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Penehyclidine hydrochloride inhibits the LPS-induced inflammatory response in microglia

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ABSTRACT

Background: Activated microglia play an important role in neuroinflammation, which contributes to the neuronal damage found in many neurodegenerative diseases. Penehyclidine hydrochloride (PHC) is an anesthetic used before surgical operations, but also exhibits anti-inflammatory effects on the respiratory and digestive system. In the present study, we investigated whether PHC produces similar anti-inflammatory effects in activated microglia in the central nervous system.

Materials and methods: Microglial cells were incubated with lipopolysaccharide (LPS) in the presence or absence of various concentrations of PHC, SB203580 (p38 mitogen-activated protein kinase [MAPK] inhibitor), and pyrrolidine dithiocarbamate (nuclear factor-kappa B [NF-κB] inhibitor). Markers of inflammation and oxidative stress were measured using enzyme-linked immunosorbent assay and quantitative real-time polymerase chain reaction. The effect of PHC on NF-κB activity was assessed with a NF-κB p50/p65 transcription factor assay kit. The involvement of p38 MAPK phosphorylation in the anti-inflammatory effects of PHC was evaluated with a specific enzyme-linked immunosorbent assay kit for phospho-p38.

Results: PHC significantly inhibited the release of nitric oxide, prostaglandin E_2, interleukin 1β, and tumor necrosis factor α while upregulating the expression of inducible nitric oxide synthase messenger RNA in LPS-activated microglia. Moreover, PHC effectively inhibited the translocation of NF-κB from the cytoplasm to the nucleus and the phosphorylation of p38 MAPK. The activities of NF-κB and p38 MAPK in LPS-treated microglia were significantly lowered after pretreatment of PHC.

Conclusions: PHC inhibited the LPS-induced release of inflammatory mediators in microglia. These inhibitory effects of PHC may be mediated by blocking p38 MAPK and NF-κB pathways in microglia. These preclinical findings may offer a novel therapeutic option to confine microglial overactivation in neurodegenerative diseases.

1. Introduction

Increasing evidence indicates that brain inflammation is a pathologic manifestation identified in several forms of brain disease [1]. The inflammatory reaction manifests within the central nervous system and is essentially characterized by the activation of parenchymal microglial cells [2]. Microglial cells are resident macrophages in the brain and spinal cord [3].

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Under normal conditions, microglial cells participate in host defense and immune surveillance in the brain [4]. However, in response to infection, inflammation or injury, microglia are rapidly activated and secrete inflammatory mediators such as nitric oxide (NO), prostaglandin E_2 (PGE_2) and inflammatory cytokines including interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) [5,6]. The uncontrolled or aberrant activation of microglia along with the release of inflammatory mediators may, in turn, mediate several neurologic diseases, such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis [7,8]. Therefore, it is essential to develop therapeutic approaches to inhibit the activation of microglia and the production of inflammatory mediators to prevent the subsequent neuronal damage.

Penehyclidine hydrochloride (PHC) is an anticholinergic drug with both antimuscarinic and antinicotinic activity. Although it has not been used in the US, it is approved by China Food and Drug Administration, and now commonly used in China as an anesthetic in the clinical setting [9,10]. The protective effect of PHC on the liver and intestine has been shown in a rat cardiopulmonary bypass (CPB) model [11,12]. PHC effectively reverses the increased alanine transaminase and aspartate transaminase levels and prevents the damage to ultramicroscopic structures of the liver during CPB in rats [11]. PHC also protects the structure and function of the intestinal mucosa from injury after CPB by reducing the levels of plasma diamine oxidase, D-lactate, and endotoxin in serum [12]. In addition, previous studies have demonstrated that PHC has strong anti-inflammatory properties in a septic mouse model by inhibiting the production of inflammatory factors, the expression of inducible nitric oxide synthase (iNOS) and lipid peroxidation [13,14]. Recent studies have reported that PHC also attenuates lipopolysaccharide (LPS) or cecal ligation and puncture-induced lung injury *via* inhibiting inflammation-related signaling pathways [15,16]. Such protective effects of PHC were associated with its inhibitory effect on the phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) and the activation of nuclear factor-kappa B (NF-κB) signaling pathway [15,16]. However, the role of PHC in the regulation of neuroinflammation has not been well defined.

In the present study, we hypothesized that PHC has the capacity to affect the inflammatory reaction of microglia, which is induced by LPS in a cell culture model. Our findings demonstrated that PHC could effectively prevent the elevated production of inflammatory mediators from cultured microglial cells. We further show that the reduced release of inflammatory mediators might be because of the inhibitory effects of PHC on the activation of NF-κB and MAPK pathways.

2. Materials and methods

2.1. Reagents

PHC was provided by Lisite Corporation (Chengdu, China). SB203580 (p38 MAPK inhibitor), pyrrolidine dithiocarbamate (PDTC; NF-κB inhibitor) and LPS (*Escherichia coli* 0111:B4) were purchased from Sigma-Aldrich (St. Louis, MO).

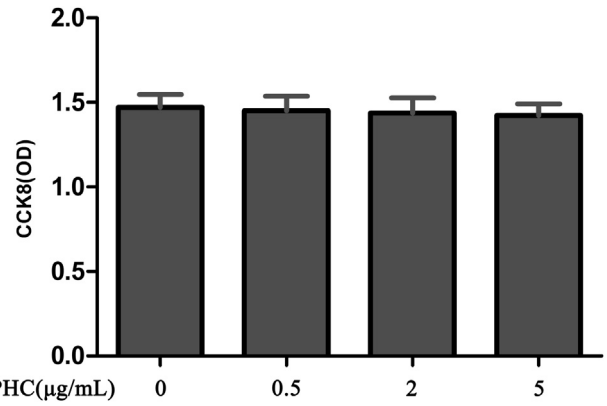

Fig. 1 – Effect of PHC on cell viability of microglia cells. Microglia cells were treated with varying concentrations of PHC (0, 0.5, 2, and 5 μg/mL) for 24 h. Cell viability was assessed using a CCK-8 assay. Data were expressed as mean ± SD from three independent experiments.

2.2. Cell culture and treatment

Sprague–Dawley rats were obtained from the Experimental Animal Center of Ningbo University (Ningbo, Zhejiang, China). Experiments were carried out according to the National Institutes of Health Guide for Care and Use of Laboratory Animals, and were approved by the Bioethics Committee of Ningbo University. Primary microglia were isolated from Sprague–Dawley rats as previously described [17]. Microglial cells were cultured in Dulbecco's Modified Eagle Medium (Gibco, Grand Island, NE) supplemented with 10% fetal calf serum (Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco) in a humidified atmosphere of 5% CO_2 at 37°C. After cells were plated overnight, they were washed three times and then transferred to six-well polystyrene culture plates at a seeding density of 1×10^5 cells/mL in 2 mL of medium per well. For PHC studies, microglial cells were incubated with LPS (1 μg/mL) in the absence or presence of PHC (0.5, 2, and 5 μg/mL). In independent studies involving inhibitors, cells were preincubated with the p38 MAPK inhibitor, SB203580 (10 μM) or the NF-κB inhibitor, PDTC (100 μM), followed by LPS treatment. The concentrations of PHC, SB203580, and PDTC used for studies were based on previous studies [18–21].

2.3. Cell viability test

The viability of microglia cells was determined using the Cell Counting Kit-8 assay kit (Beyotime, Jiangsu, China) according to the manufacturer's instructions [22]. Briefly, microglial cells were plated at a seeding density of 1×10^4 cells/well in 96-well plates in 100 μL Dulbecco's Modified Eagle Medium. Cells were incubated with various concentrations of PHC for 24 h. Next, 20 mL of Cell Counting Kit 8 reagent was added to each well and incubated for 2 h at 37°C. The absorbance of the solution was measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA) at a test wavelength of 450 nm and a reference wavelength of 630 nm.

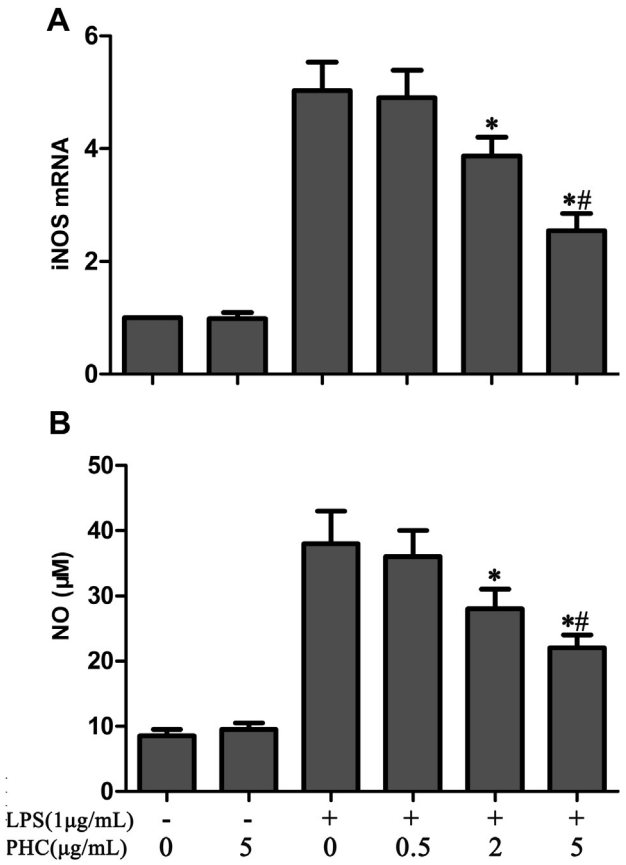

Fig. 2 – Effects of PHC on iNOS mRNA expression and NO production in LPS-activated microglia cells. For LPS treatments, microglial cells were pretreated with PHC (0, 0.5, 2, and 5 μg/mL) for 1 h followed by LPS (1 μg/mL) treatment for 23 h. Alternatively, cells were treated with 5 μg/mL PHC alone for 24 h. (A) The expression of iNOS mRNA was assessed by qPCR. (B) The NO concentration in the culture media was measured by the Griess reagent. Data were expressed as mean ± SD from three independent experiments. *P < 0.05 compared with LPS treatment alone group; #P < 0.01 compared with LPS treatment alone group.

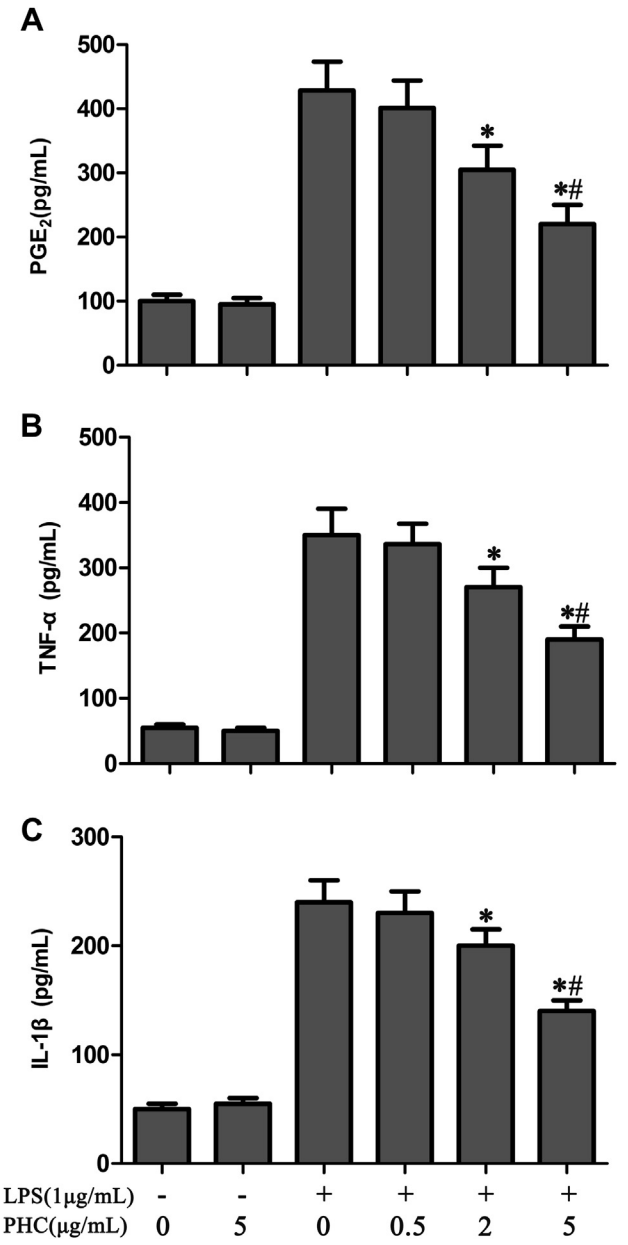

Fig. 3 – Effects of PHC on PGE_2, TNF-α, and IL-1β release in LPS-activated microglia cells. For LPS treatments, microglial cells were pretreated with PHC (0, 0.5, 2, and 5 μg/mL) for 1 h followed by LPS (1 μg/mL) for 23 h. Alternatively, cells were treated with 5 μg/mL PHC alone for 24 h. (A) The levels of PGE_2, (B) TNF-α, and (C) IL-1β in the culture media were measured using ELISA kits. Data were expressed as mean ± SD from three independent experiments. *P < 0.05 compared with LPS treatment alone group; #P < 0.01 compared with LPS treatment alone group.

2.4. *Quantitative real-time polymerase chain reaction analysis*

Total RNA was extracted from microglial cells by using the Trizol reagent (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was reverse-transcribed using moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Quantitative real-time polymerase chain reaction (qPCR) was performed using a LightCycler 2.0 Real-Time PCR System (Roche Applied Science, Indianapolis, IN). The complementary DNA was amplified by PCR using specific primers for iNOS (forward 5′-GTG TTC CAC CAG GAG ATG TTG -3'and reverse 5′- CTC CTG CCC GCT GAG TTC GTC -3′) and β-actin (forward 5′- TTG TAA CCA ACT GGG ACG ATA TGG -3'and reverse 5′- GAT CTT GAT CTT CAT GGT GCT AG -3′). The following PCR conditions were applied: 24 cycles of denaturation at 95°C for 45 s, annealing at 56°C for 30 s, and extended at 72°C for 1 min. β-actin was used as an internal control to evaluate relative expression of iNOS. The relative mean fold-change of iNOS messenger RNA (mRNA) relative to β-actin mRNA in the experimental group

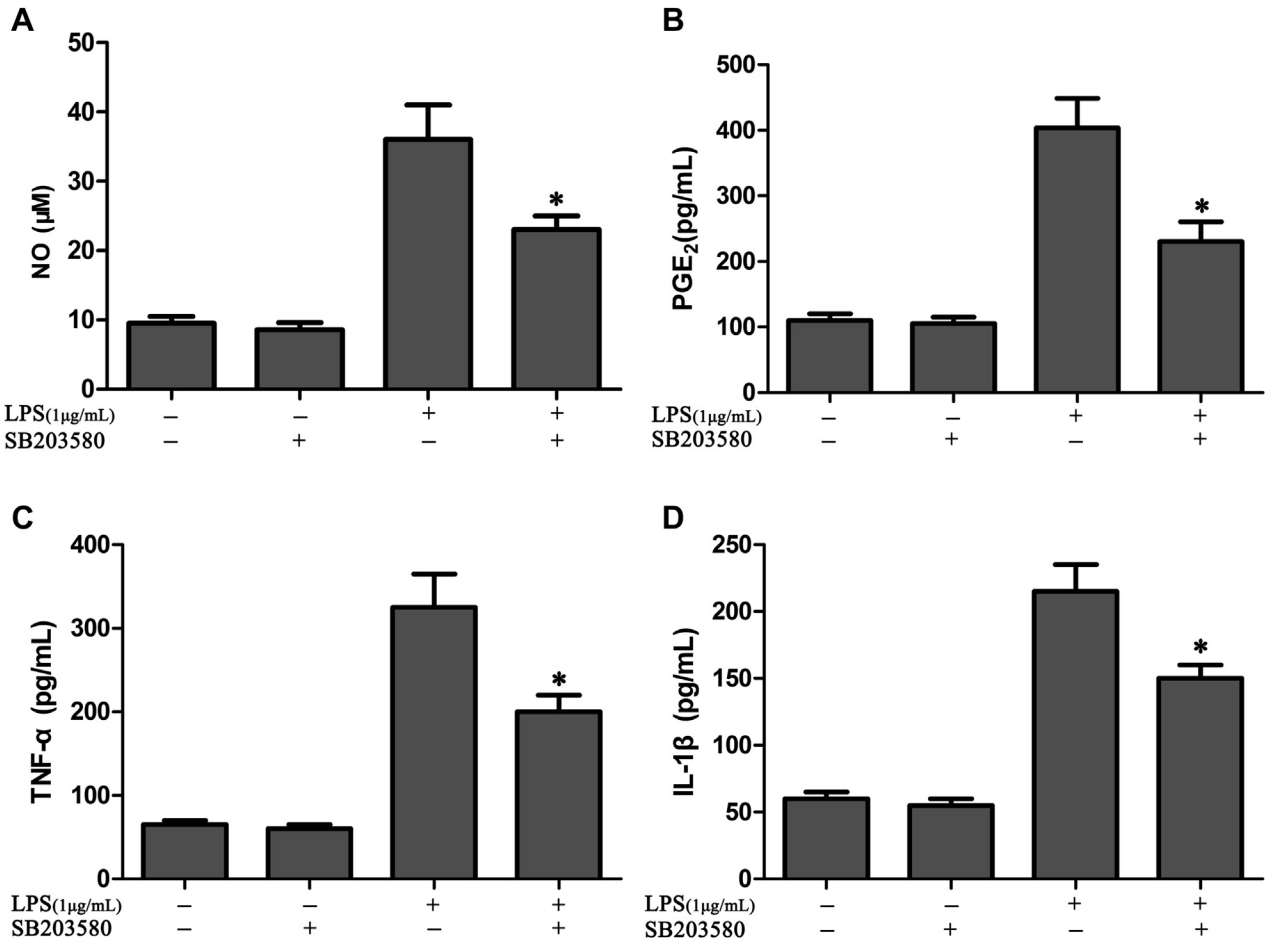

Fig. 4 – The effects of the p38 MAPK inhibitor SB203580 on LPS-induced NO, PGE_2, TNF-α, and IL-1β release. Cells were incubated with 10 μM SB203580 for 1 h and then treated with LPS (1 μg/mL) for 24 h. Data were expressed as mean ± SD from three independent experiments. *P < 0.05 compared with LPS treatment alone group.

was calculated using the $2^{\triangle\triangle}$ computed tomography method and compared with the control group [23].

2.5. NO Assay

NO concentration in culture media was determined using the Griess reagent (Sigma-Aldrich). Briefly, microglial cells were pretreated with PHC (0.5, 2, and 5 μg/mL) for 1 h followed by LPS (1 μg/mL) treatment. After 24 h, the media were collected and mixed with the same volume of Griess reagent. Samples were then incubated at room temperature for 10 min. The absorbance of samples was subsequently measured at 540 nm using a microplate reader.

2.6. PGE_2 assays

Microglial cells were seeded at a density of 4×10^5 cells/mL in six-well plates and incubated with PHC (0.5, 2, and 5 μg/mL) in the presence of LPS (1 μg/mL) for 24 h. Subsequently, 0.1 mL of the culture media was collected from wells to determine the concentration of PGE_2. The PGE_2 level in the culture media was determined using an enzyme-linked immunosorbent assay kit (ELISA; R&D Systems, Minneapolis, MN) and by following the manufacturer's instructions.

2.7. Cytokine assays

TNF-α and IL-1β levels were measured in culture media using an ELISA kit. The absorbance of culture media was measured at 450 nm using a microplate reader.

2.8. MAPKs assays

Microglial cells were pretreated with PHC (0.5, 2, and 5 μg/mL) for 1 h and subsequently treated with LPS (1 μg/mL) for 30 min. Cells were subsequently washed with PBS and lysed using a nuclear and cytoplasmic extraction reagent (Pierce Protein Research Products, Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instructions. Cell lysates were subjected to ELISA to measure phospho-p38 levels according to the manufacturer's instructions (eBioscience, Beverly, MA). Absorbance of samples was measured at 450 nm using an automated plate reader (Bio-Rad Laboratories).

2.9. NF-κB binding assay

Microglial cells were harvested and washed three times with cold PBS. Nuclear protein fractions were extracted using a nuclear and cytoplasmic extraction reagent according to the manufacturer's instructions. The DNA binding activity of NF-κB (p50/p65) in the nuclear fraction was determined using an ELISA-based nonradioactive NF-κB p50/p65 transcription factor assay kit (Chemicon, Temecula, CA), according to the manufacturer's instructions. Absorbance of samples was measured at 450 nm using an automated plate reader.

2.10. Statistical analysis

All data were presented as the mean ± standard deviation (SD) of the results obtained from three replicates. Differences between each group were assessed by one-way analysis of variance followed by Newman-Keuls tests. A P value < 0.05 was considered statistically significant. All data were analyzed using SPSS version 13.0 statistical software (SPSS, Chicago, IL).

3. Results

3.1. Effect of PHC on microglia cell viability

Cell viability was assessed in microglial cells to determine whether PHC had cytotoxic effects in microglial cells. We show that the concentrations of PHC used in the present study did not have a significant cytotoxic effect on microglial cells (Fig. 1).

3.2. Inhibition of NO release and iNOS mRNA expression by PHC

To investigate the effects of PHC on NO release in LPS-activated primary microglia, cells were treated with LPS and PHC. As shown in Figure 2, LPS significantly induced NO release compared with the control group ($P < 0.01$). PHC (2 and 5 μg/mL) significantly inhibited LPS-induced NO release in a dose-dependent manner ($P < 0.05$ and $P < 0.01$, respectively, Fig. 2). To determine whether iNOS contributed to the inhibitory effect of PHC on NO release, we examined the effects of PHC on iNOS mRNA expression in microglia. Quantitative PCR analysis showed that iNOS mRNA was barely detected in nonactivated microglia, but was expressed at a high level on stimulation with LPS (1 μg/mL). In addition, the inhibition effect of PHC on LPS-induced iNOS mRNA expression was dose-dependent (Fig. 2). These results demonstrated that PHC inhibited NO release through the downregulation of iNOS mRNA expression in LPS-activated microglia.

3.3. Suppression of PGE_2, IL-1β, and TNF-α release by PHC

The levels of PGE_2, IL-1β, and TNF-α were examined in culture media of LPS treatment groups to investigate the effect of PHC on the production of inflammatory mediators. As shown in Figure 3, LPS treatment of microglia resulted in a marked increase in PGE_2, IL-1β, and TNF-α release compared with the control group. PHC (2 and 5 μg/mL) could significantly attenuate the levels of PGE_2, IL-1β, and TNF-α in the media compared with LPS treatment alone group. However, lower levels of PHC (0.5 μg/mL) did not significantly affect these inflammatory mediators. These results suggested that the anti-inflammatory effect of PHC might be mediated *via* the suppression of inflammatory mediators, such as PGE_2, IL-1β, and TNF-α.

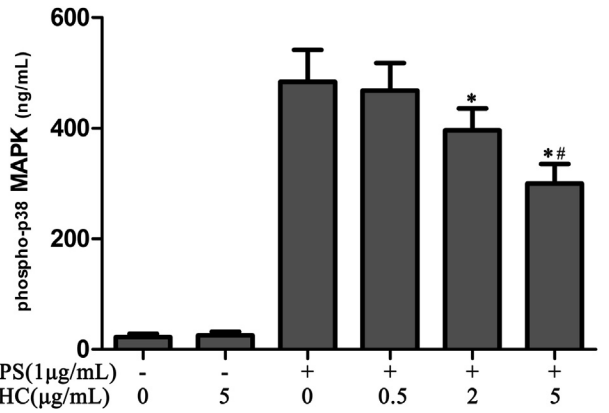

Fig. 5 – Effects of PHC on LPS-induced p38 MAPK activation in microglia cells. For LPS treatments, microglia cells were preincubated with PHC (0.5, 2, and 5 μg/mL) for 1 h and then stimulated with LPS (1 μg/mL) for 30 min. Alternatively, cells were treated with 5 μg/mL PHC alone for 1.5 h. Cell lysates were extracted and p38 MAPK activation was determined using the phospho-p38 MAPK ELISA. Data were expressed as mean ± SD from three independent experiments. *$P < 0.05$ compared with LPS treatment alone group, #$P < 0.01$ compared with LPS treatment alone group.

3.4. Role of p38 MAPK in the effect of PHC on LPS-activated microglia

The p38 MAPK pathway has been reported to be an important mediator of the LPS-induced inflammatory response [24]. To clarify the role of p38 MAPK in LPS-activated microglial cells, we examined the release of inflammatory markers, such as NO, PGE_2, IL-1β, and TNF-α, in the presence of the p38 MAPK pathway inhibitor SB203580. We show that LPS-induced NO, PGE_2, IL-1β, and TNF-α release were significantly suppressed by SB203580 treatment (Fig. 4). In addition, to determine the inhibitory effect of PHC on LPS-stimulated p38 MAPK in microglial cells, we examined the levels of phosphorylated p38 MAPK. Cells were pretreated with PHC (0.5, 2, and 5 μg/mL) for 1 h and then treated with LPS (1 μg/mL) for another 30 min. Microglia cell activation by LPS caused an increase in p38 MAPK phosphorylation, which could be significantly decreased by PHC (Fig. 5). These results indicate that the inhibition of p38 MAPK might be one of the mechanisms by which PHC can protect microglial cells against the LPS-induced inflammatory response.

3.5. Role of NF-κB in the effect of PHC on LPS-activated microglia

The NF-κB signaling pathway is acknowledged as one of the essential mediators for inflammation [25]. To clarify the role of NF-κB in LPS-induced inflammatory response in microglial

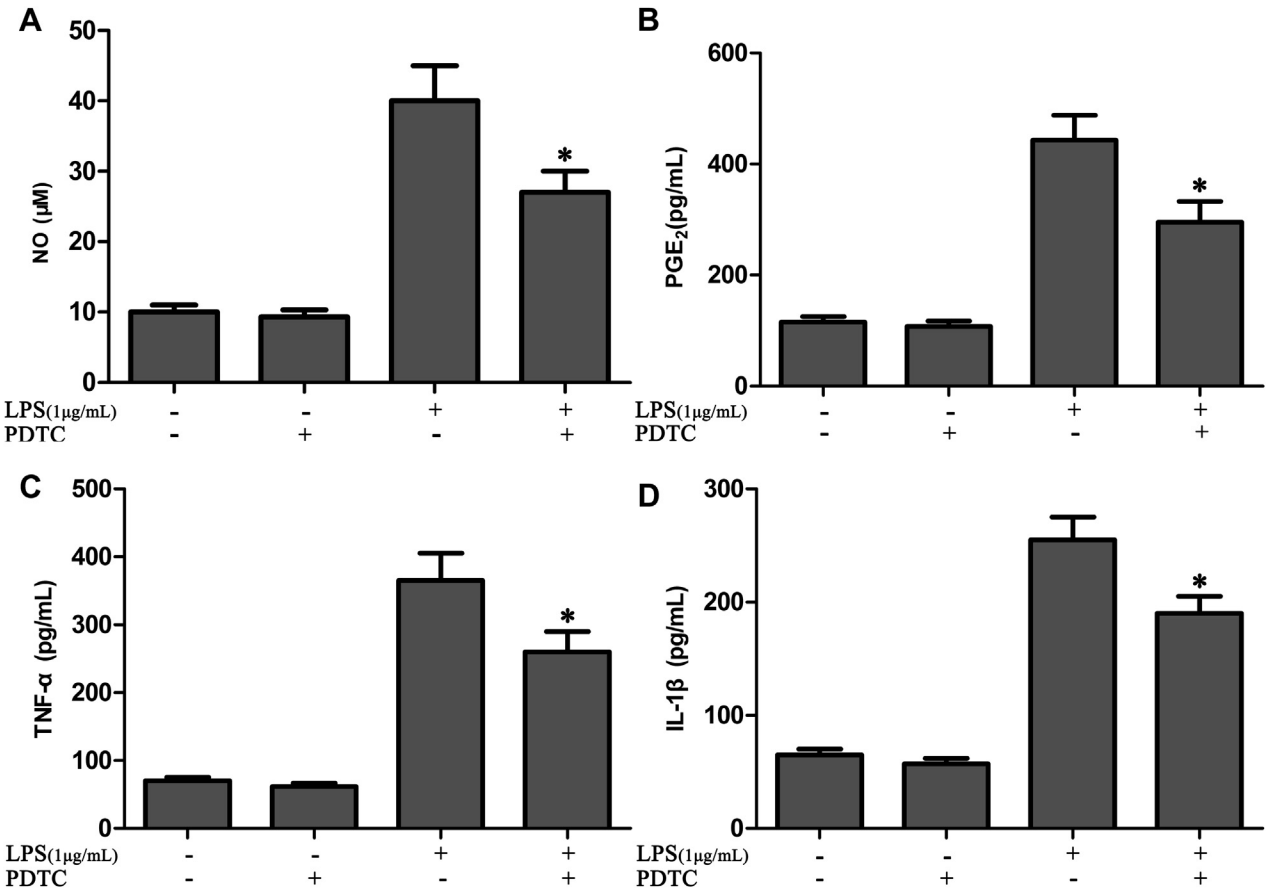

Fig. 6 – LPS-induced NO, PGE_2, TNF-α, and IL-1β release and their modulation by the NF-κB inhibitor, PDTC. Microglia cells were incubated with PDTC (100 μM) for 1 h and then treated with LPS (1 μg/mL) for 24 h. Compared with control, LPS treatment induced NO, PGE_2, TNF-α, and IL-1β release, which was attenuated by PDTC. *P < 0.05 compared with LPS treatment alone group.

cells, we examined the release of NO, PGE_2, IL-1β, and TNF-α in the presence of the NF-κB inhibitor, PDTC. We demonstrate that LPS-induced NO, PGE_2, IL-1β, and TNF-α release in microglial cells were significantly inhibited by PDTC treatment (Fig. 6). Moreover, to explore the mechanism by which PHC inhibits the inflammatory response in LPS-activated microglia, we next examined the activation of NF-κB signal pathway in microglia. Our results show that NF-κB activity was remarkable increased in LPS-activated microglia. PHC treatment significantly reduced NF-κB activation in LPS-activated microglia cells in a dose-dependent manner (Fig. 7). These results suggest that inhibition of NF-κB might be one of the mechanisms that are responsible for the protective effect of PHC on LPS-induced inflammatory response in microglia.

4. Discussion

PHC is a selective anticholinergic agent. Its anti-inflammatory and antioxidative effects in the respiratory, digestive, and kidney systems have been demonstrated in previous studies [12,15,16,26]. However, the role of PHC in the neuroinflammatory process is less known. In the present study, we use an *in vitro* cell culture model of microglia to study the effects and the possible underlying mechanisms of PHC in controlling the LPS-induced inflammatory response. Results obtained from the present study identified a novel mechanism through which PHC exerts a protective effect against the release of inflammatory mediators by LPS activation in microglial cells. PHC significantly reduced the release of inflammatory mediators such as NO, PGE_2, IL-1β, and TNF-α in LPS-activated primary microglia *in vitro*. Such effects appeared to be mediated by the inhibition of p38 MAPK and NF-κB signaling pathways in microglia.

Microglia are the primary immune effector cells in the central nervous system and their functions are similar to those of peripheral macrophages [27]. The primary role of these resident glial cells is to promote host defense in the brain by destroying invading pathogens, removing deleterious debris, promoting tissue repair, and facilitating tissue homeostasis [8]. However, sustained overactivation of microglia leads to an overproduction of various inflammatory mediators, which subsequently cause extensive neuronal injury called "secondary injury" [28,29]. These inflammatory mediators include PGE_2, NO, and inflammatory cytokines (TNF-α and IL-1β) [30–32]. Therefore, controlling the

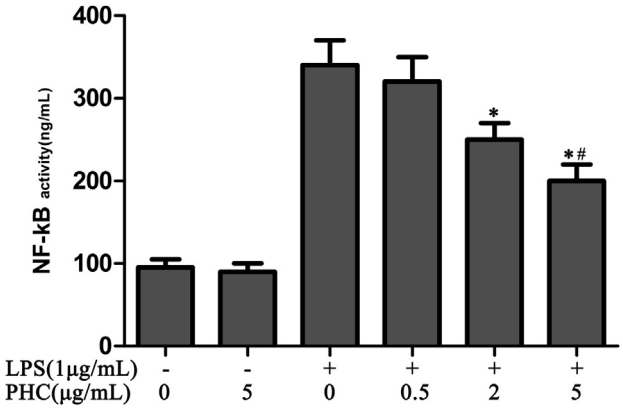

Fig. 7 – Effects of PHC on NF-κB activity in LPS-activated microglia cells. For LPS treatments, microglia cells were stimulated with or without LPS (1 μg/mL) in the absence or presence of PHC (0.5, 2, and 5 μg/mL) for 24 h. Alternatively, cells were incubated with 5 μg/mL PHC alone for 24 h. The nuclear proteins were extracted, and NF-κB activity was determined using the NF-κB p50/p65 transcription factor assay kit. Data were expressed as mean ± SD from three independent experiments. $^{*}P < 0.05$ compared with LPS treatment alone group, $^{\#}P < 0.01$ compared with LPS treatment alone group.

microglial activation process may offer a novel therapeutic option to protect neurons against the "secondary injury" phase [33,34].

Recent studies have indicated that PHC may exert anti-inflammatory effects in the liver, intestine, lung, and kidney *via* attenuating the production of inflammatory mediators [11,12,15,16,26]. Some of the effects in these studies were shown to occur by affecting the activities of NF-κB and p38 MAPK signaling pathways. However, no effort has been made to examine the possible effects of PHC on neuroinflammation, which is an important neuropathologic feature in many neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and multiple sclerosis. The goal of the present study was to examine the effects of PHC on modulating LPS-induced upregulation of inflammatory mediators and activation of NF-κB and MAPK signaling pathways in activated microglia. A primary cell culture model was set up and potential effects of PHC on cytotoxicity in these primary cultured microglial cells were tested. Our study demonstrated that PHC (0.5, 2, 5 μg/mL) treatment of microglia cells for 24 h did not affect their cell viability, which indicates that the primary effects of PHC do not include possible cytotoxic effects. Data from the present study confirmed that LPS induced a dramatic inflammatory response in primary microglia, which is essentially consistent with previous studies [22]. The production of NO, PGE_2, and proinflammatory cytokines, such as TNF-α and IL-1β, was increased in LPS-activated microglia after 24 h. Importantly, PHC (2 and 5 μg/mL) treatment significantly mitigated the LPS-induced inflammatory response in microglia in a dose-dependent manner without causing cytotoxicity.

NF-κB is a transcription factor, which is considered to be a central and critical regulator of the inflammatory process [25]. NF-κB controls the expression of genes encoding inflammatory cytokines (TNF-α and IL-1β and so forth), PGE_2 and iNOS, all of which play critical roles in controlling major inflammatory processes [35]. The MAPKs are intracellular enzymes, which allow cells to respond to stimuli, such as inflammatory cytokines, from their extracellular environment [36]. MAPKs include extracellular signal-regulated kinases, c-Jun N-terminal kinases, and p38 MAPK. The p38 MAPK signaling pathway has been shown to play a central role in the expression and activity of inflammatory cytokines, such as TNF-α and IL-1β, in many cell types [24]. MAPKs have also been shown to be important upstream modulators of PGE_2, iNOS, and inflammatory cytokines (TNF-α and IL-1β) [36,37]. In this study, we investigated potential underlying mechanisms for the effects of PHC on LPS-activated microglia by examining the MAPK and NF-κB signaling pathways. We show that PHC significantly inhibited LPS-induced phosphorylation of p38 MAPK and the nuclear translocation of the NF-κB p65 subunit in microglia cells, which could possibly account for the inhibition in inflammatory mediator production in microglial cells.

These results provided evidence to show that PHC can effectively inhibit the LPS-induced inflammatory response from microglial cells by regulating NF-κB and p38 MAPK signaling pathways. In addition, our study shows for the first time that PHC is just as potent as the NF-κB and MAPK inhibitors in inhibiting NO, PGE_2, TNF-α, and IL-1β. Altogether, our data suggest that PHC could be a promising drug candidate to effectively inhibit the overactivated microglia and release of inflammatory mediators from microglia. Because of lack of data concerning the physiologically relevant concentrations of PHC that may be effective *in vivo*, we may not be able to translate the concentrations of PHC used in our models to humans *in vivo*. Thus, future studies are needed to determine the clinically relevant concentrations of PHC to use *in vivo*, which will benefit from performing extensive investigations in animal models.

5. Conclusions

The present study demonstrated that PHC inhibited LPS-induced release of inflammatory mediators in primary microglia. The anti-inflammatory effects of PHC are achieved by inhibiting the p38 MAPK and NF-κB signaling pathways in LPS-activated microglia. In light of the promising use of PHC clinically and its fewer cardiovascular side effects [38], PHC may provide a new strategy for the treatment of neurodegenerative diseases in the future.

Acknowledgment

Disclaimer: PHC is a new anticholinergic drug, which has been approved by China Food and Drug Administration. It is used widely in China as an anesthetic. PHC has not been evaluated by U.S. Food and Drug Administration. The possibility of the usage of PHC in US should be looked at.

Author contributors: The authors are justifiably credited with authorship, according to the authorship criteria. In

detail, Changshun Huang was responsible for conception, design, analysis and interpretation of the data, writing the article; Jianguo He was responsible for conception, design, analysis and interpretation of the data, data collection, critical revision of the article, statistical expertise, obtaining funding, and supervision; Yijun Chen was responsible for data collection, critical revision of the article; Yiwei Zhang was responsible for conception and design, critical revision of the article; Chunru Chen was responsible for conception, design, analysis and interpretation of the data, writing and critical revision of the article, obtaining funding, supervision. All authors were given final approval.

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