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Research Article

Role of Nrf2 Signaling Pathway: Sulforaphane Enhances NGF-Induced Neurite Outgrowth in PC12 Cells

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Abstract

Sulforaphane (SFN) is an organosulfur compound, which is found in cruciferous vegetables, such as broccoli Brussels sprouts, and cabbage. Accumulating evidence suggested that the mechanism for the antipsychotic drugs were promoting neurite outgrowth. Our recent study found that SFN showed antipsychotic effects. Therefore, it is interesting to investigate SFN enhanced NGF-induced neurite outgrowth in PC12 cells via Nrf2 signaling pathway. First, we found that SFN significantly enhanced NGF-induced neurite outgrowth in PC12 cells via the activation of Nrf2. Second, inositol 1,4,5-triphosphate (IP3) receptors inhibitors (Xestospongin C and 2-aminoethoxydiphenyl borate (2-APB)) significantly blocked SFN enhanced NGF-induced neurite outgrowth in PC12 cells. The results suggested that SFN enhanced NGF-induced neurite outgrowth in PC12 cells via Nrf2 signaling pathway, and subsequently via IP3 receptor.

Key words: Sulforaphane; NF-E2-related factor-2 (Nrf2); inositol 1,4,5-triphosphate (IP3); Neurite Outgrowth.

Introduction

Sulforaphane (SFN: 1-isothiocyanato-4-methylsulfinylbutane) is an organosulfur compound, which is found in cruciferous vegetables, such as broccoli Brussels sprouts, and

cabbage. Recent studies found SFN showed antioxidant and anti-inflammatory effects [1, 2]. The mechanism for the antioxidant and anti-inflammatory effects of SFN is thought to be mediated by activation of the NF-E2-related factor-2 (Nrf2), resulting in Phase II detoxification enzymes and antioxidant proteins transcription [3-7]. The previous studies found that SFN showed neuroprotective effects in methamphetamine or phencyclidine (PCP) induced behavioral abnormalities in mice [8]. Subsequently, they reported that dietary intake of SFN-rich broccoli sprout extracts during juvenile and adolescence could prevent PCP-induced cognitive deficits [9]. Besides, intraperitoneally (IP) injection of SFN showed antidepressant-like effects in inflammation and chronic social defeat stress (CSDS) models of depression [2, 10]. Moreover, dietary intake of 0.1% glucoraphanin (a precursor of SFN) significantly attenuated depression-like phenotypes [10]. Taken together, SFN showed antipsychotic effects in the animal model.

Some evidence suggested that at the cellular level, neuronal plasticity demonstrated by neurite outgrowth and neuroprotection, underlie the therapeutic effects of atypical antipsychotic drugs [11]. PC12 cell line derived from a pheochromocytoma of the rat adrenal medulla, constitute a recognized model system for a nerve growth factor (NGF)-induced neurite outgrowth [11]. The traditional antipsychotic drugs such as aripiprazole and brexpiprazole showed potentiation of neurite outgrowth in PC12 cells [11, 12], suggesting the antipsychotic effects of these drugs by changing neurite outgrowth.

In the current study, we try to explore the potential mechanism for SFN enhanced NGF-induced neurite outgrowth in PC12 cells. First, we found that SFN significantly enhanced NGF-induced neurite outgrowth via activation of Nrf2. Second, we examined the role of intracellular $Ca2^{+}$ and the endoplasmic reticulum (ER) protein inositol 1,4,5-triphosphate (IP3) receptors, on the potentiation of NGF-induced neurite outgrowth by SFN administration, since Ca^{2+} signaling pathway via IP3 receptors plays an important role in NGF-induced neurite outgrowth [11, 12]. We found that SFN increased the IP3 receptor expression, and IP3 receptor antagonist (Xestospongin C (Xest C) and 2-aminoethoxydiphenyl borate (2-APB)) significantly blocked SFN enhanced NGF-induced neurite outgrowth in PC12 cells.

Materials and methods

Quantification of neurite outgrowth in PC12 cells

PC12 cells were cultured at 37°C, 5% CO2 in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. PC12 cells were plated onto 6-well tissue culture plates coated with poly-D-lysine/laminin. Cells were plated at relatively low density (0.25×104 cells cm-2) in a DMEM medium containing 10% FBS, 1% penicillin-streptomycin. Twenty-four hours after plating, the medium was replaced with DMEM medium containing 10% FBS and 1% penicillin-streptomycin with NGF (2.5 ng/ml), with or without SFN (0.1 μM) (Biorbyt) or Xest C (1 μM) (Biorbyt) or 2-APB (100 μM) (TargetMol) or siRNA-Nrf2 (Santa Cruz) for 48 hours.

Western blotting assay

The cells were lysed in RIPA buffer (20 mM pH 7.5 Tris-HCl, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) on ice for 30 min. Cell lysates were then centrifuged at 13,000×g for 30 min at 4 °C. The supernatant was collected, and protein concentration was determined using a Coomassie Brilliant Blue protein assay kit (Bio-Rad). The same amount of the supernatant was boiled in an SDS loading buffer. After SDS-PAGE, the

samples were transferred to a polyvinylidenedifluoride (PVDF) membrane. The membranes were blocked in 2% BSA for 1h at room temperature and then incubated with primary antibody (Nrf2 antibody, ab137550) at 4 °C overnight. The next day, the blots were incubated with an anti-mouse IgG secondary antibody. Images were captured with a Tanon-5200CE imaging system (Tanon, Shanghai, China), and immunoreactive bands were quantified.

Immunofluorescence staining

The immunofluorescence staining for PC12 cells. Briefly, 4% paraformaldehyde-fixed cell slides were treated with 3% hydrogen peroxide at room temperature for 10min. Manufacturer-supplied blocking buffer (Invitrogen) was used for each reaction. Then the sections were incubated with primary antibodies (anti-NeuN antibody, ab128886; Nrf2 antibody, ab137550, and IP3 antibody, ab5804) overnight at 4 °C. For double immunofluorescence staining, cell slides were incubated with an Alexa Fluor 488 or 568 conjugated isotype-specific secondary antibody for 1h at room temperature. The fluorescence staining was visualized with an Olympus confocal microscope.

Statistical analysis

The data are shown as the mean ± standard error of the mean (S.E.M.). The data were analyzed using PASW Statistics 20 (formerly SPSS statistics; SPSS). All data were analyzed using a one-way analysis of variance (ANOVA), followed by the post hoc Fisher LSD test. P values < 0.05 were considered statistically significant.

Results

SFN enhances neurite outgrowth in PC12 cells after NGF administration via activation of Nrf2 signaling pathway

Neurite outgrowth is involved in the antipsychotic drug's mechanisms, we examined *SFN enhanced neurite outgrowth in PC12 cells after NGF administration*. The NeuN immunofluorescence staining showed that SFN (1 μM) in conjunction with NGF significantly increased the number of cells with neurites (Fig. 1A, B). One-way ANOVA analysis revealed significant differences among the three groups [$F(3, 17) = 13.419$, $P < 0.001$]. The dose of SFN was selected as previously reported [10]. To explore the function of Nrf2 in SFN enhanced neurite outgrowth in PC12 cells after NGF administration. We performed western blot assay for Nrf2, we found that SFN significantly increased the Nrf2 protein expression, and SiRNA-Nrf2 significantly decreased Nrf2 protein expression in PC12 cells after NGF administration (Fig. 1C, D). One-way ANOVA analysis revealed significant differences among the three groups [$F(3, 9) = 7.072$, $P = 0.026$]. The results suggested that SFN enhanced NGF-induced neurite outgrowth in PC12 cells via Nrf2 signaling pathway.

Role of IP3 receptor signaling pathway on the potentiation of NGF-induced neurite outgrowth by SFN administration

IP3 receptors on the ERs are part of the signaling pathway that promotes NGF-induces neurite outgrowth in PC12 cells [13]. To explore the involvement of IP3 receptors in SFN's action for these effects. We examined IP3 receptor by using immunofluorescence staining for PC12 cells, we found that SFN significantly increased IP3 receptor and Nrf2 fluorescence intensity, and these effects can be blocked by siRNA-Nrf2 (Fig 2). One-way ANOVA analysis revealed significant differences among the three groups [$F(3, 17) = 36.487$, $P < 0.001$ for IP3 receptor; $F(3, 17) = 94.088$, $P < 0.001$ for Nrf2].

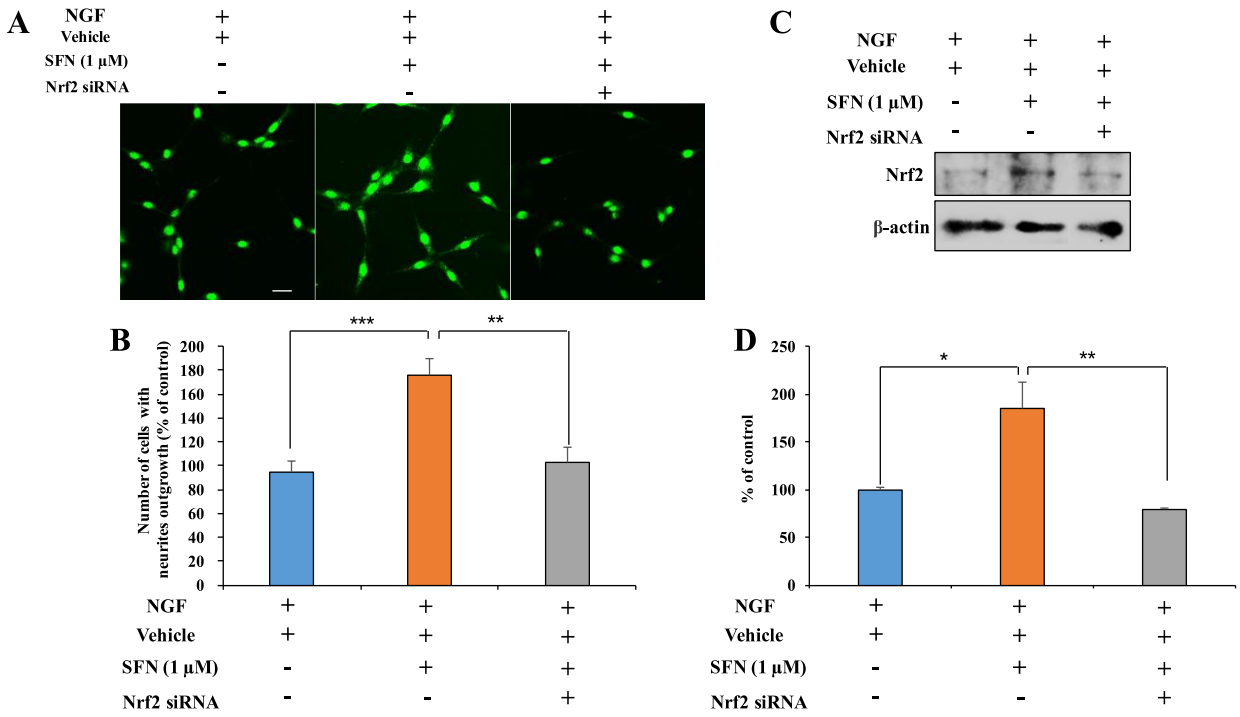

Fig. 1. The role of Nrf2 in SFN potentiates nerve growth factor (NGF)-induces neurite outgrowth in PC12 cells. (A and B): SFN potentiates NGF-induces neurite outgrowth in PC12 cells. (Mean ± SEM, n = 6 per group, one-way ANOVA, **p < 0.01, ***p < 0.001). Scar bar = 50 μm. (C and D) The western blot analysis for Nrf2. (Mean ± SEM, n = 3 per group, one-way ANOVA, *p < 0.05, **p < 0.01).

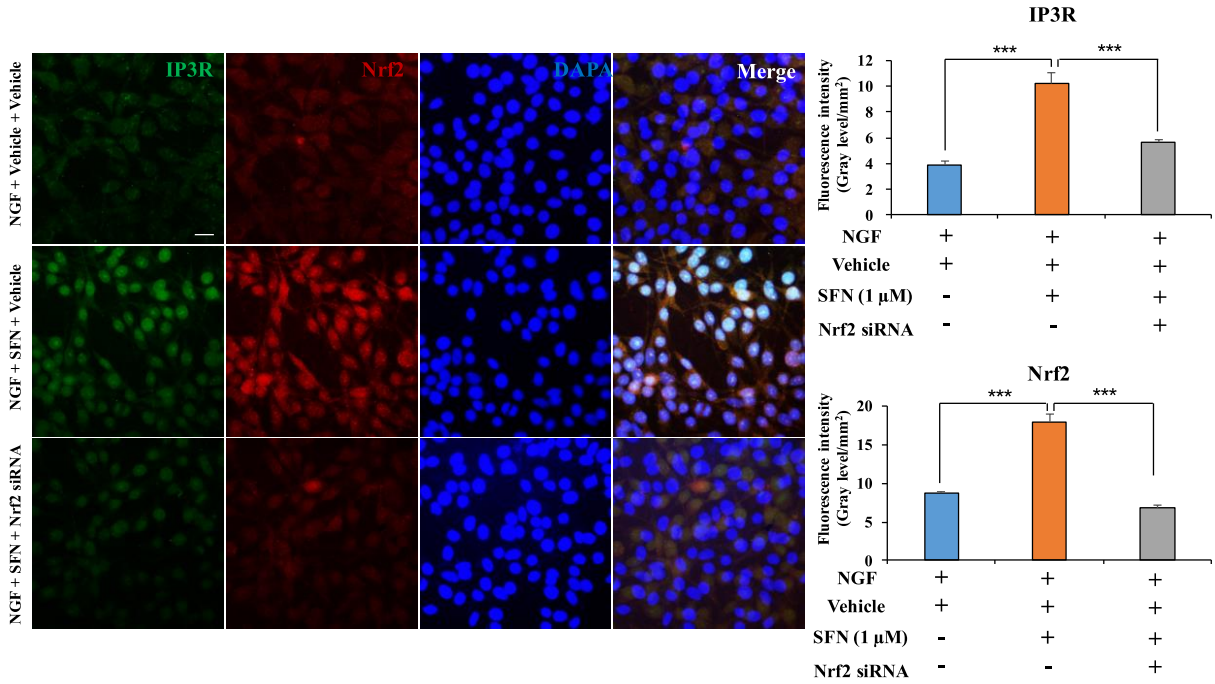

Fig. 2. The immunofluorescence for Nrf2 and IP3 receptor in SFN potentiates nerve growth factor (NGF)-induces neurite outgrowth in PC12 cells. (Mean ± SEM, n = 4 per group, one-way ANOVA, ***p < 0.001). Scale bar = 50 μm.

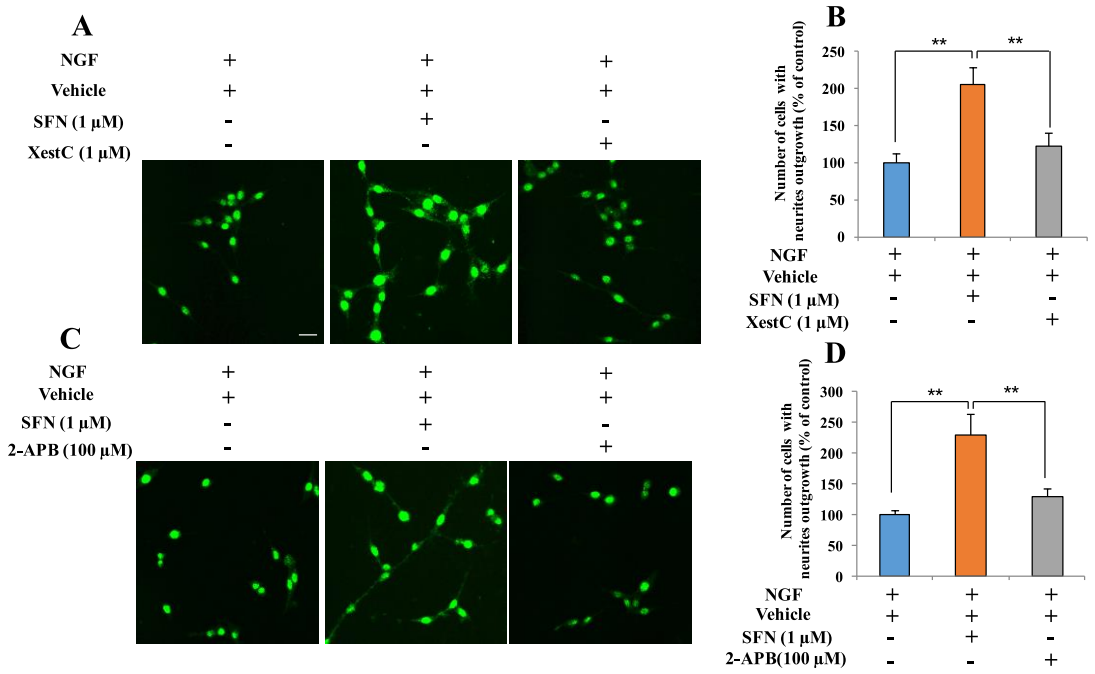

Fig. 3. The role of IP3 receptor signaling pathway in SFN potentiates NGF-induces neurite outgrowth in PC12 cells. (A and B): The immunofluorescence staining for NeuN after Xestospongin C administration. (Mean ± SEM, n = 6 per group, one-way ANOVA, *p < 0.05, **p < 0.01). (C and D): The immunofluorescence staining for NeuN after 2-aminoethoxydiphenyl borate (2-APB) administration. (Mean ± SEM, n = 6 per group, one-way ANOVA, *p < 0.05, **p < 0.01). Scale bar = 50 μm.

Moreover, we further examined the IP3 receptor inhibitor Xest C (1 μM) and 2-APB (a selective, reversible, and membrane-permeable inhibitor of IP3 receptors, 100 μM) [11, 12]. Using the NeuN immunofluorescence staining for PC12 cells, we found the addition of Xest C and 2-APB with SFN significantly decreased the potentiation of NGF-induced neurite outgrowth (Fig 3A-D). One-way ANOVA analysis revealed significant differences among the three groups [F (3, 23) = 10.087 P = 0.002 for Xest C; F (3, 23) = 9.973, P = 0.002 for 2-APB].

Discussion

In the current study, we found that SFN could enhance neurite outgrowth in PC12 cells after NGF administration, and this effect could be blocked by siRNA-Nrf2. Second, using immunofluorescence staining for IP3 receptors, we found SFN could increase IP3 receptors and Nrf2 fluorescence intensity. Third, by using Xest C and 2-APB, which is reversible and membrane-permeable inhibitor for IP3 receptors. We found that Xest C and 2-APB both could block the SFN enhanced neurite outgrowth in PC12 cells after NGF administration. The results suggested that the Nrf2, and subsequent IP3 signaling pathway play a role in SFN enhanced of NGF-induced neurite outgrowth in PC12 cells after NGF administration.

The Nrf2 activator SFN is an organosulfur compound. Recent studies found SFN showed antioxidant and anti-inflammatory effects [3, 4, 14]. The mechanism study revealed that antioxidant and anti-inflammatory effects afforded by SFN are thought to be mediated via the activation of the Nrf2 and subsequent up-regulation of Phase II detoxification enzymes and antioxidant proteins through an enhancer sequence referred to as the electrophilic-responsive element or the antioxidant-responsive element (ARE) [6, 7]. Our study found that SFN showed antipsychotic

effects (such as depression and schizophrenia). The mechanism study suggested that the antipsychotic effects of SFN by activating brain-derived neurotrophic factor (BDNF) and its receptor tropomyosin-receptor-kinase B (TrkB) in the mouse brain [2, 8, 9]. Moreover, accumulating evidence suggested that at the cellular level, neuronal plasticity, such as neurite outgrowth and neuroprotection, underlie the therapeutic effect of atypical antipsychotic drugs [15-18]. Previously, studies found that atypical antipsychotic drugs such as olanzapine, quetiapine, and clozapine could enhance NGF-induced neurite outgrowth in PC12 cells [11, 12]. In current study, we found that SFN in conjunction with NGF significantly increased the number of cells with neurites outgrowth, and these effects could be blocked by siRNA-Nrf2, suggesting that Nrf2 signaling pathway plays a role in SFN enhanced NGF-induced neurite outgrowth. Therefore, the mechanism for the antipsychotic effects of SFN are similar with olanzapine, quetiapine and clozapine via enhanced NGF-induced neurite outgrowth in PC12 cells.

IP3 is a ubiquitous second messenger responsible for the release of Ca^{2+} from the ER, a tightly controlled process that is critically important for maintaining cellular functions, including cell growth, and neurite outgrowth [13, 19]. It is reported that IP3 signaling pathway plays a key role in aripiprazole and brexpiprazole enhanced NGF-induced neurite outgrowth [11, 12]. In this study, we found that IP3 inhibitor Xest C and 2-APB both could block the potentiation of NGF-induced neurite outgrowth by the administration of SFN, suggesting the Ca^{2+} signaling via IP3 receptor plays a role in SFN enhanced NGF-induced neurite outgrowth in PC12 cells. However, the detail relationship between the Nrf2 and IP3 is also unclear. It needs further study.

In conclusion, the current data suggested that SFN enhanced NGF-induced neurite outgrowth in PC12 cells via activation of Nrf2 signaling pathway. SFN enhanced of NGF-induced neurite outgrowth in PC12 cells could be blocked by IP3 inhibitor Xest C and 2-APB, suggesting the role of IP3 signaling pathway in SFN enhanced of NGF-induced neurite outgrowth. Taken together, it is likely that Nrf2 and subsequent IP3 signaling pathway plays a crucial role in SFN enhanced of NGF-induced neurite outgrowth in PC12 cells.

Declarations

1) ***Consent to publication***

We declare that all authors agreed to publish the manuscript at this journal based on the signed Copyright Transfer Agreement and followed publication ethics.

2) ***Ethical approval and consent to participants***

Not applicable.

3) ***Disclosure of conflict of interests***

We declare that no conflict of interest exists.

4) ***Funding***

None

5) ***Availability of data and material***

We declare that the data supporting the results reported in the article are available in the published article.

6) ***Authors' Contributions***

JZ conceived the project, designed the experiments, analyzed the data, and wrote the manuscript. RT and SF performed cell culture and western blot. QC performed immunofluorescence.

7) ***Acknowledgement***

None

8) ***Authors' biography***

None

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