α decreased transcriptional activation of ACOX-1 PPRE by PPARα/RXRα [36, 55]. Importantly, our current finding that HNF4α is involved in the PPARα-induced expression of Thb in liver in mice indicates that our data of transactivation assays observed in COS-7 cells are translated in vivo.
The lingering question arises why HNF4α would enhance transactivation from TB PPRE3 and MFP-1 PPRE while decrease that from ACOX-I PPRE? The molecular mechanism by which HNF4α influences transactivation by PPARα/RXRα from TB PPRE3 likely involves interaction between HNF4α and RXRα. Furthermore, the specific association of coactivators/corepressors with PPARα/RXRα or the configuration of the chromatin might strengthen or weaken the stability of PPARα/RXRα to the different PPREs rendering them more or less susceptible to interaction with HNF4α. It is, therefore, expected that the enhanced PPARα/RXRα-mediated transactivation by HNF4α only concerns a very limited subset of PPARα target genes that display composite PPREs, similar to those identified at Thb and Mfp-1.

Lastly, the chromatin structure within the serine protease inhibitor (serpin) gene cluster was found to be orchestrated by the nuclear receptor HNF4α and HNF1α [56]. Additionally, expression of the chromatin remodeling genes Smarcd3 and Cdt-1 was found to be altered in HNF4αΔL mice suggesting that these factors are potential candidates that may contribute to the indirect effects of HNF4α [57]. In support of this idea, it can be hypothesized that a similar regulation also takes place at the Thb gene locus. Supporting this hypothesis, we found that the transactivation of PPARα/RXRα via TB PPRE3 was similarly induced by HNF4α or trichostatin A, a well-known inhibitor of histone deacetylation (data not shown).

From a physiological point of view, recent work has shed light on the critical role of HNF4α in the control of the expression of enzymes that drive fatty acid β-oxidation for energy production in Drosophila [58]. Given that PPARα also acts at the level of the β-oxidation pathway in mammals, it may come as no surprise that the PPARα and HNF4α signaling routes intersect [31]. To what degree the interaction between the PPARα/RXRα heterodimer and HNF4α influence peroxisomal lipid catabolism under more physiological conditions deserves further investigation.

In summary, this study shows that Thb is a dual target of the two liver enriched nuclear receptors HNF4α and PPARα. Our work also indicates that PPARα/RXRα likely contacts HNF4α via RXRα and in turn modulates the transcription of Thb. Hence, through interaction with other previously bound nuclear receptors to chromatin DNA, HNF4α likely facilitates the recruitment of coactivators and may enhance gene transcription. The convergence of the HNF4α, PPARα, and RXRα signalling pathways underscores the complex interplay involved for the correct transcriptional response of peroxisomal β-oxidation.

**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| DR1          | Direct Repeat-1 |
| HNF4α        | Hepatocyte nuclear factor-4 alpha |
| PPARα        | Peroxisome proliferator-activated receptor alpha |
| ThB          | 3-ketoacyl-CoA thiolase B |
| Luc          | Luciferase |

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