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PPARα agonist WY-14,643 enhances ethanol metabolism in mice: Role of catalase

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ABSTRACT

Peroxisome proliferator-activated receptor α (PPARα), a fatty acid oxidation regulator, inhibits alcohol-induced fatty liver (AFL). PPARα agonist WY-14,643 ameliorates AFL. Nicotine enhances AFL. In this study, we investigated whether PPARα activation also blocks nicotine-enhanced AFL. Mice were fed liquid diets containing ethanol in the presence or absence of nicotine, WY-14,643 was added to the above diets at 10 mg/L. The results showed that WY-14,643 blunted AFL and nicotine-enhanced AFL, which was paralleled with striking induction of PPARα target genes. However, serum ALT was dramatically increased by the ethanol/WY-14,643 feeding and was further increased by nicotine/ethanol/WY-14,643 feeding, which was confirmed by necro-inflammation and elevated oxidative stress. Interestingly, serum alcohol levels were dramatically decreased by WY-14,643. Ethanol is mainly metabolized by alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1) and catalase. ADH and CYP2E1 were not increased by WY-14,643, but catalase was induced. What is more, injection of catalase inhibitor increased serum ethanol. Decreased serum alcohol, attenuated fatty liver, and enhanced liver injury were not induced by WY-14,643 in mice lacking PPARα. In conclusion, PPARα activation by WY-14,643 attenuates alcohol/nicotine-induced fatty liver but deteriorates ethanol/nicotine-induced liver injury; WY-14,643 enhances ethanol metabolism via induction of catalase.

1. Introduction

Heavy alcohol drinking induces a spectrum of liver disorders including alcoholic fatty liver (AFL), steatohepatitis, fibrosis, cirrhosis, and even liver cancer, which are designated alcohol-related liver disease (ALD). Alcohol is mainly metabolized by alcohol dehydrogenase (ADH) to acetaldehyde in cytosol. In mitochondria, acetaldehyde is oxidized by aldehyde dehydrogenase (ALDH) to acetate. ADH-mediated ethanol oxidation consumes NAD^+ and produces NADH. The increased ratio of NADH/NAD^+ may inhibit fatty acid β-oxidation (FAO) leading to fat accumulation in liver, i.e., fatty liver [1]. Chronic ethanol consumption leads to induction of cytochrome P450s especially CYP2E1 [2]. CYP2E1 is a major enzyme in microsomal ethanol oxidation system (MEOS). Unlike ADH, CYP2E1 consumes NADPH to oxidize ethanol and produces reactive oxygen species (ROS). CYP2E1-mediated oxidative stress contributes to ALD [3,4]. On the other hand, CYP2E1-derived ROS may up-regulate nuclear factor E2-related factor 2 (Nrf2), which in turn induces mouse CYP2A5 (CYP2A6 in human) [5]. Indeed, ethanol induces CYP2A5 in a CYP2E1-dependent manner [6]. CYP2A6 and CYP2A5 are major enzymes that metabolize nicotine [7–10]. Nicotine can enhance AFL in mice, but it did not enhance alcohol-induced liver inflammation [11]. Nicotine-enhanced AFL was not observed in CYP2A5 knockout (*cyp2a5*$^{-/-}$) mice [12]. Further study indicated that nicotine metabolism by CYP2A5 generates ROS, promotes alcohol-induced oxidative stress and lipid peroxidation, and inhibits hepatocyte proliferation [12,13].

Peroxisome is one of cell organelles for fatty acid β-oxidation (FAO). Usually, very long chain fatty acids are oxidized in peroxisomes by acyl-CoA oxidase (ACOX), a rate-limiting enzyme [14]. The resultant shorter chain fatty acids will be further oxidized in mitochondria. Unlike mitochondrial FAO, peroxisomal FAO produces hydrogen peroxide (H_2O_2), which is detoxified by peroxisomal catalase [15]. Peroxisome

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proliferation is regulated by peroxisome proliferator-activated receptor α (PPARα), and many PPARα agonists are peroxisome proliferators [16,17]. PPARα is a major regulator for lipid metabolism especially for FAO [14,18]. WY-14,643, a potent peroxisome proliferator and PPARα agonist, induced FAO-related enzymes such as ACOX and liver fatty acid binding protein (L-FABP) to prevent AFL [19]. In the present study, we found that WY-14,643 also blunted nicotine-enhanced AFL.

Peroxisomes are also involved in ethanol metabolism via catalase, which is H_2O_2-dependent [20,21]. Catalase oxidizes ethanol by consuming H_2O_2 [22,23]. Peroxisomal catalase oxidation of alcohol can be stimulated by free fatty acids (FFA), because the peroxisomal FAO produces H_2O_2 [24–26]. Peroxisomal catalase can be induced by peroxisome proliferators in rats because a peroxisome proliferator responsive element (PPRE) is identified in catalase promoter [27]. Ciprofibrate, a peroxisome proliferator, can induce mRNA of catalase about 2-fold, but it can induce peroxisomal FAO activity up to 25-fold [28]. The disproportionate induction of H_2O_2-generating ACOX and H_2O_2-scavenging catalase was proposed to be responsible for peroxisome-derived oxidative stress [29]. It is unclear whether peroxisomal oxidative stress contributes to ALD.

In the present study, we examined [1] whether PPARα agonist WY-14,643 attenuates nicotine-enhanced AFL and [2] whether WY-14,643 induces oxidative stress. We found that WY-14,643 attenuated fatty liver but enhanced oxidative liver injury induced by ethanol alone or in combination with nicotine. Interestingly, WY-14,643 escalated serum ethanol clearance by inducing catalase. The WY-14,643-enhanced ethanol metabolism was not observed in mice lacking PPARα (*pparα* $^{-/-}$) but was observed in mice lacking L-FABP (*L-fabp*$^{-/-}$). The attenuated fat accumulation in liver and enhanced liver injury induced by WY-14,643 in WT mice were not observed in *pparα* $^{-/-}$ mice and *L-fabp*$^{-/-}$ mice.

2. Methods

2.1. Animals and treatment

Colonies of C57BL/6J wild type mice (#000664) and *pparα* $^{-/-}$ mice (#008154, ref. [16]) were purchased from Jackson Laboratory. *L-fabp*$^{-/-}$ mice were derived from *L-fabp*$^{+/-}$ mice (cryo recovered from Jackson Laboratory #022873, ref. [30]). All the mice were housed in temperature-controlled animal facilities with 12-h light/dark cycles and were permitted consumption of tap water and Purina standard chow *ad libitum*. The mice received humane care, and experiments were carried out according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals. The animal studies were approved by Institution Committee of Animal Use and Care (IACUC).

Eight weeks old female mice were selected for chronic ethanol feeding models. Female mice were selected because they reveal a propensity to greater ALD [31]. The mice were fed the control liquid dextrose diet (Bio-Serv, Frenchtown, NJ, USA) for 3 days followed by the liquid ethanol diet (Bio-Serv) for 3 weeks. The concentration of ethanol in the liquid diet was gradually increased from 1.7% (v/v) to 2.5%, 3.4%, 4.2%, 5.1%, each concentration for 3 days, and finally 6% for the rest of time. The control group was continually fed control diet. Nicotine hydrogen tartrate salt (From Sigma) was mixed in the liquid ethanol diet at 30 mg free base/L. WY-14,643 (from Adipogen and Selleckchem, respectively) was mixed in the liquid diet at 10 mg/L. After a 6-h fasting, the mice were sacrificed following a body weight measurement. Blood was collected for serum biochemical assays. The livers were weighted, and liver index was calculated as liver weight/100 g body weight. The liver aliquots from same lobes of different mice were put in neutral Formalin buffer for preparing paraffin sections for Hematoxylin & Eosin staining (H&E) and immunohistochemistry staining (IHC). The other liver tissue aliquots were stored at −80 °C for future assays.

In binge model, the male mice were fed control liquid diets, with or without WY-14,643 (10 mg/L), for 3 weeks, followed by a single dose of ethanol at 5 g/kg by gavage. The mice were sacrificed after 8 h of ethanol gavage. In acute-on-chronic models, male mice were fed diets containing 5% ethanol and 20 mg/L WY-14,643 for 2 weeks followed by a binge alcohol at 5 g/kg by gavage. Some mice received catalase inhibitor 3-amino-1,2,4-triazole (3-AT) by intra-peritoneal injection at 1 g/kg 30 min prior to the binge alcohol gavage. The mice were sacrificed after 8 h of binge ethanol. In binge and acute-on-chronic models, male mice were selected because they displayed a lower mortality [32].

2.2. Activities of hepatic catalase, ADH, and CYP2E1

Liver homogenates were made using 0.15 M KCl. After 20 min of 9000×*g* centrifugation, the pellets were re-suspended to be used for measuring catalase activity by monitoring the decrease in absorbance at 240 nm in 50 mM potassium phosphate containing 20 mM H_2O_2 as described before [33]. The supernatants were further centrifuged at 100,000×*g* for 1 h. The resultant pellets were suspended for measuring CYP2E1 activity by PNP hydroxylation [33]. The resultant supernatants were used for ADH assay using a commercially available assay kit.

2.3. Biochemical assays, western blotting analysis, and immunohistochemistry staining

Serum ALT, TG, FFA, alcohol and liver TG and FFA were measured using commercially available assay kits. For Western blotting, SDS-PAGE and chemiluminescence imaging was carried out as described before [7–10]. Liver paraffin sections were used for IHC and liver homogenates were used for Western blotting. For IHC, a Broad Spectrum IHC Select® HRP/DAB kit (from EMD Millipore, Cat#: DAB150) was used. Antibodies against CYP2E1 and CYP2A5 are generous gifts from Drs Jerome Lasker (Hackensack Biomedical Research Institute, Hackensack, NJ) and Risto Juvonen (Department of Pharmacology and Toxicology, University of Kuopio, Kuopio, Finland). The other primary antibodies and all assay kits are listed in Table 1.

Statistical analysis Results are expressed as mean ± S.D. Statistical evaluation was carried out by using two-way analysis of variance (ANOVA) with subsequent the Student-Newman-Keuls post hoc test. $P < 0.05$ was considered as statistical significance.

Table 1
Antibodies, assay Kit, and other material sources.

Name	Cat#	Company
Anti-CYP4A11	11688-1-AP	Proteintech
Anti-Catalase	21260-1-AP	Proteintech
Anti-ACOX1	10957-1-AP	Proteintech
Anti-ADH1C	18897-1-AP	Proteintech
Anti-GAPDH	60004-1-Ig	Proteintech
Anti-FABP1 (L-FABP)	13626-1-AP	Proteintech
Anti-PPARα	15540-1-AP	Proteintech
Anti-β-Tubulin	66240-1-Ig	Proteintech
Anti-GCS	RB-1697-P1	NeoMarkers
Anti-nitrotyrosine	sc-32757	Santa Cruz Biotechnology
Anti-CYP reductase	Sc-25270	Santa Cruz Biotechnology
Anti-XOD	55156-1-AP	Proteintech
Anti-AOD	19495-1-AP	Proteintech
Anti-SOD1	Sc-8637	Santa Cruz Biotechnology
Anti-FGF21	Ab171941	Abcam
Anti-iNOS	610328	BD Transduction Lab
Ethanol assay kit	MAK076-1 KT	Sigma
ADH assay kit	DADH-100	BioAssay Systems
Free fatty acid assay kit	EFFA-100	BioAssay Systems
Triglyceride assay kit	TR22421	Thermo
ALT endpoint assay kit	3460–08	BIOO Research Corp.
FGF21 ELISA kit	RD291108200R	BioVendor
Broad Spectrum IHC Select® HRP/DAB kit	DAB150	EMD Millipore

3. Results

3.1. WY-14,643 inhibits hepatic TG accumulation induced by ethanol alone or in combination with nicotine

In previous studies that were carried out by other scientists, WY-14,643 was fed for 2 weeks at a dose of 0.1% either mixed in liquid diets [19] or solid chows [16,17]. Here, we added WY-14,643 in liquid diets at 10 mg/L (0.001%, w/v) and fed mice for 3 weeks. As shown in Fig. 1A, PPARα and its targets such as ACOX, CYP4A, L-FABP, and FGF21 were all induced dramatically by WY-14,643. Consistent with hepatic expression, serum FGF21 was increased by WY-14,643 (Fig. 1B). Liver contents of TG were increased by ethanol feeding and were further increased by ethanol/nicotine, both of them were blunted by WY-14,643 (Fig. 1C). Consistently, liver FFA contents were also decreased by WY-14,643 although they were not increased by ethanol or ethanol/nicotine (Fig. 1D). Serum TG (Fig. 1E) and serum FFA (Fig. 1F) were also decreased by WY-14,643. Please note that ethanol feeding increased serum FFA but not serum TG, which might be due to elevated lipolysis in adipose tissues after a 6-h fast. These results suggest that ethanol-induced fatty liver and nicotine-enhanced alcoholic fatty liver were ameliorated by WY-14,643, which was paralleled with induction of PPARα pathways.

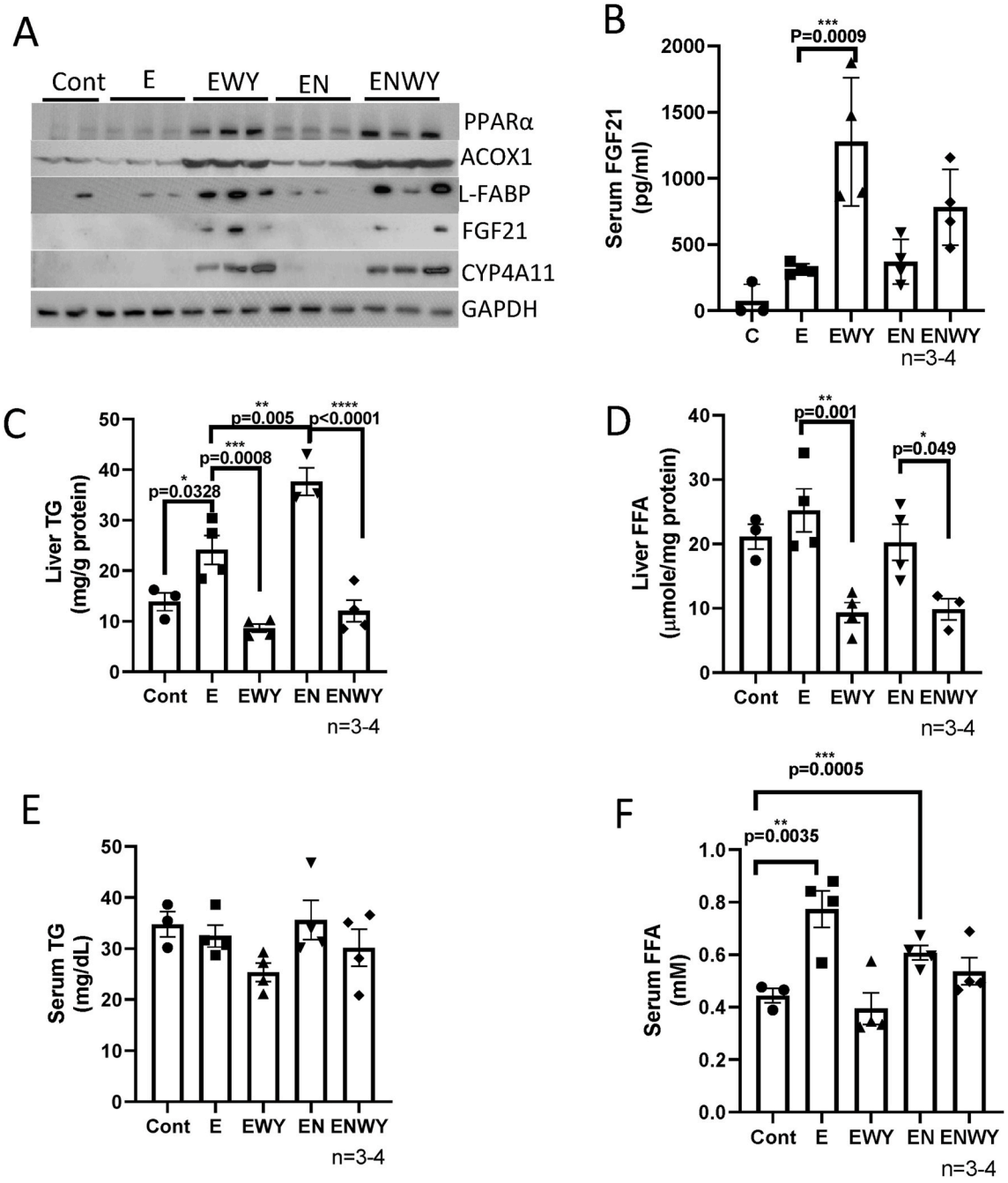

Fig. 1. WY-14,643 mixed in ethanol diets induces PPARα pathway and inhibits hepatic TG accumulation induced by ethanol alone or in combination with nicotine. (A) Western blotting analyses for PPARα-regulated ACOX, L-FABP, FGF21, and CYP4A expression in liver. (B) Serum levels of FGF21. (C) Liver contents of TG. (D) liver contents of FFA. (E) Serum levels of TG. (F) Serum levels of FFA. Cont, Control group; E, Ethanol group; EWY, Ethanol/WY-14,643 group; EN, Ethanol/nicotine; ENWY, Ethanol/nicotine/WY-14,643.

3.2. WY-14,643 enhances liver injuries induced by ethanol alone or in combination with nicotine

As a potent peroxisome proliferator, WY-14,643 can induce hepatomegaly [16]. While liver index was increased identically by ethanol and ethanol/nicotine, respectively, the liver index was further increased by WY-14,643 in both groups in the same magnitude (Fig. 2A). Serum levels of ALT were increased dramatically in ethanol/WY-14,643 group and were further increased in ethanol/nicotine/WY-14,643 group (Fig. 2B). Consistently, in the liver sections with H&E staining, necroinflammation foci were observed in both groups with WY-14,643 (Fig. 2C). Liver contents of IL-1β were not changed significantly (Fig. 2D). However, liver TNFα was decreased by WY-14,643 (Fig. 2E). CYP2A5, a nicotine metabolism enzyme, was induced by ethanol feeding, but it was inhibited by WY-14,643, although CYP reductase was still induced by WY-14,643; aldehyde oxidase (AOD), an enzyme also involved in nicotine metabolism [34,35], was induced by WY-14,643 in the ethanol/nicotine group to a greater extent than in ethanol group. (Fig. 2F). Xanthine oxidase (XOD), a source of superoxide anion, was induced by WY-14,643 only in the ethanol/nicotine group but not in ethanol group, but superoxide dismutase 1 (SOD1) was not altered by WY-14,643 (Fig. 2F). The γ-glutamyl cysteine synthetase (γ-GCS), a glutathione (GSH) synthesis rate-limiting enzyme, was inhibited by WY-14,643 (Fig. 2F), but liver total glutathione (GSH) contents were increased by WY-14,643 (Fig. 2G). The inducible nitric oxide synthase (iNOS) was induced by WY-14,643 dramatically (Fig. 2F). Consistently, 3-nitrotyrosine (3-NT) adduct formation, which was induced by ethanol and ethanol/nicotine, was further induced by WY-14,643 (Fig. 2H). Similarly, ethanol- or ethanol/nicotine-induced formation of 4-hydroxynonenal (4-HNE), a lipid peroxidation end-product, was increased by WY-14,643 (Fig. 2I). These results suggest that WY-14,643 enhances oxidative liver injuries induced by ethanol alone or in combination with nicotine.

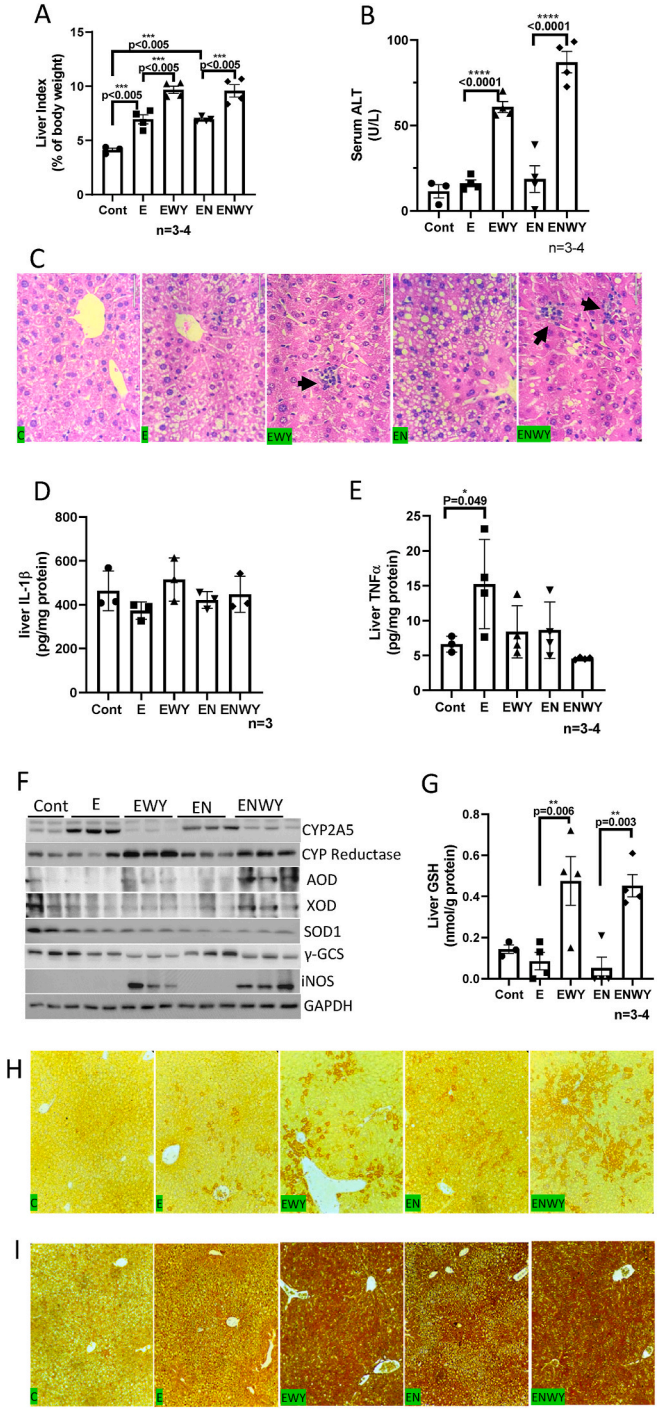

Fig. 2. WY-14,643 enhances liver injuries induced by ethanol alone or in combination with nicotine. (A) Liver index. (B) Serum levels of ALT. (C) Liver sections with H&E staining. Black arrows indicating necroinflammation foci. (D) Liver contents of IL-1β. (E) Liver contents of TNFα. (F) Western blotting analyses for hepatic expression of CYP2A5, CYP reductase, AOD, XOD, SOD1, γ-GCS and iNOS, (G) Liver contents of total glutathione (GSH). (H) IHC for 3-NT adduct formation. (I) IHC for 4-HNE adduct formation.

WY-14,643 did not synergize with ethanol to induce hepatomegaly, because WY-14,643 also increased liver index in a similar magnitude in mice fed with control diet (Fig. 3A). WY-14,643 still induced PPARα, ACOX, CYP4A, and L-FABP (Fig. 3B) and had a hypolipidemic effect (Fig. 3C). However, WY-14,643 increased serum ALT less than 2 times in mice fed with control diet (Fig. 3D), but it induced serum ALT about 4–5 times in mice fed either ethanol or ethanol/nicotine (Fig. 2B). These results suggest that WY-14,643 synergizes with ethanol to induce liver injury.

3.3. Enhancement of WY-14,643 on liver injuries induced by ethanol alone or in combination with nicotine is via a PPARα-L-FABP pathway

To examine whether the enhancing effects of WY-14,643 on alcoholic liver injuries are regulated by PPARα or L-FABP, we fed *pparα* $^{-/-}$ mice and *L-fabp*$^{-/-}$ mice ethanol diets for 3 weeks in the presence or absence of WY-14,643 as above. As expected, ACOX and CYP4A were induced by WY-14,643 in *L-fabp*$^{-/-}$ mice but not in *pparα* $^{-/-}$ mice; L-FABP was absent in *L-fabp*$^{-/-}$ mice and was not induced by WY-14,643 in *pparα* $^{-/-}$ mice (Fig. 4A). Serum FGF21, which was almost undetectable in *pparα* $^{-/-}$ mice, was induced by WY-14,643 in *L-fabp*$^{-/-}$ mice but not in *pparα* $^{-/-}$ mice (Fig. 4B). Liver contents of TG and FFA and serum levels of TG and FFA were not decreased by WY-14,643 in *pparα* $^{-/-}$ mice or *L-fabp*$^{-/-}$ mice (Fig. 4C-F), except for serum FFA was lowered by WY-14,643 in *L-fabp*$^{-/-}$ mice (Fig. 4F). Liver index in ethanol groups was higher in *pparα* $^{-/-}$ mice than in *L-fabp*$^{-/-}$ mice, but it was induced by WY-14,643 in *L-fabp*$^{-/-}$ mice but not in *pparα* $^{-/-}$ mice (Fig. 4G), suggesting that PPARα but not L-FABP is involved in hepatomegaly. Serum ALT was not increased by WY-14,643 in either *L-fabp*$^{-/-}$ mice or *pparα* $^{-/-}$ mice (Fig. 4H), suggesting that a PPARα-L-FABP pathway is involved in the WY-14,643 enhancing effects on alcoholic liver injury.

3.4. WY-14,643 escalates ethanol clearance by inducing catalase

Alcohol toxic effects are related to alcohol metabolism [1]. To examine whether WY-14,643 has an influence on ethanol metabolism, serum ethanol was measured. As shown in Fig. 5A, serum alcohol was much lower in those mice fed with ethanol diets containing WY-14,643, whereas serum alcohol was comparable in mice fed with ethanol alone

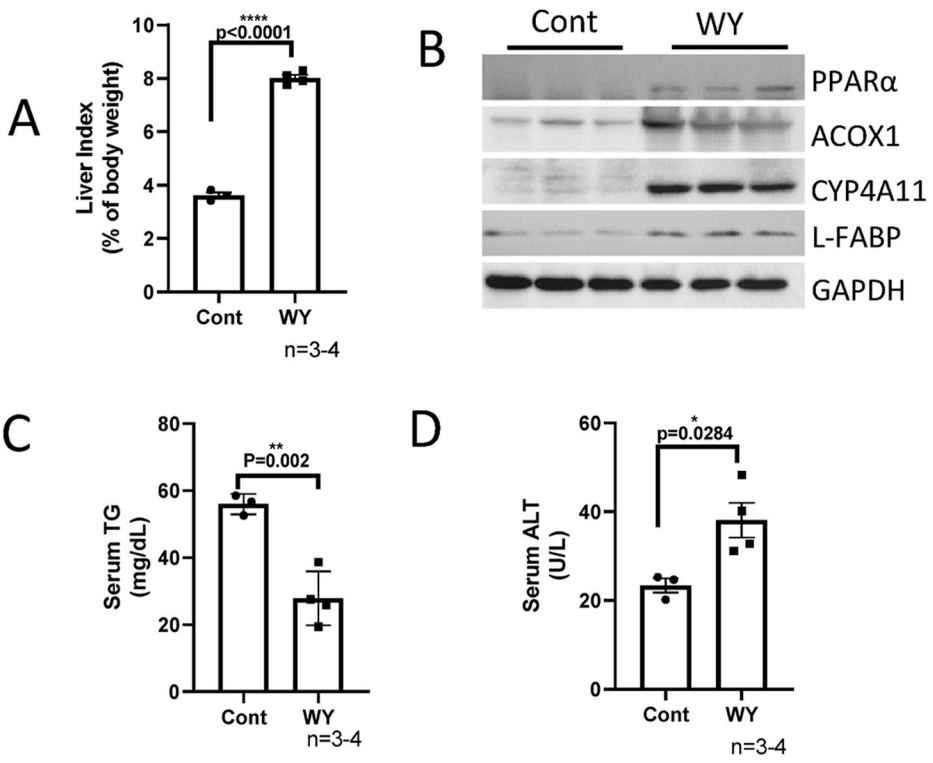

Fig. 3. WY-14,643 mixed in control diets induces similar levels of hepatomegaly but less levels of serum ALT. (A) Liver index. (B) Western blotting analyses for PPARα-regulated ACOX, L-FABP, and CYP4A expression in liver. (C) Serum levels of TG. (D) Serum levels of ALT.

or in combination with nicotine. Major ethanol metabolism enzymes in liver including ADH, CYP2E1 and catalase were measured. Liver ADH expression was not significantly altered by WY-14,643, but CYP2E1 expression was decreased by WY-14,643 (Fig. 5B). Activities of hepatic ADH and CYP2E1 reflect the changes of protein expression (Fig. 5C-D). In contrast, hepatic expression of catalase was increased by WY-14,643 (Fig. 5B) and activities of catalase were also higher in ethanol/nicotine/WY-14,643 group than in ethanol/nicotine group, although there was no significant induction in ethanol/WY-14,643 group compared with ethanol group (Fig. 5E). The induced catalase was also confirmed by IHC, which shows that the increased catalase was mainly expressed around the central veins (Fig. 5F). Interestingly, serum alcohol levels were not changed by WY-14,643 in *pparα* $^{-/-}$ mice, whereas they were dramatically decreased by WY-14,643 in *L-fabp*$^{-/-}$ mice (Fig. 6A). Consistently, catalase expression was induced by WY-14,643 in *L-fabp*$^{-/-}$ mice but not in *pparα* $^{-/-}$ mice, and CYP2E1 and ADH were not changed in either *L-fabp*$^{-/-}$ mice or *pparα* $^{-/-}$ mice (Fig. 6B). These results suggest that WY-14,643 escalates ethanol clearance by inducing catalase, which was PPARα-dependent.

To further confirm the enhancing effect of WY-14,643 on blood ethanol clearance, a binge model was applied. After 3 weeks of consumption of control liquid diet with or without WY-14,643, the mice were administrated one single dose of ethanol at 5 g/kg by gavage. Like in the chronic model, WY-14,643 decreased serum alcohol levels in the binge model (Fig. 7A). Binge alcohol did not interrupt WY-14,643 induction of PPARα pathways as indicated by induction of ACOX and CYP4A (Fig. 7B). While catalase was induced by WY-14,643, CYP2E1 and ADH were not affected by WY-14,643 (Fig. 7B), suggesting that catalase induction is responsible of the enhanced alcohol clearance. WY-14,643-induced changes in liver TG and FFA, serum TG, FFA, liver index, and ALT were like what we observed in chronic feeding model (Fig. 7C-H).

3.5. Catalase inhibitor 3-AT elevates serum alcohol and ALT in an acute-on-chronic model

To further confirm the effect of catalase on the escalated ethanol clearance, catalase inhibitor 3-AT was applied. The mice were fed ethanol diet for 2 weeks followed by a binge alcohol gavage at 5 g/kg. The catalase inhibitor 3-AT was injected i.p. at 1 g/kg prior to binge alcohol administration. As shown in Fig. 8, 3-AT increased serum ethanol levels (Fig. 8A), but it had no effect on liver index (Fig. 8B). Serum TG levels were reduced by 3-AT (Fig. 8C), but serum ALT was increased (Fig. 8D). These results suggest that 3-AT may inhibit ethanol clearance but increase liver injury.

4. Discussion

It is already known that PPARα agonist WY-14,643 reversed abnormalities in hepatic lipid metabolism in ethanol-fed mice [19]. We previously reported that nicotine enhances AFL [11]. In this study, we confirmed that WY-14,643 suppressed AFL as well as nicotine-enhanced AFL. In addition, we have new findings: 1. WY-14,643 feeding enhanced liver injury induced by ethanol in the presence or absence of nicotine; 2. WY-14,643 administration escalated blood ethanol clearance through enhancing catalase-mediated ethanol metabolism; 3. WY-14,643 exerted all effects including hepatomegaly, attenuated fatty liver, enhanced ethanol clearance and liver injury via PPARα, but as a PPARα target gene, L-FABP did not affect WY-14,643-induced hepatomegaly and ethanol clearance.

PPARα is a major regulator for hepatic lipid metabolism [14,18]. The *pparα* $^{-/-}$ mice develop more severe AFL in response to ethanol feeding [36]. Previously we found that PPARα expression was upregulated by ethanol feeding in *cyp2e1*$^{-/-}$ mice but not in WT mice, and consistently, *cyp2e1*$^{-/-}$ mice developed much less AFL than WT mice [3]. The basal level of PPARα expression was elevated in the *cyp2a5*$^{-/-}$ mice, but ethanol feeding did not upregulate PPARα in the *cyp2a5*$^{-/-}$ mice and development of AFL was enhanced in the *cyp2a5*$^{-/-}$ mice [37,38].

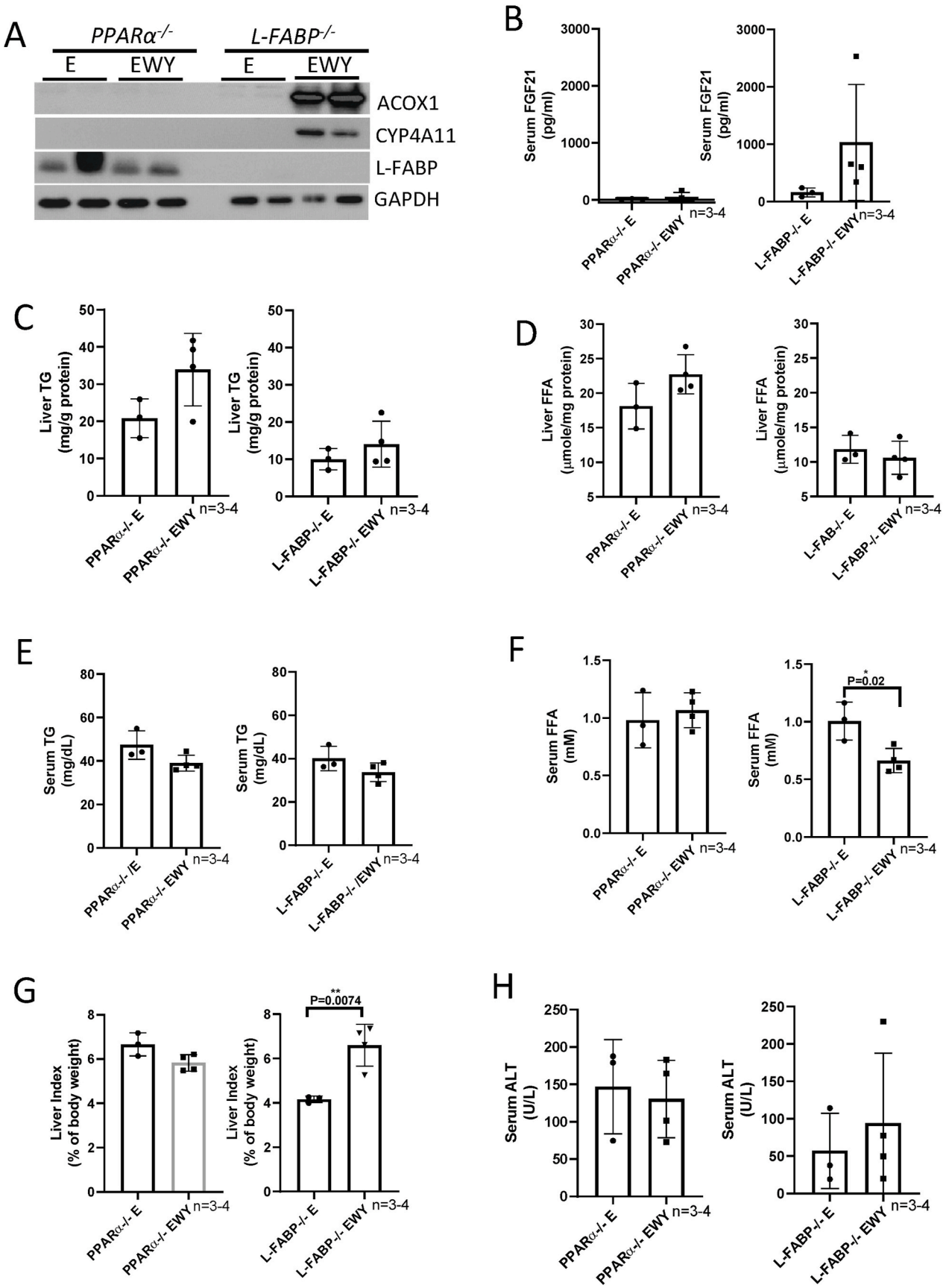

Fig. 4. WY-14,643 feeding-induced changes are not observed in *pparα*$^{-/-}$ mice and *L-fabp*$^{-/-}$ mice. (A) Western blotting analyses for PPARα-regulated ACOX, L-FABP, and CYP4A expression in liver. (B) Serum levels of FGF21. (C) Liver contents of TG. (D) Liver contents of FFA. (E) Serum levels of TG. (F) Serum levels of FFA. (G) Liver index. (H) Serum levels of ALT. E, Ethanol diets; EWY, Ethanol diets plus WY-14,643.

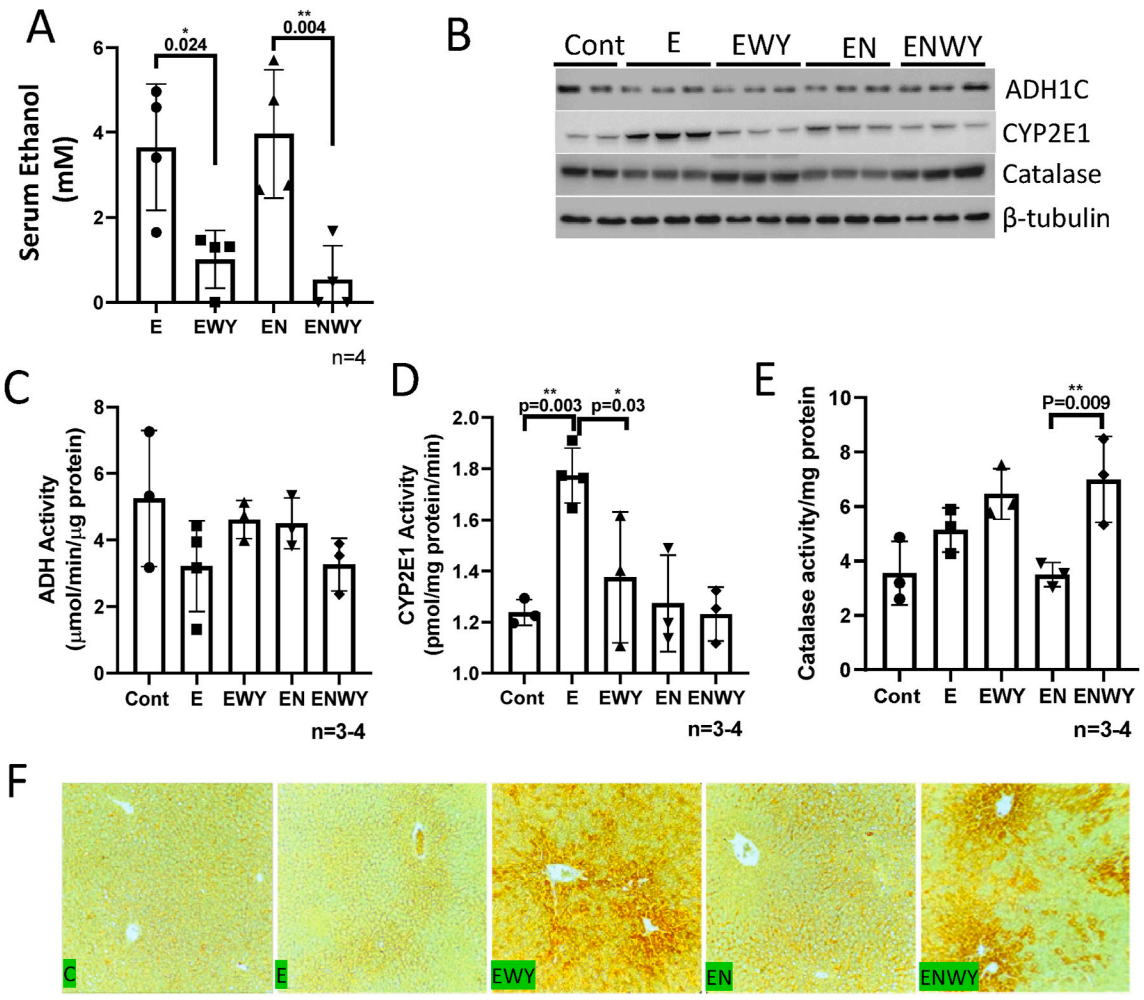

Fig. 5. WY-14,643 decreases serum levels of alcohol and induces catalase. (A) Serum levels of ethanol. (B) Western blotting analysis for expression of CYP2E1, catalase, and ADH in liver. (C) Liver ADH activities. (D) Liver CYP2E1 activities. (E) Liver catalase activities. (F) IHC for catalase in liver sections.

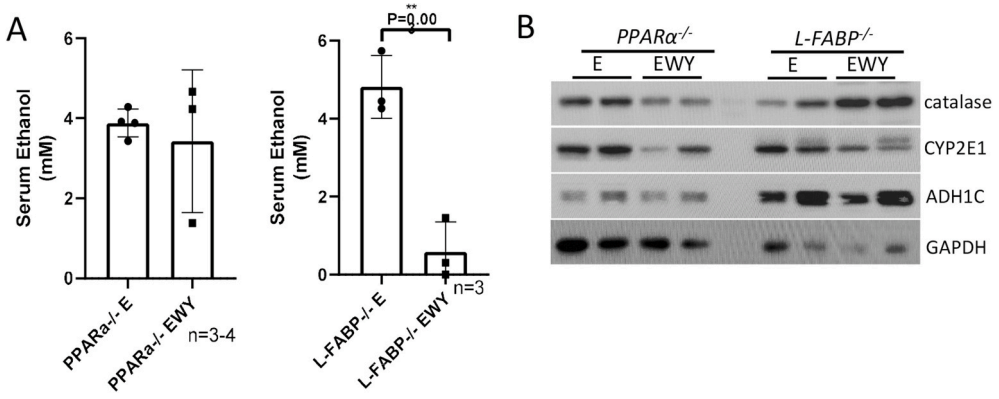

Fig. 6. WY-14,643 diet-induced alcohol metabolism is observed in *L-fabp*$^{-/-}$ mice but not in *pparα*$^{-/-}$ mice. (A) Serum levels of ethanol. (B) Western blotting analysis for liver expression of ADH, CYP2E1, and catalase.

Although WY-14,643 increased PPARα expression and its PPRE binding activity to ameliorate AFL, ethanol feeding did not alter PPARα expression including protein and mRNA but decreased the PPRE binding activity [19]. These results suggest that ethanol feeding may induce AFL via decreasing PPARα transcriptive activity but not via decreasing PPARα expression.

As a PPARα agonist, WY-14,643 up-regulated PPARα target genes, which was confirmed by the striking induction of FAO related enzymes such as ACOX, CYP4A, and L-FABP. It was suggested that WY-14,643 ameliorates ethanol-induced fat accumulation in liver via up-regulation of these FAO enzymes [19]. However, we found that in combination with ethanol, WY-14,643 also induced liver injury: serum ALT levels were dramatically increased by ethanol/WY-14,643, whereas WY-14,643 alone just slightly increased serum ALT although a similar magnitude of hepatomegaly was induced, suggesting that WY-14,643 synergizes with ethanol to induce liver injury. Nicotine only enhanced

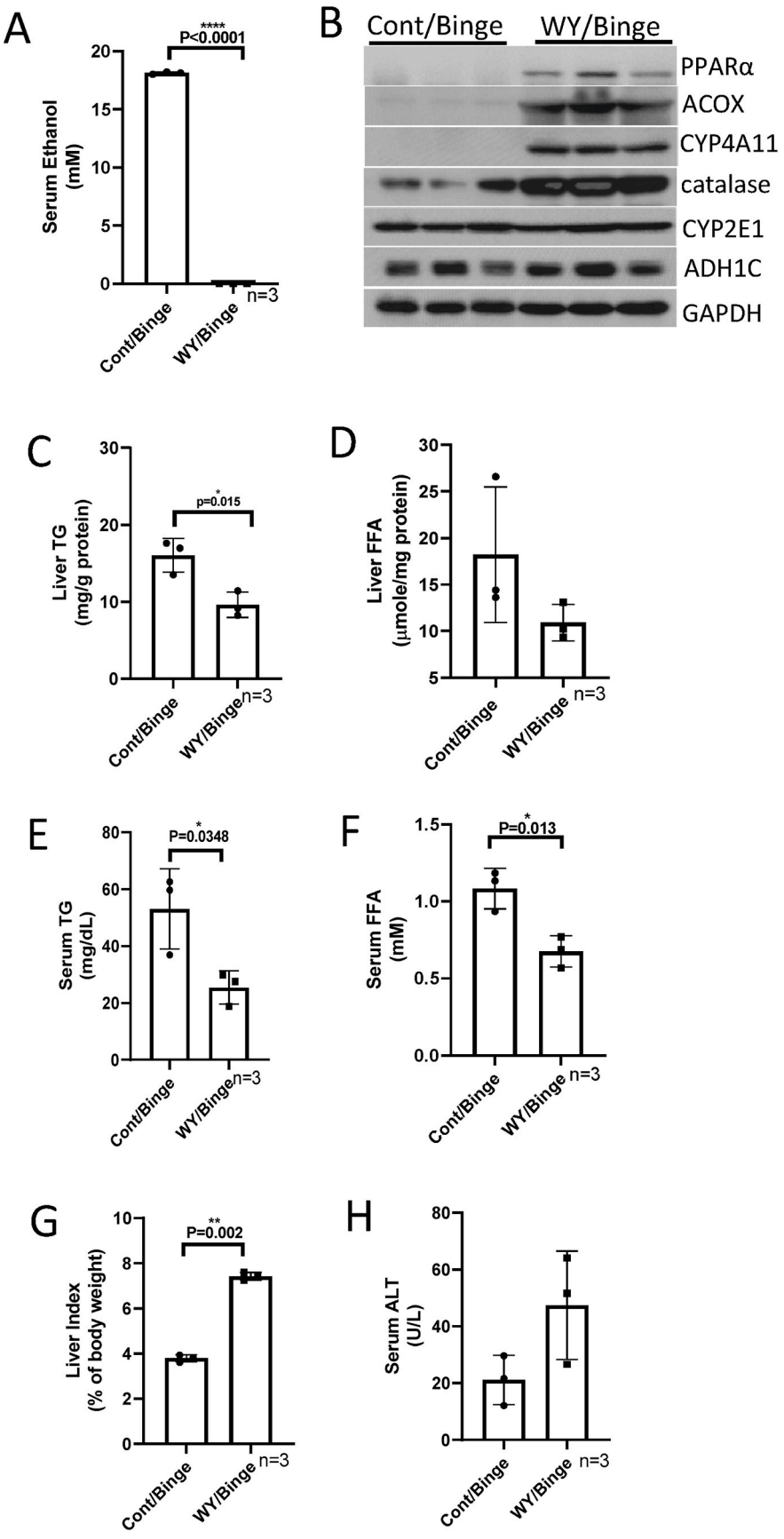

Fig. 7. WY-14,643 feeding also escalates serum alcohol clearance in a binge alcohol model. (A) Serum levels of ethanol. (B) Western blotting analyses for ACOX, CYP4A, ADH, CYP2E1, and catalase expression in liver. (C) Liver contents of TG. (D) Liver contents of FFA. (E) Serum levels of TG. (F) Serum levels of FFA. (G) Liver index. (H) Serum levels of ALT.

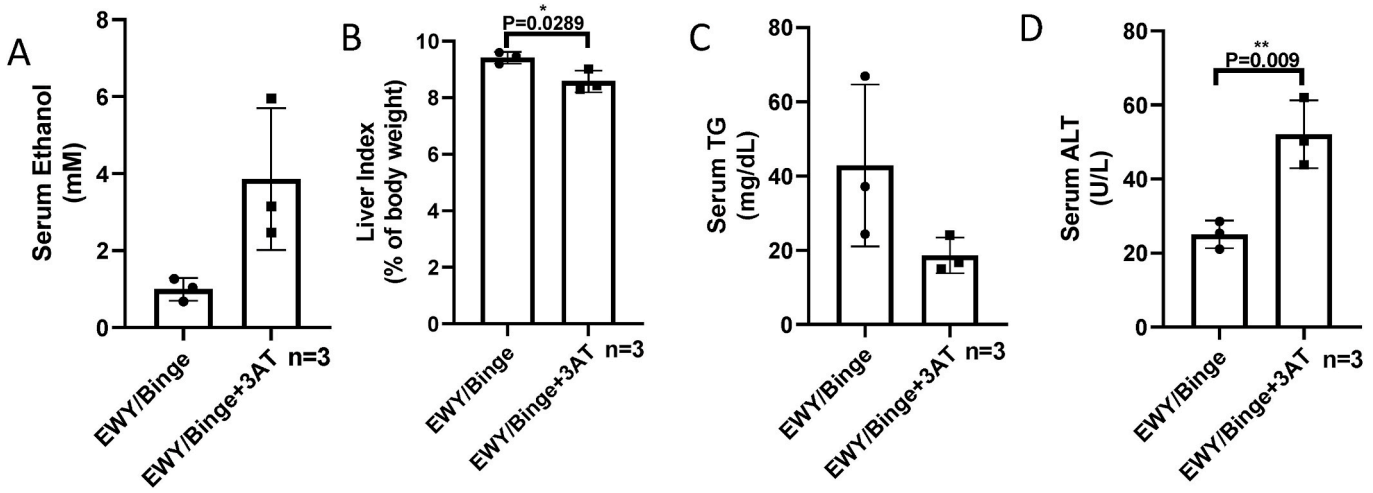

Fig. 8. Catalase inhibitor 3-AT elevates serum alcohol and ALT in an acute-on-chronic model. (A) Serum levels of ethanol. (B) Liver index. (C) Serum levels of TG. (D) Serum levels of ALT.

AFL but did not increase alcohol-induced serum ALT [11]. However, WY-14,643 further increased serum ALT in ethanol/nicotine group to a greater extent than in ethanol group. It is well known that ethanol induces ROS generation [2]. Nicotine metabolism also produces ROS [12]. ACOX-mediated fatty acid oxidation produces H_2O_2 [14]. These different sources of ROS combine together to induce oxidative liver injury as indicated by pathological observation of necro-inflammatory foci and positive staining of 3-NT and 4-HNE. Based on the 'second-hit' theory, fatty liver is vulnerable for second hits. However, we observed a minor fatty liver with a severe liver injury in mice fed with Lieber-DeCarli liquid diets containing ethanol and WY-14,643. Similar phenomenon was previously observed in CYP2E1 humanized transgenic mice fed with ethanol diets that developed a more severe liver injury with a less pronounced fatty liver [4].

WY-14,643 induced antioxidant catalase. WY-14,643-induced L-FABP and FGF21 also have antioxidant activities [39,40]. Liver contents of total GSH were also increased by WY-14,643 although the GSH synthesis rate-limiting enzyme γ-GCS was inhibited. In contrast, ROS generator CYP2E1 was inhibited by WY-14,643. All WY-14,643-induced antioxidant effects offset oxidative stress. Nrf2-regulated CYP2A5 has an antioxidant activity [5,37,38,41], but it was inhibited by WY-14,643. More importantly, nitric oxide (NO) producer iNOS was induced by WY-14,643 dramatically. NO may react with superoxide anion (O_2^-) to form peroxynitrite ($ONOO^-$), which may nitrate free and protein-associated tyrosine residues and form 3-NT [42]. In consistent with iNOS induction, 3-NT formation was increased by WY-14,643. WY-14,643-enhanced liver injury seems to be specific for ethanol since methionine-choline deficient diet (MCD)-induced serum ALT was decreased by WY-14,643 [43].

An interesting finding in this study is that WY-14,643-induced ethanol clearance. Ethanol is mainly metabolized by three pathways: ADH, MEOS mainly CYP2E1, and catalase [44]. After WY-14,643 feeding, ADH expression in liver was not changed, and CYP2E1 expression was decreased, only catalase was induced, albeit mildly. Furthermore, catalase inhibitor 3-AT elevated serum levels of ethanol. Therefore, catalase is, at least in part, responsible for the enhanced clearance of ethanol. As an antioxidant enzyme, catalase decomposes H_2O_2, which involves two steps: 1. A free catalase binds one molecule of H_2O_2 to form catalase-H_2O_2 complex; 2. The catalase-H_2O_2 complex reacts with a second H_2O_2 to decompose both H_2O_2 to 2 molecules of H_2O and release one molecule of O_2 [22]. If the second molecule of H_2O_2 is replaced by ethanol, then the catalase-H_2O_2 complex will be decomposed to free catalase and H_2O, and simultaneously ethanol is oxidized to acetaldehyde [22,23,45]. H_2O_2 production is called a primary oxidation system, and ethanol oxidation by H_2O_2 (catalyzed by catalase) is called coupled secondary oxidation. Therefore, catalase oxidizes ethanol by consuming H_2O_2. However, a solution of H_2O_2 cannot be catalyzed by catalase for coupled secondary ethanol oxidation, only "nascent" H_2O_2 gradually and continually released from a primary H_2O_2 generation system is effective for the coupled secondary ethanol oxidation [46]. Ethanol oxidation by catalase mainly depends on the ratio of the H_2O_2 generation rate to catalase concentration [22]. Fatty acids may stimulate ethanol oxidation by providing H_2O_2 generated by the peroxisomal FAO (ACOX is a rate limiting enzyme) for the catalase-catalyzed coupled secondary ethanol oxidation [26]. Lieber-DeCarli ethanol diet and control diet are high fat diets, thus ACOX-initiated FAO in peroxisomes will increase H_2O_2 generation, which will increase the ratio of the H_2O_2 generation rate to catalase concentration and promote coupled secondary ethanol oxidation (Fig. 9B).

WY-14,643 induces hepatomegaly. ADH is a major ethanol metabolism enzyme. Unlike CYP2E1, ADH expression and activities were not decreased by WY-14,643 feeding. Thus, as the liver sizes are increased, total ADH activities in liver are increased. Is it possible that the escalated alcohol clearance due to the increased sizes of liver? Our data do not support this possibility: 1. ADH-mediated ethanol oxidation consumes NAD^+ and produces NADH, which inhibits peroxisomal FAO and resultantly induces TG accumulation [1,2], but our data indicate that liver TG was decreased by WY-14,643; 2. The ADH-mediated inhibition of peroxisomal FAO should reduce the H_2O_2 generation rate and subsequently inhibit catalase-catalyzed coupled secondary oxidation of ethanol [47], but we observed a decreased serum alcohol level mediated by catalase. Further studies are needed to address this issue (Fig. 9A).

Most alcohol toxic effects are related to alcohol metabolism [1]. When ethanol metabolism was escalated by WY-14,643, oxidative liver injury was also enhanced by WY-14,643. However, when catalase-mediated ethanol oxidation was inhibited by 3-AT, serum ALT was elevated. The 3-AT produces an irreversibly inhibited enzyme by reaction with catalase-H_2O_2 complex [48]. Thus, 3-AT may inhibit H_2O_2 decomposition as well as ethanol oxidation. Therefore, 3-AT blunted ethanol metabolism and increased serum ethanol levels; 3-AT also induced liver injury and elevation of serum ALT via inhibiting catalase decomposition of H_2O_2. Further studies are needed to address the relationship between catalase-mediated ethanol metabolism and the development of liver injury.

As a peroxisome proliferator, WY-14,643 can induce peroxisome proliferation. Peroxisomes are responsible for about 20% oxygen consumption in liver [49]. Besides ACOX, other oxidases like urate oxidase

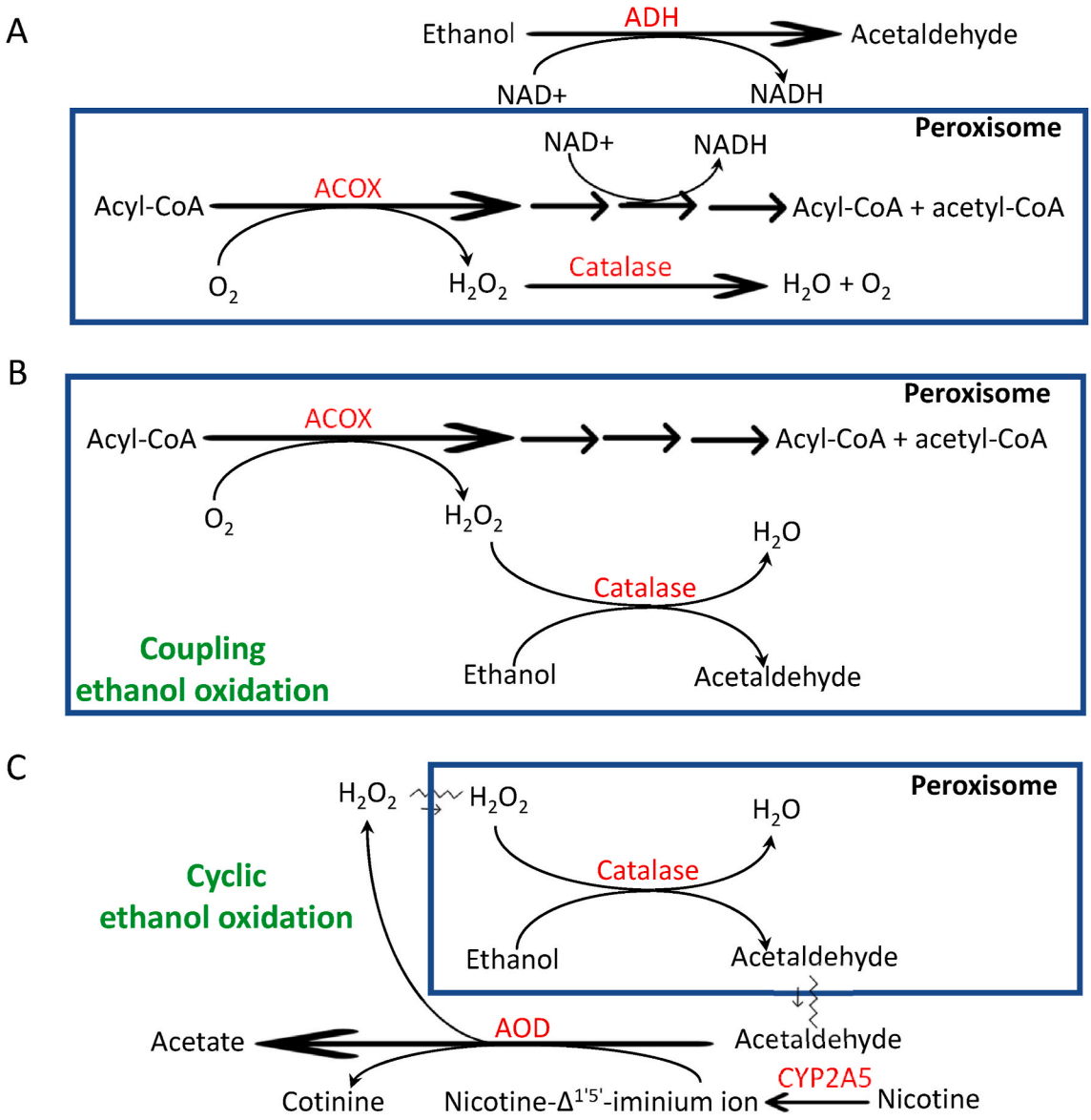

Fig. 9. Scheme for peroxisomal oxidation of ethanol. For details, please see the texts in Discussion.

and D-amino acid oxidase in peroxisomes may also generate H_2O_2 for coupled secondary ethanol oxidation [50]. Xanthine oxidase (XOD) is not located in peroxisomes, but it may oxidize ethanol metabolite acetaldehyde to acetate and generate H_2O_2, which was re-used for cyclic secondary ethanol oxidation in *in vitro* reaction systems [45,46]. Cytosolic aldehyde oxidase (AOD) can also oxidize acetaldehyde and the *Km* value of AOD for acetaldehyde is 30-fold lower than XOD for acetaldehyde [51]. Nicotine-$\Delta^{1',5''}$-iminium ion, an intermediate in the CYP2A5-mediated conversion of nicotine to cotinine, is also oxidized by AOD [34,35]. CYP2A5 was decreased, but AOD was induced by WY-14,643. AOD-mediated conversion of nicotine-$\Delta^{1',5''}$-iminium ion to cotinine also releases H_2O_2 [52], thus, nicotine oxidation via AOD may also provide H_2O_2 for "cyclic ethanol oxidation". Under PPARα induction, nicotine may enhance alcoholic liver injury through oxidative stress resulted from AOD-mediated nicotine metabolism and catalase-mediated ethanol metabolism (Fig. 9C). XOD and AOD contribute to ethanol-induced lipid peroxidation in rodents [51,53]. It will be interesting to address whether these cytosolic oxidases contribute to catalase oxidation of ethanol in *in vivo* whole organisms. In addition, WY-14,643 is not an only peroxisome proliferator. It is unclear whether other peroxisome proliferators like clofibrate also enhance ethanol metabolism and induce enhance alcoholic liver injury.

5. Conclusion

Alcohol consumption induced AFL, which is enhanced by nicotine. PPARα regulates lipid metabolism and PPARα agonist WY-14,643 blunts AFL and nicotine-enhanced AFL. On the other hand, WY-14,643 feeding also enhances catalase-mediated ethanol metabolism and alcoholic liver injury induced by ethanol in the presence or absence of nicotine. All the aforementioned effects of WY-14,643 including attenuated fatty liver, enhanced ethanol clearance and liver injury are through PPARα, but as a PPARα target gene, L-FABP did not affect WY-14,643-induced hepatomegaly and ethanol clearance.

Declaration of competing interest

None.

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