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Mouse γ-butyrobetaine dioxygenase is regulated by peroxisome proliferator-activated receptor α through a PPRE located in the proximal promoter

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A B S T R A C T

Convincing evidence from studies with peroxisome proliferator-activated receptor (PPAR)α-deficient mice suggested that the carnitine biosynthetic enzyme γ-butyrobetaine dioxygenase (BBD) is regulated by PPARα. However, the identification of BBD as a direct PPARα target gene as well as its exact regulation remained to be demonstrated. *In silico*-analysis of the mouse BBD promoter revealed seven putative peroxisome proliferator response elements (PPRE) with high similarity to the consensus PPRE. Luciferase reporter gene assays using mutated and non-mutated serial 5´-truncation BBD promoter reporter constructs revealed that one PPRE located at -75 to -87 relative to the transcription start site in the proximal BBD promoter is probably functional. Using gel shift assays we observed *in vitro*-binding of PPARα/RXRα heterodimer to this PPRE confirming that it is functional. In conclusion, the present study clearly shows that mouse BBD is a direct PPARα target gene and that transcriptional up-regulation of mouse BBD by PPARα is likely mediated by binding of the PPARα/RXR heterodimer to one PPRE located in its proximal promoter region. The results confirm emerging evidence from recent studies that PPARα plays a key role in the regulation of carnitine homeostasis by controlling genes involved in both, carnitine synthesis and carnitine uptake.

Keywords: carnitine; γ-butyrobetaine dioxygenase; peroxisome proliferator-activated receptor α; mouse.

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1. Introduction

Peroxisome proliferator-activated receptor α (PPARα) is a ligand-activated transcription factor that acts as an important regulator of lipid metabolism and energy homeostasis [1]. PPARα is abundantly expressed in tissues with high rates of fatty acid oxidation such as liver, heart muscle, skeletal muscle, and kidney [2]. Transcriptional regulation of genes by PPARα is mediated by forming a heterodimer with the retinoid X receptor (RXR) and subsequent binding of the PPARα/RXR heterodimer to a specific DNA consensus sequence, called peroxisome proliferator response element (PPRE), present in the promoter, intronic or 5´-untranslated region of target genes [2-6], thereby stimulating the expression of those genes. Proteins encoded by these genes are involved in all aspects of fatty acid catabolism, ketogenesis as well as gluconeogenesis [2]. PPARα can be activated by either endogenous ligands such as fatty acids, which are released from white adipose tissue during fasting and taken up into tissues during this state [7, 8] or exogenous ligands such as fibrates (WY-14,643, clofibrate, fenofibrate, bezafibrate, and gemfibrozil) [7, 8].

Evidence from both *in vitro*- and *in vivo*-studies clearly shows that PPARα also plays an essential role for regulating carnitine homeostasis (reviewed by [9]). For instance, activation of hepatic PPARα by fibrates or fasting increases hepatic carnitine concentrations [10-13], whereas down-regulation of PPARα as observed during lactation causes a reduction of hepatic carnitine concentrations [14]. The essential role for PPARα in regulating carnitine homeostasis is confirmed by the observation that PPARα-deficient mice have markedly reduced carnitine levels in tissues [11, 12, 15]. Carnitine is an essential metabolite that is required for the β-oxidation of long-chain fatty acids in the mitochondrial matrix [16]. Hence, all tissues that use fatty acids as a fuel source require carnitine for normal function. Carnitine is derived from dietary sources and endogenous biosynthesis [17]. Carnitine biosynthesis involves a complex series of reactions. Lysine in protein peptide linkages provides the carbon backbone of carnitine. It undergoes methylation of the ε-amino group to yield trimethyllysine,

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which is released upon protein degradation. The released trimethyllysine is further oxidised to γ-butyrobetaine which is then hydroxylated by γ-butyrobetaine dioxygenase (BBD) to form carnitine [18]. In all mammals, a significant BBD activity is found in the liver [18], and in some species such as in humans, pigs, cats, cows, hamsters, rabbits or Rhesus monkeys also in the kidney [18, 19]. Other tissues have either no or only a very low activity of BBD [18, 19], and are therefore highly dependent on active carnitine uptake from blood. Delivery of carnitine from plasma into cells is catalyzed by novel organic cation transporters (OCTN), from which the OCTN2 isoform has the highest binding affinity for carnitine and is therefore the physiologically most important carnitine transporter [20, 21].

The observation that the alterations in hepatic carnitine concentrations in response to treatment with PPARα agonists, fasting, lactation or genetic disruption of PPARα were accompanied by either increased or decreased expression of genes involved in carnitine uptake and biosynthesis like OCTN2 and BBD strongly suggested that these genes are regulated by PPARα. In fact, OCTN2 was recently identified as a direct PPARα target gene which is transcriptionally regulated by PPARα through a functional PPRE in the first intron [6]. Based on these observations we hypothesize that BBD is also a target gene of PPARα. This hypothesis is supported by the identification of a putative PPRE in the mouse BBD promoter based on sequence alignment [11]. However, the functionality of this PPRE for the regulation of mouse BBD promoter remains to be established. Furthermore, it has not been addressed whether regulation of mouse BBD by PPARα may also involve other PPRE located in the BBD promoter region. In the present study, we therefore performed *in silico*-analysis, reporter gene experiments and gel shift assays to identify functional PPRE in the mouse BBD gene. Our data indicate that mouse BBD is a direct target gene of PPARα and that transcriptional up-regulation of BBD by PPARα is mediated by one PPRE located in its promoter region.

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2. Materials and methods

2.1 Chemicals

WY-14,643 was purchased from Sigma-Aldrich (Steinheim, Germany).

2.2 Cell culture

HepG2 cells, a human hepatoma cell line (DSMZ, Braunschweig, Germany) that is commonly used for transient transfection assays [5], were cultured in RPMI1640 GlutaMax-1 medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Invitrogen) and 0.05 mg/mL gentamycin (Invitrogen). Cells were maintained at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. Medium was changed every 2 days. For reporter gene experiments, cells were seeded in 96-well culture plate at a density of 4 x 10^4 per well. Cells were used for transient transfection at a confluence of 70%.

2.3 In silico-analysis of mouse BBD promoter

Using cDNA and genomic sequences from NCBI Genbank (Accession number BC019406 and AL691416) an approximately 1.5 kb fragment of mouse BBD promoter from -1,394 to +94 relative to transcription start site was scanned and analyzed for putative PPRE using the NUBIScan (nuclear receptor binding site scanner; [22]).

2.4 Generation of mouse BBD promoter reporter constructs

Six BBD promoter-truncation constructs were designed (Fig. 1 and 2). The 1,488 bp promoter fragment from -1,394 to +94 relative to transcription start site (BBD-1,488) containing seven predicted PPRE was PCR amplified from mouse BAC clone RP23-110I8 (imaGene, Berlin Germany). The generated PCR fragment with BglII and XhoI restriction sites introduced at the 5´ and 3´ ends was subcloned into the BglII and XhoI digested

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pGL4.10 [luc2] vector (Promega, Mannheim, Germany) upstream of the luciferase reporter gene, thereby, providing the full-length construct pGL4-BBD-1488 (BBD construct 1). The BBD promoter-truncation constructs pGL4-BBD-1197 (BBD construct 2; from -1,103 to +94) containing six putative PPRE, pGL4-BBD-668 (BBD construct 3; from -574 to +94) containing five putative PPRE, pGL4-BBD-376 (BBD construct 4; from -282 to +94) containing four putative PPRE, pGL4-BBD-232 (BBD construct 5; from -138 to +94) containing three putative PPRE, and pGL4-BBD-164 (BBD construct 6; from -70 to +94) containing two overlapping PPRE were PCR amplified from parental clone pGL4.10-BBD-1488 by using different 5′-primers flanking the putative PPRE and a common 3′-primer. The primer sequences are shown in Tab. 1. The generated PCR products containing two adapters of BglII and XhoI site at the end were subcloned into the BglII and XhoI digested pGL4.10 [luc2] vector upstream of the luciferase reporter gene. The cloned DNA fragments were sequenced to confirm the integrity of the constructs.

In addition, BBD promoter constructs containing 3 copies of either PPRE6, PPRE1 or PPRE3 in front of a mini promoter of the luciferase reporter gene were designed. To generate these, the following oligonucleotides (Eurofins MWG Operon, Ebersberg, Germany) containing either 3X PPRE6, 3X PPRE1 or 3X PPRE3 and two adapters of KpnI and HindIII site were annealed: 3X mBBD_PPRE6 (forward: 5′-TCGAAATACTCTAATCAGAACAAA GGTCCCGGCATCTAATCAGAACAAAGGTCCCGGCATCTAATCAGAACAAAGGTC CCGGCATGGGGCG-3′, reverse: 5′-AGCTCGCCCCATGCCGGGACCTTTGTTCTGATT AGATGCCGGGACCTTTGTTCTGATTAGATGCCGGGACCTTTGTTCTGATTAGAGTA TT-3′), 3X mBBD_PPRE1 (forward: 5′-TCGAAGCTGGGACTGAAGTTCAACGGTAGA ATGCCGACTGAAGTTCAACGGTAGAATGCCGACTGAAGTTCAACGGTAGAATGC CTGTCTC-3′, reverse: 5′-AGCTGAGACAGGCATTCTACCGTTGAACTTCAGTCGGCA TTCTACCGTTGAACTTCAGTCGGCATTCTACCGTTGAACTTCAGTCCCAGCT-3′), 3X mBBD_PPRE3 (forward: 5′-TCGAAAGATTATTTGGTGACCCCTGACAGATTTTTA

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TTTGGTGACCCCTGACAGATTTTTATTTGGTGACCCCTGACAGATTTTTTTAAAA-3′, reverse: 5′-AGCTTTTTAAAAAAATCTGTCAGGGGTCACCAAATAAAAATCTGTCAGGGGTCACCAAATAAAAATCTGTCAGGGGTCACCAAATAATCTT-3′). After annealing, the double stranded DNA fragments were cloned into the KpnI and HindIII digested pGL4.23 [luc2/minP] vector (Promega). After cloning fragments were controlled for the identity of the 3X PPRE by DNA sequencing.

2.5 Site-directed mutagenesis of BBD promoter reporter constructs

Mutation constructs were prepared by introducing a mutation in the putative PPRE with Site-Directed Mutagenesis kit according to the manufacturer's protocol (Stratagene Europe, Amsterdam, Netherlands) using the following oligonucleotides: PPRE1mut (forward: 5′-AGACAGGCATTCTtCCGTaGAACTTCAGTCCCAGC-3′, reverse: 5′-GCTGGGACTGAAGTTCtACGGaAGAATGCCTGTCT-3′), PPRE2mut (forward: 5′-TATTTCATTAGTGACgGTATACgTGACATTCAGGA-3′, reverse: 5′-TCCTGAATGTCAcGTATACcGTCACTAATGAAATA-3′), PPRE3mut (forward: 5′-TTTAAAAAAATCTGTCtGGGGaCACCAAATAATCT-3′, reverse: 5′-AGATTATTTGGTGtCCCCaGACAGATTTTTTTAAA-3′), PPRE4mut (forward: 5′-AATACTGCAAGTGGACaCaGGCCTCTGCCTACCAC-3′, reverse: 5′-GTGGTAGGCAGAGGCCtGtGTCCACTTGCAGTATT-3′), PPRE5mut (forward: 5′-CAAGTGGACTCTGGCCaCaGCCTACCACTCAGCAG-3′, reverse: 5′-CTGCTGAGTGGTAGGCtGtGGCCAGAGTCCACTTG-3′), PPRE6mut (forward: 5′-GCCCCATGCCGGGACCaTaGTTCTGATTAGAGTAT-3′, reverse: 5′-ATACTCTAATCAGAACtAtGGTCCCGGCATGGGGC-3′), PPRE7mut (forward: 5′-ATTATAAAATAAcAAACAtGGGCAAACACAGTGAT-3′, reverse: 5′-ATCACTGTGTTTGCCCaTGTTTgTTATTTTATAAT-3′). The mutant constructs were controlled for the intended mutations and the absence of any unexpected mutations by DNA sequencing.

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2.6 Transient transfection

Transient transfection was performed as described recently in more detail [23]. In brief, HepG2 cells were transiently transfected with 50 ng of the generated reporter gene constructs and co-transfected with 50 ng of both, mouse PPARα expression plasmid (pCMX-mPPARα) and mouse RXRα expression plasmid (pCMX-mRXRα) (both, generous gifts from R.M. Evans, Salk Institute for Biological Studies, San Diego, CA, USA), or 100 ng empty vector (pCMX) using FuGENE 6 transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer´s protocol. Cells were also co-transfected with pGL4.74 Renilla luciferase (Rluc) (encoding the renilla luciferase reporter gene; Promega), which was used as internal control reporter vector to normalize for differences in transfection efficiency (Promega, Mannheim, Germany). A 3X ACO-PPRE vector (containing three copies of consensus PPRE from the ACO promoter in front of a luciferase reporter gene; a generous gift from Dr. Sander Kersten, Nutrigenomics Consortium, Top Institue (TI) Food and Nutrition, Wageningen, Netherlands) and pGL4.23 vector were transfected as positive and negative controls, respectively. Following transfection, cells were treated with either 50 µmol/L WY-14,643 to achieve activation of PPARα or vehicle only (DMSO = control) for 24 h. Afterwards, cells were washed with phosphate-buffered saline and lysed with passive lysis buffer (Promega). Luciferase activities were determined with the Dual-Luciferase Reporter Assay System from Promega according to the manufacturer´s instructions using a Mithras LB940 luminometer (Berthold Technologies, Bad Wildbad, Germany) as described recently in more detail [23].

2.7 Electrophoretic mobility shift assay (EMSA)

EMSA experiments were performed as described recently in more detail [6]. In brief, mouse PPARα and RXRα proteins were generated from the expression vectors by *in vitro* transcription/translation using TNT® Quick Coupled Transcription/Translation Systems

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(Promega) according to the manufacturer`s protocol. The following oligonucleotides were annealed with annealing buffer (10 mM Tris, 1 mM EDTA, 0.1 mM NaCl; pH 8.0): BBD-PPRE6 (forward: 5′-GACTGCCCCATGCCGGGACCTTTGTTCTGATTAGAGTAT-3′, reverse: 5′-AGCTATACTCTAATCAGAACAAAGGTCCCGGCATGGGGC-3′), BBD-PPRE6mut for mutation (forward: 5′-GACTGCCCCATGCCGGGACCaTaGTTCTGATTAGAGTAT-3′, reverse: 5′-AGCTATACTCTAATCAGAACtAtGGTCCCGGCATGGGGC-3′), OCTN2-PPRE (mouse OCTN2-PPREint-1) as specific control and for competition (forward: 5′-GACTCTGTAAGTAGGTGAAAGGGCATATAACTCTTA-3′, reverse: 5′-AGCTTAAGAGTTATATGCCCTTTCACCTACTTACAG-3′), and OCTN2-PPREmut (mouse OCTN2-PPREint-1mut) as non-specific control (forward: 5′-GACTCTGTAAGTAGGTGtAtGGGCATATAACTCTTA-3′, reverse: 5′-AGCTTAAGAGTTATATGCCCaTaCACCTACTTACAG-3′). After annealing, 100 ng double-stranded DNA-probes were labelled with 0.05 mM DIG-ddUTP in 1X labelling buffer (0.2 M potassium cacodylate, 25 mM Tris-HCl, 0.25 ng/mL bovine serum albumin; pH 6.6), 5 mM $CoCl_2$, 20 U/uL Terminal transferase (Roche) and incubated for 15 min at 37°C. Then 2 µL of each *in vitro*-translated PPARα and RXRα proteins were incubated with 1.6 ng DIG-labelled probes and 5-, 10- and 50-fold molar excess of unlabelled specific probes (OCTN2-PPRE) for competition in 1 µg poly d(I-C) and EMSA binding buffer (10 mM Tris-HCl, 120 mM KCl, 0.5 mM EDTA, 0.1% Triton-X-100, 12.5% glycerol, 0.2 mM DTT) for 20 min at RT. The protein-DNA complexes were subjected to electrophoresis on 6% native polyacrylamid gels, and transferred to a positive charged nylon membrane. The DIG-labelled DNA was detected by chemiluminescence using Anti-Digoxigenin-AP Conjugate and CSPD (both from Roche) according to the manufacturer's protocol, and a Bio-Imaging system.

2.8 Statistical analysis

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Numerical data of were analyzed by one-way ANOVA using the Minitab Statistical Software Rel. 13.0 (Minitab, State College, PA, USA). Differences of $P < 0.05$ were considered to be significant.

3. Results

3.1 In silico-analysis of mouse BBD promoter for the existence of putative PPRE

To examine, whether the mouse BBD gene contains putative PPRE, we performed *in silico*-analysis of an approximately 1.5 kb nucleotide sequence upstream of the translation start site of BBD gene using NUBIScan software. According to this, this DNA fragment contained seven putative PPRE located at positions -17 to -29 (PPRE4), -24 to -36 (PPRE5), -75 to -87 (PPRE6), -212 to -224 (PPRE7), -355 to -367 (PPRE1), -824 to -839 (PPRE2) and -1256 to -1268 (PPRE3) relative to the transcription start site. The sequence alignment between the consensus PPRE AGGTCAAAGGTCA (termed DR-1: direct repeat-1) and the predicted PPRE in the mouse BBD promoter showed high similarity. Sequence alignment of PPRE4, PPRE5, PPRE6, PPRE7, PPRE2 and PPRE3 between mouse (promoter) and rat (intron1) showed high similarity (Tab. 2). In addition, sequence alignment of PPRE4, PPRE5, PPRE6 and PPRE7 between mouse (promoter) and human (intron2) also revealed high similarity. Alignment of PPRE1 revealed only a very low sequence identity between mouse, rat and human (Tab. 2).

3.2 Transcriptional activity of putative PPRE in the mouse BBD promoter

To evaluate, whether the putative PPRE in the mouse BBD promoter are responsible for PPARα-mediated transactivation of BBD, we generated four plasmid constructs containing 5´-deleted fragments of mouse BBD promoter upstream of a firefly luciferase reporter gene. These constructs were transiently transfected into HepG2 cells with co-transfection of either pCMX-mPPARα and pCMX-mRXRα or empty vector (pCMX), and,

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subsequently, cells were treated with or without the PPARα agonist WY-14,643. As shown in Fig. 1, there was a significant increase in luciferase activity in response to co-expression of mouse PPARα and RXRα and/or WY-14,643 in cells transfected with either the full-length BBD construct 1 or the truncation constructs BBD construct 2 and BBD construct 3, in which PPRE3 and PPRE2, respectively, were serially deleted ($P < 0.05$). The fold increases in luciferase activity in response to co-expression of mouse PPARα/RXRα and/or WY-14,643 were similar between these three constructs compared to the full-length construct BBD construct 1 indicating that PPRE3 and PPRE2 are of minor importance for regulation of mouse BBD by PPARα. In cells transfected with the truncation BBD construct 4, in which PPRE1 was deleted, basal luciferase activity (without co-expression of mouse PPARα/RXRα and WY-14,643) was significantly lower when compared to the full-length BBD construct 1. However, the response of this construct to co-expression of mouse PPARα/RXRα and/or WY-14,643 was similar strong as observed with the full-length construct indicating that PPRE1 is also not of importance for PPARα-dependent regulation of mouse BBD. To further confirm our assumptions, we generated mutant constructs from the truncation reporter constructs each containing targeted mutations in PPRE3, PPRE2 and/or PPRE1. As shown in Fig. 1, cells transfected with mutant constructs harboring a single mutation in either PPRE3 or PPRE2 had a similar basal luciferase activity and showed a similar response to mouse PPARα/RXRα and/or WY-14,643 compared to cells transfected with the corresponding wild-type constructs. Cells transfected with a mutant construct with a single mutation in PPRE1 had a markedly lower basal reporter activity than cells transfected with the corresponding wild-type BBD construct 3 (Fig. 1) but the response to mouse PPARα/RXRα and/or WY-14,643 was similar as observed in cells transfected with the corresponding wild-type construct. These results confirmed that PPRE3, PPRE2 and PPRE1 probably do not contribute to the PPARα-dependent regulation of mouse BBD. However, we observed that the reporter activity of BBD construct 4, which lacks PPRE1, PPRE2 and PPRE3, was still increased by co-expression of

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mouse PPARα/RXRα and/or WY-14,643 and the response to mouse PPARα/RXRα and/or WY-14,643 was similar strong as that of BBD construct 1, BBD construct 2 and BBD construct 3 (Fig. 1). Thus, these findings suggested that a functional PPRE might be located in the more proximal region of the BBD promoter.

To identify this functional PPRE, we generated additional truncation reporter constructs in which the remaining PPRE7, PPRE6, PPRE5 and PPRE4 were either serially deleted or mutated. As shown in Fig. 2, serial deletion of PPRE1 and PPRE7 reduced basal luciferase reporter activity but did not impair the response to mouse PPARα/RXRα and/or WY-14,643 which confirms the results from the experiment shown in Fig. 1. However, when PPRE6 was either deleted (BBD construct 6) or mutated, basal reporter activity was dramatically reduced compared to BBD construct 3 and the response of the reporter activity to mouse PPARα/RXRα and/or WY-14,643 was completely abolished indicating that PPRE6 is functional, whereas PPRE7, PPRE5, PPRE4 and PPRE1 are not functional (Fig. 2). The same loss of responsiveness to mouse PPARα/RXRα and/or WY-14,643 was observed when, in addition to PPRE6, PPRE1 was mutated (Fig. 2). Confirming that PPRE7, PPRE5, PPRE4 and PPRE1 are not functional, no impairment in the reporter response to mouse PPARα/RXRα and/or WY-14,643 was found when either PPRE7, PPRE5, PPRE4 or PPRE1 were mutated (Fig. 2).

To further proof the importance of PPRE6 for PPARα-dependent regulation of mouse BBD, we generated a reporter gene construct containing 3 copies of PPRE6 in front of the mini promoter of the luciferase reporter vector. As shown in Fig. 3, cells transfected with this construct showed a pronounced response to co-expression of mouse PPARα/RXRα and WY-14,643, whereas cells transfected with reporter constructs containing multiple copies of either PPRE1 or PPRE3 showed no response at all. To provide final proof for the importance of PPRE6 for PPARα-dependent regulation of mouse BBD, we transfected cells with the full length BBD construct 1 bearing a mutation in PPRE6. The response of the reporter activity of

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this construct to mouse PPARα/RXRα and/or WY-14,643 was completely abolished indicating that PPRE6 is functional.

3.3 In vitro-binding of PPARα/RXRα heterodimer to PPRE6 of mouse BBD promoter

To finally confirm the functionality of PPRE6, we studied *in vitro*-binding of mouse PPARα/RXRα to PPRE6 of mouse BBD promoter using *in vitro*-translated mouse PPARα/RXRα and an oligonucleotide corresponding to PPRE6 in gel shift assays (EMSA). As shown in Fig. 4, a major DNA-protein complex was formed between the specific probe of mouse OCTN2-PPREint1 and the *in vitro*-translated PPARα/RXRα heterodimer as positive control (lane 4; Fig. 4), whereas no complex was formed between the mutant non-specific probe OCTN2-PPREint1_mut and *in vitro*-translated PPARα/RXRα heterodimer as negative control (lane 5; Fig. 4). A strong DNA-protein complex formation was also observed between the specific probe of mouse BBD-PPRE6 and *in vitro*-translated PPARα/RXRα heterodimer (lane 6; Fig. 4). In addition, competition experiments using the oligonucleotide corresponding to mouse BBD-PPRE6 and 5-, 10- and 50-fold molar excess of unlabelled specific probes (OCTN2-PPREint1) were performed to test the specificity of PPARα/RXRα binding (lanes 7, 8 and 9; Fig. 4). As demonstrated in Fig. 4, the complex intensity was successively reduced with increasing molar excess of unlabelled specific probe. At 50-fold molar excess of unlabelled probe, complex formation was almost completely absent being indicative of complete competition. No DNA-protein complex formation was observed between the mutant oligonucleotide PPRE6mut and *in vitro*-translated PPARα/RXRα heterodimer (lane 10; Fig. 4). These results demonstrated that PPARα/RXRα heterodimer binds specifically to PPRE6 of mouse BBD.

4. Discussion

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Although convincing evidence from studies with PPARα-deficient mice existed [11, 12, 15] suggesting that the carnitine biosynthetic enzyme BBD is regulated by PPARα, the identification of BBD as a direct PPARα target gene as well as its exact regulation remained to be demonstrated. To clarify this, we performed *in silico*-analysis of the mouse BBD promoter using NUBIScan software, and found seven putative PPRE with high similarity to the known consensus PPRE sequence in an approximately 1.5 kb fragment of mouse BBD promoter. In order to explore their functional relevance for transcriptional regulation of mouse BBD by PPARα, we created different serial 5´-truncation BBD promoter reporter constructs containing a different number of putative PPRE and tested their responsiveness to exogenous mouse PPARα/RXRα and WY-14,643 in reporter gene assays. Using this approach we found that the two PPRE most distant from the translation start site (PPRE3 and PPRE2) are not of importance for the regulation of BBD by PPARα because the responsiveness of the corresponding truncation constructs without PPRE3 and PPRE2 to exogenous mouse PPARα/RXRα and WY-14,643 did not differ from that of the full-length reporter construct (BBD construct 1). To confirm that PPRE3 and PPRE2 are not functional we also tested the responsiveness of truncation constructs harboring a selective mutation in either PPRE3 or PPRE2, and indeed found that their responsiveness to both, exogenous PPARα/RXRα and WY-14,643 did not differ from that of the non-mutated construct. When analyzing the responsiveness of the next truncation construct, in which, in addition to PPRE3 and PPRE2, PPRE1 was deleted, we observed a marked loss in the basal reporter activity (without exogenous PPARα/RXRα and WY-14,643) but the response to exogenous PPARα/RXRα and WY-14,643 was similar as that of the full-length construct indicating that PPRE1 is also not of importance for PPARα-dependent regulation of the mouse BBD promoter. Since the truncation construct without PPRE1 (BBD construct 4) and all truncation reporter constructs with a selective mutation in PPRE1 were still similar responsive to exogenous PPARα/RXRα and WY-14,643 as the full-length reporter construct and the truncation reporter constructs

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containing PPRE3 and PPRE2, we concluded that one or more of the remaining putative PPRE (PPRE4, PPRE5, PPRE6 and PPRE7) located in the more proximal region of the BBD promoter are functional. To test this, we created further truncated and mutated reporter constructs where the remaining PPRE were either serially deleted or mutated. According to this, we observed that mutating PPRE6 resulted in a complete loss of responsiveness to both, exogenous mouse PPARα/RXRα and WY-14,643 indicating that PPRE6 is a functional PPRE in the mouse BBD promoter. The same complete loss of responsiveness to exogenous mouse PPARα/RXRα and WY-14,643 was observed when both, PPRE1 and PPRE6 were mutated. The key role of PPRE6 for transcriptional regulation of the mouse BBD promoter by PPARα could be further evidenced by using reporter constructs, in which PPRE6 was either mutated (BBD construct 5) or deleted (BBD construct 6). In these constructs, the basal reporter activity was the lowest of all constructs tested and there was no response at all to exogenous mouse PPARα/RXRα and WY-14,643. Hence, these observations indicated that the two most proximal PPRE, PPRE5 and PPRE4, are not functional. This was also confirmed by testing the responsiveness of truncation constructs harboring a selective mutation in either PPRE5 or PPRE4. A further strong evidence for the key role of PPRE6 in the regulation of mouse BBD promoter was provided from experiments using a reporter construct containing multiple copies of PPRE6 in front of the luciferase reporter gene. Cells transfected with this construct showed a marked response to exogenous mouse PPARα/RXRα and WY-14,643, whereas cells transfected with constructs containing multiple copies of either PPRE1 or PPRE3 did not show any response at all. Collectively, these results from reporter gene experiments indicated that PPRE6 is essential for regulation of mouse BBD by PPARα. Moreover, these data demonstrate that the other PPRE predicted from NUBIScan analysis are obviously not functional and probably of minor importance for PPARα-dependent regulation of mouse BBD.

To further clarify whether PPRE6 in the BBD promoter is functional and binds the PPARα/RXRα heterodimer, we performed gel shift assays using *in vitro*-translated mouse

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PPARα/RXRα proteins and oligonucleotides representing PPRE6. In the presence of PPARα/RXRα, a strong band representing the DNA-PPARα/RXRα complex appeared using the PPRE6-containing oligonucleotide, whereas the bands were weaker in the presence of an excess on unlabelled specific oligonucleotide. No band for the DNA-PPARα/RXRα complex was observed when olinucleotides containing the mutated PPRE6 were used. Although we did not perform antibody supershift assays, these results strongly support our aforementioned assumption that the PPARα/RXRα heterodimer binds to PPRE6 of mouse BBD and that PPRE6 is decisive for transcriptional regulation of mouse BBD.

To investigate whether regulation of the BBD gene by PPARα might be similar between mouse, rat and human, we compared the promoter and intron sequences of these species. According to this, we found that PPRE6 shares a complete (100%) sequence identity between mouse, rat and human which is indeed indicative of a similar regulation of the BBD gene by PPARα between these species. Nevertheless, the location of the translation start site of the BBD gene differs between mouse, rat and human. In the rat, the translation start site of the BBD gene is in the second exon (cDNA sequences from FQ210746 and NM_022629, genomic DNA sequences from AC106654), whereas in mice and humans the translation start sites are in the first and third exon, respectively (cDNA sequences from NM_003986 and genomic DNA sequences AC015756). Considering these differences, the exact regulation of the BBD gene by PPARα might differ, at least partially, between mice, rats and humans. This, however, has to be clarified in future studies.

In conclusion, the present study clearly shows that mouse BBD is a direct PPARα target gene and that transcriptional up-regulation of mouse BBD by PPARα is likely mediated by binding of the PPARα/RXRα heterodimer to a PPRE located in its proximal promoter region. The results confirm emerging evidence from recent studies that PPARα plays a key role in the regulation of carnitine homeostasis by controlling genes involved in both, carnitine synthesis and carnitine uptake [11, 12, 15]. From a practical point of view, this study indicates

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that fibrates, synthetic agonists of PPARα used in the therapy of hyperlipidemia, might stimulate the synthesis of carnitine.

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Table 1

Oligonucleotides used for PCR amplification of promoter region of mouse BBD

Oligonucleotide	Oligonucleotide sequence (5′-3′)
mBBD_BglII	ATCAGATCTTGTTTCTGGTCTTTGGTGA
mBBD_164_XhoI	ATCCTCGAGATGGGGCGAACTGTAGGCT
mBBD_232_XhoI	ATCCTCGAGCTTACAGCTAGTCACTGTGC
mBBD_376_XhoI	ATCCTCGAGCCATTTGCGTAGCGGTTTTC
mBBD_668_XhoI	ATCCTCGAGTCCAGTGAGAAATATACTGTG
mBBD_1197_XhoI	ATCCTCGAGTCAGTATCGGATACAGTGAAG
mBBD_1488_XhoI	ATCCTCGAGGCACACCATGGGCTAAGAATAT

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Table 2

Sequence alignment of consensus PPRE with putative PPRE predicted from *in silico*-analysis of mouse BBD promoter using NUBIScan software and sequence alignment of putative PPRE between mouse, rat and human using 1.5 kb DNA sequence upstream of the translation start site of BBD gene

PPRE denomination	Species	Sequence and position*		
Consensus PPRE			AGGTCAnAGGTCA	
PPRE4	*M. musculus*	-29	5′AGGCCAgAGTCCA	-17prom
	R. norvegicus	85	3′AGGCCAGAGTCCA	73int1
	H. sapiens	13824	5′AGGCCAGAGTCCA	13836int2
			**********	
PPRE5	*M. musculus*	-36	5′TAGGCAgAGGCCA	-24prom
	R. norvegicus	80	3′TAGGCAGAGGCCA	92int1
	H. sapiens	13817	5′ATAGCAGAGGCCA	13829int2
			---**********	
PPRE6	*M. musculus*	-87	5′AGAACAaAGGTCC	-75prom
	R. norvegicus	131	3′AGAACAAAGGTCC	143int1
	H. sapiens	13762	5′AGAACAAAGGTCC	13774int2
			**********	
PPRE7	*M. musculus*	-212	3′AGAAACaAGGGCA	-224prom
	R. norvegicus	280	5′AGAAACAATGGCA	268int1
	H. sapiens	13632	3′AGAACCAATGACA	13620int2
			****-***-****	
PPRE1	*M. musculus*	-367	5′AGTTCAaCGGTAG	-335prom
	R. norvegicus	416	3′AGCTCAAC-----	423int1
	H. sapiens	13486	5′TTTTCAAAAG---	13495int2
			---**********	
PPRE2	*M. musculus*	-836	5′AGGTATaCGGTCA	-825prom
	R. norvegicus	886	3′AGGTATACAATCA	874int1
	H. sapiens	13210	5′AAATATATAGTCA	13022int2
			*--*******--***	
PPRE3	*M. musculus*	-1256	3′CTGTCAgGGGTCA	-1268prom
	R. norvegicus	1313	5′CTGTCAGGGATCA	1301int1
	H. sapiens	12593	3′-CATTAAG-ATAA	12603int2
			---*-*-*--*-*	

*Nucleotides of putative PPRE are underlined. Matching nucleotides between mouse, rat and human are shown by asterisks. Position of PPRE was indicated.

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Legends for figures

Figure 1

Effect of exogenous mouse PPARα/RXRα and PPARα ligand WY-14,643 on transcriptional activity of BBD promoter-truncation reporter constructs

HepG2 cells were transiently transfected with mutated and non-mutated serial 5´-truncation BBD promoter reporter constructs pGL4-BBD-1488, -1197, -668 and -376, and a renilla luciferase expression vector for normalization. Cells were also co-transfected with or without (empty vector) expression vectors for mouse PPARα and RXRα. After transfection, cells were stimulated or not with 50 µM of WY-14,643 for 24 h. Afterwards, cells were lysed, and luciferase activities of the BBD promoter-truncation reporter vectors and the renilla luciferase expression vector determined by dual luciferase assay. Results represent means ± SEM for one out of three independent experiments each performed in triplicate.

Figure 2

Effect of exogenous mouse PPARα/RXRα and PPARα ligand WY-14,643 on transcriptional activity of BBD promoter-truncation reporter constructs

HepG2 cells were transiently transfected with mutated and non-mutated serial 5´-truncation BBD promoter reporter constructs pGL4-BBD-668, -376, 232 and -164, and a renilla luciferase expression vector for normalization. Cells were also co-transfected with or without (empty vector) expression vectors for mouse PPARα and RXRα. After transfection, cells were stimulated or not with 50 µM of WY-14,643 for 24 h. Afterwards, cells were lysed, and luciferase activities of the BBD promoter-truncation reporter vectors and the renilla luciferase expression vector determined by dual luciferase assay. Results represent means ± SEM for one out of three independent experiments each performed in triplicate.

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Figure 3

Effect of exogenous mouse PPARα/RXRα and PPARα ligand WY-14,643 on transcriptional activity of 3X PPRE BBD-promoter reporter constructs

HepG2 cells were transiently transfected with BBD promoter reporter constructs containing 3 copies of either PPRE6, PPRE1 or PPRE3 in front of the TK promoter of the luciferase reporter vector, and a renilla luciferase expression vector for normalization. Cells were also co-transfected with or without (empty vector) expression vectors for mouse PPARα and RXRα. After transfection, cells were stimulated or not with 50 µM of WY-14,643 for 24 h. Afterwards, cells were lysed, and luciferase activities of the BBD promoter reporter vectors and the renilla luciferase expression vector determined by dual luciferase assay. Results represent means ± SEM for one out of three independent experiments each performed in triplicate.

Figure 4

Binding of in vitro-translated mouse PPARα/RXRα to the PPRE6 of mouse BBD promoter

EMSA was performed using *in vitro*-translated mouse PPARα/RXRα and DIG-labelled oligonucleotide corresponding to either wild-type or mutated PPRE6. Fold molar excess of unlabelled specific probe for competition (OCTN2-PPREint1 oligonucleotide) is indicated. The use of DIG-labelled specific probe (OCTN2-PPRE1int1 oligonucleotide) and non-specific probe (mutant OCTN2int1mut oligonucleotide) is also indicated.

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Figure 1

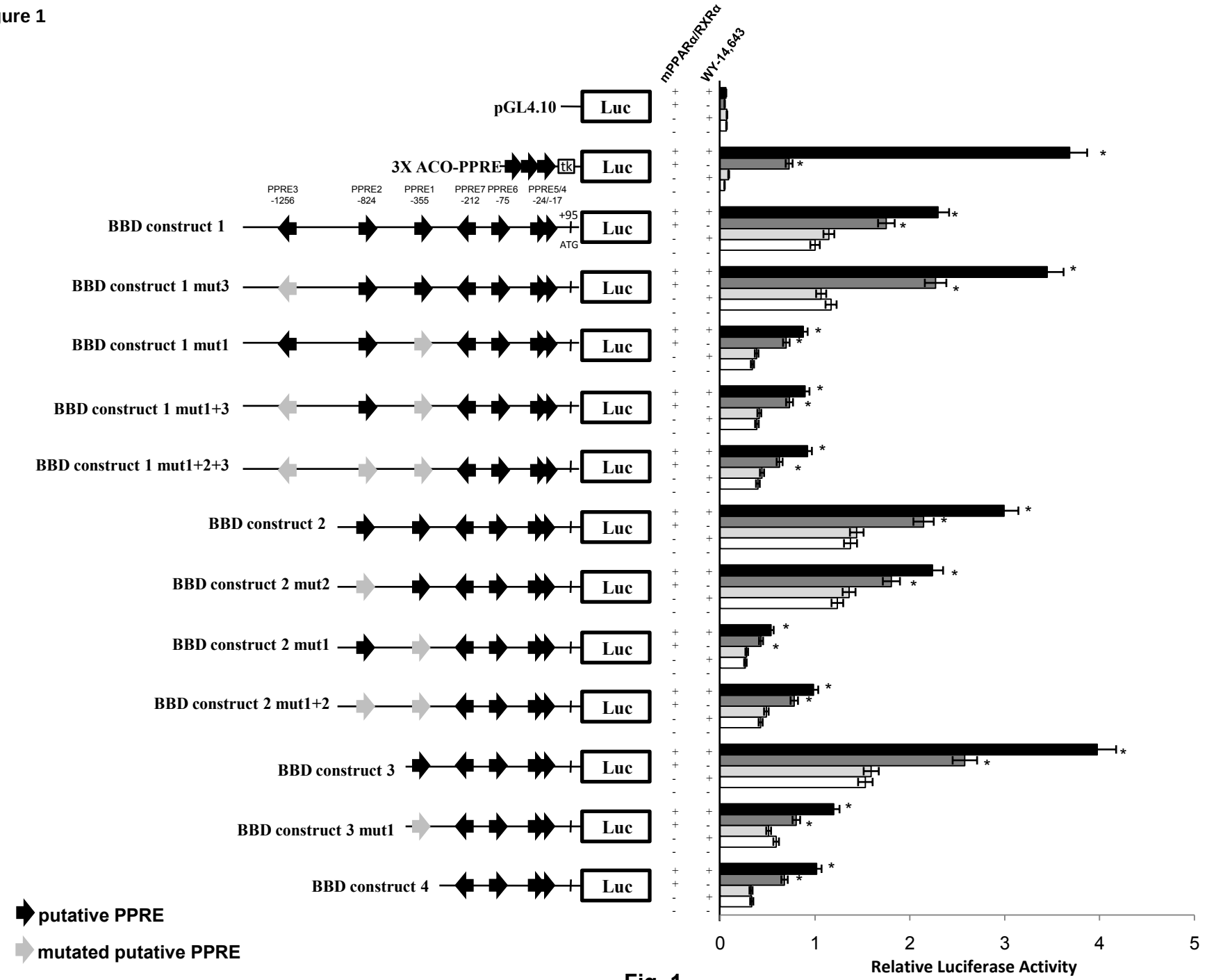


Fig. 1

Figure 2

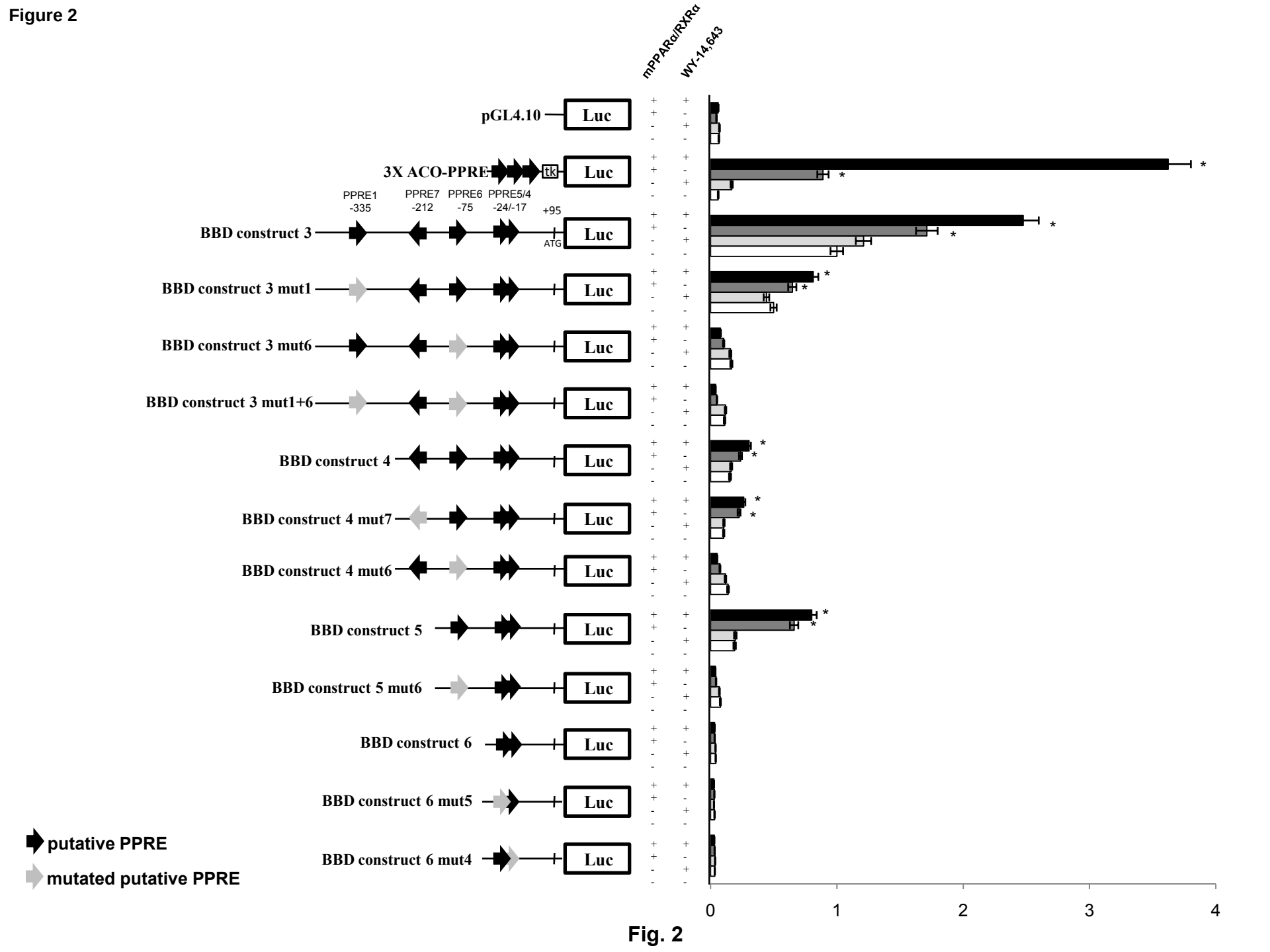
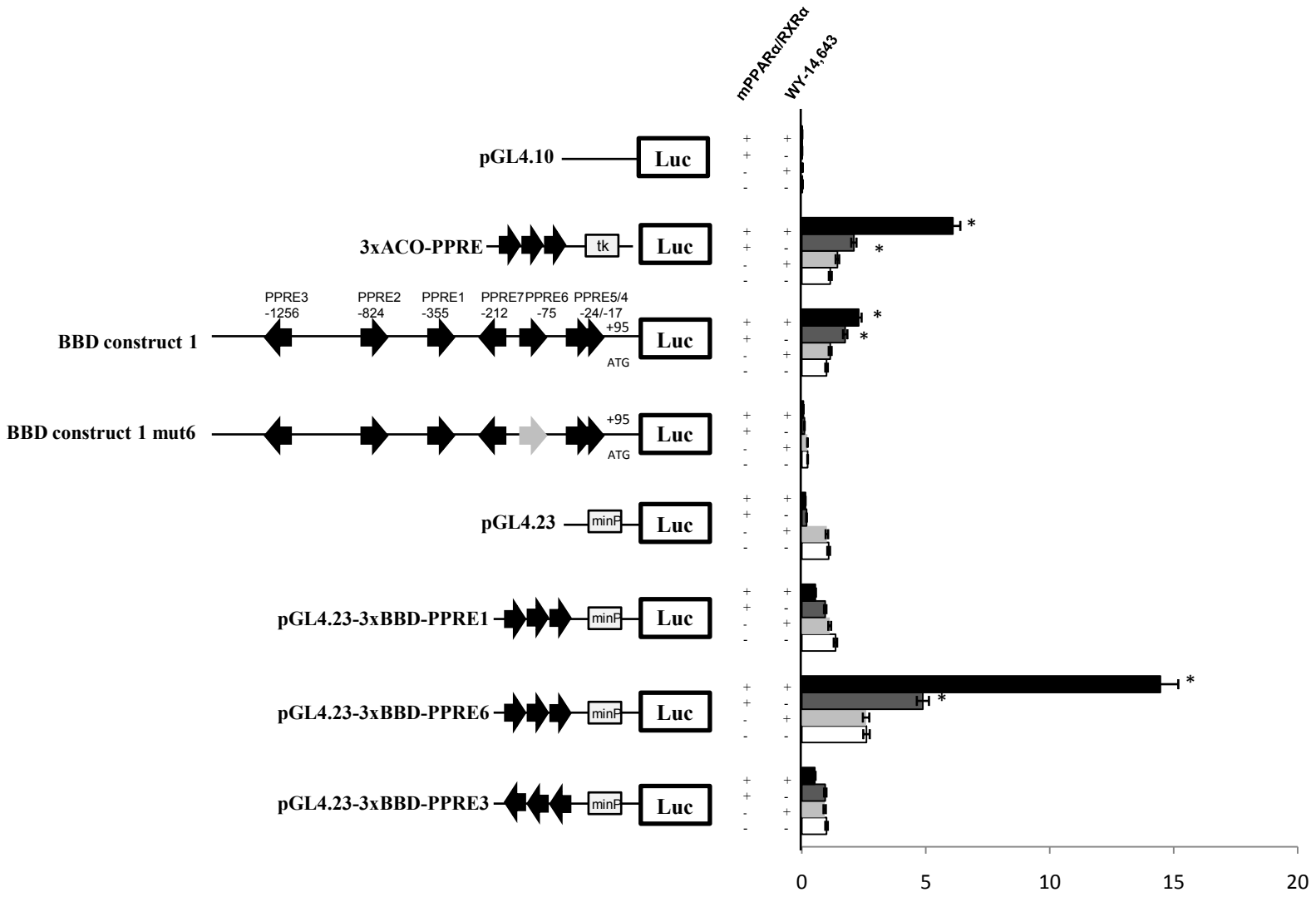


Fig. 2

Figure 3



putative PPRE

mutated putative PPRE

Fig. 3

Figure 4

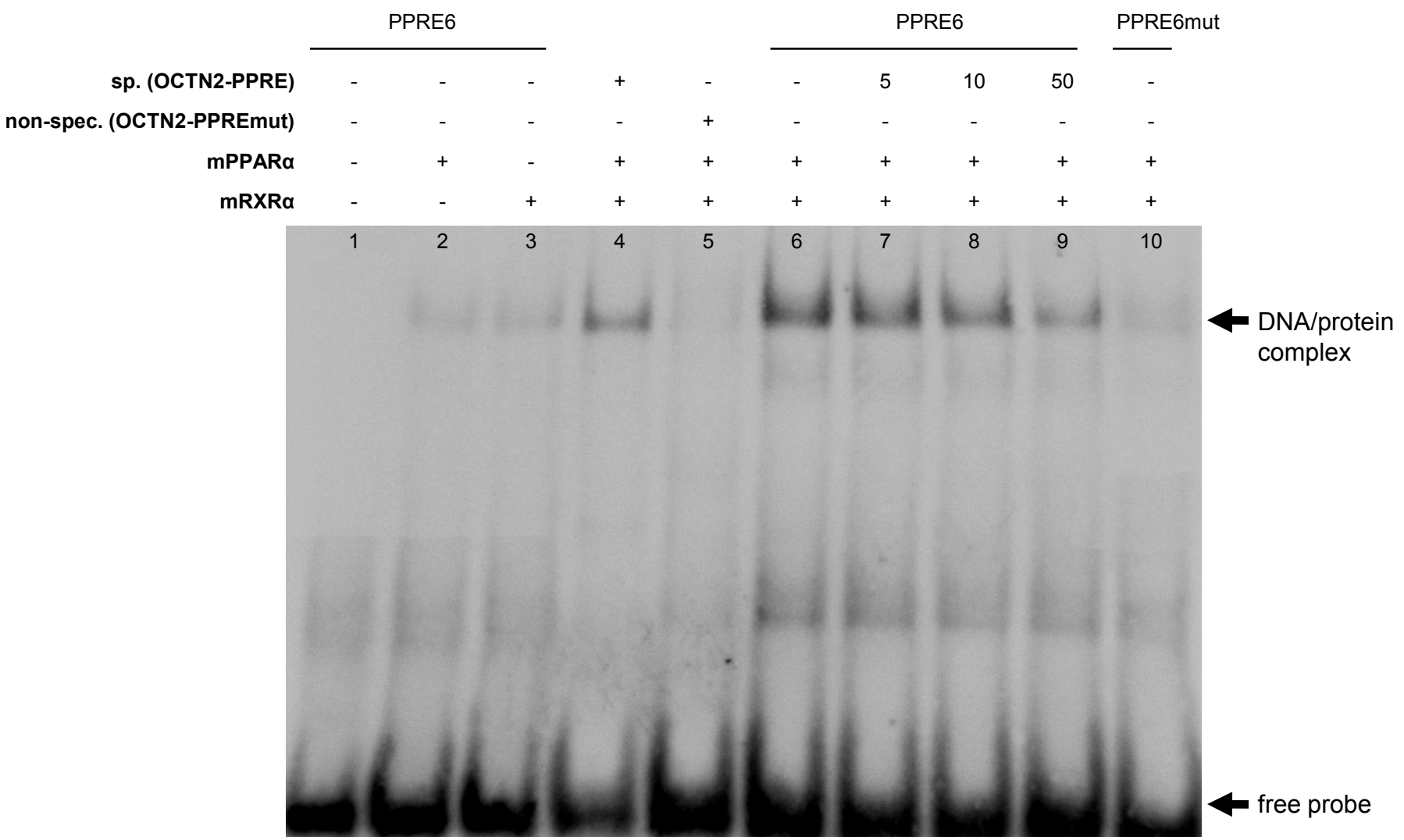


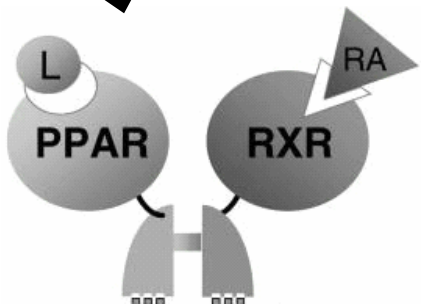
Fig. 4

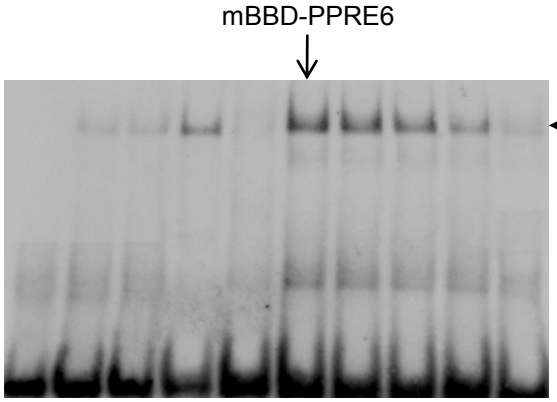
*Graphical Abstract

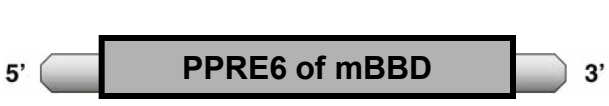
free fatty acids

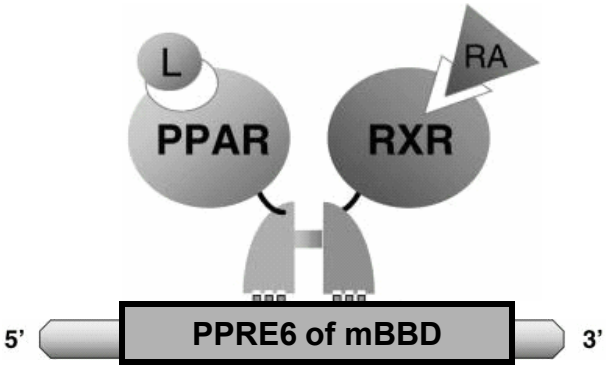
fibrates

WY-14,643









BBD mRNA ↑

Van Vlies et al. 2007
Koch et al. 2008