Protection of ghrelin postconditioning on hypoxia/reoxygenation in gastric epithelial cells

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Abstract

AIM: To investigate the protective effect and mechanisms of ghrelin postconditioning against hypoxia/reoxygenation (H/R)-induced injury in human gastric epithelial cells.

METHODS: The model of H/R injury was established in gastric epithelial cell line (GES-1) human gastric epithelial cells. Cells were divided into seven groups: normal control group (N); H/R postconditioning group; DMSO postconditioning group (DM); ghrelin postconditioning group (GH); D-Lys3-GHRP-6 + ghrelin postconditioning group (D + GH); capsazepine + ghrelin postconditioning group (C + GH); and LY294002 + ghrelin postconditioning group (L + GH). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to detect GES-1 cell viability. Hoechst 33258 fluorochrome staining and flow cytometry were conducted to determine apoptosis of GES-1 cells. Spectrophotometry was performed to determine release of lactate dehydrogenate (LDH). Protein expression of Bcl-2, Bax, Akt, and glycogen synthase kinase (GSK)-3β was determined by western blotting. Expression of vanilloid receptor subtype 1 (VR1), Akt and GSK-3β was observed by immunocytochemistry.

RESULTS: Compared with the H/R group, cell viability of the GH group was significantly increased in a dose-dependent manner (55.9% ± 10.0% vs 69.6% ± 9.6%, 71.9% ± 17.4%, and 76.3% ± 13.3%). Compared with the H/R group, the percentage of apoptotic cells in the GH group significantly decreased (12.38% ± 1.51% vs 6.88% ± 0.87%). Compared with the GH group, the percentage of apoptotic cells in the D + GH group, C + GH group and L + GH groups significantly increased (11.70% ± 0.88%, 11.93% ± 0.96%, 10.20% ± 1.05% vs 6.88% ± 0.87%). There were no significant differences in the percentage of apoptotic cells between the H/R and DM groups (12.38% ± 1.51% vs 13.00% ± 1.13%). There was a significant decrease in LDH release following ghrelin postconditioning compared with the H/R group (561.58 ± 64.01 U/L vs 1062.45 ± 105.29 U/L). There was a significant increase in LDH release in the D + GH, C + GH and L + GH groups compared with the GH group (816.89 ± 94.87 U/L, 870.95 ± 64.06 U/L, 838.95 ± 64.01 U/L). There were no significant differences in LDH release between the H/R and DM groups (1062.45 ± 105.29 U/L vs 1017.65 ± 68.90 U/L). Compared with the H/R group, expression of Bcl-2 and Akt increased in the GH group, whereas expression of Bax and GSK-3β decreased. Compared with the GH group, expression of Bcl-2 decreased and Bax increased in the D + GH, C + GH and L + GH groups, and Akt decreased and GSK-3β increased in the L + GH group. The H/R group also upregulated expression of VR1 and GSK-3β and downregulated Akt. The number of VR1-positive and Akt-positive cells in the GH group significantly increased, whereas the number of GSK-3β-positive cells significantly decreased. These effects of ghrelin were...
Ghrelin postconditioning protected against H/R-induced injury in human gastric epithelial cells, which indicated that this protection might be associated with GHS-R, VR1 and the PI3K/Akt signaling pathway.

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Key words: Human gastric epithelial cells; Ghrelin; Pharmacological postconditioning; Hypoxia/reoxygenation; Apoptosis

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INTRODUCTION

Gastric ischemia-reperfusion (GI/R) injury is a common clinical pathophysiological process. In case of clinical multiple organ dysfunction syndrome, gastrointestinal mucosal ischemia due to the redistribution of blood is the earliest to occur, and is more obvious than other organ ischemia-reperfusion (I/R) injury. Thus, the stomach is considered the earliest organ to be involved[1,2]. In recent years, ischemic postconditioning (Ipost) has been discovered to be an important endogenous protective mechanism and hypoxia postconditioning (IPost) can be elicited after hypoxia and reoxygenation in cell culture[3-5]. It has been demonstrated that IPost can effectively reduce myocardial injury[6]. Pharmacological postconditioning is the extension of Ipost, in which a drug is applied to the ischemic myocardium or hypoxic cardiomyocytes during the first few minutes of reperfusion or reoxygenation, significantly reducing organ reperfusion injury[7].

Ghrelin is a 28-amino-acid peptide, which was initially identified from rat stomach[8]. In humans, it acts as the endogenous ligand for the growth hormone secretagogue receptor (GHSR)[9]. GHSR, a seven-transmembrane-domain G-protein coupled receptor was cloned from rat stomach by endocrine cells of the fundic mucosa, formerly known as X/A-like cells[10,11,12]. Several studies have suggested that the many physiological actions are associated with ghrelin, including neuroendocrine, cardiovascular and gastrointestinal functions[13,14]. Ghrelin plays an important role in mucosal defense, such as resistance against a variety of ulcerogenic stimuli, including ethanol, stress and I/R[15-17].

Vanilloid receptor subtype 1 (VR1) is a nonselective cation channel, primarily expressed in central and peripheral terminals of nonmyelinated primary afferent neurons[18]. VR1 may be activated by physical and chemical mediators that contain noxious thermal stimulation, noxious protons, and vanilloid compounds such as capsaicin[19,20]. Our study aimed to establish whether the effect of ghrelin was mediated by VR1, and whether ghrelin served as a new activator of VR1.

The phosphoinositide 3-kinase (PI3K/Akt) signaling pathway has been implicated in the control of major cellular responses including cell proliferation, survival, development, differentiation, cell cycle, and apoptosis[21,22]. The PI3K/Akt signaling pathway is a type of significant antiapoptotic factor that plays an important protective role by reducing apoptosis-associated protein kinase caspase family, Bcl-2 and other apoptotic factors[23].

The primary aim of this study was to investigate whether ghrelin postconditioning had a protective effect on hypoxia/reoxygenation (H/R) injury in human gastric epithelial cells, and whether GHSR, VR1 and the PI3K/Akt signaling pathway are involved in this protection.

MATERIALS AND METHODS

Materials

The materials and reagent were as follows: human gastric epithelial cell line (GES-1) from Beijing Cancer Hospital; ghrelin, D-Lys3-GHRP-6, capsazepine and LY294002 (Sigma-Aldrich, United States) were dissolved in 100% DMSO and stored at -20℃; Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco Service Co., United States); fetal bovine serum (FBS) from Tianjin Hao Yang Biological Manufacture Co. Ltd. (China); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from Amresco (United States); Hoechst 33258 from Beyotime Institute of Biotechnology (China); Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Assay kit from Beijing Biosea Biotechnology (China); lactate dehydrogenase (LDH) assay kit from Nanjing Jiancheng Bioengineering Institute (China).

Cell culture

Cells were cultured in phenol-red-free DMEM containing 10% FBS at a density of 10^4 cells/mL, and placed in a humidified incubator with 95% air and 5% CO₂ at 37℃ until cells reached 70%-80% confluence.

Experimental protocol

The complete medium was replaced with DMEM containing 1% FBS 1 d before the experiment for cell synchronization. The cells were randomized into five groups and treated as follows. Cells in the normal control group (N) were kept in normoxic culture for 6 h. In the H/R group, DMEM was replaced with Krebs-Ringer bicarbonate buffer prior to hypoxia induction, and the cells
were transferred into a hypoxic incubator in a humidified atmosphere equilibrated with 94% N\textsubscript{2} + 1% O\textsubscript{2} + 5% CO\textsubscript{2} for 2 h (hypoxia). Afterwards, the Krebs-Ringer bicarbonate buffer was replaced with DMEM for reperfusion simulation, followed by 4 h normoxic culture (for reoxygenation). In the DMSO vehicle postconditioning group (DM), the Krebs-Ringer bicarbonate buffer was replaced with DMEM containing 0.05% DMSO prior to reoxygenation, with no modification of other procedures in the H/R group. In the ghrelin postconditioning group (GH), the Krebs-Ringer bicarbonate buffer was replaced with DMEM containing 10\textsuperscript{-7}, 10\textsuperscript{-8} and 10\textsuperscript{-9} mol/L ghrelin before reoxygenation, with no modification of other procedures in the H/R group. In the D-Lys3-GHRP-6 + ghrelin postconditioning group (D + GH), capsazepine + ghrelin postconditioning group (C + GH) and LY294002 + ghrelin postconditioning group (L + GH), the DMEM was replaced with Krebs-Ringer bicarbonate buffer containing D-Lys3-GHRP-6, capsazepine and LY294002, respectively, followed by 2 h hypoxia. Thereafter, the buffer was replaced with DMEM containing 10\textsuperscript{-7} mol/L ghrelin before reoxygenation, with no modification of other procedures in the H/R group.

**MTT assay**

The cells in logarithmic growth phase were cultured in a 96-well plate at a density of 10\textsuperscript{4} cells/well. Following the experiment in each group, 20 \textmu L MTT (5 mg/mL) was added to each well and incubated for 4 h. Then, 200 \textmu L DMSO was added to each well and the plate was vortexed for 10 min at 37 °C. A 96-well microplate reader (Thermo, United States) was used to determine A\textsubscript{490 nm}. Viability (%) was determined as (experimental group A\textsubscript{490 nm}/normal control group A\textsubscript{490 nm}) \times 100%. Each assay was repeated at least three times.

**Hoechst 33258 staining assay**

Cells were cultured in six-well plates at a density of 104 cells/well and incubated for 48 h. Following each experiment, cells were fixed with paraformaldehyde for 20 min and washed with 0.01 mol/L phosphate buffered solution (PBS) twice for 3 min, followed by Hoechst 33258 (0.5 mL) staining at 37 °C in darkness for 15 min, and reashed with 0.01 mol/L PBS for 2 min to remove excess background stain. Apoptotic cells were observed by fluorescence microscopy, with excitation wavelength at 350 nm and emission wavelength at 460 nm. The photographs were preserved.

**Flow cytometric analysis**

Cells were plated in 100 mL culture flasks and incubated for 48 h. Following each experiment, cells were collected, washed twice with ice-cold PBS, followed by cell density determination and dilution in 1 \times \textit{AnnexinV}-binding buffer to 10\textsuperscript{5} cells/mL. Cells were suspended in 200 \textmu L binding buffer and 5 \textmu L Annexin V-FITC for 15 min in darkness. Finally, 300 \textmu L binding buffer and 5 \textmu L propidium iodide (PI) were added to each sample. Apoptosis percentage was analyzed by flow cytometry (Becton Dickinson, United States).

**LDH assay**

The cells were cultured in 96-well plates at a density of 10\textsuperscript{4} cells/well. At the end of each experiment, 20 \textmu L supernatant was drawn from each well and transferred into test tubes, followed by determination of LDH release using the LDH assay kit.

**Western blotting**

Cells were plated in 100 mL cell culture flasks and incubated for 48 h. At the end of each experiment, the cells were washed thrice in ice-cold PBS prior to lysis with lysis buffer containing 150 mL/L protease inhibitor, followed by cell collection and centrifugation at 4 °C for 15 min. The resulting protein-containing supernatant was collected for protein quantification or storage at -80 °C. The protein contents were determined by bicinchoninic acid assay. Protein samples were adjusted to equal concentration and volume by lysis buffer and then mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The protein samples were heated at 100 °C for 5 min, and then 100 \mu g protein was loaded into each well for 12.5% SDS-PAGE, and the targeted protein was transferred onto a nitrocellulose membrane. Immunoblotting was performed with the following antibodies: mouse anti-Bcl-2 (Zhongshan Golden Bridge Biotechnology Co. Ltd., China), mouse anti-Bax (Zhongshan Golden Bridge Biotechnology), mouse anti-β-actin (Zhongshan Golden Bridge Biotechnology), rabbit anti-VR1 (Zhongshan Golden Bridge Biotechnology), rabbit anti-Akt (Zhongshan Golden Bridge Biotechnology), and rabbit anti-glycogen synthase kinase (GSK)-3β (Wuhan Boshide Biotechnology Co. Ltd., China). The secondary antibodies were alkaline phosphatase goat anti-rabbit IgG (Zhongshan Golden Bridge Biotechnology) and alkaline phosphatase horse anti-mouse IgG (Zhongshan Golden Bridge Biotechnolog). Protein expression was quantified by Image J software.

**Immunocytochemistry assay**

Exponentially growing cells were cultured in a 24-well plate. Immunohistochemistry detection reagent and DAB kit (Zhongshan Golden Bridge Biotechnology) were used to examine the expression of V\textsubscript{R1}, Akt and GSK-3β in human gastric epithelial cells. Following each experiment, cells were washed thrice in PBS and fixed with 4% paraformaldehyde at 4 °C for 30 min. Cells were incubated with 0.5% Triton X-100 for 20 min and 3% H\textsubscript{2}O\textsubscript{2} for 10 min. Cells were blocked with 10% normal goat serum for 1 h at room temperature. Primary antibodies were added and incubated at 4 °C overnight. With cells washed thrice in cold PBS, the secondary antibody was added and incubated at room temperature for 3 h. Finally, cells were washed thrice in 0.01 mol/L PBS, followed by incubation with DAB complexes for 20 min. Cells were observed for
Cells were grouped as follows: normoxic culture for 6 h (N), 2 h hypoxia/4 h reoxygenation (H/R), alcohol vehicle postconditioning (DM) and ghrelin postconditioning at three doses (10⁻⁷ mol/L, 10⁻⁸ mol/L and 10⁻⁹ mol/L). Cell viability was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. mean ± SD. n = 6. *P < 0.05, **P < 0.01 vs H/R.

photography under a phase contrast microscope.

**Statistical analysis**

All data were presented as mean ± SD. One-way analysis of variance was performed to determine differences among groups and LSD test was performed to determine differences between groups. All statistical analyses were performed with the SPSS version 13.0 (SPSS, Chicago, IL, United States). The difference was considered statistically significant at P < 0.05.

**RESULTS**

**Effects of different doses of ghrelin on cell viability in human gastric epithelial cells induced by H/R**

The MTT assay indicated that the GES-1 cells were treated with ghrelin postconditioning at 10⁻⁷ mol/L, 10⁻⁸ mol/L and 10⁻⁹ mol/L. The viability of the GH group was 69.6% ± 9.6%, 71.9% ± 17.4%, and 76.3% ± 13.3%, respectively, in a dose-dependent manner. Compared with the H/R group (55.9% ± 10.0%), the viability significantly increased (P < 0.05), suggesting that 10⁻⁷ mol/L ghrelin was the optimal protective dose, which was used in the subsequent experiments. There were no significant differences between the H/R and DM groups (55.9% ± 10.0% vs 56.1% ± 10.5%, P > 0.05, Figure 1).

**Effects of ghrelin postconditioning on viability of human gastric epithelial cells induced by H/R**

To investigate whether GHS-R, VR1 and the PI3K/Akt signaling pathway were related to this effect, their inhibitors D-Lys3-GHRP-6, capsazepine and LY294002 were administered prior to ghrelin postconditioning. The GH group had significantly increased cell viability (P < 0.01 vs H/R group), whereas the D + GH, C + GH and L + GH groups had significantly decreased cell viability (P < 0.05 vs GH group, Figure 2), which indicated that D-Lys3-GHRP, capsazepine and LY294002 could reverse the protective effect of ghrelin postconditioning on GES-1 cell viability induced by H/R.

**Effects of ghrelin postconditioning on apoptosis of human gastric epithelial cells induced by H/R**

Hoechst 33258 staining showed that apoptotic cells were apparent in normal gastric epithelial cells. Compared with the H/R group, there were fewer apoptotic cells in the GH group, whereas many apoptotic cells were observed in the D + GH, C + GH and L + GH groups compared with the GH group (Figure 3).

Flow cytometric analysis showed that the percentage of apoptotic cells in the GH group significantly decreased (12.38% ± 1.51% vs 6.88% ± 0.87%, P < 0.01) compared with the H/R group. Compared with the GH group, the D + GH, C + GH and L + GH groups had a significantly increased percentage of apoptotic cells (11.70% ± 0.88%, 11.93% ± 0.96%, 10.20% ± 1.05% vs 6.88% ± 0.87%, P < 0.05). There were no significant differences in the percentage of apoptotic cells between the H/R and DM groups (12.38% ± 1.51% vs 13.0% ± 1.13%, P > 0.05, Figure 4).

**Effects of ghrelin postconditioning on LDH release in human gastric epithelial cells induced by H/R**

There was a significant decrease in LDH release in the GH group compared with the H/R group (561.58 ± 64.01 U/L vs 1062.45 ± 105.29 U/L, P < 0.01). There was a significant increase in LDH release in the D + GH, C + GH and L + GH groups compared with the GH group (816.89 ± 94.87 U/L, 870.95 ± 64.06 U/L, 838.62 ± 118.45 U/L vs 561.58 ± 64.01 U/L, P < 0.01). There were no significant differences in LDH release between the H/R and DM groups (1062.45 ± 105.29 U/L...
Effects of ghrelin postconditioning on expression of Bcl-2, Bax, VR1, Akt and GSK-3β in human gastric epithelial cells induced by H/R.

Western blotting demonstrated that Bcl-2, Bax, Akt and GSK-3β were expressed in normal gastric epithelial cells. Compared with the H/R group, expression of Bcl-2 and Akt increased in the GH group, whereas there was a decrease in expression of Bax and GSK-3β (P < 0.01). Compared with the GH group, Bcl-2 expression decreased and Bax expression increased in the D + GH, C + GH and L + GH groups (P < 0.01). Expression of Akt decreased and expression of GSK-3β increased in the L + GH group (P < 0.01, Figure 5).

Table 1  Effects of ghrelin postconditioning on lactate dehydrogenate release

| Groups         | LDH release (U/L) |
|----------------|-------------------|
| N group        | 672.73 ± 64.79    |
| H/R group      | 1062.45 ± 105.29  |
| DM group       | 1017.65 ± 68.90   |
| GH group       | 561.58 ± 64.01    |
| D + GH group   | 816.89 ± 94.87    |
| C + GH group   | 870.95 ± 64.06    |
| L + GH group   | 838.62 ± 118.45   |

*P < 0.01 vs GH group; *P < 0.01 vs H/R group. N: Normal control group; H/R: Hypoxia/reoxygenation; DM: DMSO postconditioning; GH: Ghrelin postconditioning (10⁻⁷ mol/L); D + GH: D-Lys3-GHRP-6 + ghrelin postconditioning; C + GH: Capsazepine + ghrelin postconditioning; L + GH: LY294002 + ghrelin postconditioning; LDH: Lactate dehydrogenate.

Figure 3  Effects of D-Lys3-GHRP-6, capsazepine and LY294002 in ghrelin postconditioning on cell apoptosis in human gastric epithelial cells induced by H/R. Cells were grouped as follows: normoxic culture for 6 h (A), 2 h hypoxia/4 h reoxygenation (B), DMSO vehicle postconditioning (C), ghrelin postconditioning (10⁻⁷ mol/L) (D), D-Lys3-GHRP-6 + ghrelin postconditioning (E), capsazepine + ghrelin postconditioning (F), and LY294002 + ghrelin postconditioning (G). Mean ± SD. n = 6.

1P < 0.01 vs B; 2P < 0.01 vs D. Cells were then stained by Hoechst33258. The arrows indicate apoptotic cells.
Immunocytochemistry demonstrated expression of VR1 (Figure 6A), Akt (Figure 7A) and GSK-3β (Figure 8A) in normal human gastric epithelial cells. Two hours hypoxia followed by 4 h incubation under normoxic conditions also upregulated expression of VR1 (Figure 6B) and GSK-3β (Figure 8B) and downregulated Akt (Figure 7B). There was a significant increase in the number of VR1-positive (Figure 6D) as well as Akt-positive (Figure 7D) cells in the GH group, whereas the number of GSK-3β-positive cells significantly decreased (Figure 8D). However, these effects of ghrelin were reversed by capsaizepine and LY294002 (Figures 6E, 7E and 8E).

DISCUSSION

Gastric mucosal lesions are frequently observed in clinical situations, such as stress-induced G1/R injury, a major cause of acute gastric mucosal lesions. GI/R injury is a common clinical pathophysiological process, which is associated with such factors as excessive generation of oxygen free radicals (OFRs) in gastric mucosa, intracellular calcium overload, increased gastric acid secretion and gastric microcirculation disturbance. Excessive generation of OFRs and intracellular calcium overload may lead to apoptosis. In recent years, there...
Figure 5  Effects of D-Lys3-GHRP-6, capsazepine and LY294002 in ghrelin postconditioning on the expression of Bcl-2, Bax, Akt and glycogen synthase kinase-3β in human gastric epithelial cells induced by hypoxia/reoxygenation. Cells were grouped as follows: normoxic culture for 6 h (N), 2 h hypoxia/4 h reoxygenation (H/R), DMSO vehicle postconditioning (DM), ghrelin postconditioning (10^−7 mol/L) (GH) D-Lys3-GHRP-6 + ghrelin postconditioning (D + GH), capsazepine + ghrelin postconditioning (C + GH) and LY294002 + ghrelin postconditioning (L + GH). The expression of β-actin was detected as an internal standard. Densitometry results are expressed as ratio of test over normal group. A, B: Bcl-2 expression; C, D: Bax expression; E: Akt expression; F: Glycogen synthase kinase (GSK)-3β expression. mean ± SD. n = 6. bP < 0.01 vs H/R, dP < 0.01 vs GH.

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Figure 6  Effects of capsazepine in ghrelin postconditioning on the expression of vanilloid receptor subtype 1 in human gastric epithelial cells induced by hypoxia/reoxygenation. Cells were grouped as follows: normoxic culture for 6 h (A), 2 h hypoxia/4 h reoxygenation (B), DMSO vehicle postconditioning (C), ghrelin postconditioning (10^{-7} mol/L) (D), and capsazepine + ghrelin postconditioning (E). The expression of vanilloid receptor subtype 1 (VR1) in each group was observed by immunocytochemistry. Cells were observed for photography under a phase contrast microscope (× 400). The arrows indicate the positive cells which express VR1.

Figure 7  Effects of LY294002 in ghrelin postconditioning on the expression of Akt in human gastric epithelial cells induced by hypoxia/reoxygenation. Cells were grouped as follows: normoxic culture for 6 h (A), 2 h hypoxia/4 h reoxygenation (B), DMSO vehicle postconditioning (C), ghrelin postconditioning (10^{-7} mol/L) (D), and LY294002 + ghrelin postconditioning (E). The expression of Akt in each group was observed by immunocytochemistry. Cells were observed for photography under a phase contrast microscope (× 400). The arrows indicate the positive cells which express Akt.
has been a significant rise in the gastric mucosa cell apoptosis in gastric mucosa injury due to alcohol and nonsteroidal anti-inflammatory drugs, suggesting that there is a process of programmed cell death in acute gastric mucosal injury.[27]

Apoptosis is the main mechanism of cell death, and is mediated by a cell-intrinsic suicide program, with the relative balance of pro- and antiapoptotic signaling pathways determining the fate of the cell. There are two main pathways in mammals, one of which is the mitochondrial signaling pathway, in which Bcl-2 plays an important role and Bcl-2/Bax is the key point of apoptosis.[28] In our study, the cell viability significantly decreased and apoptosis percentage increased in the model of 2 h hypoxia/4 h reoxygenation compared with normal control group. They also show that, in the same model, the expression of an antiapoptotic protein (Bcl-2) decreased and the expression of proapoptotic protein (Bax) increased. These results suggest that we have successfully established a model of H/R-induced injury in a human GES-1. MTT assays, flow cytometric analysis, LDH assays and western blotting were used to test cell viability, apoptotic percentages, cell LDH release, and apoptosis-related protein expression.

Ghrelin is well known as a potent activator of growth hormone release.[8,9] Given the implication of growth hormone in the tissue regeneration and maintenance of integrity, ghrelin is supposed to contribute to the processes of healing and regeneration. A number of studies have confirmed the effect of ghrelin in gastroprotection. Brzozowski et al.[17] have found that ghrelin inhibits stress-induced gastric injury.[18] Ghrelin can also inhibit I/R injury. Sibilia et al.[16] have reported that ghrelin inhibits ethanol-induced gastric ulcers. Unfortunately, all of these were studies in vivo. In recent years, many studies have demonstrated that ghrelin confers protection in some cell types in vitro, but no study has clearly elucidated the effects of ghrelin in human gastric epithelial cells.[20,30]

Therefore, in our study, we demonstrated that ghrelin post-conditioning has protective effects against H/R injury in human gastric epithelial cells, and confirmed that ghrelin could increase cell viability, and decrease apoptosis and LDH release. We also found that ghrelin post-conditioning increased VR1, Akt and Bcl-2 expression, and attenuated GSK-3β and Bax expression subsequent to H/R in gastric epithelial cells in vitro. The results showed that ghrelin seems to be involved in the regulation of gastroprotection, which also supports our hypothesis that ghrelin could effectively attenuate H/R-induced injury via the mitochondrial antiapoptotic pathway in human gastric epithelial cells.

In our study, we also demonstrated that the protective effects of ghrelin against H/R-induced injury might be mediated by GHS-R, VR1 and activation of the PI3K/Akt pathway, in that the protective effects of ghrelin were reversed by the GHS-R antagonist D-Lys3-GHRP-6, VR1 antagonist capsazepine and PI3K/Akt antagonist LY294002 during H/R. The result showed that ghrelin in combination with D-Lys3-GHRP-6, capsazepine and
LY294002 decreased cell viability, and increased the percentage of apoptotic cells and LDH release compared with ghrelin post-conditioning. The result also showed a decrease in Bcl-2 expression and increase in Bax expression. In the C + GH group, expression of VR1 was downregulated, and in the L + GH group, expression of Akt decreased, and GSK-3β expression increased. These data suggested that GHS-R, VR1 and the PI3K/Akt signaling pathway might be involved in ghrelin-induced antiapoptotic effects. However, the precise underlying mechanism of their activation by ghrelin remains to be determined.

Ishii et al. showed that treatment with GHS-R antagonist, D-Lys3-GHRP-6 could partially reverse diabetic hyperphagia. GHS-R mRNA is mainly expressed in the arcuate nucleus of the brain, ventral median nucleus and the hippocampus. It has been demonstrated that GHS-R mRNA is also expressed in peripheral organs. The multifunction of ghrelin coincides with the distribution of GHS-R in various tissues. GHS-R acts as the receptor for a family of synthetic ligands known as growth hormone secretagogues. Therefore, GHS-R is involved in the regulation of physiological actions when activated by its ligands, such as ghrelin. On the grounds of its distribution in the gastrointestinal tract, GHS-R plays an important role in the regulation of gastrointestinal functions. Some studies have shown that GHS-R mediates ghrelin gastroprotection against I/R-induced injury, while the protective effect is inhibited by the GHS-R antagonist D-Lys3-GHRP-6.

Capsazepine is a specific and competitive antagonist of the VR. VR1 is a nervous-system-specific receptor. However, recent studies have indicated that VR1 is also distributed in some non-nervous tissues, such as the liver, gastric epithelial cells, bronchi, and bladder epithelium. These findings suggest that VR1 might be related to the regulation of a variety of physiological functions of different tissues and organs. Many studies have demonstrated that activation of VR1 by its agonists exerts a gastroprotective effect.

PI3K antagonists, such as LY294002, at low concentrations are considered to be selective PI3K inhibitors and valuable tools for the study of cardioprotection. Many studies have discovered that the activation of the PI3K/Akt signaling pathway is involved in the antiapoptotic effect of ghrelin in many cell types. Some studies have shown that the PI3K/Akt pathway is a significant antiapoptotic factor that has an important protective role in reducing the apoptosis-associated protein kinase caspase family, Bcl-2 and other apoptotic factors. The PI3K/Akt pathway has been implicated in the control of major cellular responses including cell proliferation, survival, development, differentiation, cell cycle, and apoptosis. GSK-3β is a multifunctional Ser/Thr kinase that plays important roles in necrosis and apoptosis of cardiomyocytes. GSK-3β activity has been associated with many cell processes, including the regulation of multiple transcription factors, the Wnt pathway, nuclear factor kB, endoplasmic reticulum stress, embryogenesis, apoptosis and cell survival, cell cycle progression, and cell migration. GSK-3β, serving as an Akt downstream effector, plays an important role during I/R-induced apoptosis of cells in the heart and brain.

In summary, our study demonstrated that ghrelin postconditioning had a protective effect against H/R-induced injury in human gastric epithelial cells in vitro, and the effect might be mediated by the receptors GHS-R and VR1 as well as activation of the PI3K/Akt signaling pathway, resulting in activation of the intracellular antiapoptotic signaling pathway, hence the inhibition of apoptosis.

**COMMENTS**

**Background**

Gastric ischemia-reperfusion injury is a common clinical pathophysiological process, and the mobilization of the endogenous protection is the most effective manner against ischemia-reperfusion (I/R) injury. In recent years, ischemic postconditioning has been regarded as important endogenous protection.

**Research frontiers**

Ghrelin is a 28-amino-acid peptide that was initially identified in rat stomach. Several studies have shown the effect of ghrelin in gastroprotection. In recent years, many studies have demonstrated that ghrelin confers protection in some cell types in vitro. Unfortunately, no study has clearly shown the effect of ghrelin in human gastric epithelial cells. In this study, the authors demonstrated that ghrelin post-conditioning has protective effect against hypoxia/reoxygenation (H/R)-induced injury in human gastric epithelial cells.

**Innovations and breakthroughs**

The injury of visceral I/R is a common pathological occurrence. Nowadays, ischemic preconditioning and ischemic postconditioning have been confirmed to be important approaches in endogenous protection. The notion of ischemic postconditioning was first proposed in 2003. Pharmacological postconditioning is the extension of ischemic postconditioning in which a drug is applied to the ischemic myocardium or hypoxic cardiomyocytes during the initial few minutes of reperfusion or reoxygenation, significantly reducing organ reperfusion injury. This study suggests that the authors have successfully established a model of H/R-induced injury in human gastric epithelial cell line (GES-1), and have demonstrated that ghrelin postconditioning has protection against H/R-induced injury in human gastric epithelial cells in vitro.

**Applications**

The results of the study suggest that the ghrelin postconditioning is a potential therapeutic approach that could be applied to the prevention of gastric mucosal lesions induced by ethanol, stress and I/R.

**Terminology**

Ghrelin, a 28-amino-acid peptide initially identified in rat stomach, is noted for its potent activation of growth hormone release. Pharmacological postconditioning is the extension of ischemic postconditioning in which a drug is applied to the ischemic myocardium or hypoxic cardiomyocytes during the initial few minutes of reperfusion or reoxygenation, significantly reducing organ reperfusion injury.

**Peer review**

The present study was well-organized and well-investigated. The authors demonstrated the antiapoptotic effects of ghrelin against H/R-induced apoptosis of human gastric epithelial cells.

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