Neuroprotective Effect of Ghrelin Is Associated with Decreased Expression of Prostate Apoptosis Response-4

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Abstract. Ghrelin is known to promote neuronal defense and survival against ischemic injury by inhibiting apoptotic processes. In the present study, we investigated the role of prostate apoptosis response-4 (Par-4), a proapoptotic gene the expression of which is increased after ischemic injury, in ghrelin-mediated neuroprotection during middle cerebral artery occlusion (MCAO). Both ghrelin and des-acyl ghrelin protected cortical neurons from ischemic injury. Ghrelin receptor specific antagonist abolished the protective effects of ghrelin, whereas those of des-acyl ghrelin were preserved, suggesting the involvement of a receptor that is distinct from GHS-R1a. The expression of Par-4 was increased by MCAO, which was attenuated by ghrelin and des-acyl ghrelin treatments. Both ghrelin and des-acyl ghrelin increased the Bcl-2/Bax ratio, prevented cytochrome c release, and inhibited caspase-3 activation. Our data indicate that des-acyl ghrelin, as well as ghrelin, protect cortical neurons against ischemic injury through the inhibition of Par-4 expression and apoptotic molecules in mitochondrial pathway.

Key words: Ghrelin, Neuroprotection, Apoptosis, Prostate apoptosis response-4 (Par-4), Bax, Bcl-2, Cytochrome c

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GHRELIN is a novel 28-amino-acid brain-gut peptide [1]. In the plasma, it exists in two major molecular forms, acylated ghrelin and des-acyl ghrelin [2]. Ghrelin is a unique peptide esterified with octanoic acid on Ser 3 [3]. This acylation is essential for the binding of ghrelin to the GH secretagogue receptor 1a (GHS-R1a) [4]. Only acylated form of ghrelin stimulates GH release and induces a positive energy balance by stimulating food intake while decreasing fat use through GH-independent mechanisms [4]. Ghrelin also exerts numerous peripheral effects, such as direct effects on exocrine and endocrine pancreatic functions, carbohydrate metabolism, the cardiovascular system, gastric secretion, stomach motility, and sleep [3]. In contrast, des-acyl ghrelin is the most abundant form of ghrelin in plasma and does not bind GHS-R1a. It has no GH stimulating activity or any effect on other anterior pituitary function. However, des-acyl ghrelin is not just a reservoir of inactive peptide [5]. Indeed, Both types of ghrelin exhibit similar biological activities, including a protective effect on cardiac and endothelial cells [6], pancreatic β-cells and human islets [7], inhibition of cell proliferation of breast and prostate carcinoma cell lines [8, 9], a stimulatory effect on proliferation of preosteoblastic as well as GH3 pituitary tumor cells [10-12], promotion of the differentiation of osteoblasts and skeletal muscle cells [11, 13], and the stimulation of adipogenesis *in vivo* [14].

Stroke is the neurological condition that develops when a portion of the brain is deprived of oxygen and glucose. The damage caused to neurons during ischemia is due to a reduction in the oxygen and glucose supply. The precise mechanism of ischemic

neuronal cell death is not clear; however, apoptosis is one of the mechanisms involved [15]. Prostate apoptosis response-4 (Par-4), a proapoptotic gene initially identified in prostate tumor cells undergoing apoptosis [16], is known to play a pivotal role in apoptotic neuronal cell death caused by transient focal cerebral ischemia [17]. Recently we have reported that Par-4 expression is increased in PC12 cells exposed to apoptotic stimuli of oxygen-glucose deprivation (OGD) insult, which was prevented by treatment of cells with insulin-like growth factor (IGF)-I [18]. In addition, it is well known that mitochondria play key roles in cell death and cell survival [19, 20]. During apoptotic process, cytochrome c is released from mitochondria through the permeability transition pore, which is regulated by the interactions of Bcl-2 family proteins [21]. Cytosolic cytochrome c leads to subsequent activation of caspase-9 and caspase-3 by forming the apoptosome with Apaf-1 [22].

Recently we have reported that ghrelin acts as a survival factor for cortical neuronal cells by inhibiting apoptotic pathways regardless of its acylation, and neuroprotective effect of ghrelin is mediated via the activation of GHS-R1a while that of des-acyl ghrelin is not [23]. The neuroprotective effects of ghrelin and des-acyl ghrelin were mediated via activation of the phosphatidylinotitol-3-kinase (PI3K)/Akt and ERK1/2 pathways, which have been implicated in the regulation of cell survival [24, 25]. Our previous results also suggest that PI3K/Akt-mediated inactivation of GSK-3β and stabilization of β-catenin contribute to the anti-apoptotic actions of ghrelin [23]. Ghrelin also has been reported to protect cortical neurons against focal ischemia/reperfusion in rats [26, 27]. However, the *in vivo* neuroprotective effect of des-acyl ghrelin against transient focal cerebral ischemic injury has been tested yet. Therefore, in the present study, to determine whether administration of des-acyl ghrelin may attenuate the severity of ischemia *in vivo*, we examined the effect of des-acyl ghrelin in middle cerebral artery occlusion (MCAO) rats. To determine the neuroprotective mechanism of ghrelin, we also investigated the effects of ghrelin or des-acyl ghrelin on the expression of Par-4. Finally, we examined the effects of ghrelin and des-acyl ghrelin on ischemia-induced cytochrome c release and caspase-3 activation, and on the Bcl-2 family of proteins.

Materials and Methods

Materials

Rat ghrelin was obtained from Peptides International (Louisville, KT). D-Lys-3-GHRP-6 was purchased from Bachem (Torrance, CA). Primary polyclonal antibodies to rabbit anti-cytochrome c and rabbit anti-Bcl-2 were obtained from Santa Cruz Biotechnology Inc. (Delaware, CA), and rabbit anti-Bax, rabbit anti-Par-4, rabbit anti-COX IV and rabbit anti-β-actin were from Abcam Inc. (Cambridge, UK). Biotinylated secondary antibody was obtained from Vector Laboratories (Burlingame, CA).

Transient middle cerebral artery occlusion

Reversible focal cerebral ischemia was generated in adult male Sprague-Dawley rats weighing 250-280 g. Animals were anesthetized by inhalation of a nitrous oxide/oxygen/halothane (69%:30%:1%) mixture. After a midline incision was made, the right MCA was occluded for 2 h as previously described [28]. Ghrelin or des-acyl ghrelin was administered intraperitoneally (80 or 160 µg/kg) 30 min before MCAO and at the beginning of reperfusion. Body temperature was maintained at 37 °C with a heating pad equipped with a thermostat until the animals began to move. The rats were sacrificed by halothane overdose after 24 h of reperfusion. Their brains were removed, cut into six 2-mm coronal slices, and stained with triphenyl tetrazolium chloride (TTC) at 37 °C for 30 min in the dark for the evaluation of infarct volume. TTC stains the undamaged tissue as red while dead cells remain white [29]. Brain slices were photographed using a digital camera and quantified using an image analyzing system (Optimas 6.5 Media Cybermetics, Silver Springs, MD). The infarct area in each slice was calculated by subtracting the normal ipsilateral area from that of the contralateral hemisphere to reduce errors due to cerebral edema and was presented as the percentage of the infarct area to that of the contralateral hemisphere. All described experiments were approved by the Kyunghee 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.

Immunohistochemical staining for Par-4

At 24 h after the reperfusion, animals were anesthetized with xylazine and ketamine and then perfused transcardially with a freshly prepared solution of 4% paraformaldehyde in phosphate-buffered saline (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 sections were incubated overnight at 4°C with anti-Par-4 antibody. The sections were incubated with biotinylated secondary antibody and then visualized using the avidin–biotin–peroxidase complex method with diaminobenzidine tetrahydrochloride as the chromogen.

Western blot analysis

Animals were anesthetized and then decapitated. The brains were rapidly removed and the ipsilateral brain cortexes were separated, weighed and protein was extracted in a lysis buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 140 mM NaCl, 1% (w/v) Nonidet P-40, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 10 μg/ml aprotinin. Cell lysates were separated by 12% SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). For the detection of Bax, Bcl-2, and cytochrome *c*, cells were fractionated into mitochondria and cytosol using the Mitochondria/Cytosol Fractionation Kit (BioVision, Mountain View, CA) according to the manufacturer's instructions. Anti-COX IV antibody was used to probe the protein COX IV as a marker in mitochondrial fraction and its absence in cytosolic fraction. The membranes were soaked in blocking buffer (1X Tris-buffered saline, 1% BSA, 1% nonfat dry milk) for 1 h and incubated overnight at 4 °C with the primary antibody. Blots were developed using a peroxidase-conjugated anti-rabbit IgG and a chemiluminescent detection system (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The bands were visualized using a ChemicDoc™ XRS system (Bio-Rad, Hercules, CA) and quantified using Quantity One imaging software (Bio-Rad).

Statistical analysis

Data are presented as the mean ± SEM (n=6-11/group). Each experiment was repeated at least twice. Statistical analysis between groups was performed using 1-way ANOVA and the Holm-Sidak method for multiple comparisons using SigmaStat for Windows Version 3.10 (Systat Software, Inc. Point Richmond, CA). $P < 0.05$ was considered statistically significant.

Results

Ghrelin and des-acyl ghrelin treatments attenuated ischemic brain damage

We investigated whether ghrelin or des-acyl ghrelin has neuroprotective effects against brain ischemia 24 h after MCAO in rats. Treatment of animals with ghrelin (80 and 160 μg/kg) significantly decreased infarct volume in a dose-dependent manner compared to vehicle-treated rats (Fig. 1) as previously reported [26]. The infarct volume of the vehicle-treated rats was $370.9 ± 26.1$ mm^3, whereas the volumes of the ghrelin-treated groups were $270.0 ± 43.6$ mm^3 (80 μg/kg, $P < 0.01$) and $136.7 ± 45.9$ mm^3 (160 μg/kg, $P < 0.01$) (Fig. 1). Furthermore, the infarct volume was significantly decreased to $232.9 ± 48.2$ mm^3 (80 μg/kg, $P < 0.01$) and $168.9 ± 64.9$ mm^3 (160 μg/kg, $P < 0.01$) by treatment of animals with des-acyl ghrelin (Fig. 1). To determine whether the neuroprotective effect of ghrelin is mediated by its receptor GHS-R1a, animals were treated with the ghrelin receptor-specific antagonist. Exposure of rats to D-Lys-3-GHRP-6 (1 mg/kg) completely abolished the protective effect of 160 μg/kg of ghrelin against ischemic brain damage (Fig. 2). In contrast, D-Lys-3-GHRP-6 did not alter the neuroprotective effect of des-acyl ghrelin (160 μg/kg).

Effects of ghrelin and des-acyl ghrelin on Par-4 expression

Next we investigated the effects of ghrelin and des-acyl ghrelin on MCAO-induced activation of Par-4 expression and found that both types of ghrelin significantly inhibited the up-regulation of the Par-4 protein levels caused by MCAO (Fig. 3A). We also examined the effects of ghrelin and des-acyl ghrelin treatments on Par-4 immunoreactivity in sections from
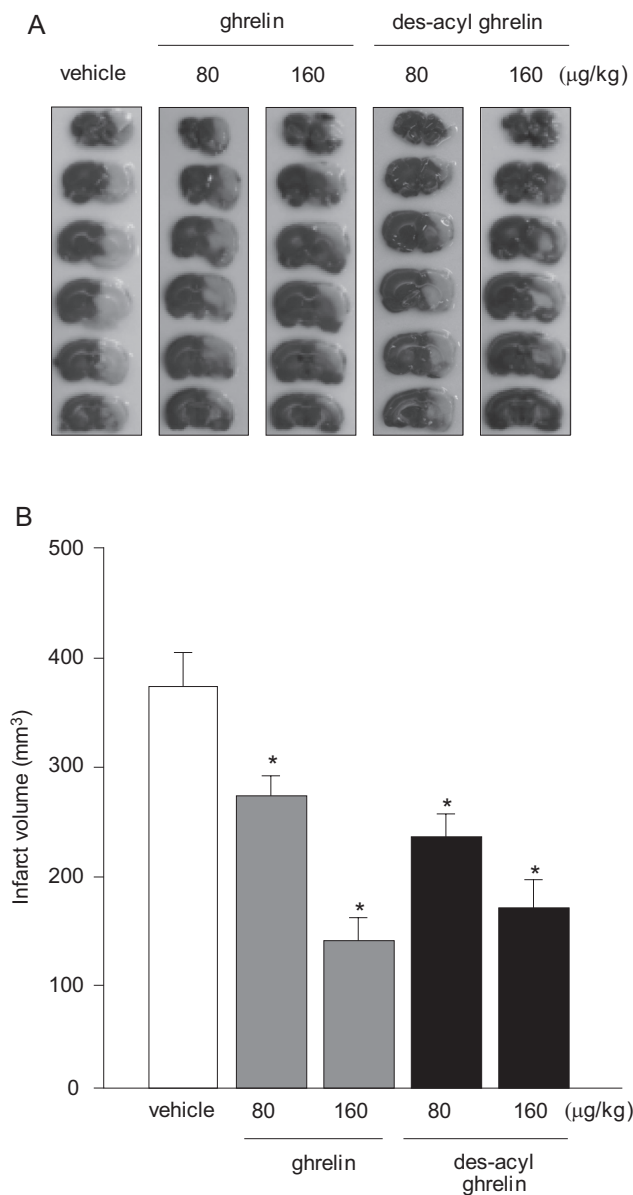
Fig. 1. Ghrelin and des-acyl ghrelin decrease infarct volume in MCAO rats. Either ghrelin or des-acyl ghrelin was administered intraperitoneally (80 or 160 μg/kg) 30 min before MCAO and at the beginning of reperfusion. The right MCA was occluded for 2 h. Brain was reperfused for 24 h and removed for the evaluation of infarct volume. A, Representative sections stained with triphenyl tetrazolium chloride. Undamaged tissue stains red, whereas dead cells do not pick up the dye and remain white. Vehicle-treated brain shows extensive damage. Ghrelin- or des-acyl ghrelin -treated brain shows a remarkable decrease of damage when compared with the vehicle-treated brain. B, Infarct volume was calculated by measuring the infarct areas on coronal brain sections. Values are the mean ± SEM (n = 6-11/group). *, $P < 0.05$ *vs.* vehicle-treated group.
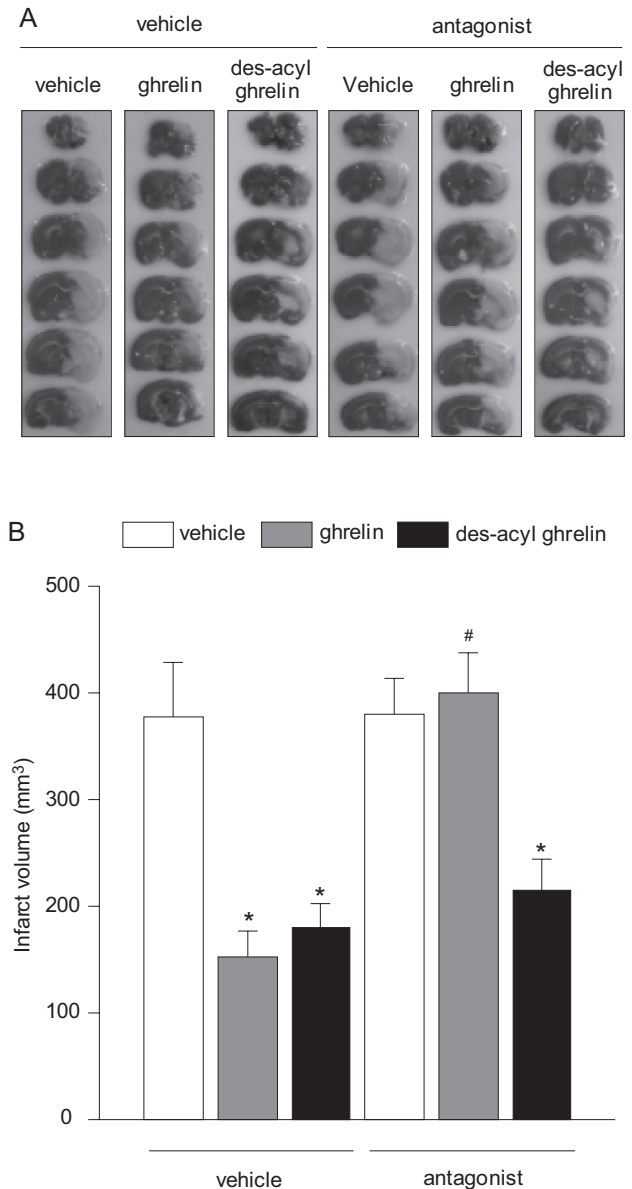
Fig. 2. The protective effect of ghrelin is mediated via the activation of its receptor, GHS-R1a, whereas that of des-acyl ghrelin is not. Rats were co-treated with the GHS-R1a antagonist D-Lys-3-GHRP-6 (1 mg/kg) and ghrelin or des-acyl ghrelin (160 μg/kg) intraperitoneally 30 min before MCAO and at the beginning of reperfusion. The right MCA was occluded for 2 h. Brain was reperfused for 24 h and removed for the evaluation of infarct volume. A, Representative sections stained with triphenyl tetrazolium chloride. B, Infarct volume was calculated by measuring the infarct areas on coronal brain sections. Values are the mean ± SEM (n = 6-8/group). *, $P < 0.05$ *vs.* vehicle-treated group; #, $P < 0.05$ *vs.* ghrelin-treated group.
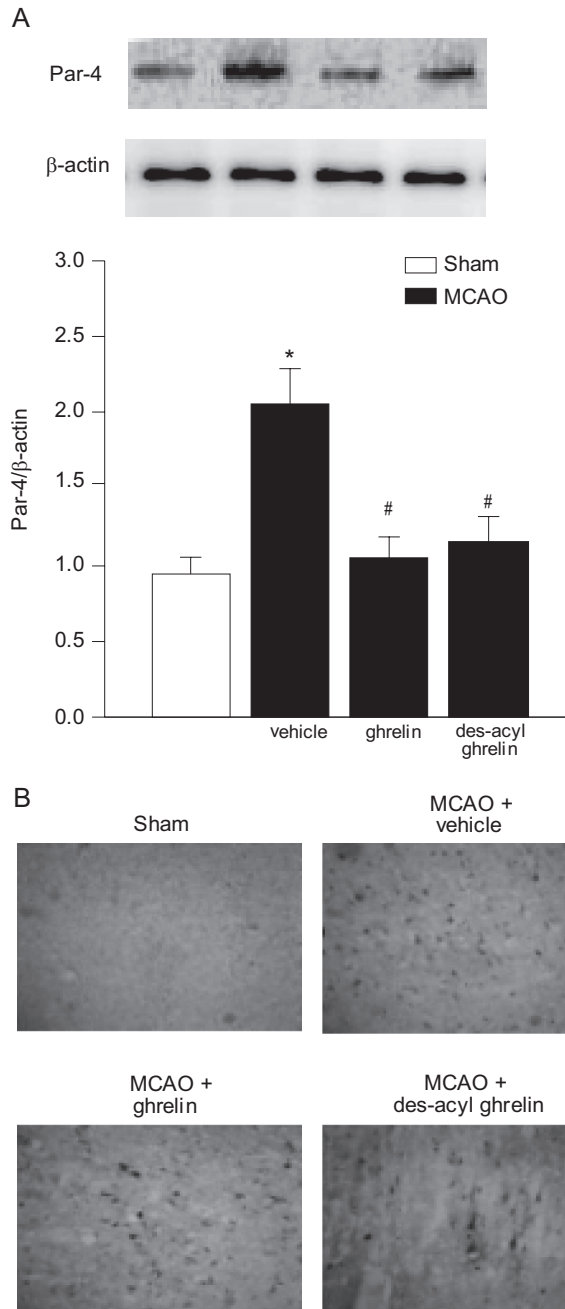
A

B

Fig. 3. Effects of ghrelin and des-acyl ghrelin on the expression of Par-4. Either ghrelin or des-acyl ghrelin was administered intraperitoneally (80 µg/kg) 30 min before MCAO and at the beginning of reperfusion. The right MCA was occluded for 2 h. A, Western blot analysis of Par-4 protein levels. B, Detection of Par-4 immunoreactivity was performed at 24 h of reperfusion. Values are the mean ± SEM (n = 6/group). *, $P < 0.05$ vs. sham-operated group. #, $P < 0.05$ vs. vehicle-treated group.

MCAO rats. As shown in Fig. 3B, there was no Par-4 immunoreactive cells in sham-operative animals. However, Par-4 exhibited a remarkable increase when animals were exposed to MCAO, which was attenuated after the animals were treated with either ghrelin or des-acyl ghrelin.

Ghrelin and des-acyl ghrelin increase Bcl-2/Bax ratio

To determine changes in protein levels of Bax and Bcl-2, a Western blot was conducted to detect Bax in mitochondrial fraction of ipsilateral cortex. Fig. 4A shows the MCAO-induced increase in Bax protein in the mitochondrial fraction, suggesting a translocation from the cytosol to the mitochondria. Treatment of animals with ghrelin or des-acyl ghrelin inhibited the MCAO-induced increase in Bax protein in the mitochondria. We also investigated the levels of Bcl-2 protein and found that Bcl-2 protein levels were decreased by MCAO, whereas ghrelin and des-acyl ghrelin increased Bcl-2, thereby significantly increasing the Bcl-2/Bax ratio.

Effects of ghrelin and des-acyl ghrelin on cytochrome c release and caspase-3 activation

To determine whether ghrelin and des-acyl ghrelin inhibit cytochrome c release, Western blots were performed on cytosolic fractions. As shown in Fig. 4B, exposure of animals to MCAO resulted in the release of cytochrome c from the mitochondria to the cytosol, which was significantly reduced by treatment with ghrelin or des-acyl ghrelin. We also observed protective effects of ghrelin and des-acyl ghrelin at the level of caspase-3 activation and found that ghrelin and des-acyl ghrelin significantly reduced the MCAO-induced increase in caspase-3 (Fig. 4C).

Discussion

In the present study, we demonstrated that des-acyl ghrelin, as well as ghrelin, protects cortical neurons from the injury induced by transient focal cerebral ischemia and reperfusion *in vivo*. The protective effect of des-acyl ghrelin appeared to be mediated by a separate receptor other than GHS-R1a. It is also shown that both forms of ghrelin attenuated ischemia-induced injury through the suppression of
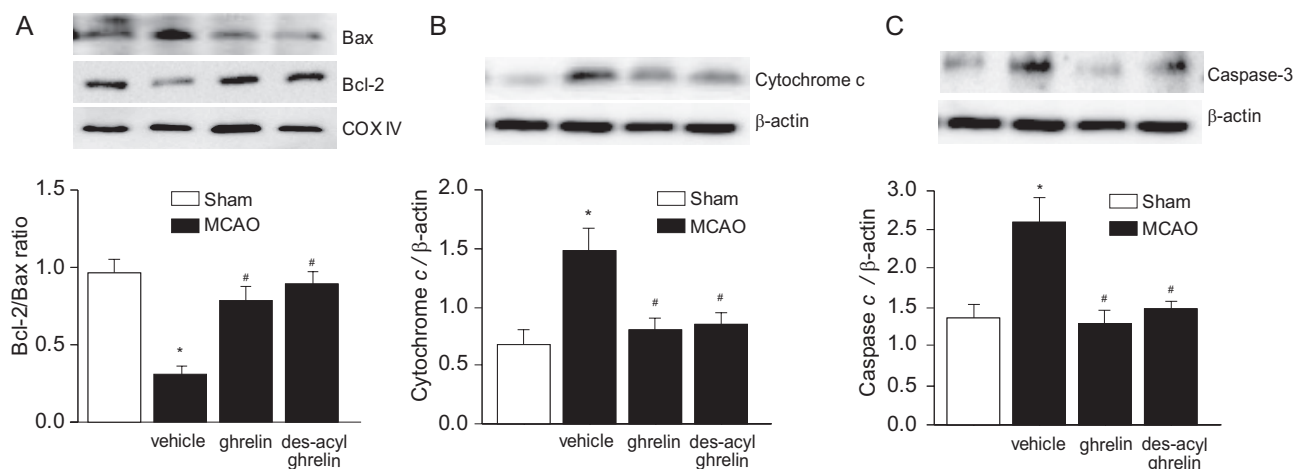
Fig. 4. Ghrelin and des-acyl ghrelin increase the Bcl-2/Bax ratio, prevent cytochrome c release, and inhibit caspase-3 activation. Either ghrelin or des-acyl ghrelin was administered intraperitoneally (80 µg/kg) 30 min before MCAO and at the beginning of reperfusion. The right MCA was occluded for 2 h. A, Effects of ghrelin and des-acyl ghrelin on Bax and Bcl-2 in the mitochondrial fraction assessed by Western blot. Bax and Bcl-2 band intensities were normalized to COX IV band intensity. B, Effects of ghrelin and des-acyl ghrelin on cytochrome c in the cytosolic fraction assessed by Western blot. Cytochrome c band intensity was normalized to β-actin intensity. C, Effects of ghrelin and des-acyl ghrelin on caspase-3 in the cytosolic fraction assessed by Western blot. Caspase-3 band intensity was normalized to β-actin intensity. Values are the mean ± SEM (n = 6/group). *, $P < 0.05$ *vs.* sham-operated group. #, $P < 0.05$ *vs.* vehicle-treated group.

Par-4 expression. The neuroprotective mechanisms of ghrelin and des-acyl ghrelin were associated with the up-regulation of Bcl-2/Bax ratio, and inhibition of cytochrome c release and caspase-3 activation.

In this study, systemic administration of both forms of ghrelin significantly reduced infarct volumes after initiation of ischemia. Given the fact that ghrelin can pass through the blood-brain barrier [30], these findings suggest that peripherally administered ghrelin enters the brain parenchyma and exerts neuroprotective effects in the central ischemic regions. Supporting evidence for this notion is that ghrelin injected intravenously has also shown neuroprotective effects in transient focal ischemia/reperfusion in rats by inhibiting apoptotic molecules of mitochondrial pathway and activating endogenous protective molecules [27]. Although these findings suggest the direct effect of ghrelin in the central nervous system (CNS), considering that blockade of the gastric vagal afferent pathway by vagotomy abolished peripheral ghrelin-induced feeding [31;32], the indirect pathway through the vagus nerve might have some significant role in conveying peripherally administered ghrelin signal to the CNS. Therefore, the precise mechanism by which ghrelin protects cortical neurons from MCAO-induced injury needs to be clarified.

In the present study, we found for the first time that intraperitoneally administered des-acyl ghrelin protected cortical neurons from MCAO-induced injury. These findings suggest that ghrelin can function as a neuroprotective agent independent of its acylation. Because the specific ghrelin receptor antagonist D-Lys-3-GHRP-6 does not block the protective effect of des-acyl ghrelin, it is unlikely that the neuroprotective effect of des-acyl ghrelin is mediated via the activation of GHS-R1a. The similar finding has been observed in cortical neurons exposed to oxygen-glucose deprivation (OGD) insult [23]. des-acyl ghrelin neither binds nor activates ghrelin receptor GHS-R1a [4]. Furthermore, des-acyl ghrelin, but not ghrelin, stimulates food intake in ghrelin receptor knockout mice [33]. Therefore, these observations suggest the existence of a different specific receptor for des-acyl ghrelin distinct from GHS-R1a.

In the present study, it is shown for the first time that treatment of rats with ghrelin or des-acyl ghrelin attenuates MCAO-induced increase in Par-4 expression. In experimental models of ischemic brain injury, rapid and robust increase of Par-4 expression has been observed and a Par-4 antisense oligonucleotide prevents neuronal cell death [17, 18]. Similar to these

observations, we found that Par-4 expression levels were significantly increased in animals exposed to MCAO. We also found that treatment of animals with ghrelin or des-acyl ghrelin attenuated MCAO-induced Par-4 expression. It is well known that Par-4 plays a critical role in ischemic brain injury. The elevation of Par-4 expression is considered to be an early event positioned upstream of mitochondrial dysfunction and caspase activation in the apoptotic pathway of neuronal cells [34]. Multiple mechanisms are involved in the ability of Par-4 to induce apoptotic cell death. Par-4 activates the Fas death receptor signaling pathway and inhibits activation of nuclear factor-κB (NF-κB) and AP-1 [35] while it down-regulates Bcl-2 expression in PC12 cells [36, 37]. Increased intracellular calcium levels due to overexpression of Par-4 in PC12 cells may be involved in the induction of apoptosis [36]. It is known that nuclear translocation of Par-4 is essential for the induction of apoptosis and inhibition of the NF-κB transcriptional activity by Par-4 [38]. The immunohistochemical findings of our previous report [18] showed that Par-4 was localized primarily in the cytoplasm, as is observed in normal tissues [39]. We also reported that OGD exposure resulted in a translocation of Par-4, which translocaton was attenuated by pretreatment of cells with IGF-I. Phosphorylation of Par-4 by Akt, which is activated by PI3K, allows 14-3-3 to bind to Par-4 and sequester it in the cytoplasm, thereby inactivating it to ensure cell survival [40]. Considering that ghrelin can activate PI3K-Akt pathways [23], we assume that ghrelin may inhibit nuclear translocation of Par-4. However, this possibility remains to be determined. Taken together, we suggest that the protective effect of ghrelin or des-acyl ghrelin is mediated, at least in part, through a suppression of Par-4 expression.

In the current study, we found that both ghrelin and des-acyl ghrelin inhibited MCAO-induced increase in the Bax protein in mitochondria and increased cytosolic Bcl-2 protein levels, resulting in the increase of the Bcl-2/Bax ratio, as previously observed in cortical neuronal cells exposed to OGD insult [23]. It is well known that the Bcl-2 family proteins play a critical role in intracellular apoptotic signal transduction by regulating the permeability of the mitochondrial membrane [41]. Mitochondrial cytochrome c is released through the permeability transition pore, which is regulated by the Bcl-2 protein family [21]. Cytosolic release of cytochrome c is increased by the pro-apoptotic protein Bax and blocked by the anti-apoptotic protein Bcl-2 [42]. The Bcl-2/Bax ratio is considered as a determining factor for cell's fate in response to apoptotic stimuli, because Bcl-2 protein inhibits apoptosis by binding to the Bax, Bcl-xs, and Bad proteins [43]. Therefore, our results suggest that both forms of ghrelin may protect cortical neurons from ischemic injury by changing the status of Bcl-2 and the Bax proteins that inhibits apoptosis and favors cell survival.

We also found in this study that cytochrome c was translocated from the mitochondria to the cytosol after MCAO, similar with previous reports [23, 26, 44]. Once released, cytochrome c forms the apoptosone, leading to activation of caspase-9, an initiator of the cytochrome c-dependent caspase cascade [22, 45, 46]. Subsequent activation of the effector caspase-3 leads to apoptosis [47]. In the present study, ghrelin & des-acyl ghrelin treatments prevented the MCAO-induced release of cytochrome c and subsequent activation of caspase-3, thereby inhibiting activation of the apoptotic cascade, in agreement with the observation in neuronal cells [23, 26]. Although our observation indicate that both types of ghrelin showed similar effects on the expression of apoptosis-related proteins, it should be noted that the neuroprotective mechanisms of des-acyl ghrelin and may be different from those of ghrelin because the specific receptor for des-acyl ghrelin has not been identified yet.

In summary, we have demonstrated that peripheral administration of acylated and unacylated ghrelin protected cortical neurons from ischemic injury caused by MCAO. The protective effects of ghrelin and des-acyl ghrelin appear to be related with an inhibition of the expression of Par-4. Another possible mechanism of ghrelin and des-acyl ghrelin is anti-apoptotic effects by targeting Bcl-2 protein family, inhibiting cytochrome c release and caspase-3 activity. Our data suggest that ghrelin, independent of its acylation, may have therapeutic potential for the treatment of stroke and can function as a neuroprotective agent.

Acknowledgements

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