OPEM
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Oriental Pharmacy and Experimental Medicine 2010 **10(3)**, 214-221
DOI 10.3742/OPEM.2010.10.3.214

Clematis chinensis suppresses lipopolysaccharide-induced expressions of inducible nitric oxide synthase and cyclooxygenase-2 in mouse BV2 microglial cells

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Received for publication August 24, 2010; accepted September 7, 2010

SUMMARY

Clematis chinensis is the root of Clematis chinensis OSBECK and is classified in Ranunculaceae. *Clematis chinensis* is a traditional medicinal herb possesses analgesic, diuretic, anti-tumorigenic, and anti-inflammatory effects. In this study, the effect of aqueous extract of *Clematis chinensis* against lipopolysaccharide-induced inflammation was investigated in mouse BV2 microglial cells. The aqueous extract of *Clematis chinensis* at the respective concentration was treated one hour before the lipopolysaccharide treatment in mouse BV2 microglial cells. From the present results, pre-treatment with the aqueous extract of *Clematis chinensis* suppressed prostaglandin E_2 synthesis and nitric oxide production by inhibiting on the lipopolysaccharide-stimulated cyclooxygenase-2 and inducible nitric oxide synthase expressions in mouse BV2 microglial cells. The present study suggests that *Clematis chinensis* may offer a valuable mean of therapy for brain inflammatory diseases.

Key words: *Clematis chinensis*; Lipopolysaccharide; Cyclooxygenase-2; Inducible nitric oxide synthase; Prostaglandin E_2; Nitric oxide

INTRODUCTION

Prostaglandins (PGs) are key inflammatory mediators that are converted from arachidonic acid by cyclooxygenase (COX). There are two isoforms of COX: COX-1 is constitutively expressed in nearly all tissues and provides PGs to maintain physiological functions such as cytoprotection of the stomach and regulation of renal blood flow (Vane and Botting, 1998; Vane *et al*., 1998). In contrast, COX-2 is inducible in the immune cells such as macrophages and synoviocytes in response to infection, injury or other stresses, and COX-2 produces excessive amount of PGs that sensitize nocieptors and induce inflammatory states (Marletta, 1993; Duval *et al*., 1996). Prostaglandin E_2 (PGE_2) is overproduced at sites of inflammation as a result of the activation of inducible COX-2 (Fu *et al*., 1990; Picot *et al*., 1994).

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Nitric oxide (NO) is a messenger and effector molecule in a variety of tissues (Palmer *et al*., 1988; Lowenstein *et al*., 1996). It has been identified as a neurotransmitter in the central nervous system and as a potent vasodilator in physiological conditions (Hibbs *et al*., 1987; Moncada *et al*., 1991). However, excessive NO production by activated inflammatory cells causes cytotoxic effect. It is well known that NO is also involved in the pathogenesis of acute and chronic inflammatory conditions. The production of NO is regulated by intracellular NO synthases (NOS), and three types of NOS have been identified: endothelial NOS (eNOS), neural NOS (nNOS), and inducible NOS (iNOS). Of these, iNOS is induced by several stimuli including bacterial lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). iNOS is present in macrophages, smooth muscle cells, and hepatocytes, and it triggers several deleterious cellular responses that induces inflammation, sepsis, and stroke (Nathan, 1992; Marletta, 1993; Duval *et al*., 1996).

LPS is a highly conserved outer membrane component of gram-negative bacteria, and LPS triggers many biological responses such as fever, septic shock, and even death (Morrison and Ryan, 1987). Murine and human macrophages exhibit a particularly vigorous response to endotoxin, and produce a variety of inflammatory modulators such as NO, interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), IL-6, and PGs.

Clematis chinensis is the root of Clematis chinensis OSBECK and is classified in Ranunculaceae. *Clematis chinensis* is a traditional medicinal herb and possesses analgesic, diuretic, anti-tumorigenic, and anti-inflammatory effects. The ethanolic extract of *Clematis mandshurica* was found to significantly block the production of the pro-inflammatory mediators (Park *et al.,* 2006), and *Clematis vitalba* dried aerial part is effective in the treatment of inflammatory diseases (Yesilada and Kupeli, 2007). Anti-inflammatory effect of *Clematis crassifolia* leaves by inhibition the iNOS induction in LPS-activated murine macrophage RAW 264.7 cells was also reported (Lee *et al.,* 2008). However, the mechanism of anti-inflammatory and analgesic actions of the *Clematis chinensis* has not been clarified yet. In the present study, we investigated the effect of the aqueous extract of *Clematis chinensis* against LPS-stimulated expressions of COX-1, COX-2, and iNOS in mouse BV2 microglial cells by using reverse transcription-polymerase chain reaction (RT-PCR), Western blot analysis, PGE_2 immunoassay, and NO detection in mouse BV2 microglial cells.

MATERIALS AND METHODS

Cell culture

Mouse BV2 microglial cells were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL) at 37 °C in 5% CO_2-95% O_2 in a humidified cell incubator.

Preparation of aqueous extract of *Clematis chinensis*

To obtain the aqueous extract of *Clematis chinensis,* 50 g of *Clematis chinensis* was added to 2 l distilled water, and extraction was performed by heating at 90 °C, concentrating with a rotary evaporator, and lyophilization. The resulting powder, weighing 7 g, was dissolved in saline solution (yield rate, 14%) and filtered through a 0.45 μm syringe before use.

The aqueous extract of *Clematis chinensis* at the respective concentration was treated one hour before the LPS treatment in mouse BV2 microglial cells.

RNA isolation and RT-PCR

To identify expressions of COX-1, COX-2, and iNOS mRNA, RT-PCR was performed. The total RNA was isolated from BV2 microglial cells using RNAzol^{TM}B (TEL-TEST, Friendswood, TX, USA). Two μg of RNA and 2 μl of random hexamers (Promega, Madison, WI, USA) were added together, and the mixture was heated at 65 °C for 10 min. One μl of AMV reverse transcriptase (Promega), 5 μl

of 10 mM dNTP (Promega), 1 μl of RNasin (Promega), and 5 μl of 10 × AMV RT buffer (Promega) were then added to the mixture, and the final volume was brought up to 50 μl with diethyl pyrocarbonate (DEPC)-treated water. The reaction mixture was then incubated at 42 °C for 1 h.

PCR amplification was performed in a reaction volume of 40 μl containing 1 μl of the appropriate cDNA, 1 μl of each set of primers at a concentration of 10 pM, 4 μl of 10 × RT buffer, 1 μl of 2.5 mM dNTP, and 2 units of *Taq* DNA polymerase (TaKaRa, Shiga, Japan). For mouse COX-1, the primer sequences were 5′-AGTGCGGTCCAACCTTATCC-3′ (a 20-mer sense oligonucleotide) and 5′-CCGCAGGTGATACTGTCGTT-3′ (a 20-mer anti-sense oligonucleotide). For mouse COX-2, the primer sequences were 5′-TGCATGTGGCTGTGG-ATGTCATCAA-3′ (a 25-mer sense oligonucleotide) and 5′-CACTAAGACAGACCCGTCATCTCCA-3′ (a 25-mer anti-sense oligonucleotide). For mouse iNOS, the primer sequences were 5′-GTGTTCCA-CCAGGAGATGTTG-3′ (a 21-mer sense oligonucleotide) and 5′-CTCCTGCCCACTGAGTTCGTC-3′ (a 21-mer anti-sense oligonucleotide). For cyclophilin, the internal control used in this study, the primer sequences were 5'-ACCCCACCGTGTTCTTCGAC-3' (a 20-mer sense oligonucleotide starting at position 52) and 5′-CATTTGCCATGGACAAGATG-3′ (a 20-mer anti-sense oligonucleotide starting at position 332). The expected size of the PCR product was 382 bp for COX-1, 583 bp for COX-2, 500 bp for iNOS, and 299 bp for cyclophilin.

For COX-1, COX-2 and iNOS, the PCR procedure was carried out using a GeneAmp 9600 PCR system (Perkin Elmer, Norwalk, CT, USA) under the following conditions: an initial denaturation at 94°C for 5 min, followed by 35 amplification cycles, each consisting of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, with an additional extension step at the end of the procedure at 72°C for 5 min. For cyclophilin, the PCR procedure was carried out under identical conditions except that 25 amplification cycles were executed. The final amount of RT-PCR product for each of the mRNA species was calculated densitometrically using Molecular Analyst^{TM} version 1.4.1 (Bio-Rad, Hercules, CA, USA).

Western blot analysis

Mouse BV2 microglial cells were lysed in a cell lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% deoxycholic acid, 1% NP40, 0.1% sodium dodecyl sulfate (SDS), 1 mM PMSF, and 100 μg was separated on SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane (Schleicher and Schuell GmbH, Dassel, Germany). Goat COX-1 antibody, goat COX-2 antibody (1:1000; Santa Cruz Biotech, Santa Cruz, CA, USA), and rabbit iNOS antibody (1:500; Santa Cruz Biotech) were used as primary antibodies. Horseradish peroxidase-conjugated anti-goat antibody (1:2000; Santa Cruz Biotech) for COX-1, COX-2, and anti-rabbit antibody (1:1000; Santa Cruz Biotech) for iNOS were used as secondary antibodies. Band detection was performed using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech GmbH, Freiburg, Germany).

Measurement of PGE_2 synthesis

In order to determine the effect of the aqueous extract of *Clematis chinensis* on PGE_2 synthesis, assessment of PGE_2 synthesis was performed using a commercially available PGE_2 competitive enzyme immunoassay kit (Amersham Pharmacia Biotech. Inc., Piscataway, NJ, USA). Supernatants (100 μl) from culture medium and standards were put into different wells on the goat anti-mouse IgG-coated microtiter plate provided in the kit. Mouse anti-PGE_2 antibody and peroxidase-conjugated PGE_2 were added to each well, and the plated was incubated at room temperature with shaking for 1 h. The wells were drained and washed, and 3,3′,5,5′-tetramethylbenzidine/hydrogen peroxide solution was added. The plate was incubated at room temperature with shaking, and the reaction was stopped after 30 min through the addition of

H_2SO_4. The absorbance of the content of each well was then measured at 405 nm.

Measurement of NO production

In order to determine the effect of aqueous extract of *Clematis chinensis* on NO production, the amount of nitrite in the supernatant was measured using a commercially available NO detection kit (iNtRON, INC., Seoul, Korea). After collection of 100 μl of supernatant, 50 μl of N1 buffer was added to each well, and the plate was incubated at room temperature for 10 min, 50 μl of N2 buffer was then added, and the plate was incubated at room temperature for 10 min. The absorbance of the content of each well was measured at 540 nm. The nitrite concentration was calculated from a nitrite standard curve.

Statistical analysis

The results are expressed as the mean ± standard error of the mean (S.E.M.). Data were analyzed by oneway analysis of variance (ANOVA) followed by Duncan's *post-hoc* test using SPSS (Version 10.1). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Effect of *Clematis chinensis* on mRNA expressions of COX-1, COX-2, and iNOS

RT-PCR analysis of the mRNA levels of COX-1 (382 bp), COX-2 (583 bp), and iNOS (500 bp) was performed in order to provide the relative level of expressions of these genes. In the present study, the mRNA levels of COX-1, COX-2, and iNOS in the control cells were set as 1.00.

The level of COX-1 mRNA following a treatment with 2 μg/ml LPS for 24 h was 1.09 ± 0.07. The level of COX-1 mRNA in the cells pre-treated with aqueous extract of *Clematis chinensis* at concentrations of 1 μg/ml, 10 μg/ml, and 50 μM acetylsalicylic acid (ASA) one hour before LPS exposure was 0.82 ± 0.04, 0.89 ± 0.03, and 0.58 ± 0.03, respectively.

The level of COX-2 mRNA following a treatment with 2 μg/ml LPS for 24 h was markedly increased to 10.17 ± 1.11. The level of COX-2 mRNA was decreased to 3.02 ± 0.61, 3.72 ± 0.62, and 2.82 ± 0.47 in the cells pre-treated with aqueous extract of *Clematis chinensis* at concentrations of 1 μg/ml, 10 μg/ml, and 50 μM ASA, respectively one hour before LPS exposure.

The level of iNOS mRNA following a treatment with 2 μg/ml LPS for 24 h was markedly increased to 8.89 ± 0.97. The level of iNOS mRNA was decreased to 3.21 ± 0.20, 4.31 ± 0.38, and 1.11 ± 0.12 in the cells pre-treated with aqueous extract of *Clematis chinensis* at concentrations of 1 μg/ml, 10 μg/ml, and 50 μM ASA, respectively one hour

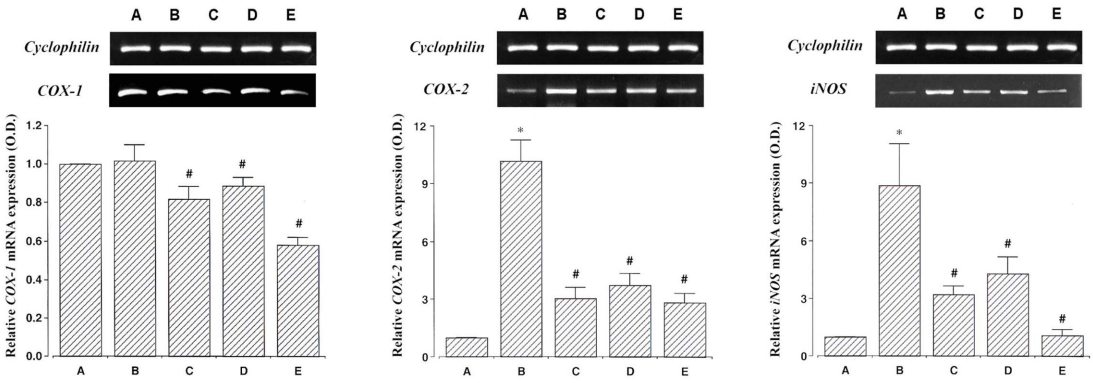

Fig. 1. RT-PCR analysis of the mRNA levels of COX-1, COX-2, and iNOS. Cyclophilin mRNA was used as the internal control. (A) Control, (B) LPS-treated group, (C) LPS- and 1 μg/ml *Clematis chinensis*-treated group, (D) LPS- and 10 μg/ml *Clematis chinensis*-treated group, (E) LPS- and 50 μM ASA-treated group. *represents $P < 0.05$ compared to the control. #represents $P < 0.05$ compared to the LPS-treated group.

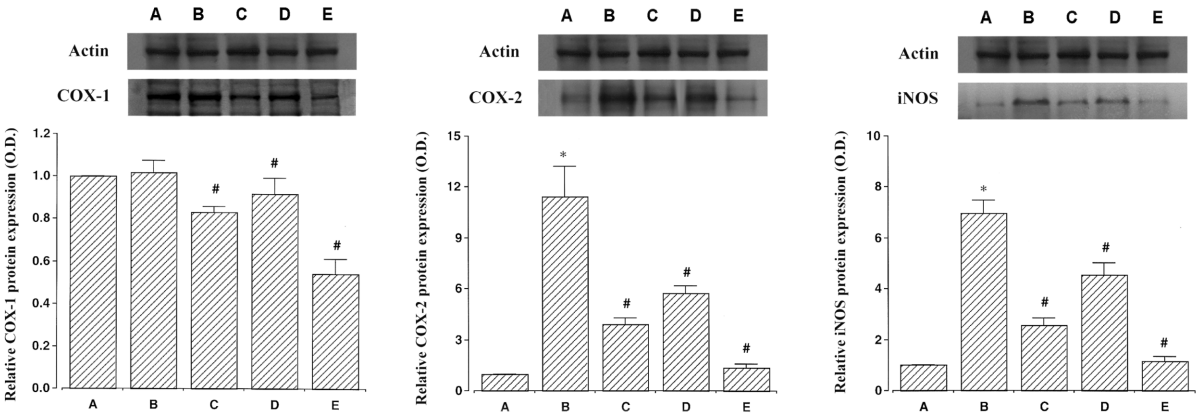

Fig. 2. Results of Western blot analysis of the protein levels of COX-1, COX-2, and iNOS. (A) Control, (B) LPS-treated group, (C) LPS- and 1 μg/ml *Clematis chinensis*-treated group, (D) LPS- and 10 μg/ml *Clematis chinensis*-treated group, (E) LPS- and 50 μM ASA-treated group. *represents $P < 0.05$ compared to the control. #represents $P < 0.05$ compared to the LPS-treated group.

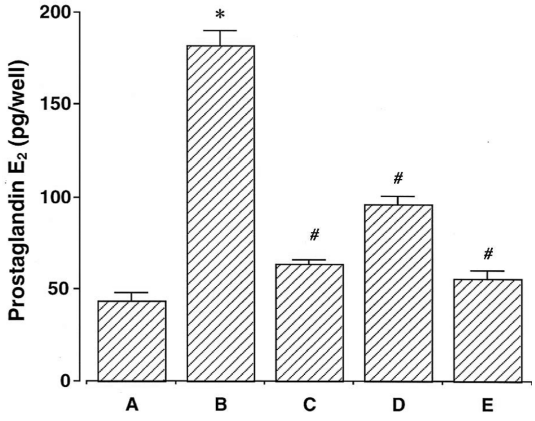

Fig. 3. Measurement of PGE_2 synthesis in BV2 cells. (A) Control, (B) LPS-treated group, (C) LPS- and 1 μg/ml *Clematis chinensis*-treated group, (D) LPS- and 10 μg/ml *Clematis chinensis*-treated group, (E) LPS- and 50 μM ASA-treated group. *represents $P < 0.05$ compared to the control. #represents $P < 0.05$ compared to the LPS-treated group.

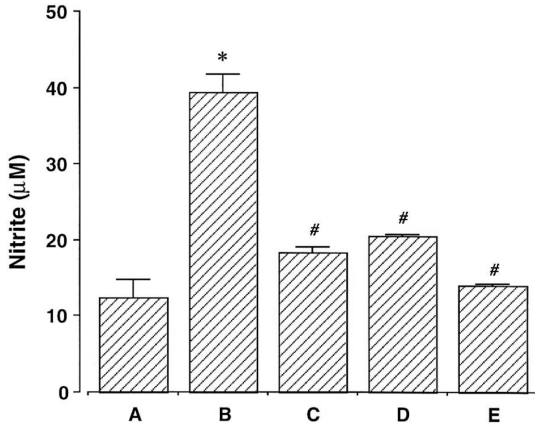

Fig. 4. Measurement of NO production in BV2 cells. (A) Control, (B) LPS-treated group, (C) LPS- and 1 μg/ml *Clematis chinensis*-treated group, (D) LPS- and 10 μg/ml *Clematis chinensis*-treated group, (E) LPS- and 50 μM ASA-treated group. *represents $P < 0.05$ compared to the control. #represents $P < 0.05$ compared to the LPS-treated group.

before LPS exposure.

In the present study, treatment with LPS did not enhance the expression of COX-1 mRNA. Pre-treatment with aqueous extract of *Clematis chinensis* slightly decreased COX-1 mRNA expression. Treatment with LPS significantly increased the levels of COX-2 mRNA and iNOS mRNA expressions. Pre-treatment with aqueous extract of *Clematis chinensis* and ASA decreased the LPS-induced the expressions of COX-2 mRNA and iNOS mRNA.

Effect of *Clematis chinensis* on protein expressions of COX-1, COX-2, and iNOS

Western blot analysis of protein levels of COX-1 (72 kDa), COX-2 (74 kDa), and iNOS (130 kDa) was performed in order to provide the relative level of expressions of these proteins. In the present study, the protein levels of COX-1, COX-2, and iNOS in

the control cells were set as 1.00.

The level of COX-1 protein following a treatment with 2 μg/ml LPS for 24 h was 1.02 ± 0.05 The level of COX-1 protein in the cells pre-treated with aqueous extract of *Clematis chinensis* at concentrations of 1 μg/ml, 10 μg/ml, and 50 μM ASA one hour before LPS exposure was 0.83 ± 0.03, 0.92 ± 0.07, and 0.54 ± 0.07, respectively.

The level of COX-2 protein following a treatment with 2 μg/ml LPS for 24 h was markedly increased to 11.45 ± 1.77. The level of COX-2 protein was decreased to 3.92 ± 0.37, 5.72 ± 0.48, and 1.37 ± 0.19 in the cells pre-treated with aqueous extract of *Clematis chinensis* at concentrations of 1 μg/ml, 10 μg/ml, and 50 μM ASA, respectively one hour before LPS exposure.

The level of iNOS protein following a treatment with 2 μg/ml LPS for 24 h was markedly increased to 6.95 ± 0.52. The level of iNOS protein was decreased to 2.58 ± 0.27, 4.55 ± 0.47, and 1.17 ± 0.33 in the cells pre-treated with aqueous extract of *Clematis chinensis* at concentrations of 1 μg/ml, 10 μg/ml, and 50 μM ASA, respectively one hour before LPS exposure.

In the present study, treatment with LPS did not enhance the expression of COX-1 protein. Pre-treatment with aqueous extract of *Clematis chinensis* slightly decreased COX-1 protein expression. Treatment with LPS significantly increased the levels of COX-2 protein and iNOS protein expressions. Pre-treatment with aqueous extract of *Clematis chinensis* and ASA decreased the LPS-induced expressions of COX-2 protein and iNOS protein.

Effect of *Clematis chinensis* on PGE_2 synthesis

From the results of the PGE_2 immunoassay, after 24 h of exposure to 2 μg/ml LPS, the amount of PGE_2 from the culture medium was increased from 43.33 ± 4.41 pg/ml (control) to 182.15 ± 7.84 pg/ml. It was decreased to 63.31 ± 3.13 pg/ml, 96.25 ± 4.36 pg/ml, and 55.36 ± 4.28 pg/ml by pre-treatment with aqueous extract of *Clematis chinensis* at concentrations of 1 μg/ml, 10 μg/ml, and 50 μM ASA, respectively, one hour before LPS exposure.

In the present study, LPS treatment increased PGE_2 synthesis in mouse BV2 microglial cells, and pre-treatment with aqueous extract of *Clematis chinensis* and ASA significantly suppressed LPS-induced PGE_2 synthesis.

Effect of *Clematis chinensis* on NO production

From the results of the NO detection assay, after 24 h of exposure to 2 μg/ml LPS, the amount of nitrite was increased from 12.43 ± 2.27 μM (control) to 39.31 ± 2.42 μM. It was decreased to 18.280 ± 0.74 μM, 20.44 ± 0.27 μM, and 14.04 ± 0.18 μM by pre-treatment with aqueous extract of *Clematis chinensis* at concentrations of 1 μg/ml, 10 μg/ml, and 50 μM ASA, respectively, one hour before LPS exposure.

In the present study, LPS treatment increased NO production in mouse BV2 microglial cells, and pre-treatment with aqueous extract of *Clematis chinensis* and ASA significantly suppressed LPS-induced NO production.

DISCUSSION

PGE_2 is a major metabolite of the COX-2 pathway and has emerged as an important lipid mediator of inflammatory and immune-regulatory processes. PGE_2 is implicated in the pathogenesis of acute and chronic inflammatory states (Hinz *et al*., 2000), and specific COX-2 inhibitor attenuate the symptoms of inflammation (Crofford *et al*., 2000). In the present results, *Clematis chinensis* inhibited LPS-induced COX-2 expression, and resulted in suppression of PGE_2 synthesis in mouse BV2 microglial cells.

Excessive NO production in response to bacterial LPS or cytokines plays an important role in endotoxemia and inflammatory conditions (Gidday *et al*., 1998: Stoclet *et al*., 1998). Inflammation is a complex process involving numerous mediators of cellular and plasma origin with elaborated and interrelated biological effects. The iNOS induced by microbial products including LPS accounts for the sustained generation of NO, and it is well known

that NO plays an important role in immunological responses such as inflammation and autoimmunity (Nathan and Xie, 1994). iNOS up-regulation induced by endotoxin, interleukin-1, TNF-α, and interferon-γ increases NO production. Inhibition of iNOS expression in murine macrophages has been suggested as another possible mechanism of nonsteroidal anti-inflammatory drugs (Amin *et al*., 1995). Drugs that inhibit NO production by suppressing iNOS gene expression and enzyme activity also showed beneficial therapeutic effects for the treatment of sepsis (Suh *et al*., 1988). In the present results, *Clematis chinensis* inhibited LPS-induced iNOS expression, and resulted in suppression of NO production in mouse BV2 microglial cells.

COX activity and subsequent production of PGE_2 are closely related to the generation of NO radicals (Pang and Hoult, 1997). Salvemini *et al*. (1993) reported that NO modulates the activity of COX-2 as a cGMP-independent fashion and that NO plays a critical role in the release of PGE_2 by direct activation of COX-2. NO and PGE_2 are involved in various pathophysiological processes including inflammation and carcinogenesis, and iNOS and inducible COX-2 are mainly responsible for the production of large amounts of these mediators (Schmidt and Walter, 1994; Simon, 1999). In the present results, the aqueous extract of *Clematis chinensis* suppressed PGE_2 synthesis and NO production by inhibiting LPS-stimulated enhancement of COX-2 and iNOS expressions in mouse BV2 microglial cells.

Clematis chinensis has traditionally been used as an ingredient of some prescriptions in Oriental Medicine for relieving pain and inflammation. *Clematis mandshurica* is known to contain three ingredients: anemonin, anemonol, and other saponins. Moreover, it was reported that anemonin significantly inhibits the production of NO induced by LPS in rat intestinal microvascular endothelial cells (Duan *et al*., 2006) and the expression of iNOS mRNA and protein in activated RAW 264.7 cells (Lee *et al*., 2008). Saponins are also known to have anti-inflammatory effect on LPS-induced RAW264.7 cells (Park *et al*., 2005). Also, vitalboside was isolated as the potent anti-inflammatory, analgesic, and antipyretic component of the *Clematis vitalba* dried aerial part (Yesilada and Kupeli, 2007). Therefore, anti-inflammatory activity of *Clematis chinensis* can be ascribed to these components.

Our present results showed that the aqueous extract of *Clematis chinensis* exerts anti-inflammatory and analgesic effects probably by suppressing COX-2 and iNOS expressions, and resulting in inhibition of PGE_2 synthesis and NO production. The present study suggests that *Clematis chinensis* may offer a valuable mean of therapy for brain inflammation.

ACKNOWLEDGEMENTS

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (No. 2005 - 0049404).

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