Sulforaphane inhibits NLRP3 inflammasome activation in microglia through Nrf2-mediated miRNA alteration

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ABSTRACT

The NLRP3 inflammasome is a multiprotein complex that activates caspase-1 and triggers the release of the proinflammatory cytokines IL-1β and IL-18 in response to diverse signals. Although inflammasome activation plays critical roles against various pathogens in host defense, overactivation of inflammasome contributes to the pathogenesis of inflammatory diseases, including acute CNS injuries and chronic neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. In the current study, we demonstrated that Sulforaphane (SFN), a dietary natural product, inhibits NLRP3 inflammasome mediated IL-1β and IL-18 secretion and pyroptosis in murine microglial cells. SFN decreased the secretion of IL-1β and IL-18, and their mRNA levels in LPS primed microglia triggered by ATP. SFN suppressed the overexpression of cleaved caspase-1 and NLRP3 protein expressions as measured by caspase activity assay and western blot, respectively. SFN also prevented caspase-1 dependent pyroptotic cell death in microglia. Our data indicate that SFN suppresses NLRP3 inflammasome via the inhibition of NF-κB nuclear translocation and Nrf2 mediated miRNAs expression modulation in murine microglia.

1. Introduction

Inflammasomes are multiprotein complexes composed of NLRs, apoptosis-associated speck-like protein (ASC), and pro-caspase-1. They are crucial to innate immunity and have roles in the protection of the host against the pathogen [1]. Inflammasomes are also essential in sterile inflammation following tissue injury. NLRP3 is an extensively studied and well-characterized type of inflammasome in innate immune responses and inflammatory diseases, like gout, arthritis, Type 2 Diabetes, and Alzheimer's disease [2]. As a result of activation, NLRP3 recruits ASC and caspase-1 (known as interleukin-1 converting enzyme, ICE) to form complexes mediating maturation and secretion of potent proinflammatory cytokines, such as Interleukin-1β (IL-1 β) and Interleukin -18 (IL-18). The release of proinflammatory cytokines amplifies the inflammatory response [3].

Sulforaphane (SFN) is one of the isothiocyanates commonly found in cruciferous vegetables. The immunomodulatory properties of SFN have led the extensive research on its use against inflammation in numerous disease models. SFN also exhibits anti-inflammatory effects in the brain [4]. In rodent microglia, SFN suppresses pro-inflammatory cytokines secretion in LPS-induced inflammation [5]. These effects of SFN are closely related to the inhibition of pro-inflammatory transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1(AP-1) [5,6]. Several other intracellular

signaling pathways may contribute anti-inflammatory effects of SFN.

Cytoprotective effects of SFN are mainly linked to its ability to modulate the Nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2) antioxidant pathway. Nrf2 is one of the significant pathways regulating homeostasis in the cell by activation of antioxidant response element (ARE) containing genes, namely Heme oxygenase-1 (HO-1) and NAD (P)H Quinone oxidoreductase 1 (Nqo1), glutathione S-transferase P (Gstp1), and glutamate-cysteine ligase catalytic subunit (Gclc) [7,8]. Nrf2 is regulated via Kelch-like ECH-associated protein 1 (Keap1), which abstains Nrf2 in the cytoplasm [9,10]. Sulforaphane acts on cysteine residues on KEAP1 and prevents polyubiquitination and degradation of Nrf2 [11]. Once translocated, it upregulates ARE containing genes expression and exerts its antioxidant, cytoprotective, and anti-inflammatory effects. SFN was shown to have an attenuating effect on NLRP3 inflammasome activation in mouse bone marrow-derived macrophages and human THP-1 monocytes. The results of SFN on microglial inflammasomes have not been characterized [12].

microRNAs (miRNAs) are 22-nucleotide long members of short endogenous non-coding RNA families. miRNAs genes are located either intergenic or intragenic and transcribed by RNA polymerase II [13]. After transcription of miRNAs genes in the nucleus, the maturation process occurs in the cytoplasm [14]. Mature miRNAs regulate gene expression post-transcriptionally by binding to 3′-UTR regions of their target mRNA and lead to suppression of translation or degradation of target mRNAs [15]. miRNAs do not have a one-to-one association with their targets; one miRNA can regulate more genes [14]. miRNAs participate in diverse processes such as cell proliferation, metabolism, apoptotic cell death, autophagy, and inflammation [16,17]. Induction or inhibition of miRNA expression upon inflammatory stimuli results in altered biological responses like a pro- or anti-inflammatory response [16,18]. Microbial induction of inflammatory responses via TLRs leads to deregulated expression of miRNA-155 [16]. Although the roles of miRNA molecules in the innate immune system have been determined primarily in the TLR4 pathway, their effects on inflammasome activation have been recently demonstrated that miRNA-223 targets the 3′-UTR region of NLRP3 mRNA [19]. miRNA-223 is mainly expressed by myeloid cells and plays a regulator role in innate immune responses [20]. SFN may exert intracellular effects by altering miRNAs' expression [21]. Modulation of miRNAs may also contribute to the anti-inflammatory effects of SFN.

We found that SFN inhibits NLRP3 inflammasome activation in LPS and ATP stimulated murine N9 microglial cells in the present study. Also, SFN attenuates ROS production, a significant inducer of inflammasome activation and pyroptotic cell death. Our results showed that SFN protects microglial cells from NLRP3 inflammasome activation by Nrf2-mediated alteration in miRNAs expression.

2. Methods

2.1. Chemicals and reagents

SFN and adenosine 5′-triphosphate (ATP) disodium hydrate were obtained from Sigma-Aldrich (St. Louis, USA), and LPS (0111: B4) were purchased from InvivoGen (San Diego, USA). Fetal bovine serum (FBS), RPMI 1640 cell culture media, L-Glutamine, penicillin/streptomycin, phosphate-buffered saline (PBS), trypsin/EDTA were purchased from Biochrom (Berlin, Germany).

2.2. Cell Culture and treatments

N9 microglial cells [22] provided by Dr. Paola Ricciardi-Castagnoli (Cellular Pharmacology Center, Milan, Italy) were cultured in RPMI 1640 supplemented with 2 mM L-Glutamine, 10 % Fetal bovine serum (FBS), 1% penicillin/streptomycin (100 U/mL penicillin and 100 µg/mL streptomycin) at 37 °C in a humidified incubator containing 5% CO_2.

2.3. Cytotoxicity assay

LDH releases were measured by Cytotoxicity Detection Kit LDH (Roche, Basel, Switzerland) according to the manufacturer's protocol. The cells were seeded in 96 well plates at a density of 1×10^5 cells/well. After treatment, cell-free supernatant was incubated with the substrate mixture for 20 min. The absorbance of each well was measured at 492 nm with a reference wavelength at 630 nm on a microplate reader, and cytotoxicity was analyzed according to formula;

Cytotoxicity (%): (exp. value-low control)/ (high control-low control) *100

2.4. PI staining for pyroptosis detection

Cells were seeded into a 48-well plate at a density of 3×10^5 cells/well. Cells were treated with SFN (5µM) for 1 h, LPS (1 µg/mL) for 4 h, and ATP (5 mM) for 1 h. Pyroptotic cell death was determined by staining cells with 50 µg/mL PI stain. After 15 min of incubation, fluorescent images were captured using the fluorescent microscopy system (Olympus IX-71). Percentage of PI-positive cells were counted by Cell Counter plugin in ImageJ Software [23].

2.5. Enzyme-linked immunosorbent assay (ELISA)

N9 microglial cells were seeded at a density of 5×10^4 in 96-well plates and were pre-incubated with the 5 µM SFN in RPMI 1640 medium containing 1% FBS for 1 h then treated with LPS (1 µg/mL) for 4 h and ATP (5 mM) for 1 h treatments. Cell culture supernatants were collected and centrifugated at 10,000 g for 5 min at 4 °C to remove insoluble material. The level of IL-1β in cell culture supernatants was measured using mice IL-1β ELISA kit (eBioscience, USA), and IL-18 levels were detected using mice IL-18 ELISA Kit (MBL International, Japan) according to the manufacturer's protocol.

2.6. Caspase-1 activity assay

Cellular caspase-1 activity was measured with Caspase-1/ICE Colorimetric Assay Kit (R&D Systems, USA) according to the manufacturer's protocol. Absorbance was read at 405 nm on a microplate reader. The data were expressed as a percentage of control.

2.7. Determination of ROS production by DCF-DA

Cells were seeded into a 96-black-well plate at a density of 5×10^4 cells/well. After a 6 h SFN incubation period, the supernatant was discarded, and the cells were incubated with 100 µl PBS containing 10 µM CM-H_2DCFDA for 1 h. Then cells were incubated with LPS (1 µg/mL) for 23 h and ATP (5 mM) for 1 h. After incubation, the cell culture plate was read using a fluorescence plate reader. Data were presented as a percentage of control.

2.8. Determination of mitochondrial ROS by MitoSOX

Cells were seeded into a 96-black-well plate at a density of 5×10^4 cells/well. Cells were treated with SFN (5µM) for 1 h, LPS (1 µg/mL) for 4 h, and ATP (5 mM) for h. Mitochondrial ROS were measured in cells by MitoSOX (Invitrogen) staining (5 µM for 15 min at 37 ℃). The MitoSOX fluorescence (Ex 530/Em 590) was measured using a fluorescence plate reader.

Cells were seeded in 6-well plates at a density of 3×10^5 cells/well. After SFN pretreatment for 1 h, LPS (1 µg/mL) for 4 h and ATP (5 mM) for 1 h incubations were performed. Localization of the MitoSOX signal was determined by treating cells with MitoSOX and 0.1 µM DAPI for 2 min. Fluorescent images were captured using the inverted fluorescent microscopy system (Olympus IX-71). Data were collected and processed using ImageJ 1.50b Software (National Institutes of Health, USA).

2.9. Mitochondria membrane potential measurement

Cells were seeded in 25 cm^2 flasks at a density of 1×10^6 cells/flask. After SFN pretreatment for 1 h, LPS (1 μg/mL) for 4 h and ATP (5 mM) for 1 h incubations were performed. After treatment, cells were pelleted and stained with 2.5 μg/mL JC-1 (Thermo, USA) for 15 min in the dark at room temperature. Mitochondria membrane potential was analyzed by FACS Canto II analyzer using 488 nm laser (Becton Dickinson, USA).

2.10. Quantitative PCR analysis of mRNAs and miRNAs

Total RNA was isolated using Nucleospin RNA II Kit (Macherey-Nagel, Germany). Reverse transcription was performed using Revert-Aid First Strand cDNA Synthesis Kit (Thermo, USA) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the SYBR-Green I kit and LightCycler ® 480 Instrument II (Roche Life Sciences, USA) following the manufacturer's protocol. The primers used in the qPCR reactions are listed in Supplementary Table 1. PCR amplification of the template cDNAs was performed for 40 cycles using the following conditions: initial denaturation at 95 °C for 10 min, temperature cycling of denaturation at 95 °C for 10 s, and annealing at 60 °C for 10 s, and extension at 72 °C for 20 s was performed. The specificity of PCR products was analyzed by the melting curve analysis. The relative expression levels of mRNAs were quantified using the $2^{-(\Delta\Delta Ct)}$ method with endogenous normalization to the average amounts of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) [24].

For miRNA determination by qPCR, total RNA was isolated using miRNeasy mini kit (Qiagen, Germany). Reverse transcription was performed using miScript II RT Kit (Qiagen, Germany) according to the manufacturer's instructions. Quantitative real-time PCR was performed using miScript SYBR® Green PCR Kit (Qiagen, Germany) on LightCycler ® 480 Instrument II (Roche Life Sciences, USA). The relative expression levels of miRNAs were quantified using the $2^{-(\Delta\Delta Ct)}$ method with endogenous normalization to the geometric mean of RNU6 (RNA, U6 Small Nuclear 1) and Small nucleolar RNA SNORD95.

2.11. Western blot analysis

Total cellular proteins were isolated from cell lines in RIPA lysis buffer (50 mM Tris−HCL, pH 7.4, 150 mM NaCl, 0.25 % deoxycholic acid, 1% Nonidet P-40, 1 mM EDTA) including protease inhibitor (Thermo Scientific, USA). Cell culture supernatant proteins were isolated using methanol/chloroform precipitation. For 500 μL supernatant, 500 μL methanol and 100 μL chloroform were added. The supernatant mixture was centrifugated at 13.000 rpm for 5 min, and supernatants were removed. Another addition of 500 μL methanol is performed, followed by centrifugation at 13.000 rpm for 5 min. Pellet was dried and resuspended with lysis buffer before protein quantification.

To check the nuclear translocation of Nrf2, the NEPER isolation method was used. The nuclear and cytoplasmic proteins were extracted and separated by NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, USA) according to the manufacturer's protocol. Extracted samples were stored at −80 °C for following western blot analysis.

Equal amounts of supernatant or lysate proteins were separated by SDS-PAGE (15 % or 8%) and blotted onto a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% non-fat dry milk or BSA in PBS-T (0.2 % Tween-20 in 1X PBS, pH 7.2) for 1 h and probed overnight at 4 °C with primary Abs (Supplementary Table 2) followed by incubation with HRP-conjugated secondary Abs for 1 h. Protein bands were detected using the enhanced chemiluminescence system (Thermo Scientific, Massachusetts, USA). The bands were scanned and analyzed using ImageJ 1.50b Software (National Institutes of Health, USA). For adjusting the density of proteins, protein bands were normalized to β-actin or lamin A/C for nuclear samples.

2.12. Nrf2 siRNA knockdown

Briefly, N9 microglial cells were plated into a 96-well plate in antibiotic-free complete RPMI 1640 medium at a density 1×10^4 cells/well. After 24 h, the cells were transfected with 50 nM Nrf2 siRNA (Dharmacon, USA) using Dharmafect I transfection reagent (Dharmacon, USA) according to the manufacturer's recommendations.

2.13. miRNA transfection

N9 microglial cells (1.5×10^5 cells) were seeded at 25cm2 cell culture flasks in antibiotic-free and serum-containing RPMI 1640 medium. The cells were transfected for 48 h with miRNA-155 mimic (50 u M) and miRNA-223 inhibitor (100 u M) by using the HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's protocol. AllStars Negative Control siRNA from QIAGEN (cat. no. 1,027,280) was used for negative control.

2.14. Statistical analysis

For each experiment, GraphPad Prism 6.0 (GraphPad Software Inc., CA, USA) was used for data analysis in triplicate and presented as means ± SEM. Comparisons between SFN-treated cells and untreated control cells were analyzed using the Mann-Whitney U test P values <0.05 were considered statistically significant.

3. Results

3.1. SFN inhibited mRNA expression and secreted levels of IL-1β and IL-18 in microglial cells

To investigate the effect of SFN on microglia NLRP3 inflammasome activation, we stimulated LPS-primed murine N9 microglial cell line with ATP both in the presence or absence of SFN. ELISA analyses of cultured cell supernatant showed that SFN pretreatment strongly inhibited the secretion of IL-1β and IL-18 (Fig. 1A-B).

We next evaluated whether the SFN effects on mRNA levels of IL-1β and IL-18. Quantitative real-time RT-PCR analyses showed that LPS and ATP treatment enhanced cytokine's mRNA expressions. Pretreatment of microglial cells with 5 μM SFN dramatically decreased both IL-1β and IL-18 mRNA expression levels (Fig. 1C-D), suggesting that SFN suppressed induced transcription of IL-1β and IL-18. We used the western blotting method for evaluating secreted and cellular IL-1β protein levels. Western blot analysis indicated that SFN pretreatment decreased secreted (Fig. 1E, G) and intracellular expression of IL-1β (Fig. 1F-G) induced by LPS and ATP.

3.2. SFN decreased caspase-1 activity and upregulated NLRP3 expression

Since IL-1β release upon inflammasome activation is dependent on caspase-1 activation, we determined caspase-1 activity and protein level by caspase-1 activation assay and Western blotting, respectively. Caspase-1 activity assay showed that SFN pretreatment reduced enzyme activation compared to LPS and ATP-induced microglial cells (Fig. 2A). SFN pretreatment decreased caspase-1 p20 subunit in the cell culture supernatant, as shown by western blotting (Fig. 2B, D). However, SFN did not affect pro-caspase-1 form in whole-cell lysates (Fig. 2B-C).

NLRP3 is the main protein that triggers proinflammatory molecules. Thus, we want to investigate whether SFN affects the induced NLRP3 mRNA expression, and we measured the mRNA expression level of NLRP3 by quantitative real-time RT-PCR analysis. As shown in Fig. 2E, SFN had a significant effect on LPS and ATP-induced NLRP3 mRNA expression. SFN also significantly reduced upregulated NLRP3 protein expression by LPS and ATP (Fig. 2F-G).
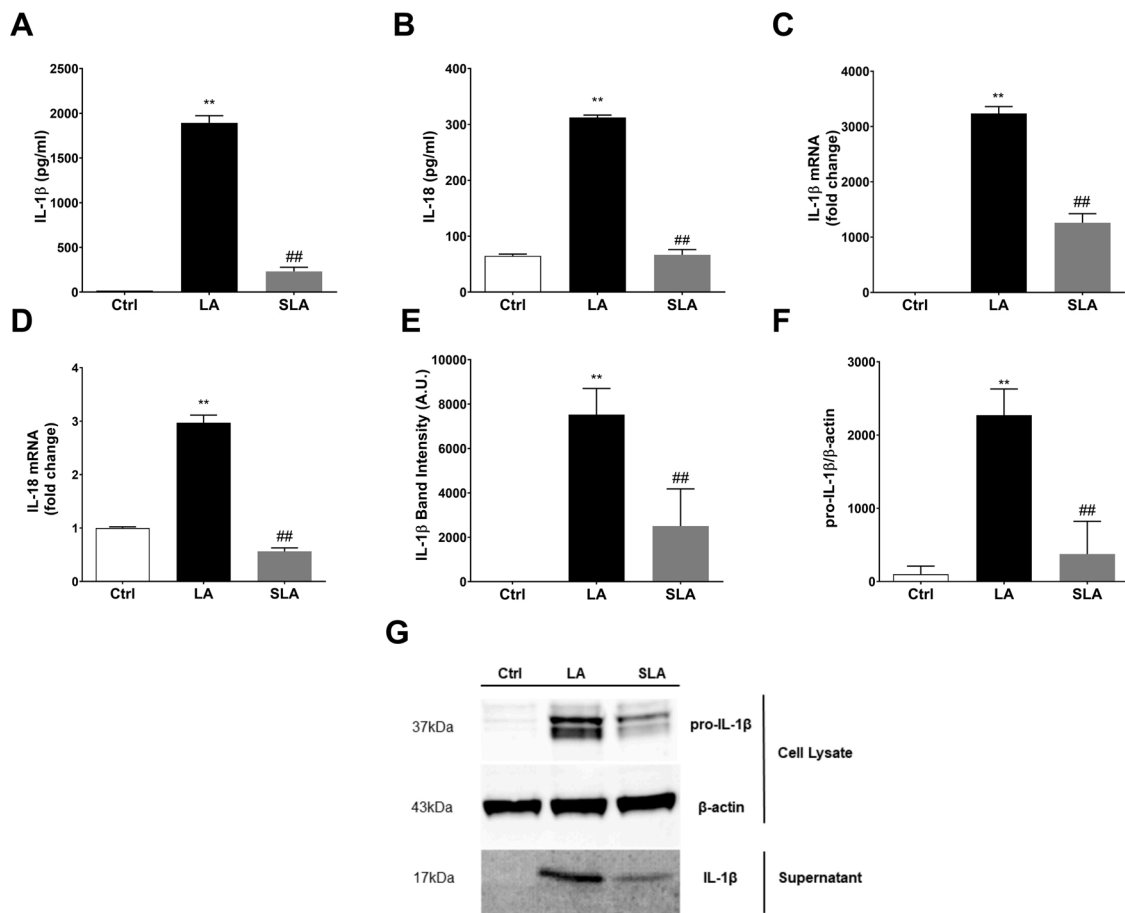
Fig. 1. Sulforaphane (SFN) pretreatment reduces inflammatory cytokine production and secretion.
N9 cells were treated with SFN (5 μM) for 1 h prior to LPS (1 μg/mL) for 4 h and ATP (5 mM) for 1 h, then cytokine secretion and mRNA expression were analyzed.
(A) IL-1β and (B) IL-18 cytokine amounts were evaluated by Enzyme-Linked ELISA. (C–D) IL-1β and IL-18 mRNA levels were detected by qPCR. (E,F,G) The IL-1β protein level in cell lysate and the supernatant was determined by western blot analysis. The results are mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01 and ***p < 0.001 compared with untreated control. #p < 0.05, ##p < 0.01 and ###p < 0.001 compared with LPS and ATP treatment.

3.3. SFN inhibited pyroptotic cell death in microglia

NLRP3 inflammasome activation causes pyroptotic cell death. Our results showed that SFN pretreatment reduced cell cytotoxicity caused by inflammasome activation in LPS and ATP-induced microglial cells (Fig. 3A). As shown in Fig. 3B-C, SFN pretreatment decreased the percent of pyroptotic cells (PI-positive).

3.4. SFN suppressed ROS production and restored mitochondrial membrane integrity

Since the cytoprotective effect of SFN is associated with its antioxidant effect, oxidative stress indicators (DCF-DA, JC-1) were analyzed. We examined ROS production induced by using CH2-DCFDA and MitoSOX fluorogenic dye. We found that LPS/ATP-induced total and mitochondrial ROS production was reversed by SFN pretreatment (Fig. 4A). LPS and ATP-induced microglial cells exhibit higher MitoSOX fluorescence than untreated control; however, the fluorescence level was decreased in SFN preincubated cells (Fig. 4B-C). The effect of LPS and ATP-induced inflammation model on mitochondrial integrity potential was also investigated using JC-1 dye with fluorometric flow cytometry measurement. SFN pretreatment significantly restored mitochondrial membrane potential, disrupted with LPS plus ATP treatment (Fig. 4D-E).

3.5. SFN pretreatment inhibited inflammasome related NF-κB translocation and ameliorated IκB-α secretion

Transcription factor NF-κB p65 has a vital role in the production of proinflammatory mediators. Western blotting results showed that while the NF-κB p65 protein level in the cytosol was increased, it was markedly reduced in the nucleus with SFN pretreatment (Fig. 5A-B-C). These opposite alterations demonstrate that pretreatment with SFN inhibited LPS and ATP-induced NF-κB nuclear translocation (Fig. 5D). IκBα is an essential protein responsible for the inhibition of NF-κB activity. While cytoplasmic NF-κB translocated to the nucleus, the IκBα protein level decreased with LPS and ATP treatment. SFN pretreatment showed a reverse effect and amended the IκBα protein level (Fig. 5E-F).

3.6. miRNA-155 overexpression diminishes the suppressive effect of SFN on NLRP3 inflammasome in microglia

Upregulated expression of miRNA-155 was detected in LPS and ATP induced N9 microglial cells, and SFN pretreatment significantly attenuated increased levels of miRNA-155 (Fig. 6A). After that, we investigated whether miRNA-155 overexpression would affect the protective effects of SFN against NLRP3 inflammasome activation. Upregulation of miRNA-155 by transfecting miRNA-155 mimics significantly reduced the protective effect of SFN against NLRP3 inflammasome activation, as seen in mRNA expression levels of inflammasome markers (Fig. 6B-D). Transfection of miRNA-155 mimics completely reversed the effect of
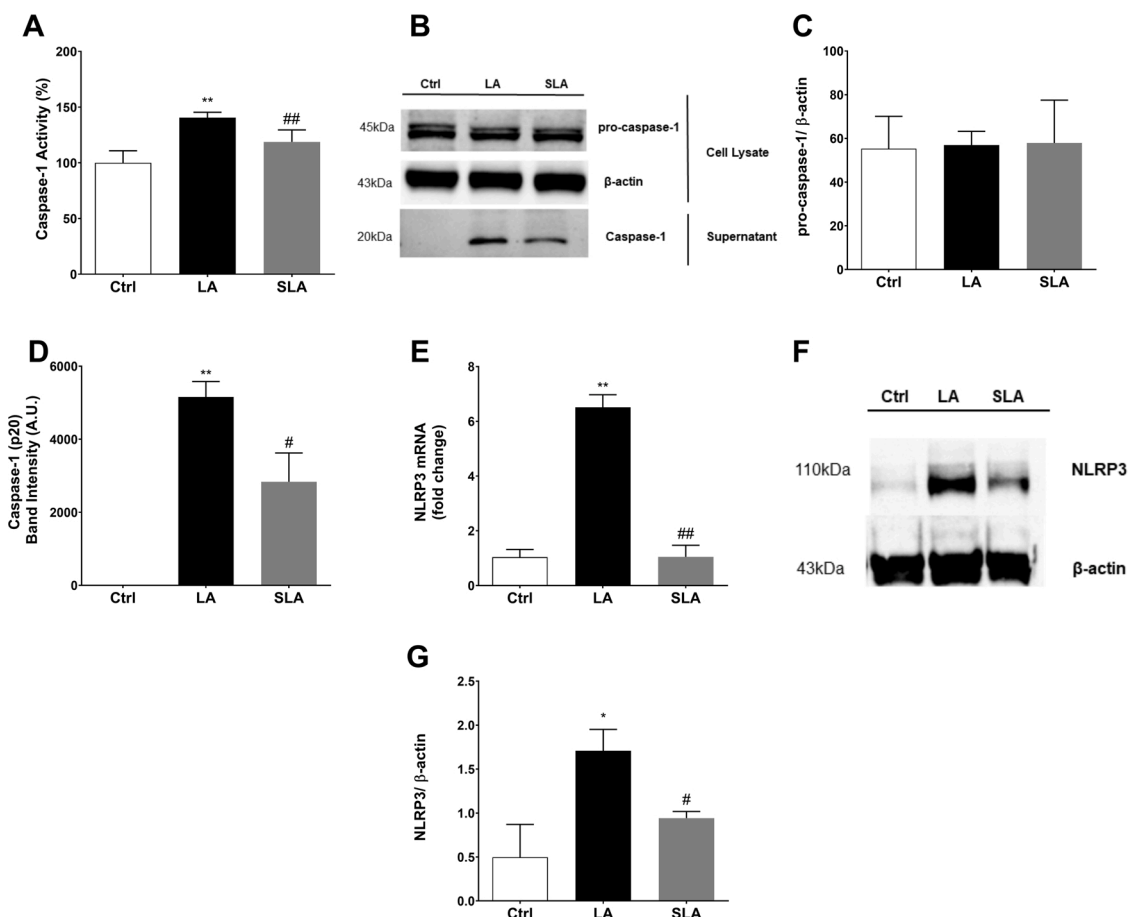
Fig. 2. SFN inhibits the active profile of inflammasome components in microglial cells.

N9 cells were treated with SFN (5 μM) for 1 h prior to LPS (1 μg/mL) for 4 h and ATP (5 mM) for 1 h to examine the alteration in inflammasome parameters. (A) SFN pretreatment reduces the activity of caspase-1. (B, C, D) Sub-units of caspase-1 protein levels were analyzed by Western Blot analysis. (E) mRNA level of NLRP3 was analyzed with qPCR at 6 h after LPS treatment. (F, G) NLRP3 protein levels in cell lysates were detected by Western blot analysis using specific NLRP3 and β-actin antibodies. The results are mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared with untreated control. #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ compared with LPS and ATP treatment.
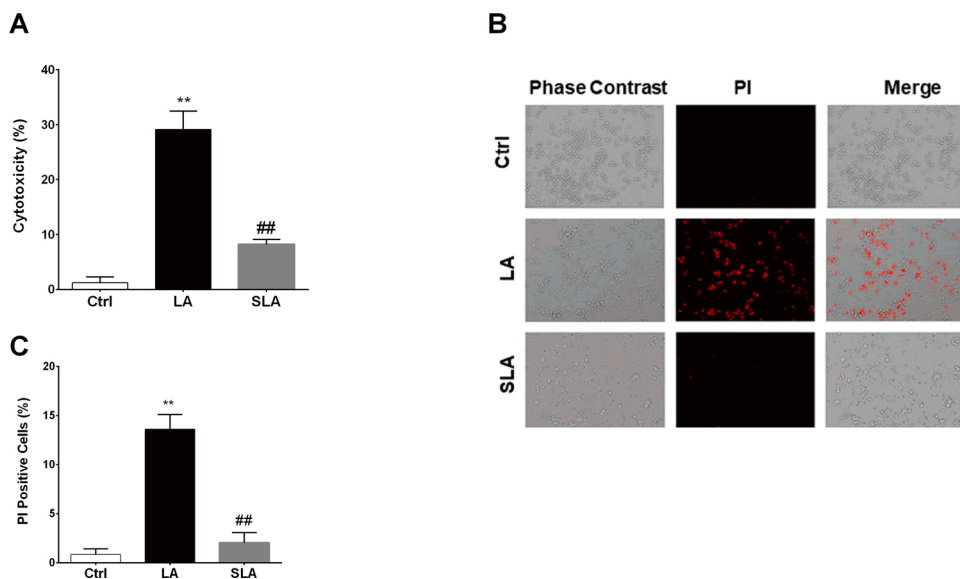

Fig. 3. SFN inhibits pyroptotic cell death in microglia attenuated by LPS and ATP.

N9 cells were treated with LPS (1 μg/mL) for 4 h and ATP (5 mM) for 1 h. (A) SFN pretreatment suppressed cytotoxicity formed by LPS and ATP. (B–C) Pyroptotic cells were stained with PI and visualized with immunofluorescence microscopy. The results are mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared with untreated control. #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ compared with LPS and ATP treatment.

untreated control. #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ compared with LPS and ATP treatment.
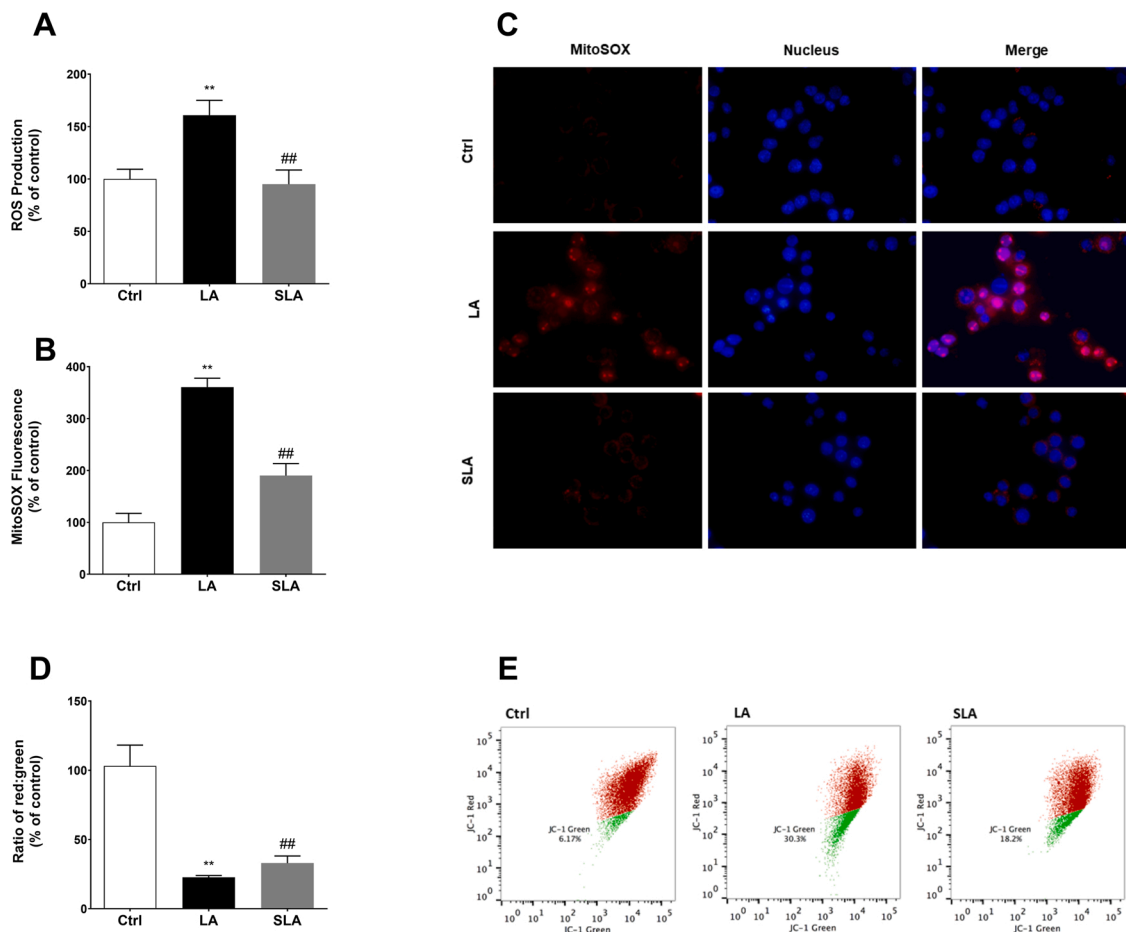
Fig. 4. SFN decreases total ROS production and mitochondrial intensity.
N9 cells were treated with SFN (5 μM) for 1 h, LPS (1 μg/mL) for 4 h, and ATP (5 mM) for 1 h. (A) Total ROS production was measured with CM2-HDCFDA fluorometric assay. Mitochondrial ROS production was determined by MitoSOX reagent with (B) fluorometric measurements, and (C) cells were visualized with immunofluorescence. Mitochondrial membrane potential analysis was performed with JC-1 dye by flow cytometry (D, E). The results are mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01 and ***p < 0.001 compared with untreated control. #p < 0.05, ##p < 0.01 and ###p < 0.001 compared with LPS and ATP treatment.

SFN on the NLRP3 protein level (Fig. 6E-F).

3.7. Increased expression of miRNA-223 by SFN is involved in the inhibitory effect of SFN on NLRP3 inflammasome in microglia

The expression of miRNA-223 was downregulated during inflammasome activation induced by LPS and ATP induction, and the level significantly increased due to SFN pretreatment (Fig. 7A). Because miRNA-223 is a negative regulator of NLRP3, the miRNA-223 inhibitor was transfected to microglial cells to define the functional effect of miRNA-223 deficiency. Antagonizing miRNA-223 function significantly reversed the SFN effect on NLRP3 inflammasome activation. mRNA levels of IL-1β, IL-18, and NLRP3 were increased (Fig. 7B–D), and the NLRP3 protein level was extremely high in SFN pretreated cells with the inhibition of miRNA-223 (Fig. 7E,F).

3.8. SFN altered miRNA-155 and miRNA-223 expression through inducing Nrf2 activation

SFN is known as a potent inducer of Nrf2. For this reason, Nrf2 translocation from cytosol to the nucleus was examined with the SFN treatment at different time points. We found that SFN treatment increased the translocation of Nrf2 over time and reached approximately 2.5-fold, according to immunoblot results (Fig. 8A,B). The effect of SFN in the regulation of Nrf-2 target genes was examined by qPCR. HO-1,

Gclc, Nqo-1, Gstp1, Gclm gene expressions were significantly upregulated with SFN treatment (Fig. 8C). We further attempted to determine whether SFN alters miRNA expression via Nrf2 activation. We transfected N9 cells with Nrf2 siRNA, then miRNA-155 and miRNA-223 expression were evaluated by qPCR. We found that Nrf2 inhibition significantly reversed the SFN effect on miRNA expression (Fig. 8D,E).

4. Discussion

The anti-inflammatory, antioxidant and cytoprotective effects of SFN are well established. Besides, SFN suppresses NLRP3 inflammasome activation in hepatic, liver, and gouty inflammation. Although it has been shown that SFN suppresses NLRP3 inflammasome activation in cerebral and retinal ischemia, its inhibitory effects on microglial NLRP3 inflammasome activation are not completely clear. In the present study, we showed that SFN protected N9 microglial cells against LPS and ATP-induced NLRP3 inflammasome activation and pyroptotic cell death. Furthermore, miRNAs contribute to the inhibitory effects of SFN on NLRP3 inflammasome activation. Our findings suggest that SFN acts on transcriptional and post-transcriptional regulation mechanisms of microglial NLRP3 inflammasome activation.

SFN inhibits proinflammatory transcription factor NF-κB, which has a prominent role in signal 1 of NLRP3 inflammasome activation [25]. It also prevents TLR4 oligomerization in LPS/TLR4/ NF-κB pathway. The priming step is necessary for macrophages and microglia, whose basal
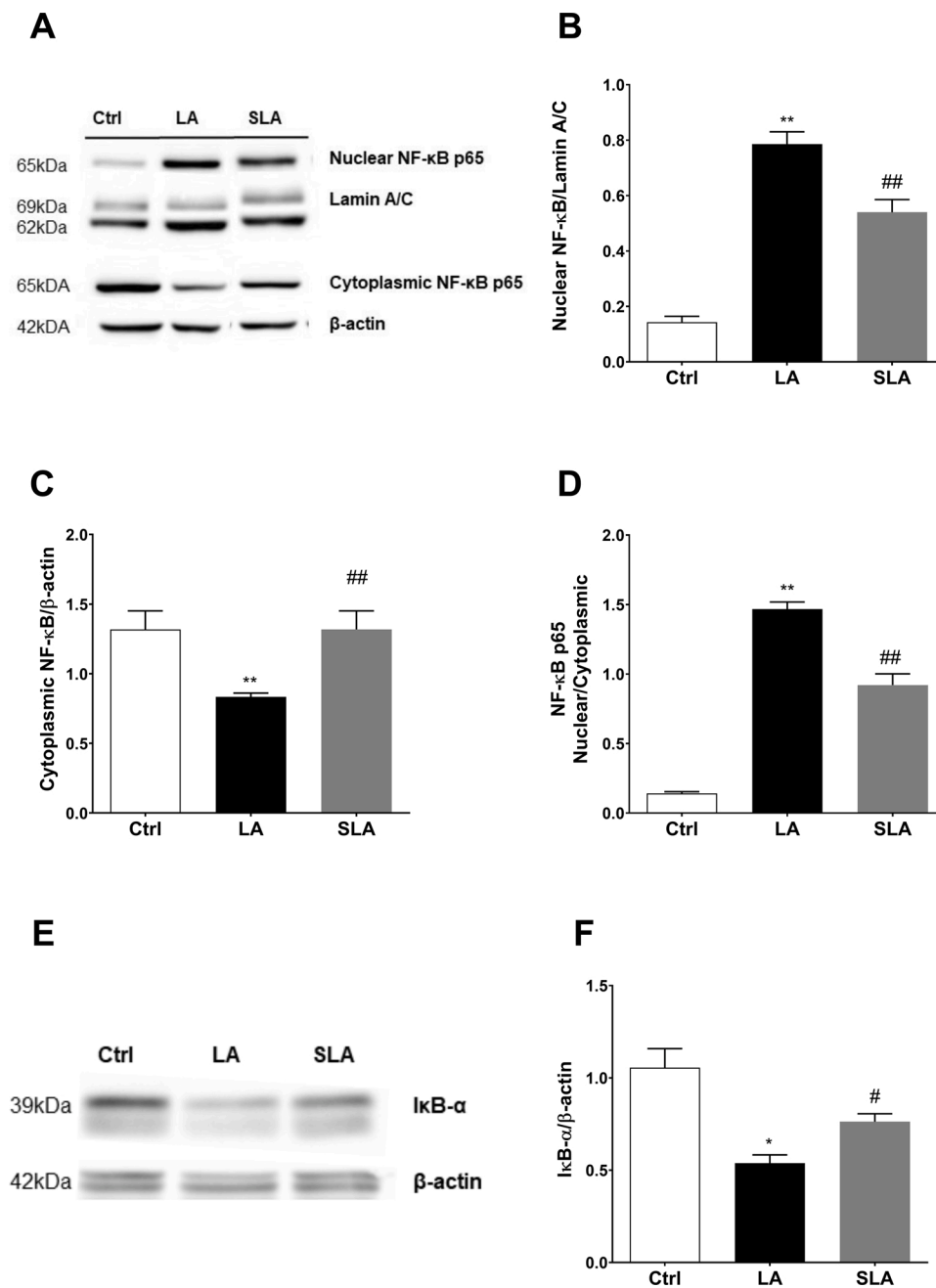
Fig. 5. NF-κB translocation is inhibited by SFN in LPS and ATP treated microglial cells.
N9 cells were treated with SFN (5 μM) for 1 h, LPS (1 μg/mL) for 4 h, and ATP (5 mM) for 1 h. (**A**-B-C-D) Nuclear NF-κB, cytoplasmic NF-κB, NF-κB translocation from cytoplasm to nucleus, and (E–F) IκB-α protein level was assessed with western blot assay. The results are mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01 and ***p < 0.001 compared with untreated control. #p < 0.05, ##p < 0.01 and ###p < 0.001 compared with LPS and ATP treatment.

IL-1β and NLRP3 expression are low to start NLRP3 inflammasome activation. Here, we showed that SFN inhibits NF-κB nuclear translocation and its target genes IL-1β and NLRP3 expression at the mRNA level. The activation of the LPS/TLR4/NF-κB pathway also results in transcriptional activation of IL-18, Gasdermin D, NEK7, NOX1, and NOX4 genes. In contrast to IL-1β, basal expression of IL-18 is not low; however, this cytokine also needs NLRP3 inflammasome activation for the cleavage of pro-IL18 and secretion of the mature form of IL-18. We found that SFN suppressed IL-18 mRNA expression in microglia. NF-κB is also involved in signal2. NEK7 provides the formation of NLRP3 oligomers and translocates the inflammasome complex to the mitochondria [26]. Finally, the interaction of TLR4 with NOX1 and NOX4 generates superoxide anions and hydrogen peroxide [27]. NOX-generated ROS crosstalk with MT-ROS sensed by NLRP3 [28].

ROS is one of the primary triggers of NLRP3 inflammasome activation [29]. Most of the studies argued that in addition to intracellular

ROS, mtROS also have a role in signaling pathways, including those regulating immune responses. Intracellular ROS activates plasma membrane cation channels and contributes to calcium influx that causes mitochondrial dysfunction. Damage of mitochondrial molecules releases and produce mitochondrial DAMPs (mtDAMPs) and increased mtROS that bind to NLRP3 [30]. It was reported that SFN inhibits the formation of mtROS in BMDMs [12]. We found that augmentation in total and mitochondrial ROS production due to LPS and ATP treatment was reversed by SFN pretreatment in N9 microglial cells. Also, we showed that mitochondrial membrane potential was protected by SFN.

The protective effect of SFN against oxidative damage in the brain is related to the activation of the redox-sensitive transcription factor Nrf2 pathway, which controls the expression of more than 200 detoxification and antioxidant defense genes Ho-1, Gstp1, Nqo1, Gpx, Srxn1, and Gclc [31]. This study showed that SFN contributes to the Nrf2 translocation into the nucleus and upregulates its target genes' expression in
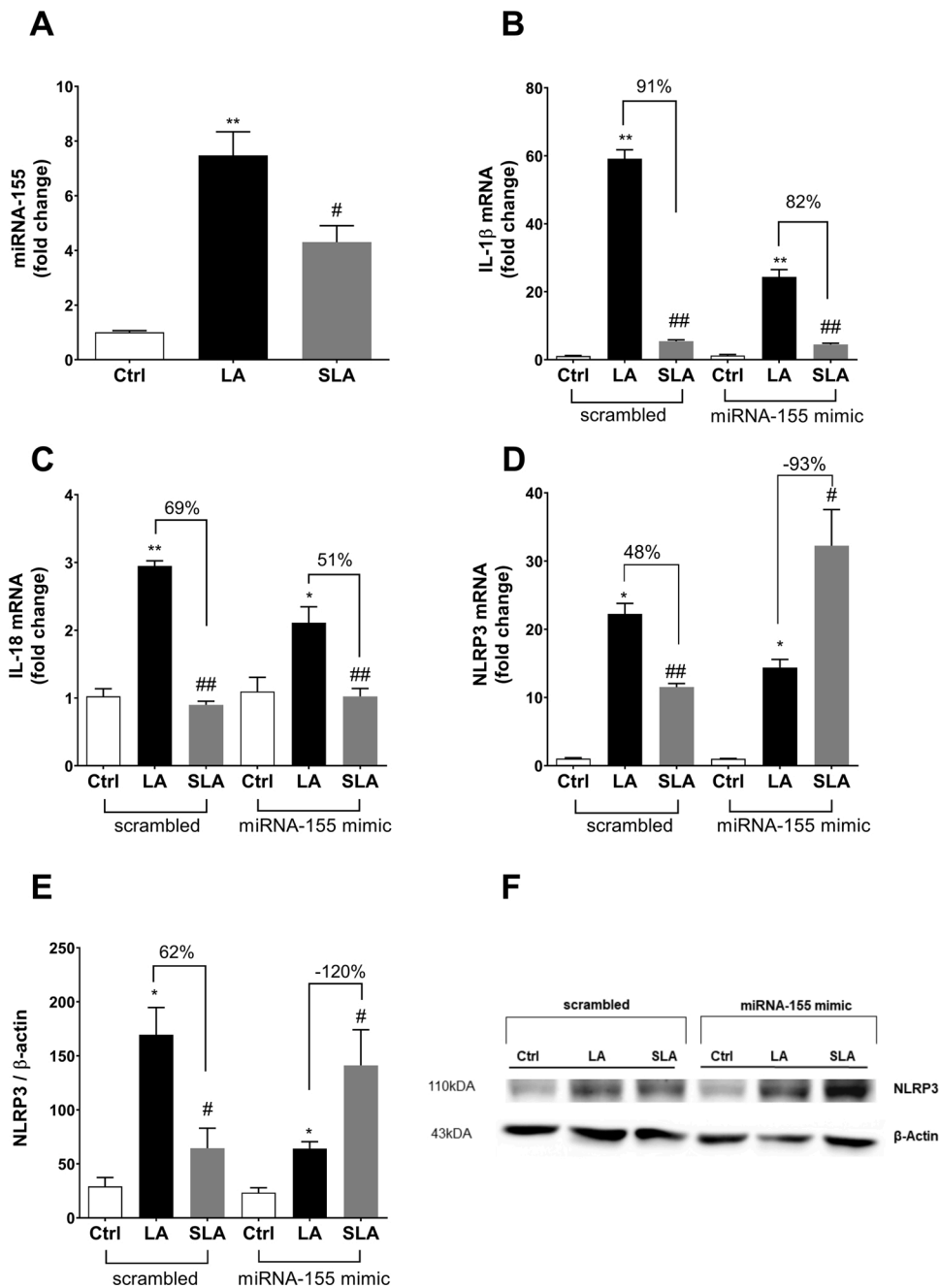
Fig. 6. miRNA-155 overexpression eliminates the effect of SFN on inflammasome components in microglial cells.

N9 cells were transfected with scrambled or miRNA-155 mimic miRNA for 48 h and then treated with SFN (5 μM) for 1 h, LPS (1 μg/mL) for 4 h, and ATP (5 mM) for 1 h. (A) The expression of the miRNA-155 level was analyzed with qPCR. (B) IL-1β (C), IL-18, and (D) NLRP3 mRNA level was determined. (E, F) The NLRP3 protein level in cell lysates was noticed by Western blot analysis. The results are mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01 and ***p < 0.001 compared with untreated control. #p < 0.05, ##p < 0.01 and ###p < 0.001 compared with LPS and ATP treatment.

microglia. Oxidative stress can also affect the IκB kinase (IKK), whose role is phosphorylating NF-κB and proteasomal degradation of IκB. Free NF-κB migrates into the nucleus and binds its target genes to activate proinflammatory mediators. Remarkably, there is a competition between Nrf2 and NF-κB at DNA binding level. Induction of expression of anti-oxidative enzymes Ho-1 by SFN suppresses the degradation of IκB and inhibits the NF-κB translocation into the nucleus [32]. Additionally, Ho-1 can directly impair NF-κB binding to the promoter of immune genes [33]. We demonstrated that SFN inhibits NF-κB nuclear trans-location and target gene's expression, including HO-1 in microglia. Our data propose that the effect of SFN is not only targeting Nrf2 via Keap1 but also uses other pathways [34].

miRNAs regulate NLRP3 inflammasome activation through targeting upstream/downstream pathways or directly proteins in the NLRP3 inflammasome complex. Numerous studies have identified the potential role of miRNA-155 in innate and adaptive immune responses. Since the

expression of miRNA-155 is dependent on TLR4/NF-κB pathways, its expression increased by TLR4 ligands such as TNF-α, IL-1β, interferons, and LPS in several cell types, including monocytes and macrophages [35,36]. Upon activation of the TLR4 receptor, proinflammatory signaling cascades cause translocation of NF-κB into the nucleus. This activation increases miRNA-155 expression and contributes to the regulation of the strength and duration of inflammation. Down-regulation of miRNA-155 in inflammation was indicated by dietary anti-inflammatory compounds such as resveratrol [37], curcumin [38], and SFN [36]. Our study found that SFN pretreatment alleviated upre-gulated miRNA-155 expression in LPS and ATP-induced microglial cells. Furthermore, we revealed that overexpression of miRNA-155 signifi-cantly decreases the protective effect of SFN against LPS and ATP in-duction. Our findings supported that the protective effects of SFN in NLRP3 inflammasome activation are associated with miRNA-155 inhi-bition. Besides, similar to our previous study [36], we confirmed the
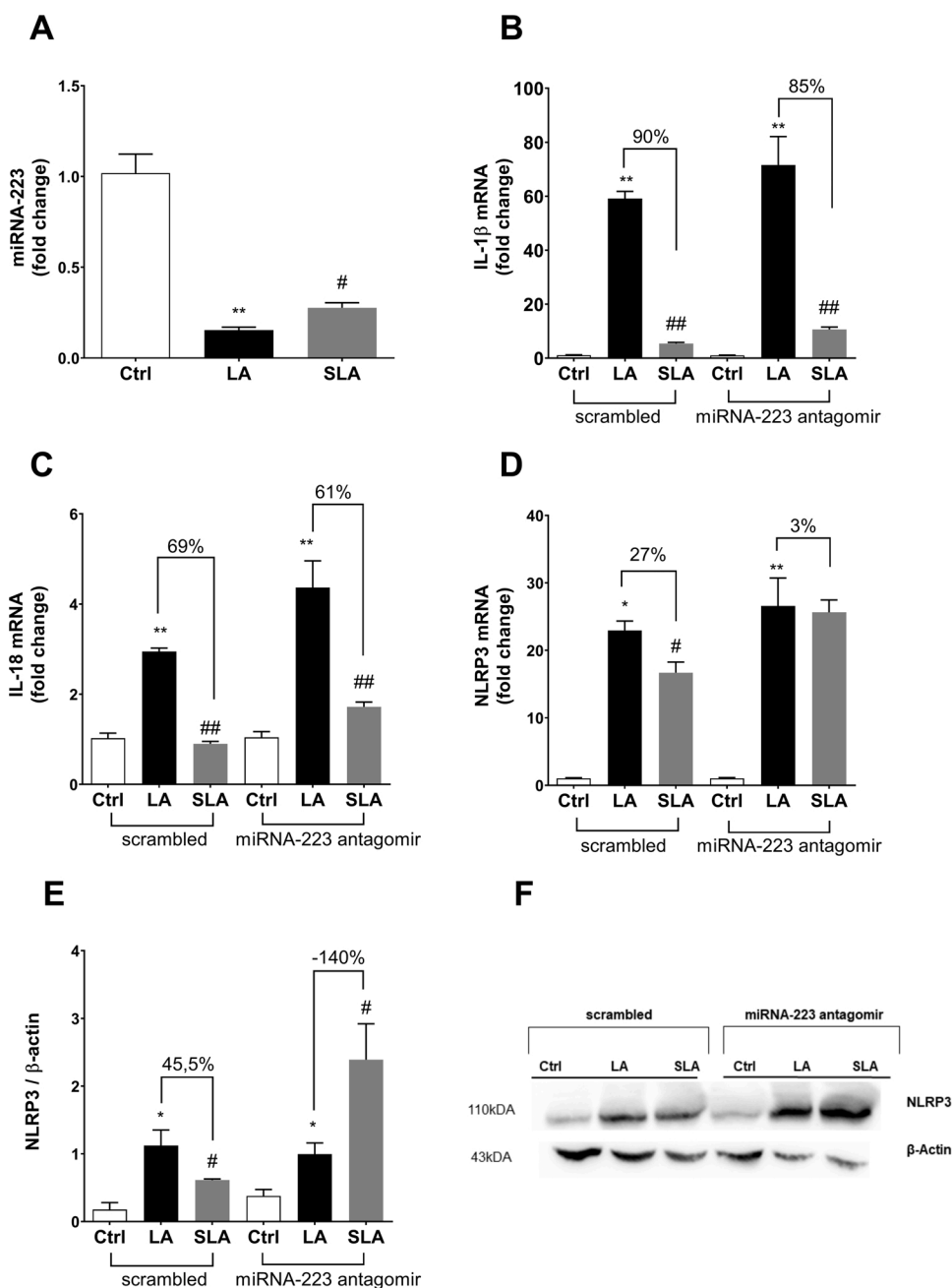
Fig. 7. miRNA-223 elimination reduces the protective effect of SFN on NLRP3 inflammasome activation in microglial cells.

N9 cells were transfected with scrambled or miRNA-223 antagomir miRNA for 48 h and then treated with SFN (5 μM) for 1 h, LPS (1 μg/mL) for 4 h, and ATP (5 mM) for 1 h. (A) the miRNA-223 expression level was determined (B, C, D) IL-1β, IL-18, and NLRP3 mRNA level was analyzed with qPCR (E, F) NLRP3 protein level in cell lysates were determined by Western blot analysis. The results are mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01 and ***p < 0.001 compared with untreated control. #p < 0.05, ##p < 0.01 and ###p < 0.001 compared with LPS and ATP treatment.

Nrf2 signaling pathway's role in miRNA-155 expression in NLRP3 inflammasome activation with the Nrf2 knockdown study. We found the inhibitory effects of SFN on miRNA-155 expression are substantially reversed after siRNA-mediated Nrf2 knockdown.

miRNA-223 is an anti-inflammatory miRNA taking place in immune responses in microglial cells by directly targeting NLRP3 protein. The upregulation of miRNA-223 expression in microglia contributes to the debris clearance via phagocytosis and CNS remyelination [39]. We showed that miRNA-223 expression levels were increased by SFN pretreatment. Interestingly, miRNA-223 knockdown reversed the beneficial effects of SFN on the activation of the NLRP3 inflammasome. Our results supported that the miRNA-223/NLRP3 axis could mediate the beneficial effect of SFN. Altered regulation of miRNA-223 by phytochemicals could be an effective treatment for various immune-related disorders [40]. Numerous transcription factors, including PU.1 and CAA-T/enhancer-binding protein (C/EBP) β, and nuclear factor I-A (NFI-A), alter miRNA-223 gene expression [41]. We also checked whether SFN

pretreatment causes differential expression of miRNA-223 dependent on the Nrf2 pathway. We demonstrated that miRNA-223 expression is altered with Nrf2 activation. Thus, we conclude that SFN protected N9 microglial cells against NLRP3 inflammasome activation via miRNA-223/NLRP3 axis-dependent manner.

In the present study, we showed for the first time the protective effects of SFN against microglial NLRP3 inflammasome activation and the pathways in which SFN modulates. However, there are some limitations to our experimental design. First, we utilized a murine N9 microglial cell line in our study, widely known and commonly used [42]. As a preliminary step to well-designed animal experiments, cell culture experiments are advantageous in cost, time, homogeneity, and yield. However, one limitation is the unavailability of animal studies. Even if the microglia cell lines have been shown to reflect similar responses to primary microglia, differences in proteome and transcriptome profiles have been reported [43]. Extrapolation of cell culture results *in vivo* conditions is still inconvenient. Despite ethical issues of animal use for
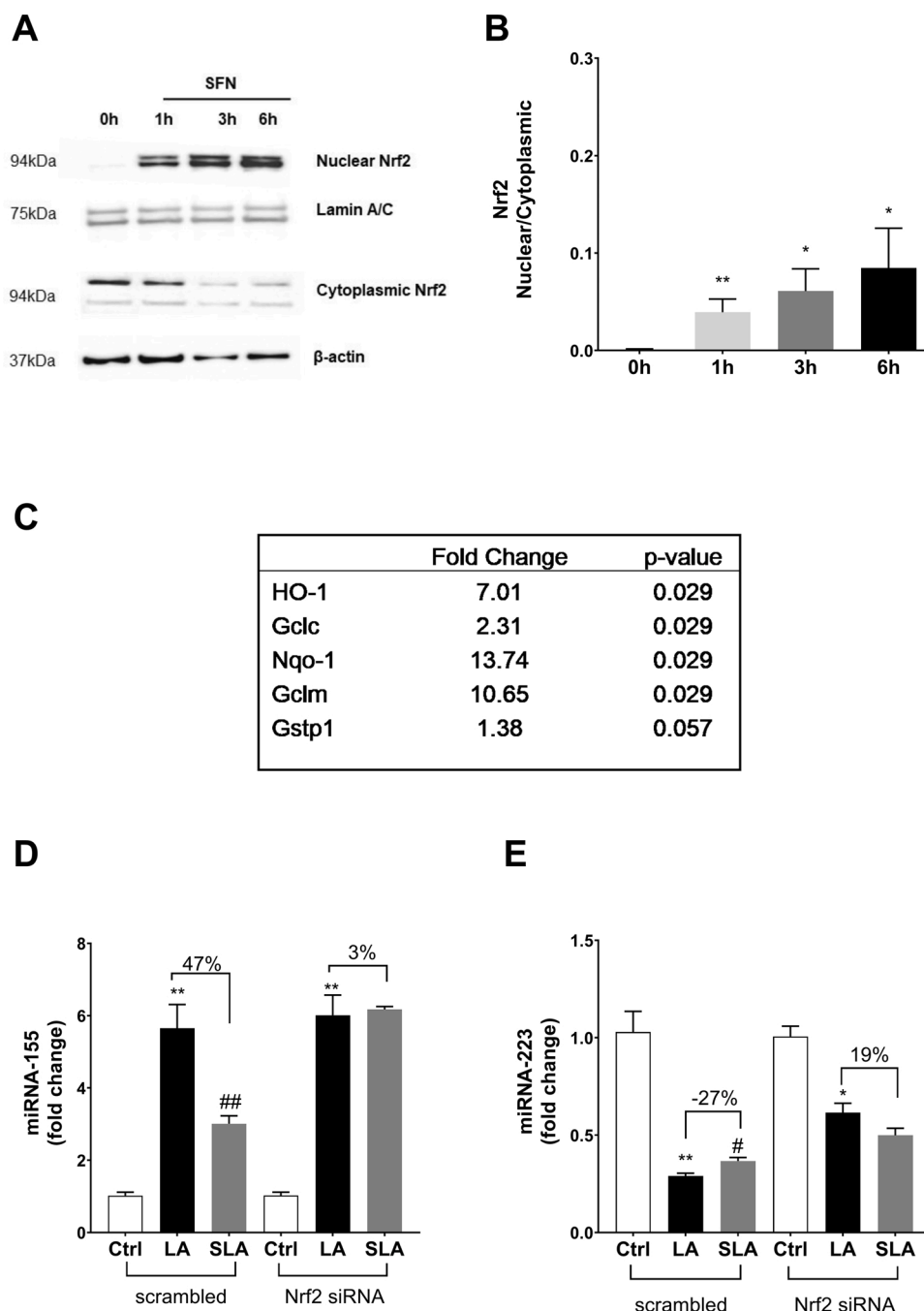
A

B

C

	Fold Change	p-value
HO-1	7.01	0.029
Gclc	2.31	0.029
Nqo-1	13.74	0.029
Gclm	10.65	0.029
Gstp1	1.38	0.057

D

E

experimentation, *in vivo* experiments are still required for clinical trials. So far, there is only one report about the effects of SFN on NLRP3 inflammasome activation by amyloid-beta treatment in microglial cells [44]. However, the underlying mechanism of protective effects SFN against NLRP3 inflammasome activation has not been focused on in that study. Furthermore, our results should also be confirmed using human microglial cells; however, one should be cautious in translating *in vitro* and *in vivo* findings to clinical studies due to the critical variances of microglial responses of humans and rodents [45]. In future studies, SFN should include *in vivo* experimental setups.

In conclusion, our findings demonstrated that SFN protected against NLRP3 inflammasome activation ad pyroptotic cell death in LPS and ATP-treated N9 microglial cells. SFN exerted anti-inflammatory and cytoprotective effects through regulating inflammatory NF-κB and anti-oxidative Nrf2 signaling axis. We furthermore showed that SFN

pretreatment change miRNA-155 and miRNA-223 expression in Nrf2 dependent manner. Altogether, our results bring new insight into the therapeutic potential of SFN in the alleviation of the immune response in CNS.

Declaration of Competing Interest

The authors declare no competing financial interests.

Acknowledgment

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2021.03.004.

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