

# Inhibition of Inflammatory Responses by *Centella asiatica* via Suppression of IRAK1-TAK1 in Mouse Macrophages

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**Abstract:** *Centella asiatica* (L.) Urb. (*C. asiatica*) has been widely treated for inflammation-related diseases in China for thousands of years. While *C. asiatica* showed relevant effects as traditional medicine, the mechanism of *C. asiatica* suppressing inflammation has not been thoroughly investigated. Therefore, this study was conducted to reveal the anti-inflammatory mechanism of methanol fraction from *C. asiatica* (MCA) at the molecular level in murine macrophages. Levels of inflammation-related mediators were observed with treatment of MCA. MCA significantly suppressed nitric oxide production and iNOS expression in RAW 264.7 macrophages. Prostaglandin E<sub>2</sub> production was alleviated by MCA via the downregulation of cyclooxygenase-2. MCA treatment also reduced pro-inflammatory tumor necrosis factor- $\alpha$  and interleukin (IL)-6 levels. LPS/D-GalN-induced acute hepatitis in mouse was alleviated by MCA treatment. In addition, MCA decreased the phosphorylation of inhibitory  $\kappa$ B $\alpha$  (I $\kappa$ B $\alpha$ ) at Ser32/36 and thereby blocked I $\kappa$ B $\alpha$  degradation. TXY motif phosphorylation in the activation loops of mitogen-activated protein kinases (MAPKs) was also suppressed by MCA treatment. Further investigation

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revealed that MCA inhibited transforming growth factor- $\beta$ -activated kinase 1 (TAK1) phosphorylation and IL-1 receptor-associated kinase (IRAK1) degradation, the upstream kinases activating nuclear factor  $\kappa$ B and MAPKs. Taken together, MCA exhibited anti-inflammatory properties via the downregulation of IRAK1-TAK1 signaling pathways.

**Keywords:** *Centella asiatica*; Anti-Inflammatory Effect; Liver Hepatitis; TAK1; IRAK1.

## Introduction

A complex biological process, inflammation, protects the organism from pathogenic agents (Lawrence *et al.*, 2002). Under normal conditions, the inflammatory response is regulated in an appropriate range since it exerts a negative feedback loop via suppression of pro-inflammatory proteins and induction of anti-inflammatory proteins (Lawrence *et al.*, 2002). However, excessive inflammatory responses result in an overproduction of inflammatory mediators and eventually lead to various inflammatory diseases, including arthritis, inflammatory bowel disease, multiple sclerosis, atherosclerosis, and asthma (Jou *et al.*, 2013). In the innate immune system, macrophages function as the first defense system against common pathogens. When macrophages are stimulated by the bacterial lipopolysaccharide (LPS), inflammation-related intracellular pathways are activated. The nuclear factor  $\kappa$ B (NF- $\kappa$ B) and mitogen-activated protein kinases (MAPKs) pathways are typical signaling pathways that regulate inflammation-related gene expressions in macrophages (Kim *et al.*, 1997). Transforming growth factor- $\beta$ -activated kinase 1 (TAK1) phosphorylates and mediates activation of MAPK, including p38 and c-Jun N-terminal kinase (JNK) or NF- $\kappa$ B pathways (Chen *et al.*, 2006). The activation of TAK1 proceeds as follows: When LPS binds to toll-like receptor 4 (TLR4), IL-1 receptor-associated kinase (IRAK) 4 is recruited by myeloid differentiation primary response 88 (MyD88); subsequently, IRAK4 phosphorylates IRAK1 at Thr-209/Thr-387, which leads to hyperphosphorylation of IRAK1 followed by a recruit of TNF receptor-associated factor 6 (TRAF6) to the receptor complex. IRAK1-TRAF6 complex dissociates from the receptor and binds to TAK1-binding proteins (TABs) complex, leading to the phosphorylation of TAK1 at Thr-184/Tyr-187. The degradation of IRAK1 in the plasma membrane facilitates TAK1 activation in the cytosol, which results in the activation of MAPKs and NF- $\kappa$ B pathways (Jiang *et al.*, 2002). Activated macrophages release a wide range of mediators and stimulate other immune cells (Aderem and Ulevitch, 2000). Nitric oxide (NO) as an inflammatory mediator is synthesized from L-arginine by three isoforms of NO synthases (NOS) and exhibits dual roles in cell-mediated immune responses (Korhonen *et al.*, 2005). Usually, NO overproduction during inflammation is due to the overexpression of inducible NOS (iNOS), which is induced by immunological or inflammatory stimulations (Korhonen *et al.*, 2005). Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), another mediator, is generated through catalysis by cyclooxygenase (COX)-2 from lipid arachidonic acid (Park *et al.*, 2006). Synthesized PGE<sub>2</sub> functions in the generation of inflammatory responses by participating in the development of typical inflammation phenomena, including pain, redness, and swelling (Ricciotti and FitzGerald, 2011).

Traditional medicinal plants have been used to treat this complex process of inflammation-related diseases (Brinkhaus *et al.*, 2000; Dahanukar *et al.*, 2000). *Centella asiatica* (L.) Urb. (*C. asiatica*, also known as Asiatic pennywort, Centella, Tiger Herb, or Gotu Kola, has been used as a folk medicine for thousands of years in China, the Philippines, Sri Lanka, Madagascar, Africa, and India (Dahanukar *et al.*, 2000). Traditionally, *C. asiatica* has been treated for lupus, ulcers, leprosy, diarrhea, fever, psoriasis, and eczema (Brinkhaus *et al.*, 2000; Gohil *et al.*, 2010). As *C. asiatica* has shown effective wound healing activity over the centuries, it is also adapted in modern pharmacology for regenerating skin (Bylka *et al.*, 2013). *C. asiatica* has been reported to possess anti-oxidative, antibacterial, and anti-inflammatory properties (Gohil *et al.*, 2010). The ethanol, methanol, and aqueous extracts of *C. asiatica* were comparatively analyzed for its inhibitory effect on COX activities and PGE<sub>2</sub> production in fibroblast cells (Nurlaily *et al.*, 2012). In addition, ethanol extracts of *C. asiatica* aerial part were reported to show anti-inflammatory effect in allergy dermatitis mouse model (Park *et al.*, 2017). Another study has shown that ethanol extract of *C. asiatica* leaves suppressed high immune responses associated with cancer cachexia (Naidoo *et al.*, 2017). Several reports have indicated that the major active compounds of *C. asiatica* extract, which are madecassic acids, madecassoside, asiatic acid, and asiaticoside, exhibit potential anti-inflammatory effects in various inflammatory mice models (Li *et al.*, 2009; Gohil *et al.*, 2010; Huang *et al.*, 2011; Chen *et al.*, 2014; Qiu *et al.*, 2015). Even though *C. asiatica* has been an excellent folk remedy for the treatment of inflammation-related diseases, and increasing evidence supports its traditional applications, the molecular mechanism underlying anti-inflammatory effects exerted by *C. asiatica* under various conditions still remains to be evaluated. Therefore, in this study, we investigated the anti-inflammatory effects of the methanol extract of *C. asiatica* leaves (MCA) and its underlying molecular mechanism in RAW 264.7 macrophages stimulated by LPS.

## Materials and Methods

### MCA Preparation

The 95% methanol extract of *Centella asiatica* (L.) Urb. was purchased from Korea Plant Extract Bank (KPEB; <https://extract.kribb.re.kr>; Daejeon, Korea). Briefly, *C. asiatica* leaves (7388 g powder which was dried and ground) were added to methanol (1500 mL), and the plant content was extracted at room temperature by implementing maceration method for 18 h. Under reduced pressure, 10.55 g of *C. asiatica* methanol extract was obtained by filtering and concentrating the total extracts using a Rotary Evaporator, Laborota 4000 (Heidolph, Schwabach, Germany). The extract stock was dissolved in dimethyl sulfoxide (DMSO) at 200 mg/mL, and subsequently diluted in culture medium before use in the following experiments.

### Cell Culture and Reagents

RAW 264.7 macrophage for quantifying inflammatory responses and HEK 293 cell for transient transfection were obtained from ATCC (Manassas, VA, USA). Cells were

maintained as previously described (Cho *et al.*, 2017). LPS and DMSO were obtained from Sigma-Aldrich (St. Louis, MO, USA). D-(+)-galactosamine hydrochloride (D-GalN) was obtained from TCI America (Portland, OR, USA). Enzyme-linked immunosorbent assay (ELISA) kits for detecting mouse aspartate aminotransferase (AST, Cat No. CSB-E12649m) and alanine aminotransferase (ALT, Cat No. CSB-E16539m) was obtained from Cusabio (Houston, TX, USA). ELISA kit for detecting IL-6 (Cat No. 88-7064) or TNF- $\alpha$  (Cat No. 88-7324) was purchased from Ebioscience (San Diego, CA, USA). PGE<sub>2</sub> ELISA kit — Monoclonal (Cat No. 514010) for detecting PGE<sub>2</sub> was from Cayman Chemical (Ann Arbor, MI, USA). Quercetin (Cat No. Q4951) was purchased from Sigma-Aldrich.

#### *Ultra-Performance Liquid Chromatography (UPLC) Analysis*

MCA and its active components, asiaticoside and madecassoside, were analyzed using UPLC under the same flow condition. An ACQUITY UPLC system (Waters, Milford, MA, USA) was used for separation. The chromatographic column used was an ACQUITY UPLC HSS T3 column (100 mm  $\times$  2.1 mm, 1.8  $\mu$ m particle size, 100 Å pore size; Waters). Mobile phase composed of 1:1 ratio of A and B (A = 1% acetic acid in nanopure water; B = 100% acetonitrile) was introduced to separate the extract and its components. Mobile phase solutions flowed with the rate of 0.3 mL/min, the injected volume of samples was 10  $\mu$ L, and samples were analyzed for 10 min.

#### *Cell Viability Assay*

Cell viability of RAW 264.7 cells ( $4.5 \times 10^4$  cells/well; 96-well plate) was observed with EZ-Cytox cell viability assay kit (DAEIL lab, Seoul, Korea) according to the manufacturer's instruction.

#### *Nitrite Assay*

RAW 264.7 cells ( $4.5 \times 10^4$  cells/well) were seeded into a 96-well plate, incubated overnight for adhesion, and then stimulated with LPS after treatment with the various concentrations of MCA. 100  $\mu$ L of culture media was transferred to and added with 100  $\mu$ L of Griess reagent in a new 96-well plate sodium nitrite diluents were used to generate a standard curve. The absorbance was measured at 540 nm, and nitrite concentration was calculated based on the standard curve.

#### *ELISA*

Culture supernatants, which were collected at the nitrite assay, were subjected to sandwich ELISA with specific monoclonal antibodies for measuring IL-6 and TNF- $\alpha$  as performed in the previous study (Cho and Cho, 2016).

PGE<sub>2</sub> level in culture media was observed with PGE<sub>2</sub> ELISA kit. ELISA buffer (from the ELISA kit) was mixed with 50 µL of sample per well. 50 µL of PGE<sub>2</sub> AChE Tracer was added to each well; subsequently, 50 µL of PGE<sub>2</sub> monoclonal antibody was added and incubated at 4°C for 18 h. After washing with wash buffer (from the ELISA kit) for five times, 200 µL of Ellman's reagent was applied to each well and incubated for 1 h in the dark. The absorbance was measured at 420 nm. PGE<sub>2</sub> concentration was calculated based on a standard curve.

### RT-qPCR

Total RNA was extracted with Accuzol solution (Bioneer Corporation, Daejeon, Korea). The same quantity of RNA was reverse transcribed to complementary DNA (cDNA) using a TOPscript cDNA synthesis kit (Enzynomics, Daejeon, Korea). qPCR was conducted using a CFX connect real-time thermal cycler (Bio-Rad, Hercules, CA, USA) with iTaq Universal SYBR Green Supermix (Bio-Rad). PCR primer sequences for qPCR were described in Table 1. cDNA was initially denatured at 94°C for 5 min and then amplified by repeating 40 cycles as follows; 94°C for 5 s and 60°C for 30 s. Fluorescence values of qPCR amplicon were normalized using multi-reference genes,  $\beta$ -actin and GAPDH, and were expressed as the ratio of gene expression levels by referring LPS-treated group as 100%, based on the  $2^{-\Delta\Delta C_q}$  method.

### Measurement of Serum AST/ALT Levels in Acute Hepatitis Mice Model

Male C57BL/6 mice (6 weeks old) were purchased from Daehan Biolink (Chungbuk, Korea) and maintained by 12-h light/12-h dark cycle with lights on at 6 A.M. The experiments were conducted according to the internationally accepted principles for laboratory animal use and care as found in the US guidelines (NIH publication #85-23, revised in 1985). Mice were repeatedly subjected to oral administration of PBS, MCA, or silibinin

**Table 1. The Primers Used for qRT-PCR**

| Gene<br>Symbol | Primer Sequences                |                       | Accession  | Product<br>Length (bp) |
|----------------|---------------------------------|-----------------------|------------|------------------------|
|                | Forward (5'-3')                 | Reverse (5'-3')       |            |                        |
| iNOS           | TGGCCAAGCTGAACT                 | TCATGATAACGTTTCTGGCTC | BC062378.1 | 79                     |
| COX-2          | GATGCTCTTCCGAGCTGTG             | GGATTGGAACAGCAAGGATTT | BC052900.1 | 75                     |
| IL-6           | TCTAATTCA-<br>TATCTTCAACCAAGAGG | TGGTCCTTAGCCACTCCTTC  | BC138766.1 | 97                     |
| TNF- $\alpha$  | CTGTAGCCCACGTCGTAGC             | TTGAGATCCATGCCGTTG    | D84199.2   | 97                     |
| IL-1 $\beta$   | TTGACGGACCCCAAAAGAT             | GATGTGCTGCGAGATT      | BC011437.1 | 95                     |
| $\beta$ -actin | CGTCATACTCTGCTTGC               | CCAGATCATTGCTCCTGA    | BC138614.1 | 98                     |
| GAPDH          | GCTCTCTGCTCCTGTTC               | ACGACCAAATCCGTTGACTC  | BC145810.1 | 115                    |

(positive control) once a day for 6 days. One hour after the final oral administration of the compound solution, mice were intraperitoneally injected with LPS (10  $\mu$ g/kg) and D-GalN (1 g/kg) to induce acute hepatitis inflammation. Control groups received the same volume of vehicle intraperitoneally. Mice were anesthetized by subjecting them to ethyl ether inhalation at 6 h after the induction of acute hepatitis. Serum was separated by centrifugation of the collected blood at  $4000 \times g$  for 15 min. Levels of serum AST/ALT were detected by ELISA.

### Immunoblot Analysis

RAW 264.7 was treated with various concentrations of MCA and stimulated with LPS for an optimal incubation period. Preparation of cell lysates and subsequent immunoblotting processes are performed as previously described (Cho *et al.*, 2017). Primary antibodies used in this study are listed in Table 2. Protein expression levels were quantified by LabWorks software (UVP Inc., Upland, CA, USA).

### Transient Transfection and Luciferase Assay

HEK 293 cells seeded in a 12-well plate were transfected with NF- $\kappa$ B firefly luciferase (pNF- $\kappa$ B-Luc) or pAP-1-Luc using polyethylenimine (PEI, Polysciences Inc., Warrington, PA, USA), followed by treatment with various concentrations of MCA in the absence or presence of phorbol 12-myristate 13-acetate (PMA) for 24 h. The cells were lysed with  $1 \times$  lysis buffer (Promega, Madison, WI, USA). Supernatants were prepared by centrifugation for 30 min at  $4^{\circ}\text{C}$ , and the luminescence was measured. Luciferase activities were normalized to the fluorescence value of green fluorescence protein.

**Table 2. The Antibodies Used in the Study**

| Name                    | Cat. No.  | Target Phospho-Residues                                                  | Host   | Brand                                                |
|-------------------------|-----------|--------------------------------------------------------------------------|--------|------------------------------------------------------|
| I $\kappa$ B $\alpha$   | sc-371    | Ser-32/36                                                                | rabbit | Santa Cruz Biotechnology<br>(Santa Cruz, CA, USA)    |
| p-I $\kappa$ B $\alpha$ | sc-101713 |                                                                          | rabbit |                                                      |
| JNK                     | sc-7345   |                                                                          | mouse  |                                                      |
| p38                     | sc-7972   |                                                                          | mouse  |                                                      |
| iNOS                    | sc-651    |                                                                          | rabbit |                                                      |
| COX-2                   | sc-1745   | Thr-202/Tyr-204<br>Thr-183/Tyr-185<br>Thr-180/Tyr-182<br>Thr-184/Tyr-187 | goat   | Cell Signaling Technology Inc.<br>(Danvers, MA, USA) |
| IRAK1                   | sc-5288   |                                                                          | rabbit |                                                      |
| $\alpha$ -tubulin       | sc-8035   |                                                                          | mouse  |                                                      |
| GAPDH                   | sc-25778  |                                                                          | rabbit |                                                      |
| ERK                     | 9102      |                                                                          | rabbit |                                                      |
| p-ERK                   | 9106      |                                                                          | mouse  |                                                      |
| p-JNK                   | 9251      |                                                                          | rabbit |                                                      |
| p-p38                   | 9211      |                                                                          | rabbit |                                                      |
| TAK1                    | 4505      |                                                                          | rabbit |                                                      |
| p-TAK1                  | 4508      |                                                                          | rabbit |                                                      |

### *Statistical Analysis and Experimental Replicates*

Experiments performed in this study were repeated three times under the same condition. The results were presented as the mean  $\pm$  standard error of the mean (SEM). Statistical significance between the experimental groups was calculated by one-way ANOVA and Dunnett's multiple comparison test using Prism 3.0 (Graph Pad Software, San Diego, CA, USA).

## **Results and Discussion**

### *UPLC Analysis of MCA*

It was reported that *C. asiatica* contains many bioactive components that inhibit inflammatory responses. Madecassoside and asiaticoside have been reported to be the major components of *C. asiatica* and exhibit anti-inflammatory properties (Sasmita *et al.*, 2018). To clarify whether MCA, which is used throughout this study, has an anti-inflammatory potential, UPLC analyses for the extract and the predominant anti-inflammatory components, including madecassoside and asiaticoside, were performed (Fig. 1). When UPLC analyses were performed using single components, including madecassoside and asiaticoside, the retention time for the major peak was 4.664 min and 5.054 min, respectively. When the extract was analyzed using UPLC under the same experimental condition, the major peaks of asiaticoside and madecassoside were observed at the same retention time, indicating that MCA contains the anti-inflammatory components. The UPLC analysis showed the possibility of elucidating the underlying mechanism regarding the anti-inflammatory effects of MCA.

### *Suppression of LPS-Induced NO Production by MCA via Transcriptional Regulation of iNOS Expression*

To establish the appropriate concentration ranges of MCA for subsequent experiments, MCA cytotoxicity was evaluated using cell viability assay. Following treatment of various concentrations of MCA on RAW 264.7 macrophages in the presence or absence of LPS, cell viability was observed by measuring the metabolic reduction of a tetrazolium salt into a formazan dye. Cell viability of MCA-treated RAW 264.7 cells was similar to that of MCA-untreated cells at MCA concentration up to 400  $\mu\text{g/mL}$  (Fig. 2A), indicating that MCA could be applied to RAW 264.7 macrophages without cytotoxicity at a dose of up to 400  $\mu\text{g/mL}$ . To investigate the effects of MCA on NO production in activated macrophages, NO level in the culture media was measured in each experimental group (Fig. 2B). The LPS-treated group showed notable increases in the NO production compared with that in the untreated group. LPS-induced NO production was suppressed by MCA treatment in a dose-dependent manner. The inhibition by MCA reached a saturation state at 400  $\mu\text{g/mL}$  (Fig. 2B), implying that higher concentration of MCA might not inhibit inflammatory responses as effectively as a dose of 300  $\mu\text{g/mL}$ . The suppression of NO production by MCA over 200  $\mu\text{g/mL}$  was more effective than 30  $\mu\text{M}$  of quercetin, a well-know anti-inflammatory agent (Li *et al.*, 2016). With these results, MCA concentration up to 300  $\mu\text{g/mL}$  was used in the following assays.

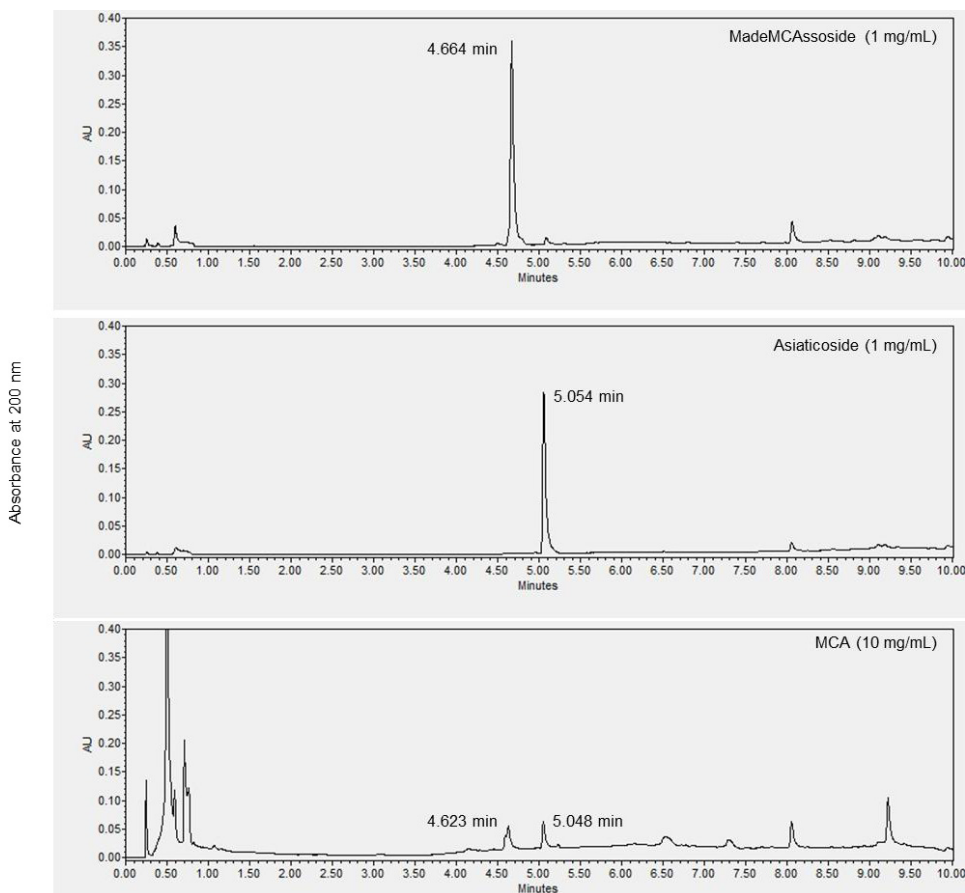


Figure 1. UPLC analysis of MCA and its major components, madecassoside, and asiaticoside. Phytochemical characteristics of MCA and its major components, madecassoside and asiaticoside, were analyzed using UPLC.

To prove whether MCA-mediated inhibition of NO levels was dependent on iNOS, an enzyme mediating NO production, expression levels of iNOS were measured via qRT-PCR and immunoblot analysis. LPS-induced iNOS gene expression levels decreased upon MCA treatment (Fig. 2C). Furthermore, the LPS-mediated increase of iNOS protein was reduced by MCA in a dose-dependent manner (Fig. 2D). MCA treatment effectively suppressed iNOS expression, almost as quercetin did. The results suggest that MCA alleviates LPS-induced NO production in macrophages by transcriptionally inhibiting iNOS expression.

#### *Inhibitory Effects of MCA on PGE<sub>2</sub> Production by Transcriptional Regulation of COX-2 Expression in Activated Macrophages*

As production of PGE<sub>2</sub> is induced by LPS in RAW 264.7 macrophages, and excessive amounts of PGE<sub>2</sub> are related to abnormal inflammatory states, including rheumatoid

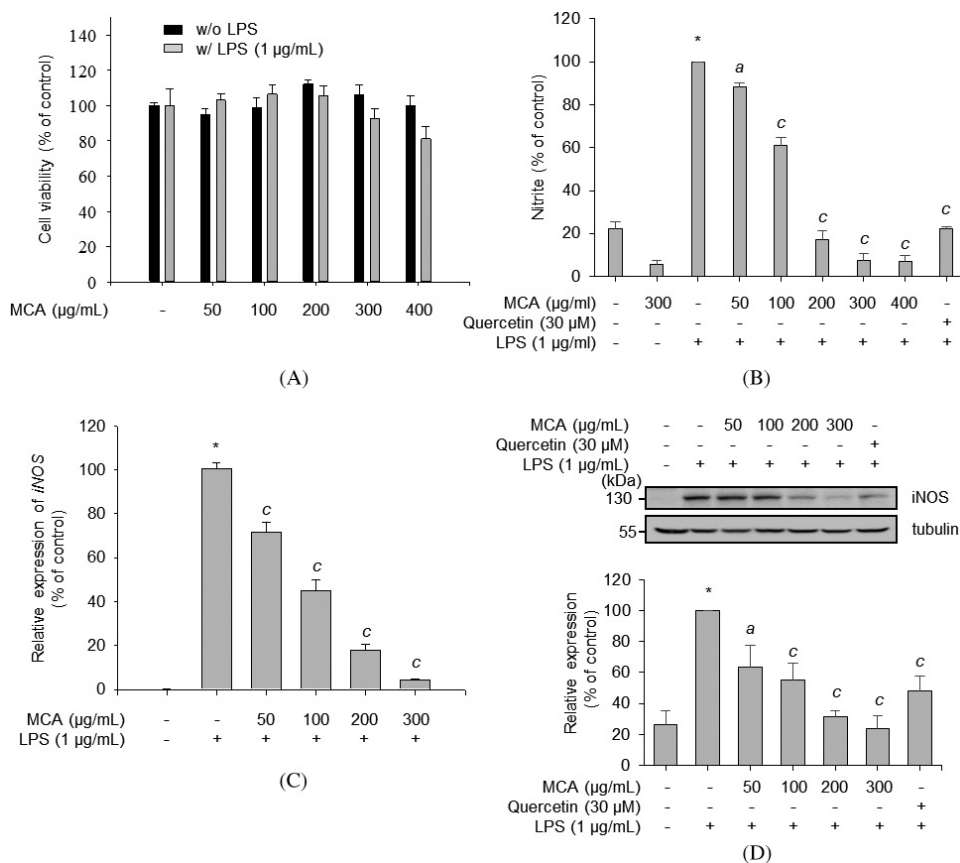


Figure 2. Inhibitory effects of MCA on NO production and iNOS expression. RAW 264.7 cells were pre-treated with the indicated concentration of MCA or quercetin for 2 h and incubated with or without LPS for an additional 24 h. (A) Cell viability was examined using the EZ-Cytox assay kits and was expressed as relative values to the MCA-untreated control group. Control in Y-axis indicates the MCA-untreated samples. (B) NO level in culture media was measured with Griess reagent and calculated by applying the absorbance values to the nitrite standard curve. (C) cDNA was amplified using iNOS primer and relative gene expression levels compared to the LPS-stimulated group were represented. (D) Immunoblot analysis using iNOS primary antibody was performed. The protein expressions of iNOS were quantified and then normalized to GAPDH levels. Representative blotting data and relative expression levels of iNOS compared with LPS-treated group were represented. Data represent the mean  $\pm$  SEM. Control in Y-axis indicates the LPS-treated and MCA-untreated samples. \* $p < 0.01$  relative to the LPS-untreated group. <sup>a</sup> $p < 0.05$  and <sup>c</sup> $p < 0.001$  relative to the LPS-treated group.

arthritis (Fattahi and Mirshafiey, 2012), the anti-inflammatory property of MCA was evaluated by observing the PGE<sub>2</sub>. PGE<sub>2</sub> levels induced by LPS in culture medium were reduced by MCA treatment (Fig. 3A). Since PGE<sub>2</sub> production is tightly determined by COX-2, expression levels of COX-2 were measured when MCA was treated to RAW264.7 cells. COX-2 expression was upregulated both at mRNA and protein levels by LPS and notably alleviated by MCA treatment (Figs. 3B and 3C). These results imply that MCA

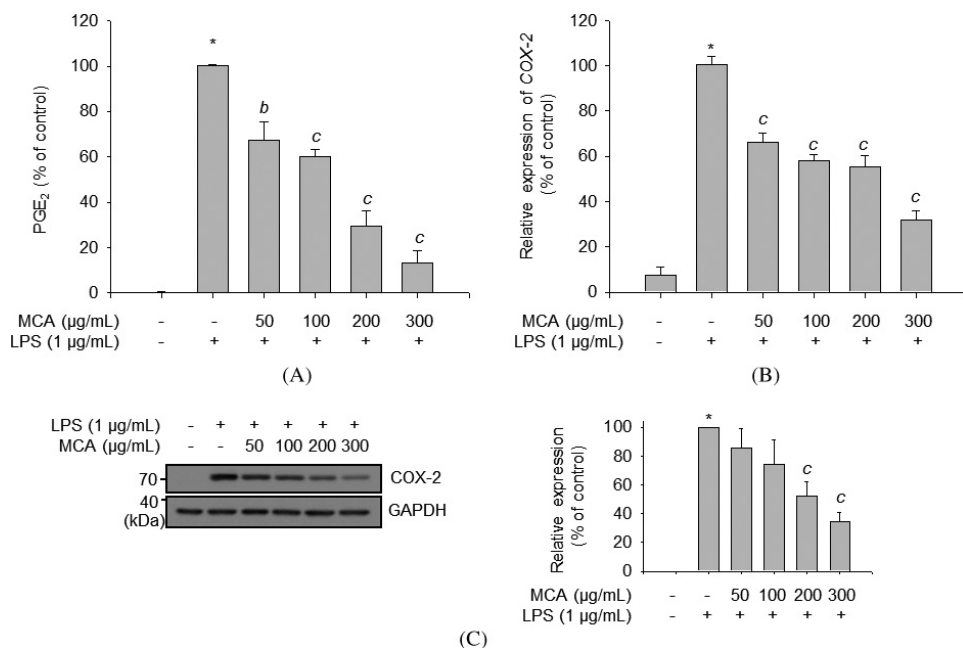


Figure 3. Inhibitory effects of MCA on PGE<sub>2</sub> production and COX2 expression. RAW 264.7 was pre-treated with MCA for 2 h and incubated with LPS for 24 h. (A) PGE<sub>2</sub> secretion was detected by ELISA. Amounts of PGE<sub>2</sub> were determined based on a standard curve. (B) cDNA was amplified using COX-2 primer and relative gene expression levels compared to LPS-stimulated group were represented. (C) Immunoblot analysis using COX-2 primary antibody was performed. The protein expressions of COX-2 were quantified and then normalized to GAPDH. Representative blotting data and relative expression levels of COX-2 compared with LPS-treated group were represented. Data represent the mean  $\pm$  SEM. Control in Y-axis indicates the LPS-treated and MCA-untreated samples. \* $p < 0.01$  relative to the LPS-untreated group. <sup>b</sup> $p < 0.01$  and <sup>c</sup> $p < 0.001$  relative to the LPS-treated group.

alleviates inflammatory responses by regulating COX-2-mediated PGE<sub>2</sub> production in LPS-activated macrophages.

The traditional use of *C. asiatica* as a folk remedy for the treatment of fever and pain was well-recognized (Brinkhaus *et al.*, 2000). The ethnopharmacological application of *C. asiatica* for fever treatment seems to be consistent with the findings that MCA inhibits the production of PGE<sub>2</sub> that induces fever and pain in inflammation by inhibiting the expression of the essential enzyme, COX-2 (Kawabata, 2011).

#### *Inhibitory Effects of MCA on Pro-Inflammatory Cytokines in Activated Macrophages*

When stimulated by LPS, macrophages produce several cytokines, such as IL-6, TNF- $\alpha$ , and IL-1 $\beta$  (Hanada and Yoshimura, 2002). Uncontrolled production of pro-inflammatory cytokines is related to the development of autoimmune diseases such as rheumatoid arthritis (Tanaka and Martin, 2014). Here, we evaluated the effects of MCA on the production of pro-inflammatory cytokines in activated macrophages. LPS-induced IL-6 and

TNF- $\alpha$  productions were notably decreased by MCA in macrophages (Figs. 4A and 4B). qPCR analysis data revealed that MCA could suppress the mRNA expression of IL-6, IL-1 $\beta$ , and TNF- $\alpha$  (Fig. 4C). These data suggest that the production of pro-inflammatory cytokines is transcriptionally regulated by MCA in macrophages.

Considering the results in Figs. 4A–4C, MCA inhibits IL-6 and IL-1 $\beta$  production more strongly than that of TNF- $\alpha$ . This effect may be the result of differences in transcription factor binding sites located in the promoter regions of IL-6, IL-1 $\beta$ , and TNF- $\alpha$ . Previous studies have shown that the promoter regions of IL-1 $\beta$  and IL-6 genes share NF- $\kappa$ B, AP-1, and STAT-binding domains, whereas that of TNF- $\alpha$  has NF- $\kappa$ B and AP-1-binding region

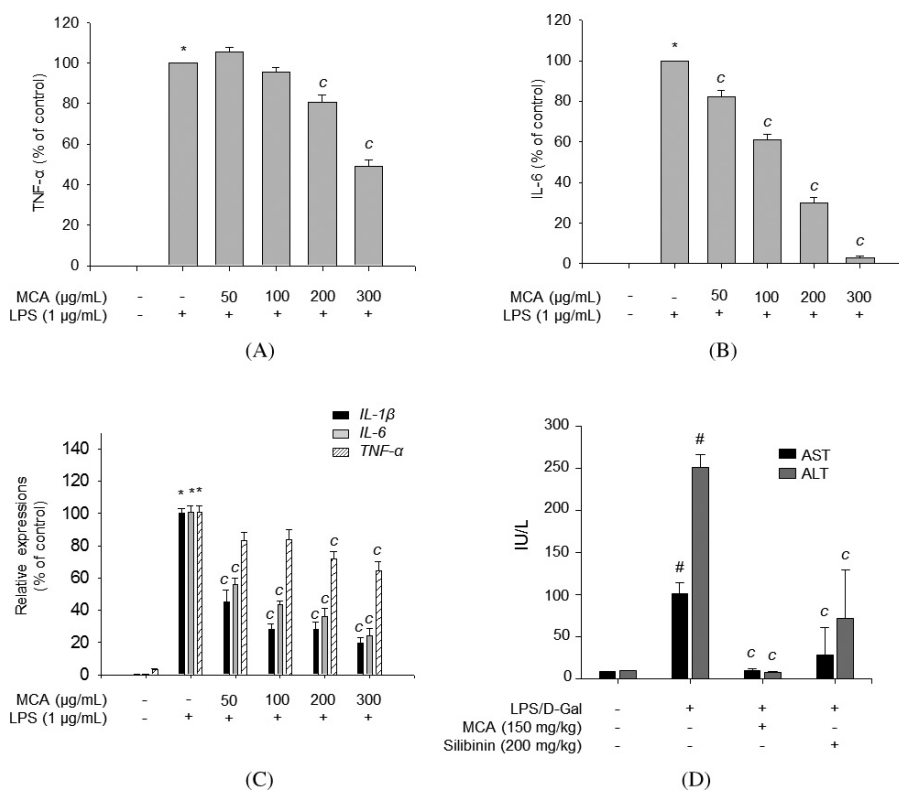


Figure 4. Inhibitory effects of MCA on the production of pro-inflammatory cytokines and acute hepatitis. RAW 264.7 cells were pre-incubated with various concentrations of MCA and then incubated with or without LPS (24 h for ELISA and 3 h for qPCR). (A) TNF- $\alpha$  or (B) IL-6 level in the culture media was quantified using ELISA. (C) cDNA was amplified using IL-1 $\beta$ , IL-6, and TNF- $\alpha$  primers and relative gene expression levels compared to LPS-stimulated group were represented. Data represent the mean  $\pm$  SEM. Control in Y-axis indicates the LPS-treated and MCA-untreated samples. \* $p$  < 0.01 relative to the LPS-untreated group. <sup>c</sup> $p$  < 0.001 relative to the LPS-treated group. (D) Mice were orally treated with MCA or silibinin for 6 days. After final administration, LPS/D-GalN was intraperitoneally injected. After 1 h, serum was prepared for analyzing biochemical parameters, namely AST and ALT levels. Data represent the mean  $\pm$  SEM. \* $p$  < 0.01 relative to LPS/D-GalN-untreated control group. <sup>c</sup> $p$  < 0.001 relative to the LPS/D-GalN-treated group.

but does not possess a STAT-binding region (Kuprash *et al.*, 1999; Lee *et al.*, 2006; Ndlovu *et al.*, 2009; Wang *et al.*, 2013; Ju *et al.*, 2015). These suggest that MCA participates in the regulation of STAT signaling pathway, which in turn regulates IL-6 and IL-1 $\beta$  genes by strongly inhibiting their expression.

#### *Inhibitory Effects of MCA in Acute Hepatitis*

The liver comprises many cell types, including hepatocyte, liver endothelial cell, stellate cell, and Kupffer cell (a specific inflammatory macrophage in the liver). Upon stimulation, Kupffer cells produce inflammatory mediators including IL-6, TNF- $\alpha$ , and IL-1 $\beta$ ; these inflammatory mediators play pivotal roles in the development of acute hepatitis (Ju and Tacke, 2016; Gao *et al.*, 2019). To evaluate whether the MCA-mediated decrease of inflammatory mediators in macrophages could be applicable in an animal model, the effect of MCA on serum AST/ALT levels in an LPS/D-GalN-induced acute hepatitis mice model was measured owing to the presence of Kupffer cells in the liver. Silibinin, a well-known hepatitis inhibitor, was used to verify the effect of MCA in acute hepatitis (Saller *et al.*, 2001; Hashem *et al.*, 2016). The repeated oral administration of MCA at a dose of 150 mg/kg notably decreased LPS/D-GalN-induced serum AST/ALT levels with a magnitude similar to the reduction observed in silibinin-treated mice (Fig. 4D). These data suggest that MCA could be applicable for treating inflammatory diseases, such as acute hepatitis, by inhibiting the production of inflammatory mediators.

A liver acute hepatitis model was developed to evaluate the further application of the inhibitory effects of MCA for the treatment of inflammatory diseases. Indeed, MCA-mediated alleviation of inflammatory responses in LPS-treated macrophages was correspondent to the inhibitory effects of MCA in a mouse liver acute hepatitis model (Fig. 4D). Specific macrophages exist in each organ, including Kupffer cell in the liver, alveolar macrophage in the lung, colonic macrophage in the colon, and microglia in the brain; these cells intervene in the development of inflammatory diseases by producing inflammatory cytokines. Therefore, the suppressive effects of MCA on the induction of inflammatory mediators might be applicable for the treatment of macrophage-mediated inflammatory diseases, such as Alzheimer's disease, alveolitis, and colitis.

#### *Inhibitory Effects of MCA on NF- $\kappa$ B and MAPK Signaling Pathways in Activated Macrophages*

We investigated the mechanism by which MCA regulates inflammation-regulating signal transduction pathways. Since NF- $\kappa$ B and AP-1 are major transcriptional regulators of the most pro-inflammatory genes, we first evaluated the activation of NF- $\kappa$ B or AP-1 with MCA. Reporter assays using the luciferase gene construct that contains NF- $\kappa$ B or AP-1-binding sites were employed. Because HEK 293 cells do not express TLR, PMA was used to induce the transcriptional activation of NF- $\kappa$ B and AP-1. PMA-induced activation of NF- $\kappa$ B and AP-1 was inhibited by MCA treatment (Figs. 5A and 5B), implying that the

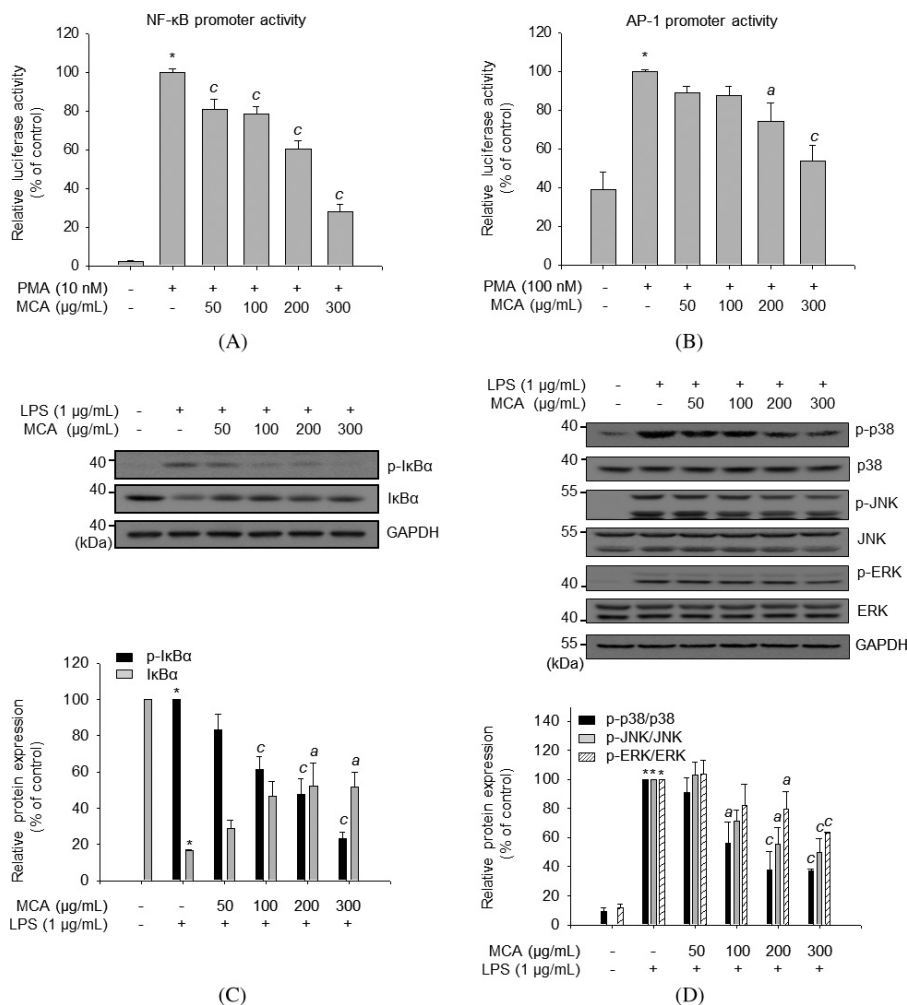


Figure 5. Inhibitory effects of MCA on NF-κB and MAPK activation. (A and B) HEK 293 was transfected with (A) pNF-κB-Luc or (B) pAP-1-Luc. Transfected cells were pre-treated with various concentrations of MCA for 2 h and incubated with or without PMA for 24 h. Luciferase activities were measured. Data represent the mean ± SEM. \* $p < 0.01$  relative to LPS-untreated group. <sup>a</sup> $p < 0.05$  and <sup>c</sup> $p < 0.001$  relative to the PMA-treated group. Control in Y-axis indicates the PMA-treated and MCA-untreated samples. (C and D) RAW 264.7 macrophages were pre-treated with different concentrations of MCA for 2 h, followed by LPS stimulation for (C) 3 min or (D) 15 min. Immunoblot analyses using primary antibodies for (C) NF-κB and (D) MAPK signaling were performed. The protein expressions were quantified and then normalized (IκB and p-IκB to GAPDH levels; p-MAPKs to total MAPKs). Representative blotting data and relative expression levels of each group compared with LPS-treated group were represented. Data represent the mean ± SEM. Control in Y-axis indicates either LPS/MCA-untreated (for the case of IκBα) or the LPS-treated and MCA-untreated samples (others). \* $p < 0.01$  relative to the LPS-untreated group. <sup>a</sup> $p < 0.05$  and <sup>c</sup> $p < 0.001$  relative to the LPS-treated group.

decreases of pro-inflammatory mediator production by MCA can be attributed to a reduction in NF- $\kappa$ B and AP-1 activities.

As MCA alleviated NF- $\kappa$ B transcriptional activity, we measured the effect of MCA on phosphorylation and degradation of I $\kappa$ B $\alpha$ , which binds and inhibits NF- $\kappa$ B in unstimulated cells (Hayden and Ghosh, 2008). I $\kappa$ B $\alpha$  was phosphorylated at Ser-32/36 and subsequently degraded by LPS stimulation; however, in MCA-treated groups, LPS-induced phosphorylation and subsequent degradation of I $\kappa$ B $\alpha$  was significantly decreased (Fig. 5C). These results strongly suggest that MCA could inhibit NF- $\kappa$ B activation.

To clarify the underlying mechanism of MCA-mediated AP-1 suppression, we evaluated whether MCA modulates the phosphorylation at the activation loop of MAPKs since such phosphorylation causes activation of transcriptional factors, including c-Fos, c-Jun, and ATF-2, which bind to and activate AP-1 (Cuevas *et al.*, 2007). When RAW264.7 were stimulated with LPS, the phosphorylation levels of p38, JNK, and ERK rapidly increased (Fig. 5D). Following MCA treatment, the phosphorylation of p38, JNK, and ERK was suppressed without altering the total protein level. These results suggest that MCA alleviates LPS-induced AP-1 activation by inhibiting MAPKs' activation.

The inhibitory effect of MCA on p-p38 and p-JNK were stronger than that on p-ERK (Fig. 5D). The involvement of ERK in inflammatory responses has been reported to be different from that of JNK and p38 (Karin, 2005). In addition, the upstream kinases that activate ERK, JNK, and p38 may differ from one another depending on various environmental stimuli or cell types. Accumulating evidence showed that TAK1 might be responsible for the phosphorylation of JNK and p38 and not for ERK activation (Chen *et al.*, 2006). Therefore, further studies for elucidating the target of MCA on differential regulation of MAPK activation were conducted by measuring TAK1 phosphorylation and IRAK1 degradation.

#### *MCA Inhibits TAK1 Phosphorylation and IRAK1 Degradation in Activated Macrophages*

TAK1 is an upstream signaling molecule of MAPK and NF- $\kappa$ B. In this study, MCA showed inhibitory effects on MAPK and NF- $\kappa$ B pathways (Fig. 5), suggesting that TAK1 might be a potential target of MCA. Therefore, the effect of MCA on TAK1 phosphorylation at Thr-184/Tyr-187 was investigated in RAW 264.7 cells activated with LPS. As expected, after LPS stimulation, an increase in TAK1 phosphorylation was detectable using immunoblot analysis, which was performed by employing an anti-p-TAK1 (Thr-184/Tyr-187). However, MCA treatment inhibited this increase in p-TAK1, suggesting that MCA regulates TAK1 signals (Fig. 6A).

We further examined whether MCA could block IRAK1 degradation, which is necessary for TAK1 phosphorylation at Thr-184/Tyr-187. As expected, LPS-induced degradation of IRAK1 was rescued by the presence of MCA, even at low concentrations (Fig. 6B). The data suggest that MCA might suppress the activation of TAK1 by blocking IRAK1 degradation, which in turn blocks downstream signaling in the MyD88-dependent pathway.

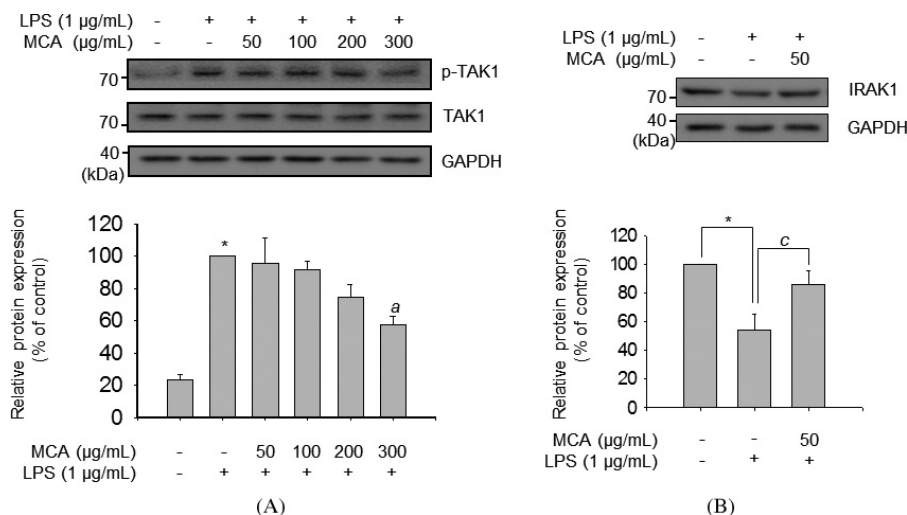


Figure 6. Effects of MCA on LPS-induced phosphorylation of TAK1 and degradation of IRAK1. RAW 264.7 cells were pre-treated with MCA for 2 h and then treated with LPS for 3 min. Immunoblot analyses using primary antibodies for (A) TAK1, p-TAK1, and (B) IRAK1 were performed. The protein expressions were quantified and then normalized to GAPDH levels. Representative blotting data and relative expression levels of each group compared with LPS-treated group were represented. Data represent the mean  $\pm$  SEM. Control in Y-axis indicates (A) the LPS-treated and MCA-untreated or (B) LPS/MCA-untreated samples. \* $p < 0.01$  relative to the LPS-untreated group. <sup>a</sup> $p < 0.05$  and <sup>c</sup> $p < 0.001$  relative to the LPS-treated group.

In this study, suppressed phosphorylation of p38 and JNK by MCA was investigated to be dependent on the MCA-mediated inhibition of TAK1 phosphorylation. However, according to the literature data, inhibition of ERK phosphorylation is not addressed by MCA-mediated TAK1 regulation (Wang *et al.*, 2001). A possible signaling pathway for the regulation of ERK by MCA treatment is Src/Syk-related Ras/Raf/MEK/ERK signaling axis (Schmid-Alliana *et al.*, 1998). This signaling pathway is another signal transduction which is activated by LPS stimulation in macrophages and separated from IRAK1-TAK1-mediated signaling axis. It might be possible to clarify the exact point of MCA action in LPS-stimulated macrophages by investigating whether MCA has inhibitory effects on the phosphorylation status of Src/Syk-related Ras/Raf/MEK/ERK signaling axis molecules.

## Conclusion

This study systemically described that MCA exerted anti-inflammatory properties by suppressing the production of several inflammatory mediators, such as NO, PGE<sub>2</sub>, IL-6, IL-1 $\beta$ , and TNF- $\alpha$ , in macrophages. The effect of MCA was manifested in acute inflammatory hepatitis. The inhibitory effects of MCA are caused by suppressing IRAK1-TAK1 signaling pathway, thereby inhibiting NF- $\kappa$ B and MAPK activation. Our findings supply a scientific basis of the traditional use of *C. asiatica* in treating various inflammatory diseases and suggest that MCA is a possible candidate for treating inflammatory diseases.

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