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Inhibitory Effects of *Coptis japonica* Alkaloids on the LPS-Induced Activation of BV2 Microglial Cells

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Abstract − *Coptis japonica (C. japonica)* is a perennial medicinal plant that has anti-inflammatory activity. *C. japonica* contains numerous biologically active alkaloids including berberine, palmatine, epi-berberine, and coptisine. The most well-known anti-inflammatory principal in *C. japonica* is berberine. For example, berberine has been implicated in the inhibition of iNOS induction by cytokines in microglial cells. However, the efficacies of other alkaloids components on microglial activation were not investigated yet. In this study, we investigated the effects of three alkaloids (palmatine, epi-berberine and coptisine) from *C. japonica* on lipopolysaccharide (LPS)-induced microglial activation. BV2 microglial cells were immunostimulated with LPS and then the production of several inflammatory mediators such as nitric oxide (NO), reactive oxygen species (ROS) and matrix metalloproteinase-9 (MMP-9) were examined as well as the phosphorylation status of Erk1/2 mitogen activated protein kinase (MAPK). Palmatine and to a lesser extent epi-berberine and coptisine, significantly reduced the release of NO, which was mediated by the inhibition of LPS-stimulated mRNA and protein induction of inducible nitric oxide synthase (iNOS) from BV2 microglia. In addition to NO, palmatine inhibited MMP-9 enzymatic activity and mRNA induction by LPS. Palmatine also inhibited the increase in the LPS-induced MMP-9 promoter activity determined by MMP-9 promoter luciferase reporter assay. LPS stimulation increased Erk1/2 phosphorylation in BV2 cells and these alkaloids inhibited the LPS-induced phosphorylation of Erk1/2. The anti-inflammatory effect of palmatine in LPS-stimulated microglia may suggest the potential use of the alkaloids in the modulation of neuroinflammatory responses, which might be important in the pathophysiological events of several neurological diseases including Alzheimer's disease (AD), multiple sclerosis (MS), Parkinson's disease (PD) and stroke.

Keywords: *Coptis japonica*, Microglia, Nitric oxide (NO), Inducible nitric oxide synthase (iNOS), Matrix-metalloproteinase-9 (MMP-9)

INTRODUCTION

Microglia is a principle immune cell in the central nervous system (CNS) (Perry and Gordon, 1988). In response to brain injury, microglia is activated and secreted various cytoactive factors including reactive nitrogen species, reactive oxygen species, and inflammatory cytokines such as tumor necrosis factor-α (TNF-α) (Cunningham *et al*., 2005; Majumdar *et al*., 2007). Excessive production of those cytoactive substances in immunostimulated microglia is generally assumed to be deleterious to neurons and has been suggested in the pathophysiology of several neurodegenerative diseases such as Alzheimer's disease (AD), traumatic brain injury, multiple sclerosis (MS), Parkinson's disease (PD), and more (McGeer *et al*., 2002;

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Mrak and Griffin, 2007), which gave microglia the position of the central regulators of pathogenesis process of various neurodegenerative diseases in terms of the modulation of neuroinflammation (Liaudet *et al*., 2000). Therefore inhibition of microglial activation could be a plausible way to modulate neuronal cell death and injury in the progression of the neurodegenerative diseases.

Lipopolysaccharide (LPS) is a cell wall component of Gram negative bacteria, which has been widely used as an experimental tool to activate microglial cells, which resulted in the production of nitric oxide (NO), an important intra- and intercellular signaling molecule involved in the regulation of diverse physiological and pathological mechanisms in cardiovascular, nervous and immunological systems (Shen *et al*., 2005; Brown, 2007; Weinstein *et al*., 2008). The production of NO is catalyzed by nitric oxide synthases (NOSs): endotherial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS). The iNOS is not present in high concentration in resting cells but could be induced by multiple cytokines as well as by infection. The excessive production of NO by iNOS works as a pro-inflammatory effector of the immune system as well as a cytotoxic agent in various pathogenesis processes (McLaughlin *et al*., 2006; Prestes-Carneiro *et al*., 2007).

In addition to iNOS, LPS-stimulation induces the expression of matrix metalloproteinases (MMPs) that are a family of zinc-dependent endopeptidases critical for the digestion of extracellular matrix (ECM). In the central nervous system, neurons and glia including oligodendrocytes, astrocytes and microglia produce MMPs (Yong *et al*., 1998), which have been implicated in the migration of neural precursors to the site of neurotrauma (Andjelkovic *et al*., 1998), oligodendrocytes-mediated myelination process (Uhm *et al*., 1998; Oh *et al*., 1999) as well as the acceleration of the outgrowth of neurite by digesting chondroitin sulfate proteoglycan that inhibits neurite outgrowth (Zuo *et al*., 1998). MMPs also participate in the pathogenesis process of several CNS diseases such as multiple sclerosis (MS), Alzheimer's disease (AD) and malignant glioma. For example, MMPs have been suggested to be crucial for leukocyte recruitment, blood-brain barrier (BBB) breakdown (Asahi *et al*., 2001), myelin destruction (Gijbels *et al*., 1993) and release of disease-promoting cytokines in inflammatory demyelinating diseases such as MS and Guillain-Barre syndrome. It has been also suggested that MMPs are upregulated in ischemia, which may underlie neural destruction observed in ischemic brain.

C. japonica is an oriental herb with anti-inflammatory characteristics, which has been used in traditional medicine (Otsuka *et al*., 1981). For example, in periodental OC2 and KB cells, treatment of berberine (BR), an alkaloid in *C. japonica* (Fukuda *et al*., 1999), reduced the level of cyclooxygenase-II enzyme and prostaglandin E-2 production (Lee *et al*., 2003). 13-methylberberine and 13-ethylberberine also inhibited NO production in LPS-stimulated macrophages. Moreover, we reported that the neural injury induced by cerebral ischemia was significantly reduced by Hwangryun-Hae-Dok-tang, an oriental medicine containing *C. japonica* as an essential ingredients. In Hwangryun-Hae-Dok-Tang, Hwangryun means *C. japonica*, while Hae-Dok means de-toxification (Hwang *et al*., 2002). Taken together, above results indicate that components of *C. japonica* would be modulators of the microglial activation as well as neuroinflammatory phenotype in various neurological diseases. At present, information about the possible anti-inflammatory role of *C. japonica* alkaloids in brain is scarce except BR. In the present study, we examined whether *C. japonica* alkaloids have an inhibitory effect in activated microglial BV2 cells.

MATERIALS AND METHODS

Materials

All four *C. japonica* compounds used in this study, i.e. palmatine (PL), epi-berberine (EB), coptisine (CP) and BR were dissolved in 0.01% DMSO (Sigma, St. Louis , MO). These compounds were kindly supplied by the National Center for Standardization of Herbal Medicine (Seoul, Korea). The chemical structure of *C. japonica* alkaloids

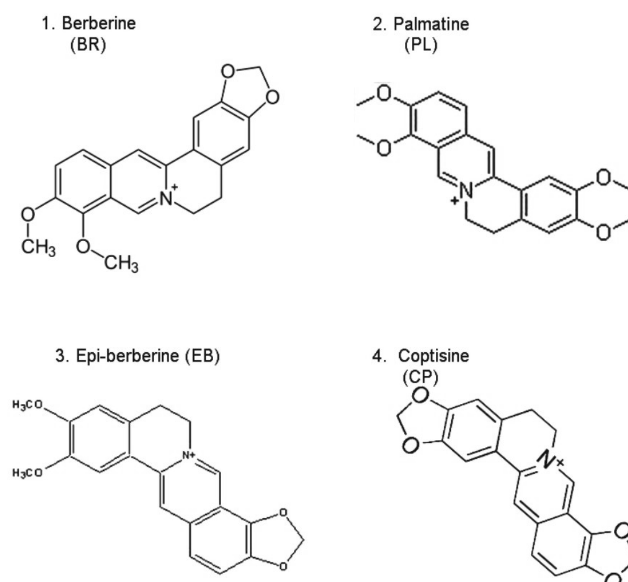


Fig. 1. The Chemical structure of *C. japonica* alkaloids.

was shown in Fig. 1. Lipopolysaccharide (LPS) (026:B6) was purchased from Sigma (St. Louis, MO). Glucose-free DMEM and fetal bovine serum were from Gibco BRL (Grand Island, NY). Reagents for transfection including lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA). Luciferase activity assay kit was purchased from Promega (Madison, WI). All other reagents were obtained from Sigma (St. Louis, MO).

Cell culture and treatment

The microglial BV2 cell line was grown and maintained in DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified incubator under 5% CO_2. For experiments, cells were washed three times with warm DMEM and then added with serum-free medium. Cells were treated with LPS (0.2 μg/ml) alone or in combination with *C. japonica* alkaloids.

Cell viability

Cell viability was determined in BV2 cell line by MTT reduction assay. In brief, cells were added with MTT dye (10 mg/ml in PBS, final concentration of 5 μg/ml) and incubated for 20 min at 37°C in lightproof condition. The reduction of MTT dye in living cell produces formazan compounds. After resolving the formazan compounds with 100% EtOH, the absorbance at 590 nm was measured using a microplate reader (TECAN, Austria).

Measurement of intracellular ROS production

Total ROS production was determined by DCF fluorescence assay as described previously (Choi *et al.*, 2004). BV2 microglial cells were loaded with 2,7-dihydrodichlorofluorescein diacetate (H_2DCF-DA, 5 μg/ml) in PBS for 10 min and then rinsed with the same solution. After 30 min incubation at 37°C in the dark, the fluorescence of DCF was measured at an excitation wavelength of 485 nm and emission wavelength of 535 nm (slit widths 20 and 25 nm, respectively) using a fluorescence microplate reader (TECAN, Austria). H_2DCF-DA diffuses through cell membranes and is subsequently deacetylated by enzymatic reaction of intracellular esterases to the non-fluorescent DCF-H. Oxidants such as $ONOO^-$ effectively convert DCF-H to the highly fluorescent. Fluorescence intensities were corrected for autofluorescence i.e., fluorescence of cells not loaded with H_2DCF-DA.

Determination of NO

NO production from the LPS-stimulated cells was determined by measuring nitrite, a stable oxidation product of NO. In brief, nitrite levels were determined by adding the Griess reagent (mixing equal volumes of 0.1% napthylethylenediamine dihydrochlroride and 1% sulfanilamide in 5% phosphoric acid). After 10 min, the absorbance at 540 nm was determined using an UV spectrophotometer (Beckman DU-650, Fullerton, CA).

Gelatin zymography

Zymography was performed for semi-quantitative analysis of MMPs levels secreted into the culture medium. In brief, samples were mixed with 5 μl of 4×SDS sample buffer (8% w/v SDS, 40% glycerol, 200 mM Tris-HCl, pH 6.8, and 0.02% bromophenol blue) in the absence of a reducing agent and resolved by electrophoresis at 180 V for 1 h on 8% polyacrylamide gel containing 0.1% SDS and gelatin (from porcine skin, Sigma) at a final concentration of 1 mg/ml. Thereafter, gels were washed twice in 2.5% Triton X-100 for 30 min each to remove the SDS and then incubated for 24 h at 37°C in reaction buffer (20 mM Tris-HCl, 166 mM $CaCl_2$, pH 7.6). After staining the gel with 0.1% Coomassie Brilliant Blue R-250, gelatinolytic activities were visualized as a clear band in the uniformly stained background. The molecular weight of the MMPs was estimated by comparing the migration distance of the clear bands with the distance migrated by markers of known molecular weight (Gibco BRL, Grand Island, NY).

Western blot

Cell extracts were prepared with 100 μl of 2 × sample buffer (4% w/v SDS, 20% glycerol, 200 mM DTT, 0.1 M Tris-HCl, pH 6.8, and 0.02% bromophenol blue). The samples were separated by 10% SDS-PAGE gel electrophoresis and electrically transferred to nitrocellulose (NC) membranes. The NC membranes were blocked with 5% Blotto and then incubated at 4°C for overnight with primary antibodies against phospho-Erk1/2 or total Erk2 (Cell Signaling Technology, Beverly, MA, USA) which were diluted 1:3,000 in 5% Blotto (Santa Cruz Biotechnology Inc., Santa Cruz, CA), and anti-Nitric Oxide Synthase II (Chemicon International, Temecula, CA, U.S.A.) diluted 1:10,000 in 5% Blotto. After three times washing with PBS containing 0.2% Tween-20 (PBS-T), the NC membranes were incubated with peroxidase-labeled goat anti-mouse IgG for pERK and peroxidase-labeled goat-anti-rabbit for iNOS or total Erk2 at room temperature for 2 h. After extensive washing with PBS-T, the membranes were developed by enhanced chemiluminescence (Amersham, Buckinghampshire, UK). As loading controls, Western blot was performed using antibodies against β-actin in 1:30,000 dilution (Sigma).

Table. I. Sequences of primers for RT-PCR and expected size of products

	MMP-9	iNOS	GAPDH
Forward Primer	5'-TAAGGTATTCAGTTACTCCTA-CTGGAA-3'	5'-CAAGAGTTTGACCAGAGGA-CC-3'	5'-TCCCTCAAGATTGTCAGCAA-3'
Reverse Primer	5'-CCTCTCTAGCACACATGCAC-TT-3'	5'-TGGAACCACTCGTACTTGG-GA-3'	5'-AGATCCACAACGGATACATT-3'
Expected size of PCR products	324 bp	654 bp	308 bp

Semiquantitative reverse transcription (RT)-PCR

Total RNA was extracted from BV2 microglial cells using Trizol reagent (GibcoBRL, Grand Island, NY) according to the manufacturer's protocol. Reverse transcription was performed for 60 min at 45°C with 1 μg of total RNA using *Maxime* RT PreMix Kit (iNtRON Biotechnology, Seoul) according to the manufacturer's protocol. Oligo $(dT)_{15}$ was used as a primer for this reaction. The samples were then heated at 94°C for 5 min to terminate the reaction. The cDNA obtained from 0.5 μg of total RNA was used as a template for PCR amplification of MMP-9 (accession number NM_031055) , iNOS (accession number U03699) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, accession number M17701) mRNA. Sequences of primers for RT-PCR and the expected size of products were shown in Table I.

PCR mixture contained 1 pmol of each forward and reverse primer and 5 μl of each cDNA and *Maxime* PCR PreMix Kit (i-Taq, iNtRON Biotechnology). For amplification, the following PCR incubation times were used: 94°C for 5 min followed by 28 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and continued to the final extension step for 10 min at 72°C . The amplified PCR products were electrophoresed and analyzed on 1% agarose-gel. For comparison, PCR products for housekeeping gene, GAPDH, were also performed.

Transient transfection and MMP-9 reporter assay

BV2 microglial cells were co-transfected with MMP-9 promoter luciferase reporter plasmid and pCMV-β-galactosidase reporter plasmid using Lipofectamine 2000 reagent according to the manufacturer's protocol as described previously. Next day, BV2 microglial cells were pretreated with *C. japonica* alkaloids (20 μM) 20 min before LPS (0.2 μg/ml) treatment in serum-free DMEM. Cells were harvested and assayed for luciferase and β-galactosidase activities by using the luciferase and β-galactosidase enzyme assay kits (Promega, Madison, WI), respectively. Luciferase activity was normalized with the β-galactosidase activity in the cell lysates.

Statistical analysis

Data are expressed as the mean ± standard error of mean (S.E.M) and analyzed for statistical significance by using one way analysis of variance (ANOVA) followed by Newman-Keuls test as a post hoc test and a p value $<$ 0.05 was considered significant.

RESULTS

Effects of *C. japonica* alkaloids on cell viability of BV2 microglial cells

We first examined whether alkaloids from *C. japonica* affects BV2 cell viability in our culture condition. In a preliminary experiment, we found that most of the alkaloids we used in this study did not show direct cell toxicity up to 50 μM concentration (data are not shown). Thereafter, we used 20 μM alkaloids throughout the study. BV2 microglial cells were stimulated with LPS (0.2 μg/ml) for 24 h, when maximal stimulation of BV2 cells was observed, in the absence or presence of *C. japonica* alkaloids and cytotoxicity was measured by MTT reduction assay. All three alkaloids used in this study, i.e. PL, EB and CP did not show significant cytotoxicity in LPS-stimulated BV2 cells (Fig. 2). In contrast, BR showed significant cell toxicity and was excluded in other experiments.

Effects of *C. japonica* alkaloids on ROS generation in LPS-stimulated microglia

We next determined the effects of *C. japonica* alkaloids on LPS-induced intracellular ROS generation using DCF-DA fluorescence methods (Fig. 3). LPS treatment increased total ROS production by 2.3 fold as compared with control. PL pretreatment reduced ROS generation by 52.1% compared to the LPS-stimulated cells. In addition, EB and CP inhibited intracellular ROS generation mediated by LPS treatment.

Effects of *C. japonica* alkaloids on LPS-stimulated NO production and iNOS mRNA and protein level in BV2 cells

It has been relatively well known that LPS induced iNOS expression and NO production in glial cells, which was confirmed in this study. LPS (0.2 μg/ml) increased NO production in BV2 microglial cells (Fig. 4A). The increase in NO production was preceded by iNOS mRNA and protein

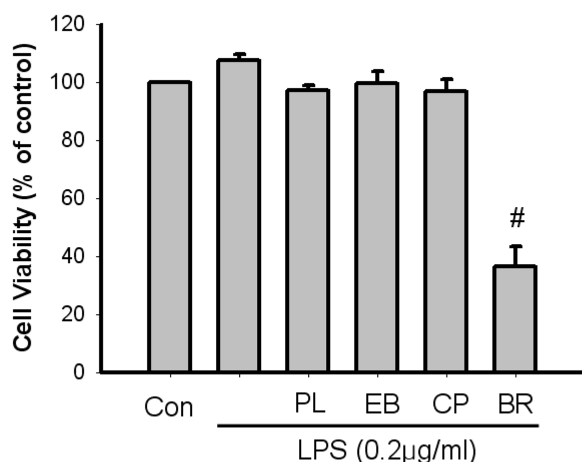


Fig. 2. Effect of *C. japonica* alkaloids on BV2 cell viability. BV2 cells were pretreated with 20 μM alkaloids as indicated, followed by stimulation with LPS (0.2 μg/ml) for 24 hrs. MTT assay was performed as indicated in materials and methods. Values represent mean ± SEM of three independent experiments (# indicates significant decrease compared with control; $^{\#}p < 0.05$ vs. LPS treatment).

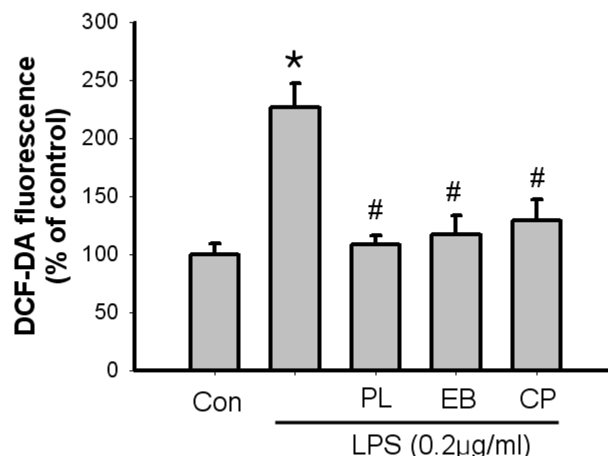
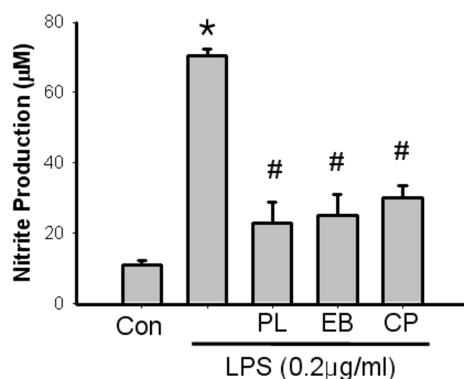
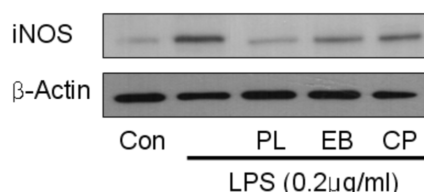


Fig. 3. Inhibitory effect *C. japonica* alkaloids on intracellular ROS production. BV2 cells were pretreated with 20 μM alkaloids as indicated, followed by stimulation with LPS (0.2 μg/ml) for 18 hrs. Cells were incubated with 5 μM DCF for 0.5 hrs and washed to remove the fluorescent dye. Fluorescence was measured using a fluorescence microplate reader and expressed as percentage increase compared with that of the control. Values represent mean ± SEM of three independent experiments (* indicates significant increase compared with control; # indicates significant decrease compared with LPS alone treated group, $p < 0.05$).





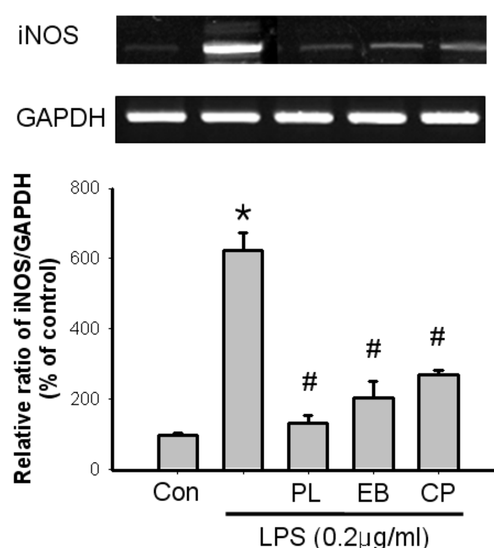


Fig. 4. Inhibition of NO (A), iNOS mRNA (B), and protein (C) expression by *C. japonica* alkaloids in LPS-stimulated BV2 cells. BV2 cells were pretreated with the alkaloids as indicated, followed by immunostimulation with LPS (0.2 μg/ml) for 18 hrs. (A) Cell-conditioned supernatants were collected and NO production was measured using Griess reagent. (B) Levels of iNOS mRNA were assayed by RT-PCR analysis. The graph represents the ratio of iNOS/GAPDH mRNA from three independent experiments. (C) Levels of iNOS and β-actin proteins were assayed by Western blot. Values correspond to the mean ± SEM of three independent experiments (* indicates significant increase compared with control; # indicates significant decrease compared with LPS alone treated group, $p < 0.05$).

expression (Fig. 4B, C). The treatment of PL, EB and CP inhibited LPS-stimulated NO production (Fig. 4A). PL reduced LPS-stimulated nitrite production (23.0 ± 5.9 μM, versus 70.4 ± 1.9), and EB and CP gave similar results (25.0 ± 5.9 μM and 30.2 ± 3.3 μM, respectively, versus 70.4 ± 1.9). Consistent with above results, co-treatment of *C. japonica* alkaloids prevented LPS-induced up-regulation of iNOS mRNA as well as proteins (Fig. 4B, C). LPS stimulated the expression of iNOS in BV2 cells by 6.2 fold compared with control, and PL significantly inhibited both iNOS mRNA and protein expression. In addition to PL, EB and CP had inhibitory effect on LPS-induced up-regulation of iNOS mRNA and proteins.

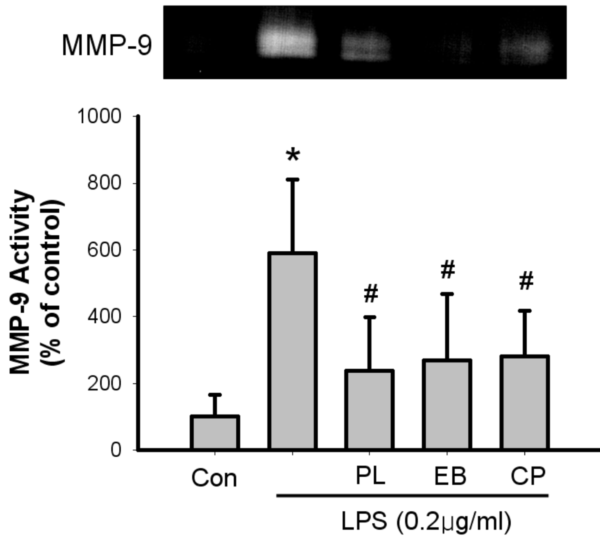


Fig. 5. *C. japonica* components reduced LPS-induced MMP-9 activity in BV2 Microglia. MMP9 activity was measured from cell-conditioned supernatants by zymography as described in materials and methods. Values correspond to the mean ± SEM of three independent experiments (* indicates significant increase compared with control; # indicates significant decrease compared with LPS alone treated group, $p<0.05$).

Effects of *C. japonica* alkaloids on LPS-stimulated MMP-9 activity

Next, we examined whether *C. japonica* alkaloids affect MMP-9 activity, which is another important cytoactive molecules up-regulated in neuroinflammatory condition. As shown in Fig. 5, we measured MMP-9 activity using gelatin zymography. LPS treatment augmented MMP-9 activity in BV2 microglial cells and co-treatment of *C. japonica* components decreased the up-regulation of MMP-9 activity. Consistent with the zymography results, mRNA encoding MMP-9 was up-regulated by LPS treatment in BV2 microglia and co-treatment of 20 μM PL, EB, and CP inhibited LPS-induced up-regulation of MMP-9 mRNA (Fig. 6A). To verify the effect of alkaloids on MMP-9 expression, we per-

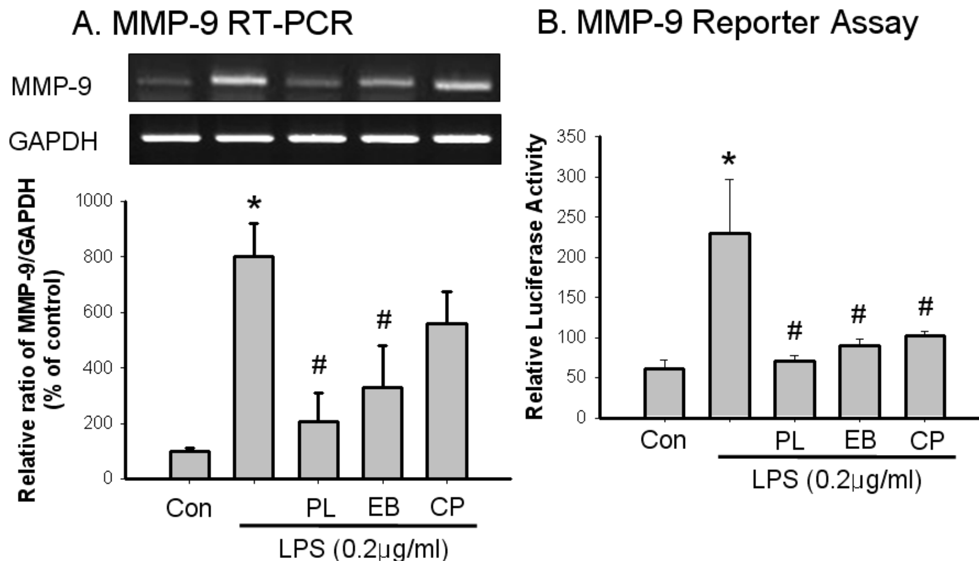


Fig. 6. MMP-9 expression by LPS-stimulated BV2 was inhibited by *C. japonica* alkaloids. (B) BV2 cells were pretreated with *C. japonica* alkaloids (20 μM), followed by LPS (0.2 μg/ml) for 24 hrs and levels of MMP-9 and GAPDH mRNA were assayed by RT-PCR analysis. (B) BV2 cells transfected with MMP9 promoter reporter construct were pretreated with *C. japonica* alkaloids (20 μM), followed by LPS (0.2 μg/ml) for 24 hrs. The luciferase activity was analyzed as described in the materials and methods. Values represent the mean ± SEM of three independent experiments. (* indicates significant increase compared with control; # indicates significant decrease compared with LPS alone treated group, $p<0.05$).

formed MMP-9 promoter luciferase reporter analysis. PL prevented the LPS-induced increase in MMP-9 promoter activity (Fig. 6B), which is consistent with zymography experiments. EB and CP also inhibited MMP-9 promoter activity, although the extent of inhibition was smaller than PL.

Inhibition of Erk1/2 phosphorylation by *C. japonica* alkaloids.

Several researchers including us reported the essential role of MAPK pathway in the regulation of MMP-9 and iNOS expression in immunostimulated glial cells (Fiebich *et al*., 2004; Koistinaho *et al*., 2005; Shin *et al*., 2007). Therefore, we investigated whether *C. japonica* alkaloids affect ERK1/2 phosphorylation by Western blot. 0.2 μg/ml LPS treatment increased the level of phosphorylated ERK1/2 levels compared with control (2.3 fold, Fig. 7). Co-treatment of PL reduced pERK1/2 level to control levels. Albeit smaller in extent, treatment of EB and CP also decreased pERK1/2 level induced by LPS stimulation. These results suggest that the observed anti-inflammatory effects of *C. japonica* components on ROS, NO and MMP-9 may be mediated by the inhibition of ERK1/2 phosphorylation.

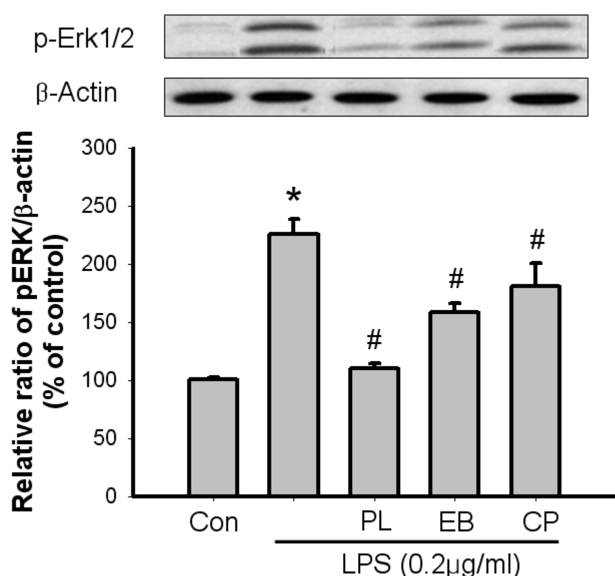


Fig. 7. Inhibition of LPS-induced ERK1/2 phosphorylation by *C. japonica* alkaloids. BV2 microglial cells were pretreated with *C. japonica* alkaloids (20 μM) 20 min before LPS treatment (0.2 μg/ml). Cells were harvested 30 min after LPS treatment and then the level of p-ERK were detected by Western blot. β-actin was used as loading control. Values represent the mean ± SEM of p-Erk/β-actin ratio from three independent experiments (* indicates significant increase compared with control; # indicates significant decrease compared with LPS alone treated group, $p < 0.05$).

DISSCUSSION

In this study, *C. japonica* alkaloids were used to investigate their efficacy under LPS stimulated BV2 cells. *C. japonica* is a perennial medicinal plant that has anti-inflammatory activity. *C. japonica* contains numerous biologically active alkaloids including BR, PL, EB, and CP (Park *et al*., 2003). And among others, *C. japonica* exhibit anti-inflammatory, sedative, antidotal, and anti-tumor properties, many of which were attributed to BR (Yesilada and Kupeli, 2002). Other alkaloids like PL, EB had inhibitory effect against edema on mouse ear induced by application of 12-O-tetradecanoylphorbol-13-acetate (Yasukawa *et al*., 1991). In addition, CP showed strong anti-photo-oxidative activity in the chlorophyll-sensitized photo-oxidation of linoleic acid (Kase *et al*., 1999).

Previously, *C. japonica* extract and its major component, BR, were reported to have NO-scavenging activity (Yokozawa *et al*., 2000). NO is synthesized from L-arginine by NOS with NADPH and oxygen. The constitutive NOS such as eNOS and nNOS synthesizes small amount of NO sufficient for wide biological function in regulating physiological processes. However, high concentrations of NO during inflammation and pathological situation have been shown to participate in multiple stages of diseases. The massive generation of NO following an inflammatory stimulation is attributed by inducible NOS (iNOS). Thus, inhibition of NO generation in inflammatory condition can be achieved by reducing iNOS induction. The inhibitory effects of PL, EB and CP on iNOS induction suggest that these alkaloids are useful to down-regulate NO production in neuroinflammatory condition.

In this study, LPS treatment significantly increased MMP-9 level and activity in BV2 cells. Many investigators reported that activated microglia induced the expression and activity of MMPs (Rosenberg, 2002). In this study, it was revealed that alkaloids such as PL, EB and CP have regulatory roles on MMP-9 activity in activated BV2 microglial cells. All the alkaloids used in this study significantly reduced MMP -9 activity as well as the expression level. MMP-9 activity is related to many neurodegeneration diseases, for example, MS (Cossins *et al*., 1997), AD (Deb and Gottschall, 1996), PD (Safciuc *et al*., 2007), and stroke (Bozdagi *et al*., 2007).

The decrease in MMP activity was attributed to the reduced MMP expression. Up-regulation of MMP-9 mRNA level by LPS was inhibited by 20 μM PL, which seems to

be regulated by transcriptional control (Fig. 6). To verify the transcriptional regulatory mechanism, we used MMP-9 reporter assay, which provided additional support for the transcriptional regulation of MMP-9 mRNA level by *C. japonica* alkaloids. Actually, the transcriptional control of MMP-9 expression has been reported in a wide variety of cells with different compounds. For instance, polyphenolic compound from *Tristaniopsis calobuxus* regulated MMP-9 expression level by interacting with promoter region and minocycline inhibited MMP-9 activity on human aortic smooth muscle cell through MMP-9 mRNA reduction (Bellosta *et al*., 2003; Yao *et al*., 2004). These results indicate that appropriate use of alkaloids like PL, EB, and CP might be helpful to treat and prevent neuroinflammatory diseases by the down-regulation of MMP-9.

To elicit inflammatory reactions in immune mediator cells like microglia, intracellular signaling pathways linked to transcription factors are activated. These include activation of protein kinases such as protein kinase C, phosphoinositide 3-kinase, protein tyrosine kinase, and MAPKs (Erk1/2, p38, SAPK/JNK). Erk1/2 activity, which plays essential roles in the regulation of NO and MMP-9 production, was inhibited by *C. japonica* alkaloids (Fig. 7). How those alkaloids prevent the activation of Erk1/2 is not clear yet. Considering the role of ROS in the activation of Erk1/2, one plausible explanation is that the inhibition of ROS production from BV2 microglial cells by *C. japonica* alkaloids mediates the observed inhibitory effects on pErk1/2.

Microglia are regarded as primary cell types responsible for inflammation-related neurotoxicity (Liu and Hong, 2003). Like peripheral immune cells, stimulated microglia release neurotoxic mediators, pro-inflammatory cytokines, and free radicals like NO after activation with LPS or interferon-γ *in vitro*. These up-regulated cytokines and mediators such as prostaglandin E2, interleukin-1,6, NO, superoxide anion, and matrix metalloproteinases (MMPs) are thought to be major cause of neuronal injuries and progression of neurodegenerative diseases (Minghetti and Levi, 1998). Activated microglia are commonly observed in injured brain and related with progression of neurodegenerative diseases. The anti-neuroinflammatory effect of *C. japonica* alkaloids may provide additional measure to control microglial activation and neuro-inflammation and the effects of them in the control of neurodegenerative diseases such as stroke, MS, AD, and PD, should be investigated further in the future study.

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