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Inhibition of cytochrome P4502E1 by chlormethiazole attenuated acute ethanol-induced fatty liver

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ABSTRACT

Cytochrome P4502E1 (CYP2E1) has been demonstrated to play crucial roles in chronic ethanol-induced fatty liver, while its role in acute ethanol-induced fatty liver remains unclear. The current study was designed to evaluate the effects of chlormethiazole (CMZ), a specific inhibitor of CYP2E1, on acute ethanol-induced fatty liver, and to explore the mechanisms. Mice were pretreated with single dose of CMZ (50 mg/kg body weight) by intraperitoneal injection or equal volume of saline, and then exposed to three doses of ethanol (5 g/kg body weight, 25%, w/v) by gavage with 12 h intervals. The mice were sacrificed at 4 h after the last ethanol dosing. It was found that CMZ significantly attenuated acute ethanol-induced increase of the hepatic and serum triglyceride levels, and reduced fat droplets accumulation in mice liver. Acute ethanol-induced increase of the hepatic malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) levels (two biomarkers for oxidative stress) and decrease of glutathione (GSH) level was significantly suppressed by CMZ. CMZ also suppressed ethanol-induced decline of serum adiponectin level, but did not significantly affect the serum tumor necrosis factor-α (TNF-α) and ethanol levels. Furthermore, a significant decline of p62 protein level was observed in CMZ/ethanol group mice liver compared with that of the ethanol group mice. However, acute ethanol-induced increase of peroxisome proliferator-activated receptor α (PPAR-α) protein level was suppressed by CMZ, while the protein levels of sterol regulatory element-binding protein-1c (SREBP-1) and diacylglycerol acyltransferase 2 (DGAT2) were not significantly affected by ethanol or CMZ. Collectively, the results of the current study demonstrated that CMZ could effectively attenuate acute ethanol-induced fatty liver possibly by suppressing oxidative stress and adiponectin decline, and activating autophagy, which suggest that CYP2E1 might also play important roles in acute ethanol-induced fatty liver.

1. Introduction

Alcoholic liver disease (ALD) represents a cluster of progressive liver diseases from steatosis to hepatitis, fibrosis, and finally cirrhosis. ALD is the second most common causes of liver cirrhosis after hepatitis C virus infection in the USA, and has been becoming an important public health issue in China due to the increasing consumption of alcoholic beverages [1–2]. Alcoholic fatty liver (AFL) is the earliest and the most common histopathological form of ALD. Although being considered benign in the past, recent studies have suggested that fat accumulation will make hepatocytes more sensitive to liver toxins such as tumor necrosis factor α (TNF-α), which plays important roles in the development of hepatitis [3].

The molecular mechanisms of AFL have been widely studied in the past few decades, and several possible factors have been revealed. Among these factors, cytochrome P4502E1 (CYP2E1) has gained great interest as it can be activated by ethanol and can lead to reactive oxygen species (ROS) production [4]. In this context, CYP2E1 inhibitors could theoretically suppress ALD. In fact, previous studies have demonstrated that CYP2E1 inhibitors such as diallyl sulfide and phenethyl isothiocyanate could attenuate chronic ethanol-induced liver injury including fatty liver [5–6]. One recently published study clearly demonstrated that CYP2E1 contributed to chronic ethanol-induced fatty liver by using CYP2E1 (−/−) mice [7]. However, it should be noticed that all these studies

Abbreviations: ACC, acyl-CoA carboxylase; ACOX, acyl-CoA oxidase; AFL, alcoholic fatty liver; ALD, alcoholic liver disease; ALT, alanine transaminase; AMPK, AMP-activated protein kinase; AST, aspartate transaminase; CMZ, chlormethiazole; CYP2E1, cytochrome P4502E1; DGAT2, diacylglycerol acyltransferase 2; FAS, fatty acid synthase; GSH, glutathione; LFABP, liver fatty acid-binding protein; MDA, malondialdehyde; PPAR-α, peroxisome proliferator-activated receptor α; ROS, reactive oxygen species; SREBP-1c, sterol regulatory element-binding protein-1c; TG, triglyceride; TNF-α, tumor necrosis factor α.

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about the roles of CYP2E1 in AFL was performed in chronic ethanol-induced animal model, and it is still unclear whether CYP2E1 also plays important roles in acute ethanol-induced fatty liver, as chronic and acute AFL might have different molecular mechanisms.

Binge drinking is on the rise at an alarming rate worldwide. A binge drinking is defined as consumption of 5 drinks for men and 4 drinks for women, respectively, in 2 h to produce a blood ethanol level over 80 mg/dl [8]. The deleterious effects of chronic alcohol consumption on liver are well known and widely described, while less attention has been paid to binge drinking-induced hepatotoxicity, although it is much more common than chronic alcoholism [9]. As the deleterious effect of binge drinking on the liver is an alarming public health issue that requires better prevention, further studies focusing on binge drinking-induced liver injury seem to be warranted [10]. Binge drinking can lead to liver injury including fatty liver [11]. It is unclear whether or not CYP2E1 plays important roles in binge drinking-induced fatty liver.

A binge drinking mice model was developed by Carson and Pruett, which was reported to achieve blood ethanol levels, behavioral effects, and physiological changes comparable with human binge drinking [12]. This model has been partially modified and has been wildly used in the literature [13–15]. In order to elucidate the roles of CYP2E1 in the pathogenesis of acute ethanol-induced fatty liver, this animal model was utilized for the evaluation of the protective effects of chlormethiazole (CMZ, an efficient non-competitive CYP2E1 inhibitor) against acute ethanol-induced fatty liver. The hepatic and serum triglyceride (TG) levels and the histopathological changes were used to evaluate the fat accumulation in the liver. Several important factors involved in fatty acid metabolism were investigated for the mechanisms exploration.

2. Materials and methods

2.1. Materials

CMZ and primary antibodies against LC3, p62, and β-actin were bought from Sigma (St. Louis, MO, USA). Primary antibodies against peroxisome proliferator- activated receptor α (PPAR-α), sterol regulatory element-binding protein-1c (SREBP-1c), and fatty acid synthase (FAS) were purchased from Santa Cruz (CA, USA). Primary antibodies against AMP-activated protein kinase α (AMPK-α), phospho-AMPK-α, acyl-CoA carboxylase (ACC), and phospho-ACC were provided by Cell Signaling Technology Inc. (Beverly, MA, USA). Rabbit acyl-CoA oxidase (ACOX) antibody and liver fatty acid-binding protein (LFABP) antibody were bought from Abcam (Cambridge, UK). Anti-4-hydroxynonenal (4-HNE) (clone HNEJ-2) monoclonal antibodies were purchased from JaICA (NOF Co., Tokyo, Japan). Rabbit polyclonal diacylglycerol acyltransferase 2 (DGAT2) antibody was bought from Novus Biologicals (Littleton, CO, USA). Sudan III and oil red O dyes were purchased from Tianjin Kermel Chemical Reagent Co., Ltd (Tianjin, China). All other reagents were purchased from Sigma, unless indicated otherwise.

2.2. Animals and ethanol treatment

Male Kun-Ming mice (18–22 g) were provided by the Laboratory Animal Center of Shandong University (Jinan, China). The mice received standard chow and tap water, and were maintained in a temperature-controlled environment (20–22 °C) with a 12-h light: 12-h dark cycle and 50%-55% humidity. After 3 days of acclimation, the mice were randomly divided into 3 groups (n = 10), i.e. control group, ethanol group, and CMZ/ethanol group. The mice in CMZ/ethanol and ethanol groups were treated with single dose of CMZ (50 mg/kg body weight) by intraperitoneal injection and equal volume of sterile saline, respectively, and then exposed to ethanol (5 g/kg body weight, 25%, w/v) by gavage in 12 h interval for a total of 3 doses [15–16]. Mice in control group received equal volume of saline and isocaloric/isovolumetric maltose-dextrin solution. The animals were sacrificed at 4 h after the last dosing of ethanol. The blood samples were collected for measurement of the serum aminotransferase activities and TG level. The livers were dissected quickly, snap-frozen in liquid nitrogen, and stored at −80 °C until analysis. The use of animals was in compliance with the guidelines established by the Animal Care Committee of Shandong University, and the experiment was approved by the Ethics Committee of Shandong University Institute of Preventive Medicine (Permit Number:20120701).

2.3. Biochemical assay

The levels of serum alanine transaminase (ALT, Catalog number: C009-2), aspartate transaminase (AST, catalogue umber: C0010-2) and TG (Catalog number: F001-2), and the levels of hepatic malondialdehyde (MDA) and glutathione (GSH) were measured using commercial assay kits provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The ALT and AST activates were determined by using alanine and aspartate as substrates, respectively, as we previously reported [17]. The MDA level was measured at 535 nm based on the reaction of thiobarbituric acid (TBA) with MDA, while the GSH levels were measured at 412 nm following the reaction of GSH with 5, 5-dithiobes- (2-ni-trobenzoic acid) (DTNB)[18].

The hepatic TG content was detected using a commercial kit (Catalog number: E1003-2) bought from Applygen Technologies Inc. (Beijing, China). The detection was based on the optimized GPO Trinder reaction. Serum ethanol concentration was determined using the Enzychrom™ ethanol assay kit (Catalog number: ECET-100) provided by Bioassay systems (Hayward, CA, USA). This assay kit is based on alcohol dehydrogenase catalyzed oxidation of ethanol, in which the formed NADH is coupled to the formazan (MTT) chromogen [19].

The serum adiponectin and TNF-α levels were measured using ELISA kits provided by Millipore (Catalog number: MRP300) and R&D Systems (Catalog number: mta00B), respectively. All the procedure was completed strictly according to the manufacturer's instructions.

2.4. Histopathological examination

Liver histopathological examination was performed as we previously reported [17,20]. Briefly, pieces of liver from the same lobes of mice were fixed in 4% formaldehyde for 24 h. Paraffin sections (5 μm) were prepared using a rotary microtome, and subjected to hematoxylin and eosin (H&E) staining [17]. For the Sudan III and oil red O staining, frozen sections (8 μm) were prepared, fixed in 4% formaldehyde for 5 min, stained in Sudan III dye or oil red O dye, and then counterstained with hematoxylin for 30 s [20]. The sections were viewed and the representative photographs were captured using a Nikon microscope (Nikon, Melville, NY, USA).

2.5. Immunohistochemical staining

Liver paraffin sections (5 μm) were deparaffinized and blocked using 3% H2O2, and 5% normal goat serum. Sections were incubated with 4-HNE antibody (1:200) at 4 °C overnight, and then incubated with poly peroxidase-anti-mouse/rabbit IgG (PV-9000, ZSGB-BIO, China) for 30 min at room temperature. The final staining was developed using 3′,5′-diaminobenzidine (DAB) detection kits. The sections were washed in tap water and counterstained with hematoxylin, and the images were viewed and captured using the light microscope at 200× magnitude [21].

Table 1
Gene-specific primers used in quantitative real-time PCR.

Gene	GeneBank ACC. No.	Forward primer (5′-3′)	Reverse primer (5′-3′)
PPAR-α	NM_001113418	TGGCAAAAGGCAAGGAGAAG	CCCTCTACATAGAACTGCAAGGTTT
ACOX-1	NM_015729	GGGAGTGCTACGGGTTACATG	CCGATATCCCCAACAGTGATG
LFABP-1	NM_017399	CAGGAGAACTTTGAGCCATTCA	GATTTCTGACACCCCCTTGATG
SREBP-1	NM_011480	GATGTGCGAACTGGACACAG	CATAGGGGGCGTCAAACAG
FAS	NM_008904	GGAGGTGGTGATAGCCGGTAT	TGGGTAATCCATAGAGCCCAG
GAPDH	NM_001001304	GCATGGCCTTCCGTGTTCC	GGGTGGTCCAGGGTTTCTTACTC

2.6. Transmission electron microscopy (TEM)

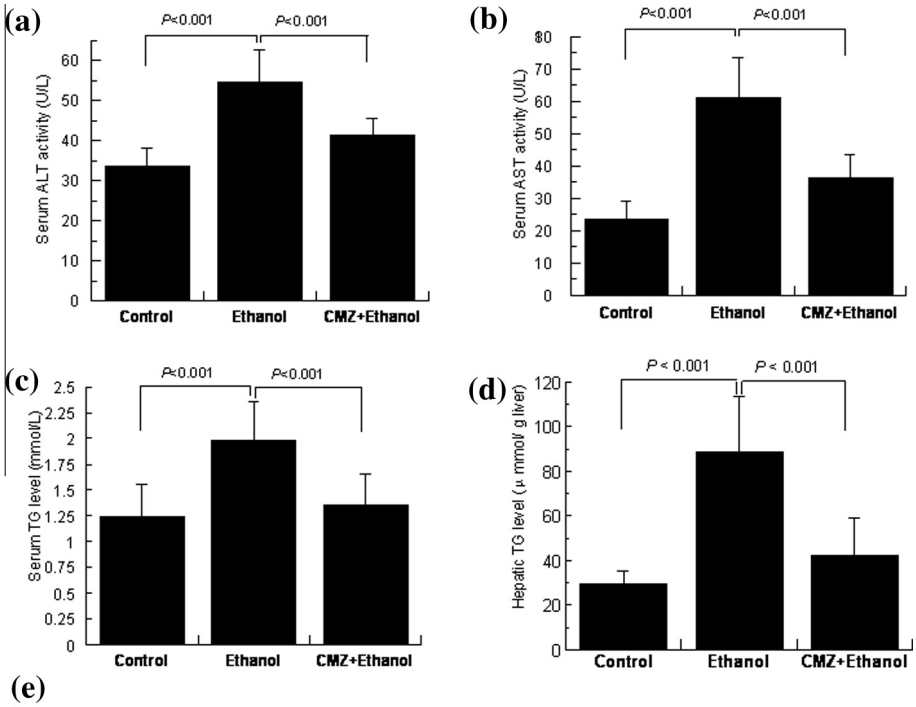
Fresh liver pieces (about 1 mm^3 cubes) were fixed in 2% glutaraldehyde overnight at 4 °C. The samples were post-fixed in 1% osmium tetroxide (OsO4) solution for 2 h, and then dehydrated through graded ethanol series. The samples were embedded in epoxy resin and were cut into 60–70 nm thick sections using LKB ultramicrotome. The sections were double-stained with uranyl acetate and lead citrate, and observed using a transmission electron microscope (JEM-2000ex, JEDL Co., Japan).

2.7. Real-time PCR analysis

Total RNA was isolated from mice liver using Trizol reagent (Invitrogen, USA). 2 μg of total RNA was reverse transcribed into complementary DNA (cDNA) at 42 °C for 1 h using Oligo dT-Adaptor primer and RevertAid™ M-MuLV Transcriptase according to the manufacturer's protocol (Fermentas, UK). Quantitative real-time PCR (qPCR) was performed using Roche lightCycler480 SYBR Green I Master (Roche, Germany) to quantify the mRNA levels of PPAR-α, ACOX and LFABP, SREBP-1, FAS, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers were synthesized by Sangon Biotech Co., Ltd (Shanghai, China), and sequences of the primers were listed in Table 1. The PCR amplification reactions were performed using Roche LightCycler 480 Instrument (Roche, Germany).

2.8. Western blot analysis

Total protein samples were prepared using RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 1 mM phenylmethylsulfonyl fluoride



(e)

Group	Initial body weight(g)	Final body weight(g)	Liver weight(g)	Liver index (%)
Control	24.25 ± 2.04	21.09 ± 1.68	0.99 ± 0.14	4.68 ± 0.50
Ethanol	24.60 ± 1.73	20.16 ± 1.80	1.06 ± 0.16	5.25 ± 0.60*
CMZ+Ethanol	24.38 ± 1.37	19.78 ± 1.27	0.99 ± 0.14	5.01 ± 0.47

*$p<0.05$, compared with the control group mice.

Fig. 1. Effects of CMZ and acute ethanol challenge on the serum ALT and AST activities, the serum and hepatic TG levels, and the liver index. (a) Serum ALT activity; (b) serum AST activity; (c) serum TG level; (d) Hepatic TG level; (e) the body weight, liver weight, and liver index (liver weight/body weight × 100%). Data were presented as mean ± SD ($n = 10$).

(PMSF), 1 mM Na3VO4, 5 mM NaF, and 1% protease inhibitors cocktail (P8340, Sigma), pH 8.0) [17]. Protein samples were separated by electrophoresis in 6%-15% denatured polyacrylamide gels, and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% nonfat milk solution for 1 h at room temperature, and then incubated with specific primary antibodies overnight at 4 °C. After washes in TBST for 3 times, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibodies for 1.5 h at room temperature. The protein bands were visualized using an enhanced chemiluminescence western blotting detection reagent. The immunoreactive bands of proteins were scanned by using Agfa Duoscan T1200 scanner, and the digitized data were quantified as integrated optical density (IOD) using Kodak Imaging Program. To ensure equal loading, the results were normalized by β-actin.

2.9. Statistical analyses

All data were expressed as mean and standard deviation (SD). SPSS13.0 statistical software was used for statistical analysis. The data were analyzed using one-way analysis of variance (ANOVA) followed by LSD's post hoc tests. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. CMZ effectively attenuated acute ethanol-induced fatty liver

Acute ethanol challenge induced significant increase of the serum ALT and AST activities, and also led to a significant increase of the serum and hepatic TG levels, which were all significantly suppressed by CMZ pretreatment (Fig. 1). In consistent with previous studies [13,22], acute ethanol challenge led to massive microvesicular steatosis in the mice liver, which was obviously alleviative in the liver of CMZ/ethanol group mice (Fig. 2a). Massive yellow- and red-stained lipid droplets in the liver sections of ethanol group mice were observed in the Sudan III staining and oil red O staining, respectively. However, no obvious fat droplets were presented in the liver sections of CMZ/ethanol group mice (Fig. 2b and c). The results of the TEM assay were parallel well with the above results. Furthermore, the fragmented, malformed and dissolved mitochondria induced by acute ethanol challenge were greatly blocked by CMZ pretreatment (Fig. 2d).

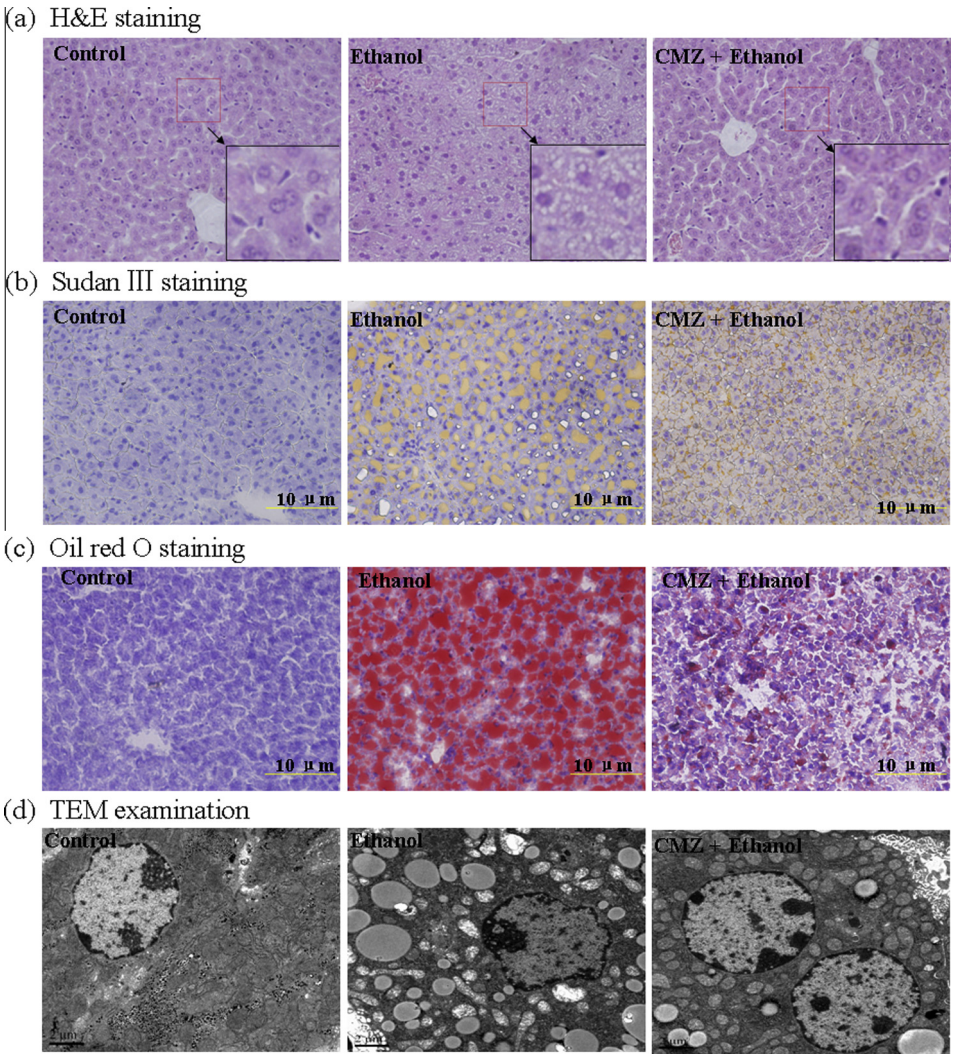


Fig. 2. CMZ effectively blocked acute ethanol-induced fat accumulation in mice liver. (a) H&E staining; (b) Sudan III staining; (c) Oil red O staining; (d) Ultrastructural examination. The mice were pretreated with a single dose of CMZ (50 mg/kg body weight) or saline, and exposed to three doses of ethanol (5 g/kg body weight) with 12 h intervals, and then sacrificed at 4 h later. The paraffin and frozen sections of liver were prepared, and subjected to H&E staining, Sudan III staining, and oil red O staining, respectively. The conventional ultrathin sections were prepared for TEM examination. The fat droplets were shown as small intracytoplasmic fat vacuoles, yellow-stained droplets, red-stained droplets, or round bulbs with homogenous content, in the H&E staining, Sudan III staining, oil red O staining, and TEM examination, respectively.

3.2. CMZ pretreatment suppressed acute ethanol-induced CYP2E1 activation and the oxidative stress

It has been well documented that oxidative stress is an important contributor to AFL; we then investigated whether CMZ pretreatment could attenuate acute ethanol-induced oxidative stress via CYP2E1 suppression. As shown in Fig. 3, the hepatic MDA and 4-HEN levels, two biomarkers for oxidative stress, were all significantly increased in ethanol group mice liver, while the GSH level was significantly decreased. CMZ significantly suppressed acute ethanol-induced activation of CYP2E1, and completely abrogated acute ethanol-induced oxidative stress, shown as the restoration of the hepatic MDA, 4-HEN, and GSH levels (Fig. 3).

3.3. Effects of CMZ and acute ethanol challenge on the PPAR-α pathway and the phosphorylation of AMPK and ACC

PPAR-α has been demonstrated to play important roles in chronic ethanol-induced fatty liver [23–24]. The mRNA and protein levels of PPAR-α and two important targets of PPAR-α including ACOX and LFABP, were detected by qPCR and western blot, respectively. As shown in Fig. 4a, compared with those of the control group mice, the mRNA levels of PPAR-α, ACOX, and LFABP were decreased by 16.35%, 29.39%, and 25.97% ($p < 0.05$), respectively. CMZ pretreatment restored the mRNA levels of PPAR-α and ACOX. Unexpectedly, the protein levels of PPAR-α, ACOX, and LFABP in the liver of ethanol group mice were all significantly increased compared with those of the control group mice, which was suppressed by CMZ pretreatment (Fig. 4b).

AMPK is a major cellular regulator of lipid and glucose metabolism by inhibiting regulatory enzymes involved in biosynthetic pathways such as ACC [25], and thus we investigated whether the protective effects of CMZ was related with AMPK activation. The results of western blot revealed no significant changes of the total or p-AMPK levels in the liver of ethanol group mice compared with those of the control group mice, while the CMZ/ethanol group mice showed slightly decreased p-AMPK protein level when compared with ethanol group mice. The protein levels of p-ACC in CMZ/ethanol group mice was also slightly decreased compared with that of the ethanol group mice (Fig. 4c).

3.4. CMZ had no effects on the protein levels of SREBP-1c and DGAT2, but led to a slight decrease of the protein levels of FAS

To investigate whether the protective effects of CMZ was related with the SREBP-1c mediated lipogenesis pathway, the mRNA and protein levels of nSREBP-1c and fatty acid synthase (FAS, a target enzyme regulated by SREBP-1c) were detected. As shown in Fig. 5a, no significant change of the protein level as well as the mRNA level of SREBP-1c was observed. However, a slight but significant decrease of FAS protein level in CMZ/ethanol group mice was observed when compared with that of the ethanol group mice. The protein levels of DGAT2, the rate-limiting enzyme in TG

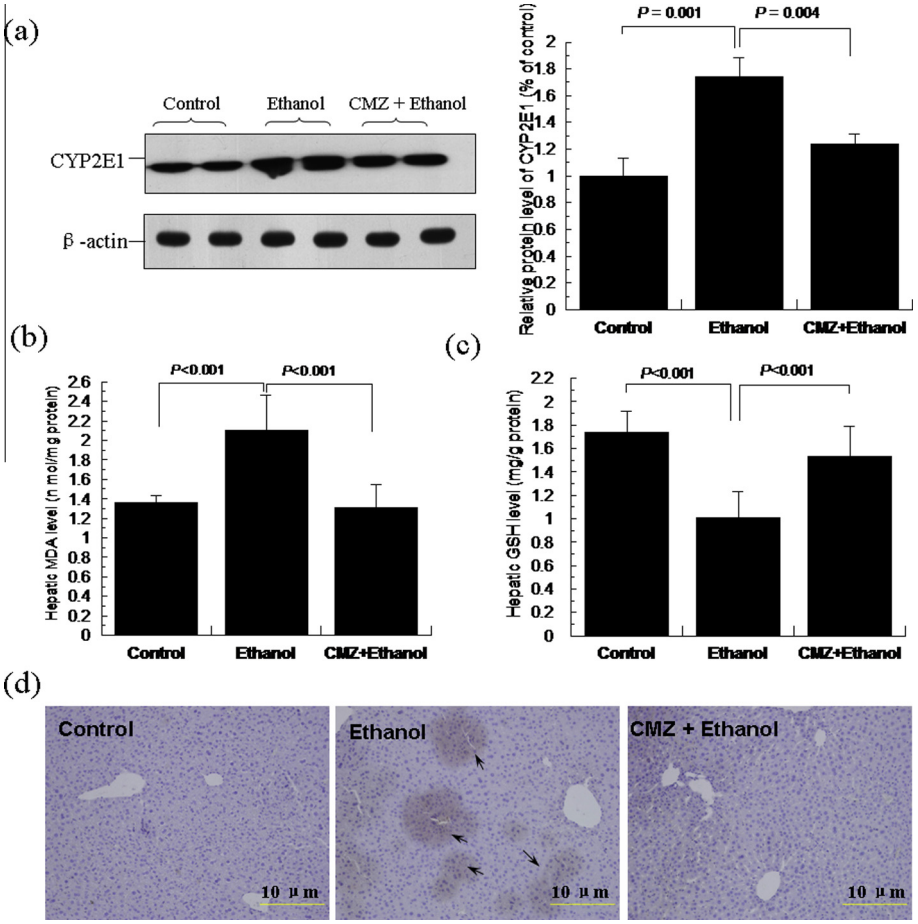


Fig. 3. CMZ pretreatment suppressed acute ethanol-induced CYP2E1 activation and suppressed oxidative stress in mice liver. (a) Protein levels of CYP2E1 were determined by western blot, and the data was shown as the percentage of the control value form 3 independent experiments; (b) The levels of hepatic MDA ($n = 10$); (c) The levels of hepatic GSH ($n = 10$); (d) Immunohistochemical staining of 4-HNE (arrows point to areas of positive staining of 4-HNE).

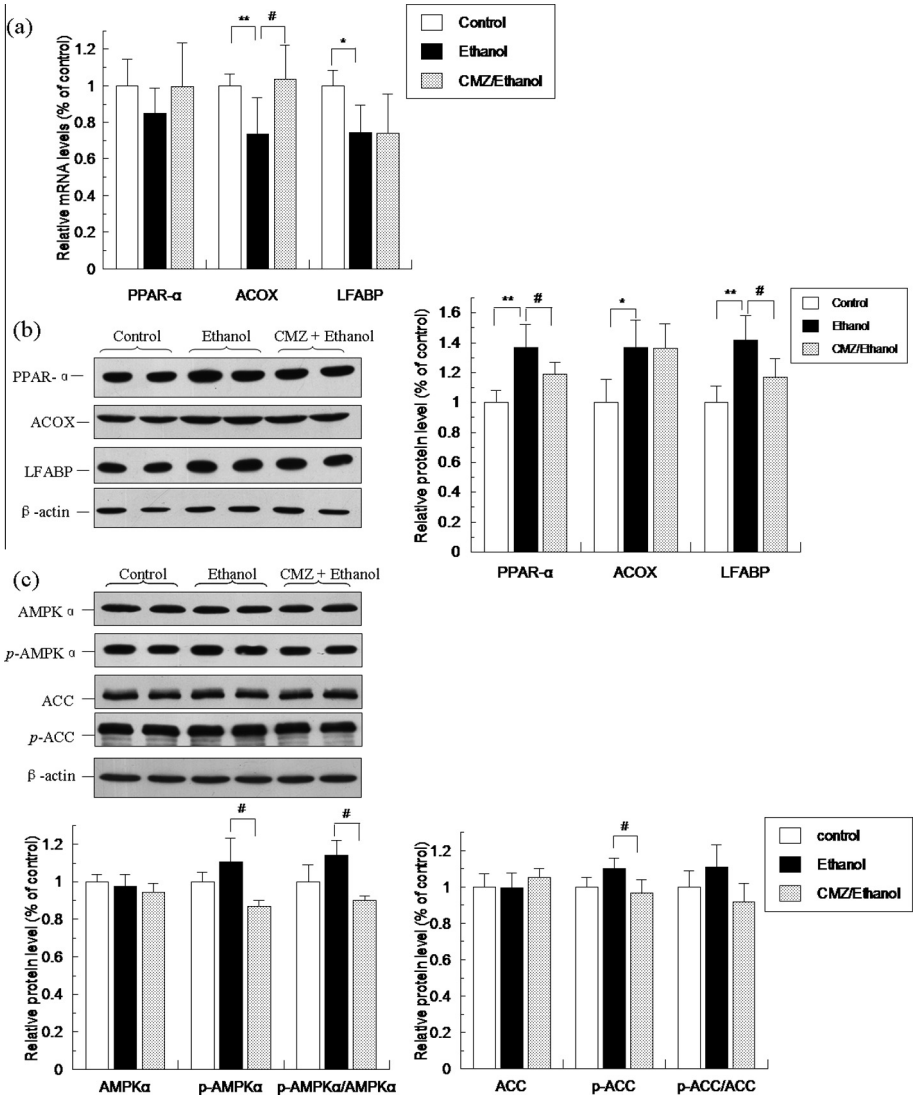


Fig. 4. Effects of CMZ and acute ethanol exposure on the PPAR-α pathway and the phosphorylation of AMPK and ACC in mice liver. (a) The mRNA levels of PPAR-α, ACOX, and LFABP were determined by real time PCR; (b) The representative western blot bands for PPAR-α, ACOX, and LFABP, and the quantitative analysis; (c) Representative western blot bands for AMPK, p-AMPK, ACC, and p-ACC. Data were presented as mean ± SD from at least 3 independent experiments, and expressed as the percentage of the control. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with control group; $^{\#}p < 0.05$, compared with ethanol group.

synthesis, were not significantly affected by acute ethanol challenge or CMZ treatment (Fig. 5b).

3.5. CMZ pretreatment led to the further activation of autophagy

Autophagy has been recognized to regulate the lipid metabolism in the liver [26–27], and therefore, we investigated the changes of two biomarkers of autophagy flux, the ratio of LC3-II/LC-I and the protein levels of p62. In consistent with other reports, the ratio of LC3II/LC3I was significantly increased in ethanol group mice liver [28], while the protein level of p62 was significantly decreased. Interestingly, the p62 protein level in CMZ/ethanol group mice was further decreased, while the ratio of LC3II/LC3I in CMZ/ethanol group mice was also slightly decreased compared with those of the ethanol group mice (Fig. 6). As autophagosome is a transient structure, and the ratio of LC3II/LC3I represents the autophagic activity at one moment but not indicate the magnitude of the flux through the autophagic pathway, while p62 is a substrate of autophagy [29], it may be speculated that the decrease of the ratio of LC3II/LC3I in CMZ/ethanol group mice might be due to the accelerated degradation of substrate.

3.6. CMZ suppressed acute ethanol-induced decline of serum adiponectin levels, but did not significantly affect the levels of serum TNF-α and ethanol

Adiponectin, a 30-kD adipokine, could alleviate AFL in mice [30], while TNF-α might induce hepatic steatosis in mice [31]. Therefore, we measured the levels of serum adiponectin and TNF-α using ELISA kits. Significant decrease of serum adiponectin level was observed in ethanol group mice compared with that of the control group mice. CMZ pretreatment significantly suppressed the decrease of serum adiponectin level (Fig. 7a). No significant difference in the serum TNF-α level was observed among three groups (Fig. 7b). And no significant difference in the serum ethanol concentrations between ethanol group and CMZ/ethanol group was observed (Fig. 7c), which might be explained by the fact that CYP2E1 only accounts for a small part of ethanol metabolism in mice.

4. Discussion

In the current study, we tested the protective effects of CMZ against acute ethanol-induced fatty liver, and demonstrated that

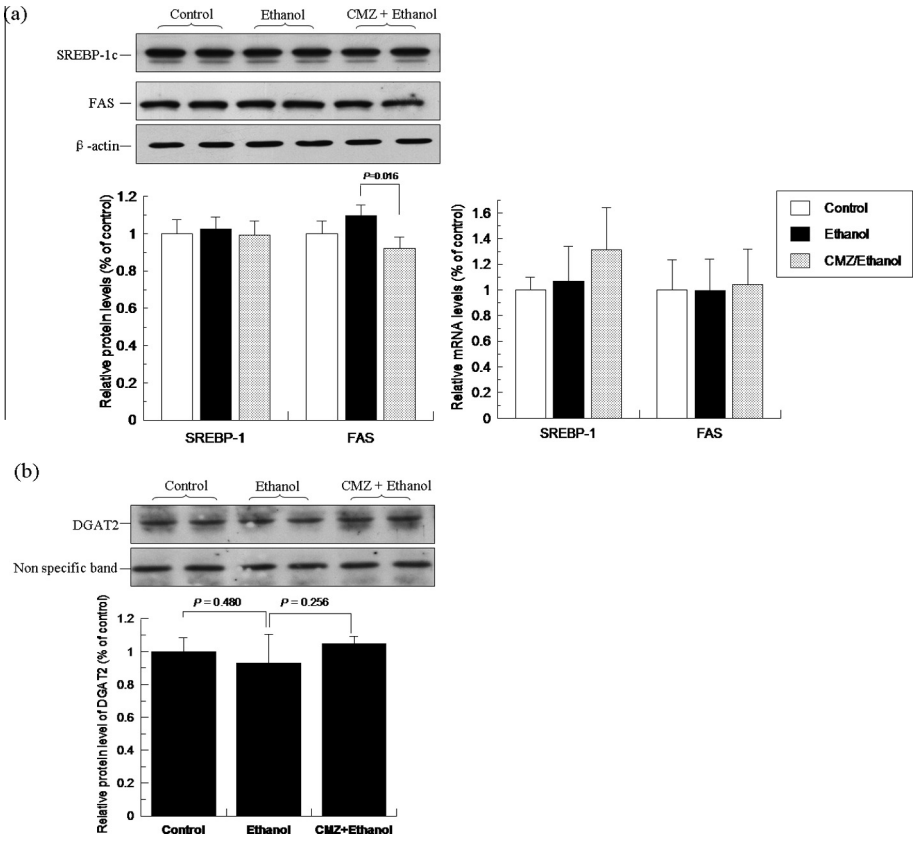


Fig. 5. CMZ had no effects on the protein levels of SREBP-1c and DGAT2, but led to a slight decrease of the protein levels of FAS in mice liver. (a) The protein levels and mRNA levels of n-SREBP-1c and FAS; (b) The protein levels of DGAT2. Data were presented as mean ± SD from at least 3 independent experiments, and expressed as the percentage of the control.

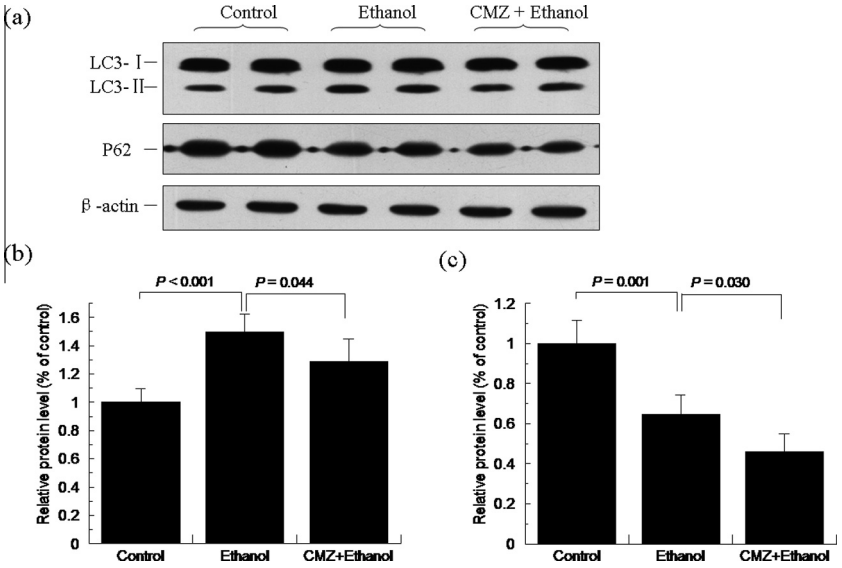


Fig. 6. Effects of acute ethanol exposure and CMZ on the protein levels of LC3 and p62 in mice liver. (a) The representative western blot bands for LC3 and p62; (b) quantitative analysis. Data were presented as mean ± SD from at least 3 independent experiments, and expressed as the percentage of the control.

single dose of CMZ (50 mg/kg body weight) could significantly suppress the increase of hepatic and serum TG levels induced by acute ethanol exposure (Fig. 1). Histopathological examination showed fewer lipid droplets accumulated in CMZ-pretreated mice liver compared with ethanol group mice (Fig. 2). These data provided strong evidences that CMZ pretreatment could effectively attenuate acute ethanol-induced fatty liver in mice.

CMZ is a non-selective CYP2E1 inhibitor, thus it theoretically could suppress acute ethanol-induced oxidative stress, which may play important roles in the pathogenesis of ALD [32]. Although it is still unclear how oxidative stress induces fat accumulation in the liver, several possible mechanisms might be involved. Firstly, oxidative stress can lead to the damage of mitochondria [33–34], which is the major organ for fatty acid oxidation.

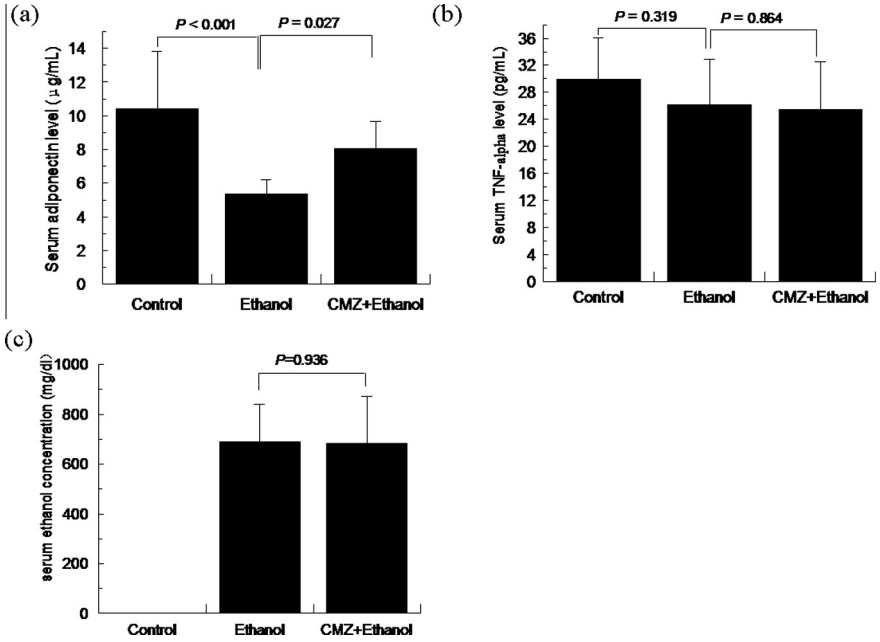


Fig. 7. Effects of acute ethanol exposure and CMZ on the serum adiponectin, TNF-α, and ethanol concentrations. (a) Serum adiponectin level; (b) serum TNF-α level; (c) serum ethanol level.

Secondly, some lipid peroxidation products such as MDA and 4-HNE may covalently bind with some important enzymes involved in fatty acid metabolism. Therefore, antioxidants might benefit for the prevention and treatment of AFL. Diluzio firstly reported that antioxidants could prevent acute ethanol-induced fatty liver [35]. Since then, a large number of studies showed that many agents with antioxidant capacity could alleviate chronic or acute ethanol-induced liver injury including fatty liver [20,22,36]. In the current study, CMZ pretreatment significantly suppressed acute ethanol-induced oxidative stress shown by the decline of hepatic MDA and 4-HNE levels (Fig. 3). TEM examination showed that the mitochondria ridge in the liver of ethanol group mice was fragmented, malformed and dissolved, which was greatly improved by CMZ pretreatment (Fig. 2d). These data suggested that the protection of CMZ against acute ethanol-induced fatty liver was closely associated with the suppression of oxidative stress.

PPAR-α and SREBP-1c are two important nuclear transcription factors controlling a battery of enzymes involved in fatty acid decomposition and synthesis, respectively [37–39]. Therefore, we investigated the changes of several important enzymes involved in the above two pathways. The mRNA levels of PPAR-α and its regulated ACOX and LFABP were all decreased in the liver of ethanol group mice, while CMZ pretreatment suppressed the decrease of mRNA levels of PPAR-α and ACOX, but not LFABP. Unexpectedly, the protein levels of PPAR-α, ACOX and LFABP were all significantly increased in ethanol group mice liver, which was suppressed by CMZ pretreatment (Fig. 4). It was difficult to understand why the changes of mRNA and protein levels of PPAR-α were opposite. However, as ethanol has been shown to suppress the proteasome activity [40–41], which is involved in the degradation of most intracellular proteins including PPAR-α [42], it may be speculated that acute ethanol challenge might impair the proteasome activity, leading to the slowing of the turnover of PPAR-α. If mice were exposed to ethanol for a longer period, down-regulation of PPAR-α, ACOX, and LFABP protein levels might be observed. In regard to the SREBP-1c mediated lipogenesis pathway, the mRNA levels of SREBP-1c and FAS was not significantly affected. Although CMZ pretreatment led to a slight decrease of the FAS protein level, the protein levels of SREBP-1c and DGAT2 were not significantly affected by acute ethanol or CMZ. These data suggested that the protective effects of CMZ might not be related with SREBP-1c mediated lipogenesis pathway.

Autophagy is a genetically programmed, evolutionarily conserved catabolic process, which has been demonstrated to play important roles in the pathogenesis of ALD [28]. In this study, we investigated whether the protective effects of CMZ against acute ethanol-induced fatty liver were related with autophagy by measuring two biomarkers for autophagy flux, the LC3-II/LC-I ratio and the protein levels of p62. Acute ethanol exposure was found to result in significant activation of autophagy evidenced by the increase of LC3-II/LC-I ratio and the decrease of p62 protein level. Although CMZ appeared to partially suppress acute ethanol-induced increase of the LC3-II/LC-I ratio, the hepatic p62 protein level in CMZ/ethanol group mice was further decreased compared with that of the ethanol group mice. Autophagosome is a transient structure, and the LC3II/LC3I ratio represents the autophagic activity at one moment, but not indicate the magnitude of the flux through the autophagic pathway, while p62 is a substrate of autophagy [29]. It might be speculated that the decrease of the LC3II/LC3I ratio in CMZ/ethanol group mice might be due to the accelerated degradation of substrate. Therefore, CMZ might lead to the further activation of autophagy, which accelerated the decomposition of TG in the liver.

Adiponectin and TNF-α are two important cytokines which might be involved in the pathogenesis of AFL [43]. Serum TNF-α level was reported to be increased in chronic ethanol-exposed rats [24,44], while the circulating adiponectin level was significantly decreased [45]. In the current study, we found that the serum adiponectin level in ethanol group mice was significantly decreased, while the serum TNF-α level was not significantly affected. Interestingly, CMZ pretreatment significantly suppressed the decline of the serum adiponectin level. As oxidative stress has been shown to decrease the adiponectin secretion from adipocytes [46], the suppression of the serum adiponectin decline by CMZ might be attributed to the inhibition of acute ethanol-induced oxidative stress.

In summary, the current study demonstrated that CMZ could alleviate acute ethanol-induced fatty liver in mice. CMZ

pretreatment significantly suppressed acute ethanol-induced activation of CYP2E1, and completely blocked ethanol-induced oxidative stress. In addition, CMZ pretreatment also inhibited acute ethanol-induced decline of serum adiponectin level, and might accelerate hepatic TG decomposition by activating autophagy. These factors may work synergistically and contribute to the protection of CMZ against acute ethanol-induced fatty liver. These results confirm that CYP2E1 also plays important roles in acute ethanol-induced fatty liver.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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