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Ghrelin contributes to protection of hepatocellular injury induced by ischaemia/reperfusion

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Keywords
AMPK – Ghrelin – ghrelin receptor – gut hormone – HIRI – liver

Abbreviations
ALT, Alanine Aminotransferase; AMPK, AMP-activated protein kinase; GHSR, growth hormone secretagogue receptor; HIRI, hepatic ischaemia-reperfusion injury; LDH, Lactate dehydrogenase.

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Abstract

Background & Aims: Ghrelin, a gut hormone with pleiotropic effects, may act as a protective signal in parenchymal cells. We investigated the protective effects of ghrelin on hepatocytes after ischaemia/reperfusion (I/R). *Methods:* Hepatic injury was assessed by measurement of plasma alanine aminotransferase (ALT) and lactate dehydrogenase (LDH), histological analysis, and TUNEL assay. Effects of exogenous ghrelin and ghrelin receptor gene deletion on I/R induced injury of liver were evaluated. *Results:* Ischaemia/reperfusion induced a profound injury to hepatocytes. This was accompanied by elevations in plasma ALT and LDH. Pretreatment with ghrelin significantly reduced elevations in plasma ALT and LDH, and attenuated tissue damage induced by hepatic I/R in mice. Hepatic injury induced by I/R was more pronounced in ghrelin receptor gene null mice. Ghrelin administration blocked the up-regulation of AMP-activated protein kinase (AMPK) activity induced by hepatic I/R. *Conclusions:* This study demonstrates that ghrelin contributes to the cytoprotection during hepatic I/R.

Hepatic ischaemia/reperfusion injury (HIRI) may occur in association with conditions ranging from liver resection, liver transplantation or generalized shock. The consequences of ischaemia/reperfusion injury vary widely from transient liver dysfunction, hepatic failure and the systemic inflammatory response syndrome with multiple organ failure (1, 2). For liver transplantation, hepatic ischaemia/reperfusion injury is a major factor in development of primary graft dysfunction and primary nonfunction, occurring in 10–30% and 5% of allografts respectively (3, 4). About 80% of allograft re-transplantation during the first week after surgery is attributable to primary nonfunction. Limiting the adverse effects of ischaemia/reperfusion injury is therefore a critical strategy in improving the success of liver transplantation.

The search for effective prevention of hepatic ischaemia/reperfusion injury has focused mainly on anti-inflammation strategies. While many investigators have demonstrated complex interactions between inflammatory mediators and hepatic injury induced by ischaemia and reperfusion, clinically useful anti-inflammatory agents are still lacking (5–7). An alternative approach suggests shifting focus from inflammatory antagonism to reduction of hepatic cell damage. As one example, activation of AMP-activated proteinkinase (AMPK), an intracellular fuel sensing molecule that protects from damage caused by depletion of ATP, significantly reduced hepatic injury induced by ischaemia and reperfusion (8).

Ghrelin, a gastric 28 amino acids peptide hormone, is the endogenous ligand for the ghrelin receptor (9), also named the growth hormone secretagogue receptor 1a (GHSR1a). Originally identified as a growth hormone secretagogue, ghrelin is now recognized to express a wide variety of physiological functions, including stimulation of food intake (10), regulation of energy homeostasis (10), gastrokinesis (11), vasodilatation (12) and modulation of immunity (13). Cytoprotective effects of ghrelin have been reported in pancreas (14), heart (12), gastrointestinal tract and liver (15). In liver, ghrelin has been reported to protect hepatocytes from acute injury induced by toxic chemical substances such as carbon

tetrachloride (16, 17), acetaminophen (15) and endotoxin lipopolysaccharide (18). Ghrelin has also been reported to ameliorate chronic injury evoked by hepatitis infection (19), inflammation (18, 20), oxidative stress (20, 21), biliary obstruction (20) or steatosis (21). These observations suggest that ghrelin can be protective for hepatocytes. We hypothesized that ghrelin may protect the liver from damage induced by hepatic ischaemia-reperfusion injury.

We report that administration of exogenous ghrelin significantly reduced liver injury following ischaemia/reperfusion as measured by plasma hepatic enzymes and histological changes such as inflammatory cell infiltration, hepatocyte ballooning and/or swelling, hepatocyte apoptosis and tissue necrosis. Deletion of ghrelin receptors exacerbated liver injury in this model.

Material and methods

Materials

Ghrelin was from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). Aprotinin was purchased from Amersham Biosciences (Pittsburgh, PA, USA). Rabbit anti-phospho-AMPKα (Thr172) and rabbit anti-AMPKα antibodies were from Cell Signalling Technology (Beverly, MA, USA). Mouse anti-β-actin was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). IRDye-conjugated affinity purified anti-rabbit and anti-mouse IgGs were purchased from Rockland (Gilbertsville, PA, USA). Alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) commercial kits were purchased from NJJC Bio Inc. (Nanjing, China). *In situ* cell death detection kit, POD was purchased from Roche Diagnostics GmbH (Roche, Mannheim, Germany).

Animals

Twelve-week-old male C57BL/6J mice, ghrelin receptor (GHSR1a) gene knockout mice and their wild-type littermates were used. GHSR1a gene knockout mice in which exon 1 and exon 2 were deleted were obtained from the Shanghai Research Center for Biomodel Organisms (22). Mice were housed in standard plastic rodent cages and maintained at a regulated environment (24°C, 12-h light, 12-h dark cycle with lights on at 07:00 hours). Regular chow and water were available *ad libitum* unless specified otherwise.

Ethical approval

The animals used in this study were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996), and all the experimental protocols were approved by the Animal Care and Use Committee of Peking University.

Hepatic ischaemia/reperfusion (I/R) procedures

All mice were anaesthetized by intraperitoneal injection of pentobarbital at a dose of 60 mg/kg bodyweight. The liver was exposed by midline laparotomy and the hepatic artery and the portal vein were clamped using an atraumatic vascular clip. This procedure has been reported to cause segmental hepatic ischaemia and to prevent mesenteric venous congestion by allowing portal decompression throughout the right and caudate lobes of the liver. Blood flow was interrupted for 45 min to induce hepatic ischaemia and reflow initiated by removal of the vascular clip to allow reperfusion for 3 h.

Experimental designs

Protocol 1: Effects of exogenous ghrelin on hepatic I/R injury. Thirty C57BL/6 mice were equally divided into three experimental groups: (i) control sham-operated group; (ii) I/R+ vehicle group; and (iii) I/R+ ghrelin group. Ghrelin at a dose of 50 μg/kg body weight was administrated intravenously via tail vein injection twice: at 20 min before ischaemia and at 15 min before reperfusion. The sham-operated and I/R groups received equal volumes of normal saline.

Protocol 2: Effects of endogenous ghrelin on hepatic I/R injury. The ghrelin receptor gene knockout mice (GHSR1a−/−) and wild-type littermates (GHSR1a+/+) were randomly divided into sham-operated and hepatic I/R groups. Hepatic ischaemia/reperfusion was performed as described above.

Both experiments in protocol 1 and protocol 2 were repeated three times.

Measurements of hepatic enzymes

Blood samples were transcardially collected after anaesthesia in the presence of aprotinin (2 μg/ml) and EDTA (1 mg/ml). Plasma was stored at −70°C before use. ALT and LDH were measured using commercial kits from NJJC Bio Inc. according to the manufacturer's instructions.

Histological examination

Liver samples were harvested, fixed in 4% paraformaldehyde, paraffin-embedded, cut into 6 μm sections and stained with haematoxylin-eosin according to standard procedures. Tissue sections were examined for hepatic injury in a double blind manner by experienced pathologists.

TUNEL assay

TUNEL assay was performed according to the manufactures' instruction (Roche, Mannheim, Germany). Briefly, paraffin-embedded liver sections were de-waxed, rehy-

drated and rinsed in PBS, followed by treatment with 3% H_2O_2 in methanol to quench the endogenous hydrogen peroxidase. Tissue sections were then treated with 20 μg/ml proteinase K at room temperature for 15 min, followed by the incubation with TUNEL reaction mixture at 37°C in a humidified atmosphere for 1 h. After washing in PBS three times, tissue sections were incubated with converter-POD (horseradish peroxidase) at 37°C in a humidified chamber for 30 min. Finally, DAB substrate was added to visualize the apoptotic signal.

Western blot analysis

Liver tissue was isolated and then homogenized in lysis buffer. Proteins were subjected to SDS-PAGE with a 10% running gel, and then transferred to a polyvinylidene fluoride membrane. Membranes were incubated for 1 h at room temperature with 5% fat-free milk in Tris buffered saline containing Tween 20, followed by incubation overnight at 4°C with primary antibodies. Specific reaction was detected using IRDye-conjugated second antibody and visualized using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analysis

Data were expressed as mean ± SEM and analysed by one-way ANOVA followed by Bonferroni's *post hoc* test or unpaired Student's *t*-test (between two groups) as appropriate, using GraphPad Prism software. $P < 0.05$ was considered significant.

Results

Effects of exogenous ghrelin on HIRI

To determine the effects of exogenous ghrelin on hepatic ischaemia/reperfusion injury, ghrelin was administrated 20 min and 15 min before ischaemia and reperfusion. As shown in Fig. 1A and B, plasma ALT and LDH levels were significantly elevated in mice with ischaemia/reperfusion relative to control mice with sham operation. Treatment of animals with exogenous ghrelin significantly reduced hepatic injury. Significant increase in levels of plasma ghrelin has been demonstrated in mice treated with exogenous ghrelin (Figure S1). In addition, hepatic ischaemia/reperfusion markedly increased the expression of ghrelin mRNA in liver tissue relative to the control treatment (Figure S1).

Consistent with the changes in plasma hepatic enzymes, histological studies revealed typical ischaemia/reperfusion alterations including hepatocyte ballooning and swelling, disruption of hepatic cords, haemorrhage, multiple areas of hepatocyte necrosis, infiltration of inflammatory cells (Fig. 2) and significant increase in hepatocyte apoptosis (Fig. 3). Pretreatment of ghrelin significantly ameliorated the hepatic injury induced by ischaemia/reperfusion. Hepatocyte apoptosis and necrosis, alterations in hepatic cords, haemorrhage and infiltration of inflammatory cells were significantly reduced in mice pretreated with ghrelin relative to animals in the ischaemia/reperfusion vehicle group.

Similar protective effect of ghrelin on hepatic ischaemia/reperfusion injury was also observed in BALB/c mice (Figure S2).

Effects of ghrelin receptor gene deletion on hepatic ischaemia-reperfusion injury

To confirm that ghrelin protects hepatocytes from injury induced by ischaemia/reperfusion, a ghrelin receptor gene deletion mouse model (22) was used. Under normal chow diet, hepatic ischaemia/reperfusion significantly increased plasma ALT and LDH levels in wild-type littermates. Significantly greater elevations in plasma ALT and LDH levels were observed in ghrelin receptor gene knockout mice relative to the wild-type littermates (Fig. 4A and B).

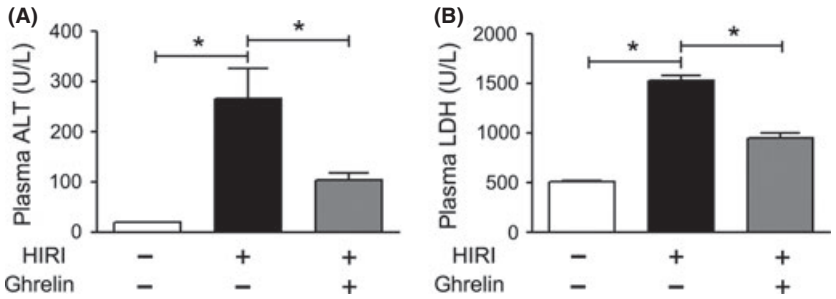

Fig. 1. Effects of exogenous ghrelin on plasma levels of hepatic enzymes after ischaemia/reperfusion. (A) Plasma levels of alanine aminotransferase (ALT) after hepatic ischaemia/reperfusion; (B) Plasma levels of lactate dehydrogenase (LDH) after hepatic ischaemia/reperfusion; Hepatic ischaemia/reperfusion injury (HIRI) was induced by clamping the hepatic artery and the portal vein using an atraumatic vascular clip to interrupt blood flow for 45 min, followed by reperfusion for 3 h. Ghrelin (50 μg/kg body weight) was administrated intravenously twice: at 20 min before ischaemia and at 15 min before reperfusion. Data are expressed as mean ± SEM; $n = 10$. *$P < 0.05$.

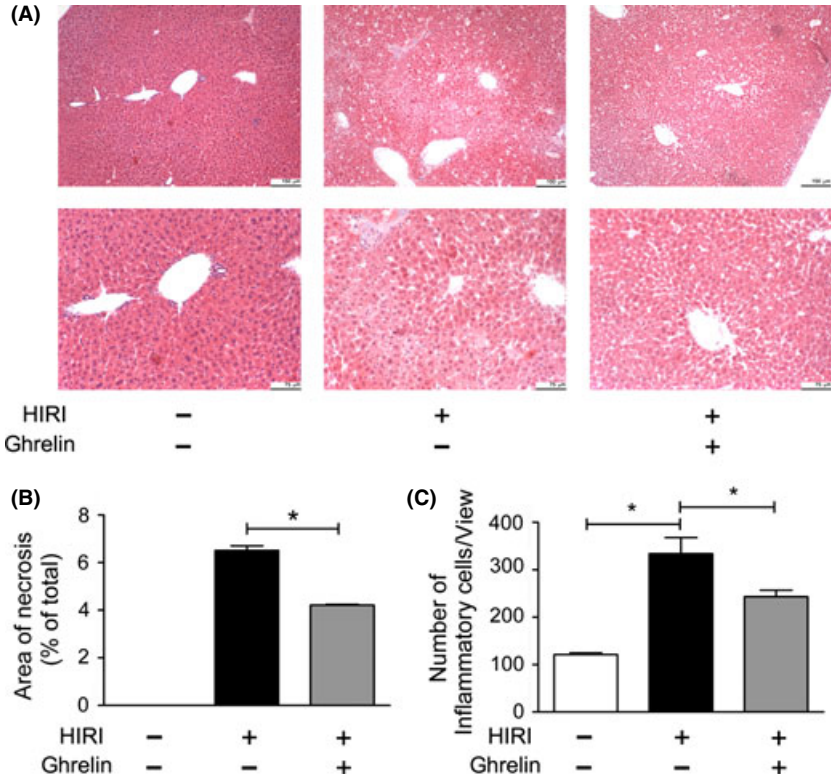

Fig. 2. Effects of exogenous ghrelin on histological lesions induced by hepatic ischaemia/reperfusion. (A) Amelioration of HIRI by ghrelin. Upper panel depicts histological changes (haematoxylin-eosin staining) in lower magnification (100×), while lower panel shows the results in higher magnification (200×) after hepatic ischaemia/reperfusion. Shown are the representative samples from 10 individual animals in each group. (B) Decrease of liver necrosis by ghrelin. Area of liver necrosis was quantified and expressed as mean ± SEM from at least 10 animals per group. *$P < 0.05$. (C) Attenuation of the infiltration of inflammatory cells by ghrelin. The number of inflammatory cells per microscopic view (400× magnification) was counted and expressed as mean ± SEM; $n = 10$. *$P < 0.05$. Statistical significance between the HIRI vs. sham treatment was determined by ANOVA with Bonferroni's multiple testing. *$P < 0.05$.

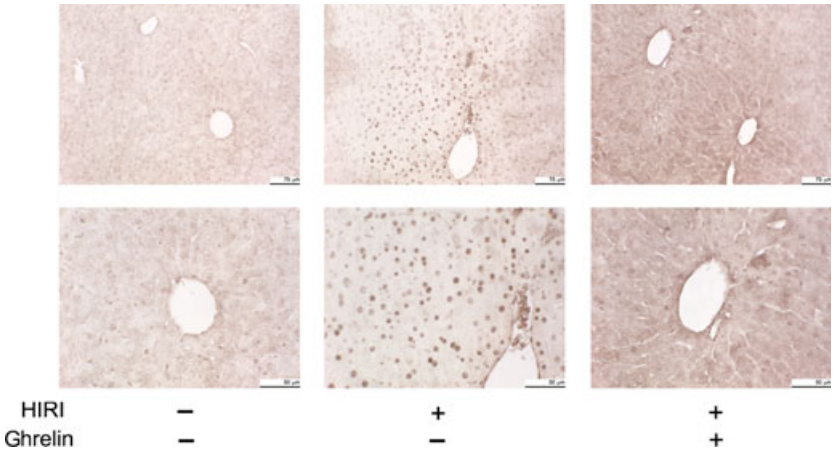

Fig. 3. Effects of exogenous ghrelin on hepatocyte apoptosis induced by hepatic ischaemia/reperfusion. Hepatic injury was induced by ischaemia/reperfusion and apoptosis in hepatocytes was detected by TUNEL assay. Shown are the representative pictures from at least 10 animals per group. Upper panel depicts TUNEL staining in lower magnification (200×), while lower panel shows the results in higher magnification (400×) after hepatic ischaemia/reperfusion.

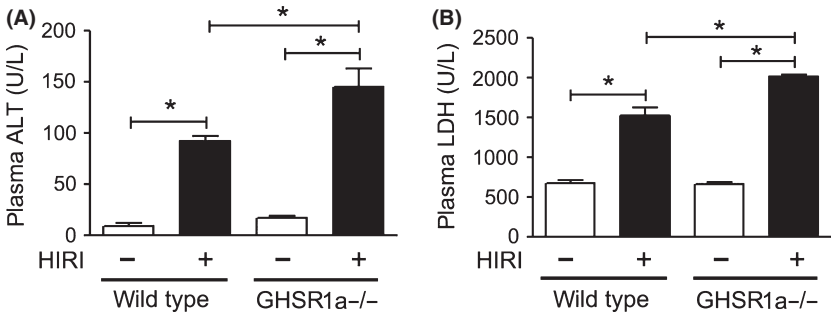

Fig. 4. Effects of ghrelin receptor gene deletion (GHSR1a−/−) on plasma levels of hepatic enzymes after ischaemia/reperfusion in mice fed normal chow diet (NCD). (A) Plasma levels of alanine aminotransferase (ALT) after hepatic ischaemia/reperfusion; (B) Plasma levels of lactate dehydrogenase (LDH) after hepatic ischaemia/reperfusion; Data expressed as mean ± SEM; Five to eight samples were examined for each condition. *$P < 0.05$.

Consistent with these observations in NCD mice, ischaemia/reperfusion induced hepatocyte ballooning and swelling, disrupted hepatic cords, haemorrhage, infiltration of inflammatory cells and tissue necrosis (Fig. 5) in wild-type littermates. A significantly greater degree of hepatic injury was observed in GHSR1a null mice with ischaemia/reperfusion (Fig. 5).

Similar results were observed in mice fed high-fat diet (HFD). Both serological (Fig. 6) and histological (Fig. 7) and apoptosis (Fig. 8) studies demonstrated that hepatic ischaemia/reperfusion also induced a profound injury to the liver tissues in mice fed HFD. A significant severer damage of hepatic tissues was detected in ghrelin receptor gene knockout mice fed HFD relative to wild-type animals (Figs 6, 7 and 8).

Effects of ghrelin on AMPK

Since the AMPK has been reported to protect hepatocytes from injury induced by ischaemia/reperfusion (8, 23), we next examined the effects of ghrelin on the phosphorylation of AMPKα in hepatic tissues derived from mice. As shown in Fig. 9A, hepatic ischaemia/reperfusion induced significant elevations in phosphorylation of AMPKα in mice relative to animals with sham operation. Pretreatment of mice with ghrelin markedly attenuated up-regulation of AMPKα phosphorylation induced by ischaemia/reperfusion. Consistent with this observation, ghrelin receptor gene knockout mice demonstrated a significant higher level of hepatic AMPKα phosphorylation induced by I/R injury relative to the wild-type littermates (Fig. 9B). In addition, exogenous ghrelin significantly reduced the phosphorylation of hepatic AMPKα in mice fed high-fat diet (Fig. 9C).

Discussion

The major finding of this study is that ghrelin contributes to protection of hepatic tissues induced by ischaemia and reperfusion injury. This general conclusion is supported by the following observations: (i) exogenous ghrelin significantly reduces hepatic damage induced by ischaemia/reperfusion as assayed by levels of plasma liver enzymes, histological assessment and apoptosis analysis of hepatic injury; (ii) in contrast, ghrelin receptor gene deletion exacerbates hepatic injury induced by ischaemia/reperfusion in mice fed NCD or HFD; and (iii) up-regulation of AMPKα phosphorylation induced by hepatic ischaemia/reperfusion is decreased by administration of ghrelin, while significantly increased in ghrelin receptor gene knockout mice. In addition, ghrelin markedly attenuates the phosphorylation of AMPKα in mice fed high-fat diet.

In addition to regulation of food intake and maintenance of energy homeostasis (10), gut hormones also regulate inflammatory and cytoprotective responses in a variety of tissues (12, 13, 15). Ghrelin, a gut hormone with pleiotropic effects, has been demonstrated to have cytoprotective effects in parenchymal cells ranging from pancreatic acinar cells (24), cardiomyocytes (12), gastrointestinal epithelia (25) to hepatocytes (19). We provide evidence in this study that ghrelin protects hepatocytes from injury induced by ischaemia/reperfusion in mice. In liver, ghrelin has been reported to be protective for hepatocytes in both acute and chronic injury models (15–17, 19). Our study expands this concept by demonstrating a protective effect of ghrelin on hepatic injury induced by ischaemia/reperfusion. Importantly, we demonstrate that both exogenous and endogenous ghrelin contribute to the protection of hepatocytes.

Two major forms of ghrelin in the circulation have been reported: acyl ghrelin and des-acyl ghrelin in which the third amino acid serine is not acylated (26). Only acyl ghrelin is able to bind and activate its receptor, GHSR1a (9). Our data indicate that endogenous ghrelin functions through GHSR1a for the protective effects in liver. This notion is supported by previous studies demonstrating the presence of GHSR1a in the liver (19).

Activation of GHSR1a triggers multiple intracellular signalling pathways including phospholipase C-inositol 1,4,5-triphosphate and AMPK signalling (27, 28). It is

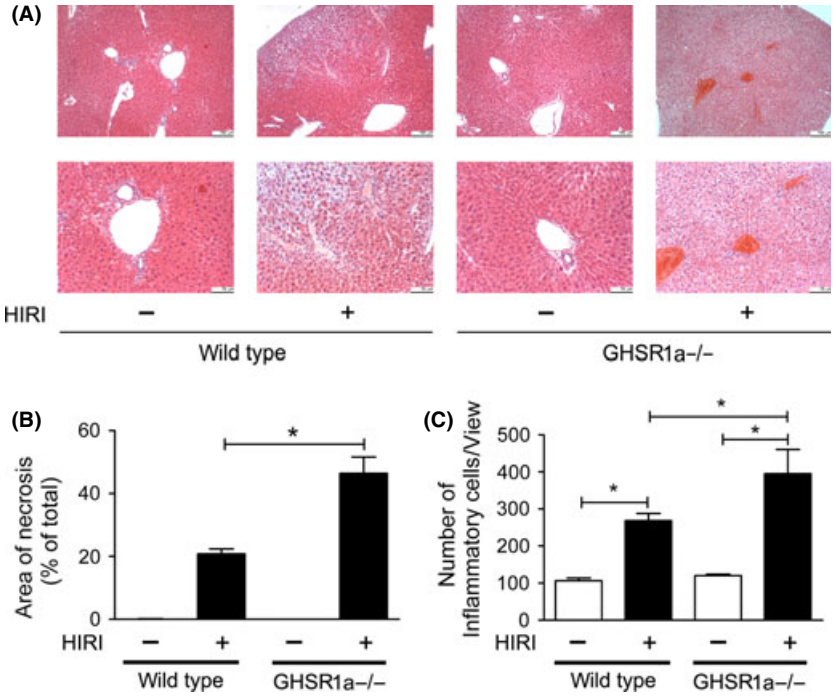

Fig. 5. Effects of ghrelin receptor gene deletion (GHSR1a−/−) on histological lesions induced by hepatic ischaemia/reperfusion in mice fed normal chow diet (NCD). (A) Upper panel depicts histological changes (haematoxylin-eosin staining) in lower magnification (100×), while lower panel shows the results in higher magnification (200×) after hepatic ischaemia/reperfusion. Shown are the representative samples of 5–8 animals per group. (B) Increase of liver necrosis in ghrelin receptor gene null mice fed chow diet. Area of liver necrosis was quantified and expressed as mean ± SEM from at least 5 animals per group. *$P < 0.05$. (C) Infiltration of inflammatory cells. The number of inflammatory cells permicroscopic view (400× magnification) was counted and expressed as mean ± SEM; $n = 5–8$. Statistical significance between the HIRI vs. sham treatment, and ghrelin null mice vs. wild-type littermates was determined by ANOVA with Bonferroni's multiple testing. *$P < 0.05$.

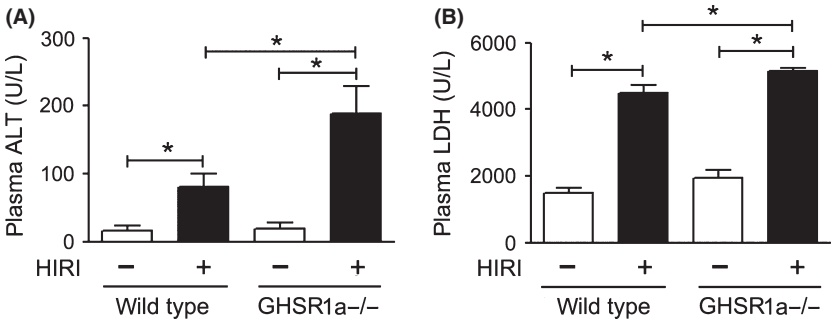

Fig. 6. Effects of ghrelin receptor gene deletion (GHSR1a−/−) on plasma levels of hepatic enzymes after ischaemia/reperfusion in mice fed high-fat diet (HFD). (A) Plasma levels of alanine aminotransferase (ALT) after hepatic ischaemia/reperfusion; (B) Plasma levels of lactate dehydrogenase (LDH) after hepatic ischaemia/reperfusion; Data expressed as mean ± SEM; Five to eight samples were examined for each condition. *$P < 0.05$.

known that AMPK, a primary fuel sensor during changes in cellular energy levels, is activated during hepatic ischaemia (8). AMPK activation is proposed to preserve ATP levels, to reduce lactate accumulation during ischaemia, and therefore to reduce hepatic injury after reperfusion (23). In this study, we have shown that hepatic AMPK activity is regulated by ghrelin. Ghrelin attenuates the up-regulation of AMPK activity during ischaemia/reperfusion, while deletion of ghrelin receptor gene significantly enhances the up-regulation of AMPK activity during ischaemia/reperfusion. In the animal fed high-fat diet, ghrelin also inhibits the AMPK activity. All these observations suggest that AMPK activity is an important signalling

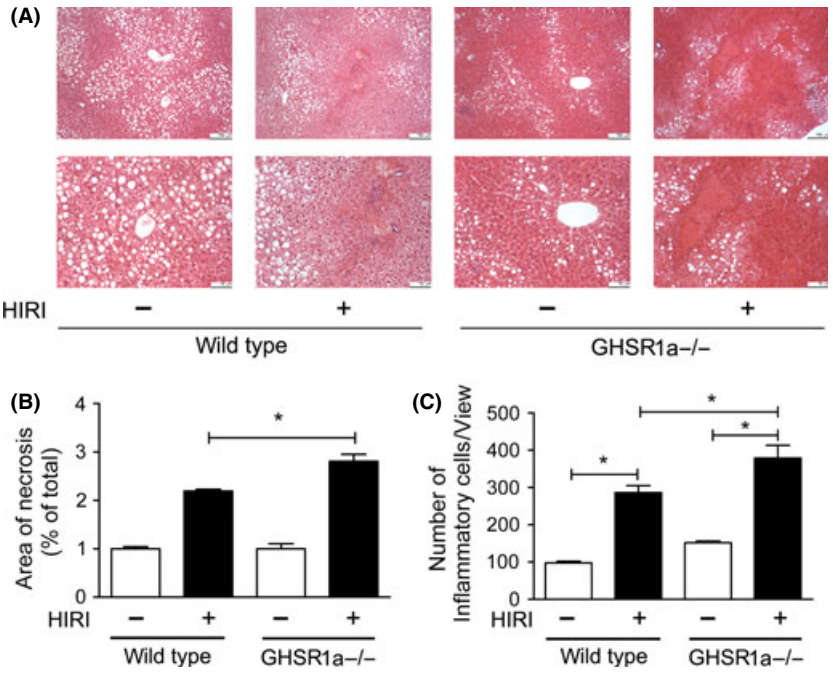

Fig. 7. Effect of ghrelin receptor gene deletion (GHSR1a−/−) on histological lesions induced by hepatic ischaemia/reperfusion in mice fed high-fat diet (HFD). (A) Upper panel depicts histological changes (haematoxylin-eosin staining) in lower magnification (100×), while lower panel shows the results in higher magnification (200×) after hepatic ischaemia/reperfusion. Shown are the representative samples of 5–8 animals per group. (B) Increase of liver necrosis in ghrelin receptor gene null mice fed high-fat diet. Area of liver necrosis was quantified and expressed as mean ± SEM from at least 5 animals per group. *$P < 0.05$. (C) Infiltration of inflammatory cells. The number of inflammatory cells *per* microscopic view (400× magnification) was counted and expressed as mean ± SEM; $n = 5–8$. Statistical significance between the HIRI vs. sham treatment, and ghrelin null mice vs. wild-type littermates was determined by ANOVA with Bonferroni's multiple testing. *$P < 0.05$.

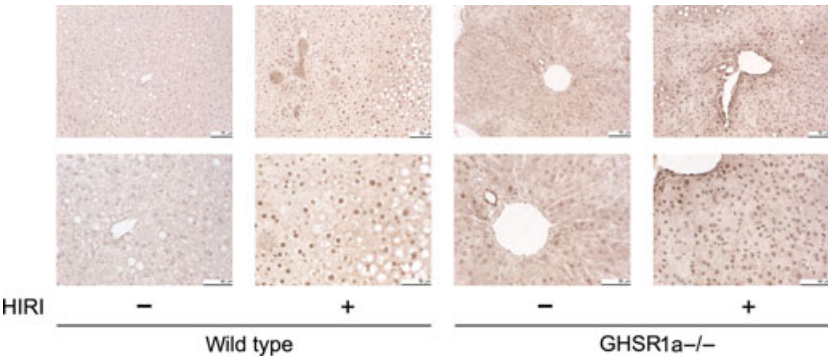

Fig. 8. Effect of ghrelin receptor gene deletion (GHSR1a−/−) on hepatocyte apoptosis induced by hepatic ischaemia/reperfusion in mice fed high-fat diet (HFD). Hepatic injury was induced by ischaemia/reperfusion and apoptosis in hepatocytes was detected by TUNEL assay. Shown are the representative pictures from at least 5 animals per group. Upper panel depicts TUNEL staining in lower magnification (200×), while lower panel shows the results in higher magnification (400×) after hepatic ischaemia/reperfusion.

molecule modulated by ghrelin in the hepatocytes. However, whether ghrelin exercises its protective effect during ischaemia/reperfusion via the AMPK signalling pathway remains to be determined.

The underlying mechanisms of hepatic ischaemia/reperfusion injury include induction of tissue necrosis (29, 30), generation of reactive oxygen species (6, 31), release of proinflammatory cytokines and recruitment and activation of immunocompetent cells (3, 32). Consistent with this notion, infiltration of inflammatory cells was significantly reduced in the ischaemia/reperfusion mice treated with ghrelin. This observation is in line with previous studies indicating that ghrelin attenuates both acute and chronic

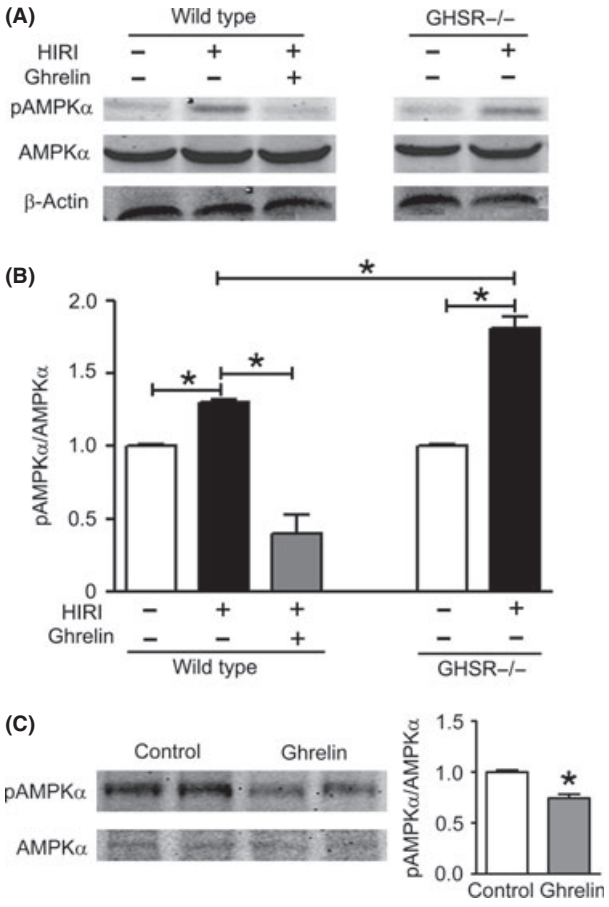

Fig. 9. Effects of ghrelin on AMP-activated protein kinase (AMPKα) phosphorylation. (A) The phosphorylation of AMPK in response to ischaemia/reperfusion and ghrelin pretreatment was shown in left panel; the phosphorylation of AMPK in response to ischaemia/reperfusion in ghrelin receptor null mice was shown in right panel. Shown are the representative Western blots from five to eight individual experiments in each group. (B) Signal intensity of pAMKPα and total AMPKα were measured to quantify the ratio of pAMKPα/AMPKα and expressed as mean ± SEM. Five to eight samples were examined for each condition. $*P < 0.05$. (C) Inhibition of hepatic AMPKα phosphorylation by ghrelin in mice fed high-fat diet. Mice fed HFD for 14 weeks were infused with ghrelin (11 nmol/kg d) for 2 weeks. Phosphor-AMPKα and AMPKα were detected by specific antibody. Shown are the representative Western blots from at least five individual experiments in each group. The ratio of pAMKPα/AMPKα signal intensity was calculated and expressed as mean ± SEM. $n = 5$. $*P < 0.05$.

inflammation by reducing the infiltration of inflammatory cells. Whether ghrelin acts directly on hepatocytes or indirectly through the modulation of inflammatory cells for its protective effect during hepatic ischaemia/reperfusion injury deserves further investigation.

In summary, our study demonstrates that ghrelin reduces hepatic injury after hepatic ischaemia/reperfusion. Our results suggest that ghrelin may provide a potential therapeutic strategy for improvement of liver function in circumstances associated with this injury.

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Conflict of interest: The authors do not have any disclosures to report.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Changes in ghrelin.
Figure S2. Effects of exogenous ghrelin on hepatic ischemia/reperfusion injury in BALB/c mice.