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Chitinase-3-like-1 deficiency attenuates ethanol-induced liver injury by inhibition of sterol regulatory element binding protein 1-dependent triglyceride synthesis

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ABSTRACT

Objective: Alcohol overconsumption and abuse lead to alcoholic liver disease (ALD), which is a major chronic liver disease worldwide. Chitinase-3-like protein 1 (CHI3L1) have an important role in the pathogenesis of inflammatory disease. However, the role of CHI3L1 in ALD has not yet been reported. In the present study, we investigated the effect of CHI3L1 on chronic plus binge ethanol-induced liver injury.
Methods: CHI3L1 knock out (KO) mice and their littermate control mice based on C57BL/6 (10–12 weeks old) were fed on a Lieber-DeCarli diet containing 6.6% ethanol for 10 days. And, CHI3L1 siRNA or CHI3L1 expressing vector was transfected HepG2 cells were treated with ethanol or without.
Results: Ethanol-induced hepatic triglyceride (TG) levels and the mRNA levels of TG synthesis-related genes such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and stearoyl-CoA desaturase-1 (SCD1) were decreased in the liver of CHI3L1 knock out (KO) mice and the HepG2 cells transfected with CHI3L1 siRNA. Increased mRNA level and activation of SREBP1 which is transcription factor of ACC, FAS and SCD1 by ethanol feeding were reduced in the liver of ethanol-fed CHI3L1 KO mice. Moreover, ethanol-induced SREBP1 luciferase activity and mRNA level of SREBP1, ACC, FAS and SCD1 were also decreased in the HepG2 cells transfected with CHI3L1 siRNA, while those were further increased in the HepG2 cells treated with recombinant human CHI3L1. Furthermore, oxidative stress and up-regulated pro-inflammatory cytokines by ethanol were recovered in the liver of ethanol-fed CHI3L1 KO mice.
Conclusion: Our finding suggest that inhibition of CHI3L1 suppressed ethanol-induced liver injury through inhibition of TG synthesis, and the blocking of oxidative stress and hepatic inflammation induced SREBP1 activity could be significant.

1. Introduction

Excessive alcohol intake is leading cause of preventable morbidity and mortality worldwide, and contributes to progress of alcoholic liver disease (ALD) [1]. In the United States, >50% of the population consumes alcohol, and three in 10 adults are heavy alcohol drinkers with a high risk for ALD [2]. The liver is primary organ of ethanol metabolism and the principal target site of alcohol-induced damage [3]. An early pathophysiological response of the liver to alcohol ingestion is the fat accumulation in hepatocytes which can lead to ALD [4]. Triglyceride (TG), a major form of fat, excessively accumulates inside liver cells and represents an important indicator for ALD [5]. During early stage of ALD, chronic alcohol exposure leads to upregulate fatty acid synthesis and accumulate hepatic TG [6,7]. Lipid accumulation leads to increase metabolic dysfunctions and hepatic sensitivity to toxins [8]. Increasing researches have demonstrated that chronic alcohol ingestion elevates lipogenesis by activating sterol regulatory element binding protein 1 (SREBP1) which is a transcription factor that promotes the expression of lipogenic genes including acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1) [9], and overexpression of these genes can lead to fatty liver by TG accumulation [6,10,11]. SREBP1 precursor is proteolytically cleaved to release the transcriptionally active form. Once the mature, the transcriptionally active form of SREPB1 is translocated into the nucleus, and then it binds to sterol regulatory elements and activates the transcription of SREBP1-responsive genes, thereby promoting lipogenesis in the liver [12]. Several studies have reported that chronic alcohol exposure to rodents resulted in a significant increase in the SREBP1 level [13–15], and TG accumulation in the liver was more

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increased in ethanol-fed SREBP1-overexpressing mice [16]. In contrast, feeding ethanol to SREBP1 knock-out mice protected development of liver steatosis [17]. In addition, reactive oxygen species (ROS) is critically involved in TG accumulation *via* regulation of the SREBP1 activation [18].

ROS leads to DNA damage and lipid peroxidation in the ALD [19]. A significant amount of ROS is generated by the catalytic reaction of cytochrome P450 2E1 (CYP2E1) that is increased during chronic alcohol abuse [1]. Increased CYP2E1 elevated the level of ROS in HepG2 cells [3], and ethanol-induced oxidative stress are reduced in CYP2E1 knock-out mice, but increased in knock-in mice [20]. In contrast, the levels of antioxidants such as superoxide dismutase (SOD), precursors of glutathione (GSH), tocopherol and vitamin E are low in patient with ALD [21]. Depletion of mitochondrial GSH by chronic ethanol feeding occurs preferentially in pericentral hepatocytes, where most of the liver injury emerges [22]. Lipid peroxidation is also involved in ethanol-induced liver injury by the formation of toxic aldehydes, including malondialdehyde (MDA) and 4-hydroxy-2,3-nonenal (4-HNE) [1]. The level of MDA was significantly increased in livers from patients with ALD, while antioxidants such as GSH, vitamins A, C and E were decreased [22]. Furthermore, hydrogen peroxide, one of ROS, treatment increased TG accumulation, SREBP1 transcriptional activity and nuclear translocation of SREBP1 were increased in HepG2 cells [18]. In histological analysis, extensive lipid droplets were observed in ethanol-fed WT mice, but a small number of tiny lipid droplets were observed in the CYP2E1 KO mice. Ethanol-induced hepatic TG level was also decreased in the CYP2E1 KO mice [23]. These data indicate that oxidative stress enhances TG accumulation and could be significant for ALD.

Ethanol-induced oxidative stress contributes to hepatic inflammation in ALD [24]. The ethanol-fed SOD1 KO mice developed liver injury that varied from mild inflammation to extensive inflammation in histological analysis [25], while elevated serum level of ALT by ethanol was reduced in SOD1 overexpressed mice [26]. In addition, overexpression of glutathione peroxidase 1 (Gpx1), an antioxidant molecule, suppressed the level of TNF-α and IL-1β in the liver of retinoic acid-related orphan receptor α (RORα) stimulated mice, which regulates Gpx1 transcription [27]. Hepatic inflammation induced by alcohol exposure produces pro-inflammatory cytokines, and recruits immune cells to the site of inflammation, and accumulate TG in the liver [28,29]. TNF-α induced cytotoxicity were also increased in primary hepatocytes by ethanol treatment [30]. Also, the levels of cytokines and chemokines involved in neutrophils infiltration were increased in the liver of patients with ALD [31]. Intracellular adhesion molecule 1 (ICAM-1) that recruit neutrophils to the site of inflammation was increased in ethanol-fed WT mice, but it was inhibited in monocyte chemoattractant protein-1 (MCP-1) KO mice [29]. Furthermore, TNF-α administration caused an upregulation of SREBP1 and TG accumulation in mice [32]. Hepatic TG level was lower in ethanol-fed MCP-1 KO mice, compared to ethanol-fed WT mice [29]. Thus, these data suggest that hepatic inflammation stimulates TG accumulation and significantly also contributes to ALD.

Chitinase-3-like protein 1 (CHI3L1) is a member of the family of mammalian chitinase-like proteins [33,34]. According to analysis from Human Protein Atlas, CHI3L1 is expressed and secreted by various cell types including hepatocyte, hepatic stellate cells, macrophages, neutrophils and chondrocytes. In addition to, the highest expression of CHI3L1 was found in the liver among the other tissues (www.proteinatlas.org) [35]. CHI3L1 plays a role in cell proliferation, differentiation, inflammation and immune responses [33]. Recently studies reported that CHI3L1 level was related to liver disease such as fibrosis, chronic hepatitis C and chronic hepatitis B [36,37]. Moreover, serum CHI3L1 level in patients with ALD (steatosis, fibrosis, cirrhosis and alcoholic hepatitis) was 5 folds higher than healthy control levels [34,38]. However, the function of CHI3L1 in ALD is still unknown. In this present study, we investigated the effects of CHI3L1 on chronic and binge ethanol-induced liver injury model.

2. Materials and methods

2.1. Animals experiment and housing condition

Male and Female CHI3L1 wild-type (WT) and knock-out (KO) mice were obtained described in the previous study [39]. The experiment was performed in accordance with the guidelines proscribed by the Chungbuk National University Animal Care Committee (CBNUA-1073-17-01). The mice were acclimatized to the laboratory environment, maintained at 22 ± 1 °C and relative humidity of 55 ± 10%, with 12 h light-dark cycles throughout the experiment. All mice were fed a standard laboratory chow diet *ad libitum*. CHI3L1 WT and KO mice were randomly divided into four groups ($n = 20$/group), each group of mice received 2 different types of liquid diets for 10 days: (1) pair-fed standard diet with water; (2) alcohol diet with ethanol. On the morning of 11th day, acute administration was performed and sacrificed 9 h post gavage.

2.2. Chronic plus binge ethanol feeding model

Male and Female CHI3L1 WT and KO mice administered a Lieber/DeCarli Regular Liquid Diet-Control (Dyets, Cat # 710027) for 5 days with *ad libitum*. Following acclimation, mice were feeding *ad libitum* with a Lieber/DeCarli Regular Liquid Diet-Ethanol (Dyets, Cat # 710260) containing 5% (vol/vol) ethanol or pair-fed a Lieber/DeCarli Regular Liquid Diet-Control for 10 days. On the morning of 11th days, mice were gavaged with a single dose of ethanol (5 g/kg bodyweight) or maltodextrin, respectively, and were sacrificed 9 h later [40].

2.3. Measurements of serum aspartate transaminase and alanine transaminase

Blood was collected at 9 h after ethanol administration. Serum was separated by centrifugation at 3000 rpm for 8 min at 21 °C. Serum aspartate transaminase (AST) and alanine transaminase (ALT) were measured using a biochemical analyzer (AU480, Beckman Coulter, CA, USA).

2.4. Histopathological analysis

For histological processing, liver tissues were fixed in 4% formalin solution. Then, liver tissues were embedded in paraffin. Specimens were sectioned 4 μm and stained with hematoxylin and eosin stain (H&E), then observed with a light microscope (Nikon, Tokyo, Japan).

2.5. Oil Red O staining

Liver tissues were fixed in formalin and cut by frozen section at 10 mm. Next, sections were rinsed with propylene glycol and stained with a 0.2% Oil Red O solution in propylene glycol for 30 min at room temperature, and subsequently washed with tap water. Cells were washed twice with phosphate-buffered saline (PBS), fixed with 0.5% glutaraldehyde for 3 h at room temperature, washed again with PBS, and allowed to dry completely. Next, fixed cells were stained with a 0.2% Oil Red O solution in isopropanol diluted in distilled water (6:4) for 1 h at room temperature, and subsequently washed twice with PBS. Stained lipid droplets were observed with a light microscope (Nikon, Tokyo, Japan).

2.6. Transcription activity test

Human hepatic HepG2 cells were obtained from the American Type Culture Collection. HepG2 cells were grown at 37 °C in 5% CO_2-humidified air in Dulbecco's modified Eagle's medium (DMEM) that contained 10% FBS and 100 units/mL antibiotics. CHI3L1 siRNA were purchased from OriGene Technologies (Rockville, MD, USA) and recombinant human CHI3L1 protein (rhCHI3L1) was purchased from Abcam (Cambridge, MA, USA). HepG2 cells were plated 12-well plates

(1×10^5 cells/well) and transiently transfected with pGL3 siRNA using Lipofectamine® RNAiMAX Reagent using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen) or treated with rhCHI3L1 (100 ng/ml). After 6 h transfection or treatment, cells were treated with H_2O_2 (1 mM) or Ascorbic acid (125 μM, Sigma-Aldrich, St. Louis, MO) or Bay 11-7082 (20 μM, Abcam, Cambridge, MA, USA). Next day, cells were treated with ethanol (100 mM) for 24 h then measured transcriptional activity.

2.7. Western blot analysis

Homogenized liver tissues were lysed by protein extraction solution (PRO-PREP, iNtRON, Sungnam, Korea) and the total protein concentration was determined using the Bradford reagent (Bio-Rad, Hercules, CA, USA). 40 μg extracted protein were separated by SDS/PAGE and transferred to Immobilon® PVDF membranes (Millipore, Bedford, MA, USA). The membrane was blocked with 5% dried skimmed milk for 1 h at room temperature, followed by incubation with specific primary antibodies for overnight at 4 °C. The membranes were washed with Tris-buffered saline containing 0.05% Tween-20 (TBST) and incubated with diluted horse radish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After washes, binding of antibodies to the PVDF membrane was detected using the Immobilon Western Chemilum HRP substrate (Millipore, Bedford, MA, USA). The band intensities were measured using the Fusion FX 7 image acquisition system (Vilber Lourmat, Eberhardzell, Germany). Specific primary antibodies were purchased from Abcam (iNOS, CYP2E1, 4-HNE; Cambridge, MA, USA), Novus Biologicals (COX-2; Inc., Littleton) and Cell Signaling Technology (pAMPK and AMPK, Danvers, MA). Secondary antibodies were purchased from Santo Cruz Biotechnology (anti-mouse and anti-rabbit; Dallas, TX, USA).

2.8. Immunohistochemistry

Paraffin-embedded ethanol-induced liver tissue sections were blocked for 60 min with 2% normal horse serum contained blocking solution diluted in 1× PBS. The sections were then blotted and incubated with specific primary antibodies in blocking solution for overnight at 4 °C. And then, the sections were washed three times for 10 min each in PBs and incubated in biotinylated anti-mouse, rabbit antibody for 90 min. The sections were washed three times for 10 min each in PBS, followed by formation of the avidinbiotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA). The slides were washed and the peroxidase reaction developed with diaminobenzidine and peroxide and then counter-stained with hematoxylin, mounted in Cytoseal XYL (Thermo Fisher Scientific, Waltham, MA, USA) and evaluated on a light microscope (Nikon, Tokyo, Japan). Specific primary antibodies were purchased from Abcam (iNOS, CYP2E1, 4-HNE; Cambridge, MA, USA) and Novus Biologicals (COX-2; Inc., Littleton).

2.9. RNA isolation and quantitative real-time RT-PCR

Total RNA from liver tissues were extracted by RiboEx™ Total RNA isolation solution (GeneAll Biotechnology, Seoul, Korea) and cDNA was synthesized using High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time RT-PCR was performed on a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) for custom-designed primers and β-actin was used for house-keeping control using HiPi Real-Time PCR 2X Master Mix (ELPIS, Daejeon, Korea). Cycling conditions consisted of a denaturation of 20 s at 94 °C, a combined annealing of 20 s at 55 °C and extension of 45 s at 72 °C by 40 cycles. The values obtained for the target gene expression were normalized to β-actin and quantified relative to the expression in control samples.

2.10. Measurement of hepatic triglycerides

A hepatic level of triglycerides (TG) was measured as described in the manufacturer's protocol using triglycerides assay kit (Abcam, Cambridge, MA, USA).

2.11. Oxidative stress assay

Hydrogen peroxides assay was performed as described in the manufacturer's protocol (Cell biolabs, San Diego, CA, USA). Hepatic levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured using GSH/GSSG Ratio Detection Assay Kit (Abcam, Cambridge, MA, USA). Lipid peroxidation was measured by determining the generation of malondialdehyde (MDA; TBARS Assay kit, Cayman, Ann Arbor, MI, USA).

2.12. Statistical analysis

The data were analyzed using the GraphPad Prism 4 version 4.03 software (Graph-Pad Software, La Jolla, CA). Data are presented as mean ± SD. The differences in all data were assessed by one-way analysis of variance. When the p value in the analysis of variance test indicated statistical significance, the differences were assessed by the Tukey's test. A value of $p \leq 0.05$ was considered to be statistically significant.

3. Result

3.1. CHI3L1 deficiency attenuated ethanol-induced liver injury in the liver of mice

We investigated the role of CHI3L1 in the chronic plus binge ethanol-feeding model using WT and CHI3L1 KO mice. Body weight and body weight change were not significantly different between in WT mice and in CHI3L1 KO mice (Fig. 1A). However, elevated serum levels of aspartate transaminase (AST) and alanine transaminase (ALT) as well as liver weight/body weight ratio by ethanol feeding were decreased in ethanol-fed CHI3L1 KO mice (Fig. 1B). Histopathological studies revealed vesicular hepatosteatosis in ethanol-fed WT mice, but its manifestation was attenuated in ethanol-fed CHI3L1 KO mice (Fig. 1C). Similarly, Oil Red O staining for neutral lipids confirmed the increased hepatosteatosis in the livers of ethanol-fed WT mice compared to ethanol-fed CHI3L1 KO mice (Fig. 1C).

3.2. Ethanol-induced hepatic steatosis was inhibited in the liver of CHI3L1 KO mice

Hepatic steatosis is the most remarkable pathological feature of ALD, which is characterized by excessive hepatic lipid accumulation [9]. To examine the degree of lipogenesis in the liver, we measured TG level in the blood and liver. The levels of total cholesterol and TG in the blood did not differ significantly between groups (Fig. 2A). However, increased hepatic TG level by ethanol (62.30 mg/g, 1.5 folds compared to pair-fed WT mice) was inhibited in the liver of ethanol-fed CHI3L1 KO mice (48.67 mg/g, 0.78 folds compared to ethanol-fed WT mice) (Fig. 2A). The mRNA level of SREBP1 and its target genes such as ACC, FAS and SCD1 involved in TG synthesis in the liver were increased by ethanol feeding but these increased levels were lowered in the liver of ethanol-fed CHI3L1 KO mice (Fig. 2B). Since activated SREBP1 is translocated into the nucleus, we investigated quantity of SREBP1 in the nuclear fraction of the liver. As showed in Fig. 2C, SREBP1 was increased in the nuclear fraction of WT mice liver by ethanol feeding, while increased SREBP1 was lowered in the nuclear fraction of ethanol-fed CHI3L1 KO mice liver compared to ethanol-fed WT mice. Furthermore, the phosphorylation of AMPK which is upstream signaling of SREBP1 was decreased by ethanol feeding in the liver of WT mice but it was not decreased in the liver of KO mice (Fig. 2D). Also, decreased the phosphorylation of AMPK by ethanol was

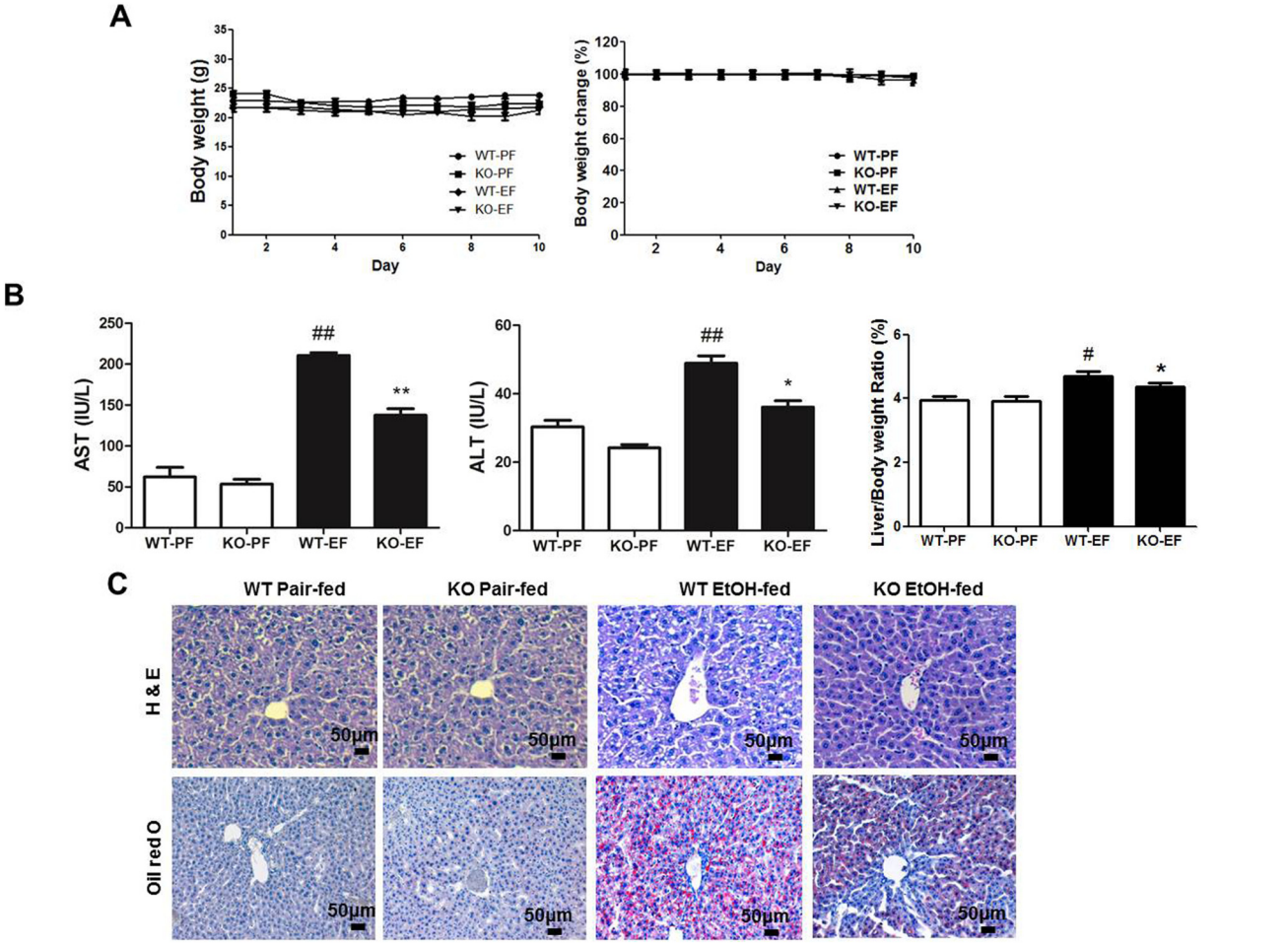

Fig. 1. Effects of CHI3L1 deficiency in chronic plus binge ethanol feeding mice model. (A) Bodyweight and liver weight/body weight ratio of pair-fed WT mice, pair-fed CHI3L1 KO mice, ethanol-fed WT mice and ethanol-fed CHI3L1 KO mice during food intake period. $n = 20$ per group; means ± SD of the mean (B) Serum aspartate transaminase (AST) and alanine transaminase (ALT) levels of pair-fed WT mice, pair-fed CHI3L1 KO mice, ethanol-fed WT mice and ethanol-fed CHI3L1 KO mice for 10 days and administration of a single dose of ethanol (5 g/kg bodyweight) or isocaloric maltodextrin. $n = 10$ per group; means ± SD of the mean. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ pair-fed WT mice *vs.* ethanol-fed WT mice, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ ethanol-fed WT mice *vs.* ethanol-fed CHI3L1 KO mice (C) Liver sections of pair-fed WT mice, pair-fed CHI3L1 KO mice, ethanol-fed WT mice and ethanol-fed CHI3L1 KO mice for 10 days and administration of a single dose of ethanol (5 g/kg bodyweight) or isocaloric maltodextrin were stained with hematoxylin and eosin (H&E) or Oil Red O (scale bars, 50 μm).

restored by CHI3L1 siRNA transfection in HepG2 cells, but it was further decreased by rhCH3L1 (Fig. 2E).

3.3. CHI3L1 regulated SREBP1 activity and TG synthesis in HepG2 cells

To elucidate whether activation of SREBP1 is correlated with CHI3L1, we measured ethanol-induced SREBP1 transcriptional activity in HepG2 cells transfected with CHI3L1 siRNA or rhCHI3L1 treated HepG2 cells using luciferase assay. The SREBP1 luciferase activity was increased by ethanol treatment in HepG2 cells, but it was decreased by CHI3L1 siRNA transfection in ethanol-treated HepG2 cells (Fig. 3A). Additionally, mRNA level of SREBP1 was reduced by CHI3L1 siRNA transfection in ethanol-treated HepG2 cells (Fig. 3A). Elevated mRNA level of ACC, FAS and SCD1 were also inhibited by CHI3L1 siRNA transfection in ethanol-treated HepG2 cells (Fig. 3A). In contrast, SREBP1 luciferase activity as well as mRNA level of SREBP1, ACC, FAS and SCD1 were further increased by rhCHI3L1 treatment in ethanol-treated HepG2 cells (Fig. 3B).

3.4. Ethanol-induced oxidative stress was reduced in the liver of CHI3L1 KO mice

The liver is the most targeted organ attacked by oxidative stress [41], and oxidative stress is critically involved in TG accumulation *via* regulation of the SREBP1 activation [18]. Moreover, ethanol could generate ROS through CYP2E1 dependent metabolism [23]. Therefore, we examined whether ethanol feeding increase TG synthesis through enhancement of ROS by ethanol metabolism. Immunohistochemically staining and Western blot analysis showed an induction of CYP2E1 in ethanol-fed WT mice. However, ethanol-induced CYP2E1 expression was reduced in the liver of CHI3L1 KO mice (Fig. 4A, B). CYP2E1 metabolize ethanol and generate ROS, and the degree of CYP2E1 induction is correlated with ROS production [42]. Ethanol-induced hepatic oxidative stress has been demonstrated by detecting ROS or by measuring lipid peroxidation [43]. When level of oxidative stress increased, GSSG accumulated and GSH/GSSG ratio decreased. As showed in Supplementary Fig. 1A, total GSH level and GSH/GSSG ratio in the liver of ethanol-fed WT mice were lower than those in the liver of ethanol-fed CHI3L1 KO mice. The level of hydrogen peroxide was elevated in the liver of ethanol-fed WT mice whereas it was reduced in the liver of ethanol-fed CHI3L1 KO mice (Fig. 4C). In addition, degree of lipid peroxidation, as assessed by expression of 4-HNE using immunohistochemistry and Western blot analysis, was elevated in the liver of WT mice by ethanol feeding, but it was decreased in the liver of ethanol-fed CHI3L1 KO mice compared to in the liver of ethanol-fed WT mice (Supplementary Fig. 1B, C). Accordingly, the level of thiobarbituric acid (TBARS), a marker of lipid peroxidation, was also increased in the liver of ethanol-fed

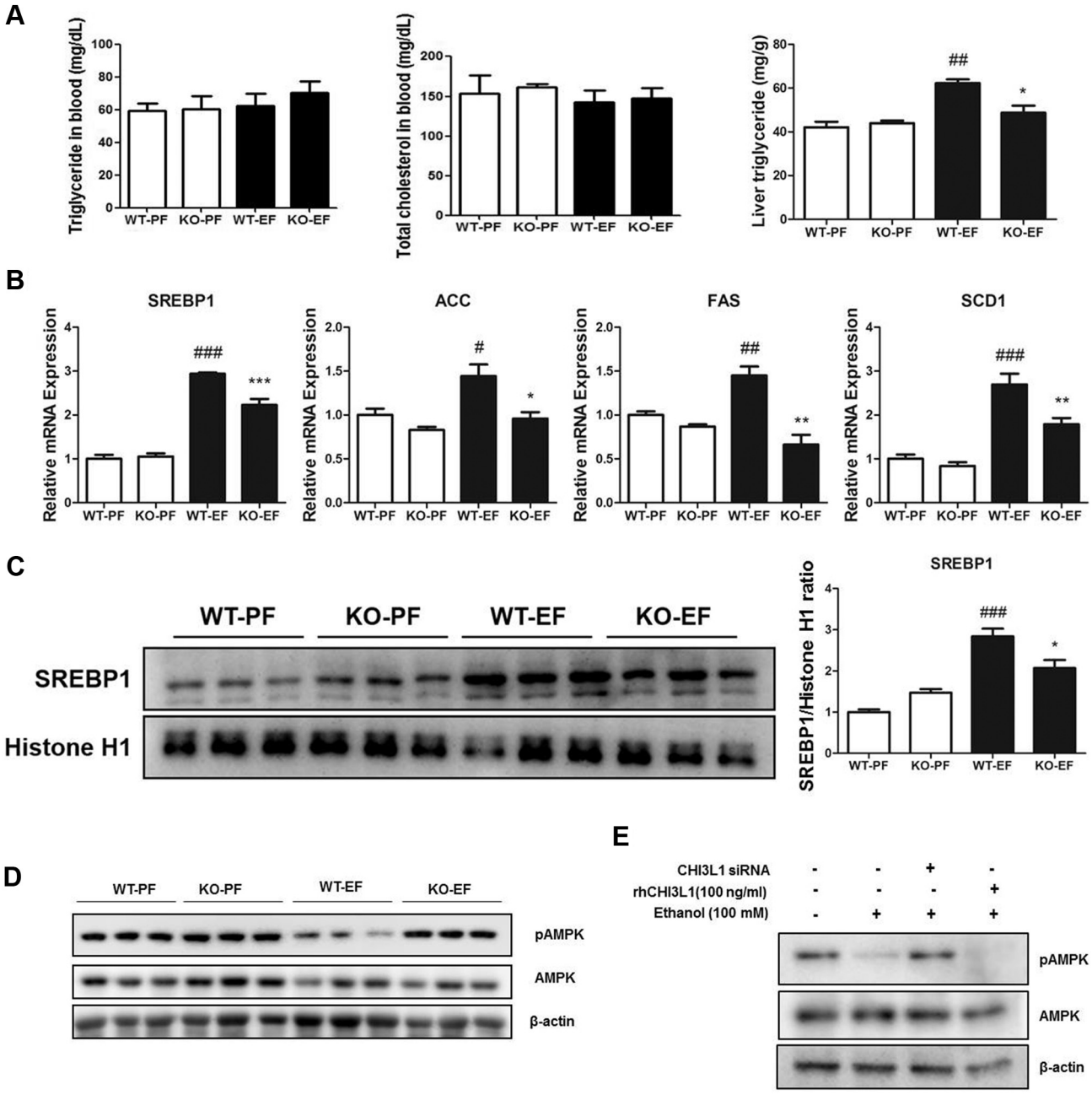

Fig. 2. Effects of CHI3L1 deficiency on hepatic steatosis in chronic plus binge ethanol feeding mice model. (A) Triglyceride level in the blood and liver tissue of pair-fed WT mice, pair-fed CHI3L1 KO mice, ethanol-fed WT mice and ethanol-fed CHI3L1 KO mice. (B) The mRNA level of triglyceride synthesis genes, SREPB1, ACC, FAS and SCD1, in the pair-fed WT mice, pair-fed CHI3L1 KO mice, ethanol-fed WT mice and ethanol-fed CHI3L1 KO mice. $n = 10$ per group; means ± SD of the mean. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ pair-fed WT mice *vs.* ethanol-fed WT mice, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ ethanol-fed WT mice *vs.* ethanol-fed CHI3L1 KO mice. (C) The expression of SREBP1 was determined in the nuclear protein extracts of mice liver tissues by Western blot analysis. $n = 3$ per group; means ± SD of the mean. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ pair-fed WT mice *vs.* ethanol-fed WT mice, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ ethanol-fed WT mice *vs.* ethanol-fed CHI3L1 KO mice. (D) The phosphorylation of AMPK was determined in the mice liver tissues by Western blot analysis. $n = 3$ per group (E) The phosphorylation of AMPK was determined in CHI3L1 siRNA transfected or rhCHI3L1 treatment HepG2 cells in the presence of ethanol or not.

WT mice. However, the increase in the level of TBARS caused by ethanol was suppressed in the liver of ethanol-fed CHI3L1 KO mice (Supplementary Fig. 1A).

3.5. CHI3L1 is associated with oxidative stress pathway which involved in ethanol induced-SREBP1 activity and TG synthesis in HepG2 cells

To analyze whether ROS stimulates SREBP1 activity, we treated H_2O_2 (1 mM) or antioxidant ascorbic acid (125 μM) in the ethanol (100 mM) pre-treated HepG2 cells transfected with CHI3L1 siRNA or in rhCHI3L1 treated HepG2 cells. Ethanol-induced SREBP1 luciferase activity was further increased by H_2O_2 treatment in HepG2 cells (Fig. 5A). H_2O_2 treatment enhanced SREBP1 activity also inhibited in CHI3L1 siRNA transfected HepG2 cells (Fig. 5A). In addition, mRNA level of ACC was more promoted by H_2O_2 treatment, and this level was also inhibited by H_2O_2 treatment in CHI3L1 siRNA transfected HepG2 cells (Fig. 5A). In contrast, rhCHI3L1 treatment more increased SREBP1 activity and mRNA level of ACC in ethanol-treated HepG2 cells, but elevated SREBP1 activity and mRNA level of ACC by ethanol was decreased by ascorbic acid which is an anti-oxidant in HepG2 cells treated with

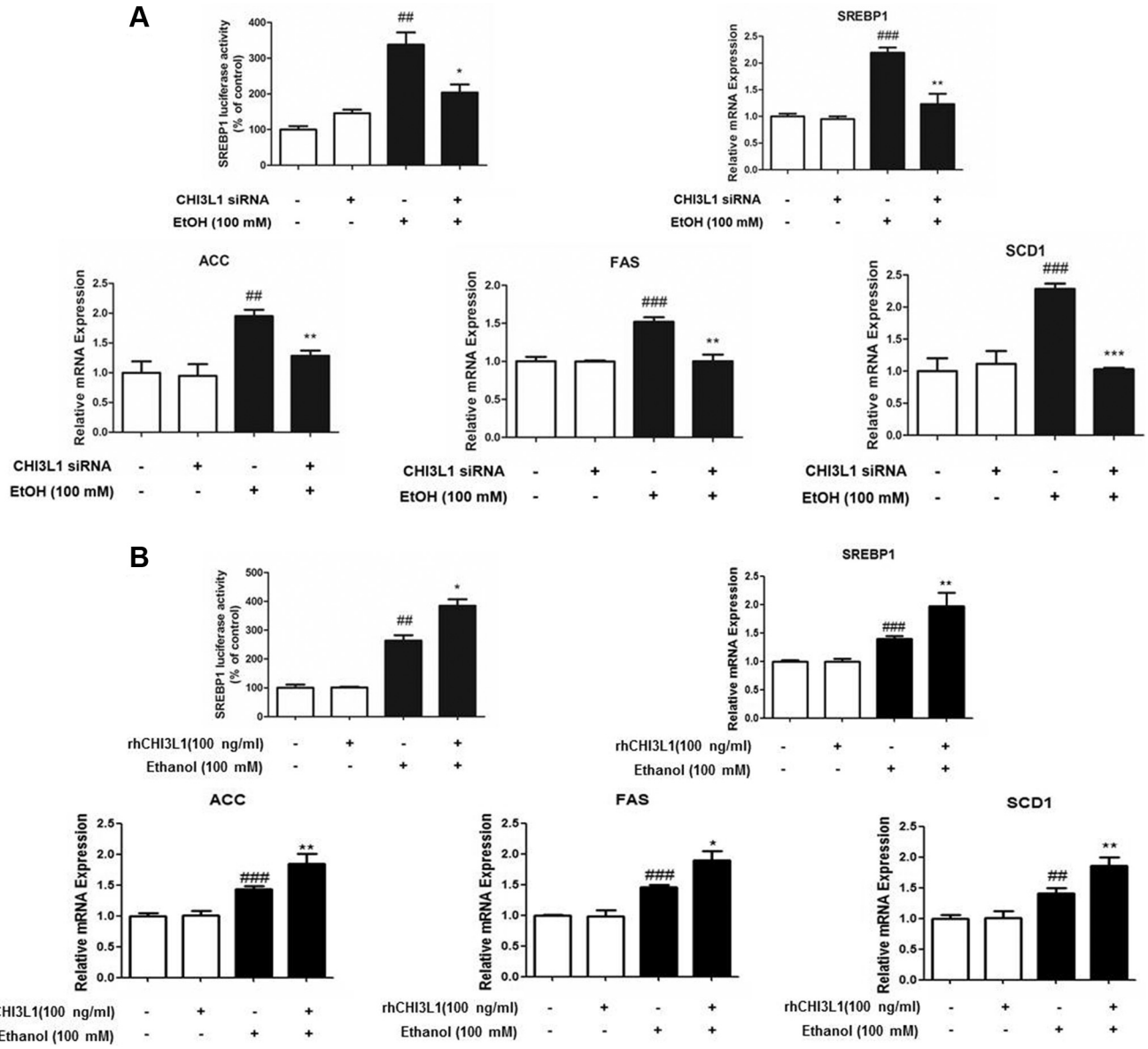

Fig. 3. A critical role of CHI3L1 in regulation of SREBP1 in human hepatic HepG2 cells. SREBP1 transcriptional activity measured by luciferase assay using transfection of luciferase-expressing SREBP1 plasmid DNA vector. Luciferase assay and mRNA level of triglyceride synthesis genes, SREPB1, ACC, FAS and SCD1, in the HepG2 cells transfected with CHI3L1 siRNA (A) or (B) treatment with rhCHI3L1 in the presence ethanol or not. $n = 3$ per group; means ± SD of the mean. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ control *vs.* ethanol treatment, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ ethanol treatment *vs.* CHI3L1 siRNA transfection or rhCHI3L1 with ethanol treatment.

rhCHI3L1-treated or not (Fig. 5B). These results suggest that CHI3L1 regulates oxidative stress stimulated SREBP1 activity and TG synthesis.

3.6. Ethanol-induced hepatic inflammation was decreased in the liver of CHI3L1 KO mice

Since chronic ethanol exposure induces liver injury through hepatic inflammation [31], we examined expression of inflammatory proteins, pro-inflammatory cytokine and chemokine levels in the liver of WT and CHI3L1 KO mice. The expression of inflammatory proteins such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2)-reactive cells were much greatly increased in the liver of ethanol-fed WT mice compared to in the liver of pair-fed WT mice, but these proteins-reactive cells were significantly decreased in the liver of ethanol-fed CHI3L1 KO mice (Fig. 6A). In consonance with the immunohistochemistry results, ethanol-fed elevated expression of iNOS and COX-2 in the liver of WT mice, but these expressions reduced in the liver of ethanol-fed CHI3L1 KO mice (Fig. 6B). In addition, mRNA levels of pro-inflammatory cytokine such as TNF-α, IL-1β and chemokine such as MIP-1α and MIP-1β were much higher in the liver of ethanol-fed WT mice compared to in the liver of pair-fed WT mice whereas these mRNA levels suppressed in the liver of ethanol-fed CHI3L1 KO mice (Fig. 6C, D). Neutrophils recruitment is considered a critical aspect of hepatic inflammation [44]. To investigate whether the inhibition of ethanol-induced inflammation in WT and CHI3L1 KO mice was associated with infiltration of neutrophils, we measured the distribution of neutrophils in the blood and liver. Blood level of neutrophils was increased in the ethanol-fed WT mice compared to pair-fed WT mice, but its level was decreased in the ethanol-fed CHI3L1 KO mice (Supplementary Fig. 2A). The mRNA level of Ly6G (a neutrophils marker) in the liver of ethanol-fed WT mice was higher than those in the liver of pair-fed WT mice whereas infiltration of neutrophils was suppressed in the liver of ethanol-fed CHI3L1 KO mice (Supplementary Fig. 2B). Immunohistochemically staining of Ly6G was increased in the liver of ethanol-fed WT mice, but it was

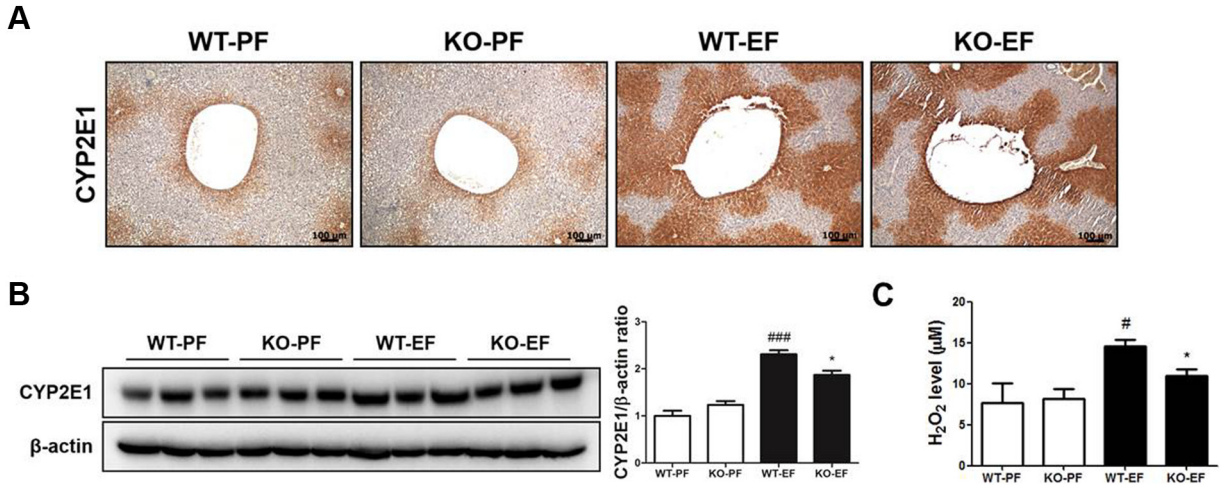

Fig. 4. Effects of CHI3L1 deficiency on ethanol-induced oxidative stress in chronic plus binge ethanol feeding mice model. (A) Immunohistochemical analysis of CYP2E1 confirmed in the pair-fed WT mice, pair-fed CHI3L1 KO mice, ethanol-fed WT mice and ethanol-fed CHI3L1 KO mice (scale bars, 100 μm). (B) The expression of CYP2E1 was determined in the total protein extracts of mice liver tissues by Western blot analysis. $n = 3$ per group; means ± SD of the mean. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ pair-fed WT mice *vs.* ethanol-fed WT mice, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ ethanol-fed WT mice *vs.* ethanol-fed CHI3L1 KO mice. (C) Hydrogen peroxide level was measured in the pair-fed WT mice, pair-fed CHI3L1 KO mice, ethanol-fed WT mice and ethanol-fed CHI3L1 KO mice. $n = 10$ per group; means ± SD of the mean. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ pair-fed WT mice *vs.* ethanol-fed WT mice, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ ethanol-fed WT mice *vs.* ethanol-fed CHI3L1 KO mice.

inhibited in the liver of ethanol-fed CHI3L1 KO mice (Supplementary Fig. 2C). In addition, the expression of ICAM-1 that is the adhesion molecule involved in leukocyte infiltration was significantly increased in the liver of ethanol-fed mice, but this expression was lower in the ethanol-fed CHI3L1 KO mice (Supplementary Fig. 2D). Thus, our result showed that decreased hepatic inflammation could contribute to down-regulation of TG synthesis in the liver of ethanol-fed CHI3L1 KO mice.

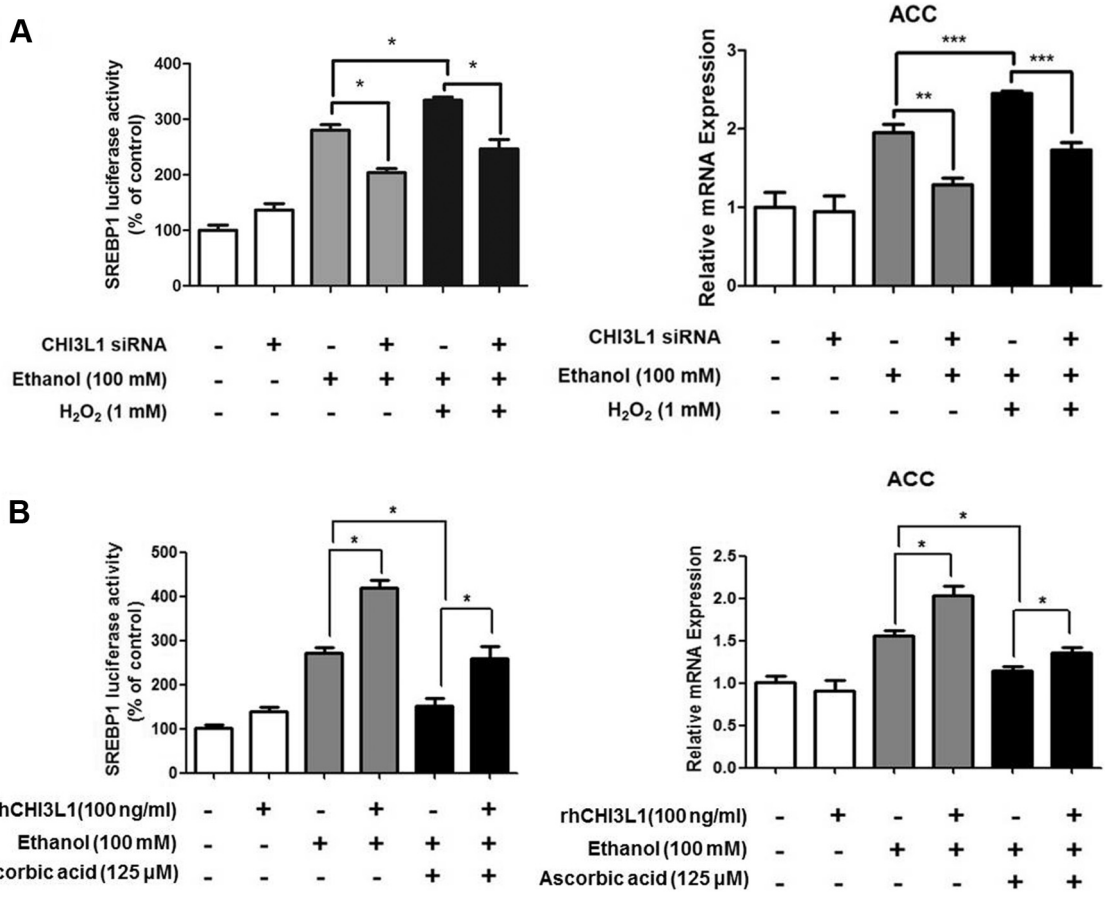

Fig. 5. Effect of H_2O_2 or antioxidant ascorbic acid on regulation of SREBP1 activity and TG synthesis in CHI3L1 siRNA transfected or rhCHI3L1 treated HepG2 cells. SREBP1 transcriptional activity measured by luciferase assay using transfection of luciferase-expressing SREBP1 plasmid DNA vector. (A) SREBP1 Luciferase activity in CHI3L1 siRNA transfected HepG2 cells after H_2O_2 or/and ethanol treatment. The mRNA level of ACC in CHI3L1 siRNA transfected HepG2 cells after H_2O_2 or/and ethanol treatment. (B) SREBP1 Luciferase activity in rhCHI3L1 treated HepG2 cells after ascorbic acid or/and ethanol treatment. The mRNA level of ACC in rhCHI3L1 treated HepG2 cells after ascorbic acid or/and ethanol treatment. $n = 3$ per group; means ± SD of the mean. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

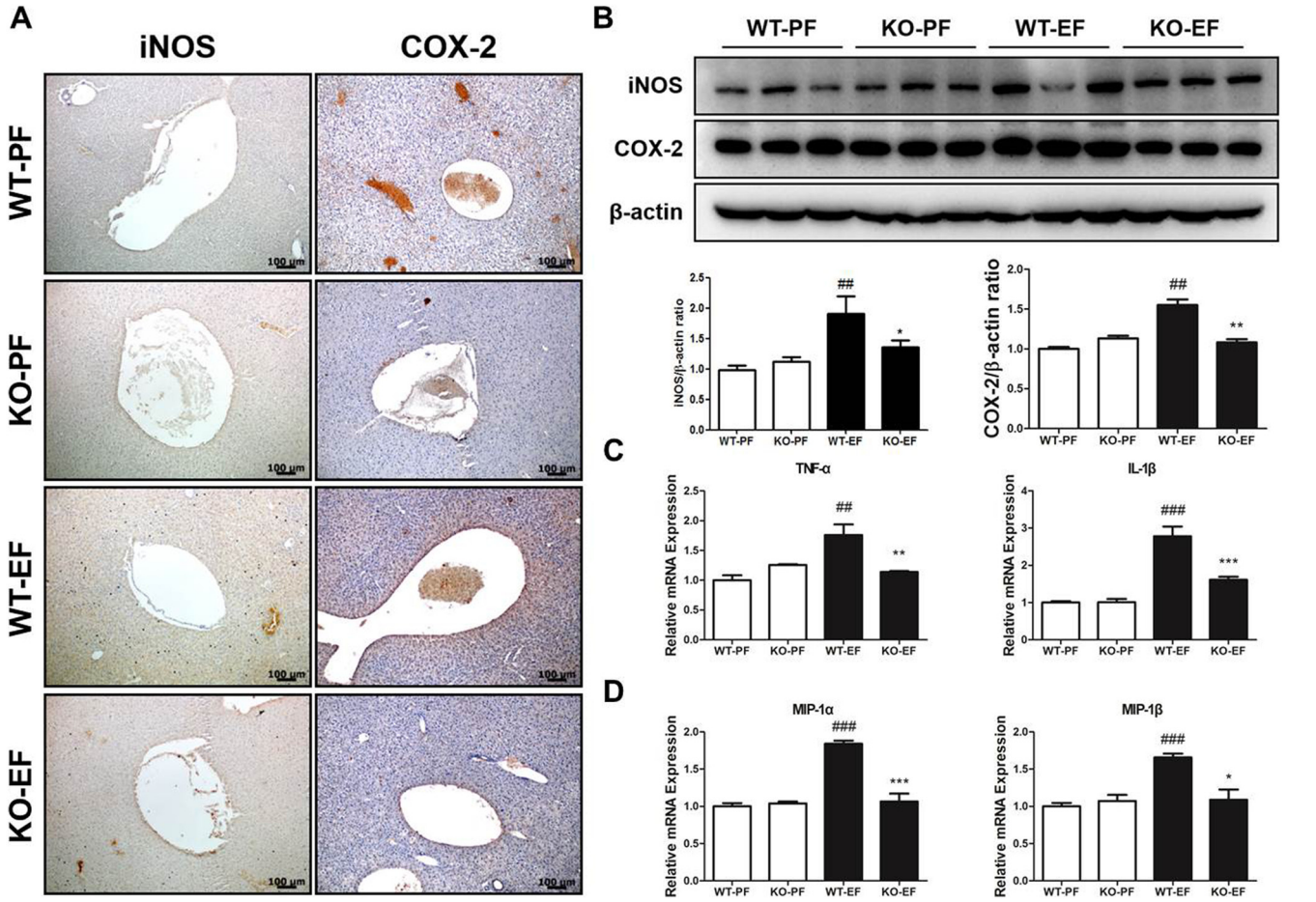

Fig. 6. Effects of CHI3L1 deficiency on inflammatory responses in chronic plus binge ethanol feeding mice model. (A) Immunohistochemical analysis of iNOS and COX-2 confirmed in the pair-fed WT mice, pair-fed CHI3L1 KO mice, ethanol-fed WT mice and ethanol-fed CHI3L1 KO mice (scale bars, 100 μm). (B) The expression of iNOS and COX-2 were determined in the total protein extracts of mice liver tissues by Western blot analysis. (C) The mRNA level of pro-inflammatory cytokines and (D) chemokines in the pair-fed WT mice, pair-fed CHI3L1 KO mice, ethanol-fed WT mice and ethanol-fed CHI3L1 KO mice. $n = 10$ per group; means ± SD of the mean. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ pair-fed WT mice *vs.* ethanol-fed WT mice, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ ethanol-fed WT mice *vs.* ethanol-fed CHI3L1 KO mice.

3.7. CHI3L1 regulates ethanol-induced SREBP1 activity and TG synthesis through inflammatory cascade in HepG2 cells

To analyze whether hepatic inflammation stimulates SREBP1 activity, we treated TNF-α (10 ng/mL) in HepG2 cells transfected with CHI3L1 siRNA and in rhCHI3L1 treated HepG2 cells. Ethanol-induced SREBP1 luciferase activity was more increased by TNF-α treatment in ethanol-treated HepG2 cells, but SREBP1 activity was decreased by TNF-α treatment in CHI3L1 siRNA transfected and ethanol-treated HepG2 cells (Fig. 7A). In addition, mRNA level of ACC was increased by TNF-α treatment in ethanol-treated HepG2 cells, but this level was inhibited by TNF-α treatment in CHI3L1 siRNA transfected HepG2 cells (Fig. 7A). In contrast, rhCHI3L1 treatment more increased SREBP1 activity and mRNA level of ACC in ethanol-treated HepG2 cells, but elevated SREBP1 activity and mRNA level of ACC by ethanol was decreased by Bay 11–7082 which is an NF-κB inhibitor in HepG2 cells treated with rhCHI3L1-treated or not (Fig. 7B). These results suggest that CHI3L1 regulated ethanol-induced inflammation which involved in SREBP1 activity and TG synthesis.

4. Discussion

TG accumulation plays an important role in the pathogenesis of chronic liver disease [45]. Increasing TG synthesis by oxidative stress and inflammatory responses through enhanced alcohol consumption is prominent feature of ALD [29]. In the current study, we have demonstrated that CHI3L1 deficiency contributes to alleviation of ethanol-induced liver injury and hepatic steatosis through inhibition of oxidative stress and hepatic inflammatory dependent TG synthesis in the chronic plus binge ethanol-feeding model, a mouse model of early stage of ALD.

Hepatic steatosis by alcohol consists of excess fat accumulation, mainly TG, within cytosolic lipid droplets [13]. Since TG level is temporarily elevated by food intake in the blood, TG level in the blood did not differ significantly between groups, but hepatic TG level was accumulated in the ethanol-fed WT mice. However, CHI3L1 deficiency reduced hepatic TG level in the ethanol-fed CHI3L1 KO mice. SREBP1 has essential function in the development of ethanol-induced fatty liver injury [10]. Lipogenesis *via* activation of SREBP1 leads to accumulate TG in rodents and human with hepatic steatosis [46]. Activation of SREBP1 by ethanol exposure elevated expression of several TG synthesis genes as well as TG accumulation in the liver [10]. TG accumulation in the SREBP1 KO mice has no difference in the WT mice [47]. In our results, hepatic mRNA level of SREBP1 and its target genes including ACC, FAS and SCD1 were increased in ethanol-fed WT mice, but it was suppressed in ethanol-fed CHI3L1 KO mice. The mRNA level of SREBP1, ACC, FAS and SCD1 were also decreased by CHI3L1 siRNA transfection in ethanol-induced HepG2 cells. In contrast, mRNA level of SREBP1, ACC, FAS and SCD1 were more increased in rhCHI3L1 treated HepG2 cells. To function as a transcription factor, activated SREBP1 is detected experimentally in nuclear fractions as a −65 kDa protein, derived

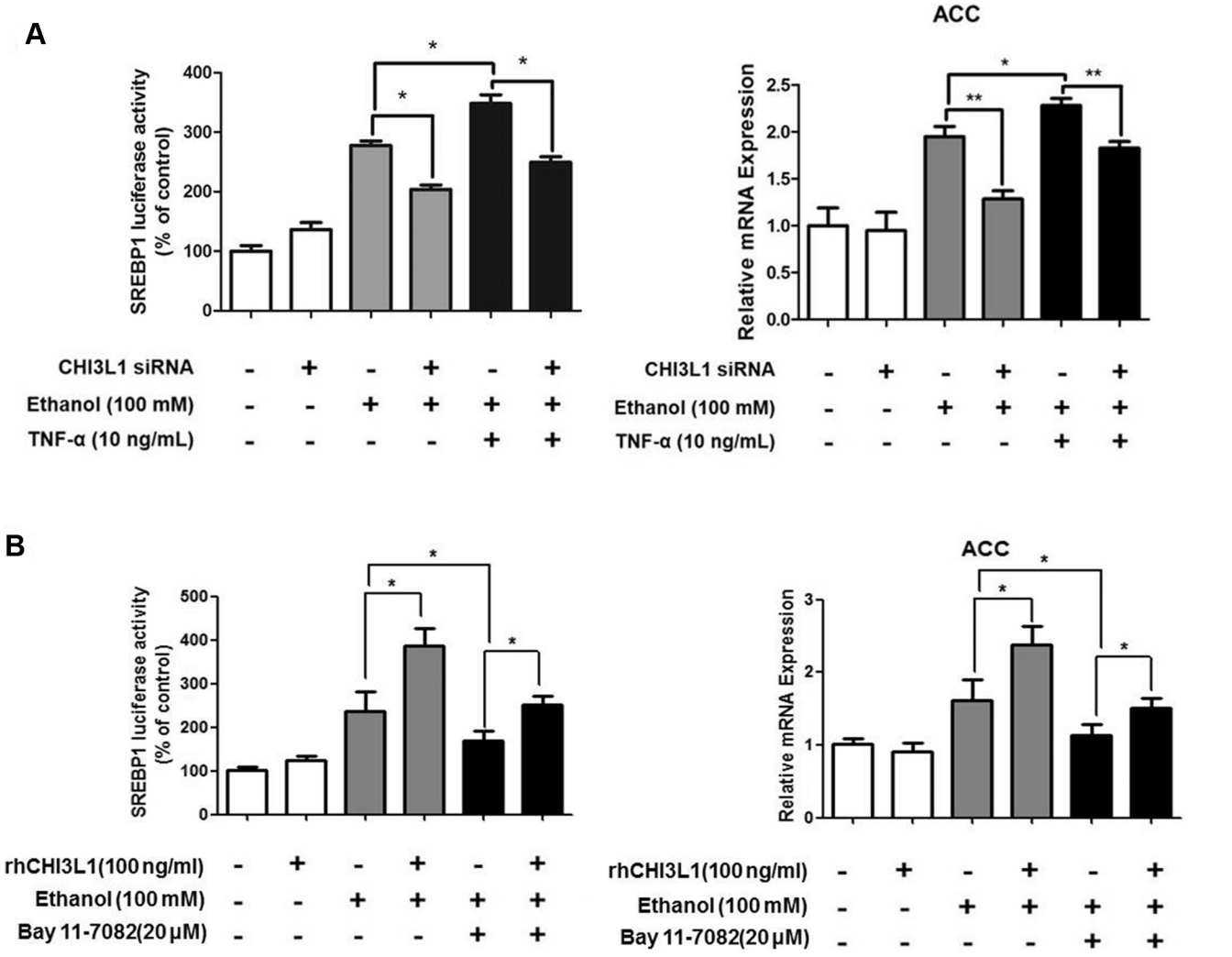

Fig. 7. Effect of TNF-α or NF-κB inhibitor Bay 11-7082 on regulation of SREBP1 activity and TG synthesis in CHI3L1 siRNA transfected or rhCHI3L1 treated HepG2 cells. (A) SREBP1 Luciferase activity in CHI3L1 siRNA transfected HepG2 cells after TNF-α or/and ethanol treatment. The mRNA level of ACC in CHI3L1 siRNA transfected HepG2 cells after TNF-α or/and ethanol treatment. (B) SREBP1 Luciferase activity in rhCHI3L1 treated HepG2 cells after NF-κB inhibitor Bay 11-7082 or/and ethanol treatment. The mRNA level of ACC in rhCHI3L1 treated HepG2 cells after NF-κB inhibitor Bay 11-7082 or/and ethanol treatment. $n = 3$ per group; means ± SD of the mean. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

originally from −125 kDa precursor [48]. As shown by Western blot analysis, the elevated expression of activated SREBP1 which translocate to nuclear was reduced in ethanol-fed CHI3L1 KO mice. In addition, SREBP1 transcriptional activity was increased by ethanol treatment in HepG2 cells, whereas it was decreased by CHI3L1 siRNA transfection in ethanol-treated HepG2 cells. However, this activity was higher in ethanol-treated rhCHI3L1 treated HepG2 cells, compared to ethanol-treated HepG2 cells. You et al. reported that ethanol feeding induces SREBP1 activation through inhibit AMPK activation [10,49]. Also, several studies reported that AMPK signaling is upstream of SREBP1 [50–52]. Thus, we investigated whether Chi3L1 involved in AMPK inactivation. We found that the phosphorylation of AMPK was decreased by ethanol feeding in the liver of WT mice but it was not decreased in the liver of KO mice. Also, decreased the phosphorylation of AMPK by ethanol was restored by CHI3L1 siRNA transfection in HepG2 cells, but it was further decreased by rhCH3L1. Our results suggest that inhibition of CHI3L1 leads to down-regulation of TG synthesis related genes in hepatocytes, and thus contributes for decrease of ALD.

The importance of oxidative stress is emphasized in the pathogenesis of various degenerative disease, such as diabetes, cancer, cardiovascular disorders and neurodegenerative disease [21,53]. It has been known for many years that ALD is associated with oxidative stress [19,54]. Chronic alcohol exposure increases the production of ROS, and lowers cellular antioxidant levels leading to oxidative stress [55]. Activated CYP2E1 induced by excessive ethanol intake causes the release of ROS, leading to oxidative stress [56]. Elevated CYP2E1 expression by ethanol was decreased in the liver of CHI3L1 KO mice. Oxidative stress depends on the balance between oxidant and anti-oxidant particles [21]. For example, a level of hydrogen peroxide is significantly elevated in glutathione peroxidase knock-out mice compared to wild-type mice [57]. Therefore, we examined several oxidant particles as well as anti-oxidant particles. Total GSH level and GSH/GSSG ratio were increased in the liver of ethanol-fed WT mice, but it was inhibited in the liver of ethanol-fed CHI3L1 KO mice. In addition, ethanol-fed CHI3L1 KO mice showed lower levels of hydrogen peroxide compared to ethanol-fed WT mice. Mouse models and patients with ALD showed lipid peroxidation levels were increased compared to normal liver [58]. Increased expression of 4-HNE and level of TBARS, a marker of lipid peroxidation, were suppressed in the liver of ethanol-fed CHI3L1 KO mice. Furthermore, H_2O_2 or/and ethanol treatment stimulated SREBP1 activity and mRNA level of ACC. CHI3L1 siRNA transfection inhibited SREBP1 activity and mRNA level of ACC. In contrast, ascorbic acid, which is an anti-oxidant, inhibits ethanol-induced SREBP1 activity and mRNA level of ACC as well as rhCHI3L1 treated group. These data suggested that

CHI3L1 deficiency might suppress SREBP1 activity and thus TG synthesis *via* decrease of oxidative stress produced by CYP2E1 expression.

Oxidative stress and hepatic inflammation are correlated in the pathogenesis of ALD. ROS sensitize hepatocytes to TNF-α toxicity [59]. Moreover, improper metabolism of ROS increases hypoxia-inducible factor-1 alpha, which up-regulates TNF-α secretion. In the present study, mRNA levels of pro-inflammatory cytokines and chemokines including TNF-α, IL-1β, MIP-1α and MIP-1β were increased in the liver of ethanol-fed WT mice whereas it was reduced in the liver of ethanol-fed CHI3L1 KO mice. Inflammatory proteins such as iNOS and COX-2 were up-regulated in the ethanol-induced mouse liver [60]. These proteins were also increased by pro-inflammatory cytokines [61]. In our results, ethanol-induced expression of iNOS and COX-2 were inhibited in the liver of CHI3L1 KO mice. These data suggested that CHI3L1 deficiency could reduce inflammatory proteins by down-regulation of pro-inflammatory cytokines and chemokines. Neutrophils infiltration is a prominent feature of ALD in patients [62]. Neutrophils, the major circulating leukocytes in human, were account for 60–70% of total white blood cells [63], and circulating neutrophils were substantially increased in alcoholics, accounting for 80% of total white blood cells [63]. Our results demonstrated that CHI3L1 plays a role in recruitment of neutrophils in the chronic plus binge ethanol-feeding mouse model. The number of neutrophils in blood was increased in the liver of ethanol-fed WT mice, but it was decreased in the liver of ethanol-fed CHI3L1 KO mice. In addition, elevated mRNA level of Ly6g, a marker of neutrophils, was also inhibited in the liver of ethanol-fed CHI3L1 KO mice. In consonance with the mRNA level of Lyg6, Lyg6 positive cells were increased in the liver of ethanol-fed WT mice, whereas its level was decreased in the liver of ethanol-fed CHI3L1 KO mice. Infiltration of neutrophils exacerbates TG accumulation *via* the generation of ROS and production of pro-inflammatory mediators such as cytokines, chemokines and adhesion molecules [64]. The histomorphological pattern of alcoholic steatosis consists of the infiltration of neutrophils and hepatocyte degeneration in patient with ALD [65]. In addition, ICAM-1 expression is typically increased by inflammatory mediators and promotes TG accumulation [66]. Steatosis, assessed by lipid droplets in histological data, were significantly less in ethanol-fed ICAM-1 KO mice than in ethanol-fed WT mice [67]. In our result, the ethanol-induced expression of ICAM-1 was decreased, and the infiltration of neutrophils was inhibited in the liver of ethanol-fed CHI3L1 KO mice. Moreover, TNF-α or/and ethanol treatment stimulated SREBP1 activity and mRNA level of ACC. CHI3L1 siRNA transfection inhibited SREBP1 activity and mRNA level of ACC. However, NF-κB inhibitor Bay 11-7082, which abrogate inflammatory cascade, prevents ethanol-induced SREBP1 activity and mRNA level of ACC as well as rhCHI3L1 treated group. Thus, our results suggest that deficiency of CHI3L1 inhibited SREBP1 activity, and thus TG synthesis through decreased of inflammatory responses.

Currently, there is no effective treatment or potential agents for the treatment of ALD. Therefore, we are required to seek novel and effective interventions for ALD. Our present study demonstrated that CHI3L1 is critical factor for the development of ALD, and suggested that CHI3L1 can be target molecules for the early stage of ALD.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2019.03.010.

Conflict of interest

The authors declare that they have no competing interest.

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Author's contributions

D.H. Lee, J.H. Han and Y.S. Lee designed experiments and performed the research, analyzed and interpreted the data, and wrote the manuscript. Y.S Roh contributed to analyze and interpreted the data. Y.S Jung, J.S Yun and S.B Han contributed to preparation and interpretation of the research. J.T Hong designed experiments, interpreted the data and edited the manuscript before submission. All authors read and approved the final manuscript.

References

[1] Ceni E, Mello T, Galli A. Pathogenesis of alcoholic liver disease: role of oxidative metabolism. World J Gastroenterol 2014;20:17756–72.
[2] Zhang D, Tong X, Nelson BB, Jin E, Sit J, Charney N, et al. The hepatic BMAL1/AKT/lipogenesis axis protects against alcoholic liver disease in mice via promoting PPARalpha pathway. Hepatology 2018;68(3):883–96.
[3] Galicia-Moreno M, Gutierrez-Reyes G. The role of oxidative stress in the development of alcoholic liver disease. Rev Gastroenterol Mex 2014;79:135–44.
[4] Kwon HJ, Won YS, Park O, Chang B, Duryee MJ, Thiele GE, et al. Aldehyde dehydrogenase 2 deficiency ameliorates alcoholic fatty liver but worsens liver inflammation and fibrosis in mice. Hepatology 2014;60:146–57.
[5] Guo R, Xu X, Babcock SA, Zhang Y, Ren J. Aldehyde dedydrogenase-2 plays a beneficial role in ameliorating chronic alcohol-induced hepatic steatosis and inflammation through regulation of autophagy. J Hepatol 2015;62:647–56.
[6] Nagy LE. Molecular aspects of alcohol metabolism: transcription factors involved in early ethanol-induced liver injury. Annu Rev Nutr 2004;24:55–78.
[7] You M, Cao Q, Liang X, Ajmo JM, Ness GC. Mammalian sirtuin 1 is involved in the protective action of dietary saturated fat against alcoholic fatty liver in mice. J Nutr 2008;138:497–501.
[8] Brandon-Warner E, Schrum LW, Schmidt CM, McKillop IH. Rodent models of alcoholic liver disease: of mice and men. Alcohol (Fayetteville, NY) 2012;46:715–25.
[9] Kawano Y, Cohen DE. Mechanisms of hepatic triglyceride accumulation in non-alcoholic fatty liver disease. J Gastroenterol 2013;48:434–41.
[10] You M, Fischer M, Deeg MA, Crabb DW. Ethanol induces fatty acid synthesis pathways by activation of sterol regulatory element-binding protein (SREBP). J Biol Chem 2002;277:29342–7.
[11] Bai T, Yang Y, Yao YL, Sun P, Lian LH, Wu YL, et al. Betulin alleviated ethanol-induced alcoholic liver injury via SIRT1/AMPK signaling pathway. Pharmacol Res 2016;105:1–12.
[12] You M, Crabb DW. Molecular mechanisms of alcoholic fatty liver: role of sterol regulatory element-binding proteins. Alcohol 2004;34:39–43.
[13] Tong X, Li P, Zhang D, VanDommelen K, Gupta N, Rui L, et al. E4BP4 is an insulin-induced stabilizer of nuclear SREBP-1c and promotes SREBP-1c-mediated lipogenesis. J Lipid Res 2016;57:1219–30.
[14] Wang Y, Viscarra J, Kim SJ, Sul HS. Transcriptional regulation of hepatic lipogenesis. Nat Rev Mol Cell Biol 2015;16:678–89.
[15] Zeng X, Li X, Xu C, Jiang F, Mo Y, Fan X, et al. Schisandra sphenanthera extract (Wuzhi Tablet) protects against chronic-binge and acute alcohol-induced liver injury by regulating the NRF2-ARE pathway in mice. Acta Pharm Sin B 2017;7:583–92.
[16] Li X, Huang W, Gu J, Du X, Lei L, Yuan X, et al. SREBP-1c overactivates ROS-mediated hepatic NF-kappaB inflammatory pathway in dairy cows with fatty liver. Cell Signal 2015;27:2099–109.
[17] Rogers CQ, Ajmo JM, You M. Adiponectin and alcoholic fatty liver disease. IUBMB Life 2008;60:790–7.
[18] Sekiya M, Hiraishi A, Touyama M, Sakamoto K. Oxidative stress induced lipid accumulation via SREBP1c activation in HepG2 cells. Biochem Biophys Res Commun 2008;375:602–7.
[19] Leung TM, Nieto N. CYP2E1 and oxidant stress in alcoholic and non-alcoholic fatty liver disease. J Hepatol 2013;58:395–8.
[20] Lu Y, Wu D, Wang X, Ward SC, Cederbaum AI. Chronic alcohol-induced liver injury and oxidant stress are decreased in cytochrome P4502E1 knockout mice and restored in humanized cytochrome P4502E1 knock-in mice. Free Radic Biol Med 2010;49:1406–16.
[21] Cichoz-Lach H, Michalak A. Oxidative stress as a crucial factor in liver diseases. World J Gastroenterol 2014;20:8082–91.
[22] Cederbaum AI, Lu Y, Wu D. Role of oxidative stress in alcohol-induced liver injury. Arch Toxicol 2009;83:519–48.
[23] Lu Y, Zhuge J, Wang X, Bai J, Cederbaum AI. Cytochrome P450 2E1 contributes to ethanol-induced fatty liver in mice. Hepatology 2008;47:1483–94.
[24] Wheeler MD, Kono H, Yin M, Rusyn I, Froh M, Connor HD, et al. Delivery of the Cu/Zn-superoxide dismutase gene with adenovirus reduces early alcohol-induced liver injury in rats. Gastroenterology 2001;120:1241–50.
[25] Kessova IG, Ho YS, Thung S, Cederbaum AI. Alcohol-induced liver injury in mice lacking Cu, Zn-superoxide dismutase. Hepatology 2003;38:1136–45.
[26] Wheeler MD, Nakagami M, Bradford BU, Uesugi T, Mason RP, Connor HD, et al. Overexpression of manganese superoxide dismutase prevents alcohol-induced liver injury in the rat. J Biol Chem 2001;276:36664–72.
[27] Han YH, Kim HJ, Kim EJ, Kim KS, Hong S, Park HG, et al. RORalpha decreases oxidative stress through the induction of SOD2 and GPx1 expression and thereby protects against nonalcoholic steatohepatitis in mice. Antioxid Redox Signal 2014;21:2083–94.

[28] Ambade A, Mandrekar P. Oxidative stress and inflammation: essential partners in alcoholic liver disease. Int J Hepatol 2012;2012:853175.
[29] Mandrekar P, Ambade A, Lim A, Szabo G, Catalano D. An essential role for monocyte chemoattractant protein-1 in alcoholic liver injury: regulation of proinflammatory cytokines and hepatic steatosis in mice. Hepatology 2011;54:2185–97.
[30] Pastorino JG, Hoek JB. Ethanol potentiates tumor necrosis factor-alpha cytotoxicity in hepatoma cells and primary rat hepatocytes by promoting induction of the mitochondrial permeability transition. Hepatology 2000;31:1141–52.
[31] Ju C, Mandrekar P. Macrophages and alcohol-related liver inflammation. Alcohol Res 2015;37:251–62.
[32] Endo M, Masaki T, Seike M, Yoshimatsu H. TNF-alpha induces hepatic steatosis in mice by enhancing gene expression of sterol regulatory element binding protein-1c (SREBP-1c). Exp Biol Med 2007;232:614–21.
[33] Roslind A, Johansen JS. YKL-40: a novel marker shared by chronic inflammation and oncogenic transformation. Methods Mol Biol 2009;511:159–84.
[34] Johansen JS, Christoffersen P, Moller S, Price PA, Henriksen JH, Garbarsch C, et al. Serum YKL-40 is increased in patients with hepatic fibrosis. J Hepatol 2000;32:911–20.
[35] Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Proteomics. Tissue-based map of the human proteome. Science 2015;347:1260419.
[36] Tao H, Yang JJ, Shi KH, Huang C, Zhang L, Lv XW, et al. The significance of YKL-40 protein in liver fibrosis. Inflamm Res 2014;63:249–54.
[37] Kumagai E, Mano Y, Yoshio S, Shoji H, Sugiyama M, Korenaga M, et al. Serum YKL-40 as a marker of liver fibrosis in patients with non-alcoholic fatty liver disease. Sci Rep 2016;6:35282.
[38] Nøjgaard C, Johansen JS, Christensen E, Skovgaard LT, Price PA, Becker U. Serum levels of YKL-40 and PIIINP as prognostic markers in patients with alcoholic liver disease. J Hepatol 2003;39:179–86.
[39] Kim KC, Yun J, Son DJ, Kim JY, Jung JK, Choi JS, et al. Suppression of metastasis through inhibition of chitinase 3-like 1 expression by miR-125a-3p-mediated up-regulation of USF1. Theranostics 2018;8:4409–28.
[40] Bertola A, Mathews S, Ki SH, Wang H, Gao B. Mouse model of chronic and binge ethanol feeding (the NIAAA model). Nat Protoc 2013;8:627–37.
[41] Arteel GE. Oxidants and antioxidants in alcohol-induced liver disease. Gastroenterology 2003;124:778–90.
[42] Sugimoto K, Takei Y. Pathogenesis of alcoholic liver disease. Hepatol Res 2017;47:70–9.
[43] Zhou Z, Wang L, Song Z, Lambert JC, McClain CJ, Kang YJ. A critical involvement of oxidative stress in acute alcohol-induced hepatic TNF-alpha production. Am J Pathol 2003;163:1137–46.
[44] Bertola A, Park O, Gao B. Chronic plus binge ethanol feeding synergistically induces neutrophil infiltration and liver injury in mice: a critical role for E-selectin. Hepatology 2013;58:1814–23.
[45] Osna NA, Donohue Jr TM, Kharbanda KK. Alcoholic liver disease: pathogenesis and current management. Alcohol Res 2017;38:147–61.
[46] Ferre P, Foufelle F. Hepatic steatosis: a role for de novo lipogenesis and the transcription factor SREBP-1c. Diabetes Obes Metab 2010;12(Suppl. 2):83–92.
[47] Ji C, Chan C, Kaplowitz N. Predominant role of sterol response element binding proteins (SREBP) lipogenic pathways in hepatic steatosis in the murine intragastric ethanol feeding model. J Hepatol 2006;45:717–24.
[48] Donohue Jr TM. Alcohol-induced steatosis in liver cells. World J Gastroenterol 2007;13:4974–8.
[49] You M, Matsumoto M, Pacold CM, Cho WK, Crabb DW. The role of AMP-activated protein kinase in the action of ethanol in the liver. Gastroenterology 2004;127:1798–808.
[50] Emerling BM, Weinberg F, Snyder C, Burgess Z, Mutlu GM, Viollet B, et al. Hypoxic activation of AMPK is dependent on mitochondrial ROS but independent of an increase in AMP/ATP ratio. Free Radic Biol Med 2009;46:1386–91.
[51] Mungai PT, Waypa GB, Jairaman A, Prakriya M, Dokic D, Ball MK, et al. Hypoxia triggers AMPK activation through reactive oxygen species-mediated activation of calcium release-activated calcium channels. Mol Cell Biol 2011;31:3531–45.
[52] Fernandez-Alvarez A, Alvarez MS, Gonzalez R, Cucarella C, Muntane J, Casado M. Human SREBP1c expression in liver is directly regulated by peroxisome proliferator-activated receptor alpha (PPARalpha). J Biol Chem 2011;286:21466–77.
[53] Apostolova N, Blas-Garcia A, Esplugues JV. Mitochondria sentencing about cellular life and death: a matter of oxidative stress. Curr Pharm Des 2011;17:4047–60.
[54] Tilg H, Moschen AR, Kaneider NC. Pathways of liver injury in alcoholic liver disease. J Hepatol 2011;55:1159–61.
[55] Ding L, Wo L, Du Z, Tang L, Song Z, Dou X. Danshen protects against early-stage alcoholic liver disease in mice via inducing PPARalpha activation and subsequent 4-HNE degradation. PLoS One 2017;12:e0186357.
[56] Wu D, Cederbaum AI. Oxidative stress and alcoholic liver disease. Semin Liver Dis 2009;29:141–54.
[57] Harrison-Findik DD, Lu S. The effect of alcohol and hydrogen peroxide on liver hepcidin gene expression in mice lacking antioxidant enzymes, glutathione peroxidase-1 or catalase. Biomolecules 2015;5:793–807.
[58] Kasdallah-Grissa A, Mornagui B, Aouani E, Hammami M, Gharbi N, Kamoun A, et al. Protective effect of resveratrol on ethanol-induced lipid peroxidation in rats. Alcohol Alcohol 2006;41:236–9.
[59] Ji C, Deng Q, Kaplowitz N. Role of TNF-alpha in ethanol-induced hyperhomocysteinemia and murine alcoholic liver injury. Hepatology 2004;40:442–51.
[60] Spitzer JA, Zheng M, Kolls JK, Vande Stouwe C, Spitzer JJ. Ethanol and LPS modulate NF-kappaB activation, inducible NO synthase and COX-2 gene expression in rat liver cells in vivo. Front Biosci 2002;7:a99–108.
[61] McKim SE, Gabele E, Isayama F, Lambert JC, Tucker LM, Wheeler MD, et al. Inducible nitric oxide synthase is required in alcohol-induced liver injury: studies with knockout mice. Gastroenterology 2003;125:1834–44.
[62] Jaeschke H. Neutrophil-mediated tissue injury in alcoholic hepatitis. Alcohol 2002;27:23–7.
[63] Li M, He Y, Zhou Z, Ramirez T, Gao Y, Gao Y, et al. MicroRNA-223 ameliorates alcoholic liver injury by inhibiting the IL-6-p47(phox)-oxidative stress pathway in neutrophils. Gut 2017;66:705–15.
[64] Ramaiah SK, Jaeschke H. Hepatic neutrophil infiltration in the pathogenesis of alcohol-induced liver injury. Toxicol Mech Methods 2007;17:431–40.
[65] Ramaiah SK, Jaeschke H. Role of neutrophils in the pathogenesis of acute inflammatory liver injury. Toxicol Pathol 2007;35:757–66.
[66] Vergani L, Vecchione G, Baldini F, Grasselli E, Voci A, Portincasa P, et al. Polyphenolic extract attenuates fatty acid-induced steatosis and oxidative stress in hepatic and endothelial cells. Eur J Nutr 2018;57:1793–805.
[67] Kono H, Uesugi T, Froh M, Rusyn I, Bradford BU, Thurman RG. ICAM-1 is involved in the mechanism of alcohol-induced liver injury: studies with knockout mice. Am J Physiol Gastrointest Liver Physiol 2001;280:G1289–95.