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Luteolin Alleviates Alcoholic Liver Disease Induced by Chronic and Binge Ethanol Feeding in Mice[1–3]

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Abstract

Ethanol consumption can lead to hepatic steatosis that contributes to late-stage liver diseases such as cirrhosis and hepatocellular carcinoma. In this study, we investigated the potential protective effect of a flavonoid, luteolin, on ethanol-induced fatty liver development and liver injury. Six-wk-old male C57BL/6 mice were divided into 3 groups: a control group; a group exposed to alcohol by using a chronic and binge ethanol feeding protocol (EtOH); and a group that was administered daily 50 mg/kg of luteolin in addition to ethanol exposure (EtOH + Lut). A chronic and binge ethanol feeding protocol was used, including chronic ethanol consumption (1%, 2%, and 4% for 3 d, and 5% for 9 d) and a binge (30% ethanol) on the last day. Compared with the control group, the EtOH group had a significant elevation in serum concentrations of alanine aminotransferase (ALT) (561%), triglyceride (TG) (47%), and LDL cholesterol (95%), together with lipid accumulation in the liver. Compared with the EtOH group, the EtOH + Lut group had significant reductions in serum concentrations of ALT (43%), TG (22%), LDL cholesterol (52%), and lipid accumulation in the liver. Ethanol elevated liver expression of lipogenic genes including sterol regulatory element-binding protein 1c (*Srebp1c*) (560%), fatty acid synthase (*Fasn*) (190%), acetyl-CoA carboxylase (*Acc*) (48%), and stearoyl-CoA desaturase 1 (*Scd1*) (286%). Luteolin reduced ethanol-induced expression of these genes in the liver: *Srebp1c* (79%), *Fasn* (80%), *Acc* (60%), and *Scd1* (89%). In cultured hepatocytes, luteolin prevented alcohol-induced lipid accumulation and increase in the expression of lipogenic genes. The transcriptional activity of the master regulator of lipid synthesis, sterol regulatory element-binding protein (SREBP), was enhanced by ethanol treatment (160%) and reduced by luteolin administration (67%). In addition, ethanol-induced reduction of AMP-activated protein kinase and SREBP-1c phosphorylation was abrogated by luteolin. Collectively, our study indicates that luteolin is effective in ameliorating ethanol-induced hepatic steatosis and injury. J. Nutr. 144: 1009–1015, 2014.

Introduction

Excessive ethanol consumption can generate alcoholic liver disease (ALD)[7], ranging from simple steatosis to severe forms of liver injury, such as steatohepatitis, cirrhosis, and hepatocellular carcinoma (1,2). Hepatic steatosis is the first manifestation of ALD, which is characterized by lipid accumulation in hepatocytes. Although hepatic steatosis is considered a benign condition because of its reversible nature, increasing evidence suggests that it is a potentially pathologic situation (3). Thus, reducing fat accumulation in the liver upon ethanol exposure may alleviate the progression of steatosis to later stages of ALD.

Although the mechanisms by which ethanol causes liver diseases seem complex and multifactorial, increasing lipogenesis in the liver is an important biochemical characteristic during the development of hepatic steatosis. Sterol regulatory element-binding proteins (SREBPs) are major transcription factors

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[3] Supplemental Table 1 is available from the ''Online Supporting Material'' link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
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[7] Abbreviations used: *Acc*, acetyl-CoA carboxylase; ALD, alcoholic liver disease; ALT, alanine aminotransferase; AMPK, AMP-activated protein kinase; AST, aspartate aminotransferase; EtOH, exposed to alcohol by using a chronic and binge ethanol feeding protocol; EtOH + Lut, administered daily 50 mg/kg of luteolin in addition to ethanol exposure; FASN, fatty acid synthase; *Hmgcr*, 3-hydroxy-3-methylglutaryl–coenzyme A reductase; *Hmgcs*, 3-hydroxy-3-methylglutaryl–coenzyme A synthase; *Il1*β, interleukin 1β; *Il6*, interleukin 6; LDLR, low density lipoprotein receptor; SCD1, stearoyl-CoA desaturase 1; SREBP, sterol regulatory element-binding protein; SREBP-1, sterol regulatory element-binding protein 1; SREBP-1a, sterol regulatory element-binding protein 1a; SREBP-1c, sterol regulatory element-binding protein 1c; SREBP-2, sterol regulatory element-binding protein 2; TC, total cholesterol; *Tnf*α, tumor necrosis factor α.

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activating the expression of genes involved in the biosynthesis of cholesterol, FAs, and TGs. There are 3 forms of SREBPs in mammals: SREBP-1a, -1c, and -2 (4). SREBP-1c, which is mainly responsible for the biosynthesis of TGs, is the predominant isoform of SREBP-1 in the liver. Transgenic mice overexpressing SREBP-1a or SREBP-1c produced massive fatty liver owing to increased accumulation of cholesterylesters and TGs (5,6). Ethanol accumulation also affects the expression concentrations of *Srebp1* and many other *Srebp* target genes, further increasing lipid synthesis (7,8).

AMP-activated protein kinase (AMPK) is an energy sensor that regulates cellular metabolism including lipid metabolism (9), and AMPK has been used as a target for the treatment of hepatic disorders (10). Activation of AMPK is modulated by changes in ATP, ADP, and AMP concentrations and phosphorylation at Thr172 by an upstream AMPK kinase (11). Phosphorylated AMPK stimulates ATP-producing catabolic pathways, such as FA oxidation, and inhibits ATP-consuming processes, such as lipogenesis. Previous studies showed an inverse correlation between AMPK activation and SREBP-1c activities in hepatocytes and in mouse liver (12–14). AMPK activation also suppresses the expression of key lipogenic enzymes such as acetyl-CoA carboxylase and fatty acid synthase (FASN) (15,16). Furthermore, recent studies demonstrate that activated AMPK specifically binds to and directly phosphorylates SREBP-1c and SREBP-2 and thereby inhibits the activities of SREBPs (17). Hence, compounds that specifically inhibit the SREBP pathway while activating AMPK can decrease the biosynthesis of both cholesterol and FA and can be useful in the treatment of alcoholic fatty liver.

Luteolin (3′,4′,5,7-tetrahydroxyflavone) is a naturally occurring flavonoid, abundant in plants worldwide such as fruits, vegetables, and certain herbal medicines. Since its isolation, various preclinical studies demonstrated that luteolin possesses a variety of biologic and pharmacologic activities, mainly involved in its antitumor, antioxidant, and anti-inflammatory functions (18–20). In addition, previous studies found that luteolin reduces lipogenesis via inhibition of FASN activation in prostate and breast cancer cells (21). Similarly, luteolin decreases TG accumulation by down-regulation of *SREBP1c* and *FASN* gene expression in HepG2 cells (22). However, it has not been reported if luteolin has a protective function in ethanol-induced liver damage and hepatic steatosis. In this study, we performed both in vivo and in vitro experiments to elucidate the activity of luteolin on ethanol-induced liver injury and steatosis.

Materials and Methods

Reagents. Luteolin (purity ≥98%) was from Ze Lang Phytoextraction Technology. Luteolin was dissolved in sterilized double-distilled water. The Lieber-Decarli diet was purchased from Dyets. The assay kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (TC), and LDL cholesterol were from ShenSuoYouFu.

Cell culture. The mouse AML-12 hepatocyte cells were cultured in DMEM/F-12 medium supplemented with 10% FBS, 1% penicillin/streptomycin, 0.1 μM dexamethasone, and insulin-transferrin-selenium (Invitrogen). Human fetal hepatocytes L-02 cells were cultured in DMEM (high-glucose concentration) supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were grown at 37°C with 5% CO_2.

Measurement of intercellular TGs. Cells were harvested by using 0.25% trypsin-EDTA solution, and total intracellular lipids were extracted from cell lysates by using a chloroform/methanol mix (2:1, v:v). Intracellular TG concentrations were measured by using a serum TG determination kit (Sigma-Aldrich). The TG concentrations were normalized to protein concentrations and expressed as μg of TGs/mg of protein.

Lipid droplet staining in cells. Cells were fixed for 10 min with 4% paraformaldehyde prepared in PBS, washed 3 times with PBS, and incubated for 1 h at room temperature in BODIPY 493/503 (at 20 μg/mL; Sigma-Aldrich) to detect intracellular lipid droplets. After washing with PBS, cells were stained with Hoechst 33342 (Molecular Probes) to detect nuclei.

Luciferase assay. The SREBP-responsive luciferase reporter pSRE-luciferase was constructed as follows: the promoter region (−588 to +92) of low density lipoprotein receptor (LDLR) was amplified by PCR and cloned into pGL3-Basic (Promega) vector by standard methods. For the luciferase assay, L-02 cells were transiently transfected with the luciferase construct and β-galactosidase (as an internal control). The cells were transfected by using PolyJet reagent (Invitrogen) according to the manufacturer's protocol. The cells were treated with ethanol (100 mM) and/or luteolin (25 μM) for 36 h, and luciferase activities were analyzed by using the luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Western blot analysis. Immunoblotting was performed as previously described (23). The primary antibodies including those against AMPKα, phosphorylated AMPKα (Thr172), and phosphorylated SREBP-1c (Ser372) were all from Cell Signaling Technology, and the tublin antibody was from Sigma-Aldrich. Western blotting was performed as previously described by using the ECL chemiluminescence kit (Thermo Scientific).

Mouse studies. The chronic and binge ethanol-fed mouse model was established as previously described (24). Six-wk-old C57BL/6 mice (male, weight: 20–23 g) were obtained from Shanghai SiLaiKe Laboratory Animal Company. The mice were randomly allocated into 3 groups: control, having no alcohol administration (n = 7); exposed to alcohol by using a chronic and binge ethanol feeding protocol (EtOH; n = 7); and administered daily 50 mg/kg of luteolin in addition to ethanol exposure (EtOH + Lut; n = 9). The entire experiment lasted 15 d. The mice were adapted to the environment by having free access to a liquid diet for the first 3 d. The diet was the Lieber-DeCarli diet (#710266, containing 53 g/L casein, 0.8 g/L DL-methionine, 85 g/L maltose dextrin, 13.25 g/L cellulose, 13.25 g/L corn oil, 9.27 g/L salt mix, 2.65 g/L vitamin mix, 0.53 g/L choline bitartrate, and 3 g/L xanthan gum; Dyets) as previously reported (25). Ethanol was introduced gradually by increasing its concentration to 1%, 2%, and 4% (v:v) for 3 consecutive days, followed by consumption of 5% (v:v) ethanol for the next 9 d. After a single dose of 30% ethanol on the last day, mice were killed 9 h later by terminal exsanguination under deep anesthesia (5% chloralhydrate, 10 mL/g body weight, intraperitoneal injection). Luteolin was administered by oral gavage with luteolin-containing water once a day at a dose of 50 mg/kg. Sterilized double-distilled water was given to the control and EtOH groups by oral gavage. The mice were maintained on a regular 12-h dark/light cycle with free access to water under a specific pathogen–free condition with temperature at 22 ± 1°C and humidity 60 ± 10%. The mouse tissues were snap-frozen in liquid nitrogen immediately after resection and stored at −80°C until further analysis. All mouse procedures and protocols were approved by the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, Chinese Academy of Sciences, Shanghai, China.

Measurement of serum and liver variables. Hepatic lipids were extracted with a previously reported method (26). Serum and liver ALT, AST, TG, TC, and LDL cholesterol concentrations were determined by colorimetric methods according to the procedure provided.

Analysis of hepatic histology. Following fixation of the livers with 4% paraformaldehyde, paraffin-embedded sections were subjected to standard hematoxylin and eosin staining and then studied by light microscopy. Hepatic steatosis was determined by staining of 8-μm thick frozen sections with Oil-Red-O (Sigma-Aldrich).

RNA isolation and real-time PCR analysis. Total RNA of liver tissues was isolated by using TRIzol Reagent (Invitrogen). Oligo (dT) primed

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RNA (1 μg) was reverse-transcribed with the Super-Script First-Strand Synthesis System (Invitrogen) to obtain cDNA. Real-time quantitative PCR was performed with the SYBR Green PCR system (Applied Biosystems) by using β-actin as an internal control for normalization. Primers used for each gene are listed in **Supplemental Table 1**.

Statistical analysis. Statistical analysis was performed by using a 1-factor ANOVA with post hoc multiple comparisons (Student-Newman-Keuls). A homogeneity test of variance was performed with all data to ensure the ANOVA could be used in the analysis. For data of unequal variance, a nonparametric test (Kruskal-Wallis H test) for 2 independent samples was performed. The data were expressed as means ± SEs, and significance was considered at $P < 0.05$. Data analyses were performed by using the statistical program SPSS 15.0 for Windows.

Results

Luteolin improves ethanol-induced hepatotoxicity and dyslipidemia. Compared with the control treatment, ethanol administration reduced body weight, and luteolin had no effect on ethanol-induced reduction of body weight (**Fig. 1**). Ethanol administration induced liver damage shown as a 561% increase in serum ALT concentration and a 73% increase in serum AST concentration upon ethanol administration (**Fig. 2***A*,*B*). The elevated serum ALT concentration in the EtOH group relative to the control group was significantly reduced by 43% upon luteolin administration but remained 277% higher than the control group (Fig. 2*A*). Ethanol consumption also induced dyslipidemia in the mice, shown as a 47% increase of serum TG, 44% increase of serum TC, and 95% increase of serum LDL cholesterol, whereas these variables in the EtOH + Lut group were not significantly different from the control group (Fig. 2*C*–*E*). Luteolin administration, on the other hand, abrogated ethanol-induced dyslipidemia in the mice. Luteolin reduced serum TG by 22%, serum TC by 39%, and serum LDL cholesterol by 52% (Fig. 2*C*–*E*). Collectively, these data indicate that luteolin possesses a protective effect on ethanol-induced liver damage in vivo.

Luteolin alleviates ethanol-induced fatty liver. Both TG and TC contents in the liver were markedly elevated to 126% and 69%, respectively, upon ethanol treatment (**Fig. 3***A*,*B*), indicating that liver steatosis was successfully induced by ethanol in our mouse model. Luteolin administration significantly reduced ethanol-induced elevation of liver TG concentration by 28% (Fig. 3*A*). However, luteolin had no significant effect on ethanol-induced increase of liver TC concentration (Fig. 3*B*).

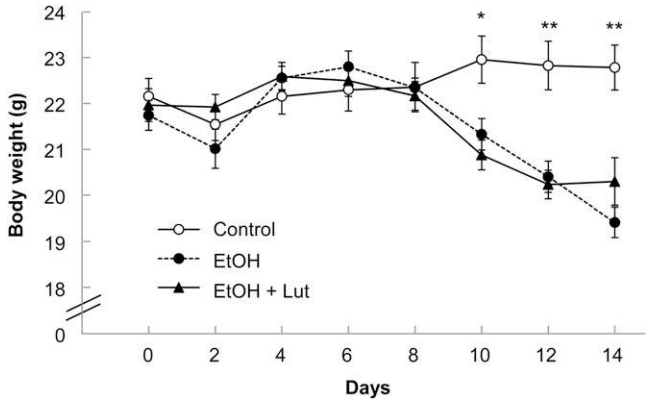

FIGURE 1 Body weight of male mice that consumed a control diet or an ethanol-containing diet with or without luteolin for 2 wk. Values are means ± SEs; n = 7–9. *$P < 0.05$ and **$P < 0.01$ between the control group and the other 2 groups. Control, mice that were not administered alcohol; EtOH, mouse group exposed to alcohol by using a chronic and binge ethanol feeding protocol; EtOH + Lut, mouse group that was administered daily 50 mg/kg of luteolin in addition to ethanol exposure.

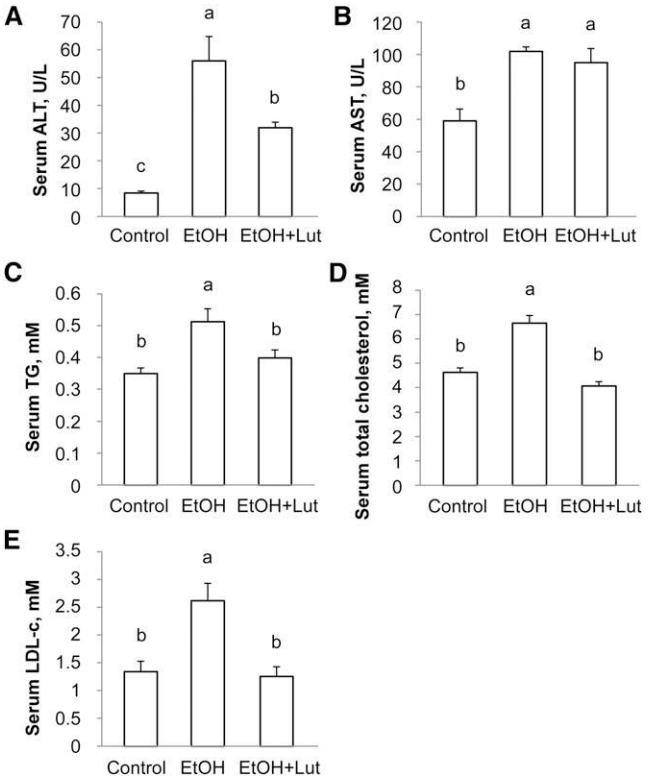

FIGURE 2 Serum concentrations of ALT (*A*), AST (*B*), TGs (*C*), total cholesterol (*D*), and LDL cholesterol (*E*) in male mice fed a control diet or an ethanol-containing diet with or without luteolin for 2 wk. Values are means ± SEs; n = 7–9. Means without a common letter differ, $P < 0.05$. ALT, alanine aminotransferase; AST, aspartate aminotransferase; control, mice that were not administered alcohol; EtOH, mouse group exposed to alcohol by using a chronic and binge ethanol feeding protocol; EtOH + Lut, mouse group that was administered daily 50 mg/kg of luteolin in addition to ethanol exposure; LDL-c, LDL cholesterol.

To confirm that luteolin inhibits ethanol-induced fat accumulation, we analyzed the histology of liver samples. Hematoxylin and eosin staining revealed that ethanol clearly caused lipid vacuoles in hepatocytes, although the ethanol-induced pathologic changes in the liver were prevented by luteolin treatment (Fig. 3*C*). Oil-Red-O staining also demonstrated that lipid accumulation was markedly enhanced by ethanol administration and prevented by luteolin treatment (Fig. 3*D*). Collectively, these histologic analyses indicate that ethanol is able to markedly induce liver steatosis that is abrogated by luteolin administration.

Luteolin relieves ethanol-induced expression of genes involved in lipid synthesis and inflammation. Ethanol significantly induced expression of TG-synthesis genes including a 560% increase of *Srebp1c*, 190% increase of *Fasn*, 48% increase of acetyl-CoA carboxylase (*Acc*), and 286% increase of stearoyl-CoA desaturase 1 (*Scd1*) in the liver (**Fig. 4***A*). Intriguingly, luteolin treatment significantly abrogated ethanol-induced expression of these genes. Compared with the EtOH group, luteolin treatment reduced the concentrations of *Srebp1c* by 79%, *Fasn* by 80%, *Acc* by 60%, and *Scd1* by 89% (Fig. 4A). On the other hand, the genes involved in cholesterol synthesis

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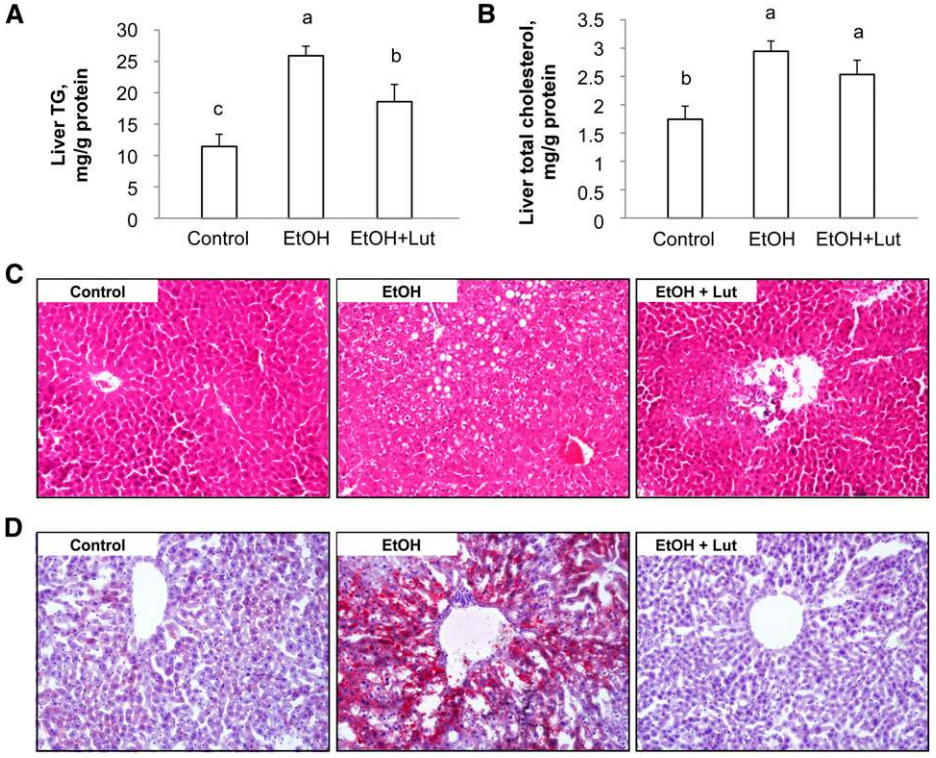

FIGURE 3 Hepatic concentrations of TGs (*A*) and total cholesterol (*B*) in male mice fed a control diet or an ethanol-containing diet with or without luteolin for 2 wk. Values are means ± SEs; n = 7–9. Means without a common letter differ, $P < 0.05$. Representative images (200×) of liver hematoxylin and eosin staining (*C*) and Oil-Red-O staining (*D*). Control, mice that were not administered alcohol; EtOH, mouse group exposed to alcohol by using a chronic and binge ethanol feeding protocol; EtOH + Lut, mouse group that was administered daily 50 mg/kg of luteolin in addition to ethanol exposure.

including *Srebp2*, 3-hydroxy-3-methylglutaryl–coenzyme A reductase (*Hmgcr*), 3-hydroxy-3-methylglutaryl–coenzyme A synthase (*Hmgcs*), and *Ldlr* were also induced by ethanol exposure, shown as a 263% increase of *Srebp2*, 203% increase of *Hmgcr*, 204% increase of *Hmgcs*, and 220% of *Ldlr* (Fig. 4*B*). However, luteolin significantly reduced *Srebp2* by 54%, *Hmgcr* by 39%, *Hmgcs* by 52%, and *Ldlr* by 74%.

We also analyzed inflammation markers including tumor necrosis factor α (*Tnfα*), interleukin 1β (*IL1β*), and interleukin-6 (*IL6*). We found that ethanol exposure significantly elevated *IL1β* by 204% and *IL6* by 173% (Fig. 4C). However, luteolin treatment reversed these ethanol-induced changes demonstrated as a 38% reduction of *IL1β* and 59% reduction of *IL6* (Fig. 4C), indicating that luteolin may relieve ethanol-induced liver damage by reducing inflammatory response in the liver.

Luteolin decreases ethanol-induced lipid accumulation in mouse hepatocytes. Ethanol treatment significantly elevated

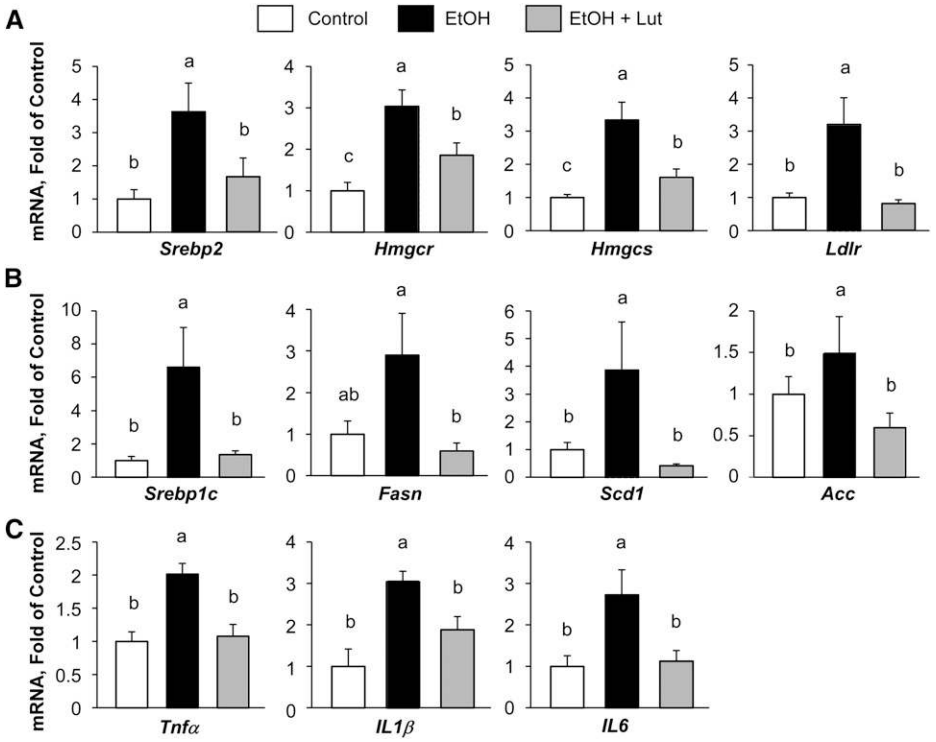

FIGURE 4 Hepatic mRNA abundances of multiple genes involved in the TG biosynthesis of TGs (*A*), cholesterol biosynthesis (*B*), and inflammation (*C*) in male mice fed a control diet or an ethanol-containing diet with or without luteolin for 2 wk. Values are means ± SEs; n = 7–9. Means without a common letter differ, $P < 0.05$. *Acc*, acetyl-CoA carboxylase; control, mice that were not administered alcohol; EtOH, mouse group exposed to alcohol by using a chronic and binge ethanol feeding protocol; EtOH + Lut, mouse group that was administered daily 50 mg/kg of luteolin in addition to ethanol exposure; *Fasn*, fatty acid synthase; *Hmgcr*, 3-hydroxy-3-methylglutaryl–coenzyme A reductase; *Hmgcs*, 3-hydroxy-3-methylglutaryl–coenzyme A synthase; *Il1β*, interleukin 1β; *Il6*, interleukin 6; *Ldlr*, low density lipoprotein receptor; *Scd1*, stearoyl-CoA desaturase 1; *Srebp1c*, sterol regulatory element-binding protein 1c; *Srebp2*, sterol regulatory element-binding protein 2; *Tnfα*, tumor necrosis factor α.

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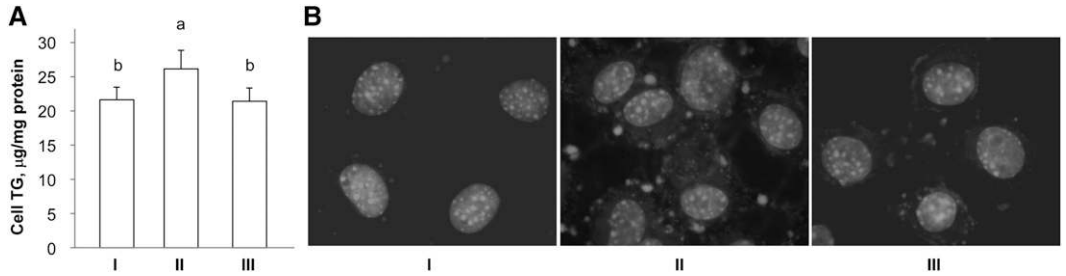

FIGURE 5 TG concentration in AML-12 cells upon different treatment (*A*). Values are means ± SEs; $n = 7–9$. Means without a common letter differ, $P < 0.05$. Representative images of BODIPY staining (*B*). Groups: I, without treatment; II, treated with ethanol (100 mM) for 36 h; III, treated with ethanol (100 mM) and luteolin (25 μM) for 36 h.

TG concentration by 21% in AML-12 cells (**Fig. 5***A*). On the other hand, luteolin administration markedly prevented an ethanol-induced increase of TG concentrations, shown as an 18% decrease upon luteolin treatment. (Fig. *5A*). We also used BODIPY staining to directly analyze lipid droplets in these cells. Ethanol exposure dramatically increased the intensity and quantity of lipid droplets in AML-12 cells, and such effect was almost completely abrogated by luteolin treatment (Fig. *5B*). Collectively, these results indicate that luteolin could reduce ethanol-induced lipid accumulation in hepatocytes, consistent with our results observed in the mouse model.

Luteolin alleviates alcoholic fatty liver via the AMPK/SREBP pathway. Ethanol treatment had a significant 160% increase in the luciferase activity of a SREBP responsive reporter in L-02 human hepatocytes (**Fig. 6***A*). Meanwhile, luteolin treatment abrogated the ethanol enhancement of the luciferase activity shown as a significant 67% reduction, indicating that luteolin may alleviate ethanol-induced lipid accumulation in hepatocytes through reduction of SREBP activity. To further confirm such hypothesis, we analyzed the expression concentrations of *Srebps* and their target genes in AML-12 hepatocytes. As expected, ethanol treatment elevated the mRNA concentrations of *Srebp1c* (70% increase) and *Srebp2* (463% increase) as well as some of their target genes including *Scd1* (110% increase), *Hmgcr* (36% increase), and *Ldlr* (326% increase) (Fig. *6B*). Luteolin treatment abrogated the effect of ethanol on these genes because it significantly reduced *Srebp1c* by 66%, *Srebp2* by 89%, *Scd1* by 51%, *Hmgcr* by 29%, and *Ldlr* by 86% (Fig. *6B*).

It was reported that SREBP-1c is negatively regulated by AMPK (13–15). In this study, we found that ethanol markedly inhibited the phosphorylation of AMPKα at Thr172, and luteolin reversed such effect (Fig. *6C*). Meanwhile, phosphorylation of SREBP-1c at Ser372 was inhibited by ethanol treatment and such effect was also abrogated by luteolin administration (Fig. *6C*). Collectively, these data indicate that ethanol may enhance the activity of SREBP-1c through reduction of SREBP-1c phosphorylation (and activation of its activity) by decreased AMPK activity. Luteolin may abrogate the effect of ethanol on SREBP-1c phosphorylation and activity via stimulation of AMPK activity.

Discussion

ALD is a major cause of morbidity and mortality in industrial countries (27). It is generally known that alcohol consumption is a leading etiologic factor in the pathogenesis of ALD, and hepatic steatosis is the precursor of more severe forms of liver injuries. A previous study showed that in humans the hepatic lipogenic pathway is activated after consumption of 24 g/d of ethanol (28). Although the biochemical and molecular mechanisms underlying ALD are complex, strategies to reduce fat accumulation in the liver would be effective to alleviate alcohol-induced liver injuries.

In this study, we used a chronic and binge ethanol feeding protocol to induce hepatic steatosis and injury in mice (24,29).

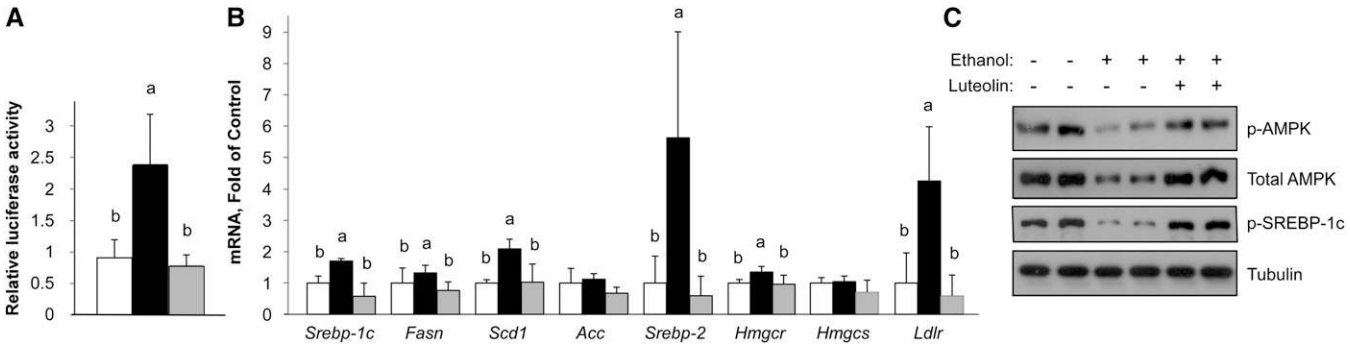

FIGURE 6 SREBP activity in L-02 hepatocytes (*A*) and the expression of lipogenic genes in AML-12 cells (*B*). Groups: open box, without treatment; black box, treated with ethanol (100 mmol/L) for 36 h; gray box, treated with ethanol (100 mmol/L) and luteolin (25 μmol/L) for 36 h. Values are means ± SEs; $n = 7–9$. Means without a common letter differ, $P < 0.05$. Phosphorylation of AMPK and SREBP-1c as analyzed by immunoblotting in AML-12 cells treated with ethanol (100 mM) and luteolin (25 μM) for 36 h as indicated (*C*). The treatment of the cells was the same as in Figure 5. *Acc*, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; *Fasn*, fatty acid synthase; *Hmgcr*, 3-hydroxy-3-methylglutaryl–coenzyme A reductase; *Hmgcs*, 3-hydroxy-3-methylglutaryl–coenzyme A synthase; *Ldlr*, low density lipoprotein receptor; p-, phosphorylated; *Scd1*, stearoyl-CoA desaturase 1; *Srebp1c*, sterol regulatory element-binding protein 1c; *Srebp2*, sterol regulatory element-binding protein 2.

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Our data indicate that oral administration of luteolin at 50 mg/kg every day significantly alleviated ethanol-induced steatosis and injury in the liver. In summary, luteolin markedly prevented ethanol-induced increases in ALT, serum TG, serum cholesterol, liver TG, and lipid accumulation in the liver as revealed by Oil-Red-O staining, inflammation markers in the liver, and expression of a number of lipogenic genes. We also used a cell model to analyze the effect of luteolin on lipid synthesis in hepatocytes. We found that luteolin reduced ethanol-induced lipid accumulation, SREBP activity, and expression of a few lipogenic genes. Collectively, these data demonstrate that luteolin effectively improved alcoholic fatty liver in vivo and in vitro. It is noteworthy that luteolin treatment did not affect hepatic cholesterol concentrations (Fig. 3*B*) but affected hepatic expression of cholesterol-related genes (Fig. 4*B*). It is likely that this discrepancy is caused by either a limitation of the number of mice used in the study or the administration of luteolin being not long enough.

Our studies indicate that luteolin may act on SREBP to relieve ethanol-induced hepatic steatosis. SREBPs belong to a major group of transcription factors that control the biosynthesis of cholesterol, FAs, and TGs (30). It was found that ethanol induces transcription of SREBP-regulated promoters via increased concentrations of mature SREBP-1 protein (7). Consistent with the previous finding, our data demonstrate that ethanol increased SREBP activity and elevated expression of *Srebp* target genes. In addition, our data indicate that the regulation of luteolin on SREBP activity is likely mediated by AMPK. It was recently demonstrated that activated AMPK specifically binds to and directly phosphorylates SREBP-1c and SREBP-2 and thereby inhibits the activities of SREBPs (17). We found that ethanol treatment markedly inhibited AMPK phosphorylation at Thr172 and phosphorylation of SREBP-1c at Ser372 (Fig. 6*C*). Consistently, luteolin treatment abrogated the inhibitory effects of ethanol on the phosphorylation of AMPK and SREBP-1c (Fig. 6*C*). Therefore, the elevated phosphorylation of SREBP-1c by luteolin could cause a reduction in SREBP activity, thereby leading to a decrease of lipogenesis in the liver.

It cannot be ruled out that other factors may contribute to the interventional effect of luteolin on ethanol-induced liver steatosis and injury. Accumulation of lipids in the liver makes it susceptible to inflammation and further injury (31), with the oxidative product contributing to inflammation by activation of NF-κB (32). The increased NF-κB activity can induce expression of proinflammatory cytokines such as TNF-α and IL-6. Consistently, we found that ethanol markedly induced expression of inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Fig. 4*C*). Interestingly, luteolin treatment significantly abrogated ethanol-induced expression of *Tnfα*, *IL1β*, and *IL6* (Fig. 4*C*), indicating that luteolin may relieve ethanol-induced liver injury through reduction of inflammation and oxidative damage in the liver. It was reported that some of these cytokines, such as IL-6 and TNF-α, play a substantial role in the induction of antioxidative genes such as metallothioneins in Kupffer cells, partly explaining the protective effect of luteolin on CCl_4-induced hepatotoxicity in mice (32). In addition, luteolin could reverse CCl_4-induced liver fibrosis by deactivating hepatic stellate cells (33). It will be of interest to investigate in the future whether luteolin also relieves alcohol-induced liver damage via Kupffer cells and stellate cells in the liver. Although the detailed molecular mechanisms underlying the protective functions of luteolin on alcoholic liver steatosis and damage remain to be fully elucidated, our studies indicate that luteolin may be an effective flavonoid for the intervention of ALD.

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