Research Communication

Blockade of Hedgehog Pathway Is Required for the Protective Effects of Magnesium Isoglycyrrhizinate Against Ethanol-induced Hepatocyte Steatosis and Apoptosis

Chunfeng Lu\textsuperscript{1,2,3,4}  
Wenxuan Xu\textsuperscript{1,2,3,4}  
Jiangjuan Shao\textsuperscript{1,2,3,4}  
Feng Zhang\textsuperscript{1,2,3,4}  
Anping Chen\textsuperscript{5}  
Shizhong Zheng\textsuperscript{1,2,3,4,*}

\textsuperscript{1}Department of Pharmacology, School of Pharmacy, Nanjing University of Chinese Medicine, Nanjing, Jiangsu, China  
\textsuperscript{2}Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Materia Medica, Nanjing University of Chinese Medicine, Nanjing, Jiangsu, China  
\textsuperscript{3}Jiangsu Key Laboratory of Therapeutic Material of Chinese Medicine, Nanjing University of Chinese Medicine, Nanjing, Jiangsu, China  
\textsuperscript{4}Jiangsu Key Laboratory of Functional Substance of Chinese Medicine, Nanjing University of Chinese Medicine, Nanjing, Jiangsu, China  
\textsuperscript{5}Department of Pathology, School of Medicine, Saint Louis University, St Louis, MO, USA

Abstract

Alcoholic liver disease (ALD), characterized by excessive deposition of lipids in hepatocytes, causes heavy health burden personally and socially. Mechanistically, hedgehog signaling was activated during the development of ALD, and exerted compelling role in regulating lipometabolism. The current promising intervention strategy is inhibition of lipid accumulation and apoptosis in hepatocytes. Magnesium isoglycyrrhizinate (MgIG) has been widely used in various liver diseases for its good hepatoprotective activities. However, the role of MgIG in ALD has not been elucidated. Therefore, this study was aimed to explore the role of MgIG and further identify the potential mechanisms. We found for the first time that MgIG reduced lipid accumulation, including triglyceride, and total cholesterol, probably via inducing peroxisome proliferator-activated receptor-alpha and inhibiting sterol regulatory element-binding protein-1c. Further, MgIG alleviated ethanol-induced oxidative stress, evidenced by reduced abundance of reactive oxygen species and increased levels of glutathione, superoxide dismutase, and mitochondrial transmembrane potential. Besides, MgIG protected hepatocytes from ethanol-induced apoptosis. In addition, MgIG dose-dependently suppressed hedgehog signaling. Of note was that disruption of hedgehog signaling could mimic the effects of MgIG, whereas activation of hedgehog signaling abrogated the effects of MgIG.

Abbreviations: ALD, alcoholic liver disease; Bax, bcl-associated X protein; Bcl-2, b-cell lymphoma 2; Bim, bcl-2-like protein 11; Cyt C, cytochrome C; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco’s modified Eagle medium; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; Gli, glioma-associated oncogene homolog; GSH, glutathione; Hhip, hedgehog-interacting protein; MgIG, magnesium isoglycyrrhizinate; MTP, mitochondrial transmembrane potential; PARP, poly ADP-ribose polymerase; PI, propidium iodide; p-p53, phospho-p53; PPAR-\(\alpha\), peroxisome proliferator-activated receptor-alpha; PUMA, p53 upregulated modulator of apoptosis; ROS, reactive oxygen species; SAG, smoothened agonist; Shh, Sonic hedgehog; Smo, smoothened; SOD, superoxide dismutase; SREBP-1c, sterol regulatory element-binding protein-1c; TC, total cholesterol; TG, triglyceride; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling

© 2017 International Union of Biochemistry and Molecular Biology

Volume 69, Number 7, July 2017, Pages 540–552

*Address correspondence to: Shizhong Zheng, Department of Pharmacology, School of Pharmacy, Nanjing University of Chinese Medicine, 138 Xianlin Avenue, Nanjing, Jiangsu 210023, China. Tel.: +86-25-88811246. Fax: +86-25-86798188.  
E-mail: nytws@163.com

Chunfeng Lu and Wenxuan Xu contributed equally to this work.  
Received 13 March 2017; Accepted 24 April 2017  
DOI 10.1002/iub.1639  
Published online 12 May 2017 in Wiley Online Library (wileyonlinelibrary.com)
Introduction

Alcoholic liver disease (ALD) is a dominantly prevalent cause for advanced liver diseases worldwide (1). It has been the most common endpoint among various types of diseases and damages correlated with alcoholism (2). ALD caused by chronic alcohol consumption is characterized by hepatocyte steatosis. Steatotic hepatocytes lose the ability of detoxification and become more sensitive to toxicants, which in turn aggravates hepatocyte injury and induces apoptosis (3). Without effective intervention on steatosis, ALD will develop from simple steatosis into hepatitis, liver fibrosis, cirrhosis, and even hepatocellular carcinoma (4). In spite of numerous studies on the pathogenesis of ALD, targeted therapies based on the mechanism by which alcohol consumption causes hepatocyte steatosis are unavailable. Compelling evidence has demonstrated that alcohol increases sterol regulatory element-binding protein-1c (SREBP-1c) expression and decreases peroxisome proliferator-activated receptor-alpha (PPAR-α) expression, implying that alcohol enhances lipogenesis and impairs lipidolysis (5,6). In addition, alcohol obviously promotes the production of reactive oxygen species (ROS) in hepatocellular mitochondria, resulting in initiation of lipid peroxidation and depletion of glutathione (GSH). Steatosis might be attributed to redox imbalance as alcohol is preferentially metabolized in liver, leading to intrahepatic lipid deposition and generation of “empty calories” (7). Hepatocytes damaged by oxidative stress and lipid overload will undergo irreversible apoptosis (intrinsic apoptosis) (8). Thus, steatosis acts as a critical pathogenic factor during ALD. It is extremely urgent to explore and illustrate the mechanisms underlying hepatocyte steatosis.

Hedgehog signaling pathway is evolutionarily conserved and plays a pivotal role in homeostasis (9). Once hedgehog ligands, especially Sonic hedgehog (Shh), leave secreted cells, they will act by binding to its receptor, patched, in the membranes of target cells via autocrine and paracrine pathways. Activated patched loses its inhibitory impacts on smoothened (Smo). Consequently, hedgehog signaling pathway is activated, triggering the nuclear translocation of glioma-associated oncogene homolog (Gli) family transcription factors (Gli1, Gli2, and Gli3) to modulate the expression of downstream target genes (10). Of note is that hedgehog-interacting protein (Hhip) prevents the engagement of hedgehog ligands to patched. Growing experimental evidence has suggested that hedgehog pathway plays an important role in modulating hepatic lipid metabolism. Basic and clinical evidence has indicated that attenuating hepatic steatosis via inhibition of hedgehog signaling is a therapeutic target in nonalcoholic fatty liver disease (11,12). However, current knowledge about the role of hedgehog pathway in ethanol-induced hepatocyte steatosis is inadequate. Preliminary evidence has indicated that ethanol facilitated the induction of high fat on hepatic steatosis, which was accompanied by enhanced activation of hedgehog pathway (13,14). These findings imply that hedgehog pathway may serve as a novel therapeutic target for ALD treatment.

Magnesium isoglycyrrhizinate (MgIG) is a magnesium salt of 18-alpha-glycyrrhizic acid stereoisomer that is a novel molecular compound extracted from the roots of plant Glycyrhiza glabra (licorice). MgIG, a safe well-tolerated drug, has been prepared as an injection for clinical uses with a characteristic of principal distribution in liver (15). Both preclinical and clinical studies have indicated its potent hepatoprotective effects (16,17). However, few studies focused on the effects of MgIG on alcohol-induced liver damages. Therefore, in the current study, we aimed to explore the effects of MgIG on ethanol-induced hepatocyte injury and further clarify the potential molecular mechanisms.

Materials and Methods

Reagents and Antibodies

MgIG was purchased from Jiangsu Chia-Tai Tianqing Pharmaceutical Co., Ltd. (Nanjing, Jiangsu, China) and dissolved in serum-free medium. Cyclopamine and smoothened agonist (SAG) were purchased from Cayman (Ann Arbor, MI) and dissolved in dimethylsulfoxide (DMSO); Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). The primary antibodies against patched (#2468), Shh (#2207), Gli1 (#3538), phospho-p53 (p-p53, #2521), p53 (#9282), p53-upregulated modulator of apoptosis (PUMA, #12450), cytochrome C (Cyt C, #4272), b-cell lymphoma 2 (Bcl-2, #15071), bcl-associated X protein (Bax, #5023), cleaved caspase-3 (#9661), cleaved caspase-7 (#8438), cleaved caspase-9 (#7237), cleaved poly ADP-ribose polymerase (PARP, #5625), β-actin (#3700), and lamin B (#13435) were purchased from Cell Signaling Technology (Danvers, MA). The primary antibodies against Smo (sc-166685), PPAR-α (sc-1982), and SREBP-1c (sc-366) were purchased from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA). The primary antibodies against Hhip (ab126147) and bcl-2-like protein 11 (Bim, ab7888) were purchased from Abcam (Cambridge, MA). The horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies (7076 and 7074) were purchased from Cell Signaling Technology.
The horseradish peroxidase-conjugated anti-goat IgG antibody (SA00001-4) was purchased from Proteintech Group, Inc. (Rosemont, IL).

Cell Culture

Human hepatocyte LO2 cells were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). LO2 cells were cultured in Dulbecco’s modified Eagle medium (DMEM; Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, Zhejiang, China), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were grown in an incubator under a controlled condition of 95% air and 5% CO2 humidified atmosphere at 37°C.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay

MTT assay was performed as we previously described to evaluate the viability of LO2 cells (18). The absorbance values were recorded by a SPECTRAmax™ microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

Nile Red Staining

LO2 cells were seeded on aseptic coverslips and incubated in 24-well culture plates. After treatment with indicated agents, cells were washed three times with ice-cold phosphate buffer saline and then fixed with 4% paraformaldehyde for 20 min at room temperature. After immobilization, intracellular lipid droplets were stained with Nile Red (3.3 μg/mL) for 15 min at room temperature. Cellular nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI; KeyGEN BioTECH, Nanjing, Jiangsu, China) for 5 min at room temperature. Nile red fluorescent images were collected using a fluorescence microscope (Nikon, Tokyo, Japan).

Biochemical Analysis

LO2 cells were incubated in six-well plates for 12 h and treated with corresponding reagents for 24 h. The levels of triacylglyceride (TG), total cholesterol (TC), and GSH, and the activity of superoxide dismutase (SOD) were measured according to the protocols from the manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). The protein concentrations were detected using a bicinchoninic acid assay kit (Pierce Biotechnology, Rockford, IL). The absorbance values were determined by co-transfection of Renilla luciferase reporter vector (pRL-TK vector; Promega) (0.5 μg/well) and expressing the luciferase reporter plasmid p11xGli1 (Promega), according to the protocol from the manufacturer (Promega), according to the protocol from the manufacturer. Luciferase activities were measured using a dual-luciferase reporter assay system (Promega), according to the protocol from manufacturer. Luciferase assays were performed as described previously (19).

Dual-luciferase Reporter Assay

Western Blot Analysis

Protein extraction and western blot analysis were performed as we previously described (18). The abundance of target protein bands was densitometrically determined using Quantity Ones 4.4.1 (Bio-Rad Laboratories, Berkeley, CA) and expressed as fold changes after normalization to the invariant control β-actin or lamin B or total proteins.

Immunofluorescence Staining

LO2 cells were seeded on coverslips in 24-well plates and treated with corresponding reagents for 24 h. The changes in mitochondrial transmembrane potential (MTP) were evaluated using a JC-1 MTP assay kit (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) in strict accordance to the protocol. LO2 cells were observed under a fluorescence microscope (Nikon).

Flow Cytometry Analyses of Apoptosis

Apoptosis was detected by fluorescein isothiocyanate (FITC)-labeled Annexin-V/propidium iodide (PI) double staining and flow cytometry analysis. LO2 cells were incubated in six-well plates, cultured for 12 h, and then treated with corresponding reagents for 24 h. An Annexin-FITC apoptosis assay kit was used in strict accordance to the protocol from the manufacturer (Nanjing KeyGen Biotech Co., Ltd.). Apoptotic cells were defined as the cells situated in the right two quadrants of each plot and the percentages were determined by flow cytometry (FACS-Cabibur; Becton, Dickinson and Company, Franklin Lakes, NJ). Data were analyzed using CELLQuest software.
Statistical Analysis
All experimental data were presented as mean ± SD, and results were analyzed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). The significance of difference was determined by one-way analysis of variance with the post hoc Dunnett’s test. Values of $P < 0.05$ were considered statistically significant.

Results

MgIG Prevents Ethanol-induced Hepatocyte Steatosis
We initially explored the role of MgIG in ethanol-induced hepatotoxicity. MTT assay indicated that ethanol obviously inhibited the cell viability, however, MgIG dose-dependently enhanced the hepatocyte viability (Fig. 1A). Nile Red staining visually showed that ethanol exposure facilitated the accumulation of intracellular lipid droplets, which was attenuated by MgIG in a dose-dependent manner (Fig. 1B). Consistently, ethanol-induced increases in intracellular TG and TC levels were abolished by MgIG (Fig. 1C,D). Taken together, these findings conformably indicated that MgIG had potent protective properties against ethanol-induced hepatocyte steatosis.

MgIG Protects Hepatocytes against Ethanol-induced Oxidative Stress and Mitochondrial Dysfunction
We next investigated the effects of MgIG on mitochondrial function in ethanol-stimulated hepatocytes. ROS fluorescence staining visually showed that ethanol significantly increased intracellular ROS levels. However, MgIG decreased ethanol-triggered ROS production (Fig. 2A). Further, it was observed that MgIG abolished the inhibitory effects of ethanol on intracellular GSH contents and SOD activities (Fig. 2B,C). In addition, as shown in Fig. 2D, hepatocytes stimulated with ethanol emitted green fluorescence. MgIG dose-dependently enhanced red fluorescence and simultaneously weakened green fluorescence, suggesting that MgIG increased the MTP of ethanol-administrated hepatocytes. Collectively, our results strongly demonstrated that MgIG ameliorated mitochondrial dysfunction and oxidative stress in ethanol-exposed hepatocytes.

MgIG Inhibits Ethanol-induced Hepatocyte Apoptosis
Based on the results above, we proposed that MgIG could inhibit ethanol-induced hepatocyte apoptosis. The morphology of apoptosis was evaluated using TUNEL staining, which could qualitatively verify our hypothesis. Results obviously showed that ethanol increased the number of TUNEL-positive apoptotic hepatocytes, whereas MgIG reduced the number of apoptotic hepatocytes in a dose-dependent manner (Fig. 3A). Additional evidence was provided by FITC-labeled Annexin V/PI staining, which quantificationally showed an increase in the number of apoptotic hepatocytes in ethanol-treated group but a decrease in the number of apoptotic hepatocytes in MgIG-treated group (Fig. 3B). In summary, these findings reinforced our hypothesis that MgIG attenuated ethanol-caused hepatocyte apoptosis.

MgIG Suppresses Ethanol-induced Activation of Hedgehog Signaling Pathway in Hepatocytes
Due to the critical role of hedgehog signaling pathway played in hepatocyte steatosis, we subsequently testified the impacts of MgIG on hedgehog signaling pathway. Western blot analyses indicated that ethanol significantly increased the expression of Patched, Smo, and Shh but decreased the expression of Hhip.
suggesting activation of hedgehog signaling in ethanol-stimulated hepatocytes. Nevertheless, MgIG abrogated the activation of hedgehog pathway in a dose-dependent manner (Fig. 4A). To further elucidate the role of MgIG in regulating the nuclear transcription factor Gli1, cytoplasmic and nuclear proteins were separated and individually detected by western blot analysis. Results suggested that MgIG dose-dependently reduced the protein abundance of Gli1 in nuclei but increased the protein abundance in cytoplasm (Fig. 4B). To investigate whether the transcriptional activity of Gli1 was affected by MgIG, LO2 cells were transiently transfected with luciferase reporter plasmid of Gli1. Luciferase assays demonstrated that ethanol significantly induced the luciferase reporter activity of Gli1, which was suppressed by MgIG (Fig. 4C). Immunofluorescence staining for Patched, Smo, Hhip, and Gli1 in hepatocytes visually confirmed the results of western blot analyses (Fig. 4D). In brief, all these data revealed that MgIG disrupted the transduction of hedgehog signaling pathway in ethanol-treated hepatocytes. It was an intriguing discovery that inspired us to further investigate whether hedgehog signaling pathway was involved in the protection of MgIG.

**Inhibition of Hedgehog Signaling Is Required for MgIG to Attenuate Steatosis in Ethanol-treated Hepatocytes**

To further explore whether hedgehog signaling was involved in the regulation of MgIG on ethanol-induced hepatocyte steatosis, gain- or loss-of-function analyses were employed in *in vitro* system. Hedgehog signaling pathway antagonist cyclopamine, similar to MgIG, abolished the induction of ethanol on lipid accumulation in hepatocytes. And cyclopamine reinforced the inhibitory effects of MgIG. However, hedgehog signaling pathway agonist SAG abrogated the suppression of MgIG on hepatocyte steatosis (Fig. 5A). At molecular levels, cyclopamine, mimicking MgIG effects, enhanced the expression of PPAR-α and reduced the expression of SREBP-1c in hepatocytes exposed to ethanol. However, SAG significantly cancelled
MgIG inhibits ethanol-induced hepatocyte apoptosis. (A) Cell apoptosis assay by TUNEL staining (original magnification, 40×). Scale bar = 20 μM. (B) Flow cytometry analyses of apoptosis. The abscissa represents FITC-Annexin V staining and the ordinate represents PI staining. For the statistics of each panel in this figure, data are expressed as mean ± SD, ###P < 0.001 compared with vehicle control, *P < 0.05, **P < 0.01, and ***P < 0.001 compared with ethanol.

<table>
<thead>
<tr>
<th>MgIG (mg/mL)</th>
<th>-</th>
<th>-</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (100 mM)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**FIG 3** Lu et al.
MgIG suppresses ethanol-induced activation of hedgehog signaling pathway in hepatocytes. (A and B) Western blot analyses of genes associated with hedgehog signaling. (C) Luciferase reporter gene assay of Gli1. Luciferase activities are expressed as relative units after Renilla luciferase reporter normalization. (D) Immunofluorescence staining for visualizing protein expression in hepatocytes (original magnification, 40×). Scale bar = 20 μM. For the statistics of each panel in this figure, data are expressed as mean ± SD, *P < 0.05, **P < 0.01, and ***P < 0.001 compared with vehicle control, *P < 0.05, **P < 0.01, and ***P < 0.001 compared with ethanol.
the regulatory effects of MgIG on these genes (Fig. 5B). Immunofluorescence analysis was further conducted. Robust PPAR-α staining and faint SREBP-1c staining were observed in hepatocytes treated with ethanol and MgIG. Of note was that the effects of MgIG were strengthened by cyclopamine but reversed by SAG (Fig. 5C). These data indicated that suppression of hedgehog signaling pathway was required for MgIG to ameliorate ethanol-induced hepatocyte steatosis possibly by inhibiting lipid synthesis and inducing lipolysis.

**Disruption of Hedgehog Signaling Is a Prerequisite for MgIG to Reduce Apoptosis in Ethanol-exposed Hepatocytes**

Finally, we investigated whether hedgehog signaling also played an important part in MgIG-led suppression on apoptosis. Results showed that the suppressive effects of MgIG on ROS production were further enhanced by cyclopamine but eliminated by SAG (Fig. 6A). Consistently, MgIG had a synergistic effect with cyclopamine on the recovery of GSH and SOD, which, however, was cancelled by SAG (Fig. 6B,C). Cyclopamine-mediated inhibition of hedgehog pathway, similar to MgIG, restored MTP, whereas SAG diminished the effects of MgIG (Fig. 6D). Of note was that MgIG treatment resulted in a significant decrease in the number of TUNEL-positive apoptotic hepatocytes, which was enhanced by cyclopamine but cancelled by SAG (Fig. 7A). MgIG apparently decreased the phosphorylation of p53, the expression of Bim, PUMA, Bax, and the release of Cyt C into cytoplasm, but increased the expression of Bcl-2, which was strengthened by cyclopamine but abolished by SAG (Fig. 7B,C). In addition, cyclopamine enhanced the inhibitory effects of MgIG on cleaved-

**FIG 5**

Inhibition of hedgehog signaling is required for MgIG to attenuate steatosis in ethanol-treated hepatocytes. (A) Nile Red staining (original magnification, 40×). Scale bar = 20 μM. (B) Western blot analyses of PPAR-α and SREBP-1c. (C) Immunofluorescence staining for visualizing expression of PPAR-α and SREBP-1c in hepatocytes (original magnification, 40×). Scale bar = 20 μM. For the statistics of each panel in this figure, data are expressed as mean ± SD, **P < 0.01** and ***P < 0.001 compared with vehicle control, *P < 0.05, **P < 0.01, and ***P < 0.001 compared with ethanol + MgIG.
caspase 3, cleaved-caspase 7, cleaved-caspase 9, and cleaved-PARP. However, SAG remarkably reversed the effects of MgIG (Fig. 7D). Taken together, these findings not only implied that hedgehog signaling modulated mitochondrial-mediated and caspase-dependent apoptosis in ethanol-stimulated hepatocytes, but also provided clear evidence that inhibition of hedgehog signaling was required for MgIG to suppress ethanol-induced hepatocyte apoptosis.

**Discussion**

In spite of great efforts have been made to clarify the pathogenesis of ALD, ALD remains a medical challenge all around the world. Advances in understanding of ALD are increasingly converted into novel therapies for ALD. Abundant studies have demonstrated that hepatocyte steatosis is the key event during the progression of ALD. Currently, an insufficient understanding of ALD pathology and deficiency in innocuous therapeutic agents result in adverse prognosis. It is extremely crucial to discover and develop effective drugs to solve this medical problem. In this study, we for the first time comprehensively expounded the protective effects and the potential molecular mechanisms of MgIG by employing a recognized *in vitro* model of ALD. Our work revealed that MgIG had potent inhibitory effects on ethanol-caused hepatocyte steatosis, oxidative stress, and apoptosis. Of note was that inhibition of hedgehog pathway was required for MgIG to function.

LO2 cells were initially isolated from healthy human and maintained the biological features and ultrastructures of normal adult hepatocytes. They were immortalized by a stable transfection with human telomerase reverse transcriptase gene (21). For years, LO2 cells were widely used in considerable studies including ours as an *in vitro* model of hepatic tissue for investigating the pathophysiology of hepatocytes including hepatotoxicity (22). Herein, according to our
Disruption of hedgehog signaling is a prerequisite for MgIG to reduce apoptosis in ethanol-exposed hepatocytes. (A) Cell apoptosis assay by TUNEL staining (original magnification, 40×). Scale bar = 20 μM. (B–D) Western blot analyses of expression of proteins associated with apoptosis. For the statistics of each panel in this figure, data are expressed as mean ± SD, ###P < 0.001 compared with vehicle control, *P < 0.05, **P < 0.01, and ***P < 0.001 compared with ethanol, &P < 0.05, &&P < 0.01, and &&&P < 0.001 compared with ethanol + MgIG.
previous studies, an in vitro model mimicking human ALD was successfully established by administrating LO2 hepatocytes with ethanol at the concentration of 100 mM for 24 h (18,23).

To our best knowledge, hedgehog pathway has been emphasized as a critical regulator involved in several liver diseases, including nonalcoholic liver disease and hepatic fibrosis (12,24), and its inhibitor could attenuate nonalcoholic liver disease and hepatic fibrosis (12,25). However, the potential role of hedgehog pathway in alcoholic animal model or even in in vitro system has not been uncovered. The major biological effects of hedgehog pathway were dependent on Gli1, a nuclear translocation factor that regulated the expression of hedgehog target genes (26). The latest data we obtained suggested that upstream signaling molecules were remarkably

**Schema of the underlying mechanism of MgIG inhibition of ethanol-induced hepatocyte injury.** MgIG inhibits the transduction of hedgehog signaling pathway possibly leading to decreased steatosis, oxidative stress, mitochondrial dysfunction, and apoptosis in hepatocytes. These actions in consequence attenuate the hepatocyte injury caused by ethanol administration. The identified mechanism probably accounts for MgIG attenuation of ethanol-triggered hepatocyte injury.
activated and the nuclear translocation of Gli1 was induced in ethanol-stimulated hepatocytes. MgIG had potent inhibitory effects on ethanol-induced activation of hedgehog signaling, implying a noticeable correlation between MgIG effects on ethanol-treated hepatocytes and inhibition of hedgehog pathway.

Hepatocytes are naturally exposed to toxins. Hepatocytes metabolize ethanol to eliminate its cytotoxicity. However, excessive ethanol causes damage to hepatocytes, which firstly results in loss of cell viability. Our compelling evidence showed that ethanol suppressed the hepatocyte viability, which was consistent with our previous finding (27). MgIG could facilitate the regaining of cell viability.

Nile Red is a kind of fluorescent oxazine dye with potent lipophilic properties. When combining with ester, triacylglycerol, and various fatty acids, Nile Red would exhibit intense red fluorescence under the ultraviolet light. By taking advantage of the variation in fluorescence colors, Nile Red can be utilized as a selective dye to detect intracellular lipid droplets in living cells by fluorescence microscopy. Results showed that ethanol stimulation obviously increased the number of lipid droplets in hepatocytes, which supported our previous conclusion (18). Clearly, MgIG diminished lipid deposition in ethanol-treated hepatocytes, which was consistent with that in *in vitro* model of nonalcoholic liver diseases (16). Given that TG and TC are major components of lipid droplets in hepatocytes, we further analyzed their contents in hepatocytes (28). Compelling evidence further confirmed that the inhibitory effects of MgIG on ethanol-caused steatosis were abolished by hedgehog agonist SAG but strengthened by its antagonist cyclopamine. As we know, lipid contents is determined jointly by lipogenesis and lipolysis. Therefore, we hypothesized that MgIG could inhibit lipogenesis and induce lipolysis. SREBP-1c has been well identified as a critical transcription factor that accelerates lipid synthesis, while PPAR-α has been recognized as a nuclear hormone receptor associated with transport and oxidation of fatty acids (29). As we previously observed, ethanol induced SREBP-1c but suppressed PPAR-α expression (27). Noteworthy, MgIG inhibited SREBP-1c but induced PPAR-α, which supported our hypothesis. Wherein, it was an interesting finding that the effects of MgIG on the expression of both genes were dependent on its inhibition of hedgehog signaling.

Oxidative stress occupied a vital status in the pathogenesis of ethanol-induced hepatocyte injury (30). ROS has been well identified as the most significant biomarker for oxidative stress. GSH, a ubiquitous antioxidant biological molecule, reduces oxidative stress by eliminating harmful free radicals (31). SOD is a well-known antioxidant enzyme facilitating cells to defend against oxidative stress. Herein, we observed that ethanol caused an obvious increase in intracellular ROS and a decrease in GSH levels and SOD activities, which was consistent with our previous findings (27). However, MgIG apparently abrogated the induction of ethanol on oxidative stress. A previous study suggested that oxidative stress may suppress Gli1 activation and abolish Gli1-induced gene expression (32). Our direct evidence for the first time showed that hedgehog signaling antagonist cycloamine, similar to MgIG, inhibited oxidative stress induced by ethanol.

Since excessive lipid accumulation triggers mitochondrial apoptosis in hepatocytes (33), we next analyzed the apoptosis of steatotic hepatocytes and the effects of MgIG. TUNEL staining visually showed that ethanol induced hepatocyte apoptosis in parallel with an obvious decline in MTP. MgIG significantly inhibited the deleterious effects of ethanol, which was abrogated by hedgehog agonist SAG. These data for the first time emphasized that inhibition of hedgehog signaling could be a prerequisite for MgIG to inhibit ethanol-caused apoptosis. Further, we observed increased levels of proapoptotic members of Bcl family, such as Bim, Bax, and PUMA, and decreased levels of antiapoptotic member Bcl-2 in hepatocytes exposed to ethanol. All of these genes are components of mitochondrial “intrinsic” machinery that precede the activation of caspase family (34). We also found that MgIG inhibited the stimulative effects of ethanol on caspase family. It was interesting that hedgehog agonist cycloamine abolished the effect of MgIG.

In conclusion, our work demonstrated that MgIG attenuated ethanol-induced hepatocyte steatosis and apoptosis. Mechanistically, inhibition of hedgehog signaling was a potential molecular basis for MgIG (Fig. 8). Our present findings not only revealed that MgIG was a potential agent for ALD treatment but also provided novel insights that hedgehog signaling could be utilized to screen active pharmaceutical ingredients for the treatment of ALD.

Acknowledgements

This study was supported by 2015 Program for Graduate Scientific Innovation of Jiangsu Higher Education Institutions (KYLX15_0999), the National Natural Science Foundation of China (81270514, 31401210, 31571455, 31600653, and 81600483), the Youth Natural Science Foundation of Jiangsu Province (BK20140955), the Open Project Program of Jiangsu Key Laboratory for Pharmacology and Safety Evaluation of Chinese Materia Medica (JKLPSE 201502), and the Project of the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). Conflict of interest: The authors declare that there are no conflicts of interest.

References


