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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