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Src and Syk are targeted to an anti-inflammatory ethanol extract of *Aralia continentalis*

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ABSTRACT

Ethnopharmacological relevance: Aralia continentalis Kitagawa (Araliaceae) is a representative ethnomedicinal herbal plant traditionally prescribed in Korea to relieve various inflammatory symptoms. However, the exact molecular mechanism of its anti-inflammatory activity has not been fully investigated.

Materials and methods: The effect of the ethanol extract from the roots of this plant (Ac-EE) on the production of the inflammatory mediator nitric oxide (NO) was studied in RAW264.7 cells. Its effect on inflammatory symptoms (gastritis and hepatitis) in mice was also examined. In particular, the molecular inhibitory mechanism was analysed by measuring the activation of transcription factors and their upstream signalling and the kinase activity of target enzymes.

Results: Ac-EE dose-dependently suppressed NO production in lipopolysaccharide (LPS)-activated RAW264.7 cells. This extract also displayed curative activity against EtOH/HCl-induced gastritis and LPS-induced hepatitis in mice. Ac-EE-mediated anti-inflammatory activity was found to be at the transcriptional level, as it blocked the activation of the nuclear factor (NF)-κB pathway composed of Syk and Src, according to immunoblotting and immunoprecipitation analyses and a kinase assay with whole and nucleus lysates from RAW264.7 cells and mice.

Conclusion: Ac-EE may be developed as a functional herbal remedy targeting Syk- and Src-mediated anti-inflammatory mechanisms. Future work using pre-clinical studies will be needed to investigate this possibility.

1. Introduction

Inflammation is one of the natural defensive pathways managed primarily by phagocytic cells such as macrophages and dendritic cells to protect the body from exogenous pathogens (Toltl et al., 2008). However, it is well known that excessive inflammation can cause serious diseases such as cancer and diabetes (McGeer and McGeer, 2008). It is now generally accepted that one of the strategies for preventing such diseases is to reduce over-activated inflammatory responses (Massarotti, 2008). To develop safe and effective drugs for this purpose, ethnopharmacological remedies have been developed as anti-inflammatory herbal medicine candidates (Lukhoba et al., 2006).

Aralia continentalis Kitagawa (Araliaceae) is a representative ethnomedicinal herbal plant traditionally prescribed in Korea to relieve pain, headache, rheumatism, lumbago, and lameness (Lee et al., 1995; Park et al., 2005). Indeed, several experimental

Abbreviations: PG, prostaglandin; NO, nitric oxide; COX, cyclooxygenase; iNOS, inducible NO synthase; (TNF)-α, tumour necrosis factor; ERK, extracellular signal-related kinase; TLR, Toll-like receptors (TLR); MAPK, mitogen activated protein kinase; NF-κB, nuclear factor-κB; AP-1, activator protein-1; JNK, c-Jun N-terminal kinase; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; MTT, 3-4,5-(Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole; PI3K, phosphoinositide 3-kinases; LPS, lipopolysaccharide; RT-PCR, reverse transcriptase-polymerase chain reaction; DSS, dextran sulphate sodium; PMA, phorbol 12-myristate 13-acetate; MyD88, myeloid differentiation primary response gene (88); IκBα, inhibitor of kappa B alpha; Iκκ, IκB kinase; Syk, spleen tyrosine kinase; CREB, cAMP response element-binding; CMC, sodium carboxymethylcellulose; *CA*, carnosic acid; PEI, Polyethylenimine

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approaches have proven its ethnopharmacological activities on inflammatory diseases or symptoms through the suppression of COX-2 and iNOS expression by ethanolic or methanolic extracts of this plant root as well as the blockade of NF-κB activation by constituents such as kaurenoic acid, a diterpenoid (Choi et al., 2011; Lim et al., 2009). Thus, the anti-inflammatory activity of this plant has been demonstrated with several *in vivo* models, such as ear and paw oedema models (Cheon et al., 2009; Lim et al., 2009) and a complete Freund's adjuvant-induced arthritis model (Park et al., 2005). Although several papers have reported that the proteins tyrosine phosphatase 1B (Na et al., 2006) and cholinesterase (Ertas et al., 2009) are directly suppressed targets of this plant or its constituents, the exact immunopharmacological target of *Aralia continentalis* regulating anti-inflammatory action has not been fully elucidated.

Therefore, in this study, we investigated the molecular anti-inflammatory mechanism of *Aralia continentalis* under the inflammatory conditions using macrophages activated by LPS. To understand the ethnopharmacological action of this plant, the ethanol extract of *Aralia continentalis* roots, which has been used for a long time, was chosen to explore the exact molecular target of its anti-inflammatory action.

2. Materials and methods

2.1. Materials

The roots of *Aralia continentalis* were purchased from Kyungdong Oriental Medicine Market (Seoul, Korea). This plant was identified by Prof. Sukchan Lee (Sungkyunkwan University, Suwon, Korea). A voucher specimen (number: SKKUMI-056-Ac-EE) was deposited in our laboratory. (3–4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT), and lipopolysaccharide (LPS, *E. coli* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PP2 and piceatannol were obtained from Calbiochem (La Jolla, *CA*, USA). Carnosic acid (*CA*) was used as reported previously (Kar et al., 2012). Luciferase constructs containing binding promoters for NF-κB and AP-1 were gifts from Prof. Chung, Hae Young (Pusan National University, Pusan, Korea) and Man Hee Rhee (Kyungpook National University, Daegu, Korea). Foetal bovine serum and RPMI1640 were obtained from GIBCO (Grand Island, NY, USA). RAW264.7 cells, a BALB/c-derived murine macrophage cell line (ATCC no.: TIB-71), and HEK293 cells, a human embryonic kidney cell line (ATCC no.: CRL-1573), were purchased from ATCC (Rockville, MD, USA). All other chemicals were purchased from Sigma. The phospho-specific or total antibodies to p65, p50, c-Fos, ATF-2, c-Jun, PDK1, Src, Syk, IκBα, lamin A/C, and β-actin were obtained from Cell Signaling (Beverly, MA, USA).

2.2. Preparation of Ac-EE and its HPLC analysis

To prepare Ac-EE, a sample of *Aralia continentalis* roots (30–40 g) was treated with 200 ml of ethanol at 70 °C (1 atm) under reflux for 8 h and repeated 3 more times. The extracted materials were concentrated to prepare Ac-EE (yield, 16%) with a speed bag (Modul spin 40; Biotron Corporation, Gangwondo, Korea) at 40 °C for 24 h. The stock solution (350 mg/ml) of Ac-EE was prepared with 100% dimethyl sulfoxide (DMSO) and diluted to 0–200 μg/ml with media for *in vitro* assays with cell lines or suspended with 1.5% sodium carboxymethylcellulose (CMC) for the *in vivo* experiment.

Phytochemical characteristics of Ac-EE and the standard compound *CA* were identified by high performance liquid chromatography (HPLC) analysis (Almela et al., 2006; Starkenmann et al., 2006). The system was equipped with a KNAUER (Wellchrom) HPLC-pump K-1001, a Wellchrom fast scanning spectrophotometer K-2600, and a 4-channel degasser K-500. Elution solvents were solvent A (0.1% H_3PO_4 in H_2O) and solvent B (acetonitrile). The gradient step of the solvent was solvent A to solvent B/min, and a phenomenex gemini C_{18} ODS (5 μm) column was used.

2.3. Animal experiments

Male C57BL/6 mice (6–8 weeks old, 17–21 g) were obtained from DAEHAN BIOLINK (Chungbuk, Korea) and maintained in plastic cages under conventional conditions. Water and pelleted diets (Samyang, Daejeon, Korea) were supplied *ad libitum*. Studies were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at Honcheon Institute of Medicinal Herb (HIMH-2012-12).

2.4. Cell culture

RAW 264.7 and HEK293 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum (Gibco, Grand Island, NY, USA), glutamine and antibiotics (penicillin and streptomycin) at 37 °C under 5% CO_2. For each experiment, the cells were detached with a cell scraper. Under our experimental cell density (2×10^6 cells/ml), the proportion of dead cells was less than 1% according to Trypan blue dye exclusion tests.

2.5. NO and PGE_2 production

After pre-incubation of the RAW264.7 cells (1×10^6 cells/ml) for 18 h, they were pre-treated with Ac-EE (0 to 200 μg/ml) for 30 min and were further incubated with LPS (1 μg/ml) for 24 h. The inhibitory effect of Ac-EE on NO and PGE_2 production was determined by analysing the NO level with Griess reagent and the PGE_2 level with an enzyme immunoassay kit (Amersham, Little Chalfont, Buckinghamshire, UK), as described previously (Cho et al., 2000a; Green et al., 1982).

2.6. Cell viability test

After pre-incubation of RAW264.7 cells (1×10^6 cells/ml) for 18 h, the Ac-EE (0–200 μg/ml) was added to the cells and incubated for 24 h. The cytotoxic effect of the Ac-EE was then evaluated by a conventional MTT assay, as reported previously (Gerlier and Thomasset, 1986; Yoe et al., 2011). At 3 h prior to culture termination, 10 μl of MTT solution (10 mg/ml in phosphate buffered saline, pH 7.4) was added, and the cells were continuously cultured until termination of the experiment. The incubation was halted by the addition of 15% sodium dodecyl sulphate to each well, solubilising the formazan (Do Kim et al., 2010). The absorbance at 570 nm ($OD_{570-630}$) was measured using a Spectramax 250 microplate reader.

2.7. LPS-induced hepatitis

Inflammation of the liver was induced by injection of LPS according to a published method (Cho et al., 2000b). Fasted C57BL/6 mice were orally treated with Ac-EE (200 mg/kg) once a day for six days. One hour after final administration, LPS (10 mg/kg) was intraperitoneally administered. Each animal was anaesthetised and sacrificed with an overdose of urethane 1 h after the administration of hepatitis inducers, and blood was drawn from the portal vein. The livers were then excised and gently rinsed under running tap water. Serum was obtained by centrifugation of blood at 890*g* for 15 min. The levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured with a Roche Modular spectrophotometric autoanalyzer.