Inhibition of 6-hydroxydopamine-induced endoplasmic reticulum stress by sulforaphane through the activation of Nrf2 nuclear translocation

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Abstract. Endoplasmic reticulum (ER) stress plays a key role in the development of neurodegenerative diseases, including Parkinson's disease (PD). Sulforaphane (SF) is a natural drug derived from isothiocyanate found in cruciferous vegetables. Although there are reports indicating that SF is a potential candidate for PD treatment, there have been no reports on the effects of SF on ER stress in PD. In this study, we investigated the cytoprotective effects of SF on 6-hydroxydopamine (6-OHDA)-induced ER stress in rat PC12 cells. Pre-treatment with SF elicited cytoprotection against 6-OHDA-induced cytotoxicity. Consistent with its cytoprotective action, SF significantly inhibited subsequent ER stress, including the expression of Bip and the C/EBP homologous protein. We also found that transfection with NF-E2-related factor-2 (*Nrf2)* siRNA reversed the inhibitory effects of SF on 6-OHDA-induced ER stress responses. In conclusion, our results show that SF can prevent ER stress response induced by 6-OHDA through the activation of Nrf2. SF may be a therapeutic candidate for the treatment of ER stress-associated neural diseases, including PD.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder and is characterized by the loss of nigrostriatal dopaminergic neurons and the presence of Lewy bodies in the neurons (1). Although PD has been widely studied, the pathogenesis of PD remains unknown. There are no effective therapies available to prevent PD progression and neurodegeneration. However, significant clues into the pathogenesis of PD have been yielded by the use of 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenylpyridinium (MPP^+), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone (2-4). 6-OHDA is a selective catecholaminergic neurotoxin that replicates most of the neuropathological hallmarks of PD (5,6). 6-OHDA is commonly used to produce an experimental model of PD *in vitro* or *in vivo*.

The endoplasmic reticulum (ER) is an important organelle involved in calcium signaling and the synthesis, folding and processing of proteins (7). Impaired function of the ER leads to ER stress, which is caused by oxidative stress, changes in Ca^{2+} homeostasis and accumulation of unfolded or misfolded proteins (8). ER stress activates signaling pathways, including the unfolded protein response (UPR) pathway (9). When ER stress occurs, the phosphorylation of eukaryotic initiation factor 2α (eIF-2α) is firstly enhanced to reduce the load of newly synthesized proteins (10,11). Then, ER chaperones, such as Bip, are induced to enhance the folding activity in the ER (12). However, if the function of the ER is severely impaired, the apoptotic pathway is activated to remove the damaged cells. One of the components of ER stress-mediated apoptosis is the C/EBP homologous protein (CHOP), also known as growth arrest and DNA damage inducible gene 153 (GADD153). CHOP is expressed at low levels under physiological conditions, but is strongly induced in response to ER stress (13,14). ER stress-induced apoptosis is suppressed in both CHOP-null cells and *CHOP*-knockout mice (15). ER stress-induced cellular dysfunction is critical in the development and progression of human diseases, including stroke, diabetes and neurodegeneration (8,16).

Sulforaphane (SF) is a natural drug derived from isothiocyanate found in cruciferous vegetables, such as broccoli, cabbage and cauliflower. SF is known to have cytoprotective effects by activating the transcription factor, NF-E2-related factor-2 (Nrf2), which binds to the anti-oxidant response element in the promoter region of a number of genes encoding anti-oxidative and phase 2 enzymes, including heme oxygenase-1, glutathione reductase and NAD(P)H:quinoine oxidoreductase 1 (17-19). SF first gained attention due to its potential as an anticancer agent (20). Recent studies have indicated that SF can serve as

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a potential candidate for the development of treatments for and/or the prevention of PD (21). SF has been shown to protect dopaminergic neurons against 6-OHDA-induced cytotoxicity (18,22,23). ER stress is a contributory factor to neuronal death and is involved in the pathogenesis of PD. A number of studies have shown how 6-OHDA induces dopaminergic neurons through mechanisms modulated by ER stress and UPR signaling components (4). However, there have been no reports on the involvement of SF in the modulation of ER stress. SF may act as a protective mechanism to attenuate ER stress, thus protecting cells from ER stress induced by 6-OHDA. In this study, to test this hypothesis, we measured the effects of SF on cell survival and ER stress responses induced by 6-OHDA in PC12 cells.

Materials and methods

Reagents. 6-OHDA, SF and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Antibodies against Bip, CHOP, β-actin, Lamin B and Nrf2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine, RPMI-1640 medium, horse serum and fetal calf serum were obtained from Gibco-BRL/Life Technologies (Grand Island, NY, USA). All other chemicals were of the highest quality commercially available.

Cell culture. PC12 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 in air at 37˚C.

MTT assay. For the determination of the cell viability, the conventional MTT assay was used. PC12 cells were pre-treated with SF for 6 h, and then incubated with 6-OHDA. After exposure to 6-OHDA for 24 h, the treated cells were incubated with 0.5 mg/ml MTT for 4 h. Isopropyl alcohol containing 0.04 N hydrochloride (HCL) was added to the culture medium and mixed to dissolve dark blue crystals. The density was measured at 570 nm using a plate reader.

Nuclear and cytosolic lysate preparation. Nuclear and cytosolic extracts were isolated as follows. Cells were incubated in ice-cold buffer A. After 15 min of incubation on ice, NP-40 was added to a final concentration of 0.6%, and then cells were vortexed and centrifuged for 1 min at 16,000 x g. The nuclear pellet was extracted with buffer B [20 mM HEPES (pH 8.0), 1 mM EDTA, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM PMSF, 0.5 mg/ml benzamidine, 0.1 mg/ml leupeptin, 1.2 mg/ml aprotinin and 20% glycerol] for 30 min on ice. The debris was removed by centrifugation at 16,000 x g for 20 min at 4˚C.

Western blot analysis. After treatment, cells were collected and washed twice with ice-cold phosphate-buffered saline (PBS). The harvested cells were then lysed with a solubilizing solution [20 mM Tris-HCl (pH 7.0), 25 mM β-glycerophosphate, 2 mM EGTA, 1% Triton X-100, 1 mM vanadate, 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride and 2 mM dithiothreitol] on ice for 40 min. The lysate was centrifuged at 15,000 rpm for 15 min and supernatants were collected. Protein concentrations were determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA). An equal quantity of proteins was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrance (Millipore Corporation, Billerica, MA, USA). The membrances were incubated with Bip, CHOP, β-actin, Lamin B and Nrf2 antibodies, washed and then incubated with peroxidase-conjugated anti-rabbit or anti-mouse IgG (KPL, Gaithersburg, MD, USA). The immunoblot was revealed with an ECL Western Blot Detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Transfection of siRNA. Rat siRNA against Nrf2 and scrambled control, non-silencing siRNA were purchased from Invitrogen. The target sequences for Nrf2-siRNA were 5'-UUA AGA CAC UGU AAC UCG GGA AUG G-3' (forward) and 5'-CCA UUC CCG AFU UAC AGU GUC UUA A-3' (reverse). The siRNA was transfected into PC12 cells at a concentration of 20 pmol/10^5 cells by Lipofectamine 24 h prior to further experiments, according to the manufacturer's instructions.

Statistical analysis. All values are expressed as the means ± SD. Data were analyzed by ANOVA followed by the Tukey-Kramer test as the post hoc test. Differences were considered statistically significant at a level of $P<0.05$.

Results

Suppression of 6-OHDA-induced cell death by SF. To assess the effects of SF on 6-OHDA-induced cytotoxicity in PC12 cells, we first used MTT assay to examine the survival of cells treated with 6-OHDA and/or SF. As shown in Fig. 1, 6-OHDA (80 μM) significantly induced cell death. Pre-treatment with SF (0.1, 1 and 5 μM) inhibited 6-OHDA-induced cell death in a dose-dependent manner (Fig. 1).

SF inhibits 6-OHDA-induced ER stress. Previous studies have demonstrated that ER stress is a contributory factor in 6-OHDA-induced cytotoxicity. Thus, we investigated whether SF attenuates the ER stress response induced by 6-OHDA. Fig. 2 shows that 6-OHDA dramatically increased the expression of Bip and CHOP. Pre-treatment with SF significantly decreased these 6-OHDA-induced ER stress responses (Fig. 2). These results indicate that SF protects cells against 6-OHDA-induced cytotoxicity through the inhibition of cell death mediated by ER stress responses.

Inhibitory effects of SF on 6-OHDA-induced ER stress are reversed by silencing Nrf2 expression. A number of studies have shown that the protective effect of SF is dependent on the activation of the Nrf2/ARE pathway. To determine the signaling pathway involved in the inhibitory effects of SF on 6-OHDA-induced ER stress responses, we examined whether Nrf2 plays a role in these effects. As shown in Fig. 3A, SF induced the translocation of Nrf2 into the nucleus. In order to further elucidate the effect of Nrf2 activation on SF-mediated protective effects against 6-OHDA-induced ER stress, siRNA methodology was employed. Western blot analysis confirmed that *Nrf2*-specific siRNA significantly decreased the protein levels of Nrf2 (Fig. 3B). Notably, transfection of the *Nrf2*

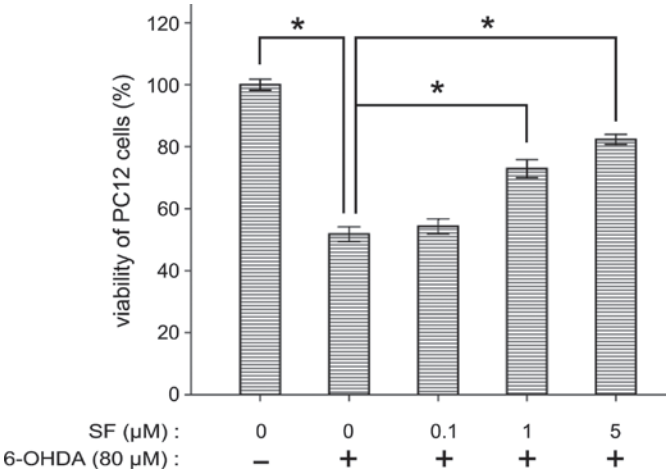

Figure 1. Effects of sulforaphane (SF) on the viability of cells treated with 6-hydroxydopamine (6-OHDA). PC12 cell were treated with SF (0.1, 1 and 5 μM) for 6 h and then incubated with 6-OHDA (80 μM) for a further 24 h. Cell viability was measured with an MTT assay. *P<0.05 indicates a statistically significant difference.

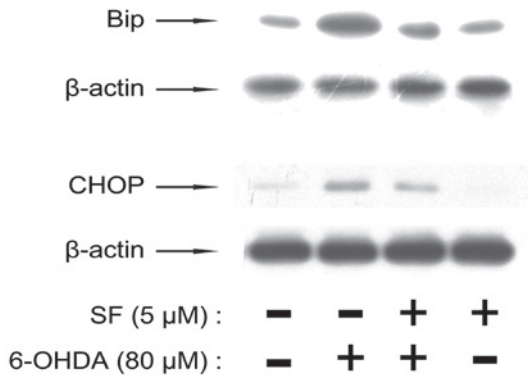

Figure 2. Expression of endoplasmic reticulum (ER) stress-related molecules in cells treated with 6-hydroxydopamine (6-OHDA) in the presence or absence of sulforaphane (SF). PC12 cells were pre-treated with 5 μM SF for 6 h and then treated with 80 μM 6-OHDA for 24 h. Cells were harvested and lysed, and an aliquot of protein was subjected to SDS-PAGE for western blot analysis, as described in Materials and methods. Representative bands are shown from triplicate experiments. CHOP, C/EBP homologous protein.

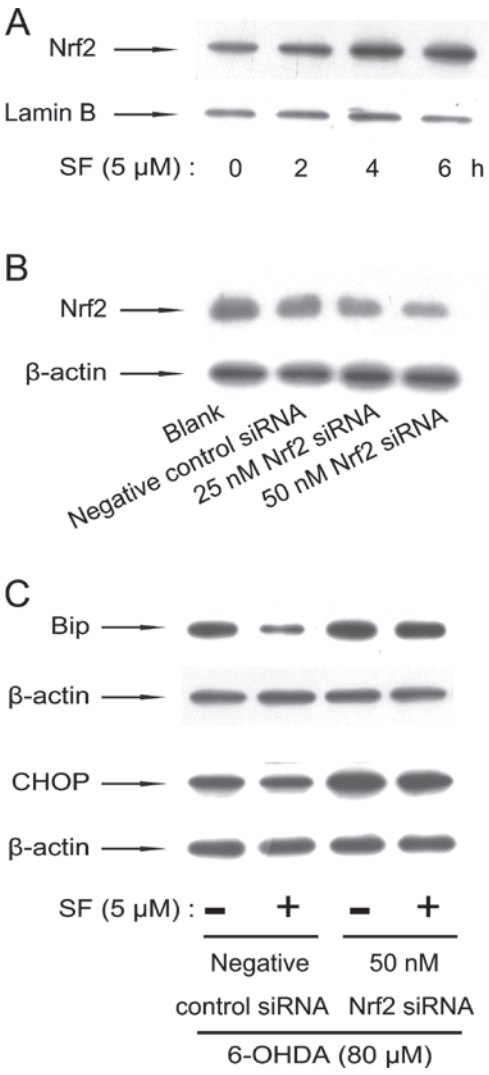

Figure 3. Effects of NF-E2-related factor-2 (Nrf2) on the protection of sulforaphane (SF) against 6-hydroxydopamine (6-OHDA)-induced endoplasmic reticulum (ER) stress in PC12 cells. (A) PC12 cells were treated with SF (5 μM) for the indicated times and nuclear extracts were prepared for the western blot analysis of Nrf2. All experiments were performed in triplicate. (B) The Nrf2 protein levels were measured in PC12 cells (Blank) and PC12 cells were transfected with *Nrf2* siRNA or negative control siRNA for 24 h. (C) PC12 cells, transfected with *Nrf2* siRNA or control siRNA for 24 h, were treated with 5 μM SF for 6 h and then challenged with 80 μM 6-OHDA. Cells were harvested and lysed, and an aliquot of protein was subjected to SDS-PAGE for western blot analysis, as described in Materials and methods. Representative bands are shown from triplicate experiments. CHOP, C/EBP homologous protein.

siRNA reversed the inhibitory effects of SF on 6-OHDA-induced ER stress responses (Fig. 3C).

Discussion

Recent studies have shown that SF exhibits a broad spectrum of biological actions. Besides an anticarcinogen, SF may serve as a neuroprotective agent. A number of *in vivo* studies have demonstrated that SF protects against acute brain injury (19). SF protects immature hippocampal neurons against death caused by exposure to hemin or to oxygen and glucose-deprivation (24). SF has been shown to induce thioredoxin expression and attenuates retinal light damage in mice (25). It has also been reported that SF may serve as a potential candidate for the treatment and/or prevention of PD. SF causes the induction of NAD(P)H:quinoine oxidoreductase 1 and glutathione, removal of intracellular dopamine quinone and protects against 6-OHDA-induced toxicity (18,22). To date, the beneficial bioactivities of SF have been attributed mainly to its anti-oxidant properties, such as radical scavenging and the induction of anti-oxidant genes (26,27). The Nrf2-ARE pathway is involved in the modulation of oxidative stress, inflammation and ER stress (28-31). It has been reported that the activation of the Nrf2-ARE pathway plays an important role in the various bioactivities of SF, suggesting that mechanisms other than anti-oxidant activities are involved in the underlying benefical effects of SF.

Previous studies have demonstrated that the activation of ER stress-mediated cell death is linked to PD (2,3). To our knowledge, there have been no reports on the involvement of SF in the modulation of ER stress. ER stress response induced by 6-OHDA or other ER stress inducers is transmitted through

the activation of three responsible proteins: inositol-required enzyme 1 (IRE1), protein kinase-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (3). PERK phosphorylates and inactivates eIF2α to reduce the protein load on the ER. These responses are followed by the increased expression of CHOP and subsequent apoptosis (32). In the present study, SF dramatically decreased the expression of early ER stress response proteins and subsequent CHOP expression by 6-OHDA. These results suggest that SF protects against 6-OHDA-induced cell death partly by inhibiting ER stress.

The generation of reactive oxygen species (ROS) is an important mediator for 6-OHDA-induced neuronal death (33,34). The production of ROS inevitably triggers stress-activated signaling pathways, including ER stress responses (35). It has been reported that anti-oxidants protect cells against ER stress (36). These results suggest that the anti-oxidant activity of SF may contribute to the inhibition of ER stress response. The mechanism underlying the anti-oxidant activity of SF relies on Nrf2 activation. Previous studies have shown that Nrf2 signaling plays a cytoprotective role in response to ER stress. The activation of Nrf2 is required for survival during UPR. $Nrf2^{-/-}$ cells are sensitive to various ER stress-inducing agents, and the overexpression of Nrf2 enhances cell survival during UPR (29,37). In the present study, we demonstrate that the inhibitory effect of SF on 6-OHDA-induced ER stress is associated with the activation of Nrf2. The transfection of *Nrf2* siRNA reversed the inhibitory effects of SF on 6-OHDA-induced ER stress responses. The mechanism of Nrf2-mediated cytoprotection relies on the ability of Nrf2 target genes. Heme oxygenase-1 and thioredoxin, two important targets of Nrf2, play roles in modulating ER stress (38-40). These results suggest that SF prevents ER stress response by 6-OHDA through the activation of Nrf2. However, further studies are required to explore the target genes of Nrf2 involved in the inhibition of ER stress by SF.

In conclusion, our results indicate that SF has a cytoprotective effect against the neurotoxin, 6-OHDA, and that this protection may be mediated by the inhibitory effect of SF on 6-OHDA-induced ER stress. The regulation of ER stress by SF is associated with the activation of Nrf2. SF may be a novel neuroprotective agent against ER stress, as well as a therapeutic candidate for the treatment of ER stress-associated neural diseases, including Parkinson's disease.

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