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Enhanced AMPK phosphorylation contributes to the beneficial effects of *Lactobacillus rhamnosus* GG supernatant on chronic alcohol-induced fatty liver disease

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Abstract

Background: We have previously demonstrated that *Lactobacillus rhamnosus* GG culture supernatant (LGGs) prevents acute alcohol exposure-induced hepatic steatosis and injury. The protective effects of LGGs were attributed to the improved intestinal barrier function leading to decreased endotoxemia. The purpose of this study was to determine whether LGGs was effective in protecting against chronic alcohol-induced hepatic steatosis and injury, and to evaluate the underlying mechanisms of LGGs on hepatic lipid metabolism.

Methods: C57BL/6N mice were fed liquid diet containing 5% alcohol or pair-fed isocaloric maltose dextrin for 4 weeks. LGGs at a dose equivalent to 10⁹ CFU/day/mouse was given in the liquid diet. Hepatic steatosis, liver enzymes and hepatic apoptosis were analyzed.

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AUTHOR CONTRIBUTIONS

M. Z., C. W., C. W., and Y. W. performed experiments and analyzed data. M. Z., C. M. and W. F. prepared the manuscript. W. F. conception and research design. C. M., Y. W and W. F. interpreted the results.

#M. Zhang and C. Wang contributed equally to this work.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

Results: LGGs prevented alcohol-mediated increase in hepatic expression of lipogenic genes, sterol regulatory element binding protein-1 and Stearoyl-CoA desaturase-1 ; and increased the expression of peroxisome proliferator activated receptor- α , peroxisome proliferator-activated receptor gamma coactivator protein-1 α and carnitine palmitoyltransferase-1 leading to increased fatty acid β -oxidation. Importantly, chronic alcohol exposure decreased AMPK phosphorylation and increased acetyl-CoA carboxylase (ACC) activity, which were attenuated by LGGs administration. LGGs also decreased Bax expression and increased Bcl-2 expression, which attenuated alcohol-induced hepatic apoptosis. These LGGs regulated molecular changes resulted in the attenuation of chronic alcohol exposure-mediated increase in hepatic fat accumulation and liver injury.

Conclusions: Probiotic LGG culture supernatant is effective in the prevention of chronic alcohol exposure-induced hepatic steatosis and injury. LGGs likely exerts its beneficial effects, at least in part, through modulation of hepatic AMPK activation and Bax/Bcl-2-mediated apoptosis.

Keywords

probiotics; alcohol; liver; lipid; AMPK

INTRODUCTION

Excessive alcohol exposure results in the development of fatty liver disease (steatosis). Although mild liver steatosis was initially considered to be generally benign, increasing evidence demonstrates that increased fat accumulation sensitizes the liver to a deleterious second “hit”, leading to progression to more advanced liver diseases, such as steatohepatitis, fibrosis, cirrhosis and even liver cancer (1, 11, 23). Eliminating or halting the hepatic fat accumulation may represent an attractive strategy for inhibition/treatment of alcoholic liver disease (ALD).

Our previous studies demonstrated that administration of probiotics improves liver enzymes in alcoholic steatohepatitis patients (19), and prevents or reverses alcohol-induced fatty liver and liver injury in experimental animals (32, 34). We further showed that *Lactobacillus rhamnosus* GG culture supernatant (LGGs) prevented acute alcohol-induced hepatic steatosis and injury (34). Mechanistic studies revealed that LGG, both the bacteria and secreted factors, attenuated alcohol-induced intestinal barrier dysfunction and endotoxemia (which may trigger a hepatic inflammatory response). However, whether LGGs can be used to prevent chronic alcohol-induced liver steatosis is unknown.

Alcohol induced fat accumulation occurs due to both increased *in situ* lipogenesis and reduced fatty acid β -oxidation (3, 5, 38). AMP-activated protein kinase (AMPK) is a key metabolic master switch which phosphorylates target enzymes involved in lipid metabolism in many tissues, including the liver (30, 39). It increases fatty acid oxidation by inactivating acetyl-CoA carboxylase (ACC). ACC is a rate limiting enzyme for fatty acid biosynthesis in the liver. It catalyzes the conversion of acetyl-CoA to malonyl-CoA, which is a precursor for the synthesis of fatty acids (31). Furthermore, AMPK also modulates sterol regulatory element binding protein-1 (SREBP-1) (39) and peroxisome proliferator activated receptor- α (PPAR- α) (20, 36), which are transcription factors playing a critical role in the regulation of

the enzymes responsible for the synthesis of cholesterol, fatty acids, and triglycerides in the liver and other tissues. Alcohol ingestion caused a reduction of AMPK activity and increased fat accumulation in the liver (39). In this study, we showed that LGGs pretreatment increased hepatic AMPK phosphorylation and PPAR- α expression, decreased SREBP-1 expression and consequently attenuated alcohol-induced hepatic steatosis and injury.

MATERIAL AND METHODS

Culture of LGG.

LGG was purchased from American Type Culture Collection (ATCC 53103; Rockville, MD) and was cultured in *Lactobacillus* De Man, Rogosa, and Sharpe broth (MRS broth; Difco; BD, Sparks, MD) at 37°C in accordance with ATCC guidelines. LGGs was prepared as described in our previous study (34).

Animal model.

Male C57BL/6N mice were obtained from Harlan Laboratories (Indianapolis, IN). The mice were pair-fed liquid diet (Lieber DeCarli) for 4 weeks. The diet contains 17% of energy as protein, 40% as corn oil, 7.5% as carbohydrate, and 35.5% as either alcohol (alcohol-fed, AF) or isocaloric maltose dextrin (pair-fed, PF). LGGs at a dose at equivalent to 10⁹ CFU/day/mouse was given to the mice in the liquid diet. Each group had 4–10 mice. Plasma and tissue samples were collected for assays. All mice were treated according to protocols reviewed and approved by the Institutional Animal Care and Use Committee of the University of Louisville.

Cell culture and treatment.

H4IIE, a hepatocarcinoma cell line, was purchased from ATCC. H4IIE cells were grown in EMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FBS, at 37°C in an atmosphere containing 5% CO₂. H4IIE cells were treated with 100 mM ethanol or 1% LGGs for 24 hours, or pre-treated with an AMPK inhibitor, Compound C (25 µM), for 2 h followed by an incubation with 100 mM ethanol or 1% LGGs for additional 24 hours.

Biochemical assays.

Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using ALT and AST Assay Kits (Thermo Fisher Scientific Inc., Middletown, VA), respectively. Plasma free fatty acids (FFA) were quantified using a commercial kit from Wako Chemicals (Richmond, VA).

Fatty liver assessment.

Liver triglyceride assay were performed as described in our previous study(34) using the Triglyceride Kit (Thermo Fisher Scientific Inc.). Liver FFA was assayed as described above. Fatty liver was also determined by Oil red O staining of frozen liver sections and then studied by light microscopy.

Liver apoptosis analysis.

Formalin-fixed paraffin liver sections were stained for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) with the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Chemicon, CA, USA), according to the manufacturer's instructions. At least five views in each slide were randomly selected and quantitated for positive staining. Five animals in each group were studied. Results were presented as TUNEL positive cells per 500 cells.

Western blot analysis.

Tissues were homogenized, and total protein was extracted using RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 40 mM NaF, 4 mM Na₃VO₄, 1 mM PMSF, 1% protease inhibitor cocktail) and centrifuged at 14,000 g for 10 min. The supernatants were collected. Aliquots containing 30 µg protein were loaded onto 4–15% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membrane. Membranes were probed using antibodies against phospho-AMPK α , phospho-ACC, AMPK, ACC (Cell Signaling, Danvers, MA), SREBP-1c, CPT-1, Bcl-2, Bax, β -actin and GAPDH (Santa Cruz Biotechnologies, Santa Cruz, CA). Protein bands were quantified by densitometry analysis with a normalization with β -actin, GAPDH or Ponceau S staining.

Quantitative Real time RT-PCR.

Total RNA was isolated from liver with Trizol according to manufacturer's protocol (Invitrogen, Carlsbad, CA) and reverse-transcribed using GenAmp RNA PCR kit (Applied Biosystems, Foster City, CA). The cDNA was amplified in 96-well reaction plates with a SYBR green PCR Master Mix (Applied Biosystems) on an ABI 7500 real-time PCR thermo cycler. The sequences of forward and reverse primers are listed in Table 1. β -actin mRNA expression was used to normalize the obtained data. Relative mRNA expression was calculated using the $\Delta\Delta C_t$ method.

Statistical analysis.

Data are expressed as means $\pm$ SE. ANOVA with Newman-Keuls multiple-comparison test was used for the determination of statistical significance. Differences between groups were considered significant at $P < 0.05$.

RESULTS

LGGs decreases chronic alcohol-induced hepatic lipid accumulation and liver injury.

To investigate the effect of LGGs on chronic alcohol-induced hepatic lipid accumulation, we measured hepatic steatosis with histological analysis and biochemical triglyceride determination. Mice exposed to chronic alcohol treatment had significantly increased hepatic lipid accumulation compared with control mice, as evaluated by Oil red O staining. However, LGGs treatment significantly attenuated fatty liver (Fig. 1A). Confirming the histological observations, we found that the hepatic triglyceride content was significantly elevated in alcohol-exposed mice compared with control mice. Pretreatment with LGGs markedly decreased the chronic alcohol-induced hepatic triglyceride accumulation (Fig. 1B).

In addition, the levels of plasma and hepatic FFA were increased by chronic alcohol exposure, and these increases were blocked by LGGs supplementation (Fig. 1C and 1D). To assess liver injury, levels of liver enzymes were measured. Plasma ALT and AST levels were significantly elevated by alcohol exposure. LGGs pretreatment significantly attenuated these elevations (Fig. 2A and 2B). Taken together, LGGs treatment attenuated chronic alcohol-induced hepatic steatosis and liver injury.

LGGs down-regulates SREBP-1 and up-regulates PPAR- α .

SREBP-1 and PPAR- α are transcription factors in the regulation of hepatic lipid metabolism, and play central roles in *de novo* lipogenesis and fatty acid oxidation. To determine the effects of LGGs on the chronic alcohol exposure-induced lipid synthesis and clearance, we measured hepatic levels of SREBP-1 c protein and PPAR- α transcript levels. As shown in Figure 3A, chronic alcohol exposure markedly increased hepatic SREBP-1 c protein expression, which was prevented by LGGs supplementation. Alcohol feeding decreased hepatic PPAR- α and peroxisome proliferator-activated receptor gamma coactivator protein-1 α (PGC-1 α) mRNA expression; this decrease was inhibited by LGGs supplementation (Fig. 3B). Furthermore, we found that LGGs treatment significantly decreased alcohol exposure-induced hepatic elevation of Stearoyl-CoA desaturase-1 (SCD-1) expression (Fig. 3C), which is a key enzyme responsible for fatty acid synthesis and a target of SREBP-1. On the other hand, LGGs pretreatment significantly increased carnitine palmitoyltransferase-1 (CPT-1) protein expression in the liver of chronic alcohol-exposed mice (Fig. 3D). Our results suggest that LGGs is effective in positively modulating chronic alcohol-induced alterations in lipogenesis and fat clearance mediated by SRBEP-1c and PPAR- α .

Effects of LGGs on hepatic AMPK signaling.

Previous studies showed that AMPK activation plays a critical role in hepatic lipid metabolism by regulating transcription factors including SREBP-1c and PPAR- α . Phosphorylation at Thr-172 of AMPK (p-AMPK α) is known to activate AMPK (15). Therefore, we measured the hepatic phosphorylation levels of AMPK in mice treated with chronic alcohol and LGGs. As expected, alcohol markedly decreased hepatic AMPK phosphorylation, and this was reversed by LGGs supplementation (Fig. 4A). It is known that activation of AMPK decreases ACC activity via phosphorylation (25, 31). As shown in Figure 4B, mice exposed to alcohol had a markedly lower level in ACC phosphorylation in the liver compared with control mice. Importantly, this decrease was completely inhibited by LGGs supplementation (Fig. 4B). To further confirm the effect of LGGs on AMPK-mediated ACC phosphorylation, we performed an *in vitro* study using H4IIE cells. Alcohol treatment decreased the levels of phosphorylated AMPK. Moreover, LGGs addition significantly inhibited this reduction (Fig. 5A). As expected, treatment with Compound C, an AMPK inhibitor, abolished LGGs-induced ACC phosphorylation (Fig. 5B), indicating an essential role of AMPK in the LGGs-mediated deactivation of hepatic lipogenic gene expression in the mice exposed to alcohol.

LGGs attenuates alcohol-induced hepatic apoptosis.

Using TUNEL staining, we found the liver sections of the alcohol-fed mice exhibited an expected increase in apoptosis compared to wild type controls. LGGs pretreatment significantly decreased the TUNEL positive cells in the hepatic tissues (Fig. 6A). To determine the molecular mechanism by which chronic ethanol exposure induces greater hepatocyte apoptosis in control mice compared with that in LGGs-pretreated mice, the expression of anti-apoptotic and pro-apoptotic proteins was examined in pair-fed mice, alcohol-fed mice and LGGs pretreated alcohol-fed mice. As shown in Figure 6B, there was no significant alteration in Bcl-2 protein expression in alcohol-fed mice, but LGGs supplementation significantly increased hepatic Bcl-2 protein levels. By comparison, alcohol-feeding increased hepatic Bax protein levels, and this elevation was attenuated by LGGs supplementation (Fig. 6C).

DISCUSSION

We previously demonstrated that the probiotic strain, LGG, in the form of culture broth (containing live bacteria, LGG) provided effective treatment for alcohol-induced fat accumulation and injury in a mouse model of chronic alcohol exposure (32). Further research showed that LGG culture supernatant (without live bacteria, LGGs) prevented acute alcohol exposure-induced hepatic steatosis and injury (34). The protective effects of LGG have been attributed to multiple factors. LGG administration prevented the alcohol-induced pathologic changes in the gut microbiome (dysbiosis) (4) and endotoxemia (32), improved the intestinal microenvironment, and corrected the pH changes in the large intestine (4). Alcohol-induced down-regulation of intestinal tight junction and mucus proteins was prevented by LGG/LGGs, which led to an improved intestinal barrier function and inhibited endotoxemia (32, 34). Increased endotoxin concentrations alter hepatic responses to multiple cellular and molecular processes, such as inflammation, oxidative stress and lipid metabolism. We have shown that LGG/LGGs supplementation attenuated alcohol-induced hepatic inflammation by inhibiting of TNF- α production (33). In this study, we further demonstrated that LGGs inhibits alcohol-induced fat accumulation, likely through improved fatty acid β -oxidation and decreased lipogenesis via AMPK activation.

Chronic alcohol intake can promote the development of hepatic steatosis, which can progress to inflammation, fibrosis and cirrhosis (3, 10, 21). Therefore, reducing or preventing fat accumulation in the liver may be an effective strategy for preventing progression of ALD. Four-week alcohol exposure induced a significant increase in hepatic FFA, triglyceride and hepatic fat accumulation. These deleterious effects were markedly reduced by LGGs supplementation. Along with a previous study in an acute alcohol exposure model, our results demonstrated that LGGs is effective in the prevention of both acute and chronic alcohol-induced fatty liver formation. Furthermore, we also demonstrated that LGGs supplementation prevented chronic-alcohol induced liver injury as evaluated by liver enzymes and apoptosis.

AMPK is a well-established cellular energy sensor that switches on catabolic pathways, including fatty acid oxidation and switches off anabolic pathways, including fatty acid synthesis (14). Several studies have suggested that AMPK might be a therapeutic target for

the treatment of alcoholic fatty liver (2, 28). In the present study, we examined whether LGGs alters cellular AMPK phosphorylation. Chronic alcohol exposure significantly reduced AMPK phosphorylation, which is in agreement with previous studies. AMPK inhibits *de novo* fatty acid synthesis by inactivating ACC and stimulates fatty acid β -oxidation by reducing malonyl-Co-A through inhibition of ACC and up-regulation of CPT-1 and PPAR- α . As expected, chronic alcohol exposure significantly reduced ACC phosphorylation, leaving the enzyme in an inactive state. Importantly, LGGs supplementation significantly attenuated chronic alcohol-decreased AMPK phosphorylation, which, in turn, attenuated the reduction of ACC phosphorylation and increased CPT-1 and PPAR- α expression. Consistent with our animal study, LGGs also increased AMPK phosphorylation in H4IIE cells in the presence or absence of alcohol. The effect of LGGs on fat metabolism was further demonstrated using an AMPK inhibitor, Compound C. Pre-incubation of the hepatic cells with Compound C significantly reduced ACC phosphorylation, supporting the role of LGGs in AMPK-mediated ACC regulation. It is worth to note that a recent study indicate that LGG treatment improves insulin sensitivity and reduces lipid accumulation by stimulating adiponectin secretion and consequent activation of AMPK (18).

SREBP-1c plays a unique role in the expression of genes involved in hepatic fatty acid synthesis. Increasing evidence shows that expression of hepatic SREBP-1c is increased by alcohol exposure (16, 17). Previous studies suggest that acetylation of SREBP-1c, owing to the alcohol-induced decreased activity of Sirtuin 1 (SIRT1), stabilizes SREBP-1c protein and up-regulates lipogenic genes in the liver, which may contribute to the development of alcoholic fatty liver (22, 37). More recent studies showed that AMPK activation inhibited SREBP-1c activity, leading to an attenuation of hepatic steatosis (6). Consistent with previous studies, chronic alcohol exposure significantly increased hepatic SREBP-1c level, which was inhibited by LGGs supplementation. Moreover, chronic alcohol exposure decreased the phosphorylation levels of hepatic AMPK, which was reversed by LGGs supplementation. These results suggested that LGGs supplementation contributes to the increase in hepatic fatty acid β -oxidation and the decrease in *de novo* lipogenesis through activation of AMPK in the chronic alcohol exposed mouse liver.

LGGs supplementation also significantly attenuated alcohol feeding-induced hepatic apoptosis. Previous studies suggested that alcohol exposure may elevate the expression of pro-apoptotic protein, Bax, in the liver (12) or increase its mitochondrial localization (7), while Bcl-2, an anti-apoptosis protein, was not changed (8) or elevated (13). The current study did not show any change in hepatic Bcl-2 protein expression, but significantly increased hepatic Bax expression, suggesting that Bax elevation during chronic ethanol exposure may contribute to alcohol-induced apoptosis in the liver. Importantly, LGGs supplementation significantly increased Bcl-2 and inhibited Bax protein expression. Therefore, LGGs may play a role in preventing alcohol-induced hepatic apoptosis by promoting anti-apoptotic and inhibiting pro-apoptotic pathways.

Several studies have showed that the secreted factors produced by probiotics were beneficial, and some active ingredients in probiotic culture supernatant have been identified, including proteins (35, 40), histamine (29), polyphosphate (27), exopolysaccharide (26), conjugated

linoleic acids (9) and polyamines (24). These active ingredients have been demonstrated to be effective in the treatment of inflammation-mediated intestinal disorders and liver disease through modulating the intestinal pH, improving the intestinal microenvironment, inhibiting cytokine-induced apoptosis, and increasing intestinal epithelial barrier function. Identifying the components that are responsible for the beneficial effects of LGGs is, therefore, important for understanding the underlying mechanisms and developing new approaches for the prevention/treatment of chronic alcohol-induced liver injury.

In summary, our findings in this study suggest that LGGs is effective in the prevention of hepatic steatosis and injury in mice chronically exposed to alcohol by increasing fatty acid β -oxidation and decreasing *de novo* lipogenesis, and by decreasing hepatic apoptosis. Recent studies have demonstrated that secreted factors from probiotic fermentation have multiple beneficial effects in gut and liver health. Thus, LGGs represents an attractive treatment/prevention strategy for alcoholic liver disease.

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Fig. 1. Effects of LGGs treatment on chronic alcohol-induced liver steatosis. Mice were fed liquid diet containing 5% alcohol (AF) or pair-fed with isocaloric maltose dextrin (PF) for 4 weeks. LGGs was added to the diet in the treatment groups. Frozen liver sections of the mice were processed for staining with Oil red O (A). Liver triglyceride (B) and liver FFA (C) levels were determined. Plasma FFA levels were also measured (D). * $P < 0.05$

Fig. 2. Effects of LGGs treatment on alcohol-induced liver injury. Plasma ALT (A) and AST (B) were measured using ALT and AST Assay Kits, respectively. *P < 0.05

Fig. 3. Effects of LGGs administration on hepatic expression in mice exposed to chronic alcohol. Protein levels of SREBP-1c were determined by Western blot, and the protein band densities were quantified by densitometry analysis (A). Hepatic mRNA levels of PPAR-α, PGC-1α (B) and SCD-1 (C) were analyzed by real-time RT-PCR. Protein levels of CPT-1 were analyzed by Western blot and quantified (D). *P<0.05

Fig. 4. Effects of LGGs administration on hepatic AMPK and ACC phosphorylation in mice exposed to chronic alcohol. AMPK (A) and ACC phosphorylation (B) levels were determined by Western blot and quantified by densitometry analysis. *P<0.05

Fig. 5.

Effects of LGGs treatment on AMPK and ACC phosphorylation in H4IIE cells. H4IIE cells were treated with 100 mM ethanol or 1% LGGs for 24 hours, or pre-treated with AMPK inhibitor, Compound C (25 μ M) for 2h followed by incubation with 100 mM ethanol or 1% LGGs for additional 24 hours. Protein levels of AMPK phosphorylation were determined by Western blot and quantified. (A) AMPK phosphorylation; (B) ACC phosphorylation (B).

*P<0.05

Fig. 6.

Effects of LGGs treatment on alcohol-induced hepatic apoptosis. Liver apoptosis was evaluated by TUNEL staining. TUNEL-positive cells were quantitated by counting 5 randomly selected view fields and expressed as total TUNEL positive cells/500 cells (A). Protein levels of Bcl-2 (B) and Bax (C) were determined by Western blot and quantified.

* $P < 0.05$

Table 1.

Primer sequences for real-time RT-PCR

Gene	Sequences (Forward/Reverse 5'–3')	
PPAR- α	AGAGCCCCATCTGTCCTCTC	ACTGGTAGTCTGCAAAACCAAA
PGC-1 α	AGACAAATGTGCTTCCAAAAAGAA	GAAGAGATAAAGTTGTTGGTTTGGC
SCD-1	CCGGAGACCCTTAGATCGA	TAGCCTGTAAAAGATTCTGCAAACC
β -actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

PPAR- α , peroxisome proliferator activated receptor- α ; PGC-1 α , peroxisome proliferator-activated receptor gamma coactivator protein-1 α ;
SCD-1, Stearoyl-CoA desaturase-1