Original Article
Propofol inhibition of microglial inflammatory processes through the toll-like receptor 4-p38 mitogen-activated protein kinase signaling pathway

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Abstract: We investigated the effects of propofol on inflammatory processes induced by lipopolysaccharide (LPS)-treated microglia and elucidated the role of the toll-like receptor 4 (TLR4)-p38 mitogen-activated protein kinase (MAPK) pathway in such effects. First, BV-2 microglia were divided into control, propofol (30 µM), LPS (1 µg/mL), and LPS+ propofol (1 µg/mL LPS+ 30 µM propofol) groups. Then, enzyme-linked immunosorbent assay (ELISA) and MTT, polymerase chain reaction (PCR), and western blot assays were utilized to determine cell viability, inflammatory factors in the cell culture supernatant, and the expression of essential proteins in the TLR4-p38 MAPK inflammatory pathway. Microglial activation was significantly stronger in the LPS and LPS+ propofol groups than in the control and propofol groups (P < 0.05). The microglial cell viability was higher in the LPS+ propofol group than in the LPS group (P < 0.05). The interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α levels were higher in culture supernatant from the LPS and LPS+ propofol groups than in the control and propofol groups; the IL-1β, IL-6, and TNF-α levels were lower in the supernatant medium from the LPS+ propofol group than they were in the LPS group (P < 0.05). PCR and western blot results showed that microglial p38MAPK and TLR4 mRNA and protein expression was significantly higher in the LPS and LPS+ propofol groups than in the control and propofol groups (P < 0.05). Microglial p38 MAPK and TLR4 mRNA and protein expression was significantly lower in the LPS+ propofol group than in the LPS group (P < 0.05). Thus, our results indicated that propofol inhibits microglial activation and inflammatory reactions by possibly downregulating the TLR4-p38 MAPK signaling pathway.

Keywords: Inflammation, lipopolysaccharide, microglia, propofol, toll-like receptor 4

Introduction
Postoperative cognitive dysfunction (POCD), a central nervous system disorder, remains a common postoperative complication, especially in elderly patients. The most obvious clinical presentations of POCD are impairment of memory and the ability to concentrate. In addition, anxiety, reduced cognitive function, and personality changes may occur, potentially resulting in death in severely affected patients. Currently, our understanding of POCD is limited, and no effective measures have been identified to prevent it. Thus, POCD and its management constitute an ongoing challenge for clinical anesthesiologists [1].

The development and progression of POCD are closely related to inflammatory factor-mediated nerve inflammation and noninfectious nerve inflammation [2, 5]. Nerve inflammation, especially excessive activated microglial-induced nerve inflammation, is one of the most important factors contributing to the high incidence and underlying development of POCD [3, 4].

Microglia are the primary immune cells in the cerebrum. Any factor that disturbs brain homeostasis, such as pathogenic invasion or brain damage, can activate microglia [6]. The activation of microglia eliminates invading pathogens and induces inflammatory reactions, thereby serving a neuroprotective role in the brain [7]. However, microglial activation is also associated with cognitive deficits in disease states, with possible long-term impairment [8].

It is widely believed that medication- and surgery-induced stress disrupts cerebral homeostasis and provokes inflammatory reactions
Propofol inhibition of microglial inflammatory processes

that lead to molecular changes in signaling pathways that impair memory [9, 11]. Thus, excessive microglial-induced inflammatory reactions can damage neurons [10].

Propofol, a sedative widely used as an intravenous anesthetic, has beneficial effects on the cardiovascular [15], respiratory [16], and urinary systems [17]. Its neuroprotective effects have also been widely investigated [18]. Our previous studies have shown that propofol has significant anti-inflammatory activity [12]. Other studies, such as those conducted by Gui Bo et al. on cell models, have demonstrated that propofol inhibits the release of inflammatory factors from activated microglia and the expression of toll-like receptor 4 (TLR4) protein [13]. In addition, van Eldik et al. revealed that p38α mitogen-activated protein kinase (MAPK) signaling is the primary functional mechanism for neurodegeneration and the secretion of large amounts of tumor necrosis factor (TNF)-α after excessive microglial activation by lipopolysaccharides (LPSs) [14].

We hypothesized that propofol may inhibit microglial overactivation through the downregulation of the TLR4-p38 MAPK signaling pathway, thereby exerting a neuroprotective effect.

Materials and methods

Cell culture and treatments

Mouse BV-2 microglial cells (CAS, China) were inoculated in Dulbecco’s modified Eagle’s medium/Ham’s nutrient mixture F-12 (DMEM/F12; Gibco, USA) culture medium containing 10% fetal bovine serum (Gibco), 10 IU/mL penicillin (Invitrogen, USA), and 100 µg/mL streptomycin (Invitrogen), and grown in an incubator at 37°C and 5% CO2. Propofol was obtained from Sigma-Aldrich (USA). When the cells reached 70-80% confluence, 0.25% trypsin digestion was used to passage or harvest the cells for experiments.

The experimental cells were divided into five groups: (1) the control group (cells were incubated in serum-free DMEM/F12 culture medium for 24 h); (2) the LPS group (cells were treated with 1 µg/mL LPS for 24 h); (3) the propofol group (cells were treated with 30 µM propofol for 24 h); (4) the LPS+ propofol group (cells were pretreated with 1 µg/mL LPS for 1 h, followed by the addition of 30 µM propofol for 24 h); (5) the dimethyl sulfoxide (DMSO) group (cells were treated with DMSO at a final concentration of 0.1% for 24 h).

After completion of their respective treatments, the cell culture media and the cells were centrifuged at 5000 rpm for 15 min and the collected pellets were stored at -80°C until use.

Morphological observation

After treatment, the cells were observed using a binocular inverted light microscope (Nikon TS100, Japan), and their morphological changes were recorded.

Cell viability

A homogeneous cell suspension was placed into a 96-well plate (5 x 103 cells/well). After adhering to the plate, the cells were treated as described earlier in “Cell culture and treatments”. An MTT (Sigma-Aldrich) colorimetric method was utilized to determine the viability of each group of BV-2 microglial cells after 24 h. The following formula was used: Viability (%) = (ODtreatment group - ODblank group)/(ODcontrol group - ODblank group) x 100, where OD is optical density.

ELISA

After 24 h of treatment, the culture medium for each group of cells was centrifuged, and interleukin (IL)-1β, IL-6, and TNF-α levels in the supernatant were measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D systems, USA).

RT-qPCR

Total RNA was extracted from each group of cells after 24 h of treatment. Using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference, we performed a reverse transcription polymerase chain reaction (RT-PCR) analysis to determine the mRNA expression of p38 MAPK and TLR4 in microglia from each treatment group.
The PCR reaction conditions were as follows: denaturation at 95°C for 4 min, denaturation at 95°C for 30 s, annealing at 55°C for 60 s, elongation at 72°C for 60 s for 42 cycles, followed by elongation at 72°C for 5 min. The RT-PCR results are presented as CT, with $2^{-\Delta\Delta CT}$ representing the relative expression of the target gene, which was calculated using the following formula: $2^{-\Delta\Delta CT} = \frac{\text{expression of the target gene in each group of BV2 microglial cells}}{\text{expression of the target gene in the control group}}$.

The sequences of the PCR primers for the target genes and internal reference GAPDH are shown in Table 1.

Western blot

After 24 h of treatment, western Blots of each group of cells were used to measure p38 MAPK and TLR4 protein expression, and the Bradford assay was used to determine the total protein concentration. A 10-µL sample of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto a polyvinylidene difluoride membrane. The membrane was blocked with nonfat milk powder and incubated with mouse anti-TLR4 (1:500 dilution) and mouse anti-p38 MAPK (1:1250 dilution) primary antibodies overnight at 4°C. Then, secondary antibodies were added (1:1000 dilution). After incubation for 2 h at room temperature, an enhanced chemiluminescence reagent was added, and the membrane was exposed to an X-ray film [13]. A Quantity 1 image analysis system (Bio-Rad, USA) was used to determine the grayscale value of p38 MAPK and TLR4 protein bands for each treatment group.

Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM). GraphPad Prism 7.0 (GraphPad Software, USA) and SPSS 20.0 statistical software (SPSS, USA) were used for data analysis and processing. The homogeneity of variance test was used for comparing the means of the sample groups. One-way analysis of variance (ANOVA) was used for comparison of data between groups. The least significant difference method was used for pairwise comparisons of data between homoscedastic groups. Dunnett’s T3 test was used for comparing data between heteroscedastic groups. Differences with $P < 0.05$ were considered statistically significant. Differences with $P < 0.01$ were considered highly statistically significant.

Results

LPS enlarges microglial cells and thickens synapses

Cells in the control group were round or elliptical. Cell bodies had a relatively regular shape, and no obvious thickening of synapses was observed (Figure 1A). In the LPS group, activation of some microglia was observed, with obvious enlargement of the cell body, thickening of synapses and extension of large processes in all four directions, which was consistent with the morphological characteristics of activated microglia (Figure 1B).
Propofol inhibition of microglial inflammatory processes

Propofol improves viability of LPS-treated M1 microglial cells

We investigated the effect of propofol on the viability of M1-activated microglial cells by using an MTT assay to determine cell viability in each treatment group. We found that microglial cell viability in the LPS and LPS+ propofol groups was lower than in the control and propofol groups, respectively ($P < 0.001, P < 0.01$). Microglial cell viability in the LPS+ propofol group was higher than that in the LPS group ($P < 0.05$; Figure 2). Cell lysate from each treatment group was subjected to western blotting.

LPS stimulates the release of large amounts of inflammatory factors

We verified that LPS stimulates microglial activation by using ELISA to measure the levels of inflammatory factors in the culture supernatant. We found that IL-1β, IL-6, and TNF-α levels in the supernatant of the LPS group were higher than they were in the control and DMSO groups ($P < 0.001$). There were no significant differences in the levels of these cytokines between the control and DMSO groups ($P > 0.05$; Figure 3A-C).

Propofol has no effect on inflammatory factors released by quiescent microglia cells

We determined the effect of propofol on inflammatory factors released by quiescent microglia by using ELISA to detect the release of inflammatory factors into the supernatant medium. ANOVA revealed no significant differences in the levels of IL-1β, IL-6, and TNF-α between the supernatants of the control and propofol groups ($P > 0.05$; Figure 3D-F).

![Figure 3](image_url). Comparison of IL-1β, IL-6, and TNF-α content in the culture supernatant from the control, LPS, DMSO (A, B, C), propofol (D, E, F), and LPS+propofol groups(G, H, I). ***$P < 0.001$, $n = 8$.  

Propofol improves viability of LPS-treated M1 microglial cells
Propofol inhibition of microglial inflammatory processes

We determined the effect of propofol on inflammatory factors released by activated microglial cells using ELISA to measure the levels of inflammatory factors in the culture supernatants of the LPS and LPS + propofol groups. We found that the levels of IL-1β, IL-6, and TNF-α in the supernatant medium of the LPS + propofol group were significantly lower than in that of the LPS group ($P < 0.05$; Figure 3G-I).

Propofol significantly inhibits the mRNA expression of the signaling pathway components TLR4 and p38 MAPK following propofol treatment in activated microglia. We found that p38MAPK and TLR4 mRNA expression in the microglia of the LPS and LPS + propofol groups was higher than it was in the control and propofol groups ($P < 0.001$). Moreover, p38MAPK and TLR4 mRNA expression in the microglia of the LPS + propofol group was lower than it was in the LPS group ($P < 0.001$; Figures 4A and 4B).

Propofol significantly inhibits TLR4 and p38 MAPK protein expression in activated microglial cells

Western blot was used to determine the protein expression of the signaling pathway components TLR4 and p38 MAPK following propofol treatment in activated microglia. We found that p38MAPK and TLR4 protein expression in microglia in the LPS and LPS + propofol groups was higher than it was in the control and propofol groups ($P < 0.001$). Protein expression of p38 MAPK and TLR4 in microglia of the LPS + propofol group was lower than it was in the LPS group ($P < 0.01$ and $P < 0.001$, respectively) (Figures 5 and 6).

Discussion

Upon activation, microglia produce inflammatory factors such as IL-1β, TNFα, IL-1, and IL-6. While IL-1β is necessary for normal learning and memory, excessively high levels of IL-1β can negatively affect cognitive function [19, 20]. When IL-1β acts synergistically with other inflammatory factors such as TNFα and IL-6, the risk of cognitive dysfunction increases [21].

Studies have shown that IL-1β plays an essential role in cognitive dysfunction by interfering with the long-term function of the hippocampus [22-25], an important brain region involved in
Propofol inhibition of microglial inflammatory processes

memory [26]. IL-1β directly or indirectly stabilizes protein synthesis required for long-term plasticity and memory by interacting with microglia. Microglia and inflammatory factors work to inhibit neuronal development in the hippocampus, thus impairing memory [27]. In our study, large amounts of pro-inflammatory factors were released after the LPS-induced activation of microglia. This effect was significantly mitigated by propofol stimulation.

The anti-inflammatory effects of propofol have gained considerable attention. Studies have shown that propofol exerts a neuroprotective effect during the acute inflammation phase of neurological and cardiovascular diseases [30, 31]. Animal studies have also shown that propofol inhibits cytokine release during sepsis and reduces neutrophil-mediated inflammation during acute lung injury [32-34]. Clinical studies have shown that propofol reduces in vitro circulating cardiac reperfusion injury and pulmonary failure by mitigating free radical release and inhibiting the inflammation process [35, 36].

Our data showed that 30 µM of propofol significantly inhibited excessive M1 activation of microglia and reduced the release of inflammatory factors to induce a protective effect and prevent cell death, which was consistent with the results of the abovementioned studies [30-36]. We noted that the dose used (30 µM) is considered a clinically relevant concentration of propofol in BV microglia [29], as human studies have demonstrated that the concentration range of propofol at the site of action (i.e., brain) is 2-6 µg/mL, which corresponds to 11-33 µM [28].

With regard to the functional mechanism underlying inhibition of excessive microglial activation by propofol, we found that LPS-induced activation of microglia was accompanied by an increase in TLR4 and p38 MAPK expression. In contrast, LPS-induced microglial activation after propofol treatment was accompanied by a decrease in TLR4 and p38 MAPK expression. Thus, propofol inhibition of excessive microglial activation may occur via the TLR4-p38 MAPK signaling pathway. Consistent with our results, a study by Yi Wang showed that within a short time after surgery in aging rats, TLR4 expression significantly increases and short-term deficits in memory and learning occurs [37]. His study found that the development of cognitive dysfunction is most significant on postoperative day 1, which coincides with the peak expression of TLR4, IL-1β, and TNFα. With the passage of time, cognitive deficits gradually recover, accompanied by a return to baseline levels of TLR4, IL-1β, and TNFα. A study by van Eldik et al. revealed that the p38α MAPK signaling is the primary functional mechanism for secretion of large amounts of TNF-α and for neurodegeneration following excessive microglial activation by LPS [14]. In addition, they found that TLR4 activates microglial cells, which then secrete large amounts of IL-1β and TNFα. However, the addition of the p38α MAPK inhibitor MW01-2-069A-SRM significantly inhibits the microglial release of inflammatory factors [38]. Additional studies on different subunits of p38 MAPK have shown that p38α MAPK plays an important role in LPS-stimulated microglial secretion of pro-inflammatory factors, whereas the role of p38β MAPK is very limited [39]. This indicates that the functional mechanism underlying propofol inhibition of microglial activation warrants further investigation at the subunit level.

Taken together, the findings of the present study indicate that clinically relevant concentrations of propofol can significantly inhibit excessive activated microglia-induced inflammatory reactions, thereby having a protective effect on cell viability. Moreover, our findings suggest that propofol’s effects are exerted primarily through the TLR4-p38 MAPK signaling pathway.

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Disclosure of conflict of interest

None.

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Propofol inhibition of microglial inflammatory processes


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