Hydrogen Sulfide Inhibits Rotenone-Induced Apoptosis via Preservation of Mitochondrial Function

Li-Fang Hu, Ming Lu, Zhi-Yuan Wu, Peter T.-H. Wong, and Jin-Song Bian

Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

Received April 15, 2008; accepted October 2, 2008

ABSTRACT

Hydrogen sulfide (H₂S) has been proposed as a novel neuromodulator, which plays critical roles in the central nervous system affecting both neurons and glial cells. However, its relationship with neurodegenerative diseases is unexplored. The present study was undertaken to investigate the effects of H₂S on cell injury induced by rotenone, a commonly used toxin in establishing in vivo and in vitro Parkinson’s disease (PD) models, in human-derived dopaminergic neuroblastoma cell line (SH-SY5Y). We report here that sodium hydrosulfide (NaHS), an H₂S donor, concentration-dependently suppressed rotenone-induced cellular injury and apoptotic cell death. NaHS also prevented rotenone-induced p38- and c-Jun NH₂-terminal kinase (JNK)-mitogen-activated protein kinase (MAPK) phosphorylation and rotenone-mediated changes in Bcl-2/Bax levels, mitochondrial membrane potential (ΔΨₘ) dissipation, cytochrome c release, caspase-9/3 activation and poly(ADP-ribose) polymerase cleavage. Furthermore, 5-hydroxydecanoate, a selective blocker of mitochondrial ATP-sensitive potassium (mitoK ATP) channel, attenuated the protective effects of NaHS against rotenone-induced cell apoptosis. Thus, we demonstrated for the first time that H₂S inhibited rotenone-induced cell apoptosis via regulation of mitoK ATP channel/p38- and JNK-MAPK pathway. Our data suggest that H₂S may have potential therapeutic value for neurodegenerative diseases, such as PD.

Although it has been the conventional view that hydrogen sulfide (H₂S) is a noxious gas, there is now accumulating evidence that it is an endogenously produced gaseous messenger and, in particular, serves as an important neuromodulator in the central nervous system (Abe and Kimura, 1996; Kimura, 2002). The production of H₂S in mammalian systems has been attributed to two pyridoxal-5'-phosphate-dependent enzymes: cystathionine β-synthase and cystathionine γ-lyase, both of which use L-cysteine as a substrate (Qu et al., 2008). In human, rat, and bovine brain, cystathionine β-synthase, which is highly expressed in hippocampus and cerebellum, has been identified to be the main enzyme responsible for the biosynthesis of H₂S (Abe and Kimura, 1996). The endogenous level of H₂S in the brain has been reported to be in micromolar concentration range, much higher than that of plasma levels. The cytoprotective and cytotoxic effects of H₂S in various model systems were summarized and reviewed by Szabo (2007).

From a physiological standpoint, H₂S regulates N-methyl-D-aspartate receptor-mediated response and thus facilitates the induction of long-term potentiation (Kimura, 2000; Eto et al., 2002b). H₂S also induces calcium waves/elevation in both astrocytes and microglia (Nagai et al., 2004; Lee et al., 2006). From a pathophysiological standpoint, disturbed H₂S synthesis in the brain has been reported in patients with Alzheimer’s disease, Down’s syndrome, and stroke (Eto et al., 2002a; Qu et al., 2006). We found recently that H₂S attenuates lipopolysaccharide-induced inflammation by inhibiting p38 mitogen-activated protein kinase (MAPK) in microglia (Hu et al., 2007). Kimura and his colleagues have demonstrated that H₂S enhances neuronal activities and protects neurons against oxidative stress via increasing intracellular glutathione levels (Kimura and Kimura, 2004; Umemura and Kimura, 2007). Since the major intracellular targets of H₂S described in the literature include ATP-sensitive potassium (K ATP) channels and a growing list of intracellular signaling molecules, notably, nuclear factor-κB and p38 MAPK. Among these, the action of H₂S on K ATP channels received more attention.
attention (Wang, 2002; Yang et al., 2005). We reported recently that H$_2$S decreases blood pressure in freely moving rats via opening K$_{ATP}$ channels in hypothalamus (Dawe et al., 2008). Thus, H$_2$S may play important roles in regulating central nervous system functions. Abnormal H$_2$S production may contribute to the pathogenesis of different brain diseases.

Parkinson’s disease (PD) is a neurodegenerative disorder characterized by symptoms including rigidity, resting tremor, and instability of gait and posture. Increasing scientific evidence has demonstrated that microglia-mediated neuroinflammation is critical for the initiation and development of neurodegenerative disorders such as PD. Chemical compounds possessing antineuroinflammatory properties are being considered as promising candidates for PD therapy. Rotenone, which is widely used as a pesticide, has been shown to induce degeneration of dopaminergic neurons in animal models, leading to behavioral and pathological symptoms similar to those of PD (Fleming et al., 2004). To follow up on our previous observation that H$_2$S produces antineuroinflammatory effects in microglia (Hu et al., 2007), we investigated the effects of H$_2$S on cell apoptosis/injury in rotenone-treated human-derived dopaminergic neuroblastoma (SH-SY5Y) cells to evaluate whether H$_2$S may be of potential therapeutic value in the treatment of neurodegenerative diseases, such as PD.

## Materials and Methods

### Reagents and Antibodies.

SB203580 and SP600125 were from Calbiochem (San Diego, CA). All other chemicals used in this study were purchased from Sigma (St. Louis, MO). Antibodies against phospho-JNK (Thr183 and Tyr185), caspase-3, phospho- and total ERK1/2, Bax, and Bcl-2 were purchased from Cell Signaling Technologies (Danvers, MA). Antibodies for detecting cytochrome c, total and phospho-p38 (Thr180 and Tyr182), and total JNK1 were obtained from Santa Cruz (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Rotenone solution in dimethyl sulfoxide (DMSO) was freshly prepared before each treatment. Final DMSO concentration in media did not exceed 0.05%. Because rotenone is lipophilic and may bind to proteins present in the serum, cells were transferred into lower-serum media (0.5% fetal bovine serum) before rotenone treatment to minimize background kinase activity. For other experiments, regular culture medium was changed immediately before treatment. Treatment groups included the following: 1) control, cells were treated with vehicle (DMSO); 2) rotenone group, cells were treated with 100 nM rotenone alone for 24 h; 3) H$_2$S + rotenone group, cells were pre-treated with NaHS at indicated concentrations for 30 min before rotenone treatment for further 24 h; and 4) 5-HD + H$_2$S + rotenone group, 5-HD (200 µM) was added into cell culture 15 min before NaHS application, followed by rotenone treatment.

### Total Sulfide Measurement.

The procedures are essentially described in the literature with modifications (Gilboa-Garber, 1971). In brief, aliquots (500 µl) of culture solution (Krebs’ buffer) were mixed with trichloroacetic acid [10% (w/v)], zinc acetate [1% (w/v)], 250 µl, N,N-dimethyl-p-phenylenediamine sulfate (20 µM, 133 µl) in 7.2 M HCl and FeCl$_3$ (30 µM, 133 µl) in 1.2 M HCl in parafilm-enveloped Eppendorf tubes. After 15 min, this mixture was centrifuged at 4000g for 10 min. The supernatant was collected, and its absorbance was measured in 96-well plates at a wavelength of 670 nm. All samples were assayed in duplicate and calculated against a calibration curve of NaHS dissolved in Krebs’ buffer: 115 mM NaCl, 2.5 mM KCl, 2.46 mM MgSO$_4$, 2 mM CaCl$_2$, 5.6 mM glucose, 1.38 mM Na$_2$HPO$_4$, and 25 mM NaHCO$_3$, pH 7.4.

### Cell Viability Assay.

Cell viability was evaluated with the MTT method as described previously with modifications (Hu et al., 2005). In brief, the medium was changed before the assay. MTT dissolved in PBS, pH 7.4, was added to the culture media to reach a final concentration of 0.5 mg/ml. After incubation at 37°C for 4 h, the culture media containing MTT were removed. DMSO was then added into each well, and the absorbance at 570 nm was measured.

### Quantification of Apoptosis.

To visualize nuclear morphology, cells were fixed in 4% paraformaldehyde and stained with 2.5 µg/ml DNA dye Hoechst 33342. Uniformly stained nuclei were scored as healthy, viable cells. Condensed or fragmented nuclei were scored as apoptotic. To obtain unbiased counting, Petri dishes were coded, and cells were scored blindly without knowledge of their prior treatment.

### Assessment of Mitochondrial Membrane Potential Loss.

Mitochondrial membrane potential ($\Delta$Ψ$_{m}$) was detected with fluorescent probe JC-1 (Sigma), which exists predominantly in monomeric form in cells with depolarized mitochondria and displays fluorescence green at 490 nm. Cells with polarized mitochondria predominantly contain JC-1 in aggregate form and show fluorescence of reddish-orange color. Loading was done by incubating SH-SY5Y cells with 2 µM JC-1 for 15 min, rinsed twice with PBS, and visualized by a lab-graded Nikon Optical TE2000-S inverted fluorescence microscope.

---

**Fig. 1.** Colorimetric assay of exogenous sulfide (NaHS) consumption in culture solution (Krebs’ buffer) of SH-SY5Y cells. Total NaHS (100 µM) was initially added into sterile Krebs’ buffer for cell culture. The total sulfide levels in culture supernatant at different time points were determined with colorimetric assay. Results show a representative time course of sulfide concentrations of three independent measurements.
concentrations from 1 to 300 M) did not show any significant effect, attenuated the stimulatory effect of rotenone on cytochrome c release (Fig. 4c). These findings indicate that cytoprotection by H2S may be mediated by raising cellular resistance against the initiating steps of apoptosis, namely the decrease of ΔΨm. In addition, Western blot analysis revealed that significant amounts of cytochrome c were released from mitochondria into the cytosol in rotenone-treated cells. NaHS (100 µM), which itself had no significant effect, attenuated the stimulatory effect of rotenone on cytochrome c release (Fig. 4c). These findings indicate that NaHS may produce antiapoptotic effects via the preservation of mitochondrial function.

H2S Suppresses Rotenone-Induced Cytotoxicity and Apoptosis. To determine the effect of H2S on the proapoptotic activity of rotenone, SH-SY5Y cells were treated with rotenone (100 nM for 24 h) in the absence or presence of NaHS, a donor of H2S. As shown in Fig. 2, rotenone substantially decreased cell viability by 43% compared with that of control. The effect of rotenone was attenuated by NaHS at concentrations from 1 to 300 µM, indicating that H2S produced protective effects against rotenone-induced cell injury. Treatment with NaHS alone (up to 300 µM) did not show any effect on cell viability (data not shown).

The beneficial effects of H2S against rotenone-induced apoptosis were further examined by Hoechst 33342 staining assay. Representative photomicrographs of nuclei morphology of SH-SY5Y cells are shown in Fig. 3, a to c. Treatment with rotenone induced condensed and fragmented nuclei, a characteristic of apoptosis. NaHS at 100 µM significantly attenuated this effect (Fig. 3c). Figure 3d shows that NaHS (1–300 µM) concentration-dependently attenuated the proapoptotic activity of rotenone (IC50 = approximately 55 µM). This confirms the protective effects of NaHS against rotenone-induced apoptosis in SH-SY5Y cells.

H2S Inhibits Rotenone-Induced Cell Apoptosis 29

Results

H2S Suppresses Rotenone-Induced Cytotoxicity and Apoptosis. To determine the effect of H2S on the proapoptotic activity of rotenone, SH-SY5Y cells were treated with rotenone (100 nM for 24 h) in the absence or presence of NaHS, a donor of H2S. As shown in Fig. 2, rotenone substantially decreased cell viability by 43% compared with that of control. The effect of rotenone was attenuated by NaHS at concentrations from 1 to 300 µM, indicating that H2S produced protective effects against rotenone-induced cell injury. Treatment with NaHS alone (up to 300 µM) did not show any effect on cell viability (data not shown).

The beneficial effects of H2S against rotenone-induced apoptosis were further examined by Hoechst 33342 staining assay. Representative photomicrographs of nuclei morphology of SH-SY5Y cells are shown in Fig. 3, a to c. Treatment with rotenone induced condensed and fragmented nuclei, a characteristic of apoptosis. NaHS at 100 µM significantly attenuated this effect (Fig. 3c). Figure 3d shows that NaHS (1–300 µM) concentration-dependently attenuated the proapoptotic activity of rotenone (IC50 = approximately 55 µM). This confirms the protective effects of NaHS against rotenone-induced apoptosis in SH-SY5Y cells.

H2S Prevents Rotenone-Induced ΔΨm Loss and Inhibits Cytochrome c Release. To investigate the mechanisms for the protective effects of NaHS on rotenone-induced apoptosis, ΔΨm was determined with molecular probe JC-1. As shown in Fig. 4a and quantified in Fig. 4b, as a result of rotenone treatment for 9 h, cells displayed a loss or collapse of ΔΨm, indicated by a shift from red-orange to greenish yellow fluorescence. Pretreatment with NaHS (100 µM) significantly prevented the loss of ΔΨm in the cells. These findings suggest that cytoprotection by H2S may be mediated by raising cellular resistance against the initiating steps of apoptosis, namely the decrease of ΔΨm. In addition, Western blot analysis revealed that significant amounts of cytochrome c were released from mitochondria into the cytosol in rotenone-treated cells. NaHS (100 µM), which itself had no significant effect, attenuated the stimulatory effect of rotenone on cytochrome c release (Fig. 4c). These findings indicate that NaHS may produce antiapoptotic effects via the preservation of mitochondrial function.

H2S Reverses Rotenone-Induced Changes of Bax and Bcl-2 Levels in SH-SY5Y Cells. Bcl-2, an antiapoptotic protein, prevents the release of cytochrome c from mitochondria, whereas Bax, a proapoptotic protein, promotes its release. During apoptosis, Bax translocates to the outer mitochondrial membrane to induce mitochondrial membrane permeabilization. This process is blocked by Bcl-2 protein. Therefore, the effects of NaHS on the protein levels of Bcl-2 and Bax were also examined. Rotenone-induced changes of protein levels of Bcl-2 and Bax in the presence and absence of NaHS (Fig. 5). Densitometric analysis indicates that rotenone significantly decreased the basal level of Bcl-2 but increased the Bax protein expression (Fig. 5a). The ratio of Bcl-2 over Bax was decreased by 40% in rotenone-treated


![Fig. 2.](image) Effects of NaHS on rotenone-induced cytotoxicity in SH-SY5Y cells. Cell viability was determined using MTT method. Values are expressed as the percentage of the untreated control and represented as mean ± S.E.M. of at least five independent experiments. †††, p < 0.001 versus control; *, p < 0.05; **, p < 0.01 versus rotenone group, respectively.
cells (Fig. 5b). This effect was markedly attenuated by NaHS (100 µM).

**H₂S Decreases Rotenone-Induced Caspase-9/3 Activation and Poly(ADP-ribose) Polymerase Cleavage.** The cytochrome c, together with Apaf-1 and pro-caspase-9, may form apoptosome, which activates caspase-9 and its downstream caspase cascades. It was found that rotenone increased the caspase-9 activity by 2.5-fold compared with that of the control group. This increase was attenuated by NaHS (100 µM) (Fig. 6a). Caspase-3 is a critical executioner of apoptosis, and the pro-caspase-3 can be cleaved by active caspase-9. As shown in Fig. 6b, treatment of the cells with rotenone significantly induced the cleavage of pro-caspase-3 (35 kDa) to its 19- and 17-kDa subunits, which was dramatically inhibited by NaHS (100 µM). Our data suggest that rotenone did activate caspase-3 in SH-SY5Y cells and that this activation could be suppressed by NaHS.

Poly(ADP-ribose) polymerase (PARP) is an enzyme implicated in DNA damage and repair mechanisms. Cleavage of PARP by active caspase-3 from native 116 to 89 kDa is a hallmark of apoptosis. We then examined the effect of H₂S on rotenone-induced apoptosis in SH-SY5Y cells by measuring PARP cleavage using Western blot analysis. As shown in Fig. 6c, treatment with rotenone increased the cleavage of PARP by 3.8-fold in SH-SY5Y cells. Pretreatment with NaHS (10–300 µM) for 30 min significantly reduced PARP cleavage induced by rotenone in a concentration-dependent manner (Fig. 6c).

**The Protective Effects of H₂S Involves mitoK<sub>ATP</sub> Channels.** To examine the involvement of mitoK<sub>ATP</sub> channels, 5-HD (200 µM), a selective mitoK<sub>ATP</sub> channel blocker, was given 15 min before NaHS application. It is interesting that 5-HD, which had no effect by itself, significantly attenuated the inhibitory effects of NaHS on rotenone-induced cytochrome c release, caspase-9 activity, cell injury, and PARP cleavage (Fig. 7, a–d). These data suggest that the initial exposure to NaHS resulted in the opening of mitoK<sub>ATP</sub> channels located in inner mitochondrial membrane, which in turn mediates the subsequent signaling cascade leading to the observed neuroprotective effects.

**Rotenone Induces p38/JNK MAPK Activation.** Both p38 and JNK signaling pathways are critical for the degeneration of dopaminergic neuronal cells (Newhouse et al., 2004). We therefore further studied the contribution of MAPK to the neuroprotective effects of H₂S. It was found that phosphorylation of p38 MAPK occurred as early as 5 min and reached the peak at 15 min after rotenone treatment. This effect gradually reduced and disappeared by 4 h after treatment (Fig. 8a). Rotenone also induced the phosphorylation of JNK but with a slower time course. The peak effect occurred at 4 h and almost disappeared by 24 h after treatment (Fig. 8b).

In addition, SB203580 (a p38 MAPK inhibitor) and SP600125 (a selective JNK inhibitor) markedly attenuated rotenone-in-
duced cell injury (Fig. 8c) and apoptosis (Fig. 8d) in SH-SY5Y cells. These data further confirmed that both p38 and JNK MAPK are involved in the mediation of the effects of rotenone.

**H$_2$S Attenuates Rotenone-Induced p38/JNK MAPK Activation.** To study the signaling mechanisms of the neuroprotective effects of H$_2$S, we also studied the concentration-dependent effects of H$_2$S on rotenone-induced stimulation of p38/JNK MAPK. As shown in Fig. 9, the maximal effects of rotenone on the phosphorylation of both p38 (at 15 min, Fig. 9a) and JNK (at 4 h, Fig. 9b) were attenuated by NaHS (1–300 µM) in a concentration-dependent manner. These data strongly suggest that the antiapoptotic effects of NaHS are associated with inhibition of the p38/JNK signaling pathways.

**Discussion**

Rotenone is a naturally occurring toxin and a commonly used pesticide to reproduce the neurochemical, neuropathological, and behavioral features of PD in rats. In the present study, we demonstrated for the first time that NaHS was able to inhibit rotenone-mediated cytotoxicity and apoptosis in a concentration-dependent manner. The underlying mechanisms may be associated with suppression of rotenone-induced events, including ΔΨ$_m$ loss, release of cytochrome c, and activation of subsequent caspase cascades via regulation of mitoK$_{ATP}$ channels/ p38- and JNK-MAPK pathway (Fig. 10).

Mitochondrial dysfunction is a prominent feature in apoptosis (Lemasters et al., 2002). Rotenone is a complex I inhibitor. The inhibition of complex I may induce ΔΨ$_m$ loss and the release of proapoptotic proteins (e.g., cytochrome c) from the mitochondrial intermembrane space to cytosol, where cytochrome c forms oligomeric complex with Apaf-1 and activates caspase-9. Our data showed that H$_2$S prevented rotenone-induced ΔΨ$_m$ loss and cytosolic accumulation of cytochrome c, suggesting that H$_2$S may produce neuroprotective effects via the preservation of mitochondrial function. The increased mitochondrial permeabilization and release of cytochrome c may also be regulated by proapoptotic proteins, such as Bax. In apoptotic cells, Bax interacts with Bid, and the resultant conformational change causes Bax to translocate from cytoplasm to the mitochondria, where it promotes the opening of permeability transition pore and thereby mediates the efflux of cytochrome c. This process could be blocked by mitochondrial protein Bcl-2. Hence, the ratio of Bcl-2/Bax may be used to indicate mitochondrial permeability. Consistent with previous findings (De Sarno et al., 2003), we found that rotenone decreased the ratio of Bcl-2/Bax protein in SH-SY5Y cells. This effect was significantly attenuated by H$_2$S. Thus, the protective effects of H$_2$S on mitochondrial function may also occur via suppression of the expression of the proapoptotic proteins.

The release of cytochrome c and apoptosis-inducing factor from mitochondria to cytosol may further stimulate caspase-9, which may lead to the activation of caspase-3 and subsequent degradation of cellular death substrates (e.g., PARP). We found in the present study that NaHS attenuated the effects of rote-

![Fig. 5. Effects of NaHS on rotenone-induced alterations of Bcl-2 and Bax level in SH-SY5Y cells. Total protein extracts were subjected to 15% SDS-PAGE for Western blot analysis. a, representative images from four different experiments. Tubulin was used as a loading control. b, densitometric analysis performed by normalizing Bcl-2 proteins to Bax proteins signals. Data were expressed as the mean ± S.E.M. †, p < 0.05 versus control; *, p < 0.05 versus rotenone group.](image)

![Fig. 6. Effects of NaHS on rotenone-induced caspase-3/9 activation and PARP cleavage in SH-SY5Y cells. a, mean data showing NaHS attenuated rotenone (rot) induced caspase-9 activation. Results are normalized by the control values. b and c, the levels of cleaved caspase-3 (b) and PARP (c) were determined by Western blot. Data were expressed by the ratio of cleaved PARP over total (uncleaved plus cleaved) PARP. Tubulin and β-actin were used as controls for equal loading. Mean ± S.E.M.; n = 4 to 8 independent experiments. †††, p < 0.001 versus control; *, p < 0.05; **, p < 0.01; †, p < 0.05 versus rotenone group, respectively.](image)
none on caspase-9/3 activation and PARP degradation and therefore protected cells against apoptotic injury. The effects of NaHS on this signaling cascade may be derived from the preservation of mitochondrial function via prevention of ΔΨm loss and inhibition of cytochrome c release.

Similar to the findings by Whitfield et al. (2008), we found that H2S concentration decayed rapidly (within 30 min) in the culture solution. Therefore, it seemed that H2S level was reaching undetectable levels before the addition of rotenone in our present experimental setting. Therefore, the obvious question is how H2S may protect cells against rotenone-induced injury. Recent studies have suggested that H2S plays a critical role in opening KATP channels (Wang, 2002; Yang et al., 2005; Dawe et al., 2008), which contributes to neuroprotective effects (Busija et al., 2004; Wu et al., 2006). The role of KATP channels in PD has been addressed as well. Activation of mitoKATP channel protects against rotenone-induced cell death and neurochemical alterations in rats (Tai et al., 2003; Zhou et al., 2007). By examining the involvement of mitoKATP channels in the neuroprotective effects of H2S,

Fig. 7. Effect of mitoKATP channels blocker 5-HD on the protective effects of NaHS against rotenone-induced cytochrome c release (a), caspase-9 activation (b), cell injury (c), and PARP cleavage (d) in SH-SY5Y cells. Cells were pretreated with NaHS for 30 min before rotenone treatment for 24 h. 5-HD (200 μM) was added into cells before NaHS treatment. Data are presented as the percentage of control value and expressed as mean ± S.E.M. ††, p < 0.01; †††, p < 0.001 versus control; *, p < 0.05; **, p < 0.01; †††, p < 0.001 versus rotenone group; #, p < 0.05; ##, p < 0.01 versus NaHS + rotenone group.

Fig. 8. Rotenone stimulates both p38 and JNK MAPK in SH-SY5Y cells. a and b, time course for the effects of rotenone on phosphorylation of p38 (a) and JNK (b). c and d, blockade of p38 MAPK with SB203580 (10 μM) and JNK with SP600125 (10 μM) attenuated rotenone-induced cell injury assessed by MTT method (c) and PARP cleavage (d). Data are presented as the percentage of control value and expressed as mean ± S.E.M. ††, p < 0.01; †††, p < 0.001 versus control; *, p < 0.05; **, p < 0.01; †††, p < 0.001 versus rotenone group, respectively.
we found that blockade of mitoK$_{ATP}$ channels with 5-HD attenuated the protective effects of H$_2$S on rotenone-induced cell apoptosis. These findings suggest that H$_2$S rapidly opens mitoK$_{ATP}$ channels, which, in turn, trigger a series of persistent intracellular responses, including inhibition of MAPK signaling pathway and preservation of mitochondrial integrity (Zhang et al., 2007). This triggering effect of H$_2$S may be similar to the mechanism for the cardioprotection conferred by H$_2$S preconditioning (Pan et al., 2008). It is interesting that it was also reported that H$_2$S can be rapidly absorbed in a kind of sulfur store in a form of bound sulfane sulfur and then gradually released upon stimulation (Warenycia et al., 1990; Ogasawara et al., 1993). Therefore, this may also explain the persistent protective effects of H$_2$S despite its short half-life.

Stimulation of MAPK (i.e., ERK1/2, p38, and JNK) leads to a wide range of cellular responses, including growth, differentiation, inflammation, and apoptosis. Because rotenone only stimulates the activities of JNK and p38, but not that of ERK1/2, in SH-SY5Y cells (Newhouse et al., 2004), we therefore only investigated the role of H$_2$S in rotenone-induced phosphorylation of p38 and JNK in the present study. We found that H$_2$S concentration-dependently inhibited the activation of these two kinases. The involvement of p38 MAPK in the neuroprotective effects has also been reported by our and other groups. Rinaldi et al. (2006) found that H$_2$S promotes the survival of the human polymorphonuclear cells via inhibition of p38 and caspase-3 (Rinaldi et al., 2006). We recently reported that H$_2$S attenuates lipopolysaccharide-stimulated p38 activation in microglial cells (Hu et al., 2007). All of these findings indicate that inhibition of p38 may contribute to the protective effects of H$_2$S. Because p38 MAPK was shown to mediate proapoptotic protein Bax-induced mitochondrial membrane permeabilization and neuro-

![Fig. 9.](image-url)

**Fig. 9.** Concentration-dependent effects of NaHS (0.1–300 μM) on rotenone-induced activation of p38 (a) and JNK (b) MAPK in SH-SY5Y cells. Data are presented as mean ± S.E.M. of 6 independent experiments. †††, p < 0.001 versus control; *, p < 0.05; **, p < 0.01 versus rotenone group, respectively.

![Fig. 10.](image-url)

**Fig. 10.** Proposed signaling mechanisms for the effects of H$_2$S on rotenone-induced apoptosis. Rotenone induces complex I inhibition, which initiates the dissipation of ΔΨ$_m$, causing the matrix swelling and promoting the opening of mitochondrial permeability transition pore. Rotenone may also stimulate intracellular p38/JNK MAPK pathway. The activation of p38/JNK MAPK, in turn, induces the recruitment of cytoplasmic apoptotic members of Bel-2 family proteins (e.g., Bax, Bid) to mitochondria and forms permeability transition pore, causing the release of cytochrome c to cytosol. The cytosolic apoptotic executors (cytochrome c, Apaf-1, and pro-caspase-9) form apoptosisome, which leads to activation of pro-caspase-9, subsequently activating caspase-3. The mechanisms underlying the antiapoptotic effects of H$_2$S may result from opening of mitoK$_{ATP}$ channels, which in turn mediates the prevention of ΔΨ$_m$ loss and the inhibition of p38/JNK MAPK pathway.
nal apoptosis (Gomez-Lazaro et al., 2007), the antiapoptotic effect of H$_2$S may be associated with the suppression of the activation of both p38 and JNK MAPK caused by rotenone.

The glutathione (GSH) redox cycle is a major endogenous protective system and an important component of the antioxidant machinery of the nervous system (Seyfried et al., 2000). Recent evidence indicates that depletion of cellular GSH results in the accumulation of ROS and loss of mitochondrial function (Bharat et al., 2002). Rotenone also induces the depletion of intracellular GSH and the accumulation of ROS, which in turn activates p38/JNK MAPK in the cells. It has previously been demonstrated that H$_2$S protects neuronal cells against oxidative stress via increasing GSH production (Kimura and Kimura, 2004). It is therefore logical to expect that GSH also contributes to the antiapoptotic effects of H$_2$S on rotenone-treated SH-SY5Y cells, but more experiments are warranted to further confirm this hypothesis.

In conclusion, the present observations identify a beneficial role of H$_2$S against rotenone-induced apoptosis in SH-SY5Y cells via regulation of mitoKATP channels and inhibition of p38 and JNK pathway. Because epidemiological studies have revealed a correlation between general pesticide (i.e., rotenone) exposure and increased risk for PD, our findings may therefore provide a potential venue to treat PD.

Acknowledgments

We thank Ester Khin Sandar Win for technical support.

References


