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Regulation of Cytochrome P450 2B10 (CYP2B10) Expression in Liver by Peroxisome Proliferator-activated Receptor-β/δ Modulation of SP1 Promoter Occupancy*

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Alcoholic liver disease is a pathological condition caused by overconsumption of alcohol. Because of the high morbidity and mortality associated with this disease, there remains a need to elucidate the molecular mechanisms underlying its etiology and to develop new treatments. Because peroxisome proliferator-activated receptor-β/δ (PPARβ/δ) modulates ethanol-induced hepatic effects, the present study examined alterations in gene expression that may contribute to this disease. Chronic ethanol treatment causes increased hepatic CYP2B10 expression in *Pparβ/δ*$^{+/+}$ mice but not in *Pparβ/δ*$^{-/-}$ mice. Nuclear and cytosolic localization of the constitutive androstane receptor (CAR), a transcription factor known to regulate *Cyp2b10* expression, was not different between genotypes. PPARγ co-activator 1α, a co-activator of both CAR and PPARβ/δ, was up-regulated in *Pparβ/δ*$^{+/+}$ liver following ethanol exposure, but not in *Pparβ/δ*$^{-/-}$ liver. Functional mapping of the *Cyp2b10* promoter and ChIP assays revealed that PPARβ/δ-dependent modulation of SP1 promoter occupancy up-regulated *Cyp2b10* expression in response to ethanol. These results suggest that PPARβ/δ regulates *Cyp2b10* expression indirectly by modulating SP1 and PPARγ co-activator 1α expression and/or activity independent of CAR activity. Ligand activation of PPARβ/δ attenuates ethanol-induced *Cyp2b10* expression in *Pparβ/δ*$^{+/+}$ liver but not in *Pparβ/δ*$^{-/-}$ liver. Strikingly, *Cyp2b10* suppression by ligand activation of PPARβ/δ following ethanol treatment occurred in hepatocytes and was mediated by paracrine signaling from Kupffer cells. Combined, results from the present study demonstrate a novel regulatory role of PPARβ/δ in modulating CYP2B10 that may contribute to the etiology of alcoholic liver disease.

Chronic consumption of ethanol causes steatosis, hepatomegaly, hepatitis, fibrosis, and cirrhosis collectively referred to as alcoholic liver disease and has become a major health issue because of high morbidity and mortality (1, 2). Nutrient deficiencies, impaired fatty acid metabolism, induction of xenobiotic-metabolizing enzymes, and increased oxidative stress are all associated with liver toxicity induced by ethanol (3). However, the precise molecular mechanisms underlying the cause of alcoholic liver disease are not well understood.

Nuclear receptors have key roles in regulating lipid homeostasis and inflammation during the pathogenesis of alcoholic liver disease (4, 5). For example, activation of constitutive androstane receptor (CAR)[3] facilitates ethanol metabolism, resulting in enhanced liver damage by increasing oxidative stress, apoptosis, and accumulation of lipids in hepatocytes (6). By contrast, peroxisome proliferator-activated receptor-α (PPARα) shows a protective role in alcoholic liver disease because ethanol-treated *Pparα*$^{-/-}$ mice exhibit marked liver damage including hepatomegaly, hepatic inflammation, fibrosis, and apoptosis as compared with ethanol-treated *Pparα*$^{+/+}$ mice (7). A recent study showed that PPARβ/δ, another PPAR subtype, prevents ethanol-induced hepatic effects by suppressing lipogenesis, modulating amino acid metabolism, and altering pyridoxal kinase activity (8). This is consistent with previous studies showing that PPARβ/δ protects against liver damage induced by various hepatotoxicants (9–11). Although the protective role of PPARβ/δ in chemically induced liver toxicity was demonstrated in several models, the detailed molecular mechanism(s) that mediate protection against alcoholic liver disease are not well understood. Thus, the present study focused on identifying and characterizing genes regulated by PPARβ/δ and their roles in alcoholic liver disease.

Results

PPARβ/δ Modulates Ethanol-induced Hepatic Cyp2b10 Expression—Microarray analysis was performed using liver RNA from *Pparβ/δ*$^{+/+}$ and *Pparβ/δ*$^{-/-}$ mice to identify genes

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[3] The abbreviations used are: CAR, constitutive androstane receptor; PPAR, peroxisome proliferator-activated receptor; PGCα, PPARγ co-activator α; LDH, lactate dehydrogenase; qPCR, quantitative real time PCR; ANOVA, analysis of variance.

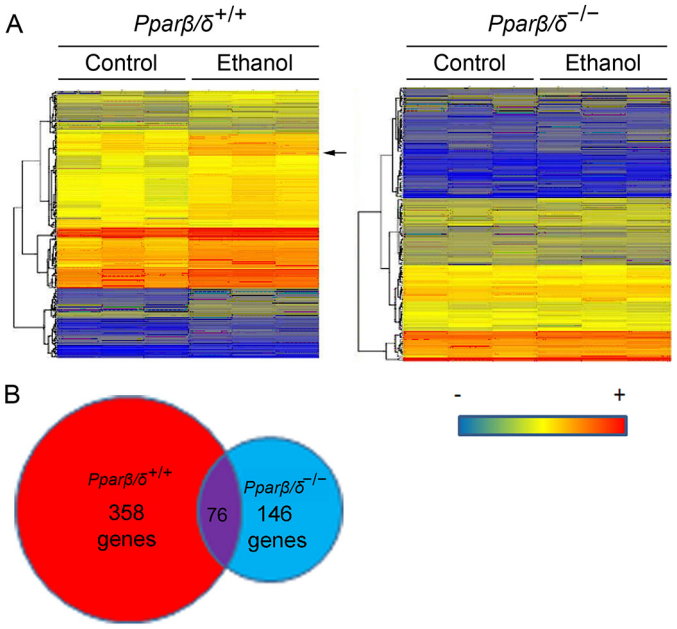

FIGURE 1. **PPARβ/δ-dependent hepatic gene expression in response to ethanol exposure.** *A*, heat map of hepatic gene expression from control and ethanol-treated *Pparβ/δ*$^{+/+}$ and *Pparβ/δ*$^{-/-}$ mice as assessed by microarray analysis. The *arrow* in the *left panel* indicates that *Cyp2b10* mRNA that was induced by ethanol in *Pparβ/δ*$^{+/+}$ mice, but the induction was not observed in *Pparβ/δ*$^{-/-}$ mice. *B*, ethanol exposure altered expression of 358 genes and 146 genes, in *Pparβ/δ*$^{+/+}$ and *Pparβ/δ*$^{-/-}$ mice, respectively. 76 genes overlapped.

that were differentially regulated by ethanol (see NCBI Gene Expression Omnibus database accession number GSE86002). The gene expression profile was markedly different between genotype with distinct changes in gene expression noted in both *Pparβ/δ*$^{+/+}$ and *Pparβ/δ*$^{-/-}$ mice (Fig. 1*A*). Of particular note was the PPARβ/δ-dependent increase in hepatic *Cyp2b10* expression in *Pparβ/δ*$^{+/+}$ but not *Pparβ/δ*$^{-/-}$ mice (Fig. 1*A*). This was of interest because previous studies demonstrated a similar PPARβ/δ-dependent effect on the hepatic expression of CYP2B10 in mice exposed to carbon tetrachloride (10). Overall, ethanol exposure significantly altered expression of 358 genes or 146 genes, in livers of *Pparβ/δ*$^{+/+}$ or *Pparβ/δ*$^{-/-}$ mice, respectively (Fig. 1*B*), with very little overlap between genotypes (76 gene products).

Ethanol-induced Cyp2b10 Expression Is Independent of CAR Signaling—Previous work revealed that ligand activation of CAR caused up-regulation of CYP2B10 in both Pparβ/δ$^{+/+}$ and Pparβ/δ$^{-/-}$ mice (10). This suggested that CAR activity was not influenced by PPARβ/δ. However, whether PPARβ/δ modulated the ability of CAR to translocate and activate transcription of *Cyp2b10* was not examined in the former study. Although expression of *Cyp3a11* mRNA in the liver of *Pparβ/δ*$^{+/+}$ and *Pparβ/δ*$^{-/-}$ mice fed ethanol was increased compared with controls, the expression of *Cyp2b10* mRNA was increased in the liver of *Pparβ/δ*$^{+/+}$ mice but not in *Pparβ/δ*$^{-/-}$ mice fed ethanol compared with controls (Fig. 2*A*). Because *Cyp2b10* and *Cyp3a11* are known target genes of CAR (12), but activating CAR with a ligand is not influenced by PPARβ/δ (10), the translocation of CAR was determined by quantitative Western blotting analysis. Interestingly, ethanol treatment did not influence the relative nuclear to cytosolic ratio of CAR expression compared with controls in either genotype (Fig. 2*B*). LAMIN and LDH were used as positive controls for nuclear and cytosolic enrichment.

Ethanol-induced Hepatic Cyp2b10 Expression Is Associated with PPARβ/δ-dependent Expression of Peroxisome Proliferator-activated Receptor γ Co-activator 1α (PGC1α)—Ethanol treatment caused increased nuclear PGC1α expression in *Pparβ/δ*$^{+/+}$ mouse liver but not in *Pparβ/δ*$^{-/-}$ mouse liver compared with controls (Fig. 3*A*). Regression analysis revealed that ethanol-induced *Cyp2b10* mRNA was positively correlated with higher nuclear PGC1α expression in *Pparβ/δ*$^{+/+}$ mouse liver following ethanol treatment (Fig. 3*B*). However, this correlation was not found in *Pparβ/δ*$^{-/-}$ mouse liver compared with controls (Fig. 3*B*). To determine whether PPARβ/δ-dependent PGC1α expression is required for ethanol-induced *Cyp2b10* expression, PGC1α was knocked down in primary hepatocytes from *Pparβ/δ*$^{+/+}$ and *Pparβ/δ*$^{-/-}$ mice using siRNA. Quantitative Western blotting analysis confirmed the knockdown of PGC1α expression in hepatocytes from both genotypes and that ethanol increased nuclear PGC1α in *Pparβ/δ*$^{+/+}$ mouse hepatocytes but not in *Pparβ/δ*$^{-/-}$ mouse hepatocytes compared with controls (Fig. 3*C*). Further, *Cyp2b10* mRNA expression was increased in primary *Pparβ/δ*$^{+/+}$ mouse hepatocytes by ethanol, but this effect was mitigated when PGC1α expression was knocked down (Fig. 3*D*). By contrast, ethanol had no effect on *Cyp2b10* expression in *Pparβ/δ*$^{-/-}$ mouse hepatocytes compared with controls, and *Cyp2b10* expression was also not influenced by knockdown of PGC1α; with or without ethanol (Fig. 3*D*). Because nuclear translocation of CAR is not influenced by PPARβ/δ expression, and previous studies demonstrated that ligand activation of CAR in mouse liver is unaffected by PPARβ/δ expression (10), these results suggest that the ethanol-induced expression of *Cyp2b10* is dependent in part on PGC1α and PPARβ/δ.

Ethanol-induced Hepatic Cyp2b10 Expression Is Regulated by PPARβ/δ-dependent Modulation of SP1 Activity—Because the previous results indicate that ethanol-induced *Cyp2b10* expression was not mediated by CAR activation, functional mapping of the 5′ upstream region of the *Cyp2b10* gene was performed to identify important regulators that may influence ethanol-induced *Cyp2b10* expression. Reporter gene assays revealed that the −1 to −500 region of the *Cyp2b10* promoter contained critical *cis*-regulatory elements that were responsive to ethanol exposure (Fig. 4*A*). The putative *trans*-acting factors in this region included the octamer-binding transcription factor 1 (OCT1), the CCAAT/enhancer binding protein β (C/EBPβ), and specificity protein 1 (SP1) (Fig. 4*B*). Mutation of the SP1-binding site in the *Cyp2b10* promoter caused decreased luciferase activity following ethanol exposure compared with controls (Fig. 4*C*). However, this effect was not observed using either mutant OCT1 or mutant C/EBPβ constructs, indicating that these transcription factors are not required for ethanol-induced *Cyp2b10* expression (Fig. 4*C*). A ChIP assay further confirmed that SP1 occupancy on the *Cyp2b10* promoter was higher in ethanol-treated *Pparβ/δ*$^{+/+}$ mouse hepatocytes compared with control (Fig. 4*D*). By contrast, SP1 occupancy of the *Cyp2b10* promoter was not signifi-

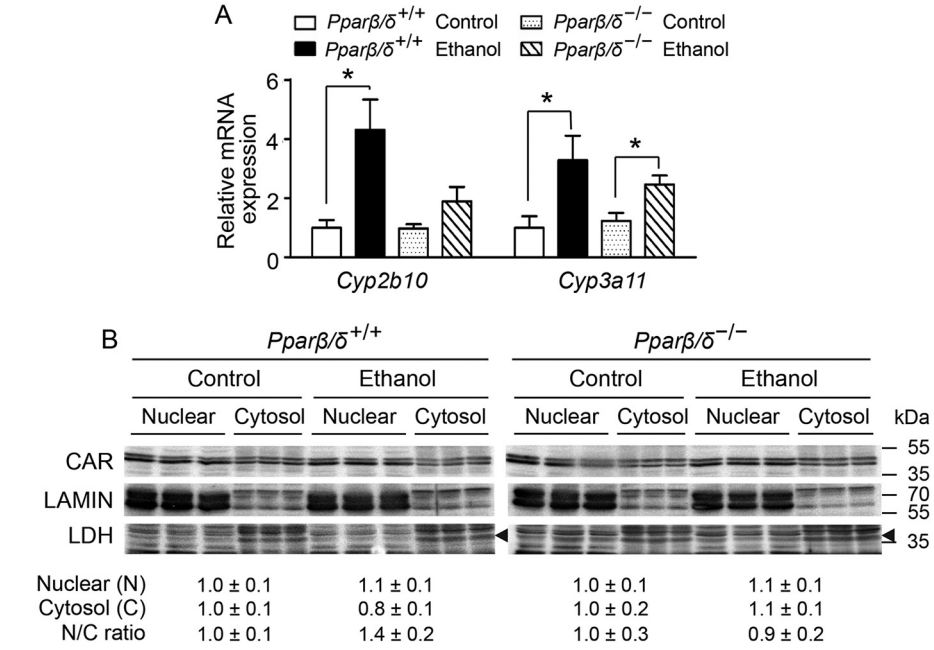

FIGURE 2. **PPARβ/δ-dependent ethanol-induced *Cyp2b10* expression is independent of CAR translocation.** *A*, relative expression of hepatic *Cyp2b10* and *Cyp3a11* mRNA in control and ethanol-treated *Pparβ/δ*$^{+/+}$ and *Pparβ/δ*$^{-/-}$ mice. *B*, quantitative Western blotting analysis of hepatic CAR expression in nuclear and cytosolic fractions of *Pparβ/δ*$^{+/+}$ and *Pparβ/δ*$^{-/-}$ mice treated with or without ethanol. *Arrowheads* mark LDH. Relative expression level of nuclear CAR was normalized to that of LAMIN, and the relative expression level of cytosolic CAR was normalized to that of LDH. The values represent the means ± S.E. *, significantly different between groups ($p \leq 0.05$).

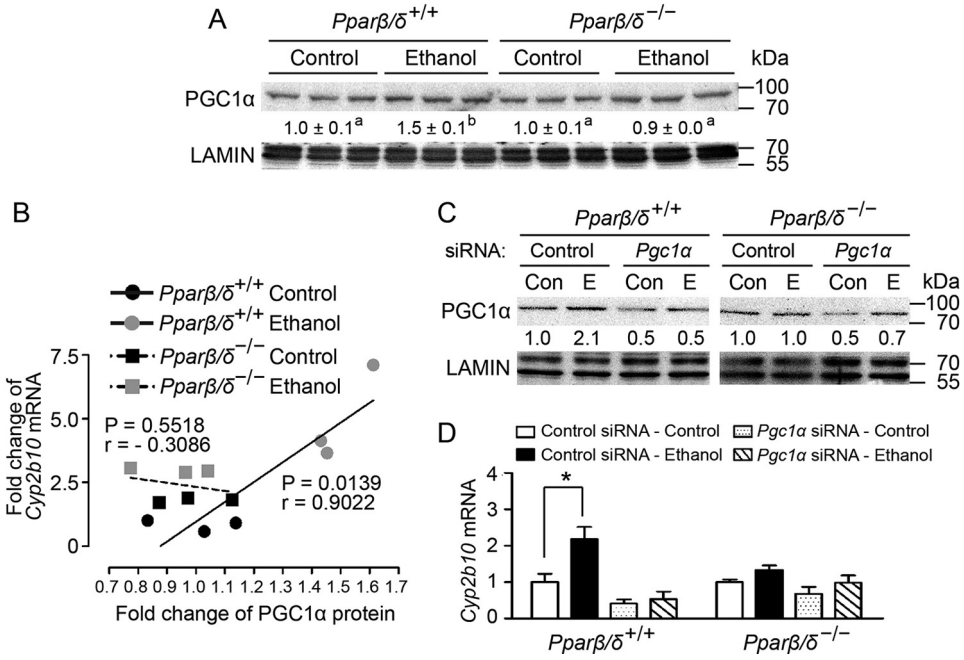

FIGURE 3. **PGC1α is required for ethanol-induced *Cyp2b10* expression in the liver.** *A*, quantitative Western blotting analysis showing ethanol-induced nuclear PGC1α expression in the livers from *Pparβ/δ*$^{+/+}$ and *Pparβ/δ*$^{-/-}$ mice. *B*, regression analysis of *Cyp2b10* mRNA expression with nuclear PGC1α protein expression in the liver ($n = 3$ independent samples/group). *C*, quantitative Western blotting analysis of nuclear PGC1α expression. *D*, knocking down PGC1α attenuates ethanol-induced *Cyp2b10* mRNA in primary hepatocytes from *Pparβ/δ*$^{+/+}$ mice. The values represent the means ± S.E. *, significantly different between groups ($p \leq 0.05$). The values with different *letters* are significantly different at $p \leq 0.05$.

cantly different in *Pparβ/δ*$^{-/-}$ mouse hepatocytes treated with or without ethanol (Fig. 4*D*).

Ligand Activation of PPARβ/δ Attenuates Ethanol-induced Hepatic CYP2B10 Expression—To further address the critical role of PPARβ/δ in modulating alcohol-induced liver injury, *Pparβ/δ*$^{+/+}$ and *Pparβ/δ*$^{-/-}$ mice were treated with the PPARβ/δ ligand GW0742 with or without ethanol exposure. Ligand activation of PPARβ/δ, with or without ethanol exposure increased expression of *Angptl4* mRNA, a well known PPARβ/δ target gene in *Pparβ/δ*$^{+/+}$ mouse liver but not in *Pparβ/δ*$^{-/-}$ mouse liver (Fig. 5*A*). Hepatic *Cyp2b10* mRNA expression in *Pparβ/δ*$^{+/+}$ mice fed the ethanol diet was higher than controls (Fig. 5*A*). Although ligand activation of PPARβ/δ with GW0742 did not influence the basal level of *Cyp2b10*

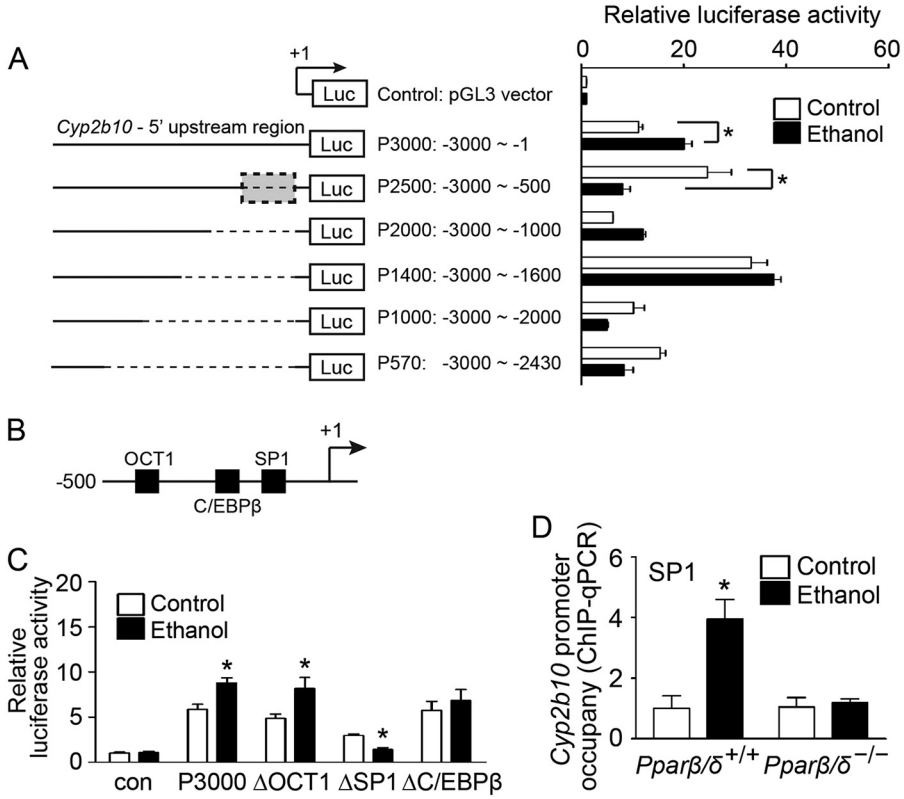

FIGURE 4. **PPARβ/δ regulates ethanol-induced *Cyp2b10* expression by modulating SP1 activity.** *A*, six luciferase reporter constructs (P3000, P2500, P2000, P1400, P1000, and P570) containing various fragments of 5′ upstream region of the mouse *Cyp2b10* gene were generated (*left panel*). Primary hepatocytes were isolated from adult male *Pparβ/δ*$^{+/+}$ mice. The cells were co-transfected with luciferase reporter constructs and pCMV-β-gal plasmid and treated with or without ethanol. Relative luciferase activity was determined and normalized to control group (*right panel*). *B*, putative *cis*-regulatory elements in the −1 to −500 bp of 5′ upstream region of *Cyp2b10* gene. *C*, site-directed mutagenesis was performed to generate luciferase reporter constructs carrying mutations in OCT1, SP1, or C/EBPβ binding sites of the *Cyp2b10* 5′ upstream region. Primary hepatocytes were transfected with mutant luciferase reporter constructs and treated with or without ethanol. Relative luciferase activity was determined and normalized to control (*Con*). *D*, ChIP-qPCR showing PPARβ/δ-dependent increased SP1 occupancy on the *Cyp2b10* promoter in response to ethanol exposure in primary hepatocytes. The values were corrected for background using rabbit IgG controls and represent the means ± S.E. *, significantly different from controls ($p \leq 0.05$).

mRNA in *Pparβ/δ*$^{+/+}$ liver, ethanol-induced *Cyp2b10* mRNA expression was significantly suppressed by ligand activation of PPARβ/δ in *Pparβ/δ*$^{+/+}$ mouse liver compared with controls (Fig. 5*A*). These observations were consistent with quantitative Western blotting analysis, showing that microsomal expression of hepatic CYP2B10 was induced by ethanol treatment in *Pparβ/δ*$^{+/+}$ mice, and this induction was diminished by ligand activation of PPARβ/δ (Fig. 5*B*). No significant changes in hepatic CYP2B10 mRNA and protein expression were observed in any groups of *Pparβ/δ*$^{-/-}$ mice (Fig. 5).

Paracrine Signaling from Intact Kupffer Cells Is Required for PPARβ/δ-dependent Cyp2b10 Expression in Hepatocytes—To determine the specific cell types that mediate attenuation of ethanol-induced *Cyp2b10* expression in liver, co-cultures of primary hepatocytes, primary non-parenchymal cells or Kupffer cells alone isolated from *Pparβ/δ*$^{+/+}$ and *Pparβ/δ*$^{-/-}$ mice were examined. In the presence or absence of ethanol treatment, ligand activation of PPARβ/δ increased *Angptl4* mRNA expression in *Pparβ/δ*$^{+/+}$ hepatocytes but not in *Pparβ/δ*$^{-/-}$ hepatocytes (Fig. 6*A*). Surprisingly, ligand activation of PPARβ/δ did not suppress ethanol-induced *Cyp2b10* mRNA expression in *Pparβ/δ*$^{+/+}$ hepatocytes (Fig. 6*B*), inconsistent with the results observed *in vivo* (Fig. 5*A*). Co-culturing primary hepatocytes with non-parenchymal cells revealed that the decrease in ethanol-induced *Cyp2b10* mRNA expression following ligand activation of PPARβ/δ was restored and that this rescue was effective when hepatocytes were co-cultured solely with Kupffer cells (Fig. 6, *C* and *D*). These changes in *Cyp2b10* mRNA expression were not observed in any groups of *Pparβ/δ*$^{-/-}$ co-cultures (Fig. 6, *C* and *D*).

Discussion

The metabolism of ethanol is mediated by enzymes including alcohol dehydrogenase and cytochrome P450s (CYP2E1 and CYP2B) (13). Oxidation of ethanol produces highly reactive oxygen species, which can cause liver damage (14, 15). Variation in the expression of these enzymes influences the sensitivity and the adaption to ethanol consumption (13). PPARβ/δ can protect against chemically induced liver injury through multiple mechanisms including inhibition of steatosis, inhibition of NF-kB-dependent signaling, and inhibition of inflammation (10, 11, 16, 17). A previous study revealed that exposure to carbon tetrachloride (CCl_4) caused an increase in hepatic *Cyp2b10* expression, and this induction was mediated by PPARβ/δ, which was not due to differences in the relative expression of CAR (10), similar to the results observed in the present study. The present study also showed that ethanol induced *Cyp2b10* expression through a mechanism that

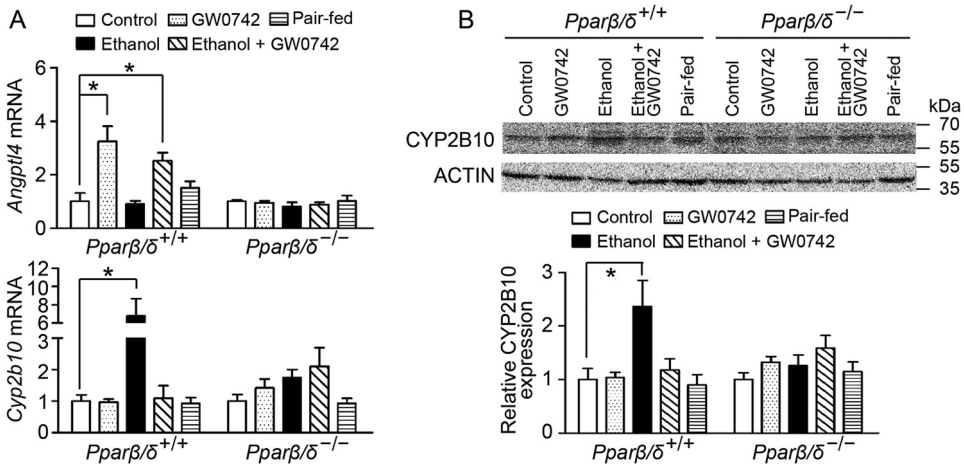

FIGURE 5. **Ligand activation of PPARβ/δ suppresses ethanol-induced hepatic CYP2B10 expression *in vivo*.** *A*, expression of *Angptl4* and *Cyp2b10* mRNA in the liver from *Pparβ/δ*$^{+/+}$ and *Pparβ/δ*$^{-/-}$ mice. *B*, quantitative Western blotting analysis of hepatic CYP2B10 expression following ligand activation of PPARβ/δ with GW0742. The values represent the means ± S.E. *, significantly different between groups ($p \leq 0.05$).

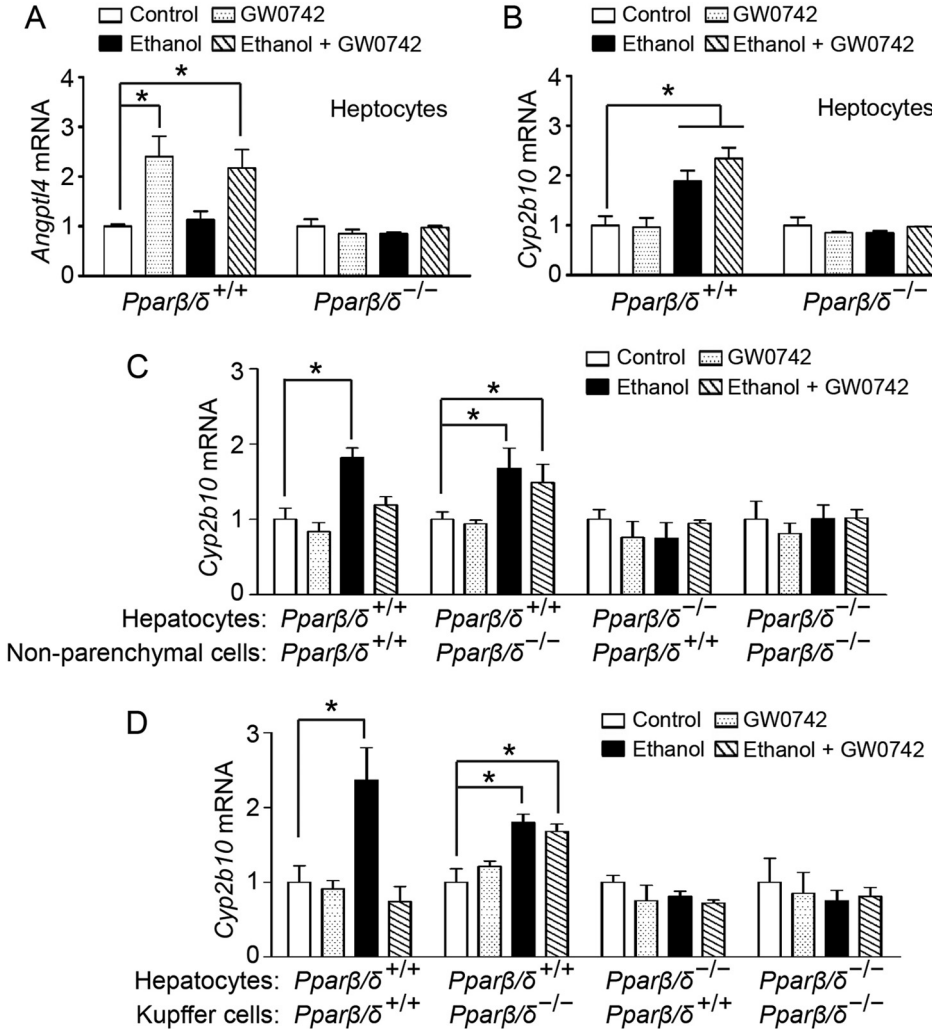

FIGURE 6. **Paracrine signaling from Kupffer cells is required to modulate PPARβ/δ-dependent expression of *Cyp2b10* in hepatocytes.** *A* and *B*, expression of *Angptl4* (*A*) and *Cyp2b10* (*B*) mRNA in primary hepatocytes from *Pparβ/δ*$^{+/+}$ and *Pparβ/δ*$^{-/-}$ mice. *C* and *D*, primary non-parenchymal cells (*C*) or primary Kupffer cells (*D*) from *Pparβ/δ*$^{+/+}$ and *Pparβ/δ*$^{-/-}$ mice were co-cultured with primary hepatocytes in Transwell® culture plates. The values represent the means ± S.E. *, significantly different between groups ($p \leq 0.05$).

required PPARβ/δ. Expression of *Cyp2b10* is thought to be mediated by CAR activation (18). However, the present study clearly showed that ethanol treatment did not influence the relative expression of CAR as also observed in a previous study (10) or enhance the nuclear translocation of CAR following treatment with ethanol. Because the translocation of CAR to the nucleus is required to initiate target gene expression (19–21), this illustrates a unique finding from the present study because the results indicate that PPARβ/δ, rather than CAR, is required for up-regulation of *Cyp2b10* in response to ethanol. This is similar to the PPARβ/δ-dependent up-regulation of hepatic *Cyp2b10* observed following treatment with CCl_4, and the fact that ligand activation of CAR caused up-regulation of *Cyp2b10* in both *Pparβ/δ*$^{+/+}$ and *Pparβ/δ*$^{-/-}$ mouse liver (10).

An alternative pathway that could be influenced by PPARβ/δ that impacts CAR activity is the relative expression of PGC1α. It is known that PPARβ/δ regulates PGC1α expression (22) and that CAR requires PGC1α as a co-activator to remodel chromatin of target genes (23). The present study demonstrated that nuclear PGC1α level was increased by ethanol treatment, and this increased expression of PGC1α requires PPARβ/δ. Indeed, PPARβ/δ-dependent expression of PGC1α is required for the increase in ethanol-induced *Cyp2b10* expression because knocking down PGC1α prevented this effect in wild-type hepatocytes. This is the first study to demonstrate the essential role of PPARβ/δ-dependent expression of PGC1α in regulating *Cyp2b10* expression in response to ethanol exposure. These findings suggest that one mechanism that may mediate the PPARβ/δ-dependent expression of *Cyp2b10* is via modulation of PGC1α, which is required for CAR activation. The present studies strongly support this notion, but further work is needed to confirm this hypothesis. Interestingly, the basal level of *Cyp2b10* mRNA expression was not altered by knocking down PGC1α compared with controls, suggesting that PGC1α is not critical for regulating constitutive *Cyp2b10* expression.

In addition to CAR, the transcription factor SP1 also regulates *Cyp2b1* expression in rat hepatocytes (24). The present study revealed that ethanol-induced increased promoter occupancy of SP1 on the *Cyp2b10* gene, and this effect required the expression of PPARβ/δ. Examination of the microarray data did not indicate an increase in the expression of *Sp1* mRNA. However, PGC1α can also increase SP1-mediated gene expression by either increasing SP1 expression or enhancing the recruitment of SP1 to the transcription complex (25, 26). Thus, it is possible that the PPARβ/δ-dependent expression of PGC1α induced by ethanol may also indirectly regulate *Cyp2b10* expression by influencing the occupancy of SP1 on the *Cyp2b10* promoter.

Because expression of CYPs is known to be involved in alcoholic liver disease in part because of the generation of metabolites such as reactive oxygen species, the finding that ligand activation of PPARβ/δ significantly suppressed ethanol-induced hepatic CYP2B10 expression suggests a protective role of PPARβ/δ in alcoholic liver disease. Indeed, recent studies have shown that PPARβ/δ protects against alcoholic liver disease by inhibiting steatosis, amino acid metabolism, and pyridoxal phosphate activity (8). Similarly, ligand activation of PPARβ/δ restores insulin sensitivity and also protects against ethanol-induced liver injury (17). However, ligand activation of PPARβ/δ did not prevent ethanol-induced *Cyp2b10* mRNA expression in primary hepatocytes alone. By contrast, ligand activation of PPARβ/δ repression of *Cyp2b10* expression induced by ethanol is restored when primary hepatocytes are co-cultured with Kupffer cells. This suggests that PPARβ/δ in the Kupffer cell may function differently than in the hepatocyte. For example, PPARβ/δ can modulate gene expression by interacting with other transcription factors and/or by directly regulating target gene expression (reviewed in Ref. 27). Indeed, PPARβ/δ expression in Kupffer cells can modulate effects observed during inflammatory insults in the liver that impact the hepatocyte (28). Further studies are needed to delineate the specific activities of PPARβ/δ in Kupffer cells and hepatocytes that underlie these differences. To date, this is the first demonstration that Kupffer cell activity is required for a protective effect in hepatocytes by facilitating repression of CYP2B10, whose expression is dependent on PPARβ/δ. This suggests that paracrine signaling between the Kupffer cell and hepatocyte may be important for preventing alcoholic liver disease through a PPARβ/δ-dependent mechanism.

It should be noted that there are advantages and disadvantages of using primary cells or co-cultures to study cell-cell interactions (29–31). For example, global transcriptional regulation is not always consistent in human primary hepatocyte cultures from different individuals (32). This suggests that there can be interindividual variability in response to chemical exposures and illustrates the need to define consistent culture condition when using primary cell cultures. The co-culture of hepatocytes with non-parenchymal cells has been shown to respond differently to chemical exposures, such as exhibiting greater drug metabolism capabilities and drug-induced inflammatory responses, compared with hepatocyte culture alone (33). Although this type of co-culture is designed to model the environmental condition *in vivo*, challenges remain required to enhance the *in vivo*-like characteristics of *in vitro* culture systems. A recent study revealed a unique and stable model because multicell cultures of parenchymal and non-parenchymal cells retain the functional ability of liver cells in response to chemical exposures (34) and may be suitable for studies similar to those performed in the present experiments.

The facts that PPARβ/δ protects against alcoholic liver disease (8, 17) and that this protection could be related to the repression of *Cyp2b10* following exposure to ethanol are of interest. Therefore, further studies are needed to determine whether this PPARβ/δ-dependent regulation of *Cyp2b10* can be used to identify novel markers of alcoholic liver progression and possibly be suitable for targeting for the prevention and/or treatment of this disease.

Experimental Procedures

Animals and Experimental Protocols—Animal usage was approved by the Pennsylvania State University Institutional Animal Care and Use Committee. Wild-type (*Pparβ/δ*$^{+/+}$) and *Pparβ/δ*-null (*Pparβ/δ*$^{-/-}$) mice (35) on a C57BL/6 genetic background were housed in a vivarium as previously described (36). Two cohorts of mice were used for the study. For the first cohort, age-matched (8–10 weeks) male *Pparβ/δ*$^{+/+}$ and

Pparβ/δ$^{-/-}$ mice were fed *ad libitum* daily with either liquid control or ethanol diets (Dyets Inc., Bethlehem, PA) for 16 weeks ($n = 10$ per group). The ethanol diet contained 4% (v/v) ethanol and was prepared as previously described (8). Samples from the first cohort of experimental mice were used for microarray analyses and hepatic gene/protein expression in response to ethanol treatment.

For the second cohort of mice, age-matched (10–12 weeks) male *Pparβ/δ*$^{+/+}$ and *Pparβ/δ*$^{-/-}$ mice were fed *ad libitum* with liquid control or ethanol diet for 16 weeks ($n = 10$/group). Mice fed with control or ethanol diet were also given a pellet made with bacon-flavored Transgenic Dough Diet (Bioserv, Inc., Prospect, CT) mixed with vehicle control (0.02% dimethyl sulfoxide) or GW0742 (5 mg/kg/day) ($n = 5$/group). Pair-fed mice ($n = 5$/genotype) were included to control for potential difference in average food intake between control and ethanol diet. However, no differences in food intake were noted, so this group was not used for all analyses. Samples from the second cohort of mice were used for analysis of CYP2B10 expression in livers.

Microarray Analysis—Total RNA was isolated as previously described (37). Purified RNA was assessed by GeneChip Mouse Gene 2.0 ST array (Affymetrix, Santa Clara, CA) following the manufacturer's recommended procedures. The robust multi-chip average approach was applied to normalize microarray data as previously described (37). The p value < 0.05 and a fold change of 1.5 in the intensity of signals were used to identify genes that were significantly regulated by ethanol treatment. The data were uploaded to the NCBI Gene Expression Omnibus database with the accession number GSE86002.

Quantitative Real Time Polymerase Chain Reaction (qPCR)—Expression of mouse *Cyp2b10*, *Cyp3a11*, and *Angptl4* in response to ethanol treatment was determined by qPCR analysis as previously described (38). Briefly, total RNA was isolated using RiboZol RNA extraction reagent (AMRESCO, Solon, OH) following the manufacturer's recommended procedures. cDNA was synthesized using 1.25 μg of total RNA as template mixed with Moloney MLV reverse transcriptase and random primers (Promega, Madison, WI). DNA amplification was carried out in 25-μl volumes containing SYBR Green PCR Supermix (Quanta Biosciences, Gaithersburg, MD) using the iCycler iQ5 PCR thermal cycler (Bio-Rad) with 45–55 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. The sequences of primers used to detect mRNA were *Cyp2b10* (NM_009999.4): forward, 5′-TTCTGCGCATGGAGAAGGAGAAGT-3′ and reverse, 5′-TGAGCATGAGCAGGAAGCCATAGT-3′; *Cyp3a11* (NM_007818.3): forward, 5′-GACAAACAAGCAGGGATGGAC-3′ and reverse, 5′-CCAAGCTGATTGCTAGGAGCA-3′; and *Angptl4* (NM_020581.2): forward, 5′-TTCTCGCCTACCAGAGAAGTTGGG-3′ and reverse, 5′-CATCCACAGCACCTACAACAGCAC-3′. The expression of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*; BC083149) was quantified as an internal control using the forward and reverse primers: 5′-GGTGGAGCCAAAAGGGTCAT-3′ and 5′-GGTTCACACCCATCACAAACAT-3′. The primers were designed that spanned exon-exon junctions, which prevents genomic DNA amplification. Each assay included a standard curve and a non-template control that were performed in triplicate. Four to six representative tissue samples/treatment group were randomly chosen for each analysis. Relative mRNA levels were normalized to *Gapdh* because there was no difference in expression between groups as determined by ANOVA and post hoc testing ($p \geq 0.05$).

Western Blotting Analysis—Hepatic microsomal protein was extracted from *Pparβ/δ*$^{+/+}$ and *Pparβ/δ*$^{-/-}$ mouse livers as previously described (39). Nuclear and cytosolic fractions were isolated from *Pparβ/δ*$^{+/+}$ and *Pparβ/δ*$^{-/-}$ mouse livers using NE-PER nuclear and cytoplasmic extraction reagents (Pierce) following the manufacturer's recommended procedures. Quantitative Western blotting analysis using radioactive detection techniques, the gold standard for quantifying protein expression, was performed as previously described (40). The following primary antibodies were used: anti-LAMIN A/C (sc7293, lot no. L0909, mouse monoclonal; Santa Cruz Biotechnologies, Santa Cruz, CA); anti-ACTIN (sc47778, lot no. K1414, mouse monoclonal, Santa Cruz Biotechnologies); anti-LDH (200-1173, lot no. 11538, goat polyclonal; Rockland Immunochemicals, Inc., Limerick, PA); anti-CYP2B1 (clone 2-66-3 (41), mouse monoclonal, produced at the National Cancer Institute as previously described (41, 42)); anti-PGC1α (ab51365, clone PPARAH6, mouse monoclonal; Abcam, Cambridge, MA), and anti-CAR (PP-N4111-00, lot no. A-1, mouse monoclonal; R & D Systems, Minneapolis, MN). Specificity of the antibodies was confirmed through different methods including: 1) confirming relative mobility for each protein (69/62 kDa for LAMIN A/C, respectively; 43 kDa for ACTIN; 36 kDa for LDH; 57 kDa for CYP2B10; 89 kDa for PGC1α; or 40 kDa for CAR); 2) confirming relative mobility and subcellular localization in the nucleus *versus* cytosol (CAR, LDH, or LAMIN A/C); 3) confirming relative mobility and knockdown of protein expression (PGC1α); or 4) confirming relative mobility and lack of inducibility in knock-out mice (CYP2B10). The relative expression level of each microsomal or cytosolic protein was normalized to the value of LDH or ACTIN. The relative expression level of each nuclear protein was normalized to the value of LAMIN. Statistical analyses of hybridization signals for LDH, ACTIN, and LAMIN revealed no significant differences between treatment groups as assessed by ANOVA and post hoc testing ($p \geq 0.05$). A minimum of three mice per group was analyzed.

Primary Hepatic Cell Isolation—Primary hepatocytes, Kupffer cells and non-parenchymal cells were isolated from adult male *Pparβ/δ*$^{+/+}$ and *Pparβ/δ*$^{-/-}$ mice as previously described (43). Primary hepatocytes (2×10^5) were seeded in 12-well collagen-coated culture plates (Becton, Dickinson and Company, Franklin Lakes, NJ) and cultured in HepatoZYME medium (Gibco) at 37 °C with 5% carbon dioxide. Primary Kupffer cells or non-parenchymal cells were seeded in normal 12-well culture plates and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen).

siRNA Knockdown of Mouse PGC1α in Primary Hepatocytes—Primary hepatocytes (2×10^5) were seeded in 12-well collagen-coated culture plates and transiently transfected with 10 μM non-targeting scrambled siRNA or mouse *Pgc1α* siRNA (Invitrogen) using Lipofectamine™ 2000 following the man-

ufacturer's recommended procedures. The cells were treated with or without ethanol (≤100 mM) for 12 h. Quantitative Western blotting analysis was performed as described above to confirm nuclear PGC1α expression.

Luciferase Assay—To identify the *cis*-regulatory element responsible for ethanol-induced *Cyp2b10* expression in hepatocytes, six reporter gene constructs driven by 5′ upstream region of *Cyp2b10* gene with serial deletion were generated by PCR. Primers were designed to contain overhanging sites for restriction enzymes, which allowed for directional cloning into the multiple cloning site of pGL3-basic luciferase reporter vector (Promega). The composition of each construct was confirmed by restriction endonuclease digestion and DNA sequencing.

Primary hepatocytes were transiently co-transfected with 0.5 μg of pCMV-β-galactosidase plasmid (Promega) and 3.5 μg of either *Cyp2b10* promoter-luciferase reporter constructs or the control plasmid (pGL3-basic vector) using Lipofectamine LTX reagent (Invitrogen) following the manufacturer's recommended procedures. The cells were treated with or without ethanol (≤100 mM) for 12 h, and cell lysates were prepared in passive lysis buffer (Promega). Luciferase and β-galactosidase activities were measured using Luciferase and Beta-Glo assay systems, respectively, following the manufacturer's recommended procedures. Relative luciferase activity was normalized to β-galactosidase activity.

Site-directed Mutagenesis—DpnI-mediated site-directed mutagenesis was performed to generate mutation in *Cyp2b10* promoter-luciferase reporter construct as previously described (44). The sequence-specific primers were overlapping with and flanking the transcription factor binding sites for identified *trans*-acting factors. The successful mutagenesis was confirmed by DNA sequencing. Primary hepatocytes were transiently transfected with mutant luciferase reporter constructs followed by ethanol treatment. Relative luciferase activity was determined as described above.

ChIP-qPCR—To confirm the occupancy of SP1 on the *Cyp2b10* promoter, ChIP-qPCR was performed to quantify relative promoter occupancy following ethanol exposure in primary hepatocytes from $Ppar\beta/\delta^{+/+}$ and $Ppar\beta/\delta^{-/-}$ mice as previously described (38). The following primary antibody was used: anti-SP1 (sc59, lot no. 0915, rabbit polyclonal; Santa Cruz Biotechnologies). Rabbit IgG was used a negative control.

Co-cultures of Primary Hepatocytes with Primary Kupffer Cells or Non-parenchymal Cells—Primary hepatocytes were seeded in 12-well collagen-coated culture plates. Primary Kupffer cells or non-parenchymal cells were seeded in Transwell® inserts (Corning Inc., Corning, NY). Primary hepatocyte-Kupffer cell co-cultures (5:1 ratio) or primary hepatocyte-non-parenchymal cell co-cultures (1:1 ratio) were pretreated with or without GW0742 (1 μM) for 6 h and then treated with or without ethanol (≤100 mM) for 12 h.

Statistical Analysis—The data were subjected to either Student's *t* test or a parametric one-way ANOVA followed by Tukey test for post hoc comparisons (Prism 5.0; GraphPad Software Inc., La Jolla, CA). The correlation between fold changes of *Cyp2b10* mRNA expression and nuclear PGC1α protein expression was determined by Pearson correlation method with a two-tailed *p* value (Prism 5.0).

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