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Original article

Isorhamnetin and hyperoside derived from water dropwort inhibits inflammasome activation

Huijeong Ahn, Geun-Shik Lee*

College of Veterinary Medicine and Institute of Veterinary Science, Kangwon National University, Chuncheon 24341, Republic of Korea

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ABSTRACT

Background: Water dropwort (*Oenanthe javanica*), an umbelliferous plant, has been reported as hypolipidemic, antiplatelet, antitumor, or immune-stimulating agents and it has been suggested to cure cardiovascular disease and cancer.

Purpose: Present study aimed to evaluate the effect of the extracts of water dropwort (EWD) and its pharmacological molecules, hyperoside and isorhamnetin, on inflammatory response, especially inflammasome activation.

Study design/Methods: The anti-inflammasome properties of EWD, isorhamnetin, and hyperoside were elucidated by human and mouse macrophages.

Results: EWD attenuated secretion of interleukin (IL)-1β and formation of Asc pyroptosome resulting from NLRP3, NLRC4, and AIM2 inflammasome activation without interruption of cytokine transcription. Isorhamnetin selectively inhibited NLRP3 and AIM2 inflammasome activation and down-regulated expression of pro-inflammatory cytokines. Hyperoside selectively interrupted NLRC4 and AIM2 inflammasome activation but did not alter cytokine expression. In addition, EWD, isorhamnetin, and hyperoside inhibited caspase-1.

Conclusion: Isorhamnetin and hyperoside, a key molecule of water dropwort, have been suggested as candidates to attenuate inflammasome inhibition.

Introduction

The search for anti-inflammatory agents from natural herbal medicines represents an area of great interest worldwide (Aggarwal et al., 2006). Nevertheless, limited knowledge of their biological actions remains a problem. Water dropwort (*Oenanthe javanica*) is a perennial herb, which is widely distributed in East Asia and Europe with distinctive aroma and taste. Water dropwort is one of the most alkaline plants, which the fresh stems and leaves are used in salads or as a seasoning in soups and stews (Seo and Baek, 2005). Water dropwort is not only consumed as a vegetable, but has also been as a medicinal agent. According to the oriental medicine, it has well known used to prevent and treat diverse diseases, such as jaundice, hypertension, fever, abdominal pain, leucorrhea, mumps, and difficult urination (Ku et al., 2013a). Also, water dropwort has shown anti-hepatitis B virus activity and anti-diabetic effects by increasing insulin release from beta-cells (Han et al., 2008; Yang et al., 2000). Recently the anti-inflammatory activities of isorhamnetic-3-O-galactoside and hyperoside, an active compound from water dropwort, have been reported (Kim et al., 2013; Yang et al., 2013), however the effects of water dropwort on inflammasome activation have not been identified.

Inflammasomes are cytoplasmic multi-protein complexes that form upon recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (Lee, 2013). Types of inflammasomes are named for the NOD-like receptors (NLR) present in the complex, such as the NLRP1, NLRP3, NLRC4, or NLRP6 inflammasome (Schroder and Tschopp, 2010). In addition, absent in melanoma 2 (AIM2), one of the PYHIN family members, is the main component of the AIM2 inflammasome

Abbreviations: EWD, the extracts of water dropwort; IL, interleukin; NLR, NOD-like receptors; PAMPs, pathogen-associated molecular patterns; DAMPs, damage-associated molecular patterns; AIM2, absent in melanoma 2; CARD, caspase activation and recruitment domain; Asc, apoptosis-associated speck-like protein containing a caspase recruitment domain; *O. javanica*, *Oenanthe javanica*; BMDMs, Bone marrow derived macrophages; LCCM, L929 cell-conditioned medium; FBS, fetal bovine serum; PMA, phorbol 12-myristate 13-acetate; LPS, lipopolysaccharide; ATP, adenosine triphosphate; Alum, aluminium potassium sulfate; NG, nigericin; dsDNA, double-stranded DNA; Sup, cellular supernatant; Lys, cellular lysate; Pellet, cross-linked pellets; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride, TNF, tumor necrosis factors; HRP, horseradish peroxidas.

* Corresponding author. Fax: +82 33 244 2367.
E-mail address: leegeun@kangwon.ac.kr (G.-S. Lee).

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(Schroder and Tschopp, 2010). Inflammasome complexes are typically comprised of an NLR which interacts directly with caspase-1 through a caspase activation and recruitment domain (CARD) or via an adapter protein, usually apoptosis-associated speck-like protein containing a caspase recruitment domain (Asc; also known as Pycard), which links the NLR to caspase-1 resulting in caspase-1 activation and subsequently processing pro-interleukin (IL)-1β and pro-IL-18 into their active forms (Lee, 2013). These cytokines, which are the first line of defense for the innate immune response, initiate a cascade of other immunological responses. Inflammasomes and the innate immune response play a key role in many infections (Franchi et al., 2012). The pro-inflammatory response initiated by inflammasomes has also been implicated in metabolic disorders such as diabetes and inflammatory diseases such as gout and arthritis (Grant and Dixit, 2013; Lamkanfi and Dixit, 2012). Furthermore, the chronic inflammation etiologically associated with numerous cancers, most notably gastric, hepatic, and colorectal cancers, has also been linked to activation of these sensors (Franchi et al., 2012). Recently molecules or compounds, which are regulating inflammasome activation, have been received attention to apply on several inflammatory diseases (Ahn et al., 2014, 2015; Coll et al., 2015; Kim et al., 2014a; Youm et al., 2015).

In this study, we show the effect of water dropwort (*O. javanica*) and its active compounds, isorhamnetin and hyperoside, on NLRP3, NLRC4, or AIM2 activation and the transcriptional expression of pro-inflammatory cytokines in macrophages. Furthermore we elucidated the inhibiting property of human recombinant caspase-1 activity.

Materials and methods

Cell culturing

Unless otherwise indicated, all materials for cell culture were purchased from PAA (GE Healthcare Bio-Sciences Co., NJ, USA). Bone marrow derived macrophages (BMDMs) were obtained by differentiating bone marrow progenitors from the tibia and femur bones from C57BL/6 mice (6–12-week-old; Narabio Co., Seoul, Republic of Korea) with L929 cell-conditioned medium (LCCM) as a source of macrophage colony-stimulating factor (Boltz-Nitulescu et al., 1987; Englen et al., 1995; Kim et al., 2014b). The progenitors were cultured in RPMI 1640 (LM 011-01, Welgene, Seoul, Republic of Korea) supplemented with 10% fetal bovine serum (FBS), 30% LCCM, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. Cells were seeded in non-tissue culture-treated Petri dishes (SPL life science Co., Gyeonggi-do, Republic of Korea) and incubated at 37 °C in a 5% CO_2 atmosphere for 7 days. THP-1 cells were obtained from Korea Cell Line Bank (KCLB No. 40202; Seoul, Republic of Korea) and maintained in RPMI 1640 medium containing 10% FBS, 100 U/ml of penicillin, and 100 µg/ml of streptomycin at 37 °C in a 5% CO_2 atmosphere. THP-1 cells were differentiated into macrophages like cells by incubating with phorbol 12-myristate 13-acetate (100 nM, PMA; tlrl-pma, InvivoGen, CA, USA) for 72 h For cytotoxicity assay, BMDMs (10,000 cells/well) were grown in 96-well plates to 90% confluence and allowed to be attached for 3 h The cells were treated with the indicated substances for 1 h and cytotoxicity was assessed by Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., MD, USA) or EZ-Cytox Enhanced cell viability assay kit (Daeilab service co., Seoul, Republic of Korea) as manufacture's protocol.

Extraction of water dropwort (EWD)

Fresh and non-dried water dropwort (*O. javanica*; 1 kg) were extracted with 1000 ml of ethanol (99.99% v/v, Daejeung Chemicals & Materials Co., Gyeonggi-do, Republic of Korea) at RT for overnight. The ethanol extraction was filtrated with filter paper (No.1, Advantec MFS, Inc., CA, USA) and evaporated ethanol in vacuo at 55–65 °C. The ethanol-free aqueous solution was further filtrated with filter paper (No. 2, Advantec MFS, Inc.) and adjusted pH to pH 7.4. The solution were dried at 105 °C and dissolved in distilled water (100 mg/ml).

Inflammasome activation and inhibition

The PMA-treated THP-1 (1.0×10^6 cells per well) or BMDMs (1.0×10^6 cells per well) were plated on 12-well plates (SPL life science Co.) and primed with 1 µg/ml of lipopolysaccharide (LPS; L4130, Sigma–Aldrich Co., MO,USA) in RPMI 1640 containing 10% FBS and antibiotics for 3 h After LPS priming, BMDMs or THP-1 cells were subjected to the following activation steps. For NLRP3 inflammasome activation, the culture medium was replaced with RPMI 1640 supplemented with adenosine triphosphate (ATP, 2 mM; tlrl-atp, InvivoGen) for 1 h, aluminium potassium sulfate (Alum, 200 µg/ml; 039-4404, Daejung Chemicals & Materials Co) for 3 h, and nigericin (NG, 40 µM; 4312, Tocris Bioscience, Bristol, UK) for 1 h The media were changed by PRMI 1649 containing purified flagellin (NLRC4 inflammasome trigger, 500 ng/ml, tlrl-stfla; InvivoGen) with Lipofectamine® 2000 (10 µl/ml; Invitrogen, CA,USA) for 1 h, or dsDNA (AIM2 inflammasome trigger, 2 µg/ml) with Lipofectamine® 2000 (4 µl/ml; Invitrogen) for 1 h To determine the inhibitory effect of EWD, isorhamnetin (sc-20668, Santa Cruz Biotechnology, CA, USA) and hyperoside (sc-215167, Santa Cruz Biotechnology; CFN98754, ChemFaces, Wuhan, Hubei, Chaina) on inflammasome activation, the extract or chemicals were co-treated with the above activators.

To elucidate the effect of EWD, isorhamnetin, or hyperoside on LPS-mediated cytokine and NLRP3 mRNA and protein expression, BMDMs (2×10^6 cells/well) in 6-well culture plates (SPL life science Co.) were treated in the absence or presence of 10 ng/ml of LPS (Sigma–Aldrich Co.) for 3 h with the indicated dosages of extract and its derived molecules. Cellular total RNA and protein were prepared for further assays.

Western blotting sample preparation

After inflammasome activation, cellular supernatant (Sup; 250 µL of RPMI 1640) was transferred into a new tube, and the remaining BMDMs were lysed with 100 µL of mild lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tri-base, pH 8.0) containing proteinase inhibitor cocktail (#M250-1, AMRESCO LLC, Solon, OH, USA). The lysate (Lys) was transferred into a new tube and collected by centrifugation at 15,000 rcf for 5 min. The remaining pellet was washed two times with PBS and then re-suspended and cross-linked with 2 mM suberic acid bis (Sigma-Aldrich Co.) for 1 h, followed by centrifugation at 15,000 rcf for 5 min. The cross-linked pellets (Pellet) were re-suspended in 50 µl of 2 X loading dye buffer (116 mM Tris, 3.4% SDS, 12% glycerol, 200 mM DTT, 0.003% bromo phenol blue) (Fernandes-Alnemri et al., 2010; Lee et al., 2012). The Sup, Lys, and Pellet were subjected to Western blot assay.

Western blot analysis

Sup, Lys, and Pellet samples were separated by SDS-PAGE (10% or 16%) using running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) and Mini-PROTEAN® Tetra Handcast Systems (BIO-RAD, Hercules, CA, USA) and transferred onto a polyvinylidene difluoride membrane (PVDF; #10849A, Pall Co., Port Washington, NY, USA) using transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol, pH 8.3) and Criterion® Blotter (BIO-RAD). The membrane