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RESEARCH PAPER

Protective effect of hydrogen sulphide against 6-OHDA-induced cell injury in SH-SY5Y cells involves PKC/PI3K/Akt pathway

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BACKGROUND AND PURPOSE
Hydrogen sulphide (H_2S) is a novel neuromodulator. The present study aimed to investigate the protective effect of H_2S against cell injury induced by 6-hydroxydopamine (6-OHDA), a selective dopaminergic neurotoxin often used to establish a model of Parkinson's disease for studying the underlying mechanisms of this condition.

EXPERIMENTAL APPROACH
Cell viability in SH-SY5Y cells was measured using MTT assay. Western blot analysis and pharmacological manipulation were employed to study the signalling mechanisms.

KEY RESULTS
Treatment of SH-SY5Y cells with 6-OHDA (50–200 μM) for 12 h decreased cell viability. Exogenous application of NaHS (an H_2S donor, 100–1000 μM) or overexpression of cystathionine β-synthase (a predominant enzyme to produce endogenous H_2S in SH-SY5Y cells) protected cells against 6-OHDA-induced cell apoptosis and death. Furthermore, NaHS reversed 6-OHDA-induced loss of tyrosine hydroxylase. Western blot analysis showed that NaHS reversed the down-regulation of PKCα, ε and Akt and the up-regulation of PKCδ in 6-OHDA-treated cells. Blockade of PKCα with Gö6976 (2 μM), PKCε with EAVSLKPT (200 μM) or PI3K with LY294002 (20 μM) reduced the protective effects of H_2S. However, inhibition of PKCδ with rottlerin (5 μM) failed to affect 6-OHDA-induced cell injury. These data suggest that the protective effects of NaHS mainly resulted from activation of PKCα, ε and PI3K/Akt pathway. In addition, NaHS-induced Akt phosphorylation was significantly attenuated by Gö6976 and EAVSLKPT, suggesting that the activation of Akt by NaHS is PKCα, ε-dependent.

CONCLUSIONS AND IMPLICATIONS
H_2S protects SH-SY5Y cells against 6-OHDA-induced cell injury by activating the PKCα, ε/PI3K/Akt pathway.

Abbreviations
3MST, 3-mercaptopyruvate sulphurtransferase; 6-OHDA, 6-hydroxydopamine; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; H_2S, hydrogen sulphide; L-dopa, levodopa; LTP, long-term potentiation; NaHS, sodium hydrosulphide; PD, Parkinson's disease; PI3K, phosphoinositol 3' kinase; PKB, protein kinase B; PKC, protein kinase C; PS, phosphatidylserine; ROS, reactive oxygen spesies; TH, tyrosine hydroxylase

Introduction

Hydrogen sulphide (H_2S), the third endogenous gaseous mediator after nitric oxide and carbon monoxide (Wang, 2002), has been reported to be produced in the brain of rat, human, bovine and other mammals (Goodwin *et al.*, 1989; Warenycia *et al.*, 1989; Savage and Gould, 1990). In mammalian cells, endogenous H_2S is formed from cysteine by pyridoxal-5'-phosphate-dependent enzymes (cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE)) (Stipanuk and Beck, 1982), and a

pyridoxal-5′-phosphate-independent enzyme (3-mercaptopyruvate sulphurtransferase (3MST)) (Shibuya *et al.*, 2009). CBS is mainly expressed in the hippocampus and cerebellum, where it is localized to astrocytes (Enokido *et al.*, 2005; Ichinohe *et al.*, 2005), whereas CSE is expressed in the ileum, portal vein and thoracic aorta (Hosoki *et al.*, 1997). 3MST is mainly located in neurones and produces bound sulphane sulphur more efficiently than CBS in the cells (Shibuya *et al.*, 2009). The expression of endogenous H_2S generating enzymes suggests that H_2S has important physiological functions.

Physically, H_2S potentiates N-methyl-D-aspartate (NMDA) receptors and improves the induction of long-term potentiation (LTP) in the hippocampus, a synaptic model of learning and memory (Abe and Kimura, 1996). In addition, we and others have reported that H_2S regulates Ca^{2+} in astrocytes and microglia, suggesting that H_2S is important for the regulation of neuronal activity (Lee *et al.*, 2006; Nagai *et al.*, 2004). Apart from that, H_2S also helps to reduce oxidative stress by inducing the production of glutathione, a kind of anti-oxidant, and suppresses oxidative stress in mitochondria (Kimura and Kimura, 2004; Kimura *et al.*, 2010). In addition, our group has recently found that H_2S protects astrocytes against oxidative stress induced by hydrogen peroxide (H_2O_2) by improving the glutamate transporters activities and therefore increases glutathione production as well as inhibits lipid oxidation (Lu *et al.*, 2008; Hu *et al.*, 2010). We also showed that H_2S has an anti-inflammatory role in lipopolysaccharide (LPS)-stimulated microglial (Hu *et al.*, 2007). In neuronal cells, we found that H_2S is able to protect cells against apoptosis via preservation of mitochondrial function (Hu *et al.*, 2009). All the above evidence suggests that H_2S plays an important role in the regulation of central nervous system functions. Hence, alterations in the endogenous H_2S concentration might contribute to the pathogenesis of certain neurodegenerative diseases.

Parkinson's disease (PD), the second most common neurodegenerative disease, is a condition called movement disorder characterized by muscle rigidity, tremor and slowing of physical movement. It usually affects the elderly and the age of onset is 60 years (Zhang *et al.*, 2007). It is caused by the progressive loss of dopaminergic neurones in the substantia nigra pars compacta, accompanied by an alteration of dopamine concentration in the striatum (Henning *et al.*, 2008). The aetiology remains unknown although oxidative stress and generation of reactive oxygen spesies (ROS) from both mitochondria impairment and dopamine metabolism might be responsible for its pathogenesis (Tian *et al.*, 2007).

6-Hydroxydopamine (6-OHDA), a neurotoxin which selectively kills dopaminergic neurones, is widely used to induce an experimental model of PD (Fornstedt *et al.*, 1986; Yuan *et al.*, 2008). 6-OHDA, which has a similar molecular structure to that of a dopamine, enters the cells via a dopamine re-uptake transporter (Ljungdahl *et al.*, 1971) and generates intracellular ROS and inhibits mitochondria to activate apoptosis cascades (Blum *et al.*, 2001). The SH-SY5Y cell line possesses many qualities of substantia nigra neurones and is thus suitable for use as an *in vitro* model to study the death of dopaminergic neurones (Takahashi *et al.*, 1994; Tian *et al.*, 2007).

In this study, we investigated the effects of H_2S on 6-OHDA-induced cell injury in SH-SY5Y cells. The involvement of various protein kinase C (PKC) isoforms and Akt signalling pathways were also examined.

Methods

All the drugs/molecular target nomenclature confirms to BJP's Guide to receptors and Channels (Alexander *et al.*, 2008).

Cell culture

The human neuroblastoma cell line, SH-SY5Y, obtained from the American Type Culture Collection (Manassas, VA, USA), was maintained in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 0.05 $U \cdot mL^{-1}$ penicillin and 0.05 $mg \cdot mL^{-1}$ streptomycin at 37°C in a humidified atmosphere containing 5% CO_2/95% air. Cultured medium was changed twice a week during cell growth.

Cell treatment

Cells were plated onto 96-well plates for viability tests or 35 mm dishes and incubated overnight as it grew into 80–90% confluency. Regular medium was replaced with low-serum medium (0.5% FBS/DMEM) immediately before treatment. Note that for LY294002 (LY) treatment, cells were deprived of serum for 4–6 h prior to treatment. After pretreatment with NaHS for 10 min or 1 h, cells were washed twice with PBS solution and incubated in fresh low-serum media with 6-OHDA. Each kinase inhibitor was added 30 min before NaHS treatment. Cells were incubated for 12 h after each exposure to toxins, and cell viability was measured using the MTT assay system.

Cell viability assay

Cell viability was measured using the MTT reduction assay as described previously (Hu *et al.*, 2009).

At the end of each treatment, MTT was added to each well at a final concentration of 0.5 mg·mL^{-1} and the cells were further incubated at 37°C for 4 h. Then, the insoluble formazan was dissolved in dimethyl sulphoxide (DMSO). Colorimetric determination of MTT reduction was measured at 570 nm with a reference wavelength at 630 nm.

Cell fractionation for determining PKC isoform translocation

Protein kinase C isoform translocation was detected with the cell fractionation method as described previously, with modifications (Pan *et al.*, 2008). Treated cells were incubated and lysed at different time points (1 h, 2 h, 6 h). SH-SY5Y cells were lysed with 200 µL ice-cold lysis buffer containing 125 mM NaCl, 25 mM Tris (pH 7.5), 5 mM EDTA, 1% Nonidet P-40 and protease inhibitors and shaken on ice for 1 h. The cell lysate was centrifuged at 500× *g* at 4°C for 10 min to discard the nuclei-rich pellet. The supernatant was recentrifuged at 20 000× *g* at 4°C for 20 min. The supernatant was collected as cytosolic fraction while the pellet was resuspended in 60 µL cell lysis buffer containing 1% Triton X-100 and shaken on ice for another 1 h and then centrifuged at 20 000× *g* at 4°C for 20 min. The second supernatant was collected as membrane fraction. Epitopes were exposed by boiling the protein samples at 90°C for 5 min. Western blots were performed to examine the translocation of the PKC isoforms.

Preparation of cell lysates for the detection of TH and phoshorylated Akt

A cell lysate technique was adopted from the literature (Yong *et al.*, 2008; Tamizhselvi *et al.*, 2009). Cells were washed twice with phosphate-buffered saline (PBS) after treatment and lysed with 200 µL ice-cold lysis buffer containing 125 mM NaCl, 25 mM Tris (pH 7.5), 5 mM EDTA, 1% Nonidet P-40, 0.4% deoxycholic acid (additional 10 mM NaF and 1 mM Na_3VO_4 are added for detection of phosphorylated Akt) and protease inhibitor cocktail tablet (Roche Diagnostics, Penzberg, Germany) and shaken on ice for 1 h. After centrifugation at 13 000× *g* at 4°C for 15 min, supernatant was collected and denatured by SDS-sample buffer. Epitopes were exposed by boiling the protein samples at 100°C for 5 min.

Western blot assays

Protein concentrations were determined with NanoDrop Spectrophotometer (ND-1000, NanoDrop technology). Equal amounts of the protein samples were separated by electrophoresis using a 10% sodium dodecyl sulphate-polyacrylamide (SDS/PAGE) gel and transferred onto a nitrocellulose membrane (Whatman®, Germany). After being blocked in 10% milk with TBST buffer (10 mM Tris-HCl, 120 mM NaCl, 0.1% Tween-20, pH 7.4) at room temperature for 1 h, the membrane was incubated with various primary antibodies (1:1000) at 4°C overnight. β-Tubulin (1:1000) or β-actin (1:10 000) was used as a loading control. Membranes were washed three times in TBST buffer, followed by incubation with 1:10 000 dilutions of horseradish peroxidase-conjugated (HRP) anti-rabbit IgG or anti-mouse IgG (β-actin) at 25°C for 1 h, and washed three times in TBST. Visualization was carried out using ECL® (plus/advanced chemiluminescence) kit (GE healthcare, UK). The density of the bands on Western blots was quantified by Image J software.

Cell transfection and apoptotic detection

SH-SY5Y cells (1×10^5) were seeded onto six-well plates and transfected with CBS-PME185-HA vector (a gift from Dr Hideo Kimura) or with empty vector alone as a control using lipofectamine 2000 transfection reagent. After transfection for 24 h, cells were washed with Krebs solution twice and then treated with 6-OHDA (50 µM) for 4 h. The apoptosis was examined with an Annexin V FITC detection kit (Calbiochem, Cat. No. PF032, Darmstadt, Germany) and analysed with fluorescence microscopy under FITC and rhodamine filter sets.

To visualize nuclear morphology, cells were also stained with 2.5 µg·mL^{-1} DNA dye Hoechst 33342. The nuclei of healthy and viable cells are usually uniformly stained, while apoptotic cells show condensed or fragmented nuclei.

H_2S 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), 250 µL), zinc acetate (1% (w/v), 250 µL), *N,N*-dimethyl-*p*-phenylenediamine sulphate (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 4000× *g* 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 NaH_2PO_4 and 25 mM $NaHCO_3$, pH 7.4.

Statistical analysis

All data are presented as mean ± SEM. Statistical significance was assessed with one-way analysis of variance (ANOVA) followed by a *post hoc* (Bonferroni) test for multiple group comparison. Differences with *P*-values less than 0.05 were considered statistically significant.

Chemicals and reagents

Sodium hydrosulphide (NaHS), 6-OHDA, methyl thiazolyl tetrazolium (MTT) were purchased from Sigma-Aldrich (St Louis, MO, USA). Gö6976 (a PKCα inhibitor) (Zeidman *et al.*, 1999), EAVSLKPT (a PKCε-selective peptide translocation inhibitor) (Chen *et al.*, 2005), rottlerin (a PKCδ inhibitor) (Maher, 2001) and LY294002 (LY) (a phosphoinositol 3' kinase (PI3K) inhibitor) (Sadidi *et al.*, 2009) were obtained from Calbiochem (Darmstadt, Germany). All chemicals were dissolved in deionized water except Gö6976, rottlerin and LY294002, which were dissolved in DMSO at a final concentration not more than 0.05%.

Primary antibody of β-tubulin and PKCε were from Santa Cruz Biotechnology while PKCα and PKCδ as well as polyclonal anti-phospho (p)-Akt rabbit IgG and polyclonal anti-total-Akt rabbit IgG were purchased from Cell Signaling Technology. Primary antibody of β-actin and anti-tyrosine hydroxylase (TH) antibody were obtained from Sigma-Aldrich (St Louis, MO, USA).

NaHS was used as an H_2S donor. When H_2S is dissolved in water at pH 7.4, HS^- is released and forms H_2S with H^+. This provides a solution of H_2S at a concentration that is approximately 33% of the original concentration of NaHS (Reiffenstein *et al.*, 1992).

Results

Protective effect of H_2S on 6-OHDA-induced cell injury

We first examined the toxic effect of 6-OHDA. As shown in Figure 1A, treatment with 6-OHDA (50–200 μM) for 12 h decreased the survival rate of SH-SY5Y cells. Pretreatment with NaHS (an H_2S donor, 100–1000 μM) for 10 min reversed the effect of 50 μM 6-OHDA (Figure 1B). These data suggest that H_2S may protect SH-SY5Y cells against 6-OHDA-induced cell injury. Figure 1C shows that the same protective effects were observed when NaHS was given 10 min or 1 h before administration of 6-OHDA.

H_2S reverses 6-OHDA-induced loss of TH

Tyrosine hydroxylase (TH) is an important rate-limiting enzyme in the conversion of amino acid tyrosine to dihydroxyphenylalanine (DOPA) for the production of dopamine (Pardridge, 2005). To confirm the neuroprotective effect of H_2S, we examined the effect of H_2S (100 μM) on TH protein level. As shown in Figure 2, treatment with 6-OHDA for 12 h significantly decreased TH level

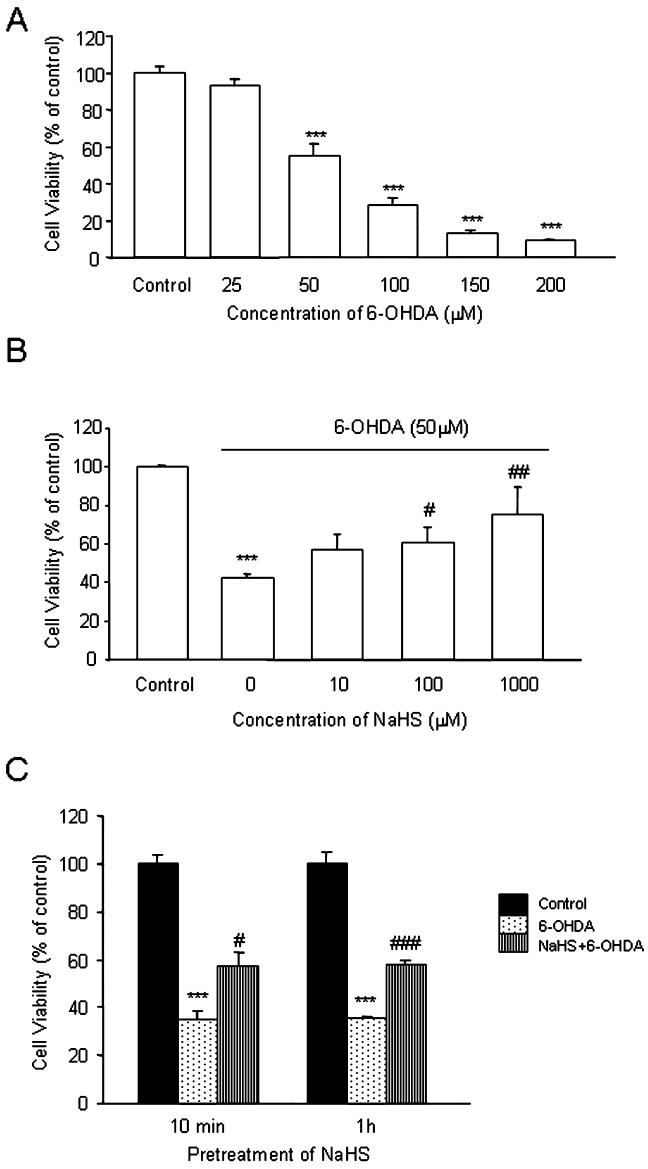

Figure 1

MTT assay showing the effect of NaHS and/or 6-OHDA on SH-SY5Y cell viability. (A) Effect of 6-OHDA on cell viability of SH-SY5Y cells. Cells were treated with 6-OHDA at different concentrations for 12 h. (B) Effect of NaHS on cell viability in SH-SY5Y cells treated with 6-OHDA. Cells were pretreated with various concentrations (10–1000 μM) of NaHS for 10 min before 6-OHDA (50 μM) was added for another 12 h. (C) Pretreatment of NaHS for 10 min and 1 h showed a similar protective effect against 6-OHDA-induced cell injury. Data are presented as mean ± SEM, $n = 5$, ***$P < 0.001$ versus control; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ versus 6-OHDA-treated cells. 6-OHDA, 6-hydroxydopamine.

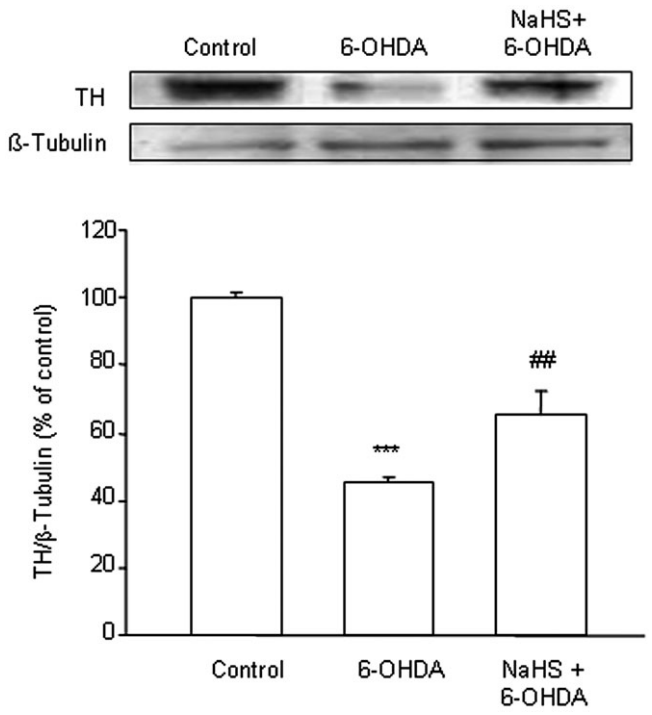

Figure 2

Effect of NaHS on TH expression in SH-SY5Y cells treated with 6-OHDA. Cells were pretreated with NaHS for 10 min before addition of 6-OHDA (50 μM) for another 12 h. Proteins were extracted and subjected to Western blot analysis using the anti-TH and β-tubulin (as a loading control) antibodies. Data are presented as mean ± SEM, $n = 5$, ***$P < 0.001$ versus control; ##$P < 0.01$ versus 6-OHDA-treated cells. 6-OHDA, 6-hydroxydopamine; TH, tyrosine hydroxylase.

in SH-SY5Y cells. This effect was reversed by H_2S, suggesting that H_2S treatment produces protective effects against 6-OHDA-induced dopaminergic neurone injury.

Effect of NaHS on translocation of PKC isoforms in 6-OHDA-treated SH-SY5Y cells

It has been reported that 6-OHDA-induced cell death is via regulating PKC activity (Tian *et al.*, 2007). In the present study, we examined the effect of 6-OHDA on the translocation of PKC isoforms. The time-courses were shown in Figure 3A. Treatment with 6-OHDA for 1–6 h inhibited the translocation of PKCα and PKCε from cytosol to membrane, but stimulated the activity of PKCδ. The maximum responses were found at 2 h for PKCδ, and 6 h for PKCα and ε. NaHS treatment significantly reversed the effect of 6-OHDA on the activities of the three PKC isoforms, suggesting that the protective effect of H_2S may involve different PKC isoforms (Figure 3B).

Effect of NaHS on cell viability in 6-OHDA-treated SH-SY5Y cells in the presence and absence of inhibitors of PKC isoforms

To further confirm the involvement of PKC, we pretreated the cells with rottlerin (5 μM, a PKCδ inhibitor), Gö6976 (2 μM, a PKCα inhibitor) or EAVSLKPT (200 μM, a specific PKCε inhibitor) for 30 min prior to administration of NaHS (100 μM). The MTT assay showed that the protective effect of H_2S was significantly diminished by Gö6976 and EAVSLKPT (Figure 4A). However, inhibition of PKCδ with rottlerin did not reverse cell injury caused by 6-OHDA (Figure 4B). This finding implies that the protective effect of H_2S may mainly originates from PKCα and PKCε, not PKCδ.

Involvement of PI3K/Akt in H_2S-induced neuroprotection

We also investigated the contribution of the PI3K/Akt pathway in the neuroprotective effects of NaHS. We first examined the time-course for the effect of 6-OHDA on Akt activity. As shown in Figure 5A, 6-OHDA significantly suppressed Akt activity after treatment for 6 h. Figure 5B shows the time-course for the effect of NaHS on Akt activity. There were two peaks (20–40 min and 4–12 h) for NaHS-induced Akt activation. Bearing the above data in mind, we examined the effect of NaHS on Akt activity in cells treated with 6-OHDA for 6 h and 12 h. As shown in Figure 5C, treatment with NaHS reversed the inhibitory effect of 6-OHDA on Akt activity at both time points.

The involvement of the PI3K/Akt pathway was further confirmed with cell viability data using MTT assay. As shown in Figure 6, pretreatment with LY294002 (a PI3K inhibitor) for 30 min, which alone had no significant effect, markedly reversed the protective effects of NaHS on cell viability. Taken together, these data clearly suggest that the protective effect of NaHS is via stimulation of the PI3K/Akt pathway.

Correlation between PKC and Akt

We further examined whether PI3K/Akt is downstream to PKC activation. As shown in Figure 7, the effect of NaHS on Akt activity was abolished by pretreatment of the cells with Gö6976 (2 μM, an inhibitor of PKCα) or EAVSLKPT (200 μM, an inhibitor of PKCε) for 30 min. These results suggest that activation of Akt by H_2S is dependent on PKCα and PKCε.

CBS overexpression attenuates 6-OHDA-induced apoptosis in SH-SY5Y cells

To confirm the role of endogenous H_2S, SH-SY5Y cells were transfected with the cDNA of CBS, the

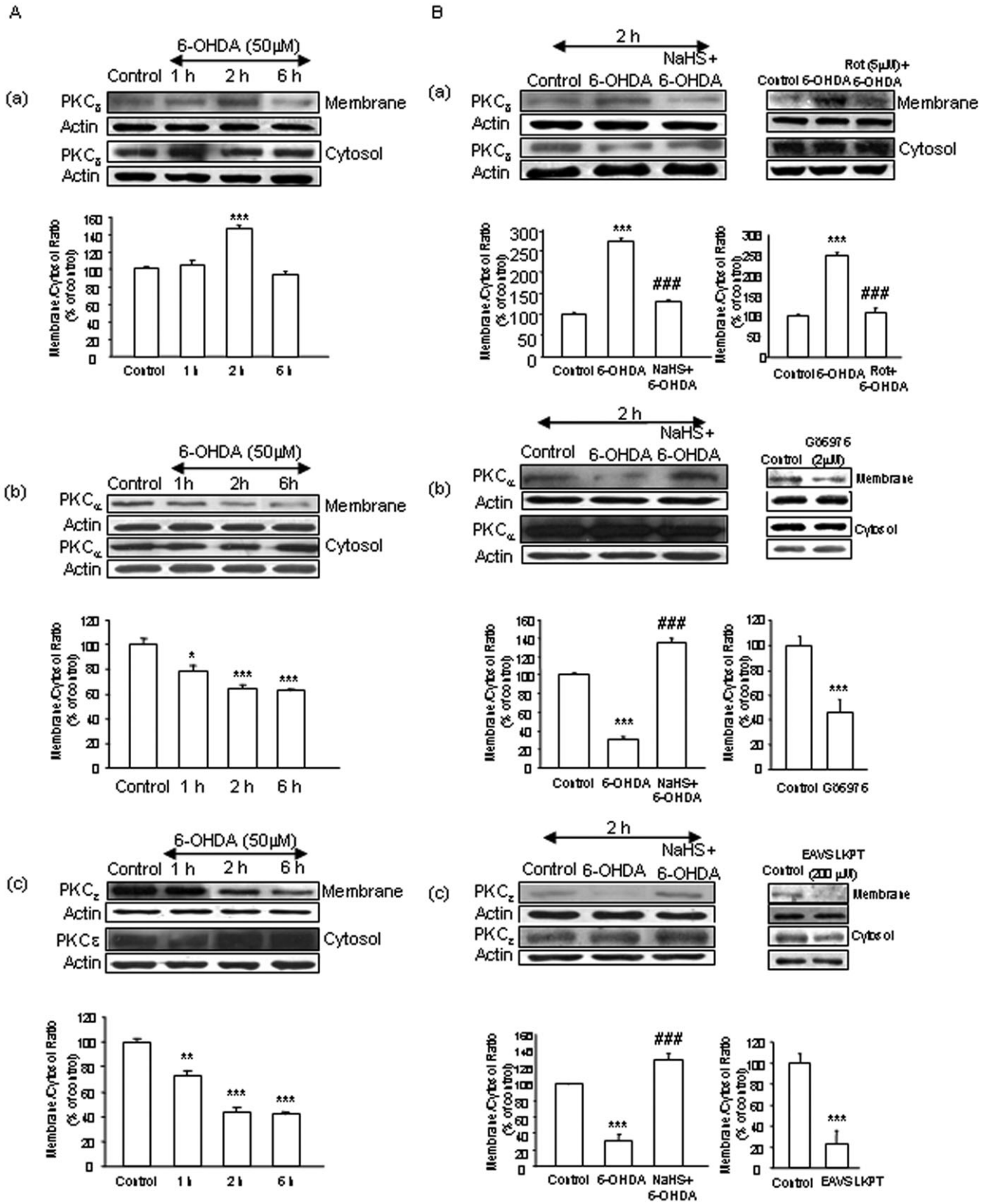
A
B
(a)
(b)
(c)
6-OHDA (50μM)
Control 1 h 2 h 6 h
PKCδ
PKCα
PKCε
PKCζ
Actin
Membrane
Cytosol
Membrane/Cytosol Ratio (% of control)
2 h
NaHS+
Control 6-OHDA 6-OHDA
Rot (5μM)+
Control 6-OHDA 6-OHDA
Control 6-OHDA NaHS+ 6-OHDA
Control 6-OHDA Rot+ 6-OHDA
G06976
Control (2μM)
Control G06976
EAVSLKPT
Control (200 μM)
Control EAVSLKPT

Figure 3

Role of PKC isoforms in the neuroprotective effects of NaHS in SH-SY5Y cells treated with 6-OHDA. (A–B) a: PKCδ, b: PKCα, c: PKCε. (A) Time course for the effect of 6-OHDA on the translocation of PKC isoforms. (B) Effect of NaHS (100 μM) on the translocation of PKC isoforms caused by 6-OHDA (50 μM, 2 h). Right panel shows the effect of various PKC isoform inhibitors on the translocation of PKC isoforms. The ratios of membrane/cytosol fraction are normalized to that of control group. β-Actin was used as a loading control. Data are presented as mean ± SEM, $n = 5$–8, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control; $^{\#\#\#}P < 0.001$ versus 6-OHDA-treated cells. 6-OHDA, 6-hydroxydopamine; PKC, protein kinase C.

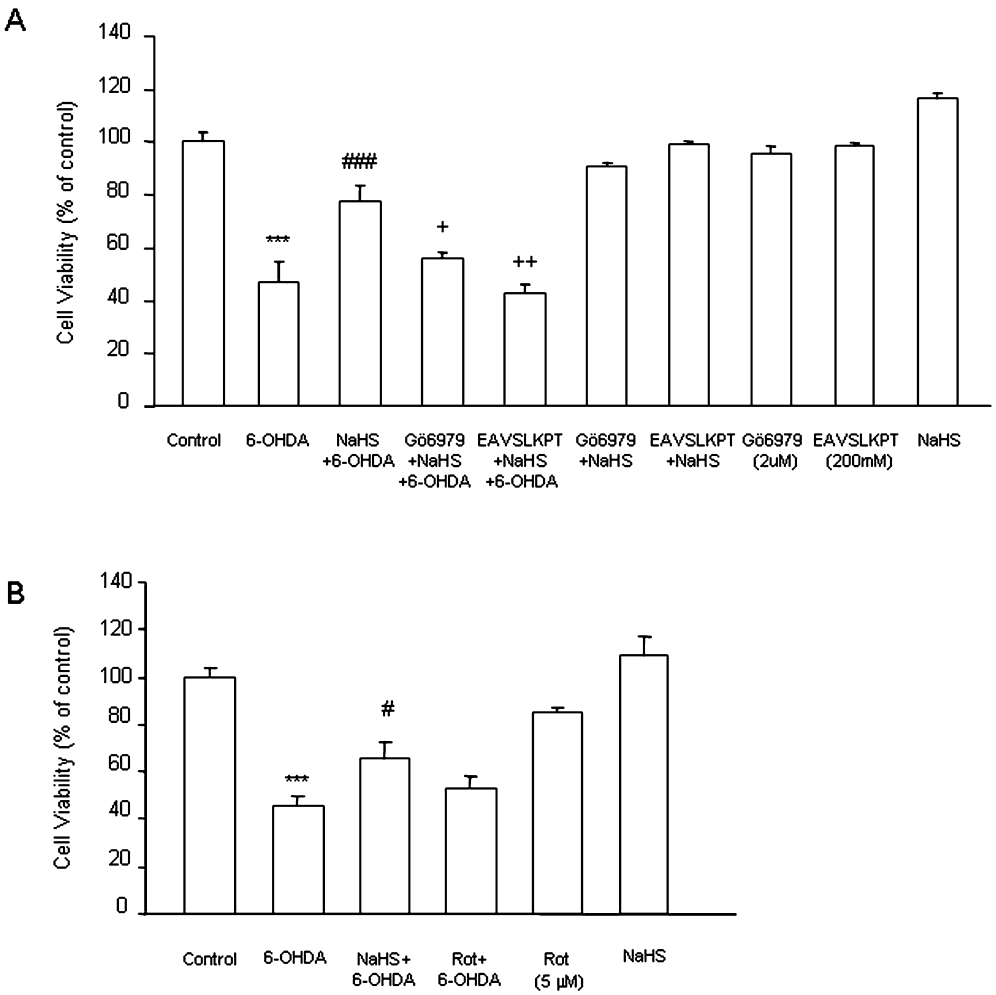

Figure 4

The protective effect of NaHS on cell viability in the presence and absence of various PKC isoform inhibitors. (A) Blockade of PKCα with Gö6976 (2 μM, 30 min pretreatment) or PKCε with EAVSLKPT (200 μM, 30 min pretreatment) attenuated the protective effect of NaHS (100 μM, 12 h) on 6-OHDA (50 μM, 12 h)-induced cell injury. (B) Blockade of PKCδ with rottlerin did not reverse cell injury caused by 6-OHDA. Data are presented as mean ± SEM, $n = 5$, $^{***}P < 0.001$ versus control; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ versus 6-OHDA-treated cells; $^{+}P < 0.05$, $^{++}P < 0.01$ versus NaHS + 6-OHDA-treated cells. 6-OHDA, 6-hydroxydopamine; PKC, protein kinase C.

main enzyme that produces H_2S in these cells. As shown in Figure 8A, CBS protein expression in SH-SY5Y cells was obviously increased 24 h after transfection. Accordingly, H_2S concentration in CBS-overexpressed cellular culture medium was significantly elevated compared with empty vector–transfected group (Figure 8B). The H_2S level increased to 13.13 ± 1.33 μM 24 h after CBS transfection compared with that in the control group of 9.93 ± 0.23 μM. After application of NaHS (100 μM) for 10 min, the H_2S level was 42.73 ± 1.92 μM in medium. Thus, the elevated H_2S level caused by overexpression of CBS is much lower than that caused by exogenous application of NaHS.

Phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane during normal

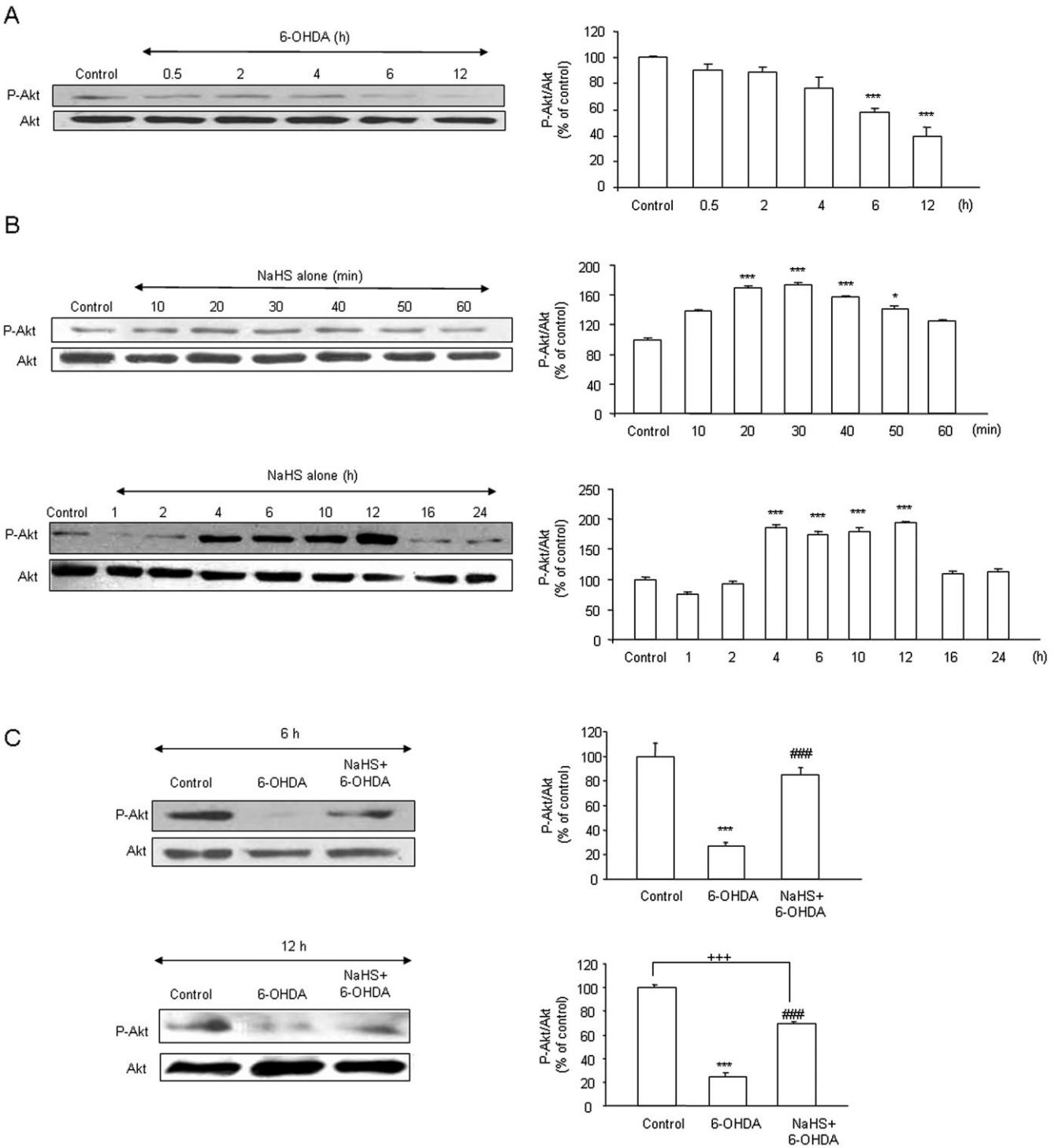

Figure 5

Involvement of PI3K/Akt pathway in the neuroprotective effects of NaHS. (A–B) Time-courses for the effect of 6-OHDA (50 μM, A) and NaHS (100 μM, B) on Akt activity. (C) NaHS reversed the inhibitory effect of 6-OHDA (6 h and 12 h) on Akt phosphorylation. The histograms represent the ratio of phosphorylated protein over total Akt. Data are presented as mean ± SEM, $n = 5$, $^{*}P < 0.05$, $^{***}P < 0.001$ versus control; $^{\#\#\#}P < 0.001$ versus 6-OHDA-treated cells. 6-OHDA, 6-hydroxydopamine.

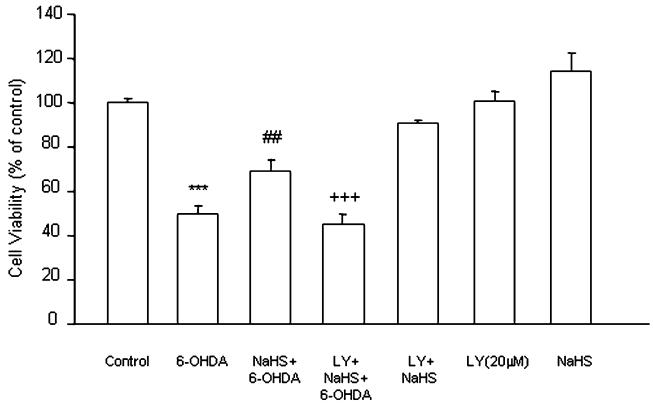

Figure 6

Blockade of PI3K with LY294002 (20 μM, 30 min pretreatment) abolished the protective effect of NaHS (100 μM, 12 h) on cell injury induced by 6-OHDA (50 μM, 12 h). Data are presented as mean ± SEM, $n = 5$, ***$P < 0.001$ versus control; ##$P < 0.01$ versus 6-OHDA-treated cells; $^{+++}P < 0.001$ versus NaHS + 6-OHDA-treated cells. 6-OHDA, 6-hydroxydopamine.

conditions. Early apoptosis leads to exposure of PS on the cell surface. The extracellular PS therefore binds with Annexin V-FITC. Late apoptotic cells not only bind with Annexin V-FITC but can also be stained with propidium iodide. As shown in Figure 8C, 6-OHDA increased the number of cells with green (AnnexinV stained by FITC) and red (stained by propidium iodide) fluorescence in the vehicle group (empty vector-transfected cells). This effect was significantly attenuated by the overexpression of CBS.

The beneficial effects of endogenous H_2S against 6-OHDA-induced apoptosis were also confirmed by Hoechst 33342 staining assay. Representative photomicrographs of nuclei morphology of SH-SY5Y cells are shown in the right panel of Figure 8C. The effect of 6-OHDA led to the condensation and fragmentation of nuclei (a characteristic of apoptosis). CBS overexpression significantly attenuated this effect. Taken together, these data suggest that 6-OHDA-induced cell apoptosis was attenuated by endogenous H_2S, which is generated by CBS overexpression.

Discussion

The cornerstone of signs and symptoms of PD are the loss of dopaminergic neurones and subsequent dopamine deficit in the brain (Zigmond *et al.*, 2002; Tian *et al.*, 2007; Henning *et al.*, 2008; Yuan *et al.*, 2008). Dopamine loss in PD is unable to be replaced directly by dopamine replacement therapy, as this monoamine does not cross the brain capillary

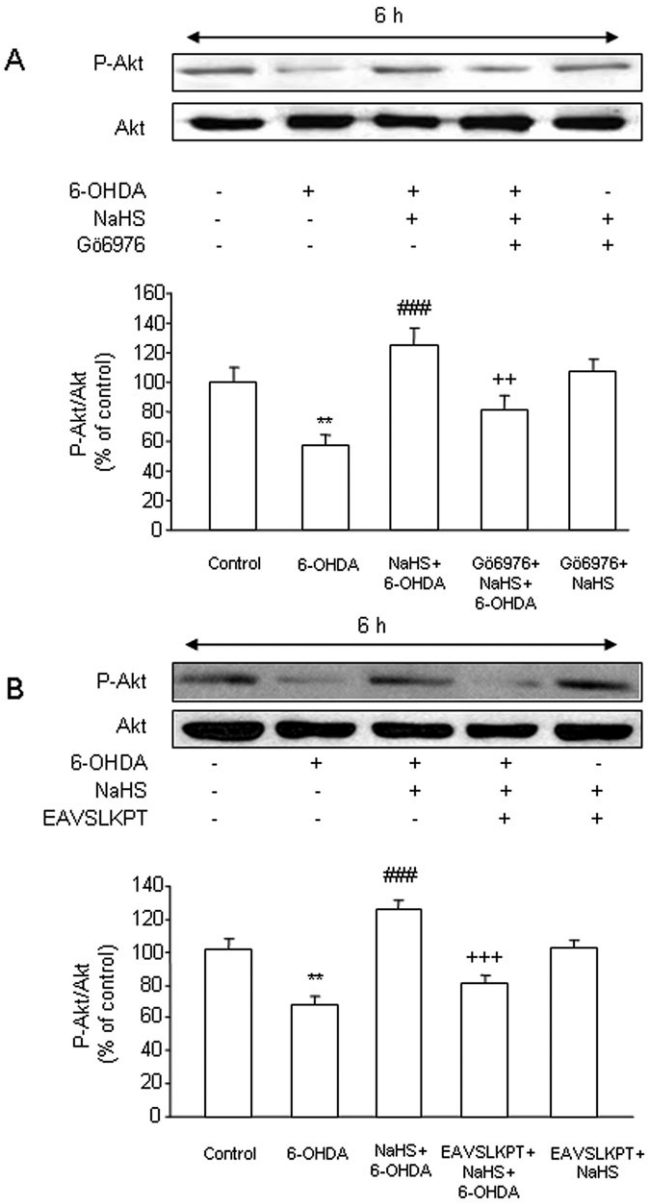

Figure 7

Effect of NaHS on Akt activation was dependent on PKC activity. Blockade of PKCα with Gö6976 (2 μM, A) or PKCε with EAVSLKPT (200 μM, B) attenuated NaHS-up-regulated Akt phosphorylation in SH-SY5Y cells treated with 6-OHDA for 6 h. Whole cell lysates were prepared for Western blot analysis of total Akt and phosphorylated Akt level. The histograms represent the ratio of phosphorylated protein to total Akt. Results shown are the mean ± SEM, $n = 5$, **$P < 0.01$ versus control; ###$P < 0.001$ versus 6-OHDA-treated cells; $^{++}P < 0.01$, $^{+++}P < 0.001$ versus NaHS + 6-OHDA-treated cells. 6-OHDA, 6-hydroxydopamine; PKC, protein kinase C.

endothelial wall, which forms the blood brain-barrier (BBB). However, the precursor to dopamine, dihydroxyphenylalanine (DOPA), is able to cross the BBB owing to its transport via the BBB large neutral amino acid transporter. DOPA is then decarboxylated to dopamine by aromatic amino acid

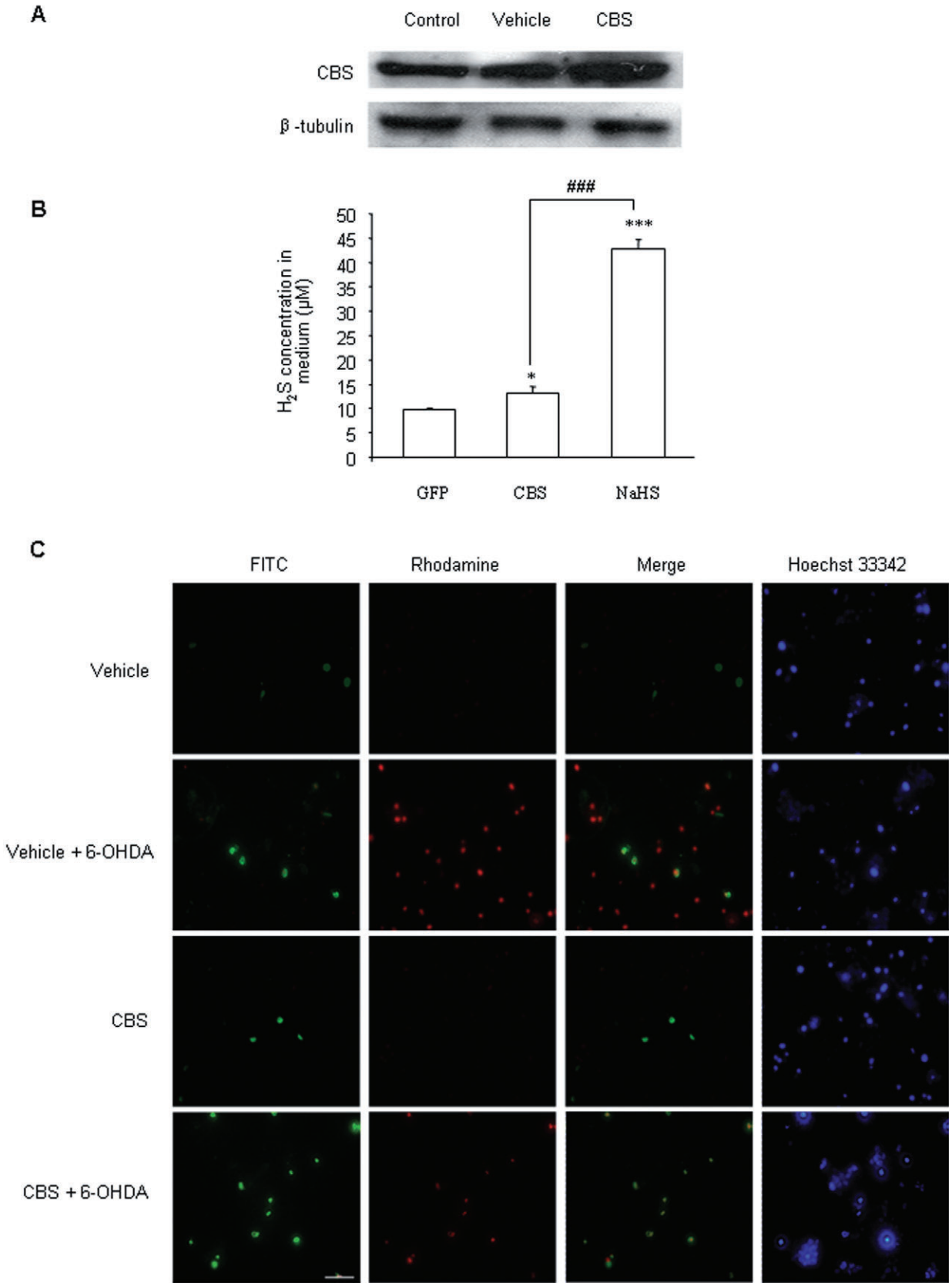
A
Control
Vehicle
CBS
CBS
β -tubulin
B
###
***
*
H_2S concentration in medium (μM)
50
45
40
35
30
25
20
15
10
5
0
GFP
CBS
NaHS
C
FITC
Rhodamine
Merge
Hoechst 33342
Vehicle
Vehicle + 6-OHDA
CBS
CBS + 6-OHDA

Figure 8

Effect of endogenous H_2S on 6-OHDA-induced cell apoptosis in SH-SY5Y cells. (A) Transfection of CBS cDNA into SH-SY5Y cells increased the protein expression of CBS. β-Tubulin was used as a loading control. (B) Effect of CBS overexpression and exogenous application of NaHS on endogenous H_2S level. Mean ± SEM, $n = 8$, $^*P < 0.05$, $^{***}P < 0.001$ versus GFP; $^{###}P < 0.001$ versus CBS. (C) CBS overexpression alleviated 6-OHDA-induced apoptosis. Green: early apoptosis indicated by FITC fluorescence; red: late phase apoptosis stained by propidium iodide; blue: nuclei stained by Hoechst 33342. Photos were taken at ×20 magnification. Scale bar: 200 nm. 6-OHDA, 6-hydroxydopamine; CBS, cystathionine β-synthase; GFP, green fluorescent protein.

decarboxylase (AAAD). The rate-limiting step in cerebral production of dopamine is the conversion of tyrosine to DOPA via tyrosine hydroxylase (TH) (Pardridge, 2005). Therefore, at present, levodopa (L-dopa) is widely used as a treatment to restore dopamine concentration in PD patients. However, studies have shown that long-term usage of L-dopa leads to pro-oxidant damage (Tian *et al.*, 2007); hence new therapy is needed. In the present study, we provide evidence that H_2S, which can easily penetrate biological membranes (Kimura *et al.*, 2005), is able to protect against 6-OHDA-induced cell injury. In addition, H_2S significantly increased the level of TH, the rate-limiting enzyme in dopamine production. Our *in vitro* findings suggest that H_2S protects dopaminergic neurones against 6-OHDA-induced injury.

We also examined the protective effect of endogenous H_2S on 6-OHDA-induced cell damage. We found that overexpression of CBS only moderately increased the H_2S level, whereas exogenous application of NaHS markedly elevated the H_2S level. However, CBS overexpression still produced obvious protective effects against cell apoptosis. This is probably because overexpression of CBS may increase H_2S level stably and persistently, whereas exogenous application of NaHS causes a transient increase in H_2S level in the buffer (Hu *et al.*, 2009). This finding reveals that endogenously produced H_2S is important to protect brain against oxidative stress-induced neurodegenerative diseases. Our observation is consistent with Kimura's finding that transfection with 3MST also showed significant resistant to oxidative stress in Neuro2a cells (Kimura *et al.*, 2010).

We further investigated the possible signalling mechanisms underlying the protective effect of H_2S on 6-OHDA-induced cell injury. We focused on PKC and Akt, because both of them are well-known pro-survival protein kinases and are hence thought to be involved in anti-oxidation effects that induce neuronal protection (Louis *et al.*, 1988; Tian *et al.*, 2007).

Protein kinase C is a family of well-studied serine-threonine kinases. It is involved in many cell functions including cell proliferation, differentiation, apoptosis and gene expression. The hallmark for PKC activation is a process called translocation, whereby PKC isoforms translocate from the cytosol to subcellular membrane regions (Mackay and Mochly-Rosen, 2001; Pan *et al.*, 2008). The PKC family consists of at least 12 isoforms, among which PKCδ, α and ε are expressed in SH-SY5Y neuroblastoma cells (Zeidman *et al.*, 1999; Mackay and Mochly-Rosen, 2001; Pan *et al.*, 2008). Various studies have shown that different isoforms play different roles in cell functions. PKCα and PKCε have been associated with cell proliferation (Dlugosz *et al.*, 1994; Kampfer *et al.*, 1998), while PKCδ activation contributes to apoptosis (Ohba *et al.*, 1998; Li *et al.*, 1999a; Maher, 2001). Weinreb *et al.* (2004) reported that PKCα phosphorylates Bcl-2 in a site which increases its anti-apoptotic function, while overexpression of PKCε elevates the expression of Bcl-2 which inhibits its apoptosis (Itano *et al.*, 1996; Akao *et al.*, 2002; Weinreb *et al.*, 2004). In view of this, we examined the effect of NaHS on translocation of these isoforms in SH-SY5Y cells treated with 6-OHDA. We found that 6-OHDA transiently (~2 h) stimulated translocation of PKCδ and sustainably (>6 h) inhibited the activities of PKCα and PKCε. These effects were inhibited by NaHS. Blockade of PKCα and PKCε with their inhibitors abolished the neuroprotection caused by H_2S. However, inhibition of PKCδ with rottlerin did not reverse the cell injury caused by 6-OHDA in SH-SY5Y cells. These findings suggest that the toxic effect of 6-OHDA may predominantly originate from sustained inhibition (>6 h) of PKCα and PKCε, but not from the transient (~2 h) activation of PKCδ. In a similar way, the neuroprotection offered by NaHS may mainly result from stimulation of PKCα and PKCε, instead of inhibition of PKCδ.

Akt, also known as protein kinase B (PKB), is a key molecule in growth factor signalling pathways mediating neuronal survival in both development and disease in multiple paradigms, including resistance against oxidative insults in the brain (Rodriguez-Blanco *et al.*, 2008). Stimulation of the PI3K pathway is necessary for Akt activation in most instances (Li *et al.*, 1999b). Once activated, Akt, in

turn, inactivates several pro-apoptotic proteins including BAD and caspase-9 (Li *et al.*, 2006) and therefore promotes cell survival. In the present study, we found that blockade of PI3K with its selective inhibitor, LY294002, abolished the protective effects of NaHS on cell viability. More importantly, NaHS reversed the down-regulated Akt activity caused by 6-OHDA. Our data clearly suggest that the protective effect of NaHS on 6-OHDA-induced cell injury is mediated by stimulation of the PI3K/Akt pathway.

A physical interaction between PKC and Akt in human vascular endothelial cells results in induction of Bcl-2 and enhancement of protection against apoptotic cell death via caspase-3 cleavage inhibition (Yonekawa and Akita, 2008). The activation sequence between PKC and PI3K/Akt has not been fully elucidated and several different mechanisms have been reported. It has been reported that activation of PI3K/Akt controls PKC activity (Le Good *et al.*, 1998). However, up-regulated Akt activity was also observed when PKCα was overexpressed in 32D myeloid progenitor cells (Li *et al.*, 1999b; Rodriguez-Blanco *et al.*, 2008). In the present study, we found that inhibition of PKCα and PKCε isoforms down-regulated Akt expression level, which led to increased cell apoptosis. Our findings suggest that Akt could be the downstream effector of PKCα and PKCε.

The results in the present study suggest that the neuroprotection offered by H_2S is a preconditioning-like effect. Our previous study showed that H_2S quickly decays to an undetectable level within 30 min after addition of NaHS into cell culture buffer (Hu *et al.*, 2009). This suggests that H_2S rapidly stimulates the PKC/PI3K/Akt pathway and triggers a series of persistent intracellular responses and therefore protects the cells. This preconditioning effect is similar to the mechanisms for the cardioprotection conferred by H_2S preconditioning (Bian *et al.*, 2006; Pan *et al.*, 2006; Hu *et al.*, 2008; Pan *et al.*, 2008; 2009). Our time-course study shows that the preconditioning period of NaHS lasts for at least 1 h. At this time point, the H_2S concentration has already decayed to an undetectable level (Hu *et al.*, 2009). This finding excludes the possibility that the neuroprotection of H_2S is caused by direct inhibition of extracellular auto-oxidation of 6-OHDA, another important mechanism for 6-OHDA-induced dopaminergic cell death (Abad *et al.*, 1995; Blum *et al.*, 2000).

In summary, the present observations identify the potential of H_2S in protecting SH-SY5Y cells against 6-OHDA-induced cell injury. The neuroprotective effect of H_2S involves the PKC-dependent PI3K/Akt pathway.

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Conflicts of interest

None.

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