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Actinidia arguta extract attenuates inflammasome activation: Potential involvement in NLRP3 ubiquitination

Kang-Hyuck Heo[a,1], Xiao Sun[a,1], Do-Wan Shim[a], Myong-Ki Kim[b], Sushruta Koppula[c], Sang–Hyeun Yu[a], Han-Bi Kim[a], Tack-Joong Kim[d], Tae-Bong Kang[a,c,*], Kwang-Ho Lee[a,c,*]

[a] *Department of Applied Life Science, Graduate School, Konkuk University, Chungju, Republic of Korea*
[b] *Department of Food Science and Engineering, Seowon University, Cheongju, Republic of Korea*
[c] *Department of Biotechnology, College of Biomedical & Health Science, Research Institute of Inflammatory Diseases, Konkuk University, 268 Chungwon-daero, Chungju, Republic of Korea*
[d] *Division of Biological Science and Technology, Institute of Biomaterials, Yonsei University, Wonju, Republic of Korea*

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ABSTRACT

Ethnopharmacological relevance: *Actinidia arguta* (*A. arguta*) has been widely used in Asian countries as a traditional medicinal herb to treat inflammation-related diseases, such as gastritis, bronchitis, and arthritis.
Aim of the study: The inhibitory effect of *A. arguta* leaves' extract (AA) on inflammasome activation was investigated to verify its traditional use in treating inflammation-related diseases.
Materials and methods: Bone marrow-derived macrophages (BMDMs) primed by lipopolysaccharide (LPS) were activated by selective inflammasome stimulators, and the effect of AA on inflammasome activation was investigated. A monosodium urate crystal (MSU)-induced peritonitis mouse model was used to study the *in vivo* efficacy of AA on inflammasome activation.
Results: In the *in vitro* study, AA regulated NLRP3 ubiquitination and apoptosis-associated speck-like protein containing a CARD (ASC) oligomerization, leading to the inhibition of NLRP3 inflammasome-mediated interleukin (IL)-1β secretion. The inhibitory effect of AA on inflammasome activation *in vitro* was further confirmed *in vivo* using an MSU-induced peritonitis mouse model.
Conclusion: AA provided scientific evidence, substantiating the traditional claims for its use in the treatment of inflammation and inflammation-mediated metabolic disorders, including gout.

1. Introduction

Inflammasome activation is a host defense system caused by stimulation from a foreign pathogens or physiological disturbance. Inflammasomes are cytosolic signaling complex molecules that consist of nucleotide-binding oligomerization domain-like receptor (NLRs) or an absent in melanoma 2 (AIM2), ASC, and caspase-1 (Davis et al., 2011). Among the NLR inflammasomes, the NLRP3 inflammasome is the most widely studied because its inordinate and continual activation contributes to harmful inflammatory syndromes, including metabolic or auto-immune diseases (Duewell et al., 2010; Martinon et al., 2006; Shaw et al., 2010; Vandanmagsar et al., 2011; Wen et al., 2011), as well as Alzheimer disease (Heneka et al., 2012) and respiratory illness (Pauwels et al., 2011; Simpson et al., 2014). NLRP3 is activated by danger-associated molecular patterns (DAMPs), such as extracellular ATP, amyloid-β, bacterial toxins including nigericin, or crystals such as silica and MSU (Latz et al., 2013; Martinon et al., 2009). The activation of the NLRP3 inflammasome induces the processing of inactive procaspase-1 into its active form, which cleaves the pro-IL-1β and pro-IL-18 to their mature forms and induces pyroptosis (Latz et al., 2013; Martinon et al., 2009). Although NLRP3 is one of the best-demonstrated inflammasome sensor molecules, its regulatory mechanisms remain elusive.

Recent studies have revealed that the post-translational modifications of NLRP3 are widely involved in its activation. Among these, ubiquitination is one of the central regulatory mechanisms in NLRP3 inflammasome activation (Juliana et al., 2012; Py et al., 2013). Hence, the regulation of NLRP3 ubiquitination is a promising therapeutic approach to inflammasome-related diseases.

Actinidia arguta (Siebold & Zucc.) Planch. ex Miq. (family, actinidiaceae) is a perennial vine that grows mainly in northeast Asian countries, including China, Japan, Korea, and Siberia (Nishiyama,

* Corresponding authors at: Department of Biotechnology, College of Biomedical & Health Science, Konkuk University, Chungju, Republic of Korea
E-mail addresses: kangtbko@gmail.com (T.-B. Kang), kwangho@kku.ac.kr (K.-H. Lee).
[1] These authors contributed equally to this work.

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2007). In China and Korea, the leaves of *A. arguta* are used traditionally as a vegetable and to manage severe conditions, such as vomiting, gastritis, bronchitis, arthritis, and other inflammatory diseases (Kim and Song, 2011; Kwak and Lee, 2014; Park et al., 2005). Previous ethnopharmacological studies revealed that the extract of *A. arguta* leaves has anti-oxidant (Jin et al., 2015), anti-inflammatory (Kwak and Lee, 2014), and anti-allergic effects (Kim et al., 2009). Additionally, the phenolic fraction of *A. arguta* leaves has been reported to possess anti-diabetic effects in experimental models (Kurakane et al., 2011; Kwon et al., 2014; Lee et al., 2015). Although the effects of *A. arguta* extract on inflammation and diabetes have been studied in pre-clinical models, the intrinsic mechanism has not been documented. In the present study, we investigated the attenuating effects of *A. arguta* extract on inflammasome activation in *in vitro* and *in vivo* experimental models and explored the underlying mechanisms.

2. Materials and methods

2.1. Reagents and antibodies

ATP, lipopolysaccharide (LPS), and MG132 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nigericin, silica crystal, and Z-VAD were purchased from Invivogen (San Diego, CA, USA). E-64-d and antibodies against ubiquitin and β-actin were purchased from Santa Cruz Biotechnology (San Diego, CA, USA). An ELISA kit of IL-1β and antibody against IL-1β (AF-401-NA) were obtained from R&D systems (Minneapolis, MN, USA). Antibodies against NLRP3 (Cryo-2), ASC (AL177), and caspase-1 (Casper-1) were obtained from Adipogen (San Diego, CA, USA). Cocktails of the protease and phosphatase inhibitor and disuccinimidyl suberate (DSS) were obtained from Thermo (Rockford IL, USA). G5 was purchased from EMD Millipore (Bedford, MA, USA).

2.2. Plant material and extraction

The methanolic extract of *A. arguta* leaves (AA) was purchased from the Korea Research Institute of Bioscience and Biotechnology (KRIBB). *A. arguta* leaves were collected from the southeastern area of Gyeongsangbuk-do in Korea during the months of April and May in 2004 and were authenticated by Dr. Shin-Ho Kang, a taxonomist at KRIBB, Korea. A voucher specimen was deposited at the herbarium of the Plant Extract Bank at KRIBB for future reference (KRIBB 024-023). The extraction procedures were done as described in our previous report (Shim et al., 2013). The final yield of the lyophilized AA was 2.97% (w/w), and was stored at 4 °C.

2.3. Animals

Female C57BL/6 mice (22–25 g, 6 weeks old) were purchased from Samtako Bio Co., Korea. Mice were housed in groups of five under standard conditions (temperature 22 ± 2 °C, humidity 55 ± 5%, 12 h light/dark cycle) with food and distilled water *ad libitum*. All experiments were performed under the guidelines of the Konkuk University Animal Care Committee, Republic of Korea (Permit No. KU16193).

2.4. Monosodium urate (MSU)-induced peritonitis mouse model and FACS analysis

The protocol for the MSU-induced peritonitis mouse model was followed as described in our previous report (Han et al., 2016). The obtained peritoneal cells were stained with Ly6G (Gr-1, clone 1A8-Ly6g), and F4/80 (clone BM8) (eBioscience San Diego, CA, USA) and analyzed using a FACS Calibur (Becton Dickinson San Diego, CA, USA). The number of neutrophils was counted by multiplying the total number of cells by the $Ly6G^+F4/80^-$ cells ratio. The supernatant of peritoneal lavage fluid was used to measure IL-1β.

2.5. Immunoprecipitation

Bone marrow-derived macrophages (BMDMs) were lysed in TTNE buffer (50 mM Tris–HCl, pH 7.4, 1% Triton X-100, 0.15 M NaCl, 2 mM EDTA) with a protease and phosphatase inhibitor cocktail. The lysates were precleared and then incubated with NLRP3 antibody overnight at 4 °C. The bound samples were precipitated with Protein G Agarose beads (Millipore Bedford, MA, USA) for 2 h at 4 °C. Beads were rinsed with TTNE buffer and eluted with 2x sample buffer (0.125 M Tris, pH 6.8, 20% glycerol, 2% sodium dodecyl sulfate, 0.355 M β-mercaptoethanol, and 0.02% bromophenol blue). Immunoprecipitated NLRP3 was detected for ubiquitinated NLRP3 by western blot analysis.

2.6. Other experimental methods

The experimental protocols for the preparation of BMDMs, cell culture and stimulation, and LDH, MTT, and ELISA assays were performed as described previously (Han et al., 2015; Shim et al., 2015). The protocols for the separation of Triton X-100 soluble and insoluble fractions, ASC oligomerization, and HPLC fingerprint analysis were followed as described previously (Shim et al., 2013; Sun et al., 2015).

2.7. Statistical analyses

All values are expressed as the mean ± S.E.M. (n = 3). The statistical analysis was performed using Student's *t*-test for two groups or a one-way ANOVA for three or more multiple groups (GraphPad Software San Diego, CA, USA). In all results, a p-value < 0.05 was considered statistically significant.

3. Results

3.1. AA inhibits NLRP3 inflammasome activation

AA at concentrations up to 100 μg/mL was not toxic to BMDMs (Fig. 1A and B), and therefore non-toxic concentrations of 12.5, 25, and 50 μg/mL were used for the following experiments. To identify the regulatory effect of AA on inflammasome activation, LPS-primed BMDMs were pretreated with AA before they were challenged by the inflammasome activators. AA inhibited NLRP3 inflammasome mediated release of cleaved IL-1β and caspase-1 in a concentration-dependent manner. AA also reduced NLRP3 inflammasome-induced pyroptotic cell death (Fig. 1C–E). However, AA did not inhibit other inflammasome activation, such as AIM2 (Fig. 1F), NLRC4 (Fig. 1G), and tumor necrosis factor (TNF)-α secretion from BMDMs stimulated with LPS and nigericin (Fig. 1H).

The results indicate that AA specifically inhibited NLRP3 inflammasome activation, thereby suppressing the secretion of caspase-1 and IL-1β.

3.2. AA treatment induced the appearance of high-molecular-weight NLRP3 and reduced ASC oligomerization

We examined whether the inhibitory effect of AA on IL-1β release was attributable to the expression of NLRP3 inflammasome components. As shown in Fig. 2A–C, AA treatment did not influence the expression of proteins, such as pro-IL-1β (p31), caspase-1 (p45), and ASC, in cell lysates from NLRP3 inflammasome-activated BMDMs. Furthermore, the TNF-α secretion was not altered (Fig. 1H). The data indicated that the inhibitory activity of AA was not influenced by the regulation of NF-κB. Interestingly, the expression level of NLRP3 was reduced in a concentration-dependent manner by AA treatment. Moreover, higher molecular weight bands of NLRP3 appeared in all AA-treated lysates (Fig. 2A–C). Therefore, we further investigated whether AA affects the expression of NLRP3 from LPS-primed BMDMs. AA did not have any effect on the mRNA expression of NLRP3 from LPS-primed BMDMs

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