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Original Article

4-methoxycinnamyl *p*-coumarate isolated from *Etlingera pavieana* rhizomes inhibits inflammatory response via suppression of NF-κB, Akt and AP-1 signaling in LPS-stimulated RAW 264.7 macrophages

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Abbreviations: MCC, 4-methoxycinnamyl *p*-coumarate
LPS, Lipopolysaccharide
NO, nitric oxide
PGE_2, prostaglandins E_2
TNF-α, tumor necrosis factor-α
IL-1β, interleukin-1β
iNOS, inducible nitric oxide synthase
COX-2, cyclooxygenase-2
NF-κB, Nuclear factor-kappa B
AP-1, activator protein-1
MAPK, mitogen-activated protein kinase
Akt, Protein Kinase B
JNK, c-Jun N-terminal Kinase
ERK, Extracellular signal-regulated kinase

ABSTRACT

Background: 4-methoxycinnamyl *p*-coumarate (MCC) was isolated from rhizomes of *Etlingera pavieana* by bioactivity-guided isolation, however, the molecular mechanism underlying its anti-inflammatory activity remains inadequately understood.
Purpose: In this study, we elucidated the suppressive effect of MCC on LPS-induced expression of inflammatory mediators and the molecular mechanisms responsible for anti-inflammatory activities in RAW 264.7 macrophages.
Methods: Cell viability of MCC-treated RAW 264.7 macrophage was measured by MTT assay. Anti-inflammatory activity was evaluated by measurement of NO, **PGE_2, and cytokine** production in LPS-stimulated cells. qRT-PCR and Western blotting analysis were used to investigate mRNA and protein levels of inflammatory responsive genes. **NF-κB** activation and transactivation activity were determined by immunofluorescence and reporter gene assay, respectively.
Results: MCC considerably suppressed both the production of NO, PGE_2, IL-1β as well as TNF-α and their expression. MCC inactivated NF-κB by reducing phosphorylation of IκBα and inhibiting NF-κB p65 nuclear translocation. Also, MCC significantly inhibited NF-κB transactivation activity. However, the inhibitory effect of MCC was independent of the MAPK signaling pathway. Furthermore, MCC significantly decreased phosphorylation of Akt and c-Jun, a main component of AP-1.
Conclusion: These findings suggest that the anti-inflammatory effect of MCC could be mediated by the inhibition of LPS-induced expression of inflammatory mediators by down-regulation of the NF-κB, Akt and AP-1 signaling pathways in murine macrophages.

Introduction

Inflammation is a complex immune response to tissue injury and infection caused by pathologic agents such as bacteria, viruses, and fungi. During infection, innate immune cells such as macrophage, fibroblast, mast cell, and neutrophil are activated (Newton and Dixit, 2012). Macrophages play a crucial role in the inflammation process by secreting a variety of pro-inflammatory mediators, including nitric oxide (NO) and prostaglandins (PGs) as well as pro-inflammatory cytokines such as tumor necrosis factor (TNF-α) and interleukin-1β (IL-1β). The function of inflammation is to eliminate infectious pathogens and repair damaged tissues. If the stimuli causing acute inflammation cannot be completely eliminated, it becomes a chronic inflammation that may persist for periods of weeks or years (Sherwood and Toliver-Kinsky, 2004). Prolonged and overproduced inflammatory mediators and cytokines in chronic inflammation are involved in the pathogenesis of many inflammatory diseases (Prasad and Aggarwal, 2014). Thus, blocking these pro-inflammatory mediators and cytokines could serve as therapeutic treatment of inflammation-related diseases.

NO, a free radical is synthesized from L-arginine by three isoforms of nitric oxide synthase (NOS): neuronal NOS (nNOS) and endothelial NOS (eNOS) which are constitutive forms, and inducible NOS (iNOS) (Kleinert et al., 2004). PGs are small molecules derived from arachidonic acid by cyclooxygenase (COX) enzymes. The most abundant PGs

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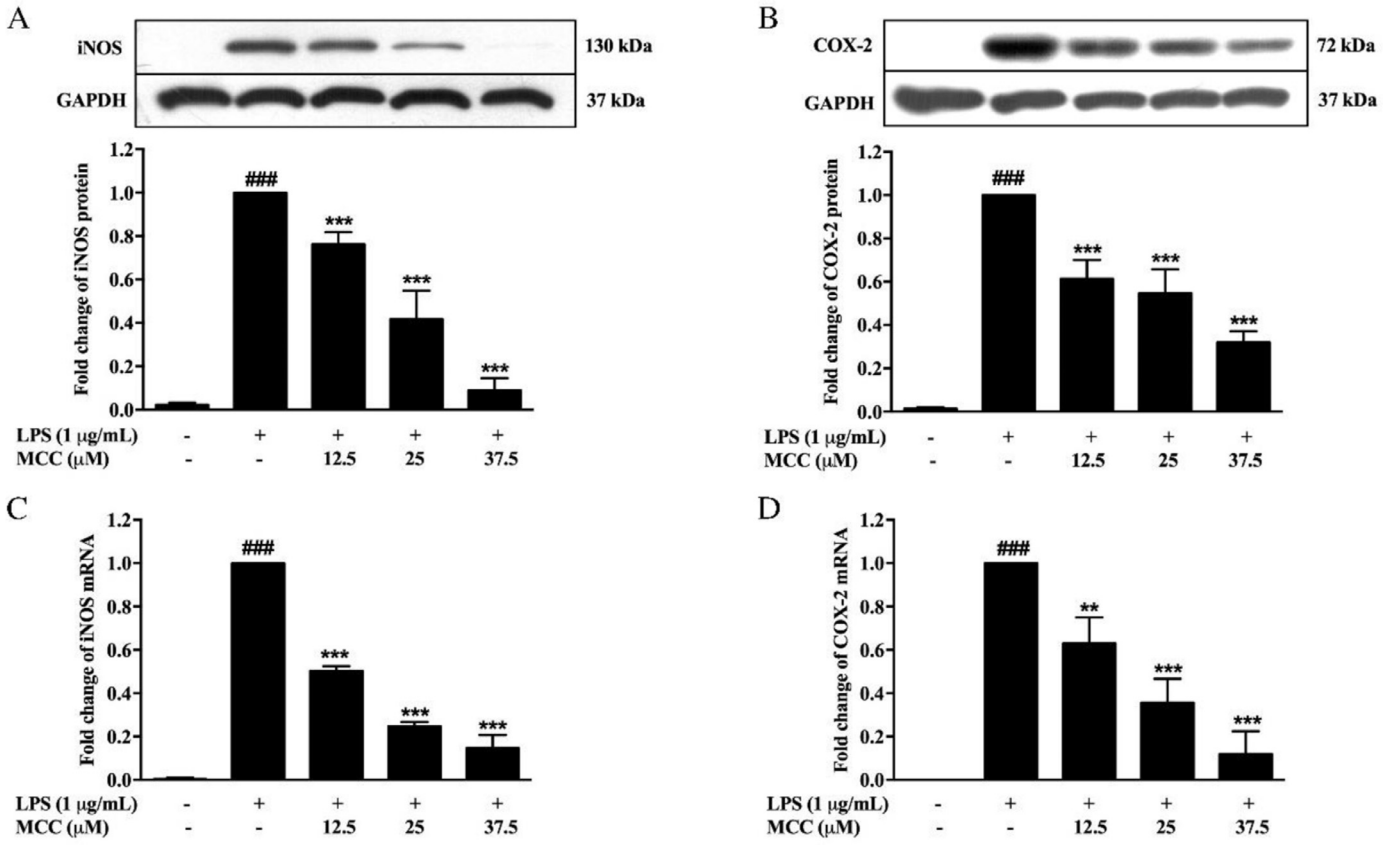

Fig. 1. Effect of MCC on iNOS and COX-2 expression in LPS-stimulated RAW 264.7 macrophages. Cells were incubated with LPS and MCC for 24 h. The level of iNOS (A) and COX-2 (B) were examined by Western blot analysis. Graph present means ± SD ($n = 3$) of densitometric value and normalized with GAPDH. Cells were incubated with LPS and MCC for 9 h. Cellular content of iNOS (C) and COX-2 (D) mRNA were examined by qRT-PCR and normalized with cothe ntent of EF-2. $^{###}p < 0.001$ compared to the unstimulated control cells, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the LPS-stimulated cells.

in a body is PGE_2. COX exists as two isoforms: COX-1, a constitutive COX, and COX-2, an isoform induced in response to a variety of stimulants and activated at an inflammation site. iNOS-catalyzed NO and COX-2-catalyzed PGE_2 are important mediators that are involved in an inflammatory response (Ricciotti and Fitzgerald, 2011).

Nuclear factor-kappa B (NF-κB) transcription factor is the main regulator of iNOS and COX-2 expression as well as pro-inflammatory cytokines in LPS-induced RAW 264.7 macrophages (Premkumar et al., 2010). Upon stimulation with LPS, NF-κB is activated via IκB-kinase (IKK) complex causing phosphorylation of the inhibitor of κBα (IκBα), the degradation of IκB and liberation of NF-κB. The liberated NF-κB dimers translocate into the nucleus to express inflammatory mediators (Ghosh and Hayden, 2008). Another transcription factor regulating inflammatory responsive genes is activator protein-1 (AP-1). It is a collection of dimers composed of Jun, Fos or ATF families that need to dimerize and bind to the AP-1 recognition sites. This transcription factor is predominantly activated through mitogen-activated protein kinases (MAPKs) signaling cascades consisting of the c-Jun NH_2-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 MAPK (Minden and Karin, 1997). Recently, several lines of evidence indicated that phosphatidylinositol 3′-kinase/Akt (PI3K/Akt) can modulate NF-κB and AP-1 activity (Dan et al., 2008; Manning and Cantley, 2007). Hence, modulation of inflammatory gene expression through these signaling pathways may represent one strategy in the development of anti-inflammatory therapeutic drugs.

In our continuing effort to find novel potential anti-inflammatory agents from Thai plants, four compounds from *Etlingera pavieana* (Pierre ex Gagnep.) R.M.Sm. rhizomes were isolated by NO inhibitory activity-guided fractionation (Srisook et al., 2017). Among them, 4-methoxycinnamyl *p*-coumarate (MCC) exhibited the most potent inhibitory effect. Moreover, MCC has some reported bioactivities such as anti-bacterial, anti-cancer and PGE_2 suppressing effects (Tachai and Nuntawong, 2016; Mankhong et al., 2017). Therefore, this compound has the potential for development as a novel anti-inflammatory agent. However, the mechanism underlying the anti-inflammatory effect of this compound has not been explored. Thus, in the current study, we investigated the anti-inflammatory effect and explored the mode of action of MCC in LPS-stimulated RAW 264.7 macrophages.

Materials and methods

Materials

Prostaglandins E_2 (PGE_2) Enzyme Immunoassay Kit was purchased from Arbor Assay (Michigan, USA). Mouse IL-1β Quantikine ELISA kit and mouse TNF-α Quantikine ELISA were purchased from R&D systems (Minnesota, USA). Antibodies of iNOS and COX-2 were obtained from BD Bioscience (California, USA). NE-PER nuclear and cytoplasmic extraction reagents, phosphatase inhibitor cocktail and protease inhibitor cocktail were purchased from Thermo Scientific (Massachusetts, USA). 2x iTaq™ Universal SYBR® Green Supermix and iScript Reverse Transcription Supermix were purchased from Bio-Rad (California, USA). Antibodies for IκB-α, p-IκBα (Ser32/36), GAPDH, secondary anti-Rabbit IgG (H + L), $F(ab')_2$ Fragment (Alexa Fluor 488 conjugate), p-SAPK/JNK (Thr183/Tyr185), p-p38 MAPK (Thr180/Tyr182), p-ERK1/2 (Thr202/Tyr204), SAPK/JNK were purchased from Cell Signaling Technology (Massachusetts, USA). Antibodies for total ERK1/2 and total p38α were purchased from Santa Cruz Biotechnology (California, USA). a pGL4.32 plasmid containing the NF-κB promoter [Luc2P/NF-κB/Hygro] vector, pGL4.74 [hRluc/TK] vector, Dual-Luciferase Assay

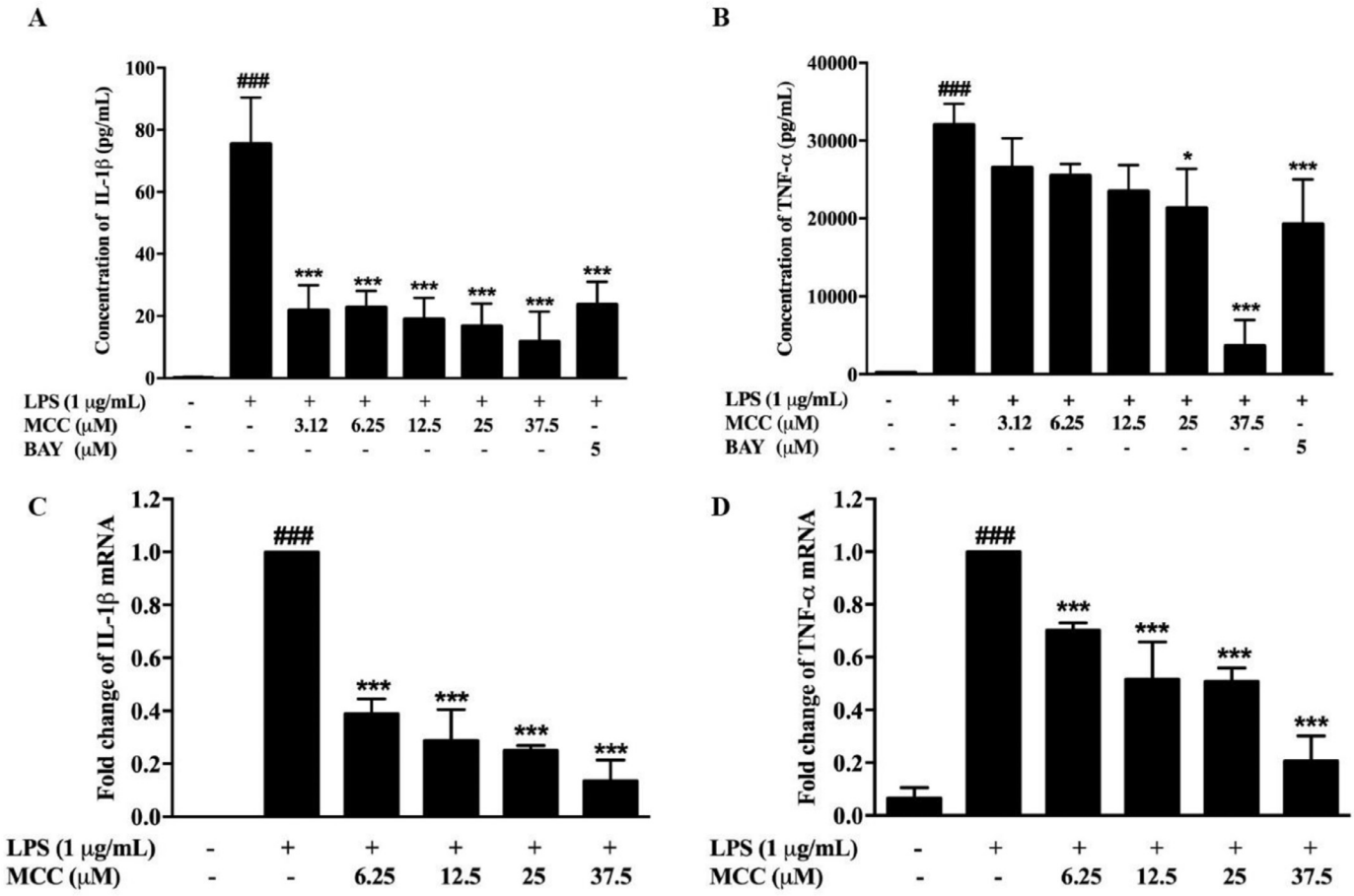

Fig. 2. The inhibitory effect of MCC on LPS-induced IL-1β, TNF-α production, and mRNA expression. Cells were incubated with MCC and LPS for 24 h. The supernatant was collected then IL-1β (A) and TNF-α (B) were analyzed by ELISA. The mRNA expression of IL-1β (C) and TNF-α (D) in LPS-induced cells determined by qRT-PCR. Cells were stimulated with MCC and LPS for 6 h. $^{###}p < 0.001$ compared to the unstimulated control cells, $^{*}p < 0.05$ and $^{***}p < 0.001$ compared to the LPS-stimulated cells.BAY = cell treated with BAY-117082.

System, and FuGENE transfection reagent were purchased from Promega (Wisconsin, USA) Prolong Gold anti-fade reagent with DAPI was obtained from Life Technologies (New York, USA).

Compound preparation

4-methoxycinnamyl *p*-coumarate (MCC) was isolated from *E. pavieana* rhizomes as described by Srisook et al. (2017). The purity of MCC was 97.7% as determined by HPLC technique and HPLC profile of MCC was shown in Supplement data Fig. S-1. It was dissolved in dimethyl sulfoxide (DMSO) and filtrated through a 0.22 μm pore size sterile filter.

Cell culture

Murine macrophage cell line RAW 264.7 was obtained from ATCC. Cells were cultured in DMEM containing 100 U/mL of penicillin, 100 μg/ml of streptomycin, and 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere at 37 °C.

Cell viability assay

The effect of MCC on cell viability was examined by MTT assay as described by Srisook et al., (2011). Briefly, cells were plated in 24-well plate and exposed to MCC for 24 h and fresh culture media containing MTT (0.1 mg/ml) was added to each well. After 2 h, culture media was removed and solubilizing formazan crystals in DMSO was measured at 550 nm using a microplate reader.

Determination of NO, PGE_2 and cytokine production

Cells were incubated with various concentration of MCC and LPS for 24 h. The concentration of nitrite, an oxidation product of NO, in the conditioned medium was used as an indicator of the amount of NO. Nitrite concentration was determined by Griess reagent as described by Srisook et al. (2011). The level of PGE_2, TNF-α, and IL-1βin culture medium were evaluated using ELISA kit according to the manufacturer's instructions.

Western blot analysis

Whole cell protein lysates were extracted with ice-cold RIPA lysis buffer as described by Buapool et al. (2013). An equal amount of protein was electrophoresed in 10% SDS-PAGE. The separated protein was transferred onto PVDF membranes. Subsequently, membranes were incubated with appropriate primary antibody followed by a secondary antibody conjugated to HRP. The specific protein bands on membranes were visualized on X-ray film activated by ECL substrate. Band signal intensities were determined by densitometry using BIOPROFIL Bio-1D version 11.9 (Vilber Lourmat, France).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Macrophages (1×10^6cells/plate) were stimulated with LPS and

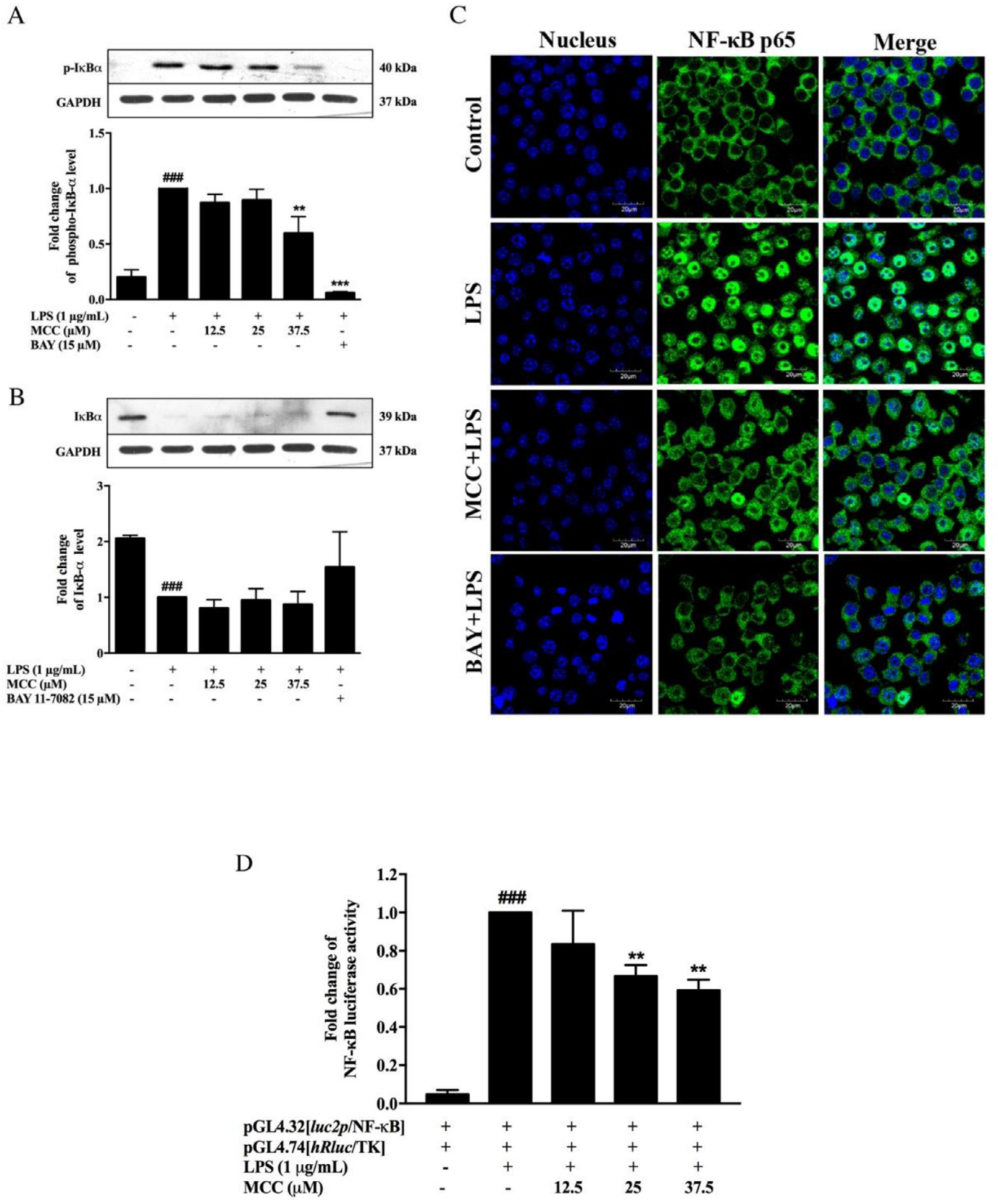

Fig. 3. Effect of MCC on LPS-induced NF-κB activation and transactivation activity. Cells were pre-incubated with MCC for 1 h, followed by stimulation with LPS for 30 min. Amounts of p-IκBα (A) and total IκBα (B) in cytoplasmic protein were determined by Western blot analysis. Data expresses the mean ± SD ($n = 3$) of densitometric value and normalized with GAPDH. NF-κB p65 translocation was performed by immunofluorescence under confocal microscopy(C). Cells were transiently co-transfected with pGL4.32[*luc2p*/NF-κB-RE/Hygro] and pGL4.74 [*hRluc*/TK] vectors, then pre-incubated with various concentration of MCC for 1 h. followed by stimulation with LPS for 6 h. The *luc2p* luciferase expression was normalized with *hRluc*luciferase expression. $^{###}p < 0.001$ compared to the un-stimulated control cells, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the LPS-stimulated cells.

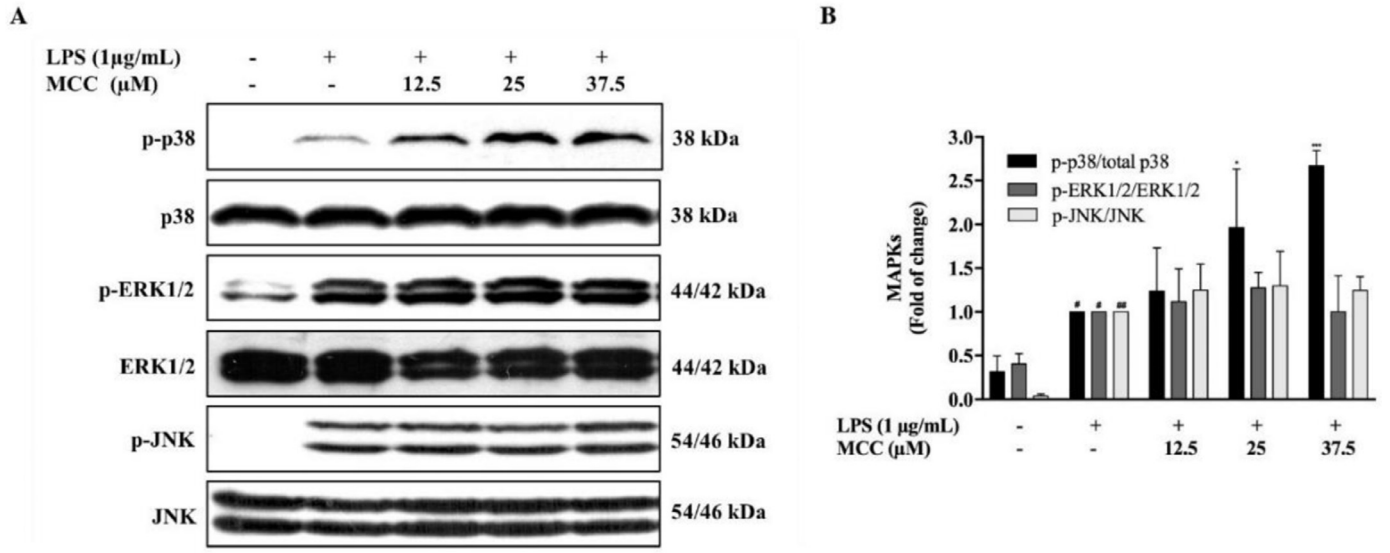

Fig. 4. Effect of MCC on LPS-induced activation of a MAP kinase signaling pathway in RAW 264.7 macrophages. The cell was pre-incubated with MCC at various concentration for 1 h. and then stimulated with LPS for 30 min. The phosphorylation level of p38 MAPK, ERK1/2, and JNK was determined by Western blot analysis (A). Graph shows mean ± SD of the densitometric value of phosphorylation level of p38 MAPK, ERK1/2, and JNK (B) which normalized with the total level of each protein. Data are expressed as fold change with respect to cells treated with LPS only. $^{\#\#\#}p < 0.001$ compared to the unstimulated control cells.$^{*}p < 0.05$ and $^{***}p < 0.001$ compared to the LPS-stimulated cells.

MCC. Total RNA was isolated from the cell pellet using a Nucleospin RNA kit according to the manufacturer's protocol. Two micrograms of RNA were reverse transcribed to cDNA using iScript Reverse Transcription Supermix. qRT-PCR reaction was amplified on CFX96 Touch Real-time PCR (Bio-Rad, USA) using 2x iTaq Universal SYBRGreen Supermix. The primer sequences used for iNOS, COX-2, EF-2, IL-1β and TNF-α were as follows: iNOS, 5′-GCACAGCACAGGAAATGTTTCAGCAC-3′ (F) and 5′-AGCCAGCGTACCGGATGAGC-3′ (R); COX-2, 5′-TGATCGAAGACTACGTGCAACACC-3′ (F) and 5′-TTCAATGTTGAAGGTGTCGGGCAG-3′ (R); EF-2, 5′-CTGAAGCGGCTGGCTAAGTCTGA-3′ (F) and 5′-GGGTCAGATTTCTTGATGGGGATG-3′ (R); IL-1β, 5′-GCCACCTTTTGACAGTGATGAG-3′ (F) and 5′-TGATACTGCCTGCCTGAAGC-3′ (R); TNF-α, 5′-CCCTCACACTCACAAACCACCA-3′ (F) and 5′-TGAGGAGCACGTAGTCGGGG-3′ (R). All relative gene expression quantifications were normalized to EF-2 as an internal control.

Immunofluorescence assay

RAW 264.7 cells were plated onto coverslips in 6-well plate and pre-incubated with MCC for 1 h, then stimulated with LPS for 30 min. Cells were washed twice with PBS followed by fixing with 4% paraformaldehyde for 30 min and thrice washed with PBS for 5 min each. Next, cells were permeated with 0.2% Triton-X100 for 10 min, thrice washed with PBS for 5 min each followed by blocking with 0.5% BSA in PBS for 1 h at room temperature. Coverslips were incubated with anti-NF-κB overnight at 4 °C then thrice washed with PBS for 5 min each before incubated with anti-rabbit IgG conjugated to Alexa Fluor 488 at room temperature for 1 h. After washing, the nuclei were stained and mounted with Prolong Gold anti-fade reagent with DAPI for 24 h. Slides were examined under a Confocal Laser Scanning Microscope FV10i-DOC (Olympus, Japan) with excitation laser beams of 488 and 358 nm. Cell images with 640 × 640 pixels resolution were captured when using 60x objective oil-immersion lens.

NF-κB luciferase reporter gene assay

Macrophages were grown in a 96-well plate and transiently co-transfected with luciferase reporter pGL4. 32 plasmid containing the NF-κB promoter vector together with 0.1 μg pGL4. 74 vector in Opti-MEM I using FuGENE transfection reagent according to manufacturer's protocol. After 24 h of transfection, the culture media was replaced with complete culture media with antibiotic and incubated for 24 h. Then, cells were pre-treated with MCC for 1 h and further treated with LPS incubated for 6 h. Total protein was extracted using lysis buffer and luciferase activity was measured with the Dual-Luciferase Assay System according to the manufacturer's instruction. All relative luciferase activity was reported as the fold of induction after being normalized by Renilla luciferase using microplate reader (TECAN Spark, Switzerland).

Statistical analysis

Results are expressed as means ± S.D. of at least three independent experiments. Data were analyzed for statistical significance by Minitab 16 for Windows. Statistical significance was determined by ANOVA followed by Tukey's method for multiple comparisons. A value of $p < 0.05$ was considered significant.

Results

Inhibitory effect of MCC on cell viability and production of NO and PGE_2 in LPS-stimulated RAW 264.7 macrophages

Our previous report has demonstrated that MCC (3.12–25 μM) significantly decreased NO and PGE_2 production (Mankhong et al., 2017). In the present study, we further investigated the inhibitory effect of MCC on NO and PGE_2 production in LPS-treated cells. MCC inhibited NO production in LPS-treated cells at an IC_{50} of 14.1 ± 2.3 μM. Aminoguanidine, an inhibitor of iNOS activity, used as a positive control, also suppressed NO production with an IC_{50} of 50.3 ± 6.3 μM. Moreover, MCC significantly decreased PGE_2 in relation to concentration with an IC_{50} of 32.7 ± 4.7 μM. The IC_{50} value of indomethacin, a COX inhibitor, was 0.05 ± 0.01 μM. MCC at 3.12–37.5 μM did not significantly change cell viability compared to the unstimulated control cells (data not shown).

MCC inhibited iNOS and COX-2 expressions in RAW 264.7 macrophages

Western blot analysis indicated a prominent induction of iNOS and

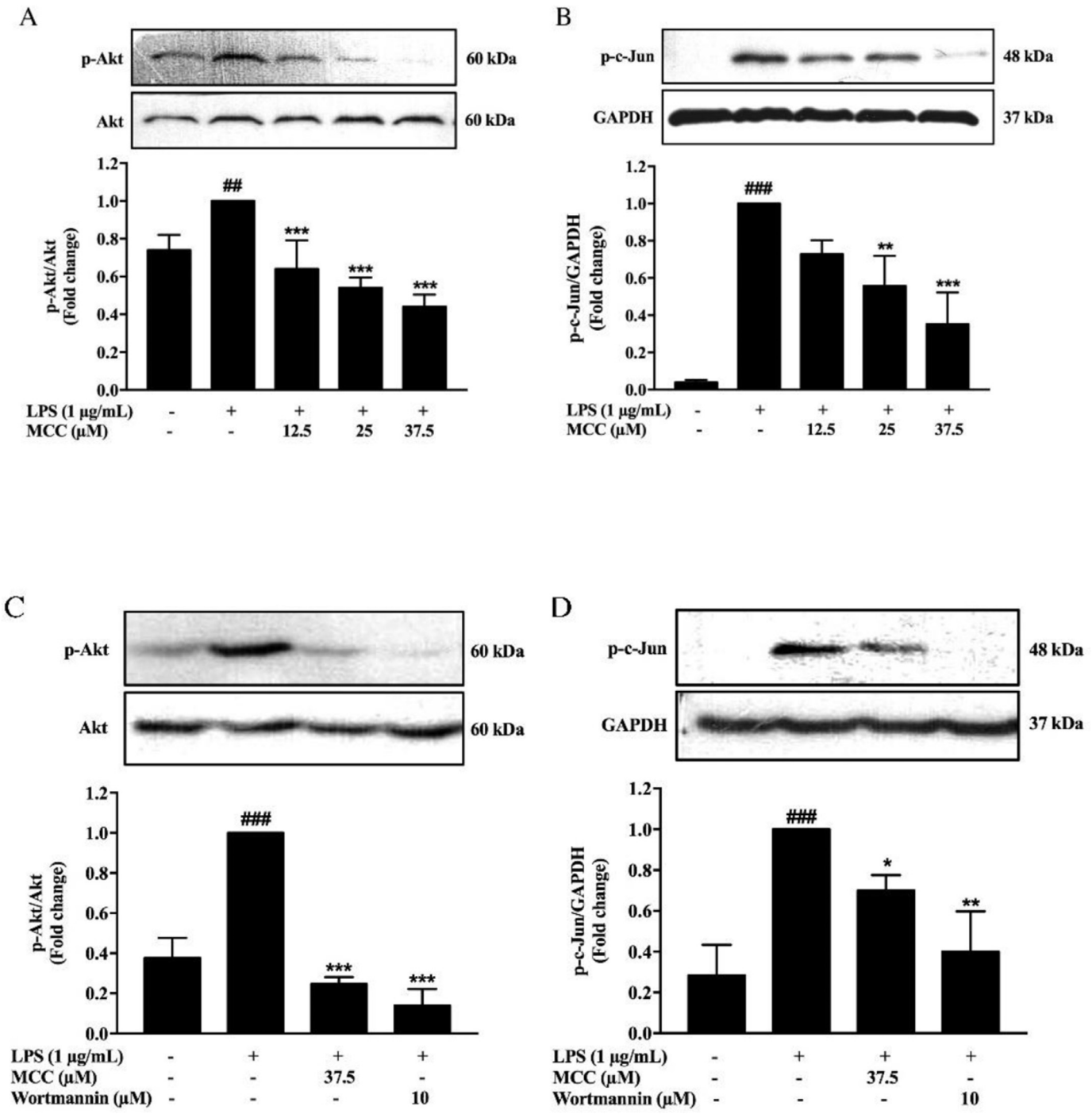

Fig. 5. Effect of MCC on LPS-induced phosphorylation of Akt and c-Jun in RAW 264.7 macrophages. Cells were pre-treated with MCC at various concentration for 1 h. followed by stimulation with 1 μg/mL of LPS for 30 min. The phosphorylation level of Akt (A) and c-Jun (B) were determined by Western blot analysis. The effect of MCC and wortmannin on phosphorylation level of Akt (C) and c-Jun (D) were determined. Graph shows mean ± SD ($n = 3$) of the densitometric value of phosphorylation level of Akt and c-Jun, which were normalized to total protein of Akt and GAPDH, respectively. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared to the unstimulated control cells. $^{*}p < 0.05$,$^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the LPS-stimulated cells.

COX-2 protein expression in cells stimulated with LPS (Fig. 1A and B). In contrast, MCC significantly suppressed iNOS and COX-2 protein expression in a dose-dependent manner. the qRT-PCR analysis also showed that iNOS and COX-2 mRNA levels were correlated with levels of the corresponding proteins (Fig. 1D and E).

Inhibitory effect of MCC on IL-1β and TNF-αexpression in LPS-stimulated macrophages

Cytokines secretions in culture media were measured by ELISA. IL-1β production by LPS-treated cells was elevated to 75.5 ± 14.7 pg/ml (Fig. 2A). MCC at an IC_{50} of 3.0 ± 0.5 μM drastically inhibited IL-1β production. LPS enhanced TNF-α level to 32,024.1 ± 2,716.2 pg/ml in RAW 264.7 macrophages (Fig. 2B). MCC significantly reduced TNF-α production at IC_{50} = 26.8 ± 5.7 μM. BAY 11–7082 (5 μM), an NF-κB

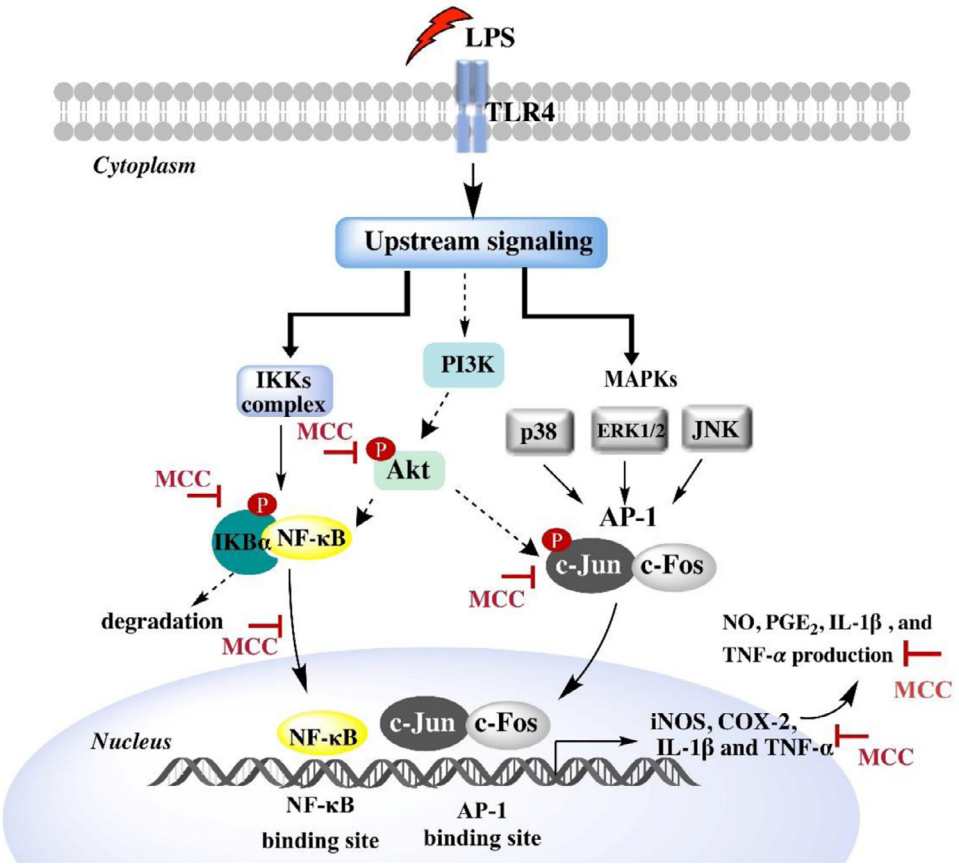

Fig. 6. Schematic diagram showing the proposed mechanism of anti-inflammatory action of MCC in LPS-induced RAW 264.7 macrophages.

inhibitor, suppressed IL-1β and TNF-α production to 59.9 ± 7.8% and 35.1 ± 6.2%, respectively. Furthermore, LPS treatment resulted in a substantial increase in IL-1β and TNF-α mRNA levels as displayed in Fig. 2C and D. MCC significantly decreased the expression of IL-1β and TNF-α mRNA in a dose-dependent manner.

Effect of MCC on LPS-induced NF-κB activation

As shown in Fig. 3A, the level of phosphorylated IκBα was markedly increased by LPS stimulation and this LPS-induced phosphorylation of IκBα was suppressed significantly by MCC. However, MCC did not restore total IκBα protein levels (Fig. 3B). The phosphorylation and degradation of IκBα declined with the addition of BAY 11-7082. NF-κB p65 was localized in the nuclei after stimulation with LPS (Fig. 3C, LPS panel) compared to the unstimulated control cells (Fig. 3C, control panel). Nuclear translocation of NF-κB p65 was reduced in the presence of MCC (Fig. 3C, MCC + LPS panel). Moreover, NF-κB luciferase activity was increased in cells treated with LPS whereas, MCC significantly reduced the level of NF-κB transactivation activity in a relation to concentration as shown in Fig. 3D.

Effect of MCC on phosphorylation of MAPKs, Akt, and c-Jun in LPS-induced macrophages

The phosphorylated level of ERK, JNK, and p38 MAPK was increased in LPS treatment as shown in Fig. 4A and was not suppressed by MCC at tested concentrations (Fig. 4A and B). Interestingly, the level of p-p38 MAPK was increased in the presence of MCC and LPS. Moreover, the phosphorylation of Akt and c-Jun were increased when cells were exposed to LPS, However, MCC treatment caused a significant decrease in phosphorylation of Akt and c-Jun in a dose-dependent manner (Fig. 5A and B). Also, the levels of p-Akt and p-c-Jun were significantly reduced in the cells that were incubated with wortmannin (PI3K inhibitor) (Fig. 5C and D).

Discussion

Macrophages are a prominent part of the host's defense in inflammatory processes. Activation of macrophages by LPS induces the release of intermediaries such as NO, PGE_2, IL-1β and TNF-α which have been implicated in inflammatory diseases (Billack, 2006; Newton and Dixit, 2012). Recently, MCC was isolated from *E. pavieana* rhizomes and demonstrated an inhibitory effect on NO and PGE_2 production in LPS-induced macrophages (Srisook et al., 2017; Mankhong et al., 2017). However, the anti-inflammatory effect of MCC on activated macrophages have not been fully described. In the present study, LPS-induced NO, PGE_2, TNF-α and IL-1β were strongly suppressed in the presence of MCC without notable cytotoxic effects indicating that reduction of these mediators was not attributed to cell death. The regulation of iNOS-produced NO and COX-2-produced PGE_2 was affected by modulation of their expression and/or activity (Aktan, 2004; Kleinert et al., 2004; Kalinski, 2012). Our previous data showed that MCC was associated with a slight but significant inhibition of iNOS activity but not COX-2 activity (Mankhong et al., 2017). However, modulation of iNOS and COX-2 expression is the most important component of regulation (Aktan, 2004; Kalinski, 2012). In the present study, we demonstrated that MCC had a strong negative modulating effect on iNOS and COX-2 expression. The data suggest that MCC inhibited NO production by decreasing LPS-induced iNOS expression and perhaps also iNOS enzyme activity. The reduction of PGE_2 by MCC could be due to the suppression of COX-2 mRNA and protein. However, our previous study indicated that this was not from the inhibition of COX-2 activity.

There is abundant evidence that excessive production of cytokines

such as IL-1β and TNF-α contributes to certain inflammatory disorders such as osteoarthritis, autoimmune diseases and rheumatoid arthritis (Matsuno et al., 2002; Prasad and Aggarwal, 2014; Wojdasiewicz et al., 2014). Our investigation of the effect of MCC on cytokine production indicated an intense inhibition of IL-1β and TNF-α production and the potential to inhibit IL-1β and TNF-α expression. Thus, a more probable mechanism for MCC inhibition of cytokine production is through transcriptional suppression. Based on these results, we suggest that MCC exerts an anti-inflammatory effect through the suppression of inflammatory mediators and cytokines in LPS-induced RAW 264.7 macrophages.

NF-κB is a transcription factor that regulates diverse inflammatory genes (Premkumar et al., 2010). Putative NF-κB sites are present in the promoter of iNOS, COX-2, IL-1β and TNF-α genes (Kordula et al., 2000; Surh et al., 2001; Lowenstein et al., 1993). NF-κB activity at inflammatory sites begins with phosphorylation of IκBα on Ser32 and Ser36 by IKK complex leading to degradation of IκBα. This liberated NF-κB dimers move into the nucleus and bind to specific κB sites at the promoter region of target genes that regulate their expression (Ghosh and Hayden, 2008). The most common NF-κB dimers found in most cells are p50 and p65 (RelA) (Billack, 2006). From our study, MCC significantly blocked activation of NF-κB through inhibition of phosphorylation IκBα and nuclear translocation of NF-κB p65 subunit regardless of IκBα degradation. Furthermore, LPS-mediated NF-κB-transactivation activity decreased significantly by MCC. Our findings demonstrated the suppression of pro-inflammatory mediators and cytokines by MCC was mediated, at least in part, via inhibition of NF-κB p65 activation and transactivation activity.

MAPKs are a highly conserved family of kinases involved in inflammatory responses. Activation of JNK, ERK1/2 and p38 MAPK by phosphorylation is a key step leading to the expression of inflammatory mediators on various stimuli (Kaminska, 2005). A number of compounds can affect the expression of iNOS, COX-2 and cytokines genes through MAPKs signaling pathway (Kaminska, 2005; Park et al., 2017). According to our results, MCC did not suppress LPS-activated MAPKs. Surprisingly, MCC evidently increased phosphorylation of p38 MAPK. The anti-inflammatory mechanism of MCC associated with this activation remains unexplained, however, our results suggest this anti-inflammatory effect of MCC might be mediated independently of MAPKs activation. A similar observation was found in the anti-inflammatory action of wedelolactone via NF-κB activation. However, it was independent of MAPKs pathways (Yuan et al., 2013).

In addition to NF-κB and MAPK signaling pathways, a PI3K/Akt cascade is reported to be involved in the expression of inflammatory genes. LPS treatment is related to activation of Akt and mediates the downstream signaling cascade including NF-κB and AP-1 (Dan et al., 2008; Manning and Cantley, 2007). However, there are conflicting observations regarding the association of PI3K/Akt pathway in both negative and positive regulation in LPS-induced inflammatory responses (Guha and Mackman, 2001; Zong et al., 2012). This conflict might be attributable to agonists and physiological differences in cells. In this study, we determined whether Akt pathway is involved in the inhibitory effect of MCC on the expression of inflammatory mediators and cytokines. As shown in our results, LPS triggered the phosphorylation of Akt but MCC considerably abolished Akt activation in LPS-stimulated macrophages. MCC significantly inhibited phosphorylation of c-Jun, a main component of an AP-1 transcription factor which is activated in part by Akt (Manning and Cantley, 2007). Moreover, our results reveal that blockade of PI3K/Akt by wortmannin leads to suppression of LPS-induced phosphorylation of Akt and c-Jun. This implies the down-regulation of inflammatory mediators by MCC might occur via interfering of NF-κB and Akt signaling pathway. This is consistent with the findings from Park et al. (2017) that ascofuranone exerts an inhibitory effect on LPS-stimulated overproduction of inflammatory mediators and cytokines involving the suppressive phosphorylation of IκBα and reduction of NF-κB translocation into the nucleus together with inactivation of an AP-1 transcription factor. Likewise, psoralidin exhibited an anti-inflammatory effect via suppression of LPS-induced overexpression of inflammatory mediators through interrupting PI3K/Akt signal cascade and IKK/IκB activation without effects on MAPK pathways (Chiou et al., 2011). However, further experiments are required to clarify the precise mechanism of action of MCC but seem to involve the crosstalk between NF-κB and PI3K/Akt pathways.

Conclusions

In conclusion, our study indicated that MCC isolated from rhizomes of *E. pavieana* exerted an anti-inflammatory action in LPS-induced RAW 264.7 macrophages via the suppression of iNOS, COX-2, IL-1β as well as TNF-α expressions. We assume the inhibitory effect of MCC might be through inactivation of NF-κB and AP-1 transcription factors via downregulation PI3K/Akt signaling pathway as shown in Fig. 6. The present study strongly supports the potential of MCC as a novel anti-inflammatory agent.

Conflict of interests

The authors have no conflict of interest to declare.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2018.09.193.

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