Abstract. Geranium thunbergii Sieb. et Zucc. (GT; which belongs to the Geraniaceae family) has been used as a traditional medicine in East Asia for the treatment of inflammatory diseases, including arthritis and diarrhea. However, the underlying mechanisms of the anti-inflammatory effects of GT remain poorly understood. In the present study, we examined the mechanisms responsible for the anti-inflammatory activity of GT in macrophages. The results revealed that GT significantly inhibited the lipopolysaccharide (LPS)- and interferon-γ (IFN-γ)-induced expression of pro-inflammatory genes, such as inducible nitric oxide synthase, tumor necrosis factor-α and interleukin-1β, as shown by RT-PCR. However, the inhibitory effects of GT on LPS- and IFN-γ-induced inflammation were associated with an enhanced nuclear factor erythroid 2-related factor 2 (Nrf2) activity, but not with the suppression of nuclear factor (NF)-κB activity, as shown by western blot analysis. In addition, in bone marrow-derived macrophages (BMDM) isolated from Nrf2 knockout mice, GT did not exert any inhibitory effect on the LPS- and IFN-γ-induced inflammation. Taken together, our findings indicate that the anti-inflammatory effects of GT may be associated with the activation of Nrf2, an anti-inflammatory transcription factor.

Introduction

Inflammation is an essential part of innate immunity that protects the host from exogenous pathogens (1). However, uncontrolled inflammation is closely associated with tissue damage, and consequently causes diseases with uncontrolled inflammation (2). Therefore, the regulation of inflammatory responses remains a challenge. Among the various types of cells comprising the innate immune system, macrophages play central roles in the initiation and resolution of inflammation, mainly through phagocytosis, the production of reactive oxygen species (ROS) and the release of inflammatory cytokines (3). Thus, the suppression of macrophage activation is considered an important strategy for the treatment of these inflammatory diseases (4).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key transcription factor that regulates the expression of various phase II detoxifying and antioxidant genes, including glutamate-cysteine ligase catalytic subunit (GCLC), NAD(P)H: quinine oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1) (5). Under normal conditions, Nrf2 is retained in the cytoplasm in low abundance. When Nrf2 is activated by external stimuli, such as oxidative stress, electrophiles and chemical activators, it translocates to the nucleus and binds to its cis-acting antioxidant response element (ARE) sequence, and induces the expression of cytoprotective proteins (6). In addition, Nrf2 seems to play an important role in ameliorating inflammation. The genetic ablation of Nrf2 in knockout mice has been shown to lead to a significantly increased susceptibility to a variety of inflammatory diseases, such as acute lung injury, inflammatory bowel disease and rheumatoid arthritis (7-9). Thus, Nrf2 may be a therapeutic target for regulating these inflammatory diseases.

Herbal extracts have long been prescribed for the treatment of a variety of inflammatory diseases in Asian countries (10).
Among the traditional herbal medicines, several herbs such as *Salvia miltiorrhiza* (11), *Amomum compactum* (12), *Gleditsia sinensis* (13) and *Alisma orientale* (14) have been found to suppress inflammation by activating Nrf2. In our ongoing effort to find novel candidate agents for the activation of Nrf2, we found that *Geranium thunbergii* Sieb. et Zucc. (GT; which belongs to the Geraniaceae family), known as Jilipul in Korean, Gennoshoko in Japanese and Oriental geranium in English, enhances the transcriptional activity of Nrf2. GT is a medicinal herb used for the treatment of diverse diseases, including arthritis (15), hemorrhaging, infection (16), diarrhea and dysentery (16). Previous studies have revealed that the extract of the GT whole plant has anti-mutagenic (17), antioxidant (18), anti-obesity (19) and anti-inflammatory activities (15,20). In addition, phytochemicals on GT have reported the extraction of tannins, lignans and flavonoids, such as geraniin, corilagin, ellagic acid, gallic acid, quercetin, kaempferol, kobusin, 4-hydroxykobusin and 7,7-dihydroxybuserhenin (16,21-23).

In this study, we investigated the inhibitory effects of GT on inflammatory responses elicited by interferon-γ (IFN-γ) and bacterial lipopolysaccharides (LPS). In addition, we determined whether these anti-inflammatory effects are dependent on Nrf2 activation using bone marrow-derived macrophages (BMDM) obtained from wild-type and Nrf2 knockout mice. The results revealed that GT suppressed the inflammatory response through Nrf2-dependent mechanisms.

Materials and methods

Preparation of GT extracts. Whole plants of GT, which were grown and collected in Gyeongsangbuk-do province in Korea in 2011, were purchased from Omniherb Co. (Daegu, Korea) and authenticated by Professor S. Lee at the College of Korean Medicine, Sangji University, Wonju, Korea. A voucher specimen (no. sjomph003) is kept at the College of Korean Medicine, Sangji University. The air-dried whole plant of GT (50 g) was cut and extracted with 50% EtOH (500 ml, twice) at 60˚C for 3 h, assisted by ultrasonic waves (40 kHz). The extract was filtered with filter paper (6 µm; Whatman PLC, Kent, UK) and concentrated using a rotary evaporator (Eyela, Tokyo, Japan). Subsequently, the extract was lyophilized using a freeze dryer (Labconco, Kansas City, MO, USA) to yield 14.2 g of powder. The solution was filtered through 0.45 µm membrane filter and HPLC was performed.

Reagents and antibodies. TLR4-specific E. coli LPS was purchased from Alexis Biochemicals (San Diego, CA, USA). Mouse INF-γ was supplied by R&D Systems (Minneapolis, MN, USA). All antibodies used in this study, including antibodies to Nrf2 (SC-13032), the p65 subunit of nuclear factor-xB (NF-xB; SC-8008), lamin B (SC-365962) and hnRNP (SC-10030R) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All chemicals and reagents, including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sulforaphane (SFN) and N-acetyl cysteine (NAC) were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless indicated otherwise.

High performance liquid chromatography (HPLC) analysis of GT. HPLC analysis was performed using an Agilent 1200 series system (Agilent Technologies, Santa Clara, CA, USA), which consisted of a solvent delivery unit, an on-line degasser, a column oven, an autosampler and a multi-wavelength detector. For data analysis, LC solution software (version 1.24) was used. The analytical column used was a Phenomenex Gemini NX-C18 column (Phenomenex Inc., Torrance, CA, USA; 4.6x250 mm; pore size, 3.5 µm). The mobile phases were solvent A [0.1% formic acid aqueous (v/v)] and solvent B (acetonitrile). The gradient flow was as follows: (A)/(B) = 95/5 (0 min) → (A)/(B) = 30/70 (60 min). The column temperature was maintained at 35˚C. The analysis was carried out at a flow rate of 0.6 ml/min with detection at 275 nm. The column injection volume was 10 µl. A standard solution, containing geraniin (ChemFaces, Wuhan, China) and quercetin (Sigma-Aldrich), was prepared by dissolving in distilled water (10 mg/100 ml). The solution was filtered through a 0.45 µm membrane filter and HPLC was performed.

Mice. Male C57BL/6 mice, inbred in a specific pathogen-free facility, were purchased from Samtako Bio Korea, Ltd. (Osan, Korea). Nrf2 knockout mice, which were generated from C57BL/6 mice, were a generous gifted from Dr Jefferson Chan (Pathology and Laboratory Medicine, University of California, Irvine, CA, USA). The animals were housed in certified, standard laboratory cages, and allowed access to food and water *ad libitum* prior to use in the experiments. All experimental procedures followed the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health of Korea, and all the experiments were approved by the Institutional Animal Care and Use Committee of Pusan National University, Pusan, Korea.

Cell culture. RAW264.7 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing L-glutamine (200 mg/l; Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen) and maintained in a humidified incubator at 37˚C and 5% CO₂ prior to use in the experiments. BMDM were prepared as previously described (24). Briefly, following the asphyxiation of the mice with CO₂, cellular material from the femurs was aspirated, pressed through a nylon mesh filter (30 µm; Merck Millipore, Billerica, MA, USA), and centrifuged at 400 x g at 4˚C for 5 min. Subsequently, the cells were re-suspended in DMEM containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen) and maintained in a humidified incubator at 37˚C and 5% CO₂ prior to use in the experiments.

Cell viability assay. The viability of the RAW264.7 cells was assessed using an MTT-based colorimetric assay. In brief, MTT solution (2.0 mg/ml) was added to each well of the cells cultured in a 96-well plate. At 4 h after incubation at 37˚C in a CO₂ cell culture incubator, the supernatants were removed and
the formazan crystals formed in viable cells were measured at 540 nm using a microplate reader (VICTOR3; Perkin Elmer, Waltham, MA, USA). The percentage of living cells was calculated against the untreated cells.

Isolation of RNA and RT-PCR. Total RNA was isolated from the tissues and cells using a Qiagen RNeasy mini kit (Qiagen, Hilden, Germany). Three micrograms of total RNA were then reverse transcribed using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and single-stranded cDNA was amplified by PCR with a set of specific primers as follows: inducible nitric oxide synthase (iNOS) forward, 5'-CTGCGACCTTGGATCAAGGAACC-3' and reverse, 5'-GGGAGTACCGCTTGTGTCACCTGGAA-3'; tumor necrosis factor-α (TNF-α) forward, 5'-CTACTCTCAGAGCCCCCAG-3' and reverse, 5'-AGGCACACTGGAAGACCTCTCC-3'; interleukin-1β (IL-1β) forward, 5'-GTGTCTTTCCGGTGACTTCT-3' and reverse, 5'-TGGTGTCTTTGCTCCCTCCT-3'; NQO1 forward, 5'-GCAATGCTTTTCCATACACC-3' and reverse, 5'-TGAGTGGCCAATGTCT-3'; HO-1 forward, 5'-TGAAAGGGCCACCAAGGAGG-3' and reverse, 5'-AGAGGTCCACCACTGAGG-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-GGAGCCAAAAAGGGTCATCAT-3' and reverse, 5'-GTGATGGCATGGACTGCCTTG-3'; IL-1α forward, 5'-CTGCAGCTGGGACCTCCT-3' and reverse, 5'-AGGCAACCTGACCACTCTCC-3'; and interleukin-1β (IL-1β) forward, 5'-CTACTCCTCAGAGCCCCCAG-3' and reverse, 5'-AGGCACACTGGAAGACCTCTCC-3'.

Results

GT reduces the INF-γ- and LPS-induced expression of inflammatory-related genes. At first, the extract was analyzed for the constituents of GT by comparing it with the known components, geraniol and quercetin (23,26) (Fig. 1A and B). Subsequently, we determined an optimal dose of GT, at which does not show any significant cytotoxicity, by MTT assay. GT showed no significant cytotoxicity to the RAW264.7 cells at concentrations of up to 100 µg/ml (Fig. 1C). The half maximal inhibitory concentration (IC_{50}) of GT to affect the viability of the RAW264.7 cells was >1,000 µg/ml. Thus, we used GT at concentrations of up to 100 µg/ml for the subsequent experiments.

In order to examine the effects of GT on INF-γ- and LPS-induced inflammation, the cells were pre-treated with GT for 4 h prior to stimulation with INF-γ- and LPS, and the expression levels of NF-xB-dependent genes were analyzed by RT-PCR. The results revealed that GT significantly reduced the levels of NF-xB-dependent pro-inflammatory genes, including iNOS, TNF-α and IL-1β (Fig. 2). The IC_{50} values of GT to affect the expression of inflammatory genes were approximately 60 µg/ml for iNOS, 63 µg/ml for TNF-α and 75 µg/ml for IL-1β.

GT enhances Nrf2 activity, but does not suppress NF-xB activity. To elucidate the mechanisms responsible for the anti-inflammatory effects of GT, we first examined the suppressive effects of GT on the INF-γ- and LPS-induced NF-xB activity. Of note, the transcriptional activity of NF-xB, measured by luciferase assay using NF-xB reporter cells, was not reduced by treatment with GT (Fig. 3A). The nuclear translocation of NF-xB was also not altered (Fig. 3B and C). However, GT induced the transcriptional activity of Nrf2 in a dose-dependent manner (Fig. 4A). The nuclear translocation of Nrf2 was also significantly increased by treatment with GT in a dose-dependent manner (Fig. 4B and C).

The time-dependent activation of Nrf2 was determined by detecting the nuclear translocation of Nrf2 by western blot
Figure 1. Chromatographic analysis and cytotoxic effects of *Geranium thunbergii* (GT). (A) HPLC chromatogram of mixed standard solutions, geraniin and quercetin. (B) HPLC chromatogram of 50% aqueous-ethanol extract of GT. (C) RAW264.7 cells were treated with the indicated concentrations of GT for 24 h, and the viability of the cells was examined by MTT assay. The data from 3 independent experiments are represented as the means ± SD. *p<0.05 compared to the controls.

Figure 2. *Geranium thunbergii* (GT) suppresses the expression of pro-inflammatory genes. RAW264.7 cells were treated with the indicated concentrations of GT for 4 h, prior to stimulation with interferon-γ (IFN-γ) and lipopolysaccharides (LPS) (10 ng/ml, respectively). The cells were cultured for 16 h. (A) The expression of pro-inflammatory genes, including inducible nitric oxide synthase (iNOS), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) was evaluated by RT-PCR. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. (B) The intensity of the bands was densitometrically quantified and calculated as the mean ± SD of 3 independent experiments. *p<0.01 compared to the negative control (first column); *p<0.05 and **p<0.01 compared to the positive control (second column).
The results revealed that the nuclear translocation of Nrf2 was significantly increased at 2 to 8 h of treatment with 100 µg/ml of GT (Fig. 5A and B). In order to exclude the possibility that ROS activates Nrf2 to protect the cells from oxidative damage (27), we examined the production of ROS by the GT-treated RAW264.7 cells. GT did not significantly increase ROS production. Taken together, these results indicate that the GT-induced transcriptional activity of Nrf2 is not mediated by ROS (Fig. 5C).

**GT enhances the expression of Nrf2-dependent genes.** The expression of Nrf2-dependent antioxidant genes may contribute to the suppression of inflammatory responses (28). Thus, we examined the expression levels of Nrf2-dependent genes, including NQO1, HO-1 and GCLC. The results revealed a dose-dependent increase in the expression of Nrf2-dependent genes, which positively correlated with Nrf2 activity (Fig. 6). The expression levels of Nrf2-dependent genes were increased by 3.4-fold for NQO1, 2.8-fold for HO-1 and by 3.1-fold for GCLC when the cells were treated with 100 µg/ml of GT.

**The anti-inflammatory effects of GT are Nrf2-dependent.** Subsequently, we investigated the possibility that Nrf2 activation has a direct association with the anti-inflammatory effects of GT. The macrophages derived from the bone marrow of wild-type C57BL/6 mice showed a similar response to GT treatment. Namely, the expression of NF-κB-dependent pro-inflammatory genes was significantly reduced by treatment with GT in a dose-dependent manner (Fig. 7A and C). However, in the macrophages derived from the bone marrow of Nrf2 knockout mice, the expression of NF-κB-dependent genes was not diminished. On the contrary, the expression of...
TNF-α was increased in accordance with the concentration of GT (Fig. 7B and D). These results clearly indicate that the anti-inflammatory effects of GT are related to Nrf2 activity.

Discussion

The regulation of macrophage activation is important for the initiation and resolution of inflammation (2). Classically activated macrophages are induced by combined stimulation with LPS and IFN-γ; these macrophages express a unique set of genes, including TNF-α, IL-1β, IL-6, cyclooxygenase-2 (COX-2) and iNOS (29). Thus, the combined stimulation of macrophages with LPS and IFN-γ is used to create in vitro models of acute and chronic inflammatory conditions, including septic shock, rheumatoid arthritis, inflammatory bowel disease and chronic obstructive pulmonary disease (30).

The high production of cytokines, such as TNF-α, IL-1β is considered to be a major factor involved in the induction and maintenance of inflammation (29). In addition, iNOS serves as a pro-inflammatory agent by producing nitric oxide, a mediator of the inflammatory response (31). The expression of these genes is mainly regulated by NF-κB; thus the classical strategy for the development of therapeutic agents against inflammation has focused on the suppression of NF-κB activity (32).

In the present study, we demonstrated that GT, a medicinal herb used for the treatment of diarrhea and dysentery (16), exerted an inhibitory effect on LPS- and IFN-γ-induced inflammation at non-toxic doses (Figs. 1 and 2). Firstly, it was assumed that the anti-inflammatory effects of GT on LPS- and IFN-γ-stimulated macrophages may be due to the suppression of NF-κB. However, surprisingly to us, GT did not reduce the transcriptional activity or nuclear translocation of NF-κB (Fig. 3).

As NF-κB also has many beneficial effects on organisms, including protection against infection, the activation of the immune response and the regulation of energy metabolism (33,34), researchers have focused on Nrf2 as an alternative molecular target for suppressing inflammation (6,35,36). The disruption of Nrf2 using a knockout technique has been shown to enhance NF-κB activity in diverse disease models (36,37).
In general, the Nrf2-mediated anti-inflammatory effects are thought to be achieved through the activation of antioxidant enzymes and the consequent suppression of the ROS-NF-κB signaling pathway, but not through direct interaction between Nrf2 and NF-κB (38,39). Thus, we also examined the effect of GT on Nrf2 activity. The results revealed that GT enhanced Nrf2 activity without inducing significant cytotoxicity or ROS production (Figs. 4 and 5). In addition, the expression of Nrf2-dependent antioxidant genes, including NQO1, HO-1 and GCLC were also increased by treatment with GT (Fig. 6). From these results, it was can by hypothesized that the inhibitory effects of GT on IFN-γ- and LPS-induced inflammatory responses may be related to Nrf2 activation.

To confirm this possibility, we examined the suppressive effects of GT on the expression of NF-κB-dependent genes in BMDM isolated from Nrf2 knockout mice. In BMDM from Nrf2 knockout mice, the mRNA levels of NF-κB-dependent genes, including TNF-α, IL-1β and iNOS, were not reduced by treatment with GT (Fig. 7A-D). Taken together, these data indicate that the inhibitory effects of GT on IFN-γ- and LPS-induced inflammation are related, at least in part, to the activation of Nrf2 (Fig. 7E). Most Nrf2 activators, such as
cafeic acid phenethyl ester (40), curcumin (41), quercetin (42) and SFN (43), have dual functions; they activate Nrf2 and inhibit NF-κB. Unlike in previous studies, our results demonstrated that GT does not directly affect NF-κB activity (Fig. 3A-C). Other previous studies (13,25,44,45) have also demonstrated that the Nrf2-dependent activation of antioxidant systems reduces inflammation without the suppression of NF-κB. The present study had the limitation of addressing Nrf2 activation in a cell type specific manner, specifically, in macrophages. Nevertheless, it is obvious that the inhibitory effects of GT on IFN-γ- and LPS-induced inflammation are dependent on Nrf2 activation. In addition, our results suggest that GT contains lead compounds which may be used for the effective treatment of macrophage-mediated inflammation.

In this study, to the best of our knowledge, we provide the first experimental evidence that GT exerts inhibitory effects on LPS- and IFN-γ-induced inflammation by enhancing Nrf2 activity without directly affecting NF-κB activity. These results suggest that the therapeutic effects of GT in traditional usage for various inflammatory diseases are, at least in a part, associated with the activation of Nrf2, a key transcription factor in anti-inflammatory systems. Our results also raise the possibility that GT may be used as a candidate agent for the further development of novel and specific therapies aiming at promoting Nrf2 activation.

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