Metformin Ameliorates Ethanol-Induced Liver Triglyceride Accumulation by LKB1/AMPK/ACC Pathway

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Abstract

Background: Hepatic lipid accumulation is one of the main pathological features of alcoholic liver disease. Metformin is an AMPK activator that has been shown to have lipid lowering effects. The purpose of this study was to investigate whether metformin had a beneficial effect on lipid accumulation in the pathogenesis of ALD.

Methods: AML12 cells and male C57BL/6 mice were used to establish ALD models *in vitro* and *in vivo*, respectively. The effects of metformin on hepatocyte lipid accumulation and ALD progression in mice were detected. The role of LKB1/AMPK/ACC axis in metformin against ethanol-induced lipid accumulation was evaluated by siRNA and AAV-shRNA interference.

Results: Metformin reduced the ethanol-induced lipid accumulation in AML12 cells through activating AMPK/ACC and SREBP1c and inhibiting PPAR α . In addition, compared with control mice, metformin treatment inhibited ethanol-induced liver adipose accumulation and the increase of ALT and AST in serum. Interference with LKB1 attenuated the effect of metformin on ethanol-induced lipid accumulation both *in vitro* and *in vivo*.

Conclusion: Metformin protects against lipid formation in ALD by activating LKB1/AMPK/ACC axis. Thus, metformin has therapeutic potential for the prevention of ALD.

Background

Alcoholic liver disease (ALD) is a global healthcare problem caused by excessive alcohol consumption, including alcoholic steatosis, hepatitis, and fibrosis/cirrhosis [1].

Ethanol metabolism is associated with oxidative stress, malnutrition, gut endotoxin leakage, S-adenosylmethionine and glutathione depletion [2]. It is estimated that more than 95 percent of heavy drinkers may develop steatosis and about 35 percent of individuals develop more severe ALD, such as alcoholic hepatitis [3]. Although current researches have greatly improved our understanding of the pathogenesis of ALD, however, the clinical main management of ALD remains abstinence, supportive care, and nutritional management as in the past few decades. Unfortunately, liver transplantation is still the only definitive treatment for advanced ALD. Indeed, many anti-oxidant, anti-inflammatory and anti-apoptotic agents have been tested to improve ALD, however, most are not effective and are concomitant with an increased risk of obesity, infection and oncogenicity [2, 4]. One of the most important features of ALD is fat accumulation in hepatocytes. Excessive alcohol consumption leads to instability of lipid metabolism, inducing fatty acid synthesis and suppressing β -oxidation. Therefore, pharmacological strategies aimed at inhibiting liver triglyceride accumulation may be effective to reduce or reverse the prevalence of ALD.

AMP-activated protein kinase (AMPK) is critical regulator of glucose and lipid metabolism. It is a heterotrimer composed of the catalytic subunit AMPK- α , and two regulatory subunits AMPK- β and AMPK-

γ [5]. Activation of AMPK can phosphorylates and inactivates acetyl-CoA (ACC) and reduce malonyl-CoA levels, and thus prompts fatty acid oxidation [6]. AMPK also can regulate cell energy homeostasis via metabolic sensors SREBP1 and PPARα, and regulates their downstream target genes [7]. Liver kinase B1 (LKB1) and Ca²⁺/Calmodulin-dependent protein kinase kinase2 (CaMKK2) are the two main upstream kinases of AMPK, promoting it phosphorylation at Thr172 [8]. LKB1 phosphorylates AMPK in response to energy stress, while CaMKK2 phosphorylates AMPK after increasing intracellular calcium levels [5, 9]. Studies have shown that ethanol consumption induces hepatic adipogenesis and weakens lipid oxidation metabolism by inhibiting AMPK [10, 11]. Hence, AMPK is expected to be a powerful therapeutic target for reversing the progression of ALD.

Metformin is a drug used to treat type 2 diabetes (T2D), which also can activate AMPK by inhibiting the mitochondrial respiratory chain in the liver [12]. Studies have shown that metformin has potential therapeutic value in a variety of diseases, including non-alcoholic fatty liver disease (NAFLD) [13], obesity [14], polycystic ovary syndrome [15] and cancer [16]. However, the role of metformin in ALD still not clear. Given the ability of metformin to activate AMPK, we hypothesize that it can counteract alcohol-induced AMPK signal suppression and reduce lipogenesis in liver. In the present study, we investigated the effects of metformin on ALD both *in vitro* and *in vivo* experiments, and we assessed whether the protective effect of metformin resisting ALD is directly related to the upstream genes, such as LKB1 or CaMKK2. Meanwhile, we have assessed the biosafety of long-term use of metformin in mice.

Materials And Methods

Chemical and reagents

Metformin was purchased from Solarbio (Beijing, China). Pure ethanol was purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China). Antibodies against AMPKα (#AF3423), phospho-AMPKα (#AF6423), ACC (#AF6421), phospho-ACC (#AF3421), LKB1 (#AF6453), phospho-LKB1 (#AF3453), CaMKK2 (#DF4793) and phospho-CaMKK2 (#AF4487) were purchased from Affinity Biosciences (OH. USA). Antibody against β-actin (#4970) was purchased from Cell Signaling Technology (Boston. USA).

Cell culture and treatment

Mouse normal hepatocyte AML12 cell line was supplied by the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM/F12 media containing 10% fetal bovine serum, ITS supplement and 40 ng/ml dexamethasone at 37 °C with 5% CO₂. For the cell experiments, the cells were treated with 200 mM ethanol or 200 mM ethanol combined with 2 mM metformin for 24 h.

Nile red staining

AML12 cells were washed by PBS, and then stained with Nile Red solution (0.1 mg/ml) at 37 °C for 20 min. After that, cells were fixed with 4% formaldehyde for 10 min, and then counterstained with DAPI for

10 min.

Animal models

Healthy adult male C57BL/6 mice (8-10 weeks old) were purchased from the Shanghai SLAC experimental animal Co., Ltd (Shanghai, China). The mice were housed in an environmentally controlled room with a 12-hour light/dark cycle and fed a normal diet for one week to acclimatize. Then, the mice were randomly divided into several groups, and NIAAA mouse model was used to establish alcoholic fatty liver disease [17]. Adeno-associated virus (AAV8) short hairpin RNAs (shRNAs) targeting LKB1, CaMKK2 were used to interfere the expression of target genes by tail intravenous injection and a scramble shRNA was injected as control. After the mice were sacrificed, their livers and serum were collected for subsequent tests. The protocols for animal care and use were approved by the animal ethics committee of Fujian Normal University.

Serum and hepatic biochemistry measurements

The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglyceride (TG) and total cholesterol (TC) in serum were measured using Hitachi LABOSPECT 008 (Hitachi High-Tech Co., Japan). Total contents of hepatic TG were measured by a colorimetric TG assay kit from Absin (Shanghai, China).

Immunoblotting

AML12 cell and liver tissues protein lysates were prepared using RIPA buffer containing complete EDTA-free protease inhibitor cocktail. A total of 50 ng of protein was subjected to 10% SDS-PAGE and transferred to a 0.22 μ m NC membrane (Millipore, Bedford, MA, USA). The membranes were incubated with specific antibodies and the density of respective bands was analyzed by the Chemi-Doc XRS imaging system (Bio-Rad, Hercules, CA).

Total RNA isolation and quantitative real-time PCR

Total cellular RNA was extracted from AML12 cells and liver tissues using RNAiso Plus kit (Takara Bio Inc., Shiga, Japan). The preparation of the first-strand cDNA was conducted following the instruction of the PrimeScript™ RT reagent Kit (Takara Bio Inc., Shiga, Japan). The mRNA expression levels of target genes were measured by Takara SYBR premix Taq quantitative PCR system (Takara Bio Inc., Shiga, Japan). The sequences of the primers used in the study are in Table 1. The levels of target genes transcript were normalized to housekeeping gene GAPDH. The $2^{-\Delta\Delta Ct}$ method was used for the data analysis.

Transfection of siRNA

Cells were transfected with the LKB1 siRNA or CaMKK2 siRNA using the Lipofectamine 2000 siRNA Transfection Reagent (Invitrogen, USA), and drug treated at 24 hours after transfection. The sequences of

LKB1 siRNA and CaMKK2 siRNA used in this study were in Table 2.

HE and oil red O staining

Liver tissue samples were fixed in 4% polyformaldehyde for 12 h, and then five-micrometre hepatic tissue sections were cut and stained with haematoxylin/eosin staining kit. For Oil red O staining, cryosections were stained with Oil red O and then counterstained with haematoxylin.

Statistical analysis

All data are reported as mean $\pm$ SEM. Statistical comparison between groups was done using Student's *t* tests or one-way ANOVA. A value of $P < 0.05$ was considered as statistically significant (Prism 5.0, Graphpad Software, San Diego, CA, USA).

Results

Metformin inhibits ethanol-induced hepatocytes lipogenesis *in vitro*

To investigate the effect of metformin on ethanol-induced lipogenesis in hepatocytes, we first evaluated the total lipid content of AML12 cell with Nile red staining. The results showed that ethanol significantly induced the lipid accumulation in AML12 cells, while metformin effectively reduced lipid content in comparison with the ethanol group (Fig. 1a). Meanwhile, the concentration of TG in AML12 also have been detected by a TG colorimetric assay kit. After ethanol treatment, TG levels rose from 40.7 ± 4.1 $\mu\text{g}/\text{mg}$ to 75.3 ± 7.1 $\mu\text{g}/\text{mg}$, while metformin reversed cellular TG levels to 49.5 ± 5.8 $\mu\text{g}/\text{mg}$ (Fig. 1b). In addition, total cellular RNA was extracted for quantitative analysis of the mRNA expression of the sterol regulatory element-binding protein 1c (SREBP-1c) and peroxisome proliferator-activated receptor α (PPAR- α). They are involved in *de novo* synthesis and β oxidation of fatty acids, respectively. Our data have shown that ethanol not only increased the expression of SREBP-1c, but also inhibited the expression of PPAR- α , thus promoting lipid accumulation in AML12 cells. As expected, metformin treatment effectively neutralized the effects of ethanol on the expression of these genes in AML12 cells (Fig. 1c-d).

The involvement of AMPK in the effect of metformin on alleviating ethanol-induced lipid synthesis in hepatocytes

AMPK is a key molecule in the regulation of biological energy metabolism and plays a crucial role in *de novo* lipid synthesis. A previous study showed that metformin alleviates TG accumulation in NAFLD via mediating AMPK-SREBP-1c signal [18]. To confirm that AMPK is involved in metformin inhibition of ethanol-induced fat accumulation in AML12 cells, we assessed the phosphorylation levels of AMPK signal-related proteins by Western blotting. Metformin increased the phosphorylation of AMPK and its downstream target acetyl-CoA carboxylase (ACC) (Fig. 2a-b). Liver kinase B1 (LKB1) and calcium/calmodulin-dependent protein kinase 2 (CaMKK2) and are upstream kinases known to activate AMPK phosphorylation [19]. To estimate the role of LKB1 and CaMKK2 in ethanol-induced triglyceride accumulation, we measured their expression by immunoblotting. The resulted showed that ethanol

significantly reduced the phosphorylation level of LKB1, but had no markedly effect on CaMKK2 phosphorylation. While co-treatment cells with metformin significantly resisted the reduction of LKB1 phosphorylation caused by ethanol (Fig. 2c-d).

Metformin ameliorates ethanol-induced hepatocytes triglyceride accumulation by LKB1/AMPK pathway

To further elucidate the effects of LKB1 and CaMKK2 on metformin resistance to ethanol-induced triglyceride accumulation in hepatocytes, we selected the one with the best interference efficiency among three siRNA which targeting LKB1 and CaMKK2 respectively for the follow-up experiments (Fig. 3a). After siRNA treatment, cellular TG levels in AML12 cells were measured. The results showed that knock-down LKB1 significantly resisted the effect of metformin, while knock-down CaMKK2 had relatively little effect (Fig. 3b). Data from qPCR for SREBP-1c and PPAR- α mRNA expression showed that LKB1-siRNA treatment attenuated the effect of metformin on these genes (Fig. 3c-d). Nile red staining also showed Knockdown LKB1 was effective in reversing metformin's effect on hepatocyte lipid droplets accumulation, but not CaMKK2 (Fig. 3e).

Meanwhile, the expression of related proteins in AMPK signaling pathway were detected by immunoblotting. Both LKB1- and CAMKK2-siRNA inhibited the expression of their target protein substrate and phosphorylation levels, respectively (Fig. 4a-b). In addition, LKB1 specific siRNA abolished the influence of metformin on the phosphorylation of AMPK and ACC, but did not affect their basal protein expression. Unlike LKB1- siRNA, CAMKK2-siRNA does not seem to have a significant effect on the phosphorylation of AMPK and ACC induced by metformin (Fig. 4c-d). These results suggested that metformin inhibited ethanol-induced hepatocytes triglyceride accumulation main through LKB1/AMPK pathway.

Metformin attenuated alcohol-induced fatty liver in chronic-binge ethanol-fed mice

Ethanol exposure is one of the major causes of hepatic steatosis. To evaluate the effects of metformin on alcohol-induced hepatic steatosis, we established alcoholic fatty liver in mice based on the chronic-binge alcohol feeding mouse model [17]. We found that metformin partially counteracted the weight loss in the mice caused by alcohol consumption (Fig. 5a). After the mice were sacrificed, we collected their livers and serum. Morphological examination showed a whiter fatty livers in EtOH group compared with the pair-fed control group, but the EtOH + Metformin group mice had relatively healthy livers. In addition, the liver index percentage in the EtOH group significantly increased when compared with the control, and it was significantly reduced by metformin administration (Fig. 5b). The levels of TG and total cholesterol in serum were also measured. The results showed that metformin reduced the increase of TG level induced by alcohol, but had no significant effect on total cholesterol (Fig. 5c). Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels are often used as important indicators of liver injury or hepatotoxicity. The results showed the levels of ALT and AST in serum were significantly elevated in the EtOH group, as compared with the control group. However, metformin treatment significantly attenuated the elevation in both these enzymes which induced by alcohol (Fig. 5c). Consistent with these results, HE and oil red O staining revealed abundant lipid droplets in ethanol

exposure group livers compared with the pair-fed control group; however, metformin administration significantly ameliorated alcohol-induced hepatic fat accumulation (Fig. 5d).

Activation of LKB1/AMPK signaling improves alcohol-induced hepatic steatosis

To further study the role of AMPK signaling pathway on metformin-mediated improvement in hepatic steatosis, we used shRNAs to interfere the expression of LKB1 and CaMKK2. The results showed that knock-down of LKB1 attenuated the effect of metformin on alcohol-induced serum TG elevation, while down-regulation of CaMKK2 did not (Fig. 6a). However, no significant effect was observed on the concentration of total cholesterol in serum when interfering with either of the two genes (Fig. 6b). In addition, interfering with LKB1 slightly reduced the effects of metformin on ALT and AST in alcoholic fatty liver, but CaMKK2 did not (Fig. 6c-d). Meanwhile, hematoxylin eosin (H&E) staining showed that compared with the EtOH+Met group, there were more lipid vacuoles in the EtOH+Met+LKB1-siRNA group, while there was no significant difference in EtOH+Met+CaMKK2-siRNA group (Fig. 6e). Consistent with this, oil red O staining showed EtOH+Met+LKB1-siRNA group had more fat deposition than EtOH+Met group (Fig. 6f). These results suggested that LKB1-siRNA inhibited the effect of metformin on alcohol induced liver lipid accumulation, while CaMKK2-siRNA had a weak influence. Then, we assessed the expression of proteins associated with the AMPK signaling pathway. Knocking down LKB1 partially inhibited metformin-induced phosphorylation of AMPK and downstream ACC phosphorylation, while knocking down CAMKK2 had less effect (Fig. 6g).

Discussion

With the global prevalence of ALD, the research on ALD has increased rapidly [20]. Currently, there are numerous studies trying to discover drugs for effective treatment of ALD, including the use of anti-inflammatory, anti-oxidant, anti-apoptotic, and modulation of gut microbiota agents [21]. However, there is still no satisfactory treatment in clinical practice. Therefore, developing an anti-ALD agent with hepatoprotective properties will be favorable to people health.

Metformin has been used to treat type 2 diabetes for more than 60 years because of its safety and low cost. We also found that there were no significant differences in histology and serum biochemistry in mice treated with metformin at 200 mg/kg for 2 months compared to the control group (supplementary Fig. 1). Recent studies have found that in addition to treating type 2 diabetes, metformin has benefits in aging and various diseases, including obesity, liver disease, cardiovascular disease and cancer [22, 23]. Previously, studies have showed that metformin inhibits high glucose and high insulin induced TG accumulation in hepatocytes via inhibiting the expression of stearyl-coenzyme A desaturase 1 (SCD1) or miR-33b [18, 24]. Song et al. suggested that metformin alleviates hepatic steatosis by mediating autophagy [25]. Meanwhile, many studies have shown that metformin can reverse fatty liver disease by activating AMPK signaling [22]. However, little is known about metformin in alcoholic liver disease. This may be because alcohol consumption may increase the risk of metformin-induced lactate poisoning. In fact, clinical data showed that metformin is unsafe only for a small subset of patients with severe liver,

kidney or heart dysfunction [26]. The benefits of metformin may outweigh the disadvantages in patients with early alcoholic liver disease.

Zhu et al. found metformin improved insulin resistance and reversed alcohol-induced liver injury in rat via the activation of AMPK and normalized adiponectin signaling [27]. Patel et al. also found that combined treatment with probiotics and metformin alleviated liver damage caused by alcohol [28].

In the present study, we evaluated whether metformin improved ethanol-induced fatty liver disease both *in vitro* and *in vivo*. We demonstrated that metformin downregulated lipogenesis genes and upregulated fatty acid oxidation genes in ethanol-treated AML12 cells, thereby reducing intracellular TG accumulation. Consistent with *in vitro* results, metformin administration protected against ethanol-induced hepatic steatosis in chronic-binge alcohol feeding mice. Furthermore, we revealed that metformin regulates AMPK signaling by activating LKB1, but not CaMKK2, to improve alcoholic fatty liver disease. Taken together, our present study demonstrated that metformin inhibits ethanol-induced TG accumulation, which might prevent the progression of ALD. Altogether, metformin could be a potential therapeutic agent for preventing ALD, and conducting further research and safety assessment for ALD in clinical might be worthwhile.

Abbreviations

ALD: Alcoholic liver disease; AMPK: AMP-activated protein kinase; ACC: Acetyl-CoA; LKB1: Liver kinase B1; CaMKK2: Ca²⁺/Calmodulin-dependent protein kinase kinase2; T2D: Type 2 diabetes; NAFLD: Nonalcoholic fatty liver disease; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; TC: Total cholesterol; TG: Triglycerides; SREBP-1c: Sterol regulatory element-binding protein 1c; PPAR- α : Peroxisome proliferator-activated receptor α ; SCD1: stearyl-coenzyme A desaturase 1.

Declarations

Ethics approval and consent to participate

The protocol was approved by the Ethics Committee of Fujian normal University.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Yi Lv and Fotian Xie conducted the experiments, Yi Lv and Dongmei Wang drafted this article. Dongmei Wang, Jia Xiao and Kwok Fai So helped to revise the manuscript. Yi Lv and Jia Xiao participated in its design and coordination. All authors read and approved the final manuscript.

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Tables

Due to technical limitations, table 1-2 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Metformin reduces lipid accumulation in AML12 cells treated by EtOH. **a** Nile red staining (scale bar = 100 μ m). **b** Quantities analyses of cellular triglyceride concentration. **c** The mRNA expression of SREBP1c. **d** The mRNA expression of PPAR- α . * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, vs. EtOH group.

Figure 2

Metformin activates LKB1/AMPK signal pathway in AML12 cells. a The protein expression of p-AMPK and AMPK. b The protein expression of p-ACC and ACC. c The protein expression of p-LKB1 and LKB1. d The protein expression of p-CaMKK2 and CaMKK2. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, vs. EtOH group.

Figure 3

Interference with LKB1 attenuated the effects of metformin on ethanol-induced TG accumulation in AML12 cells. a The mRNA expression of LKB1 and CaMKK2. b Quantities analyses of cellular triglyceride concentration. c The mRNA expression of SREBP1c. d The mRNA expression of PPAR-α. e Nile red staining (scale bar = 100 μm). *p<0.05, **p<0.01, ***p<0.001 vs. control group; #p<0.05, ##p<0.01, ###p<0.001, vs. EtOH group; &p<0.05, &&p<0.01, &&&p<0.001, vs. EtOH+Met group.

Figure 4

Interference with LKB1 attenuated the effects of metformin on AMPK/ACC signaling in AML12 cells. a The protein expression of p-LKB1 and LKB1. b The protein expression of p-CaMKK2 and CaMKK2. c The protein expression of p-AMPK and AMPK. d The protein expression of p-ACC and ACC. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, vs. EtOH group.

Figure 5

Metformin ameliorates alcohol-induced fatty liver in mice. **a** The change of body weight. **b** Liver and liver index of mice. **c** The levels of TG, TCHO, ALT and AST in serum. **d** HE and oli red O staining. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, vs. EtOH group.

Figure 6

Metformin ameliorates alcohol-induced fatty liver by LKB1/AMPK/ACC pathway. a The level of TG in serum. b c. c The level of ALT in serum. d The level of AST in serum. e HE staining (scale bar = 100 μ m). f Nile red staining (scale bar = 100 μ m). g The proteins expression of AMPK, ACC, LKB1 and CaMKK2. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, vs. EtOH group; & $p < 0.05$, && $p < 0.01$, &&& $p < 0.001$, vs. EtOH+Met group.

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