Ghrelin Inhibits Hydrogen Peroxide-Induced Apoptotic Cell Death of Oligodendrocytes Via ERK and p38MAPK Signaling

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Here, we examined the protective effect of ghrelin on apoptotic cell death induced by hydrogen peroxide (H_2O_2) in primary oligodendrocyte cultures. Ghrelin receptor, growth hormone secretagogue receptor 1a, was expressed in mature oligodendrocytes. H_2O_2 (1 mM) treatment induced apoptotic cell death of oligodendrocytes, which was significantly inhibited by ghrelin treatment. Ghrelin also reduced cytochrome c release, and caspase-3 activation increased by H_2O_2 treatment. Furthermore, the protective effect of ghrelin against H_2O_2-induced oligodendrocyte cell death was mediated through growth hormone secretagogue receptor 1a. Both ERK and p38MAPK were activated (peaked at 8 h in ERK and 1 h in p38MAPK) by H_2O_2 treatment, whereas c-Jun N-terminal kinase and Akt were not. Interestingly, ghrelin further increased ERK activation and decreased p38MAPK activation after H_2O_2 treatment. Next, we tried to elucidate the role of ERK and p38MAPK activation in H_2O_2-induced apoptotic cell death of oligodendrocytes using pharmacological inhibitors. We found that the inhibition of apoptotic cell death of oligodendrocytes by ghrelin was abolished by ERK inhibitor, PD98059 (20 μM), whereas cell survival was increased by p38MAPK inhibitor, SB203580 (10 μM). These results thus indicate that ghrelin inhibits H_2O_2-induced oligodendrocytes cell death in part by increasing ERK activation and decreasing p38MAPK activation, and ghrelin may represent a potential therapeutic agent for protecting oligodendrocytes in central nervous system injuries. **(*Endocrinology* 152: 2377–2386, 2011)**

Ghrelin, a 28-amino acid gastric hormone, has been identified as an endogenous ligand of the growth hormone secretagogue receptor 1a (GHS-R1a), often referred to as the ghrelin receptor (1). Ghrelin primarily acts on the pituitary and hypothalamus to stimulate GH release, appetite, and adiposity (1–5). Besides eliciting the release of GH, ghrelin also influences energy balance, gastric motility, and acid secretion (6–10). Moreover, ghrelin inhibits apoptosis in various cell types, including cardiocytes, endothelial cells, pancreatic β-cells, adipocytes, and neurons (11–14).

Oligodendrocyte is a myelinating cell in the central nervous system (CNS) that extends membranous processes to form multilamellar structures around axons, facilitating conduction of nervous impulses (15). Because oligodendrocytes undergo pathophysiological assault in the CNS diseases, such as multiple sclerosis and spinal cord injury (SCI), the destruction of the myelin sheath and oligodendrocytes cell death play an important role in the disease process (16, 17). Among various factors, oxidative stress has been known to contribute to a number of acute and chronic CNS injuries. Oligodendrocytes are known to be very sensitive to oxidative stress, apparently due to a low capacity for antioxidant defense and intrinsic risk factors, such as high iron content (18). Therefore, the salvation of oligodendrocytes, especially in the oxidative stress condi-

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2011-0090 Received January 26, 2011. Accepted March 18, 2011.
First Published Online April 5, 2011

Abbreviations: CNS, Central nervous system; DMSO, dimethylsulfoxide; GHRP, GH-releasing peptide; GHS-R1a, growth hormone secretagogue receptor 1a; H_2O_2, hydrogen peroxide; JNK, c-Jun N-terminal kinase; MBP, myelin basic protein; MEK, mitogen extracellular kinase-regulated pathway; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; p, phosphorylated; SCI, spinal cord injury; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling.

tion, has the potential to be of great therapeutic value in the neurodegenerative diseases.

Previously, we found that in normal spinal cord, GHS-R1a is expressed in oligodendrocytes, and SCI-induced apoptotic cell death of oligodendrocytes is inhibited by ghrelin treatment (19). Ghrelin is also known to protect hippocampal neurons from ischemic insults (20). In addition, it was known that ghrelin mitigates oxidative stress and prevents the decrease in antioxidant enzyme activities, such as superoxide dismutase and catalase in pentylenetetrazol-induced oxidative stress, in a rat seizure model (21). Recently, in hypothalamus and substantia nigra, ghrelin showed the antioxidant effect by enhancing mitochondrial proliferation and fatty acid β-oxidation (22, 23). Moreover, ghrelin inhibited hydrogen peroxide (H_2O_2)-induced proinflammatory responses in endothelial cells and lung epithelial cells (24, 25). However, whether ghrelin exerts protective effect on H_2O_2-induced oligodendrocyte cell death is not examined yet.

Here, we tried to examine whether ghrelin may protect H_2O_2-induced cell death in cultured oligodendrocytes and whether the protective effect by ghrelin may be mediated through GHS-R1a or not. As a result, we show that ghrelin significantly inhibited apoptotic cell death of oligodendrocytes in H_2O_2-treated primary oligodendrocyte cultures. Furthermore, the protective effect by ghrelin was mediated in part by activating ERK pathway and inhibiting p38MAPK pathway, which were mediated through the ghrelin receptor.

Materials and Methods

Primary oligodendrocyte cultures

Primary cultures of rat cortical oligodendrocytes were prepared as described previously (16, 26). In brief, Sprague Dawley (Samtako, Osan, Korea) rat pups (postnatal d 1) were killed by decapitation, and cortices were dissected from their meninges and dissociated by sequential trituration. Cells were plated into 75-cm^2 flasks coated with poly-D-Lysine (10 μg/ml; Sigma, St. Louis, MO) in the presence of DMEM supplemented with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD) and 1% penicillin-streptomycin. The cultures were grown for 8–9 d in humidified 5% CO_2 incubator at 37 C, with changes of media every 2 d. The flasks were then shaken for 1 h at 200 rpm at 37 C, and the supernatants containing microglia were replaced with fresh media after a wash with PBS. After 5–6 h of incubation at 37 C, the cultures were subjected to an overnight shaking at 250 rpm at 37 C. The resulting oligodendrocyte precursor cells were further purified from astrocytes by subsequent preplating steps using 10-cm uncoated dishes for 1 h. The preplating step was repeated twice. Isolated cells were resuspended in 10% fetal bovine serum in DMEM and plated on poly-D-Lysine-coated 12-well (1.2×10^5 cells/well) or 24-well (6×10^4 cells/well) culture plates. Four to five hours after seeding, the medium was changed to a differentiation medium without serum, containing Basal Medium Eagle/F12 (1:1), transferrin (100 μg/ml), putrescine (20 μg/ml), progesterone (12.8 ng/ml), selenium (10.4 ng/ml), insulin (25 μg/ml), thyroxine (0.8 μg/ml), glucose (6 mg/ml), and glutamine (6.6 mM) as previously described (16). Every 2 d, one-half of the culture media was exchanged. After 4–5 d, these cultures contained a highly enriched population of differentiated oligodendrocytes with numerous cellular processes and stained positive for myelin basic protein (MBP). Cultures did not contain neuronal cells and astrocytic contamination, and purity was more than 95%.

Oxidative stress and drug treatment

To treat mature oligodendrocytes with H_2O_2, the differentiation media in each well was replaced with fresh media containing a known concentration of H_2O_2 diluted appropriately. Acylated ghrelin (Peptides International, Louisville, KT) was dissolved in PBS and treated for 30 min before H_2O_2 treatment. The antagonist of ghrelin receptor, [D-Lys3]-GH-releasing peptide (GHRP)-6 (100 μM; Bachem, Torrance, CA) was dissolved in PBS and treated for 1 h before ghrelin treatment. For control, PBS without ghrelin or [D-Lys3]-GHRP-6 was used. For some experiments, cells were pretreated with ERK inhibitor, PD98059 (20 μM; Calbiochem, La Jolla, CA) or p38MAPK inhibitor, SB203580 (10 μM; Calbiochem) for 15 min before H_2O_2 treatment. PD98059 or SB203580 was dissolved in dimethylsulfoxide (DMSO) and then diluted in PBS (the final concentration of DMSO was 0.1%). For control, diluted DMSO (0.1%) without PD98059 or SB203580 was used.

Cell survival assay

To assess cell cytotoxicity by H_2O_2, the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay was carried out as previously described (27). In brief, oligodendrocytes were prepared as described above and plated on poly-D-Lysine-coated 24-well culture plates. The differentiated media were changed with fresh media (1 ml) with or without H_2O_2 or the other compounds at the indicated concentrations, and then cells were further incubated for 24 h. MTT solution (100 μl/well; 5 mg/ml in PBS) was added to the wells, and plates were incubated for 4 h. After remove the media, DMSO was added and incubated for 30 min at 37 C to dissolve the formazan salt. Quantification was then carried out with a microplate reader (Molecular Devices, Sunnyvale, CA) at 595 nm. Oligodendrocyte survival was expressed as a percentage relative to that in the vehicle-treated control (100%). Values represent the means $\pm$ SD of three independent experiments.

RNA isolation and RT-PCR

Total RNA isolation from cultured cells using TRIZOL Reagent (Invitrogen, Carlsbad, CA) and cDNA synthesis were performed as previously described (28). A 20 μl PCR contained 1 μl first strand cDNA, 0.5 U Ex *Taq* polymerase (Takara, Kyoto, Japan), 20 mM Tris-HCl (pH 7.9), 100 mM KCl, 1.5 mM $MgCl_2$, 250 μM deoxyribonucleotide triphosphate, and 10 pmole of GHS-R1a-specific primer. Samples were subjected to 30 cycles of 95 C 30 sec, 58–64 C 30 sec, and 72 C for 30 sec on a PCR Thermal Cycler (Takara). The primers used for this experiment were designed according to the sequences previously reported (14) and synthesized by the Genotech Corp. (Daejeon, Korea). The primer sequences were as follows: GHS-R1a sense primer, 5′-TTC GCC ATC TGC TTC CCT CTG-3′ and antisense

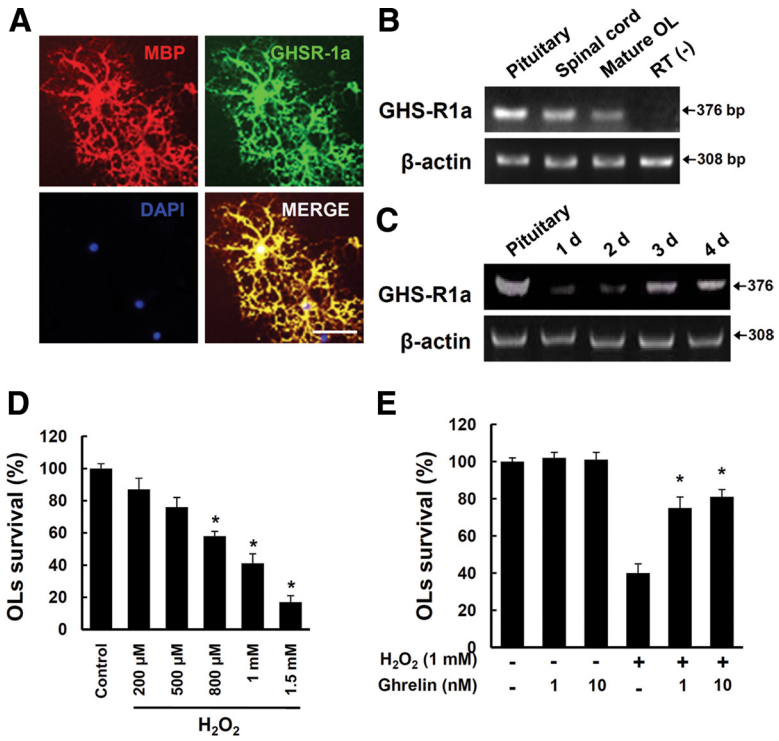

FIG. 1. The effect of ghrelin on cell death of oligodendrocytes (OL) after H_2O_2 treatment. A, Representative fluorescence microscopic photographs of MBP- and ghrelin receptor-positive mature oligodendrocytes. *Scale bar*, 20 μm. DAPI, 4′,6-diamidino-2-phenylindole. B, RT-PCR of GHS-R1a mRNA at 5 d after differentiation. Pituitary and normal spinal cord tissues were used as positive controls for GHS-R1a. C, RT-PCR of GHS-R1a mRNA during maturation. D, Cytotoxicity of H_2O_2 on mature oligodendrocyte cultures. Oligodendrocyte cultures were exposed to H_2O_2 for 24 h, and cell viability was measured by MTT reduction assay. Oligodendrocyte survival was expressed as a percentage relative to that in the vehicle-treated control (100%). Data represent the mean value ± SD of three separate experiments. E, The effect of ghrelin on oligodendrocyte survival after H_2O_2 treatment. Cells were treated with ghrelin (10 nM) for 30 min before 1 mM H_2O_2 treatment. By MTT assay, oligodendrocyte survival at 24 h after H_2O_2 treatment was expressed as a percentage relative to that in the vehicle-treated control (100%). Data represent the mean value ± SD of three separate experiments. *, $P < 0.05$.

primer, 5′-TGT CTG CTT GTG GTT CTG GTC-3′ (376 bp); β-actin sense primer, 5′-ATT TGG CAC CAC ACT TTC TAC A-3′ and antisense primer, 5′-TCA CGC ACG ATT TCC CTC TCA G-3′ (380 bp). Negative controls consisted of PCR lacking primers or reverse transcriptase. After amplification, PCR products were subjected to a 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The relative density of bands was analyzed by the ChemiImager 4400 (Alpha Innotech Corp., San Leandro, CA). β-Actin was used as an internal control. Experiments were repeated three times, and the values obtained for the relative intensity were subjected to statistical analysis. The gels shown in figures are representative of results from three separate experiments.

Preparation of total cell lysate and cellular fractionation

Total protein extraction and cellular fractionation were performed as previously described (16, 29). Whole cell lysates were homogenized in ice-cold lysis buffer containing 1% Nonidet P-40, 20 mM Tris (pH 8.0), 137 mM NaCl, 0.5 mM EDTA, 10% glycerol, 10 mM $Na_2P_2O_7$, 10 mM NaF, 1 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium vanadate, and 1 mM phenylmethylsulfonylfluoride. The homogenates were centrifuged at 4 C for 20 min at 14,000 × *g*, and the supernatant was transferred to a fresh tube. For cellular fractionation, cells were washed with PBS, and MS buffer [225 mM mannitol, 25 mM sucrose, 10 mM HEPES, 1 mM EDTA (pH 7.4), 1 μg/ml pepstatin, 2.5 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM phenylmethylsulfonylfluoride] was added directly to the dishes. Cells were harvested by scraping with a rubber policeman and homogenized with a Dounce homogenizer. Cell homogenates were centrifuged at 250 × *g* for 10 min at 4 C to remove the unbroken cells and nuclear particles, and the supernatants were further centrifuged at 8500 × *g* for 20 min (mitochondrial pellets). After transfer to a new tube, the supernatant was further centrifuged at 100,000 × *g* for 1 h at 4 C and used as the cytoplasmic fraction (S-100). Mitochondrial pellets were resuspended in lysis buffer and centrifuged at 25,000 × *g* for 30 min. Protein concentration was determined using bicinchoninic acid assay kit (Pierce, Rockford, IL).

Western blot analysis

Total (30 μg), cytosolic (20 μg), and mitochondrial (10 μg) proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Bedford, MA) by electrophoresis. The membranes were then incubated with polyclonal antibodies against cytochrome c (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), cleaved caspase-3 (1:1000; Cell Signaling Technology, Danvers, MA), ERK (1:5000; Cell Signaling Technology), phosphorylated (p)ERK (1:5000; Cell Signaling Technology), p38MAPK (1:1000; Cell Signaling Technology) and p-p38MAPK (1:1000; Cell Signaling Technology), c-Jun N-terminal kinase (JNK) (1:3000; Cell Signaling Technology), p-JNK (1:3000; Cell Signaling Technology), Akt (1:1000; Cell Signaling Technology), and p-Akt (1:1000; Cell Signaling Technology). Both β-tubulin (1:10,000; Sigma) and cytochrome c oxidase IV (1:5000; Invitrogen) were used as internal controls. Densitometric quantification of all of the bands on Western blottings was done using AlphaImager software (Alpha Innotech Corp.). Background in films was subtracted from the optical density measurements.

Immunocytochemistry and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL)

Cells were seeded on poly-D-Lysine-coated 13-mm diameter glass cover slips (1×10^5 cells in 24 well) and treated as described above. Medium was removed, and cells were washed twice with PBS. Cells were then fixed with 4% paraformaldehyde for 10 min and washed three times with PBS. The fixed oligodendrocytes were processed for immunocytochemistry with antibodies against GHS-R1a (1:300; Phoenix Pharmaceuticals, Inc., Burlingame, CA) and MBP (1:1000; Millipore) as previously described (16). TUNEL staining was performed according to the

protocol for cell culture using the apoptosis detection kit (Millipore). TUNEL- and MBP-positive cells were visualized with a microscope at ×200 magnification and analyzed by counting the number of MBP/TUNEL positive cells per field of each coverslip. Five fields of each coverslip were counted and averaged. To avoid any bias in the results, cell counts were done by the persons who did not know the treatment history of cultures; no results were revealed to the counters until the completion of data collection for the entire study. Cell images were captured with an Olympus microscope (Olympus, Tokyo, Japan) with software accompanying the CoolSNAP camera (Rhoper Scientific, Tucson, AZ).

Statistical analysis

Data were presented as the mean ± SD values. Statistical analyses were performed by the Student's *t* test or one-way ANOVA with Tukey's multiple comparison *post hoc* test. $P < 0.05$ was considered significant. All statistical analyses were performed by SPSS 15.0 (SPSS Science, Chicago, IL).

Results

Ghrelin inhibits H_2O_2-induced cytotoxicity in oligodendrocyte cultures

The stages of oligodendrocyte differentiation can be defined using antibodies recognizing some of the major developmental markers for the oligodendrocyte lineage. MBP is known to be expressed in mature myelinating oligodendrocytes (30). At 4 d after differentiation, MBP was expressed in mature oligodendrocytes (Fig. 1A), and all MBP-positive oligodendrocytes were also positive for GHS-R1a (Fig. 1A). Furthermore, RT-PCR data show that GHS-R1a mRNA was expressed in mature oligodendrocyte cultures (Fig. 1B). GHS-R1a mRNA was expressed in early differentiation stage (*e.g.* 1 or 2 d after differentiation), but the level was very low. However, the level of GHS-R1a mRNA was markedly increased according to maturation process (Fig. 1C). To assess the cytotoxicity of H_2O_2, oligodendrocytes were exposed to H_2O_2 (0.2, 0.5, 0.8, 1, and 1.5 mM), and cell viability was determined at 24 h after H_2O_2 treatment by measuring the changes in cellular reduction capacity by the MTT assay. As shown in Fig. 1C, H_2O_2 treatment induced a dose-dependent reduction in cell viability as reported (31). Incubation of oligodendrocytes with 200 or 500 μM H_2O_2 did not significantly change MTT reduction as compared with vehicle-treated control. However, exposure to 800 μM to 1.5 mM H_2O_2 significantly decreased the capacity of oligodendrocytes to reduce MTT (Fig. 1D). From this result, 1 mM H_2O_2 was used throughout experiments. Next, we examined the effect of ghrelin on H_2O_2-induced cytotoxicity in oligodendrocyte cultures. As shown in Fig. 1E, ghrelin (1 and 10 nM) significantly increased oligodendrocyte survival after H_2O_2 treatment compared with the vehicle control. In addition, cell survival was not affected when oligodendrocytes were treated only with ghrelin (Fig. 1D).

Ghrelin inhibits H_2O_2-induced apoptotic cell death of oligodendrocytes

To assess apoptotic cell death of oligodendrocytes, we employed TUNEL staining for the detection of DNA fragmentation after 1 mM H_2O_2 treatment. As shown in Fig. 2A, a number of TUNEL-positive oligodendrocytes was observed in cultures 24 h after H_2O_2 treatment, and ghre-

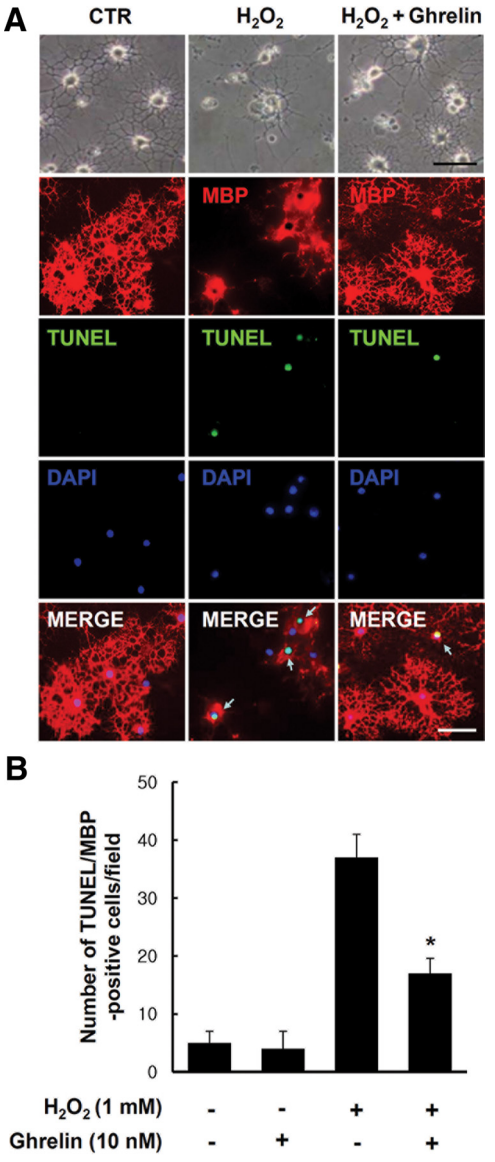

FIG. 2. The effect of ghrelin on H_2O_2-induced apoptotic cell death of oligodendrocytes. A, *upper panels*, Representative phase contrast photographs of mature oligodendrocytes after vehicle, H_2O_2, and H_2O_2 + ghrelin (10 nM) treatment. Mature oligodendrocytes were treated with 1 mM H_2O_2 for 24 h. A, *bottom panels*, TUNEL/MBP positive oligodendrocytes (*arrows*) after H_2O_2 treatment. *Scale bars*, 20 μm. CTR, control; DAPI, 4′,6-diamidino-2-phenylindole. B, Quantitative analysis of TUNEL/MBP-positive oligodendrocytes at 24 h after H_2O_2 treatment. Data represent mean ± SD obtained from five separate experiments. *, $P < 0.01$.

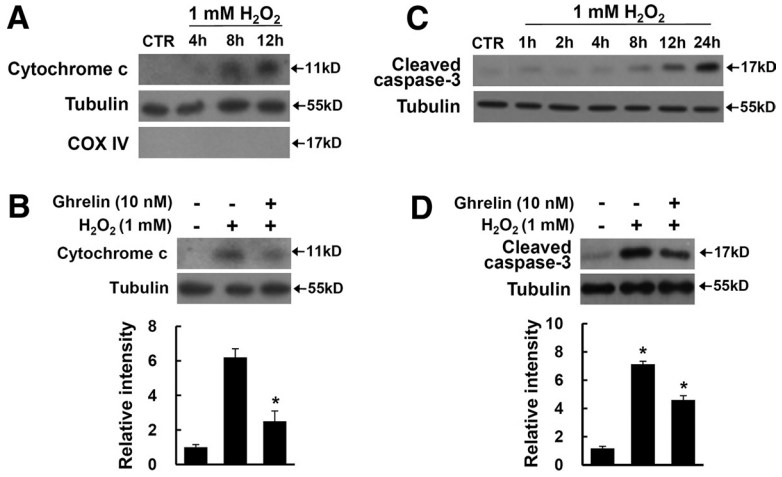

FIG. 3. The effects of ghrelin on cytochrome c release and caspase-3 activation in oligodendrocytes after H_2O_2 treatment. Cells were treated with ghrelin (10 nM) for 30 min before H_2O_2 treatment. A, Western blot analysis of cytochrome c with cytoplasmic fractions at 4, 8, and 12 h after H_2O_2 treatment. COX IV, cytochrome c oxidase IV. B, Western blot analysis showing the effect of ghrelin on cytochrome c release at 12 h after H_2O_2 treatment (*upper panel*). Quantitative analysis of Western blots shows that ghrelin treatment significantly decreased the level of cytochrome c release into cytoplasm at 12 h compared with vehicle treatment after H_2O_2 treatment (*bottom panels*). Values are mean ± SD of three separate experiments. *, $P < 0.05$. C, Western blot analysis of cleaved (activated) caspase-3 with total protein extract at 1, 2, 4, 8, 12, and 24 h after H_2O_2 treatment. CTR, control. D, Western blot analysis showing the effect of ghrelin on caspase-3 activation at 24 h after H_2O_2 treatment (*upper panel*). Quantitative analysis of Western blots shows that ghrelin treatment significantly decreased the level of activated caspase-3 at 24 h compared with vehicle treatment after H_2O_2 treatment (*bottom panel*). Values are mean ± SD of three separate experiments. *, $P < 0.05$.

lin (10 nM) treatment reduced the number of TUNEL-positive cells. Quantitative analysis of TUNEL/MBP double positive cells shows that ghrelin significantly decreased the number of TUNEL-positive oligodendrocytes after H_2O_2 treatment (H_2O_2, 37 ± 4 cells *vs.* H_2O_2 + ghrelin, 17 ± 2.6 cells) (Fig. 2B). Apoptotic cell death is known to be mediated by mitochondria-dependent or mitochondria-independent processes. In mitochondria-dependent process, the release of cytochrome c from the mitochondria to the cytosol is pivotal in the activation of caspases and the ensuing cell death (32). As shown in Fig. 3A, cytochrome c release from oligodendrocytes was increased after H_2O_2 treatment. Ghrelin (10 nM) significantly inhibited the release of cytochrome c at 12 h after H_2O_2 treatment (Fig. 3B). The level of cleaved (activated) caspase-3 (17 kDa) was also increased from 8 h and gradually increased up to 24 h after H_2O_2 treatment (Fig. 3C). Furthermore, ghrelin (10 nM) significantly decreased the level of cleaved caspase-3 at 24 h after H_2O_2 treatment (Fig. 3D). These results indicate that ghrelin inhibits mitochondria-dependent apoptotic cell death of oligodendrocytes after H_2O_2 treatment.

The effect of ghrelin is mediated through ghrelin receptor, GHS-R1a

It has been known that the endocrine activities of ghrelin are mediated by ghrelin receptor, GHS-R1a, a G protein-coupled receptor expressed mainly in the pituitary and hypothalamus (33, 34). To test a hypothesis that the antiapoptotic effect of ghrelin on oligodendrocytes is mediated via the ghrelin receptor, oligodendrocyte cultures were treated with the receptor specific antagonist [D-Lys-3]-GHRP-6 before ghrelin treatment. As shown in Fig. 4A, exposure of cells to [D-Lys-3]-GHRP-6 (100 μM) mitigated the protective effect of ghrelin on oligodendrocyte survival after H_2O_2 treatmentin the MTT assay (H_2O_2, 42 ± 5%; H_2O_2 + ghrelin, 78 ± 4%; H_2O_2 + ghrelin + [D-Lys-3]-GHRP-6, 53 ± 3.7%). TUNEL staining also shows that the ghrelin receptor antagonist treatment abolished the protective effect of ghrelin on oligodendroctye cell death induced by H_2O_2 (H_2O_2, 39 ± 3.7 cells; H_2O_2 + ghrelin, 19 ± 2.5 cells; H_2O_2 + ghrelin + [D-Lys-3]-GHRP-6, 38 ± 4.4 cells) (Fig. 4B). No cytotoxicity was detected when oligodendrocytes were treated only with the ghrelin receptor antagonist (data not shown). These results indicate that the protective effect of ghrelin against H_2O_2-induced apoptotic cell death of oligodendrocytes is mediated via the ghrelin receptor, GHS-R1a.

Ghrelin increases ERK activation and decreases p38MAPK activation in oligodendrocytes after H_2O_2 treatment

Akt and MAPK, such as ERK, JNK, and p38MAPK, are known to be associated with cell death or survival (35). Because the antiapoptotic effect of ghrelin has been known to be mediated through MAPK signaling *in vitro* and *in vivo* (11, 14, 19, 36), we investigated the effect of ghrelin on Akt and MAPK in oligodendrocyte cultures after H_2O_2 treatment. Western blot analysis revealed that the level of p-ERK was increased and peaked at 8 h, and the level of p-p38MAPK was also increased and peaked at 1 h after H_2O_2 treatment (Fig. 5A). Quantitative analysis of Western blots shows that the level of p-ERK was increased 8.4-fold at 8 h, and p-p38MAPK was increased 3.3-fold at 1 h after H_2O_2 treatment compared with vehicle control (Fig. 5, B and D). However, both the levels of p-JNK and p-Akt were not significantly changed by H_2O_2 treatment (Fig. 5, A, C, and E). The level of total ERK, p38MAPK, JNK, or Akt was not changed by H_2O_2 treatment. Next, we determined the effect of ghrelin on ERK and p38MAPK activation after H_2O_2 treatment. As shown in Fig. 6, ghrelin treatment increased the level of p-ERK and decreased the level of p-p38MAPK when compared with the vehicle-treated control. Quantitative analysis of Western blots re-

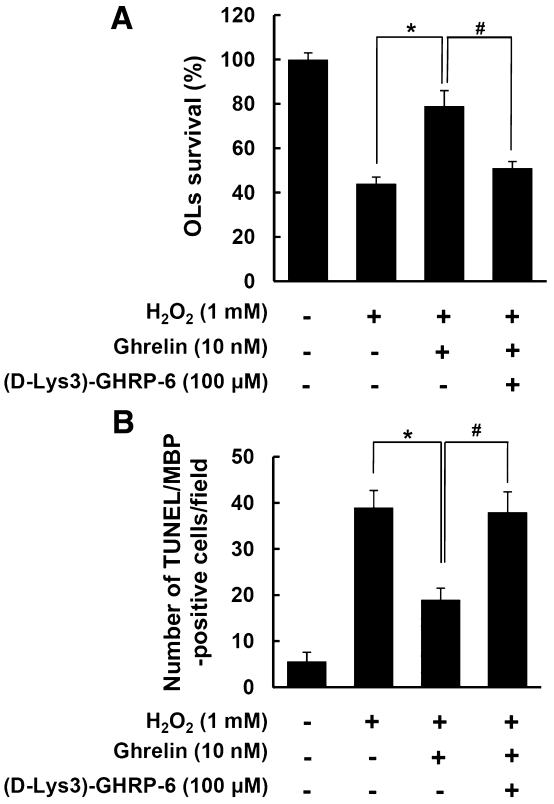

FIG. 4. The protective effect by ghrelin on apoptotic cell death of oligodendrocyte after H_2O_2 treatment is mediated through the ghrelin receptor, GHS-R1a. Mature oligodendrocytes were treated with vehicle or ghrelin (10 nM) for 30 min before H_2O_2 treatment. Cells were also preincubated with PBS or ghrelin receptor antagonist, [D-Lys3]-GHRP-6 (100 μM), 1 h before ghrelin treatment. A, Cell viability measured at 24 h after H_2O_2 treatment by MTT reduction assay. Oligodendrocyte (OL) survival was expressed as a percentage relative to that in the vehicle control (100%). B, Quantitative analysis of TUNEL/MBP-positive oligodendrocytes after H_2O_2 treatment at 24 h after H_2O_2 treatment. Data represent mean ± SD obtained from five separate experiments. *, $P <$ 0.01 compared with vehicle treatment; #, $P < 0.05$ compared with ghrelin treatment.

vealed that ghrelin treatment increased 1.5-fold in p-ERK level at 8 h and decreased 1.9- fold in p38MAPK at 1 h after H_2O_2 treatment as compared with vehicle control (Fig. 6). Furthermore, treatment with the antagonist of ghrelin receptor, [D-Lys-3]-GHRP-6 (100 μM) significantly attenuated the stimulatory effect of ghrelin on ERK activation at 8 h and the inhibitory effect of ghrelin on p38MAPK activation at 1 h after H_2O_2 treatment (Fig. 6), indicating that the effects of ghrelin on ERK and p38MAPK activation is mediated through the ghrelin receptor. Thus, these data suggest that the antiapoptotic effect of ghrelin on oligodenrocytes after H_2O_2 treatment may be mediated in part via ERK and p38MAPK signaling.

The protective effect of ghrelin is mediated by further increasing ERK activation and decreasing p38MAPK activation after H_2O_2 treatment

A conflicting body of literature exists on the potential role of MAPK in oxidative stress (37). Because ghrelin treatment increased ERK activation and inhibited p38MAPK activation after H_2O_2 treatment, we hypothesized that ghrelin would promote oligodendrocyte survival by increasing ERK activation and decreasing p38MAPK activation after H_2O_2 treatment. Oligodendrocyte cultures were pretreated for 15 min with the inhibitors of mitogen extracellular kinase-regulated pathway (MEK), the upstream molecules of ERK (PD98059) and p38MAPK (SB203580), followed by exposure to H_2O_2. At 24 h after H_2O_2 treatment, cell viability was assessed by MTT assay and TUNEL staining. As shown in Fig. 7A, ghrelin treatment significantly increased oligodendrocyte survival after H_2O_2 treatment. SB203580 (10 μM), an inhibitor of p38MAPK, also significantly increased oligodendrocyte survival after H_2O_2 treatment when compared with H_2O_2 treatment (H_2O_2, 39 ± 2.5%; H_2O_2 + SB203580, 73.4 ± 1.2%). Furthermore, the protective effect of ghrelin on oligodendrocyte survival was abolished by PD98069, an inhibitor of ERK. (H_2O_2, 39 ± 2.5%; H_2O_2 + ghrelin, 78.5 ± 5.2%; H_2O_2 + ghrelin + PD98059, 36.2 ± 3%) (Fig. 7A). However, there was no additive or synergetic effect in cells treated with ghrelin + SB203580 after H_2O_2 treatment when compared with ghrelin treatment (H_2O_2 + ghrelin, 78.5 ± 5.2%; H_2O_2 + ghrelin + SB203580, 74.1 ± 1.7). TUNEL/MBP double staining also revealed that the number of TUNEL-positive oligodendrocytes was significantly decreased by SB203580 after H_2O_2 treatment when compared with H_2O_2 treatment (H_2O_2, 44 ± 2.3 cells; H_2O_2 + SB203580, 25 ± 1.3 cells), whereas the protective effect of ghrelin on apoptotic cell death was cancelled by PD98059 after H_2O_2 treatment (H_2O_2, 44 ± 2.3 cells; H_2O_2 + ghrelin, 23 ± 3.2 cells; H_2O_2 + ghrelin + PD98059, 45 ± 5 cells) (Fig. 7B). Again, no additive or synergetic effect was observed when cells were treated with ghrelin + SB203580 after H_2O_2 treatment (H_2O_2, 44 ± 2.3 cells; H_2O_2 + ghrelin, 23 ± 3.2 cells; H_2O_2 + ghrelin + SB203580, 22 ± 3). Moreover, oligodendrocyte survival was not affected when cells were treated only with PD98059 or SB203580 (Fig. 7, A and B). These results indicate that ERK activation is involved in cell survival, and p38MAPK activation is involved in cell death in oligodendrocytes after H_2O_2 treatment. Furthermore, our data suggest that the protective effect of ghrelin on oligodendrocyte survival may be mediated in part by increasing ERK activation and decreasing p38MAPK activation after H_2O_2 treatment.

Discussion

Our previous report shows that ghrelin treatment inhibits apoptotic cell death of oligodendrocytes after SCI (19).

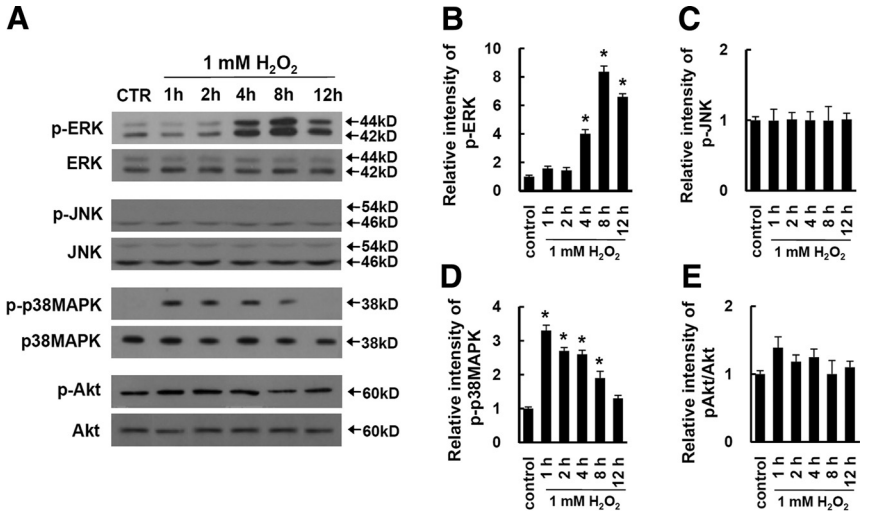

FIG. 5. The effect of H_2O_2 on ERK and p38MAPK activation in oligodendrocytes. Total cell extracts were prepared at the indicated time points (1, 2, 4, 8, and 12 h) after H_2O_2 treatment. A, Western blot analyses of p-ERK, p-JNK, p-p38MAPK, and p-Akt. CTR, control. Quantitative analyses of Western blots of p-ERK (B), p-JNK (C), p-p38MAPK (D), and p-Akt (E). Values are means ± SD of three separate experiments. *, $P < 0.01$ compared with untreated control.

However, the mechanisms underlying the antiapoptotic action of ghrelin have not been fully understood. Using *in vitro* culture system, we demonstrated that oxidative stress by H_2O_2 induced apoptotic cell death in mature oligodendrocytes as reported (31, 37), and ghrelin treatment inhibited the H_2O_2-induced apoptotic cell death by decreasing cytochrome c release and caspase-3 activation. Furthermore, we showed that the antiapoptotic effect of ghrelin may be mediated in part by increasing ERK activation and decreasing p38MAPK activation after H_2O_2 treatment. Finally, we demonstrated that the antiapoptotic effect by ghrelin is mediated through the ghrelin receptor, GHS-R1a.

It has been shown that ERK signaling is activated by phosphorylation on regulatory threonine and tyrosine residues by the upstream kinases MEK1 and MEK2 (38). Typically, the ERK pathway has been known to play important roles in proliferative and cell survival responses (39, 40). Ghrelin has also been shown to exert an antiapoptotic effect mediated by ERK signaling pathway in 3T3-L1 adipocytes, osteoblasts, cardiomyocytes, endothelial cells, and hypothalamic neurons (11, 12, 14, 36). Furthermore, Mronga *et al.* (41) show that ERK pathway in oligodendrocytes is involved in the initial survival response after exposure to oxidative stress. Our data demonstrated that ghrelin increases ERK activation in oligodendrocytes after H_2O_2 treatment, and the inhibition of ERK pathway by PD98059 abolished the protective effect by ghrelin on oligodendrocyte survival (see Fig. 7). Taken together, these results indicate that ERK signaling is involved in oligodendrocyte survival pathway, and ghrelin protects oligodendrocytes by increasing ERK activation after H_2O_2 treatment.

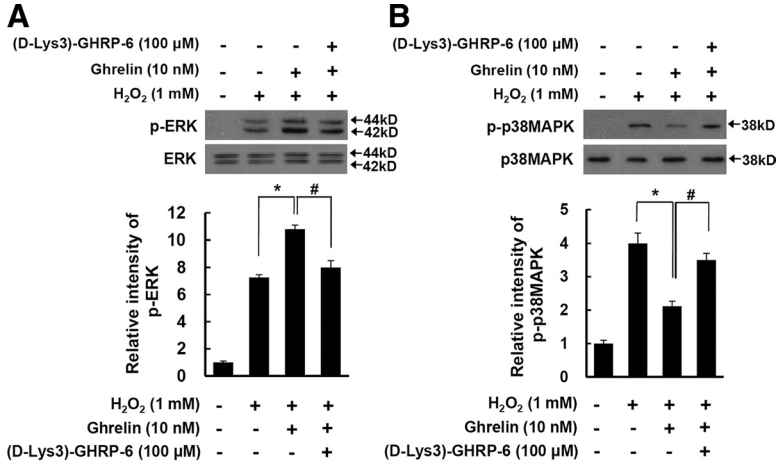

FIG. 6. The effects of ghrelin and ghrelin receptor antagonist on ERK and p38MAPK activation after H_2O_2 treatment. Mature oligodendrocytes were pretreated with vehicle or ghrelin (10 nM) for 30 min before H_2O_2 treatment. Cells were also preincubated with PBS or [D-Lys3]-GHRP-6 (100 μM) 1 h before ghrelin treatment. A, Western blot analysis of p-ERK with total extract at 8 h after H_2O_2 treatment. Quantitative analysis of Western blots shows that [D-Lys3]-GHRP-6 treatment significantly alleviated the stimulatory effect of ghrelin on p-ERK H_2O_2 treatment. Values are means ± SD of three separate experiments. *, $P < 0.05$ compared with vehicle treatment; #, $P < 0.05$ compared with ghrelin treatment. B, Western blot analysis of p-p38MAPK with total extract at 1 h after H_2O_2 treatment. Quantitative analysis of Western blots shows that [D-Lys3]-GHRP-6 treatment significantly mitigated the inhibitory effect of ghrelin on p-p38MAPK H_2O_2 treatment. Values are means ± SD of three separate experiments. *, $P < 0.05$ compared with vehicle treatment; #, $P < 0.05$ compared with ghrelin treatment.

Recent studies demonstrate that ghrelin promotes cell survival by inhibition of p38MAPK in ischemic neuronal injury *in vitro* (14) and SCI animal model (19). In the present study, we show that H_2O_2 treatment increased p38MAPK activation, which was inhibited by ghrelin treatment. Our data also show that H_2O_2-induced activation of p38MAPK was involved in oligodendrocyte cell death pathway, because SB203580 treatment, an inhibitor of p38MAPK signaling, increased the cell survival after H_2O_2 treatment. Similarly, Fragoso *et al.* (37) report that PD169316, an inhibitor of p38MAPK, reduces the toxic effect of H_2O_2 on oligodendrocytes. Thus, our data suggest that the antiapoptotic effect of ghrelin on oligodendrocytes may be mediated in part by inhibiting p38MAPK activation after H_2O_2 treatment.

Our results show that the antiapoptotic effect of ghrelin might be mediated through the mitochondrial-dependent apoptotic pathway,

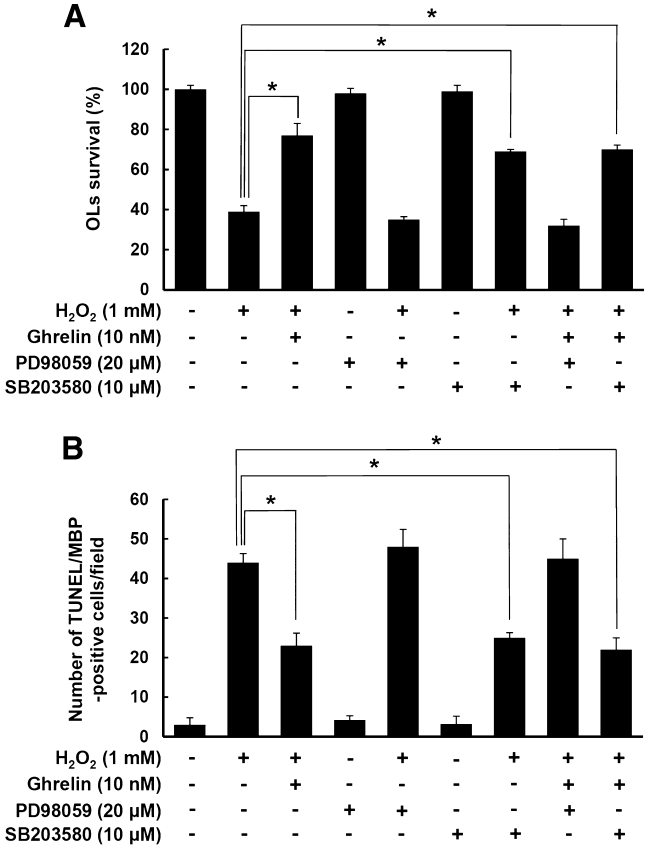

FIG. 7. The effect of ERK and p38MAPK inhibitors on oligodendrocyte (OL) cell death after H_2O_2 treatment. Both ERK inhibitor, PD98059 (20 μM), and p38MAPK inhibitor, SB203580 (10 μM), were added 15 min before the H_2O_2 (1 mM) treatment. A, Cell viability at 24 h after H_2O_2 treatment measured by MTT reduction assay. Oligodendrocyte survival was expressed as a percentage relative to that in vehicle control (100%). B, Quantitative analysis of TUNEL/MBP-positive oligodendrocytes. Cells were fixed at 24 h after H_2O_2 treatment and assessed TUNEL/MBP double staining. Quantification was analyzed by counting as described in *Materials and Methods*. Data are expressed as positive cell numbers per field and represent the mean value ± SD of three separate experiments. *, $P < 0.05$.

because ghrelin inhibited cytochrome c release and caspase-3 activation after H_2O_2 (see Fig. 3). Chung *et al.* (14) demonstrate that ghrelin inhibits the cytochrome c release via protecting mitochondrial membrane depolarization and increasing Bcl-2/Bax ratio during oxygen glucose deprivation-induced hypothalamic neuronal cell death. In addition, Andrews *et al.* (22, 23) show that ghrelin has the neuroprotective effect in substantial nigra and hypothalamus by enhancing mitochondrial respiration, proliferation, and fatty acid β-oxidation. They also demonstrate that ghrelin buffers and neutralizes reactive oxygen species while promoting fatty acid oxidation in a uncoupling protein 2-dependent manner in substantia nigra. Further study is required to determine whether ghrelin affects Bcl-2/Bax ratio, mitochondrial homeostasis, and lipid metabolism in oligodendrocyte cultures after H_2O_2 treatment.

Ghrelin binding to GHS-R1a has been shown to activate intracellular second messengers coupled to G_q, leading to phospholipase C and protein kinase C (PKC) activation and calcium mobilization (33). GHS-R1a also activates distinct systems of second messengers, including cyclic AMP/protein kinase A and extracellular Ca^{2+}, suggesting that this receptor couples to different intracellular signaling pathways, depending on the cell type and on the binding agonist (42–45). A recent report suggests that ghrelin inhibits neuronal apoptosis induced by oxygen glucose deprivation insult via the activation of PKC and protein kinase A pathways (14). Based on these reports, it is conceivable that multiple signaling pathways may be involved in the antiapoptotic effect of ghrelin on oligodendrocytes after H_2O_2 treatment.

The neuroprotective effects of ghrelin have been observed in animal models of neurological disorders, including Parkinson's disease, stroke, epilepsy, multiple sclerosis, and SCI (14, 19, 21, 23, 46–48). The neuroprotective mechanisms of ghrelin have been proposed as follows. Ghrelin activates intracellular signaling cascade (such as the activation of ERK, Akt, phosphatidylinositol 3 kinase, and PKC signaling pathways), and activation of these kinase signaling pathways leads to the inhibition of apoptotic events, via the subsequent increase in the Bcl-2/Bax ratio, the prevention of cytochrome c release, and inhibition of caspase-3 activation (14, 19, 49, 50). In addition, ghrelin inhibits activation of proapoptotic events, such as the activation of p38MAPK and JNK (14, 19). Furthermore, ghrelin prevents inflammation and microglial activation and also activates the mitochondrial protein uncoupling protein 2, which enhances neuroprotection by suppressing reactive oxygen species and promoting mitochondrial biogenesis (20, 23, 51). However, these neuroprotection mechanisms of ghrelin have been demonstrated in injury model. The endogenous role of ghrelin in neuroprotection has not been determined yet, although GHS-R1a is expressed in numerous extrahypothalamic neuronal populations, including spinal cord neurons and oligodendrocytes in normal condition (34, 52, 53). At least in the normal spinal cord, it is conceivable that ghrelin may play a role in maintenance or survival of these cells as neurotrophins do in the CNS.

In summary, our data show that ghrelin treatment can inhibit apoptosis of oligodendrocytes induced by H_2O_2-induced oxidative stress. The protective effect by ghrelin is mediated in part by activating ERK pathway and inhibiting p38MAPK pathway. Also, the antiapoptotic effect of ghrelin is mediated through the ghrelin receptor. Furthermore, the present study suggests that ghrelin can be potentially useful as a therapeutic agent for protecting oligodendrocytes after CNS injuries.

Acknowledgments

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This work was supported by the Basic Science Research Program through the National Research Foundation of Korea Grants 20100001758 (Science Research Center) and 2010K000824 and 2010K000833 from Brain Research Center of the 21st Century Frontier Research Program funded by the Ministry of Education, Science, and Technology, the Republic of Korea.

Disclosure Summary: The authors have nothing to disclose.

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