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4-methoxycinnamyl p-coumarate reduces neuroinflammation by blocking NF- κ B, MAPK, and Akt/GSK-3 β pathways and enhancing Nrf2/HO-1 signaling cascade in microglial cells

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ABSTRACT

The active compound, 4-methoxycinnamyl p-coumarate (MCC), derived from the rhizome of *Etingera pavieana* (Pierre ex Gagnep) R.M.Sm., has been shown to exert anti-inflammatory effects in several inflammatory models. However, its effects on microglial cells remain elusive. In the current study, we aimed to investigate the anti-neuroinflammatory activities of MCC and determine the potential mechanisms underlying its action on lipopolysaccharide (LPS)-induced BV2 microglial cells. Our results revealed that MCC significantly reduced the secretion of nitric oxide (NO) and prostaglandin E2, concomitantly inhibiting the expression levels of inducible NO synthase and cyclooxygenase-2 mRNA and proteins. Additionally, MCC effectively decreased the production of reactive oxygen species in LPS-induced BV2 microglial cells. MCC also attenuates the activation of NF- κ B by suppressing the phosphorylation of I κ B α and NF- κ B p65 subunits and by blocking the nuclear translocation of NF- κ B p65 subunits. Furthermore, MCC significantly reduced the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), and protein kinase B (Akt)/glycogen synthase kinase-3 β (GSK-3 β). In addition, MCC markedly increased the expression of heme oxygenase-1 (HO-1) by upregulating the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway. Collectively, our findings suggest that the anti-inflammatory activities of MCC could be attributed to its ability to suppress the activation of NF- κ B, MAPK, and Akt/GSK-3 β while enhancing that of Nrf2-mediated HO-1. Accordingly, MCC has promising therapeutic potential to treat neuroinflammation-related diseases.

1. Introduction

Neuroinflammation is the innate immune response of the brain to an inflammatory stimulus, characterized by various cellular and molecular alterations induced via the activation of resident innate immune cells in the central nervous system (CNS) [1]. Microglial cells play an important role in neuroinflammation by releasing proinflammatory mediators and cytokines such as nitric oxide (NO), prostaglandin E₂ (PGE₂), and reactive oxygen species (ROS), which function as barriers against and eliminate stimuli, thereby restoring the homeostasis in the CNS [2]. However, unregulated microglial activation can induce prolonged and excessive production of proinflammatory mediators and cytokines, key factors in neurodegenerative diseases such as Alzheimer's and

Parkinson's disease [3]. Hence, alleviating neuroinflammation by suppressing the production of proinflammatory mediators in microglia could be key in preventing neurodegenerative diseases.

NO is a small signaling molecule derived from L-arginine through the action of the NO synthase (NOS) enzyme family, which comprises three isoforms: neuronal NOS, inducible NOS (iNOS), and endothelial NOS [4]. PGE₂ is another signaling molecule generated from arachidonic acid by cyclooxygenase (COX). COX has two isoforms: COX-1, a constitutive isoform, and COX-2, induced in response to several stimuli [5]. NO and PGE₂ are key mediators of the inflammatory response. In lipopolysaccharide (LPS)-induced microglial cells, the expression of iNOS and COX-2 is primarily regulated by the nuclear factor kappa B (NF- κ B) transcription factor [6]. In response to cell stimulation, NF- κ B activation

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is induced via the degradation of inducible $\kappa\text{B}\alpha$ inhibitor ($\text{I}\kappa\text{B}\alpha$) on its site-specific phosphorylation (Ser32 and Ser36) mediated by the $\text{I}\kappa\text{B}$ -kinase (IKK) complex, resulting in transient nuclear translocation of free NF- κB dimers to enhance the transcription of target genes [7]. The mitogen-activated protein kinase (MAPK) signaling pathway, another transcription factor induced in response to inflammation, comprises three major families: extracellular signal-regulated kinase (ERK), c-Jun NH₂-terminal kinase (JNK), and p38 MAPK [8]. Moreover, the phosphatidylinositol 3'-kinase/Akt (PI3K/Akt) is also related to the regulation of the NF- κB pathway [9,10].

Heme oxygenase-1 (HO-1) is a rate-limiting enzyme triggered by cellular oxidative stress and various stimuli, subsequently catalyzing the degradation of heme to free iron, carbon monoxide (CO), and biliverdin, which reportedly exert a robust anti-inflammatory effect [11,12]. HO-1 expression is primarily regulated by the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway. Upon stimulation, Nrf2 is activated via Nrf2 dissociation from the repressor protein Keap1 and the subsequent release of Nrf2. The released Nrf2 translocates to the nucleus and binds to the antioxidant response element (ARE), which enhances the transcription of Nrf2-regulated genes such as HO-1 [13,14]. Therefore, the induction of the Nrf2/HO-1 pathway may be a useful strategy for alleviating neuroinflammation.

4-Methoxycinnamyl *p*-coumarate (MCC) is a naturally active compound extracted from *Etilingera pavieana* (Pierre ex Gagnep) R.M.Sm. rhizomes using NO inhibitory activity-guided isolation, which was found to yield the most efficient inhibitory activity [15]. The anti-inflammatory activity of MCC has been explored in several models. MCC was shown to inhibit LPS-induced inflammation by down-regulating the NF- κB , Akt, and AP-1 signaling pathways in RAW 264.7 macrophages [16]. In human endothelial cells, MCC attenuated tumor necrosis factor (TNF)- α -induced vascular adhesion molecule expression [17]. MCC has also been shown to elicit an anti-inflammatory response in vivo [18]. However, the anti-inflammatory activities of MCC compound in microglial cells remain unexplored. In the current study, we evaluated the anti-inflammatory activity of MCC and examined the underlying molecular mechanisms in LPS-induced microglial cells.

2. Materials and methods

2.1. Compound preparation

MCC was isolated from *E. pavieana* rhizomes, as described by Srisook et al. [15], and provided by Dr. E. Srisook of the Department of Chemistry, Faculty of Science, Burapha University. Subsequently, MCC was dissolved in dimethyl sulfoxide (DMSO) and filtered through a 0.22 μm nylon syringe filter.

2.2. Cell culture

The murine microglial cell line, BV2, was obtained from the Interlab Cell Line Collection (Genova, Italy). The cells were cultured at 37 °C in humidified air containing 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM), supplemented with 100 U/mL of penicillin, 100 g/mL of streptomycin, and 10% heat-inactivated fetal bovine serum.

2.3. Cell viability assays

To assess the effect of MCC on cell viability, the MTT assay was performed as described by Poonasri et al. [19]. Briefly, the cells were treated with various MCC concentrations for 24 h and then washed with PBS. Subsequently, fresh DMEM containing MTT (0.1 mg/mL) was added to each well and incubated for 2 h. The culture medium was removed, and DMSO was used to dissolve the formazan crystals. The absorbance of the solution in each well was measured at 550 nm using a microplate reader (Versamax, Molecular Devices, USA).

2.4. Determination of NO and PGE₂ levels

Briefly, BV2 cells (1.5×10^5 cells/well) were plated in 24-well plates and pretreated with various MCC concentrations for 1 h before stimulation with LPS (1 $\mu\text{g}/\text{mL}$) for 24 h. The amount of nitrite, a stable oxidation product of NO in the culture medium, was quantified using the Griess reagent. An appropriate ELISA kit was used to determine PGE₂ levels in the culture medium in accordance with the manufacturer's instructions.

2.5. Determination of intracellular ROS

Intracellular ROS levels were determined using the method described by Srisook et al. [20], with few modifications. Briefly, BV2 cells pretreated with MCC for 1 h in 24-well plates were subjected to LPS stimulation for 12 h. Then, the culture medium was removed, and cells were washed with warm PBS before incubating with DMEM containing H₂DCF-DA (50 μM) for 30 min. The cells were washed with ice-cold PBS and scraped using ice-cold PBS on ice. The fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 521 nm using a fluorescence spectrophotometer (Cary Eclipse, Agilent, USA).

2.6. Western blot analysis

Briefly, BV2 cells (1×10^6 cells/plate) pretreated with MCC for 1 h in 60-mm plates were subjected to LPS stimulation. RIPA lysis buffer was used to extract proteins from whole cells. Proteins were electrophoresed on an equal volume of 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The membranes were then incubated with the appropriate primary antibodies, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Specific protein bands were detected on an X-ray film using chemiluminescence. The band intensities were estimated using the Image Studio Lite 5.2 Quick Start Guide program for Windows.

2.7. Nuclear protein extraction

In brief, 5×10^6 BV2 cells were seeded per 100-mm plates. After treatment, cells were rinsed twice with cold PBS and collected by gently scraping on ice with cold PBS. The resulting cell homogenates were centrifuged at 9500 $\times g$ for 5 min at 4 °C. Then, the cell pellets were resuspended in 200 μL of Buffer 1 (25 mM HEPES, 5 mM KCl, 0.5 mM MgCl₂, 0.5 mM DTT, and 0.5 mM PMSF), supplemented with 1X protease inhibitor, followed by incubation on ice for 20 min. Subsequently, 200 μL of Buffer 2 (Buffer 1 with 5% Nonidet P-40) was added, and the mixture was gently shaken on ice for 15 min. Then, centrifugation was performed at 13,700 $\times g$ for 6 min at 4 °C to separate the supernatant, constituting the cytoplasmic fraction, from the nuclear pellets. The nuclear pellets were washed by adding 200 μL of Buffer 3 (1:1 mixture of Buffer 1 and Buffer 2), followed by gentle shaking, before subsequent centrifugation at 13,700 $\times g$ for 5 min at 4 °C. Then, the supernatant was discarded, and cell pellets were resuspended in 70 μL of Buffer 4 (25 mM HEPES, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 20% glycerol, 0.5 mM DTT, and 0.5 mM PMSF), supplemented with 1X protease inhibitor. The resulting suspension was incubated for 40 min on ice, with vortexing performed at 5-min intervals. Subsequently, centrifugation was performed at 13,700 $\times g$ for 20 min at 4 °C to obtain the supernatant as the nuclear fraction. This nuclear fraction was carefully transferred to a new microcentrifuge tube and stored at -80 °C until further analysis.

2.8. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the cell pellet using a Nucleospin RNA kit following the manufacturer's instructions. Then, 2 μg of extracted

RNA was converted to cDNA using the iScript Reverse Transcription Supermix. RT-qPCR was performed using 2x iTaq™ SYBR Green Supermix on a CFX96 Touch Real-Time PCR (Bio-Rad, USA). The sequences of iNOS, COX-2, and elongation factor-2 (EF-2)-specific PCR primers were as follows: iNOS, 5'-GCACAGCA-CAGGAAATGTTTCAGCAC-3' (F), and 5'-AGCCAGCGTACCGGATGAGC-3'(R); COX-2, 5'-TGATCGAAGACTACGTGCA ACACC-3' (F), and 5'-TTCAATGTTGAAGGTGTCGGGCAG-3' (R); and EF-2, 5'-CTGAAGCGGCTGGCTAAGTCTGA-3' (F), and 5'-GGGTCA-GATTTCTTGATGGG GATG-3' (R). As a housekeeping gene, EF-2 was used to normalize the relative gene expression levels.

2.9. Immunofluorescence assay

Briefly, BV2 cells (1×10^5 cells/mL) were seeded onto glass coverslips in a 6-well plate, pretreated with MCC for 1 h, and then stimulated with LPS for 30 min. Next, the cells were rinsed twice with PBS at room temperature and fixed with 4% (w/v) paraformaldehyde for 30 min at room temperature. The cells were thrice rinsed again for 5 min with PBS, then permeabilized with 0.2% Triton-X100 for 10 min at room temperature, and thrice rinsed with PBS for 5 min. Afterward, non-specific binding was blocked with 0.5% (w/v) bovine serum albumin in PBS for 1 h at room temperature, followed by overnight incubating with primary antibodies against NF- κ B p65 at 4 °C. The cells were thrice rinsed with PBS for 5 min, followed by further incubation with secondary anti-rabbit IgG (H + L) and F(ab')₂ fragments (Alexa Fluor 488 conjugate) for 1 h at room temperature. Next, cells were thrice rinsed for 5 min with PBS before staining and mounting nuclei with Prolong Gold antifade reagent with DAPI for 24 h. The slides were examined under a Nikon C2 Plus confocal microscope (New York, NY, USA) with excitation laser beams of 488 and 405 nm. The cell images were captured using a 40 × objective CFI60 plan Achromat λ lens.

2.10. Statistical analysis

Experimental data are reported as means \pm standard deviation (SD) of three independent experiments. Statistical analyses were performed using Minitab 18 for Windows. Statistical significance was determined using one-way analysis of variance (ANOVA), followed by Tukey's method for multiple comparisons. A *p*-value < 0.05 was deemed statistically significant.

3. Results

3.1. The effect of MCC on the viability of BV2 microglial cells

To evaluate the cytotoxicity of MCC, BV2 cells were treated with

various MCC concentrations, and cell viability was estimated using the MTT assay. Treatment with MCC (50–200 μ M) markedly decreased the cell viability when compared with control unstimulated cells, although MCC (6.25–25 μ M) did not notably alter cell viability (Fig. 1A). Moreover, treatment with MCC (6.25–25 μ M) did not significantly impact cell viability, even in the presence of LPS (1 μ g/mL) (Fig. 1B). Thus, up to 25 μ M MCC was used in further experiments.

3.2. MCC inhibits NO, PGE₂, and ROS production in BV2 microglial cells

Levels of NO and PGE₂ secreted in the culture media were assayed using the Griess reaction and ELISA, respectively. LPS stimulation enhanced NO and PGE₂ production to $9.43 \pm 1.72 \mu$ M (Fig. 2A) and $10,375 \pm 1809.51$ pg/mL (Fig. 2B), respectively. Pretreatment with MCC markedly reduced LPS-induced NO production ($IC_{50} = 3.32 \pm 1.13 \mu$ M). As a positive control, aminoguanidine suppressed NO production by $74.76 \pm 3.10\%$ (Fig. 2A). Additionally, pretreatment with MCC drastically reduced PGE₂ production ($IC_{50} = 13.32 \pm 2.18 \mu$ M). Indomethacin, as a positive control, inhibited PGE₂ production by $93.47 \pm 2.34\%$ (Fig. 2B). Given that excessive ROS plays a central role in the common pathophysiology of neurodegenerative diseases, we determined whether MCC exhibited inhibitory properties against ROS production. Our findings revealed that pretreatment with MCC suppressed LPS-induced ROS production in BV2 microglial cells in a concentration-dependent manner (Fig. 2C).

3.3. MCC suppresses LPS-induced iNOS and COX-2 expression in BV2 microglial cells

To examine the inhibitory effect of MCC on NO and PGE₂ production, protein expression levels of iNOS and COX-2 were determined using western blot analysis. The expression of iNOS and COX-2 protein was significantly increased in LPS-stimulated cells after 24 h (Fig. 3A and B). Pretreatment with MCC markedly and dose-dependently suppressed iNOS protein expression (Fig. 3A). In addition, 25 μ M MCC significantly suppressed COX-2 protein expression (Fig. 3B). The RT-qPCR analysis (Fig. 3C and D) revealed a correlation between expression levels of iNOS and COX-2 mRNA and their corresponding proteins.

3.4. MCC attenuates LPS-induced NF- κ B activation in BV2 microglial cells

Based on the ability of MCC to inhibit LPS-induced iNOS and COX-2 expression, we further examined the effect of MCC on the activation of the NF- κ B signaling pathway. According to western blotting data, protein levels of phosphorylated I κ B α and NF- κ B p65 were markedly enhanced upon cell exposure to LPS for 30 min, whereas pretreatment

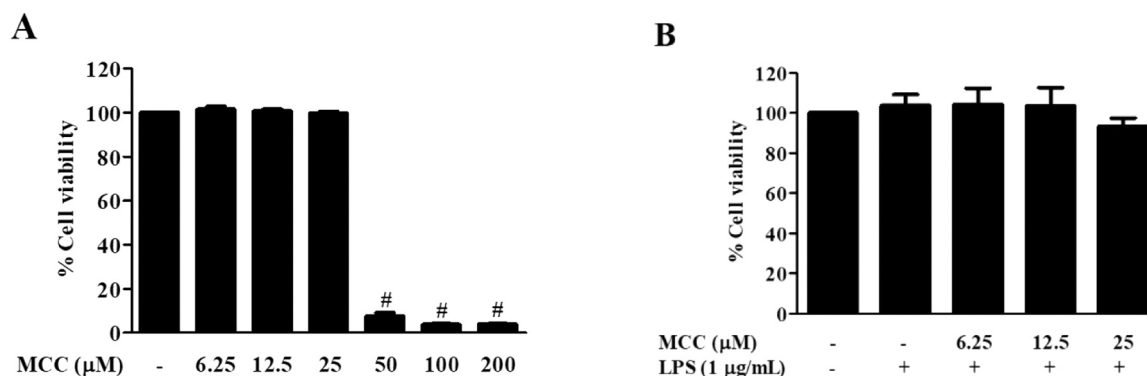


Fig. 1. Effect of MCC on the viability of BV2 microglial cells. BV2 cells were treated with the indicated concentrations of MCC alone for 24 h (A) and pretreated with nontoxic concentrations of MCC for 1 h before stimulation with LPS for 24 h (B). Cell viability was measured by using the MTT assay. Data values represent the mean \pm standard deviation (SD) of three independent experiments. ###*p* < 0.001 compared with the untreated cells. LPS, lipopolysaccharide; MCC, 4-methoxycinnamyl *p*-coumarate.

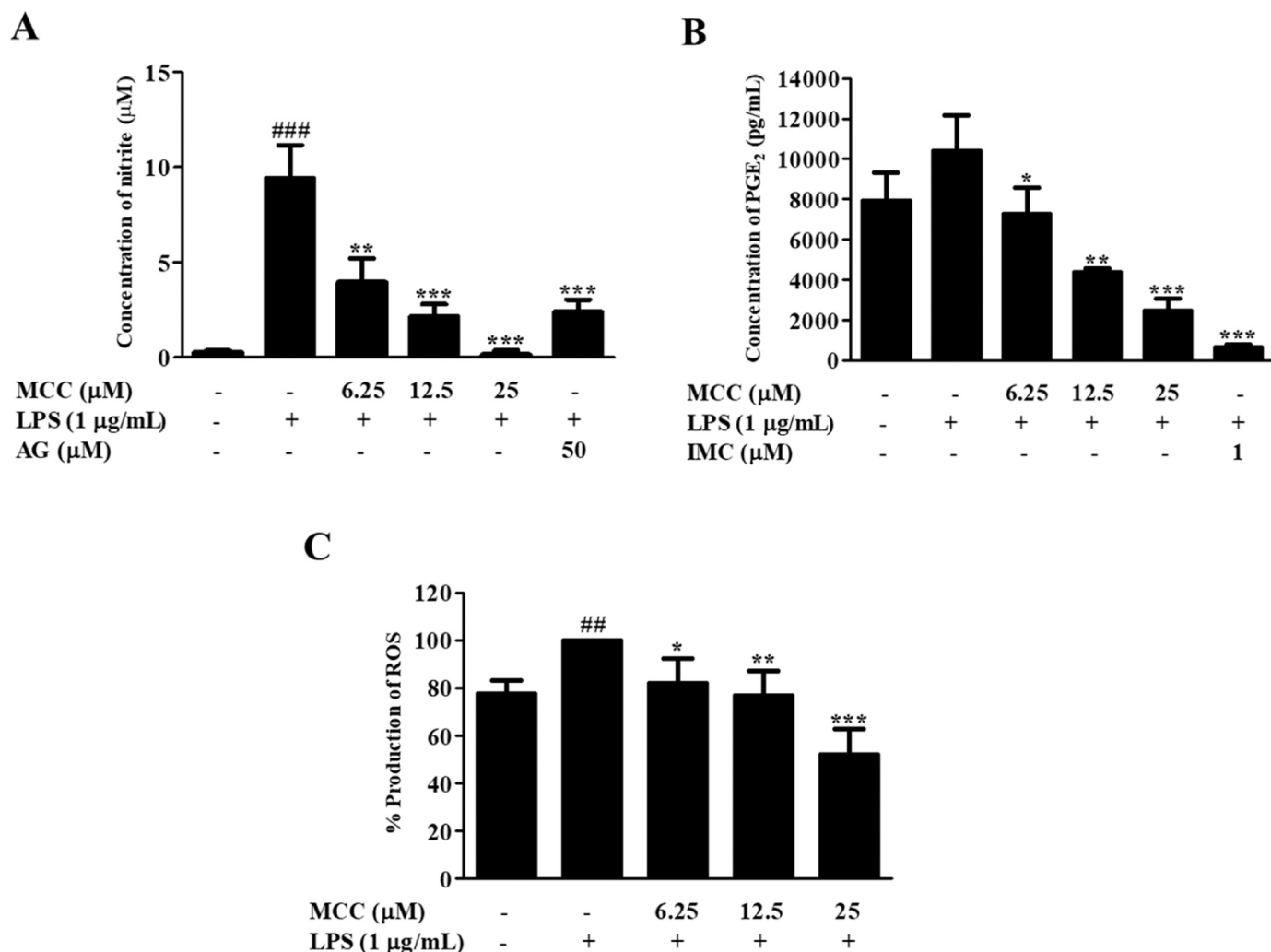


Fig. 2. Inhibitory effect of MCC on LPS-induced NO, PGE₂, and ROS production in BV2 microglial cells. BV2 cells were pretreated with MCC for 1 h and then stimulated with LPS for 24 h. The amount of nitrite (A) and PGE₂ (B) secreted in the culture medium was measured using Griess and ELISA assays, respectively. BV2 cells were pretreated with MCC for 1 h and then stimulated with LPS for 12 h. Levels of intracellular ROS production were measured using the H₂DCF-DA fluorescent probe (C). Data values represent the mean \pm standard deviation (SD) of three independent experiments. ##*p* < 0.01, ###*p* < 0.001 compared with untreated cells, **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 compared with the LPS-treated cells. AG, aminoguanidine; IMC, indomethacin; LPS, lipopolysaccharide; MCC, 4-methoxycinnamyl *p*-coumarate; NO, nitric oxide; PGE₂, prostaglandin E₂; ROS, reactive oxygen species.

with MCC significantly suppressed the LPS-enhanced phosphorylation of I κ B α and NF- κ B p65 (Fig. 4A and C). As shown in Fig. 4B, levels of total I κ B α protein were restored in MCC-treated cells when compared with levels of untreated LPS-stimulated cells. BAY 11-7082 was used as a positive control. Based on immunofluorescence analysis, cells treated with LPS exhibited nuclear translocation of NF- κ B p65 (LPS; Fig. 4D) when compared with unstimulated cells (Fig. 4D, Control). Conversely, pretreatment with MCC (LPS+MCC) substantially attenuated the nuclear translocation of NF- κ B p65 (Fig. 4D).

3.5. MCC suppresses LPS-induced MAPK and Akt phosphorylation in BV2 microglial cells

As shown in Fig. 5A, LPS stimulation for 30 min increased phosphorylation levels of p38, JNK, and ERK1/2 (Fig. 5A). Pretreatment with MCC suppressed the phosphorylation of p38 (Fig. 5B) and JNK (Fig. 5D). Conversely, pretreatment with MCC (6.25–12.5 μM) significantly increased the phosphorylation level of ERK1/2; however, this level was substantially decreased following pretreatment with 25 μM MCC (Fig. 5C). Moreover, pretreatment with MCC dose-dependently decreased the LPS-induced substantial increase in Akt phosphorylation

(Fig. 5A and E).

3.6. The effect of MCC on the activation of the Akt/Nrf2/HO-1 pathway

Next, we examined whether the anti-inflammatory action of MCC is associated with Nrf2/HO-1 activation. Based on the western blot analysis, MCC-treated cells exhibited upregulation of nuclear Nrf2 protein, which significantly reduced the cytosolic Keap1 protein compared with unstimulated cells (Figs. 6A and 6B), while markedly enhancing HO-1 expression in a concentration-dependent manner (Fig. 6C). Furthermore, in N-acetyl-L-cysteine (NAC)-treated cells, treatment with MCC reduced HO-1 protein expression (Fig. 6D). In light of our current findings, which indicate that MCC can activate Nrf2 in BV2 microglial cells, we further investigated the involvement of Nrf2 in MCC-mediated HO-1 expression using a known Nrf2 inhibitor, ML385. Inhibition of the Nrf2 signaling pathway by ML385 effectively suppressed MCC-induced HO-1 expression and nuclear translocation of Nrf2 (Figs. 6E and 6F). Next, we aimed to establish whether HO-1 activation played a pivotal role in the mechanism of action of MCC in LPS-induced BV2 microglial cells. As depicted in Fig. 6G, nitrite levels substantially increased upon treatment with SnPP, an inhibitor of HO-1 activity, when compared with

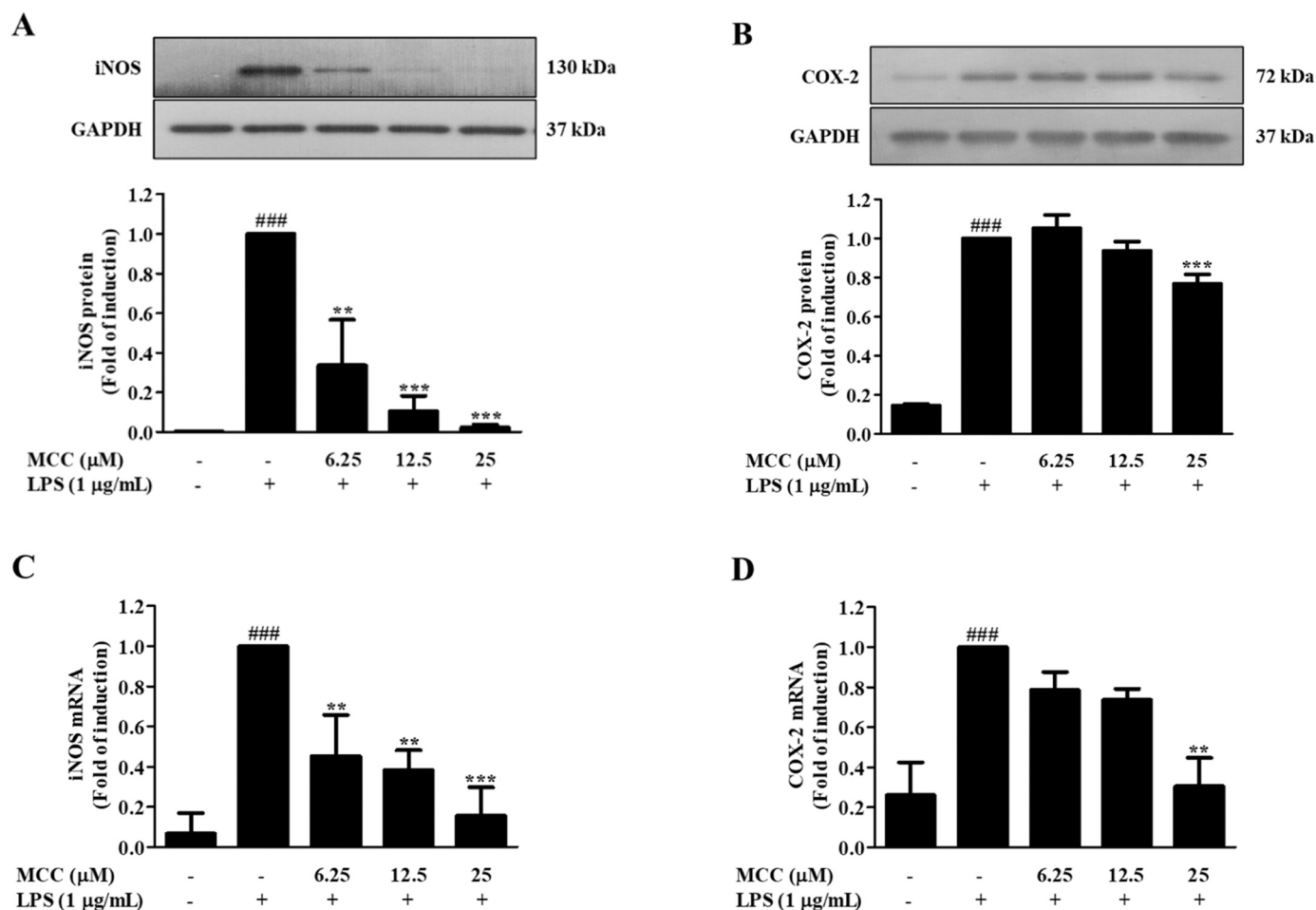


Fig. 3. Inhibitory effect of MCC on LPS-induced iNOS and COX-2 expression. BV2 cells were pretreated with MCC for 1 h, followed by LPS stimulation for 24 h and 9 h for protein and mRNA, respectively. Protein expression levels of iNOS (A) and COX-2 (B) were measured using western blot analysis. Data values represent the mean \pm standard deviation (SD; $n = 3$) of densitometric values, normalized to that of GAPDH. mRNA levels of iNOS (C) and COX-2 (D) were detected by RT-qPCR. ### $p < 0.001$ compared with the untreated cells, * $p < 0.01$, and *** $p < 0.001$ compared with the LPS-treated cells. COX-2, cyclooxygenase 2; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MCC, 4-methoxycinnamyl *p*-coumarate; NO, nitric oxide; PGE₂, prostaglandin E₂; ROS, reactive oxygen species; RT-qPCR, reverse transcription-quantitative PCR.

levels in cells treated solely with LPS. MCC inhibited nitrite production, whereas SnPP reversed this effect. This led to a statistically significant increase in nitrite levels compared with those in cells exposed to LPS and MCC.

To further investigate whether the upstream pathways that regulate MCC-mediated Nrf2/HO-1 activation occur through the PI3K/Akt pathway, we employed the PI3K inhibitor, wortmannin. First, we determined whether MCC-mediated HO-1 induction occurs via the Akt/GSK-3 β signaling pathway. As shown in Fig. 7A, cells treated with MCC alone displayed the induction of Akt and GSK-3 β phosphorylation (Fig. 7B and C), as well as HO-1 expression, when compared with unstimulated cells (Fig. 7D). However, treatment of cells with MCC in the presence of wortmannin resulted in a significant decrease in Akt and GSK3 β phosphorylation, as well as an increase in HO-1 expression, when compared with cells treated with MCC alone (25 μ M). We then investigated whether Akt activity was required for the MCC-mediated Nrf2-dependent HO-1 induction, which might underlie the suppression of iNOS and COX-2 expression. To test this hypothesis, we pretreated BV2 cells with MCC and wortmannin for 1 h, followed by LPS stimulation. As shown in Fig. 8A, pretreatment with MCC effectively induced Nrf2 nuclear translocation and HO-1 protein expression, even when cells were exposed to LPS stimulation (Fig. 8B and C); this is particularly interesting because the by-products of HO-1 exhibit anti-inflammatory effects. As expected, iNOS and COX-2 expression decreased with increased

HO-1 activity (Fig. 8D and E). In addition, pretreatment with wortmannin significantly reduced levels of nuclear Nrf2 and HO-1 protein in cells treated with MCC and LPS. Our results also revealed that inhibition of HO-1 activity slightly reversed the inhibitory effect of MCC on COX-2 protein expression, indicating a differential effect on iNOS expression.

4. Discussion

During the neuroinflammatory process, microglial activation by stimuli such as LPS is known to induce the secretion of proinflammatory mediators such as NO, PGE₂, and ROS, which have been associated with the development of neurodegenerative diseases [2,21,22]. Therefore, compounds that inhibit the production of these proinflammatory mediators may be valuable in treating inflammation-related neurodegenerative diseases.

MCC has been reported to exert anti-inflammatory effects on macrophages [16], human endothelial cells [17], and in rat models [18]. In the present study, we investigated the anti-inflammatory effects of MCC on LPS-induced neuroinflammation in a BV2 microglial cell model. First, we observed that MCC inhibited LPS-induced NO, PGE₂, and ROS production in BV2 microglial cells without inducing marked cytotoxic effects. NO and PGE₂ are produced via the regulation of iNOS and COX-2, respectively, and are primarily expressed by activated microglia [4,5]. We further demonstrated that MCC alleviated the expression of iNOS

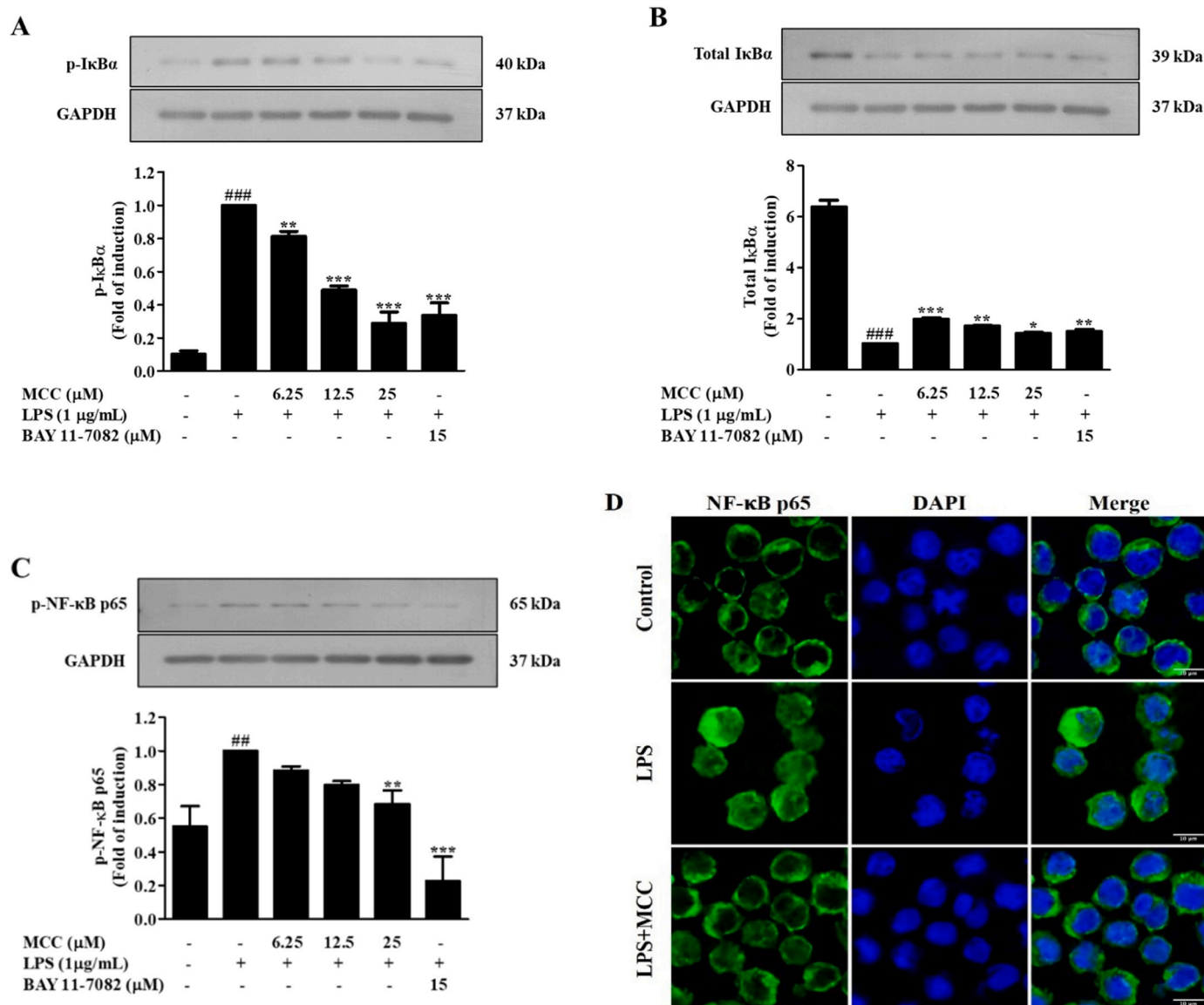


Fig. 4. Effect of MCC on LPS-induced NF- κ B activation in BV2 microglial cells. BV2 cells were pretreated with MCC for 1 h, followed by LPS exposure for 30 min. Expression levels of p-I κ B α (A), total I κ B α (B), and p-NF- κ B p65 in the whole-cell protein extract were examined by western blot analysis. Data values represent the mean \pm standard deviation (SD; $n = 3$) of densitometric values, normalized to that of GAPDH. NF- κ B p65 localization was assessed using immunofluorescence microscopy (D). ## $p < 0.01$, ### $p < 0.001$ compared with untreated cells, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with the LPS-treated group. LPS, lipopolysaccharide; MCC, 4-methoxycinnamyl *p*-coumarate.

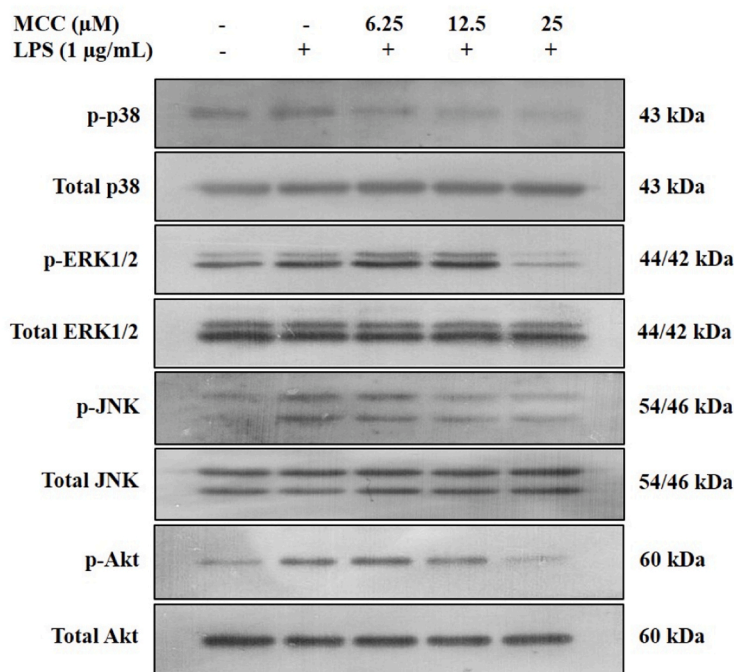
and COX-2. Consistent with our findings, treatment with MCC has been shown to reduce levels of iNOS- and COX-2-produced PGE₂ in RAW264.7 macrophages [16]. Overall, these findings indicate that MCC reduced NO and PGE₂ production by inhibiting the expression of iNOS and COX-2 genes, respectively.

In microglia-associated neuroinflammation, NF- κ B is a crucial transcription factor that regulates the expression of inflammatory genes [23]. Reportedly, the phosphorylation of the Ser536 residue of the NF- κ B subunit p65 can modulate its transcription activity [24], along with the phosphorylation of I κ B α and subsequent nuclear translocation of NF- κ B. Our study revealed that MCC could effectively inhibit the phosphorylation of I κ B α and NF- κ B p65 in BV2 microglial cells and suppress the nuclear translocation of NF- κ B p65. These results are consistent with those of previous reports that employed LPS-induced RAW 264.7 macrophages [16]. Additionally, our findings suggest that MCC can reverse the LPS-induced I κ B α degradation in BV2 microglial cells. Thus, we conclude that MCC possesses anti-inflammatory

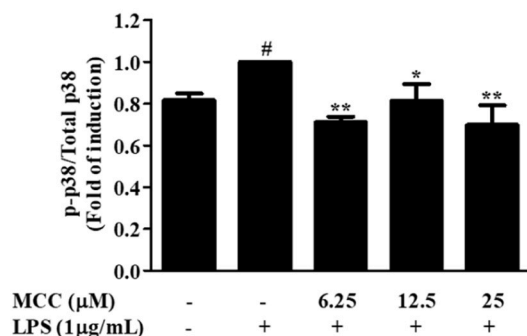
properties, mediated, at least in part, by inhibiting the NF- κ B pathway through the modulation of phosphorylated I κ B α and NF- κ B p65, ultimately preventing NF- κ B p65 nuclear translocation.

Phosphorylation of JNK, ERK1/2, and p38 MAPK correlates with the neuroinflammatory response upon stimulation [25]. Therefore, we assessed whether MCC can impact MAPK phosphorylation. We observed that MCC could reduce LPS-activated MAPK expression, suggesting the MAPK signaling pathway participates in the anti-inflammatory activity of MCC in LPS-induced BV2 microglial cells. However, this finding contradicts that observed in a previous study on RAW264.7 macrophage cells, which reported that the MCC-mediated anti-inflammatory effect was independent of the MAPK signaling pathway [16]. Our findings are consistent with recent reports indicating that hispidin exerts diverse regulatory effects on the activated signaling pathways in BV2 microglial cells and RAW264.7 [26,27]. These discrepancies in regulatory effects are most likely attributable to differences in cell origin, which result in varying expression levels of protein kinase C (PKC) protein in respective

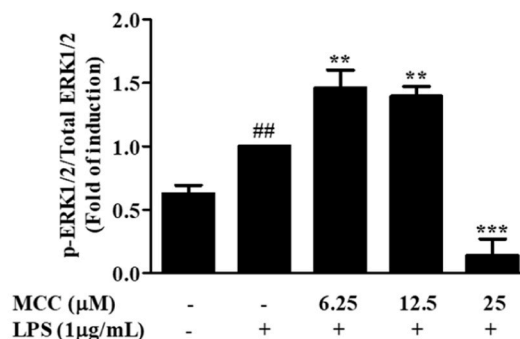
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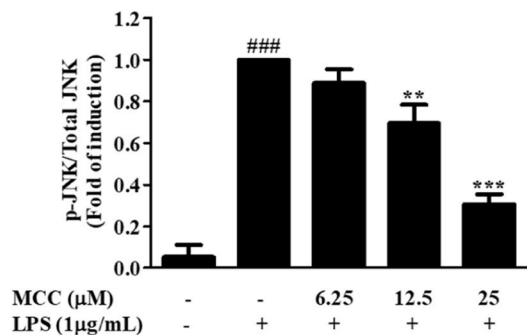
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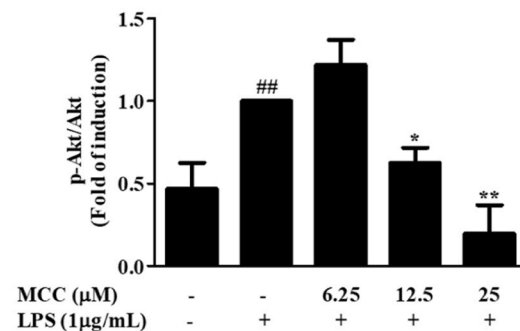
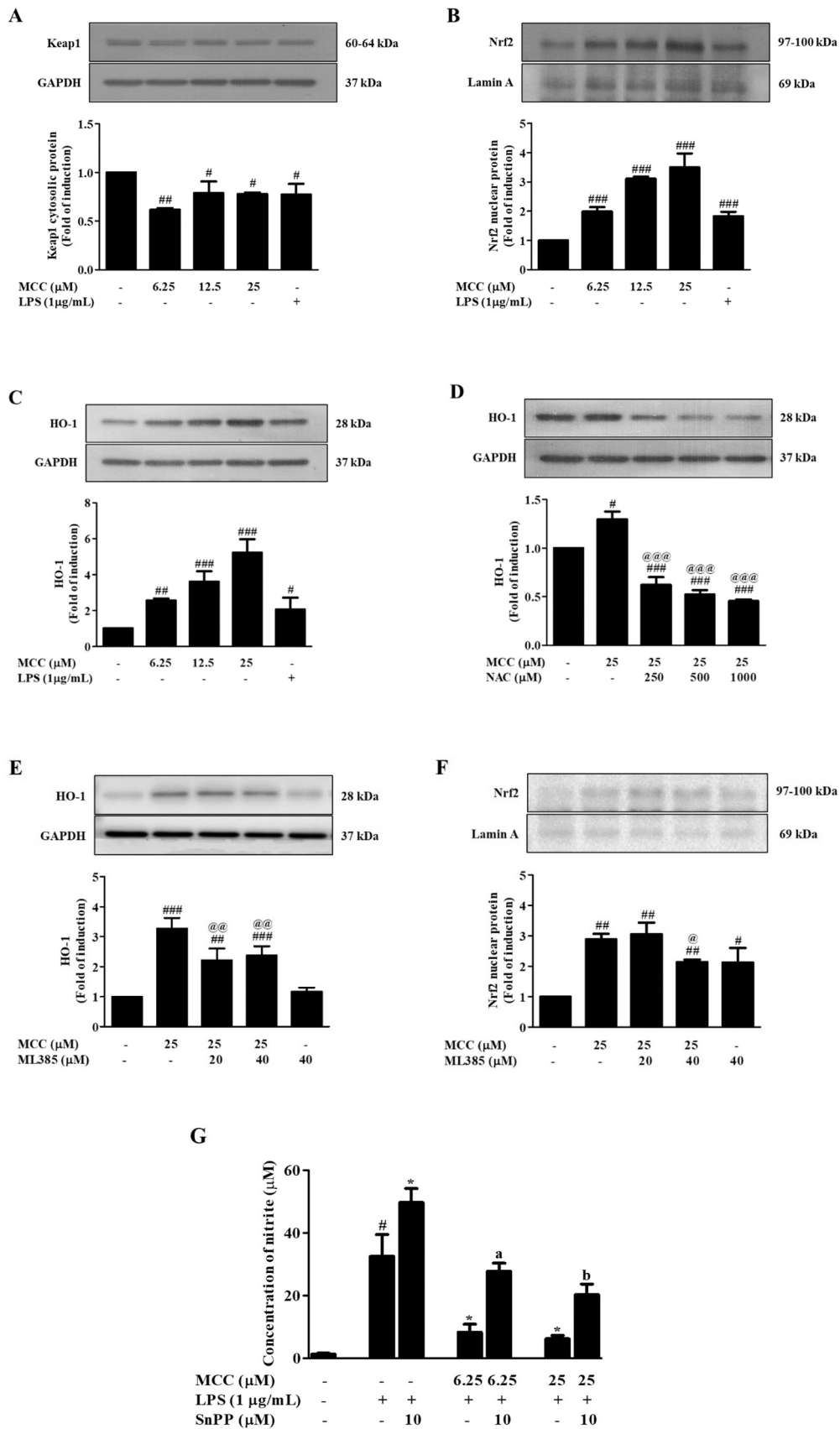


Fig. 5. Inhibitory effect of MCC on LPS-induced MAPK and Akt phosphorylation in BV2 microglial cells. BV2 cells were pretreated with MCC for 1 h, followed by LPS stimulation for 30 min. The phosphorylation levels of p38 MAPK, ERK1/2, JNK, and Akt in the whole-cell protein extract were detected by western blot analysis (A). The graphs are plotted using the mean ± standard deviation (SD) ($n = 3$) of densitometric values of phosphorylation of p38 MAPK (B), ERK1/2 (C), JNK (C), and Akt (E), normalized to the total level of each protein. [#] $p < 0.05$, ^{##} $p < 0.01$, ^{###} $p < 0.001$ compared with untreated cells, ^{*} $p < 0.05$, ^{**} $p < 0.01$, and ^{***} $p < 0.001$ compared with the LPS-treated group. ERK1/2, extracellular signal-regulated kinase 1/2; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MCC, 4-methoxycinnamyl *p*-coumarate.



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Fig. 6. Effect of MCC on LPS-induced Nrf2/HO-1 activation in microglial cells. BV2 cells were pretreated with MCC for 1 h, followed by LPS stimulation for 1 h. Expression levels of Keap1 in the cytoplasmic protein (A) and Nrf2 in the nuclear protein (B) were measured by western blot analysis. The graphs are plotted using the mean \pm standard deviation (SD; $n = 3$) of the densitometric values of Keap1 and Nrf2, normalized to those of GAPDH and Lamin A, respectively. Cells were pre-treated with MCC for 6 h (C), with NAC pretreatment performed 1 h prior to MCC (D). Levels of HO-1 in the whole-cell protein extract were assessed by western blot analysis. The graph is plotted using the mean \pm SD ($n = 3$) of the densitometric values, normalized to that of GAPDH. BV2 cells were treated with ML385 for 30 min prior to co-treatment with MCC for another 6 h for HO-1 expression (E) and 1 h for nuclear Nrf2 (F). The graph is plotted using the mean \pm SD ($n = 3$) of the densitometric values, normalized to those of GAPDH and Lamin A, respectively. Microglia were treated with MCC and SnPP for 1 h and co-stimulated by LPS for an additional 24 h. Nitrite concentrations in conditioned media were determined using the Griess reaction (G). # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared with untreated cells, and @ $p < 0.05$, @@ $p < 0.01$, @@@ $p < 0.001$ compared with cells treated with MCC (25 μM) * $p < 0.05$ compared with LPS, a $p < 0.05$ compared with LPS+MCC 6.25 μM , b $p < 0.05$ compared with LPS+MCC 25 μM . HO-1, heme oxygenase-1; LPS, lipopolysaccharide; MCC, 4-methoxycinnamyl p-coumarate.

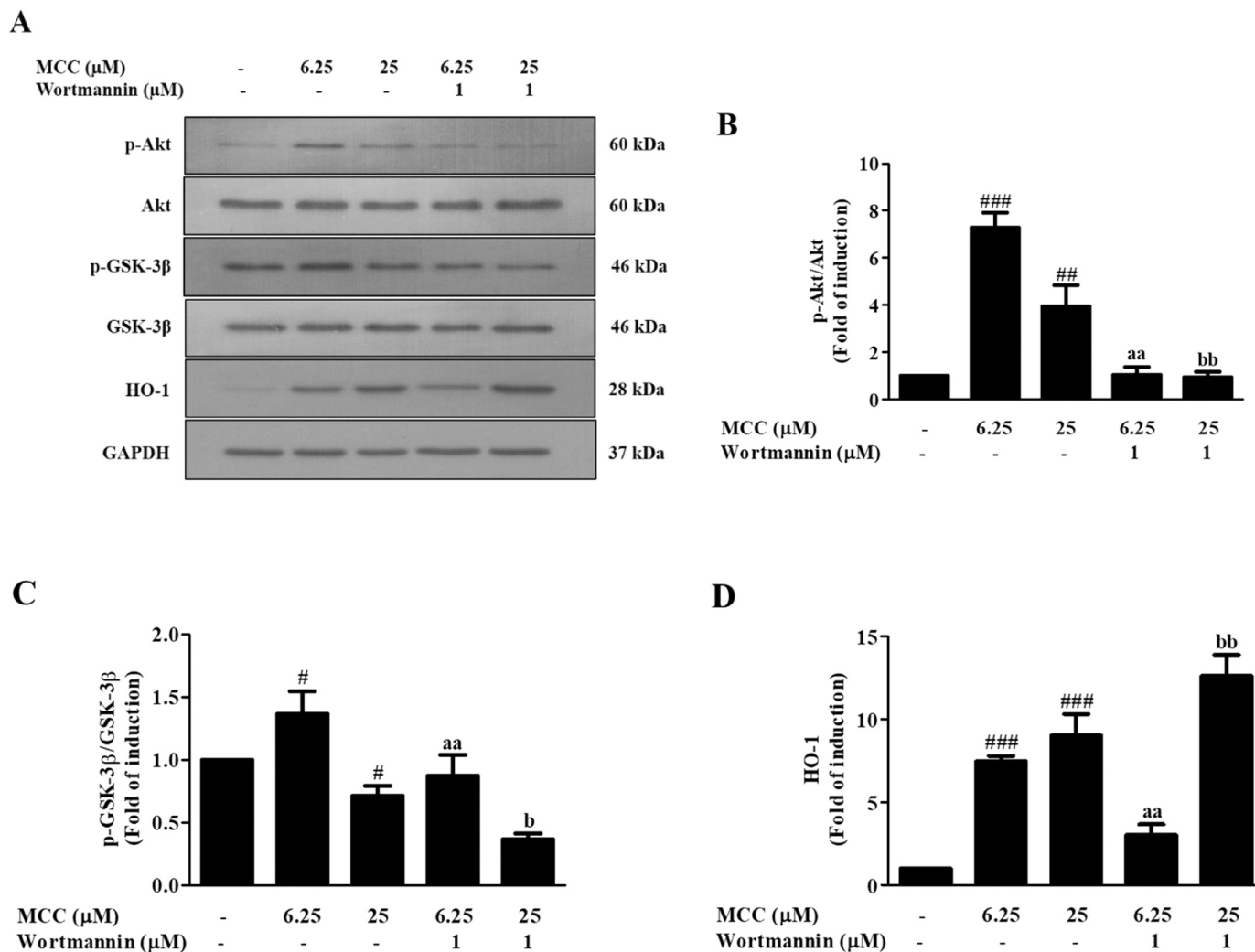


Fig. 7. Effect of PI3K/Akt inhibitor, wortmannin, on MCC-mediated HO-1 activation. BV2 cells were treated with MCC and/or wortmannin (1 μM) for 30 min. Western blotting was performed to quantify protein expression levels (A). The is plotted using the mean \pm standard deviation (SD; $n = 3$) of densitometric values of the p-Akt (B) and p-GSK3 β protein (C), normalized to the total level of each protein. BV2 cells were treated with MCC and/or wortmannin for 6 h (A). The graph is plotted using the mean \pm SD ($n = 3$) of densitometric values of HO-1 protein, normalized to those of GAPDH (D). # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared with untreated cells, aa $p < 0.01$ compared with cells treated with MCC (6.25 μM), b $p < 0.05$, and bb $p < 0.01$ compared with cells treated with MCC (25 μM). GSK-3 β , glycogen synthase kinase-3 β ; HO-1, heme oxygenase-1; LPS, lipopolysaccharide; MCC, 4-methoxycinnamyl p-coumarate.

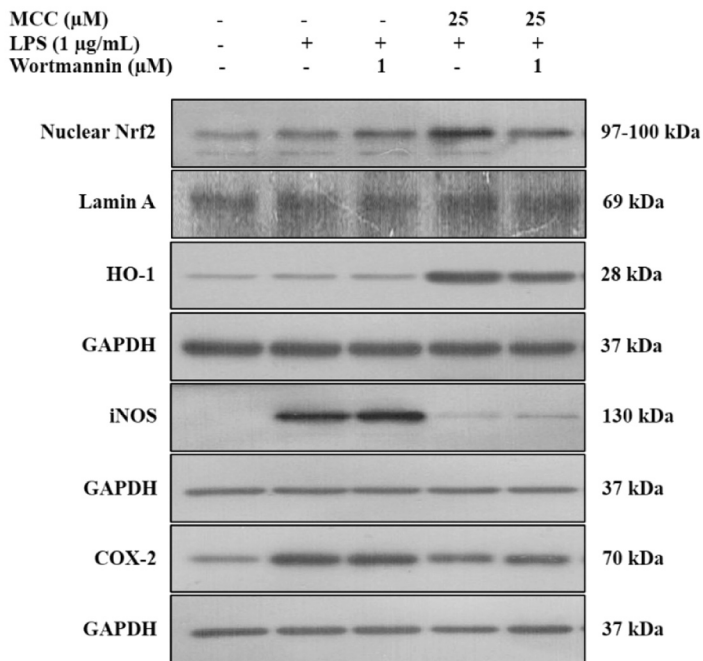
cell membranes [27,28].

Moreover, we explored the effect of MCC on PI3K/Akt, another signaling pathway involved in microglia-associated neuroinflammation [29]. In a previous report, we have shown that MCC could suppress LPS-induced inflammatory response by inactivating NF- κ B and inhibiting the PI3K/Akt signaling pathway in RAW264.7 macrophages [16]. Although several reports have demonstrated that the Akt-dependent regulation of NF- κ B on gene expression can vary depending on cell types and agonists used [9,30], our results showed that MCC is capable of suppressing both phosphorylated Akt and inactivated NF- κ B

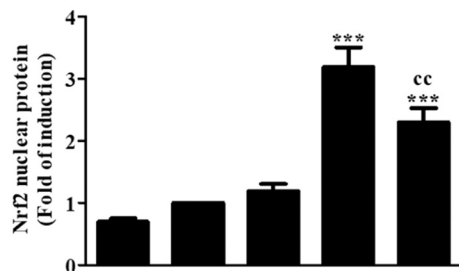
signaling. Consequently, it can be postulated that the MCC-driven NF- κ B inactivation might depend on the Akt pathway. Consistently, Cianciulli et al. [31] have shown that curcumin, a diarylheptanoid, can exert an inhibitory effect on LPS-induced neuroinflammation in BV2 microglial cells by suppressing the activation of NF- κ B and Akt signaling pathways.

HO-1 is a cytoprotective enzyme that plays a role in protecting cellular homeostasis by catalyzing the degradation of heme turnover to CO, iron, biliverdin, and ultimately bilirubin [32]. HO-1 and its end products exert antioxidant and anti-inflammatory effects [12]. To

A

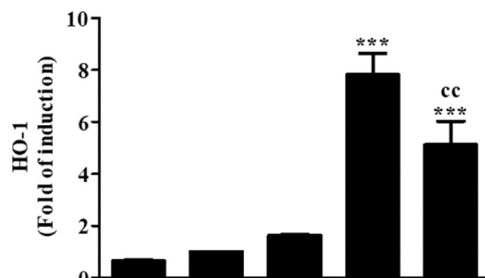


B



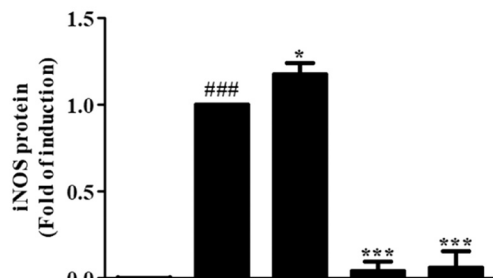
MCC (μM)	-	-	-	25	25
LPS (μg/mL)	-	+	+	+	+
Wortmannin (μM)	-	-	1	-	1

C



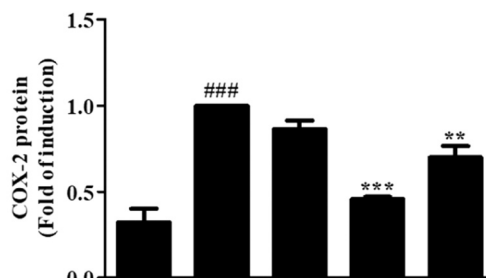
MCC (μM)	-	-	-	25	25
LPS (1 μg/mL)	-	+	+	+	+
Wortmannin (μM)	-	-	1	-	1

D



MCC (μM)	-	-	-	25	25
LPS (μg/mL)	-	+	+	+	+
Wortmannin (μM)	-	-	1	-	1

E



MCC (μM)	-	-	-	25	25
LPS (μg/mL)	-	+	+	+	+
Wortmannin (μM)	-	-	1	-	1

(caption on next page)

Fig. 8. Effect of wortmannin on MCC-mediated anti-neuroinflammatory effects. BV2 cells were pretreated with MCC and/or wortmannin for 1 h and exposed to LPS for 1 h. Western blotting was performed to quantify protein expression levels (A). The graph is plotted using the mean \pm standard deviation (SD; $n = 3$) of densitometric values of the Nrf2 protein, normalized to those of Lamin A (B). BV2 cells were pretreated with MCC and/or wortmannin for 1 h and exposed to LPS for 6 h (A). The graph is plotted using the mean \pm SD ($n = 3$) of the densitometric values of HO-1 protein, normalized to those of GAPDH (C). BV2 cells were pretreated with MCC and/or wortmannin for 1 h and exposed to LPS for 24 h (A). The graph is plotted using the mean \pm SD ($n = 3$) of the iNOS and COX-2 protein densitometric values, normalized to those of GAPDH (D and E). ### $p < 0.001$ compared with untreated cells, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with LPS-treated cells, and ^c $p < 0.01$ compared with cells treated with MCC (25 μ M). COX-2, cyclooxygenase 2; HO-1, heme oxygenase-1; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MCC, 4-methoxycinnamyl p-coumarate.

elucidate the pivotal role of HO-1 in mediating the anti-inflammatory effects of MCC in BV2 cells, we inhibited HO-1 activity using SnPP. The data illustrated in Fig. 6 indicates a reduction in the MCC-induced anti-neuroinflammatory response. These findings strongly suggest the potential involvement of HO-1 in mediating the anti-inflammatory effects of MCC in LPS-stimulated BV2 microglial cells.

Nrf2 is a well-known redox-regulated transcription factor that acts as the principal regulator governing the expression of antioxidant enzyme genes such as HO-1 and plays a critical role in regulating neuroinflammation [22]. Under cellular stress conditions, stimulation with toxins (e.g., LPS) causes the dissociation of Nrf2 from the Keap1 repressor to induce nuclear translocation and bind the ARE to enhance the transcription of target genes [14]. In the present study, we observed that MCC alone decreased the cytosolic Keap1 protein and subsequently enhanced HO-1 expression by effectively upregulating Nrf2 nuclear protein (Fig. 6). Our findings are further supported by the observation that NAC, a potent antioxidant and disulfide-breaking agent [33], suppressed HO-1 expression. Electrophilic agents have been shown to play a crucial role in activating Nrf2 by modifying the conformation of the Keap1 protein [34,35]; this is achieved by electrophilic agents that modify specific cysteine residues on the Keap1 protein, thereby inducing conformational changes that lead to Nrf2 dissociation from Keap1 and, ultimately, Nrf2 activation [36]. Based on the structure of MCC, which is characterized as an electrophilic agent, we speculate that MCC may directly induce the activation of Nrf2 by modulating Keap1 conformation, thereby promoting HO-1 expression. Building upon prior research indicating the pivotal role of Nrf2 in the anti-inflammatory activity of compounds like β -hydroxyisovalerylshikonin [10], we employed the selective Nrf2 inhibitor, ML385, to assess MCC-induced expression of HO-1. Our findings revealed a significant reversal of MCC effects; ML385 effectively decreased the nuclear translocation of Nrf2 and HO-1 expression in BV2 microglia. ML385, a compact compound, has been recognized for its potent Nrf2 inhibitory action, achieved by binding to the Neh1 domain of Nrf2, which is critical for DNA binding and dimerization of small MAF proteins. This interaction impairs the association between the MAFG-Nrf2 complex and ARE [37–39]. Consequently, our results in BV2 cells suggest that the anti-neuroinflammatory effect of MCC is partly due to the enhanced HO-1 expression facilitated by upregulated Nrf2 activation.

Several studies have suggested that the PI3K/Akt cascade is associated with Nrf2-mediated HO-1 expression [40]. The activation of Akt can regulate Nrf2 activation by modulating its downstream glycogen synthase kinase-3 β (GSK-3 β), a repressor of Nrf2 [41]. GSK-3 β , a rate-limiting step kinase enzyme that regulates glucose metabolism, is generally activated in unstressed cells and suppresses Nrf2 activation by regulating its nuclear export via Fyn phosphorylation [42]. GSK-3 β can also directly phosphorylate specific serine residues of Nrf2, leading to its ubiquitination and proteasomal degradation [41]. Conversely, Akt activation in stimulated cells inhibits GSK-3 β activity via phosphorylation on Ser9, resulting in the upregulation of nuclear Nrf2 expression [41,43]. Herein, we observed that treatment of cells with 25 μ M MCC resulted in lower levels of phosphorylated Akt and GSK-3 β than those observed following treatment with 6.25 μ M MCC (Fig. 7), which, in turn, leads to increased HO-1 expression. Furthermore, the addition of the PI3K inhibitor wortmannin augmented the MCC-induced HO-1 protein expression, indicating the involvement of Akt and GSK-3 β in MCC-mediated HO-1 expression. However, our results also revealed that

treating cells with wortmannin and 6.25 μ M MCC resulted in lower levels of HO-1 protein than those in cells treated with 25 μ M MCC (Fig. 7). It is worth noting that the reason underlying the reduced HO-1 protein levels remains elusive, warranting further investigations. Our investigation revealed that MCC could induce a substantially higher degree of LPS-induced Nrf2 nuclear translocation and HO-1 expression than LPS alone. Additionally, the obtained data demonstrated that wortmannin successfully prevented MCC-induced HO-1 protein expression in LPS-treated BV2 microglial cells, as shown in Fig. 8. Furthermore, our results indicate that inhibition of Akt signaling has a differential effect on LPS-induced iNOS and COX-2 expression. Interestingly, we also discovered that the downregulation of HO-1 expression partially but substantially reversed the inhibitory effect of MCC on COX-2 protein expression, whereas no significant impact was observed on iNOS in BV2 microglial cells. These findings suggest that the MCC-mediated activation of the Nrf2/HO-1 pathway may not solely depend on the Akt/GSK-3 β pathway in LPS-induced BV2 microglial cells. Further studies are necessary to elucidate the precise mechanisms through which MCC inhibits Akt signaling and impacts HO-1 activity in microglial cells.

Previously, we have reported the anti-inflammatory effects of MCC, highlighting its capacity to inhibit NF- κ B and Akt signaling pathways substantially in LPS-induced RAW264.7 macrophages without impacting the MAPK signaling pathway [16]. The present study provides novel insights into the potential of MCC to inhibit the neuroinflammatory responses induced by LPS-stimulated microglia. Interestingly, in contrast to our findings in RAW264.7 cells, MCC inhibited MAPK signaling pathways, and the Akt/GSK-3 β pathway in the current study. Furthermore, the compound consistently exhibited anti-inflammatory activity via activation of the Nrf2/HO-1 pathway.

Taken together, the present study revealed that MCC could exert an anti-neuroinflammatory effect in LPS-induced BV2 microglial cells by inhibiting the production of NO and PGE₂ and attenuating the expression of iNOS and COX-2. Our research suggests that the inhibitory effect of MCC is mediated via the inactivation of NF- κ B, MAPK, and Akt/GSK-3 β signaling pathways and the enhancement of Nrf2-mediated HO-1. Although further research is required to establish the anti-neuroinflammatory efficacy of MCC in vivo, the data derived from this study offer valuable insights into the molecular mechanisms underlying the anti-inflammatory action of MCC on LPS-stimulated microglia. In addition, our findings suggest that MCC is a promising therapeutic agent for treating neurodegenerative diseases.

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CRediT authorship contribution statement

MP: performed experiments, analyzed data and wrote the first draft of the manuscript. **SM:** created the figure, reviewed related papers and edited manuscript. **NC:** conceptualization and design of study. **KS:** funding acquisition, conceptualization and design of study, interpreted

data and edited manuscript.

Declaration of Competing Interest

The authors have no relevant financial or non-financial interests to disclose.

Data availability

Inquiries about data availability should be directed to the corresponding author.

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