Dipterocarpus obtusifolius attenuates the effects of lipopolysaccharide-induced inflammatory response in RAW264.7 macrophages

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Abstract. *Dipterocarpus obtusifolius* has been traditionally used as a herbal medicine and is considered to have anticancer properties. The biological activity of *D. obtusifolius* in inflammation and the underlying mechanisms of its activity remain to be elucidated. The present study investigated the effects of *D. obtusifolius* methanolic extract (DOME) on lipopolysaccharide (LPS)-stimulated inflammation in RAW264.7 cells. The effects of DOME on the production of nitric oxide, prostaglandin E_2 and pro-inflammatory cytokines were assessed by ELISA, western blot analysis and reverse transcription-quantitative polymerase chain reaction. It was demonstrated that expression of inducible nitric oxide synthase, cyclooxygenase-2, interleukin-1β and tumor necrosis factor-α was suppressed by DOME in LPS-stimulated cells. Furthermore, treatment with DOME suppressed phosphorylation of mitogen activated protein kinase (MAPK) molecules, including extracellular signal-regulated kinase, c-Jun N-terminal kinase and p38 MAPK. Translocation of the nuclear factor-κB p65 subunit into the nucleus was additionally inhibited by DOME. Phosphorylation of MAPK promoter activity was inhibited by treatment with DOME, PD98059, SB202190 and SP600125. These results demonstrated that DOME inhibits LPS-induced inflammatory responses. Therefore, DOME may be a potential therapeutic approach for the treatment of inflammatory diseases.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are typically used to treat inflammatory states and injury. Non-selective NSAIDs inhibit cyclooxygenase-1 (COX-1) mediated production of prostaglandins and are known to cause vascular and gastrointestinal toxicity, and a variety of nephrotoxic syndromes (1). Due to these adverse effects, there is a requirement for novel alternative treatments for inflammatory diseases. Meanwhile, there has been an increase in the use of herbal dietary supplements. The rise in the popularity of natural products may be attributed to dissatisfaction with conventional medicines (2-5).

The function of nitric oxide (NO) in the inflammatory reaction is controversial. During inflammation and sepsis, increased production of diverse mediators, including endotoxins, pro-inflammatory cytokines and eicosanoids, induces inducible nitric oxide synthase (iNOS) activity. Increased mucosal NO production and NOS-2 activity have been associated with active inflammatory bowel disease or pathogenesis of chronic disease in patients (6-8) and in an experimental animal model of induced or inherent inflammation (9). In addition, prostaglandin E_2 (PGE_2) is a primary inflammatory mediator and upregulates vascular permeability increasing factor, which may lead to edema, pain and fever during inflammatory disease (10). Members of the COX family are accountable for the production of prostaglandins, including thromboxanes and

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prostacyclin (11,12). COX exists as two isoforms: Inducible COX-2 and constitutive COX-1. COX-2 levels are reduced under healthy physiological conditions, but is transiently and rapidly induced by pro-inflammatory mediators, activating the biosynthesis of prostaglandin and inflammatory responses (13). Inhibition of COX-2 may provide relief from the symptoms of inflammation and pain (14). Consequently, a previous study aimed to investigate selective COX-2 inhibitors (15). Fraxinellone has been suggested to have mild but significant inhibitory effects on the stimulation of PGE_2 by LPS, and Kim *et al* (16) suggested reductions in PGE_2 may be due to the transcriptional suppression of COX-2.

D. obtusifolius is a plant genus that belongs to the family Dipterocarpaceae, which consists of ~75 species distributed in tropical regions (17). This family of plants is known to contain sesquiterpenes, triterpenes, flavonoids and resveratrol oligomers, and exhibits diverse biological anticancer, anti-human immunodeficiency virus (18), antibacterial and antioxidant activities (19). *D. obtusifolius* is a tree with a typical height of 10-15 m that grows in cleared forests in low altitude regions, and is widely distributed across Southeast Asian countries. Traditionally, the resin of this plant has been used to relieve abdominal discomfort in Cambodia, Laos and Vietnam (20). To the best of our knowledge, there have been no reports on the anti-inflammatory effects of *D. obtusifolius* methanolic extract (DOME). Therefore, the aim of the present study was to investigate the anti-inflammatory effects of DOME using LPS-stimulated RAW264.7 cells. NO, cytokines and PGE_2 levels were assessed following treatment with DOME in LPS-stimulated RAW264.7 cells. To elucidate the protective underlying mechanisms of DOME, the expression of mRNA and proteins associated with inflammatory responses were examined.

Materials and methods

Preparation of DOME from D. obtusifolius leaves. *D. obtusifolius* was collected from the Tbaeng region of Cambodia in 2009. The extract was obtained from 86 g leaves with MeOH, an extract efficiency of 9.22%. A voucher specimen (KRIB 0025367) was deposited in the herbarium of the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Republic of Korea). The *D. obtusifolius* leaves were obtained from the International Biological Material Research Center (Daejeon, Republic of Korea).

Cell culture. The RAW264.7 macrophage cell line was provided by the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in a 95% air, 5% CO_2 atmosphere in a 37°C incubator in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone; GE Healthcare, Logan, UT, USA) and 1% penicillin-streptomycin-glutamine (Invitrogen; Thermo Fisher Scientific, Inc.). RAW264.7 cells were passage weekly at 70-80% confluence. Following pre-incubation for 4 h, 0-30 μg/ml DOME was added to cells. In another set of cultures, the cells were co-incubated with 10 μM p42/44 inhibitor, PD98059, 10 μM p38 inhibitor, SB203580 and 10 μM c-Jun N-terminal kinase (JNK) inhibitor, SP600125.

Cell viability assay. Cell viability was determined by an assay based on the mitochondrial-dependent reduction of MTT (Amresco, LLC, Solon, OH, USA) to formazan with 100 μl DMSO per well. Based on the results of cell viability, nontoxic concentrations of DOME were used with RAW264.7 cells in the present study. The optical density of formazan was measured using a microplate reader (Benchmark; Bio-Rad Laboratories, Hercules, CA, USA) at a wavelength of 570 nm. The optical density of formazan generated by untreated cells was used to determine the 100% viability.

NO assay. The NO level in cell culture supernatants from centrifugation at 100 x g for 5 min at room temperature was determined using the Griess test. RAW264.7 cells were plated at a density of 5×10^4 cells/well in 96-well plates and subsequently incubated with or without 0.5 μg/ml LPS in the absence or presence of various concentrations of DOME for 24 h. Nitrite in the culture supernatants was mixed with an equal volume of Griess reagent [1% sulfanilamide and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in 5% phosphoric acid]. The absorbance was measured at a wavelength of 540 nm using a microplate reader and a series of known concentrations of $NaNO_2$ was used as a standard.

PGE_2 assay. The quantity of PGE_2 in the supernatant was determined using a PGE_2 ELISA kit (cat. no. 514010; Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's protocol. A total of 50 μl diluted standard:sample 2:1 was pipetted into a 96-well plate pre-coated with goat polyclonal anti-mouse IgG-HRP antibody (sc-2005; Santa Cruz Biotechnology, Inc.). Aliquots of a PGE_2 monoclonal antibody and PGE_2 acetylcholine esterase (AChE) conjugate were added to each well and allowed to incubate at room temperature for 18 h. The wells were washed six times with buffer containing 0.05% Tween-20, followed by the addition of 200 μl Ellman's reagent containing acetylthiocholine and 5,5'-dithio-bis-(2-nitrobenzoic acid). PGE_2 concentrations were determined by measuring the absorbance at a wavelength of 405 nm using a microplate reader (Benchmark; Bio-Rad Laboratories, Inc.).

Cytokine assays. The levels of interleukin (IL)-1β and tumor necrosis factor (TNF)-α were determined using commercial ELISA kits for IL-1β (cat. no. 559603) and TNF-α (cat. no. 558534) purchased from BD Biosciences, Inc. (San Jose, CA, USA) according to the manufacturer's protocols. The concentrations of mediators were determined by measuring the absorbance at a wavelength of 450 nm using a microplate reader (Benchmark; Bio-Rad Laboratories, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was performed for the detection of the mRNA expression of iNOS, COX-2, IL-1β, TNF-α and β-actin. Following 0.5 μg/ml LPS stimulation of RAW264.7 cells for 6 h, total RNA was isolated using TRIzol™ reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RT reactions were performed using a kit for producing cDNA (QuantiTect Reverse Transcription; 205313; Qiagen GmbH, Hilden, Germany). PCR was carried out using specific forward and reverse primers and SYBR®

Table I. Primers for reverse transcription-quantitative polymerase chain reaction.

Gene	Forward	Reverse
iNOS	CAAGAGTTTGACCAGAGGACC	TGGAACCACTCGTACTTGGGA
COX-2	GAATGCTTTGGTCTGGTGCCTG	GTCTGCTGGTTTGGAATAGTTGC
IL-1β	GTGTCTTTCCCGTGGACCTT	TCGTTGCTTGGTTCTCCTTG
TNF-α	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
β-actin	TGTTTGAGACCTTCAACACC	CGCTCATTGCCGATAGTGAT

iNOS, inducible nitric oxide synthase; COX, cyclooxygenase; IL, interleukin; TNF, tumor necrosis factor.

FAST Master Mix (KAPA, KR0389; Bioneer Corporation, Daejeon, Korea) according to the manufacturer's protocol. The following conditions were used for each PCR reaction: 94˚C for 5 min (1 cycle); 94˚C for 20 sec, 5˚C for 30 sec and 72˚C for 45 sec (30 cycles); and a final extension phase at 72˚C for 10 min. Primer sequences iNOS, COX-2, IL-1β, TNF-α and β-actin are presented in Table I. β-actin expression served as an internal housekeeping gene control. The reaction products were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and visualized using a UV Transilluminator imaging system. Gels were imaged using an C-4000 Zoom camera (Olympus America, Inc., Center Valley, PA, USA).

Western blot analysis. Following 0.5 µg/ml LPS stimulation for 30 min, 5x10^5 cells/ml cells were harvested. Cells were washed twice with ice-cold PBS, scraped off with a rubber policeman and centrifuged at 1,000 x g for 5 min at 4˚C. Cell pellets were resuspended in an appropriate volume of Protein Extraction Solution (NP-40; catalog no. EBA-1049; ELPIS-Biotech, Inc., Daejeon, Korea), and incubated for 10 min at room temperature and subsequently for 20 min on ice. Lysates were subsequently centrifuged at 30,000 x g for 10 min at 4˚C and collected for further analysis. The supernatant was stored at -87˚C until use. Protein concentrations of samples were determined by Bicinchoninic Acid assay (Thermo Fisher Scientific, Inc.) using samples equilibrated to 2 mg/ml with Protein Extraction Solution. A total of 20 µg protein was separated by 10% SDS-PAGE and subsequently transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). Each membrane was incubated for 1 h with 5% skimmed milk in TBS with Tween-20 buffer (0.1 M Tris-HCl, pH 7.4; 0.9% NaCl; 0.1% Tween-20) to block non-specific binding, followed by 4˚C overnight incubation with the following primary antibodies: Anti-iNOS (ADL-905-431; 1:1,000; Enzo Life Sciences, Farmingdale, NY, USA), anti-COX-2 (sc-1747; 1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-β-actin (4967S; 1:2,000; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-extracellular signal regulated kinase (ERK)2 (sc-154), anti-p38 mitogen activated protein kinase (MAPK) (sc-7149), anti-JNK1/3 (sc-474), anti-nuclear factor (NF)-κB p65 (sc-372), anti-proliferating cell nuclear antigen (PCNA) (sc-56) (all 1:1,000; Santa Cruz Biotechnology, Inc.), anti-phosphorylated (p)-p38 MAPK (MA 022; 1:1,000) , anti-p-JNK1/2 (both 1:1,000; Enzo Life Sciences) and anti-p-ERK (4370;
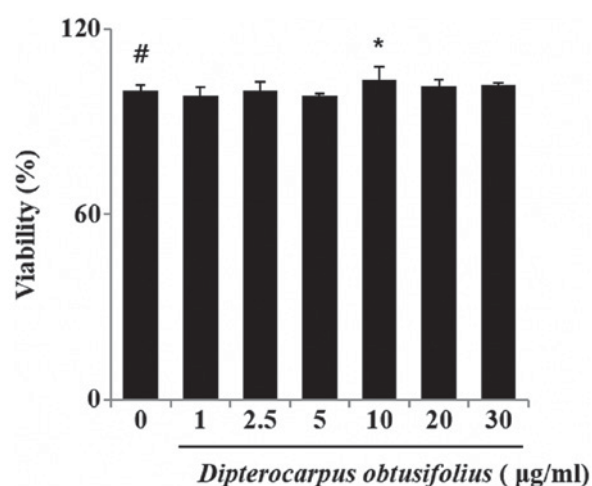

Figure 1. Effect of DOME on cell viability. RAW264.7 cells were incubated in the presence or absence of 0-30 µg/ml of DOME, and cell viability was determined using the MTT assay. Data are presented as the mean ± standard error (n=3). #P<0.01 and *P<0.05 vs. the normal control group. DOME, *Dipterocarpus obtusifolius* methanolic extract.

1:1,000; Cell Signaling Technology, Inc.). Each protein was detected using an Enhanced Chemiluminescence detection system according to the ImageQuant LAS4000 (GE Healthcare Life Sciences, Chalfont, UK).

Statistical analysis. Data are expressed as the mean ± standard error of the mean. Statistical significance between two groups was determined using the Student's t-test. Data with values of P<0.05 were considered to indicate a statistically significant difference.

Results

Evaluation of cytotoxicity of DOME on RAW264.7 cells. To determine if DOME affects cell viability, RAW264.7 cells were incubated for 24 h with the extract at a wide range of concentrations (0-30 µg/ml), and cell viability was evaluated by MTT assay. A statistically significant decrease in cell survival was detected at concentrations higher than 30 µg/ml (Fig. 1).

DOME inhibits NO production by suppressing iNOS expression in LPS-stimulated RAW264.7 macrophage cells. Nitric oxide serves a central role in the inflammatory response.
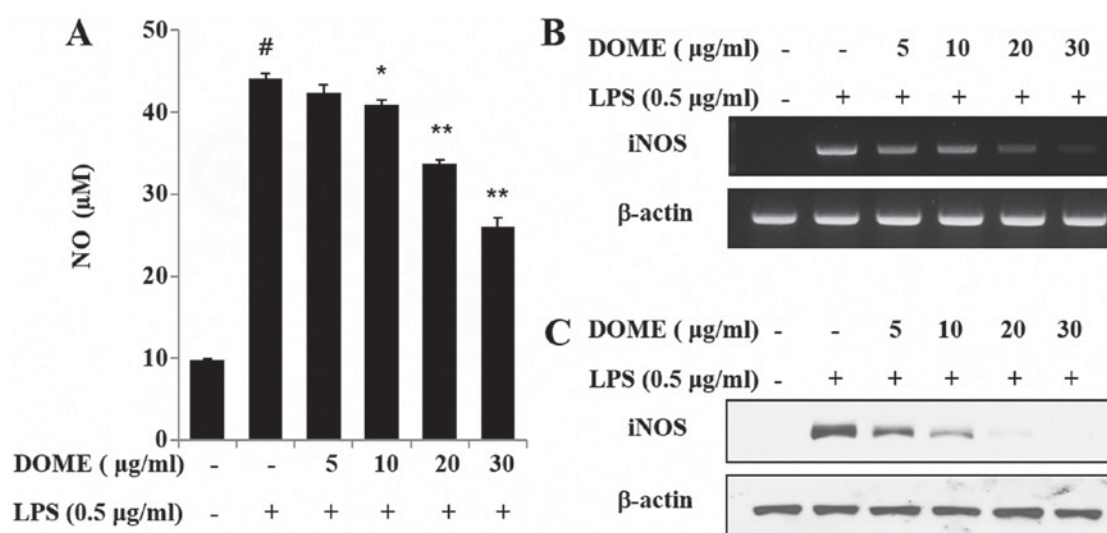
Figure 2. DOME inhibits LPS-induced NO production and iNOS expression in RAW264.7 cells. (A) RAW264.7 cells were pretreated with 5, 10, 20 or 30 μg/ml DOME for 1 h and subsequently stimulated with 0.5 μg/ml LPS. After 24 h, NO concentrations were measured using the Griess reaction. Data are presented as the mean ± standard error (n=3). $^{\#}$P<0.05 vs. normal control group, *P<0.05 and **P<0.01 vs. cells treated with LPS alone. RAW264.7 cells were pretreated with DOME for 1 h and stimulated with LPS for 6 h. (B) mRNA and (C) protein expression levels of iNOS, as assessed by reverse transcription-quantitative polymerase chain reaction (n=3) and western blot analysis (n=4), respectively. β-actin served as an internal control. DOME, *Dipterocarpus obtusifolius* methanolic extract; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; NO, nitric oxide.
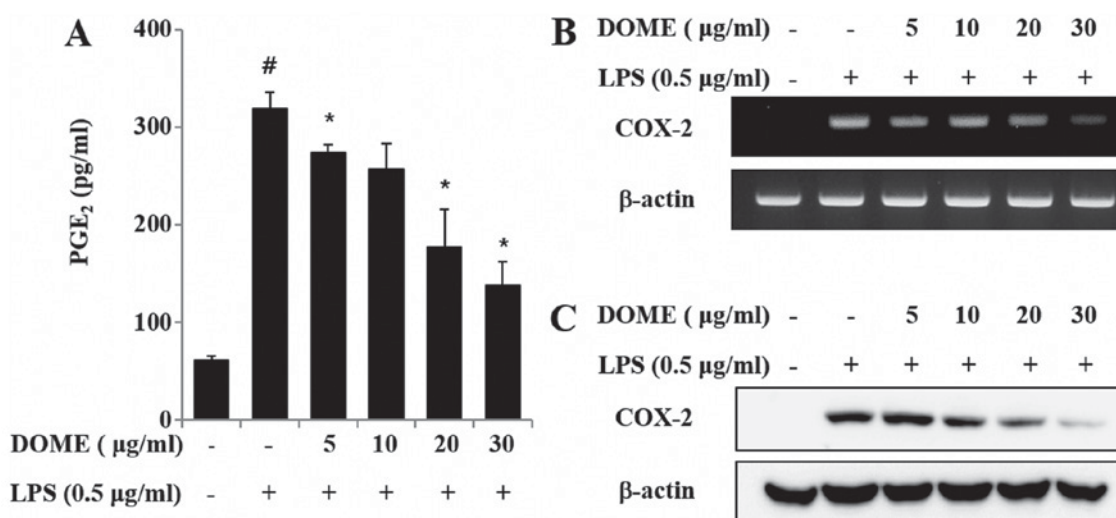

Figure 3. DOME inhibits LPS-induced PGE$_2$ production and COX-2 expression in RAW264.7 cells. (A) RAW264.7 cells were pretreated with 5, 10, 20 or 30 μg/ml DOME for 1 h and subsequently stimulated with 0.5 μg/ml LPS. After 24 h, PGE$_2$ concentrations were measured using by ELISA. Data are presented as the mean ± standard error (n=3). $^{\#}$P<0.05 vs. normal control group, *P<0.05 vs. cells treated with LPS alone. RAW264.7 cells were pretreated with DOME for 1 h and stimulated with LPS for 6 h. (B) mRNA and (C) protein expression levels of COX-2, as assessed by reverse transcription-quantitative polymerase chain reaction (n=3) and western blot analysis (n=4), respectively. β-actin served as an internal control. DOME, *Dipterocarpus obtusifolius* methanolic extract; LPS, lipopolysaccharide; PGE$_2$, prostaglandin E$_2$; COX-2, cyclooxygenase-2.

Unstimulated RAW264.7 cells secreted basal levels of NO, whereas LPS stimulation induced an increase in NO production. DOME treatment was demonstrated to inhibit NO production in LPS-induced RAW264.7 macrophages in a dose-dependent manner (Fig. 2A). To further evaluate the effects of DOME on expression of iNOS, RT-qPCR and western blot analyses were performed 6 and 18 h following LPS stimulation. Untreated cells and those pre-incubated with DOME expressed detectable levels of iNOS mRNA and protein, which were elevated upon LPS addition (data not shown). Notably, DOME reduced iNOS mRNA (Fig. 2B) and protein (Fig. 2C) expression levels,

indicating that DOME inhibits LPS-induced NO production by inhibiting iNOS.

DOME inhibits PGE$_2$ production by suppressing COX-2 expression in LPS-stimulated RAW264.7 cells. To assess whether DOME extract exerted an inhibitory effect on PGE$_2$ production, RAW264.7 cells were pretreated with the extract for 1 h and stimulated with LPS. Incubation of macrophages with LPS alone for 24 h significantly increased the secretion of the pro-inflammatory mediator PGE$_2$, as measured by ELISA (P<0.05). However, pretreatment with DOME in macrophages
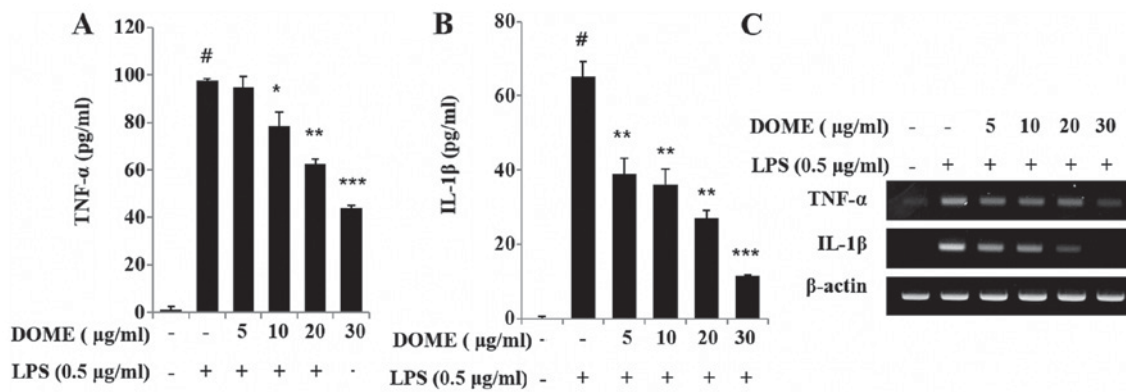
Figure 4. DOME inhibits IL-1β and TNF-α production in LPS-stimulated RAW264.7 cells. Cytokines were measured by ELISA of cultured supernatants collected from treated cells. (A) TNF-α and (B) IL-1β levels in culture media. (C) mRNA expression levels of IL-1β and TNF-α, as assessed by reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean ± standard error (n=3). #P<0.01, *P<0.05, **P<0.01 and ***P<0.001 vs. the normal control group. DOME, *Dipterocarpus obtusifolius* methanolic extract; LPS, lipopolysaccharide; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α.
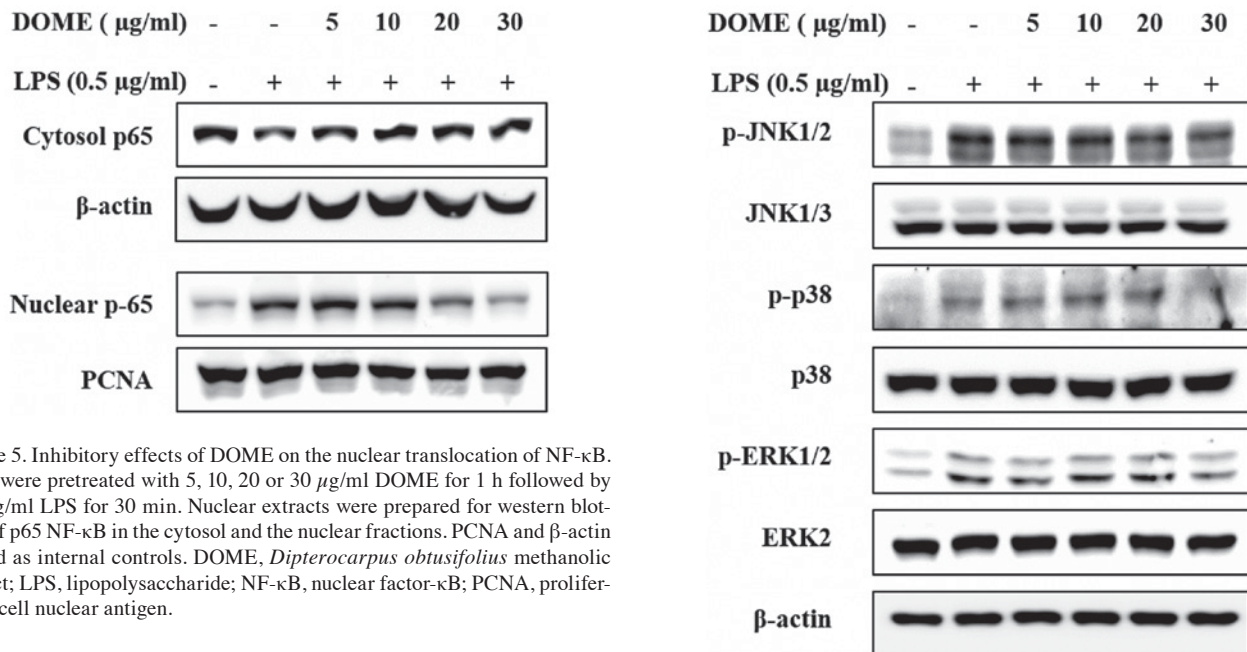

Figure 5. Inhibitory effects of DOME on the nuclear translocation of NF-κB. Cells were pretreated with 5, 10, 20 or 30 μg/ml DOME for 1 h followed by 0.5 μg/ml LPS for 30 min. Nuclear extracts were prepared for western blotting of p65 NF-κB in the cytosol and the nuclear fractions. PCNA and β-actin served as internal controls. DOME, *Dipterocarpus obtusifolius* methanolic extract; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; PCNA, proliferating cell nuclear antigen.

Figure 6. DOME suppresses phosphorylation of MAPK molecules in LPS-stimulated RAW264.7 cells. RAW264.7 cells were pretreated with 5, 10, 20 and 30 μg/ml DOME for 1 h followed by 0.5 μg/ml LPS for 30 min. Cellular proteins were evaluated for detection of total and phosphorylated forms of the MAPK signaling molecules ERK, JNK1/2 and p38 MAPK (n=3). DOME, *Dipterocarpus obtusifolius* methanolic extract; LPS, lipopolysaccharide; p, phosphorylated; JNK, c-Jun N-terminal kinase; ERK, extracellular signal regulated kinase; MAPK, mitogen activated protein kinase.

dose-dependently decreased the secretion of PGE2 compared with treatment with LPS alone (Fig. 3A; P<0.05). To further clarify the effects of DOME on COX-2 mRNA and protein expression levels, RT-qPCR and western blot analyses were performed 6 and 18 h after LPS stimulation. Cells treated with DOME alone expressed detectable levels of COX-2 mRNA and protein, which were further increased following LPS stimulation (data not shown). DOME markedly attenuated mRNA (Fig. 3B) and protein (Fig. 3C) expression levels of LPS-induced COX-2 in RAW264.7 cells. Therefore, DOME may inhibit PGE$_2$ production via suppression of COX-2 expression in LPS-stimulated RAW264.7 cells.

DOME reduces the release of pro-inflammatory cytokines in LPS-stimulated RAW264.7 cells. Secretion of pro-inflammatory mediators is a critical factor in inflammatory processes. LPS stimulation induced an increase in TNF-α (Fig. 4A) and IL-1β (Fig. 4B) levels in RAW264.7 cells. However, DOME significantly reduced the release of these LPS-stimulated mediators in a dose-dependent manner. Similarly, mRNA

expression levels of TNF-α and IL-1β were increased in LPS-stimulated RAW264.7 cells, whereas DOME suppressed this effect (Fig. 4C).

DOME inhibits the phosphorylation of MAPKs and activation of NF-κB in LPS-stimulated RAW264.7 cells. DOME-treated RAW264.7 cells exhibited a reduction in the translocation of NF-κB into the nucleus (Fig. 5). The MAPKs may be activated by LPS, a key stimulator of the inflammation response, in macrophages as well as many cell types (20). As presented in Fig. 6, DOME markedly decreased the expression levels
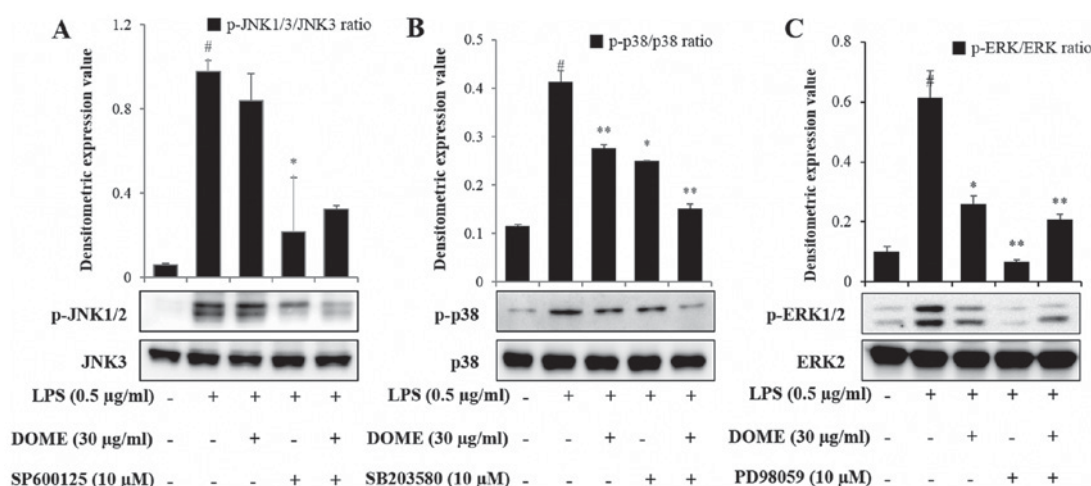
Figure 7. Effects of mitogen activated protein kinase inhibitors on JNK, p-38 and ERK phosphorylation. RAW264.7 cells were pretreated with 30 µg/ml DOME, 10 µM SB203580, 10 µM PD98059 or 10 µM SP600125 for 1 h followed by the incubation with 0.5 µg/ml LPS for 1 h. (A) p-JNK, (B) p-p38 and (C) p-ERK protein expression levels were measured by western blot analysis. Data are presented as the mean ± standard error. #P<0.01, *P<0.05 and **P<0.01 vs. the normal control group. DOME, *Dipterocarpus obtusifolius* methanolic extract; LPS, lipopolysaccharide; p, phosphorylated; JNK, c-Jun N-terminal kinase; ERK, extracellular signal regulated kinase.
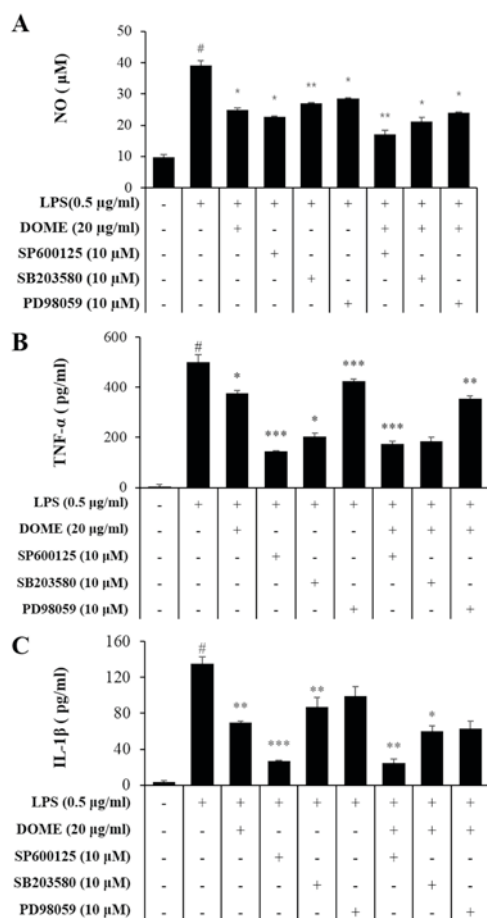

Figure 8. Effects of mitogen activated protein kinase inhibitors on NO production and proinflammatory cytokine expression. RAW264.7 cells were pretreated with 30 µg/ml DOME, and 10 µM SB203580, 10 µM PD98059 or 10 µM SP600125 for 1 h followed by incubation with 0.5 µg/ml LPS for 18 h. (A) NO levels were measured by Griess reaction. (A) NO production (B) TNF-α and (C) IL-1β levels were measured by ELISA. Data are presented as the mean ± standard error of the mean (n=3). #P<0.01, *P<0.05, **P<0.01 and ***P<0.001 vs. the normal control group. DOME, *Dipterocarpus obtusifolius* methanolic extract; LPS, lipopolysaccharide; NO, nitric oxide; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β.

of p-p38, p-JNK and p-ERK in LPS-induced RAW264.7 cells compared with untreated cells. These data suggested that DOME suppresses the inflammatory response via partial regulation of MAPK signaling. To assess the role of MAPKs in LPS-induced inflammatory mediator production, the effects of selective MAPK inhibitors on LPS-induced NO production were examined. Western blot analysis revealed that SP600125 (Fig. 7A), SB203580 (Fig. 7B) and PD98059 (Fig. 7C), markedly inhibited JNK, p38 and ERK phosphorylation, respectively. Furthermore, these inhibitors were demonstrated to markedly inhibited LPS-mediated NO (Fig. 8A), TNF-α (Fig. 8B) and IL-1β (Fig. 8C) production. These results suggested that DOME-mediated inactivation of ERK, JNK and p38 MAPK may be, at least in part, responsible for the suppression of NO, IL-1β and TNF-α in RAW264.7 cells. In addition, LPS stimulation induced an increase in the activation of NF-κB, whereas treatment with DOME inhibited this effect. The activation is consistent with the results of western blotting for NF-κB. Furthermore, LPS stimulation may cause translocation of NF-κB into nucleus.

Discussion

Inflammation is a host response against diverse injuries. Macrophage cells regulate inflammatory mediators, including NO, PGE$_2$, IL-1β, and TNF-α (21). A previous study has indicated that certain inducible enzymes, including iNOS and COX-2, cytokines (IL-1β and TNF-α), and their response products are involved in chronic inflammatory sickness (22). iNOS is involved with inflammation, and its reaction product involved in a wide range of conditions; for example, psoriasis and atopic inflammation. COX-2, an inducible isoform of COX, is upregulated in inflammation (23,24). Therefore, to assess the biological activity of DOME and to estimate its effects against allergic inflammation, the present study examined the anti-inflammatory effect of DOME in LPS-induced RAW264.7 macrophage cells. DOME inhibited the production of NO and PGE$_2$ in LPS-induced RAW264.7 cells.

Therefore, DOME may mediate iNOS and COX-2 activity. Numerous pro-inflammatory cytokines, including TNF-α and IL-1β, additionally contribute to inflammatory reactions; therefore, inhibition of their activity is a key factor to estimate the effectiveness of anti-inflammatory medicines. In the present study, DOME considerably suppressed the production of the pro-inflammatory mediators TNF-α, and IL-1β in LPS-stimulated RAW264.7 cells. Therefore, DOME may have an anti-inflammatory function (25,26).

NF-κB regulates the expression of genes encoding pro-inflammatory inducible enzymes, including COX-2, iNOS and other inflammatory-associated proteins (8,27,28). In its inactive form, NF-κB forms a complex with IκB kinase. Upon stimulation, this composite is broken down and NF-κB is activated, translocating into the nucleus and controlling transcription of a large number of inflammatory genes (29). As expression of these pro-inflammatory mediators is known to be regulated by NF-κB (30,31), the results of the present study indicated that transcriptional inhibition of these pro-inflammatory mediators by DOME are due to blockage of the NF-κB signaling pathway. In addition, it was demonstrated that translocation of activated NF-κB p65 to the nucleus is significantly inhibited by DOME. These data indicated that DOME may inhibit NF-κB activation by suppressing translocation of the p65 subunit of NF-κB from the cytosol to the nucleus in LPS-induced RAW264.7 cells. DOME, an inhibitor of MAPKs, markedly suppressed the nuclear translocation of NF-κB p65 by LPS in RAW264.7 cells, suggesting that the MAPK signaling pathway may serve a central role in upregulation of NF-κB.

MAPKs are a family of serine/threonine protein kinases responsible for the majority of cellular responses to external stress signals and cytokines, and are important for regulation of the production of inflammation mediators (32,33). The MAPK superfamily have been extensively studied due to their consistent activation by pro-inflammatory cytokines and to their involvement in nuclear signaling. This superfamily involves JNKs, ERKs, and p38 MAPKs (additionally known as cytokine suppressive binding protein). ERKs are activated by growth factors and mitogenic stimuli, whereas p38 and JNK are regulated by stress-inducing signals and pro-inflammatory cytokines (30,34). Therefore, this signaling pathway may be a potential therapeutic target for the treatment of inflammatory diseases (35). In the present study, rapid phosphorylation of JNK, ERK1/2 and p38 MAPK induced by LPS stimulation in RAW264.7 macrophage cells were suppressed by DOME in a dose-dependent manner, suggesting that DOME may inhibit the MAPK signaling cascade. Treatment with DOME, the p38 MAPK inhibitor SB203580, the JNK inhibitor SP600125 and the ERK inhibitor PD98059 blocked LPS-activated phosphorylation of MAPKs to baseline values. Furthermore, these inhibitors reduced JNK, ERK and p38 phosphorylation by ~50%.

In conclusion, treatment with DOME synergistically inhibited inflammatory mediators in LPS-induced RAW264.7 cells, which may be attributed to down regulation of iNOS and COX-2. These effects were considered to be associated with suppression of MAPK signaling pathway molecules, resulting in inhibition of NF-κB activation and its nuclear translocation. Therefore, these results implicate DOME as a potential novel therapeutic approach for the treatment of inflammatory diseases.

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