Gender Dictates the Nuclear Receptor-Mediated Regulation of CYP3A44

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ABSTRACT:

The CYP3As are broad-spectrum drug-metabolizing enzymes that are collectively responsible for more than 50% of xenobiotic metabolism. Unlike other CYP3As, murine CYP3A44 is expressed predominantly in the female liver, with much lower levels in male livers and no detectable expression in brain or kidney in either gender. In this study, we examined the role of nuclear hormone receptors in the regulation of Cyp3a44 gene expression. Interestingly, we observed differential effects of pregnane X receptor (PXR) and constitutive androstane receptor (CAR) -mediated activation of Cyp3a44 gene expression, which was gender-specific. For example, activation of PXR by pregnenolone-16α-carbonitrile (PCN) and dexamethasone (DEX) induced CYP3A44 mRNA levels in a PXR-dependent fashion in male mice, whereas no induction was detected in female mice. In contrast, PCN and DEX down-regulated CYP3A44 expression in female PXR null animals. Similar to PXR, CAR activation also showed a male-specific induction with no effect on CYP3A44 levels in females. When PXR knockout mice were challenged with the CAR activator phenobarbital, a significant up-regulation of male CYP3A44 levels was observed, whereas levels in females remained unchanged. We conclude that gender has a critical impact on PXR- and CAR-mediated effects of CYP3A44 expression.

Cytochromes P450 catalyze the initial step in the detoxification pathways of many foreign compounds, including prescription drugs (Guengerich, 1991; Coon, 2005). The CYP3A subfamily is clinically significant as it metabolizes a large number of endogenous compounds, such as steroids and bile acids, in addition to xenobiotics (Wrighton et al., 2000). Several clinical studies indicate increased clearance of many CYP3A drug substrates, such as cyclosporine, erythromycin, diazepam, and prednisolone, in women compared with men (Hunt et al., 1992; Zhu et al., 2003). One important basis for such differences is variation in the expression of CYP3A isoforms, which can vary from 5- to 20-fold among individuals (Flockhart and Rae, 2003).

The variability in CYP3A expression creates a potential for harmful drug interactions involving these isoforms, especially in patients undergoing therapies with multiple drugs (Thummel and Wilkinson, 1998). The molecular basis for this variability remains unclear, but several laboratories have reported gender-, tissue-, and age-dependent CYP3A expression profiles (Holazo et al., 1988; Kawai et al., 2000; Anakk et al., 2003b; Wolbold et al., 2003; Gandhi et al., 2004) that may account for increased metabolism and decreased therapeutic effectiveness. Current Food and Drug Administration guidelines emphasize the importance of understanding variability in drug metabolism and disposition in women, and the identification and characterization of gender-specific CYP3A isoforms is essential in this process (Anakk et al., 2003b).

CYP3A4, a major human CYP3A isoform responsible for drug metabolism, has been shown to exhibit higher expression in women than men, leading to faster drug clearance in women (Holazo et al., 1988; Kashuba et al., 1998; Schmidt et al., 2001). A similar gender difference is also found in rodents; rat CYP3A9 is expressed 28-fold higher in female rats compared with males, and the mouse enzymes CYP3A41 and CYP3A44 also show female-specific expression. Typically, this gender difference in CYP3A expression has been attributed to the regulatory influences of growth hormone, estrogen, and testosterone (Waxman et al., 1995; Sakuma et al., 2002; Cheung et al., 2006).

Apart from gender-biased regulation, CYP3A levels can also be altered by a number of compounds that are both CYP3A substrates and inducers of CYP3A expression. These include glucocorticoid...
receptor agonists and antagonists, phenobarbital, and rifampicin. The increase in CYP3A levels is mediated via the pregnane X receptor (PXR) and constitutive androstane receptor (CAR), both members of the nuclear receptor superfamily. On activation by a xenobiotic ligand, PXR and CAR bind as heterodimers with retinoid X receptor to their respective response elements and thus bring about CYP3A induction (Mangelsdorf et al., 1995; Bourguet et al., 2000; Makishima et al., 2002).

PXR and CAR can bind common response elements, resulting in cross-talk between these two receptors in CYP3A regulation (Xie et al., 2000; Seuyoshi and Neigishi, 2001). Thus, we examined the role of these three important nuclear receptors, PXR, and CAR in regulation of the female-specific CYP3A44 isoform.

We first characterized CYP3A44 expression in various tissues in both sexes. The expression pattern of CYP3A44 mRNA verified the female-specific profile, which led us to examine the role for estrogen in mediating this female specificity. Then, we analyzed the function of nuclear receptors PXR and CAR in regulating CYP3A44 expression under different xenobiotic challenges using the respective nuclear receptor gene knockout mice. Our results suggest complex effects of gender, the xenobiotic activator, and the nuclear receptor in controlling the response of CYP3A44 expression.

**Materials and Methods**

**Chemicals.** Phenobarbital (PB), pregnenolone-16α-carbonitrile (PCN), and dexamethasone (DEX) were obtained from Sigma Chemicals (St. Louis, MO). 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) was a gift from Dr. Stephen Safe.

**Animal Treatments.** C57/6NHSd mice of either sex (20–25 g body weight, 8–10 weeks old) were purchased from Harlan Laboratories (Indianapolis, IN). Animals were allowed food and water ad libitum and were subjected to a 12-h light/dark cycle. All the protocols for animal use and euthanasia were approved by the Animal Care Committee at University of Texas Medical School at Houston and were in accordance with National Institutes of Health guidelines. For conducting the nuclear receptor studies, genetically matched control (PXR+/−) and null (PXR−/−) mice in 129sv/C57BL6 mixed background were treated with DEX (100 mg/kg) (Anakk et al., 2003a), PCN (400 mg/kg), PB (100 mg/kg in saline), or vehicle to study the role of PXR on CYP3A44 expression (Staudinger et al., 2001). The inducers were dissolved in corn oil and injected i.p. once daily for 4 days. Control CAR+/− and CAR−/− mice were treated with PB (100 mg/kg), TCPOBOP (3 mg/kg), or corn oil for 3 days by i.p. injection (Wei et al., 2000). CAR inverse agonist experiments were conducted in wild-type mice injected i.p. with a single dose of androstenedione (100 mg/kg) followed by TCPOBOP (3 mg/kg). At the end of the experiments, all the mice including controls were sacrificed, and tissues were excised, immediately frozen in liquid nitrogen, and stored at −80°C until analyzed.

**RNA Isolation.** Frozen tissues were thawed on ice, and total RNA was isolated using a commercially available reagent (TRIzol, Invitrogen, Carlsbad, CA). The quality of the isolated RNA was assessed by electrophoresis on 1% agarose gels based on the integrity of 28S and 18S rRNA bands after ethidium bromide staining.

**Semiquantitative CYP3A44 Reverse Transcription-Polymerase Chain Reaction.** We designed specific primers to differentiate CYP3A44 from other CYP3As, especially CYP3A41, which shares 94% sequence identity. The reverse transcription-polymerase chain reaction (RT-PCR) of CYP3A44 RNA was followed by EcoRI or BanI restriction enzyme digest to ensure that the amplified sequence was unique. The forward primer used was 5′-GAA ACT GCA GGC AGA GAT CGA TAA-3′, and the reverse primer was 5′-TCA GAA GAA CTC CTT GAG GGA ATG-3′. To determine the linear range of amplification, PCR test reactions were performed at 25, 30, 35, and 40 cycles. The optimal RT-PCR conditions for female mice were 50°C RT (30 min), 94°C denaturing (2 min, followed by 30 s), 65°C annealing (1 min), and 72°C extension (1 min) for 25 cycles.

**Results**

**Specificity of CYP3a44 RT-PCR Technique.** Using the RT-PCR assay described, we analyzed CYP3A44 mRNA expression in the liver, kidney, and brain in both male and female mice. To ensure that the amplified product was unique to CYP3A44 mRNA and did not include CYP3A41, which shares 94% nucleotide identity with CYP3A44 mRNA, we designed a restriction digestion analysis that was performed after RT-PCR. The amplified CYP3A44 DNA fragment from either gender was cut with EcoRI, which in turn amplified the product from both genders and sequenced it to confirm that the PCR product belonged to CYP3A44. Densitometry was carried out using Alpha Ease (Alpha Innotech, San Leandro, CA) imaging software to semiquantitate the amounts of CYP3A44 between the different groups with β-actin as an internal standard. In addition, we also analyzed the ligand treatments at different PCR cycles (25, 35, and 40) to ensure that the amplifications were always in the linear range. The samples in each group were pooled, and RT-PCR was performed in duplicate to generate the S.E. value.

**CYP3A11 RT-PCR Assay.** The Cyp3a11 gene was amplified using 5′-GAG GAT CAC ACA CAC AGT TGT-3′ and 5′-TGG GAC AGC AAG GAG AGG CGT T-3′ as a forward and reverse primer, respectively. The amplifications were performed with 1 μg of total RNA using the superscript one-step RT-PCR kit (Invitrogen). The RT-PCR conditions used in the study were 50°C RT (30 min), 94°C denaturing (2 min, followed by 30 s done for 25 cycles), 56°C annealing (1 min), and 72°C extension (2 min) for 25 cycles.

**Preparation of Microsomes and High-Performance Liquid Chromatography Testosterone 6β-Hydroxylation Assay.** Liver microsomes were prepared by differential centrifugation as described previously (Saito and Strobel, 1981). Protein concentration of isolated microsomal preparations was determined with the bicinchoninic acid Protein Assay Reagent kit (Pierce Chemical, Rockford, IL) as described by the manufacturer. Microsomal testosterone 6β-hydroxylase activities were determined using high-performance liquid chromatography (Pearce et al., 1996). The data are expressed as picomole per minute per milligram of protein.

**Statistical Analysis.** Data are presented as mean ± S.E. Statistical significance for effects of the nuclear receptor ligands on CYP3A44 and differences among CYP3A44 expression between different treatment groups was determined using a two-tailed unpaired Student’s t test. Multiple groups after various treatments were compared using one-way analysis of variance. Differences were considered significant if p < 0.05.

**Fig. 1.** Tissue distribution of CYP3A44. a, RT-PCR assay standardization followed by EcoRI restriction digest specific for CYP3A44. On the other hand, BanI restriction is specific for CYP3A41, and the figure shows an uncleat CYP3A44. b, CYP3A44 expression in male mice. A 100-bp ladder is used as a marker in all the figures.
produced three bands specific to CYP3A44. Figure 1a clearly shows 250-, 200-, and 100-bp bands on EcoRI digest of CYP3A44. Also, the absence of restriction digest by BanI, which distinctly recognizes CYP3A41, establishes the fact that we have uniquely amplified CYP3A44 mRNA (Fig. 1a). Our RT-PCR results confirm a female-specific liver expression of CYP3A44 (data not shown). This is demonstrable by the fact that female CYP3A44 levels appear in 25 cycles, whereas male CYP3A44 levels are observed only after 35 cycles (Fig. 1b).

PXR Is Crucial in Contributing to CYP3A44 Expression in Males but Is Negligible for Maintaining Female Expression Levels. PCN. Wild-type and PXR\(^{+/−}\) mice (n = 3) were treated with PCN as described under Materials and Methods. Total RNA was isolated from liver and analyzed by RT-PCR for CYP3A44 and CYP3A11 expression. For the CYP3A44 expression pattern, male mice were amplified for 40 cycles, and the female mice were amplified for 25 cycles. The marker used in this study is 100-bp ladder from Promega (Madison, WI). β-Actin served as an internal standard. a, agarose gel showing the effect of PCN treatment on CYP3A11 and CYP3A44 mRNA expression. Lanes 1 and 2 show expression from wild-type animals, whereas lanes 3 and 4 show expression from PXR\(^{−/−}\) animals treated with vehicle or PCN, respectively. b, semiquantitation of CYP3A11. *, p < 0.05 compared with male control. #, p < 0.05 compared with female control. C, semiquantification of CYP3A44 mRNA after PCN treatment. #, p < 0.05 compared with female control.

Fig. 2. Role of PCN-mediated PXR activation on hepatic CYP3A44 mRNA profile. Wild-type and PXR\(^{−/−}\) mice (n = 3) were treated with DEX as described under Materials and Methods. Total RNA was isolated from liver and analyzed by RT-PCR for CYP3A44 and CYP3A11 expression. The male mice were amplified for 40 cycles, and the female mice were amplified for 25 cycles to detect CYP3A44 expression. a, agarose gel depicting changes in hepatic CYP3A11 and CYP3A44 after DEX treatment. Lanes 1 and 2 show expression from wild-type mice, whereas lanes 3 and 4 show expression from PXR\(^{−/−}\) mice treated with vehicle or DEX, respectively. b, semiquantitation of CYP3A11. *, p < 0.05 compared with male control. #, p < 0.05 compared with female control. c, Semiquantification of CYP3A44 mRNA after DEX treatment. #, p < 0.05 compared with female control.
tered PCN, a known PXR ligand. Wild-type female mice did not show any response to PCN, whereas in PXR null animals CYP3A44 levels were significantly down-regulated. Surprisingly, in male mice we observed a PXR-mediated PCN induction of CYP3A44 expression in control animals, which was lost in PXR null mice (Fig. 2, a and c). The CYP3A44 mRNA response obtained in male mice is analogous to the response seen in CYP3A11 expression (Fig. 2, b and c).

**DEX.** Treatment with DEX, a ligand that can act via PXR, led to the induction of CYP3A44 expression in control male mice, whereas female mRNA levels remained unchanged. PXR−/− animals on DEX treatment lost their ability to induce CYP3A44 expression in male mice but were able to significantly ablate expression in female mice.
This suggests that DEX induction in male mice requires the presence of PXR, whereas its absence leads to the down-regulation of female CYP3A44 mRNA levels. However, CYP3A11 was induced by DEX in both genders in a PXR-dependent fashion (Fig. 3, a and b).

**PB.** To evaluate PXR/CAR cross-talk, we tested the effect of PB, a CAR activator, in PXR null mice. To our surprise, we saw a significant PB-mediated CYP3A44 induction in male PXR knockout mice, whereas this response was absent in female mice. Surprisingly, mouse CYP3A11 also displayed a parallel response to that of CYP3A44 in either gender (Fig. 4, a–c).

**CAR Plays a Vital Role in Mediating Male CYP3A44 Expression, whereas Female Mice Show a Minor Role for CAR in Regulating CYP3A44 mRNA.** **PB.** To elucidate the role of CAR in regulating CYP3A44 levels, we treated mice with PB as described under Materials and Methods. PB treatment did not alter female CYP3A44 mRNA levels in either wild-type or CAR null mice (Fig. 5, a and c). On the other hand, CYP3A11 mRNA levels clearly showed a CAR-mediated up-regulation on PB treatment (Fig. 5, a and b).

**TCPOBOP.** Our experiments using wild-type and CAR null mice after TCPOBOP treatment show a definite role for CAR in mediating CYP3A44 induction in male mice. On the contrary, female mice exhibit no change in CYP3A44 expression in wild-type or CAR null mice (Fig. 5, a and c). To assess further the significance of CAR in dictating CYP3A44 mRNA levels, mice were administered androstanol, an inverse agonist to CAR, before ligand treatment. We did not see any appreciable change in expression profile of CYP3A44 and CYP3A11 in the presence or absence of androstanol (Fig. 6, a–c).

**Testosterone Hydroxylation: A CYP3A Activity Assay Correlates Well with the mRNA Data.** The total CYP3A activity was assessed using testosterone, a typical CYP3A substrate. Treatment of mice with 100 mg/kg DEX induced testosterone hydroxylation in a PXR-dependent manner, which was consistent with the RNA data. This increase in CYP3A activity was almost 7-fold in males. In contrast, only a 3- to 4-fold increase in activity was seen in females (Fig. 7).

**Discussion**

CYP3A44 has been identified as a female-specific gene that is primarily expressed in the liver. Sakuma et al. (2002) have shown that estradiol benzoate could positively regulate the mRNA expression for CYP3A44, which we failed to observe in our study (data not shown). Also, recent work with the transgenic human CYP3A mice has shown a crucial role for growth hormone and age rather than estrogen in establishing the female specificity of CYP3A44 expression (Cheung et al., 2006).

In this study, we have defined the role of nuclear receptors PXR and CAR in the regulation of the female-specific Cyp3a44 gene. CAR and PXR are the two principal nuclear receptors that regulate many cytochrome P450 genes during xenobiotic stress (Xie et al., 2000; Wei et al., 2002). Clear gender-specific expression of these nuclear receptors is yet to be reported. Nonetheless, during pregnancy progesterone mediates the induction of PXR up to 50-fold (Masuyama et al., 2001). On the other hand, estradiol can activate CAR by increasing nuclear expression. The male mice were amplified for 40 cycles, and the female mice were amplified for 25 cycles to detect CYP3A44 expression. A 100-bp ladder was used as a marker, and β-actin was used as an internal control. a, an agarose gel depicting CYP3A11 and CYP3A44 mRNA levels after androstanol treatment. b, semi-quantitation of CYP3A11. *, p < 0.05 compared with male control. #, p < 0.05 compared with female control. c, semi-quantification of CYP3A44 mRNA. *, p < 0.05 compared with male control.
translocation of CAR (Kawamoto et al., 2000). Because PXR and CAR are the vital nuclear receptors controlling Cyp3a gene expression, we investigated their role in regulating female-specific CYP3A44.

To determine the role of PXR in CYP3A44 induction by xenobiotics, we measured CYP3A44 mRNA levels in wild-type and PXR null mice after treatment with DEX or PC (Anakk et al., 2003a). The results from our study suggest that both PCN and DEX do not alter CYP3A44 levels in wild-type female mice; however, the two ligands were able to significantly down-regulate CYP3A44 mRNA levels in PXR null female mice. This implies that both PCN and DEX may act through a separate pathway that in the absence of PXR could suppress CYP3A44 gene expression in female mice. In contrast to females, male mice show a PXR-dependent induction response to both the ligands, which is similar to the response obtained with CYP3A11. DEX treatment induced CYP3A13, another mouse CYP3A, in the absence of PXR (Anakk et al., 2003a), whereas CYP3A44 showed a PXR-dependent DEX suppression in females. Thus, one may clearly infer that individual CYP3A isoforms show differential responses to PXR-dependent DEX suppression in females. This may be explained by the fact that in the absence of PXR, CAR dominates and can be readily accessed and activated via PB. Alternatively, in male animals the androstanol-mediated CAR inhibition may be relieved in the absence of PXR, thereby promoting nuclear translocation of CAR by PB.

In summary, the ligand treatments strengthen the fact that individual CYP3A isoforms are uniquely regulated, and there are few, if any, universal modes of regulation. Our data also emphasize the gender-specific regulation of CYP3A44. Our results suggest that CYP3A regulation may involve pathways other than the major known ones, namely, CAR and PXR. The expression pattern in PXR/CAR double knockouts should shed more light on alternate mechanisms governing xenobiotic induction of CYP3A enzymes. The availability of such a model is foreseeable in the future and will be actively pursued.

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References


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