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ARTICLE

Anti-Inflammatory Bioactivities of Honokiol through Inhibition of Protein Kinase C, Mitogen-Activated Protein Kinase, and the NF-κB Pathway To Reduce LPS-Induced TNFα and NO Expression

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Much recent research has demonstrated that honokiol, a phenolic compound originally isolated from *Magnolia officinalis*, has potent anticancer activities; however, the detailed molecular mechanism of its anti-inflammatory activity has not yet been fully addressed. In this study we demonstrated that honokiol inhibited lipopolysaccharide (LPS)-induced tumor necrosis factor-α secretion in macrophages, without affecting the activity of the tumor necrosis factor-α converting enzyme. At the same time, honokiol not only inhibited nitric oxide expression in LPS-stimulated murine macrophages but also inhibited the LPS-induced phosphorylation of ERK1/2, JNK1/2, and p38. By means of confocal microscope analysis we demonstrated that phosphorylation and membrane translocation of protein kinase C-α, as well as NF-κB activation, were inhibited by honokiol in LPS-stimulated macrophages. Furthermore, it was found that honokiol neither antagonizes the binding of LPS to cells nor alters the cell surface expression of toll-like receptor 4 and CD14. Our current results have exhaustively described the anti-inflammatory properties of honokiol, which could lead to the possibility of its future pharmaceutical application in the realm of immunomodulation.

KEYWORDS: Honokiol; LPS; cytokines; signaling

INTRODUCTION

The innate immunological response of mammalian cells is typically triggered by pathogen-associated molecular patterns that are shared by groups of different microbial pathogens, which are recognized by toll-like receptors (TLRs) expressed on the cell surface of monocytes and macrophages (*1*). Lipopolysaccharide (LPS) activates monocytes and macrophages by binding to TLR4, and stimulates the production of tumor necrosis factor-α (TNFα) and nitric oxide (NO) (*2*). TNFα- and NO-mediated signaling play various physiological processes, including immune defense and smooth muscle relaxation (*3*); however, overexpression of TNFα and NO are responsible for the origin and progression of rheumatoid arthritis and other inflammatory diseases (*4*). Development of a potential therapeutic approach to modulate inflammatory disease has become of increasingly greater concern and importance.

Several signal transduction cascades are involved in the regulation of inflammatory mediator expression in LPS-stimulated macrophages, such as protein kinases and transcription factors (*5*). Protein kinase C (PKC) is one of the signaling molecules in an LPS mediated inflammatory response (*6*). PKC is phosphorylated and translocated from cytosol when it is activated by physiological stresses; it then triggers a downstream signal transduction cascade via modulation of the mitogen-activated protein kinase (MAPK) pathways, such as extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase 1/2 (JNK1/2), and p38 MAP kinase (*7*). In addition to protein kinase, nuclear transcription factor kappa-B (NF-κB) also plays a pivotal role in the regulation of inflammatory-related gene expression (*8*).

Honokiol, a natural product with a small molecular weight—originally isolated from the Chinese medicinal herb *Magnolia officinalis*—inhibits the growth of various cancer cell lines *in vitro* (*9*) and *in vivo* (*10*). In addition to its anticancer activity, honokiol reduces *Propionibacterium acnes*-induced TNFα, as well as the expression of interleukin-8 and cyclooxygenase-2, by the reduction of NF-κB activation in human monocytic THP-1 cells (*11*). Honokiol also inhibits phorbol-12-myristate-13-acetate- or *N*-formylmethionyl-leucyl-phenylalanine-induced inflammatory responses by reducing reactive oxygen species release in neutrophils (*12*). Honokiol-containing materials have been shown

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to inhibit the production of TNFα and NO in LPS-activated murine macrophages (*13*). In this study, we demonstrate that honokiol regulates LPS-induced TNFα and NO expression by affecting MAPKs, PKCα, and NF-κB pathways. These findings provide support for the potential future pharmaceutical application of honokiol as an immune modulator.

MATERIALS AND METHODS

Materials. Honokiol was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and repurified by semipreparative HPLC (Hitachi L-7100 pump, with a UV detector model Jasco UV 975) with a Merck 250−100 column (the purity of honokiol reached 99%). LPS (from *Escherichia coli* 0111:B4), anti-MAPKs antibodies, PD98059, SP600125, and SB203580 were purchased from Sigma Co. (St. Louis, MO). Alexa Fluor 488-labeled LPS (LPS-Alexa) were purchased from Molecular Probes (Eugene, OR). PE-conjugated anti-TLR4, and PE-conjugated anti-CD14 were purchased from Imgenex Corp. (Carlsbad, CA). Anti-mouse CD14 blocking antibody was purchased from BD Pharmingen (San Diego, CA). TNFα ELISA kit was purchased from R&D Systems, Inc. (Minneapolis, MN). Anti-PKC antibody, anti-rabbit IgG-HRP, and anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell Cultures. Human primary monocytes were obtained from normal blood donor buffy coats (Taipei Blood Center, Taipei, Taiwan). Buffy coat cells were mixed with an equal volume of PBS, layered on Histopaque-1077 (Sigma, St. Louis, MO) and centrifuged at 400x*g* for 30 min at 20 °C. The interface containing mononuclear cells was collected and washed twice with PBS. Thereafter, human primary monocytes (98% CD14+, analyzed by flow cytometry) were isolated from mononuclear cells by a Monocyte Isolation Kit II (Miltenyi Biotec, Auburn, CA). Human primary monocytes were cultured in RPMI-1640 supplemented with 10% fetal calf serum (Hyclone, Logan, UT). Human primary macrophages were obtained by culturing monocytes for 7 days in RPMI-1640 supplemented with 15% fetal calf serum at a density of $1.5 \times 10^5/cm^2$. During the culturing period, nonadherent cells were removed by washing with PBS. After 7 days of incubation, the adherent cells were used as human blood monocyte-derived primary macrophages. Human THP-1 monocytes and murine J774A.1 macrophages were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Murine RAW 264.7 macrophages stably transfected with the NF-κB reporter gene (RAW-Blue cells) were purchased from InvivoGen (San Diego, CA).

NO Production Assay. RAW 264.7 cells were plated at 1×10^5 cells/well in 12-well plates, and then incubated with or without LPS (1 μg/mL) in the absence or presence of honokiol for 24 h. NO levels in culture media were determined using the Griess reaction. Briefly, 100 μL of cell culture medium was mixed with 100 μL of Griess reagent and incubated at room temperature for 10 min. The absorbance was then measured at 540 nm using a microplate reader (Perkin-Elmer Cetus, Foster City, CA). Fresh culture media were used as blanks in all experiments. NO levels in samples were read off a standard sodium nitrite curve.

NF-κB Reporter Gene Assay. RAW-Blue Cells (InvivoGen), RAW 264.7 macrophages which stably express a secreted embryonic alkaline phosphatase (SEAP) gene inducible by NF-κB, are resistant to the selectable marker Zeocin. RAW-Blue cells were seeded in 60 mm dishes at a density of 8×10^5 cells in 2 mL of medium, and grown overnight in a 5% CO_2 incubator at 37 °C. After pretreatment with test samples, followed by LPS stimulation for 24 h, the medium was harvested. Medium samples (20 μL) were then mixed with QUANTI-Blue (InvivoGen) medium (200 μL) in 96-well plates at 37 °C for 15 min. The results of SEAP activity were assessed by reading OD at 655 nm using an ELISA reader.

Flow Cytometry Analysis. To test the binding of LPS to cell surfaces, paraformadehyde-fixed J774A.1 macrophages were incubated with honokiol (3 μg/mL or 10 μg/mL), anti-CD14 antibody (10 μg/mL), or isotype control antibody (10 μg/mL) for 30 min, followed by incubation with LPS-Alexa for 30 min at 4 °C. After washing, cells were subjected to flow cytometry analysis on FACSCalibur using CellQuest software from Becton, Dickinson (Franklin Lakes, NJ). For cell surface expression experiments with TLR4 and CD14, J774A.1 macrophages were incubated with honokiol (3 μg/mL or 10 μg/mL) or LPS (1 μg/mL) for 30 min. Cells were fixed, and cell surface expressions of TLR4 and CD14 were measured by staining cells for 30 min with PE-conjugated anti-TLR4 antibody or PE-conjugated anti-CD14 antibody on ice, respectively. After washing, cells were subjected to flow cytometry analysis.

Confocal Microscope Analysis. J774A.1 macrophages were stimulated with LPS (1 μg/mL) in the presence or absence of honokiol (10 μg/mL) for 5 min, followed by fixation and permeability. Cells were stained with PE-conjugated anti-PKCα antibody for 2 h at room temperature. After washing, cells were visualized using a Leica TLS SP2 confocal microscope (Leica Lasertechnik, Heidelberg, Germany).

Analysis of TACE Activity. Cells were incubated with honokiol and NAC (10 mM) for 30 min, followed by LPS stimulation for 6 h. The cell lysates were obtained by frozen and thaw method. Enzymatic reactions were carried out in buffer containing 75 μg of protein and 2.5 mM fluorogenic peptide Mca-P-L-A-Q-A-V-Dpa-R-S-S-S-R-NH_2. The reaction mixtures were incubated at 30 °C for 30 min, and the fluorescent formation was measured at excitation 360 nm and emission 460 nm.

Microculture Tetrazolium (MTT) Assay for Cell Viability. The cytotoxicity of honokiol was assessed using a MTT assay. After culturing on 96-well plates for 24 h, the cells were washed twice and incubated with 100 μL of MTT (1 mg/mL) for 2 h at 37 °C. The medium was discarded, and 100 μL of lysis buffer was then added. After 30 min incubation, the absorbance was measured at 570 nm using a microplate reader.

Western Blotting Analysis. Whole cell lysates were separated by 12% SDS−PAGE and electrotransferred to a PVDF membrane. The membrane was incubated in blocking solution (5% nonfat milk in PBS with 0.1% Tween 20) at room temperature for 1 h. The membrane was incubated with anti-PKCα and anti-MAPKs antibody at room temperature for 2 h. After washing three times in PBS with 0.1% Tween 20, the membrane was incubated with an HRP-conjugated secondary antibody directed against the primary antibody. The membrane was developed by an enhanced chemiluminescence Western-blotting detection system (DuPont NEN Research Product Co., Boston, MA) according to the manufacturer's instructions. Phospho-p38 in the cell samples with ImageQuaNT software from Molecular Dynamics.

Enzyme Linked Immunosorbent Assay (ELISA). The J774A.1 cell, THP-1 cells and human primary cells (1×10^6/mL) were stimulated with honokiol (0−10 μg/mL) for the indicated time points within 6 h and 24 h separately. The TNFα concentration in the condition medium was analyzed by ELISA according to the protocol from the R & D mouse TNFα ELISA System (R&D Systems, Inc.). In brief, 50 μL of biotinylated antibody reagent and 50 μL of supernatant were added to an anti-mouse TNFα precoated stripwell plate and incubated at room temperature for 3 h. After washing the plate three times with washing buffer, 100 μL of diluted streptavidin-HRP concentrate was added to each well and incubated at room temperature for 30 min. The washing process was repeated, and 100 μL of a premixed TMB substrate solution was added to each well and developed at room temperature in the dark for 30 min. Following the addition of 100 μL of provided stop solution to each well to stop the reaction, the absorbance of the plate was measured by a MRX microplate reader (Dynex Tech. Inc.) at a 450−550 nm wavelength. All methods and procedures followed the previous descriptions (*14*).

Statistical Analysis. All values are given as means ± SD. Data analysis involved one-way ANOVA with subsequent Scheffé test.

RESULT

Effect of Honokiol on Cell Viability. The goal of this study was to investigate the anti-inflammatory acivity of honokiol on macrophages. In consideration of the macrophages bing normal immune cells, the dosage of honokiol used in this study should not affect the survival of macrophages. To examine the toxicity of honokiol under experimental conditions, J774A.1 macrophages were treated with various concentrations (1, 3, and 10 μg/mL) of honokiol, PD98059 (MEK1 inhibitor, positive control) or DMSO (vehicle) for 6 and 24 h. The cell viability was analyzed by MTT assay. Experimental results revealed that cell proliferation was not affected by 6 h honokiol treatment. Cell proliferation was slightly decreased when cells were treated with 10 μg/mL honokiol and PD98059 for 24 h (**Figure 1A**). In order to understand whether

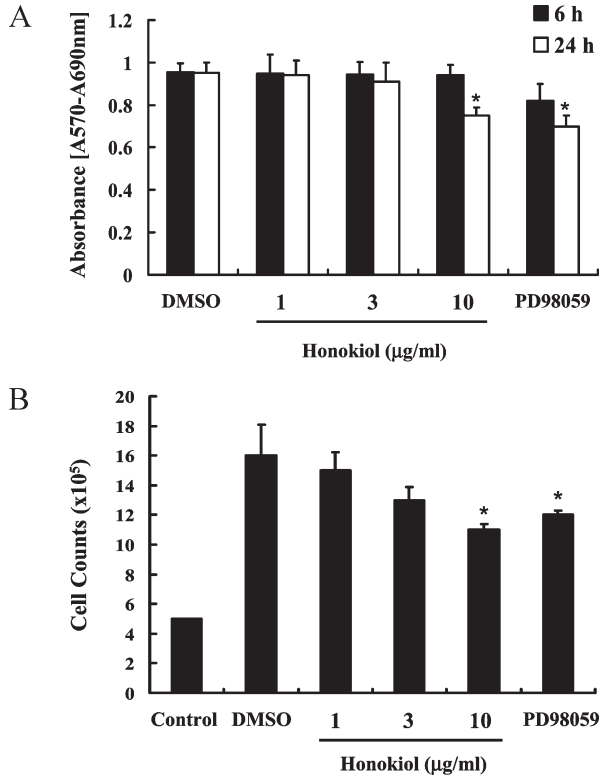

Figure 1. Effects of honokiol on cell viability and proliferation. (**A**) J774A.1 macrophages were treated with honokiol, PD98059 (50 μM), or DMSO (vehicle, the final concentration contains 0.05% DMSO) for 6 and 24 h, followed by incubating with MTT reagent. The absorbance ($A_{550} - A_{690}$) was measured by spectrophotometry. Data was expressed as means ± SD, with three separate experiments. *$p < 0.05$ versus vehicle. (**B**) J774A.1 macrophages (5×10^5, control) were treated with honokiol, PD98059 (50 μM), or DMSO (vehicle) for 24 h, followed by trypan blue staining and counting. Data was expressed as means ± SD, with three separate experiments.

10 μg/mL honokiol treatment for 24 h is toxic to cells or just reduced cell proliferation, 5×10^5 cells (control) were incubated with honokiol, PD98059, or DMSO for 24 h; during the 24 h period of cell culture at 37 °C, cell numbers in the 10 μg/mL honokiol-treated group and PD98059-treated group increased from 5×10^5 to 11×10^5 and 12×10^5, respectively, although to a lesser extent than that of DMSO-treated cells (increased from 5×10^5 to 16×10^5) (**Figure 1B**). These results indicated that 10 μg/mL honokiol slightly reduced cell proliferation, but did not exhibit a cytotoxic effect.

Honokiol Inhibits LPS-Induced TNFα Expression. To examine whether honokiol could inhibit TNFα expression in LPS-stimulated macrophages, murine macrophages were stimulated with LPS in the presence or absence of honokiol for 6 h. As can be seen in **Figure 2A**, honokiol at 10 μg/mL inhibited LPS-induced TNFα expression to around 50%. In order to test whether the inhibitory effect of honokiol on TNFα expression was cell type specific, we used human THP-1 monocytes and human peripheral blood monocyte-derived macrophages to test the hypothesis. The results showed that honokiol inhibited LPS-induced TNFα expression both in human THP-1 monocytes (**Figure 2B**) and in human peripheral blood monocyte-derived macrophages (**Figure 2C**). In addition, we also investigated whether honokiol could inhibit the induced TNFα expression of a Gram-positive bacteria cell wall component, lipoteichoic acid (LTA, a TLR2 ligand). We found that honokiol inhibited TNFα expression in LTA-stimulated cells (**Figure 2D**). TNFα is synthesized as a membrane-anchored

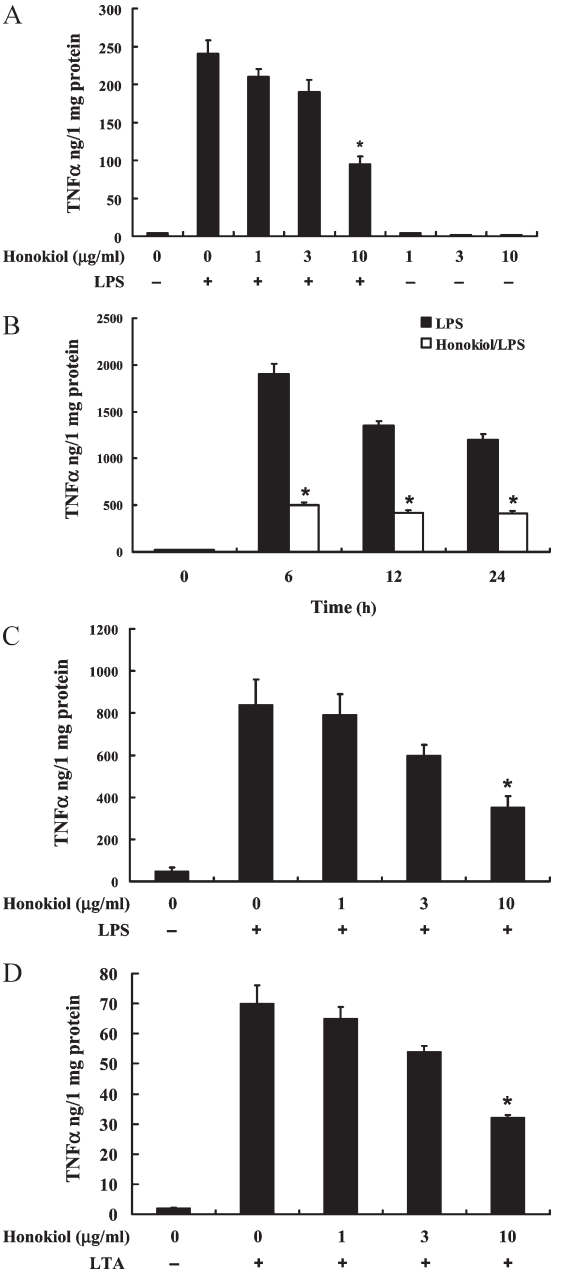

Figure 2. Effect of honokiol on TNFα expression. (**A**) J774A.1 macrophages (1×10^6/mL) were pretreated with honokiol or DMSO (vehicle) for 30 min, followed by stimulating with LPS (1 μg/mL) for 6 h. TNFα concentration in culture medium was assayed by ELISA. Data was expressed as means ± SD, with three separate experiments. *$p < 0.05$ versus LPS alone. (**B**) THP-1 monocytes (1×10^6/mL) were pretreated with honokiol (10 μg/mL) or DMSO for 30 min, followed by stimulating with LPS (1 μg/mL) for 0−24 h. TNFα concentration in culture medium was assayed by ELISA. Data was expressed as means ± SD, with three separate experiments. *$p < 0.05$ versus LPS alone. (**C**) Human primary macrophages derived from peripheral blood monocytes were pretreated with honokiol or DMSO for 30 min, followed by stimulating with LPS (1 μg/mL) for 6 h. TNFα concentration in culture media was assayed by ELISA. Data was expressed as means ± SD, with three separate experiments. *$p < 0.05$ versus LPS alone. (**D**) J774A.1 cells (1×10^6/mL) were pretreated with honokiol or DMSO for 30 min, followed by stimulating with LTA (10 μg/mL) for 6 h. TNFα concentration in culture media was assayed by ELISA. Data was expressed as means ± SD, with three separate experiments. *$p < 0.05$ versus LTA alone.

precursor, and is released into the extracellular space by TNFα converting enzyme (TACE) cleavage (*15*). The effect of honokiol

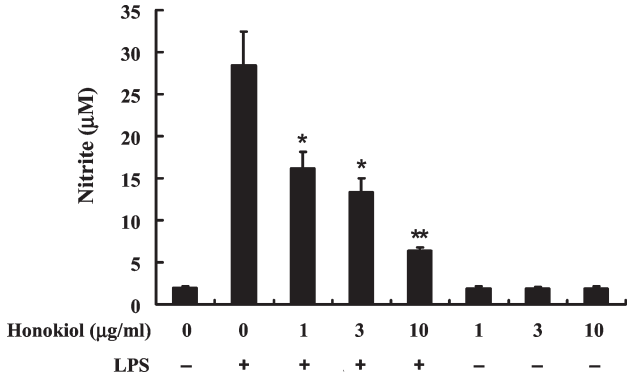

Figure 3. Effect of honokiol on NO expression. RAW 264.7 cells (1×10^6/mL) were pretreated with honokiol (10 μg/mL) or DMSO for 30 min, followed by stimulating with LPS (1 μg/mL) for 24 h. NO expression was measured by Griess reaction. Data was expressed as means ± SD, with three separate experiments. *$p < 0.05$, **$P < 0.01$ versus LPS alone.

on post-translational regulation of TNFα expression at the TACE level was also investigated. LPS-induced TACE activity was inhibited by an antioxidant, *N*-acetylcysteine (NAC) (10 mM); however, honokiol did not influence TACE activity in LPS-stimulated macrophages (data not shown).

Honokiol Inhibits LPS-Induced NO Production. In addition to TNFα expression, we investigated the inhibitory effects of honokiol on the LPS-induced production of NO. Cells were pretreated with honokiol for 30 min, followed by LPS stimulation for 24 h. NO concentrations in culture media were measured by Griess reaction. Honokiol inhibited LPS-induced NO production in a dose-dependent manner (**Figure 3**). In addition, it inhibited JNK1/2 and p38, but not ERK1/2, and reduced NO production in LPS-stimulated cells (data not shown), thus indicating that LPS-mediated NO production is via activation of JNK1/2 and p38 related pathways.

Honokiol Reduces NF-κB Activation through Inhibition of PKCα. To dissect the molecular mechanism of honokiol-mediated anti-inflammatory responses, we tested the effect of honokiol on LPS-induced PKCα activation. The phosphorylation level of PKCα peaked 15 min after LPS stimulation (data not shown). LPS-mediated PKCα phosphorylation was reduced by honokiol (**Figure 4A**). In addition, the effect of honokiol on LPS-mediated PKCα activation was confirmed by using subcellular distribution analysis. We showed that PKCα resided in the cytoplasm as a nonactive enzyme in the resting stage (**Figure 4B**, upper panel). PKCα underwent translocation from the cytosol to the cell membrane upon stimulation with LPS for a period of 5 min (**Figure 4B**, middle panel); this reflected the relative potency of LPS as an activator of PKCα. In contrast, honokiol treatment significantly inhibited LPS-induced PKCα membrane translocation (**Figure 4B**, lower panel). This result confirmed that honokiol inhibited PKCα activation in LPS-stimulated cells.

PKC is implicated in the regulation of NF-κB activation in macrophages (*16*); hence, we attempted to test whether honokiol could inhibit NF-κB activity in LPS-induced macrophages. Using NF-κB-dependent alkaline phosphatase reporter cells, we demonstrated that NF-κB transcriptional activity in LPS-stimulated cells was blocked by honokiol as well as by the PKCα inhibitor Gö6976 (**Figure 4C**). These results indicated that honokiol's inhibition of NF-κB activation may be due, at least in part, to downregulation of PKCα.

Effect of Honokiol on Protein Kinase Activation. Because LPS-induced NO production was regulated by JNK1/2 and p38 (data not shown), we investigated the influence of honokiol on LPS-induced activation of MAPKs. LPS strongly induced phosphorylation of ERK1/2, JNK1/2 and p38, whereas phosphorylations of ERK1/2 and JNK1/2, but not p38, were significantly inhibited by honokiol in LPS-stimulated cells (**Figure 5**). In addition, to test whether LPS-induced NF-κB activation is regulated by MAPKs, cells were preincubated with MAPK inhibitors PD98059 (MEK1 inhibitor), SP600125 (JNK1/2 inhibitor), and SB203580 (p38 inhibitor), followed by LPS stimulation for 24 h. We found that all of these inhibitors reduced NF-κB activation in LPS-stimulated cells (data not shown). These results indicated that LPS induced NF-κB activation through MAPKs, and that honokiol possibly mediated NF-κB down-regulation, at least in part, through inhibition of MAPKs. In addition, LPS-induced TNFα expression was inhibited by inhibition of MAPKs (data not shown).

Effect of Honokiol on LPS Binding and Cell Surface Expression of TLR4/CD14 in Macrophages. The results described above indicated that honokiol significantly inhibited TNFα and NO expression. This indicates that honokiol may influence the events in LPS signaling, or inhibit LPS binding to the cell surface receptors CD14/TLR4 of macrophages (*1*). We therefore conducted experiments to evaluate the effect of honokiol on LPS binding to macrophages and cell surface expression of TLR4 and CD14. Preincubation of cells with anti-CD14 monoclonal antibody resulted in significant inhibition of LPS binding to positive control cells; however, the binding was not blocked by isotype control antibody (negative control). Thus, the binding of LPS to cells was CD14-dependent. In contrast to the anti-CD14 monoclonal antibody, honokiol did not block the binding of LPS to cells even at a concentration of 10 μg/mL (**Figure 6A**). We then analyzed surface expression of TLR4 and CD14, essential signaling and binding receptors for LPS, upon honokiol treatment. The cell surface expression of TLR4 and CD14 was partially decreased by LPS stimulation; however, surface TLR4 and CD14 expression was not changed by honokiol treatment (**Figure 6B**). These results suggest that honokiol inhibited TNFα and NO production without reducing LPS receptor expression and without antagonizing the binding of LPS to the TLR4/CD14 complex.

DISCUSSION

It appears from our review of the literature that interest in honokiol is increasing. Honokiol induces cell death through caspase-dependent apoptosis in various cancer cells, human myeloma cells (*9*), breast cancer cells (*17*) and human gastric cancer cells (*18*). Honokiol induces necrotic cell death through the mitochondrial permeability transition pore in HL60 and MCF-7 cells (*19*). Honokiol also inhibits angiogenesis and angiosarcoma tumor growth *in vivo* (*10*). In addition to its anticancer activity, honokiol inhibits oxidized LDL-induced cytotoxicity and adhesion molecule expression in endothelial cells (*20*). A recent report shows that honokiol inhibits inflammatory signals and alleviates inflammatory arthritis induced by collagen treatment (*21*); however, the cellular mechanism involved in the honokiol-mediated down-regulation of pro-inflammatory mediators in LPS-stimulated macrophages still appears to be unclear.

TNFα and NO are produced by activated macrophages, as well as by many other cell types. The inhibitory effects of honokiol on TNFα expression were observed in both murine macrophages and human primary macrophages derived from peripheral blood monocytes, as well as in human THP-1 monocytes; this suggests that differences in species do not greatly affect the efficacy of honokiol. The concentration of honokiol required for inhibition of LPS-induced TNFα expression appears to be compatible with its antibacterial activity against *Propionibacterium acnes*

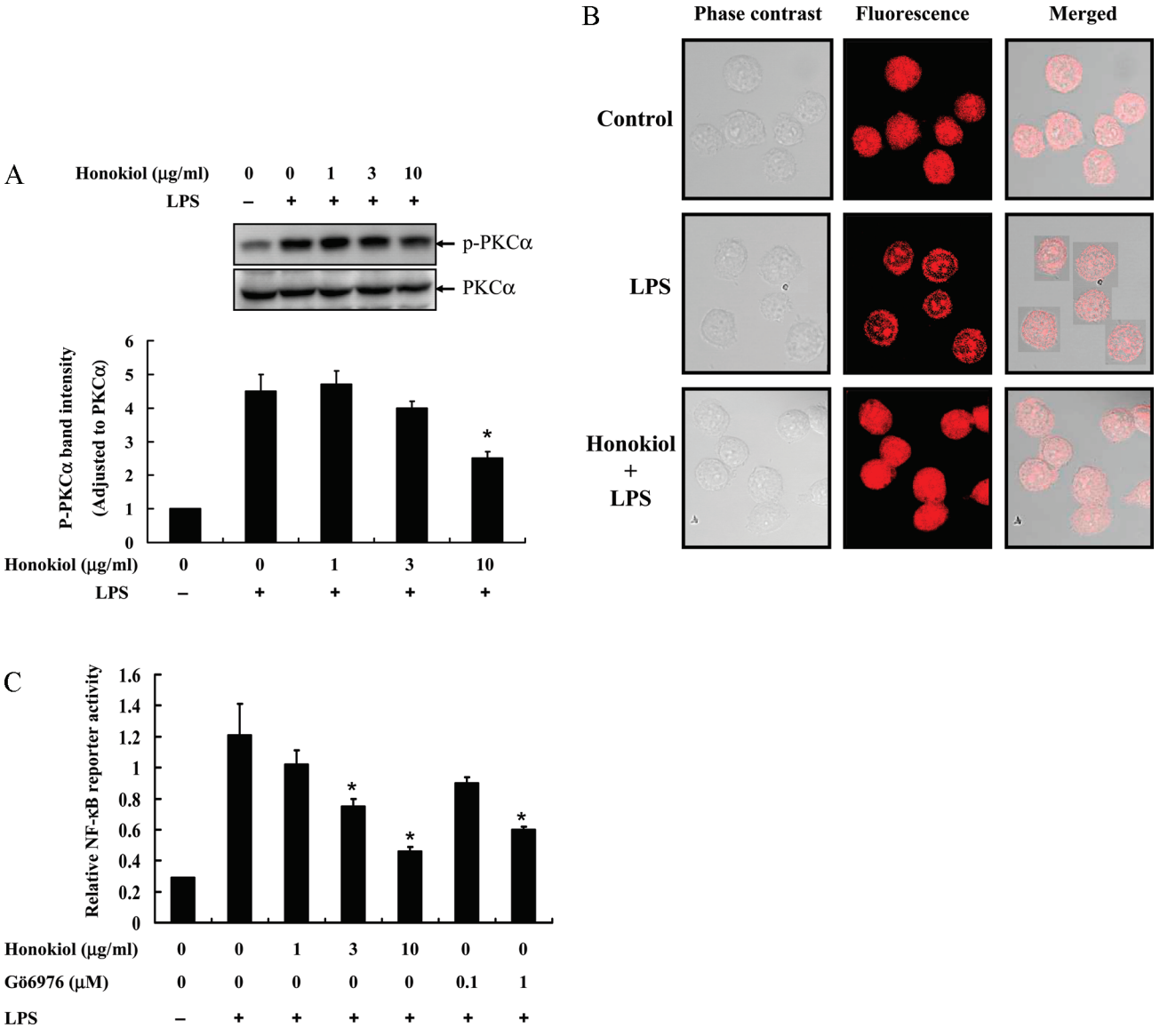

Figure 4. Effect of honokiol on PKCα activation. (**A**) RAW 264.7 cells were pretreated with honokiol (10 μg/mL) or DMSO for 30 min, followed by stimulating with LPS (1 μg/mL) for 15 min. The phosphorylation level of PKCα was measured by Western blot (n = 3). The histogram represents quantification by PhosphorImager of phosphor-PKCα (adjusted to total PKCα) using ImageQuant software. *p < 0.05 versus LPS alone. (**B**) RAW 264.7 cells were pretreated with honokiol (10 μg/mL) or DMSO for 30 min, followed by stimulating with LPS (1 μg/mL) for 5 min. Cells were fixed with 2% paraformaldehyde and permeated with 0.1% Triton X-100, followed by staining with PE-conjugated anti-PKCα antibody. Images were obtained by confocal microscope. (**C**) RAW-Blue cells were pretreated with honokiol, Gö6976 or DMSO for 30 min, followed by stimulating with LPS (1 μg/mL) for 24 h. SEAP activities were measured by QUANTI-Blue. Data was expressed as means ± SD, with three separate experiments. *p < 0.05 versus LPS alone.

and *P. granulosum*, which are acne-causing bacteria (*22*). Compared with previous research (*13*), our study demonstrated that honokiol at 10 μg/mL (equal to 37.5 μM) exhibited an obvious inhibiting effect on NO production, TNFα expression and phosphorylation of p38 in LPS-induced macrophages, a result similar to Kim et al. (*13*), but with different results regarding the phosphorylation of ERK.

Studies reveal that TLR2 and TLR4 are the key molecules for recognizing cell wall components of Gram-positive and Gram-negative bacteria, respectively, in order to elicit inflammatory responses (*2*, *5*). We found not only that honokiol inhibited TLR4/LPS-mediated TNFα expression but also that TLR2/LTA induced TNFα expression, indicating that honokiol could be developed as an anti-inflammatory agent against bacterial infections.

PKC plays an important role in LPS-mediated TNFα expression (*23*). We found that honokiol inhibited LPS-induced PKCα phosphorylation and membrane translocation, indicating that it inhibited PKCα activation upon LPS stimulation. Inhibition of PKCα by Gö6976 reduced ERK1/2, JNK1/2, and NF-κB activation as well as TNFα and NO expression in LPS-stimulated macrophages, suggesting that PKCα is one of the possible molecular targets for honokiol-mediated anti-inflammatory responses. MAPK inhibitors targeting various inflammatory cells and pathways are important treatment modalities for patients with inflammatory diseases (*24*). Downregulation of MAPK activation by anti-inflammatory natural products in LPS-stimulated macrophages is one of the cellular mechanisms that inhibit cytokine expression (*14*). We found that honokiol significantly inhibited MAPK activation, especially ERK1/2 and JNK1/2, which are involved in LPS-induced cytokine expression in macrophages (*23*). Honokiol also has been reported to reduce JNK1/2 phosphorylation and protects against glycochenodeoxycholic acid-induced apoptosis in primary cultured rat hepatocytes (*25*).

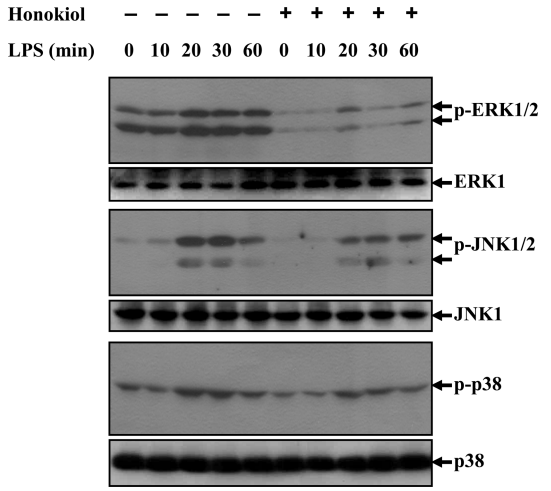

Figure 5. Effect of honokiol on MAPKs phosphorylation. J774A.1 macrophages were pretreated with honokiol (10 μg/mL) or DMSO for 30 min, followed by stimulating with LPS (1 μg/mL) for 0−60 min. The phosphorylation levels of ERK1/2, JNK1/2 and p38 were analyzed by Western blot ($n = 3$).

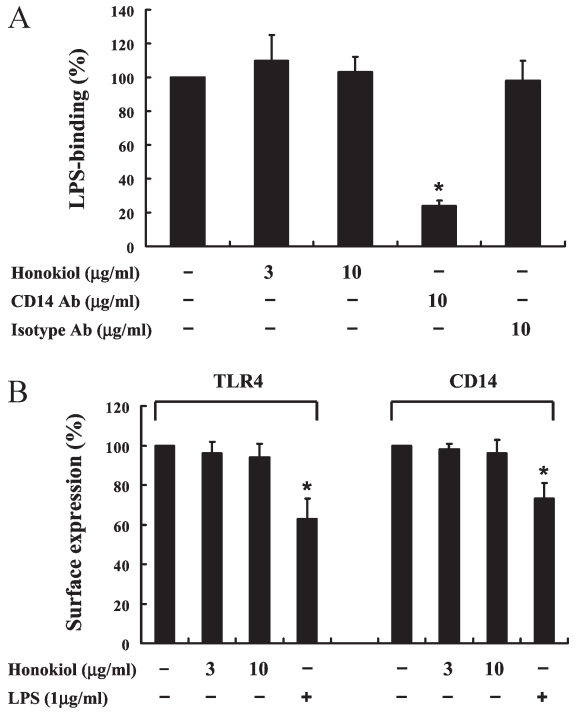

Figure 6. Effect of honokiol on LPS binding and cell surface expression of TLR4 and CD14 in macrophages. (**A**) J774A.1 macrophages were incubated with honokiol (3 μg/mL or 10 μg/mL), anti-mouse CD14 monoclonal antibody (10 μg/mL), or isotype control antibody (10 μg/mL) for 30 min at 4 °C, followed by incubating with fluorescence-conjugated LPS (LPS-Alexa) (1 μg/mL) for 30 min. After washing, the cells were analyzed by flow cytometry. Data was expressed as means ± SE, with three separate experiments. *$p < 0.05$ versus LPS alone. (**B**) J774A.1 macrophages were incubated with honokiol (3 μg/mL or 10 μg/mL), LPS (1 μg/mL) or DMSO for 30 min at 37 °C, followed by fixing with 2% paraformaldehyde. Cells were stained with PE-conjugated TLR4 or CD14 antibody for 30 min, and then analyzed by flow cytometry. Data was expressed as means ± SD, with three separate experiments. *$p < 0.05$ versus control.

In contrast, honokiol activated ERK1/2 and induced neurite outgrowth promotion (*26*); it also activated p38 MAPK in vascular smooth muscle cells and hepatocellular carcinoma cells (*27*). In addition to MAPKs, phosphoinositide 3-kinase/AKT pathways were inhibited by honokiol in LPS-stimulated macrophages (*13*).

It is well-known that NF-κB is involved in the modulation of immune-related genes and the expressions of pro-inflammatory mediators, such as TNFα and iNOS (*28*). Because the expressions of TNFα and iNOS are regulated by NF-κB, the possibility that honokiol inhibits NF-κB activity was investigated in LPS-stimulated macrophages. The present study demonstrated that honokiol effectively inhibited LPS-induced NF-κB activation. Previous studies demonstrated that honokiol inhibited NF-κB activation in *P. acnes* stimulated human THP-1 monocytes (*11*), in Epstein−Barr viral mimic latent membrane protein 1 stimulated mouse B cells (*21*), in high glucose stimulated human umbilical vein endothelial cells (*29*), and in TNFα stimulated cancer cell lines (*30*).

In conclusion, our results suggest that honokiol inhibits LPS-induced TNFα and NO expression through, at least in part, inhibition of MAPKs, PKCα, and NF-κB pathways, without interfering with LPS binding to CD14/TLR4. As a powerful anti-inflammatory, honokiol could offer an advantage in protecting the host from endotoxic shock. Comprehension of the details of such a mechanism clearly warrants further *in vivo* investigation.

ABBREVIATIONS USED

LPS, lipopolysaccharide; TLR, toll-like receptor; MAPKs, mitogen-activated protein kinases; PKCα, protein kinase C-α.

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