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# Anti-inflammatory activity of 4-methoxyhonokiol is a function of the inhibition of iNOS and COX-2 expression in RAW 264.7 macrophages via NF-κB, JNK and p38 MAPK inactivation

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ABSTRACT

The extracts or constituents from the bark of *Magnolia* (*M.*) *obovata* are known to have many pharmacological activities. 4-Methoxyhonokiol, a neolignan compound isolated from the stem bark of *M. obovata*, was found to exhibit a potent anti-inflammatory effect in different experimental models. Pretreatment with 4-methoxyhonokiol (i.p.) dose-dependently inhibited the dye leakage and paw swelling in an acetic-acid-induced vascular permeability assay and a carrageenan-induced paw edema assay in mice, respectively. In the lipopolysaccharide (LPS)-induced systemic inflammation model, 4-methoxyhonokiol significantly inhibited plasma nitric oxide (NO) release in mice. To identify the mechanisms underlying this anti-inflammatory action, we investigated the effect of 4-methoxyhonokiol on LPS-induced responses in a murine macrophage cell line, RAW 264.7. The results demonstrated that 4-methoxyhonokiol significantly inhibited LPS-induced NO production as well as the protein and mRNA expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Furthermore, 4-methoxyhonokiol inhibited LPS-mediated nuclear factor-κB (NF-κB) activation via the prevention of inhibitor κB (IκB) phosphorylation and degradation. 4-Methoxyhonokiol had no effect on the LPS-induced phosphorylation of extracellular signal-regulated kinase (ERK), whereas it attenuated the phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun $NH_2$-terminal kinase (JNK) in a concentration-dependent manner. Taken together, our data suggest that 4-methoxyhonokiol is an active anti-inflammatory constituent of the bark of *M. obovata*, and that its anti-inflammatory property might be a function of the inhibition of iNOS and COX-2 expression via down-regulation of the JNK and p38 MAP kinase signal pathways and inhibition of NF-κB activation in RAW 264.7 macrophages.



## 1. Introduction

Inflammation is a beneficial host response to external challenge or cellular injury that leads to the release of a complex array of inflammatory mediators, finalizing the restoration of tissue structure and function. However, prolonged inflammation can be harmful, contributing to the pathogenesis of many diseases. Macrophages are potent secretory cells that release an array of mediators, including proinflammatory and cytotoxic cytokines and growth factors, bioactive lipids, hydrolytic enzymes, reactive oxygen intermediates, and nitric oxide (NO), all of which have been implicated in the pathogenesis of tissue injury (Laskin and Pendino, 1995). Activated macrophages transcriptionally express inducible nitric oxide synthase (iNOS), which catalyzes the oxidative deamination of L-arginine to produce NO, and is responsible for prolonged and profound production of NO (Xie and Nathan, 1994). High-output NO by iNOS can provoke deleterious consequences such as septic shock and inflammatory diseases (Titheradge, 1999; Zamora et al., 2000). Prostaglandins also play a major role as mediators of the inflammatory response. The rate-limiting enzyme in the synthesis of prostaglandins is cyclooxygenase (COX). Two isoforms of COX have been found: COX-1 and COX-2. COX-2 is induced by several stimuli, and is responsible for the production of large amounts of pro-inflammatory prostaglandins at the inflammatory site (Lee et al., 1992). Based on these observations, it has been hypothesized that inhibiting high-output NO and prostaglandins production in macrophages, by blocking iNOS and COX-2 expressions or their activities, could serve as the basis for the potential development of anti-inflammatory drugs.

Nuclear factor-κB (NF-κB) is an important transcription factor complex that regulates the expression of many genes involved in immune and inflammatory responses (Kopp and Ghosh, 1995). In

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unstimulated cells, NF-κB is constitutively localized in the cytosol as a heterodimer by physical association with an inhibitory protein called inhibitor κB (IκB) (Baeuerle and Baltimore, 1988; Baldwin, 1996). Many stimuli, including lipopolysaccharides (LPS), cytokines, activators of protein kinase C, oxidants, and viruses, activate NF-κB via several signal transduction pathways that all lead to phosphorylation of IκB. Following activation, the NF-κB heterodimer is rapidly translocated to the nucleus, where it activates the transcription of target genes, including the genes encoding the pro-inflammatory cytokines, adhesion molecules, chemokines, and inducible enzymes such as COX-2 and iNOS (Pahl, 1999). In turn, the products regulated by NF-κB, such as tumor necrosis factor-α and interleukin-1β, also lead to the activation of NF-κB. This means that there is a complex regulatory loop that amplifies and perpetuates inflammatory responses. Because NF-κB plays such a pivotal role in the amplifying loop of the inflammatory response, it has become a logical target for new types of anti-inflammatory treatment.

The mitogen-activated protein kinases (MAPKs) are a group of signaling molecules that play a critical role in the regulation of cell growth and differentiation, as well as in the control of cellular responses to cytokines and stresses. These "classical" MAPKs are extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun $NH_2$-terminal kinase (JNK) (Davis, 1994; Han et al., 1994; Nishida and Gotoh, 1993). Phosphorylation of MAPKs is known to be a critical component of production of NO and pro-inflammatory cytokines in activated macrophages (Ajizian et al., 1999; Carter et al., 1999). Also, it has been demonstrated that the specific MAPK inhibitors suppress the expression of the iNOS gene (Chan and Riches, 1998; Chen et al., 1999). Moreover, several studies have shown that MAPKs play critical roles for the activation of NF-κB (Carter et al., 1999; Nakano et al., 1998).

The stem bark of *Magnolia obovata* Thunb as well as that of *Magnolia officinalis* Rhed et Wils have been used in traditional Chinese medicine for treatment of thrombotic stroke, typhoid fever, fever, and headache (Maruyama and Kuribara, 2000). Honokiol, a small-molecular-weight neolignan originally isolated from *M. officinalis*, has been demonstrated to exhibit anti-inflammatory and anti-cancer properties (Chiang et al., 2006; Son et al., 2000). In our ongoing screening of medicinal plants for potential anti-inflammatory compounds, 4-methoxyhonokiol, another neolignan isolated from the stem bark of *M. obovata*, was found to exhibit a potent *in vivo* anti-inflammatory effect in different experimental models. The mechanisms underlying this anti-inflammatory action was investigated in LPS-induced responses in a murine macrophage cell line, RAW 264.7. The results demonstrated that 4-methoxyhonokiol significantly inhibits LPS-induced NO production as well as the expressions of iNOS and COX-2 via down-regulation of the JNK and p38 MAP kinase signal pathways and inhibition of NF-κB activation.

## 2. Materials and methods

### 2.1. Materials

UV spectra were obtained with a Beckman Du-650 UV/VIS recording spectrophotometer. IR spectra were recorded on a Jasco Report-100 infrared spectrometer. $^1$H NMR (300 MHz) and $^{13}$C NMR (75 MHz) were recorded on Bruker DRX300 and JEOL 400 spectrometers. For column chromatography, silica gel (Kieselgel 60, 70–230 mesh and 230–400 mesh, Merck) was used. Thin layer chromatography (TLC) was performed on precoated silica gel 60 F254 (0.25 mm, Merck). LPS (*Escherichia coli* serotype 0127:B8), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), dexamethasone, Evans blue, carrageenan from seaweed (a mixture of lambda and kappa-carrageenans), 2,3-diaminonaphthalene (DAN), SB202190, SP600125, and U0126, *N*-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies against iNOS, COX-2, IκBα, phospho-p38, p38, phospho-JNK, JNK, and phospho-ERK1/2, as well as horseradish peroxidase (HRP)-conjugated anti-mouse and anti-goat IgG, were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Anti-ERK1/2 antibody was obtained from R&D Systems (Minneapolis, MN, USA). Antibody against phospho-IκBα was purchased from Calbiochem (San Diego, California, USA). HRP-conjugated anti-rabbit IgG were purchased from GE Healthcare (Buckinghamshire, UK).

### 2.2. Extraction and Isolation of 4-methoxyhonokiol

4-Methoxyhonokiol (purity ≥ 95.0%) was isolated from the stem bark of *M. obovata*. Briefly, the stem bark of *M. obovata* (20 kg) was extracted with methanol three times under reflux for 4 h. The methanol solutions were combined, filtered and concentrated to yield a dry methanol extract (4 kg). The methanol extract was suspended in distilled water and fractionated with hexane, ethyl acetate, and butanol to yield a hexane soluble fraction (600 g), an ethyl acetate soluble fraction (1000 g), and a butanol soluble fraction (800 g), respectively. The hexane soluble fraction was chromatographed over a silica gel column eluted with hexane-ethyl acetate (100:0–50:50) to afford nine fractions (H1–H9). Fraction H3 was chromatographed on a silica gel column eluted with hexane-ethyl acetate (50:1) to yield 4-methoxyhonokiol (8.5 g), and its chemical structure is shown in Fig. 1. 4-Methoxyhonokiol was freshly dissolved in olive oil in all *in vivo* experiments. In *in vitro* experiments, 4-methoxyhonokiol was dissolved in dimethyl sulfoxide (DMSO), and the final DMSO concentration was adjusted to 0.1% (v/v) in the culture media, similar amounts being added to the controls.

4-Methoxyhonokiol: Colorless oil; UV (Methanol): $\lambda_{max}$ nm (log $\varepsilon$) = 208, 253, 290; IR (KBr): $\nu_{max}$ = 3550, 1645, 1610; EIMS: *m/z* 280 $[M]^+$; $^1$H NMR (300 MHz, $CDCl_3$) δ: 3.35, 3.45 (2H, each dd, *J* = 6 Hz, H-7, 7′), 5.09, 5.15 (4H, m, H-9, 9′), 6.01 (2H, m, H-8, 8′), 6.91 (1H, d, *J* = 8 Hz, H-5), 6.96 (1H, d, *J* = 8 Hz, H-3′), 7.06 (1H, dd, *J* = 8, 2 Hz, H-6), 7.08 (1H, d, *J* = 2 Hz, H-2), 7.26 (1H, d, *J* = 2 Hz, H-6′), 7.30 (1H, dd, *J* = 8, 2 Hz, H-4′), 3.90 (3H, s, $OCH_3$); $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 29.89 (C-7), 39.61 (C-7′), 111.19 (C-3′), 115.47 (C-9), 115.75 (C-9′), 116.02 (C-5), 128.07 (C-1′), 128.10 (C-2), 128.94 (C-3), 129.30 (C-1), 129.98 (C-4′), 130.41 (C-6′), 130.71 (C-6), 132.36 (C-5′), 136.72 (C-8), 138.01 (C-8′), 151.05 (C-2′), 157.25 (C-4), 55.74 (4-$OCH_3$).

### 2.3. Animals

ICR male mice weighing 20–25 g were purchased from Samtako Bio Korea (O San, Korea) and maintained under constant conditions (temperature: 22 ± 2 °C, humidity: 40–60%, 12-h light/12-h dark cycle). All animal studies were carried out in a pathogen-free barrier zone and in accordance with the procedure outlined in the *Guide for the Care and Use of Laboratory Animals* published by the Korea National Institute of Health.

### 2.4. Acetic-acid-induced vascular permeability test in mice

This experiment was performed using a modification of a method originally devised by Whittle (Whittle, 1964). The mice were

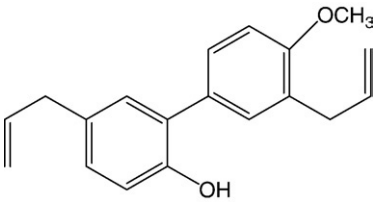


**Fig. 1.** Chemical structure of 4-methoxyhonokiol from the stem bark of *M. obovata*.

randomly divided into four groups. 4-Methoxyhonokiol (20 mg/kg and 100 mg/kg, i.p.) was administered to the test groups. The positive control group and the vehicle control group were given dexamethasone (50 mg/kg, i.p.) and the same volume of olive oil (10 ml/kg, i.p.), respectively. After 30 min, 10 ml/kg body weight of 1% Evans blue in normal saline was injected intravenously into the tail vein of each animal. Sixty minutes after administration, each mouse was injected with 10 ml/kg (i.p.) of 0.6% acetic acid in normal saline. Fifty minutes after the injection, the mice were sacrificed by cervical dislocation. Eight milliliters of normal saline was injected into the peritoneal cavity, and the washing solutions were collected in test tubes. To clear turbidity due to protein, 80 μl of 1 N NaOH solution was added to each tube, and allowed to stand overnight at 4 °C. The solution was subjected to colorimetry using a UV/VIS JASCO V-550 spectrophotometer (TS Science, Korea) at a wavelength of 610 nm. The vascular permeability effects were expressed according to the concentration of dye (μg/ml) that had leaked into the peritoneal cavity.

### 2.5. Carrageenan-induced paw edema test in mice

The method of Winter et al. was employed in this experiment (Winter et al., 1962). The mice were randomly divided into four groups. Two doses (20 mg/kg and 100 mg/kg) of 4-methoxyhonokiol were administered i.p. to the test groups, respectively. The positive control group and the vehicle control group were given dexamethasone (50 mg/kg, i.p.) and the same volume of olive oil (10 ml/kg, i.p.), respectively. Thirty minutes after the administration, acute paw edema was induced in the right hind paw by subplantar injection of 1% freshly prepared carrageenan suspension in normal saline, 0.05 ml/mouse. The thickness of the paw was measured pre-injection and at intervals of 1, 2, 3, 4 and 5 h post-injection, using a Dial Thickness Gage (Mitutoyo, Japan). The percent increase of paw thickness was calculated based on the pre-injection thickness of the paw.

### 2.6. Measurement of plasma nitrite/nitrate level in LPS-challenged mice

The mice were randomly divided into five groups, and were treated with vehicle (olive oil, 10 ml/kg, i.p.) plus vehicle for LPS (saline, 10 ml/kg, i.p.), vehicle (olive oil, 10 ml/kg, i.p.) plus LPS (10 mg/kg, i.p.), 4-methoxyhonokiol (20 mg/kg and 100 mg/kg, i.p.) plus LPS (10 mg/kg, i.p.), or dexamethasone (50 mg/kg, i.p.) plus LPS (10 mg/kg, i.p.), respectively. Olive oil, 4-methoxyhonokiol or dexamethasone was administered i.p. 30 min before the LPS (10 mg/kg, i.p.) administration. Four hours after the LPS injection, blood samples were collected by orbital puncture in the presence of heparin, and then centrifuged at 3000 rpm for 10 min at 4 °C. Plasma was obtained and stored at −80 °C until use. The concentration of plasma nitrite/nitrate was measured using the DAN assay devised by P. G. Winyard et al. (Winyard and Willoughby, 2003).

### 2.7. Cell culture

RAW 264.7 murine macrophages were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were grown in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 95% air, 5% $CO_2$ humidified atmosphere at 37 °C. The RAW 264.7 cells harboring pNF-κB-secretary alkaline phosphatase (SEAP)-neomycin phosphotransferase (NPT) reporter construct (Moon et al., 2001) were grown under the same conditions, except that the medium was supplemented with 0.5 mg/ml geneticin.

### 2.8. Cell viability assay

The effect of 4-methoxyhonokiol on cell viability was evaluated using the Cell Counting Kit-8 (CCK-8) produced by Dojindo Laboratories (Tokyo, Japan). In brief, RAW 264.7 cells were seeded onto a 96-well plate at a density of $1 \times 10^4$ cells per well and incubated at 37 °C for 24 h. The cells were then treated with various concentrations of 4-methoxyhonokiol alone or in combination with LPS (1 μg/ml). After 24 h incubation, 10 μl of the CCK-8 solution was added to the wells, and incubation was continued for another 3 h. The resulting color was assayed at 450 nm using a microplate reader (Molecular Devices, Emax, Sunnyvale, CA).

### 2.9. Nitrite assay

RAW 264.7 cells were seeded onto a 24-well culture plate at a density of $1 \times 10^5$ cells per well with 500 μl of culture medium and incubated for 24 h. The cells were then pre-treated with various concentrations of 4-methoxyhonokiol, TPCK (NF-κB inhibitor), SB202190 (p38 MAPK inhibitor), SP600125 (JNK inhibitor) or U0126 (ERK inhibitor) for 2 h before stimulation with LPS (1 μg/ml) for 18 h. The nitrite concentration in the medium was measured according to the Griess reaction, as described in our previous study (Ahn et al., 2005), and the calculated concentration was taken as the indicator of NO production.

### 2.10. Reporter gene assay

RAW 264.7 cells harboring pNF-κB-SEAP-NPT reporter construct were plated at a density of $1 \times 10^5$ cells per well in a 24-well cell culture plate with 500 μl of culture medium and incubated for 24 h. The cells were then pre-treated with 4-methoxyhonokiol for 2 h before stimulation with LPS (1 μg/ml) for 16 h. Aliquots of the cell-free culture media were heated at 65 °C for 6 min, and reacted with SEAP assay buffer [2 M diethanolamine, 1 mM $MgCl_2$, 500 μM 4-methylumbelliferyl phosphate (MUP)] in darkness at 37 °C for 1 h. The fluorescence from the product of the SEAP/MUP reaction was measured in relative fluorescence units (RFU) using a 96-well plate fluorometer (Molecular Devices, Gemini XS, Sunnyvale, CA, USA) with excitation at 360 nm and emission at 449 nm. TPCK, an inhibitor of NF-κB, were used as a positive control in this experiment.

### 2.11. Western blot analysis for iNOS, COX-2, NF-κB, IκBα, p-IκBα and MAPKs

RAW 264.7 cells were plated at a density of $1 \times 10^6$ cells per well in a 6-well cell culture plate with 2 ml of culture medium and incubated for 24 h. The cells were pre-treated with 4-methoxyhonokiol for 2 h and stimulated with LPS (1 μg/ml) for the specified time periods. The Western immunoblot conditions were as described in our previous study (Noh et al., 2006). The blots, finally, were developed for visualization using a WESTSAVEup kit (LabFrontier, Seoul, Korea) and exposed to X-ray film. The intensity of each band was quantitatively determined using UN-SCAN-IT™ software (Silk Scientific, UT, USA), the density ratio representing the relative intensity of each band against those of the controls in each experiment. TPCK (an inhibitor of NF-κB), SB202190 (an inhibitor of p38 MAPK), SP600125 (an inhibitor of JNK) and U0126 (an inhibitor of ERK) were used as positive controls for the experiment.

### 2.12. RNA extraction and reverse transcription-polymerase chain reaction

RAW 264.7 cells were pre-treated with 4-methoxyhonokiol for 2 h and stimulated with LPS (1 μg/ml) for 4 h. The total RNA was purified from the cells using Easy Blue reagent (Intron Biotechnology, Korea) and subjected to semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) using an ONE-STEP RT-PCR PreMix kit™ (Intron Biotechnology, Korea) according to the manufacturer's instructions. Oligonucleotides used for quantification of iNOS, COX-2 and β-actin transcripts, and the semi-quantitative RT-PCR conditions, were the same as previously described (Ahn et al., 2005). The amplified cDNA products were separated by 2% agarose gel electrophoresis, and stained with ethidium bromide. The gels were then viewed with UV transillumination.

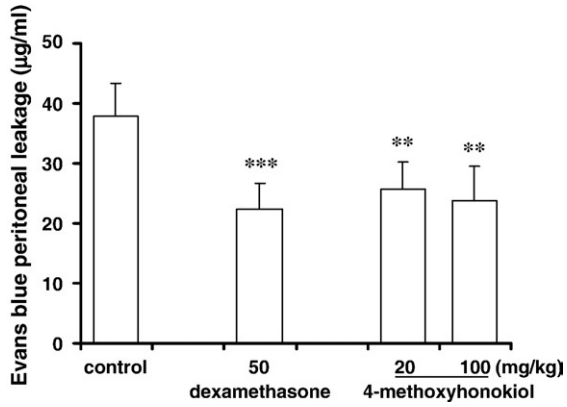


**Fig. 2.** Effect of 4-methoxyhonokiol on acetic-acid-induced vascular permeability in mice. 4-Methoxyhonokiol (20 mg/kg and 100 mg/kg, i.p.) was administered 30 min before 1% Evans blue injection. Vehicle control animals and positive control animals were treated with olive oil (10 ml/kg, i.p.) and dexamethasone (50 mg/kg, i.p.), respectively. Sixty minutes after 1% Evans blue injection, each mouse was injected i.p. with 10 ml/kg of 0.6% acetic acid. Dye leakage was determined 50 min after acetic acid injection. The concentrations of dye (μg/ml) leaked into the peritoneal cavity are shown. The values are expressed as the means±S.D. ($n$=6). $^{**}P<0.01$ and $^{***}P<0.001$ indicate significant differences from the vehicle control group.

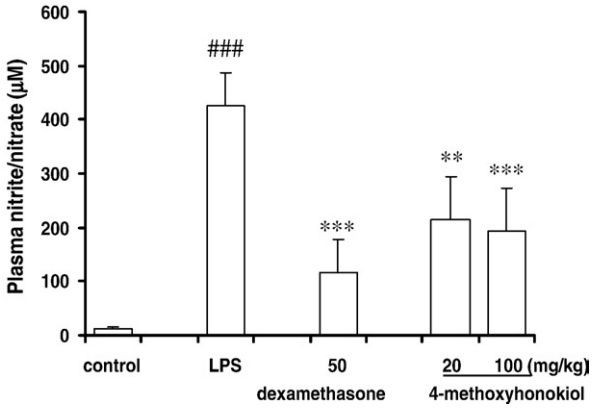


**Fig. 4.** Effect of 4-methoxyhonokiol on plasma nitrite/nitrate level in mice 4 h after LPS challenge. Different groups of mice were i.p. treated with vehicle (olive oil, 10 ml/kg) plus vehicle for LPS (saline, 10 ml/kg), vehicle (olive oil, 10 ml/kg) plus LPS (10 mg/kg), 4-methoxyhonokiol (20 mg/kg and 100 mg/kg) plus LPS (10 mg/kg), or dexamethasone (50 mg/kg) plus LPS (10 mg/kg), respectively. Four hours after LPS injection, blood samples were collected by orbital puncture in the presence of heparin, and then centrifuged at 3000 rpm for 10 min at 4 °C. Plasma was obtained and stored at −80 °C until use. The concentration of plasma nitrite/nitrate was measured using the DAN assay. The concentrations of plasma nitrite/nitrate in the LPS-challenged mice are shown. The values are expressed as the means±S.D. ($n$=5). $^{###}P<0.001$ indicates significant differences from the vehicle control group. $^{**}P<0.01$ and $^{***}P<0.001$ indicate significant differences from the LPS-treated group.

### 2.13. Statistical analysis

The values are expressed as the means±S.D. of the results of three independent experiments. A one-way analysis of variance (ANOVA) followed by Dunnett's $t$-test was applied to assess the statistical significance of the differences among the study groups. A value of $P<0.05$ was chosen as the criterion of statistical significance.

## 3. Results

### 3.1. Effect of 4-methoxyhonokiol on acetic-acid-induced vascular permeability in mice

The *in vivo* anti-inflammatory effect of 4-methoxyhonokiol was evaluated in an acetic-acid-induced vascular permeability assay, which is a typical model of first-stage inflammatory reactions. As shown in Fig. 2, at the doses of 20 mg/kg and 100 mg/kg, 4-methoxyhonokiol showed a significant and dose-dependent effect against acute inflammation induced by acetic acid, and the inhibition of dye leakage rates were 32.2% and 37.4%, respectively. As a positive control, dexamethasone (50 mg/kg, i.p.) produced a similar inhibition of dye leakage, by 40.9%.

### 3.2. Effect of 4-methoxyhonokiol on carrageenan-induced paw edema in mice

In order to further evaluate the *in vivo* anti-inflammatory effect of 4-methoxyhonokiol, a carrageenan-induced paw edema test was employed. As shown in Fig. 3, maximal edema formation was observed 1 h after 1% carrageenan injection, and treatment with 4-methoxyhonokiol dose-dependently inhibited carrageenan-induced paw swelling. In particular, treatment with 4-methoxyhonokiol at

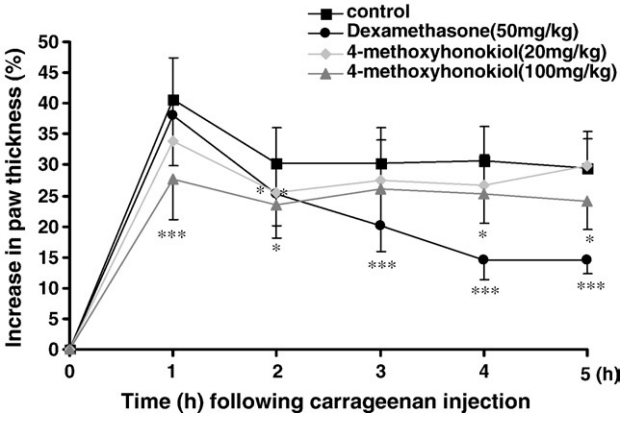


**Fig. 3.** Effect of 4-methoxyhonokiol on carrageenan-induced paw edema in mice. 4-Methoxyhonokiol (20 mg/kg and 100 mg/kg, i.p.) was administered 30 min before carrageenan injection. Vehicle control animals and positive control animals were treated with olive oil (10 ml/kg, i.p.) and dexamethasone (50 mg/kg, i.p.), respectively. Paw edema was induced 30 min later by subplantar injection of 1% carrageenan, 0.05 ml/mouse. The thickness of the paw was measured pre-injection and at intervals of 1, 2, 3, 4 and 5 h post-injection. Each point represents the percent increase of paw thickness, which was calculated based on the pre-injection thickness of the paw. The values are expressed as the means±S.D. ($n$=8). $^{*}P<0.05$ and $^{***}P<0.001$ indicate significant differences from the vehicle control group.

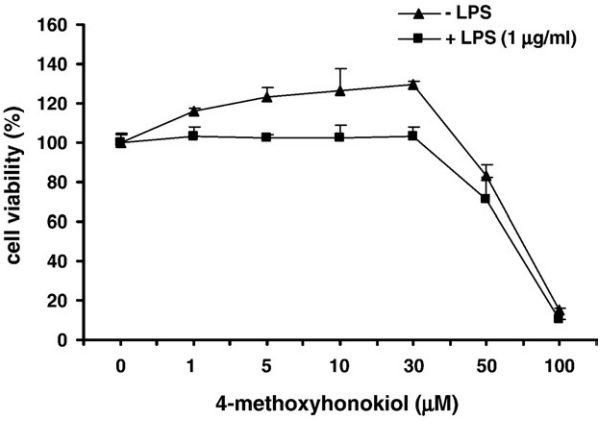


**Fig. 5.** Effect of 4-methoxyhonokiol on cell viability in RAW 264.7 macrophages. RAW 264.7 cells were cultured with the indicated concentrations of 4-methoxyhonokiol alone or in combination with LPS (1 μg/ml) at 37 °C in a 96-well plate for 24 h. Cell viability was evaluated as described in Materials and methods, and is expressed as a percentage of the control without the addition of 4-methoxyhonokiol. The values are expressed as the means±S.D. of three individual experiments.

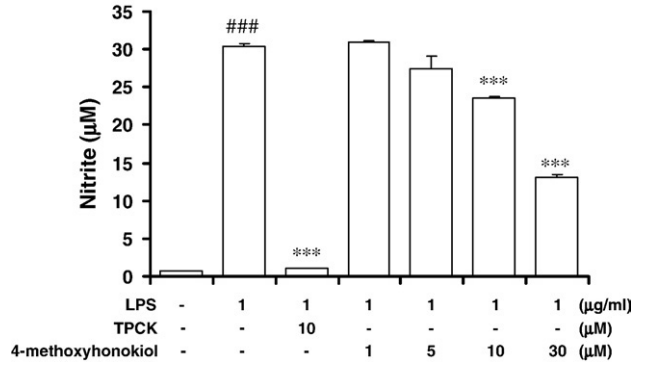


**Fig. 6.** Effect of 4-methoxyhonokiol on LPS-induced nitrite production in RAW 264.7 macrophages. RAW 264.7 cells were pretreated with the indicated concentrations of 4-methoxyhonokiol for 2 h and treated with LPS (1 μg/ml). After 18 h incubation, the amount of nitrite in the culture supernatants was measured by the Griess reaction assay, as described in Materials and methods. The values are expressed as the means ± S.D. of three individual experiments. $^{###}P<0.001$ indicates significant differences from the unstimulated control group. $^{***}P<0.001$ indicate significant differences from the LPS-treated group.

100 mg/kg (i.p.) significantly suppressed edema formation 1 h after edema induction, the inhibitory rate being 31.7%. As a positive control, dexamethasone (50 mg/kg, i.p.) produced a 33.9%–50.8% inhibition of edema development 3–5 h after the carrageenan injection (Fig. 3).

### 3.3. Effect of 4-methoxyhonokiol on plasma nitrite/nitrate level in LPS-challenged mice

As shown in Fig. 4, in the LPS group, the plasma nitrite/nitrate production 4 h after LPS (10 mg/kg, i.p.) injection was significantly higher than in the vehicle control group. Treatment with 4-methoxyhonokiol (20 mg/kg and 100 mg/kg, i.p.) significantly suppressed, in a dose-dependent manner, the increased plasma nitrite/nitrate level induced by LPS (Fig. 4).

### 3.4. Effect of 4-methoxyhonokiol on cell viability

In *in vitro* assays, we first measured the cytotoxic effect of 4-methoxyhonokiol on RAW 264.7 cells. The result showed that neither 4-methoxyhonokiol alone, at concentrations up to 30 μM, nor in the presence of LPS (1 μg/ml), affected cell viability (Fig. 5). Therefore, 4-methoxyhonokiol injections between 1 and 30 μM were used in the subsequent experiments.

### 3.5. Effect of 4-methoxyhonokiol on NO production in LPS-stimulated RAW 264.7 cells

To assess the effect of 4-methoxyhonokiol on LPS-induced NO production in RAW 264.7 cells, the cells were treated with LPS (1 μg/ml) for 18 h after treatment in the presence or absence of 4-

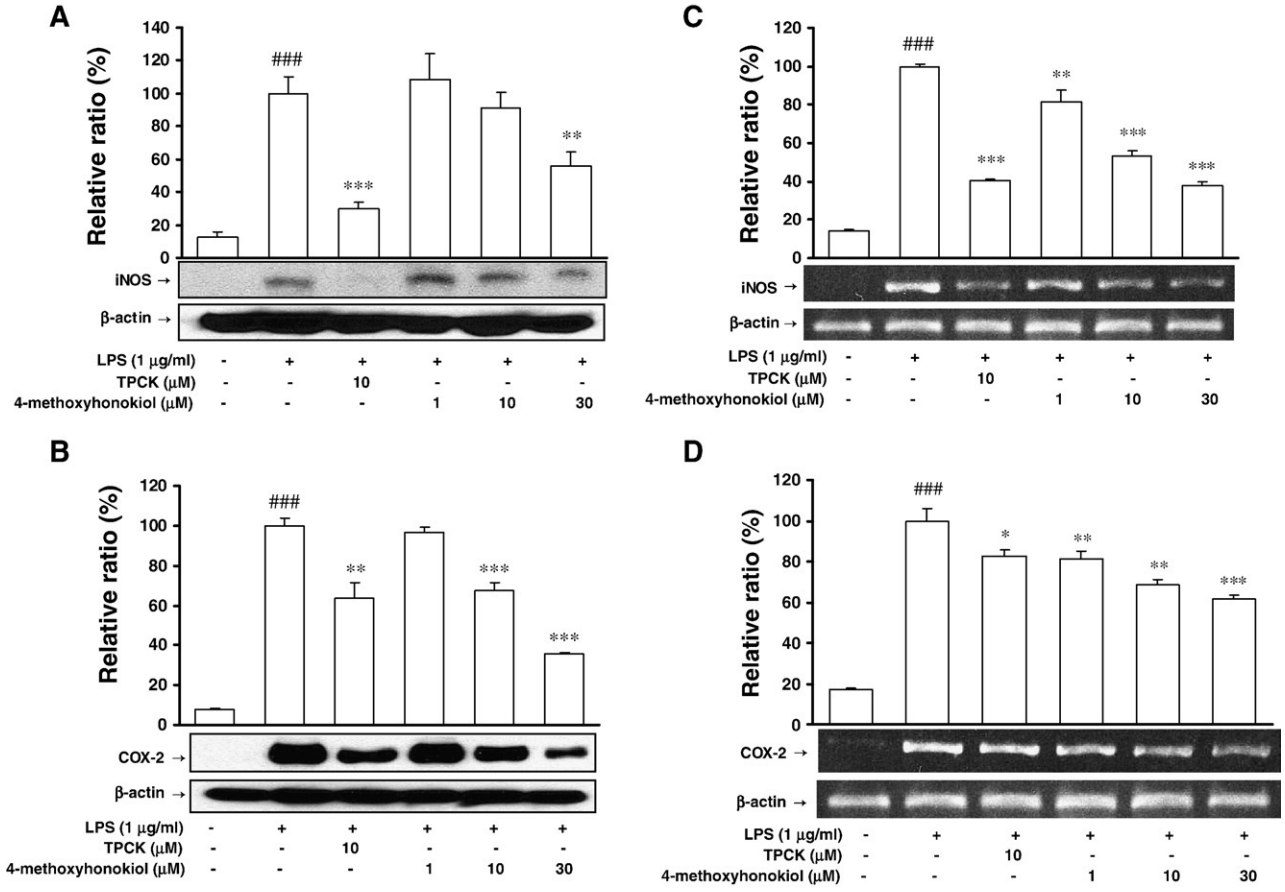


**Fig. 7.** Effect of 4-methoxyhonokiol on LPS-induced iNOS and COX-2 protein and mRNA expressions in RAW 264.7 macrophages. (A and B) RAW 264.7 cells were pretreated with the indicated concentrations of 4-methoxyhonokiol for 2 h and treated with LPS (1 μg/ml) for 18 h. Equal amounts of total protein were subjected to 8% (iNOS) and 10% (COX-2) SDS-PAGE, respectively. Expressions of iNOS and COX-2 proteins were determined by Western blot analysis. One of the similar results from three separate experiments is represented, and the relative ratio (%) is also shown, where the iNOS signal (A) and the COX-2 signal (B) were normalized to the β-actin signal. (C and D) RAW 264.7 cells were pretreated with the indicated concentrations of 4-methoxyhonokiol for 2 h and treated with LPS (1 μg/ml) for 4 h. Total RNA of the cells was subjected to semi-quantitative RT-PCR, as described in Materials and methods. One of the similar results from three separate experiments is represented, and the relative ratio (%) is also shown, where the iNOS signal (C) and the COX-2 signal (D) were normalized to the β-actin signal. The values are expressed as the means ± S.D. of three individual experiments. $^{###}P<0.001$ indicates significant differences from the unstimulated control group. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ indicate significant differences from the LPS-treated group.

methoxyhonokiol (1, 5, 10, 30 μM) for 2 h. The amount of nitrite, a stable metabolite of NO, was used as the indicator of NO production in the medium. During the 18 h incubation, RAW 264.7 macrophages in the resting state produced 0.79±0.01 μM nitrite. When LPS (1 μg/ml) was added, NO production was dramatically increased to 30.3±0.37 μM (Fig. 6). Pretreatment with 4-methoxyhonokiol inhibited LPS-induced NO production in a concentration-dependent manner, corresponding to 22.4% and 57.1% inhibition at 10 μM and 30 μM, respectively (Fig. 6).

### 3.6. Effect of 4-methoxyhonokiol on expressions of iNOS and COX-2 proteins and mRNA in LPS-stimulated RAW 264.7 cells

To investigate whether the inhibitory effect of 4-methoxyhonokiol on NO production was via inhibition of corresponding gene expression, the protein and mRNA expressions of iNOS were determined by Western blot analysis and semi-quantitative RT-PCR, respectively. Additionally, the COX-2 protein and mRNA expressions were also detected in this study. As shown in Fig. 7, in unstimulated RAW 264.7 cells, the iNOS and COX-2 protein and mRNA expressions were almost undetectable. However, upon LPS treatment, the protein and mRNA expressions of iNOS and COX-2 were markedly augmented, and pretreatment of the cells with different concentrations of 4-methoxyhonokiol attenuated LPS-induced iNOS and COX-2 protein and gene expressions in a concentration-dependent manner (Fig. 7). This data suggests that 4-methoxyhonokiol can down-regulate LPS-induced iNOS and COX-2 expression at the transcription level.

### 3.7. Effect of 4-methoxyhonokiol on LPS-mediated NF-κB transcriptional activity in RAW 264.7 cells

NF-κB transcription factor has been evidenced to play an important role in LPS-induced expression of pro-inflammatory proteins including iNOS (Xie et al., 1994). To investigate the molecular mechanism of 4-methoxyhonokiol-mediated inhibition of iNOS and COX-2 transcription, NF-κB transcription activity was investigated using a reporter gene assay system. RAW 264.7 cells were transfected stably with NF-κB-SEAP-NPT plasmid containing four copies of κB sequence fused to SEAP as the reporter (Moon et al., 2001).

LPS treatment of the transfected cells for 16 h increased the SEAP expression about 7.5-fold over the basal level (Fig. 8A). The pretreatment of cells with 4-methoxyhonokiol significantly inhibited LPS-induced SEAP expression in a concentration-dependent manner, corresponding to 22.8±0.73% at 1 μM, 30.5±1.3% at 10 μM, and 81.7±23.4% at 30 μM. As a positive control, TPCK also showed significant inhibitory effect on NF-κB transcription activity (Fig. 8A).

### 3.8. Effect of 4-methoxyhonokiol on LPS-mediated phosphorylation and degradation of IκBα in RAW 264.7 cells

Since the hyperphosphorylation of IκB and its subsequent degradation is an essential step in NF-κB activation by various stimuli, we examined, by an immunoblot analysis, the effect of 4-methoxyhonokiol on LPS-induced degradation and phosphorylation of IκBα protein. A time-course experiment showed that IκBα in the cytoplasm

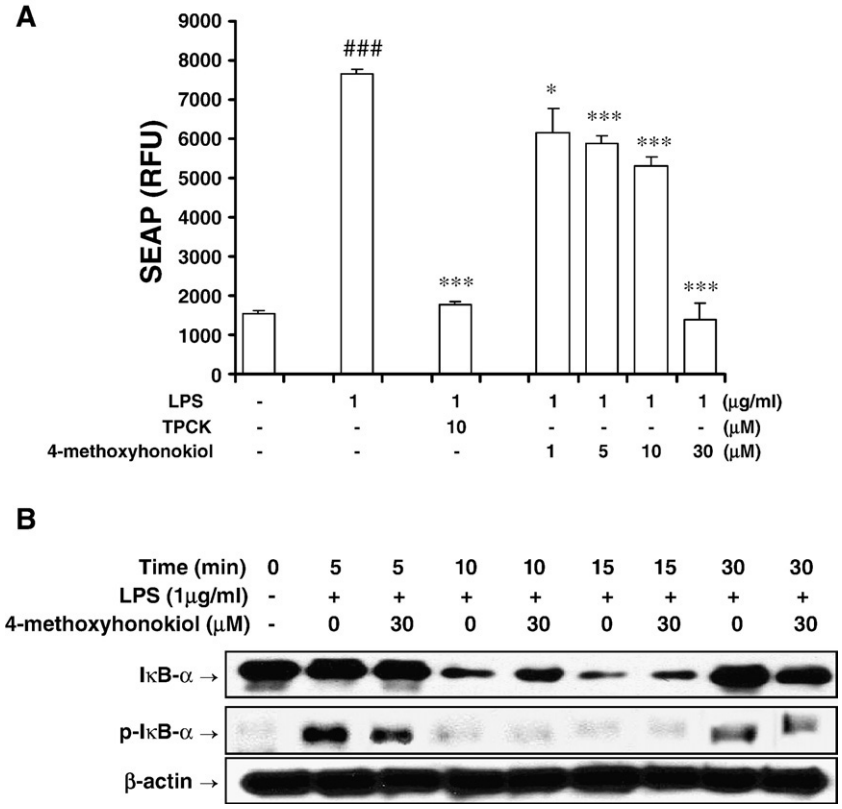


**Fig. 8.** Effect of 4-methoxyhonokiol on LPS-mediated NF-κB transcriptional activity and IκBα degradation in RAW 264.7 macrophages. (A) RAW 264.7 cells harboring NF-κB-SEAP-NPT reporter construct were pretreated with the indicated concentrations of 4-methoxyhonokiol for 2 h and treated with LPS (1 μg/ml). After 16 h incubation, the SEAP activity was measured in RFU, using a microplate fluorometer. The values are expressed as the means±S.D. of three individual experiments. $^{###}P<0.001$ indicates significant differences from the unstimulated control group. $^{*}P<0.05$ and $^{***}P<0.001$ indicate significant differences from the LPS-treated group. (B) RAW264.7 cells were pretreated with 30 μM of 4-methoxyhonokiol for 2 h and treated with LPS (1 μg/ml) for the specified time periods. The expressions of IκBα and phosphorylated IκBα protein in the cytosol extracts were determined by Western blot analysis, as described in Materials and methods. One of the similar results from three separate experiments is represented.

was almost completely degraded 15 min, and resynthesized 30 min, after LPS (1 μg/ml) stimulation (Fig. 8B). Pretreatment with 4-methoxyhonokiol prevented the induced degradation of IκBα protein at 5 and 15 min, and the resynthesis of IκBα, which is under the control of NF-κB, was also suppressed. IκBα phosphorylation was also tested by Western blot analysis. As shown in Fig. 8B, the phosphorylated forms of IκBα were hardly detectable in the resting RAW 264.7 cells. However, upon exposure to LPS (1 μg/ml) alone for 5 min, IκBα phosphorylation manifestly was initiated. At the 5 and 30 min time points after LPS stimulation, pretreatment of 4-methoxyhonokiol (30 μM) moderately inhibited LPS-mediated IκBα phosphorylation (Fig. 8B).

### 3.9. Effect of 4-methoxyhonokiol on LPS-induced activation of MAPKs in RAW 264.7 cells

The three MAPKs, ERK, p38 MAPK and JNK, are known to be activated by LPS, and several studies have demonstrated the significance of MAPKs in the transcriptional regulation of LPS-induced iNOS expression via activation of transcription factors especially NF-κB (Chen et al., 1999; Kim et al., 2004). In order to investigate whether the inhibition of NF-κB activation and NO production by 4-methoxyhonokiol is mediated through the MAPK pathway, we examined the effect of 4-methoxyhonokiol on the LPS-induced phosphorylation of JNK, p38 MAPK and ERK1/2 by using Western blot analysis. A time-course experiment showed that phosphorylation of JNK and p38 MAPK was increased from 15 min to 30 min after LPS treatment (Fig. 9A). 4-Methoxyhonokiol (30 μM) attenuated LPS-induced phosphorylation of JNK and p38 MAPK at 15 min, whereas phosphorylation of ERK was not affected (Fig. 9A). In order to further confirm this result, the cells were pretreated with different concentrations of 4-methoxyhonokiol (1, 10, 30 μM) for 2 h and stimulated with 1 μg/ml of LPS for 15 min, and the phosphorylation of MAPKs was determined by Western blot analysis. Consistent with the result of the time-course experiment, the result showed that pretreatment of the cells with 4-methoxyhonokiol inhibited LPS-induced phosphorylation of JNK and p38 MAPK in a concentration-dependent manner, whereas phosphorylation of ERK was not affected (Fig. 9). To further confirm the signaling pathway mostly involved in the inhibitory effect of 4-methoxyhonokiol on LPS-induced NO production, each specific inhibitor of MAPKs (SB202190, p38 MAPK inhibitor; SP600125, JNK inhibitor; U0126, ERK inhibitor) and the NF-κB inhibitor (TPCK) were employed. As shown in Fig. 10, pretreatment of SB202190, SP600125 and TPCK profoundly inhibited LPS-induced NO production, while pretreatment of U0126 exhibited little effect. The inhibitory effects of p38 MAPK inhibitor and JNK inhibitor significantly augmented the inhibitory effect of 4-methoxyhonokiol on the NO production (Fig. 10 A and B). The combination of 4-methoxyhonokiol with TPCK (NF-κB

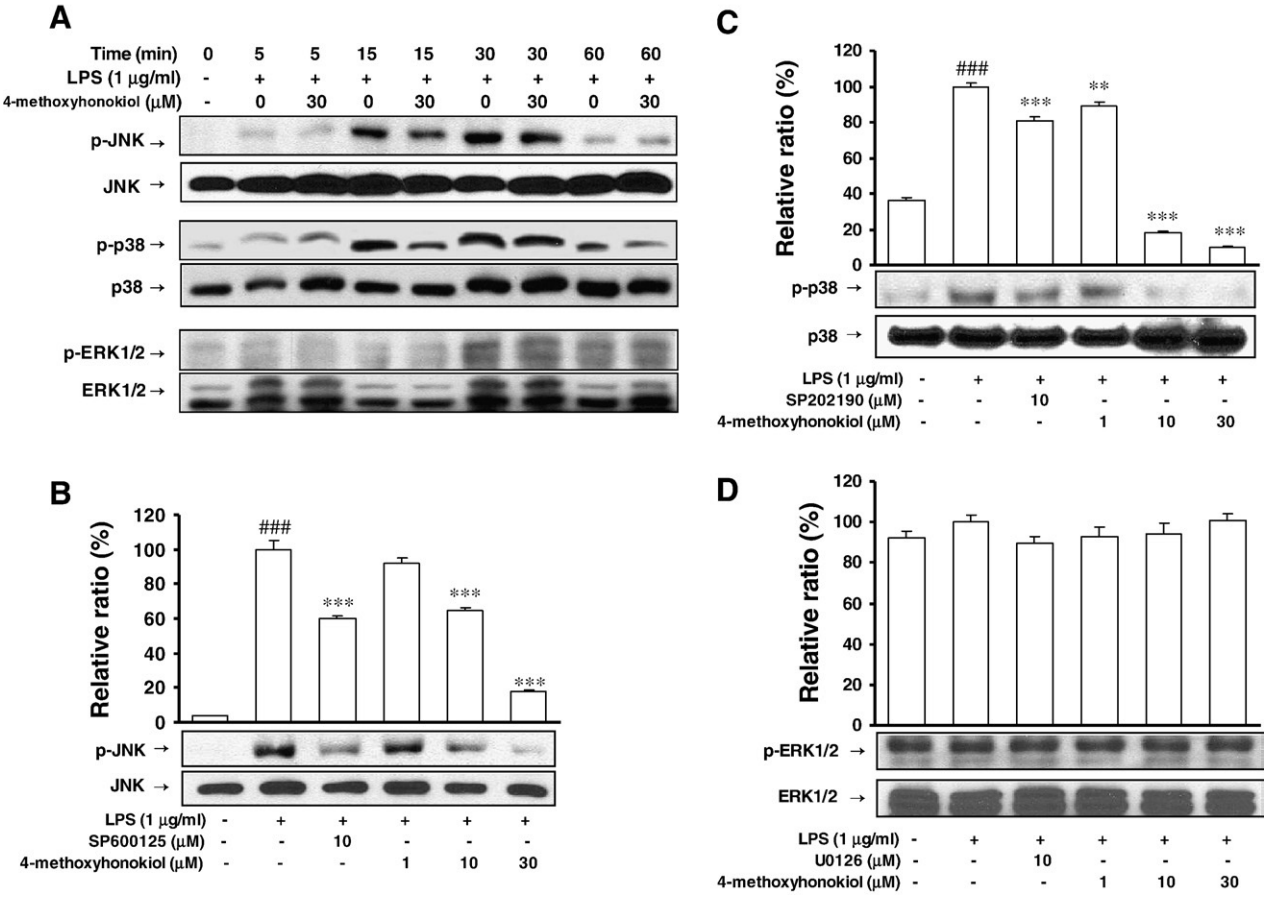


**Fig. 9.** Effect of 4-methoxyhonokiol on LPS-induced phosphorylation of MAPKs in RAW 264.7 macrophages. (A) RAW 264.7 cells were pretreated with 4-methoxyhonokiol (30 μM) for 2 h and treated with LPS (1 μg/ml) for the specified time periods. The MAPKs and phosphorylation of the MAPKs were detected by Western blot analysis using antibodies against the corresponding MAPKs and activated forms of MAPKs (phosphorylated MAPKs), as described in Materials and methods. (B, C and D) RAW 264.7 cells were pretreated with the indicated concentrations of 4-methoxyhonokiol for 2 h and treated with LPS (1 μg/ml) for 15 min. The MAPKs and phosphorylation of the MAPKs were detected by Western blot analysis using antibodies against the corresponding MAPKs and activated forms of MAPKs (phosphorylated MAPKs), as described in Materials and methods. One of the similar results from three separate experiments is represented, and the relative ratio (%) is also shown, where the p-p38, p-JNK and p-ERK signals were normalized to the p38, JNK and ERK signals, respectively. The values are expressed as the means ± S.D. of three individual experiments. $^{###}P<0.001$ indicates significant differences from the unstimulated control group. $^{**}P<0.01$ and $^{***}P<0.001$ indicate significant differences from the LPS-treated group.

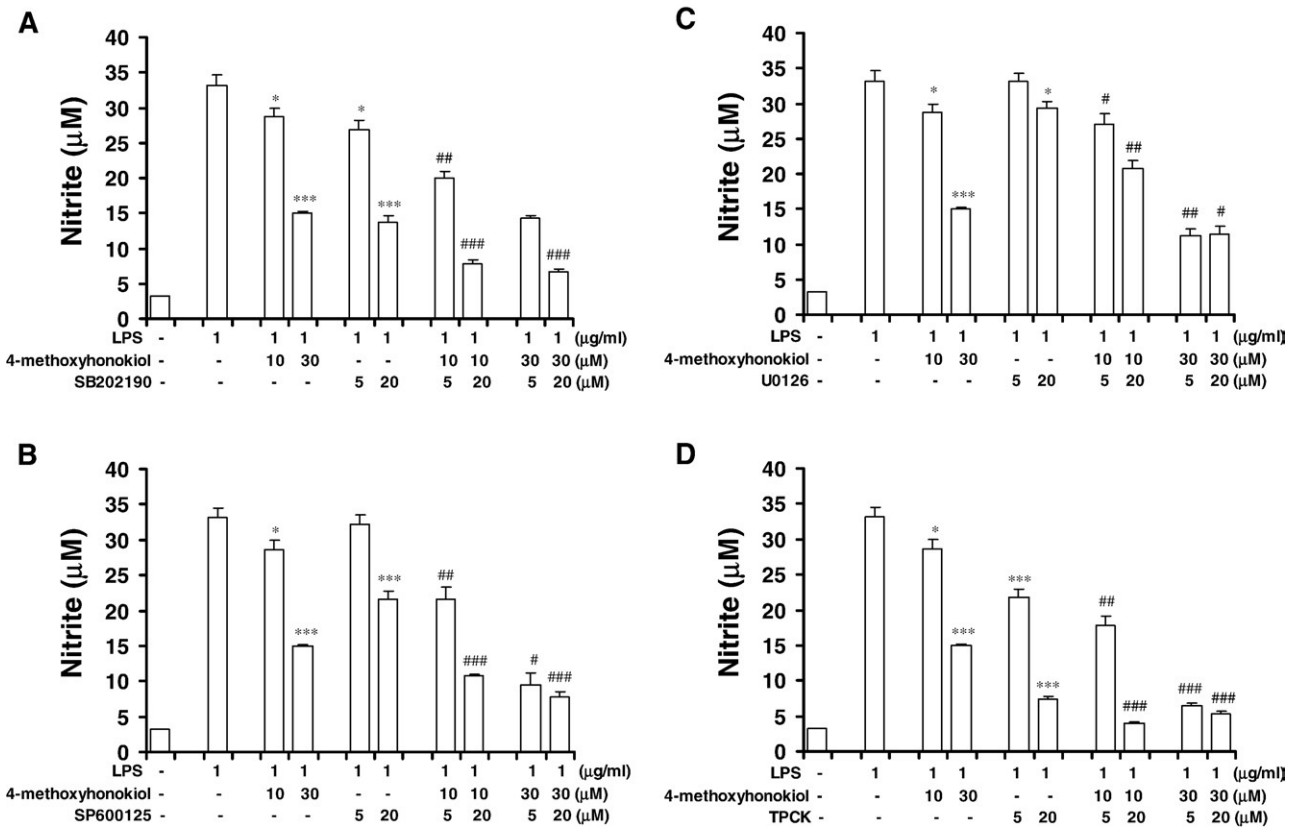


**Fig. 10.** Effects of 4-methoxyhonokiol, MAPK inhibitors and NF-κB inhibitor on LPS-induced nitrite production in RAW 264.7 macrophages. RAW 264.7 cells were pretreated with the indicated concentrations of SB202190 (A), SP600125 (B), U0126 (C) or TPCK (D) alone or with 4-methoxyhonokiol (10 or 30 μM) for 2 h and then treated with LPS (1 μg/ml). After 18 h incubation, the amount of nitrite in the culture supernatants was measured by the Griess reaction assay, as described in Materials and methods. The values are expressed as the means ± S.D. of three individual experiments. $^{*}P<0.05$ and $^{***}P<0.001$ indicate significant differences from the LPS-treated group. $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ indicates significant differences from the combination of LPS with 4-methoxyhonokiol-treated group.

inhibitor) also significantly augmented the 4-methoxyhonokiol-induced inhibition of NO production (Fig. 10 D). These results suggest that p38 MAPK and JNK conjunction with NF-κB pathway may contribute to the inhibitory effect of 4-methoxyhonol on NO production in LPS-stimulated RAW 264.7 cells.

## 4. Discussion

In our ongoing screening of medicinal plants for potential anti-inflammatory compounds, 4-methoxyhonokiol, one of the neolignans isolated from the stem bark of *M. obovata*, was found to exhibit a potent *in vivo* anti-inflammatory effect in different experimental models. Additionally, the mechanisms underlying this anti-inflammatory action were investigated in a murine macrophage cell line, RAW 264.7. Our data demonstrated that 4-methoxyhonokiol significantly inhibited NO production as well as the protein and mRNA expressions of iNOS and COX-2 in LPS-stimulated RAW 264.7 macrophages.

Increased vascular permeability is one of the essential features of the acute inflammatory response. Vascular permeability assay is a typical *in vivo* model of first-stage inflammatory reactions. Therefore, acetic-acid-induced vascular permeability was performed to investigate whether 4-methoxyhonokiol has an *in vivo* anti-inflammatory effect. The result indicated that 4-methoxyhonokiol, at the doses of 20 and 100 mg/kg (i.p.), inhibited dye leakage in vascular permeability by 32.2% and 37.4%, respectively (Fig. 2). Carrageenan-induced paw edema, a classical model of acute inflammation, has been widely used in the study of anti-inflammatory agents (Vinegar et al., 1987). The edema, or swelling, one of the cardinal signs of acute inflammation, is an important parameter to be considered when evaluating compounds with potential anti-inflammatory activity (Morris, 2003). The result obtained from the carrageenan-induced paw edema test showed that edema formation attained its maximum 1 h after edema induction, and that the treatment with 4-methoxyhonokiol dose-dependently inhibited the paw swelling induced by carrageenan (Fig. 3). These findings demonstrate that 4-methoxyhonokiol has a potent *in vivo* anti-inflammatory activity.

Septic shock is a systemic inflammatory response that develops when LPS or other microbial products stimulate the expression of various inflammatory cytokines. Recently, LPS-injected animal models have been introduced to evaluate the effects of medicine, food, or nutrition on systemic inflammation (Oberbeck et al., 2003). It has been proposed that NO production in response to LPS regulates important aspects of septic shock (MacMicking et al., 1995). Therefore, to investigate whether 4-methoxyhonokiol has a protective effect on LPS-induced systemic inflammation, the plasma nitrite/nitrate level in LPS-treated mice was examined in this study. It was found that pretreatment of mice with 4-methoxyhonokiol (20 and 100 mg/kg, i.p.) dose-dependently inhibited plasma nitrite/nitrate production in the LPS-challenged mice (Fig. 4). This finding further supports the hypothesis that 4-methoxyhonokiol has a potent anti-inflammatory activity *in vivo*.

To explore the mechanism underlying this anti-inflammatory action, the effect of 4-methoxyhonokiol on inflammation-related macrophage functions was investigated.

Macrophages play a central role in a host's defense against bacterial infection by nature of their phagocytic, cytotoxic, and intracellular killing capacities (Adams and Hamilton, 1984). Stimulation of murine

macrophages by LPS, the major component of the Gram-negative bacteria cell wall, results in the expression of iNOS (Xie and Nathan, 1994). Increased production of NO plays a critical role in the process of macrophage activation and is associated with acute and chronic inflammations. Therefore, the suppression of NO production by inhibition of iNOS expression and/or enzyme activity can be a very important therapeutic target in the development of anti-inflammatory agents. In the present study, 4-methoxyhonokiol was demonstrated to inhibit LPS-induced NO production in RAW 264.7 macrophages (Fig. 6). Similar effects have been demonstrated with other lignan compounds isolated from *Magnolial* plants (Choi et al., 2007; Matsuda et al., 2001; Son et al., 2000). The results from these studies showed that three neolignans (magnolol, honokiol and obovatol) inhibited NO production through prevention of iNOS protein expression in LPS-stimulated RAW 264.7 cells. To explore the mechanism responsible for the inhibitory effect of 4-methoxyhonokiol on LPS-induced NO production, the effect on iNOS protein and gene expressions in LPS-stimulated RAW 264.7 macrophages were examined. 4-methoxyhonokiol inhibited the expressions of iNOS protein and mRNA, as assessed by Western blot analysis and RT-PCR, respectively, in a similar dose-dependent manner (Fig. 7A and C). These results support that 4-methoxyhonokiol exerts its effect through the inhibition of the iNOS mRNA transcription step.

NO production by iNOS is regulated mainly at the transcriptional level, and the expression of the iNOS gene in macrophages is under the control of several transcription factors, which include NF-κB (Xie et al., 1994). The *cis*-acting NF-κB element's presence has been demonstrated in the 5′ flanking regions of both the COX-2 and iNOS genes (Kujubu et al., 1991; Lowenstein et al., 1993). NF-κB is functional as a hetero- or homo-dimeric form of the Rel family proteins, including RelA (p65), RelB, cRel, p50 and p52 (Baeuerle and Baltimore, 1996). In resting cells, cytoplasmic IκB proteins (IκBα, IκBβ, and IκBε) are associated with NF-κB dimmers, and some stimuli, such as IL-1 and LPS, lead to prolonged activation of IKK lasting 2 h or more. Once IκBα is phosphorylated and degraded, NF-κB translocates to the nucleus and activates gene transcription, including that of the IκBα gene. In this study, the result demonstrated that 4-methoxyhonokiol concentration-dependently inhibited LPS-mediated NF-κB transcriptional activity in a reporter gene assay (Fig. 8A). Additionally, determination of IκBα protein by Western blot analysis showed that pretreatment with 30 μM of 4-methoxyhonokiol prevented the induced degradation and resynthesis of IκBα protein. Meanwhile, pretreatment with 30 μM of 4-methoxyhonokiol moderately inhibited the phosphorylation of IκBα 5 and 30 min after LPS stimulation (Fig. 8B). Therefore, these results suggest that 4-methoxyhonokiol inhibits the expression of iNOS and COX-2, and thus NO production through inactivation of NF-κB by reducing IκBα degradation and phosphorylation.

MAPKs are a highly conserved family of protein serine/threonine kinases and include the p38, ERK1/2, and JNK subgroups (Garrington and Johnson, 1999). Several studies have shown that the activation of MAPKs are involved in LPS-induced iNOS expression and NF-κB activation (Carter et al., 1999; Nakano et al., 1998). To further investigate the mechanisms of NF-κB inactivation and NO inhibition by 4-methoxyhonokiol, the effects of 4-methoxyhonokiol on the LPS-induced phosphorylation of JNK, p38 MAPK and ERK1/2 were examined. The data demonstrated that 4-methoxyhonokiol significantly inhibited the phosphorylation of JNK and p38 MAPK but not ERK1/2 in LPS-stimulated RAW 264.7 macrophages (Fig. 9). These results suggest that the inhibition of NF-κB activation by 4-methoxyhonokiol may be due to inhibition of JNK and p38 MAPK phosphorylation. In more precise investigations by using NF-κB inhibitor and specific kinase inhibitor of each MAPK, our data further showed that the combination treatment of NF-κB inhibitor, p38 MAPK inhibitor or JNK inhibitor with 4-methoxyhonokiol resulted in a more great reduction of NO production than that by 4-methoxyhonokiol alone. Overall, our findings indicate that the inhibition of JNK and p38 MAPK phosphorylation is involved in the inhibitory effect of 4-methoxyhonokiol on LPS-induced NO production via NF-κB inactivation.

Taken together, our results suggest that 4-methoxyhonokiol is a valuable anti-inflammatory constituent of the bark of *M. obovata*. The anti-inflammatory activity of 4-methoxyhonokiol is a function of the inhibition of iNOS and COX-2 expression via down-regulation of the JNK and p38 MAP kinase signal pathways and inactivation of NF-κB. The data in the present study support the pharmacological basis of the use of the *M. obovata* plant as a traditional herbal medicine for the treatment of inflammation.

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