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Anti-inflammatory effect of honokiol is mediated by PI3K/Akt pathway suppression[1]

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Key words

honokiol; magnolol; anti-inflammatory effects; macrophages and lymphocytes; phosphoinositide 3-kinase/Akt pathway

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Abstract

Aim: In this study, we investigated the regulatory effects of honokiol on various inflammatory events mediated by monocytes/macrophages (U937/RAW264.7 cells) and lymphocytes (splenic lymphocytes and CTLL-2 cells) and their putative action mechanism. **Methods:** In order to investigate the regulatory effects, various cell lines and primary cells (U937, RAW264.7, CTLL-2 cells, and splenic lymphocytes) were employed and various inflammatory events, such as the production of inflammatory mediators, cell adhesion, cell proliferation, and the early signaling cascade, were chosen. **Results:** Honokiol strongly inhibited various inflammatory responses, such as: (i) the upregulation of nitric oxide (NO), prostaglandin E_2 and TNF-α production and costimulatory molecule CD80 induced by lipopolysaccharide (LPS); (ii) the functional activation of β1-integrin (CD29) assessed by U937 cell–cell and cell–fibronectin adhesions; (iii) the enhancement of lymphocytes and CD8+CTLL-2 cell proliferation stimulated by LPS, phytohemaglutinin A (PHA), and concanavalin A or interleukin (IL)-2; and (iv) the transcriptional upregulation of inducible NO synthase, TNF-α, cyclooxygenase-2, IL-12, and monocyte chemoattractant protein (MCP)-1. These anti-inflammatory effects of honokiol seem to be mediated by interrupting the early activated intracellular signaling molecule phosphoinositide 3-kinase (PI3K)/Akt, but not Src, the extracellular signal-regulated kinase, and p38, according to pharmacological, biochemical, and functional analyses. **Conclusion:** These results suggest that honokiol may act as a potent anti-inflammatory agent with multipotential activities due to an inhibitory effect on the PI3K/Akt pathway.

Introduction

Macrophages and lymphocytes play important roles in the host immune defense mechanism composed of innate and adaptive immunity[1]. The functional roles of these cells are known to be mediated by various soluble factors, such as cytokines and toxic molecules[2,3], having various defensive roles against pathogens and tumors[3,4]. However, certain conditions with the overproduction of inflammatory molecules can give the host severe immunopathological symptoms, such as acute and chronic inflammatory diseases[5]. Because of this, the effective modulation of the overproduction state has been considered a therapeutic target. The production of inflammatory molecules is triggered by mitogenic stimulation with various bacterial products, such as lipopolysaccharide (LPS). Owing to numerous studies, the molecular events of the inflammatory process have been widely understood. In particular, various signaling cascades composed of pattern recognition receptors [eg Toll-like receptor (TLR)-4], non-receptor type tyrosine kinases (eg Src and Syk), serine/threonine kinases [protein kinase C (PKC), protein kinase A (PKA), phosphatidylinositol-3-kinase (PI3K), and Akt], mitogen-activated protein kinases (MAPK), and various redox-sensitive transcription factors, such as NF-κB, are regarded as critical components participating in the processes[6,7].

Honokiol and magnolol (isomers of hydroxylated biphenolic compounds [$C_{18}H_{18}O_2$, molecular weight =266.33]; Figure 1) are major components isolated from the bark of the root and stem of various *Magnolia* species[8]. In particular, since these plants have been used in traditional herbal medicines against a variety of inflammatory and neuronal diseases, these compounds were initially thought of as major active compounds of the plants[9,10]. Indeed, these compounds have been demonstrated to possess various pharmacological effects, such as neuroprotective, anticancer, cardiotonic, and anti-inflammatory activities[10–13]. How these compounds are able to modulate various pharmacological actions has not yet been fully elucidated yet, but upregulating a signaling cascade composed of Ras, Raf, and the extracellular signal-regulated kinase (ERK)[14,15], downregulating the activation of NF-κB by the inhibition of its upstream kinases (IκB kinase [IKK]) under TNF-α stimulation and ERK kinase kinase-1 under LPS stimulation[16–18], and blocking NF-κB activation mediated by CD40 and latent membrane protein 1[19] are speculated to be the action mechanism of the compound.

Nonetheless, the exact pathways and molecular target of honokiol under inflammatory conditions are still controversial. Therefore, in this study, we carefully examined the inhibitory effect of honokiol (and magnolol) on various inflammatory responses mediated by mitogenic stimulation as well as its molecular target in the process of immunopharmacological actions.

Materials and methods

Materials Honokiol and magnolol (Figure 1) were obtained from Nacalai Tesque (Kyoto, Japan). Dibutyryl cyclic adenosine monophosphate (dbcAMP), phorbol 12-myrostate-13-acetate (PMA), N-monomethyl-L-arginine, curdlan, peptidoglycan (PGN), recombinant human interferon (IFN)-γ, and LPS (*Escherichia coli* 0111:B4) were purchased from Sigma (St Louis, MO, USA). U0126 and LY294002 were obtained from Calbiochem (La Jolla, CA, USA). Fetal bovine serum, penicillin, streptomycin, and RPMI-1640 were obtained from GIBCO (Grand Island, NY, USA). RAW264.7 cells, a murine macrophage cell line, U937 cells, a human promonocytic cell line, and CTLL-2 cells, a mouse cytotoxic interleukin (IL)-2-dependent T cell line, were purchased from American Type Culture Collection (Rockville, MD, USA). All other chemicals were of Sigma grade. CD29 [MEM 101A, immunoglobulin G (IgG)1], CD43 (161-46, IgG1), CD62L (Dreg56, IgG1)[20] were used for the cell–cell adhesion assays and flow cytometric analyses. Phospho-specific antibodies to Src, p85, Akt, IκBα, p38, ERK, and an antibody to β-actin were purchased from Cell Signaling (Beverly, MA, USA).

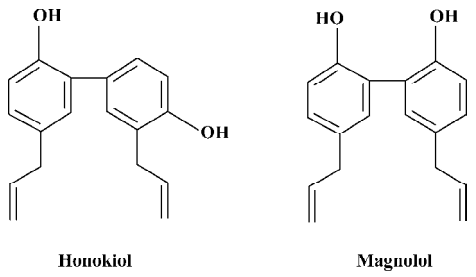

Figure 1. Chemical structure of honokiol and magnolol.

Cell culture The RAW264.7 and U937 cells were maintained in RPMI-1640 supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum. In the case of CTLL-2 cell maintenance, the cells were cultured with the same medium containing 25 U/mL IL-2. The cells were grown at 37 °C and 5% CO_2 in humidified air.

Determination of LPS-induced TNF-α production The inhibitory effects of honokiol and magnolol on TNF-α production from the LPS-treated RAW264.7 cells was determined as described previously[21]. The amount of TNF-α was assayed by ELISA.

Determination of prostaglandin E_2 production The inhibitory effects of honokiol on prostaglandin E_2 (PGE_2) production from the LPS-treated RAW264.7 cells was determined as described previously[21]. The amount of PGE_2 was assayed by ELISA.

Determination of nitric oxide production The inhibitory effects of honokiol on nitric oxide (NO) production from the LPS-treated RAW264.7 cells was determined as described previously[21]. The amount of NO was assayed by a Griess assay, as reported previously.

MTT assay (colorimetric assay) for the measurement of cell viability and proliferation Cell proliferation was measured by conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as reported previously. The culture was stopped by the addition of 15% sodium dodecyl sulfate into each well for the solubilization of formazan. The optical density (*OD*) at 570 nm ($OD_{570-630}$) was measured by a Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Determination of LPS-inducible gene expression (inducible NO synthase, TNF-α, MCP-1, IL-12, p40, cyclooxygenase-2, and GAPDH) For the evaluation of the cytokine mRNA expression levels, the total RNA from the LPS-treated RAW264.7 cells was prepared by adding TRIzol Reagent (GIBCO, USA), according to manufacturer's protocol. Semiquantitative RT reactions were conducted, as reported previously. The primers (Bioneer, Seoul, Korea)

used in this experiment are indicated in Table 1.

Flow cytometric analysis The expression of the cell surface adhesion and costimulatory molecules in U937 (CD29 and CD43) and RAW264.7 (CD80) cells was determined by a flow cytometric analysis, as reported previously[22]. Stained cells were analyzed on a FACScan (Beckton-Dickinson, San Jose, CA, USA).

Table 1. Primer sequences of the investigated genes in a RT-PCR analysis.

Gene		Primer sequences
TNF-α	F	5'-TTGACCTCAGCGCTGAGTTG-3'
	R	5'-CCTGTAGCCCACGTCGTAGC-3'
IL-12p40	F	5'-CAGGATGAGGACATGAGCACC-3'
	R	5'-CTCTGCAGACTCAAACTCCAC-3'
iNOS	F	5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3'
	R	5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'
COX-2	F	5'-CACTACATCCTGACCCACTT-3'
	R	5'-ATGCTCCTGCTTGAGTATGT-3'
MCP-1	F	5'-ACTGAAGCCAGCTCTCTCTTCCTC-3'
	R	5'-TTCCTTCTTGGGGTCAGCACAGAC-5'
GAPDH	F	5'-CACTCACGGCAAATTCAACGGCAC-3'
	R	5'-GACTCCACGACATACTCAGCAC-3'

F, forward; R, reverse.

Cell-cell or cell-extracellular matrix protein (fibronectin) adhesion assay The inhibitory effect of honokiol on cell–cell adhesion was determined by a quantitative U937 cell–cell adhesion assay with function-activating (agonistic) antibodies (1 μg/mL) as described previously[22,23]. For a cell–fibronectin adhesion assay, the U937 cells (5×10^5 cells/well) were seeded on a fibronectin (50 μg/mL)-coated plate and incubated for 3 h[24]. After removing the unbound cells with phosphate-buffered saline, the attached cells were treated with 0.1% crystal violet for 15 min. The OD value at 570 nm was measured by a Spectramax 250 microplate reader.

Lymphocyte proliferation assay The splenocytes were prepared from the spleens of mice killed by cervical dislocation under sterile conditions, as described previously[21]. Briefly, the splenocytes were released by teasing into RPMI-1640 medium supplemented with 20 mmol/L *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid] (HEPES) buffer. The splenocytes (5×10^6 cells/mL) were cultured in 96-well plates in the presence and absence of T or B lymphocyte mitogens [concanavalin A (Con A), phytohemaglutinin A (PHA), and LPS] with honokiol in a total volume of 200 μL/well at the same conditions for 48 h. The proliferation assay was performed by MTT assay.

Preparation of cell lysates and immunoblotting The total cell lysates were prepared from the cells, as reported previously[22]. The protein concentrations were determined by the Bradford method[25]. The lysates were clarified by centrifugation at 16 000×*g* for 10 min at 4 °C. Soluble cell lysates were immunoblotted, and the phospho forms of p38, ERK, Akt, and IκBα were visualized, as reported previously.

Statistical analysis Data were expressed as mean±SEM. For the statistical comparison, the results were analyzed using ANOVA and the Kruskal-Wallis test. A P-value <0.05 was considered a statistically significant difference. All statistical tests were carried out using the computer program STATISTICA version 4.5 (StatSoft, Microsoft, Oklahoma City, OK, USA).

Results

Effect of honokiol on the viability of RAW264.7 cells It was necessary to assess the cytotoxic effect of honokiol before further in vitro tests were carried out. The MTT assay using RAW264.7 and U937 cells is a suitable model for this purpose, as the cells are representative immune cells. Figure 2 shows that honokiol was significantly non-cytotoxic up to 20 μmol/L in both RAW264.7 (left panel) and U937 cells (right panel) under our 12 or 24 h incubation conditions, while more than 40 μmol/L concentrations time-dependently altered the viability of RAW264.7 and U937 cells. Thus, 40 μmol/L of this compound was non-cytotoxic in both cell lines until 3 h, but long exposure (12 and 24 h) significantly induced cytotoxic activity. Therefore, it was regarded that the anti-inflammatory effects of honokiol at less than 20 μmol/L could be due to the selective pharmacological action of the drug without any interference with normal cell function. However, for the immunoblotting and cell–fibronectin experiments, which require 30 min or 3 h incubation with honokiol, 40 μmol/L conditions were used in these experiments to obtain remarkable effects.

Effect of honokiol on LPS-mediated pro-inflammatory responses by macrophages To evaluate the inhibitory effect of honokiol (and magnolol) on LPS-mediated pro-inflammatory responses by macrophages, the production of pro-inflammatory mediators, surface levels of the costimulatory molecule CD80, and the mRNA expression of the pro-inflammatory genes were determined under LPS and honokiol treatment. Honokiol strongly inhibited the release of TNF-α (Figure 3A), PGE_2 (Figure 3B), and NO (Figure 3C) in a dose-dependent manner. However, magnolol, its structural analog, suppressed NO and TNF-α production more weakly than

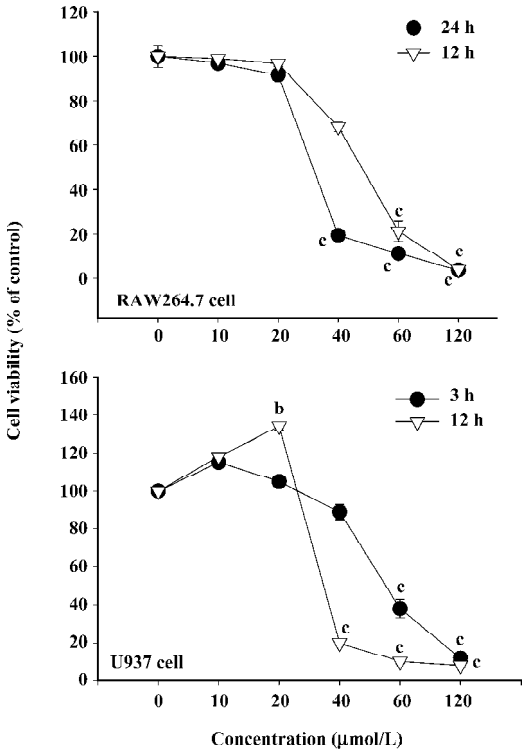

Figure 2. Effect of honokiol on the viability of RAW264.7 cells. RAW264.7 and U937 cells (1×10^6 cells/mL) were incubated with honokiol for 12 or 24 h. Cell viability was determined by conventional MTT assay. Data represent mean±SEM of 3 independent observations performed in triplicate. $^bP<0.05$, $^cP<0.01$ *vs* normal group.

honokiol. Furthermore, honokiol significantly diminished the LPS-induced surface upregulation of the costimulatory molecule CD80, indicating that honokiol may commonly affect LPS-mediated pro-inflammatory responses. Indeed, this compound blocked the mRNA expression of pro-inflammatory genes, such as inducible NO synthase (iNOS), MCP-1, cyclooxygenase (COX)-2, and TNF-α (Figure 3E), suggesting that the immunomodulatory effect of honokiol may be occurring at the transcriptional level.

Positive control drugs for TNF-α production used in this experiment, prednisolone and dbcAMP, also significantly suppressed TNF-α production in a dose-dependent manner with IC_{50} values of 28.9 and 47.4 μmol/L, respectively. Indomethacin also strongly suppressed PGE_2 production with an IC_{50} value of 3.3 μmol/L, as reported previously[26–28]. The control drug N-MMA inhibited NO release from LPS-stimulated RAW264.7 cells with an IC_{50} value of 193.3 μmol/L.

Effect of honokiol on CD29-mediated cell–cell and cell-fibronectin adhesion To assess the regulatory effect of honokiol on monocyte adhesion to other cells or the extracellular matrix (eg fibronectin) mediated by β1-integrins (CD29), quantitative homotypic U937 cell-cell and cell-fibronectin adhesion assays were employed. As shown in Figure 4A, honokiol dose-dependently blocked U937 cell–cell adhesion induced by the agonistic antibody to CD29, but not CD43. Furthermore, this compound also downregulated cell-fibronectin adhesion in a dose-dependent manner (Figure 4B). To determine whether the suppressive effect of honokiol is due to the downregulation of the surface level of CD29, a flow cytometric analysis was performed. However, there was no alteration of the surface levels of CD29 and CD43 after 3 h incubation (Figure 4C), indicating that the diminishment may occur by interrupting intracellular signaling levels, playing a critical role in cell–cell adhesion.

Effect of honokiol on mitogenic response of splenic lymphocytes and IL-2-dependent CTLL-2 cells To assess whether honokiol is able to modulate the mitogenic cellular events of other immune cells, such as lymphocytes, splenic lymphocytes, IL-2-dependent CD8+CTLL-2 cells, and mitogens (such as LPS, Con A, PHA, and IL-2) were employed. As shown in Figure 5A, honokiol clearly suppressed splenic lymphocyte proliferation induced by all mitogens tested when they were treated with 12.5 μmol/L for 48 h, whereas magnolol showed weak effects and enhanced the proliferation at 6.25 μmol/L. In particular, the inhibitory pattern was also similarly observed in the IL-2-dependent proliferation of CTLL-2 cells. Thus, honokiol, but not magnolol, strongly inhibited CD8+CTLL-2 cell proliferation triggered by IL-2 in a dose-dependent manner.

Mechanism of honokiol inhibition in inflammatory responses To understand the molecular mechanism by which honokiol blocked various inflammatory processes performed by macrophages and lymphocytes, the effects of honokiol and signaling enzyme inhibitors (LY294002 and U0126) on LPS-induced signaling and cellular events were first investigated. Since the compound has been reported to suppress a signaling cascade composed of IKK, IκB, and NF-κB, we focused on the upstream signaling events for NF-κB activation. Figure 6A shows that LPS stimulation triggered the activation of several upstream signaling molecules (Src, p85 [a regulatory subunit of PI3K p110], Akt, IκBα, p38, and ERK), as assessed by their phosphorylation levels using phospho-specific antibodies. Interestingly, honokiol only strongly blocked the phosphorylation of Akt and IκBα (at 15 and 30 min) without altering the total level of Akt, but weakly suppressed or not at all the phosphorylation of other proteins. This suggests that the Akt pathway could be regarded as a target event, although it enhanced LPS-induced Akt phosphorylation at 5 min, as shown in the cases of phosphatase

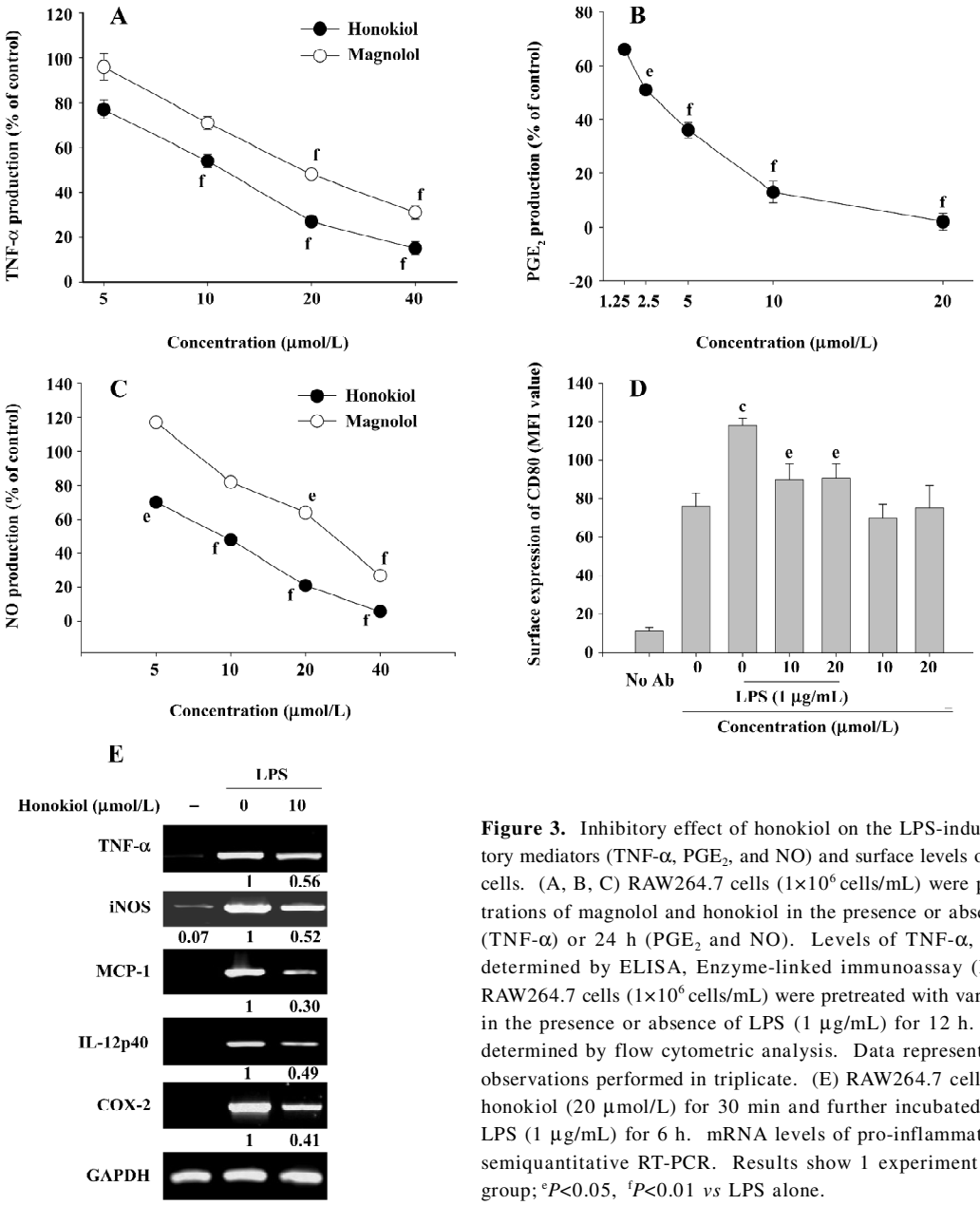

Figure 3. Inhibitory effect of honokiol on the LPS-induced upregulation of inflammatory mediators (TNF-α, PGE_2, and NO) and surface levels of CD80 in activated RAW264.7 cells. (A, B, C) RAW264.7 cells (1×10^6 cells/mL) were pretreated with various concentrations of magnolol and honokiol in the presence or absence of LPS (1 μg/mL) for 6 h (TNF-α) or 24 h (PGE_2 and NO). Levels of TNF-α, PGE_2, and NO released were determined by ELISA, Enzyme-linked immunoassay (EIA) and Griess assays. (D) RAW264.7 cells (1×10^6 cells/mL) were pretreated with various concentrations of honokiol in the presence or absence of LPS (1 μg/mL) for 12 h. Surface levels of CD80 were determined by flow cytometric analysis. Data represent mean±SEM of 3 independent observations performed in triplicate. (E) RAW264.7 cells (5×10^6) were pretreated with honokiol (20 μmol/L) for 30 min and further incubated in the presence or absence of LPS (1 μg/mL) for 6 h. mRNA levels of pro-inflammatory genes were determined by semiquantitative RT-PCR. Results show 1 experiment out of 3. $^bP<0.05$ *vs* normal group; $^eP<0.05$, $^fP<0.01$ *vs* LPS alone.

inhibitors. Furthermore, since 40 μmol/L honokiol never or weakly blocked the phosphorylation of MAPK (ERK and p38; Figure 6A), it is thought that this compound may selectively modulate the PI3K/Akt pathway.

To confirm whether the Akt signal is critical in honokiol-mediated inhibitory effects, specific inhibitors (LY294002 and U0126) blocking the activation of Akt and ERK were employed to evaluate their potency in various inflammatory responses. As depicted in Figure 6B, the PI3K/Akt inhibitor LY294002 strongly suppressed the LPS-induced production of TNF-α, NO, PGE_2, CD29-mediated cell–cell adhesion, and Con A-induced T cell proliferation, as shown in the case of honokiol, whereas U0126 only partially blocked NO production and T cell proliferation. Meanwhile, LY294002 and U0126 were shown to suppress the phosphorylation of Akt and ERK triggered by LPS at 30 min, as assessed by measuring the phosphorylation level, indicating that the inhibitors worked very well. To characterize the importance of the Akt pathway as an early signaling event, the time-dependent function of honokiol treatment and NO inhibitory effects under different Akt-inducing stimuli or Akt-regulating signals (such as PKC and PKA) were carefully evaluated. The

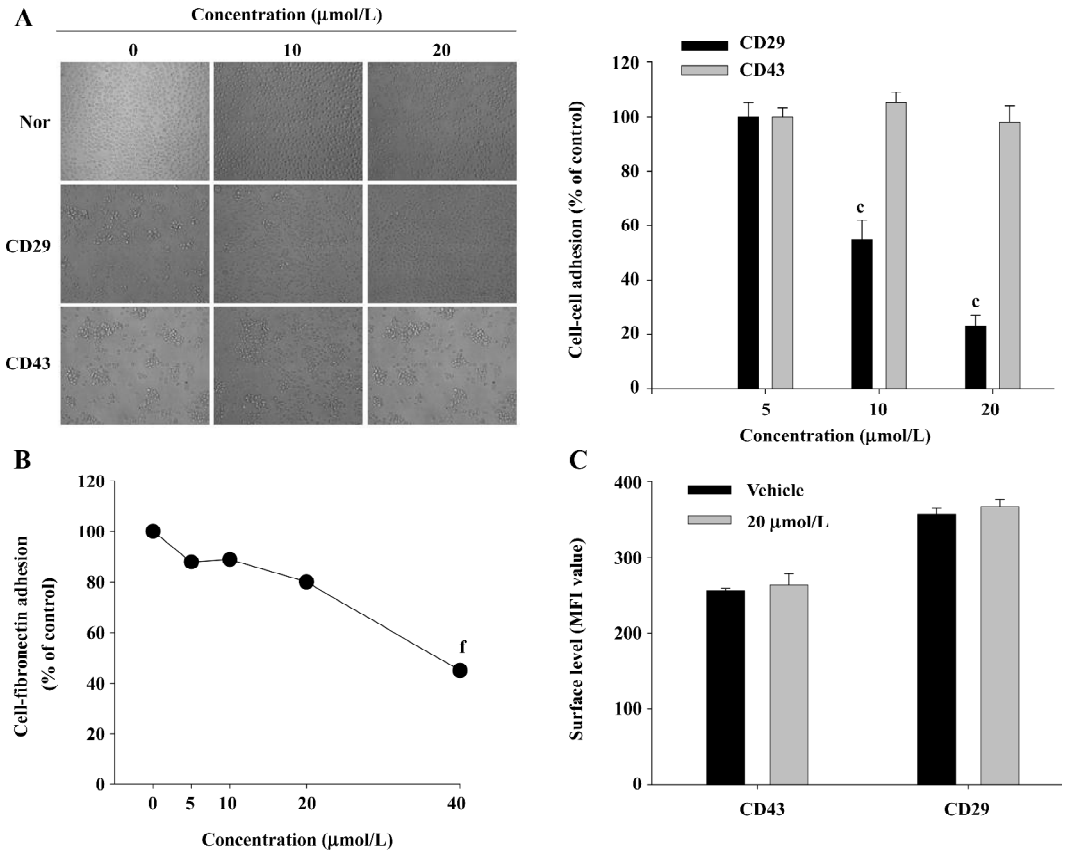

Figure 4. Inhibitory effect of honokiol on CD29-induced cell–cell and cell–fibronectin adhesions and surface levels of CD29. (A) U937 cells were incubated with indicated concentrations of honokiol in the presence or absence of pro-aggregative (activating) antibodies (antibodies [1 μg/mL each] to CD29 [MEM 101A] and CD43 [161–46]) for 2 h. Images of the cells in culture were obtained using an inverted phase contrast microscope attached to a video camera (left panel). Quantitative approach of cell–cell adhesion was determined using a quantitative U937 cell–cell adhesion assay (right panel). (B) U937 cells (1×10^6 cells/mL) pretreated with honokiol were seeded on fibronectin (50 μg/mL)-coated plates and further incubated for 3 h. Attached cells were determined by crystal violet assay, as described in Materials and Methods. (C) U937 cells (1×10^6 cells/mL) pretreated with honokiol were seeded on fibronectin (50 μg/mL)-coated plates and further incubated for 3 h. Attached cells were determined by crystal violet assay. $^cP<0.01$ *vs* CD29 antibody; $^fP<0.01$ *vs* fibronectin alone.

inhibitory potency of honokiol was dramatically reduced when honokiol treatment was delayed after exposure to LPS (Figure 6C). Honokiol also strongly suppressed NO production induced by LPS, PGN (a ligand of TLR-2), curdlan (β-glucan), and IFN-γ, which are known to induce the phosphorylation of Akt (Figure 6D). Finally, the Akt inhibitory conditions achieved through upregulating PKC (by PMA) or PKA (by dbcAMP) interestingly abrogated honokiol inhibition obtained in the LPS exposure (Figure 6E). Therefore, our data suggest that the Akt pathway may be a critical target of honokiol in its anti-inflammatory actions.

Discussion

In this study, we explored the anti-inflammatory effect of honokiol (Figure 1) and its regulating mechanism using various immune cells and their inflammatory responses under non-cytotoxic conditions (Figure 2). Thus, honokiol (and magnolol) suppressed LPS-mediated cellular responses (Figure 3), such as the production of TNF-α, PGE_2, and NO, and upregulated CD80; honokiol (and magnolol) also suppressed CD29-mediated cell–cell and cell-fibronectin adhesions (Figure 4) and mitogen-mediated lymphocyte proliferation (Figure 5) in a dose-dependent manner. The production of inflammatory mediators seems to be due to the suppression of transcriptional activation, since it blocked mRNA levels of the pro-inflammatory proteins (Figure 3E). In agreement with previous papers[29,30], honokiol blocked the pro-

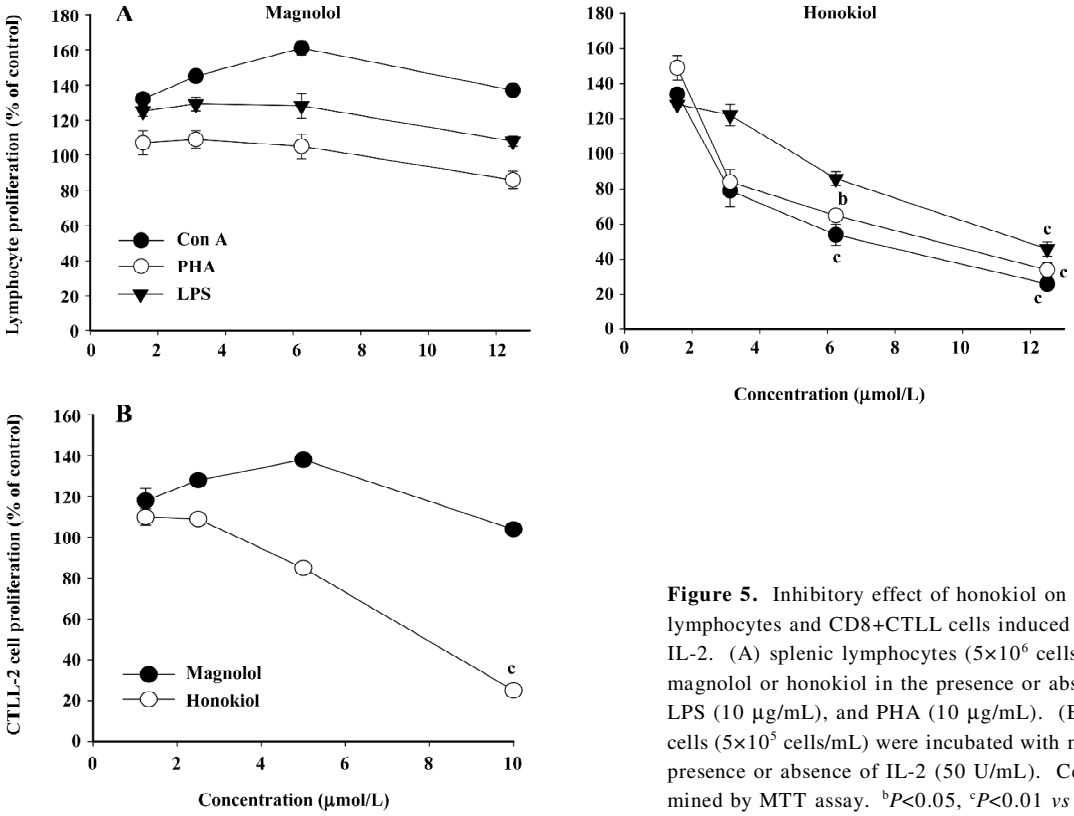

Figure 5. Inhibitory effect of honokiol on the proliferation of splenic lymphocytes and CD8+CTLL cells induced by Con A, LPS, PHA, and IL-2. (A) splenic lymphocytes (5×10^6 cells/mL) were incubated with magnolol or honokiol in the presence or absence of Con A (1 μg/mL), LPS (10 μg/mL), and PHA (10 μg/mL). (B) IL-2-dependent CTLL-2 cells (5×10^5 cells/mL) were incubated with magnolol or honokiol in the presence or absence of IL-2 (50 U/mL). Cell proliferation was determined by MTT assay. $^bP<0.05$, $^cP<0.01$ *vs* inducer alone.

duction of NO and TNF-α and the proliferation of lymphocytes and CTLL-2 cells more significantly than magnolol (Figures 3, 5). In particular, the inhibitory potency (IC_{50} values=3–15 μmol/L) of honokiol on NO, PGE_2, and TNF-α production is considerable when compared with other natural products or clinically-available drugs. The activities are similar with other sesquiterpene lactone compounds, such as cynaropicrin[21], or higher than those of previously isolated natural compounds, such as ginsenosides, flavonoids and coumarins, and pentoxifylline and prednisolone with IC_{50} values of 75–200 μmol/L[31–33]. Furthermore, honokiol was previously found to inhibit other inflammatory gene products, such as IL-8, intercellular adhesion molecule (ICAM)-1, and matrix metalloproteinase-9 induced by LPS, TNF-α, and *Propionibacterium acnes*[16,34]. It has been also reported that honokiol inhibited PMA- or formyl peptide (fMLP)-induced reactive oxygen species production by neutrophils[35]. Therefore, our data suggest that honokiol may be considered a promising drug with potent anti-inflammatory actions in addition to its strong anticancer, anti-angiogenesis, and antineurodegeneration effects.

Post-treatment with honokiol 3 and 6 h after LPS markedly reduced its inhibitory activity (Figure 6C), suggesting that the pharmacological target of honokiol may be activated early. Several research groups have reported that honokiol and magnolol strongly inhibited NF-κB translocation, a critical step found in early inflammatory events and tumor angiogenesis[36–38]. Using an immunoblotting analysis of the phosphorylation level of IκBα, we also learned that the phosphorylation of IκBα at 30 min was diminished by honokiol treatment. Indeed, the Fong group mechanistically proved current results through demonstrating that magnolol and honokiol strongly inhibited IKK activation induced by TNF-α in U937 cells via directly suppressing IKK activity[16,17], although the signal transducer and activator of transcription protein 3[39] and the c-Jun N-terminal kinase were also regarded as pharmacological targets[40]. It was however notable that the LPS induction of the phosphorylation of Akt, an upstream kinase of IKK, was strongly suppressed by honokiol exposure (Figure 6A), suggesting that the real target of honokiol may be not IKK only, but its upstream kinase. Several lines of evidence seem to support this possibility including: (i) honokiol has been reported to block the TNF-α-induced phosphorylation of Akt[41]; (ii) an inhibitor of PI3K/Akt, LY294002, but not U0126, an ERK inhibitor, as well as p38 MAPK inhibitor SB203580 (data not shown), displayed a similar inhibitory spectrum (Figure 6B); (iii) honokiol suppressed all NO production conditions induced by other Akt-

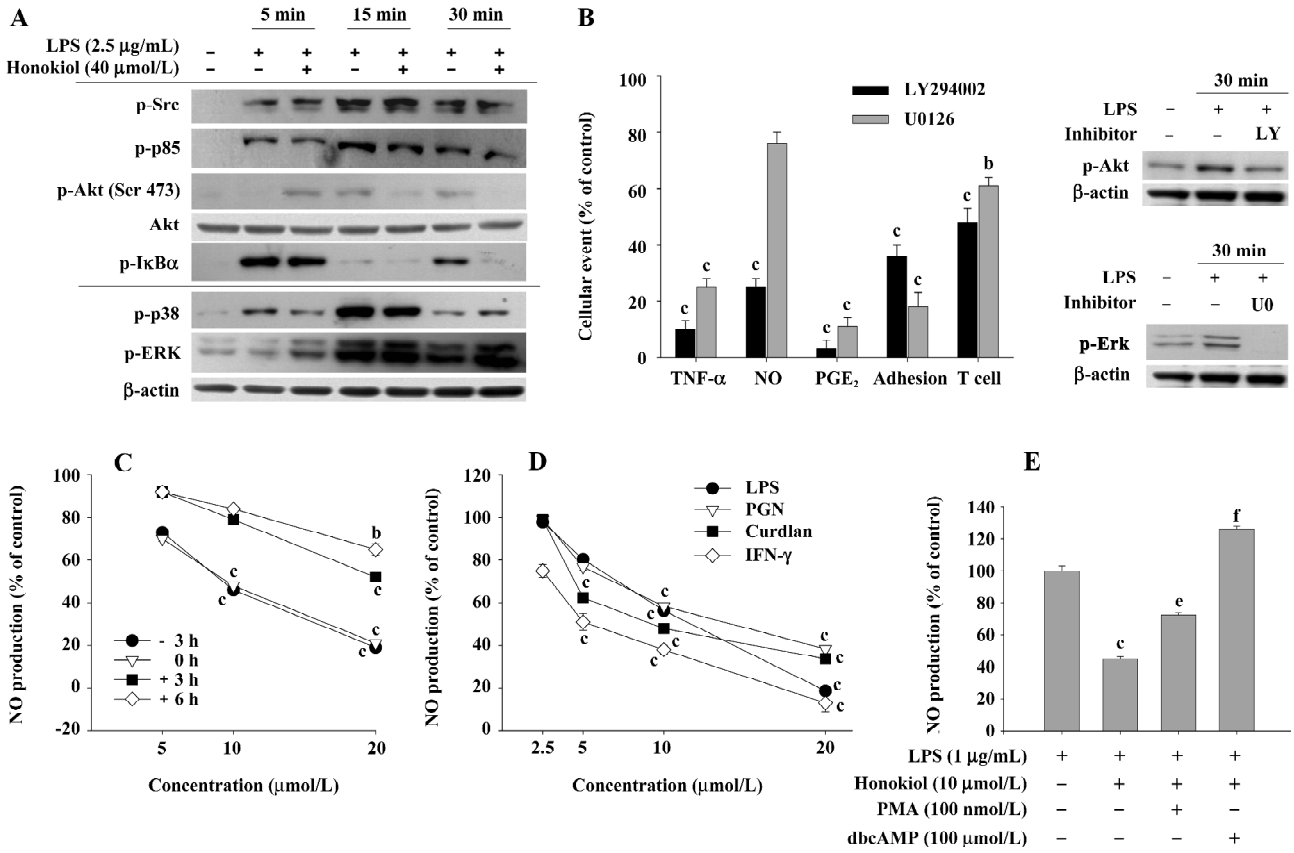

Figure 6. Effect of honokiol on LPS induced early intracellular signaling events. (A) RAW264.7 cells (5×10^6 cells/mL) pretreated with honokiol (40 μmol/L) were stimulated in the absence or presence of LPS (1 μg/mL) for the indicated times. After immunoblotting, the phosphorylation or the total levels of p38, ERK, Src, p85, Akt, and IκBα were identified by their phospho-specific or non-phospho-specific antibodies. Results show 1 experiment out of 3. (B) assays for LPS-induced TNF-α, NO, and PGE_2 production, CD29-induced cell–cell adhesion, Con A-treated T lymphocyte proliferation, and immunoblotting analysis of p-Akt and p-ERK were performed under LY294002 (25 μmol/L), a PI3K/Akt inhibitor, and U0126 (20 μmol/L), an ERK inhibitor. (C, D) RAW264.7 cells (1×10^6 cells/mL) were pretreated with honokiol (20 μmol/L) at the indicated times in the presence or absence of LPS (1 μg/mL), PGN (10 μg/mL), curdlan (300 μg/mL), and IFN-γ (100 U/mL) for 24 h. (E) RAW264.7 cells (1×10^6 cells/mL) were pretreated with honokiol (10 μmol/L) in the presence or absence of LPS (1 μg/mL) as well as PMA (100 nmol/L) or dbcAMP (100 μmol/L) for 24 h. Levels of NO released (C, D, E) were determined by a Griess assay. Data represent mean±SEM of 3 independent observations performed in triplicate. $^bP<0.05$, $^cP<0.01$ *vs* inducer alone; $^eP<0.05$, $^fP<0.01$ *vs* LPS+honokiol.

activating inducers, such as IFN-γ, PGN, and curdlan (Figure 6D); and (iv) honokiol inhibition was abolished by PKC (by PMA) or PKA (by dbcAMP) treatment (Figure 6E), similar to Akt inhibition[42,43], although we cannot exclude Akt-independent upregulation manners.

The PI3K/Akt pathway is known to play critical roles in various cellular processes, such as apoptosis, inflammatory responses, and tumor angiogenesis[36–38]. Due to its multipotential roles in various disease onsets, it is regarded as a therapeutic target protein in developing anticancer and anti-inflammatory drugs[44]. The activation of the PI3K/Akt pathway requires a series of upstream signaling cascades composed of non-receptor-type protein tyrosine kinase, such as Src, serine/threonine kinases, such as 3'-phosphoinositide-dependent protein kinase-1 (PDK1) and S473 kinase, and p85[45,46]. Thus, the Src-induced phosphorylation of p85 allows the activation of p110, a catalytic subunit of PI3K, which participates in producing phosphatidylinositol-3,4,5-triphosphate (PIP3)[47]. Then, through binding to PIP3, PDK1 is consequently able to phosphorylate threonine-308/serine-473 of Akt[47]. Whether honokiol is able to directly diminish the kinase activity of Akt is not clear yet. However, no inhibition of Src and p85 phosphorylation (Figure 6A) seems to suggest that the target of honokiol may be p110 with kinase

activity, PDK1, or even Akt itself. Since exploring the direct target of honokiol will greatly improve our understanding of its pharmacological actions, the exact molecular studies on this point will be carefully carried out in the next experiments using direct kinase assays.

Pharmacokinetic studies with honokiol revealed that this compound is effective *in vivo*. Thus, single treatment with a 250 mg/kg injection (ip) exhibited a maximum plasma concentration of more than 3.5 mmol/L with an elimination half-life of 5.2 h[48]. Multiple intraperitoneal treatments with 3 mg/mouse at 1–2 d intervals was promisingly curative of tumor generation and growth in nude mice[49]. These results indicate that various *in vitro* effects on inflammation, apoptosis, and neuroprotection can be achievable under *in vivo* conditions, and therefore honokiol seems to be applicable as an anti-inflammatory and anticancer drug.

In conclusion, honokiol strongly inhibited various inflammatory responses, such as the LPS-induced upregulation of inflammatory mediators, inflammatory genes, and CD80; the functional activation of β1-integrin (CD29); and the mitogenic proliferation of lymphocytes and CD8+CTLL-2 cells. According to pharmacological, biochemical, and functional analyses, honokiol was found to interrupt the early activated intracellular signaling molecules PI3K/Akt, but not Src, ERK, and p38. Therefore, our data suggest that honokiol may act as a potent anti-inflammatory agent to be developed with PI3K/Akt inhibitory activity.

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