Aromadendrin Inhibits Lipopolysaccharide-Induced Nuclear Translocation of NF-κB and Phosphorylation of JNK in RAW 264.7 Macrophage Cells

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Abstract
Aromadendrin, a flavonol, has been reported to possess a variety of pharmacological activities such as anti-inflammatory, antioxidant, and anti-diabetic properties. However, the underlying mechanism by which aromadendrin exerts its biological activity has not been extensively demonstrated. The objective of this study is to elucidate the anti-inflammatory mechanism of aromadendrin in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells. Aromadendrin significantly suppressed LPS-induced excessive production of pro-inflammatory mediators such as nitric oxide (NO) and PGE2. In accordance, aromadendrin attenuated LPS-induced overexpression iNOS and COX-2. In addition, aromadendrin significantly suppressed LPS-induced degradation of IκB, which sequesters NF-κB in cytoplasm, consequently inhibiting the nuclear translocation of pro-inflammatory transcription factor NF-κB. To elucidate the underlying signaling mechanism of anti-inflammatory activity of aromadendrin, MAPK signaling pathway was examined. Aromadendrin significantly attenuated LPS-induced activation of JNK, but not ERK and p38, in a concentration-dependent manner. Taken together, the present study clearly demonstrates that aromadendrin exhibits anti-inflammatory activity through the suppression of nuclear translocation of NF-κB and phosphorylation of JNK in LPS-stimulated RAW 264.7 macrophage cells.

Key Words: Aromadendrin, COX-2, iNOS, JNK, Lipopolysaccharide, NF-κB, RAW 264.7 cells

INTRODUCTION

Macrophages play essential roles in host defense against bacterial infection (Rehman et al., 2012). Macrophages orchestrate the innate immune response to infection by expressing various inflammatory cytokines such as TNF-α, IL-1, NO, and PGE2 (Kim et al., 2012). The most common cause of macrophage activation is an exposure to LPS, the principal component of the outer membrane Gram-negative bacteria (Rietschel and Brade, 1992). However, aberrantly over-activated macrophages have also been attributed to detrimental responses in inflammatory diseases including sepsis, septic shock, or systemic inflammatory response syndrome (Rackow and Astiz, 1991; Shapiro et al., 2010). Therefore, suppression of aberrant activation of macrophage may have valuable therapeutic potential for the treatment of inflammatory diseases such as sepsis.

Aromadendrin is a flavonol, which is a type of flavonoids. Flavonoids have been widely reported to exhibit various pharmacological activities including anti-inflammatory, anti-oxidant, anti-tumor, and neuroprotective properties (Havsteen, 1983; Dixon and Steele, 1999). Aromadendrin has been reported to possess a variety of biological properties such as anti-inflammatory activity (Zhang et al., 2006), radical scavenging activity (Lee et al., 2009), and anti-tumor activity (Kwak et al., 2009). Recently, it has been reported that aromadendrin possesses anti-diabetic activity by stimulating glucose uptake and ameliorating insulin resistance (Zhang et al., 2011). In addition, it has been also reported that aromadendrin derivatives exhibit various biological activities such as anti-proliferative (Duh et al., 2012) and osteogenic actions (Swarnkar et al., 2011). However, the underlying mechanism by which aromadendrin exerts its pharmacological activity, especially anti-inflammatory, has not been clearly demonstrated. Aromadendrin has been reported to be present in various medicinal herbs including Chi-onanthus retusus (Kwak et al., 2009), Cudrania tricuspidata...
(Lee et al., 2009), *Populus davidiana* (Zhang et al., 2006), and *Gelditsia sinensis* (Zhang et al., 2011). Aromadendrin, used in the present study, was isolated from the heartwood of *Hemiptelea davidii* (Chang et al., 2004).

In the present study, to provide a novel pharmacological agent that could suppress aberrantly activated macrophages, the anti-inflammatory activity of aromadendrin and its underlying mechanism were elucidated in LPS-stimulated RAW 264.7 macrophage cells.

**MATERIALS AND METHODS**

**Reagents and cell culture**

Bacterial lipopolysaccharide (LPS) from *Escherichia coli* serotype 055:B5 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Aromadendrin was isolated and identified from *Hemiptelea davidii* (Fig. 1) (Chang et al., 2004). Aromadendrin was dissolved in dimethyl sulfoxide (DMSO) and added to the cell culture at the desired concentrations. The macrophage RAW 264.7 cell line was maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL, Grand Island, NY, USA) containing 5% heat-inactivated fetal bovine serum and penicillin-streptomycin (Gibco BRL) at 37°C, 5% CO₂. In all experiments, cells were incubated in the presence of the indicated concentration of Aromadendrin before the addition of LPS (200 ng/ml).

**Cell viability**

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW 264.7 macrophage cells were seeded at 5×10⁵ cells per well and incubated with aromadendrin at various concentrations for 24 hr at 37°C. After incubation, MTT (0.5 mg/ml in PBS) was added to each well, and the cells were incubated for 3 hr at 37°C and 5% CO₂. The resulting formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Absorbance was determined at 540 nm. The results were expressed as a percentage of surviving cells over control cells.

**Nitrite quantification assay**

The production of NO was estimated by measuring the amount of nitrite, a stable metabolite of NO, using the Griess reagent before the addition of aromadendrin. After aromadendrin pretreatment, RAW 264.7 macrophage cells were stimulated with LPS in 12-well plates for 24 hr, 100 μl of the cell supernatant was mixed with an equal volume of Griess reagent. Light absorbance was read at 540 nm. The absorbance was measured using the protocol of NF-κB activation assay kits (FIVE signaling Technology), iNOS, COX-2, extracellular signal-regulated kinase (ERK), phosphorylated (p)-ERK, p38, p-p38, c-Jun N-terminal kinase (1:1,000; Cell signaling Technology), IkB-α (1:1,000; Santa Cruz Biotechnology Inc), and NFκB (1:2,500; Sigma) were diluted in 5% skim milk. After thoroughly washing with TBST, horseradish peroxidase-conjugated secondary antibodies were applied. The blots were developed by the enhanced chemiluminescence detection (Amersham Biosciences).

**Isolation of nuclear extract**

The isolation of the nuclear fraction was performed according to the protocol of NF-κB activation assay kits (FIVE photon).

**Western blot analysis**

The RAW 264.7 macrophage cells were pretreated with aromadendrin for 1 hr and stimulated with LPS. Cells were washed with PBS and lysed in PRO-PREP lysis buffer (iN-TRON Biotechnology, Seongnam, Korea). Equal amounts of protein were separated on 10% SDS-polyacrylamide gel. Proteins were transferred to Hybond PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA) and blocked in 5% skim milk in TBST for 1 hr at room temperature. Specific antibodies against inducible NO synthase (iNOS), COX-2, extracellular signal-regulated kinase (ERK), phosphorylated (p)-ERK, p38, p-p38, c-Jun N-terminal kinase (1:1,000; Cell signaling Technology), IkB-α (1:1,000; Santa Cruz Biotechnology Inc), and NFκB (1:2,500; Sigma) were diluted in 5% skim milk. After thoroughly washing with TBST, horseradish peroxidase-conjugated secondary antibodies were applied. The blots were developed by the enhanced chemiluminescence detection (Amersham Biosciences).

**RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)**

The RAW 264.7 macrophage cells were treated with aromadendrin in the absence or presence of LPS (200 ng/ml) for 6 hr. Total RNA was isolated using the Total RNA Extraction Kit (iNTRON Biotechnology, Inc, USA) according to the manufacturer’s instruction. The total RNA (2 μg) obtained from cells was reverse-transcribed using oligo-(dT) 15 primers (Promega, Madison, WI, USA). PCR amplification conditions using primer sets specific for iNOS, COX-2 and NFκB were optimized for each pair of primers. PCR primers were as follows: mouse iNOS forward, 5’-TCCTACACCAACCAAAAC-3’; iNOS reverse, 5’-CTCCAATCTCTGCTATCC-3’; COX-2 forward, 5’-CTCCAACATCAGCTTAC-3’; COX-2 reverse, 5’-TCACCATGATGAAGATTCCAC-3’; GAPDH forward, 5’-ACCCGAAGACTGTGGAT-3’; GAPDH reverse, 5’-CTTCACGCATCAGTTTTTCAAG-3’. Parallel PCR analysis was run for the housekeeping gene GAPDH to normalize data for differences in mRNA quantity and integrity. PCR products were separated on agarose gel.

**Statistical analysis**

All values shown in the figures are expressed as the mean ± SD obtained from at least three independent experiments. Statistical significance was analyzed by two-tailed Student’s t-test. Data with values of p<0.05 were considered as statistically significant. Single (*) and double (**) marks represent statistical significance in p<0.05 and p<0.01, respectively.

**RESULTS**

Aromadendrin inhibits NO and PGE₂ production in LPS-stimulated RAW 264.7 macrophage cells

Given the previous reports that pro-inflammatory mediators such as NO and PGE₂ play key roles in the progression of inflammation (Ock et al., 2009; Lee et al., 2012), the inhibitory ef-