Research Article

Roots of *Erigeron annuus* Attenuate Acute Inflammation as Mediated with the Inhibition of NF-κB-Associated Nitric Oxide and Prostaglandin E₂ production

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*Erigeron annuus* is a naturalized plant belonging to Compositae (asteraceae) family, which is called the annual fleabane, and commonly found at meadows and roadside. This study investigated the anti-inflammatory effects of the extract of *E. annuus* roots (EER), as assessed by the paw edema formation and histological analysis in rat, and the productions of nitric oxide (NO), prostaglandin E₂ (PGE₂), and pro-inflammatory cytokines in Raw264.7 murine macrophages. Carrageenan treatment promoted infiltration of inflammatory cells and caused swelling in the hind paw. Oral administrations of EER (0.3 g/kg and 1 g/kg) attenuated acute inflammation similar to the result using dexamethasone (1 mg/kg). Treatment of macrophages with lipopolysaccharide (LPS) simulated inflammatory condition: LPS significantly increased the productions of NO, PGE₂, and proinflammatory cytokines. EER suppressed activation of macrophages, preventing the induction of iNOS and COX-2 protein expressions. LPS treatment induced phosphorylation of I-κBα and increased the level of nuclear NF-κB protein, both of which were suppressed by concomitant treatment of EER. In conclusion, EER ameliorated acute inflammation in rats, and the induction of NO, PGE₂, and proinflammatory cytokines in Raw264.7 cells. EER's effects may be associated with its inhibition of NF-κB activation, suggesting its effect on inflammatory diseases.

1. Introduction

Inflammation of human body is one of the most important biological responses to harmful stimuli (e.g., pathogens or damaged cells) during the host defense mechanism. Acute inflammatory response initiates pathological process of numerous disorders such as infection, bacterial sepsis, cancer, and chronic inflammation in specific organ (i.e., hepatitis and arthritis) [1–6]. In the progression of inflammation, the representative pathological symptoms are characterized by an increased blood flow to the peripheral tissue by augmentation of vascular permeability, which leads to cellular infiltration and swelling [7].

Macrophage is one of the most important phagocytic cells and plays a crucial role in the process of inflammation by producing proinflammatory mediators. Lipopolysaccharide (LPS) is the major component of outer membrane of gram-negative bacteria and has the ability to directly activate macrophages to secrete the inflammatory mediators such as NO and PGE₂ [8–12]. Nitric oxide (NO) is produced by three distinct isoforms of nitric oxide synthases (NOS) (i.e., neural (nNOS), endothelial (eNOS), and inducible (iNOS)). Among them, iNOS is a key regulator for inducing a large quantity of NO during inflammation and is indicated in many cell types such as macrophage, endothelial, and epithelial cells [13, 14]. PGE₂ is also another important inflammatory mediator which is produced from arachidonic acid through the catalytic reaction by COX-2 [15, 16]. It has been shown that iNOS and COX-2 are transcriptionally activated by NF-κB, one of the most important transcription factors regulating the immune responses, cell adhesion, and survival [17–19].
Erigeron annuus (E. annuus) is a naturalized plant belonging to Composite (Asteraceae) family and commonly found at meadows and roadside. In traditional oriental medicine, E. annuus has been used to treat indigestion, epidemic hepatitis, enteritis, lymphadenitis, and hematuria [20, 21]. However, the effects of E. annuus roots have not been elucidated. In this study, we investigated the potential effect of the extract of E. annuus roots (EERs) on acute inflammation induced by carrageenan injection in rats. Furthermore, this study identified EER as an active component having an ability of the inhibitory effects on LPS-stimulated iNOS and COX-2 expressions as well as TNF-α and IL-1β productions in RAW264.7 macrophages. In view of importance of the inflammation in the process of human disease, these results show the pharmacological effects of EER in terms of the treatment of inflammatory diseases.

2. Materials and Methods

2.1. Preparation of the EER. E. annuus was collected by ourselves in May 2011 from Sangdong, Suseonggu, Daegu, Korea, and identified by Professor Sang Chan, Kim Ph.D. (College of Oriental Medicine, Daegu Haany University, Korea). The roots part of E. annuus were washed twice with water and then dried in the air. EERs were prepared with methanol at room temperature for 72 h. The extract was filtered through a 0.2 µm filter (Nalgene, New York, NY, USA), lyophilized, and stored at −20 °C until use. The amount of EERs was estimated by the dried weight of lyophilized EER. The yield of lyophilized EER was 0.89%.

2.2. Analysis of EER. Analysis of EER was performed on an Agilent 6890 gas chromatography equipped with a 5975 GC/MS selective detector (Agilent, CA, USA). Separations were performed in a 30 m length × 0.25 mm i.d. and 0.25 µm film thickness fused silica capillary column HP-5MS (Agilent, CA, USA). The carrier gas was ultrapure helium with a flow of 1 mL/min, and the splitless injector temperature was set as 280 °C. The column temperature program was the initial temperature of 70 °C for 4 min and increased by 2 °C/min to 100 °C (held 2 min). After that the temperature was varied from 100 to 200 °C at 5 °C/min (held 20 min) and increased to 280 °C (held 5 min) at 10 °C/min, in a total run time of 73 min. Mass spectral analyses were performed using the NIST05 library resident in the computer. Percentage composition was calculated using the area normalization method (see Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2013/297427).

2.3. Materials. Anti-NF-κB p65 and horseradish peroxidase-conjugated goat antirabbit were supplied from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antiphospho-1-κBα (p-1-κBα), anti-Lamin A, horseradish peroxidase-conjugated antimouse, and antigeno, IgGs were purchased from Cell Signaling (Beverely, MA, USA). Antimurine iNOS was purchased from BD Bioscience (San Jose, CA, USA). Anti-COX-2 antibody was purchased from Cayman (Ann Arbor, Mi, USA). Polyethylene glycol number 400 (PEG) solution, carrageenan, dexamethasone, and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.4. Animal Experiment. Animal experiments were conducted under the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Daegu Haany University [12]. Sprague-Dawley rats at 4 weeks of age (male, 80–100 g) were provided from Hyochang Sience (Daegu, Korea), acclimatized for 1 week, and maintained in a clean room at the Animal Center for Pharmaceutical Research, College of Oriental Medicine, Daegu Haany University. Animals were caged under the supply of filtered pathogen-free air, commercial rat chow (Purina, Korea), and water ad libitum at a temperature between 20 and 23 °C with 12 h light and dark cycles and relative humidity of 50%.

2.5. Carrageenan-Induced Paw Edema. Rats (N = 20) were randomly divided into four groups, and thus each group consisted of five animals. EER, dissolved in 40% polyethylene glycol (PEG) number 400, was orally administered to rats at the dose of 0.3 or 1 g/kg/day for four consecutive days. Dexamethasone (1 mg/kg/day), an anti-inflammatory drug, was used as a positive control [22]. To induce acute phase inflammation in paw, rats were injected subcutaneously into the right hind paw with a 1% solution of carrageenan dissolved in saline 30 min after last treatment of vehicle or EER. The paw volumes were measured up to 4 h after the injection at intervals of 1 h. The hind paw volume was determined volumetrically by measuring with a plethysmometer (Ugo Basile, VA, Italy). After euthanasia using ether, the hind paw samples were collected.

2.6. Histological Process. The hind paw skins ventrum pedis skins were separated and fixed in 10% neutral buffered formalin, then embedded in paraffin, sectioned (3–4 µm), and stained with hematoxylin and eosin (H and E) [23]. The histopathological profiles of each sample were observed under light microscope (Nikon, Japan).

2.7. Histomorphometry. The thicknesses of ventrum pedis skins (from epidermis to dermis, keratin layers were excluded) were measured using automated image analyzer (DMI-300 Image Processing, DMI, Korea) under magnification 40 of microscopy (Nikon, Japan) at prepared skin histological samples as mm/paw. The infiltrated inflammatory cells were also counted using automated image analyzer as cells/mm² of dermis under magnification 200 of microscopy [23].

2.8. Cell Culture. Raw264.7 cell, a murine macrophage cell line, was obtained from American Type Culture Collection (Rockville, MD, USA). The cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 mg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO₂. For all experiments, the cells were grown to 80–90% confluency and were subjected to no more than 20 cell passages. Raw264.7 cells were incubated with 1 µg/mL.
**2.1. Enzyme-Linked Immunosorbent Assay (ELISA).** Raw264.7 cells were preincubated with EER for 1 h and continuously incubated with LPS for 24 h [12]. TNF-α, IL-1β (Endogen, Woburn, MA, USA), and PGE\(_2\) (RnD Systems, Minneapolis, MN, USA) contents in the culture medium were measured by ELISA using antimouse TNF-α, IL-1β, and PGE\(_2\) antibodies and biotinylated secondary antibody according to the manufacturer's instruction.

**2.12. Immunoblot Analysis.** Cells were lysed in the buffer containing 20 mm Tris-HCl (pH 7.5), 1% Triton X-100, 137 mm sodium chloride, 10% glycerol, 2 mm EDTA, 1 mm sodium orthovanadate, 25 mm b-glycerophosphate, 2 mm sodium pyrophosphate, 1 mm phenyl methyl sulfonfl fluoride, and 1 mg/mL leupeptin [12]. Cell lysates were centrifuged at 15,000 g for 10 min to remove debris. The immunoreactive bands were visualized using enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences Corp., Piscataway, NJ, USA) according to the manufacturer's instructions. Equal loading of proteins was verified by actin or lamin immunoblottings.

**2.13. Scanning Densitometry.** Densitometric measurements of the bands were made using an image analyzing system (Ultra-Violet Products Ltd., Upland, CA, USA). Repeated experiments were separately performed to confirm changes.

**2.14. Statistical Analysis.** One-way analysis of variance (ANOVA) was used to assess statistical significance of differences among treatment groups. For each statistically significant effect of treatment, the Newman-Keuls test was used for comparisons between multiple group means. The data were expressed as means ± 95% confidence intervals (CI). All statistical tests were two sided.

### 3. Results

**3.1. Inhibition of Carrageenan-Induced Paw Edema.** To verify inhibitory effects of EER on acute inflammation in vivo, we used the carrageenan-induced paw edema model, which is a well-established model for screening the efficacy of anti-inflammatory drugs [24]. Paw edema formation by carrageenan was observed at 3 h and persisted at least up to 4 h after injection (Figure 1). Maximal induction of paw swelling by carrageenan was observed at 3 h. Administration of EER (0.3 and 1 g/kg/day, p.o., for 4 days) inhibited the ability of carrageenan to induce paw swelling. We also confirmed that dexamethasone (1 mg/kg/day, p.o., for 4 days), a positive control, inhibits the edema formation.

**3.2. Inhibition of Carrageenan-Induced Acute Inflammation.** In addition, we confirmed the effects of EER on histological profiles of ventrum pedis skin stained with H and E. As shown in Figure 2(a), carrageenan successfully induced paw...
swelling in ventrum pedis in rats. However, pretreatment of EER at the oral doses of 0.3 and 1g/kg for 4 days significantly blocked the changes of the thickness of ventrum pedis (Figure 2(b)). Moreover, injection of carrageenan markedly increased infiltration of inflammatory cells, which were inhibited by treatment of EER (Figures 2(c) and 2(d)). Dexamethasone also decreased the number of infiltrated inflammatory cells. These results suggest that repression of paw swelling and of infiltration of inflammatory cells by EER may represent an important efficacy in association with the inhibition of acute inflammation in rats.

3.3. Inhibition of LPS-Inducible NO, PGE₂, and Proinflammatory Cytokines Production. Next, our studies were extended to determine the effects of EER in vitro. First, we examined any possible toxicity of EER in Raw264.7 cells. In MTT assay, cell viability was not affected by EER
treatment up to 30 μg/mL (data not shown). We chose 3–30 μg/mL concentrations of EER to determine the effect of EER on NO accumulation in LPS-stimulated Raw264.7 cells. LPS (1 μg/mL, 24 h) significantly increased NO production, which was markedly inhibited by EER (3, 10, and 30 μg/mL) in a concentration-dependent manner (i.e., 29%–50%) (Figure 3(a)). PGE$_2$ production in LPS-treated Raw264.7 cells was also inhibited by EER (3 and 10 μg/mL) (Figure 3(b)).

Cytokine is known as one of the major mediators in inflammatory responses. To confirm the inhibition of proinflammatory cytokines, we verified the effects of EER on TNF-α and IL-1β productions in Raw264.7 cells. LPS stimulation significantly increased the productions of TNF-α and IL-1β...
in culture media of Raw264.7 cells (Figure 3(c)). Treatment of EER at 10 μg/mL inhibited the ability of LPS to induce the cytokines accumulation (Figure 3(c)). Moreover, EER treatment alone had no effects on macrophage activation. These results suggest that EER blocked the activation of macrophage cells in terms of inhibition of NO, PGE\(_2\), and cytokines.

3.4. Inhibition of LPS-Inducible iNOS and COX-2 Protein Expression. iNOS and COX-2 are key enzymes in the pathophysiological process of inflammation and the induction of inflammatory mediators such as NO and PGE\(_2\). Next, we determined the protein expressions of iNOS and COX-2 by western blot analysis. LPS treatment at 1 μg/mL for 18 h significantly induced protein levels of iNOS and COX-2 in Raw264.7 cells (Figure 4(a)). Densitometer analysis revealed that EER treatment (3 and 10 μg/mL) at 1 h prior to LPS significantly inhibited the iNOS and COX-2 protein expressions in Raw264.7 cells (Figure 4(b)).

3.5. Inhibition of LPS-Inducible NF-κB Activation. NF-κB is one of the most important transcription factors for the induction of the inflammatory genes such as iNOS and COX-2 in immune cells stimulated with inflammatory inducers including LPS [17–19]. NF-κB is translocated to the nucleus by phosphorylation of I-κBα and subsequent degradation of I-κBα subunit. Treatment of Raw264.7 cells with EER for 1 h markedly inhibited the nuclear level of NF-κB induced by LPS (Figure 5(a)). Moreover, exposure of LPS decreased the increased phosphorylation of I-κBα protein level at 30 min, which was also blocked subsequent treatment of EER (Figure 5(b)). These results suggest that EER might prevent translocation of NF-κB to the nucleus by inhibiting I-κBα phosphorylation.

4. Discussion

E. annuus is called the annual fleabane, which is a plant indigenous to Eastern North America widely found at meadows and roadside [20]. It is well known to be used as the treatment of indigestion, epistaxis, hematomas, lymphanitis, and hematuria in traditional oriental medicine [25, 26]. Recently, it has been studied that each part of E. annuus has different pharmaceutical efficacies. The flower extract E. annuus had anti-inflammatory effect through the induction of hemeoxygenase-1, and its leaf extract was shown to have neuroprotective and antioxidant effects against oxidative stress induced by H\(_2\)O\(_2\) [27, 28]. Nevertheless, the scientific proof and mechanistic basis for the effect of E. annuus roots have almost not been studied. In this study, we determined anti-inflammatory effects of EER in vivo and in vitro for the first time.

Swelling is one of the most important symptoms of acute inflammation and characterized by an increase in vascular permeability and infiltration of cells. Therefore, its induction by carrageenan is a well-established model to screen novel anti-inflammatory drug [22, 29, 30]. In our study, carrageenan injection successfully creates acute inflammation in the peripheral tissue, as indicated by increases in the number of inflammatory cells and skin thicknesses in ventrum pedis. Also, carrageenan induced paw swelling during 1–4 h (maximally 3 h) after injection. These results are consistent with previous observations, showing that carrageenan induces infiltration of immune cells and resultantly increased inflammatory response [29, 30]. Next, we assessed the effects of EER in rats injected with carrageenan. Administration of EER inhibited the infiltration of inflammatory cell in the tissue and swelling in the paw skin. These results, here, demonstrate that EER could prevent the acute inflammation in vivo.

The pathology of inflammation is started by complex processes stimulated by the endotoxins such as LPS. In this immune defense mechanism, macrophages play an important role in the progression of the human disorder [8]. LPS can directly activate macrophages, which produce the inflammatory mediators such as NO, eicosanoids, TNF-α, and ILS [9, 10]. TNF-α and IL-1β, the representative proinflammatory cytokines, are small secreted proteins mediating immunity and inflammation. TNF-α can activate macrophages and initiate immune responses by stimulating secretion of other cytokines. IL-1β, mainly synthesized by macrophages, is also inflammatory cytokines which play important roles in the acute phase response.

Here, we evaluated the effects of EER on the accumulations of NO and PGE\(_2\) in media of Raw264.7 cells stimulated by LPS. Pretreatment of EER suppressed production of LPS-inducible NO and PGE\(_2\). In addition, EER treatment significantly blocked the production of proinflammatory cytokines, TNF-α, and IL-1β. Moreover, it has been shown that carrageenan promotes the release of NO as well as PGE\(_2\) in the peripheral tissue [31]. Chuha et al. have also shown that carrageenan stimulates the release of TNF-α and ILS. In view of the inhibitory effects of EER on NO and cytokines productions in vitro and the important role of NO and PGE\(_2\) on swelling in vivo, the effects of EER on acute inflammation in rats might result, at least in part, from its inhibition of NO and PGE\(_2\) in the tissues.

NO and PGs play key roles in defense mechanisms against xenobiotic stimuli such as infection; it has been perceived that the proteins (i.e., NOS or COX) are main regulators having harmful role in the pathological process of inflammation. Coffey et al. showed that macrophages from iNOS knock-out animals are protected tissue from acute inflammation [32]. In case of eicosanoids, it has been shown that production of a large amount of PGs have detrimental effects in inflammation-related diseases, which were mainly dependent on the COX. Immunoblot analysis enables us to verify that the treatment of EER significantly suppressed the ability of LPS to induce iNOS and COX-2.

NF-κB is one of important transcription factors in terms of regulation of the genes, which were involved in inflammatory and immune responses, cell adhesion, and survival [17, 18, 33]. Degradation of I-κBα by its phosphorylation causes activation of NF-κB, which enables NF-κB itself to translocate into the nucleus to initiate transcription of the target genes. In this study, we assessed immunoblot analysis to show the effects of EER on NF-κB activation. As shown in Figure 5, pretreatment of EER inhibited phosphorylation
of I-κBα induced by LPS and resultant degradation of I-κBα (data not shown). Also, EER treatment markedly blocked the nuclear level of NF-κB, showing that the effect of EER on the expression of inflammatory genes might result from the inhibition of NF-κB activation. Moreover, it has been shown that I-κBα is phosphorylated by I-κBα kinase (IKK) complex, which may be activated by a line of kinases (e.g., tyrosine kinase family members and mitogen-activated pathway).
**Figure 5:** Inhibition of LPS-induced NF-κB activation by EER. (a) The level of NF-κB protein in nucleus. The cells were treated with LPS or LPS + EER for 1 h. Immunoblottings for lamin A verified equal loading and purity of the nuclear proteins. (b) The level of phosphorylated I-κBα (p-I-κBα) in total lysates. Raw264.7 cells were treated with LPS or LPS + EER for 30 min. Immunoblots are representative results from repeated experiments. Data represents the mean ± S.E.M. from three separate experiments (significant as compared with vehicle-treated control, *P < 0.05, **P < 0.01; significant as compared with LPS alone, #P < 0.05). EERs: extract of *Erigeron annuus* roots.
protein kinases) [34, 35]. It might have value to study the effects of EER on these upstream kinases, which may have the possibility of being a potential pharmacological target of EER.

The chemical compositions of *E. annuus* have been reported by many researchers. Flower of *E. annuus* has a large number of phenolic compounds [36]. The three quinic acid derivatives and four flavonoids were isolated from the leaves and stems of *E. annuus* [25]. The aerial parts or the roots of *E. annuus* contained sesquiterpenoids, diterpenoid, polyacetylenic compound, γ-pyrone derivatives, sterols, triterpenoids, monoterpen hydrocarbons, and monoterpen oxygenated components [37, 38]. Among them, it has been shown that sesquiterpenoids, diterpenoid, sterols, and triterpenoids had anti-inflammatory effects [39–46]. Therefore, it remains to be confirmed what is pharmacologically effective ingredient in the EER in terms of anti-inflammation.

### 5. Conclusions

In our study, we used two approaches to show the effect of EER: (1) an animal model using carrageenan-induced acute inflammation and (2) a cell model using activated macrophage induced by LPS. Oral administrations of EER attenuated acute inflammation, as indicated by the inhibition of inflammatory cells infiltration and paw swelling induced by carrageenan. Treatment of EER in Raw264.7 cells suppressed the activation of the macrophage by preventing the induction of iNOS and COX-2 protein expressions as well as the productions of NO and PGE2, which is related with its inhibition of NF-κB activation. These findings showing here might help to understand the pharmacology of the roots of *E. annuus* and offer the possibility of herbal candidate for the treatment of inflammatory disease.

### Abbreviations

- COX: Cyclooxygenase
- EER: Extract of *Erigeron annuus* root
- I-κB: Inhibitor of κB
- IL: Interleukin
- iNOS: Inducible nitric oxide synthase
- LPS: lipopolysaccharide
- NF-κB: Nuclear factor-κB
- NO: Nitric oxide
- PGE2: Prostaglandin E2
- TNF-α: Tumor necrosis factor-α.

### Conflict of Interests

The authors declare that they have no competing financial interests.

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