Cynandione A from *Cynanchum wilfordii* Attenuates the Production of Inflammatory Mediators in LPS-Induced BV-2 Microglial Cells via NF-κB Inactivation

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Received December 4, 2013; accepted May 12, 2014

*Cynanchum wilfordii* is one of most widely used medicinal plants in Oriental medicine for the treatment of various conditions. In the present study, we isolated cynandione A (CA) from an extract of *Cynanchum wilfordii* roots (CWE) and investigated the effects of CA on the expression of inducible nitric oxide synthase (iNOS) and pro-inflammatory cytokines in lipopolysaccharide (LPS)-induced BV-2 microglial cells. CWE and CA significantly decreased LPS-induced nitric oxide production and the expression of iNOS in a concentration-dependent manner, while they (CWE up to 500 μg/mL and CA up to 80 μM) did not exhibit cytotoxic activity. Results from reverse transcription-polymerase chain reaction (RT-PCR) analysis and enzyme-linked immunosorbent assay (ELISA) showed that CA significantly attenuated the expression of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and IL-1β in LPS-stimulated BV-2 cells. Furthermore, CA inhibited the phosphorylation of inhibitor kappa B-alpha (IKB-α) and translocation of nuclear factor-kappa B (NF-κB) to the BV-2 cell nucleus, indicating that CWE and CA may have effective anti-inflammatory activities via NF-κB inactivation in stimulated microglial cells.

**Key words** Cynanchum wilfordii; natural product; inflammatory cytokine; inducible nitric oxide synthase (iNOS); nuclear factor-kappa B (NF-κB)

Microglia, a type of glial cells, are the resident macrophages in the brain and spinal cord, which play a major role in an innate immune defense and inflammatory responses in the central nervous system. 1–3) The microglial cells are activated under pathological conditions such as brain injury and the resulting exposure to lipopolysaccharide (LPS), interferon-γ or oxidative stresses. 4, 5) The activated microglia cells release neurotoxic and pro-inflammatory mediators, including nitric oxide (NO) and pro-inflammatory cytokines. 6–8) Chronic neuroinflammation is considered to be potential cause of a variety of neurodegenerative diseases, including Alzheimer’s disease, Parkinson’s disease and multiple sclerosis. 9) A number of reports have demonstrated that attenuation of microglial activation and subsequent suppression of the production of neurotoxic inflammatory mediators could provide a potential therapeutic approach for the prevention and the treatment of neurodegenerative disorders. 9–12)

*Cynanchum* is a genus of about 200 species and is distributed worldwide. Most *Cynanchum* species have been traditionally used in oriental medicines in Korea for the prevention and treatment of various diseases such as rheumatic arthritis, geriatric diseases, vascular diseases and ischemia-induced diseases. 13–15) *Cynanchi wilfordii* Radix is prescribed as the roots of *Cynanchum wilfordii* in Korean Pharmacopoeia (KFDA, 2008). The bioactive constituents of *Cynanchum* species have been explored in a number of studies. 16–18) It has been reported that acetophenones, cynandione A (CA) and its derivatives, had exhibited neuroprotective and anti-tumor activities. 19–21) In view of the importance of acetophenones in the biological function of *Cynanchum* species and the fact that CA may suppress microglial activation, we investigated whether CA suppresses microglial activation in LPS-stimulated BV-2 microglia. In the present study, we demonstrate that CA strongly inhibits the production of NO and pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and IL-1β in LPS-stimulated BV-2 microglial cells. Our results showed that CA suppresses expression of the cytokine genes and inducible nitric oxide synthase (iNOS) via inhibition of nuclear factor-kappa B (NF-κB). This study explores therapeutic potential and mechanism of CA against neuroinflammation and neurodegeneration.

**MATERIALS AND METHODS**

**Materials and Cell Culture** Dulbecco’s modified Eagle’s medium (DMEM), streptomycin, and fetal bovine serum (FBS) were purchased from Invitrogen Co. (Carlsbad, CA, U.S.A.). Antibodies for iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, U.S.A.). Specific antibodies against phospho-inhibitor kappa B-alpha (IκB-α), IκB-α, NF-κB p65 and proliferating cell nuclear antigen (PCNA) were obtained from Cell Signaling Technology, Inc. (Danver, MA, U.S.A.). Other chemicals were obtained commercially from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). CA was isolated from roots of *Cynanchum wilfordii* as previously reported. 19) BV-2 microglial cells were cultured at 37°C in DMEM containing 10% FBS, 2 mM glutamate, 100 unit/mL of penicillin, and 100 μg/mL of streptomycin in humidified incubator of 5% CO2.

**Quantitative Determination of CA in Cynanchum wilfordii** Formic acid was purchased from Sigma-Aldrich Co. HPLC-grade acetonitrile, methanol (MeOH) and water were
obtained from Burdick and Jackson. HPLC analysis was achieved using a Shimadzu LC-20 (Tokyo, Japan) with a Shimadzu SPD-20AV UV detector (290 nm). The column was a Waters C18 (250 mm×4.6 mm, particle size 5 µm). The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B), which were eluted at a flow rate of 0.6 mL/min and with the following gradient elution: 0→20 min, 40→100% of solvent B. The 50 g of *Cynanchum wilfordii* roots (CWE) were extracted in MeOH (50 mL) at 75–80°C for 4 h. The extracted solution was filtered through a 0.45 µm membrane-filter and a 20 µL aliquot was injected into the HPLC system.

**Viability of BV-2 Microglial Cells** BV-2 were seeded in 96-culture well plate at a density of 5×10⁴ cells/well and allowed to attach for 24 h. After discarding the growth medium, BV-2 were treated with various concentrations of an extract of *Cynanchum wilfordii* roots (CWE) or CA in serum-free medium for 18 h. After incubation, cells were treated with 10 µg/mL of LPS for 18 h. The levels of iNOS mRNAs were determined by RT-PCR analysis. GAPDH was used as the internal control. (C) BV-2 microglial cells treated with various concentrations of CWE with 1 µg/mL of LPS for 18 h. The levels of iNOS proteins were measured by Western blot analysis using polyclonal antibodies against murine iNOS.

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis** Total RNA was prepared from BV-2 microglial cells using a Trizol Reagent kit (Invitrogen). Total RNA (5 µg) was reverse transcribed using M-MuLV reverse transcriptase (Thermo Scientific, Pittsburgh, PA, U.S.A.). The following primers were used for PCR amplification: iNOS: 5'-TCTTTCGAAAATCCACCTGAC-3' (sense) and 5'-CCAAGTGCTCATTCTGC-3' (antisense); TNF-α: 5'-ATGAGCACAAGAGATGCATC-3' (sense) and 5'-TACAGGTCTTGTCACTGATCP-3' (antisense); IL-6: 5'-GAGGATACCACTCCCCAGAG-3' (sense) and 5'-TTCTACAAGGATTACCCTCC-3' (antisense); IL-1β: 5'-TGAGGATACCCTCC-3' (sense) and 5'-GAATCTGGTGGGCACTCCTATGC-3' (antisense); GAPDH: 5'-CGAAGCTTCAAGGACCTCT-3' (antisense). The RNA samples were treated by the same procedure for RT-PCR analysis. Levels of iNOS proteins were measured by Western blot analysis using polyclonal antibodies against murine iNOS.

**Fig. 1. Effects of CWE on Nitrite Production, Cell Viability, and Expression of iNOS**

(A) BV-2 cells were treated with various concentrations of CWE with 1 µg/mL of LPS for 18 h. Nitrite level was measured by the Griess assay. (B) Cell viability was measured by the MTT assay. Data shown are the average of three independent experiments and are shown as the mean value±S.D. *p<0.05 and **p<0.01 compared to control. (C) BV-2 microglial cells treated with various concentrations of CWE with 1 µg/mL of LPS for 18 h. The levels of iNOS mRNAs were determined by RT-PCR analysis. GAPDH was used as the internal control. (D) BV-2 cells were treated by the same procedure for RT-PCR analysis. Levels of iNOS proteins were measured by Western blot analysis using polyclonal antibodies against murine iNOS.

**Western Blot Analysis** BV-2 microglial cells were treated with various concentrations of CWE or various concentrations of CA with 1 µg/mL of LPS for 18 h. The cells were lysed in radio immunoprecipitation assay (RIPA) buffer (Pierce, Rockford, IL, U.S.A.) containing protease inhibitor cocktail (Roche Diagnostics Co., Indianapolis, IN, U.S.A.). Protein concentration was quantified with a protein assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The cell extracts were prepared and were fractionated by electrophoresis on 8–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked by soaking the membrane in Tris buffered saline-Tween 20 (TBS-T) buffer (0.5 mol/L Tris–HCl (pH 7.5), 0.15 mol/L NaCl, and 0.1% (w/v) Tween 20) containing 3–5% non-fat dry milk. Rabbit anti-iNOS and anti-p65, anti-phospho-IκBα, rabbit anti-IκBα and anti-p-p65 were utilized as primary antibodies and peroxidase-conjugated antibody was used as a secondary antibody. Peroxidase activity on the membrane sheet was visualized on X-ray films (FUJIFILM Co., Tokyo, Japan) by a standard enhanced chemiluminescence procedure.

**Enzyme Linked Immunosorbent Assay (ELISA)** Levels of pro-inflammatory cytokine such as TNF-α, IL-6 and IL-1β and were measured in cell culture media and in plasma using
ELISA kits (eBioscience, Inc., San Diego, CA, U.S.A.). BV-2 microglial cells were cultured in 6-well plates for 24 h, washed with PBS, and grown in fresh medium containing various concentrations of CA. Six hours after pretreatment with the CA, BV-2 cells were stimulated with 1 µg/mL of LPS for 18 h and supernatants of the cultures were collected. The levels of TNF-α, IL-6 and IL-1β released into the culture supernatants were measured using ELISA kits according to the manufacturer’s recommendations.

Statistical Analysis

Unless otherwise stated, all experiments were performed with triplicate samples and repeated at least three times. The data are presented as means±S.D. and statistical comparisons between groups were performed using one-way ANOVA followed by Student’s t-test.

RESULTS

Effects of CWE on NO Production in LPS-Stimulated Microglial BV-2 Cells

To investigate the effects of CWE on production of inflammatory mediators in LPS-stimulated BV-2 microglial cells, we measured NO production in LPS-stimulated cells in the presence and absence of CWE. A basal level of nitrite, a stable metabolite product of NO (2.2 µM/10^4 cells, ±0.17) was detected in unstimulated BV-2 cells. Upon stimulation, NO production levels increased markedly, by up to 11.7 µM/10^4 cells ±0.16 for 18 h. CWE profoundly inhibited the LPS-induced production of NO in a dose-dependent manner (Fig. 1A). The effects of CWE on the cell viability were evaluated via MTT assay. Cell viability was over 90% up to a concentration of 500 µg/mL of CWE for 18 h (Fig. 1B), thereby indicating that the inhibition of nitrite production was not attributable to cell death.

In order to determine whether CWE inhibits NO production at gene expression level, we determined iNOS mRNAs levels in BV-2 cells treated with LPS, in the presence of various concentrations of CWE. RT-PCR analyses indicated that co-treatment of BV-2 cells with CWE resulted in a concentration-dependent reduction of iNOS mRNA levels in a similar fashion as in NO production, in LPS-stimulated cells, without affecting the mRNA expression of GAPDH, a house-keeping gene product (Fig. 1C). Our results indicate that CWE could inhibit NO production in LPS-activated BV-2 cells via the suppression of iNOS mRNA expressions. We then attempted to determine whether CWE inhibits the levels of iNOS proteins. Our results indicated that the amounts of the iNOS proteins were significantly increased in the LPS-stimulated BV-2 cells, and these increases were suppressed in a dose-dependent manner by treatment of cells with CWE (Fig. 1D).

Quantitative Determination of CA in Cynanchum wil-
**CA** were isolated from the roots of *Cynanchum wilfordii* and the structural identity of CA was verified by comparison of NMR, IR and MS spectroscopic data with those in *fordii* and *Cynanchum wilfordii* and in the MeOH extracts was determined to be 0.0885% and 3.54%, respectively (Figs. 2B, C).

**Effects of CA on NO Production in LPS-Stimulated Microglial BV-2 Cells** We examined the effect of CA on NO production in LPS-stimulated BV-2 microglial cells. CA significantly inhibited the LPS-induced production of NO in a dose-dependent manner (Fig. 3A). The IC₅₀ was determined to be 2.73±3.58 μM. To confirm that the inhibitory effect of CA on NO production was not due to cytotoxicity of CA, we examined the effects on cell viability of BV-2 cells. The cells were incubated at 37°C for 18 h with or without various concentrations of CA. MTT assays showed that CA did not affect cell viabilities of microglial BV-2 cells in these conditions (Fig. 3B).

We next determined iNOS mRNA and protein levels in BV-2 cells treated with LPS, in the presence of various concentrations of CA. RT-PCR and immunoblot analysis showed that CA attenuated the expression of iNOS in LPS-treated cells in a concentration-dependent manners, indicating that CA inhibits NO production in LPS-activated BV-2 cells via the suppression of iNOS expressions (Figs. 3C, D).

**Effects of CA on Production of Pro-inflammatory Cytokines in LPS-Stimulated BV-2 Cells** TNF-α, IL-6 and IL-1β are pro-inflammatory cytokines and released from BV-2 cells upon stimulation with LPS or other inflammatory conditions. To examine the effects of CA on inflammatory cytokine production, levels of TNF-α, IL-6 and IL-1β in culture media of LPS-stimulated BV-2 cells with or without various concentrations of CA were measured by ELISA. Stimulation of BV-2 cells with LPS increased the secretion levels of TNF-α, IL-6 and IL-1β, which were significantly reduced in the supernatants of cells treated with CA in a dose-dependent manner (Fig. 4A).

To investigate whether inhibitory effect of CA on production of TNF-α, IL-6 and IL-1β via inhibition of the inflammatory gene expression, we determined the mRNA levels of the cytokines in LPS-stimulated BV-2 cells. Upon treatment of LPS for 18 h, the mRNA expressions of TNF-α, IL-6 and IL-1β were dramatically increased in BV-2 cells, and cotreatment of cells with LPS and different concentrations of CA dose-dependently reduced LPS-induced mRNA levels of TNF-α, IL-6 and IL-1β (Fig. 4B), indicating that CA inhibits production of the inflammatory cytokines at the gene expression levels.

**CA Inhibits Phosphorylation of IκB-α, and Nuclear Translocation of NF-κB** Activation of NF-κB plays critical roles in the LPS-induced expression of inflammatory mediators and cytokines such as iNOS, TNF-α, IL-6 and IL-1β in BV-2 cells. Since nuclear translocation of NF-κB is processed by IκB phosphorylation and degradation of IκB-α, we analyzed the effects of CA on phosphorylation and degradation of IκB-α. The results demonstrated that LPS-induced phosphorylation of IκB-α were inhibited by CA (Fig. 5A). We measured translocation of NF-κB p65 subunit levels following treatment with LPS in the presence or the absence of CA. Basal levels of p65 protein, NF-κB p65 subunit, were detected in...
LPS, a major outer membrane component of Gram-negative bacteria, acts through Toll-like receptor-4 (TLR4). The experimental model of LPS stimulation has been widely used to induce microglial activation of immune cells in the central nervous system, which triggers production of large amounts of NO as well as pro-inflammatory cytokines including TNF-α, IL-6 and IL-1β. The progressive production of these mediators may result in clinical syndrome of neuroinflammation and neurodegeneration. Nitric oxide has been shown to perform a pivotal function as a neurotransmitter, vasodilator, and immune regulator at physiological concentrations. High levels of NO generated by iNOS, however, induces energy depletion-induced necrosis or apoptosis via oxidant activation. Excessive pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β produced by activated microglia play a critical role in chronic inflammatory diseases such as neurodegenerative disorders. Thus, suppression of these mediators may be an effective therapeutic strategy for preventing inflammatory reaction and diseases. Our results showed that CA inhibited the production of NO and inflammatory cytokines in LPS-activated microglial cells, suggesting that CA may be potentially effective for prevention of neuronal disorders related to inflammation.

The expression of many inflammatory genes including iNOS, TNF-α, IL-6 and IL-1β is induced via activation of the transcription factor NF-κB which is considered the major inflammatory mediator in neuronal tissue. NF-κB is bound to the inhibitory protein IκB in the cytoplasm under the resting states. NF-κB activation by external stimuli such as LPS requires a sequential cascade of kinases such as IκB kinase (IKK-α/β)-dependent IκB phosphorylation, ubiquitination, degradation and translocation of NF-κB to the nucleus, where it binds and activates target genes containing NF-κB binding sites. Since the enzyme that catalyze the ubiquitination of phospho-IκB are constitutively active, the key event in NF-κB activation is IκB phosphorylation. Our results demonstrate that CA inhibits phosphorylation of IκB-α in dose-dependent manner, indicating that CA may suppress LPS-induced expression of the iNOS and pro-inflammatory cytokine genes via inhibiting IκB kinase (IKK-α/β) or its upstream TLR signaling pathways.

CA, a plant-derived acetophenone, was isolated from the roots of Cynanchum species. The previous studies report that CA has biological activities, including neuroprotection and suppression of degranulation from immune cells. It was demonstrated that CA alleviated neurotoxicity induced by the excitotoxic neurotransmitter, L-glutamate, and by kainite. Recent report showed that CA attenuated cerebral ischemia-induced injuries in rats. It is well known that ischemic injuries is most likely related to generation of a burst of reactive oxygen species, which could activate NF-κB. It was reported that (−)-epigallocatechin-3-gallate (EGCG) from green tea, the dried leaves of Camellia sinensis (L.) mediated NF-κB inhibition via blockade of the catalytic activities of the 20S/26S proteasome complex and intracellular accumulation of IκB-α in activated T cells, and exerted neuroprotection in autoimmune encephalomyelitis. Resveratrol, a natural polyphenol, suppressed LPS-stimulated expression of iNOS and inhibited LPS-induced degradation of IκB-α and phosphorylation of p38 mitogen-activated protein (MAP) kinase in microglial cells. Resveratrol was shown to modulate some of the symptoms...
of debilitating neurological disorders, such as ischemia\textsuperscript{13} and Alzheimer’s disease.\textsuperscript{33} Nobleitin from the peel of citrus fruits was shown to suppress microglial activation \textit{via} blocking NF-\kappaB activation and MAP kinases in LPS-stimulated BV-2 microglial cells.\textsuperscript{34} In addition, there are many other natural compounds targeting NF-\kappaB pathways to block microglial activation and to ameliorate microglia-associated neuroinflammatory diseases.\textsuperscript{10} Taken together, our present data suggest that CA may exert the neuroprotective effects in ischemic conditions and in neuroinflammation \textit{via} inhibiting activation of NF-\kappaB signaling pathways.

In conclusion, we showed that CA inhibits the production of NO and pro-inflammatory cytokine, including TNF-\alpha, IL-6 and IL-1\beta in LPS-stimulated BV-2 microglial cells. This anti-inflammatory effect occurs by down-regulation of iNOS, TNF-\alpha, IL-6 and IL-1\beta gene expression \textit{via} the suppression of NF-\kappaB activation. Furthermore, CA inhibits phosphorylation and degradation of I\kappaB-\alpha, which lead to blocking NF-\kappaB activation and to suppressing expression of the pro-inflammatory cytokines and iNOS. These results provide a possibility that CA may be utilized for developing a therapeutic or chemo-preventive agent against chronic inflammatory disorders in which production of inflammatory mediators involves in their pathogenesis.

Acknowledgment This research was supported by the Grant from the Next Generation BioGreen 21 program [PJ007985], Rural Development Administration, Republic of Korea.

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