

# Ethanol extract of *Cnidium officinale* exhibits anti-inflammatory effects in BV2 microglial cells by suppressing NF- $\kappa$ B nuclear translocation and the activation of the PI3K/Akt signaling pathway

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**Abstract.** Chronic microglial activation endangers neuronal survival through the release of various toxic pro-inflammatory molecules; thus, negative regulators of microglial activation have been identified as potential therapeutic candidates for several neurological diseases. In this study, we investigated the inhibitory effects of an ethanol extract of *Cnidium officinale* rhizomes (EECO), which has been used as a herbal drug in Oriental medicine, on the production of lipopolysaccharide (LPS)-induced pro-inflammatory mediators, such as nitric oxide (NO) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), as well as that of pro-inflammatory cytokines in BV2 microglia cells. EECO significantly inhibited the excess production of NO and PGE<sub>2</sub> in LPS-stimulated BV2 microglia cells. It also attenuated the expression of inducible NO synthase, cyclooxygenase-2, as well as that of pro-inflammatory cytokines, such as interleukin-1 $\beta$  and tumor necrosis factor- $\alpha$ . Moreover, EECO exhibited anti-inflammatory properties by suppressing nuclear factor- $\kappa$ B (NF- $\kappa$ B) translocation and the activation of the phosphoinositide 3-kinase/Akt pathway in LPS-stimulated BV2 cells. These

results indicate that EECO exerts anti-inflammatory effects in LPS-stimulated BV2 microglial cells by inhibiting pro-inflammatory mediators and cytokine production by blocking the NF- $\kappa$ B pathway. These findings suggest that EECO has substantial therapeutic potential for the treatment of neurodegenerative diseases accompanied by microglial activation.

## Introduction

Microglia are important cells that are involved in initial responses to tissue damage in the central nervous system (CNS). However, abnormally overactivated microglia promote the expression of pro-inflammatory mediators, such as nitric oxide (NO) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), as well as that of pro-inflammatory cytokines, including interleukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and monocyte chemoattractant protein-1 and other factors that contribute to the development of chronic inflammatory diseases (1-3). In particular, microglia are activated by lipopolysaccharides (LPS),  $\beta$ -amyloid, thrombin, or interferon- $\gamma$ , and the secretion of inflammatory molecules by abnormally activated microglia disturbs the homeostasis of the immune system, thus inducing and promoting degenerative CNS autoimmune diseases, such as multiple sclerosis, Alzheimer's disease and Parkinsonism (4-6). Therefore, understanding congenital immune system disorders related to the overactivation of microglia and controlling the inflammation molecules secreted by abnormally activated microglia is an approach to delaying chronic inflammatory diseases.

The transcription factor, nuclear factor- $\kappa$ B (NF- $\kappa$ B), plays a central role in the regulation of several genes responsible for the generation of pro-inflammatory mediators and cytokines. In normal cells, NF- $\kappa$ B subunits are present in the cytosol bound

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to the inhibitory protein I $\kappa$ B (I $\kappa$ B), which inactivates them (7,8). However, in response to various stimuli, such as LPS, I $\kappa$ B is rapidly degraded by the ubiquitin-proteasome pathway. The degradation of I $\kappa$ B induces the translocation of NF- $\kappa$ B subunits into the nucleus, and the NF- $\kappa$ B subunits bind to the promoter regions of target genes, including inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), TNF- $\alpha$  and IL-1 $\beta$ , and stimulate their transcription. The activation of the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway plays an important role in regulating LPS-induced pro-inflammatory responses by inducing NF- $\kappa$ B activation through proteasome-dependent I $\kappa$ B degradation (9-14). Therefore, inhibiting NF- $\kappa$ B activation through the PI3K/Akt pathway results in anti-inflammatory effects.

*Cnidium officinale* (*C. officinale*) Makino, which belongs to the Umbelliferae family, is a perennial herb native to China. The dried rhizomes of *C. officinale* have been used as one of the most commonly prescribed traditional Oriental medicinal herbs in East Asian countries. In Korean traditional medicine, they are widely used in the treatment of menstrual disturbances and as a blood pressure depressant, as well as for relieving pain from headaches and rheumatic arthralgia (15-20). Although some pharmacological beneficial effects of this herb and extracts of its rhizome have recently been reported, including anticancer, anti-inflammatory and antioxidant effects (21-24), its molecular mechanisms of action have not yet been fully elucidated. Therefore, the present study was conducted to evaluate the effects of an ethanol extract of *C. officinale* rhizomes (EECO) on the production of pro-inflammatory mediators and cytokines and the respective regulatory genes with a focus on the underlying molecular mechanisms in LPS-stimulated BV2 microglial cells.

## Materials and methods

**Reagents, chemicals and preparation of EECO.** LPS and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against COX-2, iNOS, TNF- $\alpha$ , IL-1 $\beta$ , NF- $\kappa$ B p65 and I $\kappa$ B- $\alpha$  were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against phosphorylated PI3K (p-PI3K), PI3K, phosphorylated Akt (p-Akt) and Akt were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against nucleolin and actin were obtained from Sigma-Aldrich. Peroxidase-labeled goat anti-rabbit immunoglobulin was purchased from Koma Biotechnology (Seoul, Korea). Other chemicals were purchased from Sigma-Aldrich. To prepare EECO, the rhizomes of *C. officinale*, which were obtained from Dongeui University Oriental Hospital (Busan, Korea), were pulverized and extracted twice with 10 volumes of 80% ethanol at 85-90°C in a reflux condenser for 3 h, and then filtered with a 50  $\mu$ m filter and concentrated by vacuum evaporation at 60°C. The solid form of the extract was dissolved in dimethyl sulfoxide.

**Cell culture and viability assay.** BV2 microglial cells were cultured at 37°C in 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum and antibiotics (WelGENE Inc., Daegu, Korea). In all the experiments, the cells were pre-treated with the indicated concentrations of EECO for 1 h prior to the addition of LPS (500 ng/ml) in serum-free DMEM. Cell viability was measured

based on the formation of blue formazan that was metabolized from colorless MTT by mitochondrial dehydrogenases, which are active only in live cells. In brief, the BV2 cells were seeded and treated with reagents for the indicated periods of time. Following treatment, the medium was removed, and the cells were incubated with 0.5 mg/ml of MTT solution for 2 h at 37°C and 5% CO<sub>2</sub>, and then the supernatant was removed and the formation of formazan was measured at 540 nm using a microplate reader (Dynatech MR-7000; Dynatech Laboratories, Chantilly, VA, USA).

**Measurement of NO.** The concentration of NO generated by BV2 cells activated by LPS was detected using Griess reagent [1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride]. BV2 cells were cultured for 24 h in a 6-well culture plate, pre-treated with various concentrations of EECO for 1 h, and then treated again with LPS (500 ng/ml). After 24 h of culture, the cell culture medium was collected and the same quantity of Griess reagent was added to induce a reaction at room temperature. The optical density of the reaction solution was measured at 540 nm using a microplate reader and the quantity of NO generated by the cells was calculated based on the concentration of the sodium nitrite (NaNO<sub>2</sub>) standard solution (standard curve).

**Measurement of PGE<sub>2</sub>.** To measure the quantity of PGE<sub>2</sub> generated by BV2 cells, medium from the cultures under the same conditions was collected and the quantity of PGE<sub>2</sub> generated was measured using a PGE<sub>2</sub> enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Co., Ann Arbor, MI, USA). The concentration (pg/ml) of PGE<sub>2</sub> in the cell culture medium was calculated based on the concentrations of the standard solution as previously described (25).

**Measurement of cytokines.** The levels of IL-1 $\beta$  and TNF- $\alpha$  were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, BV2 cells were loaded in 24-well plates and pre-treated with the indicated EECO concentrations for 1 h prior to stimulation with 500 ng/ml LPS for 24 h. A total of 100  $\mu$ l of culture medium supernatant was collected to determine IL-1 $\beta$  and TNF- $\alpha$  concentration by ELISA.

**Isolation of total RNA and reverse transcription-polymerase chain reaction (RT-PCR).** RT-PCR was conducted to examine the effects of EECO on the expression of LPS-induced iNOS, COX-2, and inflammatory cytokines at the transcription level. Total RNA was separated from the BV2 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and reverse-transcribed using MMLV reverse transcriptase (Promega, Madison, WI, USA) to produce cDNA. The cDNA was amplified by PCR using specific primers: iNOS forward, 5'-CCT CCT CCA CCC TAC CAA GT-3' and reverse, 5'-CAC CCA AAG TGC TTC AGT CAA-3'; COX-2 forward, 5'-AAG ACT TGC CAG GCT GAA CT-3' and reverse, 5'-CTT CTG CAG TCC AGG TTC AA-3'; IL-1 $\beta$  forward, 5'-ATG GCA ACT GTT CCT GAA CTC AAC T-3' and reverse, 5'-TTT CCT TTC TTA GAT ATG GAC AGG AC-3'; TNF- $\alpha$  forward, 5'-GCG ACG TGG AAC TGG CAG AA-3' and reverse, 5'-TCC ATG CCG TTG GCC AGG AG-3'.

and GAPDH forward, 5'-ACC ACA GTC CAT GCC ATC AC-3' and reverse, 5'-TCC ACC ACC CTG TTG CTG TA-3'. The following PCR conditions were applied: iNOS, COX-2, IL-1 $\beta$  and TNF- $\alpha$ : 25 cycles of denaturation at 94°C for 30 sec, annealing at 59°C for 30 sec, and extension at 72°C for 30 sec; GAPDH, 23 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec and extension at 72°C for 30 sec. The PCR products were electrophoresed on 1.5% agarose gels and stained with ethidium bromide. GAPDH was used as an internal control to evaluate relative expression.

**Protein extraction and western blot analysis.** The cells were washed three times with phosphate-buffered saline (PBS) and lysed in lysis buffer [1% Triton X-100, 1% deoxycholate, 0.1% sodium azide (NaN<sub>3</sub>)] containing protease inhibitor cocktail tablets to isolate total protein (Roche Diagnostics GmbH, Mannheim, Germany). In a parallel experiment, cytoplasmic and nuclear extracts were prepared using NE-PER nuclear and cytosolic extraction reagents (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH, USA) by electroblotting. Proteins were detected using an enhanced chemiluminescence detection system (Pierce).

**Immunofluorescence.** The prepared cells were washed twice with PBS and fixed for 15 min at 4°C using 4% paraformaldehyde. The cells were washed again with PBS, reactions were induced for 20 min at 4°C in PBS that contained 0.3% Triton X-100, and then reactions were induced for 1 h at room temperature in PBS containing 2% bovine serum albumin (BSA) to suppress non-specific reactions. The anti-NF- $\kappa$ B p65 antibody was then diluted to 1:200 in a PBS solution that contained 2% BSA to induce reactions for 2 h at room temperature. The cells were washed three times in PBS and fluorescein isothiocyanate (FITC)-conjugated IgG (Molecular Probes, Eugene, OR, USA), which is a secondary antibody, was diluted to 1:100 to induce reactions for 1 h at room temperature. Samples of the immunofluorescence-stained cells were observed under a confocal laser scanning microscope (Olympus, Tokyo, Japan). A wavelength of 488 nm was used for FITC, and the images were reassembled into final three-dimensional images according to the manufacturer's instructions (Olympus Fluoview 300, Olympus).

**Statistical analysis.** All results are expressed as the means  $\pm$  standard errors. Each experiment was repeated at least three times. Statistical significances were identified between each treated group by the paired Student's t-test. A P-value <0.05 was considered to indicate a statistically significant difference.

## Results

**Inhibition of LPS-induced NO and PGE<sub>2</sub> production by EECO.** To determine the inhibitory effects of EECO on LPS-induced NO and PGE<sub>2</sub> production, BV2 macroglial cells were incubated with the indicated concentrations of EECO in the presence or absence of LPS for 24 h, and the levels of NO and PGE<sub>2</sub> production were measured in the culture medium using Griess reagent

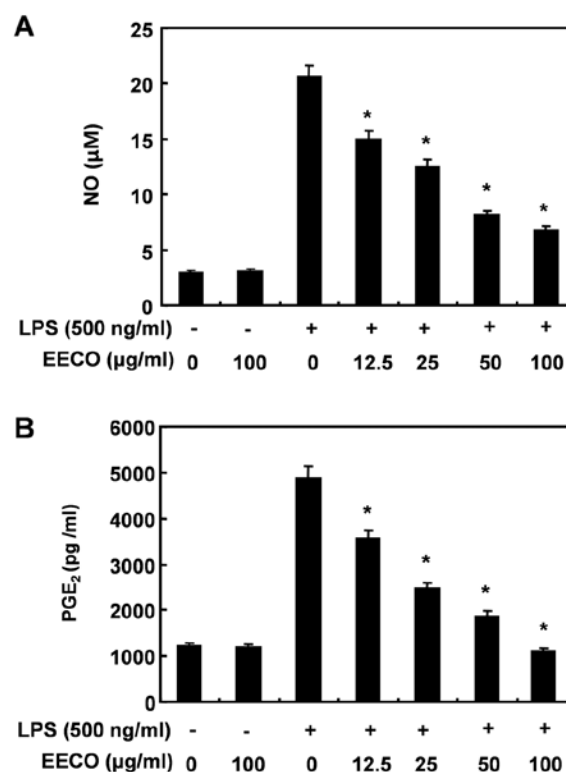


Figure 1. Inhibition of nitric oxide (NO) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production by the ethanol extract of *Cnidium officinale* rhizomes (EECO) in lipopolysaccharide (LPS)-stimulated BV2 microglia cells. BV2 cells were pre-treated with various concentrations of EECO (12.5–100  $\mu$ g/ml) for 1 h prior to incubation with LPS (500 ng/ml) for 24 h. (A) Nitrite content was measured by Griess reaction and (B) PGE<sub>2</sub> concentration was measured in the culture medium using a commercial enzyme-linked immunosorbent assay (ELISA) kit. Each value indicates the mean  $\pm$  standard deviation of results obtained from three independent experiments. \*P<0.05 indicates a significant difference from the value obtained for cells treated with LPS in the absence of EECO.

and ELISA, respectively. LPS triggered an approximate 7-fold increase in NO production compared with that in the untreated control group; however, pre-treatment with EECO reduced LPS-induced NO production in a concentration-dependent manner (Fig. 1A). The amount of PGE<sub>2</sub> present in the culture medium also increased after 24 h of exposure to LPS alone; however, a concentration-dependent decrease was observed following pre-treatment with EECO (Fig. 1B).

### Inhibition of LPS-induced NO and PGE<sub>2</sub> expression by EECO.

We then investigated whether the inhibitory effects of EECO on NO and PGE<sub>2</sub> production are associated with decreased levels of iNOS and COX-2 expression, which are known to induce NO and PGE<sub>2</sub> production, using western blot analysis and RT-PCR. The mRNA levels of iNOS and COX-2 were markedly augmented in the presence of LPS alone; however, their expression levels were markedly upregulated in the presence of EECO (Fig. 2A). Western blot analyses also revealed that treatment with LPS increased iNOS and COX-2 protein expression, whereas pre-treatment of the cells with EECO attenuated the LPS-induced iNOS and COX-2 protein expression (Fig. 3B). These results indicate that EECO inhibits the LPS-induced release of NO and PGE<sub>2</sub> by suppressing iNOS and COX-2 expression at the transcriptional level.

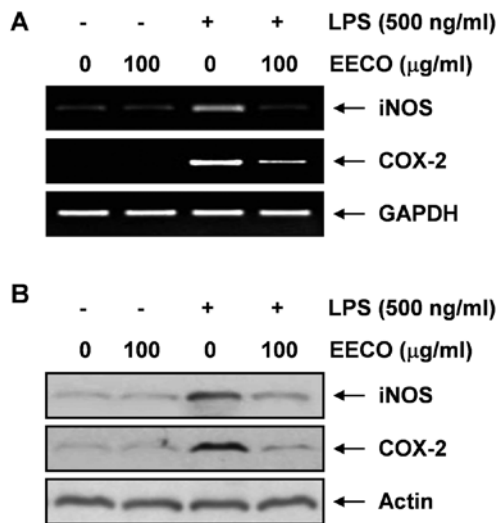


Figure 2. Inhibition of inducible nitric oxide (NO) synthase (iNOS) and cyclooxygenase-2 (COX-2) expression by the ethanol extract of *Cnidium officinale* rhizomes (EECO) in lipopolysaccharide (LPS)-stimulated BV2 microglial cells. (A) BV2 cells were treated with 100 µg/ml EECO or 500 ng/ml LPS alone, or pre-treated with 100 µg/ml EECO for 1 h prior to treatment with LPS. Following treatment with LPS for 6 h, total RNA was prepared for RT-PCR analysis of iNOS and COX-2 mRNA expression in LPS-stimulated BV2 microglial cells. (B) Cells were pre-treated with EECO 1 h prior to incubation with LPS (500 ng/ml) for 24 h. The cells were then lysed and the same amounts of cellular proteins were separated on SDS-polyacrylamide gels. After the proteins were transferred onto nitrocellulose membranes, the membranes were probed with anti-iNOS and anti-COX-2 antibodies. The proteins were visualized using an ECL detection system. GAPDH and actin were used as internal controls for the RT-PCR and western blot assays, respectively.

**Inhibition of LPS-induced TNF- $\alpha$  and IL-1 $\beta$  generation by EECO.** We then determined the potential effects of EECO on the production of pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$  by ELISA. The levels of TNF- $\alpha$  significantly increased in the culture medium of LPS-stimulated BV2 cells; however, the levels decreased significantly in a dose-dependent manner following pre-treatment with EECO (Fig. 3A). IL-1 $\beta$  production increased following stimulation with LPS and EECO significantly decreased the levels of IL-1 $\beta$  in the supernatant of LPS-stimulated BV2 cells (Fig. 3B).

**Inhibition of LPS-induced TNF- $\alpha$  and IL-1 $\beta$  expression by EECO.** RT-PCR and western blot analysis were performed in parallel experiments to determine whether EECO inhibits TNF- $\alpha$  and IL-1 $\beta$  expression. The increased expression of TNF- $\alpha$  and IL-1 $\beta$  following treatment with LPS was markedly attenuated by pre-treatment with EECO at both the transcriptional and translational levels (Fig. 4). These results indicate that EECO is effective in suppressing pro-inflammatory cytokine production by altering the transcriptional levels of TNF- $\alpha$  and IL-1 $\beta$  in LPS-activated microglia.

**Inhibition of LPS-induced NF- $\kappa$ B translocation by EECO.** As NF- $\kappa$ B is a central transcription factor that regulates the expression of a large number of inflammation-related genes (7,26), the effects of EECO on the LPS-stimulated nuclear translocation of NF- $\kappa$ B p65 subunits were examined. The western blot analysis results in Fig. 5A indicate that the levels of NF- $\kappa$ B p65 in the nucleus markedly increased within 15 min of exposure to LPS;

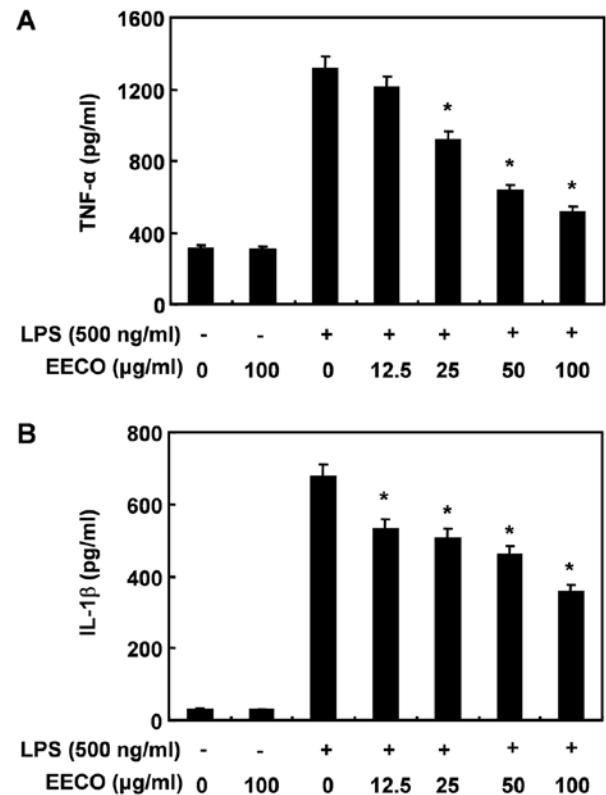


Figure 3. Effect of the ethanol extract of *Cnidium officinale* rhizomes (EECO) on lipopolysaccharide (LPS)-stimulated tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin (IL)-1 $\beta$  production in BV2 microglia cells. BV2 cells were pre-treated with various concentrations of EECO for 1 h prior to treatment with 500 ng/ml LPS. After 2 h of incubation, the levels of (A) TNF- $\alpha$  and (B) IL-1 $\beta$  present in the supernatants were measured using enzyme-linked immunosorbent assay (ELISA). Each value indicates the mean  $\pm$  standard deviation of results obtained from three independent experiments. \*P<0.05 indicates a significant difference from the value obtained for the cells treated with LPS in the absence of EECO.

however, the LPS-induced p65 levels in the nuclear fraction decreased following pre-treatment with EECO. In addition, I $\kappa$ B- $\alpha$  was markedly degraded 15 min following exposure to LPS; however, the LPS-induced I $\kappa$ B- $\alpha$  degradation was significantly reversed by EECO. We also investigated whether EECO interferes with the translocation of NF- $\kappa$ B in LPS-treated BV2 cells by immunofluorescence. The level of NF- $\kappa$ B p65 in the nucleus decreased significantly by EECO, indicating that EECO inhibits NF- $\kappa$ B activation in BV2 microglial cells by suppressing I $\kappa$ B degradation and the nuclear translocation of NF- $\kappa$ B (Fig. 4B).

**Inhibition of LPS-induced PI3K/Akt activation by EECO.** As the activation of the PI3K/Akt signaling pathway leads to the production of inflammatory mediators and cytokines through the activation of NF- $\kappa$ B (9-12), we investigated the effects of EECO on the phosphorylation of PI3K and Akt proteins in LPS-stimulated BV2 cells. Using western blot analysis with anti-phospho-specific antibodies for PI3K and Akt, we found that EECO suppressed the LPS-induced phosphorylation of PI3K and Akt (Fig. 6), whereas the levels of non-phosphorylated PI3K and Akt were unaffected by either EECO or LPS treatment. These findings strongly suggest that the anti-

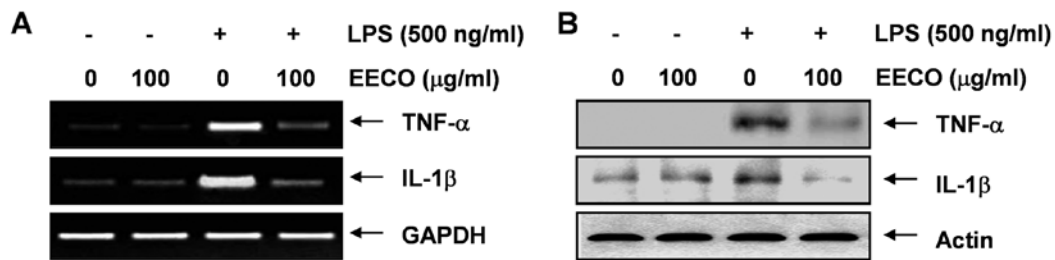


Figure 4. Inhibition of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin (IL)-1 $\beta$  expression by ethanol extract of *Cnidium officinale* rhizomes (EECO) in lipopolysaccharide (LPS)-stimulated BV2 microglia cells. (A) BV2 cells were treated with 100  $\mu$ g/ml EECO or 500 ng/ml LPS alone, or pre-treated with 100  $\mu$ g/ml EECO for 1 h prior to treatment with LPS. Following treatment with LPS for 6 h, total RNA was prepared for RT-PCR analysis of TNF- $\alpha$  and IL-1 $\beta$  mRNA expression in LPS-stimulated BV2 microglial cells. (B) Cells were pre-treated with EECO 1 h prior to incubation with LPS (500 ng/ml) for 24 h. The cells were then lysed and the same amounts of cellular proteins were separated on SDS-polyacrylamide gels. After the proteins were transferred onto nitrocellulose membranes, the membranes were probed with anti-TNF- $\alpha$  and anti-IL-1 $\beta$  antibodies. The proteins were visualized using an ECL detection system. GAPDH and actin were used as internal controls for the RT-PCR and western blot assays, respectively.

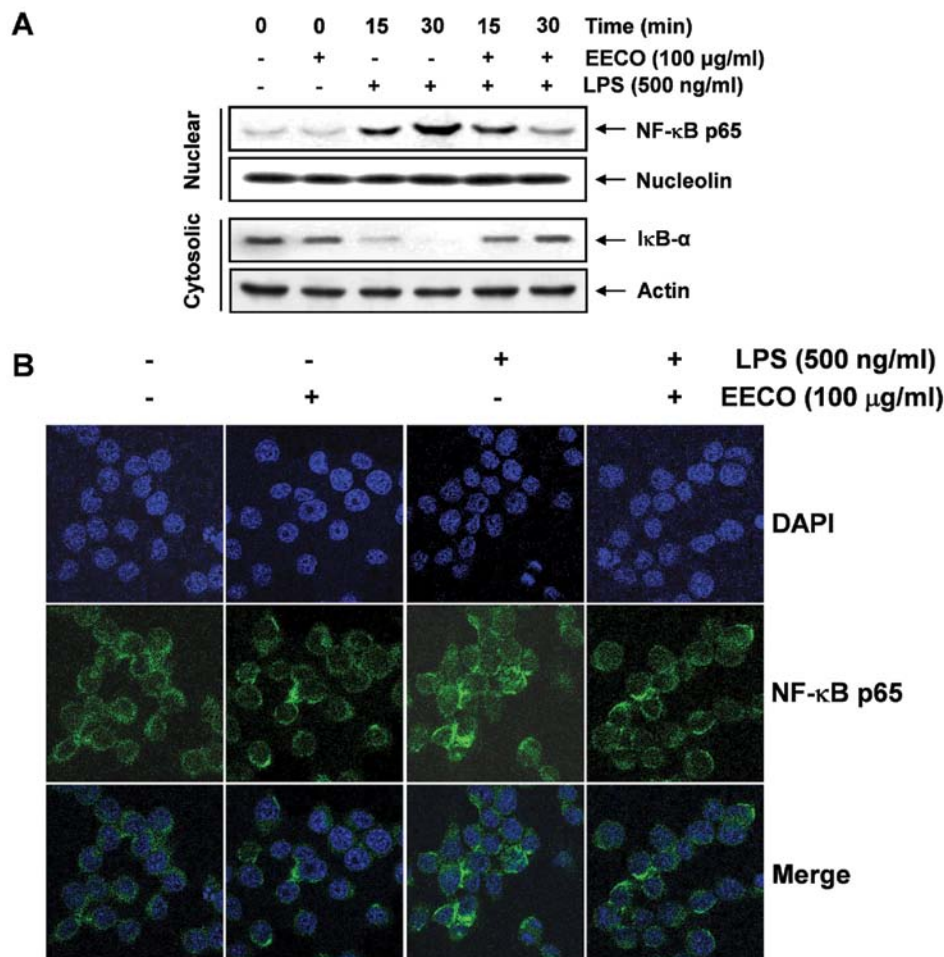


Figure 5. Effects of the ethanol extract of *Cnidium officinale* rhizomes (EECO) on lipopolysaccharide (LPS)-induced nuclear factor- $\kappa$ B (NF- $\kappa$ B) translocation in BV2 microglial cells. (A) Cells were treated with 100  $\mu$ g/ml EECO for 1 h prior to treatment with 500 ng/ml LPS for the indicated time periods of time. Nuclear and cytosolic proteins were isolated and subjected to 10% SDS-polyacrylamide gel electrophoresis followed by western blot analysis using anti-NF- $\kappa$ B p65 and anti-I $\kappa$ B- $\alpha$  antibodies. Nucleolin was used as the internal control for the nuclear fraction. (B) BV2 cells were pre-treated with EECO for 1 h prior to stimulation with LPS for 1 h. NF- $\kappa$ B p65 protein localization was determined using an anti-NF- $\kappa$ B p65 antibody and an fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG antibody, and cells were visualized under a laser confocal scanning microscope.

inflammatory effects of EECO in LPS-stimulated BV2 cells are associated with the inactivation of the PI3K/Akt pathway.

**Effect of EECO on the viability of BV2 microglial cells.** We evaluated the viability of BV2 cells incubated with or without LPS in the absence or presence of EECO by MTT assay to

determine the cytotoxic effects (if any) of EECO on BV2 microglia. The concentrations (12.5 to 100  $\mu$ M/ml) of EECO used to inhibit LPS-induced inflammatory responses did not affect cell viability, confirming that the anti-inflammatory effects of EECO in LPS-stimulated BV2 cells are not due to the cytotoxicity of EECO (Fig. 7).



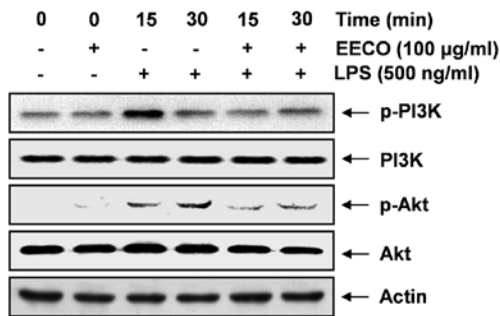


Figure 6. Effect of the ethanol extract of *Cnidium officinale* rhizomes (EECO) on lipopolysaccharide (LPS)-induced phosphorylation of phosphoinositide 3-kinase (PI3K) and Akt in BV2 microglial cells. BV2 cells were pre-treated with 100 µg/ml EECO for 1 h prior to treatment with LPS (500 ng/ml), and total proteins were isolated at the indicated times following treatment with LPS. Proteins were subjected to SDS-polyacrylamide gel electrophoresis followed by western blot analysis using the indicated antibodies, and an ECL detection system.

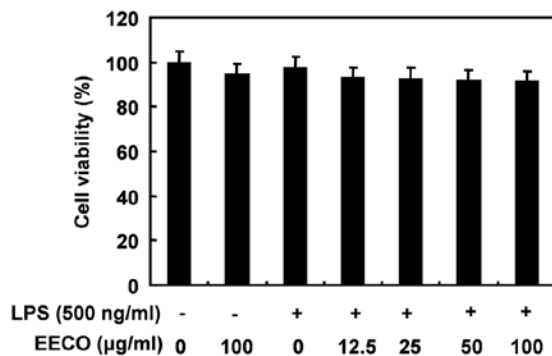


Figure 7. Effects of the ethanol extract of *Cnidium officinale* rhizomes (EECO) and lipopolysaccharide (LPS) on the viability of BV2 microglia cells. Cells were treated with 100 µg/ml EECO or 500 ng/ml LPS alone, or pre-treated with the indicated concentrations of EECO for 1 h prior to treatment with LPS. After 24 h, cell viability was assessed by MTT reduction assays. Each value indicates the mean  $\pm$  standard deviation of results obtained from three independent experiments.

## Discussion

In the present study, we found that EECO impaired LPS-induced gene expression and the secretion of pro-inflammatory mediators (NO and PGE<sub>2</sub>), as well as that of the inflammation-related genes, iNOS and COX-2, and cytokines (IL-1 $\beta$  and TNF- $\alpha$ ) in a BV2 microglia cell model. Further experiments revealed that EECO attenuated the LPS-induced NF- $\kappa$ B activation by suppressing the degradation of I $\kappa$ B- $\alpha$ , which was associated with the inactivation of the PI3K/Akt signaling pathway.

Inflammation in the brain caused by activated microglia plays an important role in the pathology of neurodegenerative disorders (5,6). iNOS and COX-2, which are responsible for synthesizing NO and PGE<sub>2</sub>, respectively, are critical enzymes that mediate inflammatory processes. The improper upregulation of iNOS and COX-2 has been associated with certain types of inflammatory disorders, including neuronal degeneration (27,28). Pro-inflammatory cytokines also play critical roles in the process of inflammation and the increased production of these cytokines is associated with neuronal dysfunction and neuronal loss (29,30). Therefore, the suppression of neuroinflammation during microglial activation would

theoretically attenuate the progression of neurodegenerative disease. Thus, the inhibition of pro-inflammatory mediators and cytokines by EECO in LPS-stimulated BV2 microglia shown in the present study, may play a beneficial role in the treatment of neurodegenerative diseases.

NF- $\kappa$ B is an important transcription factor that regulates various cellular responses required for inducing the expression of inflammation-related genes (7,26). In an inactive state, NF- $\kappa$ B exists as a heterodimer of p65 and p50 subunits in the cytoplasm, but translocates to the nucleus once activated through the phosphorylation and degradation of I $\kappa$ B, and proceeds to transcribe the majority of pro-inflammatory genes, thus contributing to the development of anaphylaxis, septic shock, multiple organ failure and even cell death (31-33). In our experiments, the majority of intracellular NF- $\kappa$ B p65 had translocated from the cytosol to the nucleus following treatment with LPS, as demonstrated by strong NF- $\kappa$ B p65 accumulation and staining in the nucleus. However, the levels of NF- $\kappa$ B p65 in the nucleus decreased significantly following pre-treatment with EECO. These results suggest that EECO inhibits LPS-induced acute pro-inflammatory responses mediated by the NF- $\kappa$ B signaling pathway.

The PI3K/AKT signaling pathway also controls a variety of cellular proliferation and survival processes. Upon stimulation, PI3K phosphorylates specific phosphoinositide lipids, which accumulate in the plasma membrane, creating docking sites for Akt. Akt undergoes phosphorylation at the plasma membrane, leading to its activation (34,35). Numerous studies have shown that the PI3K/Akt signaling pathway plays an important role in negatively regulating LPS-induced acute inflammatory responses in microglia (13,14,36-38). Although the role of PI3K/Akt signaling cascades in the regulation of NF- $\kappa$ B transactivation remains controversial (8,39,40), certain studies have demonstrated that LPS-induced NF- $\kappa$ B activation is directly regulated as a main upstream molecule of NF- $\kappa$ B via the phosphorylation of Akt (41,42). Therefore, to further confirm the inhibitory effects of NF- $\kappa$ B activation by EECO, we investigated the effects of EECO on the levels of PI3K and Akt phosphorylation in LPS-stimulated BV2 cells. As a result, we found that PI3K and Akt phosphorylation was markedly suppressed by EECO. These results suggest that EECO inhibits LPS-induced NF- $\kappa$ B activation by inhibiting the activation of the PI3K/Akt pathway.

In conclusion, in this study, we demonstrate that EECO inhibits pro-inflammatory mediator and cytokine production by suppressing the activation of NF- $\kappa$ B in LPS-stimulated BV2 microglial cells. The regulation of NF- $\kappa$ B activity by EECO was also associated with the inactivation of the PI3K/Akt signaling pathway during the LPS-induced anti-inflammatory reaction. Therefore, the present results provide a molecular basis for understanding the inhibitory effects of *C. officinale* rhizomes on endotoxin-mediated inflammation.

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