Ghrelin attenuates kainic acid-induced neuronal cell death in the mouse hippocampus

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Abstract

Ghrelin is an endogenous ligand for GH secretagogue receptor type 1a (GHSR1a), and is produced and released mainly from the stomach. It has been recently demonstrated that ghrelin can function as a neuroprotective factor by inhibiting apoptotic pathways. Kainic acid (KA), an excitatory amino acid L-glutamate analog, causes neuronal death in the hippocampus; previous studies suggest that activated microglia and astrocytes actively participate in the pathogenesis of KA-induced hippocampal neurodegeneration. However, it is unclear whether ghrelin has neuroprotective effect in KA-induced hippocampal neurodegeneration. I.p. injection of KA produced typical neuronal cell death in the CA1 and CA3 pyramidal layers of the hippocampus,

and the systemic administration of ghrelin significantly attenuated KA-induced neuronal cell death in these regions through the activation of GHSR1a. Ghrelin prevents KA-induced activation of microglia and astrocytes, and the expression of proinflammatory mediators tumor necrosis factor α , interleukin-1 β , and cyclooxygenase-2. The inhibitory effect of ghrelin on the activation of microglia and astrocytes appears to be associated with the inhibition of matrix metalloproteinase-3 expression in damaged hippocampal neurons. Our data suggest that ghrelin has a therapeutic potential for suppressing KA-induced pathogenesis in the brain.

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Introduction

Excitotoxic brain damage is one of the major mechanisms by which neurons die in the adult central nervous system (CNS; Choi 1988, Lipton & Nicotera 1998). An essential event in excitotoxicity is the direct and constant activation by kainic acid (KA) of specific glutamate receptors, which results in neuronal cell death (Choi 1988, Doble 1999, Wang *et al.* 2005). KA is an analog of the excitotoxin glutamate, which induces hippocampal cell death and seizure (Sharma *et al.* 2007). Studies using KA have provided major contributions to the understanding of neuronal cell death caused by excitotoxicity. Administration of KA is known to induce a sequence of altered behavioral events characterized by epileptiform seizures (Ben-Ari *et al.* 1980, Sperk 1994), which are followed by neurodegeneration in specific brain regions, such as the hippocampus, piriform cortex, thalamus, and amygdala. In the hippocampus, the CA3 pyramidal cells and interneurons in the hilus of the dentate gyrus are the most vulnerable, followed by CA1 pyramidal cells (Coyle 1983, Sperk *et al.* 1985, Tauck & Nadler 1985). Although the exact molecular mechanisms underlying excitotoxicity-induced cell death remain unclear, it has been shown that the activation of microglia and astrocytes in the hippocampus and consequent enhanced release of reactive oxygen species

and proinflammatory cytokine may play an important role in KA-induced neurodegenerative processes (Wang *et al.* 2005).

Ghrelin, a novel 28-amino acid hormone principally released from the stomach (Date *et al.* 2000), is a unique peptide esterified with octanoic acid on Ser3 (Kojima & Kangawa 2005). In addition to stimulating GH release from the anterior pituitary and inducing a positive energy balance by stimulating food intake while decreasing fat usage through GH-independent mechanisms (Kojima *et al.* 1999, Peino *et al.* 2000), we recently reported that ghrelin acts as a survival factor for neurons *in vivo* and *in vitro* by inhibiting apoptotic pathways (Chung *et al.* 2007, 2008, Hwang *et al.* 2009, Moon *et al.* 2009). Moreover, ghrelin receptor, GH secretagogue receptor type 1a (GHSR1a), is expressed in the hippocampal neurons (Guan *et al.* 1997), and ghrelin protects hippocampal neurons against ischemic injury (Liu *et al.* 2006, 2009). These findings prompted us to hypothesize that ghrelin could have a neuroprotective role in hippocampal neurons against KA-induced excitotoxicity. Indeed, it was very recently reported that ghrelin protected hippocampal neurons against pilocarpine-induced seizures (Xu *et al.* 2009) via a promotion of the phosphatidylinositol-3-kinase (PI3K)/AKT pathway and an inhibition of the mitochondrial apoptotic pathway. However, it is still unknown whether ghrelin exerts its neuroprotective effect in KA-induced hippocampal

neurodegeneration via inhibition of microglial and astrocyte activation. Therefore, we investigated the neuroprotective effect of ghrelin treatment on the KA-induced hippocampal neurodegeneration in the mice.

Materials and Methods

Animals and drug treatment

Male C57BL/6 mice (22–24 g, 8-week-old) were used in the present study. They were housed under controlled environmental conditions (12 h light:12 h darkness) and acclimatized for at least 1 week. Food and tap water were made available *ad libitum*. Animals were randomly divided into four groups of five animals each: controls, KA-injected mice, KA-injected mice treated with ghrelin, and KA-injected mice treated with ghrelin and its receptor antagonist. The mice received a single i.p. injection of KA (30 mg/kg) in saline. The control group received saline solution. KA injection caused overt seizure 5–10 min later, which lasted for several hours. Seizure activities were scored as follows: 1) arrest of motion; 2) myoclonic jerks of the head and neck, with brief twitching movement; 3) unilateral clonic activity; 4) bilateral forelimb tonic and clonic activity; and 5) generalized tonic-clonic activity with loss of postural tone including death from continuous convulsions, as previously described (Yang *et al.* 1997). All experiments were approved by the Kyung Hee University Animal Care Committee, and conducted according to the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of mice used. To determine the neuroprotective effects of ghrelin on KA-induced hippocampal neuronal cell death *in vivo*, mice were given i.p. injection of ghrelin (80 µg/kg) 30 min prior to KA injection, and a second ghrelin treatment was performed 24 h after the KA injection. In this study, 80 µg/kg of ghrelin was chosen on the basis of our previous studies that this dose of ghrelin exerted a potent neuroprotective action (Chung *et al.* 2007, Hwang *et al.* 2009, Moon *et al.* 2009). To examine whether the effect of ghrelin is exerted through GHSR1a, animals were co-treated with D-Lys-GHRP-6 (1 mg/kg, i.p.) and ghrelin. During the concurrent administration, ghrelin antagonist was administered first followed immediately by ghrelin.

Immunohistochemistry

Three days after the KA injection, mice were anesthetized with xylazine and ketamine, and then perfused transcardially with a freshly prepared solution of 4% paraformaldehyde in PBS. The brains were removed and post-fixed overnight in the same fixative before being immersed in a solution of 30% sucrose in PBS. Serial 30-µm-thick coronal tissue sections were cut using a microtome and immunostained as

free-floating sections. Tissue reverse transcriptase was included and sections were incubated overnight at 4 °C with one of the following primary antibodies: mouse anti-NeuN antibody (1:500, Millipore, Billerica, MA, USA), rat anti-Mac-1 antibody (1:500, Chemicon, Carlsbad, CA, USA), rabbit anti-caspase-3 antibody (1:500, Sigma), goat anti-metalloproteinase-3 (MMP3) antibody (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-tumor necrosis factor (TNF)- α antibody (1:200, R&D Systems, Minneapolis, MN, USA), goat anti-interleukin (IL)-1 β antibody (1:100, R&D Systems), or rabbit anti-cyclooxygenase-2 (COX-2) antibody (1:500, Abcam, Cambridge, MA, USA). The sections were incubated with appropriate biotinylated secondary antibody (1:200, Vector Laboratories, Burlingame, CA, USA), and then visualized using the avidin-biotin-peroxidase complex method with diaminobenzidine tetrahydrochloride as the chromogen. For fluorescent immunodetection of *Mmp3*, sections were washed and incubated with a secondary Cy2-donkey anti-goat IgG (1:400). Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) before mounting, and images were acquired by the Carl Zeiss LSM 510 Meta (Oberkochen, Germany) confocal microscope. All tissue sections from all experimental groups to be compared were processed at the same time using identical reagents.

TUNEL assay

For *in situ* detection of apoptotic cells, TUNEL assay was performed using ApopTag peroxidase *in situ* apoptosis detection kit (Chemicon) according to the manufacturer's instruction. Briefly, after quenching with endogenous peroxidase activity, sections were washed in PBS and incubated in an equilibration buffer. The sections were then incubated with digoxigenin-conjugated dUTP in a terminal deoxynucleotidyl transferase-catalyzed reaction for 60 min at 37 °C in a humidified atmosphere, and were then immersed in stop/wash buffer for 10 min at room temperature. The sections were then incubated with anti-digoxigenin antibody conjugated with peroxidase for 30 min. DNA fragments were stained using diaminobenzidine tetrahydrochloride as the substrate for the peroxidase.

RNA extraction and RT-PCR

Total RNA from the ventral midbrain was isolated using a previously reported standard procedure (Kamegai *et al.* 1998). The RNA was then precipitated with isopropanol, and the pellet was washed with 70% ethanol, air dried, and dissolved in sterile diethylpyrocarbonate (DEPC) water. The concentration and purity of RNA were determined with a NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). One microgram of total RNA was reverse transcribed using Superscript II reverse transcriptase (Life Technologies, Inc.) at 42 °C with random hexamer priming. An RNA control tube containing all the

reverse transcription reagents except as a negative control to monitor genomic DNA contamination. The resulting cDNA was amplified using the GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA) or the Light-Cycler (Roche Diagnostics Ltd). Quantitative real-time PCR analysis was carried out with SYBR Green I and specific primers for *Tnfrα* (NM_013693; sense, 5'-CCC AGA CCC TCA CAC TCA GAT-3'; antisense, 5'-AAC ACC CAT TCC CTT CAC AGA-3'), *Il1β* (*Il1b*) (NM_008361; sense, 5'-ACC TTT TGA CAG TGA GAA-3'; antisense, 5'-AAG ATG AAG GAA AAG AAG GTG-3'), *Cox2* (*Ptgs2*) (NM_011198; sense, 5'-TCT CCA ACC TCT CCT ACT AC-3'; antisense, 5'-GCA CGTAGT CTT CGA TCA CT-3'), *Mmp3* (NM_010809; sense, 5'-GAT CTC TTC ATT TTG GCC ATC TCT TC-3'; antisense, 5'-CTC CAG TAT TTG TCC TCT ACA AAG AA-3'), and β -actin (NM_031144; sense, 5'-ATG GGT CAG AAG GAC TCC TAC G-3'; antisense, 5'-AGT GGT ACG ACC AGA GGC ATA C-3'). Details of the procedure for real-time PCR were as previously described (Park *et al.* 2004).

Statistical analysis

Data are presented as mean $\pm$ S.E.M. ($n=5$ /group). Each experiment was repeated twice, giving essentially identical results. Statistical analysis between groups was performed using one-way ANOVA and Holm-Sidak method for multiple comparisons using SigmaStat for Windows Version 3.10 (Systat Software, Inc., Point Richmond, CA, USA). $P<0.05$ was considered statistically significant.

Results

Changes in seizure activity by ghrelin treatment

We first investigated the effect of ghrelin on KA-induced seizure activity according to the previously described scoring system (Yang *et al.* 1997) at the specified time points for 90 min (Table 1). Animals injected with KA first exhibited 'staring' spells, with abnormal body posture, progressed to

Table 1 Effect of ghrelin on kainic acid (KA)-induced seizure activity. Ghrelin treatment significantly decreased seizure scores. Mice were treated with i.p. injection of KA (30 mg/kg) in saline. Either vehicle or ghrelin (80 μ g/kg) was injected i.p. 30 min before KA injection in the presence or in the absence of the GHSR1a antagonist D-Lys-3-GHRP-6 (1 mg/kg). Seizure activities were scored according to the rating scale described by Yang *et al.* (1997) at the specified time points for 90 min. Temporal seizure activity was presented as means $\pm$ S.E.M.

	30 min	60 min	90 min
KA	3.9 $\pm$ 0.2	4.4 $\pm$ 0.4	3.0 $\pm$ 0.3
KA + ghrelin	2.8 $\pm$ 0.5*	3.3 $\pm$ 0.3*	0
KA + ghrelin + antagonist	4.2 $\pm$ 0.2	4.3 $\pm$ 0.3	3.6 $\pm$ 0.3

* $P<0.05$ versus KA-treated group.

head nodding (wet-dog shakes), forepaw tremor, rearing, loss of postural control, and eventually continuous convulsions, lasting for several hours. The seizure scores in KA-treated mice were progressively increased to 3.9 ± 0.2 at 30 min, 4.4 ± 0.4 at 60 min, and 3.0 ± 0.3 at 90 min. In contrast, animals treated with KA together with ghrelin showed minor behavioral symptoms with significantly decreased seizure scores when compared with KA-injected mice. Ghrelin treatment significantly decreased seizure scores to 2.8 ± 0.5 at 30 min ($P<0.05$) and 3.3 ± 0.3 at 60 min ($P<0.05$). The KA-induced seizures were completely subsided 1.5 h after injection in ghrelin-treated group. However, concurrent administration of ghrelin with GHSR1a antagonist completely abolished the suppressive effect of ghrelin on KA-induced seizure activity.

Ghrelin attenuates KA-induced cell death in the hippocampus

Next, we investigated the effect of ghrelin treatment on KA-induced hippocampal cell death. As shown in Fig. 1A, i.p. administration of 30 mg/kg of KA resulted in selective cell death in the pyramidal layer of the CA1 and CA3 hippocampal regions as assessed by cresyl violet staining. I.p. administration of ghrelin prevented the KA-induced loss of hippocampal neuronal cells (Fig. 1A). Ghrelin alone did not significantly alter the number of hippocampal neuronal cells (data not shown). The loss of hippocampal neurons was confirmed by immunohistochemical staining with anti-NeuN antibody (Fig. 1B). The numbers of NeuN-positive cells in vehicle-treated mice counted in the CA1 (0.01 mm^2 , $0.1 \times 0.1 \text{ mm}$) and CA3 (0.018 mm^2 , $0.13 \times 0.13 \text{ mm}$) were 206.9 and 202.8 respectively (Fig. 1C). In KA-injected mice, the numbers of these cells in the CA1 and CA3 were decreased to 67.6 and 58.2 respectively. In contrast, ghrelin treatment increased the number of NeuN-positive cells to 167.2 (CA1) and 184.2 (CA3) (Fig. 1C). TUNEL analysis showed that numerous TUNEL-positive cells were detected in the CA1 and CA3 of the KA-injected mice, but no TUNEL-positive cell was detected in the hippocampus of ghrelin-treated mice (Fig. 1D). We then performed an immunohistochemical analysis with anti-caspase-3 antibody, and found that 3 days after KA injection, many CA1 and CA3 pyramidal neurons were immunopositive for caspase-3, which were significantly attenuated by ghrelin treatment (Fig. 1E). To determine whether the neuroprotective effect of ghrelin is mediated by its receptor, animals were treated with the receptor-specific antagonist D-Lys-3-GHRP-6 (Smith *et al.* 1993). Treatment with itself failed to affect the number of hippocampal neurons (data not shown). However, the protective effect of ghrelin (80 μ g/kg) against KA-induced seizure behavior, hippocampal apoptotic neuronal cell death, and caspase-3 levels were significantly attenuated by concurrent treatment with D-Lys-3-GHRP-6 (1 mg/kg, i.p.; Fig. 1A–E), indicating that GHSR1a is involved in the neuroprotective effect of ghrelin.

Figure 1 Neuroprotective effect of ghrelin on KA-induced hippocampal neuron loss *in vivo*. C57BL/6 mice were treated with i.p. injection of KA (30 mg/kg) in saline and killed 3 days after the injection. The control mice received saline solution. Either vehicle or ghrelin (80 µg/kg) was injected i.p. 30 min before and 1 day after KA injection in the presence or in the absence of the GHSR1a antagonist D-Lys-3-GHRP-6 (1 mg/kg). The extents of KA-induced neuronal losses were assessed by staining with cresyl violet (A) and anti-NeuN antibody (B). Representative photomicrographs from two independent experiments are shown. (C) Numbers of NeuN-positive cells in CA1 and CA3 regions were counted, and presented as means $\pm$ S.E.M. Representative images of TUNEL staining (D) and immunolabeling of caspase-3 (E) in the hippocampus CA1 and CA3 regions were presented. Scale bars represent 100 µm. * $P < 0.05$ versus vehicle-treated control; # $P < 0.05$ versus KA-treated group. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0040>.

Ghrelin suppresses KA-induced activation of microglia and astrocytes

Sequential activation of microglia and astrocytes is one of the events that take place in the hippocampus following KA-induced excitotoxic injury. Activated microglial cells and astrocytes are believed to be key players in the induction of neuronal damage caused by excitotoxic injury. To investigate whether the neuroprotective effect of ghrelin is associated with the inhibition of KA-induced glial response, we examined the expression of Mac-1, which is a specific marker for microglial activation (Gonzalez-Scarano & Baltuch 1999). Similarly, we also determined the expression of GFAP, which is a marker protein for astrogliosis (Eng & Ghirnikar 1994). Immunohistochemical analysis showed that KA administration resulted in a dramatic increase in the number of activated microglia and the change in morphology (large cell body with poorly ramified short and thick processes) in both CA1 and CA3 regions 3 days after the KA injection (Fig. 2A). However, in KA-injected ghrelin-treated mice, CA1 and CA3 Mac-1-positive cells were significantly decreased when compared with KA-injected vehicle-treated group (Fig. 2B). With regard to astrogliosis, resting astrocytes were distributed throughout the entire hippocampus (Fig. 2C). Three days after KA injection, significant increase in GFAP immunoreactivity was detected in all regions of the hippocampus (Fig. 2C). Both the number and the intensity of GFAP-positive cells were increased. Similar to the inhibition of Mac-1 expression, ghrelin treatment strongly suppressed KA-induced increase of GFAP immunoreactivity in CA1 and CA3 regions (Fig. 2D). In addition, the inhibitory effects of ghrelin on KA-induced microglial activation and astroglial response were significantly attenuated by concurrent treatment of animals with D-Lys-3-GHRP-6 (Fig. 2A–D). Taken together, these results indicate that ghrelin inhibits the KA-induced activation of microglia and astrocytes in the hippocampus.

Ghrelin inhibits the production of proinflammatory and neurotoxic products derived from activated glial cells

As mediators of the inflammatory response, glial cells are known to play an important role in the course of KA-induced hippocampal neurodegeneration. Given the fact that ghrelin suppressed the KA-induced glial response, we investigated whether the expression of proinflammatory and neurotoxic products from activated microglial cells and astrocytes is inhibited by ghrelin treatment. Figure 3A–C shows that KA injection resulted in a significant increase in TNF- α , IL-1 β , and COX-2 immunoreactivities in both CA1 and CA3 regions. However, similar to the suppression of glial activation, ghrelin treatment significantly inhibited the KA-induced increase of these deleterious mediators. These inhibitory effects of ghrelin were completely blocked when mice were exposed to ghrelin receptor antagonist. *Tnf α* , *Il1 β* (*Il1b*), and *Cox2* (*Ptgs2*) mRNA levels assessed by RT-PCR analysis showed similar results (Fig. 3D).

Figure 2 Ghrelin suppresses KA-induced microglial and astrocyte activation in the hippocampus. Mice were treated with i.p. injection of KA (30 mg/kg) in saline and killed 3 days after the injection. The control mice received saline solution. Either vehicle or ghrelin (80 µg/kg) was injected i.p. 30 min before and 1 day after KA injection in the presence or in the absence of the GHSR1a antagonist D-Lys-3-GHRP-6 (1 mg/kg). (A and C) The number of activated microglia or astrocytes in the CA1 and CA3 regions was determined by immunostaining with antibody against Mac-1 or GFAP in brain coronal sections. Representative photomicrographs of Mac-1 (A) or GFAP (C) immunostaining in each group are shown. (B and D) Numbers of Mac-1- or GFAP-positive cells in CA1 and CA3 regions were counted and presented as means ± S.E.M. Scale bars represent 100 µm. **P* < 0.05 versus vehicle-treated control; #*P* < 0.05 versus KA-treated group. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0040>.

Ghrelin attenuates KA-induced *Mmp3* expression

It has been previously reported that *Mmp3* is up-regulated in the hippocampus of KA-treated mice (Penkowa *et al.* 2005), and *Mmp3* is known to play an important role in dopaminergic neuronal cell death and neuroinflammation

Figure 3 Ghrelin inhibits the production of deleterious mediators derived from activated glial cells in the hippocampus. Mice were treated with i.p. injection of KA (30 mg/kg) in saline and killed 3 days after the injection. The control mice received saline solution. Either vehicle or ghrelin (80 µg/kg) was injected i.p. 30 min before and 1 day after KA injection in the presence or in the absence of the GHSR1a antagonist D-Lys-3-GHRP-6 (1 mg/kg). Representative photomicrographs of *Tnfα* (A), *Il1β* (B), and *Cox2* (C) immunostaining in each group are shown. *Tnfα*, *Il1β*, and *Cox2* mRNA levels in the hippocampus were determined by RT-PCR (D) and real-time RT-PCR analysis (E). β-Actin was used as an internal control. Data represent means ± S.E.M. Scale bars represent 100 µm. **P* < 0.05 versus vehicle-treated control; #*P* < 0.05 versus KA-treated group. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0040>.

(Kim *et al.* 2005, 2007). Therefore, we performed immunohistochemistry and RT-PCR to investigate the effect of ghrelin on KA-induced *Mmp3* expression in the hippocampus. Immunohistochemical analysis revealed that *Mmp3* was barely detectable in saline-treated mice, whereas increased *Mmp3* expression was observed in animals treated with KA for 3 days (Fig. 4A). Using a NeuN antibody to detect hippocampal neurons, we found that most of *Mmp3*-positive cells colocalized to hippocampal neurons (Fig. 4B). Administration of ghrelin significantly attenuated the upregulation of *Mmp3* by KA, while the effect of ghrelin

Figure 4 Ghrelin attenuates KA-induced *Mmp3* expression in the hippocampus. Mice were treated with i.p. injection of KA (30 mg/kg) in saline and killed 3 days after the injection. The control mice received saline solution. Either vehicle or ghrelin (80 µg/kg) was injected i.p. 30 min before and 1 day after KA injection in the presence or in the absence of the GHSR1a antagonist D-Lys-3-GHRP-6 (1 mg/kg). (A) Representative photomicrographs of *Mmp3* immunostaining in each group are shown. (B) MMP-3 expression in the CA3 neurons was determined by double fluorescent immunohistochemistry against *Mmp3* (green) and NeuN (red). Representative pictures of *Mmp3* expression in NeuN-positive cells are shown. (C) *Mmp3* mRNA levels in the hippocampus were determined by RT-PCR (upper panel) and real-time RT-PCR analysis (lower panel). β -Actin was used as an internal control. Data represent means $\pm$ S.E.M. Scale bars represent 100 µm (for A) and 5 µm (for B). * $P < 0.05$ versus vehicle-treated control; # $P < 0.05$ versus KA-treated group. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0040>.

was completely blocked by co-administration of ghrelin receptor antagonist. These results indicated that ghrelin is a potent inhibitor of KA-induced MMP-3 expression. RT-PCR analysis of MMP-3 showed similar results (Fig. 4C).

Discussion

In the present study, our data clearly show that ghrelin treatment reduces KA-induced seizures and hippocampal neurodegeneration. Ghrelin-mediated protection is associated with the inhibition of glial activation and concomitant production of proinflammatory neurotoxic mediators derived from activated glial cells. Additionally, the protective effect of ghrelin seems to be mediated through the activation of its receptor GHSR1a, and ghrelin-mediated suppression of glial activation appears to be associated with the inhibition of *Mmp3* expression in the hippocampal neurons.

These results suggest that ghrelin can be a potential therapeutic agent in neurodegenerative disorders, in which excitotoxic neuronal cell death and inflammatory processes are involved.

In this study, systemic administration of KA produced well-characterized seizure activity in mice, consistent with previous report (Yang *et al.* 1997). KA, an agonist of ionotropic, non-N-methyl-D-aspartic acid (NMDA) glutamate α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and KA receptors, is one of the most commonly used chemoconvulsants used to create status epilepticus model of mesial temporal lobe epilepsy (Sharma *et al.* 2007). In the current study, we initially analyzed the effect of ghrelin on seizures induced by KA, and found that the intensity and duration of behavior seizure activity were attenuated by the administration of ghrelin. Antiepileptic effect of ghrelin was also reported in an acute experimental epilepsy model in pentylenetetrazole-injected rats (Obay *et al.* 2007). Neuropeptide Y (NPY) and γ -aminobutyric acid (GABA) may be involved in the anticonvulsant effect of ghrelin because NPY and GABA exert antiepileptic effects in animal seizure models (Czapinski *et al.* 2005, Stroud *et al.* 2005) and ghrelin not only enhances NPY but also increases GABA-ergic activity in the brain (Cowley *et al.* 2003). It is well known that a majority of epileptic seizures are due to an imbalance between the activities of inhibitory and excitatory neurotransmitters (Sharma *et al.* 2007). Therefore, the stimulatory effect of ghrelin on NPY and GABA activities may contribute to the antiepileptic properties of ghrelin. It also should be noted that the indirect pathway through the activation of vagus nerve might have significant role in conveying systemically administered ghrelin signal to the CNS because blockade of the gastric vagal afferent pathway by vagotomy completely abolished peripheral ghrelin-induced feeding (Asakawa *et al.* 2001, Date *et al.* 2002). Taken together, these findings suggest that ghrelin may have a therapeutic value as an antiepileptic drug.

Neuroprotective effects of ghrelin have been recently reported in rodent model of focal cerebral ischemia (Chung *et al.* 2007, Miao *et al.* 2007, Hwang *et al.* 2009), in which systemic administration of ghrelin augmented the expression of Bcl2/Bax and heat shock protein 70 and inhibited prostate apoptosis response-4, caspase-8, -9, and -3. In addition, we demonstrated the *in vitro* neuroprotective effect of ghrelin in neurons, which was associated with the inhibition of the apoptotic cascade and preservation of mitochondrial integrity (Chung *et al.* 2007, 2008). In this study, we demonstrated that the i.p. injection of ghrelin significantly reduced hippocampal neuronal cell death, TUNEL-positive cells, and caspase-3 expression after KA injection. Although these findings suggest that peripherally administered ghrelin can pass through the blood-brain barrier, enter the brain parenchyma, and exert the direct effects in the CNS, the brain insulin-like growth factor 1 (IGF1) system may be involved in the neuroprotective effect of ghrelin because systemic administration of ghrelin agonist GHRP6 in rats increased IGF1 expression in the hypothalamus, cerebellum, and hippocampus (Frago *et al.* 2002).

Collectively, these studies suggest that the anti-apoptotic properties of ghrelin may contribute to the neuroprotective effects of ghrelin observed in a KA-induced excitotoxicity animal model. However, the precise mechanism by which ghrelin protects hippocampal neurons against KA-induced neurotoxicity remains to be clarified.

The protective effect of ghrelin appears to be mediated through the activation of GHSR1a because simultaneous treatment of animals with the ghrelin receptor antagonist significantly attenuates the neuroprotective effect of ghrelin against KA-induced excitotoxicity. The GHSR1a belongs to a family of receptors operating via the Gq-phospholipase C signaling pathways (Howard *et al.* 1996). It was demonstrated that ghrelin could stimulate the ERK1/2 and PI3K/Akt pathways, which have been implicated in the regulation of cell survival (Datta *et al.* 1999, Pearson *et al.* 2001), in neurons through the activation of GHSR1a (Chung *et al.* 2007, 2008). In addition, ghrelin has been shown to exert its neuroprotective effects through the stimulation of protein kinase A and C pathways (Chung *et al.* 2007). Taken together, these findings suggest that multiple signaling pathways are involved in ghrelin-mediated protection in KA-induced neurotoxicity.

In the present study, we show that ghrelin has a potent anti-inflammatory effect *in vivo* after KA-induced excitotoxicity. Excitotoxic neuronal damage evoked by excessive or prolonged activation of the excitatory amino acid receptors is recognized as an important underlying mechanism in several neurodegenerative disorders, such as stroke, traumatic brain injury, amyotrophic lateral sclerosis, Parkinson's disease (PD), Huntington's disease, and Alzheimer's disease (AD; Doble 1999, Salinska *et al.* 2005). Also, an accumulating body of evidence have demonstrated a strong link between neurodegeneration and chronic neuroinflammatory processes (Skaper 2007, DeLegge & Smoke 2008). Under inflammatory conditions, activated microglia and astrocytes produce proinflammatory and neurotoxic factors, such as superoxide, nitric oxide, proinflammatory cytokines, and eicosanoids (Teismann *et al.* 2003, Teismann & Schulz 2004). Therefore, inhibition of these processes may constitute a valuable target for the discovery and development of neurodegenerative therapeutics. Indeed, previous studies reported that long-term treatment with nonsteroidal anti-inflammatory drugs reduced the risk of developing AD or PD (Chen *et al.* 2003, Hald & Lotharius 2005, Townsend & Pratico 2005). Furthermore, we have recently demonstrated that ghrelin prevented microglial activation and concomitant production of TNF- α and IL-1 β in an animal model of PD (Moon *et al.* 2009). Consistent with these findings, we show here that ghrelin significantly reduces the accumulation of reactive microglia and astrocytes in the hippocampus. The underlying mechanisms of this inhibitory effect of ghrelin on glial activation in the hippocampus may involve the suppression of certain proinflammatory mediators, such as TNF- α , IL-1 β , and COX-2. Moreover, in the present study, we found that *Mmp3* expression was significantly increased in KA-injected

mice as previously reported (Penkowa *et al.* 2005), while ghrelin treatment suppressed *Mmp3* levels through the activation of GHSR1a. Considering that MMP-3 plays a pivotal role in dopaminergic neurodegeneration in PD (Kim *et al.* 2005, 2007) and ghrelin suppresses MPTP-induced *Mmp3* expression (Moon *et al.* 2009), ghrelin-mediated inhibition of *Mmp3* expression in KA-induced neurotoxicity may provide another plausible therapeutic target to prevent progressive neurodegeneration. Taken together, these results indicate that ghrelin plays an important role in neuroinflammation, and it is able to efficiently attenuate this process when administered peripherally.

In summary, we have demonstrated that systemic administration of ghrelin in KA mouse model of seizures attenuates hippocampal neuron loss. Our data suggest that the neuroprotective effect of ghrelin is mediated by the inhibition of glial activation and the concomitant release of proinflammatory mediators. The inhibitory effect of ghrelin on glial activation seems to be associated with the inhibition of *Mmp3* expression. These findings suggest that ghrelin can function as a neuroprotective agent, and may have therapeutic value for the treatment of neurodegenerative diseases where inflammatory responses play a major role.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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