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The Peroxisome Proliferator-Activated Receptor Alpha (PPAR) regulates Bile Acid Biosynthesis.

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Abbreviations: PPARα, peroxisome proliferator-activated receptor alpha; PPRE, peroxisome proliferator-response element; DR1, direct repeat 1; ACO, acyl-CoA oxidase; EMSA, electrophoretic mobility shift assay; bp, base pair.

ABSTRACT:

Fibrates are a group of hypolipidemic agents which efficiently lower serum triglyceride levels by affecting the expression of many genes involved in lipid metabolism. These effects are exerted via the peroxisome proliferator-activated receptor alpha (PPARα). In addition, fibrates also lower serum cholesterol levels, suggesting a possible link between the PPARα and cholesterol metabolism. Bile acid formation represents an important pathway for elimination of cholesterol, and the sterol 12α-hydroxylase is a branch-point enzyme in the bile acid biosynthetic pathway, which determines the ratio of cholic acid to chenodeoxycholic acid. Treatment of mice for one week with the peroxisome proliferator WY-14,643 or fasting for 24 hours both induced the sterol 12α-hydroxylase mRNA in liver. Using the PPARα knockout mouse model, we show that the induction by both treatments was dependent on the PPARα. A reporter plasmid containing a putative peroxisome proliferator-response element (PPRE) identified in the rat sterol 12α-hydroxylase promoter region was activated by treatment with WY-14,643 in HepG2 cells, being dependent on co-transfection with a PPARα expression plasmid. The rat 12α-hydroxylase PPRE bound *in vitro* translated PPARα and RXRα, albeit weakly, in electrophoretic mobility shift assay. Treatment of wild-type mice with WY-14,643 for one week resulted in an increased relative amount of cholic acid, an effect which was abolished in the PPARα null mice, verifying the functionality of the PPRE *in vivo*.

INTRODUCTION

Fibrates and their derivatives constitute a group of hypolipidemic agents which are used in the treatment of hypertriglyceridemia and combined hyperlipidemia. These fibrates belong to a structurally diverse group of compounds known as peroxisome proliferators, which have been shown to cause liver hepatomegaly, proliferation of peroxisomes and induction of many enzymes involved in peroxisomal and mitochondrial β-oxidation and ω-oxidation of fatty acids (for review see (1)). In the past number of years, significant progress has been made in understanding the mechanism of action of these peroxisome proliferators. These compounds activate the peroxisome proliferator-

activated receptor alpha (PPARα), a member of the nuclear hormone receptor superfamily (2). The PPARα binds as a heterodimer with the retinoid X receptor (RXR) to a peroxisome proliferator-response element (PPRE) located in the promoter region of target genes e.g. peroxisomal acyl-CoA oxidase (ACO) (3), P450 4A1 (CYP4A1) (4), and the enoyl-CoA/3-hydroxyacyl-CoA hydratase/dehydrogenase multifunctional enzyme (5). Targeted disruption of the PPARα gene resulted in lack of peroxisome proliferation, no hepatomegaly and lack of induction of peroxisome proliferator-regulated genes in response to peroxisome proliferators (6-8). The PPARα has also been shown to play a critical role in the adaptive response to fasting in mice (8-11) as the induction of several genes involved in lipid catabolism is abolished in the PPARα-null mice. In addition to the triglyceride lowering effect, fibrates also lower plasma cholesterol levels in humans (12). Bezafibrate treatment of normolipidemic gallstone patients resulted in decreased levels of total, LDL- and VLDL-cholesterol, while HDL-cholesterol was unchanged. In addition, bezafibrate treatment changed the bile acid composition in bile with cholic acid being increased and chenodeoxycholic acid being decreased, suggesting an involvement of the PPARα in the regulation of expression of sterol 12α-hydroxylase and thereby affecting bile acid composition.

Bile acids are formed in the liver from cholesterol and their synthesis represents an important pathway for elimination of cholesterol from the body. The bile acid biosynthetic pathway involves a number of enzymatic modifications of the cholesterol backbone catalyzed by several P-450 enzymes, followed by β-oxidation of the cholesterol side chain and conjugation of the formed bile acid to glycine or taurine (for review see (13)). The rate limiting step in bile acid formation is generally believed to be the 7α-hydroxylation of cholesterol, catalyzed by cholesterol 7α-hydrolxylase (CYP7A1). The sterol 12α-hydroxylase (CYP8B1) is a hepatic microsomal enzyme that acts at a branch-point in the bile acid synthetic pathway by catalyzing the conversion of 7α-hydroxy-4-cholesten-3-one to 7α,12α-dihydroxy-4-cholesten-3-one. This conversion determines the ratio of cholic acid to chenodeoxycholic acid, and the balance may be important in the development of gallstones; chenodeoxycholic acid may, in contrast to cholic acid, reduce the degree of cholesterol saturation in bile, which is of importance for cholesterol gallstone formation. Sterol 12α-hydroxylase is highly regulated and many of the early studies carried out were involved in the regulation of the enzyme activity. Clofibrate treatment increases sterol 12α-hydroxylase activity and mRNA level in rat liver microsomes (14,15). The enzyme was initially purified from rabbit liver

microsomes by Ishida et al in 1992 and the activity was shown to be elevated by several treatments including starvation and administration of streptozotocin in rat (16). Other studies showed that following starvation, both sterol 12α-hydroxylase enzyme activity and mRNA levels were increased in rat and mouse, suggesting a possible transcriptional regulation in this case (17), but the mechanism of this regulation was not understood. Recently, the structure of the sterol 12α-hydroxylase gene in rat, human and mouse (15,18,19) and the cDNA in rabbit (17) have been elucidated, providing important tools for detailed studies on the regulation of this gene.

As fibrates act as ligands for the PPARα and treatment with these compounds results in an altered bile acid synthesis, we examined the effects of these compounds on the expression of sterol 12α-hydroxylase. From *in vivo* experiements using the PPARα-null mouse model and *in vitro* experiments, we show that the sterol 12α-hydroxylase gene is under regulation of the PPARα, activation of which results in a changed bile acid composition.

EXPERIMENTAL PROCEDURES

Animals and treatment - Ten to twelve week old wild-type or PPARα-null male mice on a pure Sv/129 genetic background (derived from the original colony of mixed background mice) (6) were housed in a temperature and light controlled environment. Mice were treated with a diet containing 0.1% (w/w) WY-14,643 (Calbiochem-Novabiochem Intl.) for one week or maintained on a normal chow diet. Fasting experiments were carried out for 24 hours and animals were sacrificed at 9.00 am. All mice had access to water ad libitum. Animals were euthanized by CO_2 asphyxiation followed by cervical dislocation, and liver and gallbladders were excised. The liver samples were frozen in liquid nitrogen and stored at -70^{o}C for preparation of total RNA.

Northern blot analysis - Total RNA was isolated from mouse liver samples using QuickPrep^{R} Total RNA Extraction Kit (Pharmacia Biotech, Uppsala, Sweden). Northern blot analysis was carried out as described (8) using full-length cDNA probes corresponding to the mouse sterol 12α-hydroxylase, mouse cholesterol 7α-hydroxylase, rat ACO and β-actin.

Reporter gene assay system - A fragment of the 5'-flanking region of the rat sterol 12α-hydroxylase gene containing an identified putative peroxisome proliferator-response element (PPRE) was amplified by PCR from a clone containing the rat gene. The fragment corresponded to bp -173 to +48 from the transcription start site (18). The forward primer used was CTG ACC AAG CTC

TGC TGT GTC and the reverse primer was CAG CCT CAG AGC AAG GTC CA. The primers contained restriction sites for *Kpn*I and *Mlu*I to facilitate cloning into the luciferase reporter vector pGL3 Basic (pGL3 12α). Sequences were verified using the ABI Prism Dye Terminator Ready-Reaction Kit (Perkin Elmer). Mutations were introduced into the PPRE by polymerase chain reaction using QuikChange™ Site Directed Mutagenesis kit (Stratagene) using the following primers 5'-cctcagagcaCTGTCCAAGGGCAtgggcgtttg-3' (pGL3 12α-M1) and 5'-cctcagagcaAGGTCCGCGGGCAtgggcgtttg-3' (pGL3 12α-M2) (mutations in the PPRE are underlined). The presence of the mutations were confirmed by sequencing. Cells were co-transfected with pcDNA3.1+ (Invitrogen) containing the mouse PPARα cDNA as described (8). The pSV-β-galactosidase vector was used to monitor transfection efficiency (Promega Corp., Madison, WI, USA).

Cell culture - HepG2 cells were routinely cultured in DMEM/F12 medium (Sigma Corp) with 10% fetal calf serum, penicillin and streptomycin (100 U/ml of each) in an atmosphere of 5% CO_2. The cells were cultured in 12 well plates with 10^5 cells per well. The cells were grown to approximately 80% confluence before transfection. Transfections were carried out using Tfx-20 reagent (Promega Corp.), in a ratio of 1:3 (w/v) plasmid to reagent. Cells were transfected with 0.4 µg pGL3 Basic vector containing the sterol 12α-hydroxylase promoter region or pGL3 Basic vector containing mutations in the sterol 12α-hydroxylase promoter, 0.4 µg of the PPARα expression vector and 0.2 µg of the pSV-β-galactosidase control vector. The cells were incubated for 48 hours before harvest with/without addition of 50 µM WY-14,643 in medium containing 10% delipidated calf serum (Sigma Corp.) as indicated. Cell lysates were assayed for luciferase activity using Luciferase Reporter Gene Assay (Promega Corp.) and for β-galactosidase activity using β-Galactosidase Enzyme Assay System (Promega Corp.). Four to eight individual experiments were carried out and luciferase activity was normalized to β-galactosidase activity.

Electrophoretic Mobility Shift Assay (EMSA) - Oligonucleotides (obtained from Cybergene AB, Novum, Huddinge) corresponding to the PPRE for the rat acyl-CoA oxidase gene (ACO) and the putative PPRE for rat sterol 12α-hydroxylase were as follows: ACO - 5'-tcgagactTGACCTTTGTCCTggtc-3'; sterol 12α-hydroxylase - 5'-cagagcaAGGTCCAAGGGCAtgggcgt-3', with the core sequence of the PPRE site indicated in capital letters. Mutated rat sterol 12α-hydroxylase probes were prepared containing various nucleotide substitutions (underlined) as follows: 5'-cctcagagcaCTGTCCAAGGGCAtgggcgtttg-3' (12α-M1), 5'-cctcagagcaAGGTCCGCGGGCAtgggcgtttg-3' (12α-M2) and 5'-tcagagcaAGGTCAAAGGGCAtgggt-3' (12α-M3). Ten pmol of each probe was

labeled with γ-^{32}P dATP (NEN Life Science Products) using T7 polynucleotide kinase (Boehringer Mannheim). *In vitro* translated mouse PPARα and RXRα were synthesized using the TNT coupled reticulocyte lysate system (Promega Corp., Madison, WI). Gel mobility shift assay incubation mixes (25 µl) contained 10 mM Tris (pH 7.8), 20 mM KCl, 2 µg bovine serum albumin (BSA), 10% glycerol, 500 ng of poly(dI-dC).poly(dI-dC) (Pharmacia Biotech) and 1 µl of *in vitro* translated PPARα and RXRα. In antibody supershift assays, 0.65 µl of RXRα antibody (a gift from Dr. Pierre Chambon) was added to the reaction mix. 50,000 cpm of labeled probe was added to each reaction and incubations were carried out for 45 minutes on ice. The complexes were resolved on a 5% polyacrylamide gel in 1X TBE and the gel was dried and exposed to X-ray film overnight.

Bile acid analysis - Gallbladders were removed from untreated wild-type and PPARα-null mice and mice treated with WY-14,643 for one week. The gallbladders were minced in saline and hydrolyzed in 1 M KOH for 4 hours at 110^{o}C. The hydrolyzed mixture was extracted twice with diethylether to remove neutral steroids. After acidification with hydrochloric acid, the bile acids were extracted with diethylether. Bile acids in the ether phase were methylated by treatment with 2,2-dimethoxypropane in acid methanol for 30 minutes at 55^{o}C. After removal of the solvent under N_2 the material was converted into trimethylsilyl ethers and analyzed by gas-liquid chromatography. A Hewlett Packard 6890 gas chromatograph equipped with a 30 m, 0.25 mm i.d. fused silica column coated with a 0.25 µm layer of cross-linked methyl silicone was used. Statistical analysis was performed using the SPSS version 9.0 and data was analyzed using one factor analysis of variance (ANOVA) followed by Tukey's test.

RESULTS

Sterol 12α-hydroxylase mRNA expression is regulated via PPARα - It has previously been shown that sterol 12α-hydroxylase mRNA is induced in rat liver by the peroxisome proliferator clofibrate (15). As the effects of peroxisome proliferators are mediated via the PPARα (6), we used the PPARα-null mouse model to examine the *in vivo* regulation of sterol 12α-hydroxylase. Feeding mice a WY-14,643 containing diet for one week resulted in a doubling of sterol 12α-hydroxylase mRNA (Fig. 1A). However this upregulation was not evident in the PPARα-null mice which were also treated with WY-14,643, demonstrating that the peroxisome proliferator-mediated induction of sterol 12α-hydroxylase is dependent on the PPARα.

The cholesterol 7α-hydroxylase is considered as the rate limiting enzyme in bile acid biosynthesis, and we therefore examined the effect of WY-14,643 treatment on its expression. No apparent change by this treatment was observed in wild-type mice. However, the expression was considerably lower in the PPARα-null mice, with an individual variation in expression. We also examined the regulation of the peroxisomal acyl-CoA oxidase (ACO), the rate-limiting enzyme in peroxisomal β-oxidation, which is widely used as a marker enzyme for PPARα-regulated gene expression. ACO expression was strongly elevated by treatment of wild-type mice with WY-14,643, and this effect was also mediated via the PPARα as the upregulation of mRNA expression was not evident in the PPARα-null mice after one week of treatment.

Several groups have shown that sterol 12α-hydroxylase mRNA is increased following starvation in rodents (15,17,19). Studies have shown that the PPARα is involved in mediating the fasting-induced upregulation of a number of genes in lipid metabolism (9-11) and we therefore used the PPARα-null mouse model to examine the mRNA level of sterol 12α-hydroxylase following fasting for 24 hours. Sterol 12α-hydroxylase mRNA expression was increased in wild-type animals by fasting but this upregulation was not present in the PPARα-null animals, demonstrating that the induction by fasting is also dependent on the PPARα (Fig 1B). A similar pattern of mRNA expression was also seen for ACO, with mRNA induced approximately 3-fold by fasting for 24 hours. This induction was also dependent on the PPARα, as the mRNA level was not elevated in the PPARα-knockout mice by this treatment. In contrast to WY-14,643 treatment, starvation increased the expression of the cholesterol 7α-hydroxylase mRNA levels in mouse in a PPARα-dependent manner.

In summary, the Northern blot data demonstrate that similar to ACO, the sterol 12α-hydroxylase gene is upregulated in the liver following treatment with WY-14,643 or starvation, and that this regulation is dependent on the PPARα.

Identification of a peroxisome proliferator-response element in the sterol 12α-hydroxylase promoter region - The PPAR/RXR heterodimer recognizes a response element with a 13 bp core sequence consisting of AGGTCA A/T AGGTCA (a direct repeat 1 - DR1). Sequence analysis of the promoter regions of the mouse (19) and rat sterol 12α-hydroxylase genes (18) identified a conserved but imperfect DR1 sequence in the promoters, located at -120 and -106 bp respectively upstream of the ATG start site (Fig. 2A). It also appears that additional nucleotides in the flanking region of the PPRE element are important for PPARα/RXR heterodimer binding. Four to seven nucleotides 5' (20,21) of the PPRE core sequence are important for the function of the PPARα/RXR

heterodimer and analysis of the rat 12α-hydroxylase PPRE flanking sequences identified 5 out of 7 conserved bases in the 5' extended half-site (Fig. 2B).

To determine whether the PPARα/RXR heterodimer can bind to the DR1 element identified in the rat promoter sequence, electrophoretic mobility shift assay was performed using *in vitro* translated PPARα and RXRα, together with ^{32}P-labeled oligonucleotides representing the PPRE of the ACO gene, the putative PPRE for sterol 12α-hydroxylase and mutated sterol 12α-hydroxylase PPREs, as shown in Fig. 2C. Neither PPARα nor RXR alone bound to the labeled probes (Fig. 3). In the presence of both PPARα and RXR, there was a strong binding to the PPRE of ACO, which was further retarded using the RXR antibody. Binding, although weak, was detectable also for the sterol 12α-hydroxylase PPRE, which was also retarded using the RXR antibody. In order to further characterize the binding of PPARα to the 12α-PPRE, several mutations were introduced in the half sites of the response element. Two of the mutations introduced, 12α-M1 and 12α-M2, abolished binding of the PPARα/RXR heterodimer to the sterol 12α-hydroxylase PPRE. In mutant 12α-M3, a single base substitution introduced, which resulted in an oligonucleotide that more closely resembled a perfect DR1 element, showed a very strong binding in the presence of both PPARα and RXR and which showed supershift with the RXR antibody. The strong binding by the mutated 12α-hydroxylase PPRE (12α-M3) was weakly competed using 50-fold or 150-fold molar excess of unlabeled wild-type sterol 12α-hydroxylase probe (15% and 50% competition respectively - data not shown). These data establish that the identified PPRE in the rat sterol 12α-hydroxylase promoter region is capable of binding the PPARα/RXR heterodimer, although this PPRE appears to be weak compared to e.g. the ACO PPRE.

The sterol 12α-hydroxylase promoter is activated via PPARα - A 222 bp fragment of the rat sterol 12α-hydroxylase promoter region containing the identified PPRE was cloned upstream of a luciferase reporter gene (pGL3 12α). This construct was then used to examine if the putative PPRE could be activated in a cell system by co-transfection with a PPARα expression vector and treatment of cells with the peroxisome proliferator WY-14,643. HepG2 cells were transiently transfected with the reporter constructs and as seen in Fig. 4A, reporter gene activity was not changed significantly by treatment with WY-14,643 in the absence of PPARα. Following co-transfection of the reporter plasmid containing the sterol 12α-hydroxylase promoter, together with an expression vector for PPARα, treatment of cells with WY-14,643 resulted in a 2.5-fold increase in promoter activity over non-treated cells. Reporter gene constructs were also prepared containing mutations in the PPRE, which were also used in transient transfection

experiments. Transfection of HepG2 cells with construct pGL3 12α-M1 resulted in an unchanged reporter activity following treatment with WY14,643 in the presence or absence of co-transfected PPARα, showing that the mutated PPRE could not be activated via PPARα (Fig 4B). Similar results were obtained for a second mutant prepared (pGL3-M2) (Fig. 4C) showing no induction in reporter gene activity by WY-14,643 in the presence of PPARα. The data demonstrate that the PPRE identified in the rat sterol 12α-hydroxylase is activated by peroxisome proliferators via the PPARα. Notably, co-transfection with the pGL3 12α promoter construct and PPARα resulted in a decreased promoter activity when compared to transfection with the promoter construct alone (data not shown). This reduced promoter activity may be explained by competition for binding with the α_1-fetoprotein transcription factor, which has recently been shown to be involved in expression of the sterol 12α-hydroxylase, as both PPARα/RXR and the α_1-fetoprotein bind to the same DNA binding site in the sterol 12α-hydroxylase promoter region (22).

The PPARα influences bile acid composition following treatment with WY-14,643 - The sterol 12α-hydroxylase activity influences the ratio of cholic acid to chenodeoxycholic acid, and increased sterol 12α-hydroxylase activity is expected to result in increased cholic acid formation. We therefore determined whether the observed increase in sterol 12α-hydroxylase expression would affect bile acid composition. Unfortunately it was not possible to measure bile acids quantitatively since total bile was extracted from the gallbladders, which showed highly individual bile content. Therefore, the composition of bile acids were determined in bile using gas chromatography (Fig. 5A). In control animals, the predominant bile acids were cholic acid and β-muricholic acid, but also present in smaller amounts were chenodeoxycholic, deoxycholic, ursodeoxycholic and ω-muricholic acids. β-Muricholic acid is a primary bile acid formed from chenodeoxycholic acid in the liver, whereas deoxycholic, ursodeoxycholic and ω-muricholic acids are secondary bile acids (23). Treatment of wild-type animals with WY-14,643 for one week resulted in an increase in the relative amount of cholic acid ($p<0.009$), with the PPARα-null animals showing no significant change in bile acid composition following treatment with WY-14,643. Unexpectedly the relative amount of chenodeoxycholic acid was increased approximately 4-fold by WY-14,643 treatment in wild-type animals ($p<0.001$), but this increase was accompanied by a concomitant decrease in β-muricholic acid ($p<0.001$), while these changes were not evident in similarly treated PPARα-null animals. Thus, treatment of mice with WY-14,643 increased the relative amount of cholic acid in a PPARα-dependent manner, which correlated to a decrease in

chenodeoxycholic acid plus β-muricholic acid as observed in the change in ratio of these bile acids (Fig. 5B), indicating a physiological importance of the observed PPARα-dependent regulation of sterol 12α-hydroxylase gene expression.

DISCUSSION

Fibrates are peroxisome proliferators which are widely used as hypolipidemic drugs and which act as ligands for the PPARα (2,24,25). Treatment of humans with bezafibrate causes a change in the composition of individual bile acids, with the proportion of cholic acid being increased relative to chenodeoxycholic acid (12), indicating that the human sterol 12α-hydroxylase gene may be regulated by the PPARα. To explore this possibility, we took advantage of the PPARα-null mouse model. The results from *in vivo* experiments clearly showed that the sterol 12α-hydroxylase gene is upregulated at the mRNA level by treatment with WY-14,643 and fasting, and that these effects are dependent on the PPARα. If such a regulation is of physiological relevance, an altered bile acid composition would be expected due to treatment with WY-14,643. We therefore collected gallbladders from wild-type and PPARα-null mice that had been treated with WY-14,643, and analyzed the bile acid composition. Treatment for one week with this compound changed the bile acid composition in wild-type mice; the relative amount of cholic acid increased from 61% to 76% and this effect was abolished in the PPARα-null mice. In addition, somewhat unexpectedly, the relative amount of chenodeoxycholic acid increased in the wild-type mice from about 5% to 19%. However, this change correlated with an observed decrease in β-muricholic acid, which is formed from chenodeoxycholic acid by hydroxylation at the 6β-position and an epimerization at the 7-hydroxy position (23). The relative amount of β-muricholic acid decreased from 26% to 4% in response to WY-14,643 treatment and both these effects were abolished in the PPARα-null mice. This demonstrates that the enzymes catalyzing the hydroxylation/epimerization of β-muricholic acid in mouse are down-regulated by WY-14,643 in a PPARα-dependent manner.

Our data show that treatment with WY-14,643 increased the relative amount of cholic acid, the product of the sterol 12α-hydroxylase pathway, when compared to the amounts of chenodeoxycholic acid and β-muricholic acid, demonstrating that regulation of the sterol 12α-hydroxylase gene affects the composition of the bile. The rate limiting enzyme in bile acid biosynthesis is considered to be cholesterol 7α-hydroxylase. The regulation of expression of this enzyme is apparently complex and shows species differences (13). Our data shows that WY-14,643 treatment did not alter the expression of cholesterol 7α-hydroxylase in

mouse, and it is therefore reasonable to assume that the treatment does not change total bile acid production to any larger extent, indicating that PPARα mediates a quantitative regulation of cholic acid synthesis in the mouse.

In addition to the now established involvement of the PPARα in regulating gene expression in response to peroxisome proliferators, such as the fibrates, this receptor was recently shown also to mediate the effects of fasting and diabetes on the expression of many enzymes involved in lipid metabolism (8-11). The findings here demonstrating that upregulation of expression of the sterol 12α-hydroxylase and cholesterol 7α-hydroxylase genes in response to fasting, and increased expression of sterol 12α-hydroxylase in response to WY-14,643 treatment is PPARα-dependent, suggests that the regulatory regions of these genes contain functional PPREs. A careful scrutiny of the available genomic sequences for the sterol 12α-hydroxylase revealed a putative response element for PPARα, conserved in the mouse and rat promoters. The sequence of the putative PPRE conformed well to the consensus sequence for PPRE with only one nucleotide in each half site being different from a perfect DR1. In addition, 5 out of the 7 nucleotides in the 5'-flanking region, which have been shown to be important for PPARα binding, conformed to the consensus (20,21). The functionality of the rat PPRE was verified in cell culture experiments, showing that a reporter gene containing the PPRE was activated by addition of WY-14,643 only in the presence of co-transfected PPARα. Mutations introduced into the PPRE resulted in lack of induction by WY-14,643 in the presence of PPARα.

Recently del-Castillo-Olivares et al (22) showed that the α_1-fetoprotein transcription factor is required for the expression of sterol 12α-hydroxylase. The α_1-fetoprotein binding site identified by this group is in fact the same site as the PPRE now identified by us. Notably, the mutations which we introduced into the PPRE resulted in a substantially lower promoter activity than the wild-type promoter (approximately 95% lower), which could be explained by a distrubance in the binding site for α_1-fetoprotein transcription factor. During our transfection experiments, a decreased promoter activity was obtained with co-transfection of PPARα and the sterol 12α-hydroxylase promoter, and this could also be explained by competition between PPARα and the α_1-fetoprotein transcription factor for the same binding site.

Direct binding of PPAR/RXR heterodimer to the PPRE was verified in EMSA experiments, but binding was weak compared to binding to the rat ACO PPRE probe. However, mutation of the PPRE (mutants 12α-M1 and 12α-M2) completely abolished binding. In contrast, one mutated oligonucleotide generated (12α-M3) containing a single base substitution in the PPARα-binding half-site, had a

profound effect on the binding; the mutated sterol 12α-hydroxylase PPRE was as efficient as the ACO PPRE and the wild-type sterol 12α-hydroxylase probe acted as a weak competitor for binding. The weak binding properties of the sterol 12α-hydroxylase PPRE is however in line with the *in vivo* data; expression of the sterol 12α-hydroxylase gene is only increased about 2-fold in response to WY-14,643 treatment of mice, while ACO is strongly induced. Notably, the PPARα-mediated increase in sterol 12α-hydroxylase expression by fasting is at least as strong as the effect of WY-14,643, suggesting that the 12α-hydroxylase gene may be under nutritional regulation *in vivo*. As fatty acids and fatty acid derivates are natural ligands of PPARα, a number of metabolic processes generating increased levels of non-esterified fatty acids, i.e. fasting and diabetes, may result in production of bile with a larger content of cholic acid (more hydrophobic), which could enhance resorption of sterols and long chain fatty acids from the intestine. On the other hand, administration of fibrates to humans causes a decreased activity of the cholesterol 7α-hydroxylase which may result in decreased bile acid formation and increased cholesterol saturation and a subsequent increased risk of gallstone formation (12,26,27).

The important roles of the nuclear receptors farnesoid X receptor (FXR) and the liver X receptor alpha (LXRα) in regulation of bile acid metabolism have recently been demonstrated (28,29). The transcription of the genes encoding cholesterol 7α-hydroxylase and ileal bile acid binding protein appears to be regulated by the interaction of these receptors with specific response elements in the 5'-flanking regions of the genes (30,31). Our data demonstrate the involvement of another nuclear receptor, the PPARα, in the regulation of bile acid metabolism. It appears that the PPARα plays a dual role in regulation of fatty acid metabolism as well as in regulation of cholesterol metabolism by modulating expression of enzymes involved in the biosynthesis of bile acids.

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FIGURE LEGENDS:

Fig. 1: Effects of treatment with WY-14,643 and fasting on mRNA expression in mouse liver. (A) PPARα-null mice (-/-) or age matched wild-type mice (+/+) were treated with a diet containing 0.1% WY-14,643 for one week, while control animals had access to normal chow diet ad libitum. Mice were sacrificed and total RNA was isolated from liver. Northern blot analysis was carried out on 20 μg RNA using α-^{32}P-labeled cDNA probes for sterol 12α-hydroxylase, cholesterol 7α-hydroxylase, acyl-CoA oxidase (ACO) and β-actin as described in Experimental procedures. **(B)** Groups of six PPARα-null mice (-/-) or age matched wild-type mice (+/+) were fasted for 24 hours, while control animals had access to normal chow diet ad libitum. Mice were sacrificed and total RNA was isolated from liver. Northern blot analysis was carried out on 20 μg RNA using α-^{32}P-labeled cDNA probes for sterol 12α-hydroxylase, cholesterol 7α-hydroxylase, ACO and β-actin as described in Experimental procedures. A representative blot with two samples per group is shown.

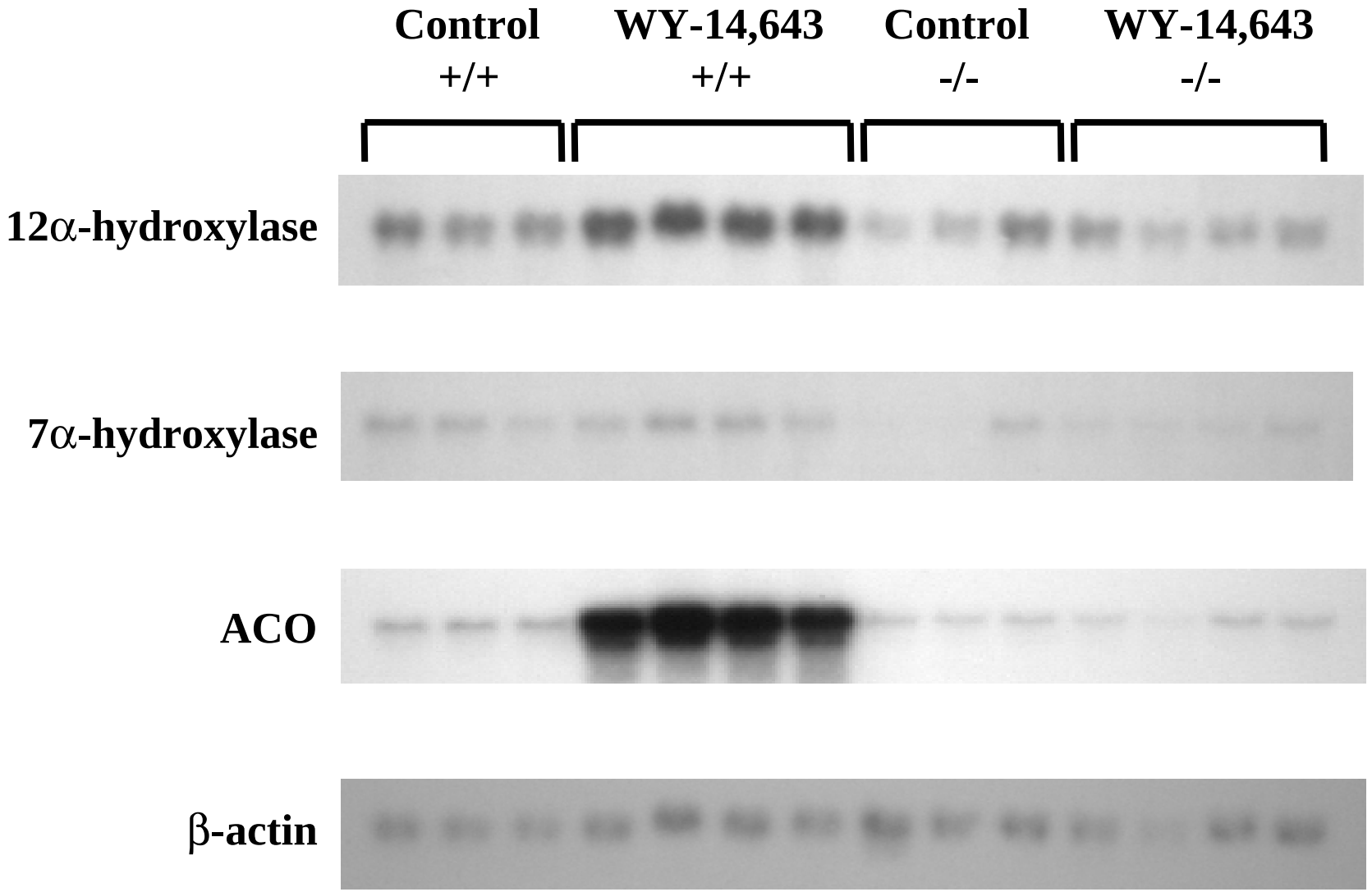
Fig. 2: The peroxisome proliferator-response element in sterol 12α-hydroxylase is conserved in rat and mouse promoter regions. (A) Alignment of 200 bp of the promoter regions of rat and mouse sterol 12α-hydroxylase gene. The identified peroxisome proliferator-response element (PPRE) is boxed and the extended half-site sequences in the 5' flanking region are underlined. The identified transcription start sites previously determined (18,19) are indicated in the rat and mouse sequences by filled triangles. The ATG start site is boxed with double lines. **(B)** The core consensus sequence for the PPRE (DR1) is indicated together with the 5'flanking region. The nucleotide sequence for the identified PPREs in rat sterol 12α-hydroxylase and rat ACO are shown. **(C)** The nucleotide sequence for the rat sterol 12α hydroxylase is shown. The mutated PPREs 12α-M1, 12α-M2 and 12α-M3 are shown with the mutated bases underlined.

Fig. 3: Electrophoretic mobility shift assay of the ACO and sterol 12α-hydroxylase peroxisome proliferator-response elements. Electrophoretic mobility shift assay (EMSA) was carried out using *in vitro* translated PPARα and RXRα. Labeled probes for the PPREs for rat acyl-CoA oxidase (ACO), rat sterol 12α-hydroxylase (12α) and three mutated PPRE probes for sterol 12α-hydroxylase (12α-M1, 12α-M2 and 12α-M3) were used. Supershift experiments were carried out using an RXR antibody (Ab). The PPARα/RXRα heterodimer is indicated by the lower arrow, while the supershift bands are indicated by the upper arrow.

Fig. 4: Activation of the sterol 12α-hydroxylase peroxisome proliferator-response element (PPRE) *in vitro*. HepG2 cells were transfected with a 222 bp fragment of the rat sterol 12α-hydroxylase 5'-flanking region containing the putative PPRE fused upstream of a luciferase reporter gene: **(A)** pGL3 12α **(B)** pGL3 12α-M1 **(C)** pGL3 12α-M2 were used for transfections. Transfected cells were treated with 50 μM WY-14,643 in the presence or absence of an expression vector for PPARα. The luciferase activity was normalized to β-galactosidase activity and the normalized activity in the absence of treatment with WY-14,643 was set to 1. Data shown are mean ± S.E.M of 4 (B and C) to 8 (A) different experiments.

Fig. 5: PPARα influences bile acid composition following treatment with WY-14,643. Wild-type mice (+/+) or PPARα-null mice (-/-) were treated with a diet containing 0.1% WY-14,643 (WY) for one week, while control animals had free access to normal chow diet. The mice were sacrificed and the gallbladders were excised. Bile acid composition of bile was determined using gas-chromatography. **(A)** Relative distribution (%) of bile acids. CA, cholic acid: CDCA, chenodeoxycholic acid: β-MCA, β-muricholic acid: DCA, deoxycholic acid: UDCA, ursodeoxycholic acid: ω-MCA, ω-muricholic acid. **(B)** Ratio of primary bile acids, cholic acid versus chenodeoxycholic + β-muricholic acid. * $p < 0.011$.

A


Control
+/+
WY-14,643
+/+
Control
-/-
WY-14,643
-/-
12α-hydroxylase
7α-hydroxylase
ACO
β-actin

B

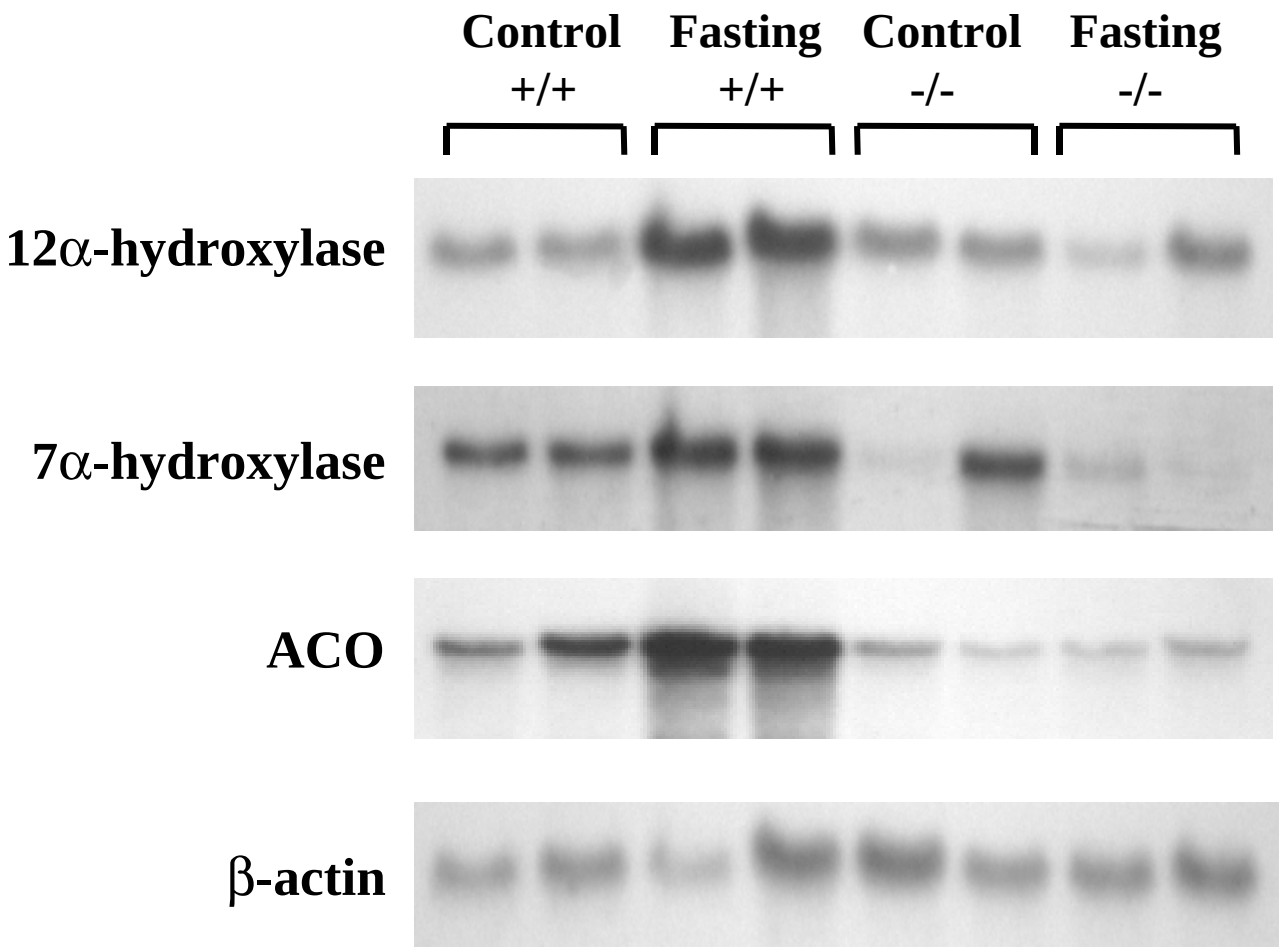
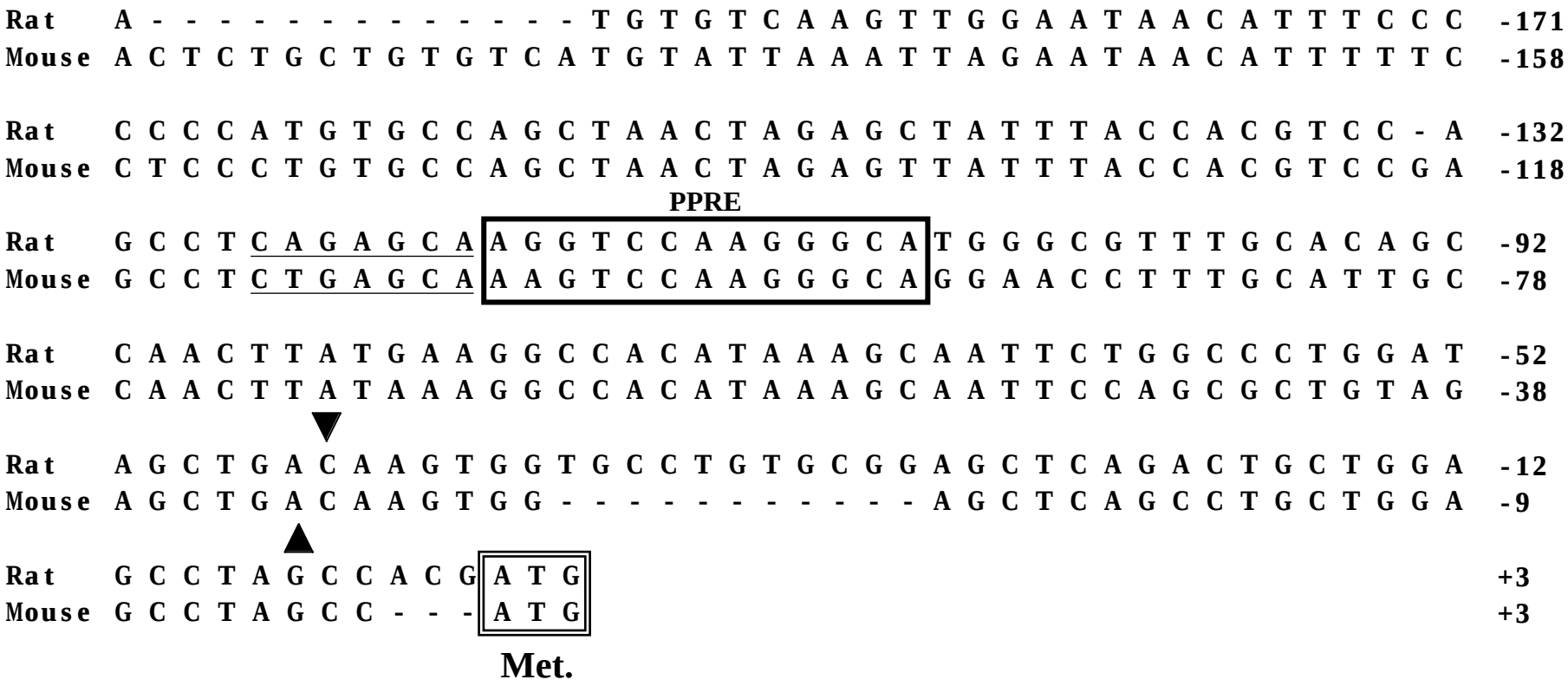

Control
+/+
Fasting
+/+
Control
-/-
Fasting
-/-
12α-hydroxylase
7α-hydroxylase
ACO
β-actin

Fig. 2a

A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Mouse A C T C T G C T G T G T C A T G T A T T A A A T T A G A A T A A C A T T T T T C -158
Rat C C C C A T G T G C C A G C T A A C T A G A G C T A T T T A C C A C G T C C - A -132
Mouse C T C C C T G T G C C A G C T A A C T A G A G T T A T T T A C C A C G T C C G A -118
PPRE
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Mouse G C C T C T G A G C A A A G T C C A A G G G C A G G A A C C T T T G C A T T G C -78
Rat C A A C T T A T G A A G G C C A C A T A A A G C A A T T C T G G C C C T G G A T -52
Mouse C A A C T T A T A A A G G C C A C A T A A A G C A A T T C C A G C G C T G T A G -38
Rat A G C T G A C A A G T G G T G C C T G T G C G G A G C T C A G A C T G C T G G A -12
Mouse A G C T G A C A A G T G G - - - - - - - - - - - A G C T C A G C C T G C T G G A -9
Rat G C C T A G C C A C G A T G +3
Mouse G C C T A G C C - - - A T G +3
Met.

B

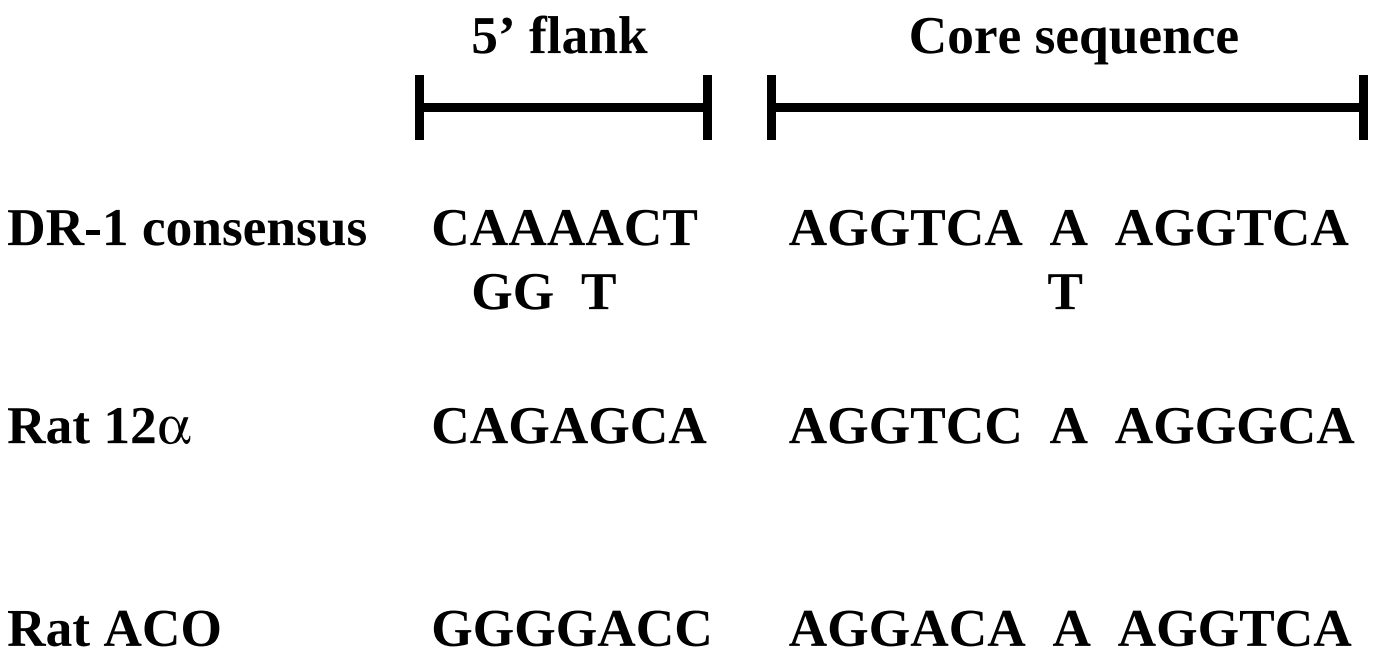

5’ flank
Core sequence
DR-1 consensus
CAAAACT
GG T
AGGTCA A AGGTCA
T
Rat 12α
CAGAGCA
AGGTCC A AGGGCA
Rat ACO
GGGGACC
AGGACA A AGGTCA

Fig. 2c

C

Rat 12α PPRE wild-type	**AGGTCC A AGGGCA**
Rat 12α-M1	**CTGTCC A AGGGCA**
Rat 12α-M2	**AGGTCC G CGGGCA**
Rat 12α -M3	**AGGTCA A AGGGCA**

Fig. 3

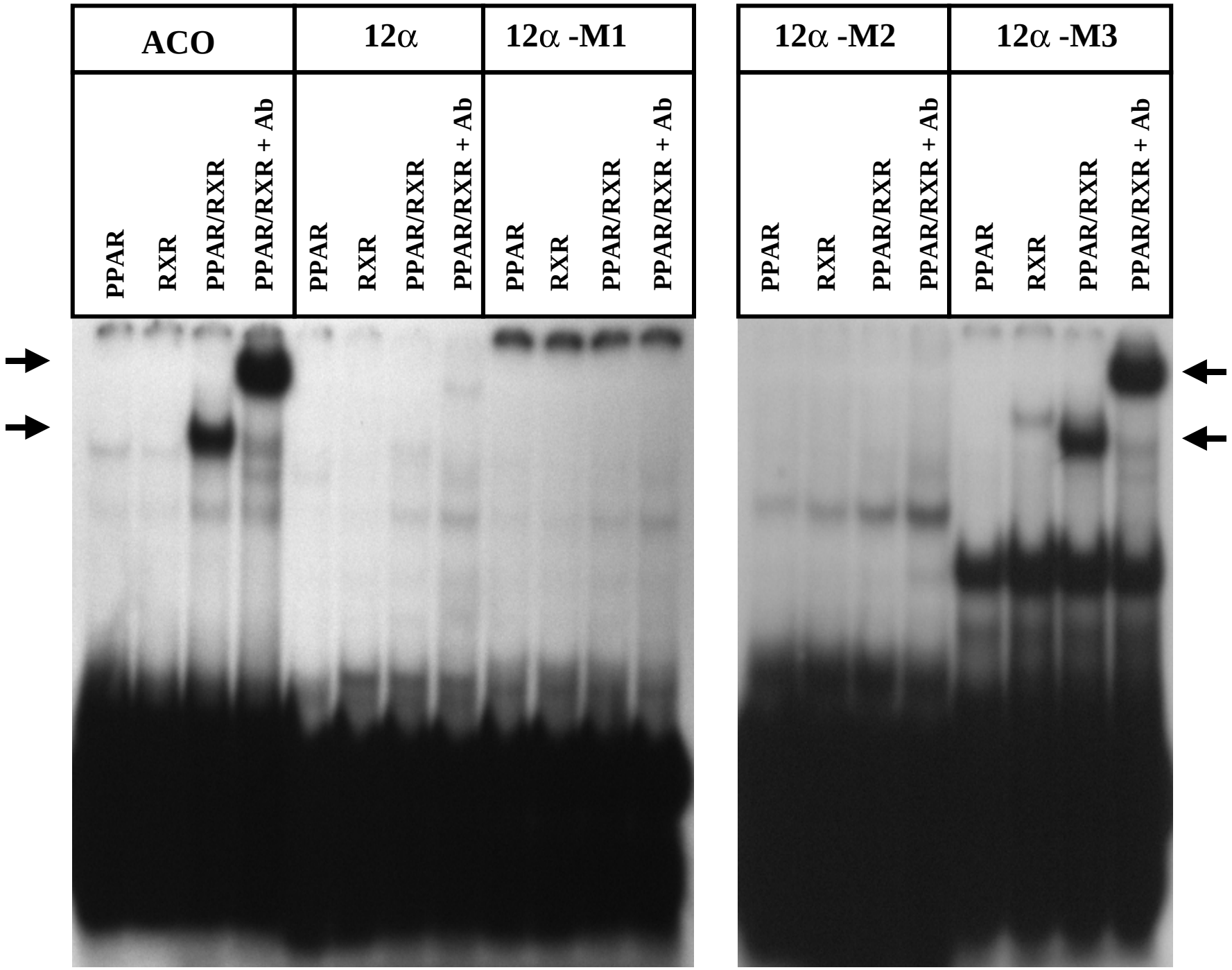


Fig. 4A

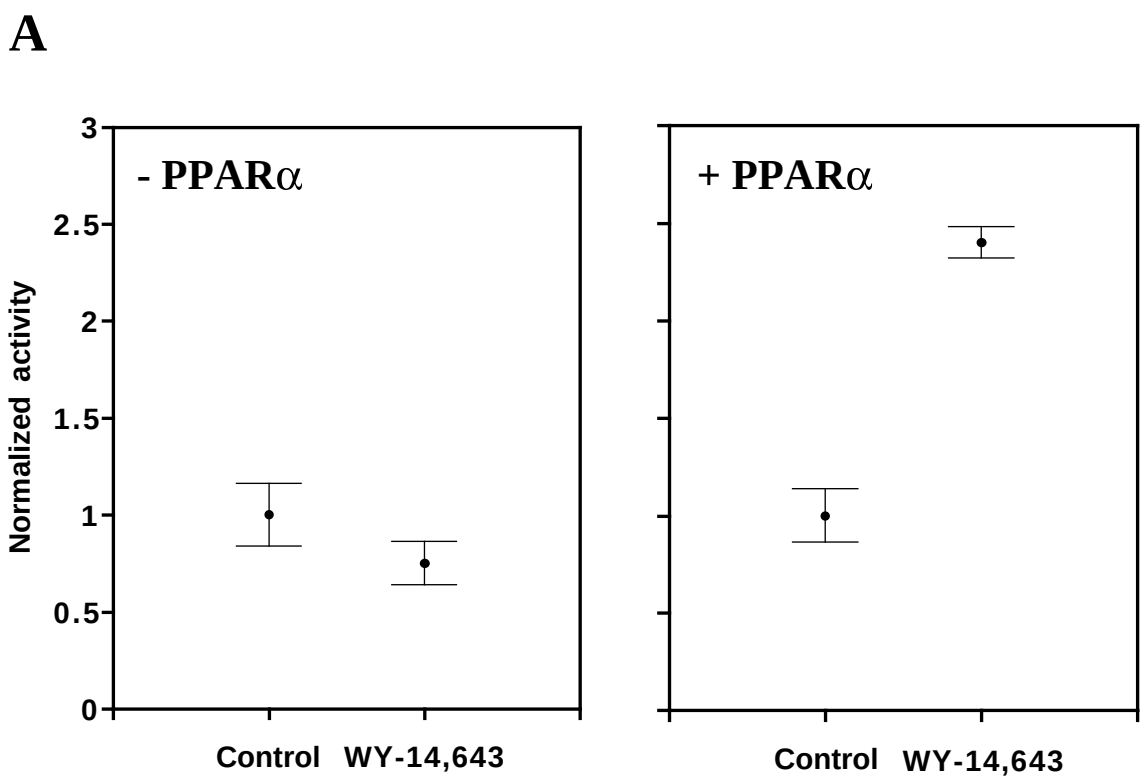

A
- PPARα
+ PPARα
Normalized activity
3
2.5
2
1.5
1
0.5
0
Control
WY-14,643
Control
WY-14,643

Fig. 4b

B

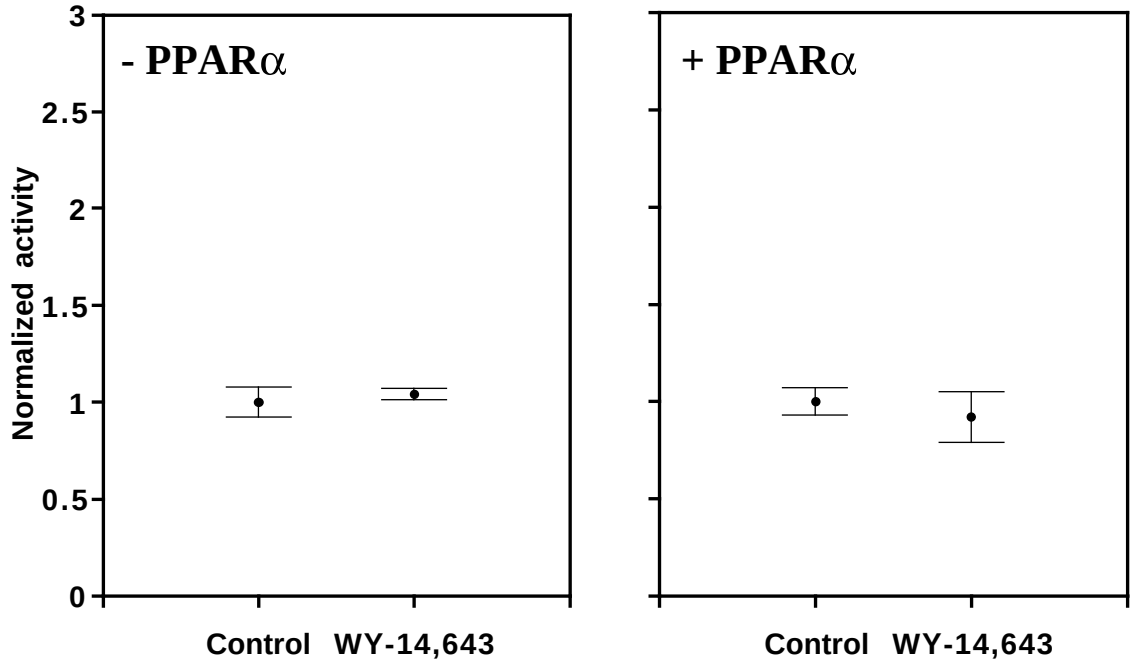
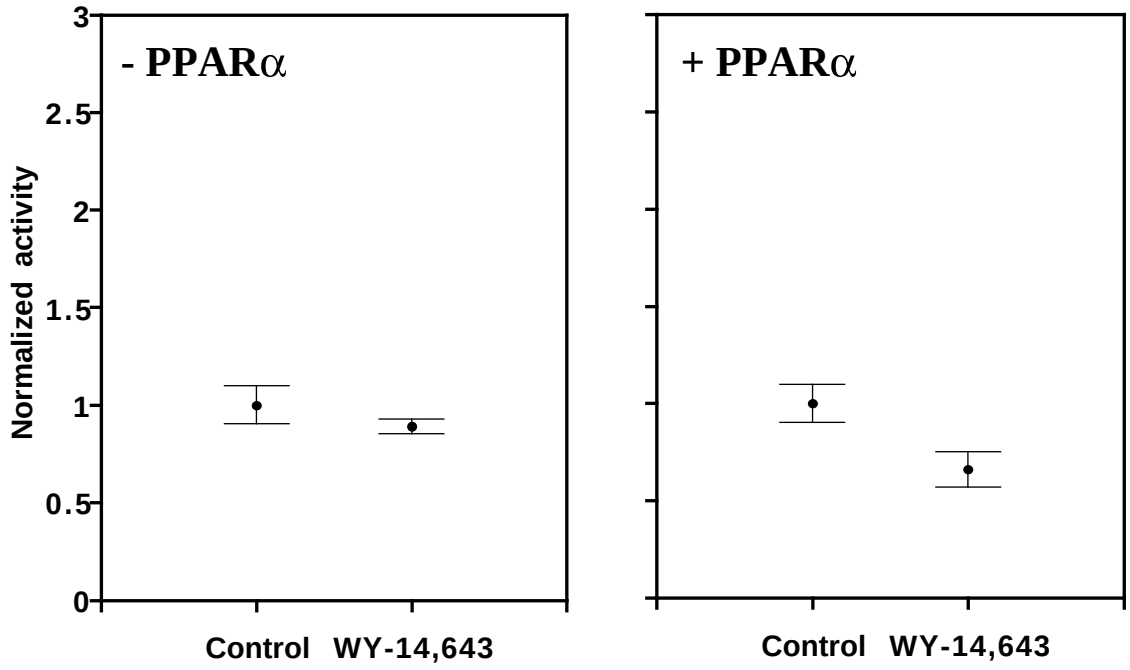

- PPARα
+ PPARα
Normalized activity
3
2.5
2
1.5
1
0.5
0
Control
WY-14,643
Control
WY-14,643

Fig. 4c

C


- PPARα
+ PPARα
Normalized activity
3
2.5
2
1.5
1
0.5
0
Control
WY-14,643
Control
WY-14,643

A

Fig. 5a

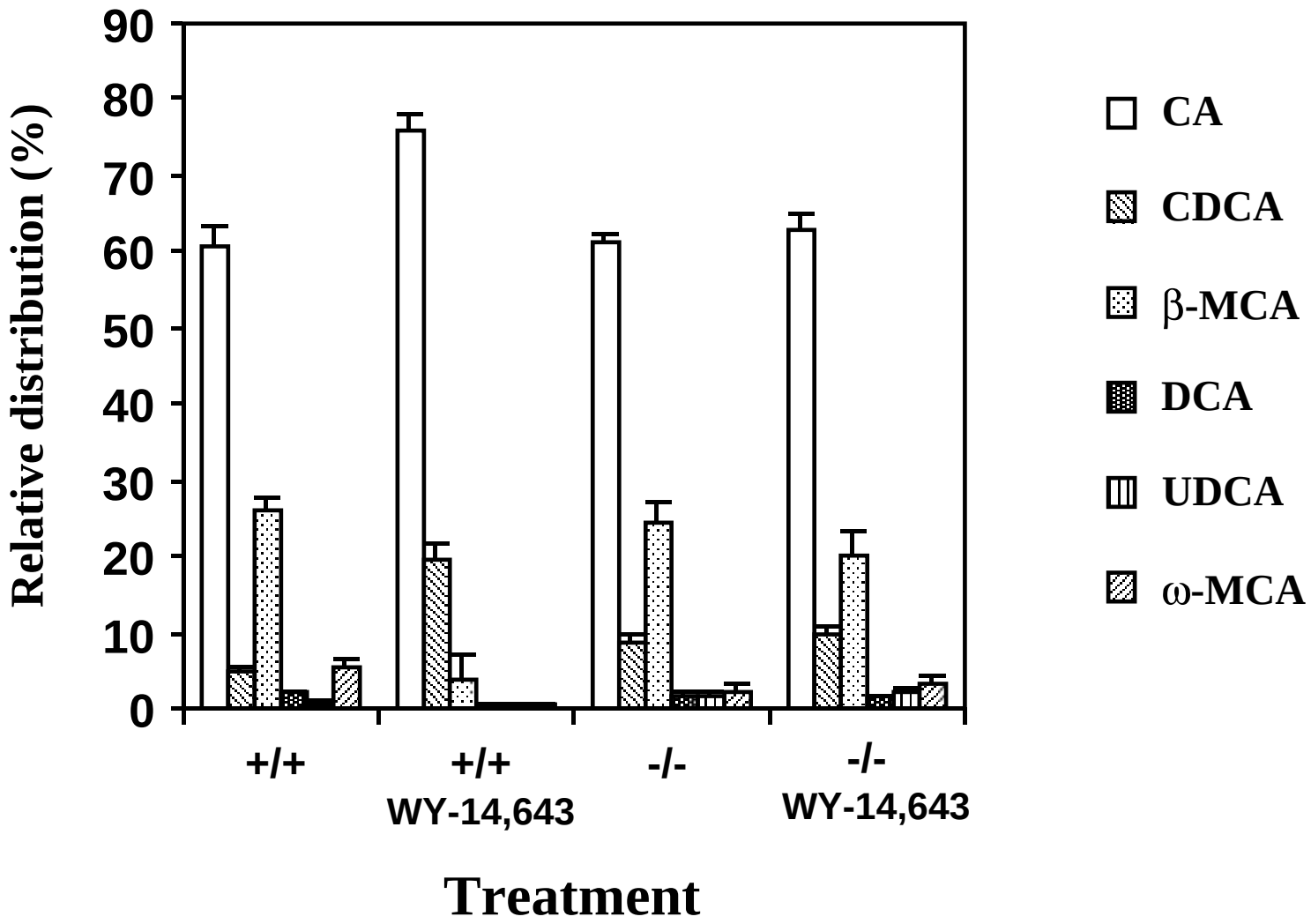

Relative distribution (%)
90
80
70
60
50
40
30
20
10
0
+/+
+/+
WY-14,643
-/-
-/-
WY-14,643
Treatment
CA
CDCA
β-MCA
DCA
UDCA
ω-MCA

Fig. 5b

B