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ORIGINAL

Ghrelin suppresses tunicamycin- or thapsigargin-triggered endoplasmic reticulum stress-mediated apoptosis in primary cultured rat cortical neuronal cells

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Abstract. Ghrelin functions as a neuroprotective agent and rescues neurons from various insults. However, the molecular mechanisms underlying ghrelin neuroprotection remains to be elucidated. An accumulation of unfolded proteins in the endoplasmic reticulum (ER) leads to ER stress and then induces ER stress-mediated cell death. Here, we report that acylated ghrelin inhibited tunicamycin- or thapsigargin-triggered ER stress-induced apoptotic cell death in primary rat cortical neurons. An analysis using a specific inhibitor of phosphatidylinositol-3-kinase (PI3K), LY294002, showed that ghrelin prevented apoptosis *via* the activation of PI3K signaling pathway. Ghrelin suppressed tunicamycin- or thapsigargin-induced upregulation and nuclear translocation of C/EBP homologous protein (CHOP). Ghrelin also inhibited tunicamycin or thapsigargin induction of PRK-like ER kinase (PERK), eukaryotic translation initiation factor-2α (eIF2α) and activating transcription factor (ATF) 4. Exposure of cells to tunicamycin or thapsigargin resulted in nuclear translocation of forkhead box protein O1 (Foxo1), which was reduced by pretreatment with ghrelin. The protective effect of ghrelin was accompanied by an increased phosphorylation of Akt and glycogen synthase kinase (GSK)-3β. Furthermore, ghrelin phosphorylated and inactivated pro-apoptotic BAD and Foxo1. In addition, phospho-Akt was translocated to the nucleus in response to ghrelin and PI3K inhibition by LY294002 prevented ghrelin-induced effect on phospho-Akt localization. Our study suggests that suppression of CHOP activation *via* the inhibition of PERK/eIF2α/ATF4 pathway and prevention of Foxo1 activation and nuclear translocation may contribute to ghrelin-mediated neuroprotection during ER stress responses. Our data also suggest that PI3K/Akt-mediated inactivation of GSK-3β, BAD and Foxo1 may be associated with the anti-apoptotic effect of ghrelin.

Key words: Ghrelin, Endoplasmic reticulum stress, Neuroprotection, PI3K, Akt

GHRELIN, initially identified as an endogenous ligand for growth hormone (GH) secreatagogue (GHS) receptor, is a unique 28-amino acid gastric hormone esterified with octanoic acid on Ser 3 [1]. This acylation is essential for the binding of ghrelin to the GHS-R1a [2]. In addition to ghrelin's activity in stimulating GH release and modulating feeding behavior and energy balance [3], recent numerous data have shown that acylated form of ghrelin shows broad-spectrum *in vivo* and *in vitro* neuroprotective effects. Ghrelin inhibits apoptosis of hypothalamic neurons during oxygen-glucose deprivation (OGD) [4]. Ghrelin also prevents cortical neuronal cell death induced by ischemia [5, 6], protects nigrostriatal dopaminergic neurons against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity [7], and attenuates kainic acid-induced excitotoxicity in the hippocampus [8]. Moreover, ghrelin improves functional recovery after traumatic spinal cord injury [9]. The neuroprotective effect of ghrelin can be attributed to its inhibition of apoptotic pathway [4], inflammatory activity [10], and oxidative stress [4]. However, the exact molecular mechanisms of ghrelin in neuroprotection remain to be further clarified.

The endoplasmic reticulum (ER) is an important organelle involved in intracellular calcium homeostasis, folding and processing of proteins, and cell death signal activation [11, 12]. Many conditions, such as glucose deprivation, perturbed calcium homeosta-

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sis, exposure to free radicals, and hypoxia can interfere with protein folding and lead to the accumulation of misfolded or unfolded proteins in the lumen of the ER, causing ER stress that activates the unfolded protein response (UPR) [13-15]. The initial aim of UPR is to restore normal function of the cell, however, if the damage is too severe to repair, the UPR ultimately initiates cell death through activation of the apoptotic pathway [16]. During UPR, PRK-like ER kinase (PERK) is phosphorylated and activated and in turn it phosphorylates the eukaryotic translation initiation factor-2α (eIF2α), resulting in inhibition of most protein synthesis and activation of activating transcription factor (ATF) 4 translation [17]. This ER stress signaling system is capable of inducing transcription of C/EBP homologous protein (CHOP), a transcription factor that binds with other transcription factors and induces pro-apoptotic genes [18, 19]. Many neurodegenerative disorders, including Alzheimer's diseases (AD), Huntington's diseases (HD), or Parkinson's diseases (PD) are associated with the accumulation and deposits of misfolded proteins, such as β-amyloid, huntingtin, or α-synuclein [20-25]. Considering that ER stress-induced apoptosis is a critical step in the pathogenesis of many neurodegenerative diseases and ghrelin can function as a survival factor for neurons, these findings prompted us to hypothesize that ghrelin could have a protective role in ER stress-induced neuronal cell death. Indeed, it was recently reported that ghrelin protected the heart against ischemia/reperfusion injury through inhibiting myocardial ER stress [26]. However, it is still unknown whether ghrelin rescues neurons from ER stress-induced apoptosis.

We have previously reported that ghrelin protects hypothalamic neurons [4] and cortical neurons [5] during OGD by inhibiting apoptotic pathways. Protection of neuronal cells is achieved by inhibition of reactive oxygen species generation, stabilization of mitochondrial transmembrane potential, increase of the Bcl-2/Bax ratio, prevention of cytochrome *c* release, and inhibition of caspase 3 activation [4]. Our previous studies also suggest that phosphatidylinositol-3-kinase (PI3K)/Akt pathways play important roles in the mechanisms of ghrelin-mediated neuroprotection. Moreover, PI3K/Akt-mediated inactivation of glycogen synthase kinase (GSK)-3β, which is a downstream effector of Akt and plays a key role in apoptosis, and subsequent stabilization of β-catenin may contribute to the anti-apoptotic effect of ghrelin [5]. BAD, a member of the Bcl-2 family of proteins, binds to Bcl-2 or Bcl-X and inhibits their anti-apoptotic potential [27]. BAD does not show pro-apoptotic activity when it is phosphorylated by Akt, therefore it is regarded as one of the direct targets of Akt. It also has been shown that activated Akt is able to translocate to the nucleus, and there phosphorylates and inactivates forkhead box transcriptional factors such as Foxo1, resulting in decreased expression of pro-apoptotic genes and increased cell survival [28, 29]. Taken together, these findings suggest that Akt downstream effectors may contribute to neuroprotection of ghrelin. However, more precise intracellular signaling mechanisms underlying ghrelin-mediated regulation of Akt downstream effectors remain to be understood.

In the present study, in order to study the role of acylated ghrelin in ER stress-induced injury, we investigated the effect of ghrelin on the survival of primary cultured cortical neurons exposed to tunicamycin or thapsigargin. Our results suggest that the neuroprotective properties of ghrelin during ER stress responses are associated with the inhibition of CHOP activation through the suppression of PERK/eIF2α/ATF4 pathway. Moreover, our data also suggest that inactivation of GSK-3β, BAD and Foxo1 by a pathway involving activation of PI3K/Akt cascades is associated with the anti-apoptotic effect of ghrelin.

Materials and Methods

Materials

Chemical ER stressors, tunicamycin and thapsigargin, were purchased from Sigma (St. Louis, MO, USA). Acylated form of rat ghrelin was obtained from Peptides International (Louisville, KY, USA). Neurobasal™ and RPMI 1640 media, and B-27 supplement were from Gibco/Invitrogen (Carlsbad, CA, USA). B-27 is an optimized serum substitute developed for low-density plating and long-term viability and growth of central nervous system neurons [30]. Primary antibodies to glucose-regulated protein (GRP) 78 (BiP), CHOP, ATF-4, phospho-eIF2α, phospho-PERK and anti-phospho-antibodies to Akt (Ser473), GSK-3β (Ser9), BAD (Ser136) and Foxo 1 (Ser256) were purchased from Cell Signaling Technology (Danvers, MA, USA). LY294002 was from Tocris (Ellisville, MO, USA). All tissue culture reagents were obtained from Gibco/Invitrogen, and all other reagents were obtained from Sigma, unless otherwise indicated.

Cell cultures

Primary rat cortical neuronal cultures were prepared as previously described [6, 30]. Briefly, cortical tissues were obtained from 1-d-old Sprague-Dawley rats. A pregnant mother for 1-d-old pups was purchased from Orient Bio (Seongnam, Korea] under IACUC approval for breeding. All tissues were quickly dissected and mechanically dispersed in Ca^{2+}- and Mg^{2+}-free buffered Hank's balanced salt solution. Then tissues were dissociated enzymatically (0.125% trypsin solution, 37 °C for 10 min] and mechanically and filtered through a nylon mesh (pore size 40 μm). Cells were plated at a density of 2×10^4 cells/cm^2 on 50 μg/mL poly-L-lysine-coated 100-mm culture dishes and grown in Neurobasal™ medium supplemented with 2% B27, 0.5 mM L-glutamine, and 2.5 ng/mL basic fibroblast growth factor (bFGF). Three days after dissociation, the medium was changed to bFGF-free Neurobasal™ medium. Cells were used between 6 and 8 d *in vitro*. More than 90% of primary cultured cortical cells were positive for neuronal marker NeuN antibodies, determined by immunohistochemistry and confocal microscopy (data not shown).

Cell survival and apoptosis

Cells viability was measured by the MTT assay, as previously described [31]. Apoptosis was also evaluated by flow cytometric analysis (FACSCalibur, Becton Dickinson, San Jose, CA, USA) of the proportion of cells stained with Annexin-V/fluorescein isothiocyanate (FITC) (BD Pharmingen™, CA, USA). Briefly, 5 x 10^5 cells were stained with 500 μL binding buffer, containing Annexin-V-FITC and propidium iodide (PI), for 10~15 min at room temperature. Data were analyzed using flow cytometry software.

Western blot analysis

Cells were lysed in a 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, USA). For the detection of CHOP and Foxo 1, cells were fractionated into nuclear and cytosolic contents, using the Nuclear/Cytoplasmic Extraction Kit (PIERCE Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. 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, USA). The bands were visualized using a ChemicDoc™ XRS system (Bio-Rad, Hercules, CA, USA) and quantified using Quantity One imaging software (Bio-Rad).

Immunocytochemical analysis for subcellular localization of CHOP, Foxo1and phospho-Akt

For the evaluation of intracellular localization of CHOP, Foxo1 and phospho-Akt, primary cultured cortical neuronal cells were fixed with 4% paraformaldehyde (Sigma) in phosphate-buffered saline for 30 min at room temperature. After blocking with 3% normal goat serum (Vector Laboratories, Burlingame, CA, USA), the slides were incubated with primary antibodies to CHOP (1:500), Foxo1 (1:200) and phosphor-Akt (1:500) overnight at 4 °C. After washes, the slides were incubated with a secondary Cy3-goat anti-rabbit IgG (1:500 dilution; Jackson Immunoresearch, West Grove, PA, USA) or Cy5-goat anti-rabbit IgG (1:500 dilution; Jackson Immunoresearch) at room temperature for 2 h. Images were acquired by the Carl Zeiss LSM 700 (Oberkochen, Germany) confocal microscope.

Statistical analysis

Data are presented as mean ± SEM (n=4 per treatment). Each experiment was repeated three times, giving essentially identical results. Statistical analysis between groups was performed using 1-way ANOVA and Holm-Sidak method for multiple comparisons using SigmaStat for Windows Version 9.0 (Systat Software, Inc. Point Richmond, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Ghrelin protects cortical neuronal cells against tunicamycin- or thapsigargin-induced ER stress

We first investigated the effect of ghrelin on tunicamycin-induced cell death in cortical neuronal cells. Tunicamycin is an inhibitor of the glycosylation of newly biosynthesized proteins and induces ER stress and subsequent apoptosis [32]. After a 24-h exposure of cells to tunicamycin (1 μg/mL), approximately 50% of cells were not viable as assessed by the MTT

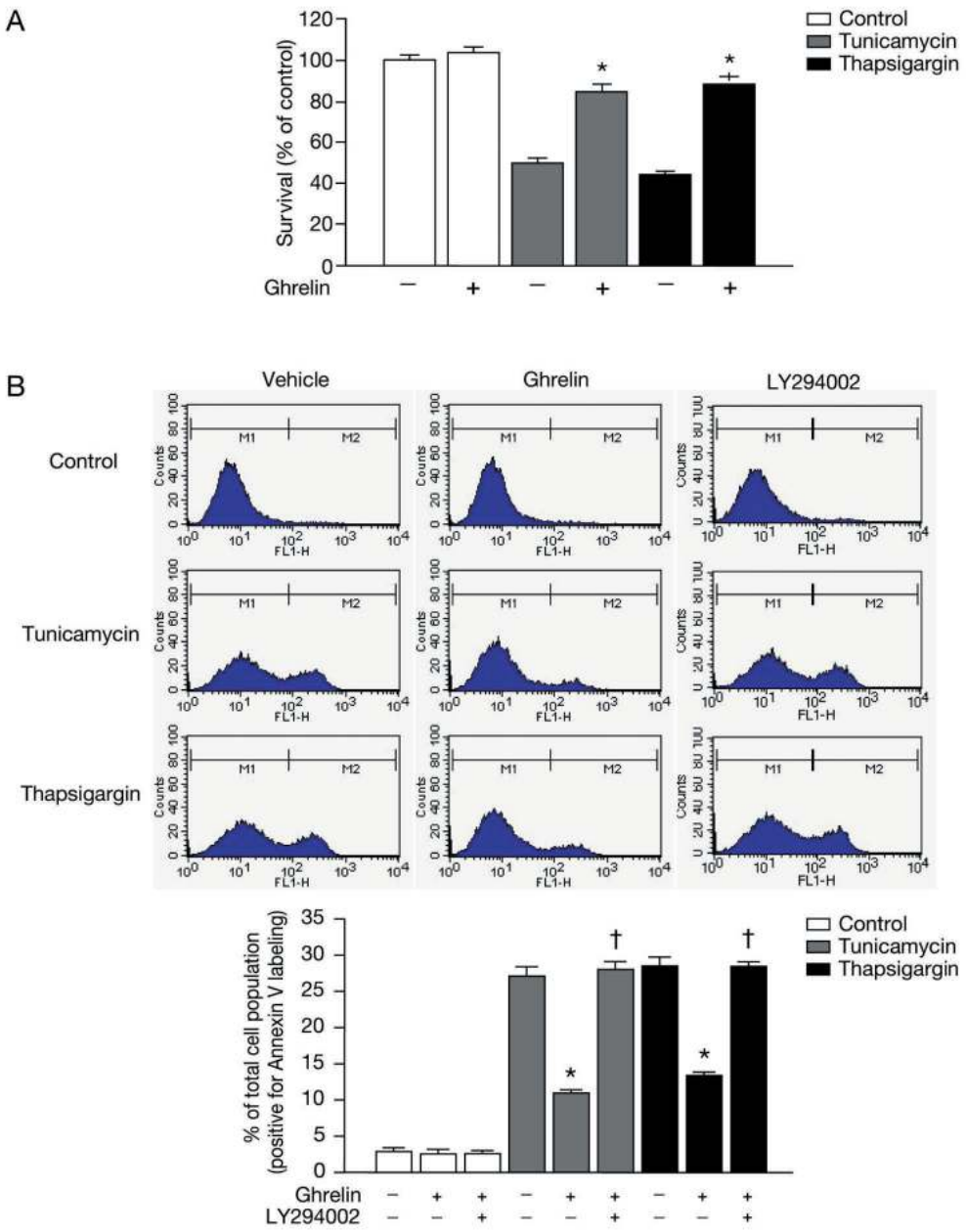

Fig. 1 Ghrelin protects cortical neuronal cells against tunicamycin- or thapsigargin-induced cell death. Cortical neuronal cells were treated with ghrelin (100 nM) for 16 h and then treated with tunicamycin (1 μg/mL) or thapsigargin (10^{-6} M) for 24 h. A. Cell viability was determined by the MTT assay. B. Cells were collected and incubated with FITC Annexin V/PI for 15 min at room temperature in the dark, and then apoptotic cells were measured by flow cytometry. The results are representative of three independent experiments. Results determined by 1-way ANOVA. *, $P < 0.05$ *vs*. tunicamycin (or thapsigargin)-treated cells, † $P < 0.05$ *vs*. tunicamycin (or thapsigargin)-treated, ghrelin-treated cells.

assay (Fig. 1A). However, tunicamycin-induced cell death was significantly reduced by a 24-h pretreatment with ghrelin (100 nM). In this study, 100 nM of ghrelin was chosen on the basis of our previous studies that this dose of ghrelin exerted a potent anti-apoptotic effect [4]. The percentage of viable cells compared to vehicle-treated controls decreased to 50.2% (tunicamycin) and was significantly increased to 77.0% by pretreatment with ghrelin. We also investigated the effect of ghrelin on tunicamycin-induced apoptosis in cortical neuronal cells as determined by flow cytometric analysis and found that the percent-

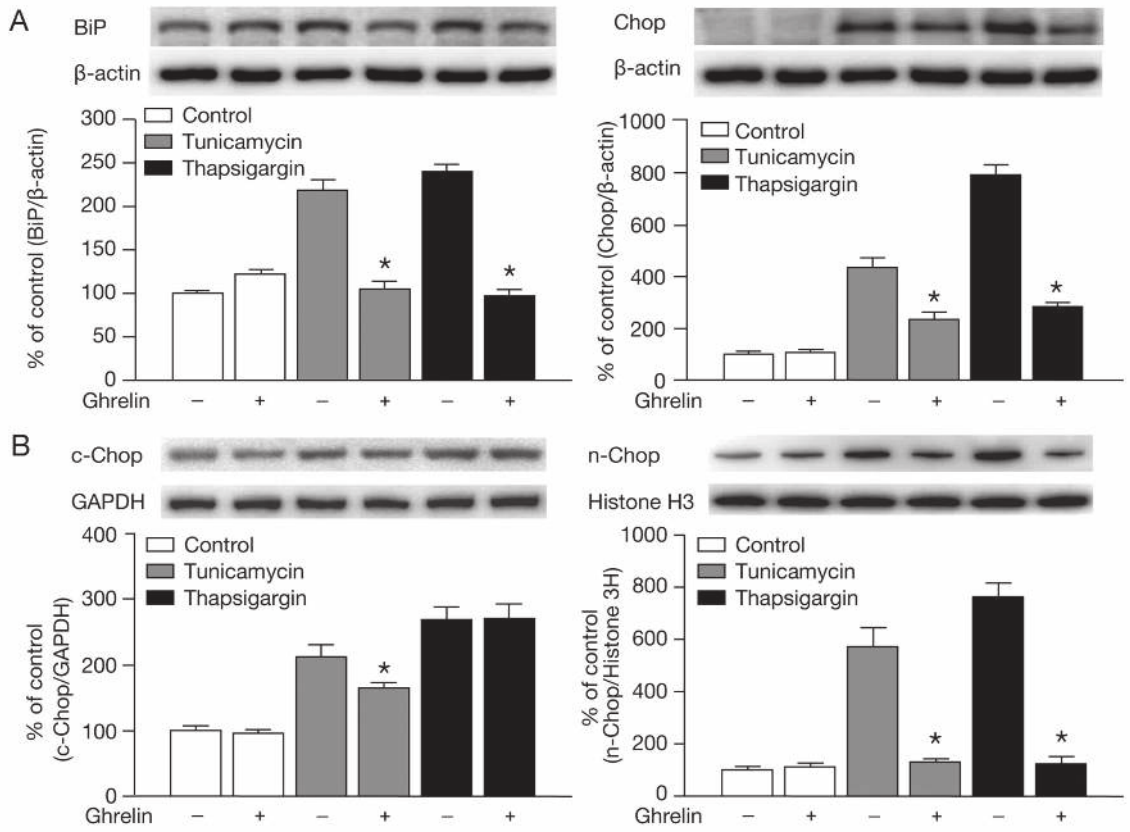

Fig. 2 Ghrelin suppresses tunicamycin-or thapsigargin-induced CHOP upregulation and nuclear translocation. Cells were incubated in B27-free medium in presence or absence of ghrelin (100 nM) and then treated with tunicamycin (2 μg/mL), or thapsigargin (2 μM) for 6 h. A. Whole cell lysates were collected and assayed by Western blot using specific anti-BiP and anti-CHOP antibodies. Anti-BiP and anti-CHOP band intensities were normalized to β-actin intensity. B. Cytoplasmic and nuclear proteins were collected, and expression of CHOP in cytoplasmic and nuclear fraction was determined by Western blotting. Cytoplasmic CHOP (c-CHOP) and nuclear CHOP (n-CHOP) band intensities were normalized to GAPDH or histone H3 intensity. The results are representative of three independent experiments. Results determined by 1-way ANOVA. *, $P < 0.05$ *vs*. tunicamycin (or thapsigargin)-treated cells.

age of Annexin V (+)/PI (-) fluorescent cells increased from 2.9% (control) to 27.1% (tunicamycin) and was significantly decreased to 10.9%, by pretreatment with ghrelin (Fig. 1B). Pretreatment of cells with the PI3K inhibitor LY294002 (10 μM) completely abolished the anti-apoptotic effect of ghrelin, suggesting that ghrelin inhibits apoptosis induced by tunicamycin *via* the activation of PI3K pathway. We also examined the effect of ghrelin on thapsigargin-induced apoptotic cell death of cortical neurons. Thapsigargin, an inhibitor of the sarcoplasmic/ER Ca^{2+} ATPase, also induces ER stress by disrupting the homeostatic balance of the Ca^{2+} concentration in the ER [33]. We found that pretreatment of cell with ghrelin showed similar protective effect against thapsigargin-induced apoptotic cell death (Fig. 1A and 1B).

Ghrelin suppresses tunicamycin- or thapsigargin-induced CHOP upregulation and nuclear translocation

It has been reported that tunicamycin or thapsigargin triggered the expression of a number of markers for ER stress, such as BiP, CHOP, p-PERK, p-eIF2α, and ATF4 in neuronal cells [17]. As shown in Fig. 2 and 3, tunicamycin induced the expression of these markers in cortical neuronal cells. Ghrelin suppressed tunicamycin-induced upregulation of BiP (Fig. 2A). In addition, ghrelin significantly attenuated tunicamycin-induced CHOP upregulation (Fig. 2A). Next, we further investigated the subcellular distributions of CHOP by using Western blot analysis of cytoplasmic and nuclear proteins. Both cytoplasmic and nuclear CHOP was significantly increased by tunicamycin (Fig. 2B). It should be noted that tunicamycin-induced upregu-

lation of nuclear CHOP was much greater than that of the cytoplasmic fraction, indicating that tunicamycin caused nuclear translocation of CHOP. Ghrelin not only decreased tunicamycin-induced cytoplasmic CHOP, but also inhibited the nuclear translocation of CHOP induced by tunicamycin (Fig. 2B). A similar effect of ghrelin on cytoplasmic and nuclear CHOP as well as BiP was also observed when cells were exposed to thapsigargin (Fig. 2A and 2B).

Ghrelin inhibits tunicamycin- or thapsigargin-induced activation of PERK/eIF2α/ATF4 pathway

ER stress activates PERK, which phosphorylates eIF2α, resulting in downregulation of the biosynthetic load of the ER by inhibiting protein synthesis and inducing ATF4 translation [17]. ATF4 is capable of inducing transcription of CHOP [18, 19]. Therefore, we examined the expression of these CHOP regulators. As shown in Fig. 3A and 3B, tunicamycin stimulated phosphorylation of PERK and eIF2α, which was significantly attenuated by pretreatment with ghrelin. Ghrelin treatment also significantly inhibited the tunicamycin-induced increase in the expression of ATF4 (Fig. 3C). Similar findings were observed in thapsigargin-treated cells.

Ghrelin promotes nuclear exclusion of pro-apoptotic transcription factor Foxo1

To identify changes in protein levels of transcription factor Foxo1, a Western blot was conducted to detect Foxo1 in cytoplasmic and nuclear fractions. Fig. 4 shows the tunicamycin-induced increase in Foxo1 protein in the nuclear fraction of cells, suggesting a translocation from the cytoplasm to the nucleus. Ghrelin treatment significantly inhibited the tunicamycin-induced increase in Foxo1 protein in the nucleus. We also found that cytoplasmic Foxo1 protein levels were decreased by tunicamycin, whereas ghrelin treatment increased Foxo1.

Ghrelin activates Akt and inactivates its downstream effectors GSK-3β, BAD and Foxo1

In consistent with our previous report [5], we found that treatment of cells with ghrelin significantly activated Akt and inactivated GSK-3β (Fig. 5 B), which was attenuated by the pretreatment of cells with LY294002. These results suggest that PI3K/Akt-mediated inactivation of GSK-3β is associated with the anti-apoptotic effect of ghrelin. We then examined the effect

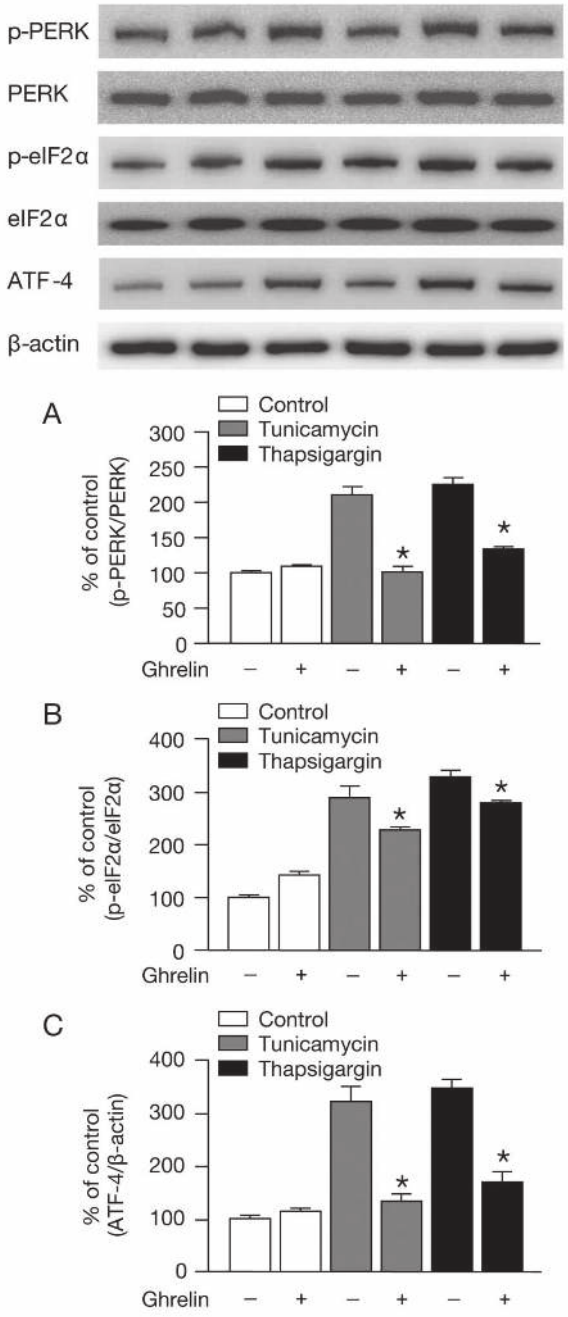

Fig. 3 Ghrelin suppresses tunicamycin- or thapsigargin-induced activation of PERK/eIF2α/ATF4 pathway. Cortical neuronal cells were incubated in B27-free medium in presence or absence of ghrelin (100 nM) and then treated with tunicamycin (2 μg/mL), or thapsigargin (2 μM) for 9 h. Whole cell lysates were collected and assayed by Western blot using specific anti-phospho-PERK (A), anti-phospho-eIF2α (B) and anti-ATF-4 (C) antibodies. Anti-phospho-PERK, anti-phospho-eIF2α and anti-ATF-4 band intensities were normalized to β-actin intensity. The results are representative of three independent experiments. Results determined by 1-way ANOVA. *, $P < 0.05$ *vs*. tunicamycin (or thapsigargin)-treated cells.

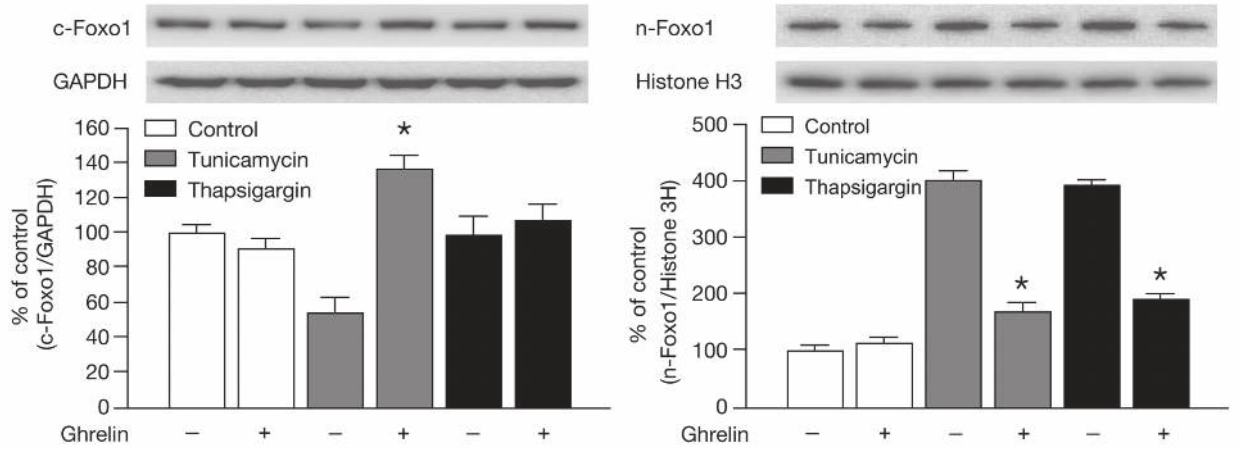

Fig. 4 Ghrelin suppresses tunicamycin- or thapsigargin-induced nuclear translocation of Foxo1. Cortical neuronal cells were treated with vehicle, ghrelin (100 nM) and then treated with tunicamycin (2 μg/mL), or thapsigargin (2 μM) for 6 h. Cytoplasmic and nuclear proteins were collected, and expression of Foxo1 in cytoplasmic and nuclear fraction was determined by Western blotting. Cytoplasmic Foxo1 (c-Foxo1) and nuclear Foxo1 (n-Foxo1) band intensities were normalized to GAPDH or histone H3 intensity. The results are representative of three independent experiments. Results were determined by 1-way ANOVA. *, $P < 0.05$ *vs*. tunicamycin (or thapsigargin)-treated cells.

of ghrelin on the intracellular compartmentalization of phospho-Akt and found that ghrelin promoted nuclear translocation of phospho-Akt (Fig. 5A). PI3K inhibition by LY294002 prevented ghrelin-induced effect on phospho-Akt localization. Moreover, Akt is known to regulate its anti-apoptotic effect by phosphorylating and inactivating pro-apoptotic protein BAD. Akt also exerts indirect control of apoptosis through regulation of transcription by phosphorylating Foxo1. Therefore, we evaluated the effect of ghrelin on BAD and Foxo1 (Fig. 5B). As shown in Fig. 5B, BAD phosphorylation was increased after 30 min of ghrelin treatment. In addition, ghrelin caused a rapid and dramatic increase in phosphorylation of Foxo1 (Fig. 5B). Pretreatment of cells with LY294002 significantly attenuated ghrelin-induced BAD and Foxo1 phosphorylation.

Discussion

In the present study, we demonstrated that acylated ghrelin protects cortical neurons from the ER stress-induced apoptotic cell death *via* the activation of PI3K/Akt-mediated signaling pathway. The neuroprotective effect of ghrelin might be associated with the suppression of ER stress-induced expression and nuclear translocation of CHOP through the inhibition of PERK/eIF2α/ATR4 pathway and nuclear accumulation of Foxo1 during ER stress responses. We also demonstrated that ghrelin-induced stimulation of PI3K/Akt results in phosphorylation and inactivation of GSK-3β, BAD, and Foxo1.

In this study, we showed for the first time that ghrelin rescues primary cortical neurons from tunicamycin- or thapsigargin-triggered ER stress-induced apoptotic cell death. This observation is comparable to a recent report of Zhang *et al*. [26], in which ghrelin protected the heart against ischemia/reperfusion injury *via* the inhibition of myocardial ER stress. The neuroprotective effect of ghrelin during ER stress responses appears to be mediated through the activation of PI3K pathway because a selective inhibitor of PI3K completely blocks the anti-apoptotic effect of ghrelin against tunicamycin- or thapsigargin-induced insult. However, the precise mechanism of PI3K activation by ghrelin remains to be determined. Ghrelin-mediated survival responses require the involvement of the PI3K/Akt pathway. Consistent with our previous report [5], other investigators also found that PI3K/Akt activation is involved in the anti-apoptotic effect of ghrelin in several numerous cell types [34-36]. Taken together, our findings provide evidence that ghrelin could function as a survival factor for neurons and offer a new perspective on the potential role of this peptide in ER stress-related neurodegenerative diseases.

In the current study, we observed that exposure of neuronal cells to pharmacological ER stress inducers produced well-characterized UPR through the activation of ER chaperone, similar to previous reports [37-39]. In agreement with previous report showing that ghrelin inhibited the expression of BiP and CHOP

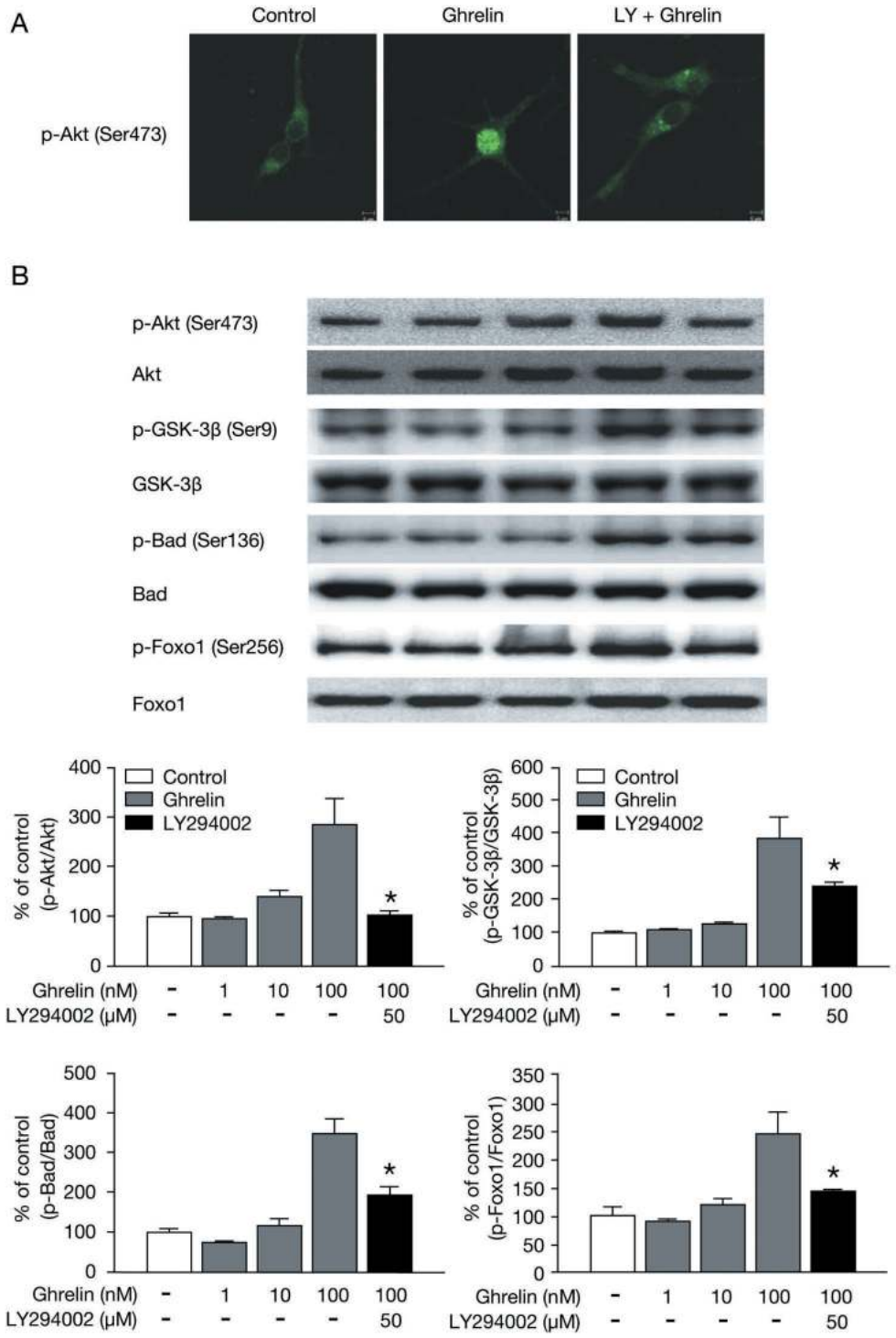

Fig. 5 Ghrelin activates Akt and inactivates its downstream effectors GSK-3β, BAD and Foxo1. Cortical neuronal cells were preincubated with vehicles or 50 μM of LY294002 for 30 min and then treated with 100 nM of ghrelin for 30 min. A. Cells were fixed and probed with phospho-Akt antibody to detect subcellular localization of phospho-Akt. Intracellular localization of phospho-Akt was visualized by confocal microscopy. B. Whole cell lysates were collected and assayed by Western blot using specific anti-phospho-Akt (Ser473), anti-phospho-GSK-3β (Ser9), anti-phospho-BAD (Ser136), and anti-phospho-Foxo1 (Ser256) antibodies. Phospho-Akt, phospho-GSK-3β, phospho-BAD and phospho-Foxo1 band intensities were normalized to β-actin intensity. These results are representative of three independent experiments. Results were determined by 1-way ANOVA. *, $P < 0.05$ *vs.* ghrelin-treated cells.

during ischemia/reperfusion injury in rat heart [26], we have shown that the enhanced levels of BiP and CHOP under ER stress were suppressed by treating cells with ghrelin. Similar findings were observed in SH-SY5Y cells, in which tunicamycin increased BiP and CHOP expression, whereas brain-derived neurotrophic factor decreased it [39]. Ghrelin also suppresses phosphorylation of PERK and eIF2α and decreases levels of ATF4 during ER stress responses. Collectively, these findings suggest that the protection by ghrelin against ER stress was not related to the activation of the UPR, which is the case for insulin-like growth factor (IGF)-I [40] and exendin-4 [41]. Our data support that ghrelin-mediated protection of neuronal cells during ER stress is, at least in part, through the suppression of CHOP expression/nuclear translocation by inhibiting PERK/eIF2α/ATF4 pathway. However, considering that CHOP is believed to be regulated *via* the activation of ATF6 and inositol requiring enzyme 1 during ER stress, it remains to be addressed whether the inhibitory effect of ghrelin on CHOP is achieved through the suppression of these pathways.

It remains unclear how ghrelin suppresses ER stress induction of CHOP. In the current study, we found that nuclear localization of Foxo1 was increased during ER stress responses. Foxo1, an inducer of apoptosis in its unphosphorylated form [42], is known to be activated through the activation of Jun NH_2-terminal kinase during ER stress [43]. Dephosphorylation of Foxo1 and its concomitant translocation into the nucleus in neurons preceded delayed neuronal death in the vulnerable hippocampal regions after ischemic brain injury [42]. IGF-I rescued neuronal cells from various insults by preventing Foxo1 activation and nuclear translocation [44]. Foxo1-mediated transcriptional activation required for apoptosis was inhibited after preventing nuclear translocation of Foxo1 [45]. Furthermore, ghrelin protects pancreatic β-cells from lipotoxicity by inhibiting the nuclear translocation of Foxo1 [46]. In agreement with these reports, in this study, ER stress-mediated accumulation of Foxo1 in the nuclei of neuronal cells was significantly attenuated by pretreatment with ghrelin. Taken together, these findings suggest that ghrelin protects neurons against ER stress by excluding Foxo1 from the nucleus. Considering that Foxo1 activity is increased during ER stress and inhibition of Foxo1 decreases CHOP expression and ER stress-induced cell death [43], we assume that ghrelin-mediated suppression of CHOP induction is mediated, at least in part, through the inhibition of Foxo1 localization in the nucleus.

It is well known that CHOP plays a crucial role in ER stress-mediated apoptosis [47]. Several lines of evidence suggest that the induction of CHOP is involved in brain ischemia and neurodegenerative diseases including AD, PD and HD. Specifically, mRNA and protein levels of CHOP were increased in the hippocampus after occlusion of common carotid arteries whereas ischemia-induced neuronal cell death was decreased in CHOP knockout mice [15]. Mutations in the presenilin-1 gene cause a marked increase in CHOP protein, and this hazardous effect is attenuated by anti-sense-mediated suppression of CHOP production [48]. CHOP expression was dramatically increased by the Parkinsonism-inducing neurotoxin 6-hydroxydopamine in neurons [49, 50]. Moreover, the expression of mutant huntingtin fragment proteins causes an increased CHOP expression and increased cell death [51]. These findings indicate that CHOP plays an important role in ER stress-induced neuronal cell death. Therefore, the molecules targeting pharmacological intervention of CHOP may be plausible candidates for neuroprotective agents in ER stress-related neurodegenerative diseases.

We previously reported that Akt downstream effector GSK-3β is involved in ghrelin mediated anti-apoptotic effect during OGD [5]. Consistent with this report, in the current study, we demonstrate ghrelin phosphorylation of GSK-3β, which was attenuated by pretreatment with LY294002. It also has been shown that hexarelin increases GSK-3β phosphorylation in post-hypoxic-ischemic animals [52]. In addition, we show ghrelin-induced BAD phosphorylation that is inhibited by LY294002. In that BAD shows pro-apoptotic property [53] and phosphorylation and inhibition of BAD by β-neuregulin reduces apoptosis induced by serum withdrawal in Schwann cell [54], our data suggest that this response (PI3K/Akt-mediated inactivation of BAD) is at least partly responsible for the anti-apoptotic effect of ghrelin. In the current study, we demonstrate that ghrelin causes phosphorylated (active) Akt in the nuclei of cortical neurons and inhibition of the PI3K/Akt pathway blocks increased accumulation of phosphorylated Akt in the nucleus. Finally, redistribution of Akt into the nucleus was correlated with the ghrelin-mediated Foxo1 phosphorylation. Akt can directly act as a transcription factor or regulate (phosphorylate) other transcription factors at the nuclear

level [55]. Akt-mediated phosphorylation of Foxo1 attenuates the DNA binding ability of Foxo1, causing it to be excluded from the nucleus [56]. Therefore, our data suggest that increased nuclear localization of active Akt followed by nuclear inhibition of Foxo1 may be related to ghrelin-mediated neuroprotection. Collectively, these data suggest that Akt downstream effectors including GSK-3β, BAD and Foxo1 may be involved in ghrelin-induced anti-apoptotic effect.

In summary, we have demonstrated that ghrelin protected against apoptotic cell death in cortical neurons exposed to ER stress. We have shown that the protective effect of ghrelin was mediated by PI3K/Akt pathway. Ghrelin targeted CHOP protein *via* the inhibition of PERK/eIF2α/ATF4 pathway. We assume that ghrelin-mediated suppression of CHOP is associated with the exclusion of Foxo1 from the nucleus. Moreover, we provide evidence that increased Akt signaling by ghrelin is associated with downstream attenuation of GSK-3β, BAD and Foxo1. These findings are important because ghrelin can function as a neuroprotective agent and may have therapeutic potential for the treatment of neurodegenerative diseases where ER stress responses play a major role.

Declaration on Conflicts of Interest

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

Acknowledgements

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