Ethyl Acetate Soluble Fraction of *Cnidium officinale* MAKINO Inhibits Neuronal Cell Death by Reduction of Excessive Nitric Oxide Production in Lipopolysaccharide-Treated Rat Hippocampal Slice Cultures and Microglia Cells

Jeong Min Kim, Dongwook Son, Pyeongjae Lee, Kang Jin Lee, Hocheol Kim, and Sun Yeou Kim*

Department of Herbal Pharmacology, Graduate School of East-West Medical Science, Kyung Hee University, #1 Hoegi-dong, Dongdaemum-ku, Seoul 130-701, Korea

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Abstract. In the present work, we found that the ethyl acetate-soluble fraction of *Cnidium officinale* MAKINO (COEA) decreased nitric oxide (NO) production in the lipopolysaccharide (LPS)-stimulated BV-2 and primary microglia and suppressed expression of inducible nitric oxide synthase (iNOS) in BV-2 cells with the same pattern of NO production. In addition, we showed that excessive NO production played an important role in neuronal cell death in LPS-treated rat hippocampal slice cultures. Our data suggest that the COEA inhibits neuronal cell death by reduction of excessive NO production in LPS-treated rat hippocampal slice cultures. The ethyl acetate-soluble fraction of *C. officinale* reduced propidium iodide uptake and NO production in cultured media at the same time.

Keywords: *Cnidium officinale* MAKINO, nitric oxide, hippocampal slice

Microglia cells can be considered as resident brain macrophages. They are diffusely present in the brain to protect it from infective, toxic, ischemic, and other type of insults, but may contribute to the establishment of brain damage when the magnitude of the microglia reaction surpasses a critical threshold level (1). Over-activated microglia cells produce excessive inflammatory substances such as nitric oxide (NO), various cytokines, and prostaglandins (2). Recently, there are many evidences that over-activated microglia cells are intensely involved in neuro-degenerative disease such as Alzheimer’s disease, Parkinson’s disease, and ischemia (3). Among numerous substances, NO produced by expression of inducible nitric oxide synthase (iNOS) is an important mediator of inflammation and neuronal cells death (4). The chemical property of NO is that it rapidly and spontaneously reacts with a superoxide anion ($O_2^-$) to form a peroxynitrite anion (ONOO⁻) and its conjugated acid, peroxynitrous acid (ONOOH), which is more toxic to a biological system than $O_2^-$ or NO alone (5). NO is biosynthesized by the oxidation of L-arginine to NO and citrulline via an intermediate $NO^\cdot$-hydroxy-L-arginine. The overall reaction is a five-electron oxidation of L-arginine using nicotinamide adenine dinucleotide phosphate (NADPH) as the source of electrons. These series of processes are catalyzed by the family of NOS isoenzymes: neuronal NOS (nNOS), endothelial NOS (eNOS), and iNOS. Generally, eNOS and nNOS isoforms are constitutively expressed and produce NO continuously and independently of intracellular Ca$^{2+}$ concentration is elevated and calmodulin is bound to the enzyme. In contrast, iNOS is not normally expressed but can be induced by selected immunological stimuli such as lipopolysaccharide (LPS), interferon-$\gamma$, tumor necrosis factor-$\alpha$, and interleukin-1$\beta$, and this enzyme produces NO continuously and independently of intracellular Ca$^{2+}$ (6).

In Korea and Japan, the root of *Cnidium officinale* MAKINO has been used as a medical plant for a long time. It is a perennial plant of the family Umbelliferae and is one of the important traditional medicines to counteract diverse diseases; it is particularly used for the treatment of a female genital inflammatory disease such as menstrual irregularity. Recently, there has been
considerable interest in this medicinal plant because of its anti-inflammatory and analgesic properties (7).

In the present work, we investigated inhibitory effect of the ethyl acetate-soluble fraction of *C. officinale* (COEA) on LPS-stimulated NO production in BV-2 and primary microglia cells and on expression of iNOS enzyme. In addition, the neuroprotective effect of this fraction was evaluated in LPS-treated organotypic rat hippocampal slice cultures.

The dried rhizome of *C. officinale* (10 kg) was extracted with 85% methanol solution using sonication and evaporated. The methanol extract (1260 g) was fractioned into ethyl acetate (169 g, yield 13.4%) (COEA) and H2O (951 g, yield 75.5%) (COW)-soluble fractions.

BV-2 cells, a murine microglia cell line from Dr. Choi EJ in Korea University, were maintained in 10 ml of Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco BRL, Renfrewshire, UK) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL) and 1% antibiotic-antimylocotic (A.A.) (Gibco BRL). Primary microglia cells were isolated from whole brains of neonatal rats (1–2-day-old). The brain tissues were chopped and dissociated by trypsinization and mechanical force. After centrifugation at 1,200 rpm, the precipitates were plated in a 75-cm² T-flask with 10 ml DMEM supplemented with 10% FBS. Primary cells were maintained for two weeks. Media were changed every five days. Microglia cells were separated by shaking at 200 rpm for 2 h and filtering with a nylon mesh (20 μm-size).

BV-2 and primary microglia cells were seeded into a 96-well plate (3 × 10⁴/well) with 100 μl DMEM supplemented 10% FBS for NO production. COEA was dissolved in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA). The final concentration of DMSO never exceeded 0.01%, so that it would not interfere with the used assay. NO production was measured by the Griess reaction (8). After treatment with LPS (100 ng/ml) in the absence or in the presence of COEA (1, 5, and 10 μg/ml), cultures were incubated for 24 h in for the BV-2 cells and 48 h for the primary microglia cells. The supernatant (50 μl) was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylethylendiamine dihydrochloride in 5% phosphoric acid). After 10 min incubation at room temperature, absorbance at 540 nm was measured by using a microplate reader (Emax; Molecular Device, Menlo Park, CA, USA). Sodium nitrite was used as the standard to calculate the concentration of NO₂⁻.

Cell viability was evaluated using the MTT assay. After removing the medium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (0.5 mg/ml) was added to each well followed by incubation at 37°C for 2 h. The supernatant was removed and DMSO was added. After the crystals were completely dissolved, absorbance at 570 nm was measured by using a microplate reader.

The suppressive effect of COEA on iNOS enzyme expression was examined by Western blot analysis. Cells were harvested and lysed in triple lysis buffer (50 mM Tris-Cl pH 8.0, 0.1% SDS, 150 mM NaCl, 1% NP-40, 0.02% sodium amide, 0.5% sodium deoxycholate, 1 mM PMSF, 1 μg aprotinin). The protein concentration was determined by using a protein assay kit (Bio-Rad, Hercules, CA, USA). An equal amount of protein was separated by 8% SDS-polyacrylamide gel electrophoresis and transferred to a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was blocked with 5% skim milk and incubated sequentially with iNOS primary (Transduction Laboratories, San Diego, CA, USA) and horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech) followed by ECL detection (Amersham Pharmacia Biotech).

Hippocampal slices cultures were performed by Norberg’s protocol (9). The hippocampi were dissected from 9-day-old Sprague-Dawley rats and cut into 400-μm-thickness slices using a microslicer (Mickle Laboratory Engineering Co., Surrey, UK). Six slices were placed on polytetrafluoroethylene insert membranes (Millicell™-CM; Millipore, Bedford, MA, USA), which were inserted into 6-well plates filled with 1.2 ml culture medium composed of 50% minimum Eagle medium (Gibco BRL) 25% horse serum, and 25% Hank’s Balanced Salt Solution (HBSS) (Gibco BRL) supplemented by d-glucose at a final concentration of 25 mM and were maintained for 14 days. After 14 days, the cultures were treated with 10 μg/ml of LPS in the absence or presence of COEA (1, 10, and 20 μg/ml), and the cultures were incubated for 48 h. N-Monomethyl-L-arginine (NMMA, 500 μM) was used as a positive control. Propidium iodide (PI) is a popular compound that only enters cells with damaged membranes and becomes brightly red fluorescent after binding to nucleic acids (10). PI fluorescent imaging analysis was performed with a confocal laser scanning microscope (LSM 510; Carl Zeiss, Goettingen, Germany) at 514 nm. Neuronal cell death of CA1, CA3, and dentate gyrus region was analyzed densitometrically using the NIH Image 1.62 analysis program (National Institute of Health, Bethesda, MD, USA). To investigate the correlation between NO production and neuronal cell death, NO accumulation was determined with each cultured medium by the Griess reaction.

Data were reported as the mean ± S.E.M. of three independent determinations. All experiments were done

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at least three times, each time with three or more independent observations. Statistical analysis was performed by analysis of variance (ANOVA) followed by Duncan's test.

COEA inhibited the NO production in LPS-stimulated BV-2 and primary microglia cells in a dose-dependent manner (Fig. 1). COEA had no toxicity in the BV-2 and primary microglia cells at the experimental concentration (Data not shown). This result indicates that inhibition of NO by COEA is due to not its toxicity but other inhibitory pathways. The mechanisms by which NO production is blocked are 1) scavenging of NO, 2) inhibition of iNOS enzyme activity without affecting the iNOS expression, and 3) suppression of effect of COEA on iNOS induction, Western blot analysis was carried out. COEA suppressed the iNOS induction in LPS-stimulated BV-2 cells in a dose-dependent manner (Fig. 2). This result was in agreement with that of inhibition of NO by COEA. The inhibitory profile of COEA on iNOS induction overlapped with NO production. As for hippocampal slice cultures, we predicted that excessive NO production in the hippocampal slice causes neuronal cell death. There was large amount of cell death in the LPS-treated group, but COEA protected the neuronal cells against death in a dose-dependant manner with inhibition of NO production at the same time (Fig. 3).

Microglia cells have “bright” and “dark” sides at the same time as to their functions. Microglia cells primarily function in host defense and neuroprotection from endogenous or exogenous stimuli. However, when the microglia cells are over-activated, they have generally become aggressive cells that are capable of inducing neuronal cell damage through their secreted products such as NO, oxygen radicals, inflammatory cytokines, glutamate, and so on (11). Among the agents released from activated microglia cells, the roles of NO have not exactly been elucidated. It was reported that NO showed a neuroprotective effect through the S-nitrosylation of cysteine in caspase, which plays an important role in apoptosis of neuronal cells (12). On the other hand, NO was reported to induce neuronal cell death following the damage of DNA and disruption of mitochondrial function. Especially, NO reacts with superoxide anion to yield peroxynitrite, which is a strong oxidative stress agent (13). Whether NO has a neuroprotective action or a neurotoxic one may depend on several factors including the amount of NO production and microenvironment surrounding it. However, it may be clear that excessive NO produced in a pathological condition contributes to neuronal cell death. Therefore,
inhibition of NO production may be an important strategy for treating neuro-degenerative diseases. COEA inhibited LPS-stimulated NO production and iNOS expression. On the basis of these results, COEA would inhibit iNOS-mediated NO production. In addition, COEA prevented neuronal cell death in a dose-dependent manner with reduction of NO production in hippocampal slice culture. These results support that COEA has potential activities as a pharmacological medicine for the treatment of brain diseases.
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References