

**Original article:****6,7-DIMETHOXY-4-METHYLCOUMARIN SUPPRESSES  
PRO-INFLAMMATORY MEDIATOR EXPRESSION THROUGH  
INACTIVATION OF THE NF- $\kappa$ B AND MAPK PATHWAYS IN  
LPS-INDUCED RAW 264.7 CELLS**

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**ABSTRACT**

In this study, we investigated the ability of 6,7-dimethoxy-4-methylcoumarin (DMC) to inhibit lipopolysaccharide (LPS)-induced expression of pro-inflammatory mediators in mouse macrophage (RAW 264.7) cells, and the molecular mechanism through which this inhibition occurred. Our results indicated that DMC downregulated LPS-induced nitric oxide (NO) synthase (iNOS) and cyclooxygenase-2 (COX-2) expression, thereby reducing the production of NO and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in LPS-activated RAW 264.7 cells. Furthermore, DMC suppressed LPS-induced production of pro-inflammatory cytokines such as interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor (TNF)- $\alpha$ . To elucidate the mechanism underlying the anti-inflammatory activity of DMC, we assessed its effects on the mitogen-activated protein kinase (MAPK) pathway and the activity and expression of nuclear transcription factor kappa-B (NF- $\kappa$ B). The experiments demonstrated that DMC inhibited LPS-induced phosphorylation of extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase (JNK), and p38. In addition, it attenuated LPS-induced NF- $\kappa$ B activation via the inhibition of I $\kappa$ B- $\alpha$  phosphorylation. Taken together, these data suggest that DMC exerts its anti-inflammatory effects in RAW 264.7 cells through the inhibition of LPS-stimulated NF- $\kappa$ B and MAPK signaling, thereby downregulating the expression of pro-inflammatory mediators.

**Keywords:** 6,7-Dimethoxy-4-methylcoumarin (DMC), anti-inflammatory, RAW 264.7 cells, NF- $\kappa$ B, MAPKs

## INTRODUCTION

Inflammation is an essential host defense mechanism that can be induced in response to a wide variety of exogenous and endogenous stimuli (Lawrence et al., 2002). Lipopolysaccharide (LPS) is a potent immune system activator, inducing local inflammation, antibody production, septic shock, fever, and death (Rietschel and Brade, 1992). LPS activation of macrophages results in the upregulation of cyclooxygenase-2 (COX-2) and inducible nitric oxide (NO) synthase (iNOS) expression and the release of NO, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), and inflammatory cytokines such as interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor (TNF)- $\alpha$ , which can in turn activate other macrophages and nearby cells (Fujiwara and Kobayashi, 2005; Lawrence et al., 2002). Overproduction of inflammatory mediators is involved in the pathophysiology of chronic inflammatory diseases, such as rheumatoid arthritis, diabetes, and cancer (Feldmann, 2008; Serhan and Savill, 2005). Therefore, understanding the pathways that regulate the release of inflammatory mediators from activated macrophages is important for the development of novel therapeutics for the treatment of inflammatory diseases.

Previous studies have shown that compared to untreated macrophages, LPS-activated macrophages have a higher level of active nuclear transcription factor kappa-B (NF- $\kappa$ B), which regulates the production of pro-inflammatory cytokines and inflammatory mediators (Kim et al., 2013a; Ruland, 2011). Further, the mitogen-activated protein kinase (MAPK) pathway is also activated in LPS-stimulated macrophages and contributes to the inflammatory response (Cargnello and Roux, 2011).

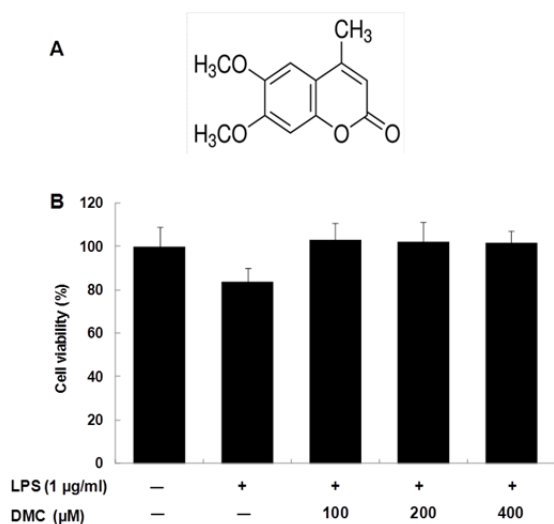
Coumarins are a group of heterocyclic compounds synthesized by numerous green plant species as well as by some bacteria and fungi, and have been widely used as fragrances in food and cosmetic products (Hoult and Paya, 1996; Lake, 1999). Numerous biological activities have been attributed to simple coumarins and coumarin deriva-

tives, including anti-coagulant (Manolov et al., 1993), anti-inflammatory (Fylaktakidou et al., 2004; Kontogiorgis and Hadjipavlou-Litina, 2003), anti-viral (Kostova et al., 2006), antimicrobial (Nagamallu and Kariyappa, 2013), and anti-oxidant effects (Nagamallu and Kariyappa, 2013; Raj et al., 1998). However, the anti-inflammatory effects of 6,7-dimethoxy-4-methylcoumarin (DMC) in RAW 264.7 macrophages and the molecular mechanisms regulating these effects remain largely unknown. The present study aimed to assess the effects of DMC on LPS-induced inflammatory responses in RAW 264.7 macrophages, and to explore the possible molecular mechanisms underlying these activities.

## MATERIALS AND METHODS

### Chemicals

DMC (Figure 1A), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen-Gibco (Grand Island, NY, USA). Enzyme-linked immunosorbent assay (ELISA) kits for PGE<sub>2</sub>, TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 were purchased from R&D Systems, Inc. (St. Louis, MO, USA). Anti-iNOS (mouse), Anti-COX-2 (rabbit), Anti-I $\kappa$ B- $\alpha$  (mouse), anti-phosphorylated I $\kappa$ B- $\alpha$  (rabbit, anti-p-I $\kappa$ B- $\alpha$ ), anti-c-Jun N-terminal kinase (rabbit, JNK), anti-phosphorylated JNK (rabbit, anti-p-JNK), anti-extracellular signal-regulated kinases (rabbit, ERK1/2), anti-phosphorylated ERK1/2 (rabbit, anti-p-ERK1/2), anti-p38 (rabbit), and anti-phosphorylated p38 (rabbit, anti-p-p38) mouse or rabbit antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). All other reagents were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).



**Figure 1:** Effect of DMC on RAW 264.7 cell viability. **(A)** Chemical structure of DMC. **(B)** RAW 264.7 cells ( $1.5 \times 10^5$  cells/ml) plated on 96-well plates were treated with DMC at 37 °C for 24 h. Cytotoxicity of DMC was assessed by MTT assay. Values are expressed as means  $\pm$  S.D. of triplicate experiments. \* $P < 0.05$  for the comparison with the LPS-stimulated group

### Cell culture

The murine RAW 264.7 macrophage cell line was obtained from the Korean Cell Line Bank (KCLB; Seoul, Korea) and cultured in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 % FBS. The cells were then incubated in an atmosphere of 5 % CO<sub>2</sub> at 37 °C and were sub-cultured every 3 days.

### Cell viability assay

The effect of DMC on RAW 264.7 cell viability was assessed using a colorimetric MTT assay. The cells ( $1.5 \times 10^5$  cells/well) were pre-incubated for 18 h and then incubated with or without LPS (1 μg/ml) in the absence or presence of various concentrations (100, 200, and 400 μM) of DMC for 24 h. MTT stock solution (50 μl; 2 mg/ml in PBS) was added to each well to achieve a total reaction volume of 250 μl. After incubation for 4 h, the plates were centrifuged for 10 min at 2000 rpm, and the supernatants were aspirated. The formazan crystals in each well were dissolved in dimethylsulfoxide. The amount of purple formazan was as-

essed by measuring the absorbance at 540 nm.

### NO determination

Cells ( $1.5 \times 10^5$  cells/well) were pre-incubated for 18 h and then incubated with or without LPS (1 μg/ml) in the absence or presence of various concentrations (100, 200, and 400 μM) of DMC for 24 h. The nitrite in the medium was measured by performing Griess assay and used as an indicator of cellular NO synthesis. Culture supernatants were mixed with Griess reagent (1:1 mixture of 1 % sulfanilamide and 0.1 % naphthylethylenediamine dihydrochloride in 2.5 % phosphoric acid) and incubated at room temperature for 10 min. The absorbance at 540 nm was then measured in a microplate reader. Samples of fresh culture media were used as blanks for all experiments. The nitrite levels in the samples were determined using a standard sodium nitrite curve.

### ELISA

The levels of PGE<sub>2</sub>, TNF-α, IL-1β, and IL-6 in the culture media were quantified using ELISA kits. Briefly, cells ( $1.5 \times 10^5$  cells/well) were pre-incubated for 18 h and then incubated with or without LPS (1 μg/ml) in the absence or presence of various concentrations (100, 200, and 400 μM) of DMC for 24 h. Cell-free supernatants were collected for ELISA determination of PGE<sub>2</sub>, IL-1β, IL-6, and TNF-α concentrations in accordance with the manufacturer's instructions.

### Western blot analysis

Cells ( $1.5 \times 10^5$  cells/well) were pre-incubated for 18 h and then incubated with or without LPS (1 μg/ml) in the absence or presence of various concentrations (100, 200, and 400 μM) of DMC for 24 h or with 400 μM DMC for 10, 20, and 30 min. After incubation, the cells were collected and washed twice with cold PBS. The cells were lysed in a lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 % Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM NaVO<sub>3</sub>,

10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 µg/ml aprotinin, and 25 µg/ml leupeptin) and maintained on ice for 30 min. The cell lysates were centrifuged, and the protein concentrations were determined using a BCA™ protein assay kit. Aliquots of the lysates (40 µg of protein) were separated on a 12 % sodium dodecyl sulfate-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (BIO-RAD) using a glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl [pH 8.8], 20 % methanol [v/v]). After blocking nonspecific binding sites with 1 % bovine serum albumin (BSA), the membrane was incubated overnight with a specific primary antibody (1:1000) at 4 °C. The membrane was then incubated for an additional 60 min with a peroxidase-conjugated secondary antibody (1:5000, Vector Laboratories, Burlingame, CA, USA) at room temperature. The immunoreactive protein bands were detected using an enhanced chemiluminescence (ECL) western blotting detection kit.

### **Statistical analysis**

All data are expressed as means ± standard deviation (S.D.). Significant differences among the groups were determined using the unpaired Student's *t*-test. A value of *\*p* < 0.05 was considered to be statistically significant.

## **RESULTS**

### **Effect of DMC on RAW 264.7 cell viability**

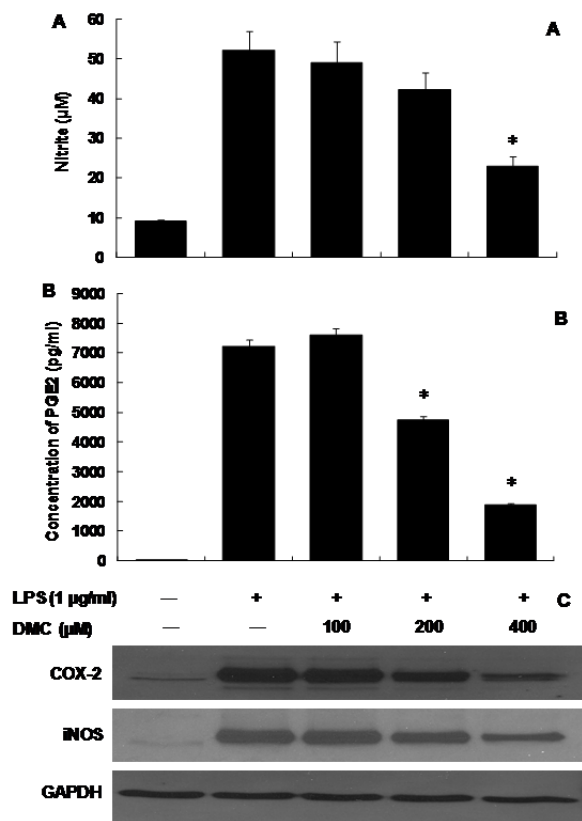
To assess whether DMC affected the viability of RAW 264.7 cells, MTT assays were performed 24 h after treatment with the indicated concentrations of DMC, in the presence or absence of LPS. The results indicated that cell viability was not affected by DMC at concentrations of up to 400 µM (Figure 1B). Thus, DMC did not show any cellular toxicity in RAW 264.7 cells.

### **DMC inhibited iNOS and COX-2 expression and the subsequent production of NO and PGE<sub>2</sub> in LPS-induced RAW 264.7 cells**

To investigate the potential anti-inflammatory effects of DMC, RAW 264.7 cells were stimulated with LPS for 24 h in the presence or absence of DMC (100, 200, and 400 µM) and NO and PGE<sub>2</sub> production was analyzed. Compared to the control group, treatment of RAW 264.7 cells with LPS alone resulted in a significant increase in NO and PGE<sub>2</sub> production. However, DMC inhibited LPS-induced production of NO and PGE<sub>2</sub> in a dose-dependent manner (Figure 2A and B). In order to determine if the inhibitory effects of DMC on NO and PGE<sub>2</sub> production were due to changes in iNOS and COX-2 levels, we examined their expression levels by western blot. This analysis identified a marked increase in the expression of iNOS and COX-2 24 h after LPS treatment. However, DMC strongly inhibited iNOS and COX-2 protein expression in a concentration-dependent manner (Figure 2C). These results suggested that DMC inhibited NO and PGE<sub>2</sub> release by reducing iNOS and COX-2 expression.

### **DMC inhibited LPS-induced production of pro-inflammatory cytokines in RAW 264.7 cells**

We next determined the effect of DMC on IL-1β, IL-6, and TNF-α production in LPS-induced RAW 264.7 cells. IL-1β, IL-6, and TNF-α levels significantly increased in the culture media of LPS-stimulated RAW 264.7 cells, and the levels of these cytokines showed significant, concentration-dependent, decreases in the presence of DMC (Figure 3).

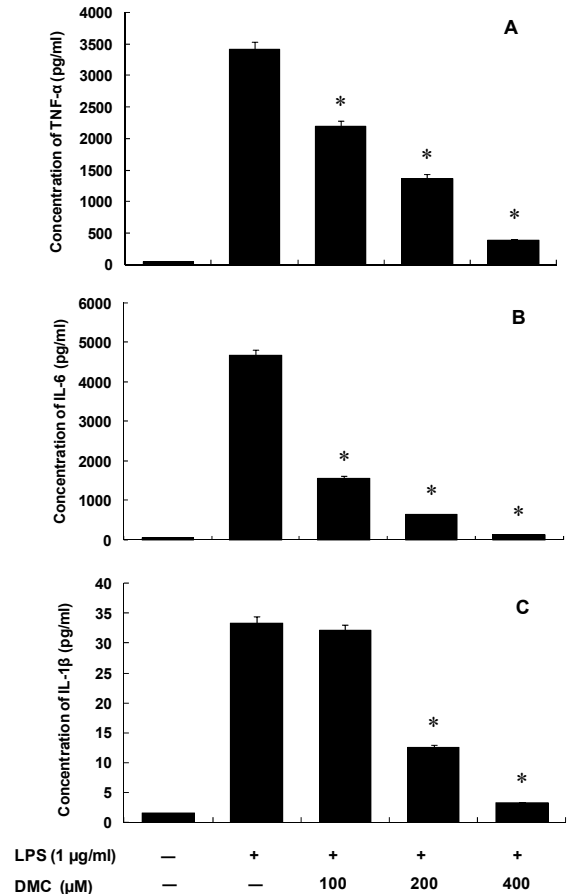


**Figure 2:** Effect of DMC on LPS-induced expression of iNOS and COX-2 and production of NO and PGE<sub>2</sub> in RAW 264.7 cells. Cells were stimulated with LPS (1 µg/ml) in the presence of DMC (100, 200, and 400 µM) for 24 h at 37 °C. **(A)** Culture media were collected in order to measure (A) NO and **(B)** PGE<sub>2</sub> production by the Griess reaction and ELISA, respectively. Values are expressed as means ± S.D. of triplicate experiments. \**P* < 0.05 for the comparison with the LPS-stimulated group. **(C)** Cells were stimulated with LPS (1 µg/ml) in the presence of DMC (100, 200, and 400 µM) for 24 h at 37 °C. The levels of iNOS and COX-2 proteins in cell lysates were analyzed by western blot.

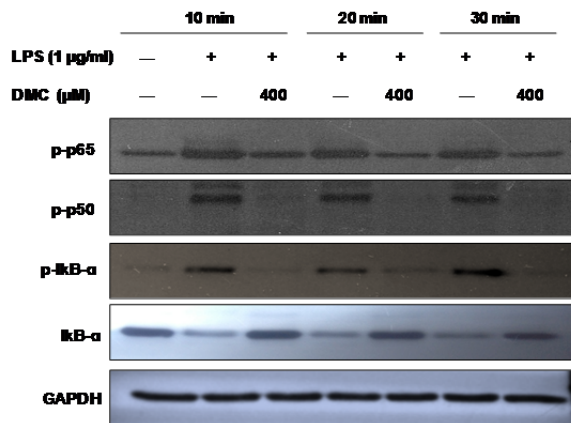
**DMC inhibited LPS-induced NF-κB activation in RAW 264.7 cells**

NF-κB has been shown to regulate the expression of iNOS and COX-2 following stimulation by LPS or other inflammatory cytokines (Gloire et al., 2006). Therefore, we investigated whether DMC inhibited the NF-κB signaling pathway in LPS-stimulated RAW 264.7 cells. Western blot analysis revealed that 400 µM DMC treatment inhibited LPS-induced NF-κB (p65 and p50) activation (Figure 4). We also found that treatment

of LPS-stimulated cells with 400 µM DMC significantly decreased IκB-α phosphorylation and degradation, compared to that observed in the cells treated with LPS alone (Figure 4).



**Figure 3:** Inhibitory effect of DMC on pro-inflammatory cytokine production in RAW 264.7 cells. The production of **(A)** TNF-α, **(B)** IL-6, and **(C)** IL-1β were assayed in the culture medium of cells stimulated with LPS (1 µg/ml) for 24 h in the presence of DMC (100, 200, and 400 µM). The concentrations of TNF-α, IL-6, and IL-1β in the supernatants were determined by ELISA. Values are expressed as means ± S.D. of triplicate experiments. \**P* < 0.05.



**Figure 4:** Inhibitory effects of DMC on LPS-induced phosphorylation of IκB-α and NF-κB p50. Cells were treated for different times (10, 20, and 30 min) with LPS (1 µg/ml), alone or with 400 µM DMC, as indicated. The levels of p-IκB-α (phosphorylated-IκB-α), IκB-α, p-p50 (phosphorylated p50) and p-p65 (phosphorylated p65) were determined by western blotting.

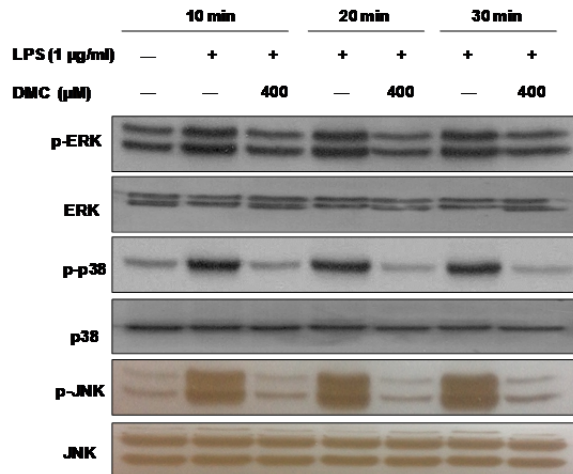
#### DMC inhibited LPS-induced MAPK phosphorylation in RAW 264.7 cells

To determine whether DMC inhibited the production of pro-inflammatory mediators through effects on the MAPK pathway, we evaluated the effect of DMC on LPS-induced phosphorylation of p38, JNK, and ERK in RAW 264.7 cells using western blotting. We found that LPS stimulation significantly increased phosphorylation of p38, JNK, and ERK. However, treatment with 400 µM DMC attenuated the phosphorylation of these proteins (Figure 5).

### DISCUSSION

Macrophages play a key role in immune responses during the inflammation process. After macrophages are activated by LPS, large amounts of inflammatory mediators are released (Lee et al., 2012). Because of this, LPS-induced macrophages have been used to assess the anti-inflammatory effects of various agents. The present study sought to elucidate the pharmacological effects of DMC on the production of pro-inflammatory mediators, and its mechanism of action, in LPS-stimulated RAW 264.7 macrophages. We found that DMC significantly inhibited LPS-

induced NO, PGE<sub>2</sub>, TNF-α, IL-1β, and IL-6 production in RAW 264.7 cells through the suppression of NF-κB and MAPK activation.



**Figure 5:** Inhibitory effects of DMC on ERK, JNK, and p38 protein levels in RAW 264.7 cells. Cells were treated for different times (10, 20, and 30 min) with LPS (1 µg/ml), alone or with 400 µM DMC, as indicated. The levels of p-ERK (phosphorylated-ERK), ERK, p-JNK (phosphorylated-JNK), JNK, p-p38 (phosphorylated-p-38) and p-38 were determined by western blotting.

Macrophages stimulated by LPS or other cytokines overexpress iNOS, which metabolizes L-arginine to L-citrulline and NO (Jiang et al., 2012). NO assists in the activation of macrophages by regulating signal transduction to enhance the ability of macrophages to kill microorganisms (Olefsky and Glass, 2010). However, overproduction of NO by macrophages could induce innate immunity and cause an inflammatory response (Yan and Hansson, 2007). In acute and chronic inflammatory diseases, including asthma, arthritis, atopic dermatitis, and fever caused by bacterial infection, macrophages are activated and release more NO, causing tissue injury (Caplin and Leiper, 2012; Yan and Hansson, 2007; Zhou et al., 2012). Therefore, regulation of NO release through the inhibition of iNOS may alleviate inflammation. Because our results indicated that DMC decreased NO and PGE<sub>2</sub> production via the suppression of iNOS and COX-2 expression in LPS-stimulated RAW 264.7 cells, it may

be useful for the treatment of NO- and PGE<sub>2</sub>-induced inflammatory responses.

Similar to NO, PGE<sub>2</sub> is a pleiotropic signaling molecule produced in considerable quantities by COX-2 at sites of inflammation. It has also been reported to be involved in the pathogenesis of inflammatory diseases (Lipsky, 1999; Zhang et al., 2011). Thus, chemopreventive agents that attenuate COX-2-mediated PGE<sub>2</sub> production have had a therapeutic effect in many inflammatory diseases (Hocherl et al., 2002). In the present study, we found that DMC inhibited PGE<sub>2</sub> production in LPS-stimulated RAW 264.7 cells by reducing the expression of COX-2. These data suggested that the inhibition of NO and PGE<sub>2</sub> production by DMC may be due to its suppression of iNOS and COX-2 upregulation during activation of macrophages by LPS. Importantly, the inhibitory effect of DMC on LPS-induced iNOS and COX-2 expression did not result from its cytotoxicity, because there was no observable loss of cell viability in our MTT assays.

Inflammatory stimuli such as LPS induce the production of cytokines, including TNF- $\alpha$ , IL-6, and IL-1 $\beta$ , during macrophage activation. These cytokines can then mediate tissue responses during different phases of inflammation (Hseu et al., 2005; Kim et al., 2013b). TNF- $\alpha$  has many physiological effects, including promotion of inflammation and apoptosis, and the overproduction of TNF- $\alpha$  may cause tissue injury, sepsis, or death (Chong and Sriskandan, 2011). IL-6 is an important cytokine released in many immunological and inflammatory responses (Liu et al., 2011). In addition, IL-1 $\beta$  is an inflammatory cytokine involved in the induction of innate immune responses by macrophages and in the secretion of other inflammatory cytokines (Dinarello, 2011). Because of their important roles in inflammatory responses, downregulation of these pro-inflammatory cytokines is of utmost importance during anti-inflammatory therapy. Here, we showed that DMC inhibited the LPS-induced production of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6.

NF- $\kappa$ B is a key transcription factor regulating the expression of inflammation-associated enzymes and cytokines, including iNOS, COX-2, TNF- $\alpha$ , and IL-1 $\beta$  (Gloire et al., 2006). Inactive NF- $\kappa$ B is located in the cytoplasm, where it complexes with I $\kappa$ B- $\alpha$ . Phosphorylation and degradation of I $\kappa$ B- $\alpha$  releases NF- $\kappa$ B, allowing it to translocate to the nucleus and initiate the transcription of its target genes (Gomez et al., 2005). Therefore, NF- $\kappa$ B activation could be assessed in RAW 264.7 cells by measuring the expression level of the I $\kappa$ B- $\alpha$  protein. In the present study, we found that DMC inhibited LPS-induced I $\kappa$ B- $\alpha$  phosphorylation and degradation, and the activation of NF- $\kappa$ B p65 and p50. These data suggested that the effects of DMC on the production of inflammatory mediators and cytokines are mediated, at least in part, via suppression of the NF- $\kappa$ B signaling pathway.

MAPK signaling pathways play important roles in many biological processes, including inflammation, apoptosis, proliferation, and differentiation. The MAPKs include ERK, JNK, and p38 kinase (Kim et al., 2010). MAPKs have been reported to promote iNOS and COX-2 expression in LPS-stimulated macrophages (Kyriakis and Avruch, 2012). In addition, the phosphorylation of MAPKs promotes the production of pro-inflammatory cytokines in LPS-induced macrophages (Ajizian et al., 1999; Carter et al., 1999). Our results indicated that DMC was a potent inhibitor of MAPK activation following LPS stimulation of RAW 264.7 cells, suggesting that its anti-inflammatory effects were also due to inhibition of MAPK signaling pathways.

In summary, the present study demonstrated that DMC was a potent inhibitor of NO, PGE<sub>2</sub>, iNOS, COX-2, IL-6, IL-1 $\beta$ , and TNF- $\alpha$  production in LPS-induced RAW 264.7 cells. These inhibitory actions of DMC were mediated through the inhibition of I $\kappa$ B- $\alpha$  degradation, which prevented the activation and translocation of NF- $\kappa$ B. Furthermore, the levels of phosphorylated MAPKs in LPS-induced RAW 264.7 cells were sig-

nificantly decreased by treatment with DMC. Taken together, the findings led us to conclude that DMC appeared to have the potential to prevent LPS-induced inflammation.

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