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Gender dictates the nuclear receptor mediated regulation of CYP3A44

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Running Title: Role of PXR & CAR in *Cyp3a44* gene expression.

Abbreviations used are: CYP, Cytochrome P450; PXR, Pregnane X Receptor; CAR, Constitute Androstane Receptor.

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Abstract

The cytochromes P4503A 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 instance, 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 female. When PXR knockout mice were challenged with the CAR activator Phenobarbital, a significant up regulation of male CYP3A44 levels was observed while levels in females remained unchanged. We conclude that gender has a critical impact on PXR and CAR mediated effects of CYP3A44 expression.

Cytochromes P450 (CYPs) 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 to men (Hunt et al., 1992; Zhu et al., 2003). One important basis for such differences stems is variation in the expression of CYP3A isoforms, which can vary from 5 to 20 fold amongst individuals (Flockhart and Rae, 2003).

The variability in CYP3A expression creates a potential for harmful drug interactions involving these isozymes, 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 FDA 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 to 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 (NR) superfamily. Upon activation by a xenobiotic ligand, PXR and CAR bind as heterodimers with Retinoid X Receptor (RXR) 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.

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-dichlorpyridyloxy)] benzene (TCPOBOP) was a gift from Dr. Stephen Safe.

Animal Treatments

C57B/6NHsd mice of either sex (20-25g 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 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) or PCN (400mg/kg) or PB (100mg/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 intraperitoneally (i.p.) once daily for 4 days. Control CAR+/+ and CAR-/- mice were treated with either PB (100mg/kg) or TCPOBOP (3mg/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 androstanol (100 mg/kg) followed by TCPOBOP (3 mg/kg). At the end of the experiments, all mice including controls were sacrificed, tissues 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.

Semi quantitative CYP3A44 RTPCR

We designed specific primers to differentiate CYP3A44 from other CYP3As especially CYP3A41, which shares 94% sequence identity. The RT-PCR of CYP3A44 RNA was followed by EcoR I or Ban I 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 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^{o}C RT (30 min), 94^{o}C denaturing (2 min, followed by 30 sec), 65^{o}C annealing (1 min) and 72^{o}C extension (1 min) for 25 cycles. For assaying CYP3A44 in male mice PCR amplifications were carried out up to 40 cycles. Further, we gel purified the amplified product from both the gender 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 semi-quantitate the amounts of CYP3A44 between the different groups with β-actin as an internal standard. Additionally, 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 RTPCR was performed in duplicates to generate the SE value.

CYP3A11 RT-PCR Assay:

The Cyp3a11 gene was amplified using 5'- GAG GAT CAC ACA CAC AGT TGT - 3' and 5'- TGT GAC AGC AAG GAG AGG CGT T -3' as a forward and reverse primer respectively. The amplifications were performed with 1µg of total RNA using the superscript one step RT-PCR kit (Invitrogen Life Technologies, Inc., Carlsbad, CA). The RT-PCR conditions used in the study were 50^{o}C RT (30 min),

94^{o}C denaturing (2 min, followed by 30 sec done for 25 cycles), 56^{o}C annealing (1 min) and 72^{o}C extension (2 min) for 25 cycles.

Preparation of Microsomes and HPLC Testosterone 6β-Hydroxylation Assay:

Liver microsomes were prepared by differential centrifugation as previously described (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 HPLC (Pearce et al., 1996). The data are expressed as picomoles 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 were determined using a two-tailed unpaired student's t-test. Multiple groups after various treatments were compared using one way analysis of variance (ANOVA). Differences were considered significant if $P < 0.05$.

Results

Specificity of Cyp3a44 RT-PCR technique.

Using the RTPCR 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 EcoR I which inturn produced three bands specific to CYP3A44. Figure 1a clearly shows 250, 200 and 100 bp bands upon EcoRI digest of CYP3A44. Also, the absence of restriction digest by Ban I which distinctively recognizes CYP3A41 establishes the fact that we have uniquely amplified CYP3A44 mRNA (Figure 1a). Our RTPCR 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 (Figure 1b).

PXR is crucial in contributing to CYP3A44 expression in males but is negligible for maintaining female expression levels.

PCN:

Wild type and PXR null mice of either sex were administered with PCN, a known PXR ligand. Wild type female mice did not show any response to PCN while 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 (Figure 2a & 2c). The CYP3A44 mRNA response obtained in male mice is analogous to the response seen in CYP3A11 expression (Figure 2b & 2c).

DEX:

Treatment with DEX, a ligand which 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 (Figure 3a & 3c). This suggests that DEX induction in male mice requires the presence of PXR while its absence leads to the down regulation of female CYP3A44 mRNA levels. However, CYP3A11 was induced by DEX in both the genders in a PXR-dependent fashion (Figure 3a & 3b).

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 while this response was absent in female mice. Surprisingly, mouse CYP3A11 also displayed a parallel response to that of CYP3A44 in either gender (Figure 4a-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 in the Materials and Methods section. PB treatment did not alter female CYP3A44 mRNA levels either in wild type or in CAR null mice (Figure 5a & c). On the other hand, CYP3A11 mRNA levels clearly showed a CAR-mediated up regulation on PB treatment (Figure 5a & 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 in CAR null mice (Figure 5a & 5c). 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 as well as CYP3A11 in the presence or absence of androstanol (Figure 6a -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 100mg/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 3-4 fold increase in activity was seen in females (Figure 7).

Discussion

CYP3A44 has been identified as a female specific gene which is primarily expressed in the liver. Sakuma et al., (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 demonstrated a crucial role for GH 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 which regulate many CYP 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 translocation of CAR (Kawamoto et al., 2000). Since 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 might 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) while CYP3A41 showed a PXR-dependent DEX suppression in females. Thus one may clearly infer that individual CYP3A isoforms show differential responses to

the same ligand challenge. Another key fact is the discrepancy observed in the response to PCN as well as DEX in male versus female mice, suggesting a principal role for gender in determining CYP3A44 regulatory processes.

Testosterone 6β hydroxylation, a classical catalytic activity test for all the CYP3As, further strengthens the mRNA data. The induction of CYP3A-catalyzed testosterone metabolism is observed in males and females in a PXR- dependent fashion. Further, the male mice show a higher induction of testosterone metabolizing capability compared to females. This may be attributed to increased expression of both CYP3A11 and CYP3A44 in male animals but in female mice only CYP3A11 is induced.

Ding and Staudinger reported recently that the ratio of CAR to PXR determines the net activity of these two receptors by regulating the amount of the steroid receptor coactivator protein-1 associated with either PXR or CAR (Ding and Staudinger, 2005). In order to evaluate the relative role of CAR in controlling CYP3A44 mRNA expression, CYP3A44 levels were assessed after CAR activation with TCPOBOP or PB. In female mice, CAR activation did not change CYP3A44 mRNA expression. In contrast CYP3A44 levels in male mice are up regulated by TCPOBOP in a CAR-dependent manner. This induction seen specifically in male mice can possibly be attributed to CAR itself as shown by Yoshinari et al. who suggest that the CAR activation process as such is higher in male than female WKY rats (Yoshinari et al., 2001). Administration of androstanol, an inverse agonist of CAR, showed little inhibition of TCPOBOP induction of CYP3A44. PB treatment of PXR null mice resulted in a significant induction of male CYP3A44 and CYP3A11. Interestingly, this induction by PB was greater in PXR-/- mice than in mice having a functional PXR. 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 might 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 might 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

Anakk, S., Kalsotra, A., Shen, Q., Vu, M.T., Staudinger, J.L., Davies, P.J. and Strobel, H.W. (2003a) Genomic characterization and regulation of CYP3a13: role of xenobiotics and nuclear receptors. *Faseb J* **17:**1736-1738.

Anakk, S., Ku, C.Y., Vore, M. and Strobel, H.W. (2003b) Insights into Gender Bias: Rat Cytochrome P450 3A9. *J Pharmacol Exp Ther* **305:**703-709.

Bourguet, W., Germain, P. and Gronemeyer, H. (2000) Nuclear receptor ligand-binding domains: three-dimensional structures, molecular interactions and pharmacological implications. *Trends Pharmacol Sci* **21:**381-388.

Cheung, C., Yu, A.M., Chen, C.S., Krausz, K.W., Byrd, L.G., Feigenbaum, L., Edwards, R.J., Waxman, D.J. and Gonzalez, F.J. (2006) Growth hormone determines sexual dimorphism of hepatic cytochrome P450 3A4 expression in transgenic mice. *J Pharmacol Exp Ther* **316:**1328-1334.

Coon, M.J. (2005) CYTOCHROME P450: Nature's Most Versatile Biological Catalyst. *Annu Rev Pharmacol Toxicol* **45:**1-25.

Ding, X. and Staudinger, J.L. (2005) The ratio of constitutive androstane receptor to pregnane X receptor determines the activity of guggulsterone against the Cyp2b10 promoter. *J Pharmacol Exp Ther* **314:**120-127.

Flockhart, D.A. and Rae, J.M. (2003) Cytochrome P450 3A pharmacogenetics: the road that needs traveled. *Pharmacogenomics J* **3:**3-5.

Gandhi, M., Aweeka, F., Greenblatt, R.M. and Blaschke, T.F. (2004) Sex differences in pharmacokinetics and pharmacodynamics. *Annu Rev Pharmacol Toxicol* **44:**499-523.

Guengerich, F.P. (1991) Reactions and significance of cytochrome P-450 enzymes. *J Biol Chem* **266:**10019-10022.

Holazo, A.A., Winkler, M.B. and Patel, I.H. (1988) Effects of age, gender and oral contraceptives on intramuscular midazolam pharmacokinetics. *J Clin Pharmacol* **28:**1040-1045.

Hunt, C.M., Westerkam, W.R. and Stave, G.M. (1992) Effect of age and gender on the activity of human hepatic CYP3A. *Biochem Pharmacol* **44:**275-283.

Kashuba, A.D., Bertino, J.S., Jr., Rocci, M.L., Jr., Kulawy, R.W., Beck, D.J. and Nafziger, A.N. (1998) Quantification of 3-month intraindividual variability and the influence of sex and menstrual cycle phase on CYP3A activity as measured by phenotyping with intravenous midazolam. *Clin Pharmacol Ther* **64:**269-277.

Kawai, M., Bandiera, S.M., Chang, T.K. and Bellward, G.D. (2000) Growth hormone regulation and developmental expression of rat hepatic CYP3A18, CYP3A9, and CYP3A2. *Biochem Pharmacol* **59:**1277-1287.

Kawamoto, T., Kakizaki, S., Yoshinari, K. and Negishi, M. (2000) Estrogen activation of the nuclear orphan receptor CAR (constitutive active receptor) in induction of the mouse Cyp2b10 gene. *Mol Endocrinol* **14:**1897-1905.

Makishima, M., Lu, T.T., Xie, W., Whitfield, G.K., Domoto, H., Evans, R.M., Haussler, M.R. and Mangelsdorf, D.J. (2002) Vitamin D receptor as an intestinal bile acid sensor. *Science* **296:**1313-1316.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. and Et Al. (1995) The nuclear receptor superfamily: the second decade. *Cell* **83:**835-839.

Masuyama, H., Hiramatsu, Y., Mizutani, Y., Inoshita, H. and Kudo, T. (2001) The expression of pregnane X receptor and its target gene, cytochrome P450 3A1, in perinatal mouse. *Mol Cell Endocrinol* **172:**47-56.

Pearce, R.E., Mcintyre, C.J., Madan, A., Sanzgiri, U., Draper, A.J., Bullock, P.L., Cook, D.C., Burton, L.A., Latham, J., Nevins, C. and Parkinson, A. (1996) Effects of freezing, thawing, and storing human liver microsomes on cytochrome P450 activity. *Arch Biochem Biophys* **331:**145-169.

Saito, T. and Strobel, H.W. (1981) Purification to homogeneity and characterization of a form of cytochrome P-450 with high specificity for benzo[alpha]pyrene from beta-naphthoflavone-pretreated rat liver microsomes. *J Biol Chem* **256:**984-988.

Sakuma, T., Endo, Y., Mashino, M., Kuroiwa, M., Ohara, A., Jarukamjorn, K. and Nemoto, N. (2002) Regulation of the expression of two female-predominant CYP3A mRNAs (CYP3A41 and CYP3A44) in mouse liver by sex and growth hormones. *Arch Biochem Biophys* **404:**234-242.

Schmidt, R., Baumann, F., Hanschmann, H., Geissler, F. and Preiss, R. (2001) Gender difference in ifosfamide metabolism by human liver microsomes. *Eur J Drug Metab Pharmacokinet* **26:**193-200.

Seuyoshi, T. and Neigishi, M. (2001) Phenobarbital response elements in cytochrome P450 genes and nuclear receptors. *Annu Rev Pharmacol Toxicol* **41:**123-143.

Staudinger, J.L., Goodwin, B., Jones, S.A., Hawkins-Brown, D., Mackenzie, K.I., Latour, A., Liu, Y., Klaassen, C.D., Brown, K.K., Reinhard, J., Willson, T.M., Koller, B.H. and Kliewer, S.A. (2001) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci U S A* **98:**3369-3374.

Thummel, K.E. and Wilkinson, G.R. (1998) In vitro and in vivo drug interactions involving human CYP3A. *Annu Rev Pharmacol Toxicol* **38:**389-430.

Waxman, D.J., Ram, P.A., Pampori, N.A. and Shapiro, B.H. (1995) Growth hormone regulation of male-specific rat liver P450s 2A2 and 3A2: induction by intermittent growth hormone pulses in male but not female rats rendered growth hormone deficient by neonatal monosodium glutamate. *Mol Pharmacol* **48:**790-797.

Wei, P., Zhang, J., Dowhan, D.H., Han, Y. and Moore, D.D. (2002) Specific and overlapping functions of the nuclear hormone receptors CAR and PXR in xenobiotic response. *Pharmacogenomics J* **2:**117-126.

Wei, P., Zhang, J., Egan-Hafley, M., Liang, S. and Moore, D.D. (2000) The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism. *Nature* **407:**920-923.

Wolbold, R., Klein, K., Burk, O., Nussler, A.K., Neuhaus, P., Eichelbaum, M., Schwab, M. and Zanger, U.M. (2003) Sex is a major determinant of CYP3A4 expression in human liver. *Hepatology* **38:**978-988.

Wrighton, S.A., Schuetz, E.G., Thummel, K.E., Shen, D.D., Korzekwa, K.R. and Watkins, P.B. (2000) The human CYP3A subfamily: practical considerations. *Drug Metab Rev* **32:**339-361.

Xie, W., Barwick, J.L., Simon, C.M., Pierce, A.M., Safe, S., Blumberg, B., Guzelian, P. and Evans, R.M. (2000) Reciprocal activation of xenobiotic response genes by nuclear receptors PXR/SXR and CAR. *Genes Dev* **14:**3014-3023.

Yoshinari, K., Sueyoshi, T., Moore, R. and Neigishi, M. (2001) Nuclear receptor CAR as regulatory factor for the sexually dimorphic of CYP2B1 gene by phenobarbital in rat livers. *Mol Pharmacol* **59:**278-283.

Zhu, B., Liu, Z.Q., Chen, G.L., Chen, X.P., Ou-Yang, D.S., Wang, L.S., Huang, S.L., Tan, Z.R. and Zhou, H.H. (2003) The distribution and gender difference of CYP3A activity in Chinese subjects. *Br J Clin Pharmacol* **55:**264-269.

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Legends to figures

Figure 1. Tissue distribution of CYP3A44

a. RT-PCR assay standardization followed by EcoR I restriction digest specific for CYP3A44. On the other hand, Ban I restriction is specific for CYP3A41 and the figure shows an uncut CYP3A44.

b. CYP3A44 expression in male mice. 100 bp ladder is used as a marker in all the figures.

Figure 2. Role of PCN mediated PXR activation on hepatic CYP3A44 mRNA profile

Wild type and PXR-/- mice (n=3) were treated with PCN as described in methods. Total RNA was isolated from liver and analyzed by RT-PCR for CYP3A44 as well as 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. β-actin served as an internal standard.

a. Agarose gel showing the effect of PCN treatment on CYP3A11 and CYP3A44 mRNA expression. Lanes 1&2 show expression from wild-type animals while Lanes 3&4 show expression from PXR-/- animals treated with vehicle or PCN, respectively. (b) Semi-quantitation of CYP3A11. *$p<0.05$ when compared to male control. $^{\#}p<0.05$ when compared with female control. (c) Semi-quantification of CYP3A44 mRNA after PCN treatment. $^{\#}p<0.05$ when compared to female control.

Figure 3 Role of DEX mediated PXR activation on hepatic CYP3A44 mRNA profile

Wild type and PXR-/- mice (n=3) were treated with DEX as described in methods. Total RNA was isolated from liver and analyzed by RT-PCR for CYP3A44 as well as CYP3A11 expression. The male mice were amplified for 40 cycles and the

female mice were amplified for 25 cycles in order to detect CYP3A44 expression.

(a) Agarose gel depicting changes in hepatic CYP3A11 and CYP3A44 after DEX treatment.

Lanes1&2 show expression from wild type mice while Lanes 3&4 show expression from PXR-/- mice treated with vehicle or DEX respectively. (b) Semi-quantitation of CYP3A11. *$p<0.05$ when compared to male control. $^{\#}p<0.05$ when compared with female control. (c) Semi-quantification of CYP3A44 mRNA after DEX treatment. $^{\#}p<0.05$ when compared to female control.

Figure 4 **Role of PB on hepatic CYP3A44 mRNA profile in the absence of PXR**

PXR-/- mice (n=3) were treated with phenobarbital as shown in methods. Total RNA was isolated from liver and analyzed by RT-PCR for CYP3A44 as well as CYP3A11 expression. The male mice were amplified for 40 cycles and the female mice were amplified for 25 cycles in order to detect CYP3A44 expression.

(a) Agarose gel depicting changes in hepatic CYP3A11 and CYP3A44 after PB treatment.

Lanes1&3 show expression from PXR-/- vehicle treated samples while Lanes 2&4 show expression from PXR-/- mice treated with PB. (b) Semi-quantitation of CYP3A11. *$p<0.05$ when compared to male control (c) Semi-quantification of CYP3A44 mRNA after PB treatment.

Figure 5 **Role of CAR and xenobiotics on hepatic CYP3A44 mRNA profile**

Wild-type and CAR -/- mice (n=3) were treated according to the protocol described in the methods section. RNA was prepared from liver (n=3) and RT-PCR was performed to analyze CYP3A44 and

CYP3A11 mRNA expression. The male mice were amplified for 40 cycles and the female mice were amplified for 25 cycles in order to detect CYP3A44 expression. 100bp ladder was used as a marker and β-actin was used as an internal control.

(a). Effect of xenobiotics on CYP3A11 and CYP3A44 expression pattern. Lanes 1, 3 &5 depict control animals while lanes 2, 4 & 6 represent CAR-/- animals treated with vehicle, PB or TCPOBOP respectively. (b) Semi-quantitation of CYP3A11. *$p<0.05$ when compared to male control. $^{\#}p<0.05$ when compared with female control. (c) Semi-quantification of CYP3A44 mRNA. *$p<0.05$ when compared to male control.

Figure 6 CYP3A44 and CYP3A11 mRNA levels after inverse agonist treatment followed by TCPOBOP induction.

This experiment was carried out using control wild type mice. Lane 1 is untreated, lane 2 represents androstanol treatment and lane 3 is Androstanol followed by TCPOBOP. RNA was prepared from liver (n=3) and RT-PCR was performed to analyze CYP3A44 and CYP3A11 mRNA expression. The male mice were amplified for 40 cycles and the female mice were amplified for 25 cycles in order to detect CYP3A44 expression. 100bp 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 *$p<0.05$ when compared to male control. $^{\#}p<0.05$ when compared with female control. (c) Semi-quantification of CYP3A44 mRNA. *$p<0.05$ when compared to male control.

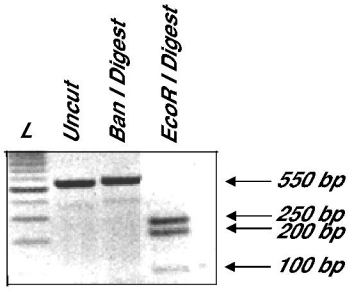
Figure 7 Analysis of CYP3A activity using testosterone hydroxylation

Microsomes were prepared from DEX treated livers of wild type and PXR -/- mice of either sex. Each of these groups was analyzed for

testosterone hydroxylation in order to evaluate total CYP3A activity. The catalytic activity is represented as picomoles/min/mg of protein. *$p<0.05$ when compared to their respective control.

Figure 1

1a.



1b.

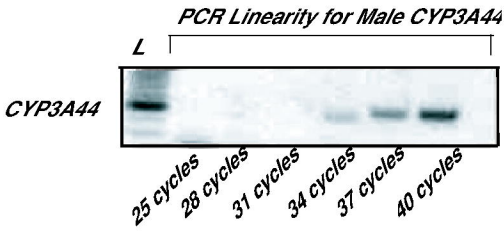
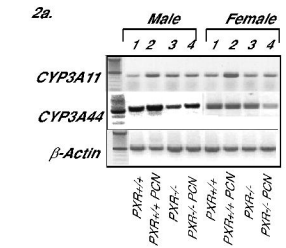
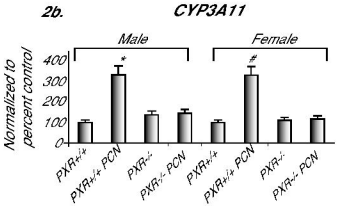


Figure 2


2a.
Male
Female
1 2 3 4
1 2 3 4
CYP3A11
CYP3A44
β-Actin
PXR+/+
PXR+/+ PCN
PXR-/-
PXR-/- PCN
PXR+/+
PXR+/+ PCN
PXR-/-
PXR-/- PCN


2b.
CYP3A11
Male
Female
Normalized to percent control
400
300
200
100
0
*
#
PXR+/+
PXR+/+ PCN
PXR-/-
PXR-/- PCN
PXR+/+
PXR+/+ PCN
PXR-/-
PXR-/- PCN

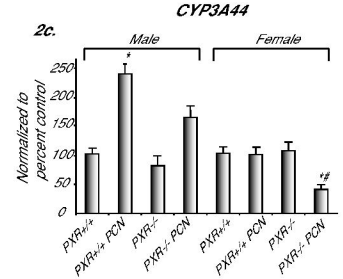
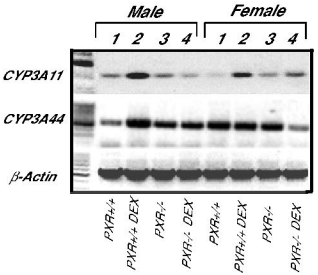
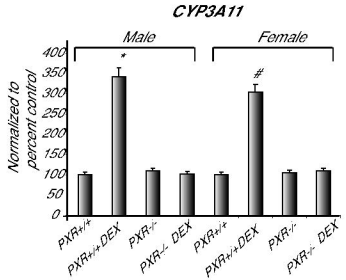

CYP3A44
2c.
Male
Female
Normalized to percent control
250
200
150
100
50
0
#
*#
PXR+/+
PXR+/+ PCN
PXR-/-
PXR-/- PCN
PXR+/+
PXR+/+ PCN
PXR-/-
PXR-/- PCN

Figure 3

3a.



3b.



3c.

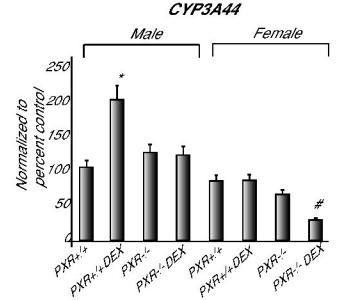
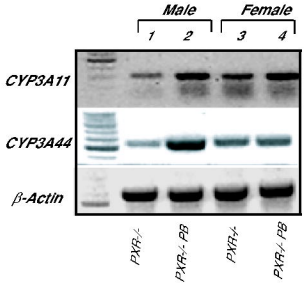
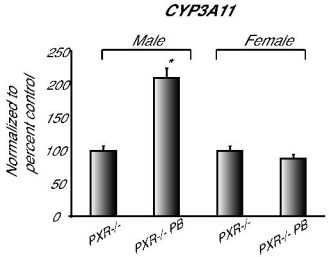


Figure 4

4a.



4b.



4c.

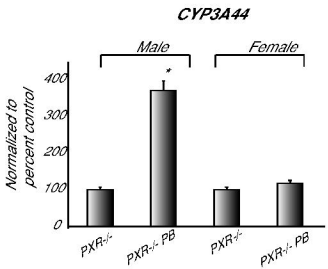
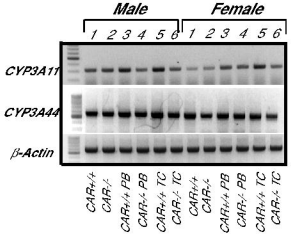


Figure 5

5a.



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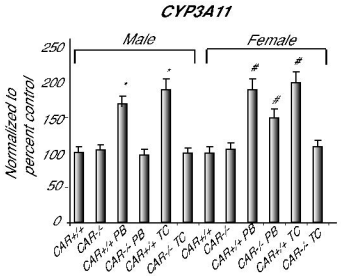


5c.

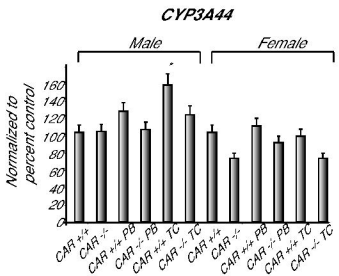
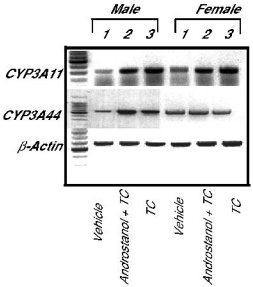
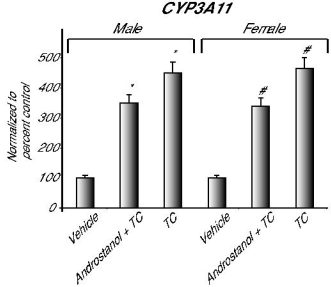


Figure 6

6a.



6b.



6c.

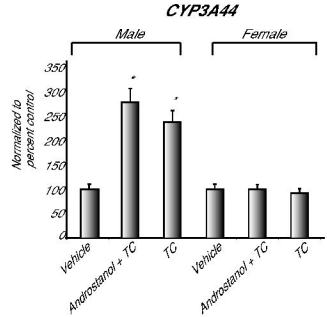
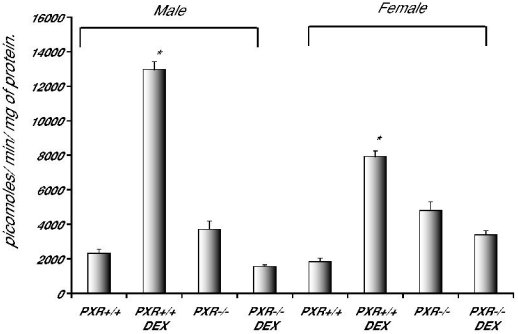


Figure 7


Male
Female
picomoles/ min/ mg of protein.
16000
14000
12000
10000
8000
6000
4000
2000
0
*
*
PXR+/+
PXR+/+ DEX
PXR-/-
PXR-/- DEX
PXR+/+
PXR+/+ DEX
PXR-/-
PXR-/- DEX