HEPATIC CYTOCHROME P450 GENE REGULATION DURING ENDOTOXIN-INDUCED INFLAMMATION IN NUCLEAR RECEPTOR KNOCKOUT MICE

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Running Title: Hepatic P450 down-regulation in nuclear receptor knockouts

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Number of text pages: 36
Number of tables: 1
Number of figures: 6
Number of references: 39
Number of words: Abstract: 234

Introduction: 703
Discussion: 1498

Abbreviations: CYP, cytochrome P450; PPARα, peroxisome proliferator activated receptor alpha; PXR, pregnane X receptor; IL, interleukin; TNFα, tumor necrosis factor alpha; LPS, lipopolysaccharide; AGP, α1-acid glycoprotein; FBG, fibrinogen alpha polypeptide; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde dehydrogenase; PCR, polymerase chain reaction
Recommended Section Assignment: Absorption, Distribution, Metabolism, & Excretion
Inflammatory agents such as lipopolysaccharide (LPS) down-regulate the hepatic expression of many cytochrome P450 (CYP) mRNAs and proteins. Previous studies suggested that suppression of some CYP mRNAs could involve the regulation or modulation of the nuclear receptors peroxisome proliferator activated receptor alpha (PPARα) or pregnane X receptor (PXR). To determine the involvement of these receptors in CYP down-regulation, PPARα knockout (KO), PXR KO, and appropriate wildtype (WT) mice were administered either saline or 1 mg/kg LPS. Hepatic mRNA and protein expression of several CYP isoforms, interleukin (IL)-1β, IL-6, tumor necrosis factor alpha (TNFα), α1-acid glycoprotein (AGP), and fibrinogen (FBG) were examined 16 hours later. LPS administration significantly decreased the hepatic expression of CYP1A2, 2A5, 2C29, 2E1, 3A11, 4A10, and 4A14 mRNAs in both groups of PPARα and PXR mice, whereas CYP3A13 mRNA was increased slightly in PPARα WT and KO mice, but not in PXR mice. Effects of LPS administration on mouse hepatic CYP proteins (probed using rat P450 2C, 3A, 4A, and 2E antibodies) were consistent with mRNA results in most cases. LPS treatment significantly increased IL-1β, IL-6, TNFα, AGP, and FBG mRNA in both PPARα and PXR mice, with the greatest effect observed with TNFα. Because decreases in CYP mRNA expression were essentially identical in both WT and KO mice for both nuclear receptors, these data indicate that down-regulation of CYP during inflammation does not require the nuclear receptors PPARα and PXR.
INTRODUCTION

Cytochromes P450 (CYP) are drug-metabolizing enzymes that oxidize numerous endogenous and foreign compounds, including the majority of therapeutic agents, resulting in drug activation or inactivation. CYP gene expression is regulated by several factors, including gender, microsomal enzyme inducers, age, diet, and hormones. During inflammation and infection both CYP expression and metabolic activities in liver and extrahepatic tissues can be down-regulated (reviewed by Morgan, 2001; Renton, 2004); however, some CYP activities are induced or unchanged. As such, alterations in CYP expression and activities during inflammation can lead to increased or decreased drug efficacy or changes in the metabolism of physiological substrates.

Injection of bacterial lipopolysaccharide (LPS) is a widely used model of inflammation, and is well characterized regarding its effects on basal and inducible hepatic CYP expression. A prominent feature of this inflammatory response is the cytokine-mediated induction of acute phase proteins. Type I acute phase proteins are induced by interleukin (IL)-1-like cytokines (IL-1α, IL-1β, tumor necrosis factor alpha [TNFα], TNFβ) and include serum amyloid A and α1-acid glycoprotein. In contrast, type II acute phase proteins are induced by IL-6-like cytokines (such as IL-6 and oncostatin M) and include fibrinogen and α1-antitrypsin (Moshage, 1997). In vitro and in vivo studies show decreased CYP mRNA and protein and induction of acute phase proteins after treatment of rodents or hepatocytes with LPS or the cytokines IL-1β, IL-6, and TNFα (Morgan,
Due to the complexity of the inflammatory response, the in vivo contributions of individual cytokines are difficult to determine. Studies using cytokine- or cytokine receptor-null mice to investigate LPS-mediated CYP down-regulation have reported differential dependence of CYP down-regulation on cytokines, contingent on the CYP subfamily or model of inflammation being studied (Warren et al., 1999; Seiwert et al., 2000; Ashino et al., 2004). As such, LPS-mediated CYP down-regulation is regulated through multiple pathways.

There is some evidence that hepatic CYP down-regulation during inflammation may be mediated by modulation of nuclear receptors. Drug-induced transcription of CYP is mediated by nuclear receptors, including the peroxisome proliferator activated receptor alpha (PPARα, NR1C1) and the pregnane X receptor (PXR, NR1I2). Reductions in mRNA levels of PPARα, PXR, retinoid X receptor (RXR), and liver X receptor (LXR) have been recently reported in liver and intestine of rodents treated with LPS (Beigneux et al., 2000; Kalitsky-Szirtes et al., 2004), and these findings have been associated with CYP down-regulation. An earlier study from our laboratory found that LPS down-regulation of hepatic CYP2A5, 2C29, and 3A11 mRNAs was attenuated in PPARα knockout (KO) mice (Barclay et al., 1999). Moreover, Beigneux et al. (2002) associated a reduction in CYP3A and 2B10 mRNA with decreases in the expression of PXR, the constitutive androstane receptor (CAR), and RXRα after LPS treatment, with similar results found for PXR and CYP3A11 (Xu et al., 2004). A recent microarray investigation of nuclear receptors indicated down-regulation of PPARα, PXR, CAR, RXRα,
LXR, and farnesoid X receptor after LPS treatment of Wistar rats (Fang et al., 2004). Most recently, Teng and Piquette-Miller (2005) studied the role of PXR in down-regulation of hepatic transporters and of CYP3A11 during LPS inflammation. They found that down-regulation of multidrug resistance associated protein 2 (mrp2) was attenuated in PXR KO mice, suggesting a role for PXR in its down-regulation during inflammation. On the other hand, down-regulation of five other drug transporters and of CYP3A11 showed no evidence of a requirement for PXR in the LPS model. Altogether these observations show down-regulation of nuclear receptors during LPS-induced inflammation and associate down-regulation of CYP with decreased receptor levels, as well as down-regulation of inducible CYP. Although the correlation of down-regulation of nuclear receptors with CYP down-regulation may be suggestive of a mechanistic connection, receptor knockout models suggest a minimal role for nuclear receptors in the constitutive expression of CYP genes. We sought to definitively determine the involvement of the nuclear receptors PPARα and PXR in mediating down-regulation of several constitutive hepatic CYP isoforms during inflammation by comparing the responses in wildtype (WT) and knockout mice after LPS treatment. Possible effects of the genetic modifications of the overall inflammatory response of the liver were studied via the expression of proinflammatory cytokines (IL-1β, IL-6, TNFα) and hepatic acute phase proteins (α1-acid glycoprotein, fibrinogen).
MATERIALS AND METHODS

Chemicals, Animals and Treatments. Unless otherwise specified, all chemicals were obtained from Sigma Chemical (St. Louis, MO). Female PPARα wildtype (129S1/SvImJ) and PPARα knockout (129S4/SvJae-Ppara\textsuperscript{tm1Gonz}/J) mice (20 g) were obtained from Jackson Laboratory (Bar Harbor, ME). PXR wildtype (C57BL/6N) mice (20 g) were obtained from Taconic (Germantown, NY) and PXR knockout mice were generously provided by Dr. Bryan J. Goodwin (GlaxoSmithKline, Inc., Research Triangle Park, NC). All mice were 8 weeks of age at the time of experimentation. Animals were acclimatized to the animal facility for 1 week, and were provided rodent chow and water ad libitum until 8 hours before injection. Because lipopolysaccharide (LPS) causes a reduction in food intake in mice (Kozak et al., 1994), which may itself modulate CYP expression, animals were fasted before and after injection to eliminate this variable. \textit{Escherichia coli} LPS, serotype 0127:B8 (Sigma Chemical, St. Louis, MO) was dissolved in sterile 0.9% saline and mice were injected intraperitoneally with 1 mg/kg LPS or saline. We have previously demonstrated that this dose of LPS produces a maximal suppression of total CYP and rat CYP2C11 (Morgan, 1989), and induces CYP4A expression in rat liver (Sewer et al., 1996, 1997; Mitchell et al., 2001). At 16 hours after injection, livers were collected and stored at -80°C until RNA or microsome preparation. This time point was chosen based on previous experiments reporting LPS-mediated down-regulation of nuclear receptors at 16 hours (Beigneux et al., 2002). The Institutional Animal Care and
Use Committee of Emory University approved these procedures. Five or six mice were used in each group (n = 5, PPARα; n = 6, PXR).

**Preparation of Total RNA.** Total RNA was prepared using RNA-Bee isolation reagent according to the manufacturer’s instructions (Tel-test, Friendswood TX). Total RNA concentration was determined spectrophotometrically by measuring absorbance at 260 nm, and RNA purity and integrity was confirmed by formaldehyde-agarose gel electrophoresis followed by visualization with ethidium bromide.

**Microsome Preparation.** Liver microsomes were prepared by differential centrifugation and stored at -80°C (Haugen and Coon, 1976). Microsomal protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

**cDNA Synthesis.** Purified total RNA was reverse-transcribed using the SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen Life Technologies, Carlsbad, CA), according to the manufacturer’s protocol. Briefly, 0.5 µg of total RNA was mixed with oligo(dT)₁₂₋₁₈ primers (0.5 µg/µl), 1 µl of dNTP mix (10 mM of each dNTP), and water to a volume of 10 µl, and incubated at 65°C for 5 min. Following an incubation on ice for 1 min, 4 µl 10X RT buffer, 2 µl of 50 mM MgCl₂, 2 µl of 0.1M dithiothreitol, 1 µl of RNAse OUT™ (recombinant RNAse inhibitor; 2 U/µl), and 0.5 µl Superscript II reverse transcriptase
(50 units/µl) was added to each vial (final volume 20 µl). Each reaction mixture was incubated at 42°C for 50 min, and then at 70°C for 15 min to inactivate the transcriptase enzyme. One µl RNase H (2 U/µl) was added, and the samples were incubated for 20 min at 37°C (to remove RNA from the final preparation).

**Primer Sequences.** Primers for mouse CYP, cytokines, acute phase proteins, and glyceraldehyde dehydrogenase (GAPDH) were designed using the Primer Select software program (Lasergene). To exclude cross-reactivity with other mouse CYP sequences, as well as other enzymes, all primers were submitted to the National Center for Biotechnological Information (NCBI) for nucleotide comparison by the basic local alignment search tool (BLASTn; Altschul et al., 1990). Oligonucleotides with a high degree of similarity (>80%) to other mouse CYP mRNA transcripts were eliminated from further consideration. Primers were custom-synthesized on a 50-nmol scale by MWG Biotech, Inc. (High Point. NC), and obtained desalted and lyophilized. Primers were diluted to 100 µM in deionized water, and stored at -80°C. Designed primer sequences are listed in Table 1; other primer sequences used (CYP1A2, 2E1, 3A11, IL-1β) were published previously (Pan et al., 2000; Overbergh et al., 2003). In addition to the GAPDH primers listed in Table 1, other GAPDH primers were designed for use at annealing temperatures corresponding to the various primer sets.

**Quantitative Reverse Transcriptase PCR (real-time PCR).** Real-time RTPCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied
Biosystems, Bedford, MA), to determine CYP mRNA expression in mouse liver. Reactions were performed in a total volume of 25 µl using SyBr Green Master Mix reagent (Applied Biosystems); 2 µl of cDNA/sample was used as template for the reaction, with 10 µM forward and reverse primers. Both CYP and GAPDH amplification was done in duplicate wells using the same sample. Thermal cycling conditions included 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 1 min at the appropriate annealing temperature for each CYP (listed in Table 1). This technique allows, by means of fluorescence emission, identification of the cycling point when PCR product is detectable (threshold cycle or C_t value). To normalize the amount of total mRNA present in each reaction, levels of the housekeeping gene GAPDH were monitored in parallel samples. Results are expressed as relative levels of CYP mRNA, referred to as control samples (the calibrator), chosen to represent 1x expression of the gene. The amount of target (CYP in treated sample), normalized to an endogenous reference (GAPDH) and relative to the calibrator (control CYP sample), was defined by the C_t method as described by Livak and Schmittgen (2001). All primer sets yielded a single PCR product of expected size by agarose gel electrophoresis, and specificity was routinely monitored by checking product melting curves (dissociation curves) in each reaction well.

**Western Immunoblotting.** CYP protein levels in mouse hepatic microsomes were measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting. Ten micrograms of protein sample was separated on a 7%
polyacrylamide gel (NuPAGE Tris-acetate gel; Invitrogen) and transferred
electrophoretically (Xcell II blotting apparatus; Invitrogen) at 40 V for 1.5 h onto a
nitrocellulose membrane (Schleicher & Schuell, Keene, NH). Blots were
incubated in PBS containing 0.05% Tween 20 (PBS-Tween) and 3% bovine
serum albumin overnight and followed by incubation with primary antibody in
PBS-Tween for 1 h at room temp. Bound antibodies were detected using
horseradish peroxidase-coupled secondary antibodies (Jackson
ImmunoResearch Laboratories, West Grove, PA) in PBS-Tween and the
enhanced chemiluminescence detection system (Amersham Biosciences,
Piscataway, NJ) according to the manufacturer’s instructions. Antibodies to rat
CYP3A2, 4A1, and 2E1 were generously provided by Dr. James Halpert
(University of Texas Medical Branch, Galveston, TX), Dr. Gordon Gibson
(University of Surrey, Guildford, UK), and Dr. Magnus Ingelman-Sundberg
(Karolinska Institute, Stockholm, Sweden), respectively. Polyclonal antibodies to
rat CYP3A2, 4A1, and 2E1 proteins were diluted 1:5000, whereas 2C11 antibody
(Morgan et al., 1994) was diluted 1:20000. Secondary antibodies were as
follows: goat anti-rabbit, 3A, 2C, and 2E; rabbit anti-sheep, 4A; dilution for each
was 1:2500, with the exception of 2C, which was 1:10000. All assays were
performed within a linear range and the intensity of stained bands was measured
by laser densitometry.

**Statistical Analysis.** Control and experimental groups were compared by
Student’s t-test (p<0.05).
RESULTS

Effect of LPS Treatment on Hepatic CYP mRNA and Protein Expression in PPARα Wildtype and Knockout Mice.

LPS administration significantly down-regulated hepatic CYP1A2, 2A5, 2C29, 2E1, and 3A11 mRNAs in PPARα WT mice. Similar responses were observed in PPARα KO mice for all these mRNAs (Fig. 1). Of the CYP isoforms studied, CYP2A5 mRNA was the most affected by LPS exposure (7% of control) and 2C29 mRNA was least affected (46% of control). PPARα KO mice had slightly higher basal levels of CYP2C29 mRNA compared to WT mice. In contrast to the other isoforms, CYP3A13 mRNA expression in PPARα WT mice increased significantly after LPS exposure (153%), and was increased in PPARα KO mice (139%) as well, although the latter comparison did not reach significance. Expression of CYP4A10 and 4A14 mRNAs was reduced by LPS treatment to 19% and 29% of control in PPARα WT mice, respectively. CYP4A mRNA expression in PPARα KO mice was barely measurable by sensitive real-time PCR methods (control levels, 0.0003 relative units).

In general, effects of LPS on CYP proteins (2C, 3A, 4A, and 2E) corresponded with real-time PCR results for the CYP mRNAs (Fig. 2). LPS administration decreased hepatic CYP2C, 3A, and 2E proteins in both PPARα WT and KO mice, although the decrease was not significant in WT mice for CYP2C (Fig. 2). LPS tended to decrease CYP4A protein expression in PPARα WT mice,
although these effects were not statistically significant, and CYP4A proteins were undetectable in KO mice.

**Effect of LPS Treatment on Hepatic Cytokine and Acute Phase Protein mRNA Expression in PPARα Wildtype and Knockout Mice.**

As expected, LPS administration increased mRNA expression of proinflammatory cytokines and acute phase proteins (Fig. 3). LPS significantly induced mRNAs for IL-1β, IL-6, TNFα, AGP, and FBG in livers of PPARα WT mice by 380%, 1013%, 2615%, 652%, and 745%, respectively. LPS also induced the same mRNAs in PPARα KO mice, although the level of induction was slightly attenuated in each case.

**Effect of LPS Treatment on Hepatic CYP mRNA and Protein Expression in PXR Wildtype and Knockout Mice.**

LPS exposure significantly decreased hepatic expression of CYP1A2, 2A5, 2C29, 2E1, 3A11, 4A10, and 4A14 mRNAs in PXR WT and KO mice (Fig. 4). CYP2A5 mRNA was most affected by LPS exposure (13% of control) and 2E1 mRNA levels were least affected (43% of control), with higher basal CYP2A5 and 2E1 mRNA in PXR KO mice as compared to WT controls. Additionally, basal 3A11 mRNA levels were 2-fold higher in PXR KO mice (Fig. 4). In contrast, there was little effect of LPS treatment on basal CYP3A13 mRNA expression in PXR WT and KO mice.
CYP protein expression in PXR WT and KO mice showed some similarities and some differences from the mRNA results. Treatment with LPS tended to decrease hepatic CYP2C proteins in both PXR WT and KO mice (Fig. 5), although these decreases were not significant. CYP3A proteins were significantly down-regulated in PXR WT mice, but this did not reach statistical significance in KO mice. There was little effect on CYP2E proteins after 16 hours of LPS exposure (Fig. 5), in contrast to the effects on CYP2E1 mRNA levels (Fig. 4). CYP4A proteins in PXR WT mice were the most significantly affected after LPS administration, although variability in individual protein samples prevented this significance in PXR KO mice.

Effect of LPS Treatment on Hepatic Cytokine and Acute Phase Protein mRNA Expression in PXR Wildtype and Knockout Mice.

LPS treatment tended to induce IL-1β and IL-6 mRNA expression in PXR WT and KO mice, although the increases were not significant (Fig. 6). LPS exposure induced TNFα mRNA in PXR WT (406%), and had a greater response in PXR KO mice (638% of control). As expected, hepatic mRNA expression of AGP was significantly increased after LPS treatment in both WT (549%) and KO (1068%) mice. Similar results were observed with FBG in PXR WT (280%) and KO (582%) mice. Overall, the responses tended to be slightly greater in the PXR KO mice.
DISCUSSION

Infection or inflammatory stimuli such as lipopolysaccharide (LPS) can alter hepatic cytochrome P450s (CYP) at the mRNA and protein levels, resulting in changes in both expression and activities (Morgan, 2001). Drug-induced CYP transcription is controlled by nuclear receptors, which may be involved in CYP down-regulation during inflammation. In this study, we sought to determine the involvement of the nuclear receptors PPARα and PXR in the down-regulation of several CYP isoforms after LPS-induced inflammation using mice deficient in these nuclear receptors. Our data show down-regulation of mRNA expression of CYPs 1A2, 2A5, 2C29, 2E1, and 3A11 during inflammation in WT mice, with essentially identical results in both PPARα and PXR KO mice, indicating that down-regulation of these CYPs during inflammation is not a consequence of down-regulation of the nuclear receptors PPARα and PXR.

The nuclear receptor PPARα has been implicated in inflammatory pathways, and induction of PPARα target genes may be important in termination of the action of inflammatory mediators (Morgan, 2001; Barbier et al., 2004). PPARα primarily regulates lipid metabolism, glucose homeostasis and stimulates the β-oxidative degradation of fatty acids (Chinetti et al., 2000). Target genes of PPARα include CYP4A subfamily enzymes, β-oxidation enzymes, and fatty acid binding proteins (Aoyama et al., 1998). During LPS-induced inflammation, both hepatic and renal CYP4A mRNAs are induced in rats (Sewer et al., 1996, 1997; Mitchell et al., 2001), whereas in this study we observed down-regulation of hepatic CYP4A10.
and 4A14 mRNAs in PPARα WT mice after LPS exposure (Fig. 1) in agreement with our previous report (Barclay et al., 1999). It could be speculated that down-regulation of CYP4A mRNA after LPS exposure could be directly linked to down-regulation of PPARα mRNA levels. Although hepatic PPARα mRNA levels were not measured in our mice, Tai et al. (2003) observed down-regulation of PPARα mRNA in female mice (50% of control) 2 hours after LPS exposure, with recovery to baseline levels at 24 hours. Therefore, it seems unlikely that the extensive down-regulation of CYP4A10 and 4A14 mRNAs after a 16-hour LPS exposure (to 19% and 29% of control) is due solely to down-regulation of PPARα mRNA.

Our previous investigation found that LPS treatment down-regulated hepatic CYP2A5, 2C29, and 3A11 mRNA in PPARα WT mice, and that the effects on these CYP isoforms were attenuated or blocked in PPARα KO mice (Barclay et al., 1999). In contrast, our current data indicate similar down-regulation of CYP2A5, 2C29, and 3A11 mRNAs after LPS exposure in both PPARα WT and KO mice (Fig. 1) as well as down-regulation of CYP proteins (Fig. 2), suggesting that PPARα is not involved in down-regulation of these CYPs during inflammation. In our previous study, mRNA levels were determined by Northern blotting, used fewer animals, and had a slightly longer LPS exposure time (24 vs 16 hours). The current findings using the more sensitive and quantitative real-time PCR method, and a larger number of animals, conclusively establish that PPARα is not involved in down-regulation of the CYPs studied here (1A2, 2A5, 2C29, 2E1, 3A11).
Little is known about PXR and inflammatory pathways. Activation of PXR regulates xenobiotic-inducible CYP3A gene expression in mice (Kliewer et al., 1998), as well as CYP2B and 2C, glutathione S-transferases, sulfotransferases, UDP-glucuronosyltransferases, organic anion transporter peptide 2, and multidrug resistance protein 3 (Staudinger et al., 2003). Studies have associated down-regulation of PXR after LPS administration with reductions in PXR-regulated CYP (Beigneux et al., 2002; Xu et al., 2004; Teng and Piquette-Miller, 2005). As shown in Fig. 4, we observed similar down-regulation of CYP1A2, 2A5, 2C29, 2E1, 3A11, 4A10, and 4A14 mRNAs in both PXR WT and KO mice, indicating that PXR is not required for CYP down-regulation during inflammation. These observations corroborate a recent finding by Teng and Piquette-Miller (2005), who also demonstrated down-regulation of CYP3A11 mRNA in both WT and PXR KO mice after a shorter exposure to a higher dose of LPS (5 mg/kg, 6 hours). Both our data and the Teng study indicate higher basal levels of CYP3A11 mRNA in PXR KO mice compared to PXR WT mice. Regardless of the higher basal levels in the PXR KO, the percent reduction of CYP3A11 by LPS was similar in both PXR WT and PXR KO mice (81.5% and 79.6% reduction, respectively). In contrast to the other CYP isoforms, CYP3A13 was not affected by LPS treatment in our PXR study (Fig. 6), although PXR is reported to be involved in its basal expression (Anakk et al., 2003). Interestingly, LPS treatment induced CYP3A13 mRNA in the PPARα experiment (Fig. 1). This difference in
regulation of CYP3A13 by LPS may be due to the different background strains used in the two studies.

Measurements of CYP proteins in PXR WT and KO mice exhibited considerable variability. CYP3A and 4A proteins in PXR WT mice most closely corresponded with mRNA results (Figs. 5 and 4). Densitometric analysis of individual protein samples indicated down-regulation of most but not all proteins, but when averaged, this decrease was negated by samples that were not affected by LPS exposure. Rat antibodies recognize several mouse proteins within a subfamily, making it difficult to associate down-regulation of a single mouse mRNA transcript with down-regulation of several related mouse proteins. Also, this study was conducted at a single time point and LPS dose, that we chose to be optimal for down-regulation of CYP mRNAs. 16 hours may not be sufficient time for significant down-regulation of CYP proteins to become fully manifested. The possibility cannot be excluded that PPARα and/or PXR could influence the time- or concentration- dependence of CYP mRNA down-regulation by LPS. This question could be resolved by future time course and dose-response studies.

Although CYP down-regulation does not require PPARα and PXR, the involvement of other transcription factors must be considered. Several laboratories have attempted to determine the involvement of basal transcription factors (TF) in CYP regulation, and many questions remain. Jover et al. (2002) demonstrated that expression of liver-enriched transcriptional inhibitory protein
(LIP) represses human CYP3A4 reporter gene expression, suggesting that LIP induction mediates the suppression of CYP3A4 during the acute phase response. However, Cheng et al. (2003) found that levels of LIP were not significantly affected in rat liver 1 hr after LPS injection, and that suppression of rat liver CYP3A2, 2C11, and 2E1 transcription at early time points is not due to elevation of nuclear LIP. Reduction of other TFs has been suggested as the reason for CYP suppression after LPS treatment, including hepatocyte nuclear factor (HNF)-1 (Roe et al., 2001), HNF3 (Park and Waxman, 2001), and HNF4 (Cheng et al., 2003). Overall, the combination of reduced activities of several TFs could contribute to the CYP suppression (Cheng et al., 2003).

The down-regulation of multiple CYP isoforms during inflammation can be mimicked by *in vivo* and *in vitro* treatment with proinflammatory cytokines such as IL-1β, IL-6, and TNFα (Morgan, 2001). It is possible that cytokines produced *in vivo* may mediate CYP down-regulation in our study, although this cannot be determined from these data because plasma cytokine levels were not determined, and the hepatic cytokine mRNAs were not measured at peak times (1-6 hours). Although our studies show that PPARα and PXR are not directly involved in CYP down-regulation, it is possible that these nuclear receptors are involved in the modulation of proinflammatory cytokines. The LPS-mediated induction of TNFα mRNA was attenuated in PPARα KO mice (Fig. 3), suggesting the involvement of PPARα in regulation of TNFα expression. Hill et al. (1999) have observed that PPARα activators up-regulate TNFα expression in mice.
during endotoxemia. In contrast to PPARα KO mice, hepatic TNFα mRNA in PXR KO mice was significantly increased after LPS treatment (Fig. 6), suggesting possible cytokine compensation or an absence of negative feedback for TNFα in PXR KO mice. Alternatively, there may simply be a difference in the time course of the inflammatory response regarding the induction of TNFα in the two strains of mice. Together these data suggest that PPARα and PXR may be involved in modulation of TNFα, which in turn can mediate CYP down-regulation.

Several studies have investigated cytokine involvement in CYP decreases during LPS-induced inflammation, using cytokine- or cytokine receptor-null mice. These studies have generally shown no significant differences between WT and KO mice (Warren et al., 1999; Ashino et al., 2004; Siewert et al., 2000), suggesting that cytokines signal redundantly to down-regulate CYPs during LPS-induced inflammation. Also, LPS may alter CYP expression by mechanisms that differ depending on the LPS dose. In addition to cytokines, reactive oxygen species have been suggested to contribute to LPS down-regulation of PXR and CYP3A11 mRNA (Xu et al., 2004). The same authors have also recently shown that the antioxidant melatonin attenuates LPS-induced down-regulation of PXR and CYP3A11 (Xu et al., 2005).

In summary, we have conclusively shown that the nuclear receptors PPARα and PXR are not required for the down-regulation of CYP isoforms during LPS-induced inflammation, as we observed similar down-regulation of several CYP
mRNAs in both wildtype and knockout mice.
Acknowledgments

The technical assistance of Kimberly L. Pierce is gratefully acknowledged. The authors are indebted to Dr. Gary W. Miller, Emory University Center for Neurodegenerative Disease and the Department of Environmental and Occupational Health for the use of equipment. The authors also thank Dr. Bryan J. Goodwin of GlaxoSmithKline for providing PXR knockout mice.
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FOOTNOTES

The National Institutes of Health Grant GM46897 provided funding for this study.

Portions of this work were previously presented at 44th Annual Society of Toxicology meeting, New Orleans, LA.

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FIGURE LEGENDS

Fig. 1. Effects of LPS on hepatic CYP mRNA expression in PPARα WT and KO mice. Animals were treated with either saline or 1 mg/kg LPS, and relative levels of mRNA expression determined at 16 hours. Values represent means ± S.E.M. for each group (n = 5), and designations denote significant differences (p<0.05) from WT control (*) or KO control (#).

Fig. 2. Western blot data of LPS effects on hepatic proteins in PPARα WT and KO mice (top panel). Quantitative analysis of Western blot data (bottom panel). Animals were treated with either saline or 1 mg/kg LPS, and relative levels of mRNA expression determined at 16 hours. Values represent means ± S.E.M. for each group (n = 5), and designations denote significant differences (p<0.05) from WT control (*) or KO control (#).

Fig. 3. Effects of LPS on hepatic cytokine and acute phase protein mRNA expression in PPARα WT and KO mice. Animals were treated with either saline or 1 mg/kg LPS, and relative levels of mRNA expression determined at 16 hours. Values represent means ± S.E.M. for each group (n = 5), and designations denote significant differences (p<0.05) from WT control (*) or KO control (#).

Fig. 4. Effects of LPS on hepatic CYP mRNA expression in PXR WT and KO mice. Animals were treated with either saline or 1 mg/kg LPS, and relative levels of mRNA expression determined at 16 hours. Values represent means ± S.E.M.
for each group (n = 6), and designations denote significant differences (p<0.05) from WT control (*) or KO control (#).

Fig. 5. Western blot data of LPS effects on hepatic proteins in PXR WT and KO mice (top panel). Quantitative analysis of Western blot data (bottom panel). Animals were treated with either saline or 1 mg/kg LPS, and relative levels of mRNA expression determined at 16 hours. Values represent means ± S.E.M. for each group (n = 5), and designations denote significant differences (p<0.05) from WT control (*) or KO control (#).

Fig. 6. Effects of LPS on hepatic cytokine and acute phase protein mRNA expression in PXR WT and KO mice. Animals were treated with either saline or 1 mg/kg LPS, and relative levels of mRNA expression determined at 16 hours. Values represent means ± S.E.M. for each group (n = 6), and designations denote significant differences (p<0.05) from WT control (*) or KO control (#).
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</table>

*Previously published in Pan et al. (2000)*

*Previously published in Overbergh et al. (2003)*
Fig. 1
Fig. 2

The figure shows immunoblots with the following labels:

- **WT CON**
- **WT LPS**
- **KO CON**
- **KO LPS**

The proteins labeled are:

- **2C**
- **3A**
- **4A**
- **2E**

A bar graph below the immunoblots illustrates the protein levels, with different symbols indicating statistical significance: * and #.
Fig. 3

The figure shows a bar graph with the y-axis labeled "mRNA (Relative levels)" and the x-axis labeled with five categories: IL-1, IL-6, TNF, AGP, and FBG. The categories are divided into two groups: WT Control and KO Con, and within each group, there are subcategories: WT LPS and KO LPS. The graph includes error bars and asterisks to indicate statistical significance.

- IL-1: WT Control and KO Con groups show a significant increase compared to WT LPS and KO LPS groups.
- IL-6: Similar pattern to IL-1.
- TNF: The KO Con group has a significant increase compared to the other groups.
- AGP: The KO Con group shows a significant increase.
- FBG: The KO Con group shows a significant increase.

The asterisks and error bars indicate statistical significance between groups.
Fig. 4

![Graph showing mRNA levels for different genes and conditions.]

Legend:
- **WT Control**
- **WT LPS**
- **KO Con**
- **KO LPS**

Key:
- * indicates a significant difference compared to WT Control.
- # indicates a significant difference compared to WT LPS.
Fig. 6

The figure shows a bar graph comparing mRNA expression levels of various genes under different conditions. The x-axis represents different genes (IL-1, IL-6, TNF, AGP, FBG) and the y-axis represents mRNA levels (Relative levels). The conditions tested are WT Control, WT LPS, KO Con, and KO LPS.

Key points:
- WT Control shows consistent mRNA levels across genes.
- WT LPS shows slight increases in mRNA levels for some genes.
- KO Con shows higher expression levels for certain genes.
- KO LPS shows the highest expression levels, especially for AGP and FBG.

Significance markers: * and # indicate statistical differences between conditions.