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Effect of 5-*O*-Methylhirsutanonol on Nuclear Factor-κB-Dependent Production of NO and Expression of iNOS in Lipopolysaccharide-Induced RAW264.7 Cells

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Diarylheptanoids are known to have anti-inflammatory and anti-atherosclerotic activities in various cell types, including macrophages. 5-*O*-Methylhirsutanonol (5-MH) isolated from the leaves of *Alnus japonica* Steud exhibited the antioxidant activities on Cu^{2+}- and AAPH-mediated low-density lipoprotein (LDL) oxidation in the thiobarbituric acid-reactive substances (TBARS) assay as well as the macrophage-mediated LDL oxidation. In the main study, we examined anti-inflammatory activities of 5-*O*-methylhirsutanonol (5-MH) on nuclear factor κB (NF-κB)-dependent nitric oxide (NO) production and expression of inducible nitric oxide synthease (iNOS) in lipopolysaccharide (LPS)-induced RAW264.7 macrophages. 5-MH inhibited NO production with an IC_{50} value of 14.5 μM and expression of both iNOS protein and iNOS mRNA in a parallel dose–response manner. Then, expression of inflammation-associated genes, such as TNF-α, COX-2, and IL-1β, was suppressed by 5-MH, as determined by reverse transcriptase polymerase chain reaction analysis. Moreover, 5-MH attenuated NF-κB activation by inhibition of hyperphosphorylation of IκB-α and its subsequent proteolytic degradation and p65 nuclear translocation, as well as preventing DNA-binding ability. In addition, 5-MH suppressed the mRNA expression of the gene reactive oxygen species (ROS) concerned in the regulation of NF-κB signaling.

KEYWORDS: Diarylheptanoids; 5-*O*-methylhirsutanonol (5-MH); nitric oxide (NO); inducible nitric oxide synthease (iNOS); nuclear factor κB (NF-κB); reactive oxygen species (ROS)

INTRODUCTION

In general, it has been known that nitric oxide (NO) and reactive oxygen species (ROS) are responsible for regulation on the transcriptional pathways of NF-κB (*1, 2*). NO is a free radical produced from L-arginine by a catalytic reaction of two types of constitutive or inducible NOS and an important intercellular signal messenger (*3*). NO plays a dual role as a beneficial or detrimental molecule in the process of inflammation; namely, a small amount of NO produced by constitutive endothelial NOS (eNOS) and neuronal NOS (nNOS) is an important regulator of physiological homeostasis in reducing blood pressure, inhibition of platelet aggregation, and regulating neuronal transmission (*4, 5*). However, high output production of NO by inducible nitric oxide synthease (iNOS) reacts with macrophage-derived superoxide to produce highly cytotoxic peroxynitrite, which contributes to a variety of diseases and inflammation (*3, 4*). The expression of genes encoding iNOS was regulated by nuclear factor κB (NF-κB) in macrophages. NF-κB is a dimer of members of the Rel family proteins, including p65, c-Rel, and p50. In most unstimulated cells, NF-κB covalently bound to inhibitor protein, IκB-α, which is sequestered in the cytoplasm (*6*). Exposure of the cells to external stimuli, such as inflammatory cytokines, oxidative stress, ultraviolet irradiation, or bacterial endotoxins, results in the activation of NF-κB through the stimulation of the phosphorylation of IκB-α and its subsequent proteolytic degradation (*7, 8*). The activated NF-κB is then translocated to the nucleus, where it binds to the *cis*-acting κB enhancer element of target genes and regulates the expression various inflammatory cytokines, such as interleukin-1, -2, -6, and -8 and TNF-α,

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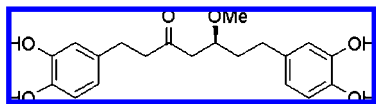

Figure 1. Chemical structure of 5-MH.

as well as proinflammatory mediators, iNOS, cyclooxygenase-2 (COX-2), and cell-adhesion molecules (*9*).

Among several NF-κB inhibitors, it was well-reported recently that NF-κB inhibitors having antioxidant activity attenuate atherosclerotic lesions by its supplementation in apoE/LDLR-double and/or apoE-deficient mice (*10, 11*). Diarylheptanoids, which are classified into linear and cyclic types, are known to have a variety of biological activities, such as PKC inhibitory, antifungal, prostaglandin biosynthesis inhibitory, antihepatotoxic, COX-2 inhibitory (*12, 13*), and iNOS inhibitory effects (*14, 15*). Among them, 5-*O*-methylhirsutanonol (5-MH) was first reported by Kuroyanagi et al. and showed simply superoxide and DPPH radical-scavenging activities (*16*). Recently, we also have shown that three diarylheptanoids, oregonin, hirsutanone, and 5-MH, isolated from *Alnus japonica* Steud, exhibited potent antioxidant activity against low-density lipoprotein (LDL) oxidation (*17, 18*). *A. japonica* Steud is a Betulaceous tree found in damp areas of mountain valleys and has been used in traditional Asian medicine as remedies against fever, hemorrhage, diarrhea, and alcoholism (*19*). In this study, we examined the effects of 5-MH (**Figure 1**) on lipopolysaccharide (LPS)-mediated NO, iNOS, and NF-κB activation in murine RAW264.7 macrophages.

MATERIALS AND METHODS

General Experimental Materials. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco-BRL (Gaithersburg, MD). LPS and ammonium pyrrolidine dithiocarbamate (PDTC) were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies against iNOS and Actin were obtained from BD Transduction Laboratories (Lexington, KY) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. 2′,7′-Dichlorofluorescein diacetate ($DCFH_2$-DA) was purchased from Molecular Probe (Eugene, OR).

Isolation of 5-MH. Dried leaves of *A. japonica* (1.0 kg) were extracted with 100% MeOH (4 L) at room temperature for 2 weeks. After filtration, the methanol was evaporated and the remaining extracts were suspended in water and further partitioned with *n*-hexane, then $CHCl_3$, and finally, EtOAc. These extracts were dried over anhydrous sodium sulfate. Filtration and evaporation gave three fractions, *n*-hexane-extracted (35.2 g), $CHCl_3$-extracted (25.4 g), and EtOAc-extracted (30.0 g) residues. Because the EtOAc extract showed the highest inhibitory activity against LDL oxidation (85% inhibition at 40 μg/mL), a part of the EtOAc extract (13 g) was subjected to column chromatography employing silica gel (70–230 mesh, Merck, 500 g) and elution with a step gradient of $CHCl_3$/MeOH (98:2, 97:3, 95:5, 10:1, 5:1, 3:1, 1:1 (v/v), and 100% MeOH, 1.5 L of each). On the basis of thin-layer chromatography analysis, fractions were monitored by the thiobarbituric acid-reactive substances (TBARS) assay. The collected active fraction (95:5–10:1 $CHCl_3$/MeOH, 193 mg) was applied to a silica gel column (70–230 mesh; Merck, 150 g) with a step gradient of $CHCl_3$/MeOH (100:0, 97:3, 96:4, 95:5, 10:1, 5:1, 3:1, 1:1 (v/v), and 100%, MeOH, 100 mL of each) to give nine fractions. The active fractions (5:1−3:1 $CHCl_3$/MeOH, fractions 6 and 7, 80 mg) with strong LDL-antioxidant activity were finally purified by preparative reverse-phase high-performance liquid chromatography [HPLC, YMC Hydrosphere C18 (5 μM, 20 × 250 mm), 50% (v/v) aqueous MeOH at 280 nm, 4 mL/min] to yield 5-MH (54 mg; t_R = 40 min). The structural identity of the 5-MH has been characterized by its spectroscopic analysis. Amorphous powder. HR FAB MS, *m/z* $[M + Na]^+$: 360.1573; calcd, 360.1573 for $C_{20}H_{24}O_6$. IR cm^{-1}: 3419 (OH), 1652 (CO). ^1H NMR (DMSO-d_6, 500 MHz) δ: 8.69 (4H, br, 3′-, 3″- 4′-, 4″-OH), 6.61 (1H, dd, J = 4.0, 7.9 Hz, H-5″), 6.59 (1H, dd, J = 4.0, 7.9 Hz, H-5′), 6.55 (1H, d, J = 2.6 Hz, H-2″), 6.53 (1H, d, J = 2.6 Hz, H-2′), 6.40 (1H, dd, J = 2.7, 7.8 Hz, H-6″), 6.39 (1H, dd, J = 2.0, 7.8 Hz, H-6′), 3.61 (1H, m, H-5), 3.22 (3H, s, H-8), 2.63 (2H, m, H-4), 2.57 (2H, m, H-2), 2.50 (2H, m, H-1), 2.38 (2H, m, H-7), 1.56−1.62 (2H, m, H-6).

Detection of LDL Oxidation. Blood was collected from normalipidaemic volunteers with permission according to the "Guidelines of Blood Donation Program for Research" of the Korea Red Cross Blood Center, and LDL was isolated from the plasma by preparative ultracentrifugation as described (*17*). The extent of LDL oxidation on copper- and 2,2′-azobis(2-amidinopropane)hydrochloride (AAPH)-mediated LDL oxidation was measured by the TBARS assay (*20*). To examine the effect of 5-MH on macrophage-mediated LDL oxidation, THP-1 macrophages, which were differentiated with phorbol 12-myristate 13-acetate (PMA, 150 ng/mL, Sigma) for 3 days, were incubated with 100 μg/mL LDL in the serum-free culture medium with or without 5-MH, supplemented with 2 μM $CuSO_4$ for 24 h at 37 °C. The content of the MDA-like products was also determined directly in the harvested medium using the TBARS assay (*21*).

Cell Culture. RAW264.7 cells (murine macrophage cell line) obtained from American Type Culture Collection (Manassas, VA) were cultured in DMEM containing 2 mM L-glutamine, 100 unit/mL of penicillin, 100 μg/mL streptomycin, and 10% (v/v) heat-inactivated FBS at 37 °C in a humidified incubator with 5% CO_2/95% air. After 18 h of incubation, cells were treated with or without 5-MH for 2 h, followed by incubation with 1 μg/mL LPS.

The cytotoxicity of 5-MH was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW264.7 ells were seeded at 5000 cells/well into 96-well culture plates (NUNC) and grown for 24 h. The cells were incubated with various concentrations of 5-MH in DMEM containing 0.4% FBS for 72 h, and then 10 μL of MTT was added to each well and further incubated at 37 °C for 4 h. The absorbance of the solubilized formazan by the addition of 100 μL of solubilization solution (0.1 N HCl, 10% SDS) was measured at 490 nm using in the model 680 Microplate reader (Bio-RAD, Hercules, CA). Cells incubated in control media were considered 100% viable.

Determination of NO Production and Expression of iNOS and TNF-α RAW264.7 cells were plated at 1 × 10^6 cells/mL and treated with or without 5-MH for 2 h, followed by incubation with 1 μg/mL LPS for 18 h. Nitrite accumulation, as an indicator of NO production, was measured in the medium using the Griess reagents (*22*). Total cell lysates were prepared by suspending 1 × 10^6 cells in 100 μL of lysis buffer [0.1 mM sodium orthovanadate, 140 mM NaCl, 15 mM ethylene glycol-bis(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 15 mM $MgCl_2$, 0.1% Triton X-100, 100 μM phenylmethylsulfonyl fluoride (PMSF), and 20 μM leupeptin at pH 7.2] and incubated for 30 min at 4 °C. The cytoplasmic extracts were centrifuged at 15000*g* for 10 min. The expression of iNOS in the cell lysates were measured as described (*23*) by Western blot analysis.

The cytoplasmic and efflux levels of TNF-α were quantified using an enzyme-linked immunosorbent assay (ELISA) kit (R&D system, MN) according to the protocol of the manufacturer. RAW264.7 cells were preincubated with or without different concentrations of 5-MH for 2 h in a 6-well plate and activated by 1 μg/mL LPS for 12 h. Total cell lysates were prepared by lysis buffer, and then cell lysate and medium were diluted as necessary prior to assay. Each sample was triplicated, and three independent measurements were performed.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis. RAW264.7 cells were treated with or without 5-MH for 2 h, followed by incubation with 1 μg/mL LPS for 10 h. The cells were harvested, and total RNA was isolated by RNeasy mini columns (Qiagen, Santa Clarita, CA) according to the instructions of the manufacturer. A sample (1 μg) of total RNA was used for the synthesis of the first strand cDNA using the Omniscript (Qiagen, Santa Clarita, CA) according to the instructions of the manufacturer. For amplification of iNOS, COX-2, TNF-α, IL-6, and IL-1β, the following primers were used: for iNOS, 5′-TTT GGA GCA GAA GTG CAA AGT CTC-3′ (sense), 5′-GAT CAG GAG GGA TTT CAA AGA CCT-3′ (antisense); for COX-2, 5′-AAC CGT GGG GAA TGT ATG AGC A-3′ (sense), 5′-AAC TCT CTC CGT AGA AGA ACC TTT TCC A-3′ (antisense);

for TNF-α, 5′-CTC AGA TCA TCT TCT CAA AAT TCG AGT GAC A-3′ (sense), 5′-CTT CAC AGA GCA ATG ACT CCA AAG T-3′ (antisense); for IL-6, 5′-TGG AGT CAC AGA AGG AGT GGC TAA G-3′ (sense), 5′-CAT CTG GCT AGG TAA CAG AAT ATT TAT ATC-3′ (antisense); for IL-1β, 5′-ATG GCA ACT GTT CCT GAA CTC AAC T-3′ (sense), 5′-ATA TTC TGT CCA TTG AGG TGG AGA GCT-3′ (antisense). For PCR amplification, the following conditions were used: 95 °C for 2 min for one cycle; 95 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min for 20–33 cycles. The amplified PCR products were separated on 1.2% agarose gel and then stained with ethidium bromide.

NF-κB Activity Assay. NF-κB activity was determined using RAW264.7 cells stably transfected with a plasmid containing 8 copies of κB elements linked to a secreted alkaline phosphatase (SEAP) gene (*24*). The cells were kindly provided by Dr. Jung Joon Lee (KRIBB, Daejeon, Korea). NF-κB activity was performed as described (*25*).

Western Blot Analysis. RAW264.7 cells were treated with or without 5-MH for 2 h and subsequently stimulated with 1 μg/mL LPS for 0–1 h. Cytosolic extracts were prepared in hypotonic buffer [10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.6), 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, and 0.1 mM PMSF]. Aliquots containing 50 μg of total protein were separated on 10% sodium dodecyl sulfate (SDS)−polyacrylamide gels and transferred to membranes. Each membrane was blocked overnight at 4 °C with blocking solution [10 mM Tris-HCl (pH 7.4), 125 mM NaCl, 0.1% Tween 20, and 5% skim milk] and then incubated with anti-IκB-α, anti-phospho-IκB-α, and NF-κB p65 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. The blots were washed 3 times with washing buffer (20 mM Tris, 160 mM NaCl, and 0.1% Tween 20), followed by 1 h of incubation with the appropriate horseradish peroxidase-conjugated secondary antibody. The peroxidase bound to the blot was detected using the ECL Western blot kit (Elipis Biotech, Korea). This immunoblot analysis was performed 3 times independently.

Nuclear Extracts and Gel Electromobility Shift Assay (EMSA). RAW264.7 cells were treated with or without 5-MH and subsequently stimulated with 1 μg/mL LPS for 2 h, and then the preparation of nuclear extracts and EMSA were performed as described (*26*). The cells were lysed with hypotonic buffer, and nuclei were pelleted by centrifugation at 3000*g* for 5 min. The nuclear lysis was performed using a hypertonic buffer [50 mM HEPES (pH 7.9), 400 mM KCl, 0.1 mM EDTA, and 10% glycerol]. After lysis, the samples were centrifuged at 14000*g* for 15 min, and the supernatant was retained for use in the DNA binding assay. DNA-binding reactions were performed for 30 min at room temperature with 5×10^4 cpm of double-strand oligonucleotide probes, 5 μg of nuclear extract, and 0.5 μg of poly(dI−dC) in a final volume of 10 μL. The samples were resolved on a 5% native polyacrylamide gel using 0.5× TBE buffer, at 150 V for 1.5 h, and the results were visualized by autoradiograpy.

Measurement of Intracellular ROS Generation. RAW264.7 cells (10^4 cells/well) in a 96-well plate were treated with or without 5-MH for 2 h, followed by incubation with 1 μg/mL LPS for 16 h. After the removal of media from wells, the cells were incubated with 10 μM $DCFH_2$−DA for 45 min. The fluorescence was measured on a spectrofluorometer (Wallac 1420, Perkin-Elmer, Turku, Finland) at 485 nm excitation and 530 nm emission wavelength, and fluorescence intensity of the cells was calibrated to 100%.

Data Analysis. All values are expressed as mean ± standard deviation (SD). Statistical analysis was performed using Student's *t* test. A value of $p < 0.05$ was accepted as statistically significant.

RESULTS AND DISCUSSION

In this study, we report that 5-MH inhibits the LPS-induced upregulation of iNOS-derived NO through suppression of NF-κB transcriptional activity in RAW264.7 macrophages. It was well-known that *A. japonica* Steud has been used as a traditional Asian medicine for the treatment of various diseases, including fever, hemorrhage, diarrhea, and alcoholism. Recently, we have reported that oregonin and hirsutanone isolated from the methanolic extracts of *A. japonica* leaves are potent antioxidants against LDL oxidation (*17*). Subsequently, 5-MH was isolated from the extracts and also showed potent Cu^{2+}-induced LDL-antioxidant activity with an IC_{50} value of 2.1 ± 0.1 μM. Under AAPH-mediated LDL oxidation, 5-MH exhibited an IC_{50} value of 2.2 ± 0.2 μM in the TBARS assay. Next, the antioxidant activities of 5-MH in the macrophage-mediated oxidation of LDL were examined (**Table 1**). The cellular oxidative modification of LDL to a form recognized by the scavenger receptor requires the presence of transition-metal ions in the medium (*27*). When the LDL was incubated in THP-1 macrophages without $CuSO_4$, the MDA-like product formation was much too low. The MDA-like product formation was increased 8-fold in the 2 μM copper-induced plus macrophage-mediated LDL oxidation (83.2 ± 1.4 nmol of MDA/mg of LDL protein) compared to only the copper-induced LDL oxidation (10.5 ± 0.7 nmol of MDA/mg of LDL protein). The content of the MDA-like product in the presence of 1 μM 5-MH was reduced to 10.4 ± 0.7 nmol of MDA/mg of LDL protein. At the same concentration of probucol, it was reduced to 48.1 ± 2.7 nmol of MDA/mg of LDL protein. As a result, the antioxidant activity of 5-MH was much higher than that of probucol on macrophage-mediated LDL oxidation.

Table 1. Effects of 5-MH on Macrophage-Mediated Oxidation of LDL[a]

incubation conditions	nmol of MDA/mg of LDL protein
LDL plus cell	2.5 ± 0.5
LDL plus Cu^{2+}	10.5 ± 0.7
LDL plus cell plus Cu^{2+} (control)	83.2 ± 1.4
LDL plus cell plus Cu^{2+} plus 1 μM 5-MH	10.4 ± 0.4[b]
LDL plus cell plus Cu^{2+} plus 1 μM probucol	48.1 ± 2.7[b]

[a] Data are shown as mean ± SD from two independent experiments performed in duplicate. [b] $p < 0.05$ versus control.

NO derived from iNOS protein plays a significant role in regulating macrophage activation and proliferation *in vitro* through regulation of NF-κB activity (*2*). The level of NO in the culture medium was increased by 6-fold as compared to the control in 1 μg/mL LPS-treated RAW264.7 cells. However, 5-MH markedly inhibited LPS-induced NO production in a dose-dependent manner, with an IC_{50} value of 14.5 μM (**Figure 2A**). Oregonin, a diarylheptanoid (*15*), and PDTC, a known NF-κB inhibitor (*28*), also significantly inhibited LPS-induced NO production (**Figure 2B**). 5-MH, which has a ketone and methoxy group, showed more potent inhibitory activity of NO production than oregonin, which has a ketone and a xylopyranosyloxy group in LPS-activated macrophages, whereas hirsutanone and 1-(4-hydroxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-4-hepten-3-one, which have only one ketone group, showed very weak inhibitory activity (IC_{50} value of > 100 μM) (*29*) compared to that of 5-MH and oregonin. To confirm the mechanism with respect to inhibition of NO production in activated RAW264.7 cells, we investigated the effect of 5-MH on the expression of iNOS protein and iNOS mRNA. The levels of iNOS protein and iNOS mRNA were markedly increased in LPS-stimulated RAW264.7 cells. Subsequently, 5-MH inhibited the induction of LPS-induced iNOS protein at 20 and 50 μM, respectively (**Figure 2C**). Then, the expression of iNOS protein exhibited very similar inhibitory levels to that for the reduction of NO generation. To investigate whether the reduction of iNOS protein expression by 5-MH depends upon suppression of iNOS mRNA expression, the cells were harvested and total mRNAs were extracted from RAW264.7 cells to performed RT-PCR analysis. Consequently, the iNOS mRNA showed similar results to the reduction of iNOS protein expression (**Figure 2D**). The potential

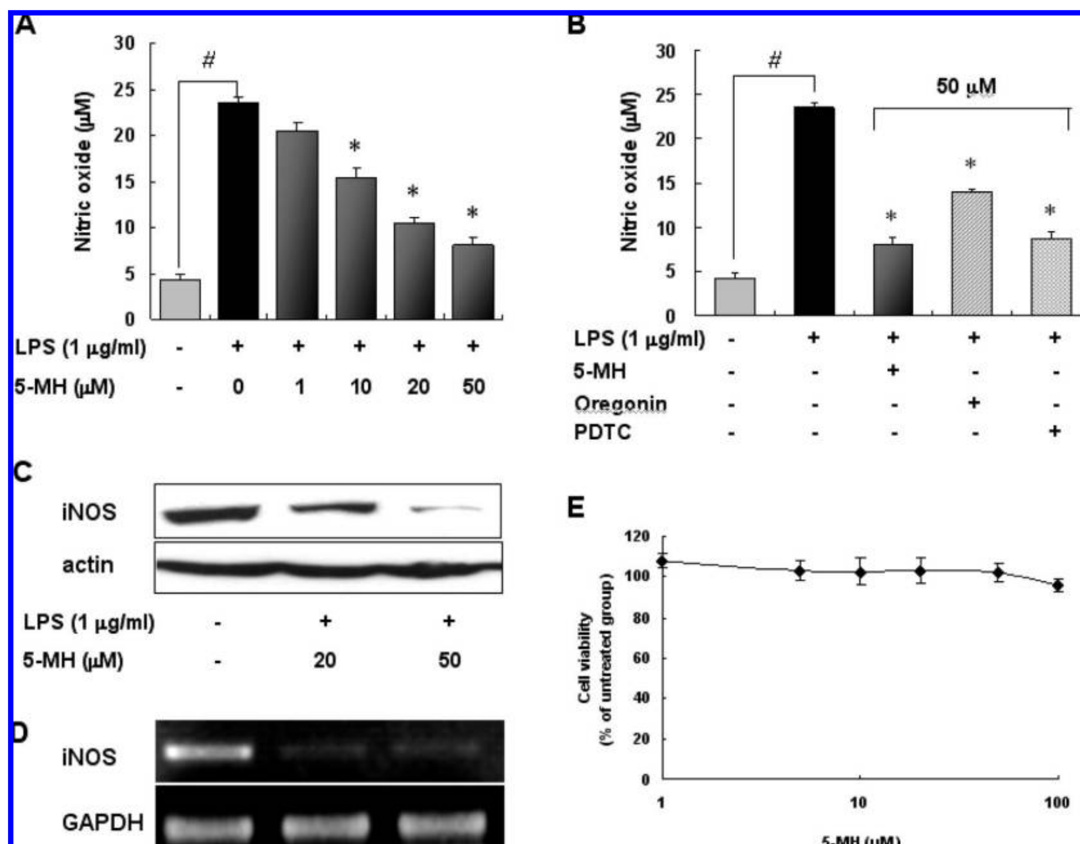

Figure 2. Dose-dependent effects of 5-MH on NO production and iNOS expressions in LPS-induced RAW264.7 cells. (**A**) 5-MH inhibited LPS-induced NO production. (**B**) 5-MH, oregonin, and PDTC at 50 μM inhibited LPS-induced NO production. (**C**) 5-MH inhibited LPS-induced iNOS protein expression. Cell lysates were then prepared and subjected to Western blotting using an antibody specific for murine iNOS. One of three representative experiments is shown. (**D**) 5-MH inhibited LPS-induced iNOS mRNA expression. iNOS mRNA expression was determined by RT-PCR. (**E**) Cell viability was measured by the MTT assay after treatment of 5-MH for 72 h. (#) $p < 0.01$ versus media-treated group. ($*$) $p < 0.01$ versus LPS-treated group.

toxicity of 5-MH to RAW264.7 cells was assessed by the MTT assay. The 5-MH at a concentration up to 50 μM had no effect on cell viability (**Figure 2E**). As a result, it has proven that 5-MH inhibited NO production and expression of both iNOS protein and iNOS mRNA in a parallel dose–response manner. These results indicate that 5-MH could downregulate LPS-induced iNOS expression at the transcription level.

A large number of cytokines and chemokines released from endothelial cells, smooth muscle cells, macrophages, and lymphocytes are involved in this chronic inflammatory and fibroproliferative process (*30*). The effect of 5-MH was evaluated on the secretion of TNF-α, which is essential in the induction of iNOS protein. The production of TNF-α dramatically increased in the culture media of LPS-induced RAW264.7 cells. The expression of TNF-α in the media is higher than in the cytoplasm. However, 5-MH at 50 μM effectively inhibited the expression of TNF-α protein (**Figure 3A**). Proinflammatory cytokines, such as COX-2, IL-1β, and IL-6, are elevated in inflammatory disease and have the important role in immune and inflammatory responses. Therefore, we investigated the effects of 5-MH on LPS-induced expression of the inflammatory genes in RAW264.7 cells by RT-PCR analysis. Stimulation of RAW 264.7 cells with LPS significantly induced the mRNA expressions of TNF-α, COX-2, IL-1β, and IL-6 (**Figures 3B** and **4**). However, 5-MH at 50 μM inhibited nearly 60% mRNA levels for TNF-α, and the result was in accordance with the efflux levels of TNF-α protein (**Figure 3**). In addition, pre-treatment with 5-MH (20 and 50 μM) suppressed the induced mRNA expressions of COX-2 and IL-1β but not IL-6 (**Figure 4**). The results indicate that 5-MH may possess the potential to

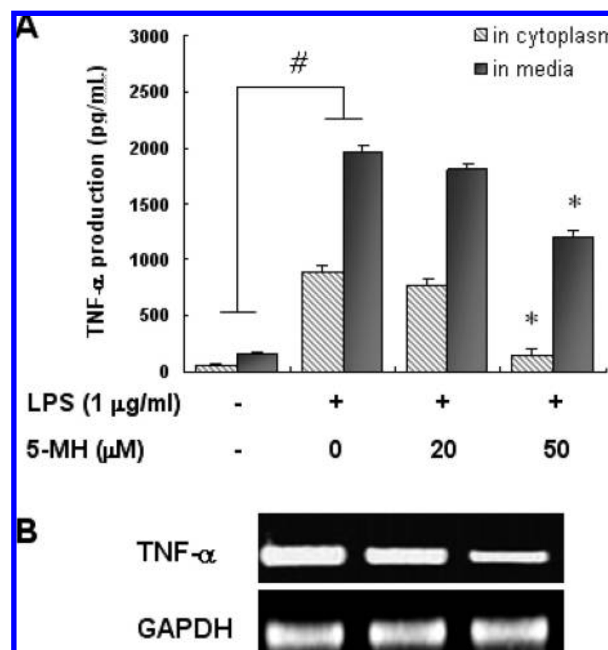

Figure 3. Effect of 5-MH on the LPS-induced expressions of both TNF-α protein and gene. (**A**) Supernatants of cytoplasmic extract and media were separated, and the protein levels of TNF-α were determined by the ELISA assay. (**B**) 5-MH inhibited LPS-induced TNF-α mRNA expression in cells. (#) $p < 0.01$ versus media-treated group. ($*$) $p < 0.01$ versus LPS-treated group.

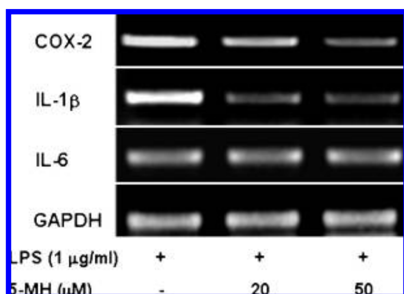

Figure 4. Effects of 5-MH on the LPS-induced expressions of inflammation-associated genes, COX-2, IL-1β, and IL-6, in RAW264.7 cells by RT-PCR analysis. RAW264.7 cells were treated with or without 5-MH for 2 h, followed by incubation with 1 μg/mL LPS for 10 h. Then, the total RNAs were isolated, and RT-PCR analysis was carried out as described in the Materials and Methods.

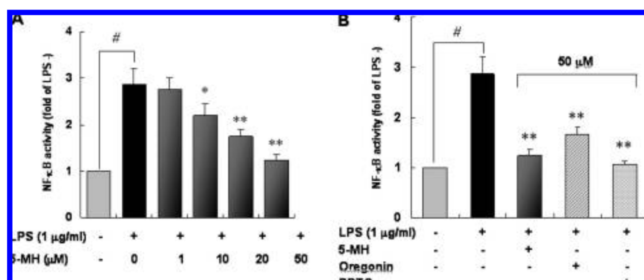

Figure 5. Inhibition of NF-κB-mediated transcription of the reporter gene by 5-MH. RAW264.7 cells transfected with a NF-κB reporter plasmid were pretreated with 5-MH (**A**), 5-MH, oregonin, and PDTC at 50 μM (**B**) for 2 h and then stimulated with LPS (1 μg/mL) for 24 h. NF-κB activity in the culture medium was measured using the SEAP assay. (#) $p < 0.01$ versus media-treated group. (*) $p < 0.05$ and (**) $p < 0.01$ versus LPS-treated group.

prevent atherosclerosis via modulation of the reduction of NF-κB target genes, such as TNF-α, COX-2, and IL-1β.

Transcription factors of the NF-κB proteins play an important role in the regulation of genes involved in proinflammatory, proliferative, and prothrombotic responses of cells, the activation of which has been observed in human and experimental atherosclerosis (*31–33*). Therefore, many authors addressed NF-κB as an important therapeutic target in atherosclerosis and thrombosis (*34, 35*). To investigate a molecular mechanism in the anti-inflammatory effect of 5-MH, NF-κB transcriptional activity was monitored using RAW264.7 cells stably transfected with a plasmid containing 8 copies of κB elements linked to the SEAP gene. The LPS-induced cells increased SEAP expression to about 3-fold over the basal levels. As shown in **Figure 5A**, 5-MH inhibited the SEAP expression in a dose-dependent manner. PDTC and oregonin, as positive controls, also significantly inhibited the SEAP expression (**Figure 5B**). Cucurmin having two ketone groups also possesses anti-inflammatory activity and is a potent inhibitor of ROS-generating enzymes, such as lipoxiganase/cyclooxygenase, xanthine dehydrogenase/oxidase, and iNOS in macrophages, by inhibiting NF-κB activation (*36, 37*). Therefore, we agree that the diarylheptanoids having a ketone and a hydroxy or 5-*O*-substututed group or diarylheptanoids having two ketone groups strongly attenuate the NF-κB activation.

The nuclear translocation of NF-κB p65 is proceeded by hyperphosphorylation of IκB-α and its subsequent proteolytic degradation (*38*). We examined the effects of 5-MH on the phosphorylation and degradation of IκB-α. In untreated RAW264.7 cells, the phosphorylation of IκB-α was not

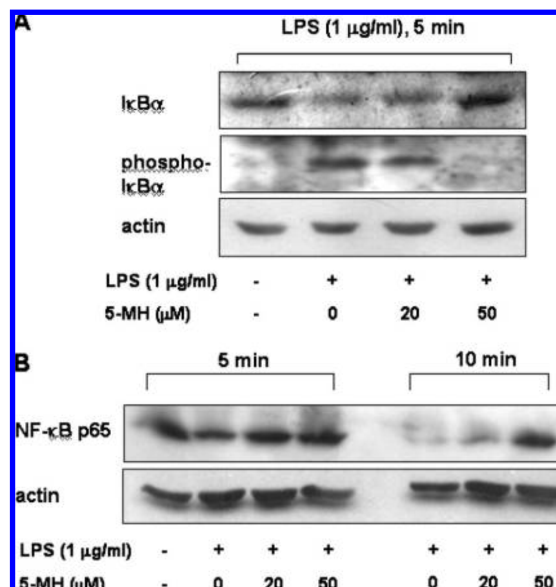

Figure 6. Inhibition of NF-κB activation by 5-MH. 5-MH treatment inhibited LPS-induced degradation and phosphorylation of IκB-α (**A**) and translocation of NF-κB p65 (**B**). Cells were incubated with LPS (1 μg/mL) in the presence or absence of 5-MH (20 and 50 μM) for 5–10 min. Cells were harvested, washed with ice-cold phosphate-buffered saline (PBS), and resuspended in nuclear extraction buffer. Cytosolic fractions were prepared as described in the Materials and Methods. Samples were separated on SDS–polyacrylamide gel electrophoresis (PAGE), and the IκB-α, phospho-IκB-α, and NF-κB p65 subunits were visualized by Western blot analysis. The blot was rehybridized with antibody for actin to verify an equal loading of protein in each lane.

observed. However, maximal phosphorylation was evident at 5 min of stimulation with 1 μg/mL LPS (**Figure 6A**). Actually, the stimulation with LPS leads to rapid phosphorylation and degradation of IκB-α, followed by complete resynthesis in 60 min (data not shown). However, 5-MH (50 μM) strongly prevents LPS-induced phosphorylation and resultant proteolytic degradation of IκB-α (**Figure 6A**). To investigate whether the inhibition of NF-κB activity by 5-MH is mediated through the modulation of p65 nuclear translocation, we determined the cytosolic NF-κB p65 subunit levels, following treatment of LPS in the presence or absence of 5-MH. The levels were time-dependently decreased after LPS treatment; however, pretreatment of the cells with 50 μM 5-MH markedly attenuated the LPS-induced nuclear translocation of p65, and consequently, the p65 subunit level in cytoplasm was almost equal to the control level (**Figure 6B**). Therefore, these results suggest that NO production and expression of iNOS were inhibited by blocking the phosphorylation as well as degradation of IκB-α and preventing the translocation of NF-κB p65 into the nucleus.

Gel electromobility shift assay (EMSA) was conducted to determine whether 5-MH inhibits induction of specific NF-κB DNA-binding activity by LPS. LPS (1 μg/mL) significantly increased the DNA-binding activity of NF-κB within 2 h in RAW264.7 cells. These increases were inhibited by 5-MH in a dose-dependent manner (parts **A** and **B** of **Figures 7**); therefore, 5-MH inhibited NF-κB activation by preventing DNA binding. Oregonin inhibits LPS-induced iNOS protein through the inhibition of NF-κB promoter activity and DNA-binding ability (*15*). However, oregonin, as a positive control, showed weak inhibitory activities on NO production (**Figure 2B**) and NF-κB DNA-binding ability (**Figure 7A**) at the same concentration of 5-MH. In addition, we showed that 5-MH has stronger inhibitory

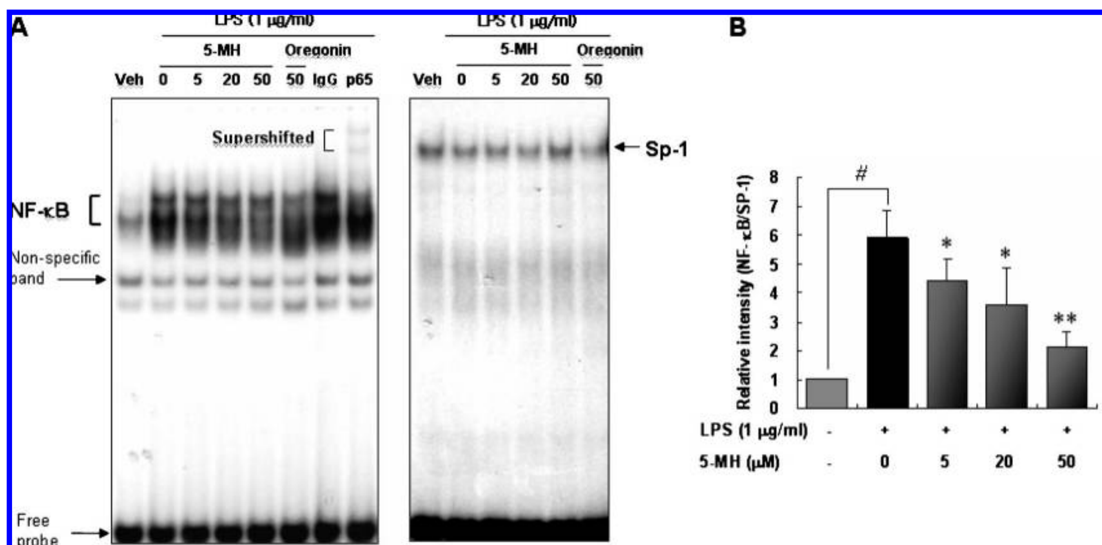

Figure 7. LPS-induced DNA-binding activity of the NF-κB complex in RAW264.7 macrophages. (**A**) (Left) DNA-binding activity of nuclear proteins to a NF-κB-specific oligonucleotide. The brackets indicate the activated NF-κB. Veh = not stimulated with LPS. The specific antibody for p65 was included in the binding solution to indicate the specific binding. (Right) Binding of nuclear proteins to an oligonucleotide containing the Sp-1 consensus sequence was also examined by EMSA in the same nuclear extracts (arrow). The EMSA autoradiograph shown is representative of three independent experiments. (**B**) Quantification of relative band intensities from three independent experimental results was determined by densitometry. Values are mean ± SD (n = 4). (#) $p < 0.01$ versus media-treated group. (∗) $p < 0.01$ versus LPS-treated group.

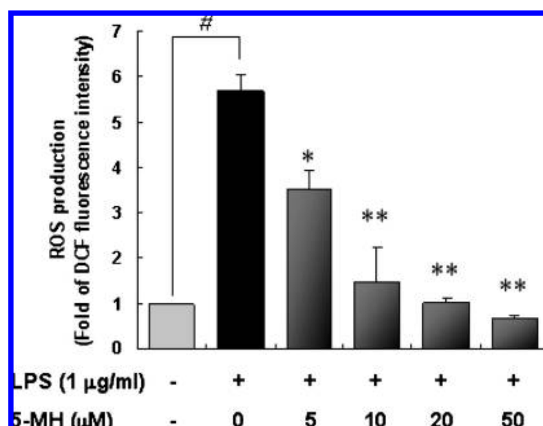

Figure 8. 5-MH suppresses the intracellular ROS level in RAW264.7 macrophages. After time-dependent incubation for 16 h, cells were incubated with $DCFH_2$−DA (10 μM) for an additional 45 min. Cells were washed twice with PBS, and the intracellular levels of ROS were analyzed by VICTOR3 (485/538 nm). Data shown are the mean ± SD (n = 4). (#) $p < 0.01$ versus media-treated group. (∗) $p < 0.01$ versus LPS-treated group.

effects than oregonin on the expression of adhesion molecules in human umbilical vein endothelial cells by inhibition of NF-κB activation (*39*). These results suggest that 5-MH may be useful for prevention and treatment of atherosclerosis by inhibiting the activation of NF-κB and may constitute an additional anti-inflammatory and anti-atherosclerotic therapeutic agent.

Oxidant damage by ROS is known to contribute to cardiovascular diseases (*40*), inflammation (*41*), and tumorigenesis. Awareness of the therapeutic action of aged garlic extract and *S*-allylcysteine (SAC) against those degenerative diseases is attributed to their antioxidant characteristics (*42*). Several lines of investigation suggest that suppression of LPS-induced ROS results in diminution of NF-κB activity and subsequent inhibition of NF-κB responded gene expression (*43*). To investigate whether 5-MH inhibits ROS generation, RAW264.7 cells were incubated time-dependently with 1 μg/mL LPS for from 3 to 16 h to increase ROS about 1.8−6-fold (data not shown). In this condition, 5-MH inhibited ROS generation in a dose-dependent manner (**Figure 8**).

In conclusion, we examined anti-inflammatory activities of 5-MH, isolated from the leaves of *A. japonica* Steud, on NF-κB-dependent NO production and expression of iNOS in LPS-induced RAW264.7 macrophages. 5-MH inhibited NO production and expression of both iNOS protein and iNOS mRNA in a parallel dose–response manner. Then, expression of inflammation-associated genes, such as TNF-α, COX-2, and IL-1β, was suppressed by 5-MH, as determined by RT-PCR analysis. Moreover, 5-MH attenuated NF-κB activation by inhibition of phosphorylation of IκB-α and its subsequent proteolytic degradation and p65 nuclear translocation, as well as preventing DNA-binding ability. In addition, ROS production concerned in the regulation of NF-κB signaling was reduced by 5-MH.

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