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Research paper

Sulforaphane attenuates activation of NLRP3 and NLRC4 inflammasomes but not AIM2 inflammasome

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ABSTRACT

Sulforaphane (SFN), a compound within the isothiocyanate group of organosulfur compounds originating from cruciferous vegetables, has gained attention for its antioxidant, anti-inflammatory, and cancer chemopreventive properties. However, the effects of SFN on inflammasomes, which are multi-protein complexes that induce maturation of interleukin (IL)-1β, have been poorly studied. In this study, we investigated the effects of SFN on the assembly of NLRP3, NLRC4, and AIM2 inflammasomes as well as on the priming step of NLRP3 inflammasome in murine macrophages. In our results, SFN attenuated activation of NLRP3 and NLRC4 inflammasomes but not AIM2 inflammasome. In addition, SFN blocked expression of the *NLRP3* gene and pro-IL-1β during the priming step. SFN further attenuated IL-1β secretion of monosodium uric acid-induced peritonitis in mice. Lastly, SFN inhibited generation of mitochondrial reactive oxygen species, which trigger NLRP3 inflammasome activation. Thus, SFN is suggested as an anti-inflammasome molecule for NLRP3 and NLRC4 inflammasome activation.

1. Introduction

Inflammasomes are large, cytosolic, multi-protein complexes that form in response to diverse pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) or danger signals by germline-encoded pattern recognition receptors (PRRs) [1,2]. The basic components of inflammasomes include a NOD-like receptor (NLR) and PYRIN protein, which recognizes danger signals or ligands, as well as pro-caspase-1, which is central to inflammasome activation [1]. Inflammasome activation comes after cytosolic signaling, which results in recruitment of the cysteine protease caspase-1 (formerly interleukin [IL]-1β-converting enzyme) to the inflammasome platform as well as its auto-proteolytic or conformational activation. Activated caspase-1 initiates maturation and secretion of the pro-inflammatory cytokines IL-1β and IL-18 as well as induction of pyroptosis, which is rapid inflammatory cell death [1]. Several inflammasomes have been identified to date, including NLRP3 (NACHT, LRR, and PYD domains-containing protein 3, also known by cryopyrin) inflammasome, which recognizes various danger signals, AIM2 (absent in melanoma 2) inflammasome, which recognizes dsDNA and *Listeria monocytogenes*, and NLRC4 (NLR family, CARD domain-containing 4) inflammasome, which recognizes flagellin and *Salmonella* [2].

Sulforaphane (SFN) is a compound within the isothiocyanate group of organosulfur compounds [3]. It is a chemopreventive photochemical and potent inducer of phase II enzyme involved in the detoxification of xenobiotics [4]. SFN is a well-characterized pharmacologic molecule in cruciferous vegetables such as broccoli, Brussels sprouts, or cabbage and has reported anti-oxidant, anti-inflammatory, and anti-cancer effects [3–5]. SFN has been reported to reduce NF-κB signaling by down-regulating p65 gene expression and interrupting toll-like receptor 4 (TLR4) signal transduction, resulting in inhibition of cytokine expression [5,6]. In addition, SFN also presented an inhibitory effect on activation of the four well-characterized inflammasomes, NLRP1, NLRP3, NLRC4, and AIM2 [7]. Based on these anti-inflammatory properties, SFN has been suggested as a natural agent to ameliorate inflammatory diseases such as inflammatory bowel diseases and aging-related neuroinflammation [8,9].

Although the anti-inflammatory effects of SFN have been widely reported, the effects of SFN on inflammasomes have not

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been well characterized. In this study, we investigated the effects of SFN on the priming and activating steps of the three well known inflammasomes. In addition, we assessed the role of SFN in monosodium uric acid (MSU)-induced peritonitis models and mitochondrial reactive oxygen species (ROS)-mediated inflammasome activation.

2. Materials and methods

2.1. Preparation of bone marrow-derived macrophages (BMDMs)

Unless otherwise indicated, all materials for cell culture were purchased from GenDEPOT Inc. (Barker, TX, USA) and Sigma-Aldrich Co. (St. Louis, MO, USA). BMDMs were obtained by differentiating bone marrow progenitors from the tibia and femur bones from C57BL/6 mice (6–12-weeks-old; Narabio Co., Seoul, Republic of Korea) with L929 cell-conditioned medium (LCCM) as a source of macrophage colony-stimulating factor (M-CSF) [10–12]. Progenitors were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 50% 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., Phcheon-si, Gyeonggi-do, Republic of Korea) and incubated at 37 °C in 5% CO_2 atmosphere for 7 days.

2.2. Inflammasome activation and inhibition

For priming of inflammasomes, BMDMs (1.0×10^6 cells per well) were plated in 12-well plates (SPL Life Science Co.) and treated with lipopolysaccharide (1 μg/mL, LPS; #4130, Sigma-Aldrich Co.) in RPMI 1640 containing 10% FBS and antibiotics for 3 h. For activation of inflammasomes, LPS-primed BMDMs were replaced by RPMI 1640 (250 μL/well in 12-well plates) containing the following inflammasome triggers: adenosine triphosphate (2 mM, ATP; #tlrl-atp, InvivoGen, San Diego, CA, USA) for 1 h, nigericin (40 μM, NG; #4312 Tocris Bioscience, Bristol, UK) for 1 h, flagellin (0.5 μg/mL; #tlrl-stfla, InvivoGen) with Lipofectamine 2000 (10 μL/mL, InvitroGen, Grand Island, NY, USA) for 1 h, *Salmonella typhimurium* (1%, OD600: 1.2) grown to log phage for 1 h, double-stranded DNA (2 μg/mL, dsDNA) with jetPRIME™ (2 μL/mL, Polyplus-transfection Inc., Illkirch, France) for 3 h, *Listeria monocytogenes* (10%, OD600: 1.2) for 3 h, monosodium uric acid (250 or 800 μg/mL, MSU, Sigma-Aldrich Co.) for 6 h, and rotenone (80 μM: #sc-203242, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 6 h. To test the inhibitory effect of D,L-Sulforaphane (SFN, #574215, EMD Millipore, Darmstadt, Germany) on inflammasome activation, SFN as co-treated with the above triggers.

2.3. 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) [13,14]. The Sup, Lys, and Pellet were subjected to Western blot assay.

2.4. 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 membranes were blocked with 3% skim milk and probed overnight at 4 °C with anti-mouse IL-1β antibody (#AF-401-NA, R&D Systems, Minneapolis, MN, USA), anti-caspase-1 antibody (#AG-20B-0042, AdipoGen Co., San Diego, CA, USA), or anti-actin antibody (#sc-1615, Santa Cruz Biotechnology). Membranes were further probed with HRP-conjugated 2nd anti-sera (#sc-2020 or #sc-2004, Santa Cruz Biotechnology) and visualized by Power-Opti ECL™ solution (BioNote Co., Hwansung-si, Gyeonggi-do, Republic of Korea) and a Cooled CCD camera System (AE-9105 EZ-Capture II, ATTO Technology, Tokyo, Japan). Band intensity of immunoblotting was measured by a CS Analyzer Version 3.00 (ATTO Technology).

2.5. Cell treatment for cytokine expression

BMDMs (2.0×10^6 cells per well) were plated in 6-well plates (SPL Life Science Co.) and treated with LPS (10 ng/mL, Sigma-Aldrich Co.) or heat-killed (HK) bacteria (1% of HKLM [*Listeria monocytogenes*], 0.01% of HKEC [*Escherichia coli*], and 0.001% of HKST [*Salmonella typhimurium*]) with/without SFN for 3 h. Total RNA was prepared for further analysis.

2.6. RT-PCR

Total RNA was extracted using TRIzol (InvitroGen) and reverse-transcribed to first-strand complementary DNA (cDNA) using M-MLV cDNA Synthesis kit (Enzynomics, Daejeon, Republic of Korea). Transcription was amplified by a SimpliAmp Thermal Cycler (Thermo Fisher Scientific Inc. Grand Island, NY, USA) and nTaq polymerase (Enzynomics). PCR products were visualized by agarose gel electrophoresis and ethidium bromide staining. Gene specific primers are below. Pro-IL-1β (*Il1b*; Genebank ID: NM_008361) primers 5′-CCC AAG CAA TAC CCA AAG AA-3′ and 5′-GCT TGT GCT CTG CTT GTG AG-3′; TNF-α (*Tnfa*; NM_013693) 5′-ACG GCA TGG ATC TCA AAG AC-3′ and 5′-GTG GGT GAG GAG CAC GTA GT-3′; IL-1α (*Il1a*; NM_010554) 5′-CCG ACC TCA TTT TCT TCT GG-3′ and 5′-GTG CAC CCG ACT TTG TTC TT-3′; IL-10 (*Il10*; NM_010548) 5′-TCA TTT CCG ATA AGG CTT GG-3′ and 5′-TGC TAT GCT GCC TGC TCT TA-3′; NLRP3 (*Nlrp3*; NM_145827) 5′-CAG GCG AGA CCT CTG GGA AA-3′ and 5′- CCC AGC AAA CCC ATC CAC TC-3′; β-actin (*Actb*; NM_007393) 5′-AGC CAT GTA CGT AGC CAT CC-3′ and 5′-CTC TCA GCT GTG GTG GTG AA-3′; GAPDH (*Gapdh*; NM_001289726) 5′-AAC TTT GGC ATT GTG GAA GG-3′ and 5′-ACA CAT TGG GGG TAG GAA CA-3′.

2.7. Animals study

Male C57BL/6 mice (8-week-old) were purchased from Narabio Co. (Seoul, Republic of Korea). All mice were maintained under a 12 h light/dark cycle at 24 °C. Animals were provided standard sterile food and water *ad libitum*, after which they were allowed to adjust to the environment for 1 week. Mice (n = 3 per group) were intraperitoneally (ip) injected MSU (5 or 10 mg/mouse) with/without SFN (0.5 [12.5] or 25 [625] mg/kg [μg/mouse]). After 6 h, mice were sacrificed by CO_2 inhalation. Peritoneal cavities were washed with 5 mL of PBS, and peritoneal exudate cells (PECs) were analyzed by a cell counter (Moxi Z™, ORFLO Technologies, Ketchum, ID, USA). Lavage fluids were collected for further

analysis. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Kangwon National University (IACUC; approval No. KW-150313-1).

2.8. IL-1β detection using ELISA

To quantitate secreted IL-1β, cell culture supernatants of BMDMs or peritoneal lavage fluids were measured by a mouse IL-1beta/IL-1F2 Quantikine ELISA Kit (R&D Systems). The ELISA plates were readout using Epoch™ microplate spectrophotometer (BioTek, Winooski, VT, USA).

2.9. Assay for mitochondrial reactive oxygen species (mROS)

BMDMs (1.25×10^5 cells per well) plated in 96-well black plates (SPL Life Science Co.) were incubated with MitoSOX™ Red mitochondrial superoxide indicator (2.5 μM, #M36008, Invitrogen) for 30 min at 37 °C. The cells were treated with rotenone (160 μM) in the presence of SFN (4, 20, or 40 μM) for 6 h at 37 °C. The plates were readout using a plate reader (510/580 nm, SpectraMax® M2^e, Molecular Devices, CA, USA).

2.10. Cytotoxicity assay

BMDMs (10,000 cells/well) were plated in a 96-well plate (SPL life science Co.) and treated with LPS (1 μg/mL) for 3 h. BMDMs were treated with the indicated dosage of ATP, NG, or SFN for 1 h and replaced with fresh media (RPMI 1640 containing 10% FBS and antibiotics), followed by further incubation for 6 h. The cytotoxicity was measured by an EZ-cytox™ Enhanced cell viability assay kit (iTSBiO, Seoul, Republic of Korea) per the manufacturer's protocol.

2.11. Statistical analyses

Statistical analyses were performed using a *t*-test (Mann-Whitney test) for the two groups or one-way ANOVA (Turkey's multiple comparisons test) for multiple groups using GraphPad Prism 6 (GraphPad Software, San Diego CA).

3. Results

3.1. Sulforaphane attenuates NLRP3 inflammasome activation

Sulforaphane (SFN, Fig. 1A) possesses several pharmacological properties such as antioxidant, anti-inflammatory, and anti-cancer effects [15–18]. Hence, we hypothesized that SFN may regulate IL-1β maturation. To test this hypothesis, murine macrophages were primed with LPS to up-regulate pro-IL-1β and then replaced with media containing SFN or ATP as a positive control for NLRP3 inflammasome activation (Fig. 1B). SFN did not induce maturation of IL-1β while ATP induced IL-1β maturation, demonstrating that SFN alone cannot directly activate inflammasomes. SFN did not show any cytotoxicity in the current concentration (Fig. 1C). Next, we determined the effect of SFN on NLRP3 inflammasome activation. Secretion of IL-1β by the well characterized NLRP3 triggers ATP and nigericin (NG) was blocked by SFN addition (Fig. 1D). Furthermore, SFN inhibited secretion of caspase-1 (Casp1; p20) and formation of insoluble ASC oligomerization (Fig. 1E), which were the other readouts of inflammasome activation like IL-1β secretion (Fig. 1F). Pyroptotic cell death, an indicator of NLRP3 inflammasome activation, was also blocked by SFN co-treatment (Fig. 1G). Taken together, SFN can be suggested as an anti-NLRP3 molecule.

3.2. SFN blocks NLRC4 but not AIM2 inflammasome activation

We further assessed the effect of SFN on NLRC4 and AIM2 inflammasome activation. For NLRC4 inflammasome activation, we applied flagellin transfection or *Salmonella* inoculation into LPS-primed macrophages and measured IL-1β secretion (Fig. 2A). Similar to NLRP3 inflammasome, SFN attenuated NLRC4-mediated IL-1β maturation. Furthermore, we applied SFN to LPS-primed BMDMs with dsDNA transfection or *Listeria* inoculation for AIM2 inflammasome activation (Fig. 2B). Unlike NLRP3 and NLRC4 inflammasomes, SFN did not affect release of IL-1β resulting from AIM2 inflammasome activation. Although *Listeria* is a trigger of AIM2 inflammasome activation, it also induces secretion of IL-1β via NLRP3 inflammasome [19]. Therefore, attenuated *Listeria*-mediated IL-1β secretion under a high concentration of SFN may be due to the inhibitory effect of SFN on NLRP3 inflammasome activation. Thus, SFN significantly attenuated flagellin-mediated IL-1β secretion but not dsDNA-mediated IL-1β release (Fig. 2C). Similar to IL-1β maturation, SFN blocked Casp1 secretion in flagellin-treated macrophages but did not alter secretion resulting from dsDNA-mediated AIM2 inflammasome activation (Fig. 2D). Taken together, our data indicate that SFN reduces activation of NLRP3 and NLRC4 inflammasomes but not that of AIM2 inflammasome.

3.3. SFN attenuates cytokines and NLRP3 expression

Based on the previous literature [16,18], SFN blocks NF-κB signaling resulting in anti-inflammatory activities. To elucidate the effect of SFN on priming of inflammasome activation [20,21], we applied increasing dosages of SFN with/without LPS to induce NF-κB signaling via interactions with toll-like receptor (TLR) 4. SFN addition without LPS to macrophages did not result in expression of pro-inflammatory cytokines (*pro-IL-1β*, *TNFα* and *IL-1α*) or anti-inflammatory cytokine (*IL-10*), demonstrating that SFN cannot directly interfere with cytokine expression (Fig. 3A). Further, SFN alone did not alter inflammasome components (NLRP3). In contrast, SFN treatment in the presence of LPS dose-dependently attenuated LPS-mediated cytokine production and NLRP3 up-regulation. To further confirm the role of SFN in the priming step mediated by other TLR ligands, we applied several heat-killed bacteria. We co-treated BMDMs with SFN and heat-killed *Listeria monocytogenes* (HKLM, agonist of TLR2), heat-killed *Escherichia coli* (HKEC, agonist of TLR2 and TLR4), and heat-killed *Salmonella typhimurium* (HKST, agonist of TLR2, TLR4 and TLR5), resulting in inhibition of *pro-IL-1β* and *TNFα* expression (Fig. 3B). To assess the role of SFN on the 1st signal (priming step) and/or 2nd signal (activating or assembling steps) of inflammasome activation, BMDMs were treated with LPS (1st signal), followed by exchange of media containing NG (2nd signal) with/without SFN (Fig. 3C). As a result, BMDMs primed by LPS and SFN did not show secretion of Casp1 (p20) or IL-1β (p17), whereas cells treated with LPS alone showed NLRP3 inflammasome activation in the presence of NG. In addition, LPS-primed macrophages showed inhibited secretion of NG-mediated Casp1 and IL-1β when co-treated with SFN. Taken together, SFN attenuates both the 1st and 2nd signals of NLRP3 inflammasome activation.

3.4. SFN ameliorates MSU-induced peritonitis

To determine whether or not SFN can inhibit inflammasomes in an NLRP3-dependent peritoneal model of acute gout [22], cell recruitment induced by MSU crystals was tested in SFN-treated mice. MSU injection induced recruitment of cells to the

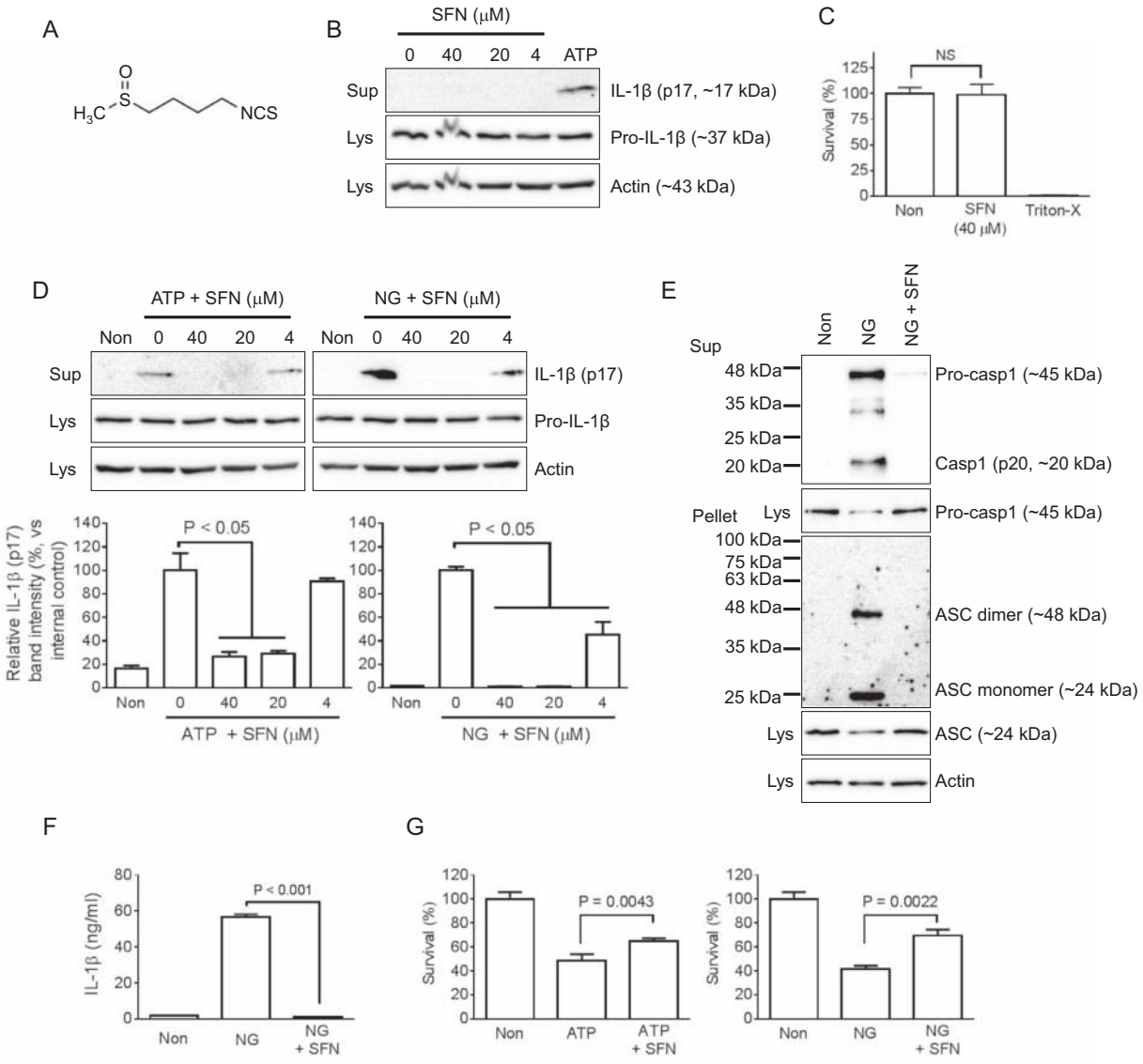

Fig. 1. Effect of SFN on NLRP3 inflammasome activation. A, Chemical structure of SFN. B, LPS-primed BMDMs were treated with the indicated concentration of SFN or ATP (2 mM) as a positive control. Secretion of active form of IL-1β was analyzed by immunoblotting. C, Cytotoxicity of SFN was measured after applying 40 μM SFN to BMDMs. Triton x-100 (0.01%, Triton) suggested by the manufacturer resulted in complete cell death. Triton-treated group was set as 0% of the survival rate and non-treated group was set as 100%. D, LPS-primed BMDMs were treated with the indicated concentration of SFN with/without ATP (2 mM) or NG (40 μM). Secretion of active form of IL-1β was analyzed by immunoblotting and the below bar graph indicates secreted IL-1β (p17) band intensity. E and F, LPS-primed BMDMs were treated with NG (40 μM) with/without SFN (40 μM). Secretion of caspase-1 (Casp1) and formation of ASC pyroptosome was analyzed by immunoblotting (E). Secretion of IL-β was analyzed by ELISA (D). G, Pyroptotic cell death was measured. LPS-primed BMDMs were treated with NLRP3 triggers (2 mM ATP or 20 μM NG) with/without 40 μM SFN for 6 h, and cytotoxicity was measured. Cellular supernatant (Sup), lysate (Lys), and cross-linked pellets (Pellet) from whole cell lysates were analyzed with the indicated anti-sera in an immunoblot assay. All immunoblot data shown are representative of at least two independent experiments. Bar graph presents the mean ± SD. Protein sizing markers (BLUEstain™ Protein ladder, #P007, GOLD Biotechnology, St. Louis, MO, USA) were indicated.

peritoneum, and this effect was reduced in SFN-treated mice (Fig. 4A). Furthermore, peritoneal IL-1β secretion in MSU-induced peritonitis was reduced by SFN co-injection (Fig. 4B). We also confirmed the inhibitory effect of SFN on MSU-mediated IL-1β secretion *in vitro* (Fig. 4C). These data demonstrate that SFN can inhibit NLRP3 inflammasome activation in a peritonitis model of acute gout.

3.5. SFN interrupts mitochondrial ROS generation

The generation of mitochondrial reactive oxygen species (mROS) is one of the most well established intracellular signals for NLRP3 inflammasome activation [23]. To elucidate the effect of SFN on mROS-mediated NLRP3 inflammasome activation, we treated LPS-primed BMDMs with rotenone, an inflammasome trigger via mROS generation [23,24], with SFN (Fig. 5A). Rotenone-induced IL-1β secretion was completely blocked by SFN treatment. Similarly, SFN attenuated production of mROS resulting from rotenone treatment (Fig. 5B). Consequentially, SFN may directly or indirectly inhibit NLRP3 inflammasome activation by attenuating mitochondrial ROS.

4. Discussion

In this study, we observed that SFN inhibited activation of NLRP3 and NLRC4 inflammasomes but not that of AIM2 inflammasome. In addition, SFN inhibited transcription of *NLRP3* and *pro-IL-1β* genes. Thus, SFN blocks both the priming and activating

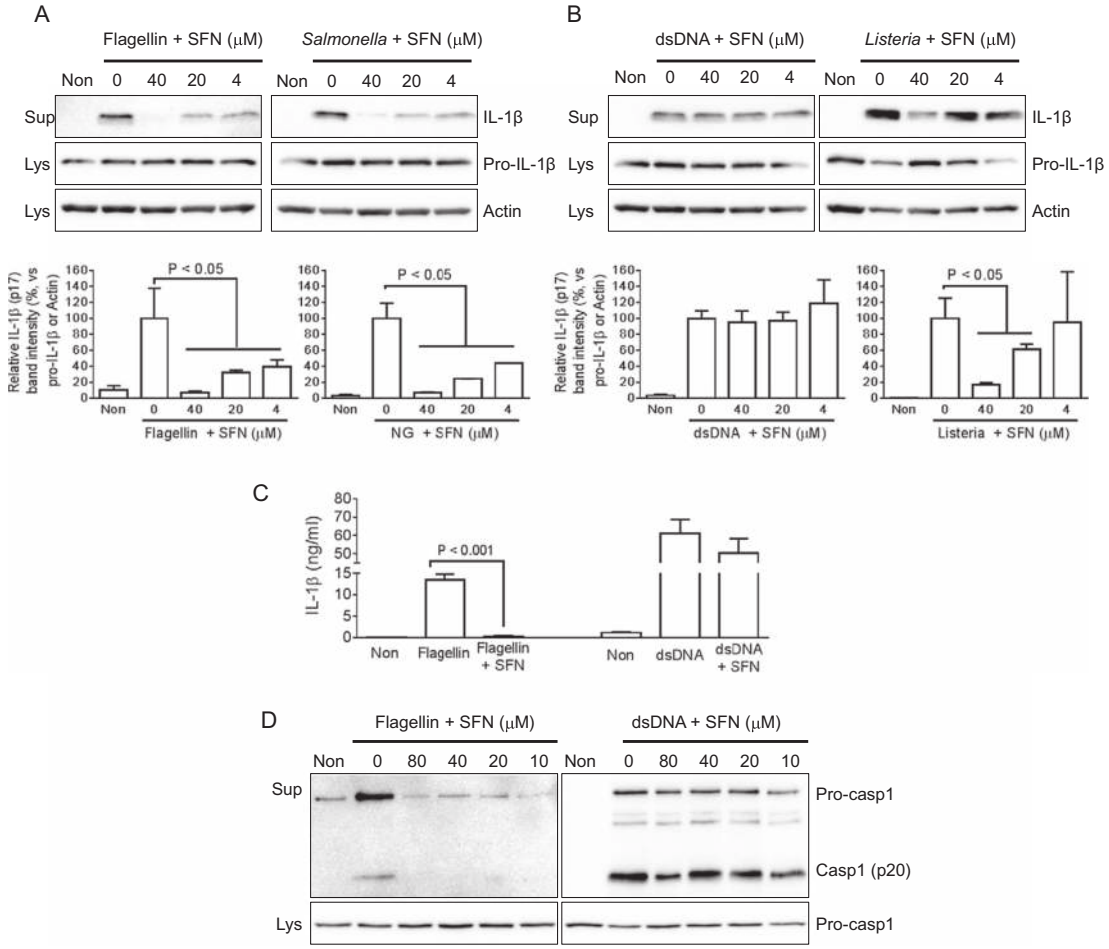

Fig. 2. Effect of SFN on NLRC4 and AIM2 inflammasome activation. A–D, LPS-primed BMDMs were treated with flagellin, *Salmonella*, dsDNA, or *Listeria*. Secretion of IL-1β was analyzed by immunoblotting (A and B) or ELISA (C). Secretion of Casp1 and formation of ASC pyroptosome was analyzed by immunoblotting. Cellular supernatant (Sup), lysate (Lys), and cross-linked pellets (Pellet) from whole cell lysates were analyzed with the indicated anti-sera in an immunoblot assay. All immunoblot data shown are representative of at least two independent experiments. Bar graph presented the mean ± SD.

steps in inflammasome activation. The anti-inflammasome effect of SFN was further confirmed in acute gout models. Furthermore, SFN might inhibit mitochondrial ROS generation, resulting in NLRP3 inflammasome activation. Taken together, SFN presents anti-inflammatory and anti-inflammasome properties.

Recently Greaney et al. reported that SFN inhibits activation of multiple inflammasomes, including NLRP1, NLPR3, NLRC4, and AIM2 [7]. They further reported that the anti-inflammasome property of SFN is independent of caspase-1 enzymatic activity, ROS modulation, and NF-E2-related factor 2 (Nrf2)-mediated protein synthesis [7]. However, some findings are different from ours. In the current study, SFN did not regulate AIM2 inflammasome activation and instead attenuated mitochondrial ROS generation, an intracellular NLRP3 inflammasome trigger [23]. Especially, cellular ROS production is a key regulator of NLRP3 inflammasome activation [20,25,26]. ROS scavengers during the priming step attenuate NLRP3 inflammasome due to lack of NLRP3 and Pro-IL-1β gene expression [21]. During the activation step, inhibition of ROS generation also inhibits NRLP3 inflammasome activation via induction of binding between thioredoxin-interacting protein and NLRP3 [27]. Thus, we suggest that SFN attenuated NLRP3 and Pro-IL-1β expression during the priming step and inhibited NLRP3 trigger-mediated IL-β and Casp1 secretion as well as ASC pyroptosome formation since SFN acts as a ROS scavenger. This conclusion is contrary to the previous report that SFN-mediated inhibition of inflammasomes is not affected by ROS modulation, as the previous study used NLRP1 inflammasome instead of NLRP3 [7]. Based on their recent paper, ROS scavengers do not impact NLRP1 inflammasome activation [28]. On the other hand, their reported inhibition of NLRP3 and NLRC4 inflammasome activation and MSU-induced peritonitis by SFN is similar to our results.

SFN has already been reported to inhibit inflammatory cytokine expression [16] as well as inflammation in mice, as indicated by reduced infiltration of immune cells and expression of pro-inflammatory cytokines in skeletal muscles of mdx mice [5]. In addition, SFN treatment also was shown to reduce expression of NF-κB (p65) in mdx mice in an Nrf2-dependent manner [5]. Another study showed that SFN can inhibit the LPS-stimulated inflammatory response in human monocytes by modulating cytokine production in an NF-κB-dependent manner [29]. Furthermore, SFN is reportedly able to covalently modify specific cysteine residues of TLR4 under non-reducing conditions as well as inhibit cytokine expression induced by LPS in THP-1 monocytes [6]. TLRs are pattern recognition receptors that detect invading microorganisms and non-microbial endogenous molecules, triggering immune and inflammatory responses during host defense and tissue repair [30]. Receptor oligomerization, which is one of the initial and critical events of TLR4 activation, was shown to be suppressed by SFN, resulting in down-regulation of NF-κB activation [30]. NF-κB signaling is crucial for NLRP3 inflammasome assembly, as TLR-NF-κB activation during the priming step up-regulates NLRP3, a limiting factor for inflammasome activation, and pro-IL-1β,

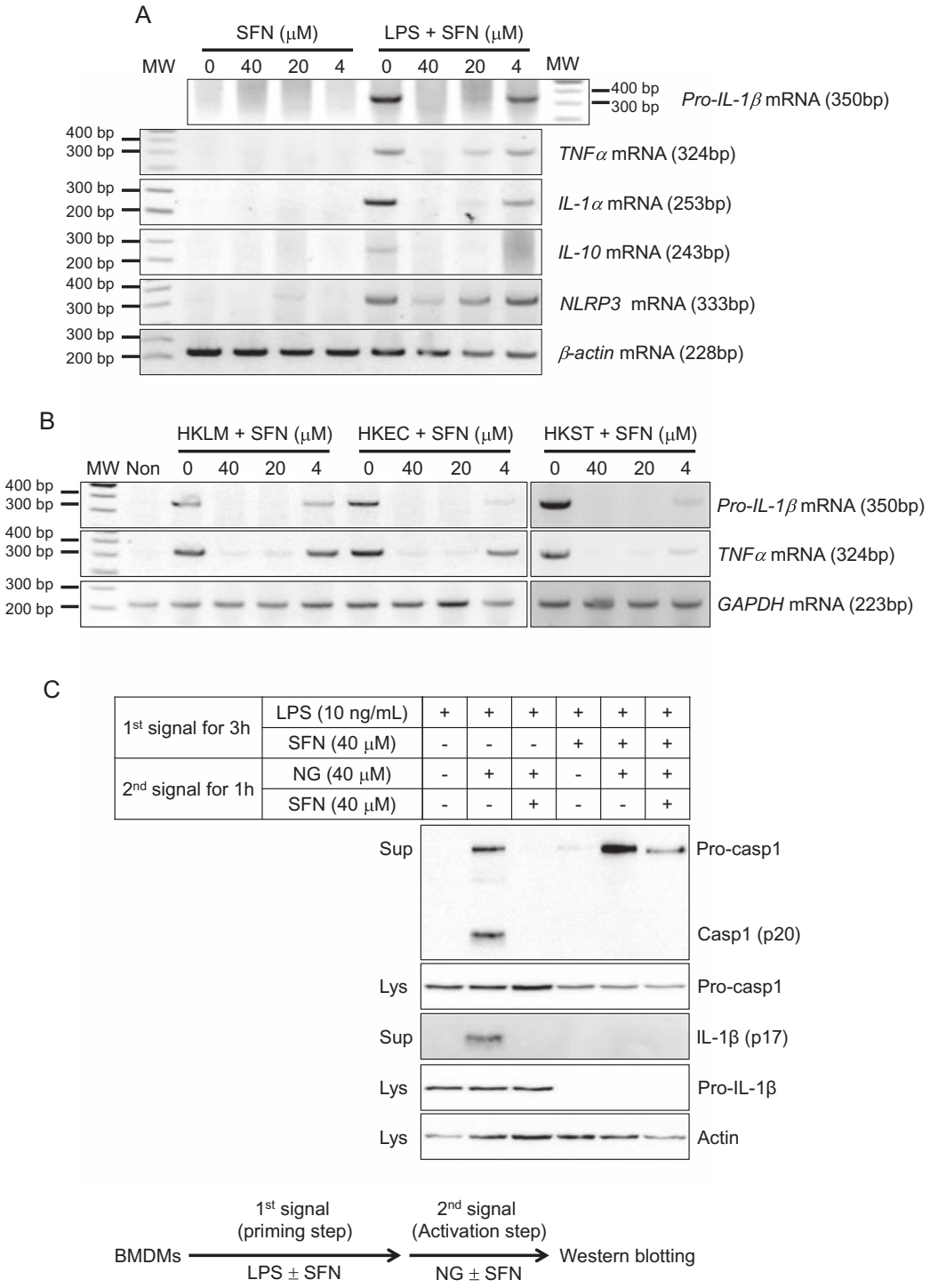

Fig. 3. Effect of SFN on cytokine and NLRP3 mRNA expressions. A, BMDMs were treated with SFN with/without LPS, and the genes were analyzed by RT-PCR. B, BMDMs were treated with heat-killed (HK) bacteria, HKLM (*Listeria monocytogenes*), HKEC (*Escherichia coli*), and HKST (*Salmonella typhimurium*). All agarose gel data shown are representative of at least two independent experiments. C, BMDMs were primed with LPS for 3 h and then treated with NG for 1 h in the presence or absent of SFN as indicated. Secretion of Casp1 and IL-1β was analyzed by immunoblotting. All immunoblot data shown are representative of at least two independent experiments. DNA sizing marker (100 bp DNA ladder marker, #DM001, Enzynomics) was indicated.

a substrate of caspase-1 [21]. The above evidence combined with our current data support the claim that SFN inhibits the priming step of inflammasome activation.

SFN has been suggested as a natural agent to ameliorate several diseases such as diabetes and its complications, inflammatory bowel diseases (IBD) or Alzheimer's diseases. In addition, NLRP3 inflammasome activation in macrophages has been shown to be a pathogenic factor for these diseases [31–33]. It has been reported that SFN prevents diabetic cardiomyopathy, high blood pressure, cardiac dysfunction, cardiac hypertrophy, fibrosis, cardiac oxidative damage, and inflammation via Nrf2 up-regulation [34,35]. However, it has been also revealed that NLRP3 inflammasome is associated with metabolic disorder and cell death, which are important triggers in diabetic cardiomyopathy [36]. *NLRP3* gene

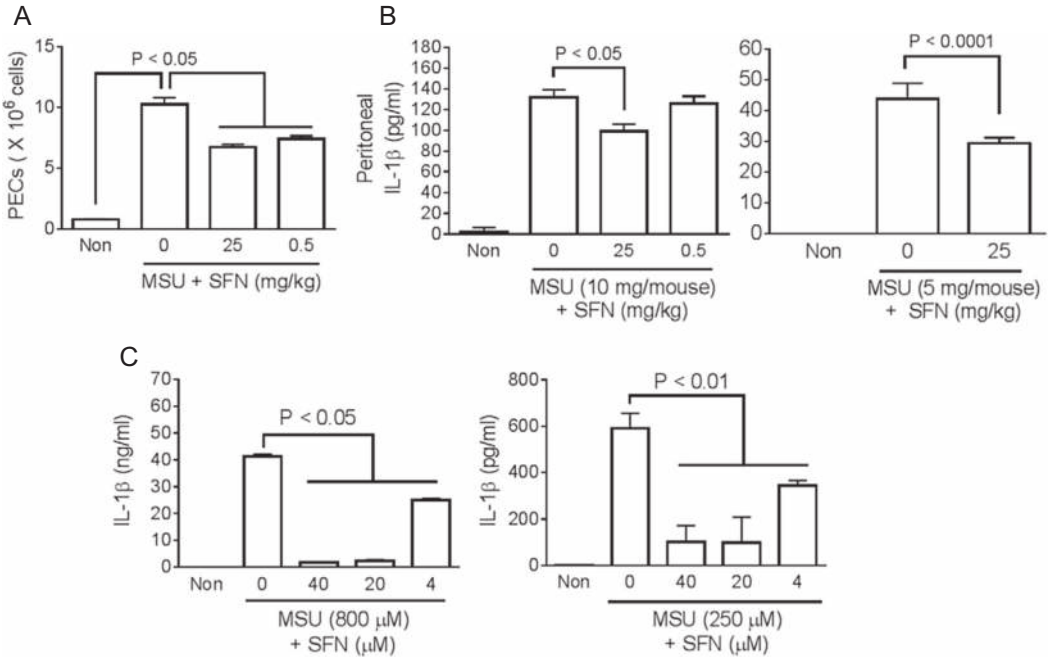

Fig. 4. Effect of SFN on MSU induced peritonitis. Mice (n = 3 per group) were intraperitoneally (ip) injected with MSU with/without SFN. The number of peritoneal exudate cells (PECs) was calculated (A), and IL-1β secretion of the peritoneal lavage fluids was measured (B). LPS-primed BMDMs were treated with MSU and SFN, and IL-1β secretion was analyzed. Bar graph presented the mean ± SD.

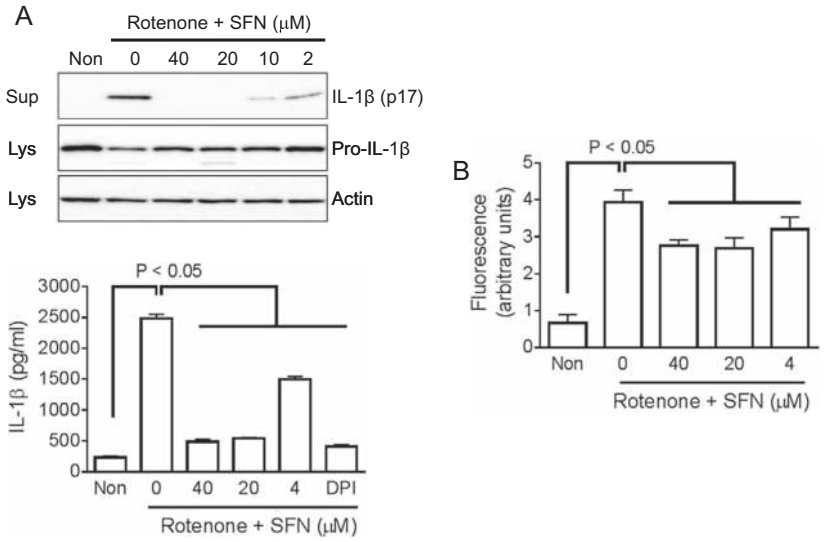

Fig. 5. Effect of SFN on mitochondrial ROS generation. A, LPS-primed BMDMs were treated with rotenone with/without SFN, and secretion of IL-1β was measured. B, BMDMs were treated with rotenone and the indicated dosages of SFN, and relative mitochondrial ROS generation was analyzed. All data shown are representative of least two independent experiments. Bar graph presents the mean ± SD.

silencing therapy has been used to inhibit cardiac inflammation, pyroptosis, fibrosis, and cardiac function [36]. In addition, inflammatory responses to cardiopulmonary bypass can be reduced by SFN, which was shown to reduce leukocyte activation and protected against renal injury [37]. In another study, NLRP3 deficiency strikingly suppressed expression of renal injury markers and inflammatory cytokines and apoptosis of renal tubular cells [38]. Dextran sodium sulfate (DSS)-induced colitis, an IBD model, is dependent on NLRP3 inflammasome activation [39]. That is, NLRP3-depleted mice showed less severe body weight loss than IBD model mice [39]. SFN also was shown to attenuate DSS-induced colitis and was suggested as a dietary supplement for relieving Crohn's disease [8,40]. Although these reports suggest that SFN blocks transcription of pro-inflammatory cytokines via NF-κB signaling or Nrf2 up-regulation [8,40], we speculate that SFN also attenuates inflammasome activation that exacerbate IBD symptoms. The other reported therapeutic property of SFN is neuroprotective activity [41]. SFN was found to ameliorate neurobehavioral deficits by reducing cholinergic neuron loss in brains of Alzheimer's diseases (AD)-like mice [41]. The role of NLRP3 inflammasome activation in the pathogenesis of AD has also been reported [42]. Taken together, the therapeutic effects of SFN are based on Nrf2-mediated anti-oxidation as well as inhibition of inflammasome activation.

Disclosures

The authors have no conflicts of interest.

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