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Gardenia jasminoides Exerts Anti-inflammatory Activity via Akt and p38-dependent Heme Oxygenase-1 Upregulation in Microglial Cells

Ji Su Song, Ji Eun Shin, Ji-Hee Kim and YoungHee Kim*

Department of Molecular Biology, College of Natural Sciences, Pusan National University, Busan 46241, Korea

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Died *Gardenia jasminoides* fruit is used as a dye in the food and clothes industries in Asia. The present study investigated the anti-inflammatory effects of aqueous extract of *G. jasminoides* fruits (GJ) in BV-2 microglial cells. GJ inhibited lipopolysaccharide-induced nitric oxide (NO) secretion, inducible nitric oxide synthase (iNOS) expression, and reactive oxygen species production, without affecting cell viability. Furthermore, GJ increased the expression of heme oxygenase-1 (HO-1) in a dose-dependent manner. Moreover, the inhibitory effect of GJ on iNOS expression was abrogated by small interfering RNA-mediated knock-down of HO-1. In addition, GJ induced nuclear translocation of nuclear factor E2-related factor 2 (Nrf2), a transcription factor that regulates HO-1 expression. GJ-mediated expression of HO-1 was suppressed by LY294002, a phosphoinositide 3-kinase (PI-3K) inhibitor, and SB203580, a p38 kinase inhibitor, but not by the extracellular signal‐regulated kinase (ERK) inhibitor PD98059 or c-Jun N-terminal kinase (JNK) inhibitor SP600125. GJ also enhanced the phosphorylation of Akt and p38. These results suggest that GJ suppresses the production of NO, a pro-inflammatory mediator, by inducing HO-1 expression via PI-3K/Akt/p38 signaling. These findings illustrate a novel molecular mechanism by which extract from *G. jasminoides* fruits inhibits neuroinflammation.

Key words : *Gardenia jasminoides*, heme oxygenase-1, microglia, nitric oxide

Introduction

Gardenia jasminoides Ellis is evergreen shrub of the family Rubiaceae and widely distributed throughout Asia and the dry fruit of *G. jasminoides* has been used as a yellow dye for food and clothes. In oriental medicine, *G. jasminoides* is known to have acopic, antipyretic, diuretic and detoxifying effects [1]. The constituents including gardenoside, gardenin, crocetin, crocin, geniposide and genipin [1, 17] have been identified from *G. jasminoides*. It has been also demonstrated that the extract of *G. jasminoides* exhibits anti-depressant [26], antiangiogenic [20], and anti-inflammatory [9, 13, 21] activities and attenuates hepatocellular [5] and gastric injury [4]. However, the mechanism responsible for anti-inflammatory effects of *G. jasminoides* fruits has not been fully clarified yet.

***Corresponding author**
Tel : +82-51-510-2526, Fax : +82-51-513-9258
E-mail : yheekim@pusan.ac.kr

The neuroinflammatory responses of the central nervous system (CNS) are well-known features of various neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease. These responses are interceded by the activation of microglia, which are the resident phagocytes of the CNS [12]. In response to extracellular stimuli, microglia can induce and release pro-inflammatory mediators such as nitric oxide (NO), prostaglandin E2 (PGE2), reactive oxygen species (ROS), and cytokines, which function to restore central nerve system (CNS) homeostasis by clearing pathogens and infected cells. However, deregulated or chronic activation of microglial cells can induce too many pro-inflammatory molecules, leading to neuronal cell death and brain injury [14]. Therefore control of microglial activation and subsequent suppression of the production of neurotoxic pro-inflammatory molecules could provide a potential therapeutic approach for the treatment of neurodegenerative diseases [25].

Heme oxygenase-1 (HO-1) is an inducible key enzyme that catalyzes the oxidative degradation of cellular heme into carbon monoxide (CO), biliverdin, and free iron [22]. HO-1 and its enzymatic by-products provide a host defense mechanism that can protect the body against oxidative injury and also contributes to the anti-inflammatory activity of cells and

tissues [22]. In activated macrophages, HO-1 expression or CO treatment inhibits the production of the pro-inflammatory mediators such as nitric oxide (NO), prostaglandin E2 (PGE2), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, monocyte chemoattractant protein-1, and macrophage inflammatory protein-1β [16]. Moreover, an increasing number of therapeutic agents have been reported to induce HO-1 expression and exert their anti-inflammatory effects through HO-1 induction. In the present study, we investigated the molecular mechanism responsible for the anti-inflammatory effects of the aqueous extract of *G. jasminoides* fruits (GJ) in microglia. We elucidated that GJ inhibits LPS-induced iNOS expression via upregulation of HO-1.

Materials and Methods

Materials and preparation of extract

Cobalt protoporphyrin (CoPP), HO-1 siRNA, and antibodies for iNOS, HO-1, Nrf-2, Akt, p38, histone deacetylase (HDAC), and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phosphorylated Akt (p-Akt) and p-p38 were purchased from Cell Signaling Technology (Beverly, MA, USA). Actinomycin D (Act. D) and cycloheximide (CHX) were purchased from Sigma-Aldrich Co. (St. Louis, MO). CM-H_2DCFDA and fetal bovine serum (FBS) were purchased from Invitrogen Corporation (San Diego, CA, USA). The dry fruits of *G. jasminoides* were purchased from a local herb store, Kwang Myoung Dang (Busan, Korea) in April 2010. The fruits of *G. jasminoides* were identified and authenticated by Professor W. S. Ko, College of Oriental Medicine, Dongeui University (Busan, Korea). A voucher specimen (number GJ-10-05) has been deposited at the Department of Molecular Biology, Pusan National University, Busan, Korea. The dry roots (300 g) were extracted with distilled water at 100°C for 2 hr. The extract was filtered through 0.45 μm filter and freeze-dried and kept at 4°C. A yield of 54.6 g (18.2%) was obtained. The dried extract was dissolved in phosphate buffered saline (PBS) and filtered through 0.22 μm filter before use.

Cell culture

Mouse BV-2 microglial cells were kindly provided by Prof. Youn-Chul Kim at Wonkwang University (Iksan, Korea), and were maintained in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated FBS and 0.1% penicillin-streptomycin at 37°C in an atmosphere of 5% CO_2.

Measurement of nitrite concentration

NO synthesis in cell cultures was measured by a microplate assay method. To measure nitrite, 100 μl aliquots were removed from conditioned medium and incubated with an equal volume of the Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min. Nitrite concentration was determined by measuring the absorbance at 540 nm with a Vmax 96-well microplate spectrophotometer (Molecular Devices, Menlo Park, CA). The sodium nitrite was used as a standard.

Cell viability assay

The cytotoxicity of GJ was assessed using the microculture tetrazolium (MTT)-based colorimetric assay. The remaining cells after Griess reaction were used for MTT assay. MTT was added to each well (final concentration is 62.5 μg/ml). After incubation for 3 hr at 37°C and 5% CO_2, the supernatant was removed and the formed formazan crystals in viable cells were solubilized with DMSO. The absorbance of each well was then read at 570 nm using microplate reader.

Western blot analysis

The cells were washed with phosphate buffered saline (PBS) three times and scraped off and lysed with lysis buffer (1% Triton X-100, 1% deoxycholate). Protein concentration of lysates was determined using Bradford reagent (Bio-Rad, Hercules, USA) and equal amounts of protein were separated electrophoretically using 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and then the gel was transferred to 0.45 μm nitrocellulose paper. The blot was incubated with primary and secondary antibody and then detected by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Co.). Alpha-tubulin or HDAC were used as protein-loading controls for each lane.

Measurement of reactive oxygen species (ROS)

To evaluate the levels of intracellular ROS, cells were treated with 10 μM of CM-H_2DCFDA (general oxidative stress indicator) for 1 hr at 37°C under 5% CO_2. The cells were then harvested and washed three times with PBS, after

which the intensity of fluorescence was measured by fluorescence microscopy.

Interference of HO-1

The siRNAs for HO-1 (GenBank accession no. NM 010442.1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cells were transfected with HO-1 siRNA or negative control siRNA using INTERFERin (Polyplus transfection, France). Then cells were incubated for 48 h until the protein expression detection.

Statistical analysis

All results were expressed as means ± SE. Each experiment was repeated at least three times. Statistical significances were compared between each treated group and analyzed by the Student's *t*-test. Data with $p<0.05$ were considered statistically significant.

Results

GJ inhibits NO synthesis and iNOS expression in microglial cells

To examine the effects of GJ on cell viability, we conducted MTT-based cell viability assay. Mouse BV-2 microglial cells were treated with various concentrations of GJ in the absence or presence of LPS. Whereas LPS induced toxic effects in BV-2 microglial cells, GJ at concentrations up to 1 mg/ml induced no cytotoxicity, and instead had a tendency to ameliorate LPS toxicity (Fig. 1A). Therefore, this concentration range of GJ was applied in all subsequent experiments.

To determine the effects of GJ on neuroinflammation, we investigated whether GJ could abrogate the production of neuroinflammatory molecule such as NO in microglial cells. BV-2 cells were incubated with GJ for 3 hr and stimulated with LPS for 24 hr. The amount of NO released into culture medium was measured by the method of Griess. As shown in Fig. 1B, GJ suppressed NO release into culture supernatant in a dose-dependent manner, whereas GJ alone has no significant effects. To determine whether the decreased nitric oxide synthesis is correlated with iNOS expression, we analyzed the amount of iNOS by Western blot analysis. The level of iNOS was dramatically reduced by GJ in a dose-dependent manner (Fig. 1C). These results suggest that GJ inhibits NO release by affecting the iNOS expression level without affecting cell viability.

GJ inhibits LPS-induced ROS production

Large amounts of ROS have been thought to contribute to neuroinflammation [10]. Furthermore, attempts to reduce

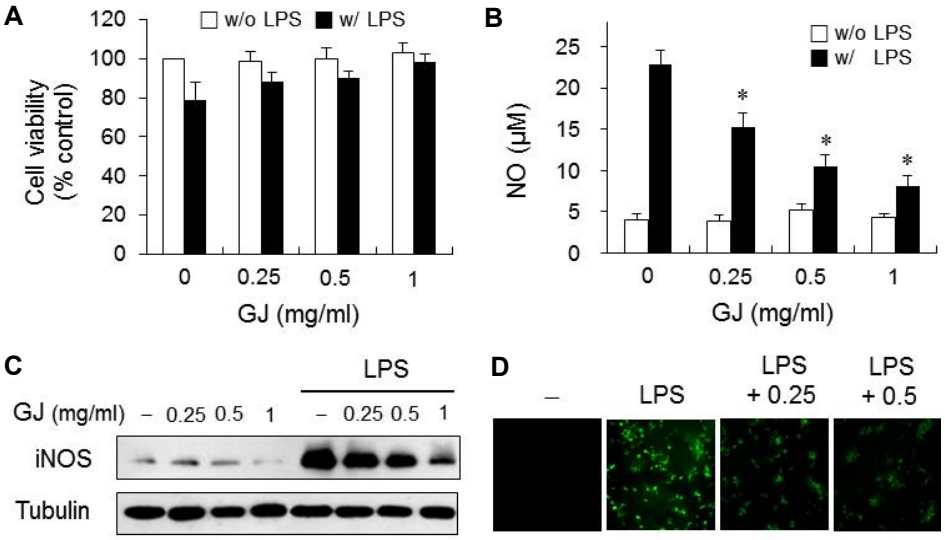

Fig. 1. Effect of GJ on the NO secretion, iNOS expression and ROS production in microglial cells. (A) BV-2 microglial cells were incubated with various concentrations of GJ in the presence or absence of LPS for 24 hr and then cell viability was measured by MTT assay. (B) Cells were incubated with various concentrations of GJ for 3 hr and then stimulated with LPS (1 μg/ml) for 24 hr. The amount of nitrite released was measured by the method of Griess. * $p<0.05$ *vs*. LPS-treated group. (C) Cells were treated as mentioned above and equal cytosolic extracts were analyzed by Western blotting. (D) Cells were treated with the indicated concentration of GJ for 1 hr and then incubated with LPS (1 μg/ml) for 16 hr. The intracellular levels of ROS were determined using fluorescence microscopy.

ROS production have been beneficial in controlling neuroinflammation-mediated neurodegenerative disorders. Therefore, we examined whether GJ reduces ROS production in microglia. BV-2 microglial cells were pre-incubated with GJ for 1 hr and then treated with LPS for 16 hr. Treatment of BV-2 microglial cells with LPS induced a significant increase in ROS levels, but pre-treatment with GJ suppressed ROS production by LPS-stimulated BV-2 cells in a dose-dependent manner (Fig. 1D). GJ did not have a significant effect on basal ROS levels (data not shown).

GJ exhibits anti-inflammatory effects through induction of HO-1

To investigate whether GJ induces HO-1 expression in microglia, BV-2 cells were incubated with various concentrations of GJ. The HO-1 protein level was significantly increased by GJ in a dose-dependent manner (Fig. 2A) and peaked at 6 hr after treatment (Fig. 2B). To confirm that GJ-induced HO-1 expression is mediated by transcription and translation, we used actinomycin D (Act. D) for an inhibitor of DNA-dependent RNA polymerase, and cycloheximide (CHX) for an inhibitor of ribosomal protein synthesis. Co- treatment of GJ and Act. D or CHX reduced the HO-1 expression (Fig. 2C).

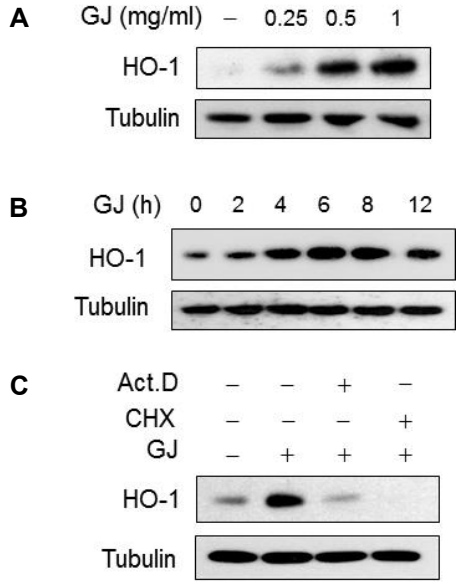

Fig. 2. Induction of HO-1 by GJ. BV-2 cells were incubated with various concentrations of GJ for 8 hr (A), or with GJ (1 mg/ml) for indicated times (B). (C) Cells were treated with GJ (1 mg/ml) for 8 hr in the absence or presence of Act D (0.25 μg/ml) or CHX (0.25 μg/ml). Cells were harvested and equal cytosolic extracts were analyzed by Western blotting with anti-HO-1 antibody.

Since by-products of HO-1 are known to have anti-inflammatory effect, we examined whether GJ exhibits its anti-inflammatory effect through the induction of HO-1 expression. BV-2 cells were pretreated with cobalt protoporphyrin (CoPP), an inducer of HO-1, and incubated with LPS for 24 hr. As shown in Fig. 3A, CoPP treatment significantly inhibited the expression of iNOS in parallel with HO-1 induction, and GJ treatment revealed similar effect. To confirm that HO-1 suppresses the expression of iNOS, we applied an HO-1 small interfering (si) RNA system to knock down HO-1 function. BV-2 cells were transfected with HO-1 siRNA or control siRNA (mock). As shown in Fig. 3B, decreased HO-1 expression blocked GJ-mediated suppression of LPS-stimulated iNOS expression, whereas transfection with control siRNA showed no effect. Taken together, these results indicate that HO-1 expression is regulated at the transcriptional level by GJ and involved in GJ-induced anti-inflammatory activity.

GJ-induced HO-1 expression is mediated by Nrf2

Since the promoter region of HO-1 gene contains binding sites for transcription factor Nrf2 and the expression of HO-1 is known to be regulated by Nrf2 [24], we examined the effect of GJ on nuclear accumulation of Nrf2 in BV-2 cells.

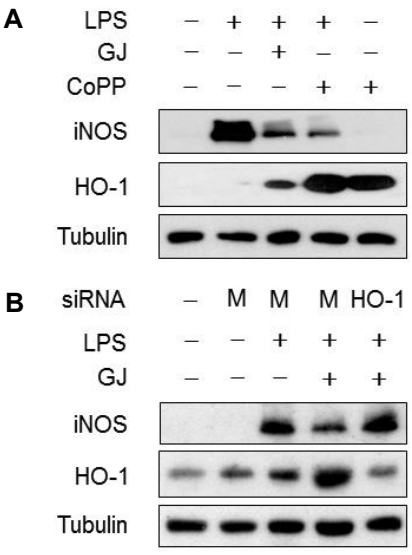

Fig. 3. Inhibitory effect of HO-1 on iNOS expression. (A) BV-2 cells were incubated with GJ (1 mg/ml) or CoPP (10 μM) for 1 hr and then stimulated with LPS (1 μg/ml) for 24 hr. (B) Cells were transfected with HO-1 siRNA or control siRNA, and were pretreated with GJ (1 mg/ml) for 3 hr and then stimulated by LPS (1 μg/ml) for 6 hr. Protein levels of iNOS and HO-1 were analyzed by Western blotting.

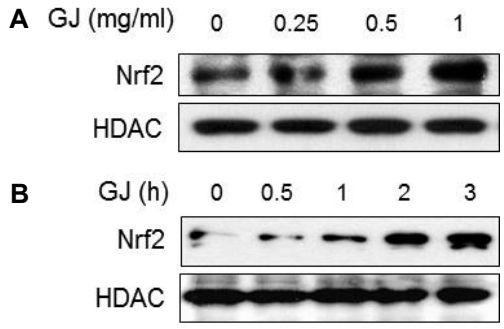

Fig. 4. Effect of GJ on Nrf2 activation. BV-2 cells were incubated with indicated concentrations of GJ for 3 hr (A), or GJ (1 mg/ml) for indicated times (B). Nuclear extracts were assayed by Western blotting with anti-Nrf2 or HDAC antibodies.

Nrf2 nuclear accumulation was increased by GJ in a dose-dependent manner and reached peak at 2 hr (Fig. 4A, Fig. 4B). These results suggest that Nrf2 is translocated into nucleus and transactivates HO-1 promoter in response to GJ.

Akt and p38 kinase mediates GJ-induced HO-1 expression

To elucidate the molecular target of GJ in further upstream signaling pathway of HO-1 expression, we examined the effect of pharmaceutical protein kinase inhibitors, LY294002 (PI-3K inhibitor), SP600125 (JNK inhibitor), PD 98059 (ERK inhibitor), and SB203580 (p38 kinase inhibitor). As shown in Fig. 5A, GJ-induced HO-1 expression was significantly inhibited by LY294002 and SB203580 but not by SP600125 and PD98059. Moreover, GJ increased the phosphorylation of Akt and p38 (Fig. 5B). These results indicate that PI-3K/Akt and p38 kinase signalings occur upstream of GJ-mediated HO-1 expression.

Discussion

The fruits of *G. jasminoides* have been used as a natural color additive in food and known to have anti-inflammatory effects. In this study, we assessed the possible molecular mechanism underlying its anti-inflammatory effect. We found that GJ significantly inhibited LPS-induced NO synthesis and iNOS expression as well as ROS production in BV-2 microglial cells without appreciable cytotoxic effects. NO is released from activated microglia, and neurons are remarkably sensitive to NO-induced cell death [2]. High levels of NO produced by iNOS from activated microglial cells are associated with the progression of various neuro-

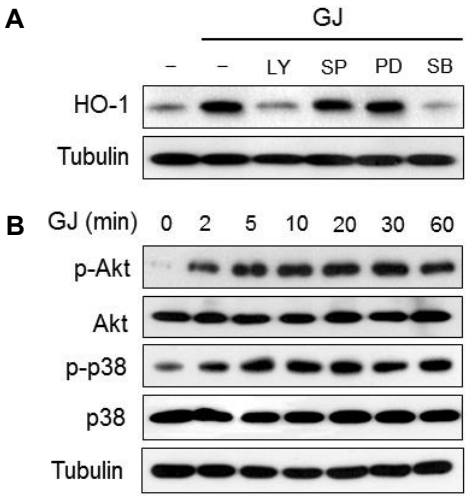

Fig. 5. Involvement of Akt and p38 kinase in GJ-mediated expression of HO-1. (A) BV-2 cells were incubated with LY294002 (10 μM), SP600125 (20 μM), PD98059 (20 μM), and SB203580 (20 μM) for 30 min and then treated with GJ (1 mg/ml) for 8 hr. (B) Cells were treated with GJ (0.5 mg/ml) for indicated times. Equal amounts of cytosolic extract were subsequently analyzed by Western blotting with HO-1, phospho-Akt or phospho-p38 antibody.

degenerative diseases [8]. ROS are also strongly associated with microglial neuroinflammatory processes in neurodegenerative diseases. Increased ROS and free radicals lead to lipid peroxidation, protein oxidation, mitochondrial dysfunction and activation of certain receptors leading to Ca^{2+} accumulation with subsequent ROS formation. These abnormal events in cells are known to lead to oxidative neuronal cell death [3]. Thus it is possible to suppose that *G. jasminoides* fruits reduce neurodegenerative diseases via inhibition of NO and ROS production in microglial cells.

Many evidences have demonstrated that HO-1 exhibits anti-inflammatory activities by inhibiting production of pro-inflammatory mediators [11], suggesting a potential therapeutic strategy for treating inflammatory diseases. Accordingly, we examined whether HO-1 expression is correlated with the inhibition of LPS-induced iNOS expression. As shown in Fig. 2, GJ increased the expression of HO-1 in a dose-dependent manner in BV-2 cells. And, treatment of CoPP, a HO-1 inducer, inhibited the expression of iNOS. Moreover, the knockdown of HO-1 expression by using siRNA markedly reversed the inhibitory effects of GJ on iNOS expression in LPS-induced macrophages. These data confirm that GJ inhibits iNOS expression via the modulation of HO-1 expression. Although the contribution of HO-1

products (ie, CO, biliverdin, and iron) has not been examined in this study, several studies point to HO-1-derived CO and biliverdin as the potential metabolite to combat neuroinflammation [11]. In particular, it has been reported that CO and biliverdin ameliorate experimental cerebral malaria, retinal ischemic injury and autoimmune neuroinflammation [7, 19, 23].

Transcription factor NF-E2-related factor 2 (Nrf2) plays a central role for inducible expression of HO-1 [24]. In basal conditions, Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) and degraded by the ubiquitin-dependent 26S proteasome system. Under activation, Nrf2 released from Keap1 inhibition, translocates to the nucleus, heterodimerizes with Maf, and binds antioxidant response elements (AREs) located in the promoter regions of many detoxifying/antioxidant genes, including HO-1 [15]. To define the molecular mechanisms underlying GJ-induced HO-1 expression in microglial cells, we examined whether GJ activate Nrf2 activity. In present study, nuclear translocation of Nrf2 was increased by treatment with GJ in a concentration-dependent manner. This result suggests that GJ up-regulates Nrf2 expression in the nucleus, which leads to up-regulation of HO-1 expression.

PI-3K/Akt and three MAPKs represented by ERK, JNK, and p38 MAPK have been reported to be involved to some extent in HO-1 expression in response to diverse stimuli [18]. To define signaling pathway of HO-1 expression, we examined the effects of inhibitors of PI-3K/Akt and three MAPKs on GJ-induced HO-1 expression. As shown in Fig. 5, GJ-mediated HO-1 expression was significantly decreased by inhibitors of PI3K/Akt and p38, not by ERK or JNK inhibitors. Moreover, the activities of Akt and p38, known from their phosphorylation, were increased by GJ. These data suggest that GJ induces HO-1 expression through PI-3K/Akt and p38 signaling in microglial cells. Recently, Cheng and Lee described that MAPK and PI-3K/Akt pathways are involved in the phosphorylation of Nrf2 to facilitate disassociation with Keap1 and nuclear translocation [6]. Thus GJ-induced activities of PI-3K/Akt and p38 might promote the disassociation from Keap1 and nuclear translocation of Nrf2, although the effects of kinase inhibitors on Nrf2 were not examined in this study.

In conclusion, we demonstrated that GJ inhibited NO release and iNOS expression in LPS-stimulated macrophages, and these effects are mediated by PI-3K/Akt and p38-dependent HO-1 expression. Our finding could help us to understand the molecular mechanism of anti-inflammatory action of GJ, although it was examined *in vitro,* and suggest that GJ may have therapeutic potential for treatment of neuroinflammatory diseases.

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초록 : 소교세포에서 heme oxygenase-1 발현 유도를 통한 치자(*Gardenia jasminoides*)의 항염증 효과

송지수 · 신지은 · 김지희 · 김영희*
(부산대학교 자연과학대학 분자생물학과)

치자 열매는 아시아에서 음식과 옷의 염료로 사용되어 왔다. 본 연구에서는 BV-2 소교세포에서 치자열매 열수 추출물(GJ)의 항염증 효과를 관찰하고 그 작용 기전을 연구하였다. GJ는 세포에 독성을 유도하지 않으면서 lipopolysaccharide로 인한 nitric oxide (NO) 분비와 inducible nitric oxide synthase (iNOS) 생성 및 활성산소 생성을 억제하였다. 또한 GJ는 농도의존적으로 heme oxygenase-1 (HO-1)의 발현을 유도하였다. 더군다나 HO-1 siRNA를 처리했을 때는 GJ가 iNOS의 발현을 억제하지 못하였다. GJ는 HO-1의 발현에 관여하는 전사인자인 nuclear factor E2-related factor 2를 핵으로 이동시켰다. 또한 GJ에 의한 HO-1의 발현은 phosphatidylinositol 3-kinase (PI-3K) 및 p38 kinase 억제제에 의해 감소되었으며, GJ가 Akt와 p38 kinase의 인산화를 유도하였다. 이상의 결과를 종합해보면, GJ는 PI3K/Akt 및 p38 신호전달과정을 통해 HO-1의 발현을 유도함으로써 NO와 같은 염증 매개물질의 생성을 억제한다는 것을 알 수 있다. 이러한 연구결과는 치자열매가 신경염증을 억제하는 새로운 기전을 밝힌 것이다.