Delayed treatment of propofol inhibits lipopolysaccharide-induced inflammation in microglia through the PI3K/PKB pathway

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Propofol is a short-acting intravenous anesthetic agent with potential neuroprotective effect. In this study, we aim to investigate whether delayed propofol treatment is protective against lipopolysaccharide (LPS)-stimulated inflammatory responses in microglial cells. Cultured BV2 microglial cells were exposed to propofol at various time points after initiation of LPS stimulation. Nitrite production and cell viability were assessed after stimulation with LPS for 24 h. The effect of propofol on mRNA levels of cyclooxygenase-2 (Cox-2), interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS), tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β) was analyzed using reverse transcription PCR (RT-PCR) 6 h after LPS stimulation. The production of TNF-α and reactive oxygen species was also studied. Propofol applied 0–4 h after the initiation of LPS dose-dependently inhibits nitric oxide production. Propofol application also decreased LPS-induced Cox-2, IL-6, iNOS, TNF-α, and IL-1β mRNA expression and induced significant protein kinase B (PKB) phosphorylation in BV2 cells. Treatment with phosphoinositide 3-kinase (PI3K)/PKB inhibitor wortmannin decreased PKB phosphorylation induced by propofol, and abolished the inhibitory effect of propofol on LPS-stimulated NO, reactive oxygen species and TNF-α production. Our results suggest that delayed propofol treatment can reduce LPS-induced activation of microglial cells. These effects may be mediated by activation of the PI3K/PKB pathway. *NeuroReport* **29:839–845**

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Introduction

Microglia cells play an important role in host defense and tissue repair in the central nervous system [1]. Under physiological conditions, microglial cells are typically found in a resting state. However, in response to injury, infection, or inflammation, microglia rapidly change to an activated state [2]. To date, at least two activation phenotypes have been suggested, consisting of a classical response (M1) and the alternative response (M2). The M1 phenotype, activated by lipopolysaccharide (LPS) and the proinflammatory cytokine interferon-γ (IFNγ), generates high levels of proinflammatory cytokines and oxidative metabolites such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), and nitric oxide (NO). In contrast, the M2 type microglia activation is associated with expression of wound repair and healing mediators such as arginase-1, and anti-inflammatory cytokines IL-4 and IL-10 [3].

Propofol (2, 6-diisopropylphenol) is an intravenous sedative-hypnotic agent that is widely used for general anesthesia and sedation. Propofol decreases cerebral oxygen consumption and reduces intracranial pressure, making it a suitable drug for neurosurgical patients [4]. The neuroprotective effect of propofol is attributed to its antioxidant property, the potentiation of γ-aminobutyric acid type A-mediated inhibition of synaptic transmission, and the inhibition of glutamate release [5–9]. However, few studies have been conducted to investigate the effects of propofol on microglia activation related to neuronal injury. Moreover, the molecular mechanisms by which propofol confers anti-inflammatory activities in the microglia remain to be elucidated.

The current in-vitro and in-vivo studies have demonstrated the protective effect under a pretreatment strategy. In the real clinical situations such as traumatic brain injury and cerebral hemorrhage, however, the onset of insult usually occurs before the administration of anesthesia. The objective of the present study was to investigate the effect of delayed treatment of propofol on the inflammatory response induced by LPS in immortalized murine BV2 microglial cells *in vitro*. LPS stimulation of the microglia has been widely used as a useful in-vitro model to study the mechanisms underlying neuronal injury by various proinflammatory and neurotoxic factors released from activated microglia [10]. We found that propofol down-regulated the production of proinflammatory mediators including NO, reactive oxygen species (ROS), and TNF-α

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through activating phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB) signaling pathways.

Materials and methods

BV2 microglial cell culture and treatment

The murine BV2 cell line was obtained from the China Center for Type Culture Collection (Wuhan, China) and cultured as previously described [11]. In summary, cells were cultivated in Dulbecco's Modified Eagle's Medium (Cat. No. 11965092; Gibco, Shanghai, China) supplemented with 10% fetal bovine serum (Cat. No. 11011-8615; Every Green, Hangzhou, China), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified incubator with 5% CO_2. For the experiments, confluent cultures were passaged by trypsinization and treated in culture medium overnight before treatments.

In the first set of experiments, propofol (Cat. No. 56931; Sigma, Shanghai, China) at final concentration of 50 μM was applied 1 h before or 0, 1, 2, 4, 6 h after the onset of LPS (10 ng/ml, Cat. No. L-4391; Sigma) stimulation to determine the treatment window for propofol. Then, different concentrations (0, 12.5, 25, 50 and 100 μM) of propofol were incubated with the cells 1 h after the addition of LPS stimulation to identify the optimal dosage of propofol. In the second set of experiments, 50 μM of propofol was incubated with the cells 1 h after the addition of LPS stimulation. In the third set of experiments, the specific PI3K inhibitor wortmannin (1 μM, Cat. No. W-1628; Sigma) was added to the incubation medium during propofol application to determine if the PI3K/PKB signaling pathway might be involved in propofol's action on BV2 cells. In the fourth set of experiments, wortmannin (1 μM) and propofol were added to the incubation medium 1 h after LPS exposure.

Measurement of nitrite production

The concentrations of NO in culture supernatants were determined by measurement of nitrite, which is a major stable product of NO, using Griess reagent (Cat. No. G-7921; Invitrogen, Shanghai, China). BV2 microglial cells (2×10^5 cells/well) were plated onto 96-well plates and treated with different concentrations of propofol plus 10 ng/ml of LPS for 24 h after LPS stimulation. The cell supernatants were collected and mixed with Griess reagent according to the manufacture's instruction, followed by incubation at room temperature for 30 min. The absorbance was measured at 540 nm on a microplate reader (Biotek, Winooski, Vermont, USA). Nitrite concentration was determined from a sodium nitrite standard curve.

Analysis of cell viability

Cell viability was assessed using a microculture 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based colorimetric assay (Cat. No. M5655; Sigma). After 24 h of incubation with propofol and LPS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to cell cultures to give a final concentration of 357 μg/ml, and the samples were incubated for 2 h at 37°C. The supernatant was then removed, and the formazan crystals produced in viable cells were solubilized with 150 μl of dimethylsulfoxide (Cat. No. C2639; Sigma). Finally, the absorbance of each well was read at 570 nm using a microplate reader. The relative cell viability (%) was expressed as a percentage of the untreated control.

Isolation of total RNA and reverse transcription polymerase chain reaction

We attempted to examine the relative expression of proinflammatory genes with RT-PCR, taking into consideration its limitations [12,13]. In summary, BV2 microglia cells were stimulated with 10 ng/ml LPS for 1 h, followed by delayed treated with propofol (50 μM) for an additional 5 h. Total RNA was isolated using TRIzol reagent (Cat. No. 15596-026; Life Technologies, Shanghai, China). The total RNA obtained from cells was used in RevertAid First Strand cDNA Synthesis Kit (Cat. No. K1621; Thermo Scientific, Shanghai, China) to produce cDNAs. The inducible nitric oxide synthase (*iNOS*), cyclooxygenase-2 (*Cox-2*), *TNF-α*, *IL-1β*, and *IL-6* genes were amplified from the cDNA by PCR. Reaction conditions were optimized in preliminary experiments so that amplifications were within the logarithmic phase and yields were approximately linear with input cDNA concentration. After amplification, PCR reactions were separated electrophoretically on 1% agarose gels and visualized by ethidium bromide staining. Band intensity was expressed as relative absorbance units. The ratio between the sample RNA to be determined and β-actin was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency.

Western blot analysis

BV2 cells were treated with propofol for 0, 30, and 60 min, respectively. The treated cells were washed with ice-cold PBS followed by homogenization in 0.2 ml ice-cold lysis buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA) containing protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM Na_3VO_4, and 1 mM NaF). Cell lysates were centrifuged at 12 000*g* for 15 min at 4°C. The supernatant was collected and protein concentration was determined using BCA protein assay kit (Biosharp, Hefei, China). Samples of the lysates were separated by 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes. After being placed in blocking buffer, the membranes were incubated with the anti-p-PKB (Cat. No. 4691; Cell Signaling Technology, Shanghai, China) and anti-PKB (Cat. No. 9271; Cell Signaling Technology, Shanghai, China) primary antibodies (1 : 1000 dilutions) followed by horseradish

peroxidase-conjugated secondary antibodies. The ECL western blotting detection reagents (Millipore, Shanghai, China) were used for visualization of the protein bands.

Measurement of intracellular ROS

Intracellular ROS levels were measured by 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate (Cat. No. C6827; Invitrogen). In brief, BV2 cells were stimulated with LPS (10 ng/ml) and propofol for 6 h. The cells were then incubated with 10-μM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate for 45 min at 37°C 5% CO_2. Fluorescence was measured on a microplate reader using excitation and emission wavelengths of 485 and 528 nm, respectively. Data are presented as raw data in arbitrary units.

Measurement of TNF-α production

BV2 microglial cells were plated at a density of 2×10^5 cells/well in a 96-well plate. Cells were then treated with propofol and 10 ng/ml of LPS. The cell-free supernatant was collected at 24 h stimulation with LPS. TNF-α was measured by ELISA kits (Cat. No. DY410; R&D Systems, Shanghai, China) according to the manufacturer's instructions. The absorbance at 450 nm was determined using a microplate reader.

Statistical analyses

Data are expressed as mean ± SEM of six independent determinations. Statistical significance was determined using analysis of variance followed by Newman–Keuls's test. A P value of less than 0.05 was considered statistically significant.

Results

Propofol attenuates LPS-stimulated nitric oxide production.

LPS stimulation of NO release has been well-established as an in-vitro model of microglia activation. To evaluate the effect of propofol on NO production, BV2 microglia cells were stimulated with 10 ng/ml LPS. Cell supernatants were collected and assayed for NO production using the Griess reaction assay. As shown in Fig. 1a, incubation with LPS alone for 24 h resulted in a marked increase in nitric oxide production by 14-fold over the basal level (0.52 ± 0.02 and 7.09 ± 0.19 μM without and with LPS, respectively; $n = 6$; $P < 0.001$). Propofol at 50 μM applied 1 h (2.93 ± 0.37 μM) before and 0 h (3.42 ± 0.38 μM), 1 h (3.38 ± 0.31 μM), 2 h (4.15 ± 0.69 μM), and 4 h (4.76 ± 0.50 μM) after LPS treatment remarkably decreased the levels of NO to 41.4, 48.3, 47.8, 58.5, and 67.1%, respectively ($n = 6$; $P < 0.001$ vs. LPS). The maximum inhibitory effect was observed at 1 h before and 0 and 1 h after LPS treatment. However, delayed application of propofol at 6 h or longer after the addition of LPS did not significantly change nitric oxide level (Fig. 1a). Concentration–response study showed that propofol, applied 1 h after the initiation of LPS stimulation, attenuated LPS-mediated nitric oxide production in a concentration-dependent manner ($n = 6$; $P < 0.001$; Fig. 1b).

To exclude the cytotoxic effect of propofol in BV2 microglia, MTT assay was performed after treatment with propofol in the presence or absence of LPS (10 ng/ml). The result demonstrated that the selected concentrations of propofol did not lead to obvious cytotoxicity. The cells viability was within the range of 94–108% by MTT assay in all cases, confirming that the inhibition of nitric oxide production in BV2 cells was not owing to a cytotoxicity action of propofol or LPS (Fig. 1c–d).

Propofol modulates LPS-stimulated proinflammatory mediators expression

To assess the effect of propofol on expression of proinflammatory mediators, BV2 microglia cells were stimulated with LPS and treated with propofol for 6 h. The mRNA levels of *Cox-2*, *IL-6*, *iNOS*, *TNF-α*, and *IL-1β* were analyzed using RT-PCR (Fig. 2). The results show that exposure of BV2 cells to LPS increased the production of *Cox-2*, *IL-6*, *iNOS*, *TNF-α*, and *IL-1β* mRNA levels by 4.1-, 4.2-, 2.0-, 2.5-, and 7.7-fold, respectively, in comparison with the untreated control ($n = 6$; $P < 0.001$). However, delayed treatment of 50-μM propofol 1 h after LPS stimulation significantly decreased the levels of *Cox-2*, *IL-6*, *iNOS*, *TNF-α*, and *IL-1β* mRNA to 68.3, 73.0, 75.8, 73.9, and 70.9%, respectively ($n = 6$; $P < 0.01$ for *Cox-2* and *iNOS*; $P < 0.001$ for *IL-6*, *TNF-α*, and *IL-1β*). These data indicated that propofol exerts anti-inflammatory properties by inhibiting LPS-stimulated production of proinflammatory mediators in BV2 cells.

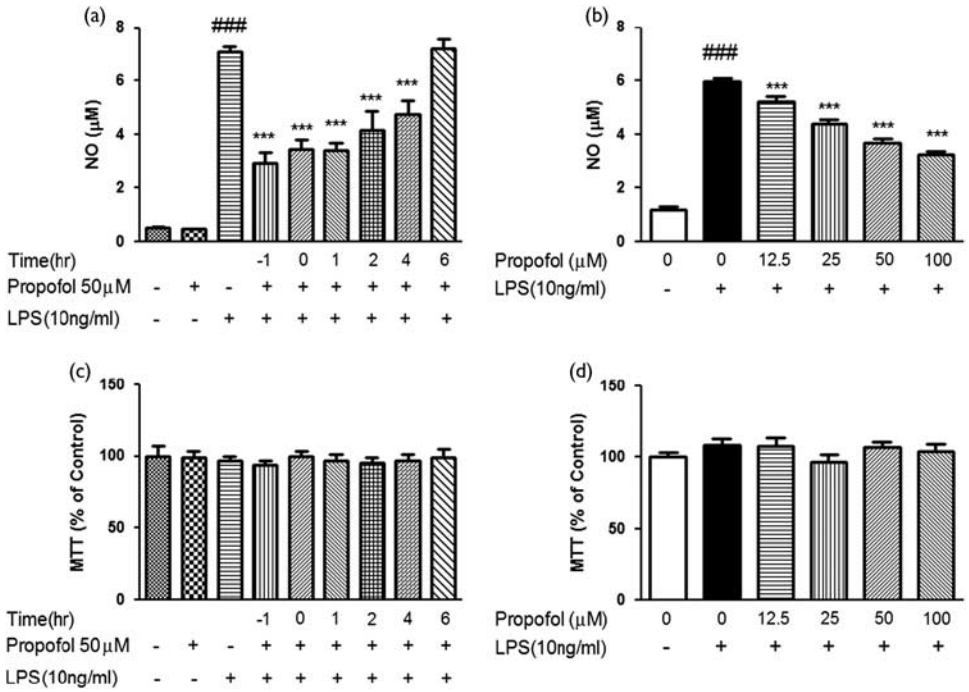
Effects of propofol on PKB phosphorylation

PI3K/PKB is a critical regulatory protein in the immunologic defense system against diseases [14]. To explore the possible intracellular mechanisms contributing to the anti-inflammatory effect of propofol, treatment of the BV2 cells with propofol (50 μM) for different time periods was conducted to assess the capacity of propofol on PKB activation. As shown in Fig. 3, propofol induced 2.1-fold increases in PKB phosphorylation beginning at 30 min after treatment (211 ± 12% of control; $n = 6$, $P < 0.01$), and the activation was still elevated by 2.3-flod till 1-h incubation (235 ± 3% of control; $n = 6$, $P < 0.01$). Treatment with wortmannin, an inhibitor of PI3K/PKB signaling pathways, abolished the propofol effects on PKB Phosphorylation. Neither propofol nor wortmannin had any effects on PKB expression at the time points that were investigated.

PI3K inhibitor blocks propofol-induced anti-inflammation

To determine whether the activation of the PI3K/PKB pathway by propofol is instrumental to the anti-inflammatory activity of propofol, we further investigated

Fig. 1



Propofol attenuates lipopolysaccharide (LPS)-stimulated nitric oxide production. (a) Effect of propofol (50 μM) on nitric oxide production when administered 1 h before and 0, 1, 2, 4, and 6 h after LPS treatment. (b) Effect of delayed propofol (0–100 μM) treatment on LPS-induced nitric oxide production. (c) Effect of propofol (50 μM) on cell viability when administered 1 h before and 0, 1, 2, 4, and 6 h after LPS treatment. (d) Effect of delayed propofol (0–100 μM) treatment on cell viability when administered 1 h after LPS treatment. Data are mean ± SEM, $n = 6$ independent measurements per group. $^{###}P < 0.001$ versus control; $^{***}P < 0.001$ versus LPS group. Statistical analyses were performed by one-way analysis of variance with Student–Newman–Keuls post-hoc test.

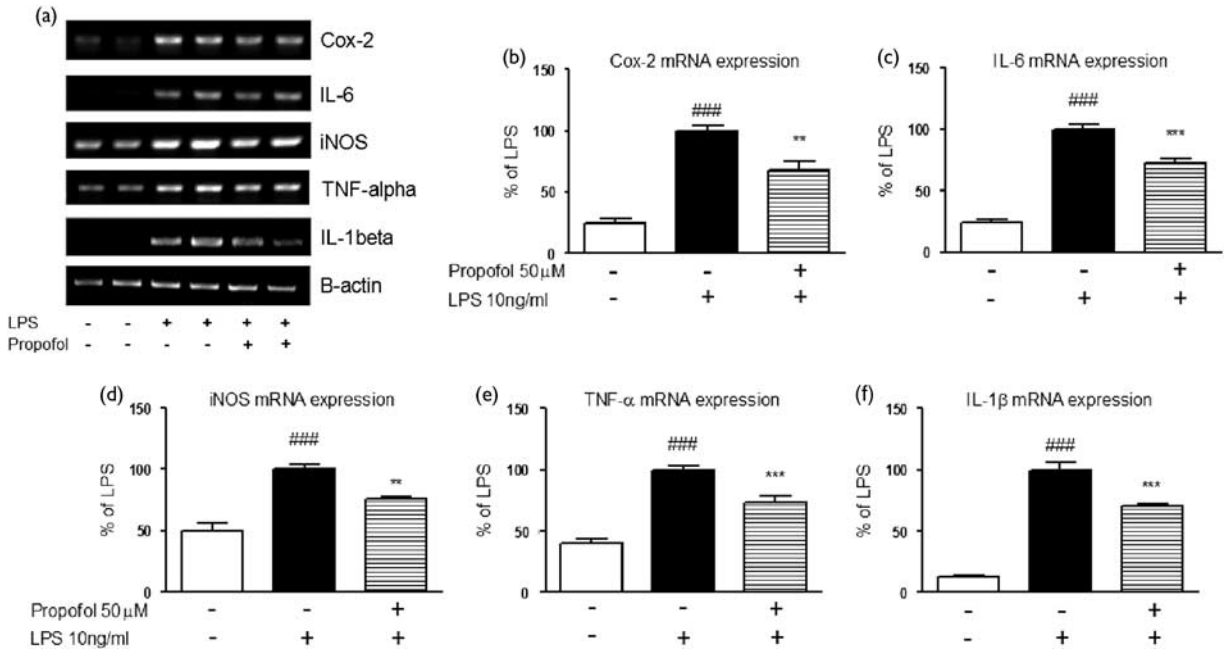
whether the inhibition of PI3K/PKB by wortmannin, could suppress the propofol effects on LPS-induced inflammatory responses in BV2 cells. As shown in Fig. 4, propofol (50 μM) markedly decreased the production of proinflammatory mediators including NO, ROS, and TNF-α when compared with the LPS-treated cells. However, the inhibitory effect of propofol on NO (3.41 ± 0.25 to 5.64 ± 0.42 μM, $n = 6$, $P < 0.001$), ROS (1432 ± 57.5 to 1773 ± 93.2 absorbance, $n = 6$, $P < 0.05$), and TNF-α (3706 ± 68.7 to 4695 ± 341.6 pg/ml, $n = 6$, $P < 0.001$) productions was almost completely restored by wortmannin. The data suggested that the effect of propofol on reducing the production of inflammatory mediators in LPS-stimulated BV2 microglia depends on activation of the PI3K/PKB pathway.

Discussion

Microglia are the principal resident immune cells in the central nervous system. They are thought to be quiescent in the healthy brain and to activate only during episodes of brain injury or disease. LPS stimulation of microglial cells to express iNOS and subsequent NO release has been well-established as an in-vitro model of microglia activation. We showed that the NO production was dose-dependently attenuated by propofol that was applied before or immediately after the application of LPS. Furthermore, propofol protection still existed when it was applied even at 4 h after the LPS treatment. To the best of our knowledge, this study demonstrated for the first time that delayed treatment with propofol can induce a protective effect in microglia. The cell viability was unaffected after treatment with propofol up to 100 μM, suggesting propofol did not have a significant cytotoxicity toward BV2 microglia cells.

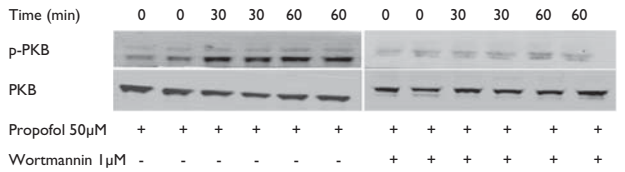
NO, prostaglandin E2, and proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 are known to be important mediators in the development of inflammation. In the central nervous system, microglia appear to rapidly express iNOS and Cox-2 in response to a wide range of injuries and pathogenic stimuli. There is also abundant evidence that cytokines such as IL-1β, TNF-α, and IL-6 are involved in the inflammatory process, and they can be indicators for microglia activation [12,13]. As expected, LPS stimulation elicits a robust proinflammatory response in our in-vitro experiment model, with the upregulation of both the levels of inflammatory mediators and the proinflammatory cytokines. We showed that propofol attenuated LPS-stimulated iNOS, IL-6 and Cox-2, TNF-α, and IL-1β expression mainly at

Fig. 2



Propofol inhibits the expression of lipopolysaccharide (LPS)-stimulated proinflammatory mediators. BV2 microglia cells were stimulated with LPS, followed by delayed treatment with propofol for a 6-h incubation period. Representative gel images of semi-quantitative RT-PCR of the proinflammatory markers (a) and quantified mRNA expression of cyclooxygenase-2 (Cox-2) (b), interleukin-6 (IL-6) (c), inducible nitric oxide synthase (iNOS) (d), tumor necrosis factor-α (TNF-α) (e), and interleukin-1β (IL-1β) (f) were measured. Data are mean ± SEM, $n = 6$ independent measurements. $^{###}P < 0.001$ versus control; $^{**}P < 0.01$, $^{***}P < 0.001$ versus LPS group. Statistical analyses were performed by one-way analysis of variance with Student–Newman–Keuls post-hoc test.

Fig. 3



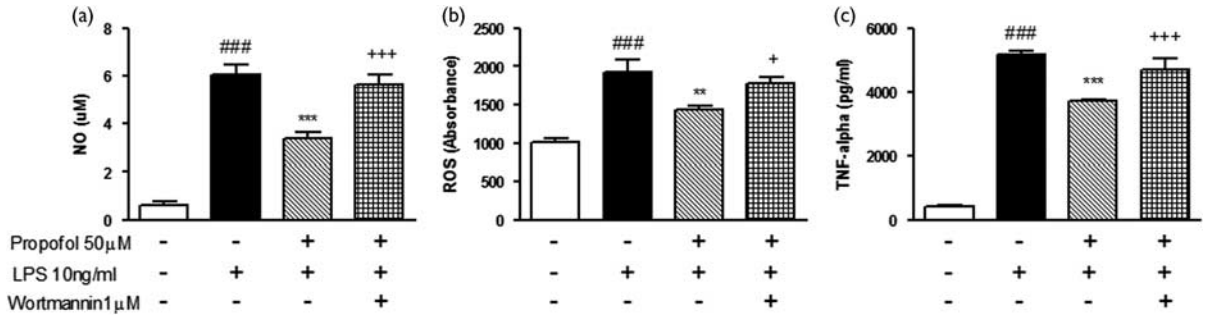
Effects of propofol on protein kinase B (PKB) phosphorylation. BV2 cells were treated with propofol (50 μM) for various times as indicated. Cell lysates were harvested, and western blot was performed to examine PKB phosphorylation. Propofol increased PKB phosphorylation, whereas it has no effects on PKB expression. Wortmannin treatment decreased PKB phosphorylation induced by propofol.

the transcriptional level, suggesting that propofol may possess potent anti-inflammatory properties.

To investigate the mechanism by which propofol inhibits LPS-induced neuroinflammation, the role of the PI3K/PKB pathway was examined. It has been reported that the PI3K/PKB pathway exists extensively in cells and is involved in the regulation of cell apoptosis, inflammatory responses, and chemotaxis [15]. Once activated through stimulation of a cell surface receptor, PI3K catalyzes the production of phosphatidylinositol-3,4,5-triphosphate, which allows for recruitment of signaling proteins, including the serine–threonine kinase PKB. Activated PKB then dissociates from the membrane to act on its targets in the cytosol and the nucleus and plays a crucial role in a wide variety of biological responses through phosphorylating a range of intracellular proteins. Recently, it has been shown that the PI3K/PKB pathway plays a pivotal role in regulating acute inflammatory responses both *in vitro* and *in vivo*. The anti-inflammatory role of PKB in inhibition of LPS signals is supported by earlier studies, which had shown that activation of the PI3K/PKB pathway suppresses LPS-activated MAPK and NF-κB pathways in dendritic cells and monocytes, resulting in decreased proinflammatory cytokines production [16,17]. Moreover, overexpression of PKB decreased sepsis-induced lymphocyte apoptosis, increased production of the Th1 cytokine IFN-γ, increased cytosolic localization of phospho-forkhead, and improved survival in sepsis [18]. On the contrary, PKB1-deficient mice injected with LPS showed increases in proinflammatory cytokine production compared with wild-type mice, suggesting that loss of PKB will render mice more sensitive to LPS [19].

We were able to demonstrate that propofol significantly increase PKB phosphorylation, and inhibition of the PI3K/PKB pathway by a major suppressor of the PI3K/PKB

Fig. 4



Blockade of phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB) inhibits propofol anti-inflammatory effect. BV2 cells were incubated with LPS (10 ng/ml) and propofol (50 μM), in the presence or absence of wortmannin. The production of nitric oxidase (NO) (a), reactive oxygen species (ROS) (b), and tumor necrosis factor-α (TNF-α) (c) was measured. Data are mean ± SEM, $n = 6$ independent measurements. $^{###}P < 0.001$ versus control, $^{**}P < 0.01$; $^{***}P < 0.001$ versus LPS group; $^{+}P < 0.05$, $^{+++}P < 0.001$ versus LPS + propofol group. Statistical analyses were performed by one-way analysis of variance with Student–Newman–Keuls post-hoc test.

pathway, wortmannin, reduced PKB phosphorylation. Furthermore, we have also found that blocking the activation of PKB was able to compromise the protective effect of propofol against LPS-induced production of inflammatory mediators and proinflammatory cytokines in microglia. Thus, the finding suggests that the PKB activation may be the molecular mechanism of decreasing inflammatory response induced by propofol.

Several reports suggest that the PI3K/PKB pathway plays a critical role in conferring the protective effects of propofol. For example, propofol was found to provide an antiapoptotic stimulus to cardiac cells through the activation of PI3K/PKB signaling pathway [20]. A similar study reported that activation of the PI3K/PKB-Bad pathway is involved in propofol cardiac protection against chemotherapeutic agent doxorubicin-induced elevation of cellular reactive oxygen and nitrogen contents [21]. In contrast, studies by Hsing *et al.* [22] reported that propofol reduces LPS-induced inflammatory responses in macrophages by inhibiting the interconnected ROS/PKB/IKKβ/NF-κB signaling pathways. The inconsistent effect of propofol on PKB function suggests that the role of the PI3K/PKB pathway in cytokine production is cell-type specific. In mammals, the PKB family consists of three isoforms, PKBα, PKBβ, and PKBγ, which are functionally distinct, despite their sequence similarities [23–25]. Further investigations will be needed to identify how these different isoforms are affect by propofol.

The current study has some clinical implications. In the event of acute central nervous system injury, such as ischemic stroke and traumatic brain injury, microglia rapidly respond to brain insults and undergo morphologic transformation from a resting state referred to as 'ramified' to an active proinflammatory M1 state. Microglial M1 polarization plays a major role in neuronal cell damage and death by releasing a variety of inflammatory and neurotoxic mediators. In contrast, pharmacological inhibition of microglial M1 activation has been suggested as an effective therapeutic approach to mitigate the progression of brain injuries and neurodegenerative disease. Our study suggested that inhibiting over-production of neuroinflammatory mediators by microglia cells could be a novel effective mechanism contributing to propofol neuroprotection. In addition, our study suggested that delayed administration with propofol may still be protective even a few hours after the insult has occurred.

Conclusion

We have shown that delayed treatment with propofol can confer a protective effect in microglial cells against LPS-induced neuroinflammation, and activation of the PI3K/PKB pathway may mediate these propofol protective effects.

Acknowledgements

Author contributions: Jun Luo and Tao Luo conceived and designed the experiments; Baoyi Huang, Zhijing Zhang, and Min Liu performed the experiments; Jun Luo and Tao Luo analyzed the data; and Jun Luo and Tao Luo wrote the paper.

Conflicts of interest

There are no conflicts of interest.

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