Inhibition of cereblon by fenofibrate ameliorates alcoholic liver disease by enhancing AMPK

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Alcohol consumption exacerbates alcoholic liver disease by attenuating the activity of AMP-activated protein kinase (AMPK). AMPK is activated by fenofibrate, a peroxisome proliferator-activated receptor α (PPARα) agonist, and inhibited by direct interaction with cereblon (CRBN), a component of an E3 ubiquitin ligase complex. Based on these preliminary findings, we investigated that CRBN would be up-regulated in the liver by alcohol consumption and that CRBN deficiency would ameliorate hepatic steatosis and pro-inflammatory responses in alcohol-fed mice by increasing AMPK activity. Wild-type, CRBN and PPARα null mice were fed an alcohol-containing liquid diet and administered with fenofibrate. Gene expression profiles and metabolic changes were measured in the liver and blood of these mice. Expression of CRBN, cytochrome P450 2E1 (CYP2E1), lipogenic genes, pro-inflammatory cytokines, serum alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were increased in the Lieber–DeCarli alcohol-challenged mice. Fenofibrate attenuated the induction of CRBN and reduced hepatic steatosis and pro-inflammatory markers in these mice. Ablation of the gene encoding CRBN produced the same effect as fenofibrate. The increase in CRBN gene expression by alcohol and the reduction of CRBN expression by fenofibrate were negated in PPARα null mice. Fenofibrate increased the recruitment of PPARα on CRBN gene promoter in WT mice but not in PPARα null mice. Silencing of AMPK prevented the beneficial effects of fenofibrate. These results demonstrate that activation of PPARα by fenofibrate alleviates alcohol-induced hepatic steatosis and pro-inflammatory effects in the liver. Silencing of AMPK is a potential therapeutic target for the alcoholic liver disease.

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1. Introduction

Alcoholic liver disease (ALD) is a metabolic disturbance caused by chronic alcohol overconsumption, often progressing from hepatic steatosis to steatohepatitis, fibrosis, cirrhosis, and even hepatocellular carcinoma [1]. It is a major cause and risk factor for chronic liver disease in developing and Western countries [1,2]. Alcohol stimulates hepatic fat accumulation by up-regulating sterol regulatory element binding protein-1c (SREBP-1c) and fatty acid synthase (FAS). At the same time, alcohol attenuates fatty acid oxidation by down-regulation of peroxisome proliferator-activated receptor α (PPARα), sirtuin 1 (SIRT1), and several nuclear receptors [1,3–5]. In addition, alcohol causes oxidative stress, lipotoxicity, endoplasmic reticulum stress, and pro-inflammatory cytokine release and eventually leads to alcoholic steatohepatitis, including severe fatty liver, inflammatory infiltrate and hepatocellular damage [1,2,6].

PPARα is a member of the nuclear receptor superfamily that functions as transcription factors for regulating metabolic processes such as inflammation, insulin sensitivity, glucose, and lipid metabolism [7].
PPARα is activated by energy deprivation, fasting, and fibrate agents. It is expressed primarily in the liver, heart, kidney and intestine [7,8]. Transcriptional regulation is achieved by the binding of PPAR/retinoid X receptor heterodimers to PPAR response elements (PPREs) in target gene promoters [7–9]. Fenofibrate, a PPARα agonist, is a potent drug used for treating dyslipidemia [10]. Moreover, fenofibrate improves hepatic steatosis and reduces pro-inflammatory cytokines in hepatic insulin resistance [11,12]. Our previous studies have shown that fenofibrate inhibits pro-inflammatory cytokines and pro-fibrotic markers by activating AMP-activated protein kinase (AMPK) in the progressive fibrotic steatohepatitis mouse model [13].

Cereblon (CRBN), known to be associated with a mild form of mental retardation, interacts with the calcium-activated potassium channels and voltage-gated chloride channel [14]. Moreover, CRBN is a major target for thalidomide-mediated teratogenicity and a component of the E3 ubiquitin ligase complex [15]. In recent studies, we demonstrated that CRBN directly interacts with AMPK and its deficiency ameliorates high-fat diet-induced obesity and insulin resistance by activating AMPK in the liver [14,16]. AMPK acts as a major sensor of cellular energy balance and is a key regulator of metabolic homeostasis [17]. In addition, AMPK is activated in response to a variety of physiological stimuli, pharmacological agents, hormones, oxidative stress, exercise, resveratrol, metformin, adiponectin, and leptin [18]. On the other hand, alcohol consumption exacerbated alcoholic liver injury by attenuating the activities of AMPK or SIRT1 [1,2,4,19]. Although CRBN regulates AMPK in vitro and CRBN deficiency preserves metabolic homeostasis by enhancing AMPK activity, a connection between the CRBN-AMPK signaling system and alcoholic liver disease has not been investigated.

In this study, we demonstrated that elevation of CRBN caused by chronic alcohol challenge is PPARα dependent. Moreover, disruption of the gene encoding CRBN and treatment with fenofibrate ameliorate alcoholic liver disease by stimulating AMPK. Our findings suggest that loss of CRBN may provide a novel approach to prevent the pathological progression of hepatic diseases caused by chronic alcohol exposure as well as the potential therapeutic strategy for the prevention of alcohol-mediated metabolic dysfunction.

2. Materials and methods

2.1. Animal experiments

Male wild-type C57BL/6J (WT) mice, PPARα null mice, and CRBN null mice were used as described previously [13,16]. For chronic alcoholic liver injury challenge, WT and CRBN null mice, weighing 20–30 g at 8 weeks of age were divided into 3 groups: 1) the control liquid diet in which alcohol was substituted isocalorically with maltose dextrin gavage for 4 weeks (n = 5), 2) Lieber–DeCarli alcohol liquid diet (Research Diets, New Brunswick, NJ, USA) with 5% alcohol (36% alcohol–contained calories) gavage for 4 weeks (n = 7), and 3) Lieber–DeCarli alcohol liquid diet–treated with daily fenofibrate (Sigma-Aldrich, St. Louis, MO, USA, 100 mg/kg body weight) gavage for last 2 weeks (n = 8), as described previously [20]. In some experiments, WT and PPARα null mice were fed with the Lieber–DeCarli alcohol liquid diet with or without 5% alcohol for 4 weeks (n = 5–8/group). For fenofibrate (100 mg/kg) stimulation experiments, WT mice and PPARα null mice were orally administered with fenofibrate (Sigma–Aldrich) for 5 days (n = 5–8/group). In other experiments, an adenoviral delivery system (Ad-DN AMPK) was injected by tail-vein into WT mice (1 × 10⁸ plaque-forming units, pfu) which were then exposed to alcohol (6 g/kg body weight, n = 7) and fenofibrate (100 mg/kg, n = 8) for 5 days. To ablation PPARα, WT mice were intravenously injected with lentivirus short hairpin PPARα (sh PPARα, a single dose of 1 × 10⁸ transducing units (TU) per ml). Mice were then fed with fenofibrate once-daily for 5 days. At the end of the liquid diet feeding periods, the mice were euthanized with CO₂ at which time liver weight, body weight, liver/body weight ratio, liver tissues, and blood samples were collected. All animal studies and protocols were approved by the Institutional Animal Use and Care Committee (IAUC) of the Gwangju Institute of Science and Technology.

2.2. Biochemical assays

Serum samples were immediately prepared after the collection of blood from the mice. Blood glucose levels of all mice were measured with Glucostix AccuCheck (Roche Diagnostics, Mannheim, Germany), and blood ethanol levels were quantified with Ethanol assay kit (Sigma–Aldrich). Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and cholesterol were determined with an automated blood analyzer (AU400; Olympus, Tokyo, Japan) [21]. Total adiponectin levels were measured using a commercial ELISA kit from R&D Systems (Minnesotapolis, MN, USA), and insulin levels were analyzed with the insulin enzyme-linked immunosorbent assay test kit (Merodia, Uppsala, Sweden). Hepatic and serum bile acid (BA) were measured with Bile acid L3K assay kit (Cell Biolabs, Inc., San Diego, CA, USA). Serum cytokine concentrations were measured using a BD Cytometry Bead Array (BD Bioscience, San Jose, CA, USA) in accordance with the manufacturer’s instructions, as described previously [21].

2.3. Measurement of hepatic and serum TG contents

Total TG levels in liver were analyzed using Infinity reagents (Thermo DMA, Louisville, CO, USA), according to the manufacturer’s protocol, as mentioned previously [21,22]. Serum TG levels were measured using a commercially available determination kits (Sigma–Aldrich).

2.4. Histological analysis

For hematoxylin and eosin (H & E) staining, liver tissue samples were fixed with 4% paraformaldehyde and embedded with paraffin, as described previously [21,22]. Liver sections (4–5 μm) were performed with H & E staining according to the standard procedures. Liver histology was measured using the Digital Pathology Service-Aperio ImageScope v12 (SeongKohn Traders’ Corp., Seoul, Republic of Korea).

2.5. Western blot analysis

Livers were harvested and extracted for Western blot analysis according to methods described previously [23]. Membranes were probed with phospho-AMPK (Thr172), AMPK, and FAS (Cell Signaling Technology, MA, USA), PPARα, β-actin (Santa Cruz Biotechnology, CA, USA), and then developed using an ECL Western blot detection kit (Amersham Bioscience, Piscataway, NJ, USA). The generation of the CRBN antibody was described previously [14].

2.6. Plasmids, cell culture, and transient transfection

The reporter gene for CRBN was described previously [24]. Expression vector of PPARα was mentioned previously [25]. The point mutant form of CRBN-Luc was generated with the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), as described previously [23]. The following primers: CRBN, forward 5′-AACAGTGCTTTACGTCTGG-3′ and reverse 5′-GTCGAGATCCACGTTGTT-3′. All constructs were confirmed by DNA sequencing. AML-12 cells (immortalized mouse hepatocytes) were cultured in DMEM/F-12 medium ( Gibco-BRL, Grand Island, NY, USA) supplemented with 10% FBS, insulin–transferrin–selenium (Gibco-BRL), dexamethasone (40 ng/ml, Sigma–Aldrich), and antibiotics in a humidified atmosphere containing 5% CO₂ at 37 °C (23). Transient transfection assays were conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instruction, as previously described [13].
2.7. Recombinant adenovirus

Adenovirus expressing dominant-negative mutant AMPK (DN-AMPK) was prepared as described previously [13,23]. Briefly, the cDNA encoding DN-AMPK (Asp157 to alanine in α1-AMPK and Lys45 to arginine in α2-AMPK) was inserted into the EcoRI/XhoI sites of the pAdTrack-CMV shuttle vector. The recombinant vector was then electroporated into BJ5183 cells containing the AdEasy adenoviral vector to exert the recombinant adenoviral plasmid. Adenovirus was purified by the Adeno-X Maxi Purification kit (Clontech, Mountain View, CA, USA). Recombinant lentiviral system of PPARα-targeted short hairpin RNA (shRNA) was purchased from Dharmacon (Lafayette, CO, USA).

2.8. RNA isolation and quantitative real-time PCR (qPCR) analysis

Total RNA, isolated by TRIzol reagent (Invitrogen), in accordance with the manufacturer’s instructions, was used for qPCR analysis as described previously [13,16,20,23,25]. cDNA was synthesized using the Maxima® First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania). The mRNA levels were measured using the Power SYBR® Green PCR Master Mix kit (Applied Biosystems, Warrington, UK) and the StepOne™ Real-time PCR system (Applied Biosystems). The sequences of all used primers were available on request. All data were normalized to ribosomal L32 expression.

2.9. Chromatin immunoprecipitation (ChiP) Assay

In vivo ChiP assay was performed as described previously [23,25]. Soluble chromatin was subjected to immunoprecipitation using anti-PPARα antibody (SantaCruz Biotechnology). Unrelated IgG was used as a negative control for immunoprecipitation. After purification, DNA samples were quantified by PCR using primers encompassing the mouse CRBN gene promoter. The specific primers used for PCR was as follows: CRBN promoter, forward 5′-TCTGCTATATCTCAACCCGCCC-3′ and reverse 5′-GGCACAGAAACCCCGGATTA-3′.

2.10. Statistical analysis

Data calculation and statistical analysis were performed using GraphPad Prism 3–5.0 software. The statistical significance of differences between groups was determined with Student’s t test and multiple comparisons were analyzed using one-way ANOVA under treatment and experiment as factors. All data are presented as means ± S.E.M. Values of P < 0.05 were considered to be statistically significant.

3. Results

3.1. Alcohol-induced CRBN gene expression and alcoholic liver disease are regulated by fenofibrate

Several studies have shown that chronic alcohol challenge elevates fat accumulation via up-regulation of lipogenic genes and increases in pro-inflammatory cytokines [1,2,6]. Our recent studies have demonstrated that disruption of the CRBN gene enhances hepatic AMPK activity and ameliorates high-fat diet-induced obesity and insulin resistance in mice [14,16]. Here, we investigated the possible link between CRBN and alcoholic liver disease with mice fed the Lieber–DeCarli alcohol liquid diet. As shown in supplementary Fig. 1, alcohol feeding significantly elevated the expression of the CRBN gene along with the expected increase in cytochrome P450 2E1 (CYP2E1), CYP7A1, lipogenic (SREBP-1c and FAS), Fyn, b-cell translocation gene 2 (BTG2), and pro-inflammatory cytokine (IL-6, TNF-α, and IL-1β) genes. Interestingly, mRNA levels of PPARα and SIRT1 are markedly reduced in the livers of mice fed with alcohol when compared with the control groups.

Fig. 1. Alcohol-mediated up-regulation of CRBN gene expression and alcoholic liver disease is regulated by fenofibrate. (A) Male C57BL/6 (WT) mice were fed the Lieber–DeCarli alcohol liquid diet for 4 weeks and also treated with fenofibrate (100 mg/kg) by oral gavage during the last 2 weeks of alcohol challenge (EtOH). Tissue extracts were analyzed by Western blot analysis with various antibodies. (B) Serum levels of pro-inflammatory cytokines and hepatic TG levels were measured. n = 5–6 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. untreated control (CON) or EtOH-fed mice.
Next, to determine whether alcoholic liver disease is affected by fenofibrate, we administered fenofibrate into alcohol-challenged WT mice. Fenofibrate markedly diminished alcohol-mediated induction of CRBN and FAS by up-regulating AMPK activity and also reduced the levels of pro-inflammatory cytokines and hepatic TG (Fig. 1B and C). Treatments with the PPARα agonists (Wy-14643) also promoted a significant increase in AMPK activation (data not shown), consistent with previous reports [26]. Overall, these findings suggest that alcohol feeding increases CRBN gene expression and that fenofibrate improves alcoholic liver disease via AMPK stimulation.

3.2. Disruption of CRBN ameliorates alcoholic liver disease

As a result of AMPK activation, CRBN deficiency produces a beneficial effect upon hepatic steatosis and insulin resistance caused by high-fat diet-fed mice [16]. Therefore, we hypothesized that CRBN may also be required for chronic alcoholic liver disease and that CRBN may regulate the expression of lipogenic genes and pro-inflammatory responses by controlling the AMPK signaling system. As shown in Fig. 2A, alcohol challenge of WT mice significantly elevated gene expression of CRBN, CYP2E1, plasminogen activator inhibitor-1 (PAI-1), pro-inflammatory markers, and lipogenic enzymes. Alcohol challenge of WT mice also markedly attenuated AMPK activity, consistent with previous findings [1,4]. In contrast, these effects of alcohol challenge were totally absent in CRBN null mice (Fig. 2B). Likewise, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), pro-inflammatory cytokines, hepatic TG, and hepatic cholesterol (CHO) levels were significantly elevated in alcohol-challenged WT mice but not in alcohol-fed CRBN null mice (Fig. 2C and D). As expected, liver weight and liver/body weight ratio were significantly elevated in alcohol-fed WT mice, whereas these effects of alcohol challenge were disrupted in alcohol-fed CRBN null mice (supplementary Fig. 2A). As shown supplementary Fig. 2B, serum levels of TG, CHO, BA, insulin, and hepatic BA contents were significantly lower in alcohol-fed CRBN null mice than in alcohol-challenged WT mice. No significant differences in blood alcohol, blood glucose concentrations, and body weight gain were observed in these mice. Surprisingly, the concentration of adiponectin was markedly reduced in alcohol-fed WT mice and was restored in alcohol-fed CRBN null mice. Overall, these results show that CRBN acts as a key player in regulating alcohol-mediated metabolic changes.

Fig. 2. CRBN null mice protect alcoholic liver disease. WT and CRBN null mice were fed the Lieber–DeCarli alcohol liquid diet for 4 weeks. (A) Total RNA was analyzed by qPCR with the indicated primers. (B) Tissue extracts were measured by Western blot analysis with various antibodies. (C) Serum levels of ALT and AST. (D) Serum levels of pro-inflammatory cytokines and hepatic TG levels. n = 5–8 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. untreated WT or EtOH-fed WT mice.
3.3. Alcohol-mediated metabolic dysfunction is altered by fenofibrate-CRBN signaling network

We evaluated the physiological effects of fenofibrate upon chronic alcohol-fed WT and CRBN null mice. The elevation of CRBN, pro-inflammatory markers, and lipogenic genes caused by alcohol feeding was attenuated in fenofibrate-fed WT mice, but remarkably, the beneficial effects of fenofibrate were abolished in CRBN null mice (Fig. 3A), consistent with data presented in Fig. 2. Thus, we further confirmed that the increase of pro-inflammatory cytokines and hepatic TG levels in response to alcohol challenge was dramatically diminished in fenofibrate-treated WT mice and this phenomenon was completely disrupted in CRBN null mice (Fig. 3B). In addition, we measured the potential role of fenofibrate on hepatic steatosis in alcohol-fed WT and

![Graphs showing relative mRNA levels of CRBN, IL-6, TNF-α, IL-1β, SREBP-1c, and FAS.](image)

**Fig. 3.** Alcohol-mediated metabolic dysfunction is altered by fenofibrate-CRBN pathway. WT and CRBN null mice were fed the Lieber–DeCarli alcohol liquid diet for 4 weeks and fenofibrate was challenged by oral gavage for the last 2 weeks of alcohol feeding. (A) Total RNA was analyzed by qPCR with the indicated primers. (B) Serum levels of pro-inflammatory cytokines and hepatic TG levels. (C) Liver histology after hematoxylin and eosin staining in mouse liver. n = 5–8 mice per group. *P < 0.05, **P < 0.01 vs. untreated WT or EtOH-fed WT mice.
CRBN null mice. Fenofibrate strikingly alleviated the elevation of hepatic fat accumulation caused by alcohol feeding in the livers of WT mice (Fig. 3C). Surprisingly, hepatic steatosis was prevented in CRBN null mice and therefore no effect of fenofibrate was observed in these mice (Fig. 3C). Collectively, these findings suggest that blockade of CRBN caused by fenofibrate plays a pivotal role in ameliorating alcoholic liver disease.

3.4. Fenofibrate regulates CRBN gene expression by a PPARα-dependent pathway

As expected, treatment of WT mice with fenofibrate markedly attenuated CRBN gene expression, and significantly stimulated AMPK activation in a dose-dependent manner (Fig. 4A). Similarly, fenofibrate enhanced the expression of PPARα and decreased CRBN gene expression in WT mice but these effects of fenofibrate were abolished in PPARα null mice (Fig. 4B and C). To confirm whether the expression of CRBN gene repressed by fenofibrate is mediated by PPARα, we introduced a knock-down system of PPARα (lentivirus-shRNA PPARα) in the liver of fenofibrate-fed mice. As expected, knockdown of PPARα using lentiviral system (sh PPARα) successfully attenuated the expression of PPARα gene when compared with the control groups. Fenofibrate markedly reduced the change of CRBN protein level, whereas this effect is rescued by silencing of PPARα (supplementary Fig. 3). Next, to verify that PPARα is involved in the regulation of CRBN gene expression, we assessed the potential role of PPARα from alcohol feeding in the liver of WT and PPARα null mice. Expression of PPARα gene was strikingly reduced in alcohol-fed WT mice and CRBN gene expression was significantly increased in these mice but this phenomenon was disrupted in PPARα null mice (Fig. 4D). Taken together, these findings demonstrate that fenofibrate-mediated inhibition of CRBN is regulated by a PPARα-dependent mechanism.

3.5. PPARα directly regulates CRBN gene transcription

Next, the fundamental molecular mechanism by which PPARα controls CRBN gene transcription was explored. As shown in Fig. 5A, the increase of CRBN gene promoter activity caused by alcohol exposure was dramatically reduced by either fenofibrate treatment or PPARα in a dose-dependent manner. We evaluated the functional significance of the PPARα-binding region on the CRBN gene promoter in hepatocytes using transient transfection assay. Alcohol-stimulated CRBN promoter activity was also markedly repressed by either fenofibrate treatment or PPARα, and this phenomenon was abolished at a mutant form of the PPARα region (Fig. 5B). To further confirm the stimulation of DNA binding of endogenous PPARα protein on the CRBN gene promoter, we performed chromatin immunoprecipitation (ChIP) assay in the livers of mice using PPARα antibody. As expected, fenofibrate strongly promoted PPARα occupancy on the CRBN gene promoter and there was no significant PPARα-occupancy observed in either alcohol-exposed or PPARα null mice (Fig. 5C and D). These findings strongly suggest that PPARα is a direct transcriptional regulator of CRBN.

3.6. Alcoholic liver disease is altered by the fenofibrate-AMPK pathway

To determine whether fenofibrate-AMPK signaling network is involved in the regulation of alcoholic liver disease, we examined the crucial role of AMPK under both alcohol exposure and fenofibrate feeding and overexpression of the dominant negative form of AMPK (Ad-DN AMPK) in mouse liver. As expected, alcohol-mediated induction of the pro-inflammatory responses and lipogenic genes was strikingly reduced by fenofibrate treatment, consistent with data presented in Fig. 3. In contrast, the beneficial effects of fenofibrate were disrupted by Ad-DN AMPK (Fig. 6A). Likewise, the increase of pro-inflammatory cytokines and hepatic TG levels caused by alcohol exposure was dramatically attenuated by fenofibrate treatment, and this phenomenon was also abolished by Ad-DN-AMPK (Fig. 6B). Overall, these results demonstrate that alcohol-mediated hepatic disease is mediated by a fenofibrate-AMPK-dependent pathway.

4. Discussion

In the present study, we delineated the potential role of CRBN on chronic alcohol challenge in mouse livers and explored the fundamental molecular mechanism responsible for alcoholic liver disease. For the first time, we provide evidence that alcohol challenge increases CRBN gene expression by attenuating the PPARα-dependent pathway, and that treatment with fenofibrate reduces hepatic steatosis and pro-

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**Fig 4.** Expression of CRBN caused by alcohol is mediated by fenofibrate-PPARα axis. (A) WT mice were administered fenofibrate (Feno) at the indicated concentrations. Six hours after the treatment, livers were harvested and extracted for Western blot analysis with the indicated antibodies. Protein levels were normalized to total form antibody and/or β-actin levels. (B) WT and PPARα null mice were orally fed with or without fenofibrate for 5 days. Total RNA was analyzed by qPCR with the indicated primers. (C) Tissue extracts were isolated from liver tissue harvested from the mice of the indicated groups and subjected to Western blot analysis with the indicated antibodies. (D) WT and PPARα null mice were fed the Lieber–DeCarli alcohol liquid diet for 4 weeks. Total RNA was analyzed by qPCR with the indicated primers. n = 5–8 mice per group. *P < 0.05, **P < 0.01 vs. untreated WT or EtOH-fed WT mice.
inflammatory modulators that are induced by chronic alcohol feeding but not in CRBN null mice. Finally, the beneficial effect of fenofibrate caused by alcohol challenge was disrupted by silencing of AMPK. Collectively, our current findings suggest that CRBN deficiency ameliorates alcoholic liver disease by attenuating hepatic steatosis and inflammation as well as enhancing AMPK activity.

Chronic alcoholic liver disease is characterized by hepatic steatosis, overproduction of pro-inflammatory cytokines, and severe liver injury [1,2]. Alcohol challenge up-regulates lipogenic genes and down-regulates PPAR-α gene expression and AMPK activity, leading to an increase of lipogenesis and inflammation and a decrease of fatty acid oxidation [1–3,6]. However, the correlation between chronic alcohol challenge and the beneficial effects of hepatic CRBN deficiency has not yet been investigated. In this study, we have observed that alcohol-induced hepatic disease is mediated by a PPAR-α-CRBN signaling system in mouse liver. Indeed, our findings demonstrated that alcohol challenge significantly elevated CRBN gene expression via the reduction of PPAR-α along with subsequent alleviation of AMPK activation (Figs. 1, 2, 4, and supplementary Fig. 1). Based on these findings, we speculate that the attenuation of CRBN by PPAR-α activators may provide protective effects on alcohol-mediated hepatic dysfunction by stimulating AMPK via the PPAR-α-CRBN signaling network.

Fyn kinase is a negative regulator of AMPK [27,28]. As a consequence of greater AMPK activity, Fyn kinase null mice display greater fatty acid oxidation, energy expenditure, and insulin sensitivity and these mice are protected against hepatic steatosis caused by chronic alcohol consumption [27,29]. Moreover, the SIRT1-AMPK network regulates hepatic steatosis and the production of pro-inflammation cytokines in alcohol-fed mice [4,30–32]. Our current study demonstrates that dysregulation of AMPK caused by the alcohol-CRBN axis (Figs 1 and 2) markedly exacerbates hepatic metabolic disease in alcohol-challenged mouse liver, consistent with previous reports. Remarkably, we observed the stimulation of Fyn and attenuation of SIRT1 in alcohol-fed mice compared to those of control (supplementary Fig. 1). Therefore, we suggest that the down-regulation of CRBN and/or the up-regulation of AMPK may provide beneficial effects in hepatic metabolic dysfunction by alcohol challenge. However, we cannot exclude the possibility that alcohol may also be associated with Fyn kinase, SIRT1, and other unknown metabolic key regulators to drive alcoholic-mediated hepatic disease.

We previously found that fenofibrate alleviates pro-fibrotic and pro-inflammatory responses by stimulating PPAR-α activity in a PPAR-α-independent manner [13,33]. In this study, we confirmed that fenofibrate regulates metabolic target genes through PPAR-α-AMPK axis in alcohol-challenged mouse liver. Indeed, our current results demonstrate that the increase of AMPK activation by fenofibrate strikingly down-regulated alcohol-induced hepatic steatosis and pro-inflammatory cytokine responses. The protective effects of fenofibrate were effectively abolished by silencing of AMPK (Fig. 6). The elevated hepatic steatosis and pro-inflammatory markers by alcohol exposure were markedly attenuated by stimulating PPAR-α-AMPK signaling pathway in fenofibrate-fed mice (Figs. 1, 4 and 6). Therefore, fenofibrate may protect against alcoholic liver disease via two different pathways. The first pathway is a PPAR-α-independent manner by activating orphan nuclear receptor small heterodimer partner, involving the progressive fibrosis model and a systemic excessive sepsis model. The second pathway is a PPAR-α-dependent manner by promoting AMPK stimulation under chronic alcoholic liver disease following the Lieber–DeCarli alcohol liquid diet. Thus, the activation of AMPK and/or other signaling pathway in response to physiological stimuli and pharmacological agents like exercise, resveratrol, and adiponectin [17,18,30,31,34] may be relevant to AMPK and alcoholic liver disease.

Previous studies have shown that PPAR-α is a ligand-activate transcription factor and stimulates the transcription of target genes involved in inflammation, glucose and lipid metabolism [7,8,25,35]. On the other
hand, PPARα also inhibits the inflammatory gene responses by attenuating the transcriptional activity of other target genes, including C/EBPβ, NF-κB, and AP-1 [36–38]. Based on these findings, we elucidated a potential link between PPARα and CRBN gene regulation in the liver of alcohol-challenged mice. As expected, CRBN gene expression was markedly diminished in fenofibrate-exposed WT mice, and this phenomenon was abolished in PPARα null mice (Fig. 4). Alcohol significantly increased CRBN gene transcription, and this effect was directly repressed by PPARα (Fig. 5), which is consistent with previous reports [36–38]. Therefore, our results suggest that the phenomenon of negative gene regulation caused by PPARα is the novel molecular mechanism responsible for alcohol-mediated induction of CRBN gene expression. However, we cannot rule out the possible molecular mechanism that links the PPARα-CRBN signaling network and alcoholic liver disease with unexplored transcriptional factors, co-regulators competition or recruitment, microRNAs, and other signaling molecules, which contribute to the regulation of CRBN gene expression.

In conclusion, our current study suggests that the PPARα-CRBN-AMPK signaling network is a critically important pathway in alcoholic liver disease. We speculate that the loss of CRBN may ameliorate alcohol-mediated hepatic steatosis and inflammation through the fenofibrate-PPARα-AMPK signaling pathway. As described in the schematic model (Fig. 7) the down-regulation of CRBN by fenofibrate and/or CRBN disruption induces beneficial effects in alcoholic liver disease, which suggest a novel therapeutic strategy for the prevention and treatment of alcohol-induced metabolic dysfunction.

Fig. 6. Alcoholic liver disease is altered by fenofibrate-AMPK signaling network. (A) WT mice were tail-vein injected with Ad-DN-AMPK. Mice were then administered alcohol and fenofibrate once-daily for 5 days. Total RNA was analyzed by qPCR with the indicated primers. (B) Serum levels of pro-inflammatory cytokines and hepatic TG levels. n = 5–8 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001, and #P < 0.05 vs. untreated control or EtOH-fed mice, and EtOH- and Feno-fed mice.

Fig. 7. Schematic model depicting the role of CRBN in alcoholic liver disease. Alcohol, a major stimulus leading to alcoholic liver disease, elevates steatosis and inflammation by up-regulating CRBN and subsequently causes alcoholic liver disease by attenuating AMPK activation. Fenofibrate, a PPARα agonist, effectively disrupts the alcohol-CRBN network and ameliorates alcoholic liver disease by enhancing AMPK activity.
Conflict of interest

There is no conflict of interest to disclose for all authors.

Author contributions

Y.D.K. contributed to the conception, design of study, and performance of experiments, analysis and interpretation of data, writing the draft; K.M.L. and S.L.H. performed experiments and interpretation of data, and critical review of the manuscript; H.W.C. and K.J.K. contributed to the design of experiments, analysis, and interpretation of data; R.A.H. and H.S.C. contributed to the analysis and interpretation of data, and critical review of the manuscript; W.S.C., S.E.L., and C.S.P. contributed to the design of study, drafting of the manuscript, interpretation of data, and critical review of the manuscript. W.S.C., S.E.L., and C.S.P. are the guarantor of this work, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2015.09.014.

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