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# Ghrelin Inhibits Oligodendrocyte Cell Death by Attenuating Microglial Activation

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**Background:** Recently, we reported the antiapoptotic effect of ghrelin in spinal cord injury-induced apoptotic cell death of oligodendrocytes. However, how ghrelin inhibits oligodendrocytes apoptosis, is still unknown. Therefore, in the present study, we examined whether ghrelin inhibits microglia activation and thereby inhibits oligodendrocyte apoptosis.

**Methods:** Using total cell extracts prepared from BV-2 cells activated by lipopolysaccharide (LPS) with or without ghrelin, the levels of p-p38 phosphor-p38 mitogen-activated protein kinase (p-p38MAPK), phospho-c-Jun N-terminal kinase (pJNK), p-c-Jun, and pro-nerve growth factor (proNGF) were examined by Western blot analysis. Reactive oxygen species (ROS) production was investigated by using dichlorodihydrofluorescein diacetate. To examine the effect of ghrelin on oligodendrocyte cell death, oligodendrocytes were cocultured in transwell chambers of 24-well plates with LPS-stimulated BV-2 cells. After 48 hours incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and terminal deoxynucleotidyl transferase 2′-deoxyuridine, 5′-triphosphate nick end labeling staining were assessed.

**Results:** Ghrelin treatment significantly decreased levels of p-p38MAPK, p-JNK, p-c-Jun, and proNGF in LPS-stimulated BV-2 cells. ROS production increased in LPS-stimulated BV-2 cells was also significantly inhibited by ghrelin treatment. In addition, ghrelin significantly inhibited oligodendrocyte cell death when cocultured with LPS-stimulated BV-2 cells.

**Conclusion:** Ghrelin inhibits oligodendrocyte cell death by decreasing proNGF and ROS production as well as p38MAPK and JNK activation in activated microglia as an anti-inflammatory hormone.

**Keywords:** Ghrelin; p38MAPK; c-Jun N-terminal kinase; Pro-nerve growth factor; Reactive oxygen species; Oligodendroglia; BV-2 microglia cell

## INTRODUCTION

After traumatic spinal cord injury (SCI), apoptotic cell death of oligodendrocytes occurs in the white matter along fiber tracts undergoing Wallerian degeneration [1]. The apoptosis of oligodendrocytes ultimately contributes to chronic demyelination and spinal cord dysfunction [2-4].

As a 28 amino-acid gastric hormone, ghrelin has multifunctional roles in appetite, adiposity, energy balance, gastric motility, and acid secretion [5-9]. The neuroprotective effect of ghrelin has also been shown in several animal models of neuronal injury such as ischemia, Parkinson disease and Alzheim-

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er disease [10-13]. In particular, we reported that SCI-induced apoptotic cell death of oligodendrocytes is inhibited by ghrelin treatment [14]. Oligodendrocytes are very sensitive to oxidative stress, apparently due to a low capacity for antioxidant defense and intrinsic risk factors such as high iron content [15]. In addition, our previous report showed that pro-nerve growth factor (proNGF) production after SCI via activation of p38 mitogen activated protein kinase (p38MAPK) in microglia is involved in oligodendrocyte cell death [16]. Furthermore, the anti-inflammatory effect of ghrelin was shown in several animal models such as arthritis, sepsis, endotoxemia, and multiple sclerosis [17-20].

Here, we examined the effect of ghrelin as an anti-inflammatory hormone on p38MAPK and c-Jun N-terminal kinase (JNK) activation as well as proNGF and reactive oxygen species (ROS) production in BV-2 microglia cells activated by lipopolysaccharides (LPSs). We also examined the effect of ghrelin on cell death of oligodendrocytes cocultured with LPS-stimulated BV-2 cells.

## METHODS

### BV-2 microglial cell culture

The BV-2 murine microglial cell line [21] was cultured in Dulbecco's Modified Eagle's Medium supplemented with 5% fetal bovine serum and 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified incubator under 5% $CO_2$. Prior to each experiment, cells were plated onto 6-well ($5\times10^5$ cells/well) or 24-well ($1\times10^5$ cells/well) plates. The next day, cells were treated with LPS (100 ng/mL; *Escherichia coli* 0111:B4, Sigma, St. Louis, MO, USA) in the presence or absence of ghrelin (1, 10, 100, or 1,000 nM). Acylated ghrelin (Peptides International, Louisville, KT, USA) was dissolved in PBS and treated for 30 minutes before LPS treatment.

### Oligodendrocyte culture

Primary oligodendrocyte cultures were prepared from rat pup (postnatal day 1) brains according to the previously reported method [22]. Isolated oligodendrocytes ($6\times10^4$ cells/well; purity, >95%) were grown on 24-well culture plates or glass coverslips coated with poly-D-lysine (10 μg/mL, Millipore, Billerica, MA, USA) with oligodendrocyte differentiation medium 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). Half of media was changed every 2 days until cells were differentiated into mature oligodendrocytes (myelin basic protein, MBP-positive) for 4 to 5 days. For oligodendrocyte/microglia cocultures, microglia ($1\times10^5$ cells per well) were grown on porous upper inserts of transwell chambers (3 μm diameter pores, BD Biosciences, San Jose, CA, USA) in 24-well plates. After treatment with LPS (100 ng/mL, Millipore) for 30 minutes, with or without ghrelin (1,000 nM), the inserts were washed and placed above oligodendrocytes (6 days in culture) growing on a coverslip in the bottom well of the transwell chambers, allowing diffusion of soluble molecules. Under this culture condition, oligodendrocytes were never exposed to LPS and ghrelin. Oligodendrocyte culture was then processed for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) and for MTT assay.

### Measurement of ROS

The production of ROS was measured fluorometrically using ROS-specific fluorescence dye, dichlorodihydrofluorescein diacetate (DCF-DA, Molecular Probes, Eugene, OR, USA), as described previously [23]. After LPS treatment, DCF-DA (10 μM, final concentration) were added to cells and cellular fluorescence (at 1 hour after treatment) was imaged using an Olympus fluorescence microscope with Metamorph software (Rhoper Scientific, Tucson, AZ, USA).

### Assessment of cytotoxicity

To assess cell cytotoxicity, the tetrazolium (MTT) assay was carried out as previously described [24]. In brief, MTT solution (100 μL/well; 5 mg/mL in PBS) was added to the oligodendrocytes culture plate, and incubated for 4 hours. After removing the media, dimethyl sulfoxide was added and incubated for 30 minutes at 37°C to dissolve the formazan salt. Quantification was then carried out with a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 595 nM. Oligodendrocyte survival was expressed as a percentage relative to that in the vehicle-treated control (100%).Values are expressed as mean±SD of three independent experiments.

### Immunocytochemistry and TUNEL

Cells were seeded on poly-D-lysine-coated glass cover slips ($1\times10^5$ cells in 24-well plates) and treated as described above. Cells were fixed with 4% paraformaldehyde for 10 minutes and washed three times with PBS. The fixed oligodendrocytes were processed for immunocytochemistry with MBP antibody

(1:1,000, Millipore) as previously described [24]. 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. Cell images were captured with an Olympus microscope with Metamorph software.

**Western blot analysis**

Cells were homogenized in a lysis buffer containing 1% nonidet P-40, 20 mM Tris, pH 8.0, 137 mM NaCl, 0.5 mM ethylenediaminetetraacetic, 10% glycerol, 10 mM $Na_2P_2O_7$, 10 mM NaF, 1 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM vanadate, and 1 mM phenylmethylsulfonyl fluoride. Cell homogenates were incubated for 20 minutes at 4°C, and centrifuged at 25,000 ×g for 30 minutes at 4°C. The protein level of the supernatant was determined using the BCA assay (Pierce, Rockford, IL, USA). Protein sample (50 μg) was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore) by electrophoresis. The membranes were blocked with 5% nonfat skim milk in Tris-buffered saline containing 0.1% tween 20 for 1 hour at room temperature and then incubated with polyclonal antibodies against p38MAPK (1:1,000; Cell Signaling Technology, Danvers, MA, USA), phosphor-p38MAPK (p-p38MAPK; 1:1,000, Cell Signaling Technology), phospho-JNK (p-JNK; 1:1,000, Cell Signaling Technology), JNK (1:1,000, Cell Signaling Technology), p-c-Jun (1:1,000, Cell Signaling Technology), c-Jun (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and proNGF (1:1,000, Alomone Labs, Jerusalem, Israel). The primary antibodies were detected with a horseradish peroxidase-conjugated goat antirabbit secondary antibody (Jackson ImmunoResearch Inc., West Grove, PA, USA). Immunoreactive bands were visualized by chemiluminescence using Thermo Scientifi SuperSigna West Pico Chemiluminescent Substrate (Pierce). β-Tubulin (1:10,000, Sigma) 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.

**Statistical analysis**

Values are expressed as mean±SD. Multiple comparisons between groups were performed with one-way analysis of variances. Tukey's multiple comparison was used as a *post hoc* analysis. Statistical significance was accepted with $P<0.05$. Statistical analyses were performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA).

## RESULTS

**Ghrelin inhibits p38MAPK and JNK activation in BV-2 cells stimulated by LPS**

Both p38MAPK and JNK activation are known to mediate inflammatory responses in microglia. LPS is also known to promote the activation of BV-2 cells that exhibit phenotypic and functional properties of activated microglial cells *in vivo*. Thus, we examined the effect of ghrelin on microglia activation in BV-2 cell stimulated with LPS (100 ng/mL) in the presence and absence of ghrelin (1, 10, 100, and 1,000 nM). Western blot and quantitative analyses show that levels of p-p38MAPK, p-JNK, and p-c-Jun, which were increased by LPS treatment, were significantly decreased by ghrelin in a dosage-dependent manner (Fig. 1). These results suggest that ghrelin inhibits microglia activation by LPS.

**Ghrelin inhibits proNGF and ROS production in LPS-induced BV-2 cells**

ProNGF is induced and plays the role of a death ligand in apoptosis of oligodendrocytes after SCI. In addition, we reported that proNGF production is dependent on p38MAPK activation in microglia. Thus, we examined the effect of ghrelin on proNGF production in LPS-induced BV-2 cells. As shown in Fig. 2A, B, proNGF production was increased by LPS, although an endogenous level of proNGF was produced in BV-2 control cells not treated with LPS. However, ghrelin treatment significantly inhibited proNGF production dose-dependently in LPS-stimulated BV-2 cells. It is also showed that JNK activation in microglia induces ROS production, leading to oxidative stress in BV-2 cells treated with fluoride. Thus, we examined whether ghrelin inhibits ROS production in LPS-activated BV-2 cells using the ROS-specific fluorescent dye, DCF-DA. Data show that the level of ROS was increased at 12 hours after LPS treatment, whereas ghrelin treatment (1,000 nM) significantly decreased ROS level induced by LPS (Fig. 2C, D).

**Ghrelin inhibits cell death of oligodendrocytes cocultured with LPS-stimulated BV-2 cells**

Since our data show that ghrelin inhibited the production of

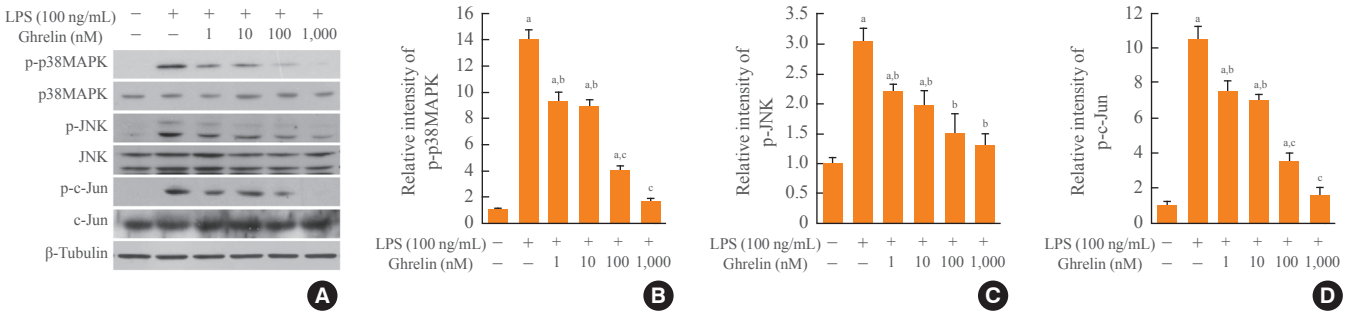


**Fig. 1.** Ghrelin inhibits p38 mitogen-activated protein kinase (p38MAPK) and c-Jun N-terminal kinase (JNK) activation in BV-2 microglia after lipopolysaccharide (LPS) stimulation. BV-2 cells were seeded in 6-well plates ($5\times10^5$ cells/well) and treated with ghrelin (1, 10, 100, and 1,000 nM) for 30 minutes prior to LPS (100 ng/mL) treatment. (A) Western blots of phosphor-p38MAPK (p-p38MAPK), p-JNK, and p-c-Jun at 30 minutes after LPS treatment. (B-D) Quantitative analysis of Western blots shows that ghrelin significantly inhibited the level of p-p38MAPK, p-JNK, and p-c-Jun as compared with LPS-treated cells. Values are expressed as mean±SD of three separate experiments. [a]$P<0.05$ vs. control; [b]$P<0.05$ vs. LPS-treated control; [c]$P<0.01$ vs. LPS-treated control.

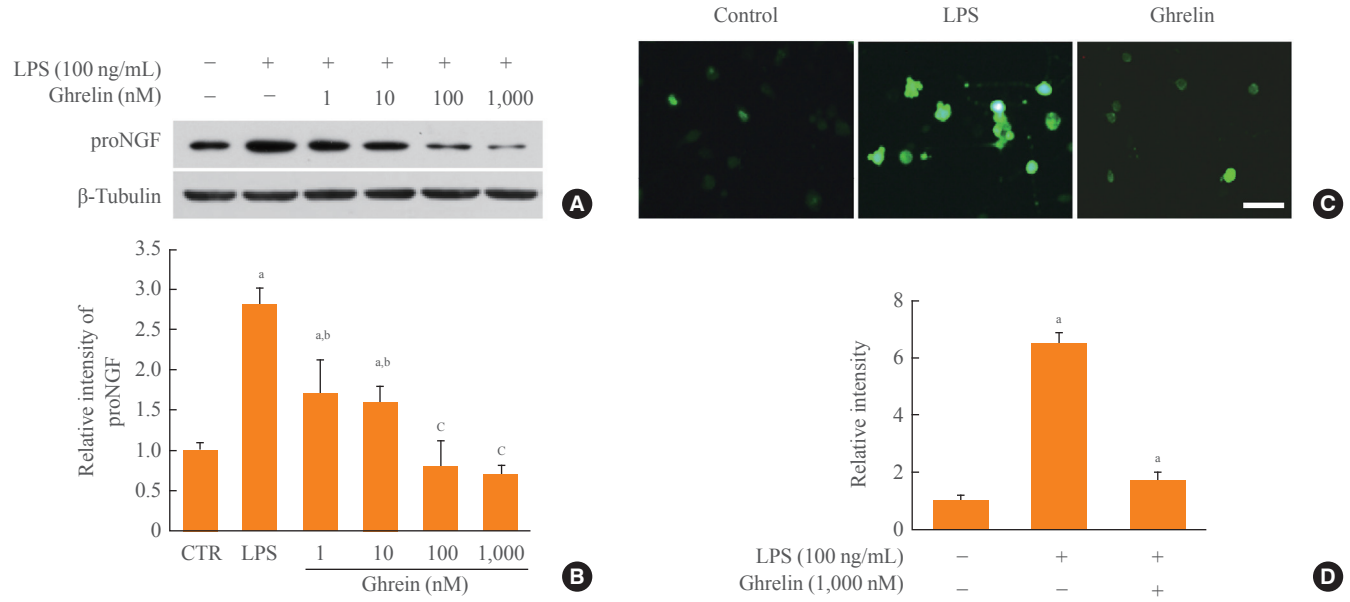


**Fig. 2.** Ghrelin inhibits pro-nerve growth factor (proNGF) and reactive oxygen species (ROS) production in BV-2 microglia after lipopolysaccharide (LPS) stimulation. BV-2 cells were seeded in 6-well or 24-well plates and treated with ghrelin (1, 10, 100, and 1,000 nM) 30 minutes before LPS (100 ng/mL) treatment. (A) Western blot of proNGF at 4 hours after LPS treatment. (B) Quantitative analysis of Western blots shows that ghrelin treatment significantly inhibited the expression of proNGF in a dose dependent manner. (C) Dichlorodihydrofluorescein (DCF) fluorescence in BV-2 at 12 hours was increased by LPS and decreased by ghrelin (1,000 nM). Scale bar, 10 μm. (D) Quantitative analysis of DCF fluorescence shows that ghrelin significantly decreased ROS production when compared to the LPS-treated control (CTR). Values are expressed as mean±SD of three separate experiments. [a]$P<0.05$ vs. control; [b]$P<0.05$ vs. LPS-treated control; [c]$P<0.01$ vs. LPS-treated control.

proNGF and ROS, which are known to lead to cell death of oligodendrocytes in experimental autoimmune encephalomyelitis and in SCI animal model, we next examined whether ghrelin protects cell death of oligodendrocytes cocultured with LPS-stimulated BV-2 cells by inhibiting proNGF and ROS production. After BV-2 cells were stimulated by LPS (100 ng/mL) with or without ghrelin (1,000 nM), primary oligodendrocytes were cocultured with BV-2 cells in transwell chambers (24-well plates). After 48 hours incubation, MTT assay and TUNEL staining were performed. When MTT assay was expressed as an oligodendrocyte cell survival (%), data show that LPS treatment significantly reduced cell survival of oligodendrocytes as compared with control cells not treated with LPS (Fig. 3A). However, ghrelin treatment significantly in-

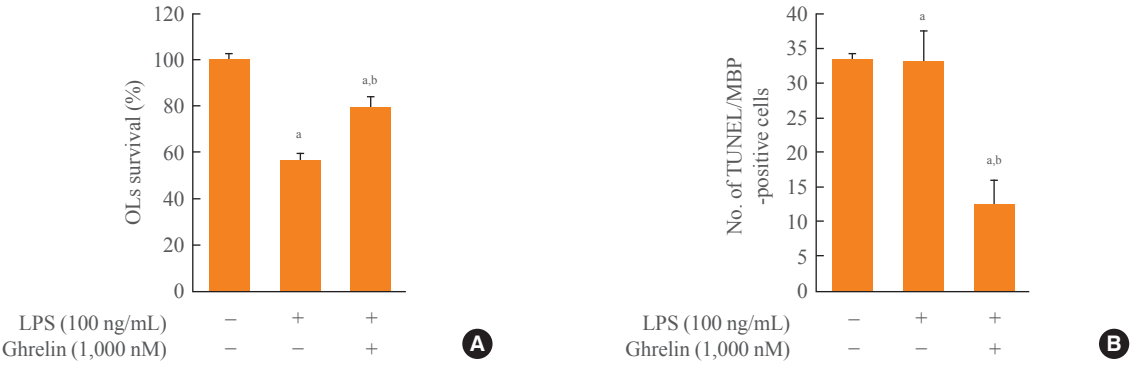


**Fig. 3.** Ghrelin inhibits cell death of oligodendrocytes cocultured with BV-2 cells activated by lipopolysaccharide (LPS). For oligodendrocyte/microglia cocultures, BV-2 cells were grown on porous upper inserts of transwell chambers in 24-well plates. After treatment with LPS (100 ng/mL) for 30 minutes, the inserts containing BV-2 were placed above mature oligodendrocyte culture in 24-well plates, allowing diffusion of soluble molecules. Treatment with ghrelin occured 30 minutes before LPS treatment. (A) Cell viability measured by MTT reduction assay at 48 hours. (B) Quantitative analysis of transferase-mediated deoxyuridine triphosphate-biotin nick end labeling: terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling/myelin basic protein (TUNEL)/MBP-positive oligodendrocytes at 48 hours. Values are expressed as mean±SD of three separate experiments. OL, oligodendrocytes. [a]$P<0.05$ vs. control; [b]$P<0.05$ vs. LPS-treated control.

creased cell survival of oligodendrocytes when compared with LPS-treated control cellsI (LPS, 56%±3.4%; LPS+ghrelin, 79%±5.0%; $P<0.05$). In addition, quantitative analysis of TUNEL-positive cells also show that ghrelin treatment significantly reduced oligodendrocyte cell death induced by LPS treatment (LPS, 33±4.5 cells; LPS+ghrelin, 12.5±3.3 cells; $P<0.05$) (Fig. 3B).

## DISCUSSION

In this study, we demonstrated that ghrelin as an anti-inflammatory hormone inhibits microglia activation, thereby attenuating oligodendrocyte cell death. Ghrelin treatment significantly inhibited LPS-induced p38MAPK and JNK activation in BV-2 cells. LPS-induced proNGF and ROS production in BV-2 cells was also significantly attenuated by ghrelin treatment, thereby reducing oligodendrocyte cell death in coculture with LPS-stimulated BV-2 cells.

Microglia are the resident macrophages in the central nervous system and play an important role in immune responses in the central nervous system. Previously we reported that minocycline treatment alleviated apoptosis of oligodendrocytes at least in part by inhibiting proNGF production in activated microglia after SCI [16]. In addition, we demonstrated that proNGF production was inhibited by treatment with SB203580, an inhibitor of p38MAPK, suggesting that production of proNGF is mediated via p38MAPK activation in microglia [16]. In this study, proNGF production in BV-2 cells activated by LPS was inhibited by ghrelin (Fig. 2A, B) and thereby oligodendrocyte cell death was significantly attenuated by ghrelin in coculture with LPS-stimulated BV-2 cells (Fig. 3). Ghrelin also inhibited p38MAPK activation in LPS-stimulated BV-2 cells (Fig. 1). Our data are in agreement with previous reports showing that proNGF induces cell death of oligodendrocytes after SCI [16,25,26]. Furthermore, these results suggest that ghrelin inhibited oligodendrocyte cell death by alleviating microglia activation in *in vitro* and can explain in part the underlying neuroprotective mechanism by ghrelin after SCI previously reported by our group [14]. Recently, the inhibitory effects of ghrelin on microglial activation in several animal models have been reported. For example, ghrelin inhibits neuronal apoptosis by inhibiting microglial activation in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease animal model [11]. In addition, ghrelin significantly reduces the level of proinflammatory cytokines such as tumor necrosis factor-α, interleukin (IL)-1β, and IL-6 in spinal cord microglia in experimental autoimmune encephalomyelitis, a representative animal model of multiple sclerosis [20]. Furthermore, our preliminary results show that ghrelin treatment significantly decreased the number of activated microglia after SCI (data not shown).

The endocrine activity of ghrelin is known to be mediated by growth hormone secretagogue receptor1a (GHS-R1a), a G protein-coupled receptor [27,28]. However, it has been reported that microglia do not express GHS-R1a in brain, spinal cord, and primary microglia cultures [11,14]. In addition, GHS-R1a

was not expressed in BV-2 cells when we determined the expression of GHS-R1a by RT-PCR and Western blot analysis (data not shown). Thus, at least in BV-2 cells, ghrelin may inhibit microglia activation via a ghrelin receptor-independent pathway in this study. The underlying mechanism how ghrelin inhibits microglia activation should be further studied.

Several studies have shown that LPS induces ROS production, including nitric oxide, via a mechanism mediated by the JNK pathway in BV-2 microglia cells [29,30]. Our data show that LPS induces JNK activation and ROS production in BV-2 cells as in previous reports (Figs. 1, 2B). In addition, ghrelin treatment significantly inhibited LPS-induced JNK activation and ROS production in BV-2 cells. It is also known that MAPK families, including JNK responsive to stress stimuli, are involved in the production of ROS [31]. Furthermore, it has been reported that nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is involved in fluoride-induced oxidative stress in BV-2 cells [32]. Thus our data suggest that ghrelin may inhibit ROS production by attenuating JNK activation in LPS-stimulated BV-2 cells, although a direct linkage between JNK and ROS by using a JNK inhibitor was not determined in this study. In addition, whether NADPH oxidase, as a source of ROS, is involved in the inhibition of LPS-induced ROS production by ghrelin should be studied.

Oligodendrocyte cell death contributes to demyelination, resulting in abnormal conductance of action potential in various pathological conditions such as a multiple sclerosis and SCI. In addition, microglia activation followed by inflammation and production of proNGF and ROS can contribute to oligodendrocyte cell death [16,33-35]. Taken together, ghrelin inhibited microglia activation, and the associated attenuation of ROS and proNGF production in this study, suggests the possibility of ghrelin as an anti-inflammatory therapeutic hormone for various pathological diseases accompanying oligodendrocyte cell death and microglia activation followed by inflammation.

## CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

## ACKNOWLEDGMENTS

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