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THE INVOLVEMENT OF PXR IN HEPATIC GENE REGULATION DURING INFLAMMATION IN MICE

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Running title: Gene regulation by PXR during inflammation

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Abbreviations:
PXR, pregnane X receptor
TNF, tumour necrosis factor
CYP, cytochrome P450
PCN, 5-pregnen-3β-ol-20-one-16α-carbonitrile
RU486, 17β-hydroxy-11β-[4-dimethylamino phenyl]-17α-[1-propynyl] estra-4,9-dien-3-one
CAR, constitutive androstane receptor
FXR, farnesoid X receptor
RXR, retinoid X receptor
BSEP, bile salt export pump
MRP, multidrug resistance associated protein
MDR, multidrug resistance
NTCP, sodium taurocholate co-transporting polypeptide
OATP, organic anion transporting polypeptide

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Abstract.

Inflammation and pro-inflammatory cytokines suppress the expression of several hepatic transporters and metabolic enzymes, often resulting in cholestatic liver disease. However, mechanism(s) of this down-regulation have not been fully elucidated. As the pregnane X receptor (PXR) is involved in inducing many of these hepatic proteins, it is possible that PXR is also involved in their down-regulation during inflammation. Thus we compared the effect of inflammation on hepatic gene regulation in wild type ($PXR^{+/+}$) versus PXR-null ($PXR^{-/-}$) mice. Treatment of $PXR^{+/+}$ but not $PXR^{-/-}$ mice with the PXR activators PCN or RU486 resulted in increased mRNA levels of *bsep*, *mdr1a*, *mrp2*, *mrp3*, *oatp2* and *cyp3a11*, indicating involvement of PXR in their regulation. Significantly lower mRNA levels of *bsep, mdr2, mrp2, mrp3, ntcp, oatp2* and *cyp3a11* were found in endotoxin-treated $PXR^{+/+}$ mice. In endotoxin-treated $PXR^{-/-}$ mice, the extent of *mrp2* suppression was significantly diminished. Changes in *mrp2* expression were supported by western blot analysis. While IL-6 imposed significant decreases in the expression of *bsep, mrp2* and *cyp3a11* in $PXR^{+/+}$ mice, this was not observed in $PXR^{-/-}$ mice. Of note, significantly lower levels of *PXR* mRNA and protein were detected in endotoxin (LPS)- and IL-6- treated $PXR^{+/+}$ mice. In addition, endotoxin and IL-6 were also able to suppress PCN-mediated induction of *bsep, mrp2, cyp3a11* and *PXR*. Taken together, our results suggest that PXR plays a role in the down-regulation of several hepatic proteins during inflammation.

Endotoxin-induced sepsis, viral infections and other inflammatory conditions are a relatively frequent cause of intra-hepatic cholestasis in patients (Trauner et al., 1999). Disruptions in the hepatic accumulation and excretion of bile salts and acids occur due to down-regulation of both basolateral uptake [Na+-taurocholate cotransporter (NTCP), organic anion transporter (OATP2)] and canalicular efflux [bile salt export pump (BSEP), multidrug resistance associated protein (MRP2), P-glycoprotein (MDR1)] transport systems. The molecular mechanisms involved in this down-regulation have not been fully elucidated. Activation of nuclear receptor networks including the liver X receptor (LXR), farnesoid X receptor (FXR), peroxisome proliferator activated receptor (PPAR) and retinoid X receptor (RXR) proteins have been found to play a key role in the induction of many genes responsible for both the transport and metabolism of bile acids (Chiang, 2002). Recently, reductions in the mRNA levels of several nuclear receptors [pregnane X receptor (PXR), constitutive androstane receptor (CAR) and FXR] were reported in rodents treated with endotoxin (Beigneux et al., 2000; Beigneux et al, 2002; Kalitsky-Szirtes et al., 2004). Moreover, it has been suggested that IL-1β mediated down-regulation of MRP2 may occur, in part, through suppression of RXR (Denson et al., 2000; 2002). Thus, it is possible that nuclear receptor networks may play a role in the down-regulation of hepatic bile acid/salt transporters. However, little is known as to the involvement of nuclear receptors in the regulation of hepatic genes during inflammation.

Interestingly, many genes that are suppressed by endotoxin or other inflammatory stimuli have been shown to be induced by activation of the PXR. It is well-established that activation of PXR by hormones, bile acids or xenobiotics leads to the induction of several genes including cytochrome P450 (CYP) 3A (Bertilsson et al., 1998), OATP2 (Staudinger et al., 2003), MRP2

(Kast et al., 2002), MRP3 (Teng et al., 2003) and MDR1 (Geick et al., 2001). Studies in our laboratory have demonstrated that MDR1 and several members of the MRP and OATP families are down-regulated during inflammation (Hartmann et al., 2001; Sukhai et al., 2001; Lee and Piquette-Miller, 2001; Hartmann et al., 2002). Moreover, expression of CYP3A and other drug metabolizing enzymes are reduced under inflammatory conditions (Renton, 2001). Although studies have suggested the contribution of several transcription factors such as C/EBP (Jover et al., 2002) and HNF-1 (Memon et al., 2001; Roe et al., 2001), the mechanisms(s) involved in the down-regulation of these genes have not been firmly established. Furthermore, the potential involvement of PXR remains to be elucidated.

Hence, the main objective of this study was to investigate the role of the nuclear receptor PXR in mediating changes in the expression of hepatic bile salt/acid transporters and CYP3A during inflammation and to examine the specific cytokines involved. Findings from the study are the first to demonstrate involvement of PXR in BSEP regulation. Moreover, administration of the pro-inflammatory cytokine IL-6 reduced the hepatic expression of *mrp2*, *bsep* and *cyp3a11* in $PXR^{+/+}$ but not $PXR^{-/-}$ mice and attenuated the PCN-mediated induction of *mrp2*, *bsep* and *cyp3a11*. Hence, these novel findings suggest that, in addition to gene induction, PXR may play a role in the inflammation-mediated down regulation of hepatic transporters and drug metabolizing enzymes.

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METHODS

Chemicals. PCN, RU486, endotoxin (LPS from *E. coli* serotype 055:B5) and cytokines (recombinant mouse cytokines IL-6, IL-1β, TNF-α) were purchased from Sigma-Aldrich Canada (Burlington, ON).

Animals. The animal studies were conducted in accordance with the guidelines of the Canadian Council on Animal Care. Wild-type ($PXR^{+/+}$) male C57BL/6 mice (8 weeks old, 25-30 g) were purchased from Charles River Canada (St. Constant, QC). PXR-null ($PXR^{-/-}$) mice were kindly provided by Dr. Christopher Sinal (Dalhousie University, Halifax, NS) with permission from Dr. Steven Kliewer (University of Texas Southwestern Medical Center, Dallas, TX). Animals were kept in a temperature-controlled facility with 12 hour light/dark cycles and were fed a standard chow diet.

To investigate the genes regulated by PXR, mice were administered daily the PXR activators PCN (50 mg/kg i.p., 4 days), RU486 (50 mg/kg i.m., 3 days) or corn oil vehicle control and were sacrificed by cervical dislocation 24 hours following the last injection. The liver was removed, snap-frozen in liquid nitrogen and stored at –80^{o}C until analysis. These doses were not associated with hepatotoxicity as determined by normal serum alanine aminotransferase levels.

For the inflammation studies, mice were injected with endotoxin (5 mg/kg or 7.5 mg/kg i.p.), IL-6 (1000 to10000U i.p.), IL-1β (1000 or 10000U i.p.), TNF-α (10000 or 25000U i.p.) or saline vehicle control and were sacrificed 6 hours later. These doses were chosen as they were previously shown by our laboratory to be effective at modulating hepatic transporter expression

in mice without causing hepatotoxicity (Hartmann et al., 2001; 2002). For studies of the reversibility of PXR-mediated transporter induction, the mice were injected each day for 4 days with PCN or corn oil as described above. On the 5th day, the mice received doses of endotoxin, IL-6 or saline vehicle control and were sacrificed 6 hours later.

Determination of mRNA expression. Total RNA was isolated from mouse liver using the QuickPrep RNA extraction kit (Amersham Biosciences, Piscataway, NJ) according to manufacturer's protocol. cDNA was synthesized from 0.5µg RNA using the First Strand cDNA Synthesis kit (MBI Fermentas, Flamborough, ON). In the PCN and RU486 activation studies, mRNA levels of various genes was determined by semi-quantitative PCR with linear conditions established from standard curves as previously described (Hartmann et al., 2002). For the inflammation and cytokine studies, mRNA levels were measured by real-time quantitative PCR using LightCycler™ technology (Roche Diagnostics, Mannheim, Germany) with LC FastStart DNA Master SYBR® Green I. All significant results from the PCN and RU486 studies were also confirmed using real-time PCR. Primers were synthesized by the DNA Synthesis Centre, Hospital for Sick Children (Toronto, ON). Primer sequences are listed in Table 1. All mRNA levels were normalized to *gapdh* mRNA. Normalisation to either *gapdh* mRNA or 18S rRNA was found to give comparable results.

Determination of transporter protein expression. The hepatic crude membrane fraction was isolated as described previously (Hartmann et al., 2001) and measured by the Bradford method using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). For the immunoblotting, 10 µg of protein was loaded and run on a 10% acrylamide gel and transferred onto a

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Hybond^{TM}ECL^{TM} nitrocellulose membrane (Amersham). Membranes containing transporter protein were then cut in half (MW > 78kD), and the upper portion was incubated with H-17 MRP2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by bovine anti-goat IgG (Santa Cruz). To control for variability in protein loading, the lower portion of the membrane (MW < 78 kD) was incubated with anti-β-actin clone AC15 antibody (Sigma-Aldrich Canada, Burlington, ON) followed by sheep anti-mouse IgG (Amersham). Bands were detected using an ECL^{TM} western blotting analysis system (Amersham), imaged on Bioflex® MSI Film (Clonex Corp., InterSciences Inc., Markham, ON) and quantified using Kodak Digital Science 1D Image Analysis Software (Eastman Kodak, Rochester, NY).

Determination of PXR protein expression. Immunodetectable levels of PXR were examined in hepatic nuclear proteins isolated from endotoxin (5 mg/kg), IL-6 (10,000 U) and saline controls, 6 h after treatment. Livers were homogenized in ice-cold buffer (10 mM HEPES, 1.5mM $MgCl_2$, 10mM KCl, 0.3mM sucrose, 0.1mM EGTA, 0.5mM dithiothreitol, 0.5mM PMSF, 1mM sodium orthovanadate, 1µg/ml pepstatin, 5µg/ml leupeptin, 10µg/ml aprotinin, 1mM sodium fluoride and 0.1% Nonidet P-40) and centrifuged at 3000rpm for 20 minutes. Pellets were incubated on ice for 1.5 h in high salt buffer and centrifuged at 14000 rpm for 20 minutes. The supernatant was dialysed for 2 h against 20mM HEPES, 100mM KCl, 0.2mM EDTA, 0.5mM dithiothreitol, 0.5mM PMSF and 20% glycerol. The dialysate was centrifuged at 14000 rpm for 20 minutes, and the supernatant was collected and stored at -70^{o}C until analysis. Immunoblotting for PXR was performed as described above using the R-14 antibody (Santa Cruz).

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Statistical analysis. All studies were performed using n=4 or more. Differences between $PXR^{+/+}$ and $PXR^{-/-}$ mice and between treatment groups were determined by ANOVA and significance was determined by the Tukey test, with $p < 0.05$ considered to be statistically significant. Analysis was performed using SigmaStat 2.03 (SPSS Inc., Chicago, IL).

RESULTS

Examination of the effect of endotoxin and cytokines on PXR. To firstly determine if PXR expression is affected during an acute phase response, the effects of endotoxin (LPS) and pro-inflammatory cytokines on *PXR* was examined *in vivo* in $PXR^{+/+}$ mice. As shown in Figure 1, administration of 5 mg/kg endotoxin caused a significant down-regulation of *PXR* mRNA levels to 48 ± 9% of controls at 6 hours. Similar changes were seen after administration of 7.5 mg/kg. IL-6 also elicited a dose-dependent down-regulation of *PXR* to 48 ± 12% of controls using 10000U. Likewise, immunodetectable levels of PXR was decreased to 38 ± 14% and 54 ± 10% of controls by endotoxin and IL-6, respectively (Fig 1B). On the other hand, *PXR* mRNA was not significantly altered after administration of increasing doses of either IL-1β nor TNF-α.

Identification of transporter genes induced by PXR activation. To identify the transporters regulated by PXR, both PXR wild-type ($PXR^{+/+}$) and knock-out ($PXR^{-/-}$) mice were treated with the PXR activators PCN or RU486. As compared to $PXR^{+/+}$, basal levels of *mdr2* (1.5 fold higher), *mrp2* (1.5 fold higher), *mrp3* (3 fold higher) *cyp3a* (2 fold higher) were significantly elevated in the $PXR^{-/-}$ mice. As shown in Figure 2, PCN and RU486 treatments imposed a significant induction in the mRNA levels of the ABC transporters *bsep, mdr1a, mrp2* and *mrp3* in $PXR^{+/+}$ but not $PXR^{-/-}$ mice. Likewise, PXR activation with PCN or RU486 was found to significantly induce mRNA levels of the basolateral transporter *oatp2* in $PXR^{+/+}$ but not $PXR^{-/-}$

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mice. This demonstrates a role for PXR in the regulation of *bsep, mdr1a, mrp2, mrp3* and *oatp2*. Western blots also confirmed induction of MRP2 protein levels in PCN-treated $PXR^{+/+}$ but not $PXR^{-/-}$ mice (Figure 3a). On the other hand, the mRNA levels of the transporters *bcrp* (breast cancer resistance protein), *mdr1b*, *mdr2*, and *ntcp* were not significantly affected by PCN or RU486 treatments in either $PXR^{+/+}$ or $PXR^{-/-}$ mice. As controls to verify our animal and experimental models, we measured the expression of *cyp3a11* and *cyp7a1,* which are known to be induced and suppressed by PXR activation, respectively. As expected, mRNA levels of *cyp3a11* were significantly induced and *cyp7a1* significantly decreased in the PCN- and RU486-treated $PXR^{+/+}$ mice, whereas changes were not detected in the $PXR^{-/-}$ mice.

Comparison of endotoxin-mediated effects in $PXR^{+/+}$ and $PXR^{-/-}$ mice. In order to determine the involvement of PXR in mechanism(s) of transporter down-regulation during inflammation, we compared the effect of endotoxin on the expression of several transporters in $PXR^{+/+}$ versus $PXR^{-/-}$ mice. As shown in Table 2, the mRNA levels of *bsep, mdr2, mrp2, mrp3, ntcp, oatp2,* and *cyp3a11* were all significantly down-regulated by endotoxin administration in $PXR^{+/+}$ mice, with declines ranging from 18 to 61% of controls. In $PXR^{-/-}$ mice treated with endotoxin, the down-regulation of *mrp2* was significantly less (52 ± 7% of controls, $p < 0.05$) than in $PXR^{+/+}$ mice (18 ± 4% of controls, $p < 0.001$). Moreover, MRP2 protein expression was suppressed in $PXR^{+/+}$ but not in $PXR^{-/-}$ mice (Figure 3b). On the other hand, *mrp3*, *oatp2*, *bsep*, *ntcp, mdr2* and *cyp3a11* mRNA were down-regulated to the same extent in $PXR^{-/-}$ mice as in $PXR^{+/+}$ mice.

Comparison of cytokine-mediated effects in $PXR^{+/+}$ and $PXR^{-/-}$ mice. Previous studies have identified IL-6 as a principle mediator in inflammation-mediated down-regulation of hepatic

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transporters (Hartmann et al., 2002). As IL-6 was found to impose a significant and substantial suppression of *PXR* mRNA in mice, we next compared the impact of IL-6 on the expression of *cyp3a11* and transporters in $PXR^{+/+}$ versus $PXR^{-/-}$ mice. Administration of IL-6 to $PXR^{+/+}$ mice imposed a 28-63% down-regulation of *bsep*, *mrp2* and *cyp3a11*, whereas significant changes were not detected in IL-6 treated $PXR^{-/-}$ mice (Table 3). Furthermore, MRP2 protein levels were down-regulated in $PXR^{+/+}$ but not in $PXR^{-/-}$ mice. Although the IL-6 mediated down-regulation of *oatp2* was somewhat attenuated in $PXR^{-/-}$ mice, differences in *oatp2* mRNA levels between $PXR^{-/-}$ mice and $PXR^{+/+}$ mice did not reach statistical significance. IL-6 did not elicit a down-regulation of *ntcp* mRNA levels in either the $PXR^{+/+}$ or $PXR^{-/-}$ mice.

Since IL-1β did not have a significant effect on *PXR* mRNA levels, we examined expression of the *bsep*, *mrp2* and *cyp3a11* genes in IL-1β-treated $PXR^{+/+}$ and $PXR^{-/-}$ mice as a control for PXR-independent regulation. Indeed, while we detected down-regulation of these genes in the IL-1β treated mice, the extent of down-regulation of *bsep*, *mrp2* and *cyp3a11* was similar in both $PXR^{+/+}$ and $PXR^{-/-}$ mice (Table 4). In addition, as the down-regulation of *ntcp* by endotoxin occurs through IL-1β -mediated pathways and is dependent on transcription factors other than PXR, we also compared the effect of IL-1β on *ntcp* levels in $PXR^{+/+}$ and $PXR^{-/-}$ mice to further confirm PXR-independent gene suppression by IL-1β. As expected, *ntcp* down-regulation was similar in both types of mice.

As differences in down-regulation could be due to differences in the severity of the inflammation induced by endotoxin or IL-6, we examined the expression of two acute phase proteins in $PXR^{+/+}$ and $PXR^{-/-}$ mice. The induction of serum amyloid A and metallothionein were measured and

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were found to be similar for both $PXR^{+/+}$ and $PXR^{-/-}$ mice (3.5- to 5.8-fold induction, $p < 0.001$). Furthermore, because the substrate specificity and target genes of PXR overlap with those of FXR and CAR and each of these receptors requires heterodimer formation with RXR, compensatory increases in the expression of these other nuclear receptors in $PXR^{-/-}$ mice could contribute to differences in down-regulation between $PXR^{+/+}$ and $PXR^{-/-}$ mice. However, this does not appear to occur as comparisons of basal expression of *FXR*, *CAR* and *RXR* mRNA in $PXR^{+/+}$ and $PXR^{-/-}$ mice did not detect any significant difference between these two groups of mice (data not shown).

Impact of endotoxin or IL-6 in PCN-treated mice. To further investigate the relationship between inflammation and PXR-mediated regulation of transporter expression, we examined the effect of IL-6 administration on gene expression following PXR activation in PCN and RU486 pre-treated mice. As shown in Figure 4, *PXR* mRNA was increased by PCN pre-treatment and this induction was attenuated by administration of either endotoxin or IL-6. Correspondingly, administration of IL-6 attenuated the induction of *bsep*, *mrp2* and *cyp3a11* but not *oatp2* in PCN pre-treated mice. While RU486 pre-treatment imposed a smaller (1.4 to 1.6 fold) but significant induction of these genes, administration of IL-6 to these animals suppressed *bsep*, *mrp2* and *cyp3a11* expression by 60%, 25% and 85% respectively. Administration of endotoxin to PCN pre-treated mice also suppressed the induction of *mrp2* and *cyp3a11* mRNA.

DISCUSSION

Reductions in the transport of bile salts and acids have been shown to play a critical role in the development of endotoxin- or inflammation-induced cholestasis. Nuclear receptors play a key role in the regulation of genes responsible for the metabolism and transport of bile acids. Hence, the goal of this study was to investigate the role of the nuclear receptor PXR in mediating changes in the expression of hepatic bile salt/acid transporters during endotoxin or cytokine-induced inflammation. In this study, we demonstrated that endotoxin imposed a down-regulation in the hepatic expression of *PXR* mRNA and protein in mice. As administration of IL-6, but not IL-1β or TNF-α , suppressed the mRNA and protein expression of *PXR* , these results suggest that IL-6 is primarily responsible for the endotoxin-mediated down-regulation of *PXR*. This is consistent with our previous studies and those of others which have observed lower *PXR* levels *in vivo* in endotoxin-treated rodents and *in vitro* in IL-6 treated hepatocytes (Beigneux et al., 2002; Fang et al., 2004; Kalitsky-Szirtes et al., 2004; Pascussi et al., 2000; Xu et al., 2004). Moreover, IL-6 has been shown to be a major contributor to the regulation of organic anion transporters during endotoxemia (Siewert et al., 2004). As PXR is involved in the transcriptional activation of CYP3A and several hepatic transporters, it is thus possible that diminished basal levels of PXR protein available for binding to promoter regions could cause decreases in the transcription and expression of these genes. Indeed, in addition to the findings that PXR protein levels are suppressed at 6 hr, we also found that the down-regulation of PXR precedes the suppression of transporters. By 3 hrs after endotoxin treatment we observed significant reductions in *PXR* mRNA levels (36 ± 4% of controls), whereas *mrp2* mRNA were slightly but not significantly diminished (data not shown). This further suggests that transporter levels are at least partially dependent on PXR expression.

To date, the involvement of PXR in gene down-regulation by endotoxin has not been directly demonstrated. As endotoxin-induced changes were examined in $PXR^{+/+}$ versus $PXR^{-/-}$ mice, our study is the first to show that PXR does, indeed, play a part in gene down-regulation during inflammation. Firstly, the reduction of *mrp2* mRNA and protein expression after endotoxin administration was significantly less extensive in $PXR^{-/-}$ mice as compared to $PXR^{+/+}$ mice. Secondly, IL-6 significantly decreased *mrp2* expression in $PXR^{+/+}$ but not $PXR^{-/-}$ mice. Overall, these results indicate that *mrp2* down-regulation during inflammation is, at least in part, PXR-dependent and that both PXR-dependent and independent factors are involved. That PXR may be one of numerous contributors is not surprising as *mrp2* expression has been shown to be regulated by several transcription factors (Kast et al, 2002; Denson et al., 2002; Geier et al., 2003). Indeed, IL-1β has been shown to play a role in *mrp2* down-regulation in rats during cholestasis and could occur, in part, through nuclear receptors such as RXR (Denson et al., 2002).

The role of PXR in the regulation of two other important hepatic bile salt/acid transporters, *bsep* and *ntcp* was also examined. The bile salt export pump, *bsep*, is a canalicular efflux transporter of bile salts. Currently, little is known about the regulation of *bsep* other than that it is highly dependent on FXR (Plass et al., 2002; Wagner et al., 2003). However it has been hypothesized that suppression of *bsep* expression may contribute to bile acid accumulation during cholestasis (Zollner et al., 2001). Results from this study demonstrated that PXR activation induces *bsep* expression in PXR wild type but not knock-out mice. What's more, we found that the suppression of *bsep* by IL-6 in $PXR^{+/+}$ mice did not occur in $PXR^{-/-}$ mice and that PXR-mediated induction of *bsep* in PCN-treated mice could be attenuated by IL-6. Overall our study is the first

to demonstrate that PXR plays a role in the regulation of *bsep* both by PXR activation and through down-regulation by IL-6. Hence, PXR likely plays a role in bile acid feed-back regulation (Staudinger et al., 2001; Xie et al., 2001). On the other hand, our study also indicated that expression of the hepatic basolateral bile acid uptake transporter, *ntcp*, is neither affected by PXR activation in wild-type nor altered in PXR knock-out mice. Although regulation of *ntcp* via PXR has not previously been examined, cholestasis-induced changes have been frequently reported (Trauner *et al*, 1999). Our findings that endotoxin and IL-1β, but not IL-6, decreases *ntcp* expression is in agreement with the findings of others (Geier et al., 2003; Siewert et al., 2004) who showed that IL-1β but not IL-6 is an important mediator of *ntcp* suppression by endotoxin.

Our results also indicate that PXR plays a role in the down-regulation of the drug metabolizing enzyme, *cyp3a11*. Although *cyp3a11* was suppressed in both endotoxin-treated $PXR^{+/+}$ and $PXR^{-/-}$ mice, administration of IL-6 resulted in the suppression of *cyp3a11* mRNA in $PXR^{+/+}$ but not $PXR^{-/-}$ mice. Likewise IL-6 treatments attenuated the PXR-mediated induction of *cyp3a11* in PCN-treated mice. This suggests involvement of PXR in IL-6 mediated suppression of *cyp3a11*. It has been reported that IL-6 but not TNF-α or IL-1β diminish rifampicin-mediated induction of CYP3A4 *in vitro* (Muntane-Relat et al., 1995). Conversely, during endotoxemia IL-6 is thought to provide minimal contribution to *cyp3a11* regulation (Siewert et al., 2000). Hence it is likely that PXR-independent pathways predominate in endotoxemia. Indeed, it has been reported that *cyp3a11* down-regulation during endotoxemia likely occurs via PPARα (Barclay et al., 1999) . In addition, Xu et al. (2004) recently reported that reactive oxygen species produced by Kupffer cells are involved in CYP3A suppression by endotoxin. Thus activity of other inflammatory

mediators induced in endotoxemia likely mask IL-6 mediated effects. These results further emphasize the complexity of the signaling pathways involved in gene regulation following exposure to endotoxin.

In addition to requiring heterodimer formation with RXR, PXR has been shown to share much functional redundancy with the nuclear hormone receptors, FXR and CAR. Hence, an enhanced expression of FXR, CAR or RXR in $PXR^{-/-}$ mice could preserve levels of regulated genes during inflammation. Likewise compensatory increases in several transporters could also occur in knockouts in order to preserve homeostasis. We found that while basal levels of FXR, CAR and RXR were not significantly different between $PXR^{-/-}$ and $PXR^{+/+}$ mice, basal levels of *mrp2* and *cyp3a11* were higher in $PXR^{-/-}$ than in $PXR^{+/+}$ mice. This could possibly obscure relative changes in the expression of these genes. On the other hand, baseline levels of *mrp3* were also higher in $PXR^{-/-}$ mice, yet the extent of down-regulation by endotoxin or IL-6 was not different from $PXR^{+/+}$ mice. This indicates that higher initial gene expression does not necessarily correlate with a lesser impact of inflammation. Moreover, we observed a comparable induction of the acute phase proteins serum amyloid A and metallothionein I in the $PXR^{+/+}$ and $PXR^{-/-}$ mice after endotoxin or cytokine administration, indicating similarity in the effect and intensity of inflammation between the two strains.

Our findings indicated that PCN- and RU486-mediated activation of PXR induced *mdr1a* mRNA levels but did not significantly affect *mdr1b* expression. This suggests differential PXR-mediated regulation of *mdr1a* and *mdr1b*. This is consistent with the distinct regulation seen with other signaling pathways (Hartmann et al., 2001). While MDR1 induction is commonly cited to occur

via PXR activation, this has only been reported *in vitro* in human cell lines (Geick et al., 2001; Synold et al., 2001). A relatively new member of the ABC transporter family, the breast cancer resistance protein (BCRP; ABCG2), is also thought to be involved in drug resistance (Miyake et al., 1999). Very little is known about the regulation of this canalicular transporter. Our results indicate that PXR does not play a role in BCRP expression as mRNA levels were not affected by PXR activation. Moreover, similar basal levels of expression were seen in PXR-/-. Clearly, further studies are necessary to characterize the regulation of this transporter.

IL-6 and endotoxin administration to wild-type mice were found to suppress hepatic expression of PXR as well as attenuate PXR-mediated gene induction. Moreover, as IL-6 mediated changes in hepatic gene expression were subdued in PXR knock-out mice, our data suggests that inflammatory-mediated changes in expression of PXR-regulated genes may be secondary to the loss of PXR. The mechanism of PXR suppression by inflammatory stimulants remains unknown. Results in our laboratory show that HNF-4α is suppressed to less than 50% of controls by IL-6 in both $PXR^{+/+}$ and $PXR^{-/-}$ mice. Furthermore, a significant correlation ($r = 0.94$, $p<0.001$) between PXR and HNF-4α mRNA levels was found in control and IL-6 treated mice (unpublished results). Others have shown that HNF-4α is an important determinant of PXR expression during development (Li et al., 2000; Kamiya et al., 2003) but can also be critical for PXR-mediated induction of CYP3A4 (Tirona et al., 2003). Thus inflammation-induced changes in the expression of HNF-4α or its gene targets could be responsible for initiating changes in PXR expression, subsequently affecting the expression of PXR target genes. Studies of HNF-4α involvement are currently ongoing.

In summary, findings from this study demonstrate that PXR regulates the expression of numerous hepatic transporters. Moreover, since inflammation-mediated down-regulation of *mrp2, bsep* and *cyp3a11* are significantly altered in the absence of functional PXR, this nuclear receptor appears to play a role in the mechanism of down-regulation of these genes during inflammation. While a decrease in PXR expression could also occur parallel to changes in hepatic genes, the fact that IL-6 mediated suppression of several of these genes did not occur in $PXR^{-/-}$ mice suggest that PXR is required for this signaling pathway. Hence, transporter down-regulation may result due to decreased binding of PXR to promoter regions and reduced transcription. Thus, PXR expression may also play a role in inflammation-mediated down-regulation of hepatic bile transporters. These results place further value on the potential of PXR as a target for developing treatments for cholestasis.

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FOOTNOTE

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LEGENDS FOR FIGURES

Figure 1. Effect of inflammation and cytokines on hepatic PXR mRNA and protein expression. $PXR^{+/+}$ mice (n=4 to 6) were injected with saline vehicle control, endotoxin (i.p.), or cytokines (i.p.) at the doses indicated and were sacrificed 6 hours later. Hepatic *PXR* mRNA and protein levels were determined by RT-PCR and western blotting, respectively, as described in Methods. Protein levels were measured from mice treated with 5mg/kg endotoxin or 10000U IL-6. *p < 0.05 versus controls.

Figure 2. Induction of transporters by the PXR activators PCN or RU486. $PXR^{+/+}$ (solid bars) and $PXR^{-/-}$ (unfilled bars) mice (n=4 to 6) were injected daily with corn oil vehicle control, 50 mg/kg PCN (i.p. for 4 days) or 50mg/kg RU486 (i.m. for 3 days) and were sacrificed 24 hours following the final injection. Livers were removed and later analysed for mRNA levels by RT-PCR as described in Methods. The values represent the ratio of treated versus control, with control=1. *p < 0.05, ** p < 0.001 versus controls.

Figure 3. Effect of PCN, endotoxin and IL-6 on hepatic MRP2 protein expression in $PXR^{+/+}$ and $PXR^{-/-}$ mice. Mice were treated with (A) 50mg/kg/day PCN (i.p., 4 days), (B) 5 mg/kg endotoxin (i.p., 6 hr) or (C) 10000U IL-6 (i.p., 6 hr). Hepatic MRP2 protein levels were analysed by western blotting as described in Methods. Values represent the ratio of treated versus control, with control=1. *p < 0.05 versus controls.

Figure 4. Effect of endotoxin or IL-6 on gene expression in PCN pre-treated mice. Mice (n=4 to 6) were injected with 50 mg/kg PCN (i.p.) each day for 4 days. 6 hours prior to sacrifice on the

5th day, mice were injected i.p. with saline, 5mg/kg endotoxin or 10000U IL-6. Livers were removed and mRNA levels were determined by RT-PCR as described in Methods. As compared to untreated controls, PCN treatments imposed a significant induction of *bsep*, *mrp2*, *oatp2* and *cyp3a11*. *$p < 0.05$, **$p < 0.001$ as compared to controls. #$p < 0.05$ as compared to PCN pre-treated mice.

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Table 1. List of primer sequences used for PCR.

Gene	Primers
mrp2	fwd: 5' – CTGAGTGCTTGGACCAGTGA – 3'
	rev: 5' – CAAAGTCTGGGGGAGTGTGT – 3'
mrp3	fwd: 5' – CGCTCTCAGCTCACCATCAT – 3'
	rev: 5' – GGTCATCCGTCTCCAAGTCA – 3'
oatp2	fwd: 5' – CCTTAAAGCCAACGCAAGAC – 3'
	rev: 5' – CACTCCTGCACAGACCAAAA – 3'
bsep	fwd: 5' – GTTCAGTTCCTCCGTTCAAA – 3'
	rev: 5' – AAGCTGCACTGTCTTTTCAC – 3'
ntcp	fwd: 5' – ACACTGCGCTCAGCGTCATTC – 3'
	rev: 5' – GCCAGTAAGTGTGGTGTCATG – 3'
mdr2	fwd: 5' – CTCGTTAACATGCAGACAGCAG – 3'
	rev: 5' – GACCAGGGAGAACATGTTACAC – 3'
mdr1a	fwd: 5' – CCCATCATTGCGATAGCTGG – 3'
	rev: 5' – TCCAACATATTCGGCTTTAGGC-3'
mdr1b	fwd: 5' – TGCTTATGGATCCCAGAGTGAC – 3'
	rev: 5' – TTGGTGAGGATCTCTCCGGCT-3'
cyp3a11	fwd: 5' – CGCCTCTCCTTGCTGTCACA – 3'
	rev: 5' – CTTTGCCTTCTGCCTCAAGT – 3'
pxr	fwd: 5'- GACGCTCAGATG CAAACCTT - 3'
	rev: 5'- TGGTCCTCAATAGGCAGGTC - 3'
gapdh	fwd: 5'- CCATCACCATCTTCCAGGAG – 3'
	rev: 5'- CCTGCTTCACCACCTTCTTG – 3'
serum amyloid A	fwd: 5'- GCGAGCCTACACTGACATGA - 3'
	rev: 5'- GGCAGTCCAGGAGGTCTGTA - 3'
metallothionein	fwd: 5' – GGTACCCCAACTGCTCCTGC – 3'
	rev: 5' – AAGCTTTGCAGACACAGCCC – 3'

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Table 2. Effect of endotoxin on transporter expression in $PXR^{+/+}$ versus $PXR^{-/-}$ mice.

	mRNA	
Gene	**$PXR^{+/+}$**	**$PXR^{-/-}$**
bsep	* 61 (6)	* 68 (3)
mdr1a	86 (6)	79 (18)
mdr1b	103 (3)	97 (11)
mdr2	* 37 (6)	* 48 (8)
mrp2	**18 (4)	* [a]52 (7)
mrp3	* 60 (3)	* 54 (2)
ntcp	**28 (11)	**19 (6)
oatp2	* 44 (7)	* 50 (5)
cyp3a11	* 43 (6)	* 49 (5)
PXR	* 48 (9)	--

Mice (n=4 to 6) were injected with saline vehicle control or 5 mg/kg endotoxin (i.p.), and were sacrificed 6 hours later. Levels of mRNA were determined by RT-PCR as described in Methods. Data is presented as % controls, mean (SEM) *$p < 0.05$, **$p < 0.001$ as compared to controls.

[a]significantly different ($p < 0.05$) versus endotoxin-treated $PXR^{+/+}$ mice.

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Table 3. Effect of IL-6 on transporter expression in $PXR^{+/+}$ versus $PXR^{-/-}$ mice.

	mRNA	
Gene	**$PXR^{+/+}$**	**$PXR^{-/-}$**
bsep	* 55 (9)	81 (8)
mdr1a	83 (8)	86 (5)
mdr1b	71 (8)	69 (13)
mdr2	68 (9)	63 (15)
mrp2	* 68 (6)	98 (7)
mrp3	70 (13)	86 (5)
ntcp	87 (11)	96 (8)
oatp2	**37 (6)	* 53 (8)
cyp3a11	* 72 (6)	88 (6)
PXR	* 48 (12)	--

Mice (n=4 to 6) were injected with saline vehicle control or 10000U IL-6 (i.p.) and were sacrificed 6 hours later. Levels of mRNA were determined by RT-PCR as described in Methods. Data is presented as % controls, mean (SEM). *$p < 0.05$, **$p < 0.001$ as compared to controls.

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Table 4. Effect of IL-1β on transporter expression in $PXR^{+/+}$ versus $PXR^{-/-}$ mice.

	MRNA	
Gene	**$PXR^{+/+}$**	**$PXR^{-/-}$**
bsep	*69 (4)	*59 (6)
mrp2	*64 (5)	*75 (3)
ntcp	*56 (5)	*65 (3)
cyp3a11	*55 (7)	*46 (6)
pxr	81 (7)	--

Mice (n=4 to 6) were injected with saline vehicle control or 10000U IL-1β (i.p.) and were sacrificed 6 hours later. Levels of mRNA were determined by RT-PCR as described in Methods. Data is presented as % controls, mean (SEM) *$p < 0.05$ as compared to controls.

Figure 1

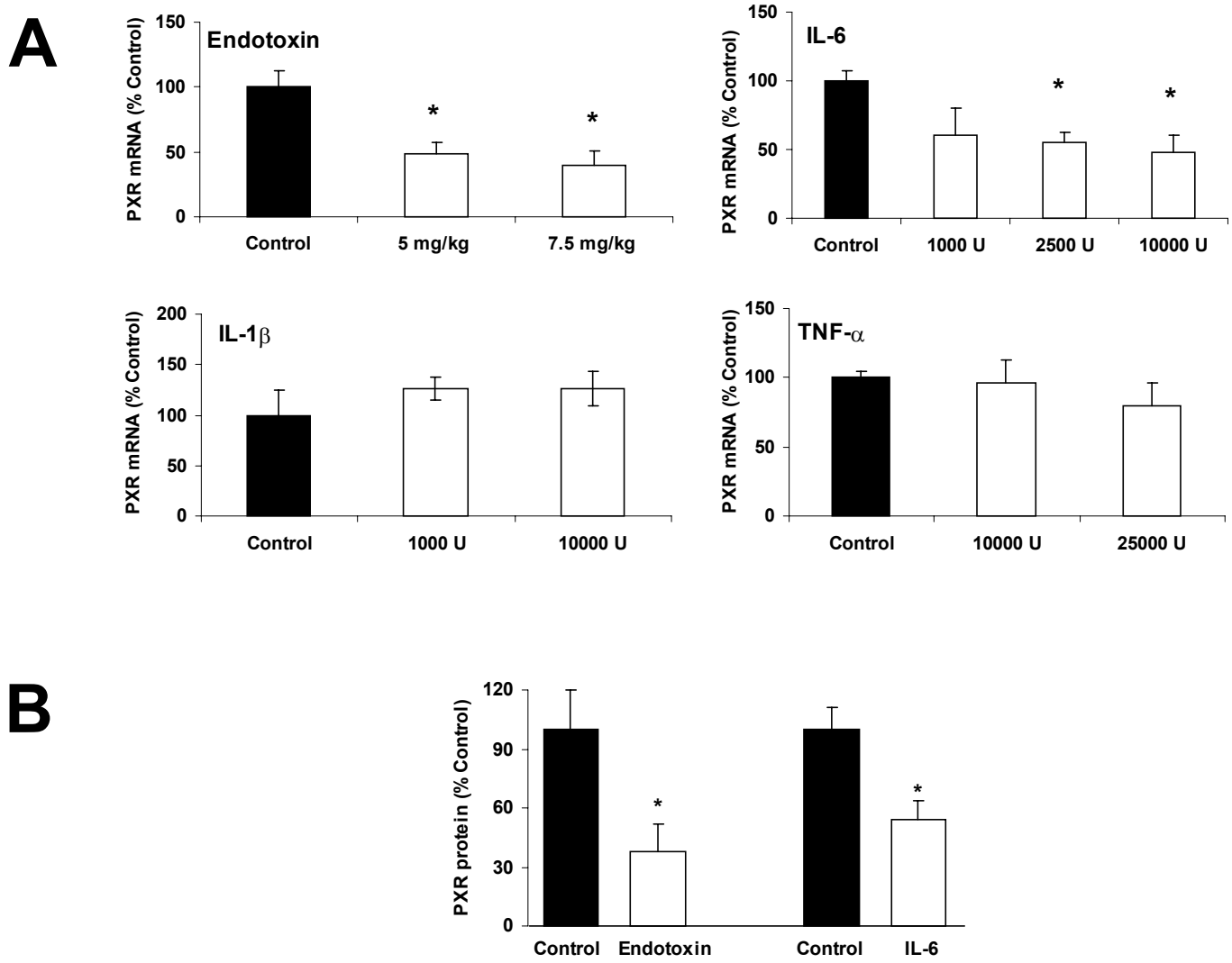

A
Endotoxin
PXR mRNA (% Control)
150
100
50
0
*
*
Control
5 mg/kg
7.5 mg/kg
IL-6
PXR mRNA (% Control)
150
100
50
0
*
*
Control
1000 U
2500 U
10000 U
IL-1β
PXR mRNA (% Control)
200
150
100
50
0
Control
1000 U
10000 U
TNF-α
PXR mRNA (% Control)
150
100
50
0
Control
10000 U
25000 U
B
PXR protein (% Control)
120
90
60
30
0
*
*
Control
Endotoxin
Control
IL-6

Figure 2

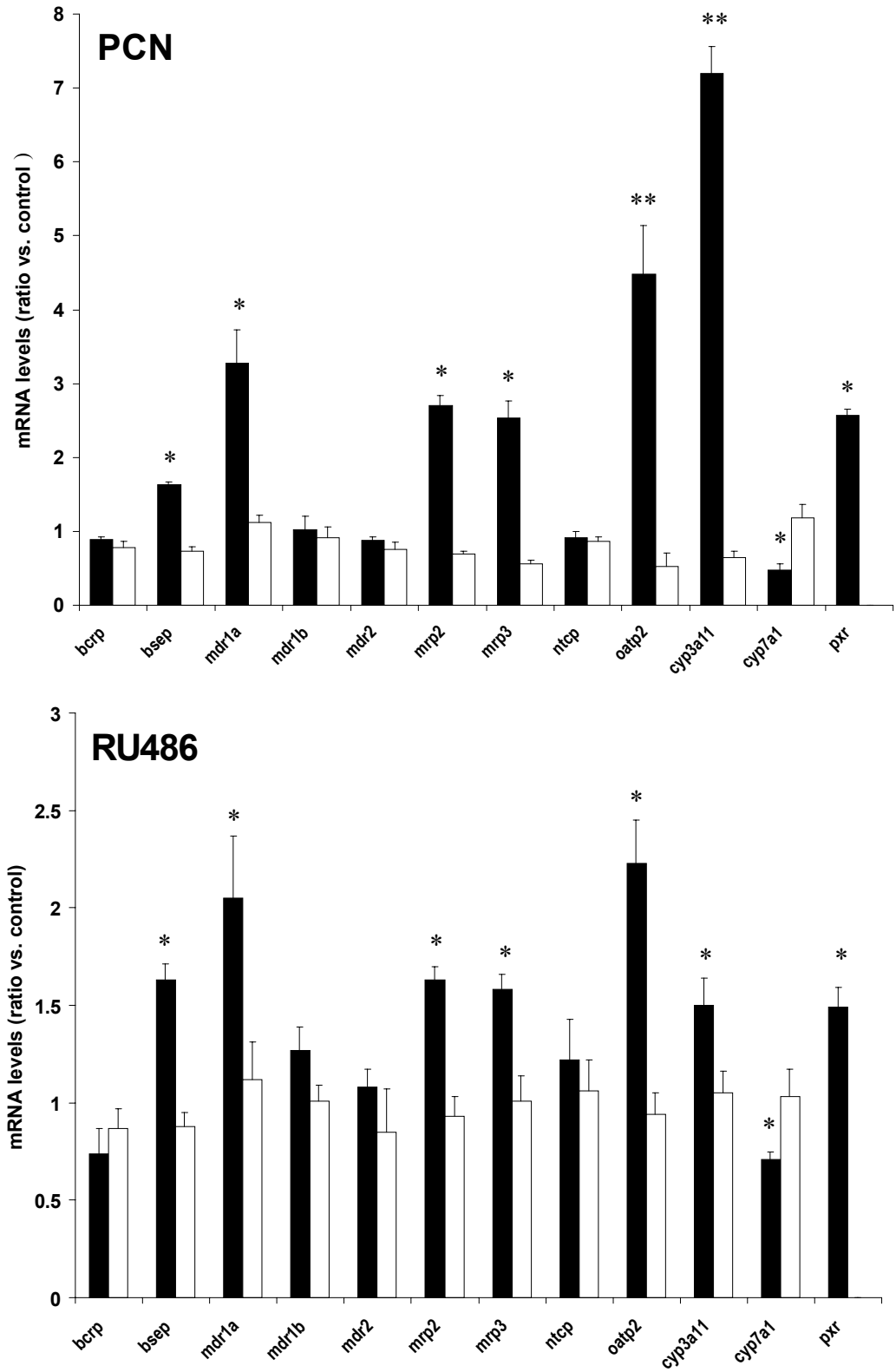
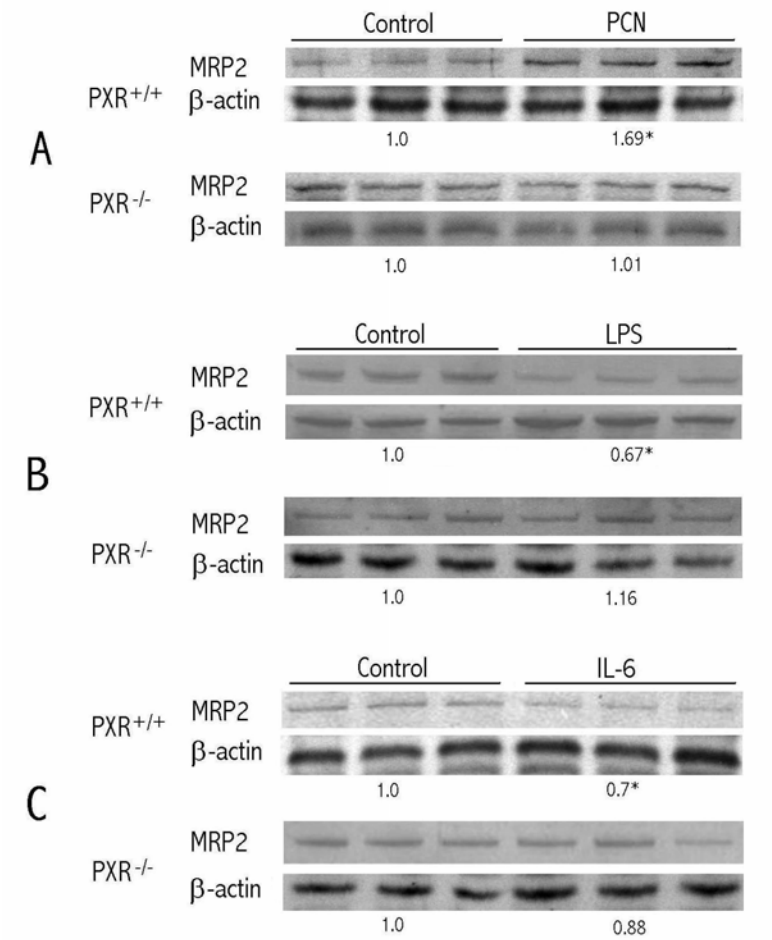

PCN
mRNA levels (ratio vs. control)
0
1
2
3
4
5
6
7
8
bcrp
bsep
mdr1a
mdr1b
mdr2
mrp2
mrp3
ntcp
oatp2
cyp3a11
cyp7a1
pxr
RU486
mRNA levels (ratio vs. control)
0
0.5
1
1.5
2
2.5
3
bcrp
bsep
mdr1a
mdr1b
mdr2
mrp2
mrp3
ntcp
oatp2
cyp3a11
cyp7a1
pxr

Figure 3


A
PXR+/+
MRP2
β-actin
Control
PCN
1.0
1.69*
PXR-/-
MRP2
β-actin
1.0
1.01
B
PXR+/+
MRP2
β-actin
Control
LPS
1.0
0.67*
PXR-/-
MRP2
β-actin
1.0
1.16
C
PXR+/+
MRP2
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Control
IL-6
1.0
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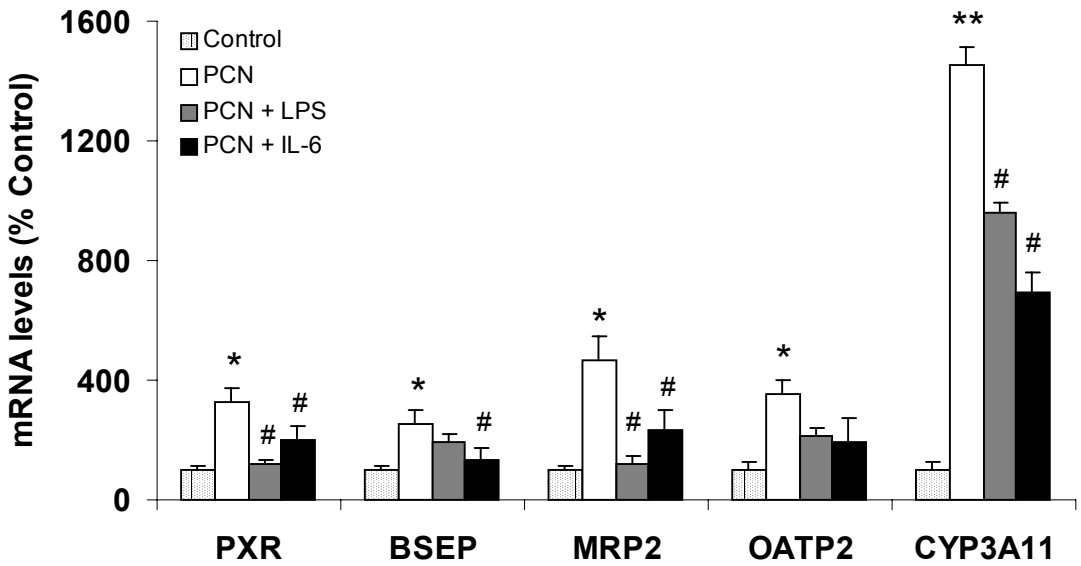
PXR-/-
MRP2
β-actin
1.0
0.88

Figure 4


1600
1200
800
400
0
mRNA levels (% Control)
Control
PCN
PCN + LPS
PCN + IL-6
*
#
#
*
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#
*
#
#
*
**
#
#
PXR
BSEP
MRP2
OATP2
CYP3A11