

Propofol attenuates intermittent hypoxia induced up-regulation of proinflammatory cytokines in microglia through inhibiting the activation of NF- κ B/p38 MAPK signalling

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Folia Neuropathol 2017; 55 (2): 124-131

DOI: <https://doi.org/10.5114/fn.2017.68579>

Abstract

As immune sentinels of the central nervous system (CNS), microglia is pivotal cellular mediator of neuroinflammatory processes. Activation of microglia might elicit the expression of proinflammatory cytokines involved in the progression of neuroinflammatory diseases. Numerous studies have demonstrated that propofol (2,6-diisopropylphenol) has an effective anti-inflammatory property. Intermittent hypoxia (IH), as a result of obstructive sleep apnoea (OSA), could lead to neuron damage and neuroinflammation in the CNS. Here, we determined the effects of propofol on the inflammatory response in microglia during IH. The levels of nuclear factor- κ B (NF- κ B) inhibitor (I κ B) and activated p38 mitogen-activated protein kinase (MAPK) exposed to IH with or without propofol treatment were detected by Western blot. The viability of cells exposed to various concentrations of propofol was monitored with MTT assay. The production and mRNA levels of tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) were evaluated by qRT-PCR and ELISA, respectively. As results, IH exposure obviously promoted the activation of NF- κ B/p38 MAPK signalling and the secretion of TNF- α and IL-6. Propofol was not toxic to microglia. Compared with the control group, propofol attenuated the IH-induced activation of NF- κ B and p38 MAPK, which accompanied with reduction of proinflammatory cytokine secretion. These data suggested that propofol down-regulated the IH-induced secretion of proinflammatory cytokine, and inhibit inflammatory responses in microglia, and might be involved in attenuation of the p38 MAPK and NF- κ B signalling pathways. Overall, propofol could contribute to alleviating IH-induced CNS diseases in patients by inhibiting p38 MAPK and NF- κ B mediated inflammation in microglia.

Key words: propofol, intermittent hypoxia, proinflammatory cytokine, microglia.

Introduction

Neuroinflammation mediated by the activation of glia has been shown to be associated with the progressive neuronal damage in a variety of neurodegenerative diseases, such as Alzheimer's disease (AD), Huntington's disease (HD), and amyotrophic lateral

sclerosis (ALS) [1,12]. Microglia are considered to be the resident immune cells in the central nervous system (CNS), which can be activated by various stimuli. Under inflammatory conditions, microglia will be activated and play a vital role in regulating inflammatory reactions by releasing diverse inflammatory

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factors including cyclooxygenase-2 (COX-2), nitric oxide (NO) and proinflammatory cytokines such as interleukin-1 beta (IL-1 β), necrosis factor- α (TNF- α) and interleukin-6 (IL-6) [5,17,20]. However, overactive microglia lead to excessive secretion of neurotoxic mediators, which can result in severely detrimental neuronal damage and neurodegenerative diseases. Consequently, blockade of the activation of microglia could reduce subsequent neuroinflammation, providing a promising therapy for the treatment of inflammation-mediated neurodegenerative diseases.

Propofol, with potent sedation/hypnotic properties, is a widely used intravenous agent for induction and maintenance of anaesthesia, as well as sedation in the intensive care unit [21]. Accumulated studies suggested that propofol exhibits anti-inflammatory effects. For instance, propofol inhibited inflammatory responses in lipopolysaccharide (LPS)-induced alveolar epithelial type II cells [18]. And it attenuated secretion of proinflammatory cytokines in a hepatocyte nuclear factor-1 α -dependent manner [19]. Emerging reports suggested that propofol effectively down-regulated inflammatory responses by inhibiting the phosphorylation of nuclear factor- κ B (NF- κ B) and p38 mitogen-activated protein kinase (MAPK) [25,26,33]. Propofol blocked astrocyte activation upon stimulation with LPS via presenting the NF- κ B, p38 MAPK and extracellular signal-regulated protein kinases (ERK) 1/2 pathways [33]. Moreover, propofol exerted effectively anti-inflammatory properties in human neuroglioma cells treated by sevoflurane through suppressing the NF- κ B signalling pathway [26]. An earlier report supported that anti-inflammatory mechanisms of propofol were shown through decreasing the level of phosphorylated p38 MAPK [25]. The anti-inflammatory property of propofol in microglia has become one of the most exciting research topics.

Intermittent hypoxia (IH), one of the pathophysiological changes resulting from obstructions of the upper airway during sleep, may play a key role in the structural neuron damage in the CNS, especially excitotoxicity in hippocampal neurons [8,9,36]. Moreover, IH would lead to the up-regulation of ROS, NO, and downstream pro-inflammatory responses [2,30]. Thus, we investigated the effects of propofol on the secretion of proinflammatory molecules in IH-exposed microglia, and evaluated the activation of NF- κ B and p38 MAPK during these processes.

Material and methods

Cell culture

BV2 microglial cells were provided by the State Key Laboratory of Brain and Cognitive Science, Institute of Biophysics, Chinese Academy of Science. The cells were seeded into six-well plates in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Scientific, Rockford, Illinois, USA) containing 10% foetal bovine serum (Gibco, Grand Island, NY, USA), 1% penicillin, and 1% streptomycin (Invitrogen, Shanghai, China), and incubated in a humidified 5% CO₂ atmosphere at 37°C. After incubation for 24 h, the cells were cultured with fresh medium and used as microglia for later experiments.

Intermittent hypoxia exposure and propofol treatment

Microglia cells in the IH exposure group were maintained at 37°C and 5% CO₂ in the hypoxic chamber with shifted O₂ levels between 1% and 21% for 400 sec/cycle. Cells in the control group were cultured at 5% CO₂ and normoxic conditions (21% O₂). Both groups were treated for 8 h.

Referring to the clinically relevant blood concentration of propofol [10,22], different concentrations of 0, 25, 50 and 100 μ M of prepared propofol were used to pre-treat the microglia 0.5 h prior to IH exposure. Cells in the control group were treated with neither propofol nor IH.

Western blot

Cells were homogenized in lysis buffer and then the total proteins were isolated through centrifuging at 12,000 \times g for 15 min at 4°C. Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (Amersham Life Science, West Chester, PA, USA). The PVDF membrane was blocked in tetrapropyl benzene sulfonate (TPBS) with 5% skimmed milk for 2 h at room temperature. Followed by incubation with primary rabbit Monoclonal Antibodies (Cell Signaling Technology, Beverly, MA, USA): anti-I κ B (cat. no. 4812, 1 : 100), anti-p38 MAPK (cat. no. 9212, 1 : 1,000), anti-phosphorylated p38 MAPK (cat. no. 9211, 1 : 500), and mouse anti- β -actin (1 : 200) for 2-4 h at room temperature, respectively. Next, the membranes were incubated with secondary anti-

bodies for 1 h at room temperature. The blotting signals were detected using enhanced chemiluminescence (ECL) reaction reagents.

MTT assay

The effect of propofol on cell viability was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kits (Sigma-Aldrich, St. Louis, MO, USA). Cells in various concentrations of the propofol pre-treated group and the control group were plated at a density of 4×10^6 per well, and incubated for 8 h. Then, 10 μ l MTT solution was added to each well and continued to incubate for 4 h. The supernatant was removed when the reaction terminated. Finally, the optical density (OD) at 570 nm was measured by a microplate reader (Bio-Rad 550, California, USA).

qRT-PCR

Total RNA was extracted from microglia with Trizol reagent (Invitrogen, Shanghai, China) and reverse transcribed to complementary DNA (cDNA) by using RNA PCR kit (Takara, Shiga, Japan) according to the instructions described by the manufacturer. qRT-PCR was performed in the 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Relative gene expression was done by the comparative CT method with β -actin gene as the internal control. The primer sequences were as follows: β -actin-F (5' CCGCCACCAGTTCGCCATG 3') and β -actin-R (5' AGGAAGAGGATGCGGCAGTGG 3'); TNF- α -F (5' GCCTCTTCTCATTCTGCTCGT 3') and TNF- α -R (5' GCTGACGGTGTGGGTGAGGA 3'); IL-6-F (5' AAATGCCAGCCTGCTGACGAAC 3') and IL-6-R (5' AACACAATCTGAGGTGCCATGCTAC 3'). The following PCR condition was applied: 95°C for 30 sec, 40 cycles at 95 °C for 5 sec, and 60°C for 30 sec. The relative expression fold change of mRNAs was calculated using the $2^{-\Delta\Delta C_t}$ methods.

ELISA

The levels of proinflammatory cytokines (TNF- α and IL-1 β) were detected using TMB enzyme-linked immunosorbent assay (ELISA) kit (Enzyme-linked Biotechnology Co., Ltd, Shanghai, China) according to the manufacturer's protocol. The absorbance was detected at 450 nm in a microplate reader (Bio-Rad 550, California, USA).

Statistical analysis

All experiments were repeated three times, and data were expressed as the means \pm standard error of the mean. Significant differences were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc analysis using GraphPad Prism 5. $P < 0.05$ was considered statistically significant.

Results

Intermittent hypoxia-induced activation of NF- κ B/p38 MAPK signalling and expressions of TNF- α and IL-6

Considering that the activity of NF- κ B was involved in phosphorylation of p38 MAPK and expression of NF- κ B inhibitor, I κ B, western blot was conducted to identify the level of phosphorylated p38 MAPK and the expression level of I κ B in IH treated microglia. The results showed that the phosphorylation level of p38 MAPK was dramatically increased with IH treatment for 8 h, while the expression level of I κ B was significantly decreased with IH treatment for 4 h, especially after treatment with IH for 8 h (Fig. 1A). These results indicated that NF- κ B signalling pathway was activated by IH treatment.

Since NF- κ B is a potent regulator of the expression of proinflammation cytokines including TNF- α and IL-6, and the expressions of these two proinflammatory cytokines are dramatically induced by IH, we detected the mRNA levels of TNF- α and IL-6 in microglia with IH treatment by qRT-PCR. Compared with normal conditions, IH exposure gave rise to obviously increased TNF- α ($p < 0.05$) and IL-6 mRNA levels ($p < 0.05$) (Fig. 1B). Furthermore, we measured secretion levels of TNF- α and IL-6 in the serum by ELISA. Consistent with the qRT-PCR, IH-induced remarkably secretions of TNF- α ($p < 0.001$) and IL-6 ($p < 0.05$) (Fig. 1C), confirming that IH could induce inflammatory response in microglia.

Non-cytotoxicity of propofol to BV2 microglia

To further investigate the effect of propofol on IH-induced activation of NF- κ B signalling and inflammatory response, cytotoxic effects of propofol were monitored by evaluating the alteration in the

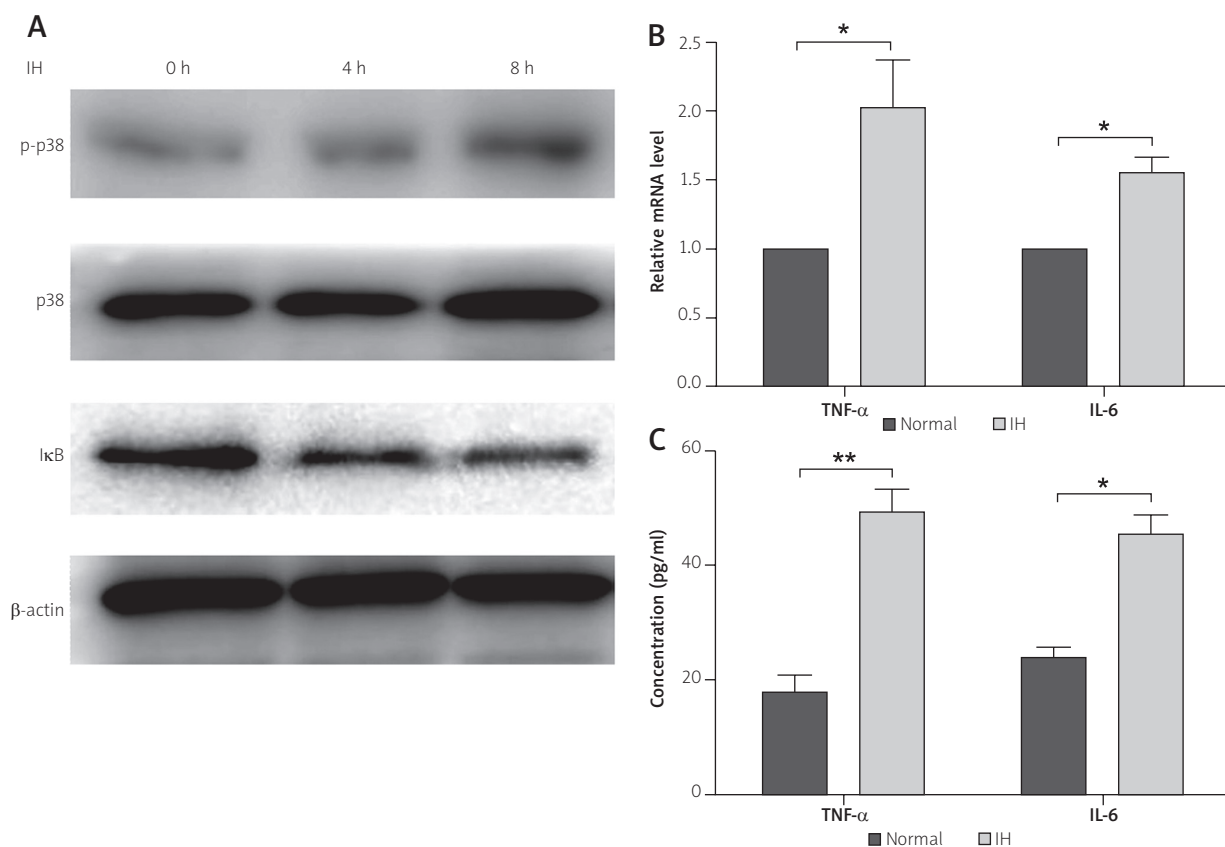


Fig. 1. Effect of intermittent hypoxia (IH) on activation of NF- κ B/p38 MAPK signalling and expressions of TNF- α and IL-6. **A)** Effects of IH on p38 MAPK phosphorylation and I κ B expression were assayed by western blot. **B)** Effects of IH on TNF- α and IL-6 mRNA levels were demonstrated by qRT-PCR. **C)** Effects of IH on TNF- α and IL-6 protein levels were demonstrated by ELISA analysis. Data are presented as the mean \pm standard error of the mean. * p < 0.05, ** p < 0.001. p-38 – p-38 MAPK, p-p38 – phosphorylated p-38 MAPK, I κ B – inhibitor of NF- κ B, TNF- α – tumor necrosis factor- α , IL-6 – interleukin-6.

viability of microglia via the MTT assay. Compared with controls, there was no significant difference in the viability of BV2 microglia with treatment of diverse concentration (0-250 μ M) of propofol (Fig. 2).

Propofol attenuated intermittent hypoxia-induced activation of NF- κ B/p38 MAPK signalling in microglia

To examine whether propofol exerts any effects on the phosphorylation level of p38 MAPK and the expression level of I κ B in microglia during IH exposure, we investigate the protein levels using western blot. Without IH exposure, the phosphorylation of p38 MAPK remains at a relatively low level, while IH obviously improved the phosphorylation level of p38 MAPK in microglia, and this effect was partially suppressed by pre-treatments with different concentrations of propofol (25 μ M, 50 μ M, 100 μ M) (Fig. 3).

Correspondingly, compared with the controls, IH dramatically decreased the level of I κ B, this effect was alleviated with propofol pre-treatment in a concentration-dependent manner. With pre-treatment of 50 μ M propofol following IH stimulation, the level of I κ B increased a little, and the effect was the most notable with pre-treatment of 100 μ M propofol (Fig. 3). These observations suggested that propofol could interfere with the IH-induced NF- κ B signalling through regulating the phosphorylation level of p38 MAPK and the expression level of I κ B.

Propofol suppressed intermittent hypoxia-induced TNF- α and IL-6 production in microglia

To investigate whether pre-treatment with propofol could inhibit IH-induced production of TNF- α and IL-6 in microglia, we measured the mRNA expression

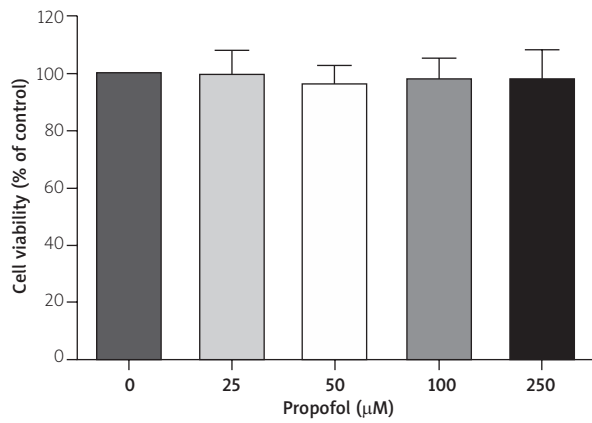


Fig. 2. Effect of propofol on cell viability of microglia was detected by MTT assay. Data are presented as the mean ± standard error of the mean.

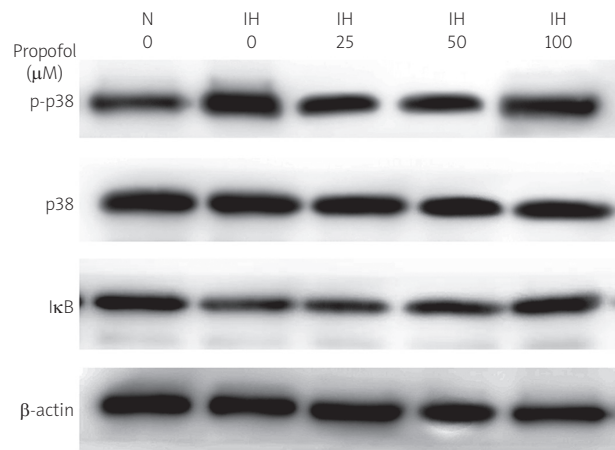


Fig. 3. Effects of propofol on IH-induced alterations of p38 MAPK phosphorylation and IκB expression were analyzed by western blot. p-38 – p38 MAPK, p-p38 – phosphorylated p-38 MAPK, IκB – inhibitor of NF-κB.

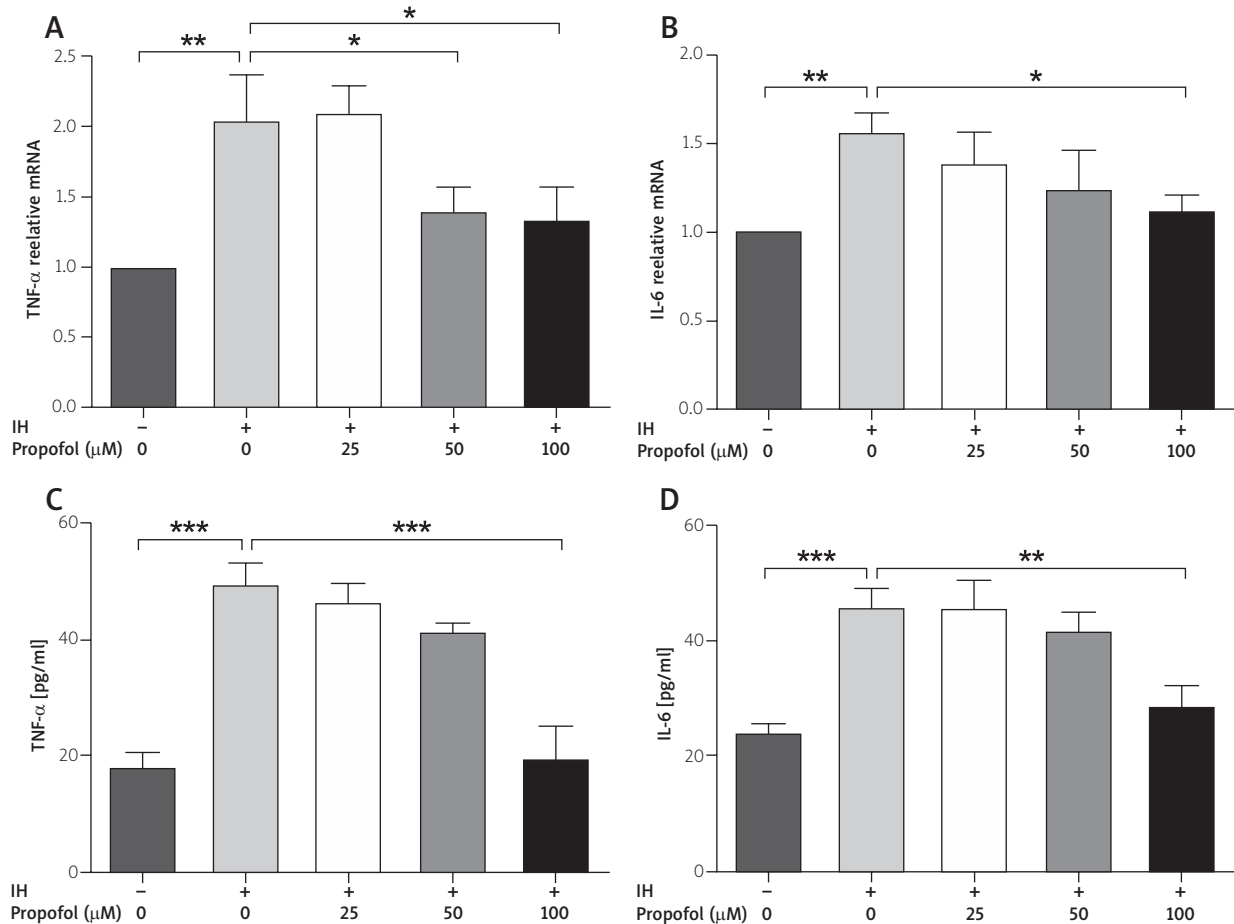


Fig. 4. Effects of propofol on IK-induced TNF-α and IL-6 expression. **A)** TNF-α mRNA level was detected by qRT-PCR. **B)** IL-6 mRNA level was detected by qRT-PCR. **C)** TNF-α protein level was detected by ELISA analysis. **D)** IL-6 protein level was detected by ELISA analysis. Data are presented as the mean ± standard error of the mean. **p* < 0.05, ***p* < 0.001, ****p* < 0.001. TNF-α – tumor necrosis factor-α, IL-6 – interleukin-6.

level of TNF- α and IL-6 after microglia treatment with IH in the presence or absence of propofol via qRT-PCR. IH exposure led to a remarkable increase in the TNF- α ($p < 0.001$) and IL-6 ($p < 0.001$) levels (Fig. 4A and B). Propofol at a concentration of 25 μ M had no effect on IH-induced mRNA levels of TNF- α and IL-6. However, propofol at a concentration of 50 or 100 μ M significantly suppressed the IH-induced TNF- α level ($p < 0.05$) (Fig. 4A), and pre-treatment with 100 μ M propofol remarkably reduced IH-induced IL-6 level ($p < 0.05$) (Fig. 4B). Furthermore, the secretion amount of TNF- α and IL-6 into serum under aforementioned conditions were determined by ELISA. We found that IH exposure gave rise to an extremely remarkable rise of TNF- α ($p < 0.0001$) and IL-6 ($p < 0.0001$) in serum, and these effects were obviously attenuated by pre-treatment with 100 μ M propofol (Fig. 4C and D).

Discussion

Microglia, as resident immune cells of the CNS, would be activated under inflammatory conditions and followed by inflammatory cascade in the CNS [4,23]. Previous reports confirmed that IH can lead to the activation of microglia and further induce neuron dysfunction and neuroinflammation [32]. Recently, the anti-inflammatory property of propofol has attracted increasing attention. A wealth of information linking that propofol prohibited inflammatory response through preventing the NF- κ B and p38 MAPK signalling and then reducing the release of inflammatory mediators is published [25,26]. In addition, emerging evidence showed that propofol selectively attenuated the activation of NF- κ B and p38 MAPK in vascular endothelium during IH exposure [16]. The effect of propofol on IH treated microglia was still unclear. Thus, in the current study, we focused on microglia and examined whether propofol exerts an efficient effect on microglia exposed to IH.

Among the inflammatory mediators induced by activated microglia, TNF- α and IL-6 are two of the main proinflammatory cytokines. TNF- α plays a core role in the proinflammatory cytokines and its temporary existence will promote the secretion of IL-1, IL-6, and other secondary inflammatory mediators. IL-6 could serve a dual function by also causing inflammation and resisting inflammation. Considering that its clearance rate is far below than that of other cytokines, the level of IL-6 can reflect the severity

of the disease and prognosis. Overproduction of TNF- α and IL-6 can cause various neurodegenerative diseases, including AD and Parkinson's disease (PD) [3,29]. It has been reported that propofol can inhibit the release of inflammatory factors such as TNF- α and IL-6 [11,27]. This study first reported that propofol significantly reduced the IH-induced secretion of proinflammatory cytokines, TNF- α and IL-6, in microglia.

NF- κ B, a pleiotropic regulator involved in microglial cell responses, can suppress the production of proinflammatory enzymes and cytokines, including NO, PGE2, TNF- α and IL-6 [13,24,34]. Under normal circumstances, NF- κ B binds to I κ B to form an inactive heterodimer in the cytoplasm. While suffering from inflammation, I κ B is phosphorylated and subjected to degradation by proteasomes. Consequently, NF- κ B will be disassociated from I κ B and translocate into nucleus, followed by the activation of NF- κ B-dependent proinflammatory molecules [6,7]. It has been demonstrated that propofol suppresses the IH-induced NF- κ B activity by preventing the phosphorylation of I κ B and accompanied by down-regulated secretion of proinflammatory cytokines in the vascular endothelial cells [16].

Additionally, MAPKs, including the extracellular signal-regulated kinase (ERK), the c-jun NH2-terminal kinases (JNK) and the p38 MAPK, can be phosphorylated and then positively regulate the expression of inflammatory genes and cytokines [31,35]. Among these, p38 MAPK acts as a crucial regulator for the production of NF- κ B-mediated proinflammatory cytokines and other mediators [15]. Glucocorticoid A (GLA) can inhibit pro-inflammatory cytokines expression in microglial cells through NF- κ B and p38 MAPK signalling pathways [14]. A recent report clarified that triroside attenuated the neuroinflammation in microglia via NF- κ B and p38 MAPK signalling pathways [28]. Furthermore, propofol suppressed the NF- κ B-mediated inflammation by prohibiting the activation of p38 MAPK in IH-treated vascular endothelium [16]. Consistently with previous reports, IH-induced activation of NF- κ B and p38 MAPK both were dramatically reduced by propofol treatment in the present study. Combined with all the results, we showed that propofol alleviates IH-induced secretion of proinflammatory cytokines in microglia probably via inhibiting NF- κ B and p38 MAPK signalling.

In conclusion, the present study highlighted the crucial role of propofol in hampering IH-induced

microglia inflammation, which would result in serious neuron damage and dysfunction and further leads to neurodegenerative diseases. Therefore, propofol with the effect of neuroprotection is a novel therapeutic objective for neurodegenerative disorders.

Acknowledgments

The study was supported by the National Natural Science Foundation of China (81670083). We are grateful to Professor Qingyun Li, the Ruijin hospital affiliated to the Shanghai Jiao Tong University School of Medicine, for providing the intermittent hypoxia cell culture chamber and intermittent hypoxia control system.

Disclosure

Authors report no conflict of interest.

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