Sulforaphane protects against 6-hydroxydopamine-induced cytotoxicity by increasing expression of heme oxygenase-1 in a PI3K/Akt-dependent manner

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Abstract. Parkinson's disease (PD) is a progressive neurodegenerative disorder with selective loss of dopaminergic neurons in the substantia nigra. Evidence suggests that oxidative stress is involved in the pathogenesis of PD. Sulforaphane (SF), a naturally occurring isothiocyanate, has been shown to protect against oxidative stress by inducing the expression of various NF-E2-related factor-2 (Nrf2) responsive genes. Previous studies have shown that SF protects dopaminergic neurons against PD-related neurotoxin 6-hydroxydopamine (6-OHDA)-induced cytotoxicity. However, the molecular mechanisms by which SF protects against 6-OHDA-induced cytotoxicity are poorly elucidated. In this study, we found that pretreatment with SF significantly reduced 6-OHDA-induced caspase-3 activation and subsequent cell death. SF also increased heme oxygenase-1 (HO-1) expression, which conferred protection against 6-OHDA-induced cytotoxicity. Furthermore, SF induced the translocation of Nrf2 into the nucleus and activated PI3K/Akt, a pathway that is involved in SF-induced Nrf2 nuclear translocation, HO-1 expression and cytoprotection. These results suggest that SF inhibits 6-OHDA-induced cytotoxicity through increasing HO-1 expression in a PI3K/Akt-dependent manner.

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by progressive degeneration and loss of dopaminergic neurons in the substantia nigra pars compacta and dopaminergic nerve terminals in the striatum (1). Although the etiology of PD remains unclear, previous studies have shown that oxidative stress is involved in the pathogenesis of PD (2,3). 6-Hydroxydopamine (6-OHDA) is commonly used to produce *in vivo* and *in vitro* models of PD (4,5). 6-OHDA can be formed from dopamine by non-enzymatic hydroxylation in the presence of Fe^{2+} and H_2O_2 (6,7). 6-OHDA stimulates the production of superoxide radicals and induces cell death (8). Numerous experiments have revealed that antioxidants exert beneficial effects in the prevention of 6-OHDA-induced cytotoxicity (9,10).

Heme oxygenase-1 (HO-1) is an enzyme that catalyzes the degradation of heme. It has been widely reported that HO-1 plays a crucial role in maintaining redox homeostasis during cellular stress (11-13). Increased HO-1 expression contributes to promoting the degradation of heme into carbon monoxide and biliverdin, the latter being the immediate precursor of the powerful antioxidant molecule bilirubin (14). The induction of HO-1 expression is primarily regulated by NF-E2-related factor 2 (Nrf2), a central transcription factor that interacts with the antioxidant response element (ARE) to activate the transcription of numerous detoxifying and antioxidant genes (11,15). Under normal conditions, Nrf2 is sequestered in the cytoplasm by its actin-bound negative regulator, Keap1. However, Nrf2 can be liberated from the Keap1-Nrf2 complex and translocates into the nucleus by various stimuli, such as electrophiles and oxidative stress (16). Recently, several studies have indicated that the translocation of Nrf2 into the nucleus requires the activation of several signal transduction pathways, including the phosphatidylinositol 3-kinase (PI3K) pathway (17,18). Increased evidence indicates that HO-1 provides protection against oxidative stress, and modulation of HO-1 expression may represent a novel target for therapeutic intervention.

Sulforaphane (SF) is a natural drug derived from isothiocyanate found in cruciferous vegetables such as broccoli, cabbage and cauliflower. SF has been used as a chemopreventive compound (19). The cytoprotective effect of SF is explained mainly by activation of the Nrf2-ARE pathway (20). *In vivo* studies have shown that SF protects the brain against hypoxic-

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ischemic injury through induction of Nrf2-dependent phase 2 enzyme (21). SF also has been reported to be a potential candidate for development of treatment and/or prevention of PD (22). SF protects dopaminergic neurons against 6-OHDA-induced cytotoxicity (23,24). However, the molecular mechanisms by which SF protects against 6-OHDA-induced cytotoxicity are poorly elucidated. In the present study, we demonstrated that SF-induced activation of PI3K/Akt increases the expression of HO-1, which in turn provides protection against 6-OHDA-induced cytotoxicity in PC12 cells.

Materials and methods

Materials. 6-Hydroxydopamine, sulforaphane, zinc protoporphyrin (znPP) and the MTT-based colorimetric assay kit were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies against phosphor-Akt, Akt, β-actin, lamin B, Nrf2 and HO-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). RPMI-1640 medium, horse serum and fetal calf serum were obtained from Gibco-BRL/Life Technologies (Grand Island, NY, USA). Caspase fluorogenic substrate for caspase-3 (Ac-DEVD-AMC) was purchased from Alexis Biochemicals (Lausanne, Switzerland). All other chemicals were reagent grade.

Cell culture and cell viability assay. 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 at 37˚C in a humid atmosphere of 5% CO_2. Cell viability was measured using MTT-based assays according to the manufacturer's instructions.

Determination of caspase-3 activity. The method described by Movsesyan *et al* was used to examine caspase-3 activity. PC12 cells were washed with PBS and extracted in lysis buffer [10 mM HEPES (pH 7.4); 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride and 20 μg/mol/l leupeptin]. The supernatants were incubated with protease assay buffer and Ac-DEVD-AMC. The change in absorbance was measured using a spectrofluorometer.

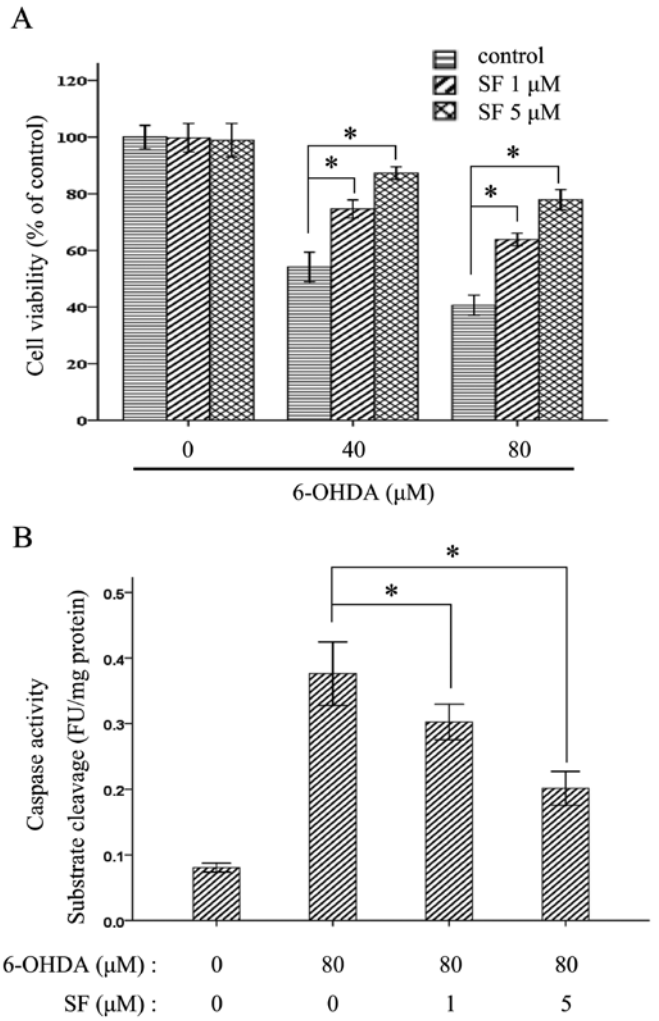

Figure 1. SF prevents 6-OHDA-induced cytotoxicity. PC12 cells were treated with SF (1 and 5 μM) for 24 h and then incubated with 6-OHDA (40 or 80 μM) for a further 24 h. (A) Cell viability was measured with an MTT assay. *Statistical significance (P<0.05). (B) PC12 cells were treated as indicated. The catalytic activity of caspase-3 in cell lysates was assayed using the synthetic peptide substrate Ac-DEVD-AMC. Values are means ± SD from three separate cultures. *Significantly different from 6-OHDA-treated cells (P<0.05).

Nuclear and cytosolic lysate preparation. Nuclear and cytosolic extracts were isolated as described previously (Huang Yuan Li, 2007). 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 supernatant was kept as the cytoplasmic extract. 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. Supernatants were collected and protein concentrations were determined using Bio-Rad protein assay reagent (Hercules, CA, USA). An equal quantity of proteins was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Millipore Corp., Billerica, MA, USA). The membranes were incubated with rabbit polyclonal Akt, phospho-Akt, Nrf2, HO-1 or mouse monoclonal β-actin 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).

Statistical analysis. All values are expressed as means ± SD. Data were analyzed by ANOVA followed by Tukey-Kramer

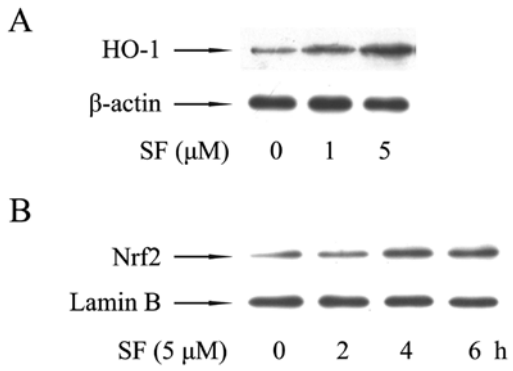

Figure 2. Effects of SF on HO-1 expression and Nrf2 translocation into the nucleus. (A) PC12 cells were exposed to SF (1 and 5 μM) for 24 h. HO-1 expression was measured by western blot analysis. All experiments were conducted in triplicate. (B) PC12 cells were treated with SF (5 μM) for the indicated times and nuclear extracts were prepared for western blotting. Nrf2 expression was assessed. All experiments were conducted in triplicate.

test as the post hoc test. Differences were considered statistically significant at a level of $P<0.05$.

Results

SF protects against 6-OHDA-induced cytotoxicity. To determine the effect of SF on 6-OHDA-induced cytotoxicity, we exposed PC12 cells to SF for 24 h prior to exposure to 6-OHDA for a further 24 h. As shown in Fig. 1A, pretreatment with SF protected cells against 6-OHDA-induced cell death in a dose-dependent manner. It has been previously demonstrated that 6-OHDA induces the activation of caspase-3, which plays an important role in the terminal execution phase of apoptosis. In the present study, we found that 6-OHDA-induced caspase-3 activation was suppressed by the pretreatment with SF (Fig. 1B). These results indicate that SF inhibits 6-OHDA-induced apoptosis in PC12 cells.

SF induces the translocation of Nrf2 into the nucleus and increases HO-1 expression. Previous studies have demonstrated that SF is a potent Nrf2 activator and exhibits antioxidative effects via the up-regulation of ARE-driven genes. To investigate the neuroprotective mechanisms of SF, we examined the effect of SF on HO-1 expression and the translocation of Nrf2 into the nucleus in PC12 cells. As shown in Fig. 2B, SF increased the amount of Nrf2 after 2 h in the nuclear fraction. In this study, we also found that the expression of HO-1, an important ARE-driven gene, was increased by SF in a dose-dependent manner (Fig. 2A).

Involvement of the PI3K/Akt pathway in HO-1 expression by SF. It has been widely reported that the PI3K/Akt pathway is involved in regulating the activation of the Nrf2-ARE pathway. To determine the upstream signaling pathway involved in SF-mediated Nrf2 activation and induction of HO-1, we examined whether SF activates the PI3K/Akt pathway. As shown in Fig. 3A, SF phosphorylated Akt in PC12 cells (Fig. 3A). Furthermore, HO-1 expression and nuclear accumulation of Nrf2 were reduced by the PI3K inhibitor LY294002 (Fig. 3B and C). These results suggest that PI3K/Akt signaling plays a

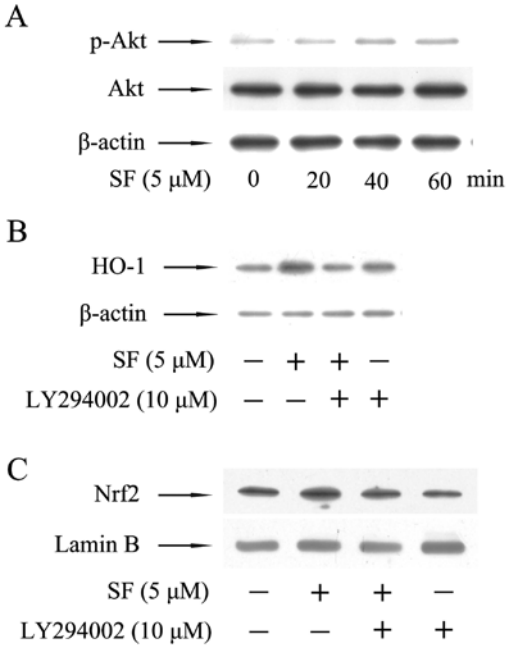

Figure 3. Induction of HO-1 and activation of Nrf2 by SF via phosphorylation. (A) Effect of SF on the phosphorylation of Akt. PC12 cells were treated with 5 μM SF for the indicated times. The level of p-Akt was measured using western blot analysis. (B) Effect of PI3K inhibitor on SF-induced HO-1 expression. PC12 cells were preincubated with 20 μM LY294002 for 30 min and then treated with 5 μM SF for 24 h. Cell lysates were subjected to western blot analysis using anti-HO-1 and anti-β-actin antibodies. (C) Effect of PI3K inhibitor on SF-induced Nrf2 translocation. PC12 cells were preincubated with 10 μM LY294002 for 30 min and then treated with 5 μM SF for 4 h. Nuclear extracts were subjected to western blot analysis by using anti-Nrf2 and anti-lamin B antibodies.

role in SF-mediated HO-1 expression through the translocation of Nrf2 into the nucleus.

PI3K/Akt pathway regulates SF-mediated protection against 6-OHDA. HO-1 plays an important role in the cellular defense against oxidative stress. Accordingly, we examined whether HO-1 induction is involved in SF-mediated protection against 6-OHDA-induced cytotoxicity. As shown in Fig. 4A, the protective effect of SF on 6-OHDA-induced cytotoxicity was reversed by zinc protoporphyrin (an inhibitor of HO-1). To examine the effects of PI3K/Akt on SF-mediated protection against 6-OHDA, cells were pretreated with LY194002 for 30 min prior to addition of SF. The MTT assays showed that LY194002 reversed the protective effects of SF against 6-OHDA-induced cell death (Fig. 4B). Furthermore, the inhibition of 6-OHDA-induced caspase-3 activation by SF was also reversed by the pretreatment with LY194002 (Fig. 4C).

Discussion

The cytoprotective role of SF has been widely studied *in vivo* and *in vitro*. SF protects immature hippocampal neurons against death caused by exposure to hemin or to oxygen and glucose-deprivation (25). SF induces thioredoxin expression and attenuates retinal light damage in mice (26). It also has been reported that SF protects against 6-OHDA-induced cytotoxicity. However, the molecular mechanisms by which

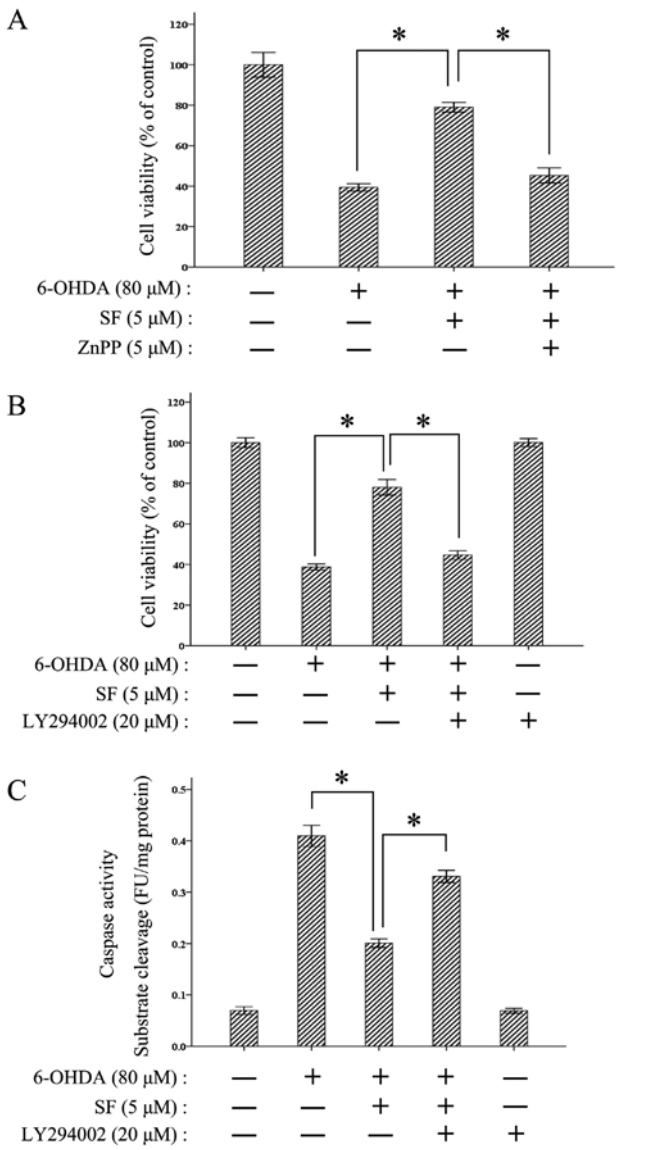

Figure 4. HO-1 and PI3K inhibitors attenuate the cytoprotective effect of SF. (A) HO-1 enzyme inhibitor ZnPP suppressed the protective effect of SF against 6-OHDA-induced cytotoxicity. PC12 cells were treated with 5 μM SF for 24 h and then incubated with ZnPP (5 μM) for 30 min and addition of 6-OHDA (80 μM) into the cells for a further 24 h. Cell viability was measured by MTT assay. (B) PC12 cells were pretreated with LY294002 for 30 min and then incubated with SF. After 24 h of incubation with SF, cells were treated with 6-OHDA (80 μM) for a further 24 h. Cell viability was measured with an MTT assay. (C) The catalytic activity of caspase-3 in cell lysates was assayed using the synthetic peptide substrate Ac-DEVD-AMC. *Statistical significance ($P<0.05$).

SF protects against 6-OHDA-induced cytotoxicity are poorly elucidated. In the present study, we demonstrated that SF modulates HO-1 induction, Nrf2 nuclear translocation and 6-OHDA-induced cytotoxicity through the PI3K/Akt pathway. To our knowledge, this is the first report demonstrating that SF protects against 6-OHDA-induced cytotoxicity through induction of HO-1 expression in a PI3K-dependent manner.

Excessive free radical formation can result in oxidative stress, a possible mechanism of PD. Mammalian cells have developed several protective mechanisms to prevent oxidative stress (27). These mechanisms employ antioxidants to augment the oxidative defense capacity (28). It has been widely demonstrated that the Nrf2-ARE pathway plays an important role in cellular defense mechanisms (29). Nrf2 binds to the ARE in the promoter region of a number of genes, encoding for phase II detoxifying enzymes and antioxidative proteins, including thioredoxin, HO-1, NAD(P)H:quinine oxidoreductase-1, glutathione reductase and glutathione peroxidase (30,31). SF is a known activator of the Nrf2-ARE pathway. It has been reported that SF protects against 6-OHDA-induced oxidative stress by increasing the levels of GSH, NAD(P)H: quinine oxidoreductase-1, GSH-transferase and reductase (23). Whether HO-1 is involved in the SF-mediated protection against 6-OHDA is still unknown. The induction of HO-1 has been considered an adaptive and benefical response to oxidative stress (11,13). Overexpression of HO-1 in transgenic mice attenuates cerebral ischemic injury and H_2O_2- or glutamate-induced oxidative stress (29,32). Several studies have demonstrated that SF markedly increases HO-1 expression in liver tissues and hippocampal neurons (25,33). In agreement with other studies, the induction of HO-1 in SF-treated PC12 cells has been demonstrated in this study. In addiction, we showed that ZnPP, an inhibitor of HO-1 activity, can partially reverse the protective effects of SF. These results suggest that the increase in HO-1 expression by SF conferred cytoprotection against 6-OHDA-induced cytotoxicity.

Activation of the PI3K/Akt pathway is a crucial step in various biological processes, such as growth, survival, anti-apoptosis and cell proliferation. It has been reported that HO-1 expression is regulated by activation of Nrf2 in a PI3K/Akt-dependent manner (17,34,35). In this study, we found that SF activates the PI3K/Akt pathway. The pretreatment of LY294002, an inhibitor of the PI3K/Akt pathway, suppressed SF-induced increase in HO-1 expression in PC12 cells. Also, the PI3K/Akt inhibitor blocked SF-induced Nrf2 nuclear translocation. Furthermore, the protective effects of SF against 6-OHDA-induced cytotoxicity were blocked by the PI3K/Akt inhibitor. These results suggest that the PI3K/Akt pathway is important for SF-induced Nrf2 nuclear translocation, HO-1 expression and protection.

In summary, SF induces HO-1 expression in PC12 cells, and this expression confers protection against 6-OHDA-induced cytotoxicity. SF also induces the activation of Nrf2 and activates the phosphorylation of Akt. The PI3k/Akt pathway is involved in SF-induced Nrf2 nuclear translocation, HO-1 expression and protection. SF may therefore serve as a useful cytoprotective agent in PD.

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