J Ginseng Res 44 (2020) 808–814

Contents lists available at ScienceDirect

Journal of Ginseng Research

journal homepage: http://www.ginsengres.org

Review Article

Fructose-arginine, a non-saponin molecule of Korean Red Ginseng, attenuates AIM2 inflammasome activation

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ARTICLE INFO

Article history:
Received 4 March 2020
Received in Revised form
22 May 2020
Accepted 26 June 2020
Available online 21 July 2020

Keywords:
Korean Red Ginseng extract
Non-saponin
Fructose-arginine
AIM2 inflammasome

ABSTRACT

Background: Korean Red Ginseng extract (RGE) has been reported to act as an inflammasome modulator. Ginsenosides, saponin molecules of RGE, selectively inhibit activation of NLRP3 and AIM2 inflammasomes, while non-saponin molecules of RGE upregulate inflammasome components associated with the initiation of NLRP3 inflammasome activation. In this study, we investigated the effect of non-saponin components of RGE on AIM2 inflammasome activation.
Methods: The role of non-saponins of RGE on AIM2 inflammasomes was tested in mouse bone marrow-derived macrophages, a human monocyte-like cell line, and a mouse animal model. Cells or mice were transfected with dsDNA or inoculated with *Listeria monocytogenes* to activate AIM2 inflammasomes. Several indices of inflammasome activation were examined via immunoblot or ELISA analysis.
Results: The non-saponin fraction and saponin-eliminating fraction (SEF) of RGE selectively attenuated the activation of AIM2 inflammasomes, but not that of NLRP3 or NLRC4 inflammasomes. Fructose-arginine, an amino-sugar, was shown to be effective against AIM2 inflammasome activation.
Conclusion: Non-saponins of RGE, such as fructose-arginine, might be effective in regulating infectious and autoimmune diseases resulting from AIM2 inflammasome activation.

1. Introduction

Korean Red Ginseng extract (RGE) has been well-characterized as an immune system modulator, including the modulation of inflammasomes [1–3]. Inflammasomes are a cytosolic multiprotein complex that harbors proteolytic enzymes, such as caspase-1, which can lead to interleukin (IL)-1β and IL-18 releases and pro-inflammatory cell death, known as pyroptosis [4]. Absent in melanoma 2 (AIM2), a member of the inflammasome complex, was named as such because it was first identified in melanoma [5]. AIM2 detects dsDNA derived from pathogens such as bacteria, viruses, and parasites, as well, it senses mislocalized or damaged host genomic DNA in the cytoplasm, and such detection leads to the initiation of inflammasome assembly [6]. We previously reported that RGE and ginsenosides attenuate IL-1β secretion resulting from activation of NLRP3 and AIM2 inflammasomes [1]. In addition, the non-saponin fraction of RGE promotes the activation of the NLRP3 inflammasome through upregulation of inflammasome components and their substrates [2]. However, ingestion of the non-saponin fractions of RGE attenuates the inflammatory response via inhibition of toll-like receptor (TLR) 4 expression [3]. In this study, we investigated the effect of non-saponin molecules of an RGE on AIM2 inflammasome assembly in macrophages and mice. Moreover, we examined the role of amino-sugars, such as fructose-arginine (FA), originating from RGE on AIM2 inflammasome activation.

2. Materials and methods

2.1. Preparation of the RGE saponin-eliminating fraction and amino-sugars

Unless otherwise indicated, chemicals were obtained from Daejung Chemicals & Metals Co., Ltd (Gyeonggi-do, Republic of Korea). The RGE and its sub-fractions were provided by the Korea Ginseng Corporation (Daejeon, Republic of Korea). Briefly, the non-

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https://doi.org/10.1016/j.jgr.2020.06.002
p1226-8453 e2093-4947/$ – see front matter

saponin fraction (NS) and the saponin fraction (SF) were prepared as follows. The RGE (2.0 kg) was subjected to sequential Diaion HP20 adsorption chromatography (Mitsubishi Chemical Co., Tokyo, Japan) using water, 20 % ethyl alcohol (EtOH), and 100 % EtOH as eluents [2,3]. The dried powder obtained from the water and 20 % EtOH treatments was regarded as the NS, and the evaporated matter from the absolute EtOH treatment was considered the SF [2,3]. Detailed information on RGE components, including the NS and the SF, is available in previous studies [2,3]. To obtain the saponin-eliminating fraction (SEF), the RGE was soaked in absolute EtOH, after which, the EtOH was removed to eliminate any saponin components. The residue was re-eluted with water, and the eluted water fraction evaporated to dryness *in vacuo*. The concentration of arginine-fructose-glucose (AFG), an index of RGE non-saponins, in the SEF was determined by performing high-performance liquid chromatography (Supplemental Fig. 1) [7].

Two amino-sugars, FA and AFG, were synthesized using the methods described in a previous study [8]. Briefly, arginine and dextrose (100 mg each) for FA synthesis and arginine and maltose (100 mg each) for AFG synthesis were dissolved in glacial acetic acid (10 mL) and the solutions incubated in a water bath at 80°C for 1 h. After completion of the Maillard reaction, the synthesized FA and AFG were dried using a vacuum evaporator and solubilized in water (up to 10 mL; 100 mg/mL).

2.2. Cell culture and treatment

Unless otherwise indicated, the materials used in cell culturing were purchased from Capricorn Scientific GmbH (Ebsdorfergrund, Germany), and the associated plastics were obtained from SPL Life Sciences (Gyeonggi-do, Republic of Korea). To obtain mouse bone marrow-derived macrophages (BMDMs), progenitor cells were harvested from the tibia and femur bones of C57BL/6 mice (Narabio Co., Seoul, Republic of Korea) and incubated for 7 d in DMEM containing 10 % fetal bovine serum (FBS), antibiotics, and 50 % L929-conditioned medium containing a macrophage colony-stimulating factor [9,10]. The human monocyte-like THP-1 cells were maintained in RPMI 1640 media containing 10 % FBS before being differentiated to macrophage-like cells by culturing with phorbol 12-myristate 13-acetate (200 nM, PMA; InvivoGen, San Diego, CA, USA) for 24 h.

For inflammasome activation (Fig. 1A), BMDMs and PMA-treated THP-1 cells (1×10^6 cells per well) were plated in 12-well plates to which 1 mL of DMEM containing 10 % FBS and lipopolysaccharide (LPS, 1 μg/mL; Sigma-Aldrich Co., St. Louis, MO, USA) were added [11,12]. After 3 h of priming, the media were replaced with 350 μL of RPMI 1640 containing an inflammasome trigger with/without RGE, NS, SF, or SEF as indicated. For AIM2 inflammasome activation, the LPS-primed cells were treated with dsDNA

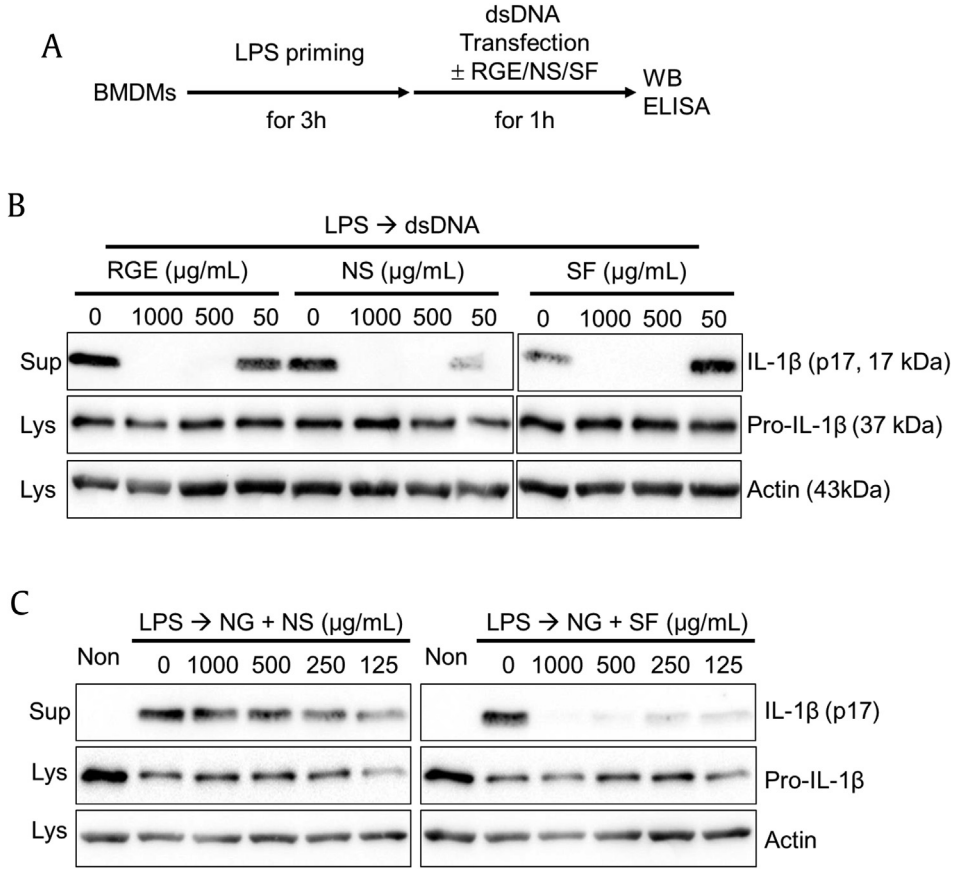

Fig. 1. Effects of RGE, NS, and SF on AIM2 inflammasome activation. **A**, Schematic diagram of the experimental process used for inflammasome activation in macrophages. **B**, Mouse BMDMs were primed with LPS and then subjected to dsDNA transfection to activate AIM2 inflammasome in the presence of RGE, NS, or SF as indicated. IL-1β secretion was analyzed by immunoblot assay. **C**, LPS-primed BMDMs were treated with NG along with increasing dosages of NS or SF, as indicated, to trigger NLRP3 inflammasome activation. The release of IL-1β was observed by immunoblot assay. All data shown are representative of at least two independent experiments. RGE, Korean Red Ginseng extracts; NS, non-saponin fraction of RGE; SF, saponin fraction of RGE; Sup, cellular supernatant; Lys, cellular lysate.

(a pcDNA3.1 plasmid, 1 μg/mL) and jetPRIME™ (2 μL/mL, Polyplus-transfection Inc., Illkirch, France) for 1 h or inoculated with *Listeria monocytogenes* (multiplicity of infection [MOI] 35) for 6 h. For NLRP3 inflammasome activation, macrophages were treated with nigericin (NG, 40 μM; Tocris Bioscience, Bristol, UK) for 1 h [13,14]. The bacteria were obtained from the Korean Culture Center of Microorganisms (Seoul, Republic of Korea) and were grown at 37°C on Luria-Bertani medium for *Salmonella* or Brain Heart Infusion medium for *Listeria*. Cellular supernatants were collected for immunoblot or ELISA analysis, and the remaining cells were lysed with 100 μL of mild lysis buffer containing a proteinase inhibitor cocktail (Sigma-Aldrich Co.) [11,15]. After harvest of the cellular lysate by centrifugation, the insoluble pellet was cross-linked by suberic acid bis (2 mM; Sigma-Aldrich Co.) for 1 h and then suspended in loading dye for immunoblot analysis [16].

2.3. Animal study

Female mice (C57BL/6, 7-week-old; Narabio Co.) were allowed free access to a standard chow diet and tap water and maintained under a 12 h light/dark cycle at 24°C. We used female mice in this study to prevent fighting among subjects. After 1 week of acclimation, the mice were allocated into one of three groups and orally administered 200 μL of water (negative control, Non) or SEF (0.3 or 1.1 mg/mouse/day) for 7 d. On the last day, mice were intraperitoneally injected with *Listeria* (1,000 cfu/mouse) and sacrificed by CO_2 inhalation 6 h after the injection. Blood and peritoneal lavage were collected from each mouse for analysis [11]. Animal experiments were conducted under the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Kangwon National University (approval KW-190319-3).

2.4. Western blot analysis

The cellular supernatant (Sup), lysate (Lys), and pellet were subjected to electrophoresis using SDS-PAGE gel (10 % or 16 %), and the products were then blotted onto polyvinylidene fluoride membranes [17]. The membranes were blocked by 3 % skim milk and probed with anti-mouse IL-1β antibody (R&D Systems, MN, USA), anti-caspase-1 antibody (AdipoGen Co., San Diego, CA, USA), anti-Asc antibody (Santa Cruz Biotechnology), anti-GSDMD antibody (Abcam plc., Cambridge, MA, USA), or anti-actin antibody (Santa Cruz Biotechnology) overnight at 4°C. The membranes were additionally probed with secondary antibody conjugated with HRP against the first anti-sera (Santa Cruz Biotechnology) for 3 h at room temperature and then visualized by applying an enhanced chemiluminescence solution (WESTSAVE STAR™, AbFrontier, Seoul, Republic of Korea) and using a chemiluminometer (EZ-Capture II, ATTO Technology, Tokyo, Japan).

2.5. Cytokine assay

The IL-1β levels in the peritoneal lavage fluids and cell culture supernatants were quantified by using a mouse IL-1beta/IL-1F2 ELISA kit (DY401, R&D Systems) or a human IL-1 beta/IL-1F2 ELISA kit (DY201, R&D Systems) as appropriate. The quantification of IL-18 of the supernatants was conducted using laboratory-developed plates with mouse IL-18/IL-1F4 antibody (D047-3, R&D Systems) and mouse IL-18/IL-1F4 biotinylated antibody (D048-6, R&D Systems). The plates were analyzed by using a microplate reader (Synergy H1™ microplate reader, BioTek, Winooski, VT, USA).

2.6. Statistical analyses

Statistical analyses were performed using the t-test (Mann-Whitney test) for two groups or the one-way ANOVA (Tukey's multiple comparisons test) for multiple groups. The applied tests were as provided in GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) software.

3. Results

3.1. Both saponin and non-saponin fractions of RGE attenuate AIM2 inflammasome activation

Macrophages were primed with LPS and then subjected to dsDNA transfection to provoke IL-1β secretion and AIM2 inflammasome activation [18]. As shown in Fig. 1A, LPS-primed BMDMs were treated with dsDNA in the presence of RGE, NS, or SF, and the level of IL-1β p17 secretion in the cellular supernatant was used as an indicator of inflammasome activation. Cytosolic dsDNA introduction lead to IL-1β secretion, which was dose-dependently decreased by RGE, NS, and SF treatments (Fig. 1B and Supplemental Fig. 2A). We expected an inhibitory effect of RGE and SF on AIM2 inflammasome activation because it was previously reported that RGE and two ginsenosides (Rh1 and Rg3) could block IL-1β p17 secretion [1]. However, the anti-AIM2 activation property of the NS was unexpected. Thus, we examined whether the NS inhibited IL-1β secretion resulting from activation of the NLRP3 inflammasome. LPS-primed BMDMs were treated with NG to activate the NLRP3 inflammasome in the presence of increasing dosages of NS or SF (Fig. 1C). The SF inhibited the IL-1β secretion resulting from the NG-mediated NLRP3 inflammasome activation, but the NS did not alter the level of IL-1β secretion. Taken together, the results suggest that non-saponin molecules might selectively attenuate AIM2 inflammasome activation.

3.2. Saponin-eliminating fraction inhibits AIM2 inflammasome activation

Because both NS and SF inhibited AIM2 inflammasome activation, we alternatively prepared a SEF to concentrate the level of non-saponin molecules in the absence of ginsenosides. To confirm the level of non-saponin molecules in SEF, we measured and compared the AFG (an index molecule of non-saponins) content in RGE (28.18 mg/g), NS (39.78 mg/g), SF (6.75 mg/g), and SEF (76.09 mg/g). The SEF contained twice the non-saponin molecule content than that of the NS. LPS-primed BMDMs were treated with SEF in the presence of an AIM2 inflammasome trigger, and the resulting caspase-1 (p20) and IL-1β levels were determined by immunoblot and ELISA analysis (Fig. 2A). SEF significantly inhibited caspase-1 (p20) and IL-1β secretions resulting from the dsDNA-mediated AIM2 inflammasome activation; however, it did not alter the NG or ATP activation of the NLRP3 inflammasome nor the flagellin transfection activation of the NLRC4 inflammasome (Supplemental Fig. 2B). We also examined the anti-AIM2 activation property of SEF on PMA-treated THP-1 cells (Fig. 2B). The results showed that SEF significantly blocked IL-1β secretion in dsDNA-activated AIM2 inflammasomes, but it did not change the level of IL-1β secretion resulting from NG- or ATP-mediated NLRP3 inflammasome activation (Supplemental Fig. 2C). Thus, molecules in the SEF can attenuate activation of the AIM2 inflammasome.

AIM2 inflammasomes are not only activated by dsDNA transfection, but also by *Listeria monocytogenes* infection, which is strongly dependent on AIM2 inflammasome activation; moreover, NLRP3 and NLRC4 inflammasomes are activated by *Listeria* [19]. As shown in Fig. 2D and Supplemental Fig. 3A, *Listeria* inoculation

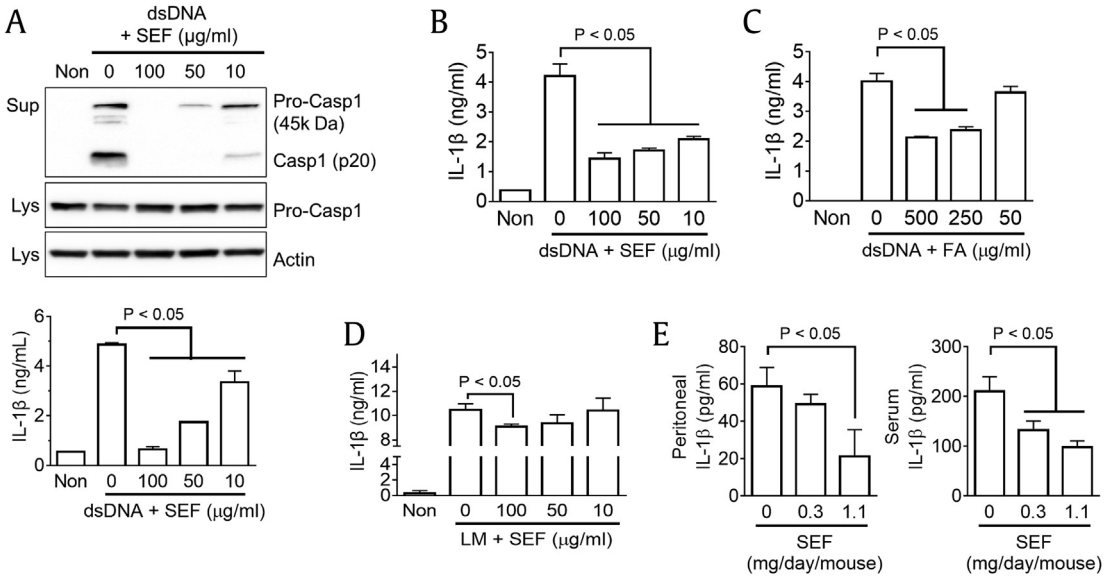

Fig. 2. Effects of SEF on AIM2 inflammasome activation in macrophages and mice. **A**, Mouse BMDMs were primed with LPS for 3 h and then treated with dsDNA for AIM2 inflammasome activation in the presence of increasing concentrations of SEF as indicated. The secretion of caspase-1 (Casp1) was analyzed by immunoblot assay and the release of IL-1β was determined by ELISA. **B** and **C**, PMA-treated THP-1 cells (human macrophage-like cell line) were primed with LPS for 3 h and then subjected to dsDNA transfection in the presence of SEF or FA (fructose-arginine) as indicated. **D**, LPS-primed BMDMs were inoculated with *Listeria monocytogenes* (LM) to activate AIM2 inflammasomes. IL-1β releases were analyzed by ELISA. **E**, Mice (n = 6 per group) were fed daily with SEF, as indicated, for 7 d, and then intraperitoneally injected with 200 μL of PBS containing LM. After 6 h, peritoneal lavage and blood serum samples were collected, and IL-1β secretions were measured by ELISA. Bar graphs indicate mean ± SD values. All data shown are representative of at least two independent experiments.

provoked IL-1β secretion in LPS-primed BMDMs. In addition, inoculation with *Salmonella* typhimurium, an NLRC4 trigger, also led to IL-1β secretion. The secretion of IL-1β resulting from the *Listeria*-mediated AIM2 inflammasome activation was significantly inhibited by a 100 μg/mL SEF treatment, but the *Salmonella*-induced NLRC4 inflammasome activation was unaffected by SEF treatment. We also tested the effect of SEF on IL-1β secretion in the mouse model. Mice were fed SEF daily for 1 week and then intraperitoneally injected with *Listeria*. As shown in Fig. 2E, SEF-fed mice exhibited significantly lower dose-dependent levels of IL-1β secretion in mouse peritoneal lavage and blood serum. Based on those results, SEF intake can reduce IL-1β secretion associated with AIM2 inflammasome activation.

3.3. Amino-sugar inhibition of AIM2 inflammasome activation

Ginseng contains various nutritional components, including glucose, fructose, maltose, and arginine, which are transformed by the Maillard reaction into amino-sugars during the steaming process used to create Korean Red Ginseng [20]. Among the non-saponin components of Korean Red Ginseng, two amino-sugars, FA and AFG, are reported to be active molecules of KRE with FA released from AFG by the actions of maltase in the intestine [20]. Thus, we tested the effect of select nutritional components and amino-sugars on inflammasome activation. LPS-primed BMDMs were treated with dsDNA (Fig. 3A) or NG (Supplemental Fig. 3B) in the presence of SEF, arginine, fructose, glucose, or maltose. The secretion levels of caspase-1 and IL-1β associated with AIM2 and NLRP3 inflammasome activations were not altered by the presence of the nutritional molecules; however, those secretions were significantly blocked by SEF as expected. Next, LPS-primed BMDMs were treated with increasing dosages of synthetic FA or AFG during the activation of AIM2 (Fig. 3B) or NLRP3 (Supplemental Fig. 3C) inflammasomes. Similar to the results of the SEF treatment, FA treatment significantly attenuated the release of IL-1β resulting from the dsDNA-mediated AIM2 inflammasome activation, but AFG did not alter the IL-1β level. In addition, neither of the two amino-sugars changed the ATP-mediated activation of the NLRP3 inflammasome. Taken together, the results suggest that, among the non-saponin components of RGE, FA might be an anti-AIM2 activation molecule.

3.4. FA inhibits AIM2 inflammasome activation

The activation of inflammasomes not only elicits IL-1β maturation and caspase-1 secretion, but also the formation of Asc pyroptosomes and the cleavage of gasdermin D (Gsdmd). Asc oligomerization provides a scaffold for interaction with caspase-1 to amplify the assembly of inflammasomes [21]. As well, cleavage of Gsdmd initiates the formation of pores at the plasma membrane, resulting in caspase-mediated cell death, pyroptosis [22]. Based on these observations, LPS-primed BMDMs were transfected with dsDNA (Fig. 3C) or treated with ATP (Supplemental Fig. 3D) in the presence of increasing levels of SEF or FA, and the effects of SEF and FA on inflammasome activation were examined. As expected, immunoblot assay results revealed that both SEF and FA attenuated the secretion of the active forms of IL-1β and caspase-1 derived via AIM2 inflammasome activation. Additionally, both SEF and FA inhibited the formation of Asc pyroptosomes and the cleavage of Gsdmd in dsDNA-transfected BMDMs. As well, ELISA results revealed that both SEF and FA significantly reduced the secretion of IL-1β and IL-18 resulting from dsDNA-mediated AIM2 inflammasome activation. However, these same indices for NLRP3 inflammasome activation were unchanged by SEF and FA treatment (Supplemental Fig. 3D). In addition, the effect of FA on human AIM2 inflammasome activation was examined. PMA-treated THP-1 cells were transfected with dsDNA in the presence of FA. The results showed that FA attenuated the level of IL-1β secretion resulting from AIM2 inflammasome activation (Fig. 2C). Thus, we suggest that FA, a component of GRE, is an anti-AIM2 activation molecule.

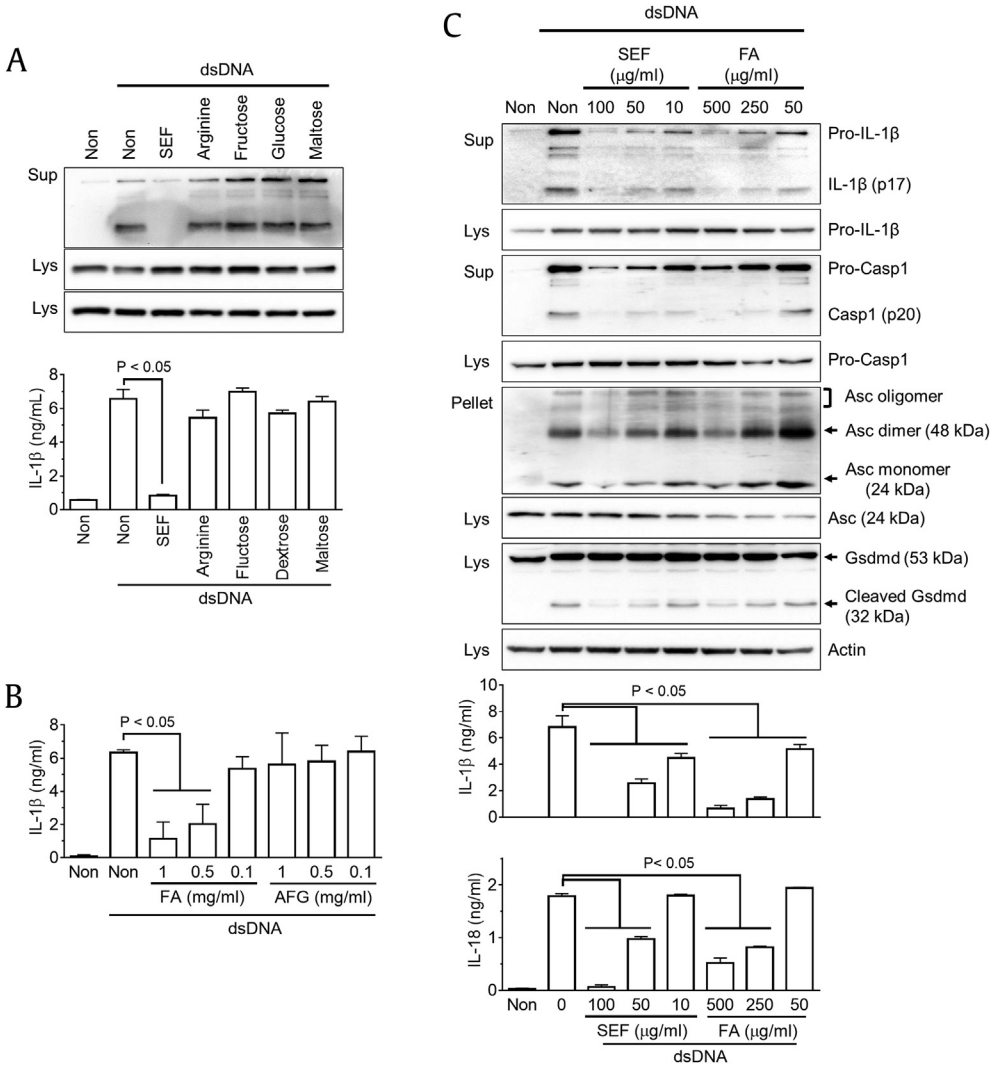

Fig. 3. Effects of fructose-arginine on AIM2 inflammasome activation. **A**, LPS-primed BMDMs were transfected with dsDNA in the presence of SEF (100 μg/mL), arginine (1 mg/mL), fructose (1 mg/ml), glucose (1 mg/mL), or maltose (1 mg/mL). Secretions of caspase-1 (Casp1) and IL-1β were measured by immunoblot assay and ELISA, respectively. **B**, BMDMs were primed with LPS, and AIM2 inflammasomes were activated by dsDNA transfection in the presence of the amino-sugars fructose-arginine (FA) or arginine-fructose-glucose (AFG). **C**, LPS-primed BMDMs were transfected with dsDNA to activate AIM2 inflammasomes. Immunoblot assays were conducted to detect secretions of IL-1β and caspase-1 (Casp1), the formation of Asc oligomerization, and the cleavage of gasdermin D (Gsdmd), as indicated. The secretions of IL-1β and IL-18 were measured by ELISA. Bar graphs indicate mean ± SD values. All data shown are representative of at least two independent experiments.

4. Discussion

Based on the results of this study, we conclude that FA, a non-saponin molecule in KRE, can inhibit the activation of AIM2 inflammasomes. Both saponins and non-saponin components of RGE exhibit anti-AIM2 activation properties. Although RGE ginsenosides have been reported to inhibit both AIM2 and NLRP3 inflammasome activation, such an effect by the NS of RGE is a novel finding. We also selectively investigated the NS from RGE by creating a SEF, which was then used in *in vitro* and *in vivo* experiments. The results showed that the SEF could selectively attenuate AIM2 inflammasome activation in both human and murine macrophages and was able to inhibit serum and peritoneal IL-1β secretion in *Listeria*-injected mice. To identify the anti-AIM2 activation molecule within the SEF, several non-saponin components (sugars, amino acids, and amino-sugars) were tested. The test results showed that FA attenuated the secretion of IL-1β, IL-18, and caspase-1, as well as Asc pyroptosome formation and Gsdmd cleavage resulting from AIM2 inflammasome activation. Thus, it is suggested that FA acts as an inhibitor of AIM2 inflammasome activation.

AIM2 has two functional domains: the pyrin domain (PYD) at the N-terminal and the hematopoietic expression, interferon-inducible nature, and nuclear localization (HIN) domain at the C-terminal. The PYD interacts with the PYD of Asc, leading to initiation of inflammasome assembly [23], whereas the HIN domain directly interacts with dsDNA [24]. AIM2 interacts with dsDNAs of various microbes and hosts and with synthetic molecules [6]. Well-characterized pathogens that promote AIM2 inflammasome assembly include *Francisella tularensis* and *Listeria monocytogenes* [25]. It has also been reported that AIM2 detects other pathogenic bacteria such as *Streptococcus pneumoniae*, *Mycobacterium tuberculosis, Mycobacterium bovis, Porphyromonas gingivalis, Legionella pneumophila, Staphylococcus aureus, Brucella abortus*, and *Chlamydia muridarum* [18]. Moreover, AIM2 detects dsDNA derived from viruses, with AIM2 inflammasome activation triggered by *Cytomegalovirus*, *Vaccinia virus*, papillomavirus, and Epstein-Bar virus [6]. In addition, endogenous DNA, which leaves the nucleus

when the nuclear envelope is perturbed by laminopathies, viral infection, or cancer, is recognized by AIM2, resulting in activation of the inflammasome [6,26]. The AIM2 inflammasome is also activated by damaged nuclear DNA and by damage resulting from exposure to ionizing radiation or chemotherapeutic medications, such as doxorubicin and etoposide that induce DNA double-strand breaks [6,27]. Moreover, AIM2 inflammasome activation is crucial for DNA vaccine-related immunization [28]. In the current study, we show that non-saponin components within RGE, especially FA, regulate the activation of AIM2 inflammasomes. This finding indicates that the NS of RGE or RGE itself might be useful in the treatment of AIM2-mediated diseases and associated health issues.

The AIM2 inflammasome activation regulatory mechanism might be considered simple compared to that of NLRP3 inflammasome activation because assembly of the AIM2 inflammasome only requires dsDNA interaction while NLRP3 inflammasome activation is initiated following the receipt of several signals [6,29]. Organisms compartmentalize their DNA into cell organelles and degrade mislocated DNA by using nucleases to control the activation of AIM2 inflammasomes [30]. Thus, the availability of DNA to AIM2 is a regulatory mechanism of AIM2 inflammasome activation. However, DNA accessibility was not considered as part of the mechanism related to anti-AIM2 activation by non-saponin and FA because the approach used for AIM2 inflammasome activation used a transient dsDNA transfection.

The assembly of inflammasomes is mediated by PYD−PYD and caspase activation and by recruitment domain (CARD)−CARD interactions [4]. In this context, hosts and pathogens can produce decoy proteins, such as PYD-only regulators (POPs) or CARD-only regulators (COPs), to regulate inflammasome assembly via the disruption of protein interactions [31,32]. Although three human POPs and two mouse POPs have been identified, only human POP3 has shown anti-AIM2 inflammasome activation properties [31]. COPs have been shown to inhibit the recruitment of caspase-1 into the inflammasome platform; however, COPs have only been identified in humans, not in mice [33]. The physiological relevance of decoy proteins involved in AIM2 inflammasome activation remains to be demonstrated, and common AIM2 selective decoy proteins in humans and mice are still uncharacterized [6]. In addition to the above mechanisms (DNA accessibility and assembly regulation) of AIM2 inflammasome activation, the expression level of AIM2 can be a regulator of AIM2 inflammasome activation. Tripartite motif 11 (TRIM11) can reduce AIM2 expression by interacting with AIM2 in the degradation of autophagosomes, an interaction that is dependent on p62, an autophagic cargo receptor [34]. Although it has been suggested that the above three mechanisms are involved in the regulation of AIM2 inflammasome activation, we speculate that FA and/or other non-saponin components of RGE may affect the upstream of Asc oligomerization and/or inflammasome assembly.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This research was supported by a grant (2017) from the Korean Society of Ginseng funded by the Korean Ginseng Corporation and by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2018R1A2B2004097 and NRF-2018R1D1A1B07048337).

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jgr.2020.06.002.

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