BRIEF ARTICLE

Glycyrrhizin attenuates HMGB1-induced hepatocyte apoptosis by inhibiting the p38-dependent mitochondrial pathway

Geum-Youn Gwak, Tae Gun Moon, Dong Ho Lee, Byung Chul Yoo

Geum-Youn Gwak, Tae Gun Moon, Dong Ho Lee, Byung Chul Yoo, Department of Medicine, Samsung Medical Center, Sugnkyunkwan University School of Medicine, 50 Irwon-Dong, Gangnam-Gu, 135-710 Seoul, South Korea

Abstract

AIM: To examine how high-mobility group box 1 (HMGB1) regulates hepatocyte apoptosis and, furthermore, to determine whether glycyrrhizin (GL), a known HMGB1 inhibitor, prevents HMGB1-induced hepatocyte apoptosis.

METHODS: A human hepatocellular carcinoma cell line stably transfected with a bile acid transporter (Huh-BAT cells), were used in this study. Apoptosis was quantified using 4',6-diamidino-2-phenylindole dihydrochloride staining and the APO Percentage apoptosis assay, and its signaling cascades were explored by immunoblot analysis. Kinase signaling was evaluated by immunoblotting and by using selective inhibitors. It is also tried to identify hepatocyte apoptosis affected by the HMGB1 inhibitor, GL.

RESULTS: HMGB1 increased cellular apoptosis in Huh-BAT cells. HMGB1 led to increased cytochrome c release from mitochondria into the cytosol, and induced the cleavage of procaspase 3. However, it did not affect the activation of caspase 8. HMGB1-induced caspase 3 activation was significantly attenuated by the p38 inhibitor SB203580. GL significantly attenuated HMGB1-induced hepatocyte apoptosis. GL also prevented HMGB1-induced cytochrome c release and p38 activation in Huh-BAT cells.

CONCLUSION: The present study demonstrated that HMGB1 promoted hepatocyte apoptosis through a p38-dependent mitochondrial pathway. In addition, GL had an anti-apoptotic effect on HMGB1-treated hepatocytes.

INTRODUCTION

High-mobility group box 1 (HMGB1) is an evolutionarily conserved protein present in the nucleus of almost all eukaryotic cells, where it is involved in DNA replication, repair and transcription[1-3]. This molecule is known to be released by cells undergoing necrosis as well as being

secreted by activated macrophages. While early studies of HMGB1 demonstrated its role as a late mediator of sepsis[4], HMGB1 has been more recently implicated as a putative danger signal involved in the pathogenesis of a variety of non-infectious inflammatory conditions including autoimmunity, cancer, trauma, and hemorrhagic shock, and ischemia-reperfusion injury (IRI)[5-11]. So far, HMGB1 has been studied in a number of organs including liver, lung, breast and prostate[7-9,11].

In the liver, the importance of HMGB1 signaling has been largely identified in cases of IRI, during which tissue levels of HMGB1 were elevated following reperfusion and neutralizing antibodies against HMGB1 ameliorated the damage resulting from IRI in a toll-like receptor (TLR)4-dependent manner[11]. The pathogenetic role of HMGB1 in liver disease was also clarified by studying the inflammatory response to viral infection[12]. Following hepatocyte death by hepatitis B virus-specific cytotoxic T lymphocytes in a mouse model of hepatitis, HMGB1 directs the intrahepatic recruitment of neutrophils and all other non-antigen specific inflammatory cells (natural killer cells, T cells, B cells, monocytes, macrophages and dendritic cells).

Apoptosis, a stereotyped morphologic form of cell death, is an event that contributes to liver injury in a wide range of acute and chronic liver diseases[13]. However, it is not clear whether HMGB1 contributes to apoptotic cell death in the liver. Furthermore, the regulatory mechanism of HMGB1 in hepatocyte apoptosis remains largely undefined.

Glycyrrhizin (GL) is a major active constituent of licorice root that is commonly used in Asia to treat patients with chronic hepatitis[14-16]. This compound has been associated with numerous pharmacologic effects, including anti-inflammatory, anti-viral, anti-tumor, and hepatoprotective activities[17]. Recently, GL was recognized by Sitia *et al*[18] as an HMGB1 inhibitor, which binds directly to both HMG boxes in HMGB1.

Thus, the aim of this study was to provide *in vitro* evidence and a potential theoretical basis for HMGB1 regulation of hepatocyte apoptosis in order to further elucidate the molecular mechanism of HMGB1 involvement in various pathologic conditions that can affect the liver. Furthermore, we attempted to determine whether GL attenuates HMGB1-induced hepatocyte apoptosis and, if so, to identify the signaling cascades responsible for this modulation.

MATERIALS AND METHODS

Cell line and culture
Several human hepatoma cell lines were chosen for this study: Huh-7 cells stably transfected with a bile acid transporter[19] derived from a well-differentiated hepatocellular carcinoma (HCC)[20] (Huh-BAT), HepG2 and SNU-475 cells derived from a poorly differentiated HCC[21]. All cells were cultured in Dulbecco's Modified Eagle medium supplemented with 10% fetal bovine se-

rum, 100 000 U/L penicillin and 100 mg/L streptomycin. In all experiments, cells were serum-starved for 12 h in order to avoid the effects of serum-induced signaling.

Materials and reagents
HMGB1 (human, recombinant expressed in *E. coli*) was synthesized by Sigma-Aldrich, Inc. (St. Louis, MO, United States) at a purity of > 90%. The MAPK inhibitors [SB203580 for p38 mitogen activated protein kinase (MAPK), U0126 for p42/44 MAPK or extracellular signal-regulated kinase, and SP600125 for c-Jun N-terminal kinase (JNK) and GL] were also obtained from Sigma-Aldrich, Inc.

Preparation of mitochondrial and cytosolic extracts
Cells were washed twice with phosphate-buffered saline, and mitochondrial and cytosolic extracts were isolated using a mitochondria/cytosol fractionation kit (BioVision, Inc., Mountain View, CA, United States) according to the manufacturer's instruction.

Immunoblot analysis
Huh-BAT cells were lysed for 20 min on ice with lysis buffer (50 mol/L Tris-HCl, pH 7.4; 1% Nonidet P-40; 0.25% sodium deoxycholate; 150 mol/L NaCl; 1 mol/L EDTA; 1 mol/L PMSF; 1 μg/mL aprotinin, leupeptin, pepstatin; 1 mol/L Na₃VO₄; and 1 mol/L NaF) and centrifuged at 14 000 × *g* for 10 min at 4 ℃. Proteins in the lysates were resolved by 10% or 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis, transferred to PVDF membranes, and probed using the following primary antibodies: mouse anti-caspase 8 (1:500 dilution) from Cell Signaling Technology (Danvers, MA, United States); rabbit anti-caspase 3 (1:1000 dilution) from Cell Signaling Technology; rabbit anti-ACTIVE® p42/p44 (1:2000 dilution), anti-ACTIVE® p38 (1:1000 dilution), and anti-ACTIVE® JNK (1:1000 dilution) specific for the phosphorylated forms of p42/p44 MAPK, p38 MAPK, and JNK, respectively, from Cell Signaling Technology; mouse anti-cytochrome c (1:500 dilution) from BD Pharmingen (San Jose, CA, United States), and goat anti-actin (1:1000 dilution) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, United States). Twenty μg of protein was used for each well in Western blotting. Primary antibody binding was detected with appropriate peroxidase-conjugated secondary antibodies (Biosource International, Camarillo, CA). Bound antibodies were visualized using a chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL, United States) and the blots were exposed to Kodak X-OMAT film.

The signals in the Western blotting were quantified by densitometric scanning and normalized by using the intensity of corresponding protein band relative to the actin band.

Quantification of apoptosis
Quantitative detection of apoptotic cells was performed using two different methods: the nuclear binding dye DAPI and fluorescence microscopy, and the APO Per-
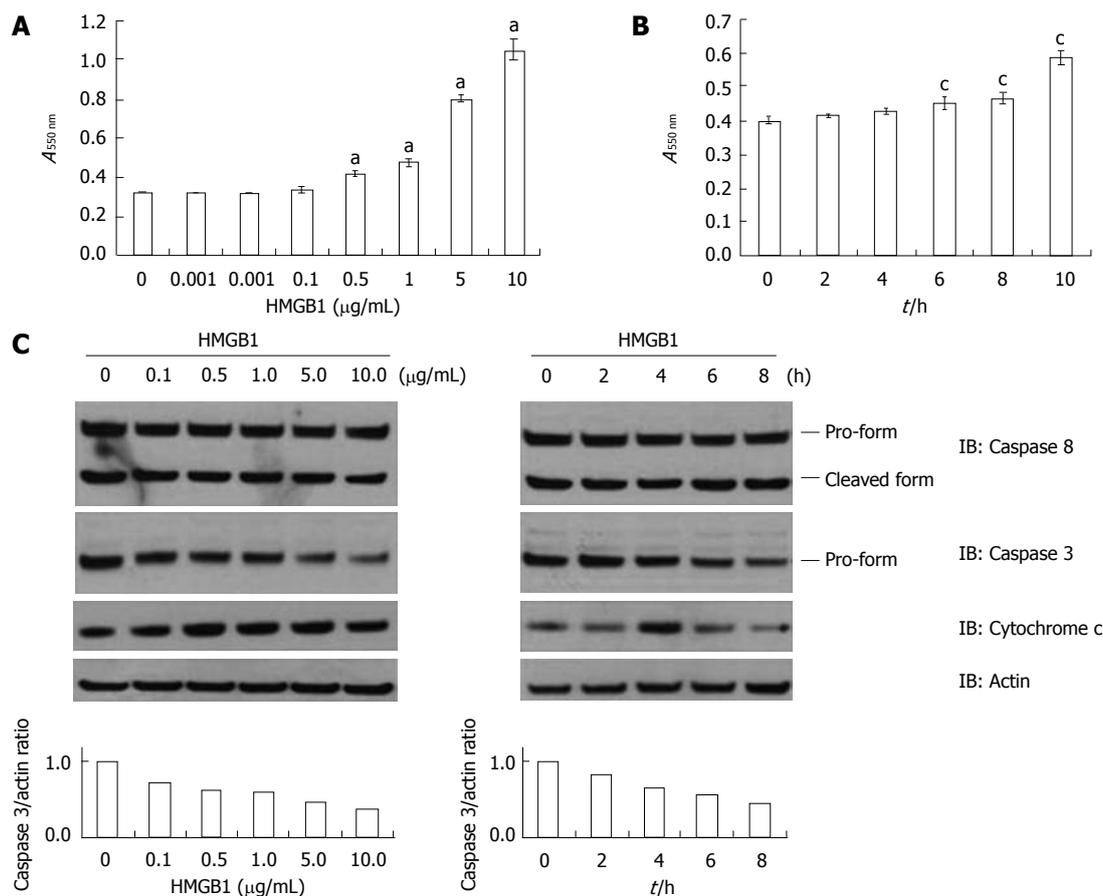
Figure 1 High-mobility group box 1 enhances hepatocyte apoptosis *via* a mitochondrial pathway. A: Huh-BAT cells were treated with high-mobility group box 1 (HMGB1) (0, 0.001, 0.01, 0.1, 0.5, 1, 5 and 10 μg/mL) for 6 h. Apoptosis was quantified using an APO Percentage apoptosis assay kit. Data are expressed as the mean ± SD of three individual experiments. [a]$P < 0.05$, *vs* HMGB1 0 μg/mL; B: Huh-BAT cells were treated with 10 μg/mL of HMGB1 for the indicated time periods. [c]$P < 0.05$, *vs* 0 h; C: Huh-BAT cells were treated with HMGB1 (0 μg/mL, 0.1 μg/mL, 0.5 μg/mL, 1, 5 μg/mL and 10 μg/mL) for 6 h (left column), or with 10 μg/mL of HMGB1 for the indicated time periods (right column). Cells were lysed at the indicated time points, and immunoblot analysis was performed using anti-caspase 8 and anti-caspase 3 antibodies. Mitochondrial and cytosolic extracts were also isolated, and equivalent amounts of cytosolic protein were immunoblotted with an anti-cytochrome c antibody. Immunoblot analysis using an anti-actin antibody was performed as a control for protein loading.

centage apoptosis assay kit (Biocolor Ltd., Belfast, Northern Ireland). For the APO Percentage apoptosis assay, the cells were seeded at 10^4 cells per well in a 96-well plate and processed according to the manufacturer's instructions.

Statistical analysis

All data were from at least three independent experiments using cells from a minimum of three separate isolations, and are expressed as the mean ± SD. Differences between the groups were compared using a two-tailed Student's *t* tests or the Mann-Whitney *U* test as appropriate. *P* values of < 0.05 were considered to be statistically significant.

RESULTS

HMGB1 significantly increased cellular apoptosis in Huh-BAT cells in a dose- and time-dependent manner (Figure 1A and B). We repeated the same experiments in the other two hepatoma cell lines (HepG2 and SNU-475 cells) and observed the same effects (data not shown). We next identified the pro-apoptotic signaling pathways

induced by HMGB1 treatment. HMGB1 increased cytochrome c release from mitochondria into cytosol and induced the cleavage of procaspase 3. However, it did not affect the activation of caspase 8, an initiator caspase downstream of death receptor activation (Figure 1C).

Since the MAPK family signaling cascades regulate apoptotic pathways, we next evaluated whether HMGB1 modulates MAPK activation. HMGB1 induced the activation of MAPKs such as p42/44, p38 MAPK, and JNK in Huh-BAT cells (Figure 2A). In order to explore the role of individual MAPKs in apoptotic signaling, the cells were then treated with HMGB1 either in the presence or absence of various inhibitors: U0126 for p42/44, SB203580 for p38, and SP600125 for JNK. When the cells were treated with the p38 inhibitor, HMGB1-induced caspase 3 activation was significantly attenuated whereas treatment with inhibitors of p42/44 or JNK did not affect caspase 3 cleavage (Figure 2B).

Pretreatment with GL significantly attenuated HMGB1-induced hepatocyte apoptosis in a dose-dependent manner (Figure 3A). GL also attenuated cytochrome c release from the mitochondria into cytosol (Figure 3B). Finally, pretreatment with GL decreased HMGB1-induced p38
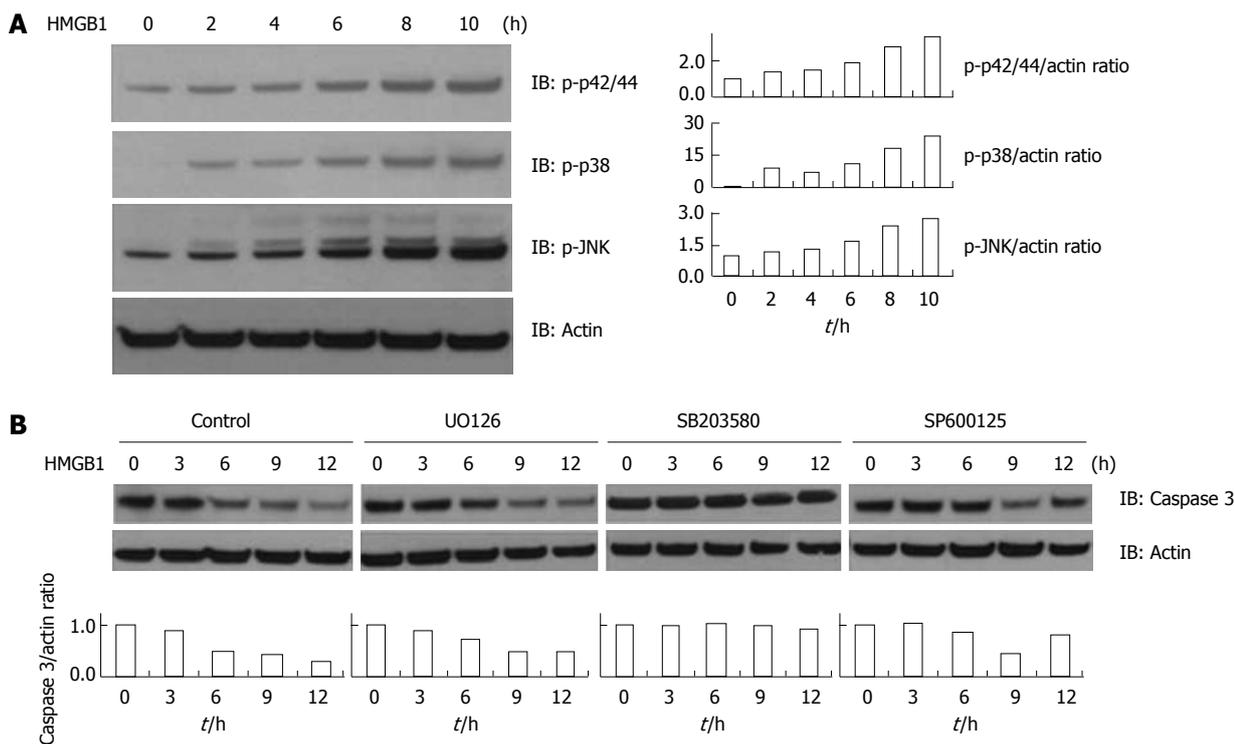
A

B

Figure 2 High-mobility group box 1-induced hepatocyte apoptosis is p38-dependent. A: Huh-BAT cells were treated with 10 μg/mL of high-mobility group box 1 (HMGB1) for the indicated time periods. Cells were lysed at the indicated time points, and immunoblot analysis was performed on cell lysates using antibodies specific for the phosphorylated forms of p42/p44, p38, or c-Jun N-terminal kinase (JNK); B: Huh-BAT cells were pretreated with mitogen activated protein kinase inhibitors U0126 (30 μmol/L), SB203580 (10 μmol/L), or SP600125 (20 μmol/L), or media (control) for 12 h. Cells were then treated with 10 μg/mL of HMGB1. Cells were lysed at the indicated time points, and immunoblot analysis was performed on cell lysates using anti-caspase 3 and anti-actin antibodies.

activation in Huh-BAT cells (Figure 3C). Taken together, all of the findings from our study indicate that HMGB1 induces hepatocyte apoptosis through a p38-dependent mitochondrial pathway which was inhibited by GL.

DISCUSSION

In virtually all human liver diseases, hepatocytes undergo cell death by apoptosis[13]. Thus, therapeutic modulation of apoptosis has the potential to alter the course of human liver disease. Apoptosis may occur *via* two fundamental pathways: (1) the death receptor or extrinsic pathway; and (2) the mitochondrial or intrinsic pathway. Caspases, representing the family of cysteine proteases, play a critical role in both pathways. Both pathways can either directly or indirectly converge to activate the "effector caspase", namely caspase-3, which induces DNA fragmentation and other morphological changes characteristic of apoptotic cell death[22]. In the present study, HMGB1 activated caspase 3 without affecting caspase 8, an initiator caspase downstream of death receptor activation. These findings suggest that the mitochondrial pathway is responsible for HMGB1-induced hepatocyte apoptosis, which was further supported by the findings that HMGB1 increased cytochrome c release from mitochondria into the cytosol.

MAPKs, which include p42/44, p38, and JNK, are involved in pro-apoptotic signal transduction as well as cell growth and differentiation[23]. It has been previously shown that the p38 MAPK cascade promotes either cell death or cell survival[23,24] depending on the cell type and the kinase isoforms activated by various stress stimuli[25]. There is abundant evidence that p38 participates in cellular apoptosis[26,27] with one mechanism being the modulation of Bcl-2 protein family members to maintain an apoptotic checkpoint for mitochondrial dysfunction and cytochrome c release[28,29]. Likewise, in the present study HMGB1-induced hepatocyte apoptosis occurred through a mitochondrial pathway which was p38-dependent.

GL, a triterpene glycoside extracted from licorice root (*Glycyrrhiza glabra*), consists of one molecule of 18b-glycyrrhetinic acid and two molecules of glucuronic acid having the structure 18-b-glycyrrhetinic acid-3-O-b-D-glucuronopyranosyl-(1/2)-b-D-glucuronide. It is known that this molecule has a variety of hepato-protective properties in terms of anti-inflammatory, antiviral, and anti-tumor effects[17]. In an animal model of acute liver injury induced by carbon tetrachloride, GL reduced the serum tumor necrosis factor-alpha (TNF-α) level and alleviates acute liver injury[30]. Moreover, GL has anti-inflammatory effects on lipopolysaccharide (LPS)-induced acute liver injury through inhibition of TNF-α release[31]. Recently, Ikeda *et al*[32] reported that GL reduced the number of TUNEL-positive cells in cases of acute hepatitis induced by LPS/D-galactosamine (GalN)-treatment. However, in a mouse model treated with LPS/D-GalN, anti-apoptotic effects of GL were found to be caspase-independent, and probably achieved
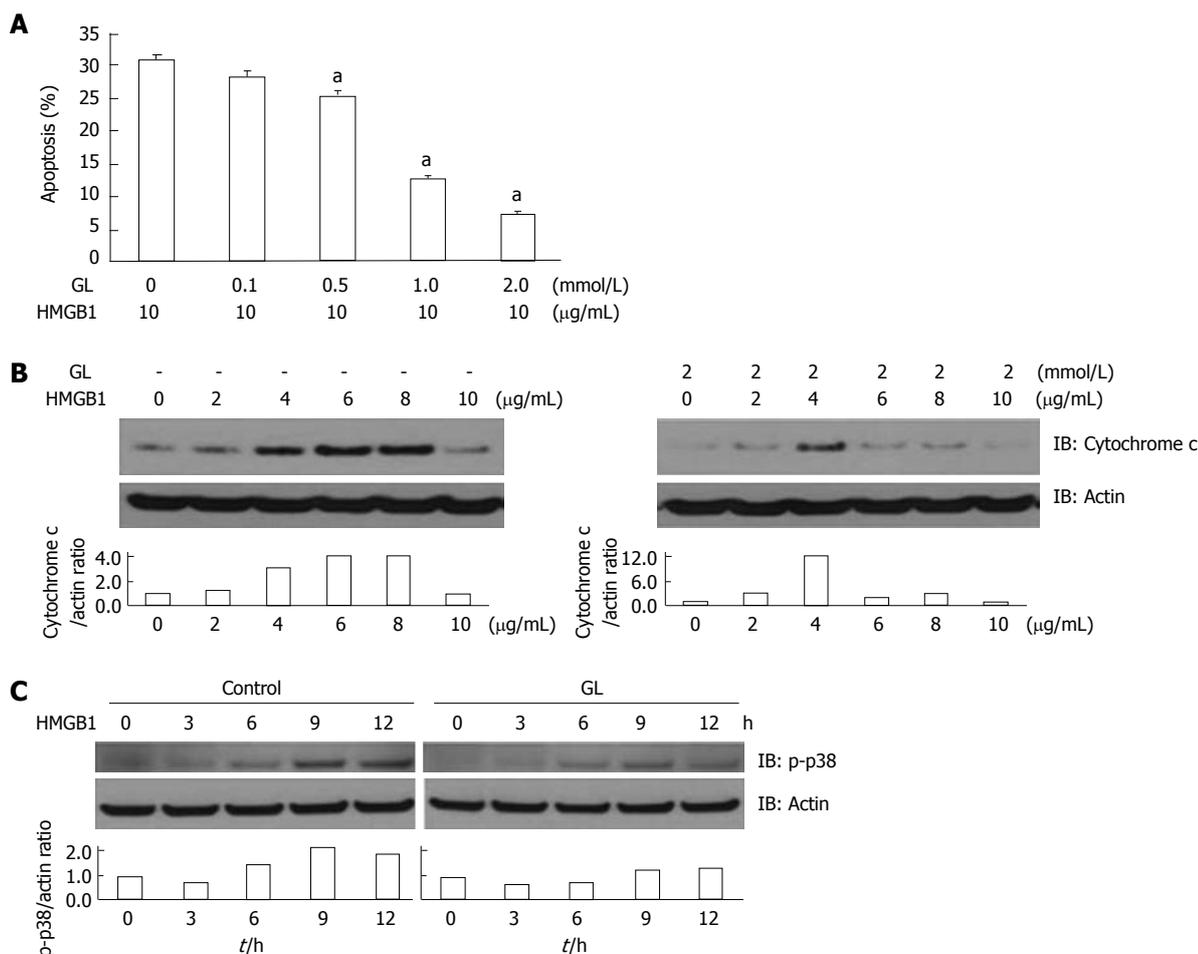
Figure 3 Glycyrrhizin attenuates high-mobility group box 1-induced hepatocyte apoptosis. A: Huh-BAT cells were pretreated with glycyrrhizin (GL) (0, 0.1, 0.5, 1.0 and 2.0 mmol/L) for 12 h. Cells were then treated with 10 μg/mL of high-mobility group box 1 (HMGB1) for 6 h. Apoptosis was quantified by 4',6-diamidino-2-phenylindole staining and fluorescence microscopy. Data are expressed as the mean ± SD of three individual experiments. [a]*P* < 0.05, *vs* GL 0 mmol/L; B: Huh-BAT cells were pretreated with GL (2.0 mmol/L) for 12 h prior to adding HMGB1 (0-10 μg/mL). Mitochondrial and cytosolic extracts were isolated after 6 h of HMGB1 treatment, and equivalent amounts of cytosolic protein were immunoblotted with an anti-cytochrome c antibody. Immunoblot analysis using an anti-actin antibody was performed as a control for protein loading; C: Huh-BAT cells were pretreated with GL (2.0 mmol/L) for 12 h prior to being incubated with HMGB1. Cells were then lysed at the indicated time points, and immunoblot analysis was performed using antibodies specific for the phosphorylated forms of p38 and actin.

through the prevention of an interleukin-18-mediated inflammatory response. In the present study, we demonstrated that GL attenuated HMGB1-induced hepatocyte apoptosis by blocking the p38-dependent mitochondrial pathway. Therefore, it is likely that the hepato-protective effects of GL are attributed to various mechanisms.

In summary, the present study demonstrated that HMGB1 participated in hepatocyte apoptosis through a p38-dependent mitochondrial pathway. In addition, GL had an anti-apoptotic effect on hepatocytes treated with HMGB1. Therefore, HMGB1 inhibitors, including GL, might be therapeutically efficacious in treating HMGB1-mediated liver injury such as viral hepatitis, hepatic ischemia-reperfusion injury and sepsis-related liver injury.

COMMENTS

Background

High mobility group box 1 (HMGB1) is an evolutionarily conserved protein present in the nucleus of almost all eukaryotic cells, where it is involved in DNA replication, repair and transcription. Glycyrrhizin (GL) is a major active constitu-

ent of licorice root that is commonly used in Asia to treat patients with chronic hepatitis. This compound has been associated with numerous pharmacologic effects, including anti-inflammatory, anti-viral, anti-tumor, and hepatoprotective activities. Recently, GL was recognized as an HMGB1 inhibitor, which binds directly to both HMG boxes in HMGB1.

Research frontiers

The authors provide *in vitro* evidence and a potential theoretical basis for HMGB1 regulation of hepatocyte apoptosis in order to further elucidate the molecular mechanism of HMGB1 involvement in various pathologic conditions that can affect the liver. Furthermore, they attempted to determine whether GL attenuates HMGB1-induced hepatocyte apoptosis and, if so, to identify the signaling cascades responsible for this modulation.

Innovations and breakthroughs

Present study demonstrated that HMGB1 participated in hepatocyte apoptosis through a p38-dependent mitochondrial pathway. In addition, GL had an anti-apoptotic effect on hepatocytes treated with HMGB1.

Applications

HMGB1 inhibitors, including GL, might be therapeutically efficacious in treating HMGB1-mediated liver injury such as viral hepatitis, hepatic ischemia-reperfusion injury and sepsis-related liver injury.

Peer review

This is an interesting study where the authors show that HMGB1 induces hepatocyte apoptosis which is mediated by p38. In addition, glycyrrhizin was shown to inhibit HMGB1-induced apoptosis as well as activation of p38 in the

cultured hepatocyte cell line. The study is well conducted and the manuscript is well written.

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