

Cytochrome P450 Genes Are Differentially Expressed in Female and Male Hepatocyte Retinoid X Receptor α -Deficient Mice

YAN CAI, TIANE DAI, YAN AO, TAMIKO KONISHI, KUANG-HSIANG CHUANG, YANHE LUE, CHAWNSHANG CHANG, AND YU-JUI YVONNE WAN

Department of Pathology (K.-H.C., C.C.), University of Rochester, Rochester, New York 14642; and Departments of Pathology (Y.C., T.D., Y.A., T.K., Y.-J.Y.W.) and Medicine (Y.L.), Harbor-University of California, Los Angeles Medical Center, Torrance, California 90509

To study the functional role of retinoid X receptor α (RXR α) in hepatocytes, hepatocyte RXR α -deficient mice have been established. Characterization has been performed on male mice. In this paper, we show that the expression of CYP450 genes is differentially expressed in male and female hepatocyte RXR α -deficient mice; male mice have reduced expression of cytochrome P450 (CYP) CYP4A, CYP3A, and CYP2B mRNAs, but females do not exhibit such phenotypes. To examine the hormonal effects on this sexual dimorphic phenotype, male and female mice were subjected to 17 β -estradiol and 5 α -dihydrotestosterone (DHT) treatment, respectively, and then the expression of the CYP450 genes was studied. Estradiol had no effect on protecting the hepatocyte RXR α -deficient mice from

reduced expression of the CYP450 genes. In contrast, DHT induced hepatocyte RXR α -deficient female mice, but not wild-type female mice, to have the reduced expression of CYP450 mRNAs. In addition, castration prevented the mutant male mice from exhibiting reduced expression of CYP450 mRNAs. Wild-type and mutant mouse livers from both genders express androgen receptors (ARs). By transient transfection, DHT-AR could inhibit RXR α -mediated transcription. Furthermore, by transfection and coimmunoprecipitation, RXR can interact with AR *in vivo*. These data suggest that testosterone has a negative impact on retinoid signaling when the level of RXR α is low, which may in turn reduce the expression of the CYP450 genes. (*Endocrinology* 144: 2311–2318, 2003)

IT HAS BEEN WELL established that retinoic acid (RA) mediates its effects through RA receptor and retinoid X receptors (RXR). RXRs and their heterodimeric partners are regulators of many CYP450 genes (for reviews see Refs. 1–3). In the liver, all three RXRs (α , β , and γ) are expressed, although RXR α has the highest expression level in the liver. Furthermore, liver expresses the highest level of RXR α among all the organs (4). Previously, we have shown that RXR α is involved in regulation of liver gene expression and hepatocyte differentiation and proliferation (5, 6). To further analyze the functional role of RXR α in the liver, we have used cre-mediated recombination to disrupt the RXR α gene specifically in mouse hepatocytes (7). Although such mice are viable, molecular, biochemical, and morphological parameters indicate that many metabolic pathways in the liver mediated by peroxisome proliferator-activated receptor, constitutive androstane receptor, pregnane X receptor (PXR), liver X receptor, and farnesoid X receptor are compromised in the mutant male mice (7–10).

Sexual differences in retinoid signaling has been identified in different experimental models. Serum retinol concentration is higher in males than females aged 20–59 yr based on analyzing data from the third National Health and Nutrition Examination Survey (11). Males are usually more sensitive to vitamin A deficiency. When rats are maintained on a vitamin

A-deficient diet, male rats develop severe manifestations of hypovitaminosis A earlier than females. Male rats also presented more extensive squamous metaplasia of the trachea than females (12). In a group of 535 vitamin A-deficient cattle, blindness is only found in steers, not in heifers (13). In addition, the metabolic rate of RA was different in male *vs.* female. When liver microsomes of male and female rats of different strains are used to investigate the *in vitro* metabolism of RA, the amounts of 4-oxo metabolites derived from all-*trans*-RA, 13-*cis*-RA, and 9-*cis*-RA are higher in male than female liver microsomes (14). Furthermore, when pregnant rats were treated with ethanol or ethanol plus vitamin A, retinyl palmitate levels in female fetuses of the group treated with ethanol plus vitamin A were significantly higher than those of the group administered vitamin A alone, and no significant differences in the level of retinyl palmitate in male fetuses were observed between these two treated groups (15). These reports indicate that there are differences in retinol level, sensitivity to retinol deficiency, and retinoids metabolic rate between males and females.

To determine if there is any sexual dimorphic difference in receptor-mediated RA signaling, we have studied female hepatocyte RXR α -deficient mice and compared the phenotype with male mice. Surprisingly, female and male mutant mice exhibit very different phenotypes in CYP450 gene expression. Male mice have reduced expression of CYP450 mRNAs, but females do not exhibit such phenotypes. However, testosterone can induce female hepatocyte RXR α -deficient mice to exhibit a full mutant phenotype. Furthermore, castration prevents male mutant mice from expressing the

Abbreviations: AR, Androgen receptor; CYP, cytochrome P450; E2, 17 β -estradiol; FBS, fetal bovine serum; DHT, 5 α -dihydrotestosterone; PXR, pregnane X receptor; RA, retinoic acid; RXR α , retinoid X receptor α ; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline with 0.5% Tween 20.

RXR α -deficient phenotype, *i.e.* reduced expression of the CYP450 genes. The data demonstrated a gender difference in RXR-mediated pathways and CYP450 gene expression. It also implicates a negative effect of testosterone on regulating CYP450 gene expression when retinoid signaling is low.

Materials and Methods

Mice

Male mice carrying the RXR α mutation in hepatocytes have been described elsewhere (7). The animals used in all the experiments were age-matched (3.5 months old) male and female mice. They were housed in groups of two or three in plastic microisolator cages at 22 C with a 12-h light, 12-h dark cycle and had free access to food and water. Mice were killed by anesthesia with pentobarbital (60 mg/kg, ip). The liver was removed immediately, weighed, frozen in liquid nitrogen, and processed for RNA extraction. For hormone treatment studies, a 3-wk release 17 β -estradiol (E2; 0.5 mg) and 5 α -dihydrotestosterone (DHT; 5 mg) pellet (Innovative Research of American, Sarasota, FL) were inserted into an sc pocket (posterior neck region) of 5-wk-old male and female mice, respectively, under anesthesia induced by ketamine/xy-lazine. Two weeks after implantation of the pellet, the mice were killed for studying the expression of nuclear receptor target genes in the liver. The dose used for E2 produces serum E2 levels physiologically equivalent to pregnant levels (~700–1500 ng/ml), and these data are consistent with others' findings (Ref. 16 and data from Innovative Research of America). The dose used for DHT also produces serum DHT levels within the physiological range (6–9 ng/ml, data from Innovative Research of America). All the animal experiments were conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals.

Northern blot hybridization

Liver RNA was extracted by the guanidinium isothiocyanate method (17). Twenty micrograms of total RNA per lane were resolved by electrophoresis on 1.2% agarose gels containing 2.2 M formaldehyde and then transferred to nylon membranes by capillary blotting. CYP4A1, CYP2E1 (provided by Dr. F. Gonzalez), CYP2B10 (M. Negishi, NIEHS, Research Triangle Park, NC), and CYP3A11 cDNA fragments were labeled by random priming and hybridized to membranes in 7% (wt/vol) sodium dodecyl sulfate (SDS), 0.5 M sodium phosphate (pH 6.5), 1 mM EDTA, and 1 mg/ml BSA at 68 C overnight. The membranes were washed twice in 1% SDS, 50 mM NaCl, and 1 mM EDTA at 68 C for 15 min each and autoradiographed using intensifying screens. At least five animals from each group were studied for each gene. The amount of mRNA expressed was quantitated by densitometry and then normalized with the level of β -actin mRNA to obtain means and sds.

Semiquantitative RT-PCR analysis

cDNA was synthesized by reverse transcription from 2.0 μ g of total RNA. Mouse β -actin, RXR β , RXR γ , and androgen receptor (AR) cDNAs were amplified by PCR using specific oligonucleotide primers. Sense GAGCTATGAGCTGCCTGACG (636–655) and antisense AGCACTT-GCGTCCACGATG (1045–1026) primers were used for amplification of β -actin. Sense CAACTCCACAGTGTCTGCTCC (225–244) and antisense CCGTTGACGCTCCTCTGAA (582–563) primers were used for amplification of RXR β . Sense CGTGCCAGTACTGTCTGCTAC (603–624) and antisense TGGCATAAACCTTCTCTCGAAGAGT (1233–1208) primers were used for amplification of RXR γ . Sense CAGCATACCA-GAATCGCGACTAC (1081–1103) and antisense TCTGGGGTG-GAAAGTAATAGTCGA (1665–1641) primers were used for amplification of AR.

To ensure the amplification was within the linear range, the correlation between the intensity of PCR-amplified products and the number of PCR cycles was studied first. For RXR β , PCR conditions used were 28 cycles of 94 C for 45 sec, 64 C for 45 sec, and 72 C for 1 min. For RXR γ , PCR conditions were 30 cycles of 94 C for 45 sec, 62 C for 45 sec, and 72 C for 1 min. For AR, PCR conditions were 30 cycles of 94 C for 45 sec, 56 C for 45 sec, and 72 C for 1 min. All PCRs were completed with a single extension cycle of 10 min at 72 C. The products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and

visualized by UV illumination. Intensity was determined using image analysis software (Quantity one; Bio-Rad Laboratories, Inc., Hercules, CA). The level of β -actin mRNA was studied as an internal control. β -Actin was amplified with 30 cycles of 94 C for 45 sec, 56 C for 45 sec, and 72 C for 1 min, followed by one cycle of 10 min at 72 C. The expression of each mRNA was normalized to β -actin mRNA level. Statistical analysis was performed with Student's *t* test, and *P* < 0.05 was considered statistically significant. Relative fold differences are shown and all the experiments were reproducible.

Cell culture and transfection

Cell line CV-1 was purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in MEM (Sigma, St. Louis, MO) supplemented with 10% charcoal-stripped fetal calf serum. Cells were seeded at 2×10^5 per well in six-well plates in 2 ml of complete medium. Sixteen hours after plating, cells were transfected using lipofectamine reagent (Life Technologies, Inc., Grand Island, NY) using the protocol provided by the manufacturer. Each well was transfected with 2 μ g of CRBP-II-luciferase reporter plasmid (18) with or without RXR α (pRS-hRXR α ; Ref. 19) and AR (pSG5-AR; Ref. 20) expression plasmid and 10 ng of pRL-TK (Promega Corp., Madison, WI), which expressed Renilla luciferase and served as an internal control. Then the transfected cells were treated with or without 9-*cis*-RA and dihydrotestosterone (DHT; Sigma) for 48 h and harvested for luciferase assay according to manufacture's protocol (Promega Corp.).

Transfection and coimmunoprecipitation

pIRES-flag-AR was constructed by inserting flag-tagged AR cDNA into pIRESneo vector (CLONTECH Laboratories, Inc., Palo Alto, CA). pCDNA3-RXR β was from Renata Polakowska (University of Lille II, Lille, France). COS-1 cells were maintained in DMEM containing penicillin (25 U/ml), streptomycin (25 μ g/ml), and 10% fetal bovine serum (FBS). Briefly, 10^6 cells were plated on 100-mm dishes 24 h before transfection. Then, cells were cotransfected with 10 μ g of pCDNA3-RXR β and/or 10 μ g of pIRES-flag-AR. Total plasmid amount was adjusted with pIRES-flag and pCDNA3 parent vector to 20 μ g for each 100-mm transfection by calcium phosphate precipitation method.

Cells plated on 100-mm dishes were incubated in DMEM 10% charcoal-depleted FBS for 24 h and then treated with or without 10 nM DHT and/or 1 μ M 9cRA for another 16 h. After washing with ice-cold $1 \times$ PBS, cells were solubilized in 1 ml RIPA buffer (10 mM NaHPO $_4$ /pH 7.0, 150 mM NaCl, 2 mM EDTA, 0.5% (wt/vol) Nonidet P-40, 0.1% (wt/vol) SDS, 1% (wt/vol) sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride). Insoluble material was removed by centrifugation (16,000 \times *g*, 10 min at 4 C). Polyclonal anti-RXR β antibodies (200 ng/ml, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added to the cell lysates and incubated for 2 h at 4 C. Immunoprecipitates were collected with protein A/G-Sepharose beads (Santa Cruz Biotechnology, Inc.), washed four times in RIPA buffer, and then analyzed by Western blotting with mouse anti-flag and anti-RXR β antibodies.

Western blot

Immunoprecipitated proteins were separated by SDS-PAGE through 10% gels, electroblotted onto polyvinylidene difluoride membrane, and Western blotted with the indicated antibodies. Blots were blocked at 4 C with Tris-buffered saline with 0.5% Tween 20 (TBST) plus 5% dry nonfat milk, and antibodies were diluted in this buffer as suggested by the manufacturers. Blots were incubated in primary antibodies for 2 h at room temperature or overnight at 4 C, washed three times in TBST. Blots were then incubated with the appropriate alkaline phosphatase-conjugated anti-rabbit IgG secondary antibodies (Bio-Rad Laboratories, Inc.) diluted in TBST plus 5% milk for 1 h at room temperature. Immunoblots were visualized using an alkaline phosphatase conjugate kit (Bio-Rad Laboratories, Inc.).

Results

The expression of CYP2B, CYP3A, and CYP4A mRNA is not reduced in female hepatocyte RXR α -deficient mice

To characterize the phenotype of female hepatocyte RXR α -deficient mice, the expression of nuclear receptor target

genes including CYP2B, CYP3A, and CYP4A was examined by Northern blot hybridization. The CYP2B and CYP3A genes can be regulated by the constitutive androstane receptor/RXR- and PXR/RXR-mediated pathways (21). The CYP4A gene is a peroxisome proliferator-activated receptor/RXR target gene (Ref. 8 and references therein). Five to six mice per group were studied and the representative Northern results showed that the expression level of CYP2B, CYP3A, and CYP4A mRNA, but not CYP2E1, was significantly reduced in the male hepatocyte RXR α -deficient mouse liver compared with the wild-type mouse liver from the same sex (Figs. 1–3). In contrast, in female mice, the level of these CYP450 mRNAs remained unchanged in the hepatocyte RXR α -deficient mouse liver compared with the wild-type mouse liver.

DHT treatment inhibits the expression of CYP2B, CYP3A, and CYP4A mRNA in female hepatocyte RXR α -deficient mice, but not in female wild-type mice

To study the effect of hormones on the phenotype of hepatocyte RXR α -deficient mice, male and female mice were subjected to E2 and DHT treatments, respectively. E2 induced the expression of CYP2B mRNA in the wild-type male mouse liver but CYP2B mRNA remained undetectable after E2 treatment of hepatocyte RXR α -deficient mice (Fig. 1). In contrast, DHT had no effect on regulating CYP2B mRNA in wild-type female mice but had an inhibitory effect on CYP2B mRNA in female hepatocyte RXR α -deficient mice. After DHT treatment, CYP2B mRNA became undetectable in female hepatocyte RXR α -deficient mice, which was similar to

FIG. 1. The expression of CYP2B and CYP2E1 mRNA in the liver of wild-type and hepatocyte RXR α -deficient mice treated with hormones. A 3-wk release E2 (0.5 mg) and DHT (5 mg) pellet were inserted into an sc pocket (posterior neck region) of 5-wk-old male and female mice, respectively, for 2 wk. Total mouse liver RNA (20 μ g) was electrophoresed and hybridized with the CYP2B and CYP2E1 cDNA probes. Representative Northern blots demonstrate the expression of CYP2B and CYP2E1 mRNA. The relative fold changes of the message levels after normalization to β -actin mRNA level are indicated below each panel. Because CYP2B mRNA is undetectable in some liver samples, the background level is set as a value of one.

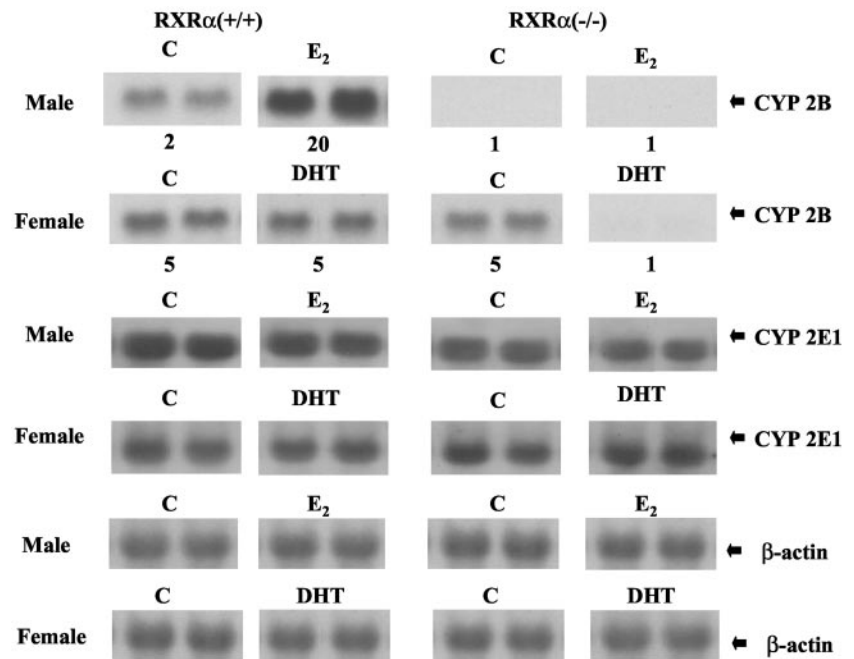
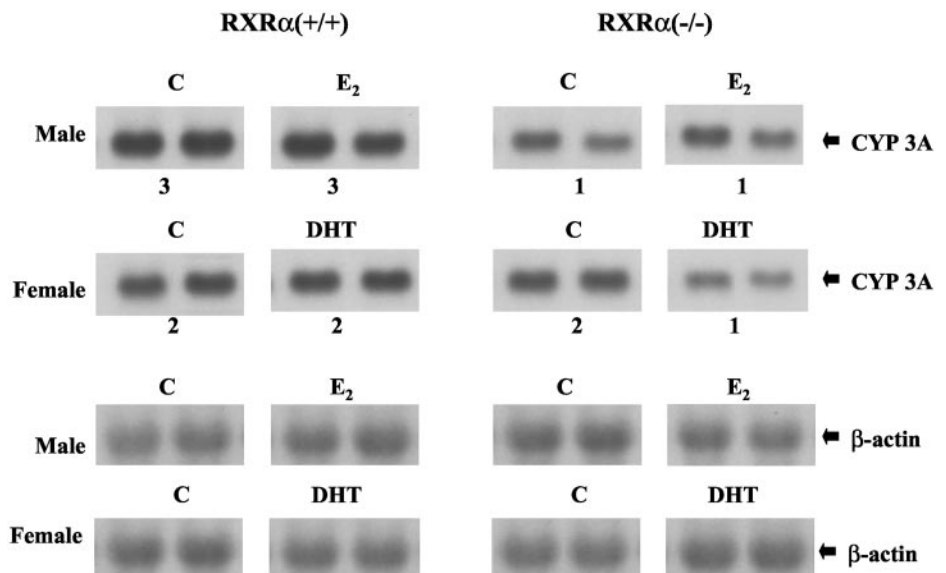


FIG. 2. The expression of CYP3A mRNA in the liver of wild-type and hepatocyte RXR α -deficient mice treated with hormones. Mice were treated as described in Fig. 1 legend. Representative Northern blots demonstrate the expression of CYP3A mRNA. The relative fold changes of the message levels after normalization to β -actin level are indicated below each panel. The lowest level within a sex is set as a value of one.



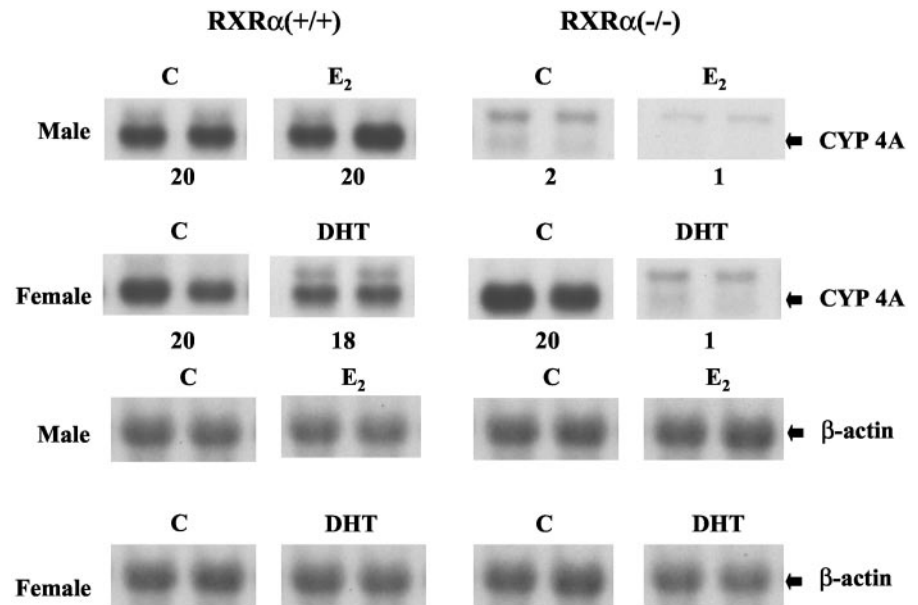


FIG. 3. The expression of CYP4A mRNA in the liver of wild-type and hepatocyte RXR α -deficient mice treated with hormones. Mice were treated as described in Fig. 1 legend. Representative Northern blots demonstrate the expression of CYP4A mRNA. The relative fold changes of the message levels after normalization to β -actin mRNA level are indicated below each panel. The lowest level within a sex is set as a value of one.

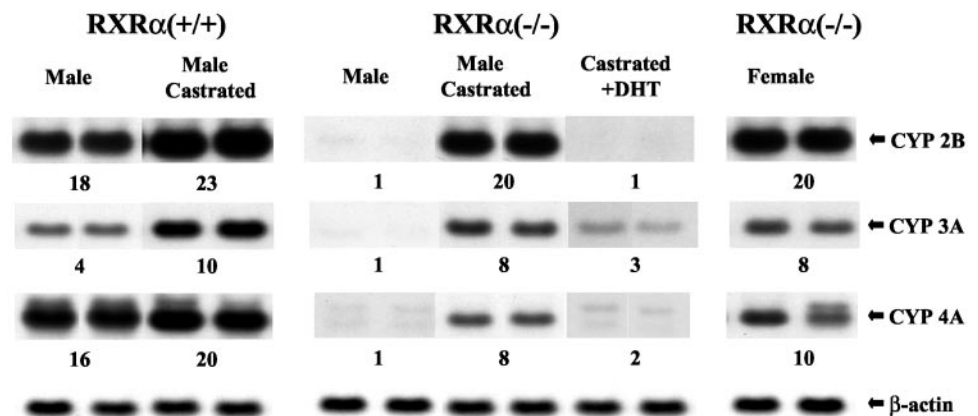


FIG. 4. Castration alters the phenotype of male hepatocyte RXR α -deficient mice. Castration was performed on 6-wk-old wild-type and hepatocyte RXR α -deficient male mice. One group of castrated hepatocyte RXR α -deficient mice received DHT (5 mg) pellet for 2 wk. Mice were killed 2 wk after castration to examine the expression of CYP2B, CYP3A, and CYP4A mRNA in the liver. Representative Northern blots demonstrate the expression of these messages. β -Actin cDNA was used as a control probe.

the phenotypic result in male hepatocyte RXR α -deficient mice (Fig. 1). Neither RXR α deficiency nor hormone treatment altered the expression of the CYP2E1 gene, which is not known, can be regulated by nuclear receptors that dimerize with RXR α (Fig. 1).

In male mice, E₂ had no effect on the CYP3A gene in wild-type and hepatocyte RXR α -deficient mice (Fig. 2). Also, in female mice, DHT did not regulate the expression of CYP3A mRNA in wild-type mice. However, DHT had an inhibitory effect on the expression of CYP3A mRNA in female hepatocyte RXR α -deficient mice (2-fold reduction, $P < 0.05$; Fig. 2). After DHT treatment, the level of CYP3A mRNA in female hepatocyte RXR α -deficient mice was similar to that in untreated male hepatocyte RXR α -deficient mice (Fig. 2).

The expression of CYP4A mRNA in wild-type male mice was not changed after E₂ treatment (Fig. 3). E₂ weakly reduced the level of CYP4A mRNA in the liver of hepatocyte RXR α -deficient male mice (Fig. 3). DHT also had a very weak effect on reducing the level of CYP4A mRNA in female wild-type mice (Fig. 3). However, DHT had a dramatic effect on inhibiting the expression of the CYP4A gene in hepatocyte RXR α -deficient female mice (20-fold reduction; Fig. 3). The

size of CYP4A transcript altered and the major transcript became undetectable when RXR α was deficient in the male mice and when the mutant female mice were treated with DHT. The possibility of alternative splicing or usage of a different promoter of the CYP4A gene remains to be investigated. Similar to CYP2B and CYP3A mRNA, the level of CYP4A mRNA in the liver was similar in DHT-treated female and untreated male hepatocyte RXR α -deficient mice. Therefore, our data indicated that E₂ could not prevent the male mutant mice from having the RXR α -deficient phenotype, whereas DHT helped the female hepatocyte RXR α -deficient mice exhibit the male mutant phenotype.

Castration prevents male hepatocyte RXR α -deficient mice from exhibiting the mutant phenotype

To further analyze the effect of male hormones on the phenotype of hepatocyte RXR α -deficient mice, castration was performed in 6-wk-old wild-type and mutant male mice. Two weeks after castration, mice were killed. The expression of CYP2B, CYP3A, and CYP4A mRNA was examined in castrated and age-matched mice. Figure 4 demonstrates that

castration had weak effect on the expression of the CYP2B, CYP3A, and CYP4A genes. In contrast, castration had a dramatic effect on the expression of the CYP2B, CYP3A, and CYP4A genes in RXR α -deficient mice. The levels of these three mRNAs increased 8- to 20-fold after castration of hepatocyte RXR α -deficient mice, and the levels of expression became similar to those of female hepatocyte RXR α -deficient mice. Furthermore, treatment of castrated hepatocyte RXR α -deficient mice with DHT restored the mutant phenotype; the expression of the CYP2B, CYP3A, and CYP4A was reduced (Fig. 4).

Neither RXR α deficiency nor hormone treatment alters the expression of RXR β and γ mRNA in the liver

One factor that may account for the differential expression of the CYP450 genes is that the level of RXR β and γ may be higher in female than in male mouse livers. We have performed quantitative RT-PCR and found no significant difference in terms of the amount of RXR β and γ mRNAs

expressed in males and females (Fig. 5, A and B). In addition, E2 or DHT treatment of male and female mice, respectively, did not change the levels of RXR β and γ mRNAs expressed in the livers.

AR is expressed in the liver of wild-type and hepatocyte RXR α -deficient male and female mice

Another possibility that may account for this sexual dimorphic CYP450 gene expression pattern may be due to the action of male hormone because DHT can induce the exhibition of hepatocyte RXR α -deficient phenotype in female mice and castration can prevent the demonstration of the mutant phenotype in male mice. To explore this possibility, the expression of AR was studied in both male and female mouse livers. Figure 6 demonstrates that the livers of wild-type and mutant mice of both genders expressed AR mRNA and that the level of AR mRNA was not affected by RXR α deficiency. These data suggest that DHT mediated through AR in the liver may have a direct effect on regulating RXR-mediated pathways.

DHT inhibits RXR α -mediated transcription

To study the molecular mechanism underlying the inhibitory effect of DHT on the RXR signaling pathways, transient transfection was performed using CRBP-II-luciferase as a reporter gene. The CRBP-II gene contains a perfect RXR α homodimer recognition site (18). CV-1 cells were used because they contain very low levels of nuclear receptors and give very low background levels for transient transfection assays. The CRBP-II-luciferase activity increased about 25-fold after 9-*cis*-RA treatment (Fig. 7A). In the presence of AR, DHT had a weak effect on CRBP-II (2-fold induction). However, DHT significantly reduced RA-induced luciferase activity. The inhibitory effect was dependent on the amount of AR present. When the ratio of RXR α and AR was 1:10, the luciferase activity was close to the basal activity. In the absence of AR, DHT had no inhibitory effect (Fig. 7B). The inhibitory effect was also dependent on the amount of DHT. At 10^{-8} M, DHT was able to reduce the RA-induced luciferase activity by half suggesting the effect might be physiologically relevant (Fig.

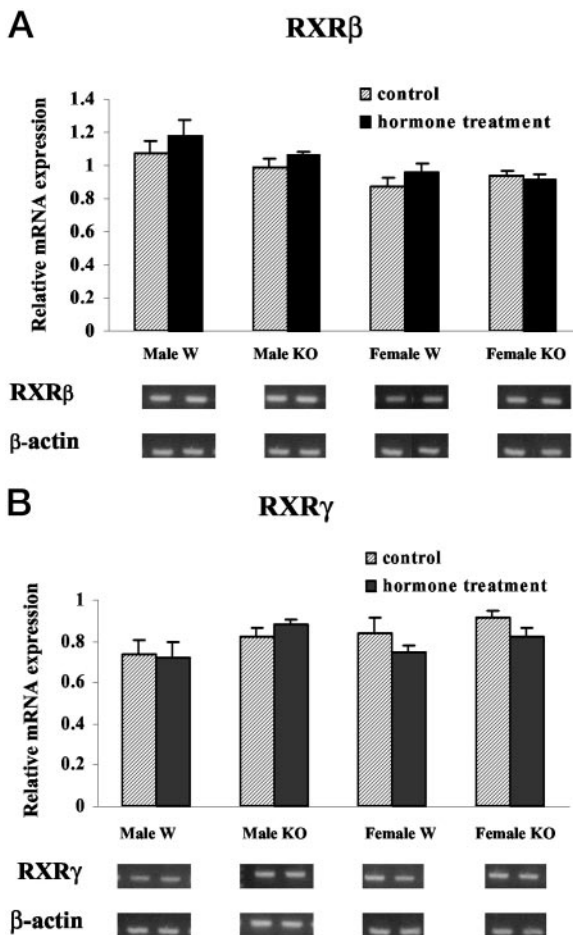


FIG. 5. The expression of RXR β (A) and γ (B) mRNA in the liver of wild-type and hepatocyte RXR α -deficient male and female mice treated with and without E2 or DHT, respectively. Mice were treated as described in Fig. 1 legend. Liver total RNA (2.0 μ g) was used for semiquantitative RT-PCR. The data were normalized by the level of β -actin mRNA and the relative amounts of RXR β and γ mRNA expressed are shown in the bar graph. Each value represents the mean \pm SD of five liver RNA samples. The representative RT-PCR results are also shown below the bars.

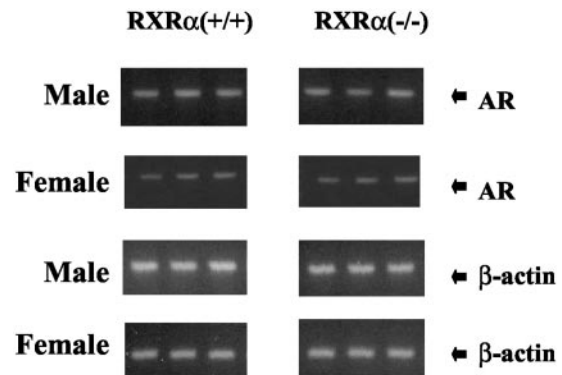


FIG. 6. The expression of AR mRNA in the liver of wild-type and hepatocyte RXR α -deficient mice measured by RT-PCR. cDNA was synthesized by reverse transcription using total liver RNA (2.0 μ g). Then semiquantitative PCR was performed using AR-specific primers. Amplification of β -actin mRNA was performed as internal controls.

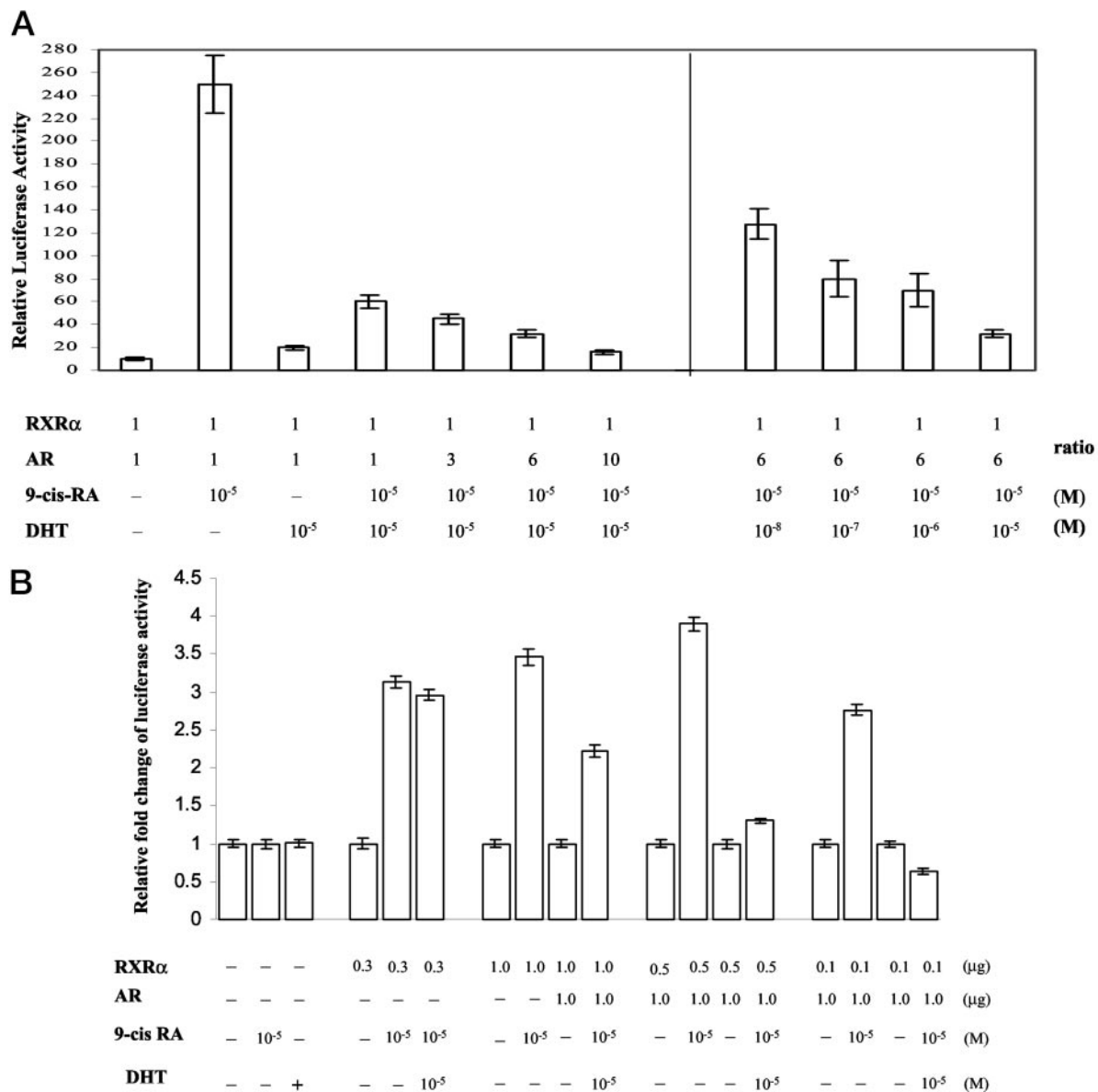


FIG. 7. DHT inhibits RXR α -mediated transcription. CV-1 cells were transiently transfected with CRBPII-luciferase (2 μ g) with cotransfection of RXR α and AR expression plasmids. The ratios of RXR α and AR used are indicated (ratio of 1:1 = 0.4 μ g : 0.4 μ g). PRL-TK (10 ng), which expresses Renilla luciferase and serves as an internal control, was also included in the transfection. The transfected cells were treated with 9-cis-RA and/or DHT for 48 h. Then cells were harvested and luciferase assays were performed. The results are expressed as ratios of firefly luciferase activities and Renilla luciferase activities (A) and relative fold changes in luciferase activity (B). Each value represents the mean \pm SD (A, n = 6; B, n = 3).

7A). Furthermore, the inhibitory effect of DHT became stronger when the amount of RXR α expression plasmid included in the transfection was reduced. These data further support the *in vivo* finding that the effect of DHT is apparent only when retinoid signaling is low (Fig. 7B).

Coimmunoprecipitation of the AR/RXR complex

The coimmunoprecipitation assay was employed to demonstrate that the AR interacts with the RXR *in vivo*. As shown in Fig. 8, AR was detected in the anti-RXR β antibody-precipitated complex from COS-1 cells cotransfected with the AR and the RXR β , but not in the complex from cells trans-

fecting only with the AR or the RXR β . This result demonstrated that the RXR could interact with the AR *in vivo*.

Discussion

We have shown that female mice are partially protected from hepatocyte RXR α deficiency. The expression of several nuclear receptor target genes including CYP2B, CYP3A, and CYP4A is retained in the liver when RXR α was deficient. This difference in CYP450 gene expression was not due to differential expression of the RXR β and γ genes in male and female mice, because neither gender nor RXR α deficiency affected the expression of the RXR β and γ genes.

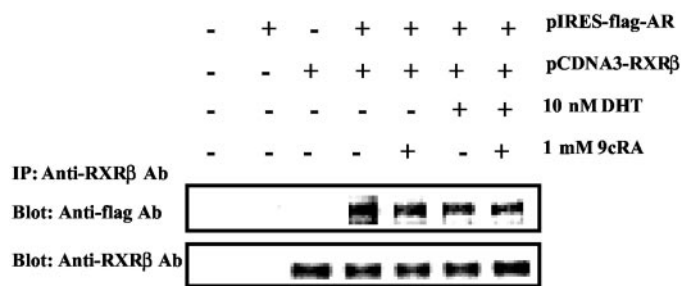


FIG. 8. *In vivo* interaction between the AR and the RXR. COS-1 cells were cotransfected with 10 μ g of pCDNA3-RXR β and/or 10 μ g of pIRES-flag-AR. The total plasmid amount was adjusted with pIRES-flag or pCDNA3 parent vector to 20 μ g for each 100-mm transfection by calcium phosphate precipitation method. Cells were incubated in DMEM 10% charcoal-depleted FBS for 24 h and then treated with or without 10 nM DHT and/or 1 μ M 9-*cis*-RA for another 16 h. Cell extracts were prepared, and immunoprecipitations were performed using an anti-RXR β antibody, followed by immunoblotting using an antibody to the flag-tag or RXR β .

The other obvious factor that might explain the sexual dimorphic phenotype is a hormonal effect. Using hormone treatment, we have shown that E2 was not able to reverse the phenotype of male hepatocyte RXR α -deficient mice, *i.e.* down-regulation of CYP2B, CYP3A, and CYP4A mRNA in the liver. In contrast, DHT could help female mutant mice to present the mutant phenotype. The effect of DHT was specific for hepatocyte RXR α -deficient mice only because DHT had no significant effect on regulating the CYP2B, CYP3A, and CYP4A genes in wild-type mice. These data clearly indicated that male hormones might have an antagonizing effect on RXR-mediated pathways. In our model, only the RXR α gene has been knocked out, and only in the hepatocytes. The presence of RXR β and γ in the liver and of RXR α in other types of liver cells can still mediate the effect of RXR within the liver. This might explain why the female hepatocyte RXR α -deficient mice do not have an apparent change in CYP450 gene expression. In male mutant mice, the remaining effects of RXR β and γ might be inhibited by male hormones because the liver does express AR and because AR and RXR can interact with each other *in vivo*. Therefore, the phenotype was apparent. This notion was further demonstrated by castration experiments. Castration reversed the phenotype of male RXR α -deficient mice, but the effect of castration on wild-type mice was not so dramatic. Furthermore, treating castrated hepatocyte RXR α -deficient mice with DHT restored the phenotype of hepatocyte RXR α -deficient mice. The level of CYP2B, CYP3A, and CYP4A mRNA expressed in the liver was very similar in castrated hepatocyte RXR α -deficient male and nonoperated female hepatocyte RXR α -deficient mice. These findings strongly indicate that male hormones can play a negative role in the RXR-mediated pathway particularly when the RXR or RA level is low or when the male hormone or AR level is high. This observation may also explain why males are more sensitive to vitamin A deficiency. It is interesting that the effect of DHT is only observed in the context of low retinoid signaling. Because RXR α is a very promiscuous receptor capable of heterodimerizing with a wide range of receptor partners, it is possible that there may be a competition for limiting

amounts of coregulators in wild-type mice, thus making AR signaling less effective in wild-type than in hepatocyte RXR α -deficient mice. It is also possible that the heightened retinoid signaling and high CYP450 activity in wild-type mice contribute to metabolism and inactivation of DHT and therefore the effect of DHT is not apparent in the wild-type mice.

RXRs play an important role in regulation of CYP450 gene expression (for reviews see Refs. 1–3). It appears that CYP450-dependent metabolism can produce virtually an unlimited number of ligands (both endogenous hormones and exogenous chemicals) for the nuclear receptors, and nuclear receptors can regulate CYP450 genes. Under normal conditions, sexual dimorphism in the expression of many rodent hepatic genes, including the CYP450 genes (22), is primarily mediated via the gender-specific profile of pituitary GH secretion. It would be interesting to determine if there is any interaction between retinoid-mediated signaling and the GH regulatory pathway.

Acknowledgments

We thank Drs. Thomas Magee and Nancy Berman for critically reading this manuscript.

Received December 11, 2002. Accepted February 4, 2003.

Address all correspondence and requests for reprints to: Yu-Jui Yvonne Wan, Ph.D., Department of Pathology, Harbor-University of California, Los Angeles Medical Center, 1000 West Carson Street, Torrance, California 90509. E-mail: agarose@ucla.edu.

This work was supported by NIH Grant CA-53596.

References

- Blumberg B, Evans RM 1998 Orphan nuclear receptors—new ligands and new possibilities. *Genes Dev* 12:3149–3155
- Kliwer SA, Lehmann JM, Willson TM 1999 Orphan nuclear receptors: shifting endocrinology into reverse. *Science* 284:757–760
- Gustafsson JA 1999 Seeking ligands for lonely orphan receptors. *Science* 284:1285–1286
- Mangelsdorf DJ, Borgmeyer U, Heyman RA, Zhou JY, Ong ES, Oro AE, Kakizuka A, Evans RM 1992 Characterization of three RXR genes that mediate the action of 9-*cis* retinoic acid. *Genes Dev* 6:329–344
- Wan Y-JY, Pan T, Wang L, Locker J, Wu T-C 1995 9-*Cis*-retinoic acid is more effective than all-trans-retinoic acid in up-regulating expression of the α -fetoprotein gene. *J Mol Endocrinol* 14:101–108
- Chen L, Wan Y-JY 1998 Differentiation and antiproliferation effects of retinoic acid receptor β in hepatoma cells. *Cancer Lett* 124:205–211
- Wan Y-JY, An D, Cai Y, Repa JJ, Chen TH-P, Flores M, Postic C, Magnuson MA, Chen J, Chien KR, French S, Mangelsdorf DJ, Sucov HM 2000 Hepatocyte-specific mutation establishes RXR α as a heterodimeric integrator of multiple physiological processes in the liver. *Mol Cell Biol* 20:4436–4444
- Wan Y-JY, Cai Y, Lungo W, Fu P, Locker J, French S, Sucov HM 2000 Peroxisome proliferator-activated receptor α -mediated pathways are altered in hepatocyte-specific retinoid x receptor α -deficient mice. *J Biol Chem* 275:28285–28290
- Cai Y, Konishi T, Han G, Campwala KH, French SW, Wan Y-JY 2002 The role of hepatocyte RXR α in xenobiotic-sensing nuclear receptor-mediated pathways. *Eur J Pharm Sci* 15:89–96
- Wan Y-J, Han G, Cai Y, Dai T, Konishi T, Leng A-S. Hepatocyte RXR 2003 α -deficient mice have reduced food intake, increased body weight, and improved glucose tolerance. *Endocrinology* 144:605–611
- Stephens CB, Gildengorin G 2000 Serum retinol, the acute phase response, and the apparent misclassification of vitamin A status in the third National Health and Nutrition Examination Survey. *Am J Clin Nutr* 72:1170–1178
- Klein-Szanto AJ, Clark JN, Martin DH 1980 Sexual differences in the distribution of epithelial alterations in vitamin A-deficient rats. *Int J Vitam Nutr Res* 50:61–69
- Paulsen ME, Johnson L, Young S, Norrdin RW, Severin GA, Knight AP, King V 1989 Blindness and sexual dimorphism associated with vitamin A deficiency in feedlot cattle. *J Am Vet Med Assoc* 194:933–937
- Marchetti MN, Sampol E, Bun H, Scoma H, Lacarelle B, Durand A 1997 In

- vitro metabolism of three major isomers of retinoic acid in rats. Intersex and interstrain comparison. *Drug Metab Dispos* 25:637–646
15. Sundaresan PR, Collins TF, Whitby KE, Welsh JJ, Black TN, Shackelford M, Flynn T, Newell RF, O'Donnell MW 1994 Effect of ethanol and vitamin A excess on vitamin A status in the liver, plasma and fetuses of pregnant rats. *Food Chem Toxicol* 32:247–254
 16. Morales DE, McGowan KA, Grant DS, Maheshwari S, Bhartiya D, Cid MC, Kleinman HK, Schnaper HW 1995 Estrogen promotes angiogenic activity in human umbilical vein endothelial cells in vitro and in a murine model. *Circulation* 91:755–763
 17. Chomczynski P, Sacchi N 1987 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159
 18. Mangelsdorf DJ, Umesono K, Kliewer SA, Borgmeyer U, Ong ES, Evans RM 1991 A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR. *Cell* 66:555–561
 19. Mangelsdorf DJ, Ong ES, Dyck JA, Evans RM 1990 Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* 345:224–229
 20. Lee YF, Young WJ, Burbach JP, Chang C 1998 Negative feedback control of the retinoid-retinoic acid/retinoid X receptor pathway by the human TR4 orphan receptor, a member of the steroid receptor superfamily. *J Biol Chem* 273:13427–13443
 21. Xie W, Barwick JL, Simon CM, Pierce AM, Safe S, Blumberg B, Guzelian PS, Evans RM 2000 Reciprocal activation of xenobiotic response genes by nuclear receptors SXR/PXR and CAR. *Genes Dev* 14:3014–3023
 22. Delesque-Touchard N, Park S-H, Waxman DJ 2000 Synergistic action of hepatocyte nuclear factors 3 and 6 on CYP2C12 gene expression and suppression by growth hormone-activated STAT5b. *J Biol Chem* 275:34173–34182

NOTICE TO AUTHORS

Beginning July 1, 2003, there will be a \$200 fee assessed for all manuscripts submitted **by mail** to *Molecular Endocrinology* and *Endocrinology*. There will be no submission fee for electronic submissions.

For complete instructions to authors, please go to <http://mend.endojournals.org/misc/itoa.shtml> or <http://endo.endojournals.org/misc/itoa.shtml>.