Ghrelin reduces liver impairment in a model of concanavalin A-induced acute hepatitis in mice

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Background and aims: Ghrelin is a 28-amino-acid gut hormone that was first discovered as a potent growth hormone secretagogue. Recently, it has been shown to exert a strong anti-inflammatory effect. The purpose of the study reported here was to explore the effect and mechanism of ghrelin on concanavalin (Con) A-induced acute hepatitis.

Methods: BALB/C mice were divided into four groups: normal control (NC) (mice injected with vehicle [saline]); Con A (25 mg/kg); Con A + 10 μg/kg ghrelin; and Con A + 50 μg/kg ghrelin (1 hour before Con A injection). Pro-inflammatory cytokine levels were detected. Protein levels of phosphoinositide 3-kinase (PI3K); phosphorylated Akt (p-Akt); caspase 3, 8, and 9; and microtubule-associated protein 1 light chain 3 (LC3) were also detected. Perifosine (25 mM) (an Akt inhibitor) was used to investigate whether the protective effect of ghrelin was interrupted by an Akt inhibitor. Protein levels of p-Akt; Bcl-2; Bax; and caspase 3, 8, and 9 were also detected.

Results: Aspartate aminotransferase, alanine aminotransferase, and pathological damage were significantly ameliorated by ghrelin pretreatment in Con A-induced hepatitis. Inflammatory cytokines were significantly reduced by ghrelin pretreatment. Bcl-2; Bax; and caspase 3, 8, and 9 expression were also clearly affected by ghrelin pretreatment, compared with the Con A-treated group. However, the Akt kinase inhibitor reversed the decrease of Bax and caspase 3, 8, 9, and reduced the protein level of p-Akt and Bcl-2. Ghrelin activated the PI3K/Akt pathway and inhibited activation of autophagy.

Conclusion: Our results demonstrate that ghrelin attenuates Con A-induced acute immune hepatitis by activating the PI3K/Akt pathway and inhibiting the process of autophagy, which might be related to inhibition of inflammatory cytokine release, and prevention of hepatocyte apoptosis. These effects could be interrupted by an Akt kinase inhibitor.

Keywords: PI3K/Akt pathway, autophagy, inflammation, apoptosis

Introduction

Hepatitis is an increasingly serious human health problem worldwide, characterized by increased levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and inflammatory cytokines, accompanied with hepatocyte apoptosis and necrosis. Hepatitis can be caused by various factors such as viruses, immune dysfunction, drugs, alcohol, lipids, genetics, and idiopathic factors. Although significant progress has been made in elucidating the mechanisms of hepatitis, effective therapeutic agents are still lacking.

Concanavalin A (Con A) is a plant lectin and T-cell mitogen, which rapidly induces T-helper (Th) cell activation, mostly CD4+ T-cells, leading to inflammatory liver injury in mice.1-3 Natural killer (NK) T-cells and macrophages are also considered to play key roles in the development of liver damage induced by Con A.4 Pro-inflammatory cytokines – including interleukin (IL)-1, IL-2, IL-6, tumor necrosis factor (TNF)-α, and...
interferon-γ are significantly increased in Con A-induced liver injury (Figure 1). Therefore, Con A-induced liver injury is an ideal model of T-cell-induced hepatitis, especially immune hepatitis.

Several signaling pathways are involved in the mechanism of Con A-induced acute hepatitis. Recent studies demonstrated that activating phosphorylated Akt (p-Akt) significantly attenuated Con A-induced liver injury. Apoptosis, known as type I programmed cell death, is also involved in Con A-induced hepatitis. Members of the Bcl-2 family of proteins have an essential role in the apoptotic pathway: Bcl-2 and Bcl-xl as anti-apoptotic proteins, and Bax and Bad as pro-apoptotic proteins. Modulation of the balance between Bax and Bcl-2 proteins determines the survival status of cells challenged with various stimuli.

Autophagy is an intracellular process that targets defective organelles, surrounding them with autophagosomes, and delivering them to the lysosomes for degradation. Autophagy, usually activated by energy restriction during inflammation, plays crucial roles in development, differentiation, survival, and homeostasis, and has attracted considerable concern due to its implications in human disease. However, autophagy is characterized as type II programmed cell death, and can also cause cell death, therefore, we regard it as a double-edged sword. Microtubule-associated protein 1 light chain 3 (LC3), is widely used as a marker for monitoring autophagy.

Ghrelin, a newly discovered 28-amino-acid gastric peptide hormone first purified from stomach of rats, is the endogenous ligand for growth hormone secretagogue receptor 1a. Originally regarded as a growth hormone, ghrelin has recently been shown to have various physiological effects, including on energy metabolism, glucose homeostasis, and food intake. Ghrelin is involved in the regulation of inflammation and shows strong anti-inflammatory effects.

The study reported here investigated whether ghrelin exerted any effect on Con A-induced immune hepatitis and its mechanism of action. We report that ghrelin significantly reduced liver injury, as measured by plasma hepatic enzymes, inflammatory cytokines, and histological changes, such as inflammatory cell infiltration and hepatocyte apoptosis and

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**Figure 1** Mechanism of the protective effect of ghrelin on Con A-induced hepatitis.

**Notes:** Ghrelin activates PI3K and promotes the phosphorylation of its downstream Akt, leading to the proliferation of injured hepatocytes and the inhibition of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α. Phosphorylated Akt promotes the expression of Bcl-2, which inhibits Bax and downregulates caspase 3, 8, and 9. PI3K/Akt may lead to the inhibition of the process of autophagy, which is activated by Con A treatment in uncertain mechanisms.

**Abbreviations:** Con, concanavalin; IL, interleukin; PI3K, phosphoinositide 3-kinase; TNF, tumor necrosis factor.
necrosis, which may be partly associated with PI3K/Akt/ Bcl-2 pathway activation and autophagy inhibition.

Materials and methods

Reagents

Ghrelin was purchased from ProSpec (Ness-Ziona, Israel). Con A was purchased from Sigma-Aldrich (St Louis, MO, USA). Perifosine was provided by Keryx Biopharmaceuticals (New York, NY, USA). Antibodies against IL-1β, IL-6, TNF-α, total Akt, and p-Akt were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). LC3, Beclin 1, Bcl-2, and Bax were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against caspase 3, caspase 8, and caspase 9 were purchased from Proteintech (Chicago, IL, USA).

Animals and treatment

Balb/c mice (8 weeks old, 23±2 g) were purchased from Shanghai Laboratory Animal Co Ltd (Shanghai, People’s Republic of China). The mice were housed in a clean room maintained at 24°C±2°C with an alternating 12-hour light and dark cycle, with free access to food and water. The study was approved by the Animal Care and Use Committee and the Ethics Committee of Fudan University. Mice were randomly divided into four groups, with seven animals per group: (1) normal control (NC): mice injected with vehicle (saline), (2) Con A, (3) Con A + 10 μg/kg ghrelin, and (4) Con A + 50 μg/kg ghrelin. Ghrelin was diluted with normal saline and injected intraperitoneally 1 hour before injection of Con A. Con A was dissolved in normal saline solution at a concentration of 5 mg/mL and injected via the tail vein at a dose of 25 mg/kg to induce acute hepatic injury. Each group of mice was sacrificed randomly at 4, 8, and 24 hours.

To investigate whether the protective effect could be interrupted by an Akt kinase inhibitor, we created another four groups of mice: NC, Con A, Con A + 10 μg/kg ghrelin, and Con A + 10 μg/kg ghrelin + 25 mM perifosine. The choice and dose of Akt inhibitor was determined based on a high-quality study.17 Mice were killed at 8 hours. This was because the preliminary experiment showed that Con A-induced hepatitis was most serious at 8 hours, and ghrelin exerted its best therapeutic effect at that time. Liver samples and blood were acquired for histological and molecular analysis.

Biochemical assays

Serum levels of ALT and AST were measured with microplate test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, People’s Republic of China). Serum pro-inflammatory cytokines TNF-α and IL-6 were measured by enzyme-linked immunosorbsent assay (R&D Systems, Minneapolis, MN, USA).

Histopathology

The middle portion of the left lobe of the liver was excised and sectioned and then perfused in 4% paraformaldehyde for at least 24 hours. After fixation, the tissues were embedded in paraffin, and 5 μm thick sections were stained with hematoxylin and eosin (H&E) to observe the tissue damage by light microscopy.

Immunohistochemistry

Liver sections (3 μm) were dewaxed, rehydrated regularly, and treated with 3% H2O2. The sections were pretreated with a microwave antigen-retrieval technique. The nonspecific sites were blocked with 10% goat serum for 30 minutes at room temperature. The liver slices were incubated overnight with antibody directed against p-Akt (1:500), IL-1β (1:500), IL-6 (1:500), TNF-α (1:500), LC3II (1:500), Bcl-2 (1:500), and Bax (1:500). On the second day after incubation with a secondary antibody, an antibody binding assay was performed using a diaminobenzidine kit. At last, the slides were counterstained with hematoxylin and observed under a light microscope. Three different fields of vision were randomly selected in one slide, and the ratios of brown staining areas and total areas were acquired with Image-Pro® Plus (v 6.0) software. We calculated the average of these three ratios. The described method was applied in all groups.

Western blotting

Total protein was extracted according to standard procedures. Equivalent amounts of total protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Nonspecific binding was blocked with 5% nonfat milk (dissolved in phosphate-buffered saline) for 1 hour and then incubated overnight at 4°C with different primary antibodies. After incubation with peroxidase-conjugated secondary antibodies for 1 hour at 37°C, membranes were developed with the Odyssey two-color infrared laser imaging system (fluorescence detection).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

TUNEL assay was performed according to the manufacturer’s instructions (Roche, Mannheim, Germany). Liver sections
were treated with 20 μg/mL proteinase K at room temperature for 15 minutes, followed by incubation with the TUNEL reaction mixture at 37°C in a humidified atmosphere for 1 hour. Three different fields of vision were randomly selected in one slide, the brown nucleus cells, regarded as positive cells, were visualized and counted, and the percentage of apoptotic cells to total cell numbers was calculated. We calculated the average of these three ratios.

RNA isolation and real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)
Liver tissue was analyzed with real-time qRT-PCR. Total RNA was extracted from frozen liver tissues or cells with TRIzol® reagent (Tiangen Biotech [Beijing] Co, Ltd, Beijing, People’s Republic of China). SYBR Green qRT-PCR was performed with an Applied Biosystems® 7900HT Fast Real-Time PCR [polymerase chain reaction] system (Thermo Fisher Scientific, Waltham, MA, USA) to determine expression of the target genes, according to the instructions for SYBR Premix EX Taq (TaKaRa Biotechnology [Dalian] Co, Ltd, Dalian, People’s Republic of China). The primers used in the PCR reactions are listed in Table 1.

Statistical analysis
All results are expressed as means ± standard deviation. Comparisons between two groups were made with Student’s t-test. Statistical differences in multiple groups were determined by multiple comparisons with analysis of variance, followed by Tukey’s post-hoc test. All statistical analyses were performed with Graphpad Prism (v 6.0) software. P-values <0.05 were considered statistically significant.

Table 1 Nucleotide sequences of primers used for PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
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| IL-1β | Forward: ATAAGCCCACCTCTACACCT  
Reverse: ATGGGCCCTGAAAGGAGGA |
| IL-6  | Forward: CTGCAAGAGAATCCATCCAG  
Reverse: AGTTGTATAGACAGGTCTGG |
| TNF-α | Forward: CAGGGCGTGCATATGCTC  
Reverse: CGATACACCCCAAGACTCATAG |
| Bcl-2 | Forward: GCCAATGCATGACTGCTG  
Reverse: CCCCAACGAATCTAAAGAAGG |
| Bax   | Forward: AGACAGGGGCTTTTCTTAC  
Reverse: AATTGCACCCAGACACTCG |
| β-actin| Forward: GGCTGTATCCCTCCCTCATCG  
Reverse: CCAGTGGTGAAACAATGCCCATGT |

Abbreviation: PCR, polymerase chain reaction.

Results

Ghrelin pretreatment ameliorates Con A-induced hepatitis
Con A significantly increased serum ALT and AST levels, with a peak at 8 hours after Con A injection, whereas both 10 and 50 μg/kg doses of ghrelin clearly reduced them, as shown in Figure 2A. Histopathological changes in the three groups were examined after H&E staining (Figure 2B). Massive areas of hepatic tissue inflammation and necrosis were observed in the mice treated with Con A alone, which was significantly ameliorated in the ghrelin and Con A co-treated group. Image-Pro Plus software showed that there were significant differences of hepatic injury between the Con A-treated and Con A and ghrelin co-treated groups.

Ghrelin pretreatment inhibits release of inflammatory cytokines during Con A-induced hepatitis
Hepatitis is associated with changes in the levels of inflammatory cytokines. Serum levels of IL-1β, IL-6, and TNF-α were significantly increased in the Con A-treated group and peaked at 8 hours after Con A injection. As expected, pretreatment with both doses of ghrelin significantly lowered the Con A-induced increase of serum levels of IL-1β, IL-6, and TNF-α, especially at 8 hours (Figure 3A). To confirm our observations, mRNA transcription and protein expression of the cytokines were also reduced by ghrelin pretreatment (Figures 3B and C). In addition, immunohistochemical staining was used to detect the expression of the inflammatory cytokines in liver tissues in the control, Con A, Con A + 10 μg/kg ghrelin, and Con A + 50 μg/kg ghrelin groups (Figure 3D). These results showed that ghrelin pretreatment reduced inflammatory cytokines in Con A-induced hepatitis.

Ghrelin attenuates hepatocyte apoptosis in Con A-induced hepatitis
We investigated expression of caspase 3, 8, and 9. All three enzymes were activated by Con A injection and reduced by ghrelin treatment (Figure 4B). Likewise, as expected, TUNEL assay showed clear improvement of apoptosis in ghrelin-pretreated compared with Con A-treated mice (Figure 4C). Furthermore, ghrelin pretreatment significantly increased mRNA and protein expression of Bcl-2 and reduced expression of Bax at all three time points, especially at 8 hours (Figure 4A). In addition, the location and expression of Bcl-2 by immunohistochemical staining was reduced in the Con A group compared with the normal group at 8 hours, while 10 μg/kg ghrelin significantly increased...
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Figure 2: Ghrelin pretreatment ameliorates Con A-induced hepatitis.

Notes: (A) Serum ALT and AST levels are expressed as the mean ± SD of six animals per group at 4, 8, and 24 h after Con A injection in mice and the effects of both low (10 μg/kg) and high (50 μg/kg) dose ghrelin pretreatment at the same time. (B) Hematoxylin and eosin staining of liver sections. The cellular necrotic or edematous areas were analyzed with Image-Pro® Plus (v 6.0); (n=6). *P<0.05 for NC vs Con A; #P<0.05 for Con A vs Con A + 10 μg/kg ghrelin; †P<0.05 for Con A + 50 μg/kg ghrelin vs Con A + 10 μg/kg ghrelin, and ‡P<0.05 for Con A + 50 μg/kg ghrelin vs Con A + 10 μg/kg ghrelin.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; Con, concanavalin; h, hours; NC, normal control.
Figure 3 Ghrelin pretreatment inhibits release of IL-1β, IL-6, and TNF-α in Con A-induced hepatitis.

Notes: (A) The serum IL-1β, IL-6, and TNF-α levels are expressed as mean ± SD of six animals per group at 4, 8, and 24 h after Con A injection in mice and the effects of both low (10 μg/kg) and high (50 μg/kg) dose ghrelin pretreatment at the same time. (B) mRNA expression of IL-1β, IL-6, and TNF-α in the NC, Con A, Con A + 10 μg/kg ghrelin, and Con A + 50 μg/kg ghrelin groups was evaluated by real-time PCR. (C) Western blots showing the expression of IL-1β, IL-6, and TNF-α in liver tissues. (D) Immunohistochemistry staining (200×) showing the expression of IL-1β, IL-6, and TNF-α in liver tissue at 8 h. The ratio of brown area to total area was analyzed with Image-Pro® Plus (v 6.0); (n=6). *P<0.05 for NC vs Con A, †P<0.05 for Con A vs Con A + 10 μg/kg ghrelin, ‡P<0.05 for Con A + 50 μg/kg ghrelin vs Con A + 10 μg/kg ghrelin, and †, *P<0.05 for Con A + 50 μg/kg ghrelin vs Con A + 10 μg/kg ghrelin.

Abbreviations: Con, concanavalin; h, hours; IL, interleukin; NC, normal control; TNF, tumor necrosis factor; PCR, polymerase chain reaction.
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Figure 4 Ghrelin attenuates hepatocyte apoptosis in Con A-induced hepatitis.

Notes: (A) Western blots and real-time PCR evaluation of expression of Bcl-2 and Bax in liver tissues. (B) Western blotting of expression of caspase 3, 8, and 9 in liver tissues. (C) TUNEL staining showed apoptotic cells in three groups at 8 h (200×). (D) Immunohistochemistry staining (200×) showed the expression of Bcl-2 and Bax protein in liver tissue at 8 h. *P<0.05 for NC vs Con A, †P<0.05 for Con A vs Con A + 10 μg/kg ghrelin, and †P<0.05 for Con A + 50 μg/kg ghrelin vs Con A + 10 μg/kg ghrelin.

Abbreviations: Con, concanavalin; h, hours; NC, normal control; PCR, polymerase chain reaction.
it. Immunohistochemical staining of Bax expression was stronger in the Con A-treated group, which was clearly reduced by 10 μg/kg ghrelin pretreatment at 8 hours (Figure 4D).

**Ghrelin activates PI3K/Akt signaling in Con A-induced hepatitis**

The protein level of PI3K was slightly elevated in the Con A-treated group but clearly increased in the ghrelin-pretreated group at three time points. Moreover, Con A impaired the expression of p-Akt at 8 hours and slightly increased the expression of p-Akt at 4 hours and 24 hours compared with the normal group. Both doses of ghrelin significantly increased the expression of p-Akt (Figure 5A). Expression of p-Akt in the liver tissues of mice in the control, Con A, and Con A + 10 μg/kg ghrelin groups at 24 hours was detected using immunohistochemistry (Figure 5B).

We conclude that ghrelin treatment can ameliorate Con A-induced hepatitis at least in part through the PI3K/Akt pathway.

**The protective effect of ghrelin could be interrupted by an Akt kinase inhibitor**

The protein levels of p-Akt; Bcl2; Bax; and caspase 3, 8, and 9 were detected. Perifosine, an Akt kinase inhibitor, reversed the decrease of Bax and caspase 3, 8, and 9, and reduced the protein level of p-Akt and Bcl2 in treated mice compared with those treated with Con A + ghrelin only (Figure 6).

This result supports our speculation that ghrelin exerts its protective effect at least partly via PI3K/Akt/Bcl2 pathway.

**Ghrelin attenuates autophagy in Con A-induced hepatitis**

LC3 is an important marker of autophagy. We assessed the activation of autophagy in each group of mice. The protein level of LC3 was increased by Con A treatment but reduced by ghrelin, indicating that autophagy was activated by Con A but inhibited by ghrelin (Figure 5A).

**Discussion**

Hepatitis can be caused by virus infection, drugs, alcohol, fatty acids, and autoimmunity, and remains a global health problem for which effective drug therapies are still lacking. Hepatitis is critical in many liver diseases that progress to end-stage liver disease, even hepatocellular carcinoma. Therefore, more effective drugs are urgently needed for intervention at the hepatitis stage. Con A-induced hepatitis is a well-established experimental model of immune hepatitis, cell apoptosis, and necrosis, and possibly ultimately liver failure.
Ghrelin reduces liver impairment in mouse model of acute hepatitis

Figure 5 Ghrelin activates PI3K/Akt signaling and inhibits autophagy in Con A-induced hepatitis.

Notes: (A) Western blotting and quantitative evaluation of PI3K, p-Akt, and LC3 expression in liver tissues. (B) Immunohistochemistry staining (200×) showed the expression of p-Akt protein in liver tissue at 24 h. *P<0.05 for NC vs Con A, *P<0.05 for Con A vs Con A + 10 μg/kg ghrelin, †P<0.05 for Con A + 50 μg/kg ghrelin vs Con A + 10 μg/kg ghrelin, and ††P>0.05 for Con A + 50 μg/kg ghrelin vs Con A + 10 μg/kg ghrelin.

Abbreviations: Con, concanavalin; h, hours; LC3, microtubule-associated protein 1 light chain 3; NC, normal control; p-Akt, phosphorylated Akt; PI3K, phosphoinositide 3-kinase.
Figure 6 The protective effect of ghrelin could be interrupted by Akt kinase inhibitor.

Notes: Western blotting of expression of p-Akt; Bcl-2; Bax; and caspase 3, 8, and 9 in liver tissues. Real-time PCR evaluation of expression of Bcl-2 and Bax, and density quantification of p-Akt. *P<0.05 for NC vs Con A, †P<0.05 for Con A vs Con A + 10 μg/kg ghrelin, and ‡P<0.05 for Con A + 10 μg/kg ghrelin + perifosine vs Con A + 10 μg/kg ghrelin.

Abbreviations: Con, concanavalin; p-Akt, phosphorylated Akt; NC, normal control; PCR, polymerase chain reaction.
increased Bax and decreased Bcl-2, resulting in cell death. However, ghrelin pretreatment significantly increased the expression Bcl-2, reduced the expression of Bax, and maintained the balance of Bcl-2 and Bax disrupted by Con A treatment (Figures 4A and D). The anti-apoptotic protein Bcl-2 inhibits apoptosis by preventing the release of cytochrome c, while Bax promotes mitochondrial membrane damage and cytochrome c release, leading to the activation of caspase 9 and 3 and cellular apoptosis (Figure 1). We supposed that ghrelin increases expression of Bcl-2 via the PI3K/Akt pathway, which may become a novel anti-inflammatory and anti-apoptotic agent for the treatment of liver injury. Moreover, we showed that the protective effect of ghrelin on hepatitis could be partly interrupted by an Akt kinase inhibitor, which supports our conclusion that ghrelin exerts its protective effect at least partly via the PI3K/Akt/Bcl-2 pathway (Figure 6). We observed a significant difference in effect between 10 and 50 μg/kg ghrelin-treated groups with reference to some but not all indicators and time points. We speculate that this effect is dose dependent, but the concentration gradient was not large enough; a greater concentration gradient is needed to investigate its dose-dependent effect.

The role of autophagy in hepatitis is worthy of further exploration. Autophagic cell death is involved in Con A-induced hepatitis. Several studies have reported that autophagy was activated in Con A-induced hepatitis, while autophagy inhibition was accompanied with hepatic injury attenuation. Therefore, we investigated whether ghrelin affected autophagy during Con A-induced hepatitis and whether it was mediated by the PI3K/Akt pathway. We showed that LC3II was inhibited in hepatocytes. This indicates that the PI3K/Akt pathway may be involved in the autophagic processes of Con A-induced hepatitis (Figure 1).

Conclusion
Our research demonstrates that ghrelin attenuated Con A-induced acute immune hepatitis, at least partly by activating the PI3K/Akt/Bcl-2 pathway and inhibiting autophagy. This is believed to be the first study to demonstrate that ghrelin attenuates Con A-induced liver injury and shows potential as a new therapeutic strategy for liver disease. However, the mechanism underlying ghrelin’s amelioration of liver injury is not clear and more research is needed.

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Disclosure
The authors declare no conflicts of interest in this work.

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