원저

Effects of Anemarrhenae Rhizoma on LPS-stimulated Expression of COX-2 and iNOS in mouse BV2 microglial cells

Seo Bong-won, Kim Ee-hwa[*], Park Se-keun[**], Jang Mi-hyeon[***], Choi Sun-mi[****] and Lee Eun-yong

Department of Acupuncture & Moxibustion,
[*]Department of Acupoint & Meridian, [**]Department of Oriental Food & Nutrition, College of Oriental Medicine, Semyung University
[***]Department of Physiology, College of Medicine, Kyung-Hee University
[****]Department of Medical Research, Korea Institute of Oriental Medicine

Abstract

Anemarrhenae Rhizoma (AR) has been widely used for the treatment of various diseases in Oriental medicine. To investigate whether AR possesses anti-inflammatory effects against lipopolysaccharide (LPS)-induced inflammation in the BV2 microglial cells, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay, reverse transcription -polymerase chain reaction (RT-PCR), and prostaglandin E2 (PGE2) assay, and nitric oxide (NO) detection assay were performed. From the present results, AR was shown to suppress PGE2 synthesis and NO production by inhibiting the LPS-stimulated enhancement of cyclooxygenase-2 and inducible nitric oxide synthase expression in BV2 microglial cells. These results suggest that AR may offer a valuable means of therapy in the treatment of inflammatory diseases by attenuating LPS-induced PGE2 synthesis and NO production.

Key words : Anemarrhenae Rhizoma, lipopolysaccharide, prostaglandin E_2, nitric oxide, inducible nitric oxide synthase, inflammation

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· **Corresponding author** : Lee Eun-yong, San 21-11, Shinwol-dong, Jecheon-city Choong Cheong Buk-do, 390-711 Korea, Department of Acupuncture & Moxibustion, Semyung University
Tel. 82-43-649-1816 E-mail : acupley@netian.com

Ⅰ. Introduction

Over the last thirty years, various clinical trBrain inflammation has been implicated in the pathogenesis of several neurodegenerative disorders including Alzheimer's disease, Parkinson's disease and ischemic brain injury. Anti-inflammatory drugs reduce the risk and progression of Alzheimer's disease, and the neuronal damage in animal models of Parkinson's disease. Microglia are the major inflammatory cells in the brain that are activated by brain injury[1-3].

Lipopolysaccharide (LPS) initiates a number of major cellular responses that play vital roles in the pathogenesis of inflammatory responses including activation of inflammatory cells and the production of cytokines and other mediators. Prostaglandin E2 (PGE2) is a key inflammatory mediator that is converted from arachidonic acid by cyclooxygenase. There are two isoforms of cyclooxygenase(COX): COX-1 and COX-2. COX-1 is a constitutively expressed form in normal physiologic functions, while COX-2 is expressed only in response to inflammatory signals such as cytokines and the bacterial endotoxin, LPS. COX-2 produces a large amount of PGE2 and this induces inflammation[4-5].

Nitric oxide (NO), endogenously generated from L-arginine by NO synthase (NOS), plays an important role in the regulation of many physiological processes. Several isoforms of NOS exist and these isoforms fall into three major classes: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). Of these, iNOS is an important enzyme involved in regulation of inflammation. It was reported that LPS up-regulates iNOS expression in macrophages[6] and microglial cells[7].

It is reported that the rhizome of Anemarrhena asphodeloides Bunge (Liliaceae), widely used as traditional medicine in Korea, contains, as one of its various constituent, saponin and xanthone. Saponin is known to partly hold timosaponin A-1-Ⅳ, B-Ⅰ, B-Ⅱ and xanthone, mangiferin as one of its separated elements[8].

Anemarrhenae Rhizoma(AR) has been known to have an anti-diabetic activity[9], anti-platelet aggregation activity[10] and diuretic activity[11].

In these study, the effects of AR against LPS-stimulated expressions of COX-2 and iNOS in the mouse microglial BV2 cells was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5 -diphenyltetrazolium bromide (MTT) assay, reverse transcription-polymerase chain reaction (RT-PCR), PGE2 immunoassay, and NO detection.

Ⅱ. Materials and Aterials and Methods

1. Cell culture

Bee venom was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The mouse BV2 microglial 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) at 37 ℃ in 5 % CO2 and 95 % O2 in a humidified cell incubator.

2. Preparation of Anemarrhenae Rhizoma

To obtain the water extract of AR, 200 g of AR was added to distilled water, and

extraction was performed by heating at 80 ℃, concentrated with a rotary evaporator and lyophilized. The resulting powder, weighing 30 g, was dissolved in saline.

3. MTT cytotoxicity assay

Cell viability was determined using the MTT assay kit (Boehringer Mannheim GmbH, Mannheim, Germany) as per the manufacturer's protocols. In order to determine the cytotoxicity of AR, cells were treated with AR at concentrations of 0.1 mg/mℓ, 0.5 mg/mℓ, 1 mg/mℓ, 5 mg/mℓ, and 10 mg/mℓ for 24 h. Cultures of the control group were left untreated. Ten μℓ of the MTT labeling reagent was added to each well, and the plates were incubated for 4 h. Solubilization solution of 100 μℓ was then added to each well, and the cells were incubated for another 12 h. The absorbance was then measured with a microtiter plate reader (Bio-Tek, Winooski, VT, USA) at a test wavelength of 595 nm and a reference wavelength of 690 nm. Optical density (O.D.) was calculated as the difference between the absorbance at the reference wavelength and that at the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample/control O.D.) × 100.

4. RNA isolation and RT-PCR

To identify expressions of COX-2 and iNOS mRNA, reverse transcription-polymerase chain reaction (RT-PCR) was performed, and the exact primer sequences used in this study were designed according to Jang et al.[12]. For mouse COX-2, the primer sequences were 5′-TGCATGTGGCTGTGGATGTCATCAA -3′ (a 25-mer sense oligonucleotide) and 5′-CACTAAGACAGAC CCGTCATCTCCA-3′ (a 25-mer anti-sense oligonucleotide). For mouse iNOS, the primer sequences were 5′-GTGTTCCACCA GGAGATGTTG-3′ (a 21-mer sense oligonucleotide) and 5′-CTCCTG CCCACTGAGTTCGTC-3′ (a 21-mer anti-sense oligonucleotide). For cyclophilin, the internal control used in the study, the primer sequences were 5′-ACCCCACCGTGTTCT TCGAC-3′ (a 20-mer sense oligonucleotide starting at position 52) and 5′-CATTTGC CATGGACAAGATG-3′ (a 20-mer anti- sense oligonucleotide starting at position 332). The expected sizes of the PCR products were 583 bp for COX-2 and 500 bp for iNOS, and 299 bp for cyclophilin.

For 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 ℃ for 5 min, followed by 40 amplification cycles, each consisting of denaturation at 94 ℃ for 30 sec, annealing at 58 ℃ for 30 sec, and extension at 72 ℃ for 30 sec, with an additional extension step at the end of the procedure at 72 ℃ 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 by using Molecular AnalystTM version 1.4.1 (Bio-Rad, Hercules, CA, USA).

5. Measurement of PGE2 synthesis

Assessment of PGE2 synthesis was performed using a commercially available PGE2 competitive enzyme immunoassay kit (Amersham Pharmacia Biotech. Inc.). Cells were lysed and cell lysates and standards were put into different wells on the goat anti-mouse IgG-coated microtiter plate provided in the kit. Mouse anti-PGE2 antibody and peroxidase-

conjugated PGE2 were added to each well, and the plate was incubated at room temperature and shook 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 450 nm.

6. Measurement of NO synthesis

In order to determine the effect of AR on NO synthesis, the amount of nitrite in cell-free culture supernatant was measured using a commercially available NO detection kit (Intron Biotech., Seoul, Korea). After collection of 100 ㎕ of supernatant, 50 ㎕ of N1 buffer was added to each well, and the plate was incubated at room temperature for 10 min. 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 450 nm. The nitrite concentration was calculated from a nitrite standard curve.

7. Statistical analyses

The results are expressed as the mean ± standard error mean (SEM). The data were analyzed by one-way ANOVA followed by Duncan's post-hoc test using SPSS. Differences were considered statistically significant at $p < 0.05$.

Ⅲ. Results

1. Effects of Anemarrhenae Rhizoma on BV2 microglial cells viability

The viabilities of cells incubated with AR at 0.003 ㎎/㎖, 0.03 ㎎/㎖, 0.3 ㎎/㎖, 3 ㎎/㎖, and 30 ㎎/㎖ for 24 h were 78.36 ± 6.57%, 78.20 ± 7.45 %, 84.43 ± 5.59 %, 91.07 ± 4.57 %, and 80.66 ± 9.95 % of the control value, respectively. The assay revealed that AR exerted no significant cytotoxicity in the BV2 microglial cells (Fig. 1).

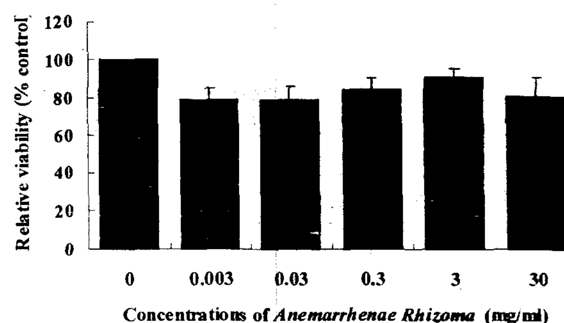

Fig. 1. Effects of Anemarrhenae Rhizoma(AR) on viability in the BV2 microglial cells. Viability was determined using 3-(4,5-dimethylthiazol -2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

2. Effects of Anemarrhenae Rhizoma on mRNA Expressions of COX-2 and iNOS

RT-PCR analyses of the mRNA levels of COX-2 and iNOS were performed in order to provide an estimate of the relative levels of expressions of these genes. In the present study, the mRNA levels of COX-2 and iNOS in the control cells were used as a control value 1.00. The level of COX-2 mRNA was markedly increased to 3.58 ± 0.09 following treatment with 5 ㎍/㎖ LPS for 24 h, while decreased to 1.04 ± 0.08, and 1.53 ± 0.05 in cells treated with AR at 0.3 ㎎/㎖, and 3 ㎎/㎖, respectively. The level of iNOS mRNA was markedly increased to 5.04 ± 0.07 following treatment with 5 ㎍/㎖ LPS for 24 h, while decreased to 2.03 ± 0.04, and 2.84 ± 0.07 in cells treated with AR at 0.3 ㎎/㎖, and 3 ㎎/㎖, respectively (Fig. 2).

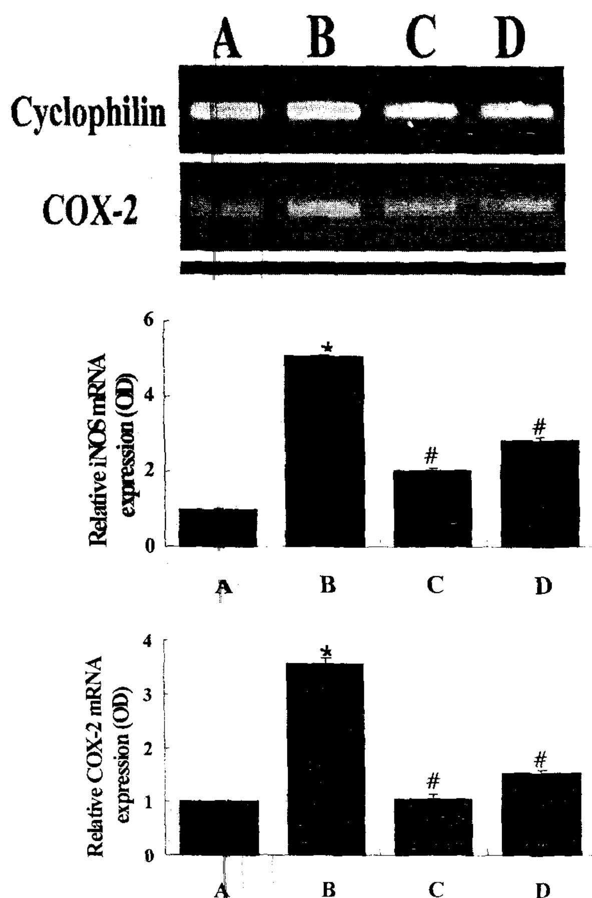

Fig. 2. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the mRNA expressions of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Cells were pre-treated with 0.3 mg/mℓ AR, 3 mg/mℓ AR, and 100 μg/mℓ Acetylsalicylic acid for 1 h followed by 5 μg/mℓ lipopolysaccharide (LPS) treatment for 24 h. Cyclophilin was used as the internal control.
* represents $p < 0.05$ compared to the control. # represents $p < 0.05$ compared to the LPS-treated group. (A) Control, (B) LPS-treated group, (C) LPS- and 0.3 mg/mℓ AR-treated group, (D) LPS- and 3 mg/mℓ AR-treated group.

3. Effects of Anemarrhenae Rhizoma on PGE2 synthesis

From PGE2 immunoassay, after 24 h of exposure to LPS, the amount of PGE2 was increased from 10.50 ± 2.84 pg/mℓ to 57.57 ± 9.48 pg/mℓ, while decreased to 51.28 ± 8.14 pg/mℓ, 45.25 ± 6.95 pg/mℓ and 38.68 ± 5.74 by the treatment with 0.3 mg/mℓ AR, and 3 mg/mℓ AR, respectively (Fig. 3).

4. Effects of Anemarrhenae Rhizoma on NO synthesis

From the NO detection assay, after 24 h of exposure to LPS, the amount of nitrite was increased from 3.00 ± 0.01 μM to 15.52 ± 3.97 μM, and it was decreased to 14.47 ± 2.82 μM, 9.54 ± 1.87 μM, and 8.04 ± 1.52 μM by treatment with 0.3 mg/mℓ AR, and 3 mg/mℓ AR, respectively (Fig. 4).

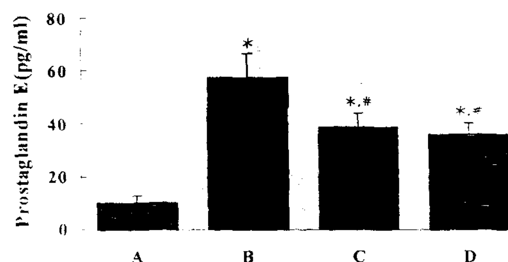

Fig. 3. Measurement of prostaglandin E2 (PGE2) in BV2 microglial cells. Cells were pre-treated with 0.3 mg/mℓ AR, and 3 mg/mℓ AR for 1 h followed by 5 μg/mℓ LPS treatment for 24 h. Cyclophilin was used as the internal control.
* represents $p < 0.05$ compared to the control. # represents $p < 0.05$ compared to the LPS-treated group. (A) Control, (B) LPS-treated group, (C) LPS- and 0.3 mg/mℓ AR-treated group, (D) LPS- and 3 mg/mℓ AR-treated group.

Ⅳ. Discussion

The purpose of the present study was to find out whether AR exerts inflammatory effects against LPS-induced COX-2 and iNOS expressions in the BV2 microglial cells. Microglia are macrophage-like cells of the central nervous system (CNS), and they generally considered being immunologically

quiescent under normal conditions. The activation of microglia that is induced by CNS injury or infection is associated with neurodegenerative disorders[1-3]. Inflammation is a complex process involving numerous mediators of a cellular and plasma origin, and these mediators have elaborate and interrelated biological effects.

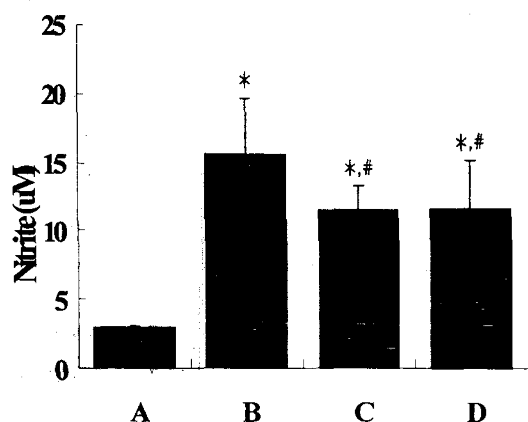

Fig. 4. Measurement of nitric oxide (NO) in BV2 microglial cells. Cells were pre-treated with 0.3 ㎎/㎖ AR, and 3 ㎎/㎖ AR for 1 h followed by 5 ㎍/㎖ LPS treatment for 24 h. Cyclophilin was used as the internal control. * represents $p < 0.05$ compared to the control. # represents $p < 0.05$ compared to the LPS-treated group. (A) Control, (B) LPS-treated group, (C) LPS- and 0.3 ㎎/㎖ AR-treated group, (D) LPS- and 3 ㎎/㎖ AR-treated group.

PGE2 and NO are involved in various pathophysiological processes including inflammation and carcinogenesis, and iNOS and COX-2 are known as the main enzymes for the production of these mediators[13]. Elevation of COX-2 activity is closely associated with the occurrence of cancer, arthritis, and several types of neurodegenerative disorders. Specific COX-2 inhibitors can attenuate the symptoms of inflammation[5,14]. NO exerts diverse and multifunctional effects in the host cells. After an exposure to endogenous and exogenous stimulators such as LPS and viral infection, iNOS is induced quantitatively in the various cells, and it triggers several deleterious cellular responses inducing inflammation, sepsis, and stroke[6-7]. In addition, COX activity and the subsequent production of PGE2 are closely related to the generation of NO radicals. Salvemini et al.[15] reported that NO modulates the activity of COX-2 in a cGMP-independent manner, and NO plays a critical role in the release of PGE2 by the direct activation of COX-2. Inhibition on the iNOS expression in murine macrophages has been suggested as another possible mechanism for the effect of non-steroidal anti-inflammatory drugs[16].

A research and development on the new medicine materials that is aimed to be applied to the treatment of inflammatory on human body is recently in brisk process. Natural substance, especially, is the most main subject among them. Genistin, bacalin and wogonin, the natural substance on the flavonoid line are known to have the effects on restraining inflammatory mediator such as pro-inflammatory cytokine, NO and COX-2[17-21].

Herbs in the Liliaceae family have mostly been used for increasing the health effects of traditional medicine in Korea. Recently, Park et al.[22] reported that compounds from AR possess anti-fungal activity. The chemical constituents of the AR have been studied, and the isolation and identification of steroidal saponins have also been reported by several groups[8,10,23]. Niwa et al.[10] have reported that timosaponin A-III and markogenin glycoside inhibited ADP-induced aggregation as well as 5-HT or arachidonic acid-induced aggregation of human platelet. However, the molecular mechanisms for the anti-inflammatory effect of AR have not yet been clarified. In the present study, AR was shown to suppress PGE2 and NO production by inhibiting LPS-stimulated enhancement of COX-2 enzyme activity and

iNOS expression in mouse BV2 microglia cells. The present results suggest that AR can exert its anti-inflammatory and analgesic effects probably by suppressing COX-2 and iNOS expressions, resulting in the inhibition of PGE2 and NO synthesis.

V. Conclusion

From above study the results can summarized as followings.

1. AR was shown to suppress PGE2 and NO production by inhibiting LPS-stimulated enhancement of COX-2 enzyme activity and iNOS expression.
2. It is very possible that AR can offer a valuable mode of therapy for the treatment of brain inflammatory diseases by attenuating LPS-induced PGE2 and NO synthesis.

VI. References

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