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Full paper

Piperlongumine inhibits neuroinflammation via regulating NF-κB signaling pathways in lipopolysaccharide-stimulated BV2 microglia cells

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ABSTRACT

Inflammatory processes in the central nervous system are feature among biological reactions to harmful stimuli such as pathogens and damaged cells. In resting conditions, microglia are involved in immune surveillance and brain homeostasis. However, the activation of abnormal microglia can be detrimental to neurons, even resulting in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease. Therefore, normalization of microglial activation is considered a promising strategy for developing drugs that can treat or prevent inflammation-related brain diseases. In the present study, we investigated the effects of piperlongumine, an active component of *Piper longum*, on lipopolysaccharide (LPS)-induced neuroinflammation using BV2 microglial cells. We found that piperlongumine significantly inhibited the production of nitric oxide and prostaglandin E_2 induced by LPS. Piperlongumine also reduced the expression of inducible nitric oxide synthase and cyclooxygenase-2 as well as proinflammatory cytokines such as tumor necrosis factor-α and interleukin-6. Piperlongumine exerted its anti-neuroinflammatory effects by suppressing the nuclear factor kappa B signaling pathway. These findings suggest that piperlongumine could be a candidate agent for the treatment of inflammation-related neurodegenerative diseases.

1. Introduction

Neuroinflammation is part of the immune response to harmful stimuli within the central nervous system (CNS). Its function is to remove necrotic cells and tissues induced by pathogens. Excessive neuroinflammation contributes to the progression of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD).[1] Microglia, resident macrophages of the CNS, play key roles in inflammatory responses and brain homeostasis.[2] Under normal conditions, microglia are scavenge debris in the CNS such as damaged neurons and pathogens and also maintain synaptic homeostasis.[3] However, abnormal activation of microglia releases neurotoxic molecules such as nitric oxide (NO) and prostaglandin E_2 (PGE_2), and proinflammatory cytokines including tumor

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necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β). Overexpression of these factors can lead to damage to neuronal cells, resulting in neurodegenerative diseases.[4–6] Thus, many studies are attempting to develop novel agent that can control microglial activation to treat or prevent inflammation-related brain diseases.

Recently, several lines of evidence have shown that compounds isolated from medicinal herbs strongly inhibit the inflammatory response. Galangin, a polyphenolic component of *Alpinia officinarum*, was shown to inhibit inflammation by regulating the nuclear factor-kappa B (NF-κB), PI3K/Akt and peroxisome proliferator activated receptor-γ signaling in polyinosinic-polycytidylic acid-stimulated microglia.[7] Moreover, lucenin-2, vicenin-2 and stellarin-2 isolated from *Korthalsella japonica* showed inhibitory effects on NO production in lipopolysaccharide (LPS)-stimulated murine macrophage RAW264.7 cells.[8]

Piperlongumine is an alkaloid extracted from *Piper longum* L (*P. longum*) that has anti-tumor, anti-platelet aggregation, analgesic, anti-fungal, anti-schistosomiasis, anti-anxiety and anti-depression effects.[9] Piperlongumine and its derivatives also showed anti-inflammatory effects by inhibiting NO and NF-κB signaling in systemic inflammatory disease models.[10–12] However, it is unknown whether piperlongumine has similar effects against neuroinflammatory conditions. To address this issue, the present study was focused on investigating anti-neuroinflammatory effects of piperlongumine and related mechanisms by which it might counteract the effects of LPS stimulation in BV2 microglial cells.

2. Materials and methods

2.1. Materials

Piperlongumine (SML0221, Fig. 1A) and LPS from *Escherichia coli* serotype O55:B5 (L6529) were purchased from Sigma–Aldrich (MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, phosphate-buffered saline (PBS pH 7.4), and other cell culture reagents were purchased from HyClone Laboratories (UT, USA). Rabbit antibodies against p38 (#9212S), phosphor-p38 (#9215S), c-Jun N-terminal kinase (JNK) (#9252S), phosphor-JNK (#9251S), extracellular-signal-regulated kinase (ERK) (#9101L), phosphor-ERK (#9102), inducible nitric oxide synthase (iNOS, #2982S), and cyclooxygenase-2 (COX-2, #4842S) were purchased from Cell Signaling Technology. Antibodies against β-actin (SC-47778 HRP) and NF-κB (rabbit, SC-372) were purchased from SantaCruz Biotechnology. Secondary antibodies were purchased from Bio-Rad, SantaCruz Biotechnology and Invitrogen. Mouse TNF-α and IL-6 ELISA kits were purchased from BD. A mouse PGE_2 kit was purchased from Enzo Life Sciences (NY, USA).

2.2. Cell culture and cytotoxicity measurement

BV2 microglial cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. All

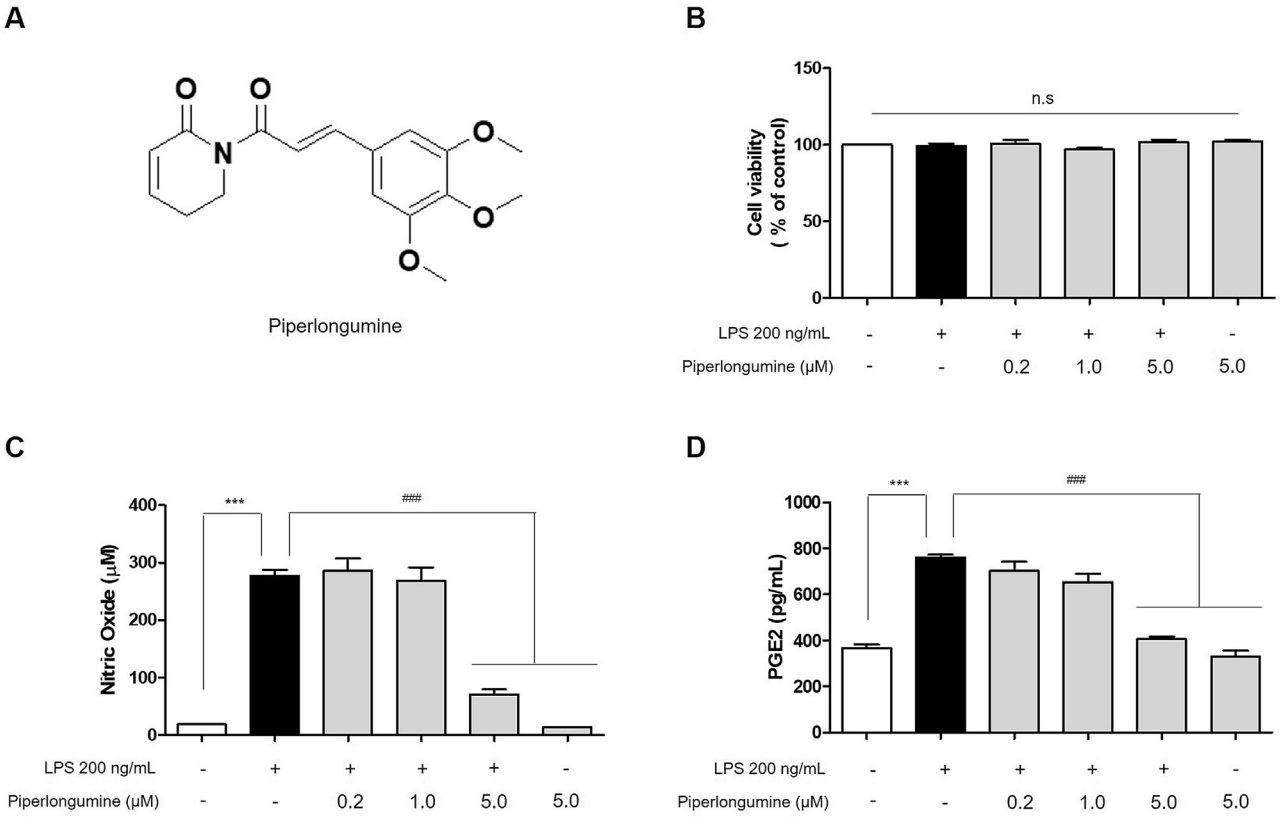

Fig. 1. Piperlongumine inhibited production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) induced by LPS in BV2 microglial cells. (A) Structure of piperlongumine. The cells were pretreated with piperlongumine (0.2, 1.0, or 5.0 μM) for 2 h prior to LPS treatment (200 ng/mL) for 22 h. (B) Cell viability was measured by WST-1 assay (n = 10 per group). The expression levels of NO (C) and PGE_2 (D) were measured by the Griess reaction assay and ELISA kit, respectively (n = 4–6 per group). Statistical analysis included one-way analysis of variance and Tukey's post hoc test. $^{***}p < 0.001$ compared with the control group, $^{\#\#\#}p < 0.001$ compared with the LPS-treated only group. N.S indicated no significant difference. Values are the mean ± SEM.

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experiments were performed 24 h after BV2 cell seeding in plates. Cell toxicity was determined by the WST-1 assay (Roche, Germany).

2.3. Measurement of NO and PGE_2

The analysis of NO and PGE_2 was performed in accordance with a previously described method.[13] After BV2 microglial cells (4.0×10^4 cells/well) were seeded in a 96-well plate, cells were pretreated with piperlongumine (0.2, 1.0 or 5.0 μM) for 2 h prior to stimulation with LPS (200 ng/mL) for 22 h. The NO and PGE_2 concentrations were assessed using Griess reagent and enzyme immune assay kit (#ADI-900-001).

2.4. Quantitative RT-PCR analysis

mRNA transcription of cytokines was analyzed by qRT-PCR. Total RNA was extracted from BV2 microglial cells using the RNeasy Plus Mini kit (Qiagen), according to the manufacturer's instructions. RNA samples (5 μg) were subjected to cDNA synthesis using an RNA to cDNA EcoDry Premix kit (Takara). cDNA was subjected to qRT-PCR using SYBR Green Mix (Toyobo, Osaka, Japan) and the CFX Connect real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Primers, synthesized at Cosmo Genetech, were as follows: TNF-α: forward, 5′-GATTATGGCTCAGGGTCCAA-3′, reverse, 5′-GCTCCAGTGAATTCGGAAAG-3′; IL-6: forward, 5′−CCGGAGAGGAGACTTCACAG-3′, reverse, 5′-TTGCCATTGCACAACTCTTT-3′; IL-10: forward, 5′-AAGGCCATGAATGAATTTGA-3′, reverse, 5′-TTCGGAGAGAGGTACAAACG-3′; and GAPDH: forward, 5′-TGAATACGGCTACAGCAACA-3′, reverse, 5′-AGGCCCCTCCTGTTATTATG-3′.

2.5. Enzyme-linked immunosorbent assay

After BV2 microglial cells (4.0×10^4 cells/well) were seeded in 96-well plates, cells were pretreated with piperlongumine (0.2, 1.0, or 5.0 μM) for 2 h prior to stimulation with LPS (200 ng/mL) for 22 h. Then the culture medium was collected and centrifuged at 4 °C at 2000 × g for 10 min. TNF-α, IL-6, and IL-10 concentrations were assessed using ELISA kits according to the manufacturer's protocols.

2.6. Western blotting

Western blot analysis was performed as previously described.[14] Briefly, BV2 microglial cells (4×10^5 cells/well) were seeded in 6-well plate and pretreated with piperlongumine at different concentrations (0.2, 1.0 or 5.0 μM) for 2 h prior to expose to LPS (200 ng/mL) for 4 or 22 h. Cells were harvested, washed in ice-cold PBS, and then lysed in 1 × RIPA buffer (Cell Signaling Technology). Lysates were sonicated for 10 s and centrifuged at 12,000 × g for 10 min at 4 °C. Proteins (30 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into polyvinylidene fluoride membranes. These membranes were washed with Tris-buffered saline (10 mM Tris−HCl, 150 mM NaCl, pH 7.5) supplemented with 0.05% Tween 20 (TBST), followed by blocking with TBST containing 5% skim milk and incubated overnight with primary antibodies. After washing three times with TBST, the membranes were then exposed to secondary antibodies coupled to horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG secondary antibody for 1 h at room temperature. The membranes were washed three times with TBST at room temperature. The detection of β-actin was performed in the same blot as an internal control for the normalization of protein loading. Protein detection was carried out using an ECL reagent (Bio-Rad Laboratories, Hercules, CA, USA) and visualized by ChemiDoc (Bio-Rad Laboratories, Hercules, CA, USA). The intensity of bands was quantified using ImageJ (NIH, USA). All Western blot assays were performed at least three times.

2.7. Luciferase assay

BV2 microglial cells were cultured in 24-well plates for 24 h and then transfected with TK-renilla and NF-κB firefly luciferase constructs (Stratagene, La Jolla, CA, USA) using polyethylenimine according to the manufacturer's instructions. Briefly, transfected cells were pretreated with piperlongumine (5.0 μM) for 2 h and then stimulated with LPS (200 ng/mL) for 12 h. The cells were then washed twice with ice-cold PBS, and 100 μL of 1 × passive lysis buffer was added. After centrifugation at 12,000 × g for 5 min at 4 °C, a 20 μL aliquot of the supernatant was analyzed using a luminometer (Molecular Devices). Luciferase activity was normalized to renilla luciferase activity.

2.8. Immunofluorescence

After BV2 microglial cells (1.0×10^4 cells/well) were seeded in a cover glass of 24-well plates, cells were pretreated with piperlongumine (5.0 μM) for 2 h prior to stimulation with LPS (200 ng/mL) for 4 h. To detect the intracellular location of NF-κB/p65, BV2 microglial cells were fixed with 4% PFA in PBS. The cells were rinsed with PBS and then incubated with NF-κB/p65 antibody (1:1000 dilution in 1% BSA) for 24 h at 4 °C. For visualization, the primary antibody was developed by incubating with Alexa Fluor 488-conjugated secondary antibodies for 1 h at room temperature. Images were captured using an Olympus BX51 microscope.

2.9. Statistical analysis

All data are expressed as the mean ± standard error of the mean (S.E.M.) using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). The results were analyzed statistically by one-way analysis of variance, followed by Tukey's *post hoc* test. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of piperlongumine on cytotoxicity and neurotoxic factors in LPS-stimulated BV2 microglial cells

To examine the cytotoxic effect of piperlongumine on BV2 microglial cells, we performed the WST-1 assay. As shown in Fig. 1B, cell viability following treatment with piperlongumine for 24 h with or without LPS stimulation did not differ between groups. Next, we measured the levels of NO and PGE_2 production to evaluate the inhibitory effects of piperlongumine on LPS-induced neuroinflammation in BV2 microglial cells. LPS significantly increased the production of NO and PGE_2 when compared with the levels in the control group. However, pretreatment with piperlongumine at 5 μM inhibited the increases of NO and PGE_2 levels induced by LPS (Fig. 1C,D). These findings indicate that piperlongumine has anti-neuroinflammatory effects in LPS-stimulated microglia without cell toxicity.

3.2. Effects of piperlongumine on inflammatory mediator expression in LPS-stimulated BV2 microglial cells

After confirming the inhibitory effects of piperlongumine on NO and PGE_2 production in BV2 microglial cells, we investigated its effects of piperlongumine on the expression of iNOS and COX-2, which are known to regulate the production of NO and PGE_2, respectively. We found that piperlongumine at 5 μM

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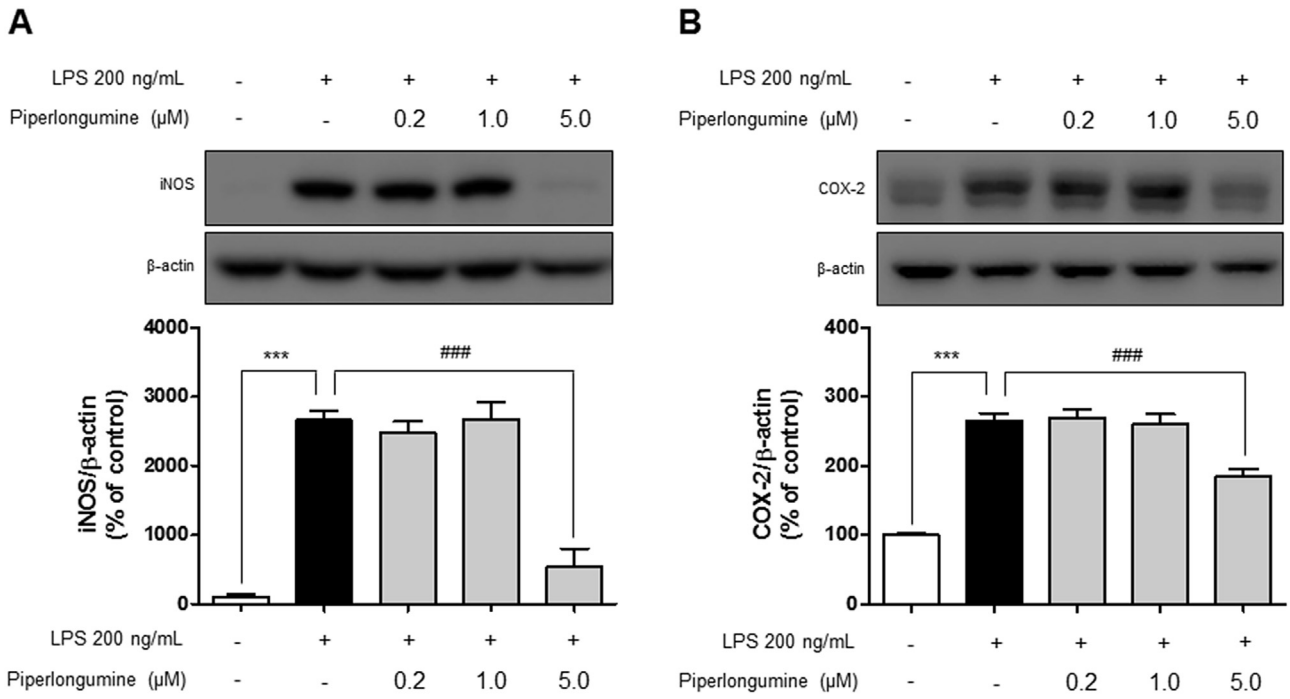

Fig. 2. Piperlongumine inhibited expression of iNOS and COX-2 induced by LPS in BV2 microglial cells. The cells were pretreated with piperlongumine (0.2, 1.0, or 5.0 μM) for 2 h prior to LPS treatment (200 ng/mL) for 22 h. The protein levels of iNOS (A) and COX-2 (B) were determined by Western blot analysis (n = 4 per group). Statistical analysis included one-way analysis of variance and Tukey's post hoc test. $^{***}p < 0.001$ compared with the control group, $^{###}p < 0.001$ compared with the LPS-treated only group. Values are the mean ± SEM.

strongly suppressed the increase in iNOS protein level induced by LPS (Fig. 2A). Similarly, the COX-2 expression level was also reduced by piperlongumine pretreatment (Fig. 2B). When microglia were exposed to external stimuli, they released proinflammatory cytokines such as TNF-α and IL-6.[6] Excessive levels of these factors can cause an inflammation-mediated disease.[15] We determined the effects of piperlongumine on the levels of proinflammatory cytokines. LPS-treated BV2 microglial cells presented significantly increased mRNA (Fig. 3A,B) and protein (Fig. 3D,E) levels of TNF-α and IL-6, but those pretreated with piperlongumine showed dramatically reduced production of these cytokines. IL-10, known as an anti-inflammatory cytokine and resolution factor of inflammation,[16] was significantly increased in piperlongumine pretreated BV2 cells compared

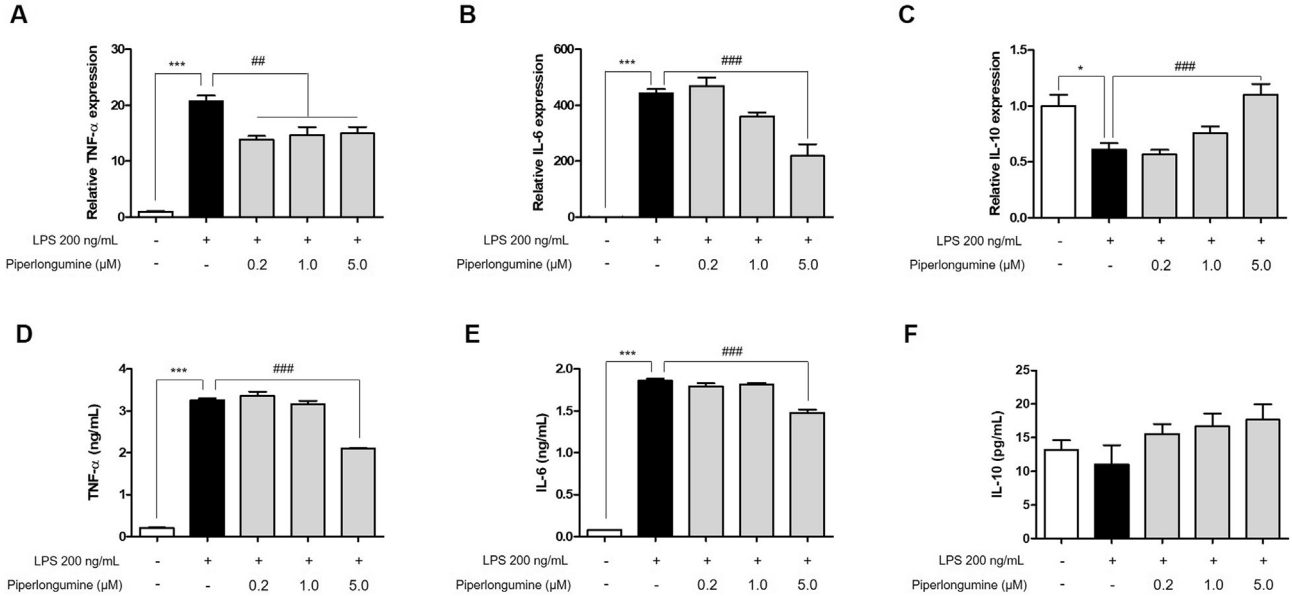

Fig. 3. Piperlongumine regulated expression of proinflammatory and anti-inflammatory cytokines induced by LPS in BV2 microglial cells. The cells were pretreated with piperlongumine (0.2, 1.0, or 5.0 μM) for 2 h prior to LPS treatment (200 ng/mL) for 22 h. Total mRNA was harvested and mRNA expression levels of TNF-α (A), IL-6 (B) and IL-10 (C) were measured by qRT-PCR (n = 4–5 per group). GAPDH was used as an internal control. The protein levels of TNF-α (D), IL-6 (E) and IL-10 (F) were determined by ELISA kits (n = 4–5 per group). Statistical analysis included one-way analysis of variance and Tukey's post hoc test. $^{*}p < 0.05$ and $^{***}p < 0.001$ compared with the control group, $^{##}p < 0.01$ and $^{###}p < 0.001$ compared with the LPS-treated only group. Values are the mean ± SEM.

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with the level in LPS-treated ones (Fig. 3C,F). These findings imply that piperlongumine suppresses the LPS-triggered inflammatory response in BV2 microglial cells.

3.3. Effects of piperlongumine on the MAPKs/NF-κB signaling activation induced by LPS in BV2 microglial cells

The mitogen-activated protein kinase (MAPK) signaling pathways such as p38, ERK1/2 and JNK 1/2 are major pathways causing inflammatory response.[17] Many studies have measured these signals to assess the mechanisms related to the anti-inflammatory effects of various drugs.[18,19] Therefore, we investigated whether the anti-inflammatory effects of piperlongumine on LPS-stimulated BV2 microglial cells were related to the MAPK signaling pathways. Our data showed that LPS increased the phosphorylation of MAPK signals, but piperlongumine did not affect these signals (Fig. 4). NF-κB signaling is another major pathway in the LPS-mediated inflammatory response. This signaling coordinates the expression of proinflammatory proteins and cytokines.[20] To evaluate the effect of piperlongumine on NF-κB transcriptional activity, we performed NF-κB luciferase assay in LPS-stimulated BV2 microglial cells. Piperlongumine at 5 μM significantly reduced the luciferase activity of NF-κB compared with LPS-treated only group (Fig. 5A). In immunocytochemistry analysis, we also observed that piperlongumine inhibited the LPS-induced translocation of p65 from the cytosol to the nucleus (Fig. 5B). These results suggest that piperlongumine has anti-neuroinflammatory effects by regulating not the MAPK signaling but the NF-κB signaling in LPS-stimulated BV2 microglial cells.

4. Discussion

Many previous studies confirmed that *P. longum L.*, including piperlongumine, has therapeutic effects on various cellular and animal models of disease, but the effects of piperlongumine on neuroinflammation and related mechanisms have remained largely unknown. Here we showed for the first time that piperlongumine has anti-neuroinflammatory effects in LPS-treated BV2 microglial cells.

P. longum, called Indian long pepper, has various pharmacological activities, including anti-cancer, anti-oxidant, hepatoprotection and anti-inflammation.[9] These activities are related to piperlongumine, the major component of *P. longum*. Piperlongumine showed barrier protective effects via the inhibition of inflammatory factors in LPS-treated human umbilical vein endothelial cells and mice.[10] Furthermore, piperlongumine and its derivative have been shown to inhibit LPS-induced production of NO and PGE_2 as well as the expression of iNOS and COX-2 in macrophage RAW264.7 cells.[21]

Neuroinflammation is a reaction of the CNS to destroy infected neurons, viruses and bacteria, but an excessive microglia-mediated inflammatory response damages neuronal cells, resulting in neurodegenerative diseases such as PD, AD and HD.[1] Microglia exhibit different phenotypes, namely, either M1 or M2, depending on the microenvironment. M1 microglia activated by harmful stimuli release excessive inflammatory factors that negatively affect neurons, such as NO, PGE_2, TNF-α, and IL-6 via the activation of NF-κB and MAPK signalings. On the other hand, M2 microglia secrete anti-inflammatory cytokines such as IL-10 and transforming growth factor-β to inhibit M1 microglia-mediated inflammation, resulting in the resolution of inflammation.[22–24] First, to evaluate the effect of piperlongumine on M1 microglia-mediated inflammation, we assessed the proinflammatory cytokines and mediators. LPS stimulation of BV2 microglia l cells caused significant increases in TNF-α, IL-1β, and IL-6 expression, an effect that was clearly decreased by piperlongumine. To confirm the related mechanisms, we evaluated signal transduction pathways induced by LPS on microglia, including MAPKs (including p38, JNK and ERK). Although piperlongumine inhibited the production of neurotoxic factors induced by LPS, it did not prevent the phosphorylation of ERK, JNK, and p38. Next, we examined the effect of piperlongumine on NF-κB signaling, known as another type of major inflammatory signal. NF-κB is a major transcription factor for iNOS and COX-2, enzymes that produce NO and PGE_2. Under normal conditions, NF-κB is located in the cytosol as a complex with IκB. When IκB is degraded by specific stimuli such as LPS, NF-κB migrates from the cytosol to the nucleus and promotes the transcription of inflammatory genes.[15,20,25] Therefore, NF-κB is recognized as an important signal to regulate M1- or M2-mediated inflammatory responses. We found that piperlongumine markedly inhibits the LPS-induced expression of iNOS and COX-2, and increases the expression of IL-10 by inhibiting the translocation of NF-κB induced by LPS. Taken together, these results suggest that piperlongumine has anti-neuroinflammatory effects through regulating NF-κB signaling rather than MAPKs signaling.

In conclusion, we demonstrated the anti-neuroinflammatory effects of piperlongumine in LPS-stimulated BV2 microglial cells. The compound increased the level of anti-inflammatory factor and reduced the levels of LPS-induced inflammatory mediators and

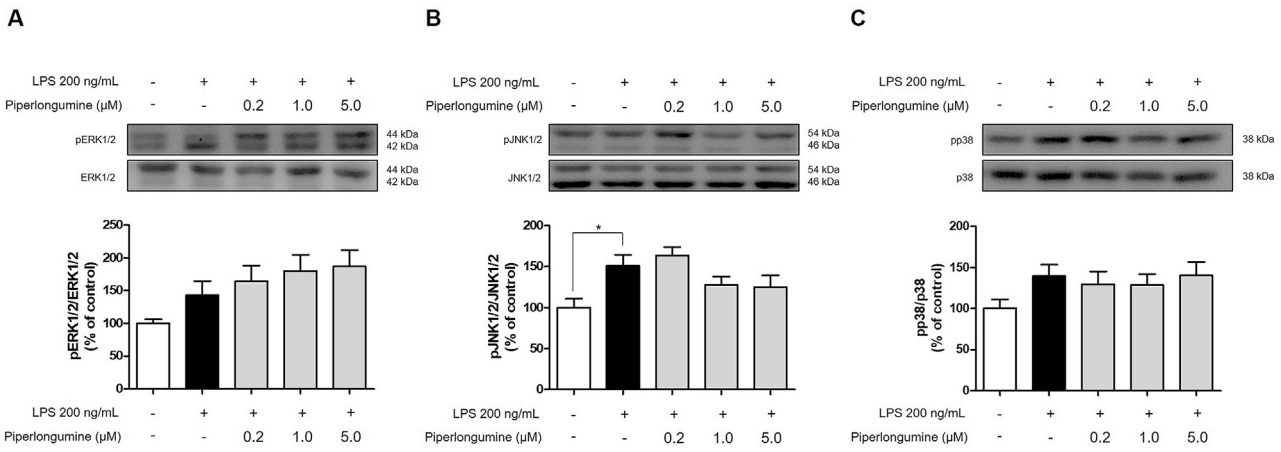

Fig. 4. Piperlongumine did not affect expression of pERK, pJNK and pp38 induced by LPS in BV2 microglial cells. The cells were pretreated with piperlongumine (0.2, 1.0, or 5.0 μM) for 2 h prior to LPS treatment (200 ng/mL) for 4 h. The protein levels of pERK1/2, ERK1/2 (A), pJNK1/2, JNK1/2 (B), and pp38, p38 (C) were determined by Western blot analysis (n = 4 per group). Statistical analysis included one-way analysis of variance and Tukey's post hoc test. $^{*}p < 0.05$ compared with the control group. Values are the mean ± SEM.

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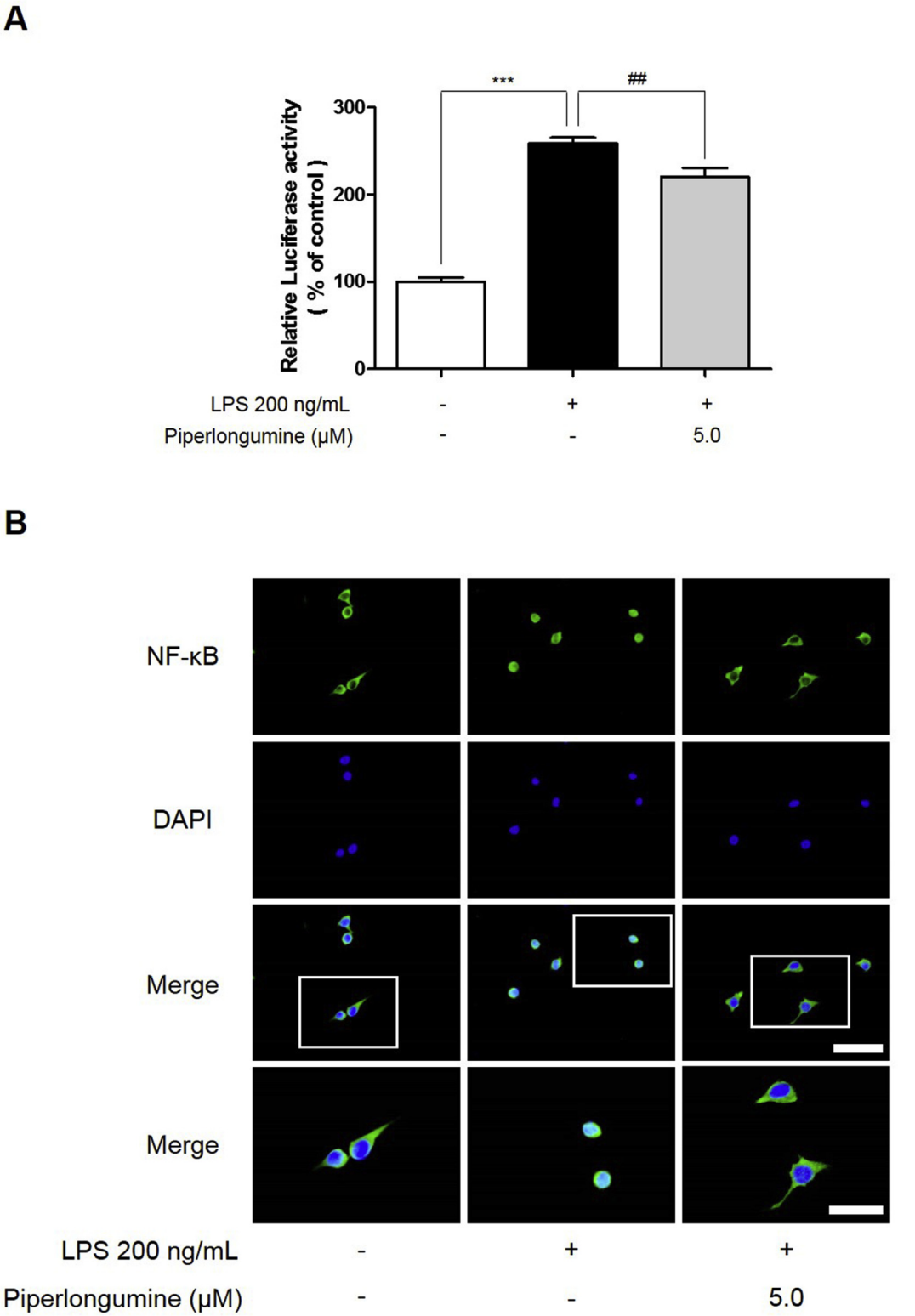

Fig. 5. Piperlongumine regulated NF-κB signaling in LPS-stimulated BV2 microglial cells. (A) NF-κB luciferase assay. Cells were transfected with TK-renilla (pRL-TK) and NF-κB firefly luciferase (pNF-κB-Luc) constructs. Transfected cells were pretreated with 5 μM of piperlongumine for 2 h and then further stimulated with LPS (200 ng/mL) for 12 h. NF-κB-dependent transcriptional activity was determined (n = 4 per group). (B) Representative images of NF-κB localization. Cells were stained by anti-p65 antibody and DAPI. Low magnification, 100 μm, High magnification, 50 μm. Statistical analysis included one-way analysis of variance and Tukey's post hoc test. $^{***}p < 0.001$ compared with the control group, $^{\#\#}p < 0.01$ compared with the LPS-treated only group. Values are the mean ± SEM.

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cytokines in BV2 microglial cells via regulating NF-κB signaling. Collectively, these results indicate that the active compound piperlongumine of *P. longum* has potential in the treatment of neuroinflammatory diseases mediated by microglial activation.

Conflict of interest

The authors declare that there are no conflicts of interest.

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