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Ghrelin Reduces Injury of Hippocampal Neurons in a Rat Model of Cerebral Ischemia/Reperfusion

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Abstract

Ghrelin, an acyl-peptide gastric hormone and an endogenous ligand for growth hormone secretagogue (GHS) receptor 1a (GHS-R 1a) exerts multiple functions. It has been reported that synthetic GHS-hexarelin reduces injury of cerebral cortex and hippocampus after brain hypoxia-ischemia in neonatal rats. However, the effect of ghrelin in tolerance of the brain tissues to cerebral ischemia/reperfusion (I/R) injury has not been studied. The aim of the present study was to examine whether ghrelin have potential protective effect on hippocampal neurons of rats against I/R injury. I/R injury was induced by a modified four-vessel occlusion model. Ghrelin was administered intraperitoneally after the insult. Histological damage of the neurons was determined with hematoxylin-eosin (H&E) staining and assay of the neuronal apoptosis was performed by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL). The results showed that I/R decreased the number of surviving neurons and induced apoptosis of the neurons in CA1 area of the hippocampus in rats. In contrast, administration of ghrelin significantly increased the number of surviving neurons and reduced the number of TUNEL-positive apoptotic neurons in the equivalent areas after I/R. In conclusion, the present data provide evidence for the first time that ghrelin can exert a neuroprotective role *in vivo* in the tolerance of hippocampal neurons to I/R injury, and that the mechanism underlying this effect involves an anti-apoptotic property of ghrelin.

Key Words: ghrelin, ischemia/reperfusion injury, apoptosis, hippocampal neuron, rat

Introduction

Ghrelin, a novel 28-amino residue polypeptide predominantly isolated from the stomach, was identified as an endogenous ligand for growth hormone secretagogue (GHS) receptor 1a (GHS-R 1a) (17). It has been demonstrated that ghrelin and the GHS receptors are widely expressed not only in the hypothalamus and the pituitary gland, but also in the hippocampus and other areas of the central nervous system as well as several peripheral tissues (2, 14, 17, 18, 20, 27). Therefore, ghrelin is more than simply a

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natural GHS. A series of elaborate studies recently showed that, apart from potently stimulating growth hormone (GH) secretion both in animals and in humans, ghrelin and many synthetic (hexarelin) GHSs exhibit several other neuroendocrine, metabolic and nonendocrine actions. These include stimulation of appetite (5), increasing energy metabolism (8, 20, 32), stimulation of gastric motility and acid secretion (24), influence on pancreatic endocrine (21) or other endocrine activities (13, 19, 20, 32), control of behavior (4), and hemodynamic actions (20, 31, 32, 33).

Recent evidence indicates that ghrelin and synthetic GHSs exert a variety of cardiovascular activities such as improvement of cardiac function (7, 16, 20, 32), inhibition of cardiomyocyte apoptosis (15, 20, 32), and protection from myocardial I/R injury *in vivo* (6, 12).

It is already known that ghrelin may promote neural proliferation in the dorsal motor nucleus of the vagus (34), and bind to neurons of the hippocampal formation where it controls dendritic spine synapse density and memory performance (10). Very recently, Brywe and co-workers demonstrated for the first time that synthetic GHS-hexarelin reduces injury of cerebral cortex, hippocampus, and thalamus after brain hypoxia-ischemia in neonatal rats (3). Thus ghrelin has attracted considerable interest as a new endogenous survival factor with multiple activities in recent years.

The currently intriguing data raise the possibility that ghrelin could be involved in neuroprotection of the central nervous system against cerebral I/R damage. The aim of the present study was to examine whether ghrelin might have a protective effect on hippocampal neurons against I/R injury in adult rats, using patho-histological examination and TUNEL labeling. The data reported here support such a possibility by providing evidence for the first time that it can reduce I/R neuronal loss in the hippocampus by inhibiting apoptosis.

Materials and Methods

Animal and Peptide Preparation

Male Wistar rats weighing 250 g ~ 320 g were provided by the center of animal laboratory in Shandong University.

Rat ghrelin, purchased from AnaSpec, Inc. (San Jose, CA, USA) was dissolved in distilled water (1 mg/ml), and stored at -20°C until the time of preparation for administration. Immediately before administration by intraperitoneal (i.p.) injection, ghrelin was diluted again with 0.9% saline to the final concentration of 0.1 mg/ml.

Forebrain Ischemia/Reperfusion Model

Transient forebrain I/R was induced by the modified four-vessel occlusion model (4-VO) as described by Schmidt-Kastner (9, 23, 30). Briefly, on day 1 the rats were anesthetized with 1% sodium pentobarbital (40 mg/kg, i.p.) and both common carotid arteries were exposed *via* a ventral, midline cervical incision. Threads were placed around each common carotid artery without interrupting the blood flow and then the incision was closed with sutures. A second incision was made behind the occipital bone directly overlying the first two cervical vertebrate. The vertebral arteries traveling *via* the vertebrate canal and passing beneath the alar formation were electrocauterized and permanently occluded. After surgery, the rats were returned to their cages and allowed free access to food and water. After 24 h, the rats were re-anesthetized with ether. The common carotid arteries were occluded with aneurysm clips for 8 min, followed by 3 days of reperfusion. Three days later the rats were anesthetized and their brains were perfused with 4% paraformaldehyde, then removed and post-fixed for histological and biochemical assessments.

The criteria of successful ischemia included bilateral pupil dilution and loss of righting and pain reflexes. The EEG became isoelectric within 1 min after 4-VO. The body temperature of the rats was measured and maintained above 37.0°C.

Experimental Protocol

The rats were randomly divided into three groups (n = 6 per group). [1] Ischemia/reperfusion + ghrelin group (I/R+G group): 24 h after vertebral artery electrocauterization, the rats underwent an 8-min lethal ischemia and then reperfusion for three days. After the ischemic insult the rats received a daily i.p. injection of ghrelin at a dose of 0.4 mg/kg for 3 days. The injections were given at the same time on each day. [2] Ischemia/reperfusion + saline group (I/R group): the rats were subjected to I/R in the same way as the I/R +G group, but received daily i.p. injection of the same volume of vehicle (saline). [3] Control group: sham surgery was performed without occlusion of both the vertebral and carotid arteries. The rats received a daily i.p. injection of the same volume of saline.

Neuropathological Examination

Pathological damage of hippocampal cells in the CA1 region was examined with standard H&E histological staining of brain paraffin sections (5 μm). The morphological changes and the pyramidal

Table 1. Comparison of neuronal counting in the control, I/R and I/R+G groups.

Groups	n	Neuronal density (number/mm)
Control group	6	251.17 ± 3.99
I/R group	6	28.50 ± 1.51$^{\#}$
I/R+G group	6	179.50 ± 5.05*

Neuronal counting is expressed (mean ± SE) as the number of surviving neurons/mm length of the CA1 pyramidal layer. Differences between groups are significant, $P \leq 0.001$, one-way ANOVA. Between-group differences were determined by Tukey's test: $^{\#}P < 0.001$ *vs*. control, $^{*}P < 0.001$ *vs*. I/R, and control.

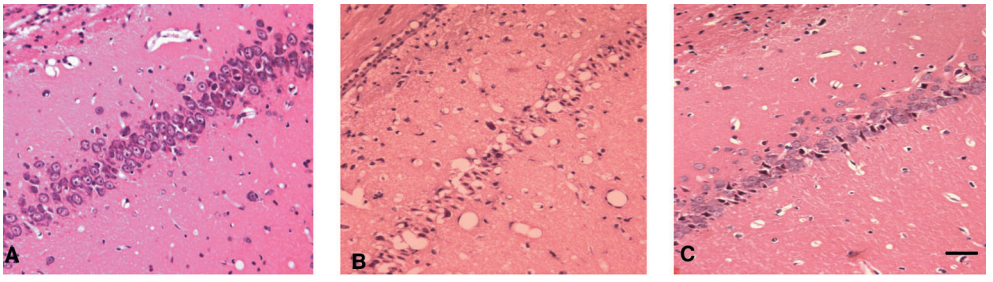

Fig 1. Hematoxylin and eosin staining of the pyramidal layer of sector CA1 of the hippocampus in (A) control group, (B) I/R group, (C) I/R+G group. After fixation by perfusion *in situ* with formalin, standard 5 μm paraffin sections of the brains were made and treated by a standard histological method. There is a dramatic loss of large cells in the pyramidal layer in (B) compared to (A), whereas many more survive in (C). Scale bar 30 μm.

neuron density were observed in the CA1 sector by counting the number of surviving pyramidal neurons per mm length of the pyramidal layer.

TUNEL Labeling

To detect cells undergoing apoptosis, brain sections were stained *via* the TUNEL labeling technique. The chemicals for labeling were purchased from R&D SYSTEMS, Inc. Switzerland. According to the protocol, after deparaffinization and rehydration, the sections were digested for 15 min in proteinase K (20 mg/ml; Sigma, St Louis, MO, USA). The reaction was terminated with tap water, and the tissue was treated with buffer A (25 mM Tris, pH 6.6, containing 200 mM potassium cacodylate and 0.25 mg/ml bovine serum albumin) for at least 5 min. Sections were then incubated at 37°C with labeling solution containing terminal deoxynucleotidyl transferase (TdT), biotinylated-16-dUTP and 1.5 mM cobalt chloride in buffer A for 1 h in a humidified chamber. The reaction was terminated with 2 × SSC (300 mM sodium chloride and 30 mM sodium citrate, pH 7.4). After vigorous washing with 0.1 M Tris, pH 7.4, the sections were blocked with 10% goat serum in 0.1 M Tris for 15 min. The DNA was visualized by treating the tissue with a 1:40 dilution of streptavidin-conjugated alkaline phosphatase and reacted with 0.05% 3-3'-diaminobenzidine tetrahydrochloride (DAB) for 10 min. Then the sections were stained with hematoxylin.

Statistical Analysis

Quantitative data were processed by Sigma Stat. The data are expressed as mean ± SE. Quantitative data on cell counting was obtained by first calculating a mean count from 4 hippocampal fields per rat, then using these figures to perform the statistical comparison with n = 6 for all groups. The comparisons were made by one-way ANOVA followed by Tukey's test to determine which differences were significant. $P < 0.05$ was considered to be statistically significant.

Results

Effects of Ghrelin on I/R-induced Cell Death In Vivo

Figure 1 shows a typical example of morphological changes of the hippocampal neurons in the CA1 area. A quantitative comparison of the pyramidal neuronal density in the CA1 area in the three groups is shown in Table 1.

Compared with the I/R+G group and control group, there were few intact neurons in the CA1 area in the I/R group (Fig. 1B) and the number of the surviving neurons was significantly decreased ($P < 0.001$). The dying cells showed shrunken cytoplasm and degeneration of the nucleus. In ghrelin-treated rats (I/R+G group), many neurons in the same area survived (Fig. 1C), and the number of intact neurons

Table 2. Comparison of the number of TUNEL-positive cells in the control, I/R and I/R+G groups.

Groups	n	TUNEL-positive neurons (number/mm)
Control group	6	7.46 ± 0.88
I/R group	6	135.01 ± 5.74$^{\#}$
I/R+G group	6	28.71 ± 1.96*

TUNEL-positive cells are expressed (mean ± SE) as the number of stained cells/mm length of the CA1 pyramidal layers. Differences between groups are significant, $P \leq 0.001$, one-way ANOVA. Between-group differences were determined by Tukey's test: $^{\#}P < 0.001$ *vs*. control, $^{*}P < 0.001$ *vs*. I/R, and control.

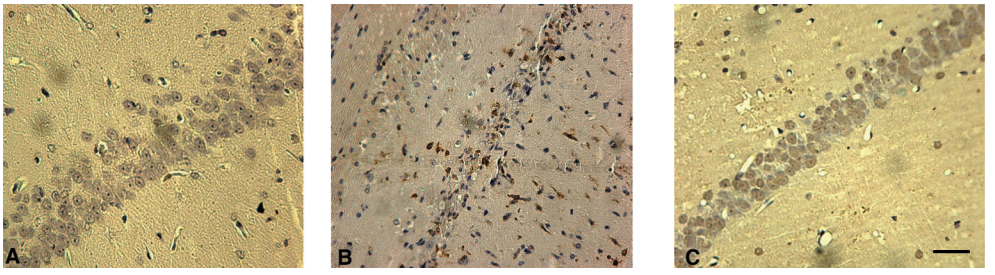

Fig 2. TUNEL staining to demonstrate apoptosis in the pyramidal layer of sector CA1 of the hippocampus in (A) control group, (B) I/R group, (C) I/R+G group. After fixation by perfusion *in situ* with formalin, standard 5 μm paraffin sections of the brains were first made. Following deparaffinization and rehydration, the standard protocol of R&D systems for staining the dislocated DNA was applied (see methods), with revelation by DAB (dark brown staining). Counterstaining was performed with hematoxylin. There were no stained apoptotic cells in the pyramidal layer in (A) a large number in (B) and very clearly a reduced number in (C). Scale bar 30 μm.

was much greater than that in the I/R group ($P < 0.001$) , but still less than that in the control group ($P < 0.001$) (Fig. 1A).

Anti-Apoptotic Activity of Ghrelin

TUNEL staining results in the three groups are shown in Figure 2. No TUNEL-positive neurons were detected in the CA1 region of normal control rats (Fig. 2A). The majority of TUNEL-positive neurons in the CA1 region were found in I/R group (Fig. 2B). These TUNEL-positive neurons showed condensed, pyknotic nuclei and chromatin condensation in the perinuclear regions, suggesting apoptosis, whereas TUNEL-positive neurons were very few in the equivalent areas in ghrelin-treated rats. (Fig. 2C). The numerical data on TUNEL staining is shown in Table 2, and reveals significant differences between the three groups.

These results strongly suggest that I/R may induce apoptosis of the hippocampal neurons in CA1 area, and that ghrelin may protect the neurons from this injury.

Discussion

Ghrelin is a peptide of 28 amino acids with a molecular weight of 3314. Kojima *et al*. first succeeded in isolating the gene coding for ghrelin. The gene is expressed in both the stomach and the hypothalamic arcuate nucleus. Human ghrelin is homologous to rat ghrelin apart from two amino acids (17, 18). Ghrelin can be rendered biologically active by post-translational acylation with octanoic acid at its serine residue along with an L-configuration of the third residue. Acylation of ghrelin is crucial to facilitate the highly regulated bidirectional transport across the blood brain barrier and to bind to the GHS-R1a receptors. Non-acylated ghrelin is present in a greater proportion in the circulation and may exert cardiovascular and anti-proliferative effects (1). Ghrelin has attracted considerable interest, as in recent years significant advances have been made in the understanding of its multiple activities.

In the present experiment, the hippocampal pyramidal neurons in the CA1 area were examined because of their high vulnerability to I/R damage. Although the pyramidal cell layer contains some non-neuronal cells, our counting was applied to clearly identifiable pyramidal cells which, forming the large majority of cells in this layer, are thus fairly represented by the data obtained, even if a small error in the exact number may have occurred. The results showed that I/R induced apoptosis of the neurons in the CA1 area

of the hippocampus, which is consistent with our previous observations in a rat I/R model (9, 22, 23). Another finding in the current study is that intraperitoneal administration of natural ghrelin significantly increased the number of surviving neurons in the CA1 area after I/R. A direct implication of this finding is that natural ghrelin has neuroprotective properties capable of rescuing hippocampal pyramidal neurons from I/R damage. When we assayed the antiapoptotic activity of ghrelin, results indicated that many representative TUNEL-positive neurons in the CA1 region were found in the I/R group, while such staining almost completely absent from the equivalent areas in ghrelin-treated rats. The results strongly support the notion that the neuroprotective effect of ghrelin is associated with its antiapoptotic action. The present work is a preliminary study and no attempt was made to clarify the possible mechanism of the antiapoptotic action of ghrelin. We previously reported that apoptosis of the pyramidal cells in the rat CA1 area was induced after I/R, in which bcl-2, bax and p53 play a key role (9, 22, 23), so that it could be postulated that the antiapoptotic effect of ghrelin may be mediated by activation of a specific mitochondrial signaling pathway.

To our knowledge, no studies regarding the neuroprotective role of ghrelin or GHS in cerebral I/R injury in rats have been reported previously. However, a very recent *in vivo* experiment demonstrates for the first time that intracerebroventricular administration of hexarelin, a synthetic peptide GHS, significantly reduces brain damage in the cerebral cortex, hippocampus and thalamus after neonatal hypoxia-ischemia insult induced by unilateral carotid ligation and hypoxic exposure, and the neuroprotective effect is accompanied by phosphorylation of Akt and GSK3β, indicating possible involvement of the PI3K pathway (3). Collectively, the currently available data suggest that ghrelin may play a protective role against cerebral I/R damage in rats. However, further studies are needed to elucidate the possible underlying mechanisms behind all the observations on ghrelin by which it exerts a neuroprotective effect.

Although the effects of ghrelin in the brain are largely unknown, possibly the most pressing questions concern whether the neuroprotective effect of ghrelin is growth hormone/insulin-like growth factor-1(GH/IGF-1)-dependent or whether it is related to binding to ghrelin receptors. In regard to the first possibility, it is known that many effects of GH are mediated *via* stimulation of IGF-1 production (11, 28). GH significantly increases IGF-1 mRNA levels in the hypothalamus and hippocampus. In hippocampus, Bcl-2 protein abundance was increased to 200% of control levels and decreased TUNEL labeling was also seen in GH-treated rats. GH treatment enhanced the expression of both IGF-1 receptor mRNA and GH receptor mRNA, and provided a moderate degree of cerebral protection in the neonatal rat brain. The GH/IGF-1 axis is involved in the chemical process leading to hypoxia-ischemia brain injury. Since ghrelin may strongly stimulate secretion of GH and increase the serum level of GH and IGF-1, it has been postulated that the beneficial effects of ghrelin could result from stimulation of GH and IGF-1 secretion. However, this hypothesis is put in doubt by many other observations. Brywe *et al*. demonstrated that the amelioration of I/R-damaged hippocampal CA1 neurons by administration of the synthetic GHS-hexarelin is IGF-1 independent (3). Many other cardiovascular investigations also suggest that the protection from myocardial I/R injury by ghrelin or GHS is GH/IGF-1-independent. Although marked organ-specific differences between brain and heart exist, some mechanisms of tolerance in I/R injury may be universal. For instance, intra-arterial infusion of ghrelin increased forearm blood flow in a dose dependent manner with an unchanged serum level of IGF-1 (1). Administration of ghrelin caused vasodilatory effects on arteries without alteration of the serum IGF-1 level, suggesting an effect through GH/IGF-1-independent mechanisms (26). Moreover, it has been reported that a prolonged treatment with hexarelin could protect the hearts of rats with experimental GH deficiency from I/R damage through a mechanism independent of GH secretion, because the effect was also present and even magnified in hypophysectomized rats (29). Numerous studies have shown that the cardioprotective effects of ghrelin are independent of GH/IGF-1 (2, 20) and that ghrelin may act as a survival factor by directly acting on the cardiovascular system through binding to a novel, as yet unidentified receptor, which is distinct from GHSR-1a (2, 15, 20).

Regarding the ghrelin receptors (GHS-R), two isoforms, GHS-R1a and 1b have been identified. GHS-R 1a is widely expressed not only in the pituitary and the hypothalamus, but also in other brain areas including the hippocampus, the substantia nigra, the NTS, and outside the nervous system in the pancreas, the thyroid gland, the spleen, the myocardium and the adrenal cortex (2, 10, 14, 32). GHS-R1a transduces the GH-releasing effects of GHS. GHS-R1b seems almost ubiquitous, having been demonstrated in the stomach, the oesophagus, the intestine, the liver, the lung, muscle and the testis, and it appears not to bind ghrelin, and is a non-functional receptor mRNA variant (2, 10, 14, 20, 32). Recent evidence indicates that circulating ghrelin can enter the hippocampus across the blood brain barrier (1, 10, 17, 18, 25) and bind to the hippocampal neurons, where it promotes dendritic spine synapse formation and generation of long-term potentiation to enhance spatial learning and memory (10). These accumulating experimental results imply that GHS-R may be involved in the neuroprotective

effects of ghrelin and GHS, though additional studies are necessary to establish clear evidence providing a possible link between GHS-R and the neuroprotective effects of ghrelin and GHS.

In conclusion, the available data reported here indicate for the first time that administration of natural ghrelin significantly increases the number of surviving neurons in the CA1 area of the rat hippocampus after I/R associated with inhibition of apoptosis of the neurons in the equivalent area. Therefore, the present results provide evidence that ghrelin features some neuroprotective activity, and that the mechanism underlying this effect may involve an anti-apoptotic property of ghrelin. Further studies are needed to elucidate the anti-apoptotic signaling mechanism of ghrelin.

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