Neuroscience Letters 453 (2009) 58–61

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Ghrelin protects against cell death of hippocampal neurons in pilocarpine-induced seizures in rats

Jingjing Xu[a], Shuzhen Wang[a], Youting Lin[a], Lili Cao[a], Rong Wang[b], Zhaofu Chi[a,*]

[a] Department of Neurology, Qilu Hospital, Shandong University, 44#, Wenhua Xi Road, Jinan 250012, PR China
[b] Key Laboratory of Cardiovascular Remodeling and Function Research, Chinese Ministry of Education and Chinese Ministry of Public Health, Shandong University, 107#, Wenhua Xi Road, Jinan 250012, PR China

ARTICLE INFO

Article history:
Received 16 December 2008
Received in revised form 25 January 2009
Accepted 26 January 2009

Keywords:
Ghrelin
Neuronal death
Neuroprotective effect
Seizure
Hippocampus
Pilocarpine

ABSTRACT

Ghrelin, a 28-amino-acid peptide, is mainly secreted by the stomach. Evidence has shown ghrelin to have neuroprotective effects. However, whether ghrelin protects hippocampal neurons against cell death in pilocarpine-induced seizures is unknown. We used Nissl staining to show that ghrelin attenuated the neuronal loss caused by pilocarpine-induced seizures in the hippocampus. Ghrelin exerted the protective action through regulating the phosphatidylinositol-3-kinase and Akt pathway. Moreover, ghrelin treatment reversed the decreased ratio of Bcl-2 to Bax induced by seizures while inhibiting the activated caspase-3. Ghrelin can inhibit hippocampal neuronal damage caused by pilocarpine-induced seizures, which might have therapeutic value in seizures.

Ghrelin, a 28-amino-acid peptide secreted mainly by the stomach is the endogenous ligand of growth hormone secretagogue receptor type 1α (GHSR-1α) [13]. GHSR-1α is a G protein-coupled receptor. GHSR-1α is expressed in peripheral tissues and extensively in the central nervous system, such as the pituitary gland, hypothalamus, thalamus, cortex and hippocampus [7,10]. Ghrelin is secreted to regulate the release of growth hormone and promote adiposity and appetite [16,20,23]. Moreover, ghrelin could inhibit the neuronal damage induced by glucose–oxygen deprivation in hypothalamus and protect neurons in the hippocampus and cortex against cerebral ischemia/reperfusion [3,14,15]. The phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway plays a central role in intracellular processes such as cell survival and proliferation. Cumulative data showed that the anti-apoptotic effects of ghrelin might be related to activation of not only the PI3K/Akt signaling pathway, but also the mitochondrial pathway [15,24]. Recently, serum ghrelin level was found to be up-regulated in the epileptic patients [2]. Ghrelin also suppressed the onset time of seizures induced by pentylenetetrazole and inhibited oxidative stress in the brain of rats [18,19].

These results implied that ghrelin had neuroprotective effects. However, whether ghrelin exerts biological effects on hippocampal neuronal injury in pilocarpine-induced seizures is unknown. In this study, we detected the protective effects of ghrelin in pilocarpine-induced seizures and the possible underlying mechanisms such as the PI3K/Akt signaling pathway and mitochondrial apoptosis pathway.

The study was performed in accordance with international guidelines and approved by the Chinese Institutional Animal Care Committee. Adult male Wistar rats (Experimental Animal Center of Shandong University) weighing 200–250 g were given lithium chloride intraperitoneally (3 mEq/kg, i.p.). Twenty hours after lithium chloride treatment, experimental rats received pilocarpine (30 mg/kg, i.p. Sigma, St. Louis, MO, USA) and control animals received normal saline at the same volume. Scopolamine methylnitrate (1 mg/kg) was injected subcutaneously 30 min before pilocarpine administration to prevent peripheral cholinergic effects. The rats showing stage 4 or 5 convulsive seizures according to Racine [21] were included in the experimental groups. Seizures were allowed to last for 60 min and then were terminated by administration of diazepam (10 mg/kg, i.p.). Rats were decapitated at 2, 8, 16, 24 and 72 h after seizures.

In order to verify the neuroprotective effects of ghrelin on pilocarpine-induced seizures, rats were randomly divided into 4 groups for treatment: control, pilocarpine, pilocarpine + saline and ghrelin. Rats in control group and pilocarpine group were treated as what have mentioned above. In ghrelin group, ghrelin (Anaspec, San Jose, CA, USA) dissolved in normal saline was injected intraperitoneally at 80 μg/kg 30 min before pilocarpine

* Corresponding author at: Department of Neurology, Qilu Hospital of Shandong University, 107#, Wenhua Xi Road, Jinan 250012, PR China. Tel.: +86 531 82169428; fax: +86 531 86927544.
E-mail address: chizhaofu0618@126.com (Z. Chi).

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doi:10.1016/j.neulet.2009.01.067

treatment and saline at the same volume in the pilocarpine + saline group.

Rats under anesthesia were intracardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffered saline and the brains were removed. The trimmed brains were fixed with paraffin, cut into coronal sections 10 μm thickness and underwent Nissl staining with toluidine blue. For every tenth section (six sections per animal), we counted in a blinded manner the number of surviving hippocampal CA1 and CA3 pyramidal cells per 1-mm length of the bilateral hemispheres by use of a microscope with high magnification (×400). The detailed procedure was carried out as previously described [11].

Total RNA was extracted from hippocampi by use of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Oligo (DT)-primed cDNA was prepared with use of M-MLV reverse transcriptase (Fermentas, Glen Burnie, MD, USA). LightCycler 2.0 (Roche, Mannheim, Germany) was used for detecting the real-time PCR products. The primers for GHSR-1α were forward, 5′-CAGCGTCTTCTTCTTTCTACCG-3′; and reverse, 5′-CACCACCACAGCAAGCATCT-3′. PCR involved 45 cycles at 95 °C for 10 s, 5 s at 58 °C and 15 s at 72 °C. The expression of the target gene was normalized to that of β-actin.

Hippocampi were homogenized in 10 volumes of ice-cold homogenization buffer and centrifuged at 15,000 rpm for 10 min at 4 °C. Supernatants were collected and the protein concentrations were measured with use of a BCA protein assay kit (Beyotime, Jiangsu, China). Thirty micrograms of protein were size-separated by SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were incubated with the primary antibodies anti-GHSR-1α (1:200), anti-Bax (1:200), and anti-Bcl-2 (1:500; all Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho (Tyr458) p85 PI3K (1:250), anti-PI3K p85 (1:250), anti-Akt (1:1000) and anti-phospho-Akt (Ser-473) (1:250; all Cell Signal Technology, Beverly, MA, USA), and anti-caspase-3 (1:200, Chemicon, Temecula, CA, USA) at 4 °C overnight. Then the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000; Jingmei, Beijing, China) for 1 h at 37 °C. Immunoreactivity was detected by an enhanced chemiluminescence kit (Millipore, Billerica, MA, USA), then the images underwent analysis by use of an image analyzer (Alpha Innotech, San Leandro, CA, USA).

Table 1
Effects of ghrelin against neuronal loss in hippocampus after seizures.

Group	Neuron number (mean ± SD)	
	CA1	CA3
Control	241.5 ± 24.5	265.5 ± 24.3
Pilocarpine	105.8 ± 10.8*	116.5 ± 12.9*
Pilocarpine + ghrelin	201.3 ± 17.7#	217.5 ± 20.0#

The number of surviving pyramidal cells per 1 mm length of the CA1 and CA3 subfields of the hippocampus was counted under light microscopy. Data were mean ± SD (n = 4/group).

* $p < 0.05$ vs. control.

\# $p < 0.05$ vs. pilocarpine.

Data were expressed as mean ± standard deviation (SD). One-way ANOVA and the Newman–Keuls test were used for statistical analysis of the result as appropriate. Significance level was set at $p < 0.05$.

Nissl staining to examine the neuronal loss in hippocampal CA1 and CA3 regions after pilocarpine-induced seizures revealed that seizures led to severe cell death at 72 h after seizures. The number of surviving neurons was decreased significantly as compared with control ($p < 0.05$), moreover, ghrelin treatment significantly attenuated the neuronal loss induced by seizures ($p < 0.05$) (Fig. 1; Table 1), with no significant difference in neuronal loss between the pilocarpine and pilocarpine + saline groups (data not shown).

GHSR-1α mRNA and protein levels showed no significant changes at 24 h after seizures as compared with control and ghrelin treatment had no effect on the expression ($p > 0.05$) (Fig. 2).

Immunoreactivity of phospho-PI3K p85 (Tyr458) and phospho-Akt (Ser473) was significantly higher at 2 and 8 h after seizures ($p < 0.05$), and lower 8 h later than that in control ($p < 0.05$) (Fig. 3A and C). After ghrelin administration, the decrease in phospho-PI3K p85 and phospho-Akt levels induced by seizures was reversed significantly ($p < 0.05$) (Fig. 3B and D), with no effect by saline treatment (data not shown). Levels of both total PI3K p85 and Akt did not significantly differ (data not shown).

Immunoreactivity of Bcl-2 in the hippocampus was decreased at 2 h and continued to decrease up to 72 h after seizures ($p < 0.05$). In contrast, Bax showed a significantly higher expression in pilocarpine groups than in control ($p < 0.05$). Moreover, the active

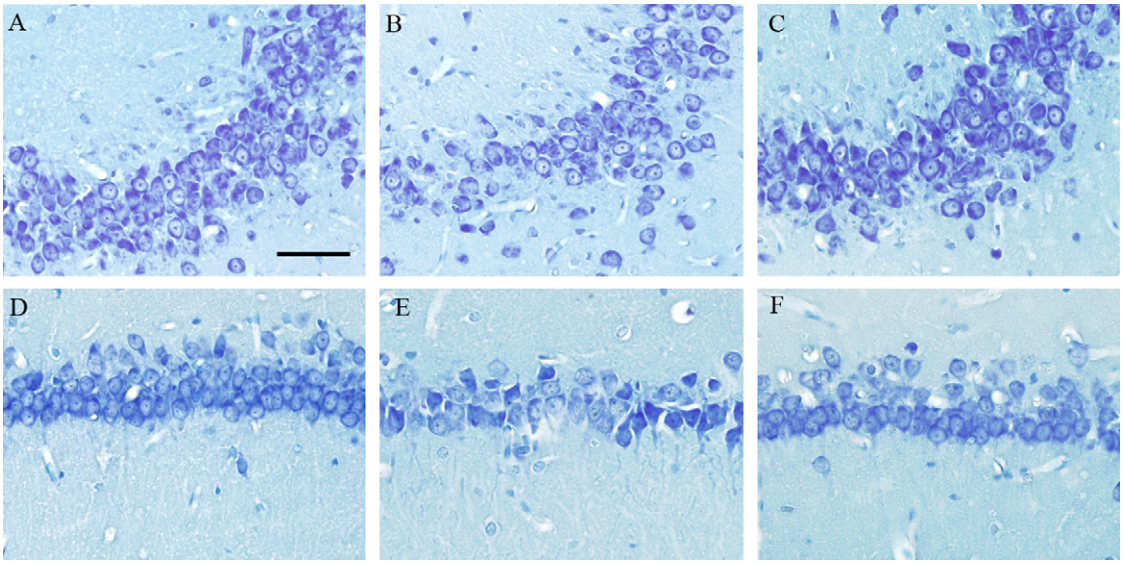

Fig. 1. Nissl staining with toluidine blue of the hippocampal CA3 (Panels A–C) and CA1 (Panels D–F) pyramidal neurons at 72 h after seizures was shown with high power (400×). (A and D) Control group, showing normal pyramidal neurons. (B and E) Pilocarpine (pilo) group, showing neuronal death (shrunken neurons with pyknotic nuclei). (C and F) Pilo + ghrelin group, showing the effect of ghrelin on neuronal loss. Bar = 50 μm, n = 4/group.

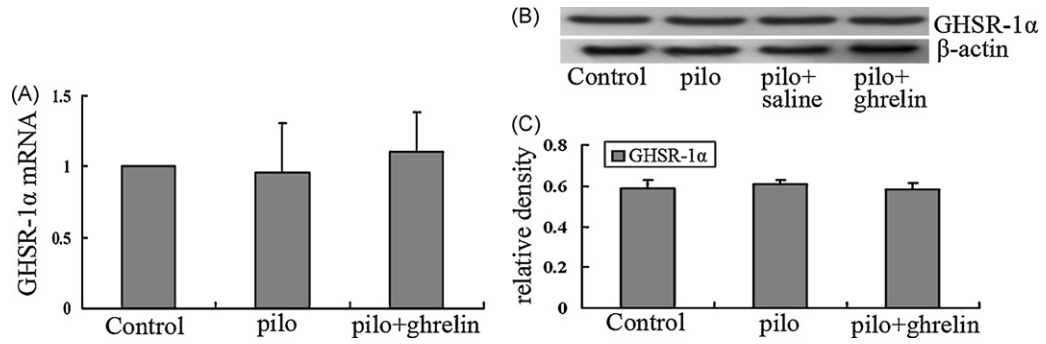

Fig. 2. The expression of GHSR-1α in the rat hippocampus after seizures and the effect of ghrelin. (A) Relative expression of GHSR-1α mRNA to β-actin in the hippocampus of the rats. (B and C) Immunoblot analysis and relative density of GHSR-1α protein. Data were mean ± SD (n = 4/group). Pilo = pilocarpine.

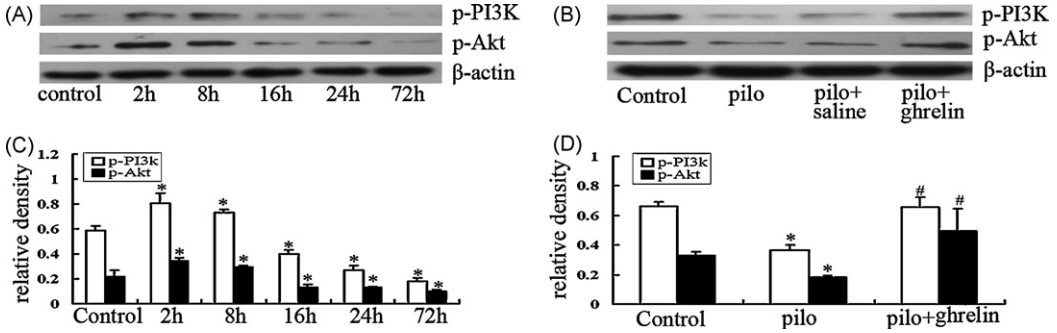

Fig. 3. The protein levels of phospho-PI3K p85 (Tyr458) and phospho-Akt (Ser473). (A and C) Immunoblot analysis and relative density of phospho-PI3K p85 and phospho-Akt at various time points after seizures. (B and D) Effect of ghrelin on seizure-induced inhibition of phospho-PI3K p85 and phospho-Akt. β-Actin was used as an internal standard. Data were mean ± SD (n = 4/group). $^{*}p < 0.05$ vs. control and $^{\#}p < 0.05$ vs. pilo. Pilo = pilocarpine.

cleavage product of caspase-3 appeared at 24 h after seizures, and increased persistently until 72 h ($p < 0.05$) (Fig. 4A and C). Ghrelin pretreatment reversed the decreased Bcl-2 level and the increased Bax level at 24 h after pilocarpine treatment ($p < 0.05$). Ghrelin also sharply reduced the increased caspase-3 activity induced by seizures ($p < 0.05$) (Fig. 4B and D) with no effect by saline (data not shown).

In this study, we showed that pilocarpine-induced seizures caused prominent neuronal loss in the hippocampus and ghrelin could significantly rescue neurons from death induced by seizures.

Ghrelin reversed the down-regulated levels of GHSR-1α mRNA and protein in the rat cerebral cortex on ischemia/reperfusion injury [15]. The expression of GHSR-1α mRNA in the hypothalamus was also up-regulated with intraventricular injection of ghrelin in rats [17]. However, our data showed that both pilocarpine treatment and ghrelin preconditioning did not regulate the expression of GHSR-1α in the rat hippocampus, which suggested that ghrelin might not up-regulate GHSR-1α in the hippocampus directly.

We extended the investigation to gain insight into the signaling pathway and apoptosis-related proteins modulated by ghrelin. PI3K, composed of an 85-kDa regulatory subunit and a 110 kDa catalytic subunit, played a critical role in preventing apoptosis, and tyrosine phosphorylation of p85 was critical for the activation of PI3K [5,8]. Akt, a downstream effector of PI3K, was a critical mediator of neuronal survival in pathological neuronal cell death [9]. In agreement with the previous study reporting that ghrelin activated the PI3K/Akt pathway in hypothalamic neuronal cells [3], we showed that ghrelin strongly up-regulated the seizure-induced decreased levels of phospho-PI3K p85 and phospho-Akt in the hippocampus, which was believed to be an important mechanism to eliminate neuronal damage in hippocampus.

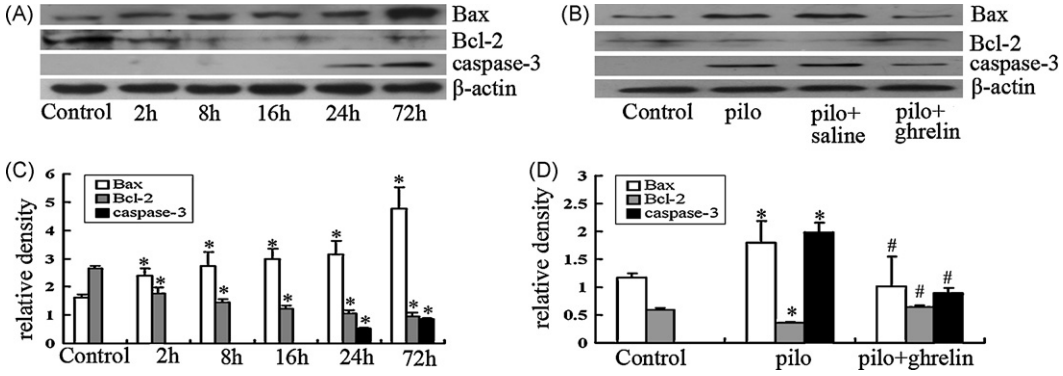

Fig. 4. The levels of Bcl-2, Bax and activated fragment of caspase-3. (A and C) Immunoblot analysis and relative density of Bcl-2, Bax and caspase-3 in the hippocampus of the rats. (B and D) Effect of ghrelin on the levels of Bcl-2, Bax and activated caspase-3. Relative density was the ratio of the target band to β-actin band. Values were mean ± SD (n = 4/group). $^{*}p < 0.05$ vs. control and $^{\#}p < 0.05$ vs. pilo. Pilo = piloarpine.

Recent study indicated that the activation of the PI3K/Akt pathway led to increased expression of Bcl-2 [1], so we examined the effects of ghrelin on the mitochondrial apoptosis pathway to confirm our points. Pilocarpine-induced seizures resulted in increased Bax and decreased Bcl-2 levels. With an increased ratio of Bcl-2 to Bax, the release of cytochrome c from mitochondria was suppressed and the activation of caspase-3 was prevented [12]. Caspase-3 in the hippocampus began to be activated at 24 h after pilocarpine treatment, whereas ghrelin pretreatment increased the decreased ratio of Bcl-2 to Bax induced by seizures and inhibited caspase-3 activation. Similar mechanisms of ghrelin protecting the neurons in the cortex against ischemia/reperfusion were observed [15]. The current data suggested that the mitochondrial pathway participated in the protective effect of ghrelin against pilocarpine-evoked prolonged seizures.

We verified the neuroprotective effects of exogenous ghrelin on pilocarpine-induced seizures in that GHSR-1α mRNA and protein levels in the hippocampus were not regulated by ghrelin. Rak et al. showed that the anti-apoptotic effect of ghrelin was independent of GHSR-1α level [22]. Some studies also reported that the proliferative and anti-apoptotic actions of ghrelin in some cell types lacking the ghrelin receptor were mediated by a distinct and yet unidentified receptor [4,6]. However, we could not confirm whether the neuroprotective effect of ghrelin was due to the classic GHSR-1α or a novel unknown receptor in neurons of the hippocampus. Perhaps, only additional exogenous ghrelin binding to GHSR-1α amplified the protective effect. The gap between ghrelin and the PI3K-Akt-mitochondrial apoptosis pathway needs further exploration.

In conclusion, our results showed that the neuroprotective effect of ghrelin was associated with promoting the PI3K/Akt signaling pathway and inhibiting the mitochondrial apoptosis pathway. Ghrelin could be a potential benefit treatment for the hippocampal neuron demise caused by pilocarpine-induced prolonged seizures.

Acknowledgement

This study was supported by a grant from National Natural Science Foundation of China (No. 30870838).

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