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Combined metadoxine and garlic oil treatment efficaciously abrogates alcoholic steatosis and CYP2E1 induction in rat liver with restoration of AMPK activity

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Abstract

Alcoholic steatosis is the earliest and most common response to heavy alcohol intake, and may precede more severe forms of liver injury. Accumulation of fat, largely triglyceride, in hepatocytes results from the inhibition of fatty acid oxidation and excessive oxidative stress involving CYP2E1. This study evaluated the therapeutic effects of metadoxine, garlic oil or their combination on alcoholic steatosis. Feeding rats an alcohol-containing diet for 4 weeks elicited an increase in hepatic triglyceride content and induced CYP2E1. The concurrent administration of metadoxine and garlic oil (MG) to rats during the last week of the diet feeding efficaciously abrogated both fat accumulation and CYP2E1 induction as compared to the individual treatment at higher doses. Histopathology confirmed the ability of MG combination to inhibit lipid accumulation. Blood biochemistry verified improvement of liver function in rats treated with MG. Alcohol administration resulted in a decrease in AMP-activated protein kinase-α (AMPKα) phosphorylation, which was restored by MG treatments. Recovery of AMPK activity by MG was supported by an increase in acetyl-CoA carboxylase phosphorylation. Hepatic fatty acid synthase (FAS) expression was markedly decreased after alcohol consumption, which correlated with a decrease in AMPK activity and a commensurate increase in lipid content. Combined MG treatments caused restoration of the FAS level. These results demonstrate that the combination of MG effectively treats alcoholic steatosis with CYP2E1 inhibition, which may be associated with the recovery of AMPK activity, promising that the combination therapy may constitute an advance in the development of clinical candidates for alcoholic steatosis.

Keywords: Metadoxine; Garlic oil; Alcoholic steatosis; CYP2E1; AMPK

1. Introduction

Chronic alcohol consumption increases hepatic accumulation of fat, largely triglyceride (TG), and lead to hepatic steatosis, which is the earliest and most common response to heavy alcohol intake. The mechanisms by which alcohol ingestion causes fatty liver seem to be complex. Alcohol-induced fat accumulation in hepatocytes may result from increase of TG synthesis, inhibition of fatty acid oxidation and excessive oxidative stress. A decrease in the activity of AMP-activated

Abbreviations: ACC, acetyl-CoA carboxylase; ALT, alanine aminotransferase; AMPK, AMP-activated protein kinase; AST, aspartate aminotransferase; CYP2E1, cytochome P450 2E1; FAS, fatty acid synthase; GGT, γ-glutamyl transferase; metadoxine, pyridoxol L-2-pyrrolidone-5-carboxylate; MG, metadoxine and garlic oil; TG, triglyceride

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protein kinase (AMPK), a conserved intracellular energy sensor, may be responsible for the disruption of fatty acid oxidation pathway [1]. Hepatic steatosis may render the liver more susceptible to oxidative damages, in particular to endotoxins [2], which promote the pathogenesis of alcoholic hepatitis or liver fibrosis [3,4]. Therefore, recovery from fatty liver would help decrease susceptibility to liver fibrosis. However, there is as yet no clearly established pharmacologic therapy for reversing fatty liver.

Cytochome P450 (CYP2E1) is a specific alcohol-inducible form of cytochrome P450, and primarily renders liver injuries after alcohol ingestion [5]. CYP2E1 induction is considered to be an early and necessary step in the pathogenesis of alcohol-induced steatosis. It has been shown that CYP2E1 expression levels were increased during the alcohol-dependent metabolic changes or the early liver injuries such as steatosis and steatohepatitis [6]. CYP2E1 not only catalyses alcohol metabolism, but also metabolizes other xenobiotics including acetaminophen and carbon tetrachloride [7,8], resulting in oxidative stress. The oxidants produced by CYP2E1 can promote toxicities such as protein oxidation, damage to the DNA and lipid peroxidation [5]. Increase in oxidative stress by alcohol contributes to fat accumulation in the liver due to redox state changes and production of reactive products such as acetaldehyde.

Both pyrrolidone carboxylate and pyridoxine are the well-known agents that are useful for the treatment of alcoholics and alcoholic liver diseases [9,10]. Metadoxine is pyridoxol L-2-pyrrolidone-5-carboxylate, which is the ion-pair between pyrrolidone carboxylate and pyridoxine. In an animal model, metadoxine treatment increases the clearance of alcohol and acetaldehyde, reduces the damaging effect of free radicals, and thereby restores cellular ATP and GSH levels [11–13]. Nevertheless, the molecular target of metadoxine therapy has not been clearly defined. Although clinical trials partly support the effect of metadoxine for steatosis [12], the therapeutic efficacy needs to be verified by other experimental approaches [14]. Hence, successful treatment of hepatic steatosis or steatohepatitis may require combination of metadoxine with other agents.

Allium species comprises organosulfur compounds. The organosulfur ingredients existing in garlic oil (GO) exert chemoprotective effects against chemical-induced carcinogenesis in rats [15,16]. The chemoprotective effect of GO is explained in part by the induction of phase II detoxifying enzymes [17,18]. Moreover, studies have shown that GO or garlic extracts exert protective effects through the inhibition of CYP2E1 responsible for the production of reactive metabolic intermediates from a wide variety of organic molecules [19]. For example, the induction of CYP2E1 by pyrazine was blocked in animals by concomitant treatment with a relatively high dose of GO (e.g., 500 mg/kg body weight) [20]. Previously, we also showed that feeding mice a diet containing a GO extract inhibited the ability of carbon tetrachloride to increase hepatic TG and cholesterol contents [21]. In spite of the findings that CYP2E1 is involved in the pathogenesis of alcohol-induced liver injury and that chemical inhibition of CYP2E1 is plausible, GO has never been tried to treat alcoholic steatosis. Furthermore, the fact that CYP2E1 inhibition necessitates high doses of GO intake may deter its application for alcoholic liver injuries.

In view of the previous reports showing the differential mechanistic basis of metadoxine and GO for hepatic effects, we attempted to evaluate the potential therapeutic efficacy of their combination on alcoholic steatosis and CYP2E1 induction under the assumption that the two agents might act on sequential steps in the obligate pathologic progress to hepatic steatosis. We found that the combination of metadoxine and GO (MG) efficaciously inhibits alcohol-induced hepatic TG accumulation and improves liver function as compared to individual agent alone. Moreover, the combination of MG recovered the activity of AMPK in alcohol-fed rats, which might account for the pharmacologic efficacy of MG for steatosis.

2. Materials and methods

2.1. Materials

Metadoxine and GO were provided from PharmaKing Pharmaceutical Co. (Seoul, Korea). Lieber-DeCarli liquid diet was purchased from Dyets, Inc. (Bethlehem, PA). Anti-CYP2E1 antibody was supplied form Oxford Biomedical Research (Oxford, MI). Antibodies that specifically recognize phosphorylated AMPK and phosphorylated acetyl-CoA carboxylase (ACC) were obtained from Cell Signaling (Beverely, MA). An antibody directed against fatty acid synthase (FAS) was supplied from BD BioSciences (San Diego, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were provided from Zymed Laboratories Inc. (San Francisco, CA).

2.2. Animals and diets

Animal studies were conducted in accordance with the institutional guidelines for care and use of labora-

tory animals. Sprague–Dawley rats at 6 weeks of age (140–160 g) were provided from Samtako Co. (Osan, Korea), acclimatized for 1 week, and maintained in a clean room at the Animal Care Center, College of Pharmacy, Seoul National University. Animals were caged under the supply of filtered pathogen-free air at a temperature between 20 and 23 °C with 12 h light and dark cycles and relative humidity of 50%. The rats were given free access to control diet or alcohol Lieber-DeCarli liquid diet (diet consumption, 12–18 g/kg/day). The body weight and general conditions of the animals were monitored at least once a week. The rats were randomly assigned to the groups specified. Each group included 10 animals. The liquid diet was prepared according to the method described by Lieber-DeCarli [22] and provided 1 kcal/ml, containing 28% carbohydrate, 20% protein, 15% fat plus 37% ethanol (alcohol diet) or isocaloric maltose dextrin (control diet). The diet was kept refrigerated in the dark. Ethanol was incorporated into the diet just before the supply. A previous study showed that the blood ethanol content in animals fed the ethanol-containing diet was 100–150 mg/dL [22].

2.3. Animal treatments

Rats were fed pair-fed control or alcohol-containing diet (EtOH) for 4 weeks. During the last week of diet feeding, the rats were daily treated with metadoxine (50, 100 or 200 mg/kg/day, $n = 10$) dissolved in 40% polyethyleneglycol 400, GO (50, 100 or 200 mg/kg/day, $n = 10$) suspended in corn oil, and MG combination (15, 50 or 100 mg/kg/day each, $n = 10$) for 6 consecutive days. Control animals ($n = 10$) received vehicle only.

2.4. Blood chemistry

Plasma alanine aminotransferase (ALT, EC 2.6.1.2), aspartate aminotransferase (AST, EC 2.6.1.1) and albumin were analyzed using Spectrum®, an automatic blood chemistry analyzer (Abbott Laboratories, Abbott Park, IL).

2.5. Histopathology

Hepatic morphology was assessed by light microscopy. The left lateral lobe of the liver was sliced (three slices per rat), and tissue slices were fixed in 10% buffered-neutral formalin for 6 h. Fixed liver tissue slices were processed and embedded in a paraplast automatic tissue processor, Citadel 2000 (Shandon Scientific, Cheshire, UK). Sections of 4 μm in thickness were subjected to hematoxylin and eosin (H&E) staining before examinations [23]. For Oil Red O (Sigma, St. Louis, MO) staining, 4 μm sections were cut from frozen optimal cutting temperature samples, affixed to microscope slide, and allowed to air-dry overnight at room temperature. The liver sections were stained in fresh Oil Red O for 10 min, rinsed in water, and then counterstained with hematoxylin.

2.6. TG analysis

Samples of rat liver were homogenized in 0.1 M Tris–acetate buffer (pH 7.4) containing 0.1 M potassium chloride and 1 mM EDTA. Six volumes of chloroform:methanol (2:1) were then added. After vigorous stirring, the mixture was incubated on ice for 1 h and then centrifuged at $800 \times g$ for 3 min. The resulting lower phase was aspirated. TG contents were determined using Sigma Diagnostic Triglyceride Reagents (Sigma, St. Louis, MO).

2.7. Immunoblot analysis

Samples were individually prepared from randomly selected 4 or 7 animals in each treatment group comprising 10 animals and subjected to immunoblot analyses (three determinations per sample). Immunoblot analyses were performed according to the previously published procedures [24]. Proteins of interest in liver homogenates were resolved using a 9% or 12% gel and developed using ECL chemiluminescence system (Amersham, Buckinghamshire, UK). A representative blot was shown in each figure and just two different samples per treatment were included in the blot.

2.8. Data analysis

One-way analysis of variance was used to assess significant differences among treatment groups. The Newman–Keuls test was used for comparisons of group means. Data represent the mean ± S.E. The criterion for statistical significance was set at $P < 0.05$ or $P < 0.01$.

3. Results

3.1. Decreases in hepatic TG content

We first evaluated the therapeutic effects of metadoxine and/or GO at the daily dose from 50 to 200 mg/kg for 6 days on alcohol-induced steatosis. Either metadoxine or GO treatment alone at the dose of 50 mg/kg

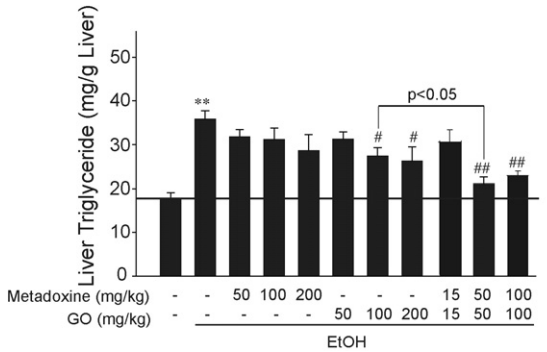

Fig. 1. TG contents in the liver. Rats were fed pair-fed control or alcohol-containing diet (EtOH) for 4 weeks. During the last week of diet feeding, the animals were daily treated with metadoxine and/or GO for 6 consecutive days. Hepatic TG contents were measured in the lipid extracts, as described in Section 2. Data represent mean ± S.E. from 10 animals (significantly different from vehicle-treated control, $^{**}p < 0.01$, or ethanol alone, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$).

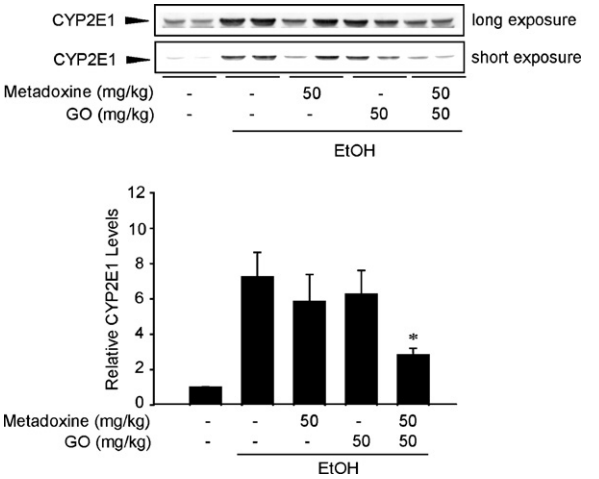

Fig. 2. Hepatic CYP2E1 expression. Metadoxine and/or GO was (were) orally administered to rats ($N = 10$ animals per group) at the daily dose of 50 mg/kg for 6 days during the last week of diet feeding (EtOH). CYP2E1 was immunoblotted in the hepatic microsomal samples prepared from randomly selected four animals per treatment. A representative blot was shown in the figure accnd just two different samples per treatment were included in the blot. Equal loading of proteins was verified by staining the replicate blots with coomassie. The relative CYP2E1 levels were assessed by scanning densitometry of the immunoblots. Data represent mean ± S.E. from four animals (significantly different from ethanol alone, $^{*}p < 0.05$).

failed to significantly inhibit hepatic TG accumulation caused by alcohol administration (Fig. 1). Metadoxine treatments at the daily dose of 100 or 200 mg/kg resulted in no significant decreases in the TG content, whereas GO treatments at the doses caused 17–27% decreases. It was noteworthy that simultaneous treatment of the alcohol-fed rats with both metadoxine and GO at the daily dose of 50 mg/kg each notably (~60%) decreased the content of TG accumulated by alcohol feeding. Also, we analyzed the increasing dose effects of MG combination at the 1:1 ratio on the TG content. Concurrent treatment of the rats with MG at the respective dose of 50 or 100 mg/kg almost completely abrogated the lipid accumulation (Fig. 1). However, the effect of MG at the respective dose of 15 mg/kg was statistically insignificant. These results indicated that the combination of MG at the respective dose of 50 mg/kg or above might be effective against alcoholic steatosis.

3.2. CYP2E1 expression

In the subsequent study, we were interested in whether the combination treatment of MG was capable of inhibiting CYP2E1 induction caused by alcohol. Exposure of alcohol-fed rats to either metadoxine or GO alone at the daily dose of 50 mg/kg for 6 days led to only marginal decreases in hepatic CYP2E1 expression (Fig. 2). As expected, concomitant MG treatments of the rats (50 mg/kg each, for 6 days) significantly repressed CYP2E1 induction (Fig. 2). Our data showed that MG combination effectively abrogated CYP2E1 induction by alcohol.

3.3. Histopathological improvements

Next, to verify the therapeutic effects of MG on alcoholic steatosis, our studies were extended to histopathologically examine the extents of fat accumulation. Healthy control rats showed no pathological changes in the liver (Fig. 3A-a). Exposure of rats to ethanol for 4 weeks caused lipid accumulation (Fig. 3A-b), which was characterized by appearance of microvesicular and macrovesicular fat droplets as usually seen after repetitive alcohol administration [25,26]. Treatments of the rats with metadoxine (50 mg/kg, for 6 days) resulted in a therapeutic effect against alcohol-induced steatosis (Fig. 3A-c). Increase in fat accumulation by alcohol was also blocked to a minor extent by 6-day treatments with GO, which was in line with the decreases in TG content (Fig. 3A-d). It was noted that concurrent treatments of alcohol-fed rats with MG (50 mg/kg each, for 6 days) completely inhibited fat infiltration (Fig. 3A-e and f), confirming the ability of MG combination to inhibit lipid accumulation in hepatocytes.

To firmly establish the therapeutic effects of MG on alcoholic steatosis, lipid accumulation was also assessed by Oil Red O staining, which generally mon-

itors lipid droplets in cells or tissues [28]. Healthy control rats did not show steatosis (Fig. 3B-a), whereas alcohol-fed rats exhibited substantial increases in lipid droplets (Fig. 3B-b), which was consistent with the results of H&E microscopy. Although either metadoxine or GO treatment alone (50 mg/kg) minimally decreased lipid infiltration in the livers (Fig. 3B-c and d), combined MG treatments completely abolished it (Fig. 3B-e). Our data verified that the combination of MG virtually treats alcohol-induced steatosis.

3.4. Blood biochemistry

After 4-weeks of alcohol administration, ALT and AST activities were increased (Table 1). ALT activity elevated by alcohol administration tended to decrease after treatment with metadoxine, GO or MG combination (i.e., statistically insignificant). MG combination treatment allowed AST activity to return to that of healthy control although each agent alone at much higher doses was ineffective. Chronic alcohol administration to rats significantly decreased the albumin level, which was

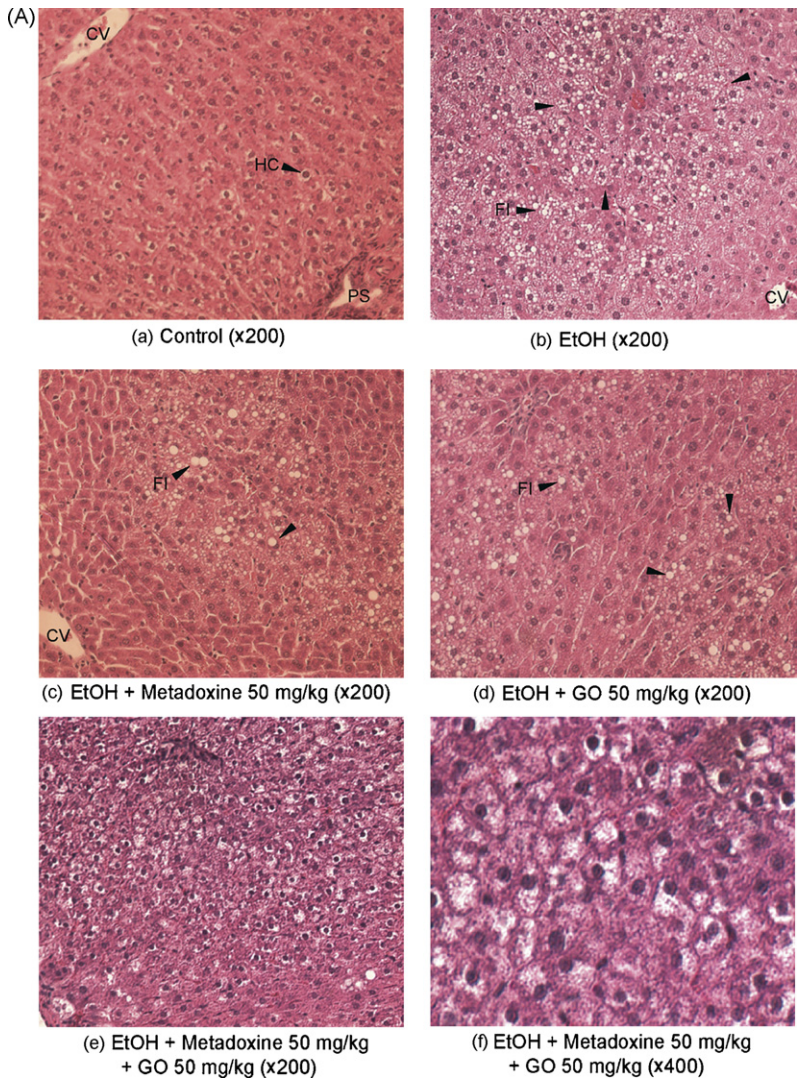

Fig. 3. Hepatic histopathology. (A) Effects of metadoxine and/or GO treatments on liver histopathology in rats. H&E-stained sections represent liver samples of rats fed control diet (control), alcohol-containing diet (EtOH), and alcohol-containing diet with metadoxine and/or GO treatments. Closed arrowheads identify the examples of fat infiltration (FI). (B) Morphology in frozen liver sections stained with Oil Red O. Microphotographs show views of the liver sections: central vein (CV), hepatocytes (HC) and portal space (PS).

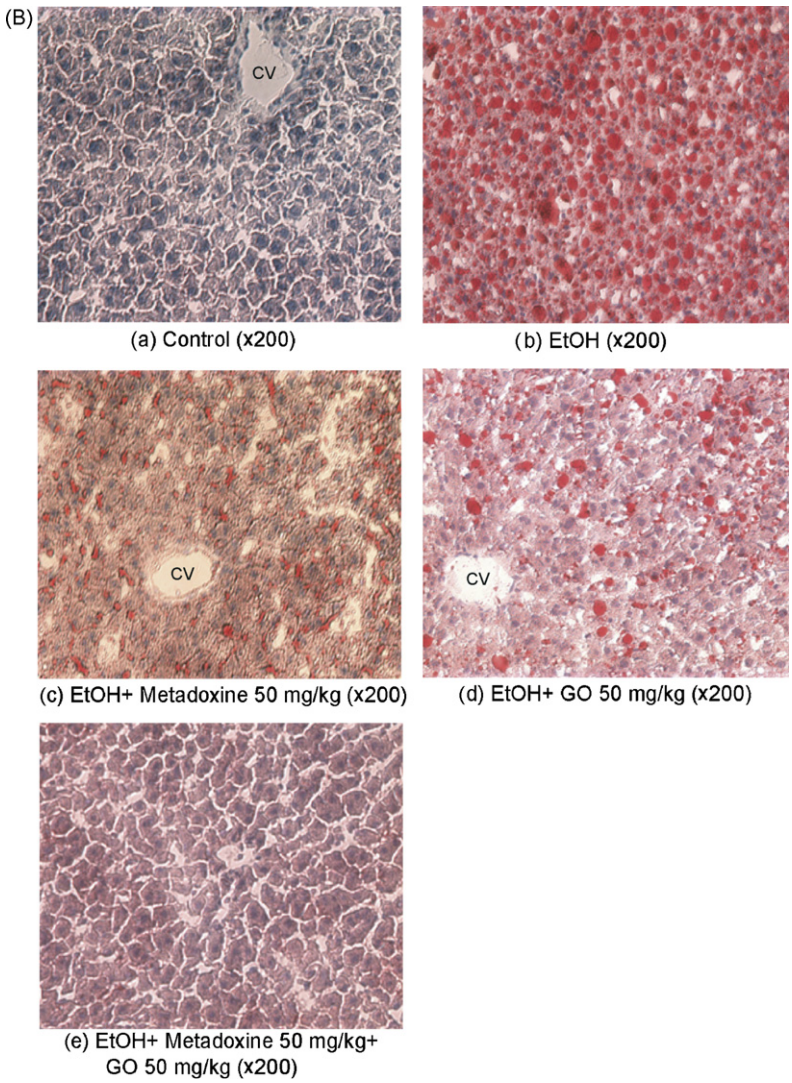

Fig. 3. (*Continued*).

Table 1
Blood biochemistry

Treatment ($n = 10$)	ALT (U/L)	AST (U/L)	Albumin (g/dL)
Pair-fed control	18.4 ± 5.5	83.6 ± 9.3	4.46 ± 0.2
EtOH (vehicle)	51.1 ± 15.4**	104.1 ± 9.8**	3.88 ± 0.23**
EtOH + metadoxine 100 (mg/kg, p.o.)	43.1 ± 12.5	106.8 ± 17.7	4.17 ± 0.2
EtOH + metadoxine 200 (mg/kg, p.o.)	43.6 ± 13.1	106.5 ± 16.3	4.15 ± 0.2
EtOH + garlic oil 100 (mg/kg, p.o.)	39.5 ± 6.0	102 ± 22.6	4.13 ± 0.3
EtOH + garlic oil 200 (mg/kg, p.o.)	40.1 ± 11.1	98.8 ± 12.3	4.28 ± 0.47
EtOH + metadoxine 50 + garlic oil 50 (mg/kg, p.o.)	37.5 ± 8.0	79.6 ± 12.8##	4.06 ± 0.25

Rats were fed pair-fed control or alcohol-containing diet (EtOH) for 4 weeks. During the last week of diet feeding, the animals were daily treated with metadoxine and/or GO for 6 consecutive days. AST and ALT activities or albumin levels were monitored in the plasma of the rats. Values represent mean ± S.E. from 10 animals (significantly different from vehicle-treated control, $^{**}p < 0.01$, or ethanol alone, $^{\#\#}p < 0.01$).

only marginally changed by metadoxine, GO or MG treatment (Table 1).

3.5. Phosphorylation of AMPK

AMPK has been implicated in the regulation of glucose and lipid homeostasis [27,28]. In view of the fact that AMPK regulates lipid metabolism in the liver [29], we examined whether the therapeutic effect of MG on steatosis is associated with AMPK. Immunoblot analysis revealed that the levels of phosphorylated AMPKα in liver homogenates were notably decreased after 4 weeks of alcohol administration (Fig. 4A). Treatment of alcohol-fed rats with metadoxine or GO (50 mg/kg, for 6 days) did not increase phosphorylated AMPKα levels. Interestingly, exposure of alcohol-fed rats to MG combi-

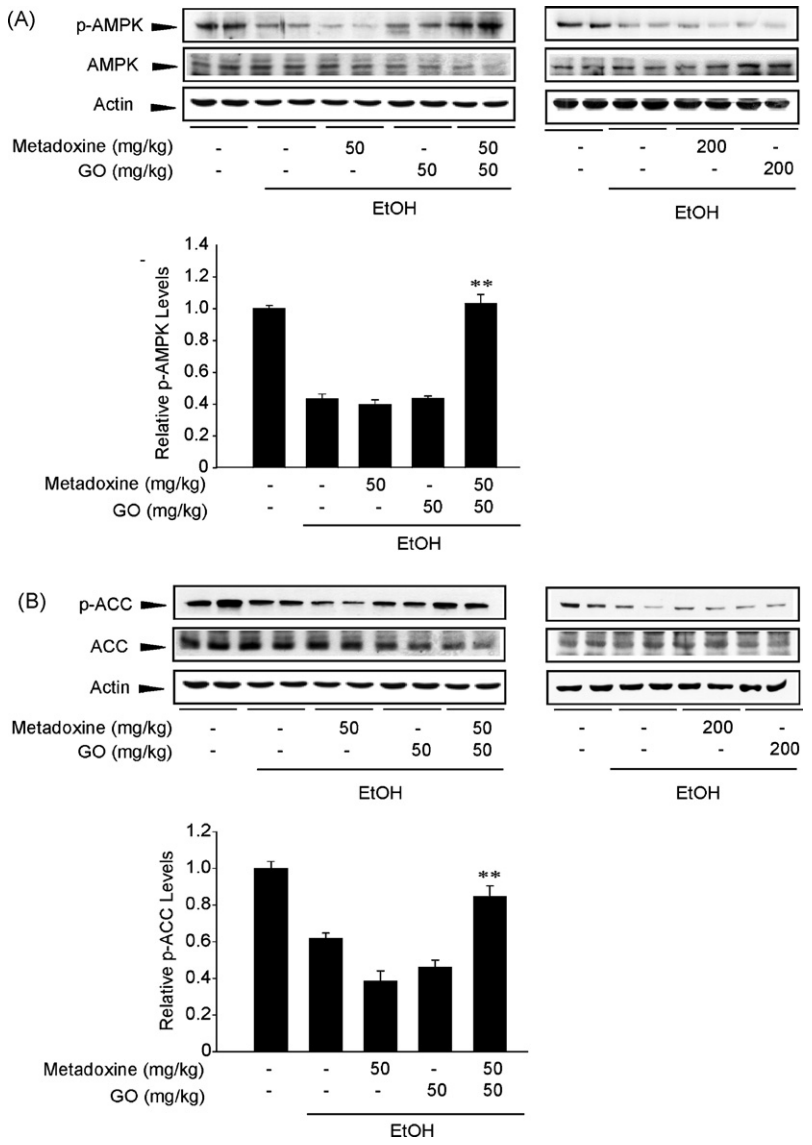

Fig. 4. Hepatic AMPK phosphorylation. (A) AMPK phosphorylation. Rats were fed pair-fed control or alcohol-containing diet (EtOH) for 4 weeks. During the last week of diet feeding, the animals were daily treated with metadoxine and/or GO for 6 consecutive days. Phosphorylated AMPK was immunoblotted in the liver homogenates prepared from randomly selected four animals per treatment. (B) ACC phosphorylation. The levels of phosphorylated ACC were determined in the liver homogenates prepared from randomly selected four animals per treatment. A representative blot was shown in the figure and just two different samples per treatment were included in the blot. Equal loading of proteins was verified by probing the replicate blots for actin. The relative protein levels were assessed by scanning densitometry of the immunoblots. Data represent mean ± S.E. from four animals (significantly different from ethanol alone, $^{**}p < 0.01$).

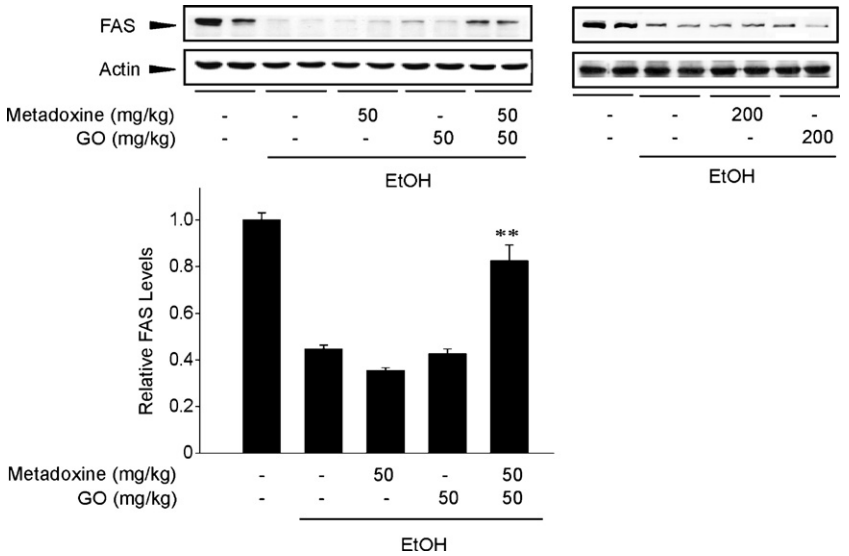

Fig. 5. Hepatic FAS expression. Rats were fed pair-fed control or alcohol-containing diet (EtOH) for 4 weeks. During the last week of diet feeding, the animals were daily treated with metadoxine and/or GO for 6 consecutive days. FAS was immunoblotted in the liver homogenates prepared from randomly selected seven animals per treatment. A representative blot was shown in the figure and just two different samples per treatment were included in the blot. Equal loading of proteins was verified by probing the replicate blots for actin. The FAS protein levels were assessed by scanning densitometry of the immunoblots. Data represent mean ± S.E. from seven animals (significantly different from ethanol alone, $^{**}p < 0.01$).

nation (50 mg/kg each, for 6 days) resulted in complete recovery of AMPKα phosphorylation. Either metadoxine or GO treatment at the dose of 200 mg/kg for 6 days resulted in only marginal effects (Fig. 4A).

We further measured the levels of total and phosphorylated ACC, a rate-limiting enzyme involved in fatty acid biosynthesis in the liver [29]. Alcohol administration inhibited ACC phosphorylation in the liver, which was not improved by metadoxine or GO treatment alone (50 or 200 mg/kg/day, for 6 days)(Fig. 4B). However, MG combination treatments (50 mg/kg each, for 6 days) led to complete restoration of ACC phosphorylation. Our results demonstrated that chronic alcohol administration resulted in a decrease in AMPK activity, which was obviously recovered after a week of MG combination treatments.

3.6. Hepatic FAS expression

Lastly, we determined the effect of MG combination on FAS expression. Hepatic FAS was markedly repressed after 4-weeks of alcohol consumption (Fig. 5), which was in parallel with the reported decrease in fatty acid synthesis after 4-weeks of alcohol consumption [30,31]. Our data also correlated with decreases in AMPK activity and in commensurate lipid accumulation. Either metadoxine or GO treatment alone (50 or 200 mg/kg/day, for 6 days) failed to recover hepatic FAS expression. By contrast, MG treatments (50 mg/kg each, for 6 days) restored FAS content. Our finding showing the recovery of FAS by MG lends support to the notion that the MG composition has the ability to recover homeostatic lipid metabolism in the liver.

4. Discussion

Accumulation of fat in the liver in response to chronic alcohol ingestion can lead to steatosis. Hepatic steatosis is characterized by accumulation of lipid droplets and of TG in the liver and has been defined as both more than 5% of hepatocytes containing fat droplets or total lipid exceeding 5% of liver weight [32]. Recent clinical studies provided evidence that such an accumulation of TG in the liver is not benign but could lead to fibrosis [33,34]. Understanding the pathogenesis of steatosis would provide promising prospects for more effective treatments of alcoholic liver diseases. It is known that long-term alcohol drinking promotes synthesis of fatty acid and TG in the liver by inducing lipid synthetic enzymes such as glycerol-3-phosphate acyltransferase, fatty acid synthase and malic enzyme. Moreover, chronic alcohol intake leads to a shift in the cytosolic [NAD+]/[NADH] ratio to reduction, a requiring step for tricarboxylic cycle and β-oxidation [35], and a decrease in AMPK activity that controls the pathway of fatty acid oxidation [1].

Metadoxine treatment improves alcoholic steatosis and liver function through the metabolic effect associated with the accelerated elimination of alcohol from the blood and acetaldehyde catabolism [12]. Metadoxine may act as an antioxidant, preventing the redox imbalance, and reducing collagen synthesis in hepatic stellate cells [13]. Other antioxidants such as ebselen [36], diphenyleneiodonium [37], or allopurinol [38] also prevent the accumulation of lipid droplets in hepatocytes, supporting the link between oxidative stress and steatosis. Nevertheless, the current pharmacologic therapy for the treatment of hepatic steatosis has limitations in its therapeutic efficacies or emergence of adverse effects.

CYP2E1 is known to metabolize ethanol and elicits oxidative stress. In addition, CYP2E1 catalyzes metabolic conversion of other small organic molecules to the reactive intermediates, which are frequently capable of covalently binding to tissue macromolecules, and thereby cause tissue injuries. Hence, exposure of cells or animals to CYP2E1 inducers in combination with the toxic substrates of CYP2E1 would elicit deleterious effects, which would increase the risk of organ toxicity and possibly chemical carcinogenesis. Moreover, an increase in CYP2E1 activity could impair mitochondrial function [5] and inhibit fatty acid oxidation [39]. Hence, CYP2E1 induction may promote not only the metabolism of alcohol to a toxic intermediate, but also fat accumulation in the liver. In view of the importance of CYP2E1 for the bioactivation of toxicants, the selective inhibition of CYP2E1 may represent protective effects against toxic injuries and possibly fat accumulation. It has been shown that organosulfur components comprised in GO exhibit hepatoprotective effects against toxicants [20]. Among the components, diallyl disulfide and diallyl sulfide possess both suppressive activities of CYP2E1 and its expression levels, therefore inhibiting CYP2E1-mediated metabolic activation of toxicants. The results of the present study indicate that GO alone at the dose of 50 mg/kg failed to inhibit hepatic CYP2E1 expression probably because the dose was marginal, which agrees with the result of the previous observation [20]. In the previous study, the metabolic activities known to be catalyzed primarily by CYP2E1 were inhibited by GO treatments at the dose of 500 mg/kg, but not 200 mg/kg. Given the previous observations that alcoholic liver disease resulted in a decrease in cellular GSH content, it is predictable that the efficacious treating effect of MG would allow the liver to restore GSH content, which remained to be clarified.

Here, we investigated the potential therapeutic effects of MG combination on alcohol-induced steatosis with the hypothesis that the combination acts on sequential steps in the obligate pathologic progress of hepatic steatosis. As anticipated, we observed that alcohol-containing diet feeding induced lipid deposition in the livers of rats and that treatment of the animals with MG resulted in significant inhibitory effects on the lipid accumulation. In this study, the combination treatment of metadoxine and GO at relatively low doses (e.g. 50 mg/kg each) was superior to that exerted by either metadoxine or GO alone at much higher doses. Histopathology confirmed the *bona fide* inhibition by MG treatments of alcohol-induced fat infiltration into hepatocytes. Oil Red O staining, a general method to identify exogenous or endogenous lipid deposit, also supported that simultaneous MG treatments almost completely abolished alcohol-induced fat accumulation. These results render us to predict that a pharmaceutical composition comprising metadoxine and GO may exert efficacious treating effects for alcoholic steatosis. Furthermore, our data showing that combined MG treatments exert antagonistic effects against CYP2E1 induction by chronic alcohol treatments suggests that the MG combination may be the effective therapeutic regimen that has the capability of improving alcoholic liver diseases.

Although γ-glutamyl transferase (GGT) is used as a clinical marker of heavy drinking, notable variations have been reported in GGT contents in patients [40]. Thus, we chose ALT and AST as inflammatory markers, whose activities are increased by liver injury or inflammation. In this study, the plasma ALT level slightly elevated by 4-weeks of alcohol consumption in rats was not significantly ameliorated by MG treatments. Rather, MG treatments significantly reduced the plasma activity of AST, which is an enzyme expressed in many body tissues such as liver, heart, red blood cells and muscles. In the patients of alcoholic hepatitis, the ratio of AST to ALT is often greater than 2:1 [41]. We observed that alcohol-containing diet feeding to rats caused an increase in the AST to ALT ratio (Table 1), which was reversed by MG treatments. The alterations in plasma aminotransferase activities in our model may support the treatment effect of MG against alcoholic steatosis.

AMPK is implicated in the regulation of glucose and lipid homeostasis [27,28]. Phosphorylation of the AMPK catalytic subunit is required for AMPK activation [42]. Once activated, AMPK phosphorylates downstream substrates to reduce ATP-consuming anabolic pathways including cholesterol, fatty acid and TG synthesis, while increasing ATP-generating catabolic pathways such as lipolysis and fatty acid oxidation [43]. AMPK phosphorylates target enzymes involved in lipid metabolism [29]. Among them, ACC is the

rate limiting enzyme involved in fatty acid synthesis in the liver and its product, malonyl-CoA, is a precursor of fatty acid synthesis and a potent inhibitor of fatty acid oxidation in mitochondria. AMPK, when activated, inhibits ACC and malonyl-CoA activities, which results in the repression of fatty acid oxidation. Moreover, AMPK activation decreases hepatic sterol regulatory factor binding protein-1c and therefore regulates FAS expression during alcohol-induced steatosis [44].

In the present study, we report that AMPK phosphorylation was substantially decreased after 4-weeks of alcohol consumption and that the inhibition of AMPK by alcohol accompanied a decrease in ACC phosphorylation (i.e., increased ACC activity). This would be responsible for the increased lipogenesis and the reduced rate of fatty acid oxidation. Because alcohol-dependent alteration in AMPK activity is likely to affect fat accumulation, the pharmacologic intervention that enables AMPK activity to increase might serve a way to treat alcoholic steatosis [45]. Here, we found that combination treatment of MG, but not each treatment alone, enhanced hepatic AMPK phosphorylation in rats fed alcohol-containing diet, which as expected led to an increase in ACC phosphorylation. Our data supports the concept that the MG combination inhibits alcohol-dependent TG accumulation in hepatocytes presumably due to AMPK-dependent fatty acid oxidation. Other studies showed that chronic alcohol consumption repressed FAS expression as hepatic TG content progressively increased [30,31]. Our results showing that MG combination caused FAS expression level to be restored might be due to the recovery of homeostatic lipid metabolism. This hypothesis agrees with the previous observation that alcohol-dependent TG accumulation feedback-inhibits TG production from fatty acids in the liver [30,31]. The present findings support the concept that the combination therapeutically treats hepatic steatosis by restoring normal fat metabolism. Since AMPK affects FAS expression presumably via activation of the hepatic sterol regulatory factor binding protein-1c [44], the beneficial effect of MG against alcohol-induced fat infiltration in the liver may be mediated at least in part with AMPK activation.

In summary, the results presented here in this study demonstrate that the combination of MG efficaciously abrogates alcohol-dependent hepatic fat accumulation with CYP2E1 repression in an animal model, and that decrease in hepatic steatosis by the combination may result from the recovery of normal fat metabolism mediated with AMPK. Our results showing the effectiveness of MG against chronic alcohol consumption promise that the combination therapy may constitute an advance in the development of clinical candidates for alcoholic steatosis.

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