JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Miyabenol A Inhibits LPS-Induced NO Production via IKK/IκB Inactivation in RAW 264.7 Macrophages: Possible Involvement of the p38 and PI3K Pathways

KUEI-TING KU,[†] YU-LING HUANG,[‡] YU-JOU HUANG,[†] AND WEN-FEI CHIOU*[,†,‡]

Institute of Life Science, College of Science and Engineering, National Taitung University, Taitung, and National Research Institute of Chinese Medicine, Taipei, Taiwan

The anti-inflammatory effect of miyabenol A, a stilbene isolated from *Vitis thunbergii*, on lipopolysaccaride (LPS)-induced nitric oxide (NO) production in RAW264.7 macrophages was studied. Miyabenol A inhibited NO production (EC_{50}: 2.7 μM) and iNOS protein and mRNA expression in a parallel concentration-dependent manner. LPS-evoked NF-κB nuclear translocation and associated IκB degradation were abrogated by miyabenol A treatment. Phosphorylations of IKKα/β, ERK1/2, JNK p38 MAPK, and Akt were observed in LPS-stimulated cells; nevertheless, miyabenol A selectively blocked IKKα/β, p38, and Akt phosphorylation. Furthermore, LPS-stimulated IKKα/β and Akt phosphorylation was abolished by p38 inhibitor SB203580. Wortmannin (a PI3K inhibitor) also attenuated LPS-induced IKKα/β phosphorylation, although to a less extent than SB203580, but failed to affect p38 phosphorylation. These observations suggested that PI3K/Akt might lie downstream of p38 MAPK to coregulate LPS-induced IKKα/β phosphorylation. Taken together, miyabenol A acted via interfering with p38 MAPK-related signal pathways to down-regulate IKK/IκB activation and NO production.

KEYWORDS: ***Vitis thunbergii*; miyabenol A; NO; iNOS; IKKα/β; IκB; NF-κB; p38 MAPK; PI3K/Akt**

INTRODUCTION

The roots of *Vitis thunbergii* Sieb. & Zucc. (*Vitis ficifolia* Bge. Vitaceae) are traditionally used for the treatment of diarrhea, fracture and injury, jaundice, and hepatitis in Taiwan (*1*). Plants in the genus *Vitis* commonly contain oligomers of resveratrol. A plethora of *in vitro* studies demonstrated various anti-inflammatory activities of resveratrol (*trans*-3,4′,5-trihydroxystilbene), including inhibition of the release of pro-inflammatory cytokines and nitric oxide (NO) from macrophages and lung epithelial cells (*2, 3*). In our investigation on the constituents of *V. thunbergii*, many stilbenes belonging to resveratrol oligomers were isolated (*4*). However, whether these resveratrol oligomers displayed NO inhibitory property remained unclear.

Macrophages play significant roles in inflammation and host defense. Although multiple molecular mechanisms mediate inflammatory processes, one of the most prominent mechanisms is the production of NO by inducible nitric oxide synthase (iNOS). Stimulation of macrophages by various stimuli, e.g., lipopolysaccharide (LPS), induces the expression of iNOS, which results in high-level NO production (*5*). NO mediates diverse physiological functions, such as nonspecific host defense, antimicrobial defense, and antitumor activities, as well as pathophysiological functions, which include the pathogenesis of septic shock and organ destruction in certain inflammatory and autoimmune diseases (*6*). Therefore, the inhibition of NO production by blocking iNOS expression may be a useful strategy for the treatment of various inflammatory disorders.

The expression of the iNOS gene in macrophages is regulated mainly at the transcriptional level. The well-known transcription factor nuclear factor-κB (NF-κB) is a pivotal regulator of important immunoregulatory genes that are involved in immune and inflammatory responses, including iNOS (*7*). NF-κB exists primarily in the cytoplasm as homo- or heterodimers complexed with inhibitory IκB proteins. Upon activation with signaling molecules, such as LPS, IκB undergoes phosphorylation, ubiquitination, and proteasome-mediated degradation, and the NF-κB released from IκB is translocated into the nucleus, where it binds to the promoter of iNOS gene and activates transcription (*8*).

In this study, five stilbenes isolated from *V. thunbergii* were employed to evaluate their effects on LPS-induced NO production in RAW264.7 macrophage and compared with resveratrol. Results showed that miyabenol A (**Figure 1**) displayed the most potent NO inhibition; thus, we further investigated its detailed mechanisms of action.

* To whom correspondence should be addressed. Division of Basic Chinese Medical Research, National Research Institute of Chinese Medicine, No. 155-1, Sec. 2, Li-Nung St., Shipai, Taipei 112, Taiwan, ROC. Tel: 886-2-2820199(4481). Fax: 886-2-28250743. E-mail: wfchiou@cma23.nricm.edu.tw.

[†] National Taitung University.

[‡] National Research Institute of Chinese Medicine.

10.1021/jf8019369 CCC: $40.75
Published on Web 09/11/2008

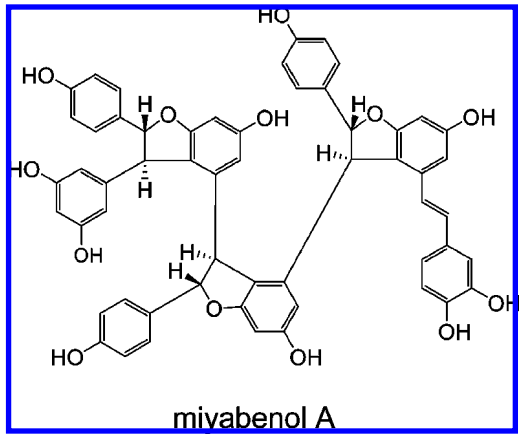

Figure 1. Chemical structure of miyabenol A.

MATERIALS AND METHODS

Isolation of Five Stilbenes from *Vitis thunbergii*. The roots of *Vitis thunbergii* were purchased in Taipei, Taiwan, in July 1998 and identified by Mr. Jun-Chih Ou, a taxonomist retired from our institute. A voucher specimen (NRICM-98-010) is deposited at the Herbarium of National Research Institute of Chinese Medicine, Republic of China. Briefly, the dried roots of *Vitis thunbergii* (3 kg) were extracted exhaustively with 95% ethanol. After a series of separation and individual chromatography on a silica gel column eluting with CH_2Cl_2-MeOH (10:1) and on Sephadex LH-20 column eluting with MeOH-H_2O (3:1), (+)-ε-viniferin, (−)-viniferal, ampelopsin C, miyabenol A, and (+)-vitisin A were obtained. More detailed information on the HPLC analysis was described in our previous work (*4*). The purity of these compounds determined by high-performance liquid chromatography with an UV detector (254 nm) ranged between 99.1 and 99.8%.

Cell Culture and Sample Treatment. The RAW 264.7 macrophage cell line was obtained from American type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY 14072, USA) supplemented with antibiotics (100 U/mL of penicillin A and 100 U/mL of streptomycin) and 10% heat inactivated fetal bovine serum (Biological Industries, Kibbutz Beit Haemek 25115, Israel), and maintained at 37 °C in 5% CO2 humidified air (*9, 10*). Cells were incubated with the tested samples at increasing concentrations or positive chemical for 1 h and then stimulated with 1 μg/mL LPS.

Measurement of Cell Viability. Cell viability was monitored by the Alamar Blue Assay kit (Serotec Ltd., 22 Bankside, Kidlington, Oxford, UK) as described previously (*10*). The change in color could be monitored with an ELISA reader at 620 nm. Cell viability correlates with optical density.

Nitrite Assay. Cells were plated at a density of 10^5 cells/mL in 96-well plates for 12 h, followed by treatment with LPS and different concentrations of the indicated compounds for a further 18 h. Nitrite (NO_2^-) accumulation was used as an indicator of NO production in the cell culture medium by the Griess reaction. One hundred microliters of each supernatant were mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylendiamine dihydrochloride in water). The absorbance of each sample after Griess reaction was determined by an ELISA plate reader (Dynatech MR-7000; Dynatech Laboratories) at 550 nm (*11*). The nitrite concentration is evaluated by means of a calibration curve (3.125 to 100 μM), using sodium nitrite as a standard.

Western Blot Analysis. Cellular proteins were extracted from control and miyabenol A treated RAW 264.7 cells. Cells were collected by centrifugation and washed once with phosphate-buffered saline (PBS). The washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES, pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM Na fluoride, and 0.5 mM Na orthovanadate) containing 5 μg/mL each of leupeptin and aprotinin and incubated for 20 min at 4 °C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacturer's instructions. Forty micrograms of cellular protein from treated and untreated cell extracts was electroblotted onto a nitrocellulose membrane following separation on 8% SDS−polyacrylamide gel electrophoresis (SDS−PAGE). The immunoblot was incubated overnight with blocking solution (5% skim milk) at 4 °C, followed by incubation for 2 h with a primary antibody. Blots were washed four times with Tween 20/Tris-buffered saline (TTBS) and incubated with appropriate dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Blots were again washed three times with TTBS and then developed by enhanced chemiluminescence (Amersham Life Science). The results were evaluated by densitometry analysis and expressed as fold change.

Preparation of Nuclear Extraction for Translocation Assay. Nuclear extracts of RAW264.7 macrophages before and after miyabenol A treatment were prepared using NE-PER nuclear and cytoplasmic extraction reagent (Pierce, 3747 N. Meridian Road, Rockford, IL 61105, USA) according to the manufacturer's instructions and supplemented with a set of protease inhibitors (Pierce). Thirty micrograms of nuclear protein extract was denatured in Laemmli buffer and separated using 8% SDS−PAGE. After transferring, the membrane was detected by antibodies against NF-κB p65 (purchased from Santa Cruz Biotechnology, 2145 Delaware Avenue, CA 95060, USA) and detected by ECL (Amersham) (*11*). Nucleolin (Chemicon International Inc.) served as a loading control for nuclear extracts.

RNA Preparation and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The total cellular RNA was isolated using RNAspin Mini RNA Isolation Kit (GE Healthcare, UK), and RT-PCR was performed using SuperScrip One-Step RT-PCR with Platinum *Taq* (Invitrogen) according to the manufacturer's instructions. From each sample, 1 μg of RNA was reverse-transcribed (RT) using the RT/Platinum *Taq* mixture. Then PCR analyses were performed on the aliquots of the cDNA preparations to detect iNOS and GAPDH (as an internal standard) gene expression using a thermal cycler (Strategen, Foster City, CA, USA). The reactions were carried out in a volume of 50 μL containing (final concentration) 1 U of Taq DNA polymerase, 0.2 mM dNTP, ×10 reaction buffer, and 100 pmol of 5′ and 3′ primers. After initial denaturation for 2 min at 94 °C, 30 amplification cycles were performed for iNOS (15 s of 94 °C denaturation, 30 s of 55 °C annealing, and 1 min of 72 °C extension) and GAPDH (15 s of 94 °C denaturation, 30 s of 55 °C annealing, and 1 min of 72 °C extension). The PCR primers used in this study are listed below and were purchased from Invitrogen (Shanghai, China): sense strand iNOS, 5′-CCCTTC-CGAAGTTTCTGGCAGCAGC-3′, antisense strand iNOS, 5′-GGCT-GTCAGAGAGCCTCGTGGCTTTGG-3′; sense strand GAPDH, 5′-GCCATCAACGACCCCTTCATTGAC-3′, antisense strand GAPDH, 5′-ACGGAAGGCCATGCCAGTGAGCTT-3′. After amplification, portions of the PCR reactions were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining and UV irradiation.

Chemicals and Antibodies. Lipopolysaccharide (LPS) (*Escherichia coli*, serotype 055:B5) and all chemicals were purchased from Sigma Chemical (St. Louis, MO, USA). The antibodies for detecting the expression of IKKα/β, p-IKKα/β, IκB-α, iNOS, NF-κB p65, and β-Actin protein were obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, 2145 Delaware Avenue, CA 95060, USA). The antibodies for total and phosphorylated MAPKs (ERK, JNK, and p38) and Akt were obtained from Cell Signaling (Cell Signaling Biotechnology, Beverly, MA, U.S.A.). All solvents were purchased from Merck KGaA (Darmstadt, Germany).

Statistical Analysis. All values are expressed as the mean ± SE. Statistical analysis was performed using Student's *t*-test. A value of $p < 0.05$ or 0.01 was accepted as statistically significant.

RESULTS

Miyabenol A Inhibits LPS-Induced NO Production and iNOS Expression. In this study, five stilbenes isolated from the roots of *Vitis thunbergii* including miyabenol A, (+)-ε-viniferin, (−)-viniferal, ampelopsin C, and (+)-vitisin A were used to evaluate their effects on LPS-induced NO production and compared with resveratrol. As shown in **Figure 2**,

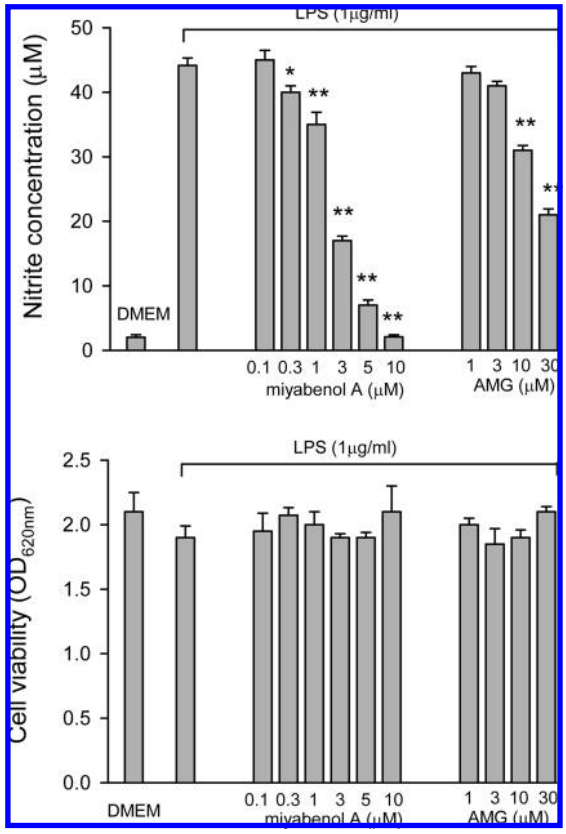

Figure 2. Effects of miyabenol A on lipopolysaccharide (LPS)-induced NO production and cell viability in RAW264.7 macrophages. Cells were treated with different concentrations of miyabenol A or aminoguanidine (AMG, an iNOS inhibitor) for 1 h followed by the addition of LPS (1 μg/mL) for an additional 18 h. Cells incubated with medium alone were represented as DMEM. NO in the medium was detected by the Griess reaction. Data reported are the mean ± SE of six independent experiments, each performed in triplicate. *$p < 0.05$ and **$p < 0.01$, indicate significance of difference as compared with samples receiving LPS alone.

Table 1. Comparison of the Inhibitory Effects of Five Stilbenes Isolated from the Roots of *Vitis thunbergii* and Resveratrol on Lipopolysaccharide-Induced Nitric Oxide (NO) Production in RAW264 Macrophages[a]

compounds	EC_{50} (μM)
miyabenol A	2.7 ± 0.2
(+)-ε-viniferin	38.5 ± 4.9
(−)-viniferal	17.1 ± 2.2
ampelopsin C	31.5 ± 1.6
(+)-vitisin A	16.5 ± 3.4
resveratrol	28.3 ± 3.5

[a] Results were expressed as the mean ± SE of triplicate tests. EC_{50} represented the 50% effective concentration to inhibit NO production.

miyabenol A (0.1−10 μM) inhibited NO production in a concentration-dependent manner without significant harmful effects on cell viability. A well-known iNOS inhibitor aminoguanidine (AMG) was employed to the assay system and displayed less potent effect than miyabenol A. According to the 50% effective concentration (EC_{50}) for inhibiting NO production, we identified miyabenol A as the most potent one (**Table 1**). Resveratrol, however, displayed a mild effect and required higher concentration than miyabenol A to inhibit LPS-induced NO production.

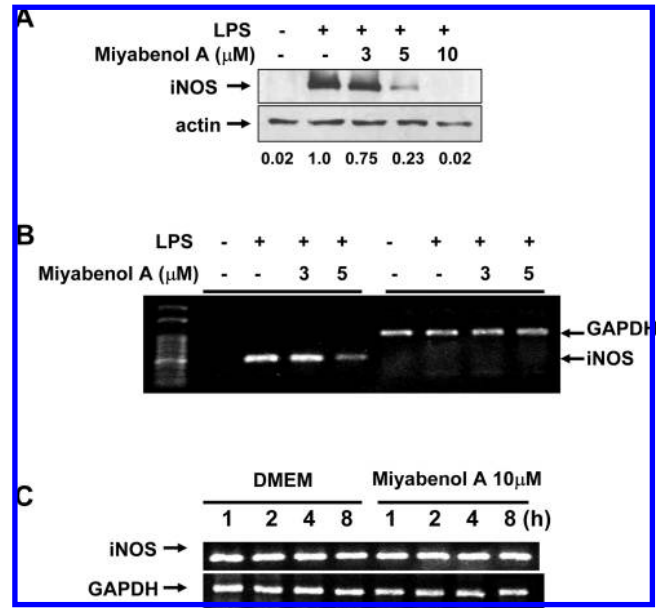

Figure 3. Effects of miyabenol A on the expression of iNOS protein and mRNA in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. In **A** and **B**, cells stimulated with LPS for 18 and 4 h were harvested for protein and mRNA analysis, respectively. In **C**, actinomycin D (0.1 μg/mL) was added to the cell culture at 4 h after LPS stimulation, then total RNA were extracted in the absence or presence of miyabenol A for further 8 h treatment to assess the effect of miyabenol A on mRNA stability. An equal amount of GAPDH mRNA was used as an internal standard. Similar results were observed in four independent experiments, and the results shown are from one of them.

Western blot analysis was carried out to determine whether the inhibition of NO by miyabenol A was due to suppression of iNOS protein expression. The concentration response for inhibition of 130-kDa iNOS protein expression by miyabenol A is shown in **Figure 3**A. Detectable reduction of iNOS protein expression by miyabenol A was observed starting at 3 μM and significantly suppressed at 5 μM, and complete abolition of iNOS expression was observed at 10 μM. The same expression of Actin suggested that inhibition by miyabenol A was due to a specific effect. Subsequently, we assessed the expression of iNOS mRNA in RAW264.7 macrophages before and after miyabenol A treatment. As shown in **Figure 3B**, the expression of iNOS mRNA was increased significantly after LPS stimulation for 4 h. When cells were pretreated with 3 μM miyabenol A, a moderate attenuation of iNOS mRNA induction was observed, and this induction was blocked further by 5 μM miyabenol A treatment. An equal amount of GAPDH mRNA was used as an internal standard. To elucidate whether miyabenol A depressed iNOS level due to loss of mRNA stability, actinomycin D (0.1 μg/mL) was added at 4 h after LPS stimulation to block further RNA synthesis, then total RNA was extracted in the absence or presence of miyabenol A for further 8 h of treatment (**Figure 3C**). Results showed that iNOS mRNA expression was stable within 8 h and that its stability was not affected in the presence of miyabenol A.

Inhibitory Effect of Miyabenol A on LPS-Induced NF-κB Translocation, IκB-α Degradation, and IKK Phosphorylation. In unstimulated cells, NF-κB is sequestered in the cytosol by its inhibitor, IκB, which is phosphorylated by LPS stimulation, and then ubiquitinated and rapidly degraded via the 26S proteosome, to release NF-κB (*12*). Here, we investigated whether miyabenol A inhibits LPS-induced NO production via blocking the degradation of IκB in RAW 264.7 macrophage.

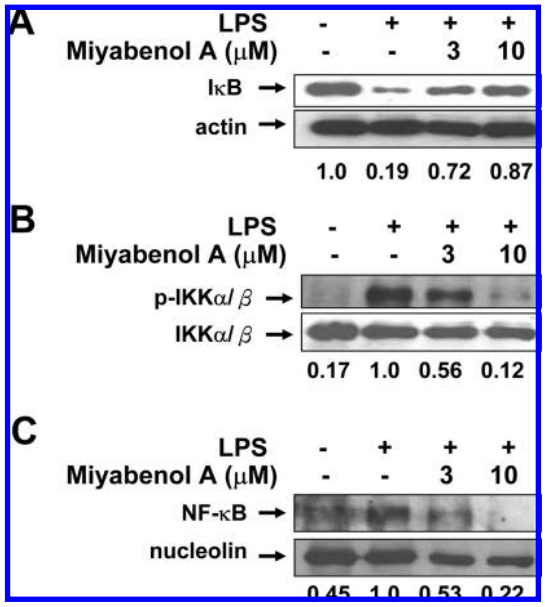

Figure 4. Effects of miyabenol A on lipopolysaccharide (LPS)-stimulated IκB degradation, IKKα/β phosphorylation, and NF-κB nuclear translocation in RAW264.7 macrophages. Cells were treated with miyabenol A (3 and 10 μM) for 1 h, and then exposed to LPS (1 μg/mL) for 30 min. Cytosol and nuclear extracts were assessed by Western blotting, and protein signals were detected with antibody against IκB-α, IKKα/β and NF-κB p65, Actin and nucleolin. Similar results were observed in three independent experiments, and the results shown are from one of them.

Figure 4A shows that LPS-induced IκB degradation was significantly attenuated by pretreatment with miyabenol A. Since IKKα/β are the upstream kinases of IκB in the NF-κB signal pathway and phosphorylate IκBα and IκBβ (*13*), we next examined the effect of miyabenol A on LPS-induced IKKα/β activations by immunoblotting using a phosphorylated IKKα/β antibody. Cells were pretreated with miyabenol A (3 and 10 μM) for 1 h and then stimulated with LPS (1 μg/mL) for 30 min. LPS was found to strongly induce IKKα/β phosphorylation, whereas miyabenol A markedly inhibited this phosphorylation (**Figure 4B**). We also investigated whether miyabenol A prevents the translocations of the p65 subunits of NF-κB from the cytosol to the nucleus after their release from IκBs. It was found that the p65 level in nuclear fractions was significantly attenuated by 3 μM miyabenol A treatment and was almost abolished by 10 μM miyabenol A (**Figure 4C**). Nuclear protein nucleolin was used as an internal control.

Effects of Miyabenol A on LPS-Induced Phosphorylation of MAPKs and Akt. MAPK signaling pathways play a critical role in the regulation of inflammatory response and coordinate the induction of many genes encoding inflammatory mediators. To investigate the molecular mechanism of NF-κB inhibition by miyabenol A in LPS-stimulated RAW 264.7 cells, we observed its inhibitory effect on the activations of ERK1/2, JNK, and p38 MAPK. Maximal MAPK (ERK1/2, p38, and JNK) phosphoprotein expression levels are known to occur 10−30 min after LPS treatment in human and murine monocytes/macrophages (*14*). Therefore, we are carried out Western blotting for MAPKs after stimulating with LPS (1 μg/mL) for 10 min. As shown in **Figure 5A**, the phosphorylations of ERK1/2, JNK, and p38 were increased in cells stimulated with LPS, but the total forms of the three MAPKs remained constant. Pretreated cells with miyabenol A (3, 5, and 10 μM) selectively and significantly suppressed only LPS-induced p38 MAPK phosphorylation, while they were without effect on JNK and ERK responsiveness.

We also used the phosphor-antibodies to determine the activation status of Akt (a downstream target protein for PI3K). As shown in **Figure 5B**, an unobvious protein band appeared in lysates of unstimulated cells; however, a marked induction of phosphorylated Akt was observed after LPS stimulation for 15 min. The lower trace indicates the gel images using antibody against control Akt. It was consistently noted that a PI3K inhibitor Wortmannin abolished LPS-induced Akt phosphorylation near the base level. A significant suppression of LPS-stimulated Akt phosphorylation was also observed in the presence of miyabenol A (**Figure 5B**, lower trace).

Interaction between p38 MAPK, Akt, and IKKα/β. Since MAPKs and PI3K/Akt were reported to play an important role in regulating the activation of the IKK signaling pathway (*15, 16*), this prompted us to study the effect of SB203580 (a p38 MAPK inhibitor) and Wortmannin on LPS-induced IKKα/β phosphorylation and IκB degradation in RAW264.7 macrophages. Results showed that pretreatment with SB203580 at 10 μM markedly reduced, while at 30 μM, it almost abolished IKKα/β phosphorylation (**Figure 6A**). SB203580 pretreatment also apparently reversed LPS-induced IκB degradation. Nonetheless, the effect of Wortmannin on IKKα/β phosphorylation and the subsequent IκB degradation in response to LPS was not so obvious. As shown in **Figure 6B**, Wortmannin (10 and 30 μM) moderately but significantly attenuated LPS-induced IKKα/β phosphorylation and IκB degradation (**Figure 6B**). This provided evidence that p38 MAPK played a more important role than PI3K/Akt in regulating IKK/IκB activation. After further evaluation of the interaction between p38 MAPK and Akt in LPS-stimulated RAW264.7 macrophages, results showed that Akt phosphorylation was strongly suppressed in the presence of SB203580. Contrarily, p38 phosphorylation was not significantly altered after Wortmannin challenge (**Figure 6C**). These findings suggested that p38 MAPK located upstream of PI3K/Akt and that its phosphorylation affected the activation state of PI3K/Akt.

DISCUSSION

Nitric oxide (NO) plays a central role in the physiology and pathology of diverse tissues including the immune system. It is clear that the levels of nitric oxide must be carefully regulated to maintain homeostasis. For example, overproduction of NO has been implicated in the pathogenesis of many disorders, including atherosclerosis, neurodegenerative diseases, cancer, and inflammatory diseases (*17*−*19*). Therefore, down-regulation of iNOS expression and/or activity to suppress the level of NO generated became a new pharmacological strategy for the treatment of the above diseases (*19*). Herein, among the five tested stilbenes isolated from *Vitis thunbergii*, miyabenol A displayed the most potent effect compared to the others and the reference compound resveratrol in suppressing LPS-induced NO production in RAW264.7 macrophages. In the tested compounds used, miyabenol A and (+)-vitisin A belong to a tetramer, ampelopsin C is a trimeric stilbene, while (+)-ε-viniferin and (−)-viniferal are stilbene dimers. We had previously reported that the increase in the resveratrol unit for tested compounds led to a more potent effect in inhibiting influenza A virus-induced RANTES formation (*20*). But this did not happen in this case. In the present study, we found that the NO inhibitory potency is not relative to the oligomeric unit.

The use of plant extracts and natural products to alleviate inflammatory diseases is centuries old and continues to this

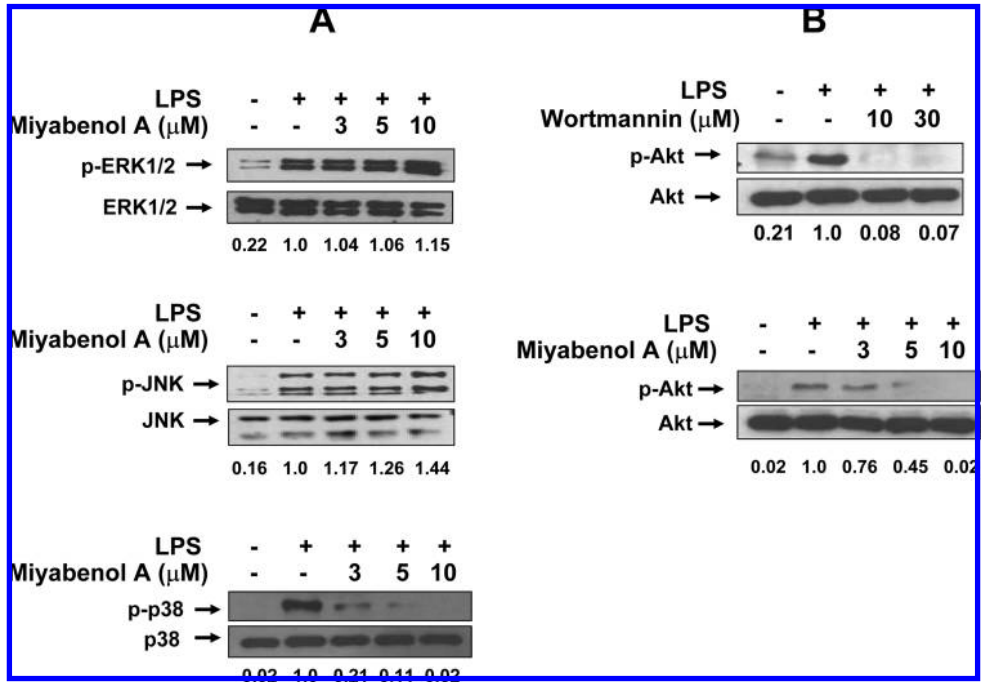

Figure 5. Effects of miyabenol A on lipopolysaccharide (LPS)-stimulated MAPKs (ERK1/2, JNK, and p38) and Akt phosphorylation in RAW264.7 macrophages. Cells were treated with miyabenol A (3, 5 and 10 μM) for 1 h and then exposed to LPS (1 μg/mL) for 10 min. Total cell lysates were assessed by Western blotting, and protein signals were detected with antibody against control and phosphorylated MAPKs and Akt, respectively. Similar results were observed in three independent experiments, and the results shown are from one of them.

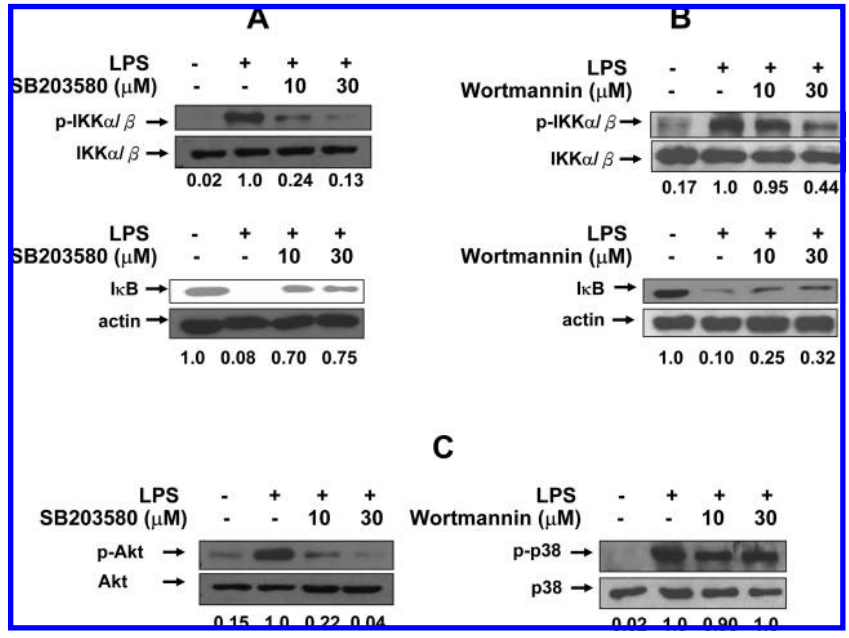

Figure 6. Effects of SB203580 and Wortmannin on lipopolysaccharide (LPS)-stimulated IKKα/β phosphorylation and IκB degradation (**A** and **B**) as well as on the phosphorylation of individual protein kinase (**C**) in RAW264.7 macrophages. Cells were treated with SB203580 (10 and 30 μM) and Wortmannin (10 and 30 μM) for 1 h and then exposed to LPS (1 μg/mL) for various time periods (as described in **Figures 4** and **5**). Total protein extracts were assessed by Western blotting, and protein signals were detected with antibody against control or phosphorylated IκB-α, IKKα/β, Akt, p38, and Actin, respectively. Similar results were observed in four independent experiments, and the results shown are from one of them.

day. Current understanding of the use of such plants and natural products isolated from them concerns their action against the ubiquitous transcription factor, NF-κB. As an activator of many pro-inflammatory cytokines and inflammatory processes, the modulation of the NF-κB transduction pathway is a principal target to alleviate the symptoms of inflammatory diseases. The nuclear translocation and DNA binding of NF-κB is preceded by the degradation of IκB. Our study indicates that miyabenol A inhibited LPS-induced iNOS expression via blocking the degradation of IκB-α and the subsequent nuclear translocation of NF-κB. Recent studies have shown that the phosphorylation of IκB is regulated by α and β isoforms of IKK (*21*). These kinases may represent a novel site for pharmacological intervention in a number of inflammatory conditions (*22*), and therefore, we observed the inhibitory effect of miyabenol A on IKKα/β activation in RAW264.7 cells stimulated with LPS. In the present study, IKKα/β phosphorylation was found to be increased in cells

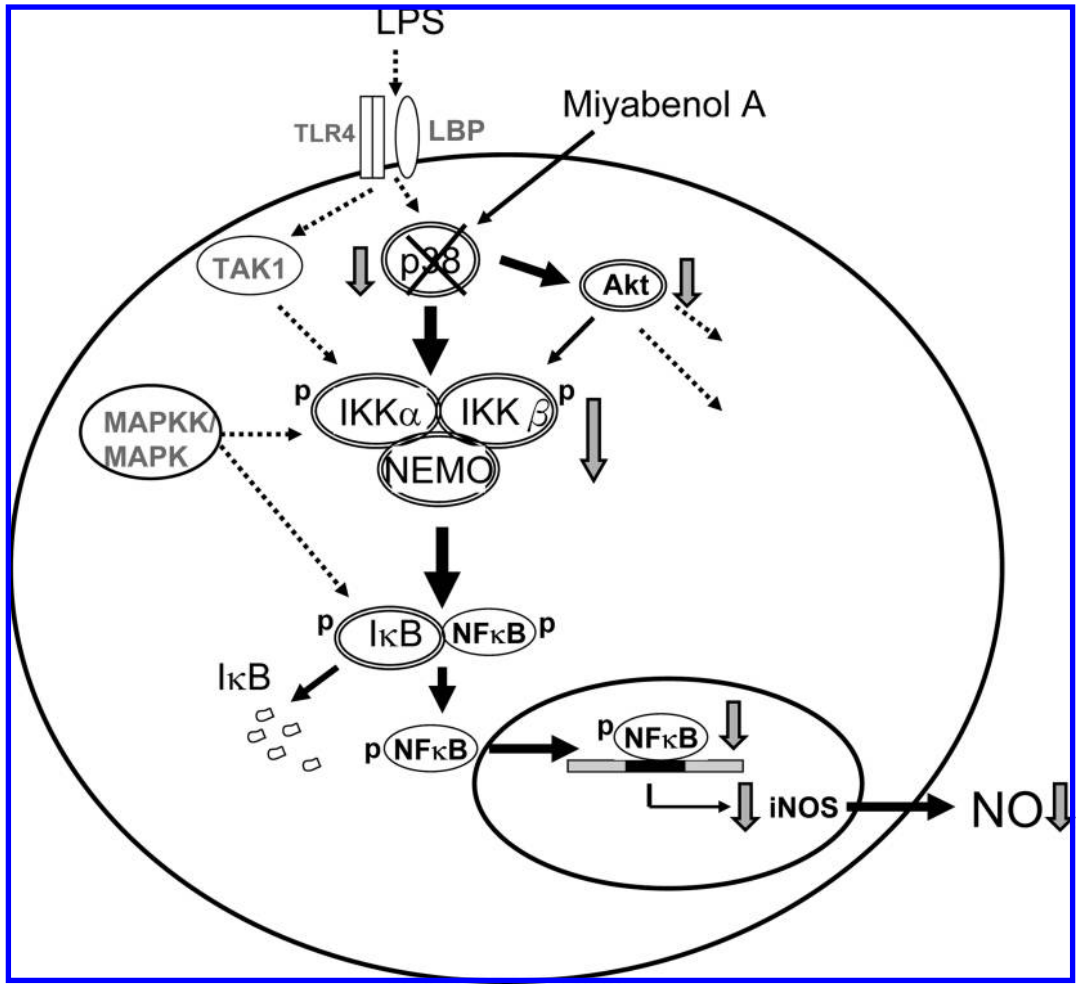

Figure 7. Schematic diagram illustrating the signaling pathways involved in miyabenol A's inhibition of LPS-induced iNOS expression through inactivation of IKK/IκB signaling cascade in RAW 264.7 macrophages. Miyabenol A acts via direct down-regulation of p38 MAPK phosphorylation and indirect influence of Akt phosphorylation, which subsequently results in suppression of LPS-induced iNOS expression and NO synthesis. LBP, LPS binding protein; TLR4, Toll-like receptor 4; TAK1, transforming growth factor-β(TGF-β)-activated kinase 1.

stimulated with LPS, and miyabenol A significantly inhibited its phosphorylation.

MAPK signaling pathways can regulate the expression of many genes. However, whether MAPK participates in regulating iNOS expression remains controversial. For example, Paul et al. showed that p38 and ERK pathways are not essential for NO production and iNOS expression because LPS-induced iNOS expression could not be blocked by SB203580 and PD98059 (*23*). By contrast, Kim et al. reported that activation of ERK and p38 is involved in LPS-induced iNOS expression (*24*). However, Choi et al. had reported that LPS stimulation of murine macrophages leads to increased phosphorylation and activation of ERK1/ 2, JNK, and p38 MAPKs (*25*). The reason for such inconsistency is still unknown because the durations and concentrations of the treatment were almost the same. Our previous finding is consistent with the results reported by Kim et al. (*24*) and Choi et al. (*25*) that LPS stimulated the phosphorylation of JNK, ERK1/2 and p38 MAPKs, and the data from MAPK-specific inhibitors also revealed that JNK, ERK, and p38 MAPKs all participated in LPS-induced iNOS expression and NO production (*9*). In the investigation of how miyabenol A affected MAPK-mediated activation of iNOS expression in RAW264.7 cells, we found that miyabenol A selectively suppressed LPS-induced p38 MAPK phosphorylation without affecting either JNK or ERK phosphorylation. Except IKK, the activation of NF-κB signal is also regulated by other cellular kinases, such as MAPKs. Thus, we further elucidated the signal linkage between p38 MAPK and NF-κB. We found that LPS-induced IκB degradation was abolished in the presence of SB203580 (a p38 MAPK inhibitor). An interesting finding is that SB203580 also strongly suppressed upstream IKKα/β phosphorylation, suggesting that p38 MAPK indeed played an important role in mediating IKK/IκB pathway activation in LPS-stimulated RAW264.7 macrophages.

Additionally, the present result showed that miyabenol A inhibits the LPS-induced phosphorylation of Akt, which is a critical step in PI3K activation. It has been suggested that Akt may also facilitate the phosphorylation of NF-κB p65 on serines 529 and 536 and subsequent nuclear translocation. Akt also phosphorylates a number of substrates at a RXRXXS/T motif; one such substrate identified is IKKα (*26*). Li and Stark have suggested that Akt activation of IKKα could be upstream of the phosphorylation of p65 by IKK (*27*). On the contrary, Dhawan et al. reported that inhibitors of PI3K blocked the endogenous NF-κB luciferase activity of malignant melanoma cells; however these inhibitors did not block IKK phosphorylation of IκBα substrate, thus indicating that Akt-mediated NF-κB activation is downstream of IKK or separate from the IKKα mediated phosphorylation of IκBα. Our result obtained from **Figure 6B** demonstrated that Wortmannin mildly but indeed significantly blunted LPS-induced IKKα/β phosphorylation and IκB degradation. Akt

activation has been reported to induce modest IκB degradation by enhancing IKK phosphorylation as well as regulating the MEK/ERK signaling cascade (*16*). On the basis of our finding that SB203580 attenuated LPS-induced Akt phosphorylation whereas Wortmannin could not affect LPS-inducedp38 phosphorylation, we predict that PI3K/Akt may lie downstream of p38 MAPK and act as another signal molecule to reciprocally mediate the activation of the IKK/IκB pathway.

In conclusion, the present study revealed that miyabenol A inhibits LPS-induced NO production through inactivation of IKK/IκB and the NF-κB signaling cascade in RAW 264.7 macrophages. The proposed action mechanism by miyabenol A is summarized in **Figure 7**. Miyabenol A acts primarily through down-regulation of p38 MAPK phosphorylation and en passant influences the phosphorylation of Akt, which subsequently results in the suppression of the IKK/IκB signaling cascade. Whether miyabenol A activates mitogen-activated protein kinase phosphatase-1 (MKP-1) to dephosphorylate and inactivate LPS-stimulated p38 MAPK activation remains to be studied. Except NO, p38 MAPK pathway function is critical for the production and activity of multiple other pro-inflammatory cytokines, including TNFα, IL-1, IL-6, and IL-8, in cells such as macrophages, monocytes, synovial cells, and endothelial cells (*28, 29*). Preclinical studies with p38 inhibitors have repeatedly demonstrated significant efficacy in many disease models, including chronic inflammation, arthritis, pain, and airway disease (*30*). Our findings provide the first molecular basis for the anti-inflammatory action of miyabenol A and demonstrated that miyabenol A is one of the major components of *Vitis thunbergii*, which appears to have the potential to prevent inflammatory diseases. Further exploration of miyabenol A from biological, cellular, and/or *in vivo* perspectives is still to be determined.

ABBREVIATIONS USED

DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; IκB, inhibitory factor κB; IKK, IκB kinase; iNOS, inducible nitric oxide synthase; JNK, c-Jun NH2-terminal kinase; LPS, lipopolysaccharide; MAPKs, mitogen-activated protein kinases; NF-κB, nuclear factor-κB; NO, nitric oxide; p38, p38 MAP kinase; PI3K, phosphatidylinositol 3-kinase.

LITERATURE CITED

(1) Chiu, N. Y.; Chang, K. H. *The Illustrated Medicinal Plants of Taiwan*; SMC Publisher: Taipei, 1995; Vol. 4, pp 147−149.

(2) Birrell, M. A.; McCluskie, K.; Wong, S.; Donnelly, L. E.; Barnes, P. J.; Belvisi, M. G. Resveratrol, an extract of red wine, inhibits lipopolysaccharide induced airway neutrophilia and inflammatory mediators through an NF-κB-independent mechanism. *FASEB J.* **2005**, *19*, 840–841.

(3) Leiro, J.; lvarez, E.A′.; Arranz, J. A.; Laguna, R.; Uriarte, E.; Orallo, F. Effects of *cis*-resveratrol on inflammatory murine macrophages: antioxidant activity and down-regulation of inflammatory genes. *J. Leukocyte Biol* **2004**, *75*, 1156–1165.

(4) Huang, Y. L.; Tsai, W. J.; Shen, C. C.; Chen, C. C. Resveratrol derivatives from the roots of *Vitis thunbergii*. *J. Nat. Prod.* **2005**, *68*, 217–220.

(5) Xie, Q. W.; Whisnant, R.; Nathan, C. Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide. *J. Exp. Med.* **1993**, *177*, 1779–1784.

(6) Petros, A.; Bennett, D.; Vallance, P. Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. *Lancet* **1991**, *338*, 1557–1558.

(7) Giuliani, C.; Napolitano, G.; Bucci, I.; Montani, V.; Monaco, F. NF-κB transcription factor: role in the pathogenesis of inflammatory, autoimmune and neoplastic diseases and therapy implications. *Clin. Ther.* **2001**, *152*, 249–253.

(8) Hayden, M. S.; Ghosh, S. Signaling to NF-kappaB. *Genes Dev.* **2004**, *18*, 2195–2224.

(9) Chen, C. C.; Tsai, P. C.; Wei, B. L.; Chiou, W. F. 8-Prenylkaempferol suppresses inducible nitric oxide synthase expression through interfering with JNK-mediated AP-1 pathway in murine macrophages. *Eur. J. Pharmacol.* **2008**, *590*, 430–436.

(10) Don, M. J.; Liao, J. F.; Lin, L. Y.; Chiou, W. F. Cryptotanshinone inhibits chemotactic migration in macrophages through negative regulation of PI3K signaling pathway. *Br. J. Pharmacol.* **2007**, *151*, 638–646.

(11) Ko, H. C.; Kuo, Y. H.; Wei, B. L.; Chiou, W. F. Laxifolone A suppresses LPS/IFN-γ-induced NO synthesis by attenuating NF-κB translocation: role of NF-κB p105 level in RAW264.7 macrophages. *Planta Med.* **2005**, *71*, 514–519.

(12) Janssen-Heininger, Y. M.; Poynter, M. E.; Baeuerle, P. A. Recent advances towards understanding redox mechanisms in the activation of nuclear factor kappaB. *Free Radical Biol. Med* **2000**, *28*, 1317–1327.

(13) Ghosh, S.; Karin, M. Missing pieces in the NF-κB puzzle. *Cell* **2002**, *109*, S81−S96.

(14) Bian, Z. M.; Elner, S. G.; Yoshida, A.; Elner, V. M. Human RPE-monocyte coculture induces chemokine gene expression through activation of MAPK and NIK cascade. *Exp. Eye Res.* **2003**, *76*, 573–583.

(15) Vanden Berghe, W.; Plaisance, S.; Boone, E.; De Bosscher, K.; Schmitz, M. L.; Fiers, W.; Haegeman, G. p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-kappaB p65 transactivation mediated by tumor necrosis factor. *J. Biol. Chem.* **1998**, *273*, 3285–3290.

(16) Sizemore, N.; Leung, S.; Stark, G. R. Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit. *Mol. Cell. Biol.* **1999**, *19*, 4798–4805.

(17) Naseem, K. M. The role of nitric oxide in cardiovascular diseases. *Mol. Aspects Med.* **2005**, *26*, 33–65.

(18) Pannu, R.; Singh, I. Pharmacological strategies for the regulation of inducible nitric oxide synthase: Neurodegenerative versus neuroprotective mechanisms. *Neurochem. Int.* **2006**, *49*, 170–182.

(19) Tinker, A. C.; Wallace, A. V. Selective inhibitors of inducible nitric oxide synthase: potential agents for the treatment of inflammatory diseases. *Curr. Top. Med. Chem.* **2006**, *6*, 77–92.

(20) Huang, Y. L.; Loke, S. H.; Hsu, C. C.; Chiou, W. F. (+)-Vitisin A inhibits influenza A virus-induced RANTES production in A549 alveolar epithelial cells through interfering with Akt and $STAT_1$ phosphorylation. *Planta Med.* **2008**, *74*, 156–162.

(21) Zandi, E.; Rothwarf, D. M.; Delhase, M.; Hayakawa, M.; Karin, M. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF kappaB activation. *Cell* **1997**, *91*, 243–252.

(22) Yun, K. J.; Kim, J. Y.; Kim, J. B.; Lee, K. W.; Jeong, S. Y.; Park, H. J.; Jung, H. J.; Cho, Y. W.; Yun, K.; Lee, K. T. Inhibition of LPS-induced NO and PGE2 production by asiatic acid via NF-κB inactivation in RAW 264.7 macrophages: Possible involvement of the IKK and MAPK pathways. *Int. Immunopharmacol.* **2008**, *8*, 431–441.

(23) Paul, A.; Cuenda, A.; Bryant, C. E.; Murray, J.; Chilvers, E. R.; Cohen, P. Involvement of mitogen-activated protein kinase homologues in the regulation of lipopolysaccharide-mediated induction of cyclo-oxygenase-2 but not nitric oxide synthase in RAW 264.7 macrophages. *Cell. Signalling* **1999**, *11*, 491–497.

(24) Kim, S. H.; Kim, J.; Sharma, R. P. Inhibition of p38 and ERK MAP kinases blocks endotoxin-induced nitric oxide production and differentially modulates cytokine expression. *Pharmacol. Res.* **2004**, *49*, 433–439.

(25) Choi, C. Y.; Park, K. R.; Lee, J. H.; Jeon, Y. J.; Liu, K. H.; Oh, S.; Kim, D. E.; Yea, S. S. 2007 Isoeugenol suppression of inducible nitric oxide synthase expression is mediated by down-regulation of NF-κB, ERK1/2, and p38 kinase. *Eur. J. Pharmacol.* **2007**, *576*, 151–159.

(26) Madrid, L. V.; Mayo, M. W.; Reuther, J. Y.; Baldwin, A. S. Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogenactivated protein kinase p38. *J. Biol. Chem.* **2001**, *276*, 18934–18940.

(27) Li, X.; Stark, G. R. NF-κB-dependent signaling pathways. *Exp. Hematol.* **2002**, *30*, 285–296.

(28) Ajizian, S. J.; English, B. K.; Meals, E. A. Specific inhibitors of p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways block inducible nitric oxide synthase and tumor necrosis factor accumulation in murine macrophages stimulated with lipopolysaccharide and interferon-gamma. *J. Infect. Dis.* **1999**, *179*, 939–944.

(29) Underwood, D. C.; Osborn, R. R.; Bochnowicz, S.; Webb, E. F.; Rieman, D. J.; Lee, J. C. SB 239063, a p38 MAPK inhibitor, reduces neutrophilia, inflammatory cytokines, MMP-9, and fibrosis in lung. *Am. J. Physiol. Lung Cell Mol. Physiol* **2000**, *279*, L895–L902.

(30) Schindler, J. F.; Monahan, J. B.; Smith, W. G. p38 pathway kinases as anti-inflammatory drug targets. *J. Dent. Res.* **2007**, *86*, 800–811.

Received for review June 24, 2008. Revised manuscript received August 6, 2008. Accepted August 6, 2008. This work was supported by grants from National Research Institute of Chinese Medicine to Y.-L.C.H. and W.F.C.

JF8019369