The pregnane X receptor (PXR, NR1I2) is a xenobiotic-sensing nuclear receptor that modulates the metabolic response to drugs and toxic agents. Both PXR activation and deficiency promote hepatic triglyceride accumulation, a hallmark feature of alcoholic liver disease. However, the molecular mechanism of PXR-mediated activation of ethanol (EtOH)-induced steatosis is unclear. Here, using male wild-type (WT) and Pxr-null mice, we examined PXR-mediated regulation of chronic EtOH-induced hepatic lipid accumulation and hepatotoxicity. EtOH ingestion for 8 weeks significantly (1.8-fold) up-regulated Pxr mRNA levels in WT mice. The EtOH exposure also increased mRNAs encoding hepatic constitutive androstane receptor (CYP3A11) and its target, Cypl2b10 (220-fold), in a PXR-dependent manner. Furthermore, WT mice had higher serum EtOH levels and developed hepatic steatosis characterized by micro- and macrovesicular lipid accumulation. Consistent with the development of steatosis, lipogenic gene induction was significantly increased in rovesicular lipid accumulation. Consistent with the development and developed hepatic steatosis characterized by micro- and macrovesicular lipid accumulation. Consistent with the development of steatosis, lipogenic gene induction was significantly increased in WT mice, including sterol regulatory element–binding protein 1c target gene fatty-acid synthase (3.0-fold), early growth response-1 (3.2-fold), and TNFα (3.0-fold), whereas the expression of peroxisome proliferator–activated receptor α target genes was suppressed. Of note, PXR deficiency suppressed these changes and steatosis. Protein levels, but not mRNAs levels, of EtOH-metabolizing enzymes, including alcohol dehydrogenase 1, aldehyde dehydrogenase 1A1, and catalase, as well as the microsomal triglyceride transfer protein, involved in regulating lipid output were higher in Pxr-null than in WT mice. These findings establish that PXR signaling contributes to ALD development and suggest that PXR antagonists may provide a new approach for ALD therapy.

Alcoholic liver disease (ALD) is a common pathology of excessive alcohol use. About 90% of individuals who chronically consume ethanol (EtOH) develop steatosis (fatty liver), the first hit, which can advance to inflammation, fibrosis, cirrhosis, and even liver cancer (1). Thus, it was suggested that the prevention or suppression of EtOH–induced steatosis may block or delay the progression of fatty liver to hepatitis and fibrosis (2). Despite intense research efforts in the past decade to understand the pathogenesis of EtOH–induced liver damage, there are no Food and Drug Administration-approved treatments for ALD. The development of an ALD treatment is hindered both by a limited understanding of the molecular mechanisms involved in EtOH–induced liver damage and the lack of animal models that recapitulate advanced ALD. Although cumulative alcohol intake correlates with liver disease, only 35% of alcohol abusers develop more severe forms of ALD, such as advanced fibrosis and cirrhosis, suggesting the contribution of genetic factors (1, 3). To date, only genes encoding the principal alcohol-metabolizing enzymes alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) have been linked to alcoholism and ALD (4–7). Interestingly, a recent report indicates that the nuclear receptors (NRs) peroxisome proliferator–activated receptor (PPAR) α and γ are linked with alcohol consumption in mice and withdrawal and dependence in humans (8).

The involvement of NRs in the pathogenesis of ALD has been demonstrated (9–11). Specifically, knock-out mice lacking retinoid X receptor (RXRα) in hepatocytes, PPARα, constitutive androstane receptor (CAR), and the farnesoid X receptor (FXR) are all highly susceptible to liver injury induced by EtOH (9, 10, 12–14). Furthermore, ligands of the NRs, including PPARα, PPARγ, PPARβ/δ, and hepatocyte nuclear factor-4α (HNF4α) were reported to attenuate EtOH–induced hepatic steatosis, insulin resistance, and hepatotoxicity (16–19). Unexpectedly, previous reports indicate that pharmacological activation of mouse CAR by its potent activator, 1,4-bis[2-[(3,5-dichloropyridyl)-oxy]] benzene (TCPOBOP), enhances both acute and chronic EtOH–induced lipid accumulation, oxidative stress, and liver injury (12). Furthermore, CAR activation also increased both methionine- and choline-deficient diets and acetaminophen-induced hepatotoxicities (20, 21). Interestingly, although pregnane X receptor (PXR) and CAR are considered to be sister...
NRs with overlapping functions, it is not known whether PXR deficiency also promotes EtOH-induced hepatotoxicity (22).

The PXR, a xenobiotic-sensing nuclear receptor expressed primarily in the liver and intestine, is involved in the detoxification of drugs and other harmful substances (23). The major target of genes of PXR are those involved in xenobiotic metabolism of drugs and other harmful substances (23). The major target of genes of PXR are those involved in xenobiotic metabolism and transport, including the cytochrome P450 (CYP) enzymes and the ATP-binding cassette drug transporters (24).

Recent reports indicate that activation of PXR inhibits nuclear factor κB, inflammation, and fibrosis (25–28). In addition to these beneficial effects, PXR activation has also been implicated in gut dysbiosis and metabolic abnormalities, tumor promotion, chemoresistance, and response of cancer cells to chemotherapeutic agents (29–31). Furthermore, PXR activation can also result in hepatic triglyceride accumulation and reactive metabolite formation leading to drug-induced liver injury (32, 33). Finally, a recent report indicates that human PXR gene variants are associated with liver injury in non-alcoholic fatty liver disease (NAFLD), in which PXR polymorphisms increase the risk of susceptibility to a more severe form of NAFLD (34).

Valid animal models are a prerequisite for developing new drug treatment for ALD. Recently, two independent groups have generated mice that lack PXR to study the physiological or functional significance of PXR in xenobiotic regulation (35, 36). Although none of the two lines of Pxr-null mice has been evaluated for EtOH-induced hepatotoxicity, recent reports indicate that PXR deficiency protects mice against high-fat diet (HFD)-induced obesity (37–39). In addition, HFD-fed male obese wildtype (WT) mice had more severe liver injury than male obese Pxr-null mice (38). Thus, PXR may potentiate the transition from fatty liver to steatohepatitis. Accumulating evidence indicates that PXR may have roles in lipid metabolism (33, 40, 41). Interestingly, loss of PXR and PXR activation leads to hepatic lipid accumulation, an important feature of ALD (41, 42). Furthermore, reports indicate that EtOH can induce both PXR and its target Cyp3a11/Cyp3A gene in rodents, human primary hepatocytes, and human liver samples of alcoholics (43–45). Currently, little is known regarding the molecular mechanism of PXR-mediated activation of EtOH–induced steatosis.

In this study, male WT and Pxr-null mice were fed control (pair-fed) or the standard Lieber-DeCarli liquid diet containing 5% EtOH for 8 weeks and were evaluated for changes in hepatic gene expression, histology, and hepatotoxicity.

**Results**

**EtOH induces lipid accumulation in WT but not Pxr-null mice**

During the 8-week feeding period, whereas food intake was similar in all groups, a significant decrease in body weight was observed in both EtOH-fed WT and Pxr-null mice, compared with their respective controls (Table 1) and consistent with what was observed by others (46). However, liver weight and liver-to-body weight ratios were not significantly different between the control and EtOH-fed groups of both genotypes (Table 1). Hematoxylin and eosin (H&E) staining revealed that the genotype influenced EtOH–induced hepatic lipid droplet accumulation, histology score, triglycerides, and NEFA levels, which were higher in WT mice but absent in Pxr-null mice (Table 1 and Fig. 1, A–C). Histologic scoring via H&E staining of lipid accumulation showed that both microvesicular (multiple smaller vacuoles in each hepatic cell without significant nuclear displacement) and macrovesicular steatosis (observed as a single vacuole displacing the nucleus in each hepatic cell) as well as mild necrosis were present in EtOH-fed WT mice (Table 1 and Fig. 1, A–C). In contrast, lipid droplets and necrosis were absent in the livers of EtOH-fed Pxr-null mice (Fig. 1A). In agreement with the morphology data, after EtOH treatment, hepatic triglyceride and NEFA levels were significantly higher only in WT mice and not in EtOH-fed Pxr-null mice (Fig. 1, B and C). However, inflammation and fibrosis were absent in both EtOH-fed genotypes (data not shown). These data indicate that the PXR promotes chronic EtOH–induced steatosis and hepatic triglyceride accumulation.

**Chronic EtOH ingestion significantly up-regulated mRNA levels of PXR and CAR and CAR target gene Cyp2b10 in WT mice**

Chronic EtOH ingestion significantly up-regulated Pxr mRNA expression (1.8-fold) in WT mice, but not in Pxr-null mice, confirming the absence of Pxr mRNA in the Pxr-null mice (Fig. 2A). The basal hepatic Car mRNA levels did not vary between WT and Pxr-null mice suggesting that Car gene expression does not appear to be dependent on PXR but that EtOH–induced up-regulation of Car mRNA might be. Chronic EtOH exposure induced the hepatic Car mRNA 2.9-fold only in WT mice (Fig. 2B). The Fxr and small heterodimer partner (Shp) basal mRNA levels did not differ between the two genotypes (Fig. 2, C and D). However, chronic EtOH significantly reduced both Fxr and Shp mRNA levels, each by 61% in WT mice compared with their respective control-fed mice. In contrast, EtOH decreased Shp mRNA by 40% but not Fxr mRNA levels in Pxr-null mice (Fig. 2, C and D). The basal hepatic gene expression of the PXR target gene, Cyp3a11, was significantly increased in Pxr-null mice (3.0-fold) compared with WT controls (Fig. 2E), as reported previously (35). Chronic EtOH ingestion non-significantly induced the Cyp3a11 gene (1.6-fold) in WT mice and had no effect in Pxr-null mice (Fig. 2E). Furthermore, the basal hepatic mRNA levels of the specific PXR target genes Slco1a4 (Oatp2) and Cyp7a1 (47) were lower, 39 and 57%, respectively, compared with their respective WT controls (Fig. 2, F and G). EtOH had no effect on Cyp7a1 and Slco1a4 mRNA

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**Table 1**

**Effect of chronic EtOH ingestion on body weight, liver weight, and pathology score**

| Parameter          | Wildtype (C57BL/6) | PXR-KO (n = 6) |
|--------------------|-------------------|---------------|
| Control (n = 7)    | Control (n = 7)   | Control (n = 7) |
| Initial body weight (g) | 23.2 ± 1.6 | 24.2 ± 1.7 | 25.4 ± 1.3 |
| Week 8 body weight (g) | 34.1 ± 4.6 | 28.2 ± 1.8*  | 34.3 ± 1.4 |
| Absolute liver weight (g) | 1.6 ± 0.3 | 1.4 ± 0.2 | 1.7 ± 0.2 |
| Liver/body weight (%) | 4.6 ± 0.5 | 4.9 ± 0.4 | 4.9 ± 0.5 |
| Macrovessel steatosis | 1.0 ± 0.8 | 2.4 ± 1.1* | 0.3 ± 0.5 |
| Microvesicular steatosis | 0.0 ± 0.0 | 2.1 ± 1.1* | 0.3 ± 0.5 |
| Necrosis score | 0.1 ± 0.4 | 0.7 ± 1.1 | 0.0 ± 0.0 |

*p < 0.05 was between mice fed control diet and EtOH.

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* p < 0.05 was between mice fed EtOH.
levels in Pxr-null mice but decreased Cyp7a1 mRNA levels by 69% in WT mice without any effect on Sclo1A4 mRNA levels (Fig. 2, F and G). The basal Cyp2b10 mRNA levels did not differ between the two genotypes (Fig. 2H). Even though EtOH-induced Car mRNA expression by about 3-fold in WT mice, the mRNA of its target gene Cyp2b10 was increased dramatically (about 220-fold) in WT mice but not in EtOH-fed Pxr-null mice (Fig. 2, B and H). In agreement with the increased Cyp2b10 gene expression by EtOH, CYP2B10 protein levels were significantly higher in EtOH-fed WT mice (27-fold) compared with WT controls (Fig. 2I).

**PXR deficiency suppresses chronic EtOH–induced lipogenic gene induction**

The steatosis observed in EtOH-fed WT mice prompted us to examine the expression of Srebp-1c and early growth response-1 (Egr-1), both of which have been implicated in EtOH–induced steatosis (48, 49). Basal hepatic Srebp-1c mRNA levels were higher (1.9-fold) in control-fed Pxr-null mice compared with their WT counterparts (Fig. 3A). EtOH non-significantly increased Srebp-1c mRNA levels in WT mice (1.6-fold) compared with control-fed WT mice (Fig. 3A). In contrast, EtOH decreased Srebp-1c mRNA levels by 48% in Pxr-null mice (Fig. 3A). The basal hepatic mRNA levels of the SREBP-1C target genes fatty acid synthase (Fas), acetyl-CoA carboxylase 1a (Acc-1a), and stearoyl-CoA desaturase 1 (Scd1) were not different between the two genotypes, and EtOH did not have any significant effects on Acc-1a and Scd1 mRNAs in both genotypes (Fig. 3, B–D). Constitutive Srebp2 mRNA levels did not vary between the two genotypes, and their mRNA levels were not different after EtOH treatment (Fig. 3E). Similarly, basal Egr-1 mRNA and protein levels did not vary between the two genotypes. However, EtOH treatment significantly increased Egr-1 mRNA (3.2-fold) and protein (12.3-fold) levels only in WT mice (Fig. 3, F and G).

**PXR deficiency protects against chronic EtOH–induced suppression of hepatic fatty acid β-oxidation genes**

Ppara mRNA and its target genes carnitine palmitoyltransferase 1 (Cpt1), acyl-CoA oxidase 1 (Acox-1), liver fatty acid-binding protein 1 (Lfabp-1), and microsomal triglyceride transfer protein (Mtp) mRNA levels were comparable between control WT and Pxr-null mice (Fig. 4, A–E). EtOH did not significantly affect Ppara and Mtp mRNA levels in both WT
PXR and chronic ethanol-induced steatosis

(A) Pxr mRNA
(B) Car mRNA
(C) Fxr mRNA
(D) Shp mRNA
(E) Cyp3a11 mRNA
(F) Sico1a4 (Gapt2) mRNA
(G) Cyp7a1 mRNA
(H) Cyp2b10 mRNA
(I) CYP2B10 Protein

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CYP2B10
α-tubulin

Control Ethanol
WT
PXR-KO

50 KDa
37 KDa
75 KDa
50 KDa
and Pxr-null mice (Fig. 4A). However, chronic EtOH ingestion significantly decreased Cpt-1, Acox-1, and Lfabp-1 mRNA levels by 38, 64, and 56%, respectively, only in WT mice (Fig. 4, B–D). Even though the basal hepatic Mtp mRNA levels were not different between the two genotypes, the constitutive MTP protein levels were significantly higher in Pxr-null mice (2.4-fold) (Fig. 4, E and F). Furthermore, after EtOH ingestion, hepatic MTP protein levels were significantly higher in Pxr-null mice than in WT mice (Fig. 4F).

Protein but not gene expression of EtOH-metabolizing enzymes is altered by EtOH in mice with differential PXR expression

The basal hepatic alcohol dehydrogenase (Adh1), aldehyde dehydrogenase 2 (Aldh2), Aldh1b1 and Aldh1a1, and catalase (Cat) mRNA levels did not vary significantly between the two genotypes, and EtOH did not significantly affect their mRNA levels (Fig. 5, A–E). However, after EtOH treatment, Aldh1a1 mRNA levels in Pxr-null mouse livers were significantly higher than in WT mice (Fig. 5D). CYP2E1 protein expression in the liver did not differ between control WT and Pxr-null mice (Fig. 6A). However, CYP2E1 protein expression was up-regulated by EtOH treatment in WT mice (2.4-fold) and Pxr-null mice (2.6-fold) (Fig. 6A). Similar to their gene expression, the basal hepatic ALDH2 and ALDH1B1 protein levels did not differ between the two genotypes; however, the basal hepatic ADH1, ALDH1A1, and CAT protein levels were significantly higher, 20-, 46-, and 2.8-fold in Pxr-null than in WT mice (Fig. 6, B, E, and F). Although EtOH significantly increased hepatic ALDH2 protein levels in WT mice (1.4-fold), it had no significant effect on the levels in Pxr-null mice (Fig. 6C). In contrast, EtOH maintained the significantly higher hepatic ADH1, ALDH1A1, and CAT protein levels in Pxr-null mice, and both ADH1 and ALDH1A1 protein levels were significantly higher in EtOH-fed Pxr-null mice than in EtOH-fed WT mice (Fig. 6, B, E, and F).

Pro-inflammatory cytokines and cellular stress markers are up-regulated in EtOH-fed WT mice but not in Pxr-null mice

To determine the effect of PXR deficiency on the expression of inflammatory and endoplasmic reticulum (ER) stress mediators during EtOH exposure, mRNA levels of several pro-inflammatory cytokines and ER stress markers were measured. Ethanol increased mRNAs encoding tumor necrosis factor α (Tnfa, 3.0-fold) and toll-like receptor 7 (Tlr7, 3.0-fold) in WT mice but not in Pxr-null mice (Fig. 7, A and B). After EtOH treatment, both hepatic Tnfa and Tlr7 mRNA levels were significantly higher in WT mice than in Pxr-null mice (Fig. 7, A and B). The basal hepatic levels of the oxidative stress marker uncoupling protein 2 (Ucp2) mRNA were not different between both control-fed WT and Pxr-null mice, whereas EtOH significantly increased Ucp2 mRNA levels only in WT mice (3.3-fold) (Fig. 7C). Expression of hepatic ER stress marker GRP78 protein levels was significantly up-regulated 1.7-fold in EtOH-fed WT mice but not in Pxr-null mice (Fig. 7D). Interestingly, after EtOH treatment, hepatic GRP78 protein levels were significantly higher in WT mice than in Pxr-null mice (Fig. 7D). Basal expression of hepatic apoptotic protein Bax was elevated in Pxr-null mice (4.2-fold) compared with control-fed WT mice (Fig. 7E). Although EtOH significantly decreased Bax protein levels in Pxr-null mice, it up-regulated the Bax protein in WT mice (2.9-fold), although the increase was not statistically significant (Fig. 7E).

Residual EtOH concentrations are significantly higher in WT mice compared with their Pxr-null counterparts

The basal serum triglyceride concentration tended to be higher in Pxr-null mice than in WT mice (Fig. 8A). However, EtOH did not have any significant effect on the serum triglyceride levels in both genotypes (Fig. 8A). Serum ALT activity was measured as an index of hepatocyte injury. Serum ALT activity was not induced in EtOH-fed Pxr-null mice, but the levels were increased 2.3-fold in EtOH-fed WT mice, although not statistically significantly (Fig. 8B). However, after EtOH treatment, serum ALT levels were significantly higher in WT mice than in Pxr-null mice (Fig. 8B). To determine the impact on EtOH metabolism of elevated levels of ADH1, ALDH1A1, and CAT protein expression observed in Pxr-null mice, residual concentrations of EtOH were measured after the 8 weeks of chronic EtOH ingestion. The residual EtOH concentration was significantly higher in WT mice compared with their Pxr-null counterparts (Fig. 8C).

Discussion

This study investigated the effect of chronic EtOH administration on steatosis, hepatotoxicity, hepatic lipid profiles, lipid and alcohol metabolism pathways, PXR target genes, and inflammatory and cellular stress gene and protein expression in Pxr-null mice. The results demonstrated that when exposed to chronic EtOH, mice that lacked PXR showed dramatically reduced levels of hepatic steatosis; the reduced levels of histologic score for steatosis, hepatic triglycerides, and NEFA and serum ALT were consistent with this. Also, dramatically reduced were serum EtOH levels in Pxr-null mice, suggesting that compensatory mechanisms exist in the absence of PXR that counteract EtOH–induced steatosis and reduce blood EtOH concentration (BEC). Among 27 genes and 11 associated proteins known to function in hepatotoxicity, lipogenesis, and PXR, the expression of a limited number was significantly different between WT and Pxr-null mice. This observation suggests that other factors, such as inflammatory cytokines and ER stress markers, might be involved in the development of hepatic steatosis and other parameters studied.
EtOH. †, Pxr and chronic ethanol-induced steatosis

Figure 3. Gene expression and immunoblot analysis of mouse hepatic PXR and chronic ethanol-induced steatosis

EtOH metabolism, and inflammation, five were strongly up-regulated in response to chronic EtOH exposure in a PXR-dependent manner; these included the Car and its target Cyp2b10, Egr-1, Tnfα, and Thr7. Furthermore, the PPARα target genes Cpt-1, AcOX-1, and Lfabp-1 as well as Fxr were suppressed only in WT mice. The protein (but not the gene) expression of ADH1, ALDH1A1, and CAT, three enzymes involved in alcohol metabolism, as well as the lipid transfer protein MTP, was significantly higher in Pxr-null mice, suggesting post-transcriptional suppression of these enzyme levels by a PXR-dependent mechanism. These results establish for the first time that PXR signaling during chronic EtOH ingestion promotes steatosis, triglyceride accumulation, and inflammation, while inhibiting EtOH oxidation, suggesting that PXR deficiency is protective against chronic EtOH-induced hepatotoxicity.

Reports indicate that PXR and the closely related CAR cross-talk by sharing response elements and overlapping affinities for some ligands resulting in co-regulation of their target genes (22, 47). We first determined the effect of chronic EtOH on several nuclear receptors and their target genes, including PXR. In this study, no Pxr expression was in Pxr-null mice. Surprisingly, although the Pxr gene was up-regulated by EtOH in WT mice, the specific PXR target genes Slco1a4 (Oatp2) and Cyp7a1 were not increased. Furthermore, the prototypical PXR target gene Cyp3a11 (50), which can also be up-regulated by CAR, was mildly induced (1.6-fold) but not significantly by EtOH (47). Previous reports indicate that both Cyp2b10 and its human ortholog, CYP2B6, as well as murine CAR, can be directly regulated by PXR (51–53). It was hypothesized that PXR-mediated activation of CAR could be involved in EtOH–induced hepatotoxicity observed in this study. Indeed, Car mRNA expression was up-regulated in WT mice exposed to chronic EtOH but not in Pxr-null mice. Besides, this study also demonstrates a robust Pxr-dependent induction of the mRNA and protein of the CAR target gene Cyp2b10 in EtOH-fed WT mice. Expression of Cyp2b10 is thought to be mediated by CAR activation (54). The PXR-dependent effect of EtOH in WT mice may have clinical relevance to ALD. Interestingly, previous reports have implicated pharmacological activation of Car/Cyp2b10 and EtOH infusion in suppression of EtOH metabolism, enhanced steatosis, oxidative stress, and hepatotoxicity (12). Furthermore, in a recent study, while activation of CAR by its potent agonist, TCPBOB reversed EtOH–induced hyperbilirubinemia, it was associated with marked hepatotoxicity (55). In contrast, Car-null mice with dampened Cyp2b10 expression completely inhibited jet-lag–induced steatosis and inflammatory gene expression (56), suggesting that the Cyp2b10 induction by EtOH in WT mice seen in this study as a result of PXR/CAR cross-talk could be a major pathway for EtOH–induced hepatotoxicity.

Although the Pxr-null mice used in this study constitutively express CAR, an intriguing and more unexpected finding was the PXR-dependent robust increases in hepatic expression of Cyp2b10 (220-fold) in WT mice but not in Pxr-null mice by EtOH. The current results are consistent with an in vitro study showing Cyp2b10 up-regulation is observed when the ratio of PXR to CAR is high (57). However, Cyp2b10 repression...
occurred when the PXR-to-CAR ratio was low, as found in Pxr-null mice (57). Furthermore, other factors, including PXR, were found to be required for optimal CYP2B activation (47). Similar to the current findings, up-regulation of Cyp2b10 expression by EtOH and by other toxicants has been reported (58, 59). Furthermore, not only CYP2E1 but other CYPs, including CYP2B, can be induced by alcohol hepatitis (60). It is also possible that the marked induction of Cyp2b10 by EtOH in WT mice, may be a source of reactive oxygen species and increased oxidative stress as reported by others (61). Oxidative stress is known to play a pivotal role in the pathogenesis of ALD (62). Because Cyp2b10 is a prototypical PXR/CAR target gene, it is important to clarify whether PXR and/or CAR regulates EtOH-induced Cyp2b10 activation. Thus, further studies are warranted using Car-null mice together with Pxr/Car double-null mice to provide further insight into the precise roles of both PXR and CAR in ALD pathogenesis.

Chronic alcohol ingestion contributes to the development of fatty liver through multiple mechanisms. Furthermore, ligand-dependent activation and knockdown of PXR both resulted in
enhanced steatosis of HepG2 cells (42). Although numerous reports have shown that clinically relevant PXR ligands can increase plasma lipid levels in patients (40), it was unclear whether PXR signaling specifically contributes to EtOH–induced lipid accumulation. Furthermore, the effect of EtOH on livers of Pxr-null mice has not been reported. Our results show that the expression of hepatic PXR was significantly enhanced in EtOH–induced steatotic livers of WT mice fed EtOH. Importantly, expression of fatty acid oxidation genes, including Ppara, Cpt1, Acac-1, and Lfabp-1, were suppressed in EtOH-fed WT mice but not in Pxr-null mice. These results are consistent with a previous report in which PXR activation by its ligand, pregnenolone 16α-carbonitrile, was found to suppress PPARα target genes by binding directly to forkhead box A2 (FoxA2) while up-regulating SCD1, a key enzyme in lipogenesis (41). Although the effect of chronic EtOH ingestion on lipid accumulation has not been examined in PXR-humanized (hPXR) mice, previous reports revealed that hepatic lipid composition and pathology are differentially affected by mouse and human PXR by obesity induction and human PXR ligand treatment (33, 38, 63). For example, whereas HFD feeding resulted in mainly macrovesicular steatosis in WT mice, in contrast, microvesicular steatosis was most prominent in the livers of hPXR mice. Furthermore, long-term treatment of rifaximin, a potent gut-specific human PXR agonist, caused hepatic triglyceride accumulation in hPXR mice but not in WT mice (63). Moreover, hepatic lipid accumulations have also been reported in hPXR mice treated with the human PXR activa-
Figure 6. Immunoblot analysis of mouse hepatic enzymes involved in EtOH metabolism. Male WT and Pxr-null mice were pair-fed control diets or standard Lieber-Decarli liquid diet containing 5% EtOH (representing 27.5% of the total caloric intake) for 8 weeks. Western blottings of liver homogenate (40 μg/lane) were probed with antibodies to CYP2E1 (A), ADH1 (B), ALDH2 (C), ALDH1B1 (D), ALDH1A1 (E), and catalase (F). The blot used to probe for the CYP2E1 protein was initially used to also probe for the MTP protein in Fig. 4F. The membrane used to probe for CYP2B10 protein expression in Fig. 2f was stripped and reprobed with primary antibodies against ALDH2 and ALDH1A1. Bands were quantified and normalized to α-tubulin. Data represent mean ± S.D. (n = 3–4). *, p < 0.05 between WT and Pxr-null mice fed control. #, p < 0.05 between mice fed control diet and EtOH. †, p < 0.05 between mice fed EtOH.
tor, rifampicin (33). Because PXR activation is species-specific in terms of ligand binding, there may be important differences in PXR actions between mice and humans. Thus, whether human PXR activation is involved in steatosis and liver injury in human alcoholics is important and remains to be investigated.

Previous reports also linked other nuclear receptors FXR, CAR, and SHP to lipid homeostasis (64). Specifically, EtOH ingestion suppresses FXR activity leading to hepatic steatosis and liver injury, whereas pharmacological activation of FXR ameliorates EtOH–induced hepatic steatosis (65). Furthermore, FXR inhibits hepatic lipogenesis through up-regulation of SHP (66). In this study, both Fxr and Shp mRNAs were inhibited by EtOH in WT mice, but only Shp mRNA in Pxr-null mice. These findings are in agreement with a recent report indicating that ablation of mouse PXR suppresses hepatic lipid accumulation (37, 39, 67), whereas PXR activation increases triglyceride formation and steatosis (33, 68, 69). Expression of an activated PXR in the livers of transgenic mice resulted in hepatic triglyceride accumulation and steatosis (33). In this study, PXR activation in the liver by EtOH also resulted in hepatic steatosis and increased cytokine gene expression; however, contrary to the notion that PXR activation promotes hepatotoxicity, a significant down-regulation of PXR has been identified in the pathogenesis of inflammatory bowel disease (IBD) in humans (70–72). IBD refers to either ulcerative colitis or Crohn’s disease with chronic inflammation of the intestinal tract. Besides the liver, PXR is highly expressed in the intestinal tract (73, 74). Interestingly, PXR activation in the intestinal tract has been found to ameliorate experimentally-induced IBD in rodents, suggesting that tissue-specific activation of PXR may have different outcomes (74, 75).

Recent studies have shown that preventing or reducing EtOH–induced fatty liver may inhibit or delay the transition from fatty liver to steatohepatitis and then fibrosis (76). In this study, massive steatosis in EtOH-fed WT mice but not in Pxr-null mice was observed. Meanwhile, EtOH inhibition of MTP, a key enzyme regulating lipid export and very low density lipoprotein (VLDL) synthesis, contributes to alcoholic fatty liver; in contrast, MTP up-regulation reverses alcoholic steatosis (77). In this study EtOH up-regulated the MTP protein in livers of EtOH-fed Pxr-null mice but not in similarly treated WT mice.
suggesting the involvement of increased MTP activity in the protection against EtOH–induced steatosis in Pxr-null mice. Taken together, the present results revealed increased hepatic expression of SREBP-1C target gene Fas in association with decreased mRNA levels of PPARα target genes, Cpt-1, Acox-1, and Lfabp-1, Fxr and Shp in EtOH-fed WT mice. Furthermore, these data suggest that increased de novo synthesis together with inhibition of the transport of fatty acids into mitochondria for fatty acid β-oxidation contribute to steatosis in WT mice.

Accumulating evidence indicates that EGR-1, a zinc finger transcription factor, contributes to the development of EtOH–induced steatosis (48, 78). Interestingly, recent reports indicate that EGR-1 is an essential factor required for CAR to activate the CYP2B6 gene, suggesting that the up-regulation of Egr-1 expression by EtOH in WT mice may play a role in the Cyp2b10 activation seen in this study (79, 80). Furthermore, Egr-1 has also been implicated in the regulation of inflammatory gene expression, including TNFα (48). Egr-1-null mice are protected against chronic EtOH–induced steatosis, indicating that Egr-1 is required for the induction of alcoholic fatty liver and inflammatory gene expression (48, 81). EtOH induced both Egr-1 mRNA and protein as well as mRNA levels of Tnfa, Tlr7, and oxidative stress marker Ucp2 and the endoplasmic reticulum stress marker GRP78 only in WT mice, suggesting that PXR deficiency is protective against EtOH–induced Egr-1 activation and its associated Cyp2b10 up-regulation, steatosis, oxidative stress, and inflammatory cytokine activation.

EtOH is metabolized primarily via oxidation to acetaldehyde through the enzymatic activity of ADH, CAT, and CYP2E1 (7). Among the ADH isozymes, ADH1 is known to play a major role in hepatic EtOH clearance due to its low Km value for EtOH (7). Acetaldehyde generated from EtOH oxidation is then converted to acetate by the ALDHs, in which three isoforms ALDH2, ALDH1B1, and ALDH1A1 are responsible for metabolizing acetaldehyde (82–84). In the human liver, mitochondrial (low Km) ALDH2 oxidizes most of the EtOH-derived acetaldehyde (83). However, in rodents both mitochondrial and cytosolic ALDH isozymes are important in acetaldehyde oxidation (83). Previous reports have shown that mouse cytosolic ALDH1A1 has a high affinity for acetaldehyde, and it is an important mediator of alcohol and acetaldehyde metabolism (83, 85). Furthermore, cytosolic ALDH1A1 with micromolar Km values also contributes to oxidation of acetaldehyde, particularly in individuals who lack active ALDH2 (86). Another novel finding observed in this study was that protein expression but not the mRNAs of major genes regulating EtOH metabolism were up-regulated in the livers of Pxr-null mice compared with their WT counterparts. Specifically, both the basal and EtOH-treated hepatic ADH1, CAT, and ALDH1A1 protein levels were significantly higher in Pxr-null mice than in EtOH-fed WT mice. These findings are consistent with previous reports showing that hepatic ADH regulation is post-transcriptional and that ADH1 protein expression increases without a corre-

Figure 8. Serum triglycerides, alanine aminotransferase, and EtOH concentration. Male WT and Pxr-null mice were pair-fed control diets (control) or standard Lieber-Decarli liquid diet containing 5% EtOH (representing 27.5% of the total caloric intake) for 8 weeks. Serum was assayed for triglycerides (A), alanine aminotransferase (B), and EtOH concentration (C) as described under experimental procedures. Data represent mean ± S.D. (n = 4–6). #, p < 0.05 between mice fed control diet and EtOH. †, p < 0.05 between mice fed EtOH.
ADH1 protein expression may protect Pxr-null mice against EtOH–induced steatosis, because ADH1 deficiency is a determining factor for increased EtOH–induced lipid accumulation and hepatotoxicity (88).

Recent evidence indicates that PXR and its agonists, including those used in the clinic such as rifampicin, rifaximin, clofazimine, and lovastatin, play a role in drug-induced liver injury, a leading cause of acute liver failure (32, 89, 90). Compared with PXR agonists, relatively few PXR antagonists have been reported (91, 92). Similar to our current results in which Pxr-null mice are resistant to EtOH–induced steatosis, both cholic acid and HFD-induced hepatotoxicity are more severe in WT mice than in their Pxr-null counterparts (38, 93), suggesting that PXR deficiency protects against drug-induced hepatotoxicity and that inhibition of PXR signaling could be a means to prevent or treat ALD. Indeed, a recent report indicates that a novel human PXR antagonist, 1-(4-(4-((2R,4S)-2-(2,4-difluorophenyl)-2-methyl-1,3-dioxolan-4-yl) methoxy)phenyl)piperazin-1-yl)ethanone (FLB-12) that specifically disrupts the function of activated PXR decreased acetaminophen-induced hepatotoxicity in mice (92, 94).

In summary, these findings suggest that PXR ablation prevented EtOH induced up-regulation of the CAR target gene, Cyp2b10, implicated in EtOH hepatotoxicity. The results of this study clearly revealed the protective effects of PXR deficiency on EtOH–induced steatosis, lipid accumulation, inflammation, and ER stress as evidenced by inhibition of lipogenic transcription factor induction, increased MTP protein expression, and induction of major EtOH catabolism enzymes leading to increased EtOH clearance and low BEC. Thus, these results demonstrate that PXR aggravates chronic EtOH–induced steatosis, inflammation, and endoplasmic reticulum stress as depicted in Fig. 9. To our knowledge, this is the first report to explore the effects of PXR deficiency on chronic EtOH–induced hepatotoxicity. This work suggests the possibility that inhibition of PXR signaling could be a means to prevent or treat ALD.

**Experimental procedures**

**Animal care and treatment**

Genetically identical male C57BL/6 mice (which served as WT) and Pxr-null mice on the C57BL/6 genetic background were used in this study (35). Although the influence of gut microbiota was not examined in this study, all mice (3–5 mice/cage) were housed under equivalent housing conditions in a specific pathogen-free animal facility within a single holding room in polycarbonate cages on racks directly vented via the facility’s exhaust system at 22 °C with a 12/12-h light/dark cycle at the Animal Resources Complex at North Carolina Central University. After 2 days of feeding a control liquid diet, age-matched (8–10 weeks of age) male WT and Pxr-null mice were each randomly separated into two groups (n = 6–7), pair-fed (control) or EtOH groups. The EtOH-fed group was allowed free access to EtOH-containing diets with increasing concentrations of EtOH (1–5%) over a 7-day period as described previously (9). The EtOH concentration was kept thereafter at 5% for an additional 7 weeks. EtOH made up 27.5% of the total
caloric intake of mice in this group. Liquid diets were based upon the Lieber-DeCarli EtOH formulation and provide 1 kcal/ml, which was purchased from a single source (DYETS Inc., Bethlehem, PA). Protein content accounted for 18.9% of total calories and fat comprised 16.5% of total calories, whereas 64.5% of calories were derived from carbohydrate. The Lieber-DeCarli liquid diet model with 5% EtOH in the diet used in this study provides an excellent means to reproduce the early stages of chronic human ALD, including steatosis, oxidative stress, and liver injury (95, 96). Control mice were pair-fed diets that isocalorically substituted cornstarch for EtOH during the entire study period. After 8 weeks, mice were anesthetized with iso-flurane and killed. Sections of liver were rapidly dissected, weighed, snap-frozen in liquid nitrogen, and kept at −80°C. Blood samples collected by cardiac puncture from anesthetized mice were centrifuged at 3000 rpm for 15 min to collect serum and stored at −80°C to determine EtOH concentration, liver enzymes, and triglycerides. All procedures were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the North Carolina Central University Institutional Animal Care and Use Committee.

**H&E staining of liver sections and morphology analysis**

Liver slices were fixed in 10% formalin/phosphate-buffered saline, and then stained with H&E for histological examination. Slides were viewed blindly and scored for steatosis, inflammation, necrosis, and fibrosis using our previously described criteria (97). The liver pathology was scored as follows: steatosis (the percentage of liver cells containing fat), <25% = 1+, <50% = 2+, <75% = 3+, >75% = 4+; inflammation, necrosis, and fibrosis, 1 focus = 1+, 2 or more foci = 2+.

**Serum ALT and triglyceride measurements**

Serum was processed from blood and stored at −80°C. Serum ALT activity was determined using a commercially available kit (Sigma). Serum triglyceride levels were quantified using assay test kits (Wako Pure Chemical Industries, Richmond, VA).

**Determination of serum alcohol concentration**

Blood samples were collected after 8 weeks of treatment with either control or EtOH diet and centrifuged at 3000 rpm for 15 min to collect serum for BEC. The EtOH L3K assay kit for quantitative measurement of EtOH concentration (Sekisui Diagnostics P.E.I. Inc., Charlottetown, Canada) was used according to the manufacturer’s instructions as reported previously (98). The reaction is based on the enzymatic conversion of EtOH by ADH to acetaldehyde and NADH. EtOH concentration in the serum was quantified as the rate of increase in NADH absorbance due to the reduction of NAD⁺ at 380 nm.

**Hepatic triglyceride and NEFA levels**

Total liver lipids were extracted from 100 mg of liver homogenate using methanol and chloroform as described previously (9). Hepatic triglyceride and NEFA levels were quantified using triglyceride and NEFA test kits (Wako Pure Chemical Industries, Richmond, VA).

**Preparation of liver extracts for Western blot analyses**

Frozen livers were homogenized at 4°C, and Western blot analysis was performed on extracts as described previously (99). Protein contents in liver homogenates were determined by the BCA™ protein assay kit (Thermo Fisher Scientific, Rockford, IL). Liver homogenates (40 µg/lane) were diluted in Laemmli loading buffer containing β-mercaptoethanol, boiled for 5 min, separated on 10 or 15% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were probed overnight with rabbit polyclonal primary antibodies from Sigma at a concentration of 1:5000 for anti-CYP2B10 (AB9916, lot no. 2794496). Rabbit polyclonal primary antibodies from Abcam (Cambridge, MA) were used at a concentration of 1:1000 for anti-CYP2E1 (ab28146, lot no. GR182611-20) and anti-catalase (ab16731, lot no. GR1693791). Primary antibodies from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), were used as follows: concentration of 1:200 for mouse monoclonal anti-ADH1 (Sc-137078 lot no. L3015); goat polyclonal antibody at a concentration of 1:1000 for anti-ALDH2 (Sc-48837, lot no. D1709); mouse monoclonal antibody at a concentration of 1:500 for anti-ALDH1A1 (Sc-393583, lot no. K2613); mouse monoclonal antibody at a concentration of 1:200 for anti-MTP (Sc-135994, lot no. G2115); or rabbit polyclonal antibody at a concentration of 1:200 for anti-GRP78 (Sc-13968, lot no. H1809). Rabbit monoclonal primary antibodies from Novus Biologicals (Littleton, CO) were used at a concentration of 1:1000 for anti-ALDH1A1 (NB110-55451, lot no. GR182706-7). Primary antibodies from Cell Signaling Technology (Boston, MA) were used at a concentration of 1:1000 for rabbit polyclonal anti-Bax (no. 2772, lot no.: 10) or were used at a concentration of 1:1000 for rabbit monoclonal anti-EGR-1 (no. 4154, lot no. 3). Blots were then incubated with the appropriate peroxidase-conjugated secondary antibodies from Cell Signaling Technology or Santa Cruz Biotechnology, Inc., and diluted in TBST plus 1% milk for 60 min at room temperature. Following initial probing, blots were stripped and reprobed with rabbit monoclonal antibody from Cell Signaling Technology at a concentration of 1:1000 for anti-α-tubulin (no. 2125, lot no. 9). Proteins were visualized using enhanced chemiluminescence, and band intensity was quantified using ImageJ software (National Institutes of Health, Bethesda).

**Quantification of mRNA levels using real-time PCR**

Total RNA was isolated from frozen liver tissues using the TRIzol reagent according to the manufacturer’s protocol (Invitrogen). Total RNA (5 µg) was reverse-transcribed into cDNA with random hexamer primers using Tetro cDNA synthesis kit (Bioline, Taunton, MA) as we described previously (38). The cDNA was then diluted 20-fold with water and subjected to real-time quantitative PCR by the SensiFast SYBR Hi-ROX Kit (Bioline, Taunton, MA) to quantify the mRNA levels of Ppara, Cpt1, Acox-1, Lfabp-1, Mtp, Srebp-1c, Scd1, Acc-1α, Fas, Srebp2, Egr-1, Cyp2b10, Cyp7a1, Tnfo, Trl7, Fxr, Shp, and GAPDH. The primers for the SYBR Green assay (Table 2) were
designed using Primer Express 2.0 (Applied BioSystems, Foster City, CA). The primer sequence for TLR7 was previously published (15). Furthermore, the following proprietary TaqMan Gene Expression Assays were purchased from Applied Biosystems/Life Technologies, Inc., and used for real-time quantitative PCR:

- **Adh1** (No. Mm00507711_m1);
- **Aldh2** (No. Mm00477463_m1);
- **Aldh1a1** (No. Mm00657317_m1);
- **Aldh1b1** (No. Mm00728303_s1);
- **Pxr** (No. Mm01344139_m1);
- **Cyp3a11** (No. Mm00731567_m1);
- **Car** (No. Mm01283978_m1);
- **Ucp2** (No. Mm00627599_m1);
- **Slco1a4** (Oatp2, No. Mm01267407_m1);
- **Cat** (No. Mm00437992_m1); and
- **Gapdh** (housekeeping gene, No. 99999915_g1).

The amplification reactions were performed in the ABI QuantStudio™ 3 Systems (Applied Biosystems, Foster City, CA). Results were presented as levels of expression relative to that of controls after normalizing with Gapdh mRNA using the comparative \( \Delta C_T \) method.

**Statistical analysis**

Data are presented as means ± S.D. (\( n = 6 – 7 \)). Statistical analysis was performed using one-way analysis of variance followed by Tukey HSD post hoc test. A p value of < 0.05 was considered statistically significant. Statistical analyses were performed using IBM SPSS Statistics 20 software (Armonk, NY).

**Author contributions**—M. A. G. conceived and designed experiments. S. C., P. N., and M. A. G. conducted experiments. M. A. G. and F. J. G. contributed new reagents and materials. S. C., M. A. G., and S. W. F. performed data analysis. M. A. G., F. J. G., and S. W. F. wrote or contributed to the writing of the manuscript.

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**Table 2**

Sequences of primers used for a SYBR Green real-time quantitative PCR

| Name     | Sequence | Accession no. |
|----------|----------|---------------|
| PPARα Sense | GATTCCAGAAGAGAAGGACGGGCAACA | NM011144 |
| Antisense | TGGCTTTTACGAACCTGGCACCTTCC | |
| CPT-1 Sense | GCAAGCTATAGAATAGACGCTACT | NM014394 |
| Antisense | GCCGCTTTTCTGCAAGACATC | |
| ACOX1 Sense | TTGTTGTTCCCTCTCAGGTTAAG | NM015729 |
| Antisense | GCCGATATCCCCAACAGGTAAG | |
| l-FABP Sense | TGGCGAAGGAGAGAAGGACGGGCAACA | NM017399 |
| Antisense | CCCCAAGGTTAATGTATGCATC | |
| MTP Sense | CCCCTCTCTTCTGCAAGGTAAG | NM008642 |
| Antisense | TGGTACACTATTGTTGCTCTATG | |
| EGR1 Sense | GCCCTGCTGACGCTGACAAT | NM007913 |
| Antisense | GCAAGGAGGACGGGCAACA | |
| SREBP2 Sense | ATGCACACTGCAAGGTAATC | NM032318 |
| Antisense | GCTGGCTTTTCTGCAAGACATC | |
| SREBP-1c Sense | CAGGAATTGAGGTAATGCAAA | NM014880 |
| Antisense | TGGTACACTATTGTTGCTCTATG | |
| SCD1 Sense | CTTCCGAGAAGTAATGCAAA | NM009127 |
| Antisense | AGGCGGGGCTGTTGCTCTATG | |
| TNFa Sense | ACAGGGGCCCTGCACGCTAC | NM013693 |
| Antisense | TTTCTGCTTCTGCAAGGTAATC | |
| ACC-1α Sense | AGGGCGAGATGAGGTAATGCAAA | NM133630 |
| Antisense | TGGTACACTATTGTTGCTCTATG | |
| FAS Sense | GCCGAGATCAGGTAATGCAAA | NM007988 |
| Antisense | GACCGGGGCTGTTGCTCTATG | |
| CYP7a1 Sense | CAGGAGATCAGGTAATGCAAA | NM007824 |
| Antisense | GACCGGGGCTGTTGCTCTATG | |
| CYP2B10 Sense | TCTTGCTTCTTCTGCAAGGTAATC | NM009999 |
| Antisense | ATGGACGTGAAGAGGGAACAGGA | |
| FXR Sense | CAAGGAGATCAGGTAATGCAAA | NM009108 |
| Antisense | GCCGAGATCAGGTAATGCAAA | |
| SHP Sense | CAAGGAGATCAGGTAATGCAAA | NM011850 |
| Antisense | GCCGAGATCAGGTAATGCAAA | |
| GAPDH Sense | TGTTCCGCTTCTGCAAGGTAATC | NM001001303 |
| Antisense | GCCGAGATCAGGTAATGCAAA | |
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