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Synergistic activation of lipopolysaccharide-stimulated glial cells by propofol

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ABSTRACT

Despite the extensive use of propofol in general anesthetic procedures, the effects of propofol on glial cell were not completely understood. In lipopolysaccharide (LPS)-stimulated rat primary astrocytes and BV2 microglial cell lines, co-treatment of propofol synergistically induced inflammatory activation as evidenced by the increased production of NO, ROS and expression of iNOS, MMP-9 and several cytokines. Propofol augmented the activation of JNK and p38 MAPKs induced by LPS and the synergistic activation of glial cells by propofol was prevented by pretreatment of JNK and p38 inhibitors. When we treated BV2 cell culture supernatants treated with LPS plus propofol on cultured rat primary neuron, it induced a significant neuronal cell death. The results suggest that the repeated use of propofol in immunologically challenged situation may induce glial activation in brain.

1. Introduction

Propofol is an intravenous hypnotic agent used in the induction and maintenance of general anesthesia and procedural sedation. In addition to the anesthetic action, propofol has been implicated in the variety of CNS action including neuroprotection, damage to developing brain and induction of addictive behaviors [1–3].

Mechanical and immunological stimulation of brain induces activation of glial cells, which then secrete various cytotoxic/cytoactive molecules including ROS, RNS, proteainases, cytokines and extracellular matrix proteins. The activated glial cells, namely astrocytes and microglia, modulates overall inflammatory responses and may also provide trophic and regenerative supports. The final outcome of the inflammatory responses of the glial cells is extremely diverse and complicated in a way dependent on the stimulus modalities and activation status of the cells and surrounding environment, which make it important to understand the responses of the glial cells against a plethora of external stimuli. In spite of the widespread use in clinical situations, relatively few things are known regarding the role of propofol on the immunological activation of glial cells.

In a study using cultured rat glial cells, it has been suggested that propofol did not modulate inflammatory response of astrocytes stimulated with LPS [4]. Although production or release of inflammatory mediators was not measured, it was reported that intraperitoneal injection of propofol during induction of intracerabral hemorrhage in rats prevented activation of astrocytes and microglia as evidenced by GFAP and OX64 immunoreactivity along the 21 day follow up observation periods [5]. Based on the fact that propofol may confer neuroprotective effects [6–8], it was investigated whether propofol regulate P2X7 receptor activity and cytokine release [9]. Although propofol increased the activity of P2X7 receptors in activated astrocytes in clinically relevant concentration (30 μM), it did not contribute to the downregulation of the secretion of TNF-α [9]. Instead, enzyme-linked immunosorbent assay showed that propofol increased the secretion of TNF-α from astrocytes in high concentration (300 μM) [9]. These results suggest that propofol may increase inflammatory responses or activation of astrocytes either alone or in the condition of immunologically challenged situation. However, no clear demonstration of inflammatory activity of propofol is available at the moment. In this study, using cultured rat primary astrocytes or BV2 microglial cell lines, we investigated the effects of propofol on the several parameters of inflammatory activation of glial cells such as NO, ROS, cytokine and MMP-9 production.

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2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle medium (DMEM)/F12, fetal bovine serum (FBS) and other reagents for culture were obtained from Gibco BRL (Grand Island, NY, USA). Lipopolysaccharide (LPS, serotype O26:B6) and other chemicals were purchased from Sigma (St. Louis, MO, USA). Propofol was obtained from Myungmoon Pharm. (Seoul, Korea). Rabbit polyclonal iNOS antibody was obtained from Millipore (Billerica, MA, USA). Phospho-specific or total antibodies against p38 and JNK were obtained from Cell Signaling (Boston, MA, USA).

2.2. Rat primary cortical neuron and astrocyte culture

All animal experimental procedures were carried out using protocols approved by the Institutional Animal Care and Use Committee of the Konkuk University. Sprague–Dawley (SD) rat pups were obtained from SamTaKo (Seoul, Korea). Rat primary astrocytes and cortical neuron were prepared and cultured as we described previously [10]. Culture condition for BV2 microglial cell line was also described elsewhere [11].

2.3. Drug treatment

Cells were washed twice with serum-free media and then co-treated with LPS and propofol for 24 h under serum-free conditions to prevent the contamination of casein-digesting activity from serum. After treatment, the astrocyte or BV2 culture supernatants and cell lysates were harvested for further analysis. In all assay conditions used in this study, no cellular toxicity was observed as determined by morphological examination and MTT assay.

2.4. Measurement of nitric oxide (NO)

Nitric oxide production was determined by measuring nitrite, a stable oxidation product of NO using Griess reaction as described previously [12]. The absorbance was read at 550 nm with a microplate reader (Spectramax 190; Molecular Devices, Palo Alto, CA, USA).

2.5. Measurement of cell viability

Cell viability was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. MTT is a water-soluble tetrazolium salt that is reduced by metabolically viable cells to a colored, water-insoluble formazan salt. MTT (1 mg/ml) was added to the cell culture medium. After incubating the plates at 37 °C for 2 h in a 5% CO_2 atmosphere, the MTT-containing medium was replaced with dimethylsulfoxide (DMSO). The absorbance was read at 570 nm with a microplate reader (Spectramax 190; Molecular Devices, Palo Alto, CA, USA).

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

The expression of mRNAs encoding *iNOS*, *Il6*, *Il1β*, *Mmp9*, and *Gapdh* was determined by RT-PCR. The cells were washed twice with ice-cold PBS, and the total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was performed using 1 μg total RNA and MMuLV reverse transcriptase (MBI Fermentas, Glen Burnie, MD). The reaction was performed at 60 °C for 60 min and heated at 97 °C for 5 min; 1 μl from each RT reaction mixture was used for PCR amplification. The primer sequences for *iNOS*, *Il6*, *Il1β*, *Mmp9*, and *Gapdh* were described in supplementary Table 1. All the PCR products were resolved by 1.2% agarose gel electrophoresis and visualized with ethidium bromide. For quantification, the gels were photographed, and the pixel intensity for each band was determined using ImageJ (NIH) and was normalized to the band intensity of *Gapdh* mRNA.

2.7. Western blot analysis

Cells were harvested and homogenized in 100 μl/well SDS sample buffer containing 62.5 mM Tris–HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% (w/v) bromophenol blue, and 1 mM sodium orthovanadate. After boiling for 5 min, equal amounts of protein, determined by BCA protein assay (Thermo Scientific, Rockford, IL, USA), were subjected to 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and the separated proteins were electrophoretically transferred to nitrocellulose membranes (Whatman, Piscataway, NJ, USA) for 90 min. The blot was blocked with 5% nonfat dried milk at room temperature for 60 min and subsequently incubated overnight with primary antibodies described in materials section, which were diluted 1:2000 in 5% nonfat dried milk at 4 °C. After incubation with horseradish peroxidase–conjugated secondary antibodies at room temperature for 60 min, bands were detected using the enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA) and exposed to LAS-3000 image detection system (Fuji, Tokyo, Japan). Western blotting with a monoclonal antibody against β-actin (Sigma; 1:50,000 dilution) was used as a loading control.

2.8. Immunocytochemistry

Cultured rat primary cortical neuron on cover glass (Fisher Scientific, Nazareth, PA, USA), were washed and fixed with 4% paraformaldehyde at 4 °C for 1 h. The cells were treated with 0.3% Triton X-100 for 15 min at room temperature and blocked for 30 min with blocking buffer (3% BSA, 5% FBS in PBS) at room temperature. The cells were incubated overnight at 4 °C with primary antibodies against Tuj-1 (mouse, 1:500) and washed in PBS. Secondary antibodies were diluted in blocking buffer and incubated for 1 h at room temperature. Nuclei were labeled with DAPI or PI for counter fluorescence staining. The cover glass was mounted in Vectashield (Vector laboratories, Burlingame, CA) and was visualized using an immunofluorescence deconvolution microscope (Olympus, PLACE) equipped with image analysis software (MetaMorph).

2.9. Statistical Analysis

Results are expressed as mean ± SEM. Statistical comparisons were performed by using one-way ANOVA followed by Tukey's *post hoc* test using GraphPad Prism Version 5 software (California, USA), and a value of $P < 0.05$ was considered significant.

3. Results

3.1. Activation of astrocytes by propofol and LPS

We first treated cultured rat primary astrocytes with 1–10 μM concentrations of propofol for 24 h, which did not produce a significant effect on the production of NO in rat primary astrocytes (Fig. 1A) suggesting propofol alone does not stimulate rat primary astrocytes. As reported previously by many researchers including us, treatment with low concentration of lipopolysaccharide (LPS, 10 ng/ml) produced a significant increase in NO production in rat primary astrocytes (Fig. 1B). In this condition, co-treatment of propofol with LPS synergistically increased the production of NO in rat

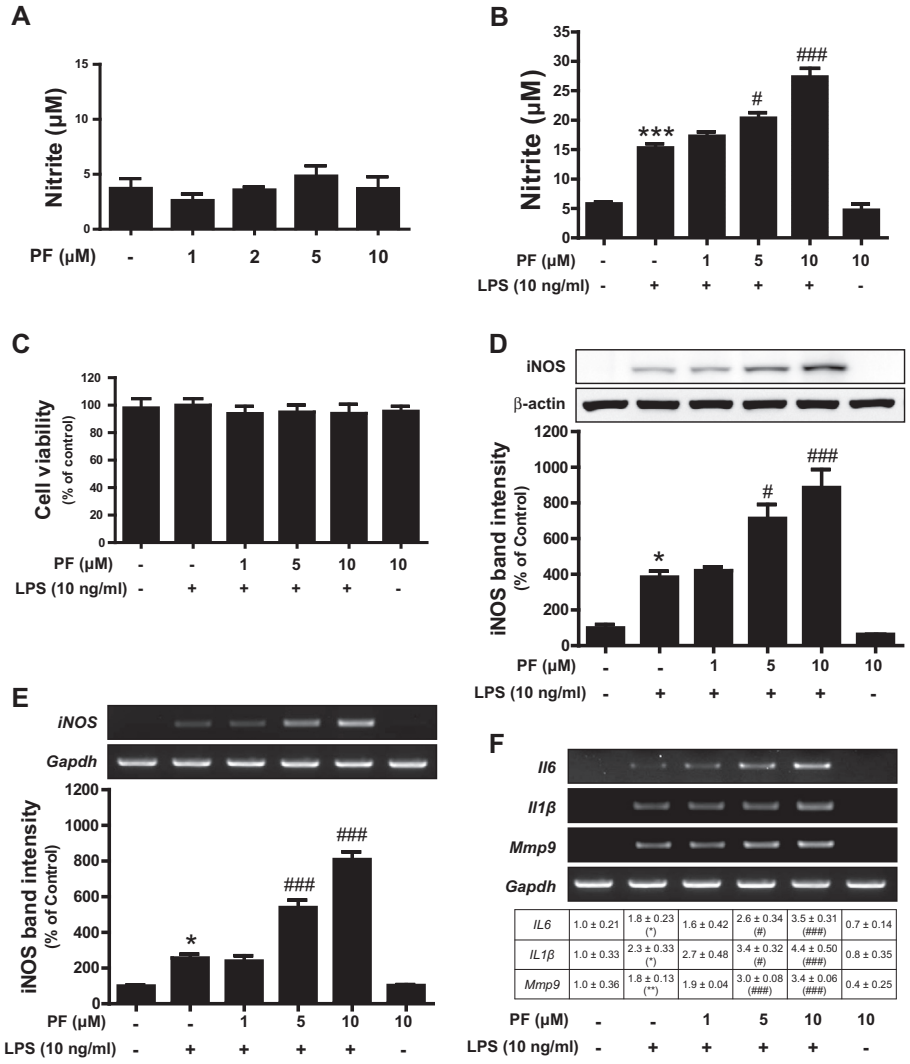

Fig. 1. Effect of LPS and propofol (PF) on Nitric oxide production in astrocytes. Rat primary astrocytes in serum-free DMEM/F12 were co-treated LPS (10 ng/ml) with indicated concentrations of PF (1, 5, and 10 μM). (A and B) The culture media were collected at 24 h after LPS and PF treatment and analyzed for nitric oxide production. (C) Cell viability was measured by MTT reduction assay. (D) Cell extracts were collected after 24 h and analyzed for *iNOS* protein expression by Western blot. (E and F) The cultured cells were harvested after 24 h and analyzed for *iNOS*, *Il6*, *Il1β*, *Mmp9*, and *Gapdh* mRNA expression by RT-PCR. The graph is the densitometric quantification data of iNOS band intensity. Values are expressed as mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.005$ vs. control and $^{\#}P < 0.05$, and $^{\#\#\#}P < 0.005$ vs. LPS ($n = 4$).

primary astrocytes in a concentration-dependent manner without deleterious effects on astrocytes survival as evidenced by MTT assay (Fig. 1C). Consistent with the synergistic increase in NO production, co-treatment of propofol with LPS increased LPS-induced protein and mRNA expression of iNOS, which was determined by Western blot and RT-PCR, respectively (Fig. 1D,E). Similarly, co-treatment of propofol with LPS increased ROS production in rat primary astrocytes (Supplementary Fig. 1). As we reported previously [13], LPS stimulation also induced *Mmp9* mRNA expression, a metalloproteinase which plays an important role in cell migration and BBB breakdown during brain injury (Fig. 1F). Co-treatment of propofol also increased *Mmp9* induction by LPS (Fig. 1F). Similarly, LPS induced expression of cytokines such as *Il-6* and *Il-1β*, and co-treatment of propofol with LPS augmented the expression of *Il-6* and *Il-1β* mRNA (Fig. 1F).

3.2. Signaling pathway mediating synergistic activation of astrocytes by propofol in LPS-stimulated condition

To investigate the signaling pathway with which propofol synergistically activates LPS-stimulated astrocytes, we examined the effects of propofol on LPS-induced JNK and p38 phosphorylation, the two signaling pathway implicated in inflammatory activation of LPS-stimulated astrocytes [14]. Consistent with previous reports [14], LPS induced phosphorylation of both JNK and p38 in astrocytes (Fig. 2A,B). Although propofol alone did not induce phosphorylation of JNK and p38, treatment with propofol synergistically increased the phosphorylation of JNK and p38 in LPS-stimulated astrocytes (Fig. 2A,B). When we treated LPS and propofol-stimulated astrocytes with SP600125 and SB203580, a JNK and p38 inhibitor, respectively, the synergistic production of nitrite was significantly inhibited (Fig. 2C,D) suggesting the essential role of JNK and p38 activation on propofol induced synergistic increase in NO production.

3.3. Activation of BV2 microglia by propofol

Next, we treated cultured mice microglial cell line BV2 with LPS and propofol. Similar to astrocytes, propofol synergistically induced mRNA and protein expression of *iNOS* in LPS-stimulated BV2 cells without effect on its own (Fig. 3A, B). Consistently, propo-

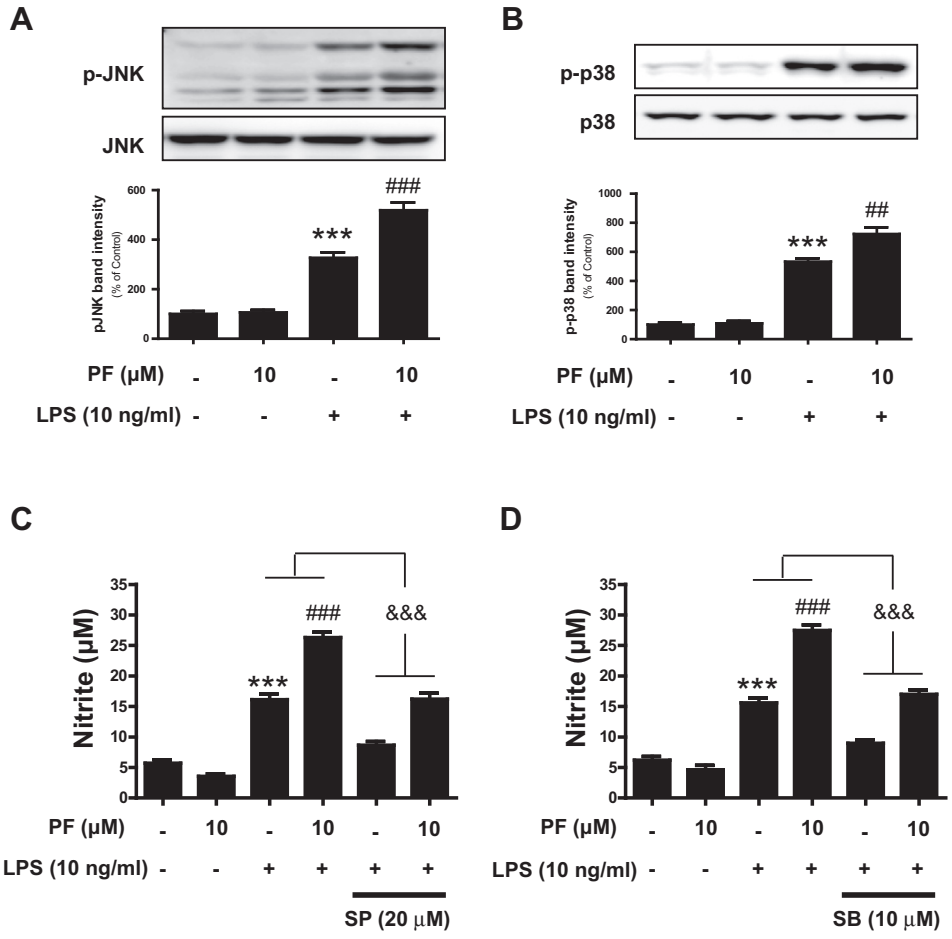

Fig. 2. Activation of p38, and JNK in rat primary astrocytes. Primary astrocytes in serum-free DMEM/F12 were co-treated LPS (10 ng/ml) with PF (10 μM). (A and B) Cells were harvested after 2 h and were analyzed for phosphorylated and total p38, and JNK by Western blot. (C and D) SB203580 (a p38 MAPK inhibitor, 10 μM), SP600125 (a JNK inhibitor, 20 μM) were pre-treated 30 min before LPS with PF application. The culture media were collected at 24 h after LPS and PF treatment and analyzed for nitric oxide production. The graph is the densitometric quantification data of phosphorylated and total p38, and JNK. Values are expressed as mean ± SEM. $^{***}P < 0.005$ vs. control, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.005$ vs. LPS, and $^{\&\&\&}P < 0.005$ vs. inhibitor, ($n = 4$).

fol augmented nitrite production in LPS-stimulated BV2 cells (Fig. 3C). We also examined the expression of *Il6*, *Il-1β*, *Mmp9*, and *Gapdh*, all of which were synergistically induced by propofol in LPS-stimulated BV2 cells (Fig. 3D). Even in BV2 cells treated with both LPS and propofol, we did not observe direct cellular toxicity, which was determined by MTT analysis (Fig. 3E).

3.4. Neuronal cell death induced by activated BV2 microglia

Overt activation of microglia may induce neuronal cell death and we examined this possibility by adding the culture supernatants obtained from BV2 cell culture to rat primary cortical neuron (Fig. 4). Rat primary cortical neuron was stained with Tuj-1 and cell death was morphologically examined by staining with propidium iodide. Although addition of BV2 conditioned culture media itself did not induce a significant cell death of rat primary cortical neuron, BV2 conditioned media obtained from LPS-stimulated cells induced a significant increase in propidium iodide positive cells. Interestingly, addition of BV2 culture conditioned media obtained from both LPS and propofol treated cells induced a synergistic increase in propidium iodide-positive cells (Fig. 4). These results suggest that synergistic activation of microglia by propofol in immunologically challenged condition may adversely affects the neuronal cell survival.

4. Discussion

In this study, propofol synergistically increased several endpoints of LPS-induced inflammatory activation of astrocytes and microglial cells in clinically relevant concentration without cellular toxicity. We used up to 10 μM concentration of propofol in this study and 30 μM of propofol in total blood is a concentration enough for inducing a loss of consciousness in more than 95% of patients [15].

Regarding the role of propofol on production of several inflammatory mediators, mixed results were reported in different cell types. For example, propofol did not affect LPS-induced nitric oxide or TNF-α production in mixed glial cells [4] but increased LPS-stimulated TNF-α production at supra clinical concentration in human whole blood [16]. In contrast, propofol inhibited nitric oxide and TNF-α release from alveolar macrophages in an intravenous endotoxin injection-induced acute lung injury model [17]. Propofol also reduced the induction of iNOS protein and mRNA in LPS-stimulated macrophages [18]. Several differences in experimental condition such as different concentrations of propofol and different types of cells used in those studies such as mixed vs. pure astrocytes or microglia and central vs. peripheral cells as well as differences *in vivo* and *in vitro* condition may explain the different results. Nevertheless, our results provide the need for more extensive investigation on the role of propofol on the production of

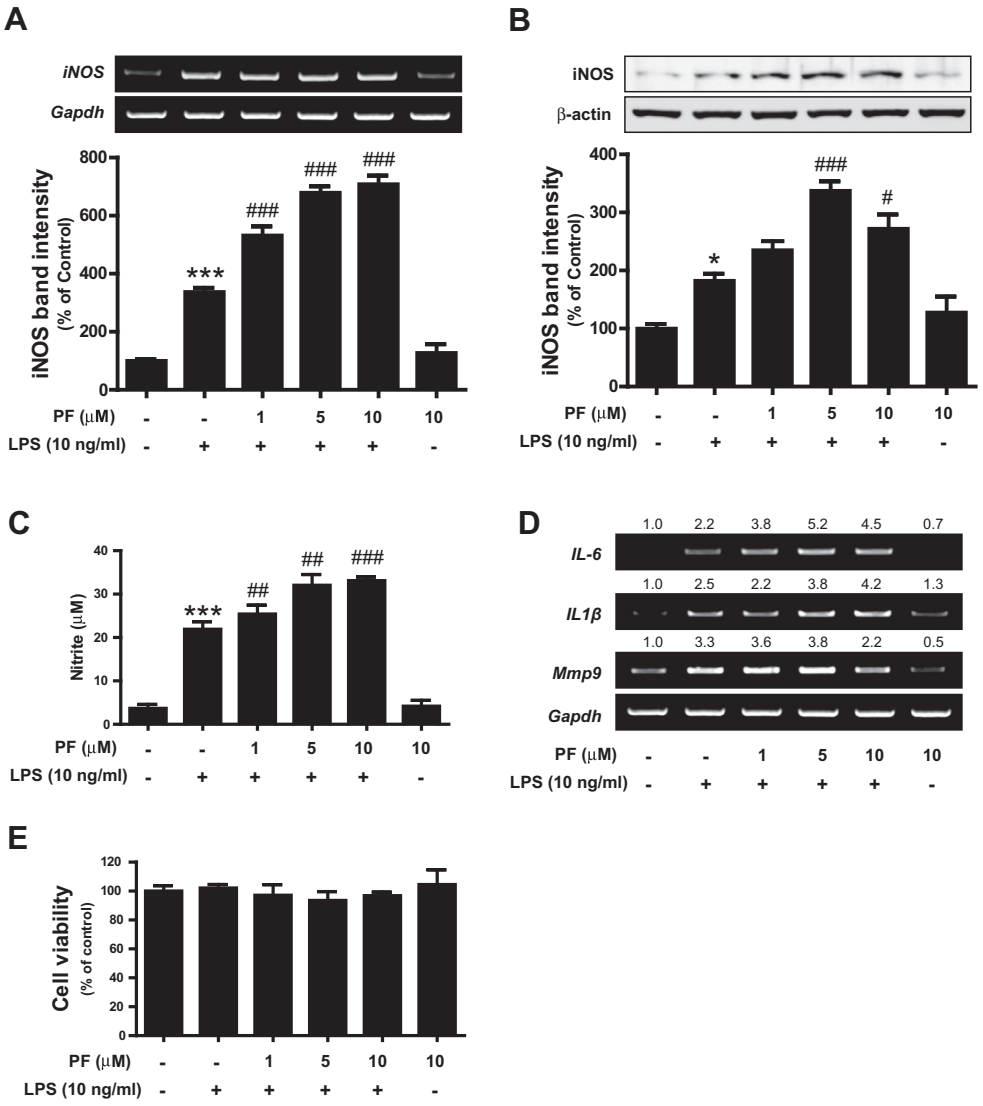

Fig. 3. Effects of LPS and PF on nitric oxide production in BV2 microglial cells. BV2 cells in serum-free DMEM were co-treated LPS (10 ng/ml) with indicated concentrations of PF (1, 5, and 10 μM). (A and D) The cultured cells were harvested after 24 h and analyzed for *iNOS*, *Il6*, *Il1β*, *Mmp9*, and *Gapdh* mRNA expression by RT-PCR. (B) Cell extracts were collected after 24 h and analyzed for iNOS protein expression by Western blot. (D) The culture media were collected at 24 h after LPS and PF treatment and analyzed for nitric oxide production. The graph is the densitometric quantification data of iNOS band intensity. Values are expressed as mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.005$ vs. control and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.005$ vs. LPS ($n = 4$).

inflammatory mediators in astrocytes or microglia in clinical or subclinical concentration.

In this study, the synergistic activation of LPS-stimulated glial cells by propofol was mediated by the activation of JNK and p38. Although different results were reported among alveolar epithelial cells, A549 human lung adenocarcinoma epithelial cells or cultured rat hepatocyte cell lines [19–21], it has been reported that propofol induced the expression of c-fos and egr-1 in MAPK activation-dependent manner in rat hippocampal slices [22].

The classical mechanism of propofol action includes the potentiation of GABAA receptor activity and blockade of sodium channel, none of which is directly involved in the activation of MAPK pathways or inflammatory stimulation in astrocytes. Recently, it has been suggested that endocannabinoid system is involved in the anesthetic and other unique features of propofol [23]. It has been suggested that propofol increases brain N-arachidonylethanolamine (anandamide) content and inhibits fatty acid amide hydrolase [24–26], which might be associated with the propofol's effects on memory formation and anti-nociceptive effects. Although activation of CB1 receptor induces activation of MAPK systems and initiates inflammatory responses including nitrosative and oxidative stress in a mouse model of type 1 diabetic cardiomyopathy as well as in cisplatin-induced model of nephropathy [27,28], most of the results obtained in astrocytes and microglia suggest that CB1 and CB2 receptor may decrease proinflammatory response either alone or in combination with LPS [29–33] suggesting endocannabinoid system in glial cells may acts differentially compared with peripheral cells. Further study might be required to unequivocally answer to these questions.

Considering a plethora of channels and receptors as well as signaling pathways modulated by propofol, physiological consequences of administration of different concentration of propofol at specific time window will be governed by the context of stimulus and involved cell types requiring more careful investigation on the final outcome of the effects. Moreover, the multiple use of propofol in a relatively short period of time may also have a profound effect on the modulation of inflammatory response of glial cells. *In vivo* animal experiments with immunologically challenged

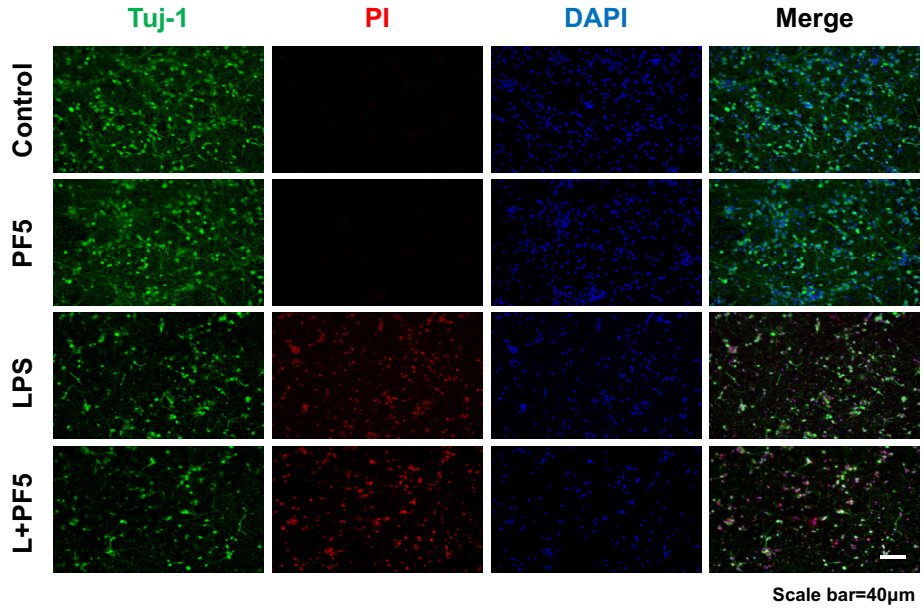

Fig. 4. Effects of BV2 microglial cell conditioned media treated with LPS plus PF on neuronal cell survival. Rat primary cortical neurons (DIV 9) were incubated for 24 h with BV2 microglial cells conditioned medium (BCM) diluted 1/3 in neurobasal medium. Neuronal cell death was determined by PI (red) staining after immunocytochemical staining of cultured neuron using an antibody against Tuj-1 (green). Inset is the representative magnified picture of each group ($n = 3$). (scale bar 40 μm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

situation such as chronic neurodegenerative diseases or cerebral ischemia may provide more relevant results in this regard.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.089.

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