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Original Article

A novel synthetic compound MCAP suppresses LPS-induced murine microglial activation *in vitro* via inhibiting NF-kB and p38 MAPK pathways

Byung-Wook KIM[1, 2], Sandeep Vasant MORE[1], Yo-Sep YUN[1], Hyun-Myung KO[1], Jae-Hwan KWAK[3], Heesoon LEE[4], Kyoungho SUK[2], In-Su KIM[1, *], Dong-Kug CHOI[1, *]

[1]Department of Biotechnology, College of Biomedical and Health Science, Konkuk University, ChungJu 27478, Republic of Korea; [2]Department of Pharmacology, Brain Science and Engineering Institute, Kyungpook National University School of Medicine, Daegu 41944, Republic of Korea; [3]College of Pharmacy, Kyungsung University, Busan 48434, Republic of Korea; [4]College of Pharmacy, Chungbuk National University, Cheongju 28644, Republic of Korea

Aim: To investigate the anti-neuroinflammatory activity of a novel synthetic compound, 7-methylchroman-2-carboxylic acid *N*-(2-trifluoromethyl) phenylamide (MCAP) against LPS-induced microglial activation *in vitro*.
Methods: Primary mouse microglia and BV2 microglia cells were exposed to LPS (50 or 100 ng/mL). The expression of iNOS and COX-2, proinflammatory cytokines, NF-κB and p38 MAPK signaling molecules were analyzed by RT-PCR, Western blot and ELISA. The morphological changes of microglia and nuclear translocation of NF-κB were visualized using phase contrast and fluorescence microscopy, respectively.
Results: Pretreatment with MCAP (0.1, 1, 10 μmol/L) dose-dependently inhibited LPS-induced expression of iNOS and COX-2 in BV2 microglia cells. Similar results were obtained in primary microglia pretreated with MCAP (0.1, 0.5 μmol/L). MCAP dose-dependently abated LPS-induced release of TNF-α, IL-6 and IL-1β, and mitigated LPS-induced activation of NF-κB by reducing the phosphorylation of IκBα in BV2 microglia cells. Moreover, MCAP attenuated LPS-induced phosphorylation of p38 MAPK, whereas SB203580, a p38 MAPK inhibitor, significantly potentiated MCAP-caused inhibition on the expression of MEF-2 (a transcription factor downstream of p38 MAPK).
Conclusion: MCAP exerts anti-inflammatory effects in murine microglia *in vitro* by inhibiting the p38 MAPK and NF-κB signaling pathways and proinflammatory responses. MCAP may be developed as a novel agent for treating diseases involving activated microglial cells.

Keywords: 7-methylchroman-2-carboxylic acid *N*-(2-trifluoromethyl) phenylamide (MCAP); microglia; NO; NF-κB; MAPKs; LPS; SB203580; neuroinflammation

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Introduction

Microglial cells are the prime effector cells that participate in immune and inflammatory responses of host defense and tissue repair in the central nervous system (CNS). The over-activation of microglia in various neuropathological conditions, such as Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis and cerebral ischemia, leads to increased neural cell death and further deteriorates the on-going disease process[1-3]. In addition to lipopolysaccharides (LPS), BV2 microglia cells also respond to other inflammatory triggers, such as nitric oxide (NO), prostaglandins and proinflammatory cytokines. These microglial products are thought to be responsible for neuroglia-mediated neurotoxicity[4]. Thus, the suppression of microglial activation could be an effective therapeutic approach for alleviating the progression of neurodegenerative diseases involving excessive microglial activation[5, 6]. Despite the fact that many of the leading anti-inflammatory compounds are analogues of natural products that are not readily amenable to high throughput chemical screening and that the inhibition of multiple protein kinases is involved in the regulation of interleukin-1β (IL-1β) and induc-

* To whom correspondence should be addressed.
E-mail choidk@kku.ac.kr (Dong-Kug CHOI);
kis5497@kku.ac.kr (In-Su KIM)
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ible nitric oxide synthase (iNOS) induction[7], there is a need for developing a new chemical class of compounds that are cell-permeable inhibitors of glial activation and are more specific inhibitors of the protein kinases involved in the regulation of proinflammatory mediators.

Nuclear factor kappa B (NF-κB) is a transcription factor that regulates the expression of numerous proinflammatory genes[8-11] and has also been demonstrated to play an essential role in the LPS-induced expression of iNOS, cyclooxygenase-2 (COX-2) and proinflammatory cytokines. Therefore, it has been suggested that inhibition of NF-κB activation might help to mitigate on-going inflammation.

In the present study, we synthesized a novel MCAP [7-methylchroman-2-carboxylic acid *N*-(2-trifluoromethyl) phenylamide] and then investigated its possible anti-inflammatory effects to an LPS-induced inflammatory response in primary microglia and BV2 microglia cells. MCAP significantly inhibited the release of NO in LPS-induced primary microglia and BV2 microglia cells. Moreover, MCAP significantly suppressed the release of inflammatory mediators, such as iNOS, COX-2 and cytokines, in LPS-stimulated BV2 cells. In addition, we also demonstrated that MCAP reduced the phosphorylation of p38 mitogen-activated protein kinase (MAPK) when it was accompanied by the inhibition of myocyte-enhancer factor (MEF-2), a transcription factor downstream of p38 MAPK[12-14]. Furthermore, MCAP inhibited the nuclear translocation of NF-κB, which ultimately contributes to the inflammatory milieu. In conclusion, our findings suggest that MCAP is a therapeutic candidate for the treatment of various neurodegenerative diseases wherein microglial activation plays an important role in the pathogenesis of the disease.

Materials and methods

Reagents

MCAP (chemical structure and synthesis are shown in Figure 1), lipopolysaccharide (LPS, *E. coli* 0111:B4), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sulfanilamide, *N*-(1-naphthyl)-ethylenediamine dihydrochloride and Tween-20 were purchased from Sigma–Aldrich (St Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin (P–S), trypsin/EDTA (TE) and TRIzol, as well as other cell culture plates, were obtained from Gibco-BRL (Rockville, MD, USA). The protease inhibitor cocktail tablets and the phosphatase inhibitor cocktail tablets were supplied by Roche (Mannheim, Germany). The antibodies for COX-2, NF-κB p65, MEF-2 and nucleolin were obtained from Santa Cruz (Santa Cruz, CA, USA). iNOS, IκBα, phosphor(p)-IκBα, p38, p-p38, SB203580 (p38 specific inhibitor) and β-actin were procured from Cell Signaling (Danvers, MA, USA). The nuclear extract kit was purchased from Thermo Scientific (Rockford, IL, USA). The nitric oxide synthase 2, inducible (NOS2) enzyme-linked immunosorbent assay (ELISA) kit was from USCNLIFE™ (Wuhan, China), and the prostaglandin E2 (PGE_2) ELISA kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

Cell culture, morphological examination, NO release assay and cell viability

Primary microglial were obtained from a 2 to 3-d old mice by chopping and mechanically disrupting the whole brain using a 70 μmol/L nylon mesh. The cells obtained were seeded in culture flasks and grown at 37 °C in a 5% CO_2 atmosphere in DMEM supplemented with 10% FBS and 100 U/mL P–S. The culture medium was changed initially after 5 days and then afterward every 3 days, and the cells were used after being in culture for 21 days. Primary microglial cells were obtained by shaking the mixed glial cultures at 200 rounds/min for 4 h. The culture medium was then centrifuged at 1200 rounds/min for 10 min. Finally, the primary microglia cultures were grown and maintained in DMEM supplemented with 10% FBS and P–S. The BV2 microglia cells were acquired as described previously[15]. The BV2 cells were cultured in DMEM supplemented with 5% FBS and 100 U/mL P–S and maintained in a humidified incubator in an atmosphere of 5% CO_2.

In all of the experiments, the cells were seeded at a density of 5×10^5 cells/mL and pretreated for 1 h with the indicated concentrations of MCAP before incubation in a medium containing LPS (50 or 100 ng/mL). In the cell morphology experiment, the primary microglial were pretreated with 0.5 μmol/L of MCAP followed by LPS (50 ng/mL) induction for 24 h, and the cellular morphology images were observed by using phase contrast microscopy (Axio; Carl Zeiss, Germany). The LPS-induced release of NO in the culture supernatants was determined by measuring nitrite, a major stable product of NO, using the Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4). The cell viability of the cultured cells was determined by measuring the reduction of MTT to formazan. Briefly, the cells (5×10^4 cells/well) were seeded and treated with different concentrations of MCAP (0.1 and 0.5 or 0.1, 1 and 10 μmol/L) followed by an incubation with LPS for 24 h. Following the 24-h LPS incubation, 50 μL of each culture medium was mixed with an equal volume of Griess reagent. Nitrite levels were determined using a microplate reader at 540 nm (Tecan Trading AG, Basel, Switzerland), and nitrite concentrations were calculated by reference to a standard curve generated by known concentrations of sodium nitrite. Cell viability was measured by adding 0.5 mg/mL of MTT to each well. After incubation for another 4 h at 37 °C in 5% CO_2, the supernatants were removed from each well and the formazan crystals formed were dissolved in DMSO. The absorbance was determined at 540 nm using a microplate reader (Tecan Trading AG).

Reverse transcription–polymerase chain reaction (RT-PCR)

The BV2 microglia cells were plated overnight in 6-well culture plates at a density of 5×10^5 cells/mL and pretreated for 1 h with various concentrations of MCAP, including 0.1, 1 and 10 μmol/L, before incubation in a medium containing LPS (100 ng/mL). Total RNA was extracted using TRIZOL (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA (2.5 μg) was reverse-transcribed using the Superscript™ III kit (Invitrogen) according to the manufacturer's instructions. The

iNOS, COX-2, tumor necrosis factor-α (TNF-α), IL-1β, IL-6 and GAPDH genes were amplified from the cDNA by polymerase chain reaction (PCR). The cDNA was amplified by PCR using the specific primers presented in Table 1. The PCR was performed using an initial step of denaturation (5 min at 94 °C), 20–28 cycles of amplification (94 °C for 30 s, 54−58 °C for 1 min and 72 °C for 1 min) and an extension (72 °C for 5 min). The PCR products were analyzed on 1% agarose gels. The GAPDH mRNA served as the internal control for sample loading and mRNA integrity. The band intensity was quantified by densitometry analysis using Multi-gauge software V3.1 (Fujifilm, Tokyo, Japan).

Western blot analysis

The BV2 cells were plated overnight in 6-well culture plates at a density of 5×10^5 cells/mL and further incubated in serum-free medium for at least 3 h before treatment. The cells were lysed using RIPA buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS, containing fresh protease inhibitor cocktail). The electrophoresis and immunoblotting procedures were performed according to a previous report[16]. The membranes were probed with primary antibodies to iNOS, IκBα, p-IκBα, p38, p-p38 (1:1000; Cell Signaling), β-actin (1:2000; Cell Signaling), COX-2, p65, MEF-2 (1:1000; Santa Cruz) and nucleolin (1:500; Santa Cruz). After incubation with the appropriate primary antibody, the membranes were incubated for 1 h at room temperature with a secondary antibody conjugated to horseradish peroxidase (1:2000 dilution; rabbit, Cell Signaling, goat; Santa Cruz). The blots were visualized by a PowerOpti-ECL (Animal Genetics Inc, Tallahassee, FL, USA) detection system according to the recommended procedure. In a parallel experiment, the nuclear protein was prepared using a nuclear extraction kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's protocol.

Inducible NOS activity assay

To obtain the total cell lysate, 70 μL of RIPA buffer was added to the BV2 cells (1×10^6 cells/mL) cultured in 6-well plates. The cells were scraped, incubated for 10 min on ice and centrifuged at 14000 rounds/min for 10 min at 4 °C. The protein concentration was determined by the DC protein assay from Bio-Rad (Hercules, CA, USA), and 10 μg of the whole cell lysate was used for inducible (NOS2) ELISA kit. The iNOS enzyme activity in lysate was determined spectrophotometrically (Tecan Trading AG) at 450 nm. Each experiment was performed in triplicate.

Table 1. PCR primers used in this study.

Gene target	Accession	Primer sequence	Size (bp)
iNOS	NM_010927	F 5′-CTTGCAAGTCCAAGTCTTGC-3′ R 5′-GTATGTGTCTGCAGATGTGCTG-3′	369
COX-2	NM_011198	F 5′-ACATCCCTGAGAACCTGCAGT-3′ R 5′-CCAGGAGGATGGAGTTGTTGT-3′	414
TNF-α	NM_01369	F 5′-TTCGAGTGACAAGCCTGTAGC-3′ R 5′-AGATTGACCTCAGCGCTGAGT-3′	390
IL-1β	NM_008361	F 5′-CATATGAGCTGAAAGCTCTCCA-3′ R 5′-GACACAGATTCCATGGTGAAGTC-3′	385
IL-6	NM_031168	F 5′-CATATGAGCTGAAAGCTCTCCA-3′ R 5′-GACACAGATTCCATGGTGAAGTC-3′	435
GAPDH	GU214026	F 5′-CCAGTATGACTCCACTCACG-3′ R 5′-CCTTCCACAATGCCAAAGTT-3′	378

Prostaglandin E2 (PGE_2) measurement

The BV2 microglia cells cultured in 24-well plates were pretreated for 1 h with the indicated doses of MCAP followed by an incubation with LPS for 24 h. The PGE_2 level in the culture supernatant was measured by a commercial kit (Cayman Chemicals) according to the manufacturer's instructions. Briefly, 50 μL of the PGE_2 acetylcholinesterase conjugate (as tracer), 50 μL of the PGE_2 monoclonal antibody and 50 μL of the culture supernatant were combined in each well of a plate pre-coated with a goat polyclonal mouse immunoglobulin G (IgG) and was incubated for 18 h at 4 °C. The wells were emptied and rinsed five times with wash buffer. Ellman's reagent (200 mL) was added to each well to develop in the dark for 60–90 min at room temperature. The PGE_2 concentrations were determined using a PGE_2 standard. The absorbance at 420 nm was determined using a microplate reader (Tecan Trading AG).

Immunofluorescence (IF) assay

For the detection of the intracellular location of the p65 subunit of NF-κB, the BV2 cells (1×10^5 cells/well in 12-well plate) were cultured on sterile cover slips placed in 24-well plates and treated with the compound followed by LPS. Sixty min after the LPS treatment, the cells were fixed with methanol for 20 min at −20 °C and washed with PBS for 5 min. The fixed cells were then permeabilized with 1% Triton X-100 in PBS for 10 min at room temperature and washed with 0.02% Tween-20 in PBS for 5 min. The permeabilized cells were then treated with 1:200 dilution of NF-κB (p65) (Santa Cruz) overnight at 4 °C and washed with 0.02% Tween-20 in PBS for 5 min. The cells were then incubated in a 1:100 dilution of an Alexa Fluor 568-labeled antibody (Invitrogen) for 1 h at room temperature and washed with 0.02% Tween-20 in PBS for 5 min. The cells were then stained with 1 μmol/L of a Hoechst (Invitrogen) staining solution for 5 min at room temperature and then washed. Finally, all of the images were captured with a fluorescence microscope (Axio; Carl Zeiss)

Statistical analysis

The values given are the means±SEM of at least three separate experiments conducted in triplicate. Comparisons between groups were analyzed using a One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using the software Graphpad Prism V5.01 (GraphPad Software Inc, San Diego, CA, USA).

Results

Synthesis of MCAP

The synthetic strategy used to prepare the target compound, MCAP, is outlined in Figure 1. Initially, 2′-hydroxy-4′-methylacetophenone was treated with diethyl oxalate in sodium ethoxide and then with a hydrochloride solution to give the cyclized intermediate ethyl 7-methyl-4-oxo-4*H*-chromene-2-carboxylate in a 98% yield. The catalytic hydrogenation of this ester was carried out with 10% Pd/C in ethanol in the presence of a catalytic amount of acetic acid under a H_2 gas atmosphere to give the ethyl 7-methylchroman-2-carboxylate in a 99% yield. Treatment of the intermediate with potassium hydroxide in water afforded 7-methylchroman-2-carboxylic acid in a 96% yield. The coupling reaction of the acid with 2-(trifluoromethyl) aniline using *N,N*-carbonyldiimidazole (CDI) in anhydrous THF provided the MCAP. The oil product was obtained in a 25% yield after flash column chromatography[17]. IR (KBr): 3421, 1710, 1535, 634 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ8.39 (d, 1H, *J*=8.1 Hz, Ar-H), 7.63 (d, 1H, *J*=8.1 Hz, Ar-H), 7.58 (t, 1H, *J*=8.1 Hz, Ar-H), 7.24 (t, 1H, *J*=8.1 Hz, Ar-H), 6.98 (d, 1H, *J*=7.7 Hz, Ar-H), 6.78 (s, 1H, Ar-H), 6.76 (d, 1H, *J*=7.7 Hz, Ar-H), 4.65 (dd, 1H, *J*=9.8, 2.9 Hz, OCH), 2.95-2.75 (m, 2H, CH2), 2.52-2.06 (m, 2H, CH2), 2.32 (s, 3H, Ar-CH3). MCAP (purity>99%) was dissolved in DMSO.

Effect of MCAP on NO production in LPS-stimulated primary microglia and BV2 microglia cells

Initially, we aimed to evaluate the effect of MCAP on NO production in LPS-stimulated primary microglia and BV2 microglia cells. For this, the primary microglia were pretreated with MCAP (0.1 or 0.5 μmol/L), while the BV2 cells were treated with 0.1, 1 or 10 μmol/L of MCAP for 1 h before the addition of LPS (50 and 100 ng/mL, respectively), Incubation with LPS alone markedly increased NO production in the primary microglia (14.1±1.2 μmol/L; Figure 2A; $P<0.01$ *vs* the control group) and BV2 cells (25.6±2.9 μmol/L; Figure 2B; $P<0.01$ *vs* the control group) compared to the control. However, pretreatment with MCAP prevented the increased NO production levels in both LPS-stimulated microglial cells in a concentration-dependent manner ($P<0.05$ and $P<0.01$ *vs* the LPS group in Figure 2A; $P<0.01$ *vs* the LPS group in Figure 2B). We observed a significant inhibition of NO after treatment with 0.5 μmol/L and 10 μmol/L of MCAP. To further evaluate the cytotoxic effects of MCAP and/or LPS in primary microglia and BV2 microglia cells, cell viability was determined by an MTT assay. MCAP- and LPS-treated microglial cells individually did not elicit any signs of toxicity at the selected concentrations. We also found that MCAP alone at doses of 0.5 μmol/L and 10 μmol/L had no toxic effects on primary microglia and BV2 microglia cells, respectively (Figure 2C−D). In addition, we examined the cell morphology of the primary microglial cells that were incubated with MCAP (0.5 μmol/L) in the presence or absence of LPS (50 ng/mL). Bright field images were obtained after 24 h using the inverted microscope. The shape of the LPS-treated microglial cells was ramified compared to the control group, indicating activation of the microglial cells. This morphological change induced by LPS treatment was successfully inhibited by pretreatment with 0.5 μmol/L of MCAP (Figure 2E).

MCAP regulates LPS-induced iNOS and COX-2 production in BV2 microglia cells

Because MCAP, at the indicated concentrations (0.1, 1 and 10 μmol/L), attenuated NO production, we further examined the effect of MCAP on the mRNA and protein expressions of iNOS and COX-2 in the BV2 cells. The inhibitory effects of MCAP on the mRNA and protein expressions of iNOS and COX-2 were determined by RT-PCR and Western blot analysis, respectively. The levels of iNOS and COX-2 mRNA were markedly increased after 24 h of LPS (100 ng/mL) treatment, and MCAP significantly inhibited iNOS and COX-2 mRNA expression in the LPS-stimulated BV2 cells in a concentration-dependent manner (Figure 3A−B; $P<0.05$, $P<0.01$ *vs* LPS group). LPS-stimulated BV2 cells showed a significant increase in iNOS and COX-2 protein levels when compared to the controls ($P<0.01$ *vs* the control group). Pre-treatment with MCAP at various concentrations (0.1, 1 and 10 μmol/L) significantly attenuated the LPS-stimulated increase in iNOS and COX-2 levels (Figure 3C−D; $P<0.01$ *vs* the LPS group). A Western blot analysis showed that the reduction in iNOS

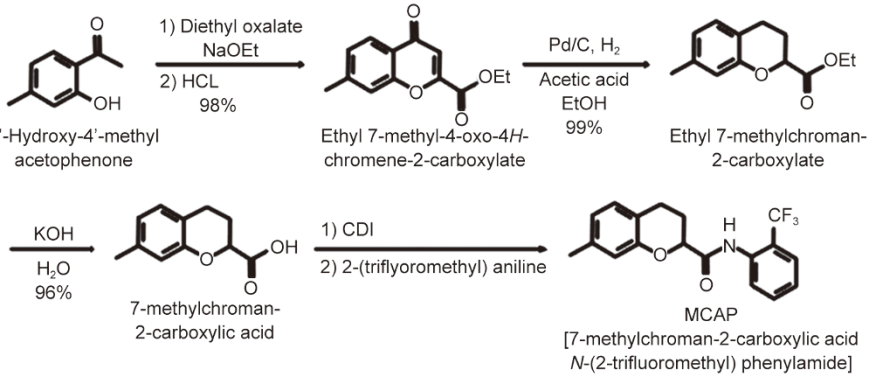

Figure 1. The structure and synthesis of MCAP. NaOEt, sodium ethoxide; EtOH, ethanol; CDI, *N*,*N*-carbonyldiimidazole.

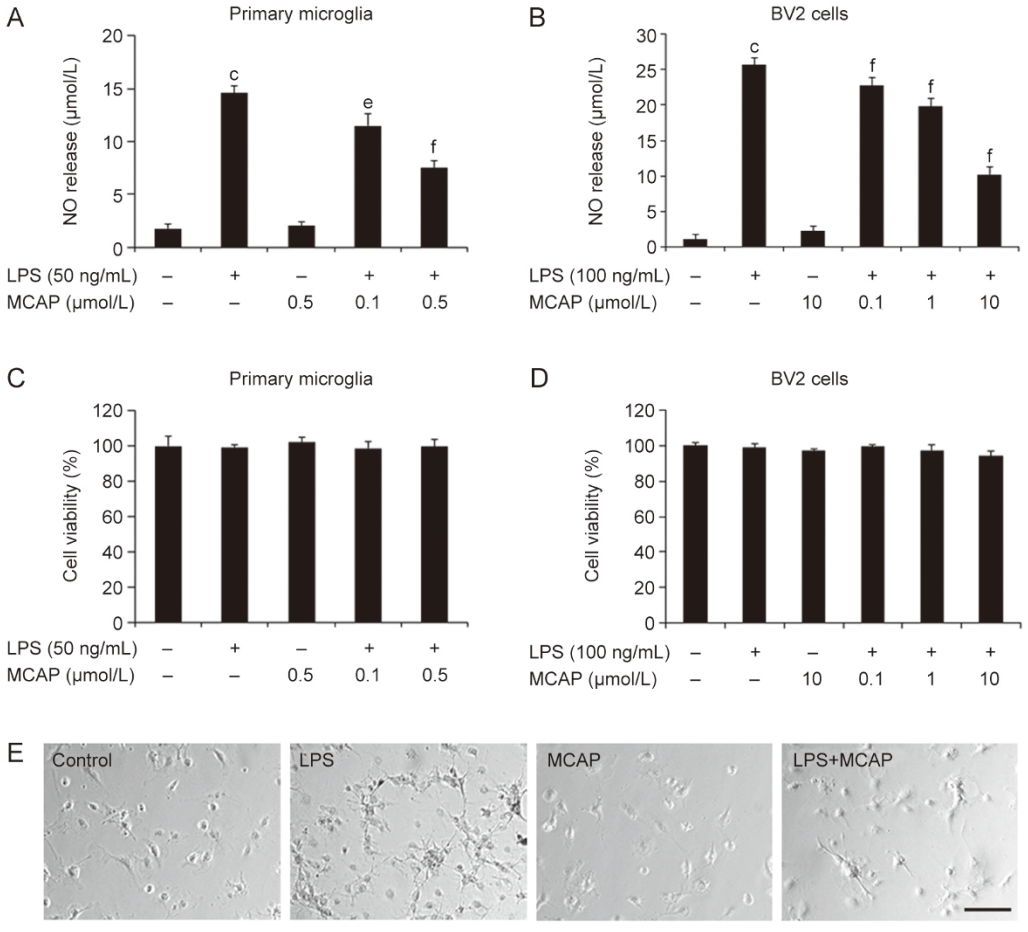

Figure 2. Effect of MCAP on cell viability and NO production in LPS-stimulated microglia. Mouse primary microglia (A, C) and BV2 microglia (B, D) cells were pretreated with various concentrations of MCAP (0.1 and 0.5 μmol/L for the primary microglia and 0.1, 1 and 10 μmol/L for the BV2 cells) for 1 h before incubation with LPS (50 and 100 ng/mL, respectively) for 24 h. The nitrite content was measured using the Griess reaction (A, B). The viability in MCAP-treated cells was evaluated using an MTT assay (C, D). The results are displayed as a percentage of the control samples. The morphological changes are represented in the primary microglia cells (E). Scale bar, 50 μmol/L. The data represent the mean±SEM from three independent experiments. $^{c}P<0.01$ *vs* the control group; $^{e}P<0.05$, $^{f}P<0.01$ *vs* the LPS alone group by a one-way ANOVA followed by Tukey's multiple comparison test.

and COX-2 protein levels was correlated with the reduction in their corresponding mRNA levels. In addition, MCAP reduced the LPS-stimulated iNOS enzyme activity in the BV2 cells in a dose-dependent manner. The data showed a significant reduction in the enzyme activity by MCAP treatment at a 10 μmol/L concentration in the LPS-treated BV2 cells (Figure 3E; $P<0.01$ *vs* the LPS group). PGE_2 represents the most important inflammatory product of COX-2 activity; therefore, we quantified the PGE_2 levels present in the supernatant of the LPS-exposed BV2 cells. To assess whether MCAP inhibits LPS-induced PGE_2 production in the BV2 cells, the cells were pretreated with MCAP for 1 h and then stimulated with LPS (100 ng/mL). After incubation for 24 h, the cell culture medium was harvested and the production of PGE_2 was measured using an ELISA. As shown in Figure 2F, the amount of PGE_2 present in the culture medium increased to approximately 221.8±4.3 pg/mL after a 24-h exposure to LPS alone ($P<0.01$ *vs* the control group). Pretreatment with MCAP (0.1, 1 or 10 μmol/L) concentration-dependently decreased the PGE_2 synthesis (Figure 3F; $P<0.01$ *vs* the LPS group).

MCAP inhibited the production of proinflammatory cytokines in the LPS-induced BV2 microglia cells

Proinflammatory cytokines, such as TNF-α, IL-1β and IL-6, play central roles in microglia-mediated inflammation. Therefore, we analyzed the effects of MCAP on proinflammatory cytokines (TNF-α, IL-1β and IL-6). The BV2 cells were incubated with MCAP (0.1, 1 and 10 μmol/L) in the presence or absence of LPS (100 ng/mL). The RT-PCR results demonstrated that the mRNA levels of these cytokines were elevated 6 h after the LPS treatment. Pre-treatment with MCAP significantly inhibited LPS-induced TNF-α, IL-1β and IL-6 production, compared to the LPS-treated cells (Figure 4A). The representative quantification data revealed that LPS-stimulated proinflammatory cytokine mRNA levels (TNF-α, IL-1β and IL-6) were significantly decreased following MCAP treatment

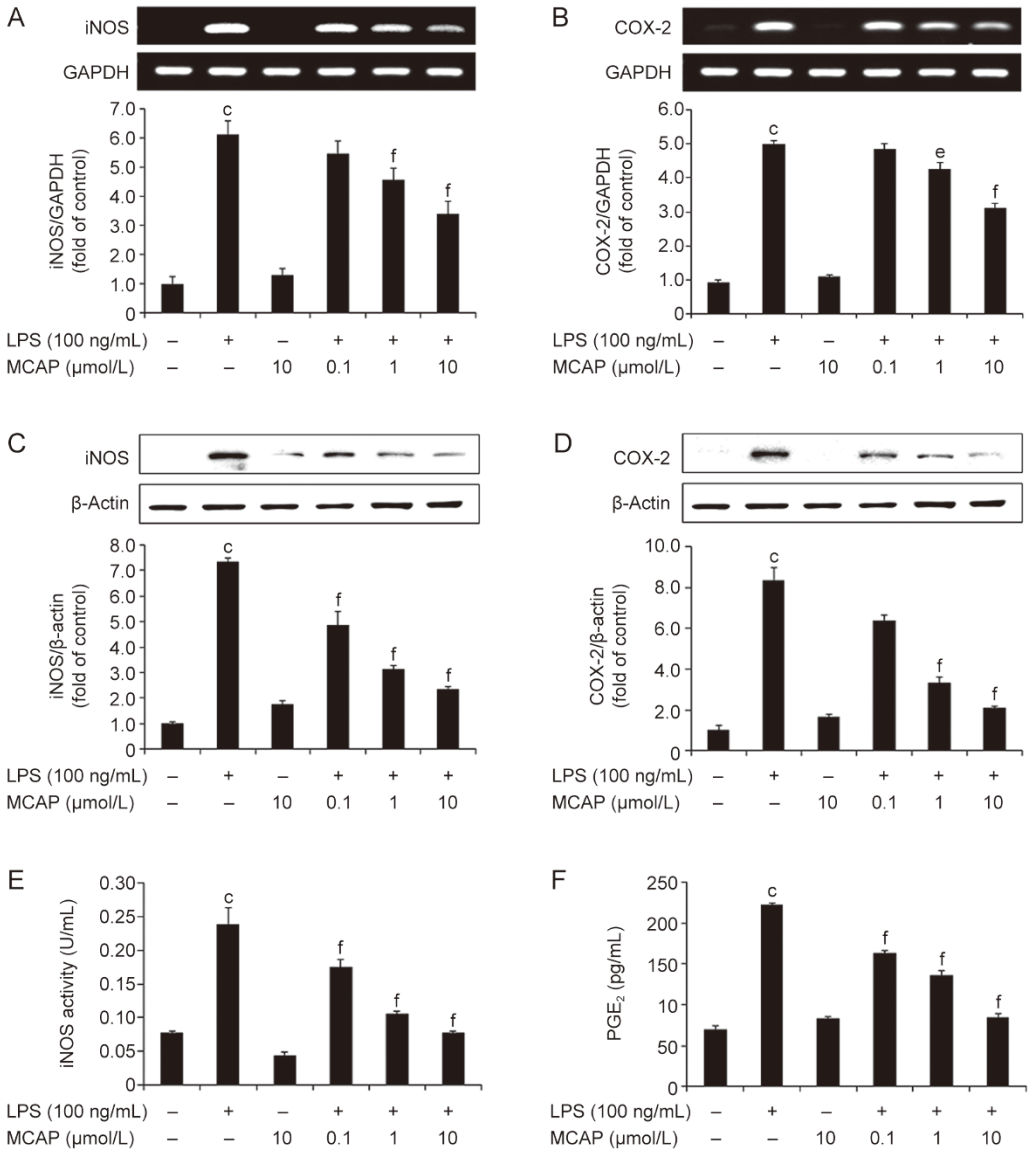

Figure 3. MCAP attenuates expression of iNOS and COX-2 levels in LPS-stimulated BV2 microglia cells. The BV2 cells were pre-treated with the indicated concentrations of MCAP for 1 h before incubating them with LPS (100 ng/mL) for 6 h (RT-PCR) and 18 h (immunoblotting). Total RNA was prepared and analyzed for iNOS (A) and COX-2 (B) gene expression by RT-PCR. The lysates were analyzed by immunoblotting with iNOS (C) and COX-2 (D) antibodies. The quantification of the data are shown in the lower panel. The BV2 cells were pre-treated with the indicated concentrations of MCAP for 1 h before incubating with LPS (100 ng/mL) for 24 h. The lysates were analyzed by an inducible (NOS2) ELISA kit. The absorbance was determined spectrophotometrically at 450 nm (E). The PGE_2 concentration in the culture medium was measured using a commercial ELISA kit. The absorbance was measured at 420 nm spectrophotometrically (F). The data represent the mean±SEM from three independent experiments. $^{c}P<0.01$ *vs* the control group; $^{e}P<0.05$, $^{f}P<0.01$ *vs* the LPS alone group by a one-way ANOVA followed by Tukey's multiple comparison test.

(Figure 4B–D; $P<0.05$ and $P<0.01$ *vs* the LPS group). Thus, our results indicate that MCAP inhibited the expression of cytokines involved in the inflammatory process.

MCAP suppressed NF-κB activity in the LPS-stimulated BV2 microglia cells

NF-κB is one of the principal factors for iNOS and COX-2 expression mediated by LPS or proinflammatory mediators[9, 18]. To elucidate the molecular mechanism by which MCAP inhibits iNOS and COX-2 expression and proinflammatory cytokine expression, the effect of MCAP on the blockade of NF-κB activity was determined by an immunocytochemistry-immunofluorescence (ICC-IF) assay. LPS treatment caused a significant increase in the translocation of the NF-κB p65 subunit into the nucleus within 30 min after LPS stimulation. In contrast, treatment with MCAP markedly suppressed the translocation of NF-κB p65 induced by LPS (Figure 5A). We also investigated the effect of MCAP on LPS-induced NF-κB p65 nuclear translocation as measured by a Western blot analysis. LPS-stimulated BV2 cells were pre-treated with MCAP (0.1, 1 or

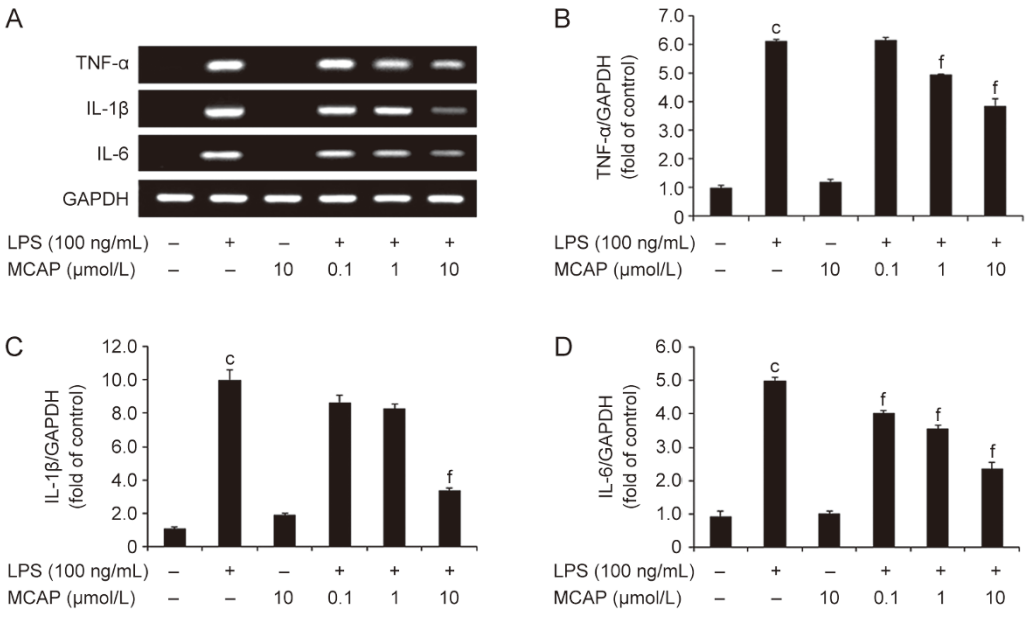

Figure 4. MCAP decreases the production of proinflammatory cytokines. (A) The cells were pre-treated with the indicated doses of MCAP for 1 h before LPS (100 ng/mL) treatment. The levels of TNF-α, IL-1β, IL-6 and GAPDH mRNA were determined by RT–PCR. The representative densitometry analyses of TNF-α (B), IL-1β (C) and IL-6 (D) compared with GAPDH mRNA. The data represent the mean±SEM from three independent experiments. $^{c}P<0.01$ *vs* the control group; $^{e}P<0.05$, $^{f}P<0.01$ *vs* the LPS alone group by a one-way ANOVA followed by Tukey's multiple comparison test.

10 μmol/L) for 1 h, and NF-κB activity was evaluated according to the nuclear translocation of the p65 subunit of NF-κB (Figure 5B; $P<0.05$ and $P<0.01$ *vs* LPS group). Furthermore, the phosphorylation of IκBα was also significantly inhibited by MCAP treatment of the BV2 cells (Figure 5C; $P<0.05$ and $P<0.01$ *vs* LPS group). Taken together, these results indicate that inhibition of NF-κB activation by MCAP may be the mechanism responsible for the suppression of iNOS, COX-2 and other proinflammatory cytokine expression in the LPS-stimulated BV2 cells.

MCAP inhibited the phosphorylation of p38 MAPK and the expression of MEF-2 in the LPS-induced BV2 cells

The phosphorylation of p38 MAPK induces the expression of proinflammatory molecules and is one of the important signaling pathways during inflammation[19, 20]. MEF-2 is a transcription factor that is activated by p38 MAPK during inflammation[12, 21, 22]. Therefore, we examined the effect of MCAP on the LPS-induced phosphorylation of p38 MAPK in the BV2 cells by a Western blot analysis. Pretreatment with MCAP at the indicated concentrations (0.1, 1 and 10 μmol/L) significantly inhibited the LPS-stimulated p38 phosphorylation in the BV2 cells (Figure 6A; $P<0.05$ and $P<0.01$ *vs* the LPS group). Furthermore, the effect of MCAP on the inhibition of MEF-2 activation was consistent with the effect of a specific p38 inhibitor SB203580[23] when examined simultaneously in LPS-stimulated BV2 cells (Figure 6B; $P<0.05$ and $P<0.01$ *vs* LPS group). These results suggest that MCAP is indeed capable of disrupting p38 MAPK, the key signal transduction pathway elicited by LPS in the BV2 cells, and subsequently preventing the production of proinflammatory mediators.

Discussion

In the present study, we demonstrated that a synthetic compound, named 7-methylchroman-2-carboxylic acid *N*-(2-trifluoromethyl) phenylamide (MCAP), exhibits a significant inhibitory action by blocking the synthesis of proinflammatory mediators in LPS-stimulated BV2 microglia cells. The functional role of microglia in the diseased brain is controversial because it has been observed to protect as well as to destroy or exacerbate the neurotoxicity[24]. Activated microglia produces several neurotoxic molecules, such as iNOS, COX-2 and cytokines, such as TNF-α, IL-1β and IL-6[25-27]. Based on the strong inhibitory action of MCAP on NO, iNOS activity and PGE_2 production, MCAP was further investigated for its effect on iNOS and COX-2 expression along with its effect on the release of proinflammatory cytokines. Additionally, we only included the primary microglia results concerning cell viability, cell morphology and NO production because they also might reflect the result of our compound in an animal study. However, due to time constraints and cell availability, we decided to carry out the rest of the experiments in the BV2 cells[15, 28]. We observed that MCAP satisfactorily inhibited the mRNA expressions of TNF-α, IL-1β and IL-6, with the most significant inhibition at 1 and 10 μmol/L for TNF-α and IL-6 secretion. MCAP was also observed to dose-dependently reduce the mRNA and protein expressions of iNOS and COX-2. In addition, 0.5 μmol/L of MCAP restored the morphological changes in LPS- (50 ng/mL) stimulated primary microglia cells. Our results are consistent with previous reports on LPS-induced morphological changes in microglia cells[29, 30].

The MAPK signaling pathways fine-tune a variety of cellular activities, such as survival, differentiation, proliferation

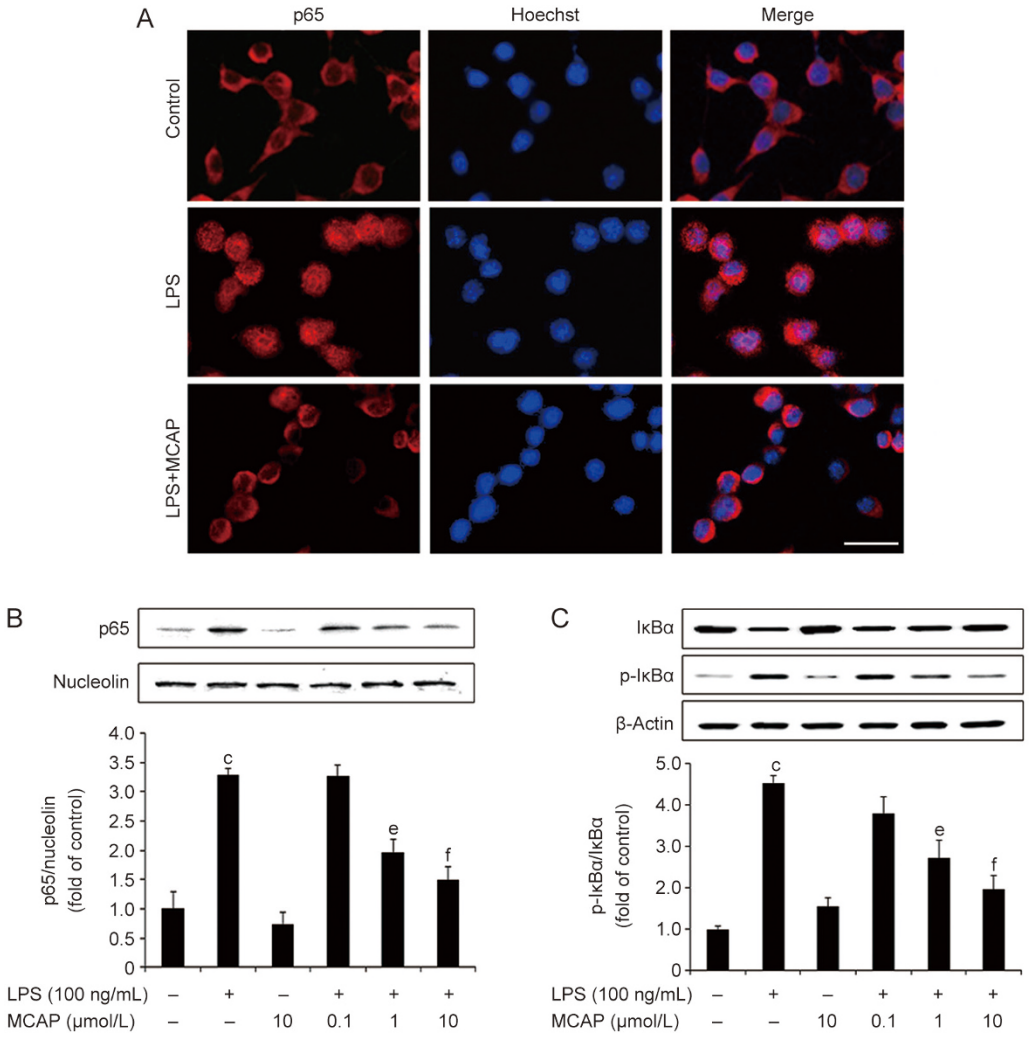

Figure 5. Inhibition of LPS-induced NF-κB activity by MCAP. (A) The BV2 cells were treated with LPS (100 ng/mL) in the absence or presence of MCAP for the indicated times. The nuclear translocation of the NF-κB p65 subunit was determined by an immunofluorescence assay. Scale bar, 20 μmol/L. (B) Nuclear extracts were prepared and analyzed by a Western blot to asses NF-κB p65 and nucleolin. (C) The cytosolic extracts were prepared and analyzed by a Western blot using IκBα, p-IκBα and β-actin antibodies. The densitometry analysis of NF-κB p65 and p-IκBα is presented in the lower panel. The data represent the mean±SEM from three independent experiments. $^{c}P<0.01$ *vs* the control group; $^{e}P<0.05$ and $^{f}P<0.01$ *vs* the LPS alone group by a one-way ANOVA followed by Tukey's multiple comparison test.

and neuronal death. Deviation from the normal MAPK signaling pathways has been implicated in the development of many neurodegenerative diseases, such as AD, PD, and amyotrophic lateral sclerosis (ALS)[31, 32]. Hyper-phosphorylation of MAPK molecules ultimately activates the transcription factor NF-κB and the subsequent production of inflammatory molecules[33]. Furthermore, several others studies[34, 35] have reported that the NF-κB and p38 MAPK pathways are important targets for therapeutic drugs designed to control inflammation in the CNS. We also found that MCAP significantly attenuated the LPS-stimulated phosphorylation of p38 MAPK in a dose-dependent manner. However, there was no effect of MCAP on LPS-induced ERK and JNK phosphorylation (data not shown). MEF-2 is the downstream signaling transcription factor involved in mediating the inflammatory effects of p38 MAPK signaling[12, 14, 36]. Therefore, in our study, we investigated the effect of MCAP on the protein expression of MEF-2 in the nuclear extracts of the LPS-stimulated microglial cells. MCAP significantly inhibited the expression of MEF-2. We also assessed the synergistic effect of MCAP and SB203580, a specific inhibitor of p38 MAPK. Our primary goal in performing this experiment was to observe the synergistic effect of MCAP and SB203580 in combination with LPS to inhibit MEF-2 levels. Additionally, some evidence[37] shows that SB203580 does not affect MEF-2 expression *per se*. Furthermore, we found a significant decrease in MEF-2 expression in the BV2 cells treated with SB203580 and MCAP.

In addition, several others studies have reported that the NF-κB and p38 MAPK pathways are important targets for therapeutic drugs (involved in the control of proinflammatory gene expression) designed to control inflammation in the CNS[34, 38]. In our study, MCAP decreased the phosphorylation

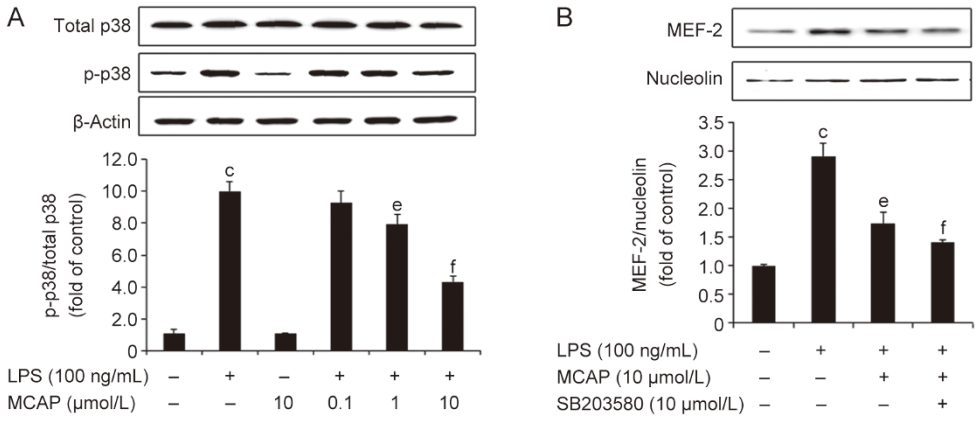

Figure 6. Inhibitory effects of MCAP on the LPS-induced activation of p38 MAPK and the expression of MEF-2 in the BV2 cells. (A) The BV2 cells were treated with the indicated doses of MCAP for 1 h before LPS treatment (100 ng/mL) for 30 min. The densitometry analysis of the p38 and p-p38 bands is presented in the lower panel. (B) The cells were pre-treated with SB203580 (10 μmol/L) and MCAP (10 μmol/L) for 1 h before LPS (100 ng/mL) treatment. The level of MEF-2 protein was determined by immunoblotting. The quantification data are shown in the lower panel. The data represent the mean±SEM from three independent experiments. $^{c}P<0.01$ *vs* the control group; $^{e}P<0.05$, $^{f}P<0.01$ *vs* the LPS alone group by a one-way ANOVA followed by Tukey's multiple comparison test.

of IκBα in a dose-dependent manner, and this effect was also correlated to a parallel decrease in the nuclear translocation of the p65 subunit of NF-κB as confirmed by immunocytochemistry and a Western blot analysis. Our results are in accordance with previous reports demonstrating that the inhibition of IκBα phosphorylation leads to the diminished nuclear translocation of the p65 subunit of NF-κB[16, 39].

In conclusion, the results of this study suggest that pre-treatment with MCAP mitigates LPS-induced iNOS, COX-2 and cytokine production in activated microglia. Apart from the inhibitory effects on microglial p38 MAPK and NF-κB, further research is needed to affirm the current findings that MCAP may have therapeutic potential for ameliorating microglia-mediated neuronal damage in many neuroinflammatory conditions.

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Author contribution

Byung-Wook KIM, In-Su KIM and Dong-Kug CHOI designed the experiments; Byung-Wook KIM, Sandeep Vasant MORE, Jae-Hwan KWAK and Yo-Sep YUN performed parts of the experiments; Byung-Wook KIM, Hyun-Myung KO, Heesoon LEE, Kyoungho SUK and In-Su KIM analyzed the data; Byung-Wook KIM, Sandeep Vasant MORE, In-Su KIM and Dong-Kug CHOI wrote the manuscript.

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