Phosphatidylinositol-3-kinase/Akt/glycogen synthase kinase-3 β and ERK1/2 pathways mediate protective effects of acylated and unacylated ghrelin against oxygen–glucose deprivation-induced apoptosis in primary rat cortical neuronal cells

Hyunju Chung, Sanghee Seo, Minho Moon and Seungjoon Park

Department of Pharmacology and Medical Research Center for Bioreaction to ROS and Biomedical Science Institute, Kyunghee University School of Medicine, Seoul 130-701, South Korea

(Correspondence should be addressed to S Park; Email: sjpark@khu.ac.kr)

Abstract

Only acylated ghrelin (AG) binds GH secretagog receptor 1a (GHS-R1a) and has central endocrine activities. An anti-apoptotic effect of AG in neuronal cells has recently been reported. However, whether there is a neuroprotective effect of unacylated ghrelin (UAG), the most abundant form of ghrelin in plasma, is still unknown. Therefore, we investigated whether UAG was neuroprotective against ischemic neuronal injury using primary cultured rat cortical neurons exposed to oxygen and glucose deprivation (OGD). Both AG and UAG inhibited OGD-induced apoptosis. Exposure of cells to the receptor-specific antagonist D-Lys-3-GHRH-6 abolished the protective effects of AG against OGD, whereas those of UAG were preserved, suggesting the involvement of a receptor that is distinct from GHS-R1a. Chemical inhibition of MAPK and phosphatidylinositol-3-kinase (PI3K) blocked the anti-apoptotic effects of AG and UAG. Ghrelin siRNA

enhanced apoptosis either during OGD or even in normoxic conditions. The protective effects of AG and UAG were accompanied by an increased phosphorylation of extracellular signal-regulated kinase (ERK)1/2, Akt, and glycogen synthase kinase-3 β (GSK-3 β). Furthermore, treatment of cells with AG or UAG resulted in nuclear translocation of β -catenin. In addition, both AG and UAG increased the Bcl-2/Bax ratio, prevented cytochrome *c* release, and inhibited caspase-3 activation. The data indicate that, independent of acylation, ghrelin can function as a neuroprotective agent that inhibits apoptotic pathways. These effects may be mediated via activation of the MAPK and PI3K/Akt pathways. Our data also suggest that PI3K/Akt-mediated inactivation of GSK-3 β and stabilization of β -catenin contribute to the anti-apoptotic effects of ghrelin.

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Introduction

Ghrelin, a novel 28-amino acid peptide that is principally released from the stomach (Date *et al.* 2000), exists in two major molecular forms, acylated ghrelin (AG) and unacylated ghrelin (UAG; Hosoda *et al.* 2000). Ghrelin is a unique peptide esterified with octanoic acid on Ser 3 (Kojima & Kangawa 2005). This acylation is essential for the binding of ghrelin to the growth hormone (GH) secretagog receptor 1a (GHS-R1a; Kojima *et al.* 1999). AG stimulates GH release and induces a positive energy balance by stimulating food intake while decreasing fat use through GH-independent mechanisms (Kojima *et al.* 1999, Peino *et al.* 2000). AG also exerts numerous peripheral effects including direct effects on exocrine and endocrine pancreatic functions, carbohydrate metabolism, the cardiovascular system, gastric secretion, stomach motility, and sleep (Van der Lely *et al.* 2004, Ghigo *et al.* 2005, Kojima & Kangawa 2005).

Recently, we have reported that the acylated form of ghrelin acts as a survival factor for hypothalamic neuronal cells by inhibiting apoptotic pathways, and this neuroprotective effect is mediated via the activation of GHS-R1a (Chung *et al.* 2007). AG has also been reported to protect cortical neurons against focal ischemia/reperfusion in rats (Miao *et al.* 2007). The phosphatidylinositol-3-kinase (PI3K)/Akt and ERK1/2 pathways have been implicated in the regulation of cell survival (Datta *et al.* 1999, Pearson *et al.* 2001). Previous studies suggest that these pathways play important roles in the mechanisms of AG-mediated protection (Baldanzi *et al.* 2002, Kim *et al.* 2004, Chung *et al.* 2007, Granata *et al.* 2007, Zhang *et al.* 2007, Zhao *et al.* 2007). However, the intracellular signaling mechanisms underlying this AG-induced protection remain elusive. The mitochondria are known to be key determinants of cell death and cell survival (Green & Reed 1998, Chan 2004). During apoptosis, cytochrome *c* is released from mitochondria through the permeability transition pore,

which is regulated by the interactions of Bcl-2 family proteins (Shi 2001). The anti-apoptotic protein Bcl-2 inhibits apoptosis by preventing the mitochondrial membrane depolarization and blocking the release of cytochrome *c* (Zamzami *et al.* 1996), whereas the pro-apoptotic protein Bax promotes apoptosis by inducing mitochondrial membrane depolarization and cytochrome *c* release (Narita *et al.* 1998). Cytosolic cytochrome *c* binds to Apaf-1 to form the apoptosome, leading to subsequent activation of caspase-9 and caspase-3 (Li *et al.* 1997, Zou *et al.* 1997, Kuida *et al.* 1998, Yoshida *et al.* 1998), which play a key role in neuronal death after ischemia (Chen *et al.* 1998, Namura *et al.* 1998). It has been reported that AG regulates the mitochondrial pathways of apoptosis and it has been shown to increase Bcl-2/Bax ratio, prevention of cytochrome *c* release, and inhibition of caspase-3 activation in hypothalamic neuronal cells (Chung *et al.* 2007) and pancreatic β -cell line hamster insulinoma tumor (HIT)-T15 (Zhang *et al.* 2007).

By contrast, UAG, the most abundant form of ghrelin in plasma, which does not bind GHS-R1a and has no GH-stimulating activity or any effect on the other anterior pituitary function, is not just a reservoir of inactive peptide (Van der Lely *et al.* 2004). Indeed, AG and UAG exhibit similar biological activities, including a protective effect on cardiac and endothelial cells (Baldanzi *et al.* 2002), pancreatic β -cells and human islets (Granata *et al.* 2007), inhibition of cell proliferation of breast and prostate carcinoma cell lines (Cassoni *et al.* 2001, 2004), a stimulatory effect on proliferation of preosteoblastic as well as GH3 pituitary tumor cells (Nanzer *et al.* 2004, Maccarinelli *et al.* 2005, Delhanty *et al.* 2006), promotion of the differentiation of osteoblasts and skeletal muscle cells (Delhanty *et al.* 2006, Filigheddu *et al.* 2007), and the stimulation of adipogenesis (Choi *et al.* 2003) and food intake (Toshinai *et al.* 2006) *in vivo*. These actions of UAG appear to be mediated by a specific receptor distinct from GHS-R1a. To date, the anti-apoptotic effect of UAG in neuronal cells is still unknown.

During ischemic brain injury, the lack of oxygen and glucose supply leads to neuronal damage. Although the cellular mechanisms of ischemic neuronal injury are not completely understood, apoptosis plays an important role in the development of ischemic neuronal cell death (Mattson *et al.* 2000). To mimic an ischemic insult, oxygen and glucose deprivation (OGD) of brain cortical neuronal cells has been used as an *in vitro* model of ischemia. In the present study, in order to pursue further the biological effects of ghrelin in the central nervous system, we investigated the effect of either AG or UAG on the survival of cultured cortical neuronal cells exposed to OGD insult. We also studied the effect of antagonist of GHS-R1a on the effects of AG and UAG on OGD-induced apoptosis. To determine the mechanisms of AG- or UAG-induced neuroprotection, selective inhibitors of PI3K and MAPK were employed. We also investigated the potential role of PI3K/Akt downstream effector glycogen synthase kinase-3 β (GSK-3 β), which plays a critical role in apoptosis (Pap & Cooper 1998), for the protective effects of

ghrelin. Finally, we investigated the effects of AG or UAG on OGD-induced cytochrome *c* release and caspase-3 activation, and on the Bcl-2 family of proteins.

Materials and Methods

Materials

Rat AG and UAG were obtained from Peptides International (Louisville, KY, USA). D-Lys-3-GHRP-6 was purchased from Bachem (Torrance, CA, USA). Human recombinant IGF-I was obtained from Sigma Chemical Co. Neurobasal and RPMI 1640 media, and B-27 supplement were from Gibco/Invitrogen. B-27 is an optimized serum substitute developed for low-density plating and long-term viability and growth of central nervous system (CNS) neurons (Brewer *et al.* 1993). Primary polyclonal antibodies to rabbit anti-cytochrome *c*, rabbit anti-Bcl-2 and rabbit anti- β -catenin were obtained from Santa Cruz Biotechnology, Inc. (Delaware, CA, USA), and rabbit anti-Bax and goat anti-GHS-R1a were from Abcam, Inc. (Cambridge, UK). Primary antibody to rabbit anti-ghrelin was purchased from Chemicon International, Inc. (Temecula, CA, USA). Polyclonal rabbit anti-phospho-antibodies to ERK1/2 (Thr202/Tyr204) and Akt (Ser473), and monoclonal rabbit GSK-3 β (Ser9) were purchased from Cell Signaling Technology (Danvers, MA, USA). PD98059, wortmannin and LY294002 were 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 and treatments

Primary rat cortical neuronal cultures were prepared as previously described (Joo *et al.* 2002, Miao *et al.* 2007). Briefly, cortical tissues were obtained from 1-day-old Sprague-Dawley rats. Pregnant mother for 1-day-old pups was purchased from Orient Bio (Seongnam, Korea) under Institutional Animal Care and Use Committee (IACUC) approval for breeding. The entire tissues were quickly dissected and mechanically dispersed in Ca²⁺- and Mg²⁺-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 μ m). Cells were plated at a density of 2×10^4 cells/cm² on 50 μ g/ml poly-L-lysine-coated 100 mm culture dishes and grown in Neurobasal medium supplemented with 2% B-27, 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 days *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). To determine whether AG or UAG inhibits

OGD-induced apoptosis in cortical neuronal cells, cells were pretreated with AG (100 nM), UAG (100 nM), or vehicle (saline) for 24 h. Then cells were exposed to OGD insult or maintained under normoxic conditions. To examine whether the anti-apoptotic effect of ghrelin was mediated via its receptor GHS-R1a, cells were pretreated with D-Lys-3-GHRP-6 (100 µM) or vehicle (saline) for 1 h before the treatment with AG or UAG. To evaluate whether ghrelin exerts an anti-apoptotic effect after the induction of OGD, the cells were treated with either AG (100 nM) or UAG (100 nM) for 0, 2, or 4 h after OGD insult. Experiments were also performed by incubating the cells with the following pharmacological inhibitors: 50 µM PD98059 for 1 h, 200 nM wortmannin for 30 min, or 10 µM LY294002 for 30 min. Apoptosis was determined by DNA fragmentation ELISA described below. To investigate the effect of AG and UAG on the ERK1/2 and Akt/GSK-3β pathways, 1) cells were treated with either AG (100 nM) or UAG (100 nM) for 5, 10, 30, and 60 min, or for 15, 30, 60, and 120 min and 2) cells were preincubated with PD98059, wortmannin, or LY294002, then treated with 100 nM AG or UAG for 10 or 30 min and assayed by western blot analysis as described below. All experiments were performed at least two times.

Oxygen–glucose deprivation

To induce ischemia, cells were exposed to OGD as previously described (Chung *et al.* 2007). On the day of the experiment, the regular Neurobasal culture medium was replaced with OGD medium (glucose-free RPMI supplemented with 1% fetal bovine serum). Cultures were then placed in a humidified 37 °C incubator within the Hypoxic Workstation (Daiki Sciences Co. Ltd by Ruskinn Technology, Bridgend, UK) containing a gas mixture of 0.1% O₂, 5% CO₂, and 94.9% N₂ for 30 min to initiate the ischemic insult. OGD was terminated by replacing the OGD medium with Neurobasal medium containing 4.5 mg/ml glucose, and cultures were incubated for an additional 24 h under normoxic conditions.

Apoptosis quantification

We used the Cell Death Detection ELISA (Roche) to evaluate the extent of apoptosis by measuring the histone-complexed DNA fragments according to the manufacturer's instructions.

Small interfering RNA knockdown experiments

For all transfection studies, primary cortical neurons were seeded in 24-well plates until 80% confluent (or cultured for 7 days). Rat preproghrelin (NM_021669) small interfering RNA (siRNA, ON-TARGETplus SMARTpool siRNA reagent (L-095230-01)) was purchased from Dharmacon, Inc. (Lafayette, CO, USA). Four different strands of siRNA were pooled in this siRNA reagent in order to target different parts of the preproghrelin mRNA to improve knockdown

efficiency. The siRNA sequences targeting preproghrelin, as the manufacturer provided, were the following. 5'-UCA AAG AGG CGC CAG CUA A-3', 5'-CAG AGG AGG AGC UGG AAA U-3', 5'-CUG CUG ACU UAC AAA UAA A-3', and 5'-CCA AGA AGC CAC CAG CUA A-3'. Scrambled siRNA (Dharmacon, Inc.) was used as a control. Cortical neuronal cells were transfected using DharmaFECT 3 siRNA Transfection kit (Dharmacon, Inc.), after which these cells were utilized for the functional studies 48 h later, and knockdown efficiency was assessed by western blot analysis using anti-ghrelin and anti-β-actin antibodies.

Immunohistochemical detection of ghrelin and GHS-R1a

Primary cultured cortical neuronal cells were fixed with 4% paraformaldehyde (Sigma) in PBS for 30 min at room temperature. After blocking with 3% normal goat serum (Vector Laboratories, Burlingame, CA, USA) and 1% BSA (Sigma), the slides were incubated with primary antibodies to neuron-specific nuclear protein (1:500 dilution; Chemicon International), ghrelin (1:1000 dilution; Chemicon International) and 5-(and-6)-carboxyfluorescein (FAM)-labeled GHS-R1a (1:500 dilution; Phoenix Pharmaceuticals, Belmont, CA, USA) overnight at 4 °C. After washes, the slides were incubated with a secondary Cy3-goat anti-mouse IgG (1:400 dilution; Jackson ImmunoResearch, West Grove, PA, USA) at room temperature for 1.5 h. Cells 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.

Immunohistochemical analysis for subcellular localization of β-catenin

Previous studies have reported that the inhibition of GSK-3β stabilizes β-catenin, leading to accumulation and translocation from the cytoplasm to the nucleus (Hashimoto *et al.* 2002). For the evaluation of intracellular localization of β-catenin, cells were fixed and probed with antibodies against β-catenin, and a secondary antibody conjugated with the fluorescent dye. Nucleus was visualized by staining with DAPI. Fluorescence was captured using a 40× objective lens on a Carl Zeiss LSM 510 Meta confocal microscope (excitation at 485 nm and emission at 535 nm).

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 Na₃VO₄, 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). 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, USA) according to the manufacturer's instructions. The membranes were soaked in blocking buffer (1× 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) and quantified using Quantity One imaging software (Bio-Rad).

Reverse transcription (RT)-PCR

Total RNA from cortical neuronal cells was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Inc.) according to the manufacturer's instructions and was reverse transcribed using the Superscript II reverse transcriptase (Life Technologies, Inc.) at 42 °C with random hexamer priming. An RNA control tube containing all RT reagents except reverse transcriptase was included as a negative control to monitor genomic DNA contamination. To confirm the GHS-R1a and ghrelin expressions in cultured cortical neuronal cells, the resultant cDNA was amplified using primers specific for GHS-R1a (sense: 5'-TTC GCC ATC TGC TTC CCT CTG-3' and antisense: 5'-TGT CTG CTT GTG GTT CTG GTC-3'), ghrelin (sense: 5'-TTC AGC CCA GAG CAC CAG AAA-3' and antisense: 5'-AGT TGC AGA GGA GGC AGA AGC T-3'), and β -actin (sense: 5'-ATG GGT CAG AAG GAC TCC TAC G-3' and antisense: 5'-AGT GGT ACG ACC AGA GGC ATA C-3') and the GeneAmp PCR System 2700 (Applied Biosystems, Helios, Singapore). The thermal cycling profile was as follows: 95 °C for 10 min, 35 (GHS-R1a), 38 (ghrelin) or 22 (β -actin) cycles of 95 °C for 30 s, 61 (GHS-R1a) or 60 (ghrelin and β -actin) °C for 1 min, and 72 °C for 1 min. The final extension was 72 °C for 10 min.

Caspase-3 activity

A caspase-3 fluorescent assay kit from Peptron (Daejeon, Korea) was used for the determination of caspase-3 activity in cells according to the manufacturer's instructions. Briefly, cell lysates were incubated with the reaction buffer containing 2.5 mM Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) at 37 °C for 120 min, and fluorescence was measured by a fluorometer (Perkin-Elmer, Wellesley, MA, USA) using excitation at 360 nm and emission at 460 nm.

Statistical analysis

Data are presented as mean $\pm$ S.E.M. 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

Expression of ghrelin and GHS-R1a in cortical neuronal cells

Figure 1A shows that ghrelin immunoreactivity was detected in primary cultured cortical neuronal cells. Moreover, RT-PCR analysis revealed that ghrelin mRNA was expressed in these cells (Fig. 1C). We also identified the expression of GHS-R1a either at the protein (Fig. 1B) or at the mRNA (Fig. 1D) level.

AG and UAG protect cortical neuronal cells against OGD insult

We investigated the effects of AG or UAG on OGD-induced apoptosis in cortical neuronal cells. The apoptotic DNA fraction was significantly increased from $6.8 \pm 0.4\%$ (normoxia) to $47.2 \pm 0.9\%$ by OGD insult (Fig. 2A). Pretreatment of cells with AG (100 nM) prevented the apoptosis induced by OGD insult ($22.8 \pm 1.1\%$; Fig. 2A) as previously reported in hypothalamic neuronal cells (Chung *et al.* 2007). Furthermore, the percentage of apoptotic DNA was significantly decreased to $28.1 \pm 0.7\%$ by 24-h pretreatment with unacylated form of ghrelin. To determine whether the anti-apoptotic effect of ghrelin is mediated by its receptor GHS-R1a, cortical neuronal cells were treated with the ghrelin receptor-specific antagonist. The exposure of cells to D-Lys-3-GHRP-6 (100 μ M) completely abolished the

Figure 1 Expression of ghrelin and GHS-R1a in cortical neuronal cells. (A) Ghrelin and (B) GHS-R1a immunoreactivity in cortical neuronal cells. Cells were incubated with primary antibodies to ghrelin, GHS-R1a, and NeuN. Cells were counterstained with DAPI and images were captured using confocal microscopy. (C) Ghrelin and (D) GHS-R1a mRNA expression assessed by RT-PCR. β -actin was used as an internal control. Total RNA was extracted from the primary cultured cortical and hypothalamic neuronal cells (as a positive control). RT (–) reaction containing all RT reagents except reverse transcriptase was included as a negative control to monitor genomic DNA contamination.

Figure 2 AG and UAG protect cortical neuronal cells from OGD-induced apoptosis. (A) Cells were preincubated with vehicle or D-Lys-3-GHRP-6 (100 μ M) for 1 h and then treated with vehicle, AG (100 nM), or UAG (100 nM) for 24 h. (B) Cells were exposed to OGD for 30 min followed by 24 h of reoxygenation, or were maintained under normoxic conditions (normoxia). Cells were treated with 100 M AG or UAG for 0, 2, or 4 h after OGD insult. (C) and (D) Cells were transfected with control scrambled siRNA or preproghrelin siRNA and incubated for 48 h. Then cells were exposed to OGD for 30 min followed by 24-h reoxygenation, or maintained under normoxic conditions. Knockdown efficiency was assessed by western blot using anti-ghrelin and anti- β -actin antibodies. The ghrelin band intensity was normalized to β -actin band intensity. Representative western blot images of siRNA transfection experiments are shown in the upper insets. DNA fragmentation, a marker of apoptosis, was measured by ELISA. Values are the mean $\pm$ S.E.M. of four separate experiments performed in triplicate. * P < 0.05 versus OGD-insulted, vehicle-treated cells.

protective effect of AG against OGD insult (Fig. 2A). By contrast, D-Lys-3-GHRP-6 did not alter the anti-apoptotic effect of UAG.

As shown in Fig. 2B, both AG and UAG significantly decreased OGD-induced apoptosis when administered after OGD insult. Specifically, the percentage of apoptotic DNA was significantly decreased to 31.1% (AG) and 30.2% (UAG) by treatment with ghrelin 2 h after OGD insult.

In this study, it was demonstrated that cortical neuronal cells express ghrelin at the mRNA and protein levels, suggesting that it may act through autocrine/paracrine mechanisms. To investigate this possibility, endogenous ghrelin expression was suppressed using an siRNA technique. Compared with control scrambled siRNA, preproghrelin siRNA resulted in 80% inhibition of ghrelin expression 48 h after transfection as assessed by western blot analysis (Fig. 2C). Knockdown of

ghrelin not only significantly increased OGD-induced apoptosis but also induced apoptosis in cells maintained under normoxic conditions (Fig. 2D).

AG and UAG inhibit OGD-induced apoptosis by activating PI3K/Akt and ERK1/2 signaling pathways

Treatment of cells with either AG or UAG rapidly activated Akt in a time-dependent manner (Fig. 3A). Moreover, AG and UAG caused a rapid and strong activation of ERK1/2 phosphorylation (Fig. 3B). AG- and UAG-induced activation of Akt and ERK1/2 peaked between 10 and 30 min and lasted for 60 min.

Figure 3 AG and UAG activate Akt and ERK1/2 in cortical neuronal cells. Time course of AG- (upper) or UAG- (lower) induced phosphorylation of (A) Akt and (B) ERK1/2. Cells were treated with 100 M AG or UAG for 5, 10, 30, and 60 min and assayed by western blot using specific anti-phospho-Akt (Ser473) and anti-Akt antibodies (A), and specific anti-phospho-ERK1/2 (Thr202/Tyr204) and anti-ERK1/2 antibodies. (B) MAPK and PI3K pathways mediate AG- and UAG-induced phosphorylation of (C) Akt and (D) ERK1/2. Cells were preincubated with 50 μ M PD98059 for 1 h, 200 nM wortmannin for 30 min, or 10 μ M LY294002 for 30 min and then treated with 100 nM AG or UAG for 10 min and assayed by western blot as above. The phospho-Akt and the phospho-ERK1/2 band intensities were normalized to Akt and ERK1/2 band intensities respectively and expressed as relative band intensities. Values are the mean $\pm$ S.E.M. (n = 4). Each experiment was repeated twice. * P < 0.05 versus untreated cells; † P < 0.05 versus AG- or UAG-treated cells.

We further determined which signaling pathways are involved in AG- or UAG-induced Akt and ERK1/2 activation. We found that pretreatment of cells with PD98059 (50 μ M) blocked AG- or UAG-induced phosphorylation of ERK1/2 (Fig. 3D, upper panel) without affecting Akt (Fig. 3C, upper panel) activation. However, AG- or UAG-induced phosphorylation of Akt (Fig. 3C, middle and lower panels) and ERK1/2 (Fig. 4D, middle and lower panels) was attenuated by pretreatment of cells with wortmannin (200 nM) or LY294002 (10 μ M). To examine this hypothesis, we tested whether pretreatment of cells with PD98059, wortmannin, or LY294002 impaired the anti-apoptotic activity of AG or UAG against OGD insult. We found that all of these inhibitors significantly blocked the anti-apoptotic effects of AG ($P<0.05$) or UAG ($P<0.05$; Fig. 4). Treatment of cells with inhibitor alone had no effect (data not shown). These data suggest that AG and UAG inhibit apoptosis induced by OGD insult via the activation of the PI3K/Akt and MAPK pathways.

Effects of AG and UAG on the regulation of Akt effectors
GSK-3 β

Our data demonstrating AG- and UAG-induced anti-apoptotic effects through the PI3K/Akt pathway suggest that Akt downstream effectors may contribute to neuroprotection of these peptides. The way by which Akt regulates its

Figure 4 The MAPK and PI3K pathways mediate the anti-apoptotic effects of AG or UAG. Cortical neuronal cells were preincubated with 50 μ M PD98059 for 1 h, 200 nM wortmannin for 30 min, or 10 μ M LY294002 for 30 min and then treated with 100 M AG or UAG for 24 h. Then cells were exposed to OGD for 30 min followed by 24-h reoxygenation, or maintained under normal conditions (normoxia). IGF-I was used as a positive control. DNA fragmentation was measured by ELISA. Values are the mean $\pm$ S.E.M. of four independent experiments run in triplicate. * $P<0.05$ versus OGD-insulted, vehicle-treated cells. † $P<0.05$ versus OGD-insulted, AG- or UAG-treated cells.

anti-apoptotic effect is by phosphorylating many effector proteins, including the cytoplasmic protein kinase GSK-3 β . GSK-3 β phosphorylation was increased after 15 min of AG or UAG treatment and lasted for 120 min (Fig. 5A). The inhibition of PI3K/Akt pathway with LY294002 reduced AG- or

Figure 5 AG and UAG regulate Akt effector GSK-3 β in cortical neuronal cells. Time course of (A) AG- or (B) UAG-induced phosphorylation of GSK-3 β . Cells were treated with 100 nM AG or UAG for 15, 30, 60, and 120 min and assayed by western blot using specific anti-phospho-GSK-3 β (Ser9) and anti-GSK-3 β antibodies. (C) PI3K pathways mediate AG- and UAG-induced phosphorylation of GSK-3 β . Cells were preincubated with vehicle or 10 μ M LY294002 for 30 min and then treated with 100 M AG or UAG for 30 min and assayed by western blot as above. The phospho-GSK-3 β band intensity was normalized to GSK-3 β band intensity and expressed as relative band intensities. Values are the mean $\pm$ S.E.M. ($n=4$). Each experiment was repeated twice. * $P<0.05$ versus untreated cells; † $P<0.05$ versus AG- or UAG-treated cells.

UAG-induced GSK-3 β phosphorylation to the control levels (Fig. 5B).

AG- or UAG-induced phosphorylation of GSK-3 β is associated with β -catenin translocation to the nucleus

We found that β -catenin immunoreactivity was primarily observed in the cytoplasm under basal conditions (Fig. 6A). However, β -catenin exhibited an increased staining pattern when cells were treated with AG or UAG, suggesting that β -catenin has translocated to the nucleus (Fig. 6B and C). By contrast, AG- or UAG-induced nuclear translocation of β -catenin was inhibited after the cells were pretreated with PI3K inhibitor LY294002 (Fig. 6D and E).

Figure 6 AG and UAG increase translocation of β -catenin from the cytoplasm to the nucleus in cortical neuronal cells. Cortical neuronal cells were preincubated with vehicle or 10 μ M LY294002 for 30 min and then treated with 100 nM AG or UAG for 30 min. Cells were fixed and probed with β -catenin antibody to detect subcellular localization of β -catenin. Nuclei were visualized by staining with DAPI. Intracellular localization of β -catenin was visualized by confocal microscopy. Images shown in the boxes are overlay of β -catenin and DAPI images, and β -catenin images are shown in the upper right insets. The results are representative of three independent experiments.

Effects of AG and UAG on the Bcl-2/Bax ratio, cytochrome c release, and caspase-3 activation

Both AG and UAG treatments significantly inhibited the OGD-induced increase in the Bax protein in mitochondria and increased the OGD-induced decrease in the Bcl-2 protein levels in the cytosol, thereby significantly increasing the Bcl-2/Bax ratio (Fig. 7A). The OGD-induced release of cytochrome c (Fig. 7B) and activation of caspase-3 (Fig. 7C) were also significantly inhibited by either AG or UAG treatment.

Discussion

In the present study, we demonstrated that ghrelin, in either the acylated or unacylated form, protects cortical neurons from the apoptotic stimuli induced by OGD insult. The protective effects of AG and UAG were dependent on the activities of the MAPK and PI3K/Akt signaling pathways. AG- and UAG-induced stimulation of PI3K/Akt pathways resulted in inactivation of GSK-3 β and translocation of β -catenin to the nucleus. It is evident that even endogenous ghrelin exhibited cytoprotective effects. We also demonstrated that AG and UAG alter the status of the Bcl-2 family of proteins, inhibiting cytochrome c release and caspase-3 activity and promoting the survival of cortical neuronal cells.

We observed that AG exerted strong inhibition of OGD-induced apoptosis in cortical neuronal cells through the activation of GHS-R1a, as previously reported in hypothalamic cells (Chung *et al.* 2007). In the present study, we report for the first time that UAG protects cortical neuronal cells from OGD-induced cell death by inhibiting apoptosis, which greatly extends previous reports on the effects of UAG on systemic tissues (Cassoni *et al.* 2001, 2004, Baldanzi *et al.* 2002, Nanzer *et al.* 2004, Maccarinelli *et al.* 2005, Delhanty *et al.* 2006, Filigheddu *et al.* 2007, Granata *et al.* 2007). The neuroprotective effect of UAG does not appear to be mediated through the activation of GHS-R1a because the specific antagonist of GHS-R1a fails to block the protective effect of UAG against OGD insult. Given the fact that UAG neither activates nor binds GHS-R1a (Kojima *et al.* 1999), we strongly suspect the existence of a separate specific receptor for UAG distinct from GHS-R1a. This hypothesis is supported by a recent observation by Toshinai *et al.* (2006), in which UAG, but not AG, stimulated food intake in GHS-R1a-deficient mice. Previous studies also suggest the existence of a different novel ghrelin receptor than GHS-R1a (Baldanzi *et al.* 2002, Muccioli *et al.* 2004, Gauna *et al.* 2006, Filigheddu *et al.* 2007, Granata *et al.* 2007). Furthermore, both types of ghrelin when administered 4 h after OGD insult still inhibited OGD-induced apoptosis, suggesting that these peptides may have the ability of attenuating disease progression. Taken together, the findings provide evidence that ghrelin, regardless of its acylation, may

function as a survival factor for neuronal cells and offer a new perspective on the potential role of these peptides in ischemic neuronal injury.

Ghrelin expression was detected in cortical neuronal cells at the mRNA and protein levels, suggesting an autocrine/paracrine mode of action of ghrelin in the inhibition of apoptosis. We tested this possibility by transfecting cells with the siRNA against preproghrelin. The suppression of ghrelin expression significantly increased the apoptosis during OGD insult and even in normoxic conditions. This observation is comparable with a report by *Granata et al. (2007)*, in which an antibody against ghrelin significantly inhibited apoptosis in pancreatic β -cells. It should be noted that the survival effect of endogenous AG and UAG could not be distinguished because the siRNA used in this study was directed against preproghrelin. However, we suggest that endogenous ghrelin in either the acylated or unacylated form has autocrine/paracrine cytoprotective effects since both AG and UAG exhibited similar anti-apoptotic activity and stimulated the identical intracellular signaling pathways.

It was recently reported that both AG and UAG can activate the PI3K/Akt and ERK1/2 pathways in HIT-T15 cells (*Granata et al. 2007*), cardiomyocytes, and endothelial cells (*Baldanzi et al. 2002*). We have shown in this study that both AG and UAG strongly induce the activation of Akt and ERK1/2, which are believed to play important roles in regulating cell survival (*Datta et al. 1999*, *Pearson et al. 2001*). Chemical inhibition of both AG- and UAG-induced phosphorylation of Akt and ERK1/2 resulted in the complete blockade of the ghrelin anti-apoptotic effect, indicating that these peptides suppressed OGD-induced apoptosis in cortical neuronal cells through the activation of Akt and ERK1/2. These data suggest that PI3K/Akt and ERK1/2 activation may be involved in the anti-apoptotic effects of either AG or UAG, or both, in cortical neuronal cells exposed to OGD insult. Ghrelin acts on the GHS-R1a to increase intracellular Ca^{2+} via the guanine nucleotide binding protein q phospholipase C (Gq-PLC) pathway (*Kojima & Kangawa 2005*). Through the α -subunit of GHS-R1a, the activation of PLC-protein kinase C pathway and Raf-MEK-MAPK occurs. Ghrelin also transactivates tyrosine kinase receptor via the β - and γ -subunits, leading to the activation of MAPK via the Ras-Raf-MEK pathway (*Nanzer et al. 2004*).

Figure 7 Effect of AG or UAG on Bax and Bcl-2 protein levels in cortical neuronal cells exposed to OGD assessed by western blot analysis. Bax and Bcl-2 band intensities were normalized to β -actin band intensity. (B) Effect of AG or UAG on cytochrome c protein levels in the cytosolic fraction of cortical cells exposed to OGD assessed by western blot. Cytochrome c band intensities were normalized to β -actin band intensity. (C) Changes in caspase-3 protein levels (upper panel) assessed by western blot and caspase-3 activity (lower panel) assessed using the cell permeable caspase-3 substrate Ac-DEVD-AMC. Results are representative of three separate experiments with similar results. * $P < 0.05$ versus normoxia; † $P < 0.05$ versus OGD-insulted, vehicle-treated cells.

Furthermore, AG and UAG have been shown to exert their effects through stimulation of cAMP-mediated PKA pathways (Granata *et al.* 2007). Taken together, the data suggest that multiple signaling pathways are involved in AG- and UAG-induced Akt and ERK1/2 activation, and the anti-apoptotic effects of AG and UAG are mediated via the MAPK and PI3K signaling pathways.

Our data suggest that Akt downstream effectors may be involved in AG- and UAG-mediated anti-apoptotic effects. Akt can phosphorylate effector proteins at the membrane or cytoplasmic levels, or is able to act as a transcription factor or phosphorylate other transcription factors at the nuclear level (Song *et al.* 2005). Akt activation controls the cell survival through phosphorylation of downstream targets, such as protein kinase GSK-3 β . In the current study, we have shown that GSK-3 β phosphorylation was increased by both AG and UAG treatments. Similarly, GH secretagog hexarelin has been shown to increase GSK-3 β phosphorylation in post-hypoxic-ischemic animals (Brywe *et al.* 2005). In that, GSK-3 β is pro-apoptotic protein (Eldar-Finkelman 2002), and the inhibitors of GSK-3 β reduce infarct size following focal cerebral ischemia *in vivo* (Kelly *et al.* 2004) and improve neuronal survival *in vitro* (Cross *et al.* 2001), our data suggest that this response (PI3K/Akt-mediated inactivation of GSK-3 β) is at least partly responsible for the anti-apoptotic effects of AG and UAG. Several transcription factors, such as cAMP-response element-binding protein (D'Amico *et al.* 2000), nuclear factor- κ B (Madrid *et al.* 2000, Sanchez *et al.* 2003), and β -catenin (Haq *et al.* 2003), were known to be regulated by GSK-3 β . These transcription factors are involved in cell survival mechanism downstream of GSK-3 β . Inactivation of GSK-3 β by phosphorylation of serine 9 is known to result in translocation of β -catenin to the nucleus (Hart *et al.* 1998). Nuclear translocation of β -catenin is required for its survival-promoting effect (Yuan *et al.* 2005). We found in this study that both AG and UAG increase the nuclear translocation of β -catenin, which is known to inhibit apoptosis, in agreement with previous reports demonstrating that the neuroprotective effects of FGF1 (Hashimoto *et al.* 2002) and lovastatin (Bergmann *et al.* 2004) are dependent on the GSK-3 β / β -catenin signaling pathway. Taken together, these results suggest that the anti-apoptotic effect of AG and UAG involve a PI3K/Akt-mediated inactivation of GSK-3 β and subsequent stabilization of β -catenin. To the best of our knowledge, this is the first report showing that ghrelin is able to act on GSK-3 β / β -catenin pathways to enhance cell survival.

We recently reported that AG inhibited the OGD-induced increase in the Bax protein in mitochondria and increased Bcl-2 levels in hypothalamic neuronal cells, thereby significantly increasing the Bcl-2/Bax ratio (Chung *et al.* 2007). Bax remains mainly in the cytosol under unstressed normoxic conditions but translocates to mitochondrial outer membranes in response to apoptotic stimuli including OGD insult (Banasiak *et al.* 2000). To identify the changes in Bax protein levels by UAG, we performed a western blot analysis using the

cytosolic and mitochondrial fractions. Bax proteins were translocated from the cytosol to the mitochondria by OGD insult while UAG treatment significantly inhibited the OGD-induced increase in the Bax protein in mitochondria. Cytosolic Bcl-2 protein levels were decreased by OGD insult, whereas UAG treatment increased Bcl-2, resulting in complete restoration of the Bcl-2/Bax ratio to normal levels. Cells are protected when Bcl-2 is in excess and Bcl-2 homodimers predominate, whereas cells are vulnerable to apoptosis when Bax is in excess and Bax homodimers predominate. Therefore, the ratio of Bcl-2/Bax is regarded as a factor that determines a cell's fate in response to apoptotic stimuli (Korsmeyer 1995). The change in the status of Bcl-2 and the Bax proteins caused by either AG or UAG treatment appears to be a change that inhibits apoptosis and favors cell survival.

It is known that the Bcl-2 protein family tightly regulates cytochrome *c* release from the mitochondria into the cytosol (Merry & Korsmeyer 1997). In this study, cytochrome *c* was found to be translocated from the mitochondria to the cytosolic compartment after OGD insult, as previously reported (Perez-Pinzon *et al.* 1999). After release from the mitochondrial intermembrane space, cytochrome *c* forms the apoptosome together with apoptosis-activating factor Apaf-1 and procaspase-9, leading to the activation of initiator caspase-9 (Li *et al.* 1997, Zou *et al.* 1997, Yoshida *et al.* 1998). Subsequent activation of downstream members of the caspase family, including effector caspase-3, leads to apoptosis (Slee *et al.* 1999). In the present study, both AG and UAG treatments prevented the OGD-induced release of cytochrome *c* and the subsequent activation of caspase-3, inhibiting activation of the apoptotic cascade, in agreement with the observation in hypothalamic neuronal cells (Chung *et al.* 2007).

In summary, we provide evidence that acylated and UAG have direct regulatory effects on apoptosis in cortical neuronal cells. The data suggest the existence of a separate specific receptor for UAG distinct from GHS-R1a. It is also shown that both types of ghrelin strongly activated Akt and ERK1/2, and that the neuroprotective effects of these peptides were mediated by the PI3K and MAPK pathways. Moreover, we provide evidence for the first time that increased Akt signaling by AG and UAG is associated with downstream attenuation of GSK-3 β and nuclear translocation of β -catenin. AG and UAG targeted the Bcl-2 protein family, inhibiting cytochrome *c* release and caspase-3 activity, and inducing changes that favored cell survival and inhibited the apoptotic cascade. These findings are significant because UAG as well as AG can function as a neuroprotective agent, and ghrelin may have therapeutic potential for the treatment of stroke.

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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