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Activation of farnesoid X receptor attenuates hepatic injury in a murine model of alcoholic liver disease

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ABSTRACT

Alcoholic liver disease (ALD) is a common cause of advanced liver disease, and considered as a major risk factor of morbidity and mortality worldwide. Hepatic cholestasis is a pathophysiological feature observed in all stages of ALD. The farnesoid X receptor (FXR) is a member of the nuclear hormone receptor superfamily, and plays an essential role in the regulation of bile acid, lipid and glucose homeostasis. However, the role of FXR in the pathogenesis and progression of ALD remains largely unknown. Mice were fed *Lieber-DeCarli* ethanol diet or an isocaloric control diet. We used a specific agonist of FXR WAY-362450 to study the effect of pharmacological activation of FXR in alcoholic liver disease. In this study, we demonstrated that FXR activity was impaired by chronic ethanol ingestion in a murine model of ALD. Activation of FXR by specific agonist WAY-362450 protected mice from the development of ALD. We also found that WAY-362450 treatment rescued FXR activity, suppressed ethanol-induced *Cyp2e1* up-regulation and attenuated oxidative stress in liver. Our results highlight a key role of FXR in the modulation of ALD development, and propose specific FXR agonists for the treatment of ALD patients.

1. Introduction

Excessive alcohol consumption leads to alcoholic liver disease (ALD), which is a major cause of morbidity and mortality worldwide [1,2]. ALD refers to a spectrum of liver disorders, ranging from alcoholic fatty liver (simple steatosis) to alcoholic steatohepatitis (ASH), fibrosis, cirrhosis and hepatocellular carcinoma (HCC) [1,2]. Approximately 90% of heavy drinkers develop fatty liver, whereas approximately 35% of heavy drinkers develop advanced ALD [1,3,4]. Despite significant advances in the understanding of the pathogenesis of alcohol-related liver injury, few treatments have been proved effective for ALD [5].

Metabolism of alcohol predominantly involves two well-characterized pathways in the liver. Generally, alcohol is oxidized by alcohol dehydrogenase (ADH) to acetaldehyde and, subsequently, to acetate by aldehyde dehydrogenase (ALDH) [6]. An alternative pathway takes place via the microsomal cytochrome P450 2E1 enzyme (CYP2E1), which is up-regulated during chronic alcohol consumption [7]. CYP2E1 has been shown to be the major contributor to alcohol-induced oxidative stress and liver injury [8–10].

The farnesoid X receptor (FXR; NR1H4) is a member of the nuclear receptor superfamily and is primarily expressed in the liver, gastrointestinal tract and kidneys [11,12]. FXR heterodimerizes with the retinoid X receptor α (RXRα; NR2B1), binds to FXR response element (FXRE) and regulates the transcription of target genes [13]. Previous study has shown acetylation of FXR is dynamically regulated by p300 and SIRT1 under normal conditions, and inhibits FXR/RXRα heterodimerization and FXR activity [14]. Bile acids are recognized as endogenous ligands of FXR [15,16]. Potent synthetic FXR ligands, such as GW4064, 6a-ethyl-chenodeoxy-cholic acid (6ECDCA) and WAY-362450, also activate FXR with higher affinity and efficiency [17–19]. FXR plays a central role in bile acid homeostasis by regulating genes involved in bile acid synthesis, secretion and re-absorption, including small heterodimer partner

Abbreviations: FXR, farnesoid X receptor; ALD, alcoholic liver disease; SHP, small heterodimer partner; BSEP, bile salt export pump; *CYP7A1*, cholesterol 7α-hydroxylase; *CYP8B1*, sterol 12α-hydroxylase; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; *CYP2E1*, cytochrome P450 2E1 enzyme; RXRα, Retinoid X receptor α; 6ECDCA, 6α-ethyl-chenodeoxy-cholic acid; FXR-/-, FXR deficient; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBARS, thiobarbituric acid reactive substances; 4-HNE, 4-hydroxynonenal; Co-IP, co-immunoprecipitation; MCP-1, monocyte chemotactic protein-1; OCT, optimal cutting temperature; SREBP-1c, sterol regulatory element-binding protein 1c.

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(SHP), cholesterol 7α-hydroxylase (CYP7A1), sterol 12α-hydroxylase (CYP8B1), and bile salt export pump (BSEP) [20–22].

Hepatic cholestasis in patients with alcoholic fatty liver disease without bile duct obstruction has been reported to appear in all stages of ALD, and accumulated bile acid in the liver further aggravates liver injury and promotes liver fibrosis [23–25]. However, the role of bile acid receptor FXR in the pathogenesis of ALD remains largely unknown. In the present work, we addressed the role of FXR in a mouse model of ALD by using the *Lieber-DeCarli* ethanol diet. We found that the activity of FXR is functionally impaired during chronic alcohol intake, and activation of FXR attenuates ethanol-induced hepatic liver injury, steatosis and cholestasis, thus prompting specific FXR agonists for the treatment of ALD.

2. Materials and methods

Methods are described in detail in Supplementary Methods.

2.1. Animals and treatments

Fxr-knockout ($Fxr^{-/-}$) mice on a C57BL/6 J background (#004144) were obtained from the Jackson Laboratories (BarHarbor, ME). Wild-type C57BL/6 J mice were from Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China). The mice received human care, and all animal procedures were performed according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals and with approval of the Animal Care and Use Committee of Fudan University.

Male mice were fed either a *Lieber-DeCarli* liquid diet or an isocaloric control diet (Bio-Serv, Frenchtown, NJ) as previously described [6,9]. This diet provides 35.5% of calories from ethanol (or maltose), 35% of calories from fat, 18% of calories from protein, and 11.5% of calories from carbohydrate. For the first week, animals were gradually introduced to the ethanol-containing liquid diet, including liquid diet containing 0.75% ethanol (w/v) for 2 days, 1.50% ethanol (w/v) for 2 days, 3.75% ethanol (w/v) for 3 days, and then 5% ethanol (w/v) diet for 4 weeks. FXR agonist WAY-362450 (30 mg/kg/d, IG) was administered to ethanol liquid diet feeding WT and $Fxr^{-/-}$ mice, and all animals had free access to the liquid diet throughout the experimental period. There was no significant difference in food intake and weight gain among the different groups of mice (data not shown). At sacrifice, animals were anesthetized with sodium pentobarbital (75 mg/kg, IP). Blood was collected from the *vena cava* just prior to sacrifice by exsanguination and plasma was stored at −80 °C until further analysis. Portions of liver tissue were either frozen immediately in liquid nitrogen, fixed in 10% neutral buffered formalin, or frozen-fixed in OCT mounting media (Tissue Tek, Hatfield, PA) for subsequent sectioning.

2.2. Serum and tissue biochemical assays, liver histopathological examination

See the Supplementary Methods for details.

2.3. Total RNA isolation and quantitative RT-PCR assay

See the Supplementary Methods for details.

2.4. Co-immunoprecipitation, western blotting assay, and immunofluorescence assay

See the Supplementary Methods for details.

2.5. Statistics

Data are shown as means ± SEM. Statistical analysis was determined by two-tailed Student's *t* test or two-way ANOVA. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. FXR activity was impaired in murine alcoholic liver diseases

To investigate the role of FXR in alcoholic liver disease, we fed wild-type mice with *Lieber-DeCarli* liquid diet (EtOH) or an isocaloric control diet (Pair-fed) for 4 weeks. Histopathological and biochemical examination revealed that chronic alcohol ingestion induced liver steatosis, as well as the increase in serum levels of ALT and alcohol (Supplementary Fig. 1). Moreover, the hepatic total bile acid level was significantly elevated after alcohol ingestion (Fig. 1A). We next examined key factors that are involved in the regulation of bile acid homeostasis. We found that alcohol intake up-regulated mRNA levels of *Cyp7a1* and *Cyp8b1* mRNA, whereas decreased *Bsep* expression (Fig. 1B). In addition, the expressions of *Mrp2, Mdr2, Mdr3* and *Oatp1* were also affected by chronic ethanol ingestion (Supplementary Fig. 2). Previous reports have suggested that gene expressions of *Cyp7a1*/*Cyp8b1* and *Bsep* are under FXR-negative and -positive control, respectively [20–22]. However, little change in *Fxr* mRNA or protein level was detected in the livers of alcohol-fed mice (Fig. 1B and C).

We next determined whether FXR activity was affected upon alcohol ingestion. We found that chronic alcohol intake significantly suppressed the FXR/RXRα interaction and remarkably elevated the level of FXR acetylation in the liver tissues of alcohol-fed mice (Fig. 1C and D). However, the interaction between LXR and RXRα was not obviously changed upon ethanol feeding (Fig. 1C). Moreover, ethanol ingestion promoted global protein hyperacetylation, as well as induced the down-regulation of deacetylase SIRT1 and the up-regulation of acetyltransferase p300 (Fig. 1D), which are responsible for the incremental FXR acetylation [26–28]. These findings suggest that chronic ethanol feeding impairs FXR activity and disrupts the expression of bile acid metabolism genes in the mouse liver.

3.2. Activation of FXR by WAY-362450 attenuated alcohol-induced liver injury, steatosis and cholestasis

To evaluate whether activation of FXR had a protective effect on alcoholic liver disease, we utilized a highly potent and selective FXR agonist, WAY-362450 [19]. As shown in Fig. 2A and B, WAY-362450 administration prevented liver injury and lipid accumulation induced by ethanol ingestion, as evidenced by a significant decline in the increase of serum ALT level and hepatic triglyceride level. However, the protective effect of WAY-362450 against serum ALT and hepatic triglyceride elevation was abolished in $Fxr^{-/-}$ mice, suggesting that WAY-362450 acted specifically through FXR (Fig. 2A and B). Moreover, histological evaluation of liver specimens demonstrated administration of WAY-362450 dramatically decreased lipid accumulation to the background level (Fig. 2C). In consistence with histological observation, the expressions of key genes involved in fatty acid synthesis, *Srebp-1c* and *Fasn*, were markedly repressed by WAY-362450 treatment (Supplementary Fig. 3).

We also determined whether FXR activation attenuated intrahepatic cholestasis in ALD mice. As expected, WAY-362450 administration induced *Bsep* mRNA expression as well as suppressed the expression of *Cyp7a1* and *Cyp8b1* in the liver of ALD mice (Fig. 2D). Hepatic bile acid levels were also decreased after WAY-362450

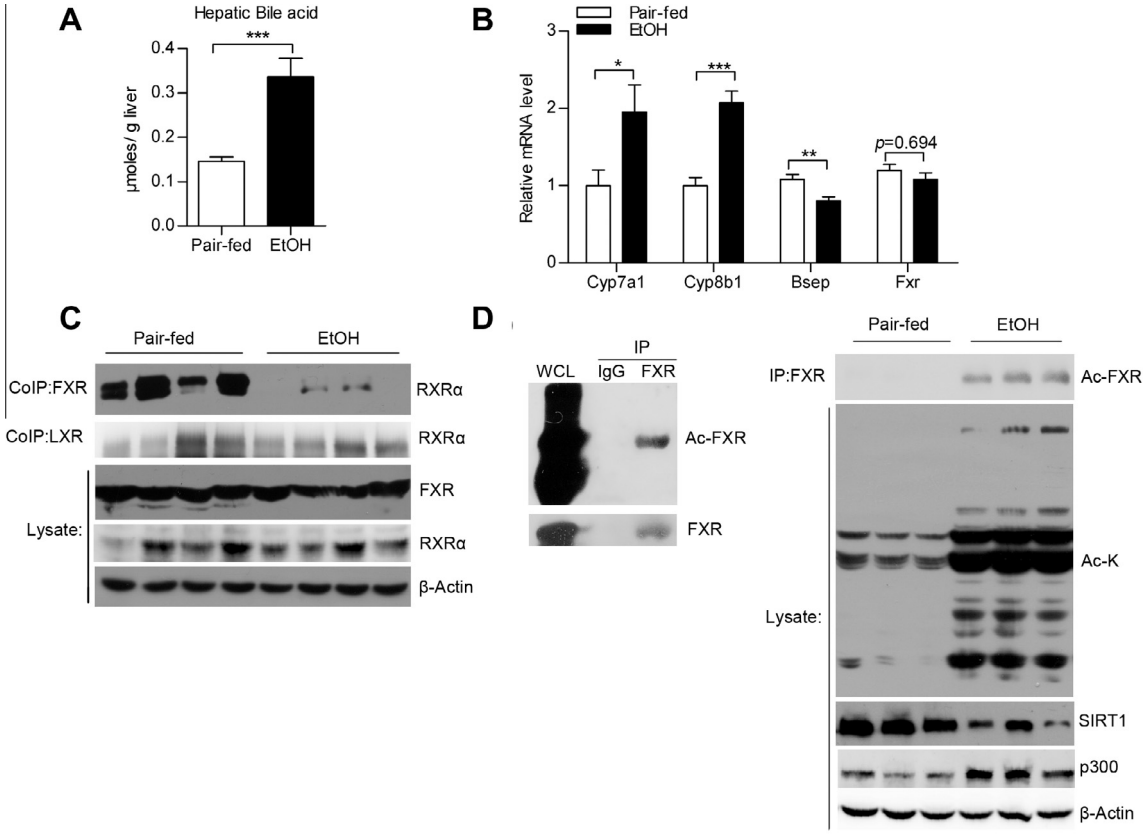

Fig. 1. FXR activity was impaired in murine model of alcoholic liver disease. Wild type mice were fed with *Leiber-DeCarli* ethanol diet (EtOH, $n = 8$) or isocaloric control diet (Pair-fed, $n = 10$) for 4 weeks. (A) Hepatic bile acid content was assayed. (B) *Cyp7a1, Cyp8b1, Bsep* and *Fxr* mRNA levels in mice livers were determined. (C) Liver FXR protein, FXR/RXRα and LXR/RXRα heterodimer levels were analyzed by immunoprecipitation and western blotting assay. (D) Acetylated endogenous FXR levels, global acetylation levels, SIRT1 and p300 protein levels in mouse liver were detected by western blotting ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

treatment, indicating ameliorated cholestasis in ethanol-fed WT mice (Fig. 2D). WAY-362450 treatment also largely diminished inflammation infiltration (as assessed by the absence of inflammatory foci under microscopy) (Fig. 2C) and reduced the hepatic levels of inflammatory factors *Tnf-α* and *Mcp-1* (Fig. 2E). Due to enhanced expressions of FXR target genes by WAY-362450 treatment in mice fed ethanol, we tested whether FXR/RXRα heterodimer was rescued by FXR agonist treatment. As indicated, WAY-362450 enhanced FXR/RXRα interaction in livers from mice fed with ethanol (Fig. 2F). Furthermore, WAY-362450 induced the expression of *Sirt1* at both mRNA and protein level (Fig. 2F and Supplementary Fig. 4). Together, these results demonstrated a potential protective role of FXR agonist WAY-362450 in alcohol-induced liver disease in mice.

3.3. WAY-362450 repressed CYP2E1 expression and attenuated oxidative stress in the liver

Excessive consumption and metabolism of ethanol plays a critical role in the pathogenesis of ALD [1,8,9]. To better understand how FXR agonist ameliorates the development of ALD in mice, we next measured the effect of FXR agonist on the expression of the major enzymes responsible for hepatic ethanol metabolism. In consistence with previous reports, ethanol feeding increased CYP2E1 protein levels in mouse livers [8–10], both at the level of protein and mRNA; however, WAY-362450 treatment evidently suppressed ethanol-induced up-regulation of *Cyp2e1* (Fig. 3A and B). Ethanol or WAY-362450 treatment showed little impact on the protein levels of ADH1 and ALDH2 in liver (Fig. 3A). Induction of *Cyp2e1* expression by chronic alcohol ingestion can promote liver injury by inducing oxidative stress [8–10]. We next examined the effect of FXR agonist on the levels of hepatic TBARS and 4-hydroxynonenal (4-HNE) adduct, markers of oxidative stress. As shown in Fig. 3C–E, mice fed the ethanol diet exhibited elevated hepatic TBARS and 4-HNE adducts levels, which were both attenuated by WAY-362450 administration. We also demonstrated that WAY-362450 induced expression of enzymes involved in anti-oxidative response, *Gsta4* and *Gstm3* (Supplementary Fig. 5). These data suggest that activation of FXR by WAY-362450 suppressed CYP2E1 expression and oxidative stress in the liver of ALD mice.

4. Discussion

FXR plays a key role in bile acid metabolism, lipid and lipoprotein metabolism, liver regeneration, glucose metabolism and protection from hepatotoxic agents [29]. As reported, cholestasis is a histological feature observed in all stages of ALD and mainly related to alcoholic hepatitis [23–25,30]. It has been suggested that ethanol may inhibit bile acid uptake by hepatocytes and disrupt steps in bile secretion [25]. At high concentrations, endogenous bile acids, especially chenodeoxycholic acid and deoxycholic acid, are hepatotoxic [25]. Endogenous toxic bile acid accumulation could partially mediate the hepatotoxicity of alcohol and promote fibrosis progression [31]. However, the mechanisms of alcohol ingestion-induced cholestasis are not thoroughly understood. Herein, we report that the activity of bile acid receptor FXR is functionally impaired during chronic alcohol intake, and activation of FXR attenuates ethanol-induced hepatic liver injury, steatosis and cholestasis in a murine model of ALD.

It has been reported that FXR acetylation levels were constitutively elevated in mouse models of metabolic disorders using

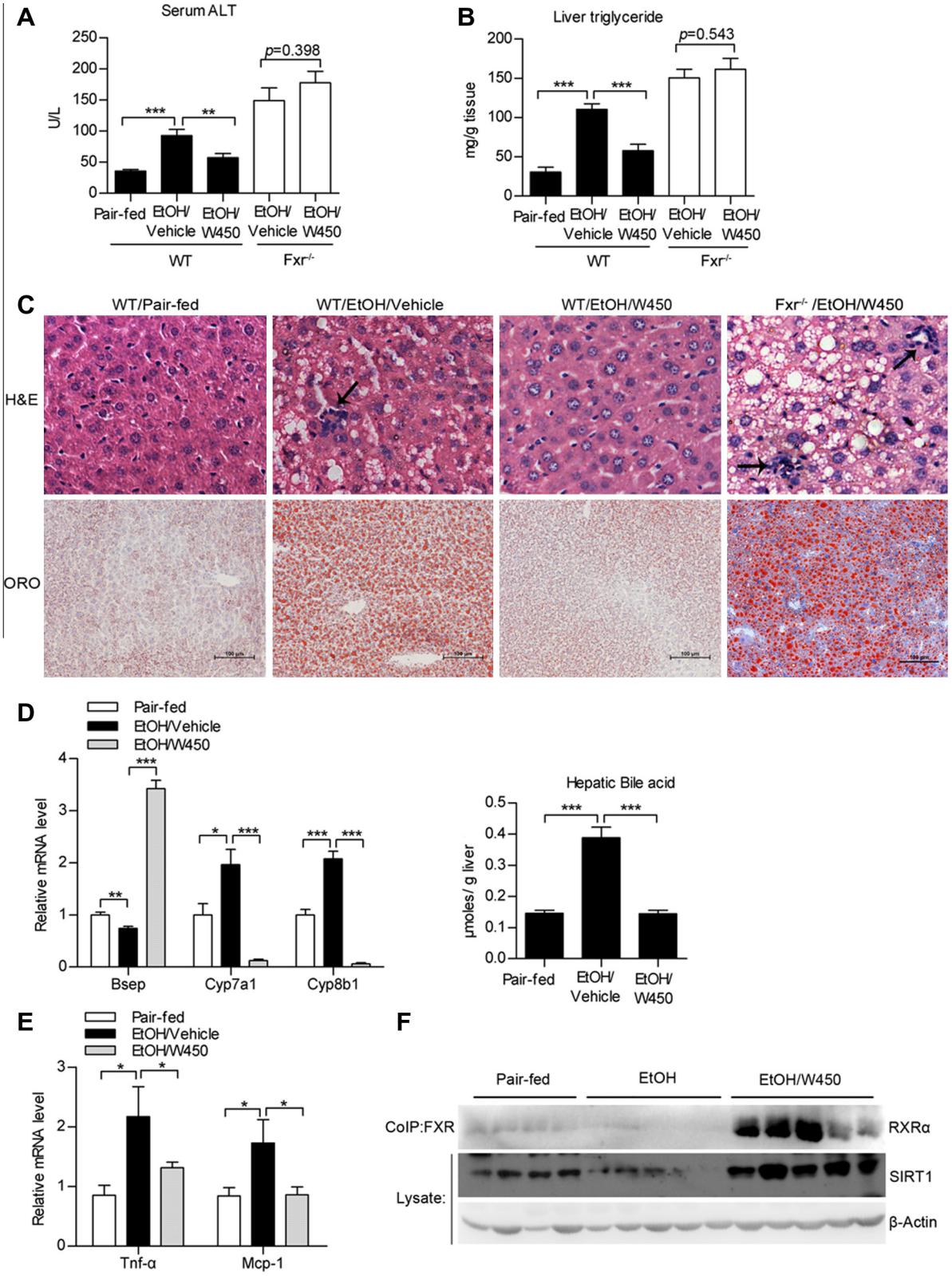

Fig. 2. WAY-362450 ameliorates alcoholic liver disease in wild-type but not FXR$^{-/-}$ mice. Wild type and FXR$^{-/-}$ mice fed with EtOH diet were treated with either vehicle (EtOH/Vehicle) or WAY-362450 (EtOH/W450) once daily for 4 weeks. Serum ALT activities (A) and hepatic triglyceride content (B) were determined. (C) Liver sections were stained with either H&E or Oil Red O (ORO) (original magnification, ×200). Arrow, inflammation foci. (D) Liver tissue was subjected to real-time RT-PCR assay for determination of *Bsep, Cyp7a1* and *Cyp8b1* mRNA levels (left panel), or quantification of hepatic bile acid content (right panel). (E) Total RNA from liver tissue was subjected to real-time RT-PCR for the determination of *Tnf-α* and *Mcp-1* mRNA. ($n = 12$ in WT/EtOH/W450 group, $n = 10$ in other groups, and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). (F) Liver samples were applied to immunoprecipitation analysis of FXR/RXRα heterodimer level or western blotting analysis of SIRT1 proteins in the liver; β-actin was used as an endogenous reference.

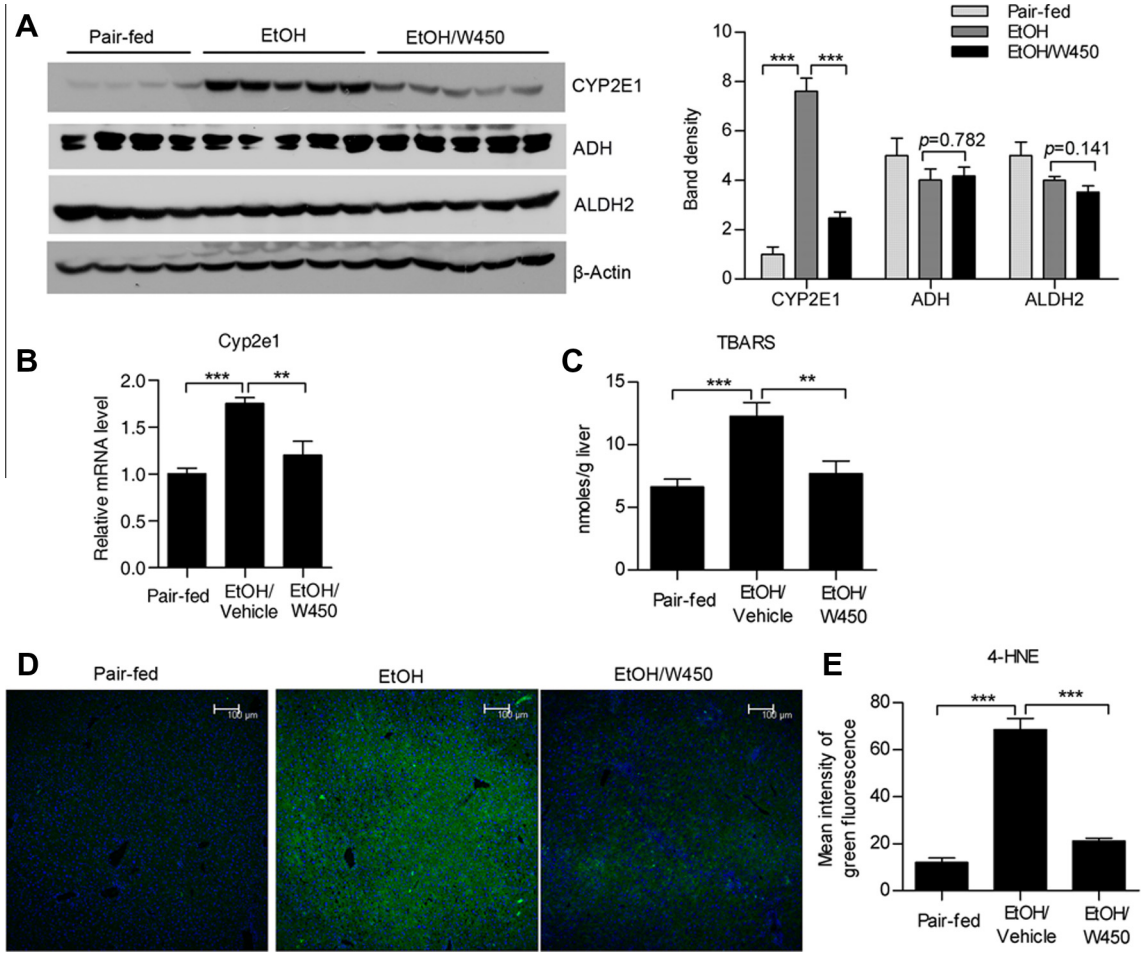

Fig. 3. FXR activation repressed alcohol-induced CYP2E1 expression and oxidative stress in murine model of alcoholic liver disease. (A) Wild-type mice were treated for 4 weeks as indicated, followed by western blotting analysis of CYP2E1, ADH and ALDH2 proteins in the liver; right panel, densitometric analysis of western blotting in left panel with normalization to β-actin. (B) Effect of WAY-362450 treatment on *Cyp2e1* mRNA level in mice livers was assayed. (C–E) Wild-type mice were treated as in (A), followed by detection of TBARS level (C) and immunofluorescence assay of 4-HNE adducts level (D and E) in mouse livers ($n = 12$ in WT/EtOH/W450 group, $n = 10$ in other groups, and $^{**}p < 0.01$, $^{***}p < 0.001$).

ob/ob mice or mice fed Western diet [14]. Acetylation of FXR inhibits its FXR/RXRα heterodimerization and its activity and is dynamically regulated by p300 and SIRT1 under normal conditions [14]. Our results demonstrated that in the murine model of ALD, ethanol ingestion also reduced the level of FXR/RXRα heterodimer and enhanced the level of acetylation of FXR in the liver. Constitutively elevated acetylation further induced the impairment of FXR transcriptional activity, which deregulates expression of *Fxr* target genes (*Cyp7a1, Cyp8b1* and *Bsep*) and disrupts bile acid metabolism in alcoholic liver diseases (Fig. 1). In contrast, activation of FXR by WAY-362450 induced *Sirt1* expression which would result in reduced FXR acetylation level, and further promoted FXR/RXRα interaction. As a result, administration of WAY-362450 prevented chronic alcohol consumption-induced hepatic bile acid accumulation (Fig. 2D) and attenuated intra-hepatic cholestasis-induced hepatic injury.

In addition to releasing bile acid overload in alcoholic livers, activation of FXR also attenuates hepatic triglyceride accumulation in wild-type mice fed an ethanol-containing diet. Previous data have suggested that ethanol induces the fatty acid synthesis pathway by activation of sterol regulatory element-binding protein 1c (*Srebp-1c*) [32], which is negatively regulated by FXR [33]. Therefore, it is likely that FXR activation prevents excessive hepatic triglyceride accumulation by inhibiting lipogenesis through decreasing *Srebp-1c* and other possible lipogenic genes. Additionally, WAY-362450 treatment reduced the expression levels of inflammatory factors (*Tnf-α* and *Mcp-1*) and decreased liver inflammatory cell infiltration (Fig. 2C). It has been demonstrated that compared with pair-fed mice, mice with ALD exhibit significantly elevated hepatic levels of *Tnf-α* and *Mcp-1*, which play important roles in the pathogenesis of alcohol-induced liver injury [34–36]. Moreover, FXR activation was reported to repress inflammation in a mouse model of non-alcoholic fatty liver disease and antagonizes the NF-κB signaling pathway and inhibits the expression of NF-κB regulated proinflammatory genes [37,38]. Therefore, inhibition of NF-κB signaling pathway may be involved in FXR agonist-mediated inflammation suppression in ALD mice.

We also demonstrated that FXR agonist, WAY-362450 reduced the levels of CYP2E1 and lowered oxidative stress in ethanol feeding mice. Hepatic CYP2E1 is highly induced after ethanol ingestion; ethanol can stabilize the enzyme by protecting it from degradation and enhancing the gene transcription. Induction of CYP2E1 induces oxidative stress and causes lipid peroxidation, which contributes to the development of liver injury [8–10]. Knock-out of *Cyp2e1* or administration of CYP2E1 inhibitors have been shown to reduce the severity of ALD significantly [9,39]. In contrast, presence of the *Cyp2e1* transgene increased liver injury and increased expression of stress related genes in mice [8]. Therefore, CYP2E1 down-regulation may play a critical role in FXR agonist-induced suppression of oxidative stress and liver injury. In addition, Lee et al. reported that the induction of xenobiotic metabolism enzymes, such as *Gsta3, Sult1a1* and *Ugt1a1*, by FXR activation

may play a protective role in acetaminophen-induced hepatotoxicity [40], suggesting other mechanisms may also be involved in the anti-oxidative effect of FXR agonist.

Currently, despite significant advances in the understanding of the pathogenesis of alcohol-related liver injury, few treatments have been proved effective for ALD [5]. Our findings suggest a possible molecular mechanism by which ethanol ingestion impaired FXR activity and induced liver injury, steatosis and cholestasis. Our study demonstrates that FXR activation ameliorates murine alcoholic liver disease, suggesting a possible therapeutic value for FXR agonist in treating ALD patients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.057.

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