

# Anti-neuroinflammatory effect of curcumin on Pam3CSK4-stimulated microglial cells

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**Abstract.** Curcumin is the main curcuminoid present in *Curcuma longa* and it has been previously reported to exhibit a wide range of pharmacological activities. In the present study, the inhibitory effects of curcumin on the inflammatory mediators released by Pam3CSK4-stimulated BV-2 microglial cells were investigated. The production of pro-inflammatory mediators and cytokines, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), were measured by enzyme-linked immunosorbent assay (ELISA). The expression of inflammatory genes, including inducible nitric oxide synthase and cyclooxygenase-2, were further investigated using reverse transcription-quantitative polymerase chain reaction. The effects of curcumin on heme oxygenase-1 (HO-1), nuclear factor (erythroid-derived 2)-like 2 (Nrf2), mitogen-activated protein kinase (MAPK) and nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling pathways were analyzed by western blotting. The results revealed that curcumin dose-dependently inhibited Pam3CSK4-induced nitric oxide, PGE<sub>2</sub>, and TNF- $\alpha$  secretion. Curcumin suppressed the secretion of inflammatory

mediators through an increase in the expression of HO-1. Curcumin induced HO-1 transcription and translation through the Nrf2/antioxidant response element signaling pathway. Inhibitory experiments revealed that HO-1 was required for the anti-inflammatory effects of curcumin. Further mechanistic studies demonstrated that curcumin inhibited neuroinflammation by suppressing NF- $\kappa$ B and MAPK signaling pathways in Pam3CSK4-activated microglial cells. The results of the present study suggest that curcumin may be a novel treatment for neuroinflammation-mediated neurodegenerative disorders.

## Introduction

Chronic neuroinflammation serves an important role in progressive neurodegenerative disorders, including Alzheimer's disease (AD) and Parkinson's disease (PD) (1). Neuroinflammation may occur as a result of endotoxins, injury or infection. Microglial cells are macrophages specific to the central nervous system (CNS), which serve a crucial role in host defenses against toxins in the CNS (2). Specific stimulators may activate the microglial cells by binding with pattern-recognition receptors (PRR) on their surface (3). Microglial cells may be activated into two distinct types, which are designated as classically activated M1 and alternatively activated M2 cells. The activation of M1 microglial cells initiates inflammatory responses via the production of inflammatory mediators, including nitric oxide (NO), reactive oxygen species (ROS), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), which may promote neuronal injury (4). Activated M2 microglial cells produce anti-inflammatory molecules that reduce inflammation and repair injured tissue (5). The maintenance of microglial cell activation is important for brain development and the repair of injured sites within the brain (6). However, under pathological conditions, extensive classically activated microglia-mediated inflammation contributes to neuronal injury through the release of cytotoxic agents, including NO, ROS and other inflammatory mediators (7).

During chronic neuroinflammation, intracellular signaling cascades, including nuclear factor- $\kappa$ B (NF- $\kappa$ B) and mitogen-activated protein kinases (MAPKs), become activated (8). This subsequently increases inflammatory

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**Abbreviations:** AD, Alzheimer's disease; CNS, central nervous system; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; TLR, Toll-like receptor; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH2-terminal protein kinase; ERK, extracellular signal-regulated kinase; NF- $\kappa$ B, nuclear factor- $\kappa$ B; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; HO-1, heme oxygenase-1; Nrf2, nuclear factor (erythroid-derived 2)-like 2

**Key words:** curcumin, anti-inflammation, Toll-like receptor 2, heme oxygenase-1

mediators, including TNF- $\alpha$ , interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6 and cyclooxygenase-2 (COX-2), as well as increases the expression of genes that regulate cell survival and growth (9). Additionally, the p38 MAPK signaling pathway in activated microglia has been associated with several inflammatory diseases, including rheumatoid arthritis, AD and inflammatory bowel disease (10). Inflammatory signaling pathways following microglial activation are considered a prime target for inhibition of neuroinflammation-mediated neurodegeneration (2). A recent study suggested that the activation of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/antioxidant response element (ARE) signaling pathway was an important target in blocking neuroinflammation and neurodegenerative diseases (11). Another previous study indicated that Nrf2 inhibited microglial hyper-activation by suppressing the p38 MAPK and NF- $\kappa$ B signaling pathways (12).

Under normal conditions, Nrf2 activation is suppressed by the Kelch-like ECH-associated protein 1, protein dimer, which acts as an adaptor molecule of the cullin3 ubiquitin protein ligase complex. However, in response to stimuli, Nrf2 translocates to the nucleus and binds with the ARE sequence located in the promoter region of the oxidative stress response genes, NAD(P)H dehydrogenase [quinone] (NQO)-1 and heme oxygenase-1 (HO-1) (13). The HO-1 gene has been previously studied for its potential neuroprotective and anti-inflammatory effects (14). The hippocampi of Nrf2 and HO-1 knockout mice were demonstrated to be hypersensitive to neuroinflammation induced by lipopolysaccharide (LPS), as indicated by an increase in the secretion of inflammatory markers, including inducible nitric oxide synthase (iNOS), IL-6 and TNF- $\alpha$  (15,16). The results of these previous studies suggest that targets to activate Nrf2 or HO-1 in the microglia would be a beneficial therapeutic strategy to reduce neuroinflammation.

Curcumin is one of the main components of the rhizomes of *Curcuma longa*. It has been previously used in oriental medicine as a sedative, analgesic and antipyretic, as well as a treatment for pancreatic cancer, AD and epilepsy (16,17). Curcumin is a highly lipophilic natural compound that is able to pass through the blood-brain barrier (18). Within the brain curcumin appears primarily in the hippocampus (18). A previous study has reported that curcumin may inhibit amyloid  $\beta$  oligomers and rescue neuronal injury in models of AD (19). Furthermore, curcumin has also been reported to promote the development of the M2 microglial phenotype in an HO-1-dependent manner and reduce iNOS induction, thus protecting microglial cells against oxidative stress (16). The anti-inflammatory effects of curcumin occur through several different mechanisms (16,17). Curcumin is able to regulate the activation of transcription factors, such as activator protein-1 and NF- $\kappa$ B and it may block COX-2 and iNOS (20). It has also been reported that curcumin has an inhibitory effect on the LPS-induced production of inflammatory mediators, including IL-1 $\beta$ , IL-6, TNF- $\alpha$  and IFN- $\alpha$  (21). However, the effect of curcumin on neuroinflammation is not thoroughly understood. Therefore, the present study investigated the anti-inflammatory effects of curcumin and the underlying molecular mechanisms by which it affects microglial cells and may confer neuroprotection.

## Materials and methods

**Materials.** Curcumin was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Protoporphyrin IX (SnPP) and antibodies directed against HO-1 (sc-390991), Nrf2 (sc-722), TATA-binding protein (TBP; sc-74595),  $\alpha$ -tubulin (sc-134237) and  $\beta$ -actin (sc-130065) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies directed against COX-2 (4842S), iNOS (13120), phosphorylated (p)-MAPK (9910s), MAPK (9926), protein kinase B (Akt; 4685), p-Akt (13038), and NF- $\kappa$ B pathway kit (9936) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Pam3CSK4, Dulbecco's modified Eagle's medium (DMEM; 11995-065) and fetal bovine serum (FBS; 10099-1) were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). c-Jun NH2-terminal protein kinase (JNK) inhibitor (JNK inhibitor II; 420119); Akt inhibitor (wortmannin; 12-338), extracellular signal-regulated kinase (ERK) inhibitor (PD98059, 513000) and p38 inhibitor (SB230580, 559395) were purchased from EMD Millipore (Billerica, MA, USA).

**Cell culture.** Mouse BV-2 microglial cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37°C in a 5% CO<sub>2</sub> humidified atmosphere.

**Cell viability assay.** The cytotoxicity of curcumin was assessed using a MTT-based colorimetric assay. BV-2 cells were seeded in 24-well plates at a density of 5x10<sup>5</sup> cells/well. The cells were treated with different concentrations of curcumin (5, 10 and 20  $\mu$ M) or co-treated with Pam3CSK4 (0.1  $\mu$ g/ml) for 24 h at 37°C. Subsequently, MTT solution (5  $\mu$ l of 5 mg/ml) was added to each well (final concentration, 62.5  $\mu$ g/ml). Following incubation for 3 h at 37°C in 5% CO<sub>2</sub>, the supernatant was removed and the formazan crystals produced in viable cells were solubilized with 150  $\mu$ l dimethyl sulfoxide. The absorbance of each well was read at 570 nm using a microplate reader.

**Measurement of TNF- $\alpha$  and PGE<sub>2</sub> concentration.** BV-2 cells were firstly incubated with various concentrations (0–20  $\mu$ M) of curcumin for 1 h (37°C) and subsequently treated with Pam3CSK4 (0.1  $\mu$ g/ml) for 16 h at 37°C. The release of TNF- $\alpha$  and PGE<sub>2</sub> were determined. Cells were pretreated with SnPP (HO-1 inhibitor, 20  $\mu$ M) for 30 min at 37°C, and then treated with curcumin in the presence or absence of Pam3CSK4 for 16 h. The release of TNF- $\alpha$  was determined. Following 16 h incubation at 37°C, TNF- $\alpha$  (MTA00B) and/or PGE<sub>2</sub> (KGE004B) secretion were quantified in the culture media using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol.

**Preparation of nuclear extract.** Cells were treated with 20  $\mu$ M curcumin for the indicated times (0, 1, 2 and 4 h) or various doses of curcumin (0, 1, 5, 10 or 20  $\mu$ M) for 2 h at 37°C. Nuclear extracts were prepared and examined for Nrf2 expression using western blot analysis. BV-2 cells were treated with curcumin followed by treatment with Pam3CSK4 for 0.5 h at 37°C. The nucleus and cytosol extracts of BV-2 cells were harvested and

analyzed by western blot analysis to detect the degree of p65 translocation and I $\kappa$ B $\alpha$  degradation. BV-2 microglial cells were washed three times with cold phosphate-buffered saline (PBS) and collected in 300  $\mu$ l PBS using centrifugation at 800 x g for 5 min (4°C). The cell pellets were suspended in buffer A [10 mM HEPES-KOH (pH 7.9); 1.5 mM MgCl<sub>2</sub>; 10 mM KCl; 0.5 mM dithiothreitol (DTT); 0.2 mM protease inhibitor (PI)] and incubated for 5 min on ice. Buffer B [10 mM HEPES-KOH (pH 7.9); 1.5 mM MgCl<sub>2</sub>; 420 mM NaCl; 0.2 mM EDTA; glycerol 25% v/v; 0.1 mM DTT; 0.2 mM PI] was added to the cell extract and it was incubated on ice for 5 min prior to centrifugation at 11,000 x g for 1 min at 4°C. Nuclear proteins were extracted by the addition of complete lysis buffer B [10 mM HEPES-KOH (pH 7.9); 1.5 mM MgCl<sub>2</sub>; 10 mM KCl; 0.5 mM DTT; 0.2 mM PI; 25% (w/v) glycerol; 420 mM NaCl; 0.2 mM EDTA] for 30 min at 4°C with occasional vortexing. Following centrifugation at 11,000 x g for 5 min at 4°C, the supernatants were collected and stored at -70°C.

**Western blot analysis.** BV-2 cells were treated with various concentrations of curcumin (1, 5, 10 or 20  $\mu$ M) for 1 h at 37°C, and subsequently treated with Pam3CSK4 (0.1  $\mu$ g/ml) for 8 h at 37°C, and the COX-2 expression level was determined. Cells were treated with 20  $\mu$ M curcumin for the indicated times (0-24 h) or various doses of curcumin (0-20  $\mu$ M) for 8 h at 37°C. Total cellular extracts were harvested and examined for HO-1 expression using western blot analysis. BV-2 cells were treated with curcumin for 1 h followed by stimulation with Pam3CSK4 for 0.5 h at 37°C. Cell extracts were collected and subjected to western blot analysis to determine p-Akt and p-MAPK expression levels. BV-2 cells were harvested in ice-cold lysis buffer (1% Triton X-100; 1% deoxycholate; 0.1% sodium dodecyl sulfate). The protein content of the cell lysates was subsequently determined using Bradford reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Total proteins in each sample (50  $\mu$ g) were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. Following blocking of the non-specific binding sites with 5% non-fat milk at room temperature for 30 min, the membranes were incubated with primary antibodies directed against COX-2 (1:500), iNOS (1:500), p-Akt (1:1,000), p-MAPK (1:1,000), MAPK (1:1,000), p-p65, p65 (1:500) p-I $\kappa$ B $\alpha$ , I $\kappa$ B $\alpha$  (1:1,000), HO-1 (1:1,000), Nrf2 (1:1,000), TBP (1:3,000),  $\alpha$ -tubulin (1:3,000) and  $\beta$ -actin (1:3,000) for 16 h at 4°C. This was followed by incubation with horseradish peroxidase-conjugated anti-rabbit (sc-2768; 1:5,000) or anti-mouse (sc-2371; 1:5,000) secondary antibodies (Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. Tubulin was used as the loading control for each lane. The proteins were visualized using an enhanced chemiluminescence detection kit (GE Healthcare, Chicago, IL, USA). Following washing with PBS with Tween-20, the protein bands were visualized using the Gel Doc™ EZ Imaging system (Bio-Rad Laboratories, Inc.) and analyzed using an ImageQuant 350 analyzer (GE Healthcare).

**Measurement of nitrite concentration.** BV-2 cells were treated with various concentrations of curcumin (5, 10 or 20  $\mu$ M) for 1 h and subsequently treated with Pam3CSK4 (0.1  $\mu$ g/ml) for 16 h at 37°C. NO synthesis in cell cultures was measured by a microplate assay. Cells were pretreated with SnPP (HO-1

inhibitor, 20  $\mu$ M) for 30 min at 37°C, and then treated with curcumin in the presence or absence of Pam3CSK4 for 16 h at 37°C. The release of NO was determined. BV-2 cells were treated with the JNK inhibitor (JNK II, 10  $\mu$ M), Akt inhibitor (Wor, 5  $\mu$ M), ERK inhibitor (PD98059, 10  $\mu$ M) or p38 inhibitor (SB230580, 10  $\mu$ M) for 1 h at 37°C, following treatment with Pam3CSK4 (0.1  $\mu$ g/ml) for 16 h at 37°C. The release of NO was determined. To measure nitrite, 100- $\mu$ l aliquots were removed from the supernatant and incubated with an equal volume of the Griess reagent [1% sulfanilamide; 0.1% N-(1-naphthyl)-ethylenediaminedihydrochloride; 2.5% H<sub>3</sub>PO<sub>4</sub>] at room temperature for 10 min. Nitrite concentration was determined by measuring the absorbance at 540 nm with a Vmax 96-well microplate spectrophotometer. Sodium nitrite was used as a standard.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** BV-2 cells were treated with various concentrations of curcumin (0, 5, 10 or 20  $\mu$ M) for 1 h at 37°C, and subsequently treated with Pam3CSK4 (0.1  $\mu$ g/ml) for 4 h at 37°C, the mRNA expression levels of iNOS and COX-2 were determined. BV-2 cells were incubated with 20  $\mu$ M curcumin for the indicated times (2, 4, 6, 8 and 10 h) or cells were incubated with increasing doses of curcumin (1, 5, 10 and 20  $\mu$ M) for 4 h at 37°C and the relative HO-1 mRNA expression level was measured. BV-2 cells were then treated with the JNK inhibitor (10  $\mu$ M), Akt inhibitor (5  $\mu$ M), ERK inhibitor (10  $\mu$ M) or p38 inhibitor (10  $\mu$ M) for 1 h at 37°C, followed by treatment with Pam3CSK4 (0.1  $\mu$ g/ml) for 4 h at 37°C. The expression levels of iNOS were determined. Total RNA was isolated from cells using an Axyprep multisource total RNA miniprep kit (AP-MN-MS-RNA; Corning, Inc., Corning, NY, USA) according to the manufacturer's protocol. cDNA was synthesized from 1  $\mu$ g total RNA using a Maxime RT-PCR PreMix kit (Takara Bio, Inc., Otsu, Japan) and anchored oligo(dT)<sub>15</sub>-primers. qPCR was performed using a Chromo 4™ system (Bio-Rad Laboratories, Inc.) and SYBR®-Green PCR Master Mix (Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used: 40 cycles of 95°C for 5 min, 95°C for 15 sec and 62°C for 30 sec. Relative amounts of target mRNA were determined using the 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method (22) by normalizing target mRNA comparative threshold values to those of  $\beta$ -actin. The PCR primers were designed using Premier-E version 6 software (Premier Group of Companies, Vancouver, BC, Canada) and are listed in Table I. The relative amount of mRNA was presented as the fold-change value compared to the control value.

**Statistical analysis.** Data were expressed as the mean  $\pm$  standard deviation. Each experiment was repeated a minimum of three times. Statistical analysis was performed using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA) to determine significant differences. Either a Student's t-test or one-way analysis of variance, followed by Dunn's post hoc test was used for analyses. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Toxicity of curcumin and PamCSK4 on BV-2 cells.** To assess the cytotoxic effect of curcumin on BV-2 cells, they were

Table I. Name and sequence of primers used for reverse transcription-quantitative polymerase chain reaction.

Gene name	Primer sequence (5'-3')	Gene ID	Product size (bp)
Inducible nitric oxide synthase	F: GGCACCGAGATTGGAGTTC R: GGTACATTCTGCTTCT	NM001313921	174
Cyclooxygenase-2	F: TCAGGTCATTGGTGGAGAGG R: ATGGTGGCATAATCATCAGAC	NM011198.4	150
Heme oxygenase-1	F: AGGTCTGAAGAAGATTGC R: TCTCCAGAGTGTTCATTCG	NM010442.2	175
$\beta$ -actin	F: GCACCACACCTTCTACAA R: TACGACCAGAGGCATACA	NM007393.5	156

F, forward; R, reverse.

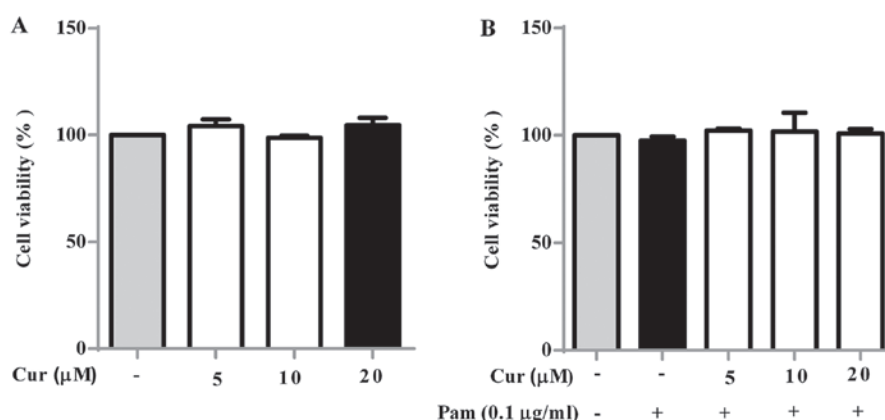


Figure 1. Cur has no significant effect on cell viability. (A) BV-2 cells were treated with various concentrations of Cur (5, 10 and 20  $\mu$ M) for 24 h. (B) BV-2 cells were pretreated with Cur (5, 10 and 20  $\mu$ M) for 1 h and subsequently exposed to Pam (0.1  $\mu$ g/ml) for 24 h. Cell viability was measured by an MTT assay. Cur, curcumin; Pam, Pam3CSK4.

treated with increasing concentrations (0–20  $\mu$ M) of curcumin for 24 h and their viability was measured (Fig. 1A). BV-2 cells without any treatment were used as the negative control. The results revealed that curcumin at concentrations of 5–20  $\mu$ M had no notable cytotoxic effect on BV-2 microglial cells. Thus, 5–20  $\mu$ M curcumin was selected for subsequent experiments. The cytotoxic effect of treatment of BV-2 cells with curcumin and Pam3CSK4 (0.1  $\mu$ g/ml) in combination was also investigated (Fig. 1B). No notable cytotoxic effects were observed at all concentrations.

**Inhibitory effect of curcumin on Pam3CSK4-induced inflammatory mediators in BV-2 microglial cells.** NO, TNF- $\alpha$ , PGE<sub>2</sub>, iNOS and COX-2 are inflammatory mediators present in activated microglial cells (7). Treatment of BV-2 cells with Pam3CSK4 alone notably increased the production of NO (Fig. 2A), PGE<sub>2</sub> (Fig. 2B) and TNF- $\alpha$  (Fig. 2C) and the expression of iNOS (Fig. 2D) and COX-2 (Fig. 2E) compared with the negative control. Curcumin had significant inhibitory effects on the secretion of NO and PGE<sub>2</sub> in Pam3CSK4-stimulated BV-2 microglial cells at higher concentrations (10 and 20  $\mu$ M) (Fig. 2A and B) compared with the Pam3CSK4-treated control cells. The secretion of TNF- $\alpha$  was also significantly suppressed by curcumin (Fig. 2C) compared

with the level in the Pam3CSK4-treated control cells, however only at the highest concentration of 20  $\mu$ M. The mRNA expression levels of iNOS and COX-2 were significantly increased following exposure to Pam3CSK4 compared with the negative control group, but significantly decreased compared with Pam3CSK4-treated control cells following treatment with 10 and 20  $\mu$ M curcumin (Fig. 2D and E). Curcumin also notably decreased the protein expression levels of COX-2 in Pam3CSK4-induced BV-2 cells (Fig. 2F).

**Treatment with curcumin causes an increase of HO-1 in microglial cells.** To assess the association of HO-1 with the anti-neuroinflammatory properties of curcumin, the effect of curcumin on HO-1 induction at the transcriptional and translational levels was investigated. Treatment of BV-2 cells with curcumin significantly increased HO-1 mRNA levels compared with the untreated cells, with a maximal effect being observed at 4 h (Fig. 3A). Curcumin increased the HO-1 mRNA in a dose-dependent manner with significant increases observed at 10 and 20  $\mu$ M (Fig. 3B) compared with the untreated cells. The protein expression level of HO-1 was measured by western blot analysis and similar results were observed (Fig. 3C and D). The protein expression of HO-1 was notably increased at 8 h following treatment and at the higher concentrations of curcumin.

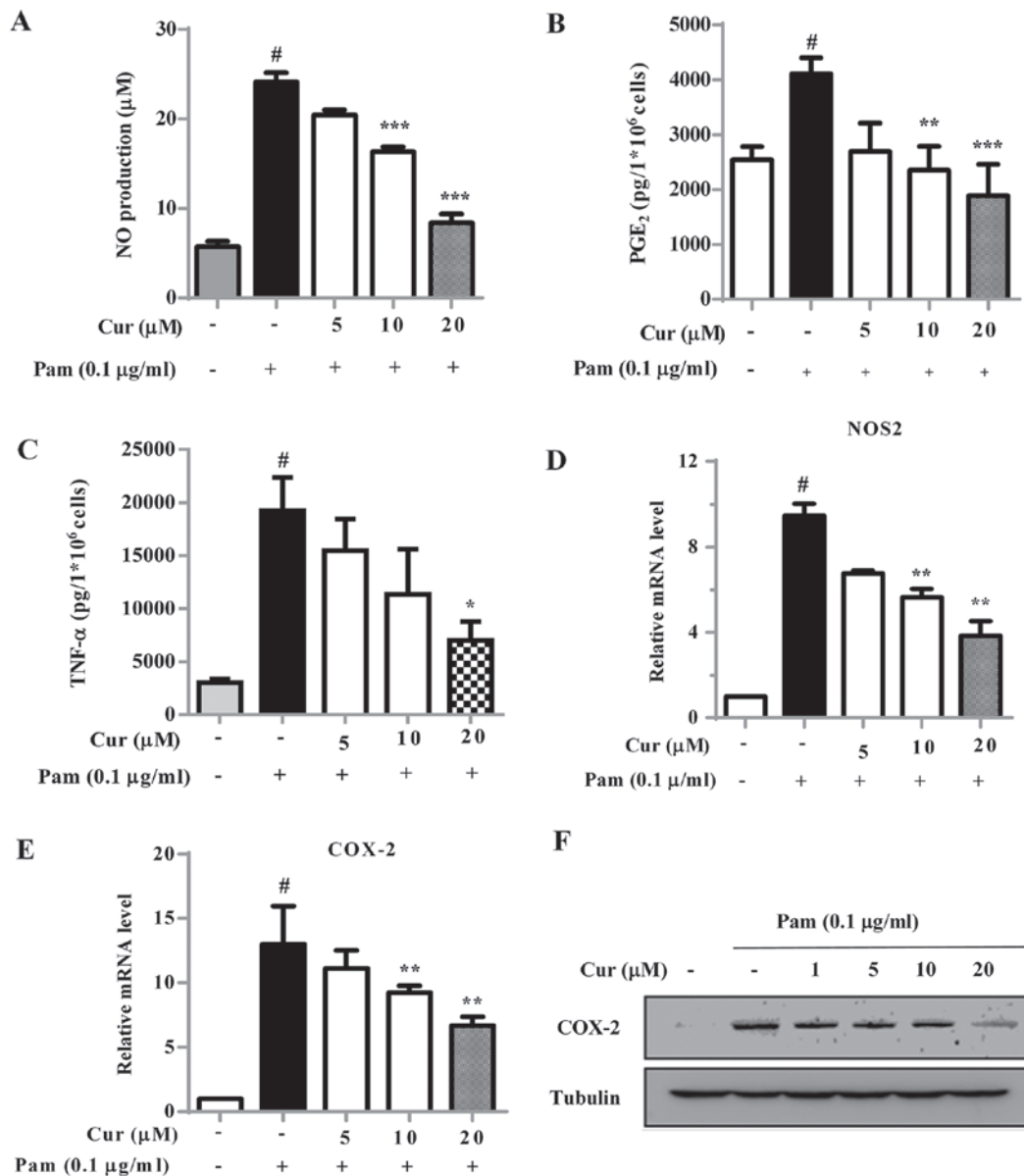


Figure 2. Effect of Cur on Pam-induced NO, PGE<sub>2</sub>, TNF- $\alpha$  secretion, iNOS and COX-2 expression in BV-2 cells. Cells were treated with Cur at increasing doses (0-20  $\mu\text{M}$ ) and stimulated with Pam (0.1  $\mu\text{g/ml}$ ). (A) Nitrite levels were measured in the collected media supernatant using the Griess method. The secretion of (B) PGE<sub>2</sub> and (C) TNF- $\alpha$  were determined by ELISA. The mRNA expression levels of (D) iNOS, encoded by NOS2, and (E) COX-2 were detected using reverse transcription-quantitative polymerase chain reaction analysis. (F) The expression of COX-2 was detected using western blot analysis. Tubulin was used as a control. Data were presented as the mean  $\pm$  standard deviation from three independent experiments. <sup>#</sup>P<0.01 vs. negative control; <sup>\*</sup>P<0.05, <sup>\*\*</sup>P<0.01 and <sup>\*\*\*</sup>P<0.001 vs. the Pam3CSK4-treated control. Pam, Pam3CSK4; Cur, curcumin; TNF, tumor necrosis factor; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; NO, nitric oxide; NOS2, nitric oxide synthase 2; ELISA, enzyme-linked immunosorbent assay.

To investigate the effect of curcumin on Nrf2 nuclear translocation, which is associated with HO-1 induction, the accumulation of Nrf2 in the nucleus was measured following treatment with curcumin (Fig. 3E and F). The quantity of Nrf2 that translocated into the nucleus notably increased following treatment with curcumin, compared with the control group. The maximum effect was observed at 2 h following curcumin treatment and was most notable at the higher concentrations of curcumin. These results suggest that curcumin increased the transcription and translation of HO-1 through activation of the Nrf2 transcription factor.

To confirm the anti-neuroinflammatory effects of curcumin in HO-1 induction, the level of inflammatory mediators, NO and TNF- $\alpha$ , were measured in Pam3CSK4-stimulated BV-2

microglial cells incubated with or without the HO-1 inhibitor SnPP (20  $\mu\text{M}$ ) (Fig. 3G and H). The cells were incubated with curcumin with or without SnPP for 0.5 h, and then exposed to Pam3CSK4 for 16 h. The secretion of NO and TNF- $\alpha$  were significantly increased following treatment with Pam3CSK4 compared with the negative control; whereas there was a significant decrease in NO and TNF- $\alpha$  production following treatment with curcumin (20  $\mu\text{M}$ ) compared with cells treated with Pam3CSK4 only. However, NO and TNF- $\alpha$  secretion were significantly increased in cells co-treated with SnPP (20  $\mu\text{M}$ ) and curcumin, compared with cells treated with curcumin only. These results suggest that HO-1 is associated with the inhibitory effect of curcumin on the Pam3CSK4-mediated release of inflammatory mediators.

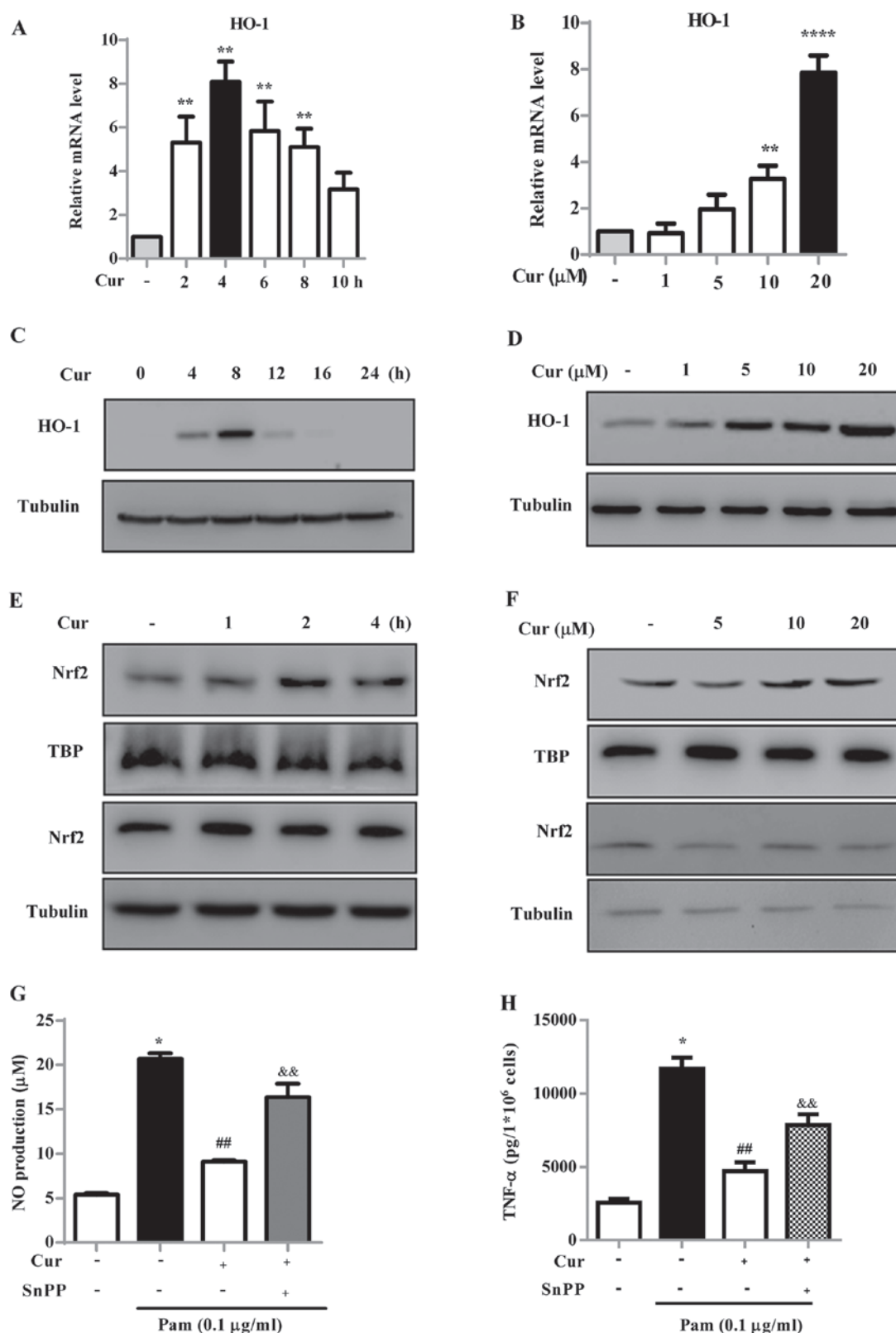


Figure 3. Cur induces HO-1 production through the Nrf2/antioxidant response element signaling pathway in BV-2 microglial cells. (A) BV-2 cells were incubated with 20  $\mu$ M Cur for the indicated times (2, 4, 6, 8 and 10 h) or (B) cells were incubated with increasing doses of Cur (5, 10 and 20  $\mu$ M) for 4 h and the relative HO-1 mRNA expression level was measured using reverse transcription-quantitative polymerase chain reaction. (C) Cells were treated with 20  $\mu$ M Cur for the indicated times (0-24 h) or (D) various doses of Cur (0-20  $\mu$ M) for 8 h. Total cellular extracts were harvested and examined for HO-1 expression using western blot analysis. Tubulin was used as the loading control for each lane. (E) Cells were treated with 20  $\mu$ M Cur for the indicated times (0-24 h) or (F) various doses of Cur (0-20  $\mu$ M) for 2 h. Nuclear extracts were prepared and examined for Nrf2 expression using western blot analysis. TBP and tubulin was detected as a loading control. Cells were pretreated with SnPP (HO-1 inhibitor, 20  $\mu$ M) for 30 min, and then treated with Cur in the presence or absence of Pam for 16 h. The release of (G) NO and (H) TNF- $\alpha$  were determined. Three independent experiments were performed and the data are presented as the mean  $\pm$  standard deviation. \*P<0.05, \*\*P<0.01 and \*\*\*\*P<0.0001 vs. negative control; ##P<0.01 vs. Pam-treated control; &&P<0.01 vs. Cur + Pam-treated group. Cur, curcumin; HO, heme oxygenase; TBP, TATA-binding protein; NO, nitric oxide; SnPP, protoporphyrin IX; Nrf2, nuclear factor (erythroid-derived 2)-like 2; TNF, tumor necrosis factor; Pam, Pam3CSK4.

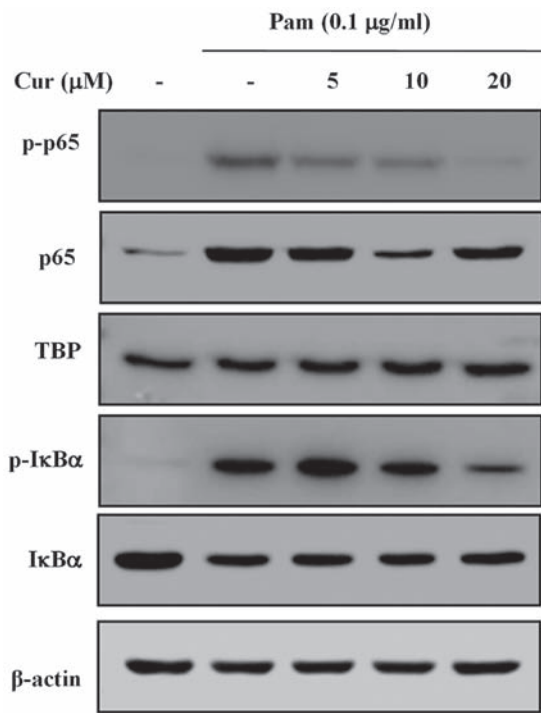


Figure 4. Cur has an inhibitory effect on NF-κB activation in Pam-activated BV-2 microglial cells. BV-2 cells were treated with Cur followed by treatment with Pam for 0.5 h. The nucleus and cytosol extracts of BV-2 cells were harvested and analyzed by western blot analysis to detect the degree of p65 translocation and IκBα degradation. Cur, curcumin; Pam, Pam3CSK4; NF, nuclear factor; IκBα, NF-κB inhibitor α; p-, phosphorylated; TBP, TATA-binding protein.

*Treatment with curcumin reduces p65 translocation and IκBα degradation.* Major cell signaling pathways, including those associated with MAPKs, are activated by Toll-like receptors (TLRs) in macrophage cells, which in turn leads to the induction of transcription factors, such as NF-κB (23). The translocation of p65 and the phosphorylation of IκBα in Pam3CSK4-stimulated BV-2 microglial cells was investigated (Fig. 4). The cells were stimulated with Pam3CSK4 (0.1 μg/ml) and treated with curcumin at the indicated doses (0-20 μM). The phosphorylation of p65 was markedly increased following stimulation with Pam3CSK4, but gradually and dose-dependently decreased following treatment with curcumin. Similarly, the phosphorylation of IκBα was induced by treatment with Pam3CSK4, but recovered dose-dependently when treated with curcumin. These results indicate that treatment with curcumin reduces the translocation of p65 into the nucleus and also reduces the degradation of IκBα.

Activation of MAPKs and the Akt signaling pathway are crucial for the regulation of inflammatory mediators in activated microglia (24). The effect of curcumin on Pam3CSK4-induced phosphorylation of JNK, ERK1/2, p38 and Akt in BV-2 cells was also investigated (Fig. 5). Exposure of BV-2 cells to Pam3CSK4 for 30 min markedly increased the phosphorylation of JNK, ERK, p38 and Akt. Treatment with curcumin notably reduced the Pam3CSK4-induced phosphorylation of Akt and p38, which are important upstream signaling molecules in inflammatory responses mediated by activated microglial cells (25). The inhibition of p38 and JNK with specific inhibitors significantly decreased the mRNA expression levels

of iNOS compared with the Pam3CSK4-treated cells, and reduced the secretion of NO (Fig. 5B and C).

## Discussion

TLR2 is a transmembrane PRR that responds to exogenous and endogenous ligands (3). TLRs are expressed in a variety of immune cells and mediate neuroimmunity (26). The majority of exogenous ligands recognized by TLR2 are lipoproteins or lipoteichoic acid from gram-positive bacteria, whereas TLR4 predominantly identifies LPS endotoxins from gram-negative bacteria (27). Bacterial endotoxins, endogenous compounds and synthesized compounds, including peptidoglycan, Pam3CSK4, lipoteichoic acid (LTA) and β-amyloid are collectively known as danger-associated molecular patterns (DAMPs) (28). DAMPs are able to activate TLR2 under chronic pain conditions and neurodegenerative disorders (28). A previous study using TLR2 knock-out mice revealed that the expression of the TLR2 receptor is required for the development and persistence of α-synuclein accumulation, α-synuclein-mediated neurodegeneration and experimental spontaneous pain due to peripheral nerve injury (26). Additionally, TLR2 activation is critical in the promoting brain injury (26). A previous study has revealed that the activation of TLR2 in microglia lead to the development of diseases associated with hypothalamic inflammation through the NF-κB and COX-2 signaling pathways (29). Therefore, controlling the activation of TLR2 and its downstream signaling pathways may be beneficial for the attenuation of neuroinflammation associated neuro-injuries. Activation of TLR2 heterodimers (with TLR1 or TLR6) primarily requires the myeloid differentiation primary response gene 88-dependent signaling pathways, including NF-κB and MAPKs (28).

In the present study, the anti-neuroinflammatory properties of curcumin were investigated in Pam3CSK4-induced microglial cells. The activation of microglia by Pam3CSK4 markedly mediated the M1/M2 ratio to imitate the M1 phenotype of microglial cells identified in AD murine models (4). The present study revealed that Pam3CSK4 notably increased the secretion of NO and TNF-α, and the mRNA expression levels of iNOS in microglial cells, which are all known markers of the M1 subtype of microglial cells (30). In addition, NO and PGE<sub>2</sub> are considered to contribute to a number of physiological and pathological processes, including ischemia, trauma, multiple sclerosis, PD and AD (31). Overexpression of NO and PGE<sub>2</sub> is also associated with the upregulation of iNOS and COX-2 (31). Inflammatory cytokines, including TNF-α and IL-1β, have been demonstrated to be significantly increased in neurodegenerative disorders (28). Recent study suggested that the inhibition of these cytokines was beneficial for the treatment of neurodegenerative diseases (28). In the present study, it was identified that curcumin had a significant anti-inflammatory effect through the inhibition of inflammatory mediators. Furthermore, the suppression of inflammatory molecules by curcumin was reversed following treatment with SnPP, an HO-1 inhibitor. Curcumin reduced the secretion of inflammatory molecules by upregulating HO-1 and Nrf2 expression.

The immunomodulatory role of HO-1 was first described in peripheral macrophages, where HO-1 by-products, biliverdin

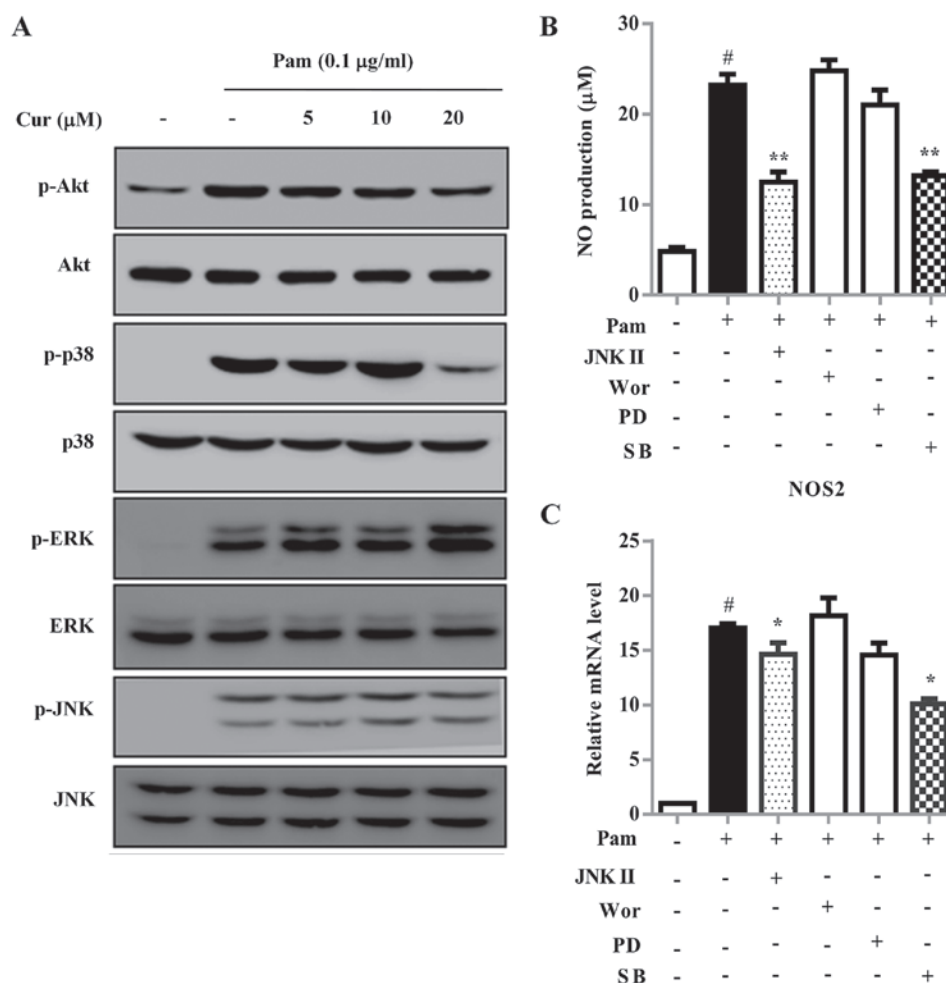


Figure 5. Cur inhibits neuroinflammation by targeting the p38 mitogen-activated protein kinase signaling pathway. (A) BV-2 cells were treated with Cur for 1 h followed by stimulation with Pam for 0.5 h. Cell extracts were collected and subjected to western blot analysis. BV-2 cells were treated with JNK inhibitor (JNK II, 10  $\mu$ M), Akt inhibitor (Wor, 5  $\mu$ M), ERK inhibitor (PD98059, 10  $\mu$ M) or p38 inhibitor (SB203580, 10  $\mu$ M) for 1 h, following treatment with Pam (0.1  $\mu$ g/ml) for 16 h. The release of (B) NO and the expression levels of inducible (C) NO synthase, encoded by NOS2, were determined. Each bar represents the mean  $\pm$  standard deviation from three independent experiments. <sup>#</sup> $P < 0.01$  vs. negative control; <sup>\*</sup> $P < 0.05$  and <sup>\*\*</sup> $P < 0.01$  vs. Pam-treated control. NO, nitric oxide; PD, PD98059; SB, SB203580; Wor, Wortmannin; Pam, Pam3CSK4; Cur, curcumin; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal protein kinase; p-, phosphorylated; NOS2, nitric oxide synthase 2.

and carbon monoxide, inhibited NAD(P)H oxidase and TLR4 signaling, thus suppressed macrophage activation (16). Several previous studies have employed various models to demonstrate the neuroprotective and anti-neuroinflammatory effects of HO-1 against oxidative stress in the CNS (14,16). Overexpression of HO-1 in microglia and macrophages was identified as providing anti-inflammatory and neuroprotective properties against secondary insults, including oxidative stress in human and rat traumatic brain injuries (32). Furthermore, a previous study has also suggested a positive correlation between HO-1 expression and IL-10 release (33). In the present study, curcumin induced the expression of HO-1 at the mRNA and protein level in a dose-dependent manner, with maximal effects at 4 and 8 h, respectively. HO-1 is a phase II antioxidant enzyme induced by translocation of the transcription factor Nrf2 (34). The effect of Nrf2 has been described in various *in vivo* models where Nrf2-knockout mice demonstrated significant activation of microglial cells compared with wild-type mice (35). In addition, LPS induced an enhanced inflammatory response in Nrf2 knockout mice compared with the control mice (36). The results of the present

study demonstrated that curcumin increased the nuclear accumulation of Nrf2 at 1 h following treatment and it reached a maximum at 2 h. Therefore, the results indicated that HO-1 upregulation by the Nrf2/ARE signaling pathway was associated with the inhibitory effects of curcumin on the release of inflammatory mediators by Pam3CSK4.

Curcumin also suppressed signaling pathways upstream of the inflammatory mediators, such as NF- $\kappa$ B, which were associated with the inflammatory responses triggered by TLR2 in BV-2 cells. NF- $\kappa$ B is a master regulator of microglial inflammatory responses (34). A previous study has revealed crosstalk between NF- $\kappa$ B and Nrf2, which has a circular regulatory effect (37). In Nrf2<sup>-/-</sup> mouse embryonic fibroblast cells, the absence of Nrf2 exacerbates NF- $\kappa$ B activity by enhancing the degradation of I $\kappa$ B $\alpha$ , which leads to an increase in cytokine production (15,35). Additionally, the NF- $\kappa$ B subunit p65 may exert a negative effect on Nrf2 activity and ARE-linked gene expression (37). During neuroinflammation, NF- $\kappa$ B activation induces the expression of inflammatory cytokines, including iNOS, NO, PGE<sub>2</sub>, COX-2 and ROS to trigger an over-activated inflammatory response, which leads to progressive neuronal

damage (31). Therefore, modulation of NF- $\kappa$ B activation is considered to be a useful method of controlling microglial activation. It has been previously reported that curcumin inhibits NF- $\kappa$ B activation in various immune cell types, thus it inhibits the production of inflammatory mediators (17). The results of the present study also indicated that curcumin decreased nuclear translocation and transactivation of NF- $\kappa$ B following Pam3CSK4 stimulation in microglial cells.

Previous studies suggested that activation of the phosphoinositide 3-kinase (PI3K)/Akt-dependent signaling pathway promoted activation of M1 microglia, leading to the expression of inflammatory mediators and neuronal injury (34,38). In addition, the MAPK signaling pathway is also stimulated by increased oxidative stress, including LPS, LTA and other agonists of TLR2 or TLR4 (24,39). The PI3K/Akt and MAPK signaling pathways have been revealed to upregulate the gene expression of iNOS and COX-2 in microglia activated by different stimuli, such as LPS (24). A previous study revealed that casticin inhibited the expression of COX-2 and iNOS through inhibition of MAPK signaling pathways in macrophage (39). The results of the present study are consistent with these previous findings. Pretreatment of microglial cells with curcumin notably decreased the phosphorylated MAPKs and Akt, particularly p-p38 and Akt, whereas it did not change the total MAPK and Akt levels. Furthermore, the p38 and JNK inhibitors suppressed the secretion of NO and the mRNA expression of iNOS. These findings illuminate the fundamental role of p38 MAPK in the anti-neuroinflammatory effect of curcumin.

In conclusion, the results of the present study revealed that curcumin exerted anti-neuroinflammatory effects in Pam3CSK4-stimulated microglial cells. These results suggest that the anti-neuroinflammatory role of curcumin is primarily through inhibition of the p38 MAPK and NF- $\kappa$ B signaling pathways, and induction of HO-1 by the Nrf2/ARE signaling pathway, thereby decreasing the production of inflammatory mediators. These results suggest that curcumin may be a novel candidate for the treatment of chronic neuroinflammatory diseases by reducing the over-activation of microglial cells.

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