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원 저

Effects of *Liriope Platyphylla* on LPS-stimulated Expression of COX-2 and iNOS in Mouse BV2 Microglial Cells

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Abstract

Objective : In this study, the effects of Liriope Platyphylla against LPS-induced inflammation was investigated.

Methods : Cell viability was determined using the MTT assay. To identify expressions of COX-2 and iNOS mRNA, RT-PCR was performed. Assessment of PGE2 synthesis was performed using the PGE2 immunoassay. Measurement of NO synthesis was performed using the NO detection.

Result : The MTT assay revealed that Liriope Platyphylla exerted no significant cytotoxicity in the microglial BV2 cells. RT-PCR analysis revealed that the mRNA levels of COX-2 and iNOS were significantly decreased in the LPS- and 5 ㎎/㎖ Liriope Platyphylla treated group. From the PGE2 immunoassay and NO detection, PGE2 and NO synthesis was significantly suppressed in the LPS- and 5 ㎎/㎖ Liriope Platyphylla treated group.

Conclusion : In these study, Liriope Platyphylla was shown to suppress PGE2 and NO production by inhibiting LPS-stimulated enhancement of COX-2 enzyme activity and iNOS expression. It is very possible that Liriope Platyphylla can offer a valuable mode of therapy for the treatment of brain inflammatory diseases.

Key words : *Liriope Platyphylla*, lipopolysaccharide, prostaglandin E_2, nitric oxide, inflammation, inducible NOS

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Ⅰ. Introduction

Brain 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: cyclooxygenase-1 (COX-1) and cyclooxygease-2 (COX-2). While COX-1 is a constitutively expressed form in normal physiologic functions, 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].

Liriope Platyphylla(LP) has been widely used for the treatment of various diseases such as a tonic, antitussive, and expectorant in oriental medicine[8].

In addition, LP has also been used for diabetes and cardiovascular disease[9]. Active components of LP has been identified as homoisoflavonoids (ophiopogone, methylopiogonone, etc), sterols (β-sistosterol etc), and steroidal glycosides (ruscogenin, ophiopogonin, etc)[9-10].

However, the effects of LP have not been reported yet. In the present study, the effects of LP against LPS-stimulated expressions of COX-2 and iNOS in the mouse BV2 microglial 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. Material and Metjods

1. Cell culture

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 % CO_2 and 95 % O2 in a humidified cell incubator.

2. Preparation of Liriope Platyphylla

LP was obtained from the Oriental Medical Hospital, Semyung University and kindly authentic cated by professor Kim Jeung-Beum. Dept. of Oriental Pathology, college of a Oriental Medicine, Semyung University. To obtain the water extract of LP, 200 g of LP

was added to distilled water, and extraction was performed by heating at 80℃, concentrated with a rotary evaporator and lyophilized. The resulting powder, weighing 30g, 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 LP, cells were treated with LP at concentrations of 0.1 ㎎/㎖, 0.5 ㎎/㎖, 1 ㎎/㎖, 5 ㎎/㎖, and 10 ㎎/㎖ for 24 h. Cultures of the control group were left untreated. Ten ㎕ of the MTT labeling reagent was added to each well, and the plates were incubated for 4 h. Solubilization solution of 100㎕ was then added to each well, and the cells were incubated for another 12h. The absorbance was then measured with a microtiter plate reader (Bio-Tek, Winooski, VT, USA) at a test wavelength of 595㎚ and a reference wavelength of 690㎚. 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, RT-PCR was performed, and the exact primer sequences used in this study were designed according to Jang et al[11].

For mouse COX-2, the primer sequences were 5′-TGCATGTGGCTG TGGATGTCAT CAA-3′ a 25-mer sense oligonucleotide) and 5′-CACT AAGACAGACCCGTCATCTCCA-3′ (a 25-mer anti-sense oligonucleotide). For mouse iNOS, the primer sequences were 5′-GTGTTCCACCAG GAGATGTTG-3′ (a 21-mer sense oligonucleotide) and 5′-CTCCTG CCCACT GAGTTCGTC-3′ (a 21-mer anti-sense oligonucleotide). For cyclophilin, the internal control used in the study, the primer sequences were 5′-ACCCCACCGTGTTCTTC GAC-3′ (a 20-mer sense oligonucleotide starting at position 52) and 5′-CATTTGCCATGGACAA GATG-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 Analyst TM 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'- tetramethyl-benzidine/ 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 H2SO4. The absorbance of the content of each well was then measured at 450 ㎚.

6. Measurement of NO synthesis

In order to determine the effects of LP 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㎚. 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. Difference was considered statistically significant at $p < 0.05$.

Ⅲ. Results

1. Effect of LP on BV2 microglial cells viability

The viabilities of cells incubated with LP at 0.1 ㎎/㎖, 0.5 ㎎/㎖, 1 ㎎/㎖, 5 ㎎/㎖, and 10 ㎎/㎖ for 24 h were 99.18 ± 11.41 %, 106.49 ± 7.58 %, 102.45 ± 8.81 %, 94.11 ± 10.24 %, and 98.46 ± 8.54 % of the control value, respectively. The MTT assay revealed that LP exerted no significant cytotoxicity in the microglial BV2 cells(Fig. 1).

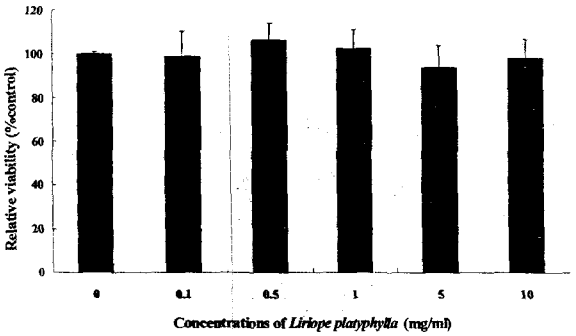

Fig. 1. Effects of Liriope Platyphylla(LP) on viability in the BV2 microglial cell. Viability was determined via 3- (4,5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

2. Effect of LP on mRNA Expressions of COX-2 and iNOS

RT-PCR analysis of the mRNA levels of COX-2 and iNOS was 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 18.84 ± 3.28 following treatment with 5 ㎍/㎖ LPS for 24 h, while decreased to 17.64 ± 2.58, 3.54 ± 0.95, and 3.60 ± 0.84 in cells treated with LP at 1 ㎎/㎖, 5 ㎎/㎖, and acetylsalicylic acid (ASA) at a concentration of 100 ㎍/㎖, respectively. The level of iNOS mRNA was markedly increased to 15.72 ± 2.94 following treatment with 5 ㎍/㎖ LPS for 24 h, while decreased to 12.41 ± 2.97, 3.28 ± 0.87, and 3.04 ± 0.59 in cells treated with LP at 1 ㎎/㎖, 5 ㎎/㎖, and ASA at a concentration of 100 ㎍/㎖, respectively (Fig. 2).

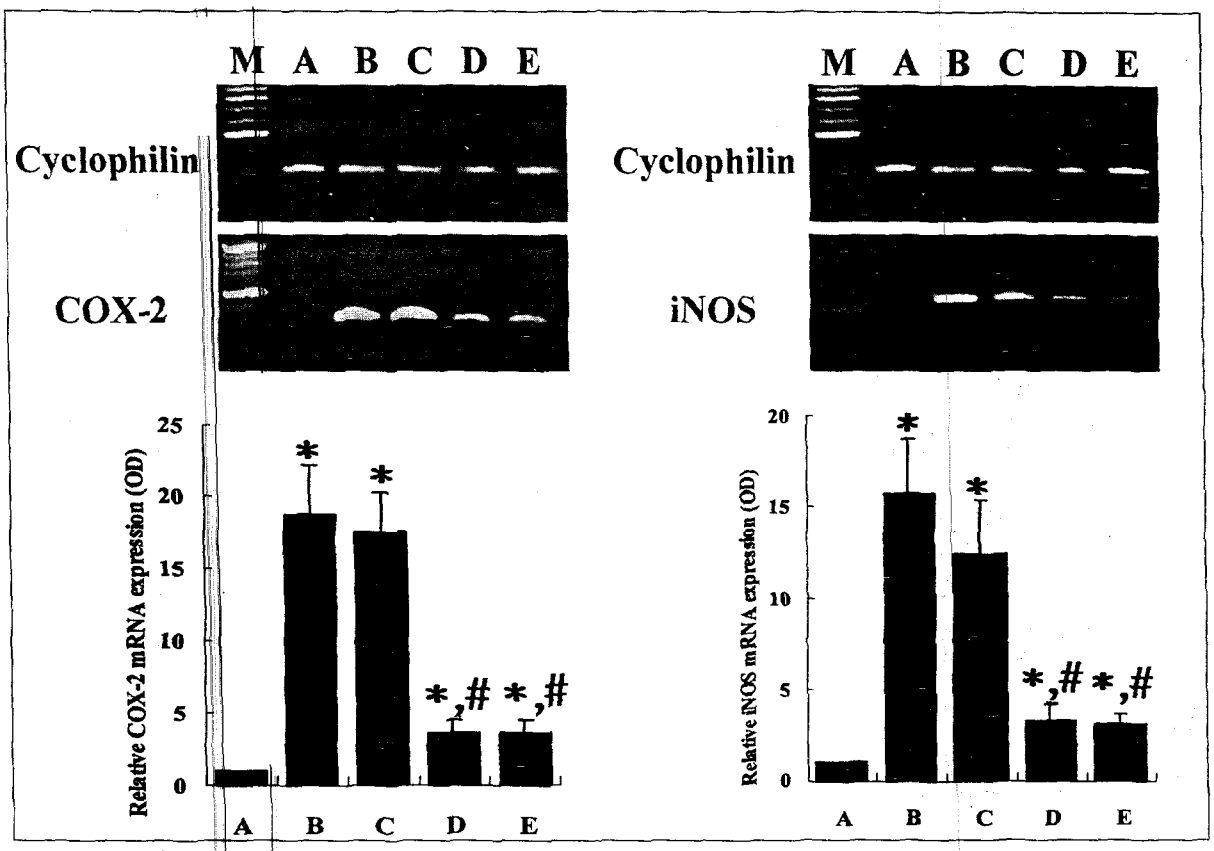

Fig. 2. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the mRNA expressions of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). Cells were pre-treated with 1 ㎎/㎖ LP, 5 ㎎/㎖ LP, and 100 ㎍/㎖ ASA for 1 h followed by 5 ㎍/㎖ lipopolysaccharide (LPS) treatment for 24 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. (M) Marker. (A) Control. (B) LPS-treated group. (C) LPS- and 1 ㎎/㎖ LP-treated group. (D) LPS- and 5 ㎎/㎖ LP-treated group. (E) LPS- and 100 ㎍/㎖ acetylsalicylic acid (ASA)-treated group.

3. Effect of LP 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/㎖ to 57.57 ± 9.48 pg/㎖, while decreased to 51.28 ± 8.14 pg/㎖, 45.25 ± 6.95 pg/㎖ and 38.68 ± 5.74 by the treatment with 1 ㎎/㎖ LP, 5 ㎎/㎖ LP, and 100 ㎍/㎖ ASA, respectively(Fig. 3).

4. Effect of LP 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,

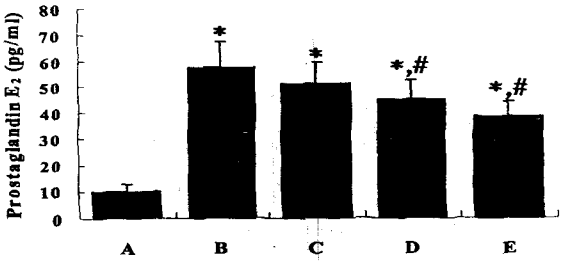

Fig. 3. Measurement of prostaglandin E2 (PGE2) in microglial BV2 cells. Cells were pre-treated with 1 ㎎/㎖ LP, 5 ㎎/㎖ LP, and 100 ㎎/㎖ ASA for 1 h followed by 5 ㎍/㎖ LPS treatment for 24 h. Cyclophilin was used as the internal control.
* represents $p < 0.05$ compared to the control. # represents $p < 0.05$ compared to the LPS-treated group. (A) Control. (B) LPS-treated group. (C) LPS- and 1 ㎎/㎖ LP-treated group. (D) LPS- and 5 ㎎/㎖ LP-treated group. (E) LPS- and 100 ㎍/㎖ acetylsalicylic acid (ASA)-treated group.

9.54 ± 1.87 μM, and 8.04 ± 1.52 μM by treatment with 1 ㎎/㎖ LP, 1 ㎎/㎖ LP, and 100 ㎍/㎖ ASA, respectively(Fig. 4).

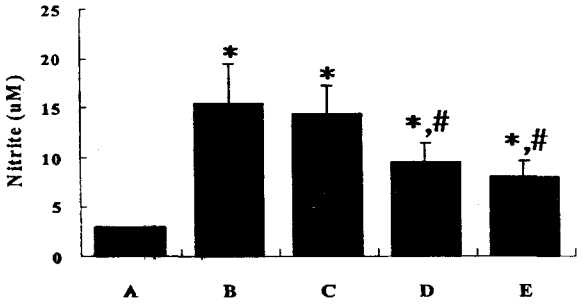

Fig. 4. Measurement of Nitric oxide (NO) in BV2 microglial cells. Cells were pre-treated with 1 ㎎/㎖ LP, 5 ㎎/㎖ LP, and 100 ㎍/㎖ ASA for 1 h followed by 5 ㎍/㎖ LPS treatment for 24 h. Cyclophilin was used as the internal control.

* 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 1 ㎎/㎖ LP-treated group. (D) LPS- and 5 ㎎/㎖ LP-treated group. (E) LPS- and 100 ㎍/㎖ acetylsalicylic acid (ASA)-treated group.

Ⅳ. Discussion

Microglia are macrophage-like cells of the central nervous system (CNS), and they are 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.

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[12]. 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[5,13]. 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.[14] 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 effects of non-steroidal anti-inflammatory drug[15].

Herbs in the Liliaceae family have mostly been used for increasing the health effects of traditional medicine in Korea. Recently, Hur et al.[8] suggested that astroglial nerve growth factor (NGF) enhanced by butanol fraction of LP in a PKC-dependent pathway contributed to the induction of neurite outgrowth of PC12 cells. Liu et al.[16] suggested that ruscogenin glycoside isolated from Liriope muscari possesses anti-inflammatory activity through protein kinase C pathway. However, the molecular mechanisms for the anti-inflammatory effects of LP have not yet been clarified.

In these study, LP was shown to suppress PGE2 and NO production by inhibiting LPS-stimulated enhancement of COX-2 enzyme activity and iNOS expression in mouse BV2 microglial cells. The present results

suggest that LP 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

In this study, the effects of LP against LPS-induced inflammation was investigated. From the these results, LP was shown to suppress prostaglandin E2 synthesis and nitric oxide production by inhibiting the LPS-stimulated enhancement of cyclooxygenase-2 and inducible nitric oxide synthase expression in BV2 microglial cells.

It is very possible that LP can offer a valuable mode of therapy for the treatment of brain inflammatory diseases by attenuating LPS-induced PGE2 and NO synthesis.

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