Cynandione A attenuates lipopolysaccharide-induced production of inflammatory mediators via MAPK inhibition and NF- κ B inactivation in RAW264.7 macrophages and protects mice against endotoxin shock

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Abstract

Cynanchum wilfordii has been traditionally used in eastern Asia for the treatment of various diseases such as gastrointestinal diseases and arteriosclerosis. Cynandione A (CA), an acetophenone, is one of major constituents from roots of *C. wilfordii*. In the present study, the anti-inflammatory activities of CA were investigated in lipopolysaccharide (LPS)-treated RAW264.7 macrophages and LPS-administered C57BL/6 N mice. CA significantly decreased LPS-induced production of nitric oxide and prostaglandin E_2 in a dose-dependent manner, while CA up to 200 μ M did not exhibit cytotoxic activity. Our data also showed that CA significantly attenuated expression of iNOS and COX-2 in LPS-stimulated macrophages. CA inhibited phosphorylation of I κ B- α and MAP kinases such as ERK and p38. Furthermore, we demonstrated that CA inhibited translocation of NF- κ B to the nucleus, transcription of the NF- κ B minimal promoter and NF- κ B DNA binding activity. Administration of CA significantly decreased the plasma levels of pro-inflammatory cytokines such as TNF- α , IL-6, and IL-1 β in LPS-injected mice and improved survival of septic mice with lethal endotoxemia. These results demonstrate that CA has effective inhibitory effects on production of inflammatory mediators via suppressing activation of NF- κ B and MAPK signaling pathways, suggesting that CA may be used as a potential anti-inflammatory agent for the prevention and treatment of inflammatory diseases.

Keywords: Inflammation, NF- κ B, MAPK pathway, cynandione A, *Cynanchum wilfordii*

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Introduction

Inflammation is a protective response of the host against injuries, irritation, and viral and bacterial pathogens. However, chronic inflammation is an undesirable phenomenon that can lead to development of various diseases such as cancer, cardiovascular, and autoimmune diseases.^{1,2} Macrophages play major roles in the response to physical, chemical stimuli, or invading pathogens and release many pro-inflammatory mediators, including nitric oxide (NO), prostaglandin E_2 (PGE₂), and cytokines such as TNF- α , IL-1 β , and IL-6.³ The progressive production of these inflammatory mediators may cause clinical syndrome of sepsis and septic shock, and life-threatening disorders triggered by systemic infection.⁴ Thus, effective inhibitors for inflammation and sepsis are expected to possess a therapeutic potential.

LPS, a major outer membrane component of Gram-negative bacteria, is the main contributing factor to the

development of inflammation, which activates inflammatory and immune cells. TLR4 is thought to be the principal LPS receptor in inflammatory response to LPS.⁵ Binding of LPS to TLR4 activates several signaling cascade including the mitogen activated protein kinases (MAPKs) such as ERK, p38, and JNK kinases and NF- κ B.^{6,7} NF- κ B is sequestered by I κ B in the cytoplasm under the normal states. NF- κ B activation by external stimuli such as LPS requires a sequential cascade of reactions such as I κ B kinase (IKK- α / β)-dependent phosphorylation, ubiquitination, and degradation of I κ B.⁸ The liberated NF- κ B translocate into nucleus, where it activates transcription of pro-inflammatory genes.⁹

A variety of herbs and plants have been traditionally used in oriental folk medicine for the treatment of various diseases. *Cynanchum wilfordii* is widely distributed in far eastern Asia and has been used in oriental medicines for the prevention and treatment of various diseases such as gastrointestinal diseases and arteriosclerosis.^{10,11} CA, an

acetophenone from *C. wilfordii*, has been reported to have protective activities of rat hepatocytes and cortical neurons from toxicity by various agents.^{12,13} However, the molecular mechanisms that underlie the anti-inflammatory effects of CA have not been reported. In the present study, we have demonstrated that CA inhibits production of NO and PGE₂ in LPS-induced RAW264.7 cells. Our results showed that CA suppresses expression of iNOS and COX-2 via inhibition of NF- κ B and MAPK signaling pathways. These effects appear to be due to inhibition of MAPKs activation and subsequently down-regulating NF- κ B. Moreover, we demonstrated its anti-inflammatory activity *in vivo* using LPS-administered mouse models.

Materials and methods

Materials and cell culture

Antibodies for iNOS, COX-2, and β -actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Specific antibodies against phospho-ERK, ERK, phospho-p38, p38, phospho-I κ B- α , I κ B- α , p65, and proliferating cell nuclear antigen (PCNA) were obtained from Cell Signaling Technology (Beverly, MA, USA). Other chemicals were purchased from Sigma Aldrich co. (St. Louis, MO, USA). CA was isolated from the roots of *C. wilfordii* as previously reported¹⁴. RAW264.7 cells (ATCC) were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM, WelGENE Inc., Korea) containing 10% fetal bovine serum (FBS, WelGENE Inc., Korea), 2 mM glutamate, 100 unit/mL of penicillin, and 100 μ g/mL of streptomycin in a humidified incubator with 5% CO₂. Cells were incubated with 1 μ g/mL LPS (Sigma Aldrich Co.), along with various concentrations of CA for 2 h to 24 h as indicated.

Measurement of NO production and cell viability assay

RAW264.7 cells (1×10^4 cells/well) were grown in serum-free medium for 18 h and then incubated with LPS (1 μ g/mL) in the presence of various concentrations of CA for 24 h. Nitric oxide (NO) was determined by measuring the amount of nitrite, a stable oxidized product of NO, in cell culture supernatant using Griess reagent (Sigma Aldrich Co.) as previously described.¹⁵ The amount of NO was determined by measuring the absorbance at 550 nm. The cell viability was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT)-based colorimetric assay as previously described.¹⁵

Reverse transcriptase-polymerase chain reaction analysis

The total cellular RNA was isolated from RAW264.7 cells using a Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (4 μ g) was reverse-transcribed using M-MuLV reverse transcriptase (Fermentas Life Science, Pittsburgh, PA, USA). The following primers (Bioneer, Daejeon, Korea) were used for PCR amplification: iNOS, forward 5'-TCT TCG AAA TCC CAC CTG AC-3' and reverse 5'-CCA TGA TGG TCA CAT TCT GC-3'. COX-2, forward 5'-TCT CCA ACC TCT CCT ACT AC-3' and reverse 5'-GCA CGT AGT CTT

CGA TCA CT-3'. β -actin mRNA levels were used as internal controls.

Quantitative real-time polymerase chain reaction

RAW264.7 cells (5×10^5 cells/well in six-well plates) were treated with LPS in presence or absence of CA for indicated concentrations for 18 h. To evaluate the expression levels of iNOS, COX-2, IL-1 β , IL-6, and TNF- α mRNA, total cellular RNA was extracted with a Trizol reagent kit (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed by using the ABI 7500 FAST real-time PCR instrument (Applied Biosystems, Foster City, CA, USA) and a reaction mixture that consisted of SYBR Green PCR Master Mix, cDNA template, and forward and reverse primers. The primer sequences for real-time PCR were as follows: iNOS, forward 5'-GGC AGC CTG TGA GAC CTT TG-3' and reverse 5'-GCA TTG GAA GTG AAG CGT TTC-3'. COX-2, forward 5'-TGA GTA CCG CAA ACG CTT CTC-3' and reverse 5'-TGG ACG AGG TTT TTC CAC CAG-3'. IL-1 β , forward 5'-CGC AGC AGC ACA TCA ACA AGA GC-3' and reverse 5'-TGT CCT CAT CCT GGA AGG TCC ACG-3'. IL-6, forward 5'-TCC AGT TGC CTT CTT GGG AC-3' and reverse 5'-GTG TAA TTA AGC CTC CGA CTT G-3'. TNF- α , forward 5'-TTC TGT CTA CTG AAC TTC GGG GTG ATC GGT CC-3' and reverse 5'-GTA TGA GAT AGC AAA TCG GCT GAC GGT GTG GG-3'. β -actin, forward 5'-AGA GGG AAA TCG TGC GTG AC-3' and reverse 5'-CAA TAG TGA TGA CCT GGC CGT-3'. A negative control without cDNA template was performed to assess the overall specificity. The PCR cycle was follows: 95°C for 10 min for one cycle and 95°C for 15 s and 60°C for 1 min for 40 cycles to allow for extension and amplification of the target sequence. The fold increase or decrease was determined relative to a blank control after normalized to a housekeeping β -actin gene using 2- $\Delta\Delta$ CT method.¹⁶

Western blot analysis

RAW264.7 cells were stimulated with LPS in presence or absence of CA for indicated times. The cells were harvested and lysed in RIPA buffer containing proteinase inhibitors. Protein concentration was determined by using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Proteins (40 μ g/lane) were resolved with SDS-polyacrylamide gel electrophoresis, and Western blot analysis was performed as described previously.¹⁵ The membranes were developed with an enhanced chemiluminescence system from Amersham and exposed for 30 s to X-ray film (FUJI Photo Film Co., Ltd., Japan).

Enzyme linked immunosorbent assay

Levels of prostaglandin E₂ were measured by using enzyme linked immunosorbent assay (ELISA) kits (Invitrogen). RAW264.7 cells were grown in 48-well plates, treated with various concentrations of CA for 24 h, and stimulated with 1 μ g/mL of LPS for 18 h. Supernatants of the cultures were collected, and PGE₂ levels were determined. Levels of

pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 were measured in mouse plasma using ELISA kits (Bender MedSystems, Inc., San Diego, CA, USA) according to the manufacturer's instructions.

Transient transfection and luciferase reporter assay

Transient transfection was performed by the lipofectamine method according to the manufacturer's instructions (Gibco-BRL, Carlsbad, CA, USA). NF- κ B promoter sequence was fused with luciferase gene in pGL3-basic (Promega, San Luis Obispo, CA, USA), which was designated as pNF- κ B/Luc. RAW264.7 cells were transfected with pGL3-basic or with pNF- κ B/Luc. The transfected cells were co-treated with LPS (1 μ g/mL) and various concentrations of CA for 12 h, harvested and lysed in Report lysis buffer (Promega). Luciferase activities were measured by using a luciferase assay kit (Promega) and a luminometer.

Electrophoretic mobility shift assay

RAW264.7 cells were treated with LPS (1 μ g/mL) in the presence or absence of CA for 2 h. Nuclear extracts from RAW264.7 cells were prepared as previously described.¹⁵ Double-stranded NF- κ B oligonucleotide (5'-AGT TGA GGG GAC TTT CCC AGG C-3') and a non-specific deoxyoligomer (5'-CGT GGG AAA ATC CAG T-3') were used as a probe and competitors. Oligomer probe was end-labeled by kination using [γ -³²P]-dATP (Bio-Rad Laboratories, Hercules, CA) and T4 polynucleotide kinase. Nuclear extracts (5 μ g) were mixed with [γ -³²P]-labelled probe and incubated at room temperature for 30 min. The reaction products were separated on 6% polyacrylamide gel. The gel was dried and subjected to autoradiography.

Animal experiments

Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC), Kyung Hee University (KHUASP(SU) 11-012). Male C57BL/6N mice (7–10 weeks) were housed in an environmentally controlled room with a 12 h light/dark cycle and allowed free access to food and water. Mice were divided into four groups, and treated with control (only PBS), LPS (4 mg/kg), or LPS treated with CA (1, 10 mg/kg). Mice were intraperitoneally (IP) injected with CA or PBS twice at 24 h intervals. After 12 h of the second injection, LPS or PBS was IP injected. Twelve hours later, a whole blood sample was collected by cardiac puncture and plasma was prepared by centrifugation at 12,000 $\times$ g for 20 min at 4°C. For evaluation of CA in the LPS model of endotoxemia, 8-week-old C57BL/6N mice were used. CA was intraperitoneally injected at a dose of 10 mg/kg for 24 h before LPS challenge, which was injected intraperitoneally at a dose of 30 mg/kg. The death of animals was monitored by vigorous physical examination. The survival rate was studied in $n = 14$ in each group, and recorded for over the next 14 days.

Statistical analysis

Unless otherwise stated, all experiments were performed with triplicate samples and repeated at least three times. The data are presented as means $\pm$ S.D. and statistical comparisons between groups were performed using one-way ANOVA followed by Student's *t*-test. Survival curves were calculated with Kaplan–Meier survival analysis using MedCal software (Broekstraat, Mariakerke, Belgium).

Figure 1 Effects of CA on nitric oxide and PGE₂ production in murine macrophages. (a) Viability in CA-treated cells was evaluated using the MTT assay. Cells were incubated with 5 to 200 μ M of CA for 24 h. The results are presented in percentage of control samples. Each value represents the mean $\pm$ S.D. and is representative of results obtained from three independent experiments. * $P < 0.05$ and ** $P < 0.001$ compared to medium alone. (b) RAW264.7 cells were co-treated with indicated concentrations of CA and LPS (1 μ g/mL) for 24 h. The culture supernatants were subsequently isolated and analyzed for nitrite levels. The values for nitrite are mean $\pm$ S.D. from three independent experiments. * $P < 0.05$ compared to treatment with LPS alone. (c) RAW264.7 cells were treated with various concentrations of CA for 24 h, and stimulated with 1 μ g/mL of LPS for 18 h. PGE₂ concentration was measured using a commercial PGE₂ ELISA kit. Data shown are the mean $\pm$ S.D. ($n = 3$). * $P < 0.05$ compared to treatment with LPS alone

Results

Cynandione A inhibits LPS-induced NO and PGE₂ production in RAW264.7 cells

First, we examined the effects of CA on the cell viability by MTT assay. Cell viability was over 90% up to the concentration of 200 μ M of CA during 24 h treatment (Figure 1a), thereby indicating that CA is not cytotoxic at the concentrations used in the present study. We next evaluated the effects of CA on production of NO and PGE₂ in LPS-stimulated RAW264.7 cells. RAW264.7 cells released detectable basal levels of nitrite, a stable metabolite product of NO (5.21 μ M/10⁴ cells, $\pm$ 0.18) and PGE₂ (47.25 pg/mL/10⁴ cells, $\pm$ 1.42). Upon stimulation by LPS, the levels of NO and PGE₂ increased markedly, by up to about five-fold and six-fold for 24 h, respectively. CA profoundly inhibited the LPS-induced production of NO and PGE₂ in a dose-dependent manner with IC₅₀ values of 18.5 μ M and 8.9 μ M, respectively (Figure 1(b) and (c)).

In order to determine whether CA inhibits production of NO and PGE₂ at gene expression level, we examined mRNA expression of iNOS and COX-2 in RAW264.7 cells treated with LPS, in the presence of CA. Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses indicated

that the CA resulted in a concentration-dependent reduction in iNOS and COX-2 mRNA levels in LPS-stimulated cells, without affecting the mRNA expression of β -actin (Figure 2a). Real-time qPCR analysis also demonstrated that iNOS and COX-2 mRNA levels increased markedly in response to stimulation with LPS, compared to that of basal level (media alone), which were significantly inhibited by CA in a dose-dependent manner (Figure 2(b) and (c)). Western blot analysis showed that the amounts of the iNOS and COX-2 proteins were significantly increased in RAW264.7 cells by stimulation with LPS, and these increases were suppressed in a dose-dependent manner by treatment of cells with CA (Figure 2d). These results indicate that the inhibitory effect of CA on LPS-induced NO and PGE₂ production may be due to the suppression of iNOS and COX-2 gene expression at the transcriptional level.

CA inhibited LPS-induced expression of pro-inflammatory cytokines in RAW264.7 macrophages

To evaluate the effects of CA on expression of pro-inflammatory cytokines in RAW264.7 macrophages, we measured the mRNA levels of IL-1 β , IL-6, and TNF- α in RAW264.7 cells treated with LPS in the presence or absence of CA.

Figure 2 Effects of CA on mRNA and protein expression levels of iNOS and COX-2 in murine macrophages. (a) For the RT-PCR of iNOS and COX-2 gene expression, total RNA was prepared for RT-PCR from RAW264.7 cells treated with LPS (1 μ g/mL) with different concentrations (5, 10, 20, 40 μ M) of CA for 24 h. iNOS-specific sequences and COX-2-specific sequences were detected by agarose gel electrophoresis, as described in the Materials and methods section. PCR of β -actin was performed to verify that the initial cDNA contents of samples were similar. (b and c) mRNA expression levels of iNOS and COX-2 were determined by real-time PCR analysis. The expression level of β -actin mRNA served as the internal control for the normalization iNOS and COX-2 mRNA expression. Data shown are the average of three independent experiments and are shown as the mean values $\pm$ S.D. * P < 0.01 versus medium alone. ** P < 0.05 versus LPS-induced cell culture medium. (d) For the Western blotting of iNOS and COX-2 protein, lysates were prepared from control cells, from cells stimulated for 24 h with 1 μ g/mL LPS, or from cells treated with LPS plus CA. Total cellular proteins (40 μ g) were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and detected with specific antibodies, as described in the Materials and methods section.

RT-PCR analysis demonstrated that the LPS-induced mRNA levels of IL-1 β , IL-6, and TNF- α were suppressed markedly by treatment of macrophages with CA in dose-dependent manner (Figure 3a). These data were further confirmed by real-time qPCR, showing that the significant and dose-dependent decrease of IL-1 β , IL-6, and TNF- α mRNAs upon treatment of cells with CA (Figure 3(b) to (d)).

Cynandione A inhibits LPS-induced activation of MAPKs in RAW264.7 cells

To elucidate molecular basis of inhibitory effects by CA on expression of the genes involved in inflammation, we examined the levels of LPS-induced phosphorylation of MAP kinases in RAW264.7 cells treated with CA. The results showed that CA significantly inhibited LPS-induced phosphorylation of ERK1/2 and p38 MAPKs in a dose-dependent manner (Figure 4a), while it barely affected phosphorylation of JNK (data not shown). These results suggest that ERK1/2 and p38 kinase pathways may contribute to the inhibitory effects of CA on the production of NO and PGE₂ in LPS-stimulated RAW264.7 cells.

In order to further elucidate mechanisms of action by CA, we investigated the effects of CA on NF- κ B activation by LPS. We measured the protein levels and phosphorylation of I κ B- α protein, an inhibitor protein which is associated with NF- κ B in the cytoplasm. The results showed

that upon LPS treatment, I κ B- α protein was diminished from the cytosolic fractions, whereas phosphorylated I κ B- α was markedly increased. LPS-induced phosphorylation and degradation of I κ B- α were significantly inhibited by CA (Figure 4b), indicating that CA inhibits phosphorylation and degradation of I κ B- α . We next measured translocation of NF- κ B p65 subunit levels following treatment with LPS in the presence of CA. Basal levels of p65, an NF- κ B subunit, were detected in nuclei of unstimulated cells, but treatment of cells with LPS for 2 h induced the nuclear translocation of p65 dramatically. The amount of p65 in the nucleus was reduced in the cells treated with CA dose-dependently, compared with those of cells treated with LPS alone (Figure 4c). Our results suggest that CA may inhibit the expression of iNOS and COX-2 via inhibition of phosphorylation and degradation of I κ B- α , thereby inhibiting nuclear translocation of NF- κ B and the expression of NF- κ B regulated genes.

Cynandione A reduces LPS-induced activation of NF- κ B

We next investigated the effects of CA on the LPS-induced transcriptional activation of NF- κ B/Luc reporter gene. CA dose-dependently inhibited the LPS-induced expression of the NF- κ B/Luc reporter gene construct in LPS-stimulated macrophages, indicating that CA down-regulates the expression of NF- κ B-regulated genes at the transcriptional

Figure 3 Effect of CA on expression of LPS-induced pro-inflammatory cytokines in RAW264.7 cells. (a) The mRNA levels of IL-1 β , IL-6, and TNF- α were determined by using RT-PCR. (b, c, and d) Cells were pretreated with CA at the indicated concentration for 2 h, and then exposed to CA with LPS (1 μ g/ml) or LPS alone for 18 h. The mRNA expression levels of IL-1 β , IL-6, and TNF- α mRNAs using 2- $\Delta\Delta$ CT method. Data shown are the average of three independent experiments and are shown as the mean values $\pm$ S.D. * P < 0.01 versus medium alone. ** P < 0.05 versus LPS-induced cell culture medium

Figure 4 Inhibitory effects of CA on LPS-induced phosphorylation of MAPKs, IκB-α and nuclear translocation of NF-κB in RAW264.7 cells. (a) RAW264.7 cells were starved for 6 h and then co-treated LPS (1 μg/ml) indicated doses of CA for 45 min. Whole-cell lysates were analyzed by Western blot analysis using specific phospho-ERK1/2, p38 antibodies. (b) RAW264.7 cells were co-treated with different concentrations (5, 10, 20, 40 μM) of CA and LPS (1 μg/ml) for 3 h. The expression levels of phospho-IκB-α and IκB-α were determined by Western blot analysis. (c) RAW264.7 cells were starved for 6 h and then co-treated LPS (1 μg/ml) indicated doses of CA for 2 h. Nuclear protein extracts were prepared as described in the Materials and methods section. The translocation levels of NF-κB p65 subunit were determined by Western blot analysis

levels (Figure 5a). We further examined the effects of CA on the DNA binding activity of NF-κB to its consensus sequence by electrophoretic mobility shift assay (EMSA). Our data demonstrated that nuclear extract from LPS-stimulated cells formed strong NF-κB-DNA complex band and that DNA binding activities of NF-κB in nuclear extracts obtained from LPS-stimulated cells in the presence of CA were reduced in a dose-dependent manner (Figure 5b). These results indicate that CA down-regulates the NF-κB target genes due to reduced binding of NF-κB to the binding sites of the target gene promoters.

Cynandione A inhibits production of pro-inflammatory cytokines and mediators *in vivo*

In order to demonstrate anti-inflammatory activity of CA *in vivo*, we examined the effects of CA on expression of iNOS and COX-2 in spleen as well as production of

Figure 5 Inhibitory effect of CA on LPS-induced activation of luciferase reporter genes and NF-κB DNA binding in RAW264.7 cells. (a) RAW264.7 cells were transiently transfected with an NF-κB dependent reporter gene, grown for 24 h, and treated for 12 h with the indicated concentrations of CA along with 1 μg/ml LPS. Data shown are the mean values ± S.D. (n = 3). **P* < 0.05 compared to treatment with LPS alone. (b) Nuclear extracts were prepared from controls or co-treated with the indicated doses of CA and LPS (1 μg/ml) of 2 h and analyzed for NF-κB binding by EMSA. The arrow indicates the position of the NF-κB band. The specificity of binding was examined by competition with the 10- and 50-fold unlabeled NF-κB oligonucleotide. F.P.: free probe; comp.: cold probe competitors; Ab: antibodies; p50: NF-κB p50 subunit antibody; p65: NF-κB p65 subunit antibody

inflammatory cytokines in mice administered with LPS. First, we performed Western blotting to determine whether administration of CA inhibits expressions of iNOS and COX-2 in spleen. LPS (4 mg/kg) markedly increased the protein levels of iNOS and COX-2 in spleen, and CA significantly inhibited the LPS-induced iNOS and COX-2 protein levels (Figure 6b). We next examined the effect of CA on *in vivo* pro-inflammatory cytokine production in LPS-treated mice. LPS-administered mice exhibited high levels of serum TNF-α, IL-1β and IL-6, which were about 4.3-, 5.4- and 6.7-fold, respectively, compared to saline control. Pretreatment of the animals with CA at 10 mg/kg significantly reduced the LPS-induced cytokine release by 89.1%, 55.3% and 74.3%, respectively (Figure 6c). These results suggest that CA inhibits the production of inflammatory mediators in septic animal models.

Figure 6 Effect of CA on expression of iNOS, COX-2, and pro-inflammatory cytokine *in vivo*. (a) Time line for injections. Mice were IP injected twice with CA or PBS with a 24 h interval. At 12 h following the second injection, LPS or PBS was IP injected. After 12 h, spleen and blood were collected. (b) For the Western blotting of iNOS and COX-2 protein levels, mouse spleen was grinded on the cell strainer. Total spleen proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and detected with specific iNOS and COX-2 antibodies, as described in the Materials and methods section. (c) The plasma levels of TNF- α , IL-1 β , and IL-6 were measured using ELISA kits. Data are mean $\pm$ S.D. from the data of five animals per group. * $P < 0.05$ compared to treatment with LPS alone

LPS administration resulted in the increase in weights and sizes of lymph nodes and spleen due to proliferation of various immune cells in these organs. Our results showed that treatment with CA reduced the size of lymph node and spleens compared to those of the control (Figure 7a). To test the protective effect of CA from LPS-induced septic shock in mice, we monitored its effect on the mortality in mice with lethal endotoxemia. We found that the LPS-injected mice without a CA treatment were all dead in the first 78 h after LPS injection, while those administered with CA (10 mg/kg) had a 74% survival rate compared with only LPS-injected group for 14 days (Figure 7b). These results suggest that CA has a protective effect related to septic shock in mice.

Discussion

In the present study, we demonstrated that CA isolated from the roots of *C. wilfordii* has effective anti-inflammatory activities by inhibiting the production of pro-inflammatory mediators in LPS-stimulated macrophages and in LPS-administered septic animals. We showed that it suppressed expression of iNOS and COX-2 at mRNA as well as protein levels. CA inhibited NF- κ B activation and LPS-induced nuclear translocation of NF- κ B through the inhibition of LPS-induced phosphorylation and degradation of I κ B α . CA had inhibitory effects on LPS-induced activation of MAP kinases ERK1/2, and p38, which may account for its anti-inflammatory activity by suppressing NF- κ B activation.

LPS is one of the pro-inflammatory factors in bacterial infection, leading to inflammatory reaction *in vivo*. Macrophages play a key role in the specific or nonspecific immune responses during inflammation processes. The addition of bacterial LPS to RAW264.7 cells or to animals greatly increases the production of inflammatory mediators such as NO, TNF- α , IL-1 β , and IL-6.⁹ NO has been shown to perform a pivotal function as a neurotransmitter, vasodilator, and immune regulator in a variety of tissues at physiological concentrations.¹⁷ High levels of NO generated by iNOS, however, has been defined as cytotoxic in studies of inflammation and endotoxemia.¹⁸ Excessive production of these mediators such as NO, TNF- α , IL-6, and IL-1 β may play a critical role in septic hypotension and other chronic inflammatory diseases such as atherosclerosis and neurodegenerative disorders.^{19,20} In this study, high levels of TNF- α , IL-6, and IL-1 β in RAW264.7 supernatant suggest that macrophages have been activated by LPS via TLR receptors and released numerous pro-inflammatory cytokines. It has been reported that TNF- α plays an important role in regulating inflammation, mostly through the induction of other inflammatory cytokines such as IL-1 β and IL-6.²¹ Our results showed that CA dose-dependently reduced expression of inflammatory mediators such as iNOS, COX-2, and pro-inflammatory cytokines TNF- α , IL-6, and IL-1 β , suggesting that CA may have an anti-inflammatory effect through inhibiting the expression of inflammatory mediators in macrophages. In septic animal models, the increased levels of TNF- α , IL-1 β , and IL-6 by LPS were significantly inhibited by co-injection with CA.

Figure 7 Effect of CA on LPS-induced septic shock mouse model. (a) Spleen and lymph nodes were collected after sacrifice of the mice after 14 days. The sizes of the organs were compared by photo-image. The weights of these organs and whole body were measured and the organ weight/body weight ratios were presented. * $P < 0.05$ compared to treatment with LPS alone. (b) Kaplan-Meier survival curves were used for the comparison of survival of septic mice between the groups (black dots) treated with LPS (30 mg/kg) alone and the group (white dots) treated with LPS (30 mg/kg) along with CA (10 mg/kg) ($n = 14$). (A color version of this figure is available in the online journal.)

Our results also demonstrated that CA improved survival of animal models with lethal endotoxemia, suggesting that CA has very effective anti-inflammatory activity *in vitro* as well as *in vivo*, and could relieve LPS-induced systemic inflammation.

LPS induces iNOS, COX-2, and pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 by activating mitogen-activated proteins kinases (MAPKs) superfamily, and the transcription factors NF- κ B.^{5,22} NF- κ B plays essential roles in inflammatory responses to external stimuli including bacterial infection. The genes for inflammatory mediators contain different combinations of transcription factor binding elements including NF- κ B, which are known to be activated by LPS.^{23,24} To further characterize the nature of the inhibitory effect of CA on production of inflammatory mediators, MAPKs and NF- κ B were examined. Our data indicated that CA inhibited phosphorylation of ERK1/2 and p38 MAPKs in a dose-dependent manner (Figure 4) but not JNK MAPK (data not shown). Previous studies report that NF- κ B activation is regulated by several cellular signaling pathways including ERK1/2 and p38 MAPKs.²⁵ Our study showed that CA dose-dependently inhibited nuclear translocation of NF- κ B and the LPS-induced expression of the NF- κ B/Luc reporter gene in

macrophages, indicating that CA down-regulates the expression of NF- κ B-regulated genes at the transcriptional levels. Thus, the anti-inflammatory activity of CA appears to be mediated via inhibition of NF- κ B activity, although we cannot exclude a possibility that the compound might suppress activation of other transcription factors activated by other signaling pathways such as JAK/STAT. Our results showed that CA inhibited expression of the inflammatory mediators via blocking phosphorylation of I κ B- α and suppressing p38 and ERK MAP kinases activation in LPS-stimulated RAW264.7 cells. These results suggest that CA function as an inhibitor of upstream mediators including I κ B kinase (IKK- α/β) in NF- κ B pathway as well as inhibitors of upstream molecules including MAPKs.

It will be valuable to identify natural compounds from edible and medicinal plants that possess effective anti-inflammatory activities. Most natural compounds that have anti-inflammatory and antioxidant activities are phenolics, such as epigallocatechin gallate (EGCG) and curcumin.²⁶⁻²⁸ CA was isolated from the roots of medicinal plants *Cynanchum* species, which belongs to plant-derived acetophenones. The previous studies report that CA has various biological activities, including protective effects of rat hepatocytes and protection of ischemic injuries in rats.^{12,29} It is known that hypoxia or ischemia is most likely related to generation of a burst of reactive oxygen species, which could activate NF- κ B.³⁰ Given that many anti-inflammatory drugs interfere with NF- κ B signaling pathway, inhibition of NF- κ B activation by CA may provide a highly attractive agent for the therapeutic development. There are large numbers of natural compounds that inhibit upstream of NF- κ B, including MAP kinases and IKK, and ameliorate inflammatory diseases.³¹

In conclusion, our results reported that CA from *C. wilfordii* significantly inhibited LPS-induced expression of TNF- α , IL-1 β , and IL-6 as well as iNOS and COX-2, which reduced release of pro-inflammatory cytokines, NO and PGE₂ production in macrophages. Furthermore, CA inhibited the activation of NF- κ B via blocking the phosphorylation of I κ B- α , and suppressing p38 and ERK MAP kinases. The anti-inflammatory activity was demonstrated in animal models with lethal endotoxemia. These results provide a possibility that CA may be utilized for developing a new therapeutic or chemopreventive agent against chronic and acute inflammatory disorders.

Authors' contributions: SHK, THL, and JK designed the project; SHK, SML, HJ, KHP, MJ, and MM performed the experiments; JHP and NIB isolated cynandione A from *C. wilfordii* and determined the structures of the compound. THL, ISC and JK analyzed the data and wrote the manuscript. SHK and THL contributed equally to this work.

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REFERENCES

- Medzhitov R. Origin and physiological roles of inflammation. *Nature* 2008;**454**:428–35
- Tabas I, Glass CK. Anti-inflammatory therapy in chronic disease: challenges and opportunities. *Science* 2013;**339**:166–72
- Fujiwara N, Kobayashi K. Macrophages in inflammation. *Curr Drug Targets Inflamm Allergy* 2005;**4**:281–86.
- Cohen J. The immunopathogenesis of sepsis. *Nature* 2002;**420**:885–91
- Lu YC, Yeh WC, Ohashi PS. LPS/TLR4 signal transduction pathway. *Cytokine* 2008;**42**:145–51
- Yang H, Young DW, Gusovsky F, Chow JC. Cellular events mediated by lipopolysaccharide-stimulated toll-like receptor 4. MD-2 is required for activation of mitogen-activated protein kinases and Elk-1. *J Biol Chem* 2000;**275**:20861–6
- Weinstein SL, Sanghera JS, Lemke K, DeFranco AL, Pelech SL. Bacterial lipopolysaccharide induces tyrosine phosphorylation and activation of mitogen-activated protein kinases in macrophages. *J Biol Chem* 1992;**267**:14955–62
- Lawrence T. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol* 2009;**1**:a001651
- Liang Y, Zhou Y, Shen P. NF-kappaB and its regulation on the immune system. *Cell Mol Immunol* 2004;**1**:343–50
- Shan L, Liu RH, Shen YH, Zhang WD, Zhang C, Wu DZ, Min L, Su J, Xu XK. Gastroprotective effect of a traditional Chinese herbal drug "Baishouwu" on experimental gastric lesions in rats. *J Ethnopharmacol* 2006;**107**:389–94
- Choi DH, Lee YJ, Kim JS, Kang DG, Lee HS. *Cynanchum wilfordii* ameliorates hypertension and endothelial dysfunction in rats fed with high fat/cholesterol diets. *Immunopharmacol Immunotoxicol* 2012;**34**:4–11
- Lee MK, Yeo H, Kim J, Kim YC. Protection of rat hepatocytes exposed to CCL4 *in-vitro* by cynandione A, a biacetophenone from *Cynanchum wilfordii*. *J Pharm Pharmacol* 2000;**52**:341–5
- Lee MK, Yeo H, Kim J, Markelonis GJ, Oh TH, Kim YC. Cynandione A from *Cynanchum wilfordii* protects cultured cortical neurons from toxicity induced by H₂O₂, L-glutamate, and kainite. *J Neurosci Res* 2000;**59**:259–64
- Jiang Y, Choi HG, Li Y, Park YM, Lee JH, Kim H, Lee JH, Son JK, Na M, Lee SH. Chemical constituents of *Cynanchum wilfordii* and the chemotaxonomy of two species of the family Asclepiadaceae, *C. wilfordii* and *C. auriculatum*. *Arch Pharm Res* 2011;**34**:2021–7
- Lee YH, Jung M, Bang MH, Chung DK, Kim J. Inhibitory effects of a spinasterol glycoside on lipopolysaccharide-induced production of nitric oxide and proinflammatory cytokines *via* down-regulating MAP kinase pathways and NF-kB activation in RAW264.7 macrophage cells. *Int Immunopharmacol* 2012;**13**:264–70
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;**25**:402–8
- Kobayashi Y. The regulatory role of nitric oxide in proinflammatory cytokine expression during the induction and resolution of inflammation. *J Leukoc Biol* 2010;**88**:1157–62
- Hickey MJ, Sihota E, Amrani A, Santamaria P, Zbytniuk LD, Ng ES, Ho W, Sharkey KA, Kubes P. Inducible nitric oxide synthase (iNOS) in endotoxemia: chimeric mice reveal different cellular sources in various tissues. *FASEB J* 2002;**16**:1141–3
- Libby P, Ridker RM, Maseri A. Inflammation and atherosclerosis. *Circulation* 2002;**105**:1135–43
- Glass CK, Saijo K, Winner B, Marchetto MC, Gage FG. Mechanisms underlying inflammation in neurodegeneration. *Cell* 2010;**140**:918–34
- Yi PF, Wu YC, Dong HB, Guo Y, Wei Q, Zhang C, Song Z, Qin QQ, Lv S, Wu SC, Fu BD. Peimine impairs pro-inflammatory cytokine secretion through the inhibition of the activation of NF-kB and MAPK in LPS-induced RAW264.7 macrophages. *Immunopharmacol Immunotoxicol* 2013;**35**:567–72
- Guha M, Mackman N. LPS induction of gene expression in human monocytes. *Cell Signal* 2001;**13**:85–94
- Surh YJ, Chun KS, Cha HH, Han SS, Keum YS, Park KK, Lee SS. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappaB activation. *Mutat Res* 2001;**480–481**:243–68
- Kundu JK, Shin YK, Surh YJ. Resveratrol modulates phorbol ester-induced pro-inflammatory signal transduction pathways in mouse skin *in vivo*: NF-kappaB and AP-1 as prime targets. *Biochem Pharmacol* 2006;**72**:1506–15
- May MJ, Ghosh S. Signal transduction through NF-kB. *Immunol Today* 2001;**19**:80–8
- Murakami A, Ohigashi H. Targeting NOX, iNOS and COX-2 in inflammatory cells: chemoprevention using food phytochemicals. *Int J Cancer* 2007;**121**:2357–63
- Lin YL, Lin JK. (-)-Epigallocatechin-3-gallate blocks the induction of nitric oxide synthase by down-regulating lipopolysaccharide-induced activity of transcription factor nuclear factor-kappaB. *Mol Pharmacol* 1997;**52**:465–72
- Brouet I, Ohshima H. Curcumin, an anti-tumour promoter and anti-inflammatory agent, inhibits induction of nitric oxide synthase in activated macrophages. *Biochem Biophys Res Commun* 1995;**206**:533–40
- Yue R, Yuan X, Liu X, Zhang J, Jiang P, He C, Shan L, Yu Y, Zhang W. Cynandione A mitigates ischemic injuries in rats with cerebral ischemia. *J Neurochem* 2012;**121**:451–64
- Nie Z, Mei Y, Ford M, Rybak L, Marcuzzi A, Ren H, Stiles GL, Ramkumar V. Oxidative stress increases A1 adenosine receptor expression by activating nuclear factor kappa B. *Mol Pharmacol* 1998;**53**:663–9
- Gupta SC, Sundaram C, Reuter S, Aggarwal BB. Inhibiting NF-kB activation by small molecules as a therapeutic strategy. *Biochim Biophys Acta* 2010;**1779**:775–87

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