Enhanced Acetaminophen Toxicity by Activation of the Pregnane X Receptor

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The pregnane X receptor (PXR) is a ligand-activated transcription factor and member of the nuclear receptor superfamily. Activation of PXR represents an important mechanism for the induction of cytochrome P450 3A (CYP3A) enzymes that can convert acetaminophen (APAP) to its toxic intermediate metabolite, N-acetyl-p-benzoquinone imine (NAPQI). Therefore, it was hypothesized that activation of PXR plays a major role in APAP-induced hepatotoxicity. Pretreatment with the PXR activator, pregnenolone 16α-carbonitrile (PCN), markedly enhanced APAP-induced hepatic injury, as revealed by increased serum ALT levels and hepatic centrilobular necrosis, in wild-type but not in PXR-null mice. Further analysis showed that following PCN treatment, PXR-null mice had lower CYP3A11 expression, inactivation of CYP3A by acting as ligands for the pregnane X receptor (PXR) in rodents or for the steroid xenobiotic receptor (SXR), the PXR homologue in humans (Moore and Kliewer, 2000). PXR is a ligand activated transcription factor and member of the nuclear receptor superfamily. activation of PXR represents an important mechanism for the induction of cytochrome P450 3A (CYP3A) enzymes that can convert acetaminophen (APAP) to its toxic intermediate metabolite, N-acetyl-p-benzoquinone imine (NAPQI). Therefore, it was hypothesized that activation of PXR plays a major role in APAP-induced hepatotoxicity. Pretreatment with the PXR activator, pregnenolone 16α-carbonitrile (PCN), markedly enhanced APAP-induced hepatic injury, as revealed by increased serum ALT levels and hepatic centrilobular necrosis, in wild-type but not in PXR-null mice. Further analysis showed that following PCN treatment, PXR-null mice had lower CYP3A11 expression, inactivation of CYP3A by acting as ligands for the pregnane X receptor (PXR) in rodents or for the steroid xenobiotic receptor (SXR), the PXR homologue in humans (Moore and Kliewer, 2000). PXR is a ligand activated transcription factor and member of the nuclear receptor superfamily. When short half-life and under normal physiological conditions is rapidly excreted after conjugation with glutathione (GSH), a reaction carried in part by glutathione S-transferase (GST), such as GSTπ (Mitchell et al., 1973). The resulting conjugate (APAP-GSH) can be further metabolized to APAP-cysteinylglycine (APAP-Cys/Gly) and APAP-mercapturate (APAP-Nac) (Gregus et al., 1988). However, when an overdose occurs, the major metabolic pathways of elimination become saturated, leading to both increased bioactivation of APAP and formation of NAPQI, as well as depletion of cellular GSH. In the absence of prevention, excess NAPQI may result in cell death and hepatotoxicity due to covalent binding to essential cellular macromolecules and/or other mechanisms such as oxidative stress (Jollow et al., 1974).

Isoforms of CYP3A may play an important role in metabolizing acetaminophen to its toxic intermediate metabolite, NAPQI. The kinetics of NAPQI formation by reconstituted human liver CYP3A4 is compatible with the therapeutic doses of this drug (Thummel et al., 1993), indicating that CYP3A4 is involved in APAP metabolism. Inducers of CYP3A potentiate, while inhibitors of CYP3A prevent, APAP toxicity (DiPetrillo et al., 2002; Madhu et al., 1992; Sinclair et al., 1998; van Bree et al., 1989). However, the role of CYP3A in acetaminophen metabolism still remains controversial as revealed by other studies in which CYP3A is negligible in APAP metabolism in human, and CYP3A inducers protect hamsters from APAP induced hepatic injury (Madhu and Klaassen, 1991; Manyike et al., 2000). The CYP3A enzymes metabolize over half of the pharmaceuticals on the current market, and the expression levels of CYP3A enzymes are modulated by a variety of xenobiotics, including numerous chemicals and dietary compounds. Many of these chemicals induce CYP3A by acting as ligands for the pregnane X receptor (PXR) in rodents or for the steroid xenobiotic receptor (SXR), the PXR homologue in humans (Moore and Kliewer, 2000). PXR is a ligand activated transcription factor and belongs to the orphan nuclear receptor superfamily. When...
activated, PXR induces a network of genes that encode phase I and phase II xenobiotic metabolizing enzymes and drug transporters by forming a heterodimer with the universal partner for class II nuclear receptors, retinoic X receptor (RXR), which subsequently binds to the promoter or enhancer regions of PXR target genes (Guo et al., 2002; Kliwer, 2003; Kliwer et al., 1998; Lehmann et al., 1998). The activation of PXR results in enhanced metabolism and/or excretion of many endogenous chemicals or xenobiotics. The role of orphan nuclear receptors in APAP metabolism was explored and recent studies show that peroxisome proliferators-activated receptor α (PPARα) and the constitutive androstane receptor (CAR) play roles in APAP induced hepatic injury (Chen et al., 2000; Zhang et al., 2002).

In order to investigate the role of PXR in APAP-induced hepatic toxicity, wild-type or PXR-null mice were pretreated with pregnenalone 16α-carbonitrile prior to the administration of a toxic dose of APAP. Pretreatment with PCN dramatically augmented APAP-induced hepatic injury in wild-type, but not in PXR-null mice. The severity of APAP toxicity correlated very well with the levels of CYP3A11 expression. In summary, the current study demonstrates an important role for PXR in APAP-induced hepatic toxicity via induction of CYP3A11, and therefore confirms that CYP3A11 is critical in APAP reactive intermediate metabolite formation in mice.

MATERIALS AND METHODS

Chemicals. Acetaminophen and PCN were purchased from Sigma (St. Louis, MO). The kit to measure alanine aminotransferase (ALT) was from ThermoTrace (Melbourn, Australia) and the assay was carried out according to the manufacturer’s instruction. Ten percent phosphate buffered formalin was purchased from Fisher Scientific (Fair Lawn, NJ). Other reagents, unless otherwise indicated, were obtained from Sigma.

Animals and treatments. Mice were housed in a pathogen-free animal facility under standard 12-h light/12-h dark cycle with access to chow and water ad libitum. All protocols and procedures were approved by the NCI Animal Care and Use Committee and are in accordance with the National Institute of Health and ALAC Guidelines. The 8- to 12-week-old male B6; 129-Pxrtm1Glaxo–Wellcome (PXR-null) mice or their corresponding wild-type controls were used throughout the study (n = 5 to 7 per group). The animals were pretreated with vehicle (corn oil, ip) or PCN (75 mg/kg, ip) for two days before one APAP administration. APAP was dissolved in alkaline solution, and therefore confirms that CYP3A11 is critical in APAP reactive intermediate metabolite formation in mice.

RESULTS

Activation of PXR Potentiated APAP Hepatotoxicity

To determine the role of PXR in APAP-induced hepatic toxicity, wild-type or PXR-null mice were pretreated with PCN (75 mg/kg, ip for two days) following APAP administration (350 mg/kg, ip). The degree of hepatic injury was assessed by serum ALT levels obtained 6 and 24 h following APAP administration (Fig. 1). In both wild-type and PXR-null mice, the administration of APAP caused a time-dependent elevation of ALT levels that was more severe in PXR-null mice. However, APAP dramatically enhanced ALT levels in wild-type mice (about 2-fold at 6 h and 6-fold at 24 h after APAP administration) following pretreatment with PCN. In contrast, following the APAP administration, the PXR-null mice pretreated with PCN exhibited reduced ALT levels compared to those pretreated with vehicles.

The degree of APAP-induced hepatic injury in wild-type and PXR-null mice were also analyzed by histological analysis (Table 1). Typical acetaminophen toxicity in the liver is manifested by centrilobular necrosis. Consistent with the results obtained from analysis of serum ALT, pretreatment with PCN dramatically enhanced the acetaminophen induced-hepatic injury (degeneration in the early stage and necrosis in the late stage) in wild-type, but not in PXR-null null
mice. Since it has been well established that APAP may cause renal injury, a histological analysis was also performed on kidney samples from the aforementioned groups; however, no morphological differences were identified (data not shown).

### Activation of PXR Enhanced Urinary Excretion of APAP Metabolites

The APAP-metabolites are removed mainly by biliary and urinary excretion (Gregus et al., 1988). Urinary excretion of APAP metabolites not only reflects the fate of APAP, the non-invasive nature of this procedure also makes urinary analysis of APAP metabolites a very attractive means to assess acetaminophen metabolism, especially in the clinic. HPLC analysis of pooled urine was performed to identify any differences in the profiles of APAP metabolites among wild-type and PXR-null mice (Fig. 2). Consistent with the patterns observed with serum ALT levels, PCN treatment of wild-type mice increased GSH-derived APAP metabolites (APAP-Glu, APAP-Sulf, APAP-Cys/CysGly, and APAP-Nac) compared to respective APAP alone groups (Fig. 2). However, pretreated of PXR-null mice with PCN reduced the GSH-derived APAP metabolites excreted into the urine comparable to, or lower than, those seen among vehicle pretreated wild-type mice. Interestingly, the urinary excretion of detoxification metabolites APAP- Gluc and APAP-Sulf was also increased in PCN pretreated wild-type mice despite enhanced toxicity. This is probably reflective of changes in APAP glucuronidation,

### TABLE 1

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**Note.** Histological analysis of wild-type and PXR-null livers after PCN and APAP treatment. The livers of wild-type and PXR-null mice with aforementioned treatments were harvested and histological evaluation was performed following H & E staining. Key: –, none; +/−, minimal degree of lesion; +, mild; ++, moderate; ++++, severe.
sulfation and/or transport-mediated basolateral efflux of these conjugates by PCN.

**Pretreatment of PCN Enhanced Glutathione Depletion in Wild-Type Mice, but Not in PXR-Null Mice**

The APAP-induced hepatic injury is tightly associated with hepatic glutathione depletion. Therefore, hepatic glutathione content was measured in wild-type and PXR-null mice after PCN and APAP treatment (Fig. 3). There were no differences in endogenous hepatic GSH levels between control wild-type and PXR-null mice. However, compared to respective control mice, pretreatment with PCN significantly increased hepatic GSH content of wild-type (*p* < 0.05) but not PXR-null mice. In addition, 6 h after APAP administration, the hepatic GSH content of PCN-pretreated wild-type (*p* < 0.05) but not PXR-null mice was significantly reduced compared to respective vehicle-pretreated or control groups. By 24 h after APAP administration, the hepatic GSH content of PCN-pretreated wild-type mice had returned to baseline levels.

**PXR-Mediated Induction of CYP3A11 Results in APAP-Induced Hepatic Toxicity**

The toxicity of APAP overdose is exerted by formation of its intermediate metabolite, NAPQI, by CYP2E1, isoforms of CYP3A and CYP1A2; whereas the detoxification of NAPQI may be via conjugation with GSH conducted by glutathione S-transferase π (GSTπ), even the role of GSTπ in NAPQI detoxification is not certain (Henderson et al., 2000). To investigate the molecular mechanisms by which activation of PXR enhanced APAP-induced hepatic toxicity, the hepatic mRNA levels of the genes encoding these key enzymes were determined by Northern blot analysis (Fig. 4). Compared with wild-type mice, basal levels of CYP3A11 expression were higher in PXR-null mice; however, they were resistant to the PCN-induced dramatic induction of CYP3A11 as seen in the wild-type mice. The expression levels of CYP2E1 were moderately reduced by APAP administration at both 6 h and 24 h in both genotypes; however, they virtually disappeared at 24 h after the administration of APAP in wild-type mice pretreated with PCN, but not in PXR-null mice. The expression levels of CYP1A2 were induced to a moderate degree by PCN and were inhibited by APAP administration regardless of the genotypes or the combination with PCN. The expression levels of hepatic GSTπ were induced by both APAP administration and PCN treatment irrespective of genotype. Moreover, in PXR-null mice, although the basal levels of GSTπ expression were low, it was induced by PCN to levels comparable to those detected in wild-type livers.

**DISCUSSION**

The bioactivation of overdosed APAP by several phase I P450 enzymes (CYP2E1, isoforms of CYP3A and CYP1A2) to its reactive intermediate metabolite, NAPQI, leads to hepatic toxicity. The role of CYP2E1 and CYP1A2 in NAPQI production has been explored comprehensively, but that of CYP3A11 is uncertain. The expression levels of CYP3A are induced by numerous endogenous and exogenous chemicals, many of which are ligands of the orphan nuclear receptor, PXR. To investigate the role of PXR and CYP3A in APAP-induced hepatic injury, wild-type or PXR-null mice were pretreated with a potent PXR activator, PCN, followed by acute administration of APAP. This study demonstrates for the first time that PXR plays a critical role in APAP metabolism, mainly via induction of CYP3A11 resulting in enhanced APAP reactive metabolite formation, thereby dramatically increasing the extent of APAP-induced hepatic injury in mice. Moreover, this study confirms a pivotal role for CYP3A in metabolizing APAP to its intermediate metabolite NAPQI in mice.

This study clearly shows that PXR plays a pivotal role in modulating APAP metabolism. Several lines of evidence have suggested that hepatic nuclear receptors are involved in APAP-induced hepatotoxicity. For example, activation of peroxisomal proliferator-activated receptor α (PPARα) appears to reduce the severity of APAP-induced hepatic injury through induction of numerous genes involved in pathways such as stress response, DNA damage repair, and cell cycle regulation (Chen et al., 2000; Shankar et al., 2003). Similarly, APAP toxicity appears to be enhanced by phenobarbital (PB), which may be attributable to the activation of CAR, which induces expression
of CYP3A and CYP1A2 that metabolize APAP to its reactive intermediate metabolite (Zhang et al., 2002). Interestingly, treatment with androstanol, a specific CAR inhibitor but a modest PXR ligand, abrogated APAP induced-hepatotoxicity in wild-type mice, but produced an even greater toxicity in the CAR-null mice. This former study may be interpreted to suggest that while CAR activation critically affects APAP induced-hepatotoxicity, activation of PXR activity influences APAP hepatotoxicity consistent with the results seen here. Modulating PXR activity can result in a markedly different outcome for drug metabolism. PXR is activated by numerous structurally unrelated compounds (Kliewer et al., 1998) and its activation affects the expression of a network of genes encoding critical enzymes or transporters involved in hepatic and enteric drug metabolism (Kliewer, 2003). Thus it is reasonable to predict that drug–drug interactions are one of the consequences after modulating PXR activity by endogenous or xenobiotic factors.

The data presented here clearly suggests that CYP3A11 is involved in APAP-induced hepatic toxicity in mice. The expression levels of CYP3A11 correlated well with the severity of APAP-induced hepatic injury. For example, the basal levels of CYP3A11 expression were higher in PXR-null mice compared to wild-type mice, and correlated with a more severe APAP-induced hepatic toxicity as revealed by serum ALT levels and the appearance of hepatic centrilobular necrosis. Although the role of GST\(\gamma\) in NAPQI detoxification remains controversial, the reduced levels of GST\(\gamma\) in the livers of PXR-null mice may also contribute to the more severe APAP-induced hepatic injury. The CYP2E1 and CYP1A2 enzymes are reported the two major forms of P450s for the generation of NAPQI (Lee et al., 1996; Tonge et al., 1998; Zaher et al., 1998), but there are studies suggesting that other mechanisms of bioactivation exist. For example, the CYP2E1-null mice are resistant to APAP toxicity even at high doses of APAP (Lee et al., 1996). The CYP1A2-null mice, on the other hand, fail to demonstrate that they are less sensitive to APAP toxicity, even within a low dose range (Tonge et al., 1998). Furthermore, despite

![FIG. 4. The effect of PCN on the hepatic gene expression levels in the wild-type and PXR-null mice after PCN and APAP administration. Hepatic tissue was obtained at 6 and 24 h after APAP administration. Total RNA was isolated, the expression levels of CYP3A11, CYP2E1, CYP1A2 and GST\(\gamma\) were determined by Northern Blot analysis. Each group consisted of data from three individual animals. CON represents for corn oil treatment, PCN for PCN treatment, AP/6 hr for 6 h following APAP administration, P/AP/6 hr for pretreatment with PCN followed by 6 h after APAP administration, AP/24 hr for 24 h after APAP administration, and P/AP/24 hr for pretreatment with PCN followed by 24 h after APAP administration.](https://academic.oup.com/toxsci/article-abstract/82/2/374/1656991)
relatively lower basal levels of CYP1A2 and similar levels of CYP2E1 in PXR-null mouse livers compared to those in the wild-type mice, the APAP toxicity was more severe in the PXR-nulls, indicating that other P450 enzymes, probably CYP3A11, contributed significantly to NAPQI formation. The role of the CYP3A family in APAP metabolism has been long speculated upon, and is confirmed to a certain degree by studies using the so-called CYP3A-specific inhibitors; however, the lack of a CYP3A family gene knockout model prohibits the direct investigation of this issue. Nevertheless, by using an indirect approach in the study here, a PXR-null mouse model confirmed the critical role of PXR-regulated CYP3A11 in APAP metabolism.

PXR also plays a critical role in regulating phase II xenobiotic metabolizing enzymes and transporters that are important to detoxifying xenobiotics. Although the role of GSTm in NAPQI detoxification is not completely clear, it is reported to be the major phase II enzyme responsible for detoxifying NAPQI with APAP overdose. However, our study showed that GSTm was induced by PCN in both wild-type and PXR-null mice; this is probably due to overlapping activation of other nuclear receptors, such as CAR that induces or stabilizes GSTm mRNA. Therefore the effect of GSTm by PCN in both wild-type and PXR-null mice on APAP detoxification is similar and can not count for the more severe APAP-induced hepatic injury in the PXR-null mice prior to PCN pretreatment. Possible activation of other nuclear receptors was also suggested by CYP1A2 expression, which was induced to a moderate degree by PCN and inhibited by APAP administration regardless of the genotypes or the combination with PCN, indicating that other nuclear receptors, such as CAR that cross-talks with PXR (Zhang et al., 2002), might be involved in regulating CYP1A2 expression in these animals. The APAP metabolites are excreted out of the liver into the circulation or bile by hepatic transporters, such as Mrp3 and/or Mrp2 (Chen et al., 2003). The expression of Mrp2 and Mrp3 is induced by PXR activation (Kast et al., 2002; Teng et al., 2003). Thus, activation of PXR enhances APAP-induced hepatic injury by induction of CYP3A isoforms, but on the other hand, also aids in the detoxification of APAP overdose by induction of phase II enzymes and transporters. However, data from this study showed that bioactivation of APAP to its reactive intermediate metabolite, NAPQI, by CYP3A11 appeared to overcome the detoxification process mediated by phase II enzymes and transporters, resulting in more severe APAP-induced hepatic injury.

In summary, this study demonstrated that activation of PXR dramatically enhanced APAP-induced hepatic injury mainly via induction of CYP3A. This finding implies that chemicals that modulate PXR activity will have a significant effect on the outcome following APAP administration, and it adds another layer of complexity in understanding APAP metabolism and treatment for APAP overdose patients.

**REFERENCES**


