Abstract. Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver diseases worldwide and there is an urgent need to identify effective pharmacological strategies to treat NAFLD. For this purpose, in the present study, we examined the possible molecular mechanisms responsible for the effects of MgIG and the protective effects of MgIG in a model of NAFLD. The human hepatic L02 cell line and oleic acid were employed to establish an in vitro model of NAFLD. The CCK-8 assay, Hoechst 33258 staining and Annexin V-PI staining were performed in order to evaluate cell viability and apoptosis. Oil red O staining was used to detect lipid accumulation within the L02 cells. We found that MgIG significantly inhibited lipid accumulation and protected the L02 cells against lipid accumulation-induced apoptosis. Key molecules involved in unfolded protein response (UPR) signaling were upregulated in lipid-overloaded hepatic cells whereas MgIG suppressed the activation of the UPR. Furthermore, MgIG significantly inhibited the expression of the downstream inflammatory cytokines which had been induced by lipid accumulation. Taken together, these findings suggest that the activation of UPR signaling induces the expression of inflammatory cytokines through the activation of nuclear factor-κB (NF-κB) in lipid-overloaded hepatic cells. In addition, MgIG may suppress the activation of UPR signaling thereby protecting hepatic cells from NAFLD-induced injury.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by the accumulation of fat in the livers of individuals who are abstinent from alcohol or drink infrequently and do not have other liver diseases such as hepatitis B and C, alcoholic disease, metabolic liver disease and autoimmune diseases. The histological pattern of NAFLD may progress from non-alcoholic fatty liver into non-alcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis and even hepatocellular carcinoma (HCC) (1). It is now one of the most common liver diseases worldwide. Approximately 10 to 40% of adults in the USA and 20% in other developed countries suffer from NAFLD (2).

The pathogenesis and underlying mechanisms of NAFLD remain to be elucidated. Accumulating evidence has verified that NAFLD is associated with obesity, type 2 diabetes, hyperlipidemia and other comorbid conditions. The most widely supported theory proposes that insulin resistance is the key mechanism responsible for the metabolic abnormalities and may cause NAFLD. Current treatment relies on lifestyle modifications including long-term weight management and regular physical exercise. Studies have been performed for the purpose of developing novel treatments for NAFLD including insulin-sensitizers, antioxidants, lipid-lowering drugs, pentoxifylline and cannabinoid receptor antagonists. However, the current pharmacological treatment options for the disease remain unsatisfactory (3-6).

The endoplasmic reticulum (ER) is an organelle in cells which is important for the folding of protein molecules and the transport of synthesized proteins. Failure of the ER's adaptive capacity results in the activation of the unfolded protein response (UPR), which intersects with many different inflammatory and stress signaling pathways. Increasing evidence has demonstrated that the UPR is activated in NAFLD and may play an important role in the development and progression of...
this disease (7-9). 4-Phenylbutyric acid (4-PBA), a chemical chaperone, is used to suppress the activation of the UPR (10).

Previous studies have indicated that lipid accumulation in the liver may regulate the hepatic production of proinflammatory cytokines, including interleukin (IL)-6, transforming growth factor (TGF)-β and basic fibroblast growth factor (bFGF) through nuclear factor-κB (NF-κB) activation and downstream cytokine production (11-13).

Magnesium isoglycyrrhizinate (MgIG), a traditional herbal remedy extracted from the roots of the plant Glycyrrhiza glabra, is a magnesium salt of 18α-glycyrrhizic acid stereoisomer. Increasing evidence indicates that MgIG acts as an anti-inflammatory and hepatoprotective agent as it protects hepatic cells against tissue injury, improves liver function and suppresses inflammatory responses in the liver (14-16). A previous study demonstrated that MgIG maintained cell viability and markedly decreased oleic acid (OA)-induced cell apoptosis and lipid accumulation (17). It has also been demonstrated that MgIG provided protection against various organ injuries and diseases, including alcoholic liver disease and lung injury induced by paraquat poisoning as a clinical medication (18-20). However, the effect of MgIG in the treatment of NAFLD, particularly with regard to the effect of MgIG on lipid-overloaded hepatic cells as well as the underlying molecular mechanisms remain unknown.

The aim of the study was to explore the possible molecular mechanisms responsible for the effects of MgIG including activation of the UPR, NF-κB activation and associated inflammatory factor expression in hepatic L02 cells, as well as to examine the protective effects of MgIG in a model of NAFLD.

Materials and methods

Cell line and OA/MgIG treatment. The human hepatic cell line L02 was obtained from the Peking University Health Science Center (Beijing, China). The L02 cells were cultured in RPMI-1640 medium (Biological Industries, Beit-Haemek, Israel), at 37°C in a 5% CO₂ humidified atmosphere. All media were supplemented with 10% fetal bovine serum and 1% Pen-Strep solution (both from Biological Industries).

The cells were seeded at either 5x10⁴ in 2 ml complete medium in a 6-well plate, or at 1x10⁵ in 200 µl medium in a 96-well plate. The cells were grown in fresh medium until 50-60% confluent and treated with medium alone or supplemented with either OA (2 mM) (Sigma, St. Louis, MO, USA) or OA coupled with MgIG (Chia-tai Tianqing Pharmaceutical Co. Ltd, Lianyungang, China) or 4-PBA (Sigma) for 24 h. The cells were then incubated for another 3 h in a incubator at 37°C and 5% CO₂. The OD values of the cells from the five different groups were measured at 450 nm using an ELISA reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). Each assay was performed in triplicate.

Oil red O staining. The L02 cells were seeded in 6-well plates and cultured in RPMI-1640 medium or supplemented with either OA and/or MgIG as described above. The culture medium was discarded and the cells were washed with phosphate-buffered saline (PBS) twice and then fixed with 4% paraformaldehyde for 20 min. Oil red O working solution (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated for 15 min at room temperature. The cells were then rinsed with 60% isopropyl alcohol once and then rinsed with PBS twice. Images were then captured under an upright microscope (magnification, x400; BX53; Olympus, Tokyo, Japan).

Hoechst 33258 staining. The effect of MgIG and OA on apoptosis were determined by fluorescence microscopy following staining with the DNA binding fluorophore Hoechst 33258. Briefly, the L02 cells were seeded in 6-well plates for 24 h and treated with either growth medium or medium containing OA with or without MgIG. The cells were washed twice with PBS, fixed with 4% paraformaldehyde for 10 min, and stained with 5 mM Hoechst 33258 (Dojindo Molecular Technologies, Inc.) for 10 min at room temperature. The cells were washed with PBS twice before images were captured under a fluorescence microscope (Olympus) at 340 nm excitation (magnification, x400).

Annexin V-FITC/prodipidium iodide (PI) staining and flow cytometric analysis. Following the OA and MgIG treatment for 24 h as described above, apoptosis was detected using the Annexin V-FITC Apoptosis Detection kit (Dojindo Molecular Technologies, Inc.) according to the manufacturer’s instructions. Individually treated L02 cells were collected by removing the medium and washed twice with PBS. The cells were gently resuspended in 100 µl binding solution. Annexin V-FITC (5 µl) was added to the cells, followed by 5 µl PI solution. The L02 cells were then incubated at room temperature in the dark for 15 min. Subsequently, 400 µl 1X Annexin V binding solution was added. The cells were analyzed by flow cytometry using a BD FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) within 1 h. The number of apoptotic cells was then calculated using FlowJo software.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Following a 24-h incubation, total RNA was extracted from the L02 cells using TRIzol reagent (Sigma) and reverse transcribed according to the manufacturer’s instructions. Briefly, the SuperReal PreMix RT-PCR kit (Tiangen Biotech Co., Ltd.,Beijing, China) was used to perform Real-Time PCR and the Golden Fast PCR kit (Tiangen Biotech Co., Ltd.) was used to perform PCR. Isolated total RNA (2 µg) was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). The primers used for IL-6, TGF-β, bFGF, NF-κB, BIP, C/EBP homologous protein (CHOP), X-box binding protein 1 (XBP-1), activating transcription factor 6 (ATF-6) and β-actin are presented in Table I. The primers were selected
using the NCBI/Primer-BLAST program (www.ncbi.nlm.nih.gov/tools/primer-blast/) and were synthesized by Sangon Biotech (Shanghai, China). In addition, the PCR products were also separated in a 2% agarose gel and analyzed using Image J software. Quantitative (real-time) PCR was performed on a 7300 Real-Time PCR system (Applied Biosystems). Cycle threshold (Ct) values were normalized to β-actin and PCR system reactions were run and calculated in triplicates.

**Protein extraction and western blot analysis.** After individual treatments, the L02 cells were harvested, rinsed with PBS twice and lysed in RIPA buffer containing 10 mM phosphate buffer, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mM sodium orthovanadate and protease inhibitors for 30 min on ice. Protein concentrations were determined using a BCA Protein Assay kit (Beyotime Institute of Biotechnology, Shanghai, China). In addition, the PCR products were also separated in a 2% agarose gel and analyzed using Image J software. Quantitative (real-time) PCR was performed on a 7300 Real-Time PCR system (Applied Biosystems). Cycle threshold (Ct) values were normalized to β-actin and PCR system reactions were run and calculated in triplicates.

**Statistical analysis.** All quantitative data are presented as the means ± SD. Statistical analysis was performed using the ANOVA and Student's t-test with SPSS 17.0 software. A P-value <0.05 was considered to indicate a statistically significant difference.

### Results

**MgIG increases the viability of L02 cells following exposure to OA.** Lipotoxicity in hepatocytes induced by OA has been implicated in the development of NAFLD. OA and hepatic L02 cells have been previously used to establish a model of NAFLD in vitro (21).

In the present study, we first treated the L02 cells with OA and different doses of MgIG (0.2, 1 and 5 mg/ml) for 24 h. Compared with the untreated control group, OA significantly reduced the viability of the L02 cells (Fig. 1A). Furthermore, MgIG increased cell viability. The optimal concentration of MgIG for protecting L02 cell viability was identified as 1 mg/ml therefore this dose was selected for subsequent experiments (Fig. 1A).

**MgIG decreases the accumulation of lipid droplets in L02 cells exposed to OA.** The Oil red O staining assay was performed in order to detect the accumulation of intracellular lipid droplets in L02 cells. After incubation with OA, the number of Oil Red O-positive droplets was significantly increased in the L02 cells (Fig. 1B). MgIG treatment significantly reduced the accumulation of Oil Red O-positive droplets in the OA-treated L02 cells (Fig. 1B).

**MgIG protects against the OA-induced apoptosis of L02 cells.** We performed Hoechst 33258 staining to evaluate the effect of MgIG on the apoptosis of lipid-overloaded L02 cells. The results indicated that L02 cells exposed to OA for 24 h exhibited membrane dissolution as well as the formation of debris and nuclear fragmentation, which was indicated by bright blue fluorescence (Fig. 2A). The number of typical apoptotic cells was decreased following treatment with MgIG compared with that in the cells incubated with OA alone (Fig. 2A).

**Enzyme-linked immunosorbent assay (ELISA).** The expression of IL-6, TGF-β and bFGF in the L02 cells was detected by ELISA. After a 24-h treatment period, the supernatants were collected and added into the human ELISA kit (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions. The OD value was measured at 450 nm using an ELISA reader (Bio-Tek Instruments). The expression values were calculated using a standard curve and ELISA was performed with supernatants with each independent culture run in triplicate.

### Table I. Primer sequences used for RT-qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5′→3′)</th>
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<tbody>
<tr>
<td>BiP</td>
<td>Forward: CTAATGGTTGAAACCCACAACG Reverse: TATGGCAAGTTGGTGTG</td>
</tr>
<tr>
<td>CHOP</td>
<td>Forward: GCTCAGAAGAAGGAGGAGA Reverse: CCTGCTTAGCCGCGTTTCTT</td>
</tr>
<tr>
<td>XBP-1</td>
<td>Forward: CAGACTACGTGACACTTTG Reverse: GCGTTGAAGAAGGGTAC</td>
</tr>
<tr>
<td>ATF-6</td>
<td>Forward: TCCTCGGTGCTGAGCTTTA Reverse: CTTGGCGTGAATGAGGGTTG</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Forward: GAACACGAATGACAGAGGC Reverse: GCTTGCGGAGATTAGCTTTTTT</td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward: GCCATCGGCAAGAAAACACC Reverse: TGGCAATTGTGTGGTGTTCA</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Forward: CTAATGGTTGAAACCCACAACG Reverse: ATACGCGAAGAATGAGGGTTG</td>
</tr>
<tr>
<td>bFGF</td>
<td>Forward: AGTTGTGCTGACCTTTGAACCATC Reverse: ACTGGCCAGTGTGTGGTTGCA</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: GTCACCAACTGGGACGACAT Reverse: AGGGATAGCACAGGCTGGAT</td>
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CHOP, C/EBP homologous protein; XBP-1, X-box binding protein 1; ATF-6, activating transcription factor 6; NF-κB, nuclear factor-κB; IL-6, interleukin-6; TGF-β, transforming growth factor-β; bFGF, basic fibroblast growth factor.
The results of Annexin V-PI staining showed that the portion of early- and late-stage apoptotic cells increased markedly following exposure to OA (Fig. 2B). However, MgIG significantly suppressed the percentage of apoptotic cells (Fig. 2B).

**MgIG suppresses the lipotoxic effects of OA through UPR signaling activation.** BiP is an ER stress marker that reflects the activation of UPR. The UPR signaling pathway consists of three branches, namely PERK, IRE1 and ATF-6. The activation...
of PERK and IRE1 promoted the activation of CHOP and XBP-1, respectively (9,10). RT-qPCR and western blot analysis were performed to evaluate the mRNA and protein expression of BiP, CHOP, XBP-1 and ATF-6, respectively.

Compared with the control group, the expression of BiP, CHOP and XBP-1 was significantly increased in lipid-overloaded L02 cells, and MgIG effectively reversed their expression back to normal both at the mRNA and the protein level (Fig. 3A and B). Compared with the other endoplasmic reticulum stress-related genes, ATF-6 expression was not significantly changed by either OA or OA with MgIG treatment (Fig. 3A and B). These data suggested that OA may induce the activation of UPR signaling mainly through downstream PERK and IRE1 pathways whereas MgIG may greatly suppress OA-induced UPR activation.

mRNA and protein expression of NF-κB is altered in lipid-overloaded L02 cells in the presence or absence of MgIG. Accumulating evidence indicates that the NF-κB pathway in the liver is activated and NF-κB targets were accordingly elevated by lipid accumulation (11). Our results showed that OA markedly raised the mRNA and protein expression of NF-κB (Fig. 4). However, these levels was significantly reduced when the cells were co-treated with MgIG (Fig. 4). The UPR inhibitor 4-PBA decreased the mRNA and protein expression levels of NF-κB as the positive control (Fig. 4).

**Expression of IL-6, TGF-β and bFGF is partly regulated in lipid-overloaded L02 cells in the presence or absence of MgIG.** The mRNA and protein expression of IL-6, TGF-β and bFGF were significantly increased in the lipid-overloaded L02 cells compared with the control groups (Fig. 5A and B). A marked decrease in the mRNA and protein expression of IL-6, TGF-β and bFGF was observed following treatment with OA and MgIG or 4-PBA.

**Discussion**

In the present study, we established a model of NAFLD by incubating hepatic L02 cells with OA in vitro. Using this cell model, we examined the role of OA and MgIG in liver cells and the associated molecular signaling events.

A previous study indicated that OA was capable of decreasing cell viability and increasing cell apoptosis markedly (22). It has also been demonstrated that OA may induce the accumulation of lipid droplets in lipid-overloaded L02 cells in the presence or absence of MgIG (MgIG). (A) RT-qPCR analysis was performed to detect the mRNA expression levels of NF-κB after MgIG or 4-phenylbutyric acid (4-PBA) treatment with or without oleic acid (OA). β-actin was used as a loading control. (B) Western blot analysis was performed to evaluate NF-κB protein expression levels after individual treatments. Expression of NF-κB protein was normalized to β-actin. Data are presented as the means ± SD. *P<0.05 vs. control (Ctrl) group, #P<0.05 vs. OA group. All the experiments were repeated at least 3 times.
Our current data are consistent with these findings. In addition, our study suggested that MgIG reduced cell death and decreased the apoptosis rate in lipid-overloaded L02 cells significantly. Moreover, MgIG treatment inhibited OA-induced lipid accumulation in L02 cells.

The UPR is regulated by three ER stress transducer proteins: PERK, IRE1, and ATF-6. BiP is an ER stress marker and a central regulator of ER homeostasis as it plays multiple roles in protein folding and the activation of transmembrane ER stress sensors. The accumulation of unfolded proteins sequesters BiP so it dissociates from transmembrane transducers thereby leading to the activation of these three proteins. Thus, the expression of BiP is thought to be an ER stress marker bound to these three pathways. The activation of PERK promotes the activation of CHOP after dissociating from BiP (25). Activated IRE1α induces the splicing of the mRNA encoding XBP-1 after dissociating from BiP, which is a transcriptional activator that has many targets including ER chaperones and genes involved in ER-associated degradation (ERAD) (26).

After dissociating from BiP, ATF-6 is activated and translocates to the nucleus where it transcriptionally activates ER chaperones and genes associated with ERAD (27).

In the present study, the exposure of L02 cells to OA, markedly upregulated the mRNA and protein expression of BiP, CHOP, and XBP-1, which was similar to the findings of a previous study using sodium palmitate (28). However, we were surprised to observe that the mRNA and protein expression of these endoplasmic reticulum stress-related genes were significantly downregulated when treated with MgIG whereas there was no significant change in ATF-6 levels. The expression of ATF-6 was upregulated slightly in the lipid-overloaded L02 cells, and suppressed slightly after MgIG treatment. These results suggested that the lipid accumulation caused by OA may activate the UPR and be involved in the activation of the IRE1-XBP-1 and PERK-CHOP pathways. Furthermore, MgIG suppresses the expression of these two pathways.

Previous findings have showed that the activation of NF-κB pathway may be induced by lipid accumulation (11). A

Figure 5. Expression of interleukin (IL)-6, transforming growth factor (TGF)-β and basic fibroblast growth factor (bFGF) is partly regulated in oleic acid (OA)-exposed L02 cells in the presence or absence of magnesium isoglycyrrhizinate (MgIG). (A, panels a to c) RT-PCR analysis was performed to evaluate the mRNA expression of IL-6, TGF-β and bFGF after OA, MgIG and 4-phenylbutyric acid (4-PBA) treatment. mRNA expression was normalized to β-actin. (B, panels a to c) Protein expression levels of IL-6, TGF-β and bFGF were measured by ELISA. Data are presented as the means ± SD. *P<0.05 vs. control group, #P<0.05 vs. OA group. All the experiments were repeated at least 3 times.
References


