Hydrogen sulfide protects PC12 cells against reactive oxygen species and extracellular signal-regulated kinase 1/2-mediated downregulation of glutamate transporter-1 expression induced by chemical hypoxia

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Abstract. Hypoxia and/or ischemia are implicated in neurodegenerative disorders. In these diseases, hypoxia/ischemia may induce oxidative stress, including production of reactive oxygen species (ROS), which result in a decrease in glutamate transporter expression. Hydrogen sulfide (H_2S), as the third gasotransmitter, has neuroprotective effects and potent antioxidant properties. In the present study, we investigated the role of glutamate transporter-1 (GLT-1) in the protection of H_2S against chemical hypoxia-induced injury in PC12 cells. We found that cobalt chloride ($CoCl_2$), a chemical hypoxia agent, reduced the expression of GLT-1 in a time-dependent manner. Pretreatment with NaHS (a donor of H_2S) reversed the $CoCl_2$-induced downregulation of GLT-1 expression. Pretreatment with DHK (a selective inhibitor of GLT-1) for 30 min prior to NaHS preconditioning significantly inhibited the cytoprotection of H_2S against $CoCl_2$-induced injuries, leading to an increase in cytotoxicity and apoptosis as well as to a loss of mitochondrial membrane potential (MMP). In addition, we found that similar to the effect of NaHS, pretreatment with NAC (a ROS scavenger) or U0126 (a MEK1/2 inhibitor) blocked the downregulation of GLT-1 expression induced by $CoCl_2$. Collectively, we demonstrated for the first time that ROS and extracellular signal-regulated kinase 1/2 (ERK1/2)-mediated reduction of GLT-1 expression may be involved in chemical hypoxia-induced neural injury and that H_2S attenuates this injury partly by upregulating GLT-1 expression in PC12 cells.

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Introduction

It is well documented that hypoxia and/or ischemia can elicit the release of several neurotransmitters (1,2), including glutamate (3). Such elevated levels of glutamate, and the subsequent activation of ionotropic NMDA receptors, can trigger the neuronal damage during hypoxia and/or ischemia (4,5). Glutamate homeostasis is therefore crucial to prevent neuronal death after a hypoxic/ischemic episode. Glutamate transport is the only mechanism for the removal of glutamate from the extracellular fluid in the brain (6,7), and it is essential for maintaining extracellular glutamate below neurotoxic levels in the normal brain (8). Therefore, glutamate transporters are considered to play a key role in the process of increase in extracellular glutamate during hypoxia/ischemia.

To date, 5 distinguishing high-affinity, Na^+-dependent glutamate transporters have been identified: excitatory amino acid transporter (EAAT)1, glutamate-aspartate transporter (GLAST), EAAT2, glutamate transporter-1 (GLT-1), EAAT3, excitatory amino acid carrier 1 (EAAC1), EAAT4 and EAAT5. These transporters are present throughout the central nervous system (CNS), with GLT-1 being highly abundant in astroglial cells, whereas GLAST exists at higher levels in Bergmann glia in the cerebellum (9,10). GLT-1 plays a critical role in CNS homeostasis, accounting for up to 70% of glutamate clearance (10,11).

The roles of GLT-1 in hypoxia/ischemia-induced injury and neuroprotection have attracted extensive attention. However, the findings are controversial. A pharmacological study indicated that the GLT-1 blocker reduces the ischemia-

induced glutamate release in rat cortical superfusates (12), revealing that GLT-1 releases glutamate during ischemia. By contrast, Rao *et al* (13) reported that the antisense knockdown of GLT-1 exacerbates ischemia-triggered neuronal damage in the rat brain, suggesting that GLT-1 takes up glutamate to protect neurons during ischemia. In addition, ischemic preconditioning upregulates the GLT-1 protein which may play a role in the neuroprotective mechanism of preconditioning (14). In neonatal rats, it was shown that the neuroprotection of ceftriaxone preconditioning against hypoxia/ischemia-induced neuronal injury is associated with upregulation of GLT-1 expression (15). On the other hand, an association between change in GLT-1 expression and hypoxia/ischemia has been reported by several studies (16,17). Raghavendra *et al* (16) observed that the expression of GLT-1 is reduced following transient global ischemia. Conversely, chronic hypoxia upregulates the expression of EAAC1 and GLT-1, but not GLAST (17). These findings support the theory that GLT-1 has a complicated function (cytoprotective vs. cytotoxic effects) after hypoxic/ischemic episodes. Thus, it is necessary to explore the roles of GLT-1 in neuronal injury or the neuroprotective effects in different hypoxic/ischemic models.

Hydrogen sulfide (H_2S), recently considered a novel neuromodulator in the CNS, has been shown to protect astrocytes against H_2O_2-induced neural damage by enhancing glutamate uptake (18), suggesting an impact of H_2S on glutamate transporters. We have also demonstrated that H_2S protects PC12 cells against chemical hypoxia-induced injury by inhibiting reactive oxygen species (ROS) overproduction, extracellular signal-regulated kinase 1/2 (ERK1/2) and the p38 mitogen-activated protein kinase (MAPK) signaling pathways (19,20). Since it is reported that ROS and the activation of the ERK1/2 pathway are involved in the downregulation of GLT-1 protein expression induced by H_2O_2 or amyloid-β (Aβ) in astrocytes (19,21), we hypothesized that ROS and ERK1/2-mediated downregulation of GLT-1 might be implicated in chemical hypoxia-induced neuronal injury and that H_2S might confer neuroprotection by enhancing GLT-1 expression. To test this hypothesis, PC12 cells, which are derived from chromafin cells of the adrenal medulla, were exposed to cobalt chloride ($CoCl_2$), a well-known hypoxia mimetic agent, to establish a model of chemical hypoxia injury. The effects of $CoCl_2$ and pretreatment with NaHS (a donor of H_2S) on GLT-1 expression were observed. We found that: i) $CoCl_2$ significantly inhibits the expression of GLT-1, ROS and the ERK1/2 pathway contribute to this inhibitory effect; ii) NaHS pretreatment clearly attenuates the inhibitory effect of $CoCl_2$ on GLT-1 expression; iii) DHK, a selective inhibitor of GLT-1, blocks the neuroprotection of H_2S against $CoCl_2$-induced injury in PC12 cells.

Materials and methods

Materials. NaHS, $CoCl_2$, N-acetyl-L-cysteine (NAC), Hoechst 33258, propidium iodide (PI), RNase and Rhodamine 123 (Rh123) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The cell counter kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). The DMEM medium and fetal bovine serum (FBS) were supplied by Gibco-BRL (Grand Island, NY, USA). Anti-GLT-1 antibody was purchased from Abcam (Cambridge, UK). DHK was purchased from Merck Co. Anti-β-actin antibody, horseradish peroxidase (HRP)-conjugated secondary antibody and the BCA protein assay kit were purchased from KangChen Bio-tech, Inc. (Shanghai, China). Enhanced chemiluminescence (ECL) solution was purchased from Nanjing KeyGen Biotech Co., Inc. (Nanjing, China).

Cell culture and treatments. The rat pheochromocytoma cell line PC12 cells were purchased from the Sun Yat-Sen University Experimental Animal Center, and were grown in DMEM medium supplemented with 10% FBS at 37˚C under an atmosphere of 5% CO_2 and 95% air. According to our previous study (20), chemical hypoxia was achieved by adding $CoCl_2$ at 600 μM into the medium and cells were incubated in the presence of $CoCl_2$ for the indicated times. The cytoprotective effects of H_2S were observed by administering 400 μM NaHS (a donor of H_2S) for 30 min prior to exposure to $CoCl_2$ for 24 h. NAC (a scavenger of ROS) or U0126 (a MEK1/2 inhibitor) was administered 60 or 120 min prior to exposure of the PC12 cells to 600 μM $CoCl_2$ for 24 h.

Cell viability assay. The CCK-8 assay was employed to investigate the cell viability of PC12 cells cultured in 96-well plates. After the indicated treatments, 10 μl CCK-8 solution was added to each well of the plate and the cells in the plate were incubated for 4 h in the incubator. The absorbance at 450 nm was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Means of 4 well optical density (OD) in the indicated groups were used to calculate the percentage of cell viability according to the formula below: Percentage of cell viability (%) = ($OD_{treatment\ group}/OD_{control\ group}$) x 100%. The experiment was repeated 3 times.

Nuclear staining for assessment of apoptosis with Hoechst 33258. Morphological changes, such as chromosomal condensation and fragmentation in the nuclei of PC12 cells, were observed by Hoechst 33258 staining followed by photofluorography. Cells were plated at a density of $1x10^6$ cells/well in 35 mm dishes. Cells were preconditioned with 400 μM NaHS for 30 min, and subsequently exposed to 600 μM $CoCl_2$ for 48 h. To test the role of GLT-1 in H_2S-induced cytoprotection against chemical hypoxia-induced apoptosis, cells were treated with the GLT-1 inhibitor DHK for 30 min prior to preconditioning with NaHS. At the end of the indicated treatments, cells were harvested and fixed with 4% paraformaldehyde in 0.1 mol/l phosphate-buffered saline (PBS, pH 7.4) for 10 min. After rinsing with PBS, the nuclear DNA was stained with 5 mg/ml Hoechst 33258 solution for 10 min before being rinsed briefly with PBS and then visualized under a fluorescence microscope (Bx50-FLA; Olympus, Tokyo, Japan). Viable cells displayed a uniform blue fluorescence throughout the nucleus, whereas apoptotic cells showed condensed and fragmented nuclei.

Flow cytometric analysis of apoptosis. Treated PC12 cells were digested with trypsin (2.5 mg/ml), centrifuged at 350 x g for 10 min and the supernatant was removed. Cells were washed twice with PBS and fixed with 70% ice-cold ethanol. Cells were then centrifuged at 350 x g for 10 min, washed twice with PBS and adjusted to a concentration of

$1x10^6$ cells/ml. Subsequently, 0.5 ml RNase (1 mg/ml in PBS) was added to a 0.5 ml cell sample. After gentle mixing with PI (at a terminal concentration of 50 mg/l), mixed cells were filtered and incubated in the dark at 4˚C for 30 min before flow cytometric analysis (FCM). The PI fluorescence of individual nuclei was measured by a flow cytometer (Beckman-Coulter, Los Angeles, CA, USA). Excitation, 488 nm; emission, 615 nm. The research software matched with FCM was used to analyze all the data of DNA labeling. In the DNA histogram, the amplitude of the sub-G1 DNA peak, which is lower than the G1 DNA peak, represents the number of apoptotic cells. The experiment was repeated 3 times.

Measurement of MMP. Mitochondrial membrane potential (MMP) was monitored using the fluorescent dye Rh123, a cell-permeable cationic dye that preferentially enters into the mitochondria based on the highly negative MMP. Depolarization of MMP results in loss of Rh123 from the mitochondria and a decrease in intracellular fluorescence. In the present study, PC12 cells were cultured in 24-well plates and treated with 400 μM NaHS for 30 min prior to the administration of 600 μM $CoCl_2$ for 24 h. DHK was administered 30 min prior to NaHS preconditioning. To evaluate MMP, Rh123 (100 μg/l) was added to cell cultures for 45 min at 37˚C and fluorescence was measured over the entire field of vision using a fluorescent microscope connected to an imaging system (BX50-FLA; Olympus). The mean fluorescence intensity (MFI) of Rh123 from 5 random fields was analyzed using ImageJ 1.410 software (National Institutes of Health, Bethesda, MD, USA), and the MFI was taken as an index of the MMP. The experiment was repeated 3 times.

Western blot assay for protein expression. After the cells were subjected to the indicated treatments, they were harvested and lysed with cell lysis solution. Total protein in the cell lysate was quantified using the BCA protein assay kit. Sample buffer was added to cytosolic extracts, and after boiling for 5 min, equal amounts of supernatant from each sample were fractionated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Total protein in the gel was transferred into polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for 1.5 h at room temperature in fresh blocking buffer [0.1% Tween-20 in Tris-buffered saline (TBS-T) containing 5% fat-free milk] and then incubated with either anti-GLT-1 (1:2,500 dilution), or anti-β-actin antibodies (1:5,000 dilution) in freshly prepared TBS-T with 3% free-fat milk overnight with gentle agitation at 4˚C. After 3 washes with TBS-T, membranes were incubated with HRP-conjugated goat anti-rabbit secondary antibodies (1:3,000 dilution; KangChen Bio-tech, Inc.) in TBS-T with 3% fat-free milk for 1.5 h at room temperature. Membranes were washed 3 times with TBS-T, developed in ECL solution and visualized with X-ray film. Each experiment was repeated at least 3 times. For quantification, the film were scanned and analyzed using ImageJ 1.410 software. The density of specific bands was measured and normalized with the bands of Action. The experiment was repeated 3 times.

Statistical analysis. Data are representative of experiments performed in triplicate and are expressed as the mean ± SE.

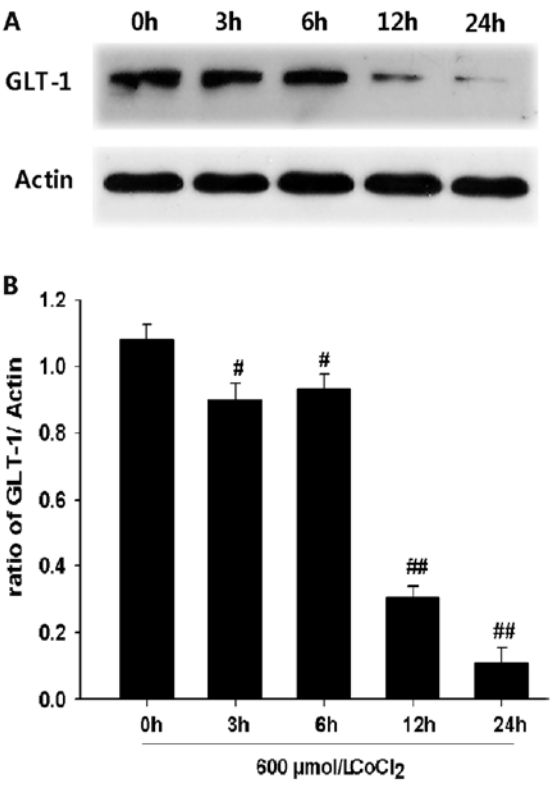

Figure 1. $CoCl_2$ induces downregulation of GLT-1 expression in PC12 cells in a time-dependent manner. (A) Time course for the effects of $CoCl_2$ on the expression of GLT-1 detected by western blot analysis. (B) Densitometric analysis for the results in (A). $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. the control group.

Differences between groups were analyzed by one-way analysis of variance (ANOVA) using SPSS 13.0 software, followed by the LSD post hoc comparison test. $P<0.05$ was considered to indicate statistically significant differences.

Results

$CoCl_2$ reduces the level of GLT-1 expression in PC12 cells. In order to explore the effect of $CoCl_2$ on the GLT-1 expression level in PC12 cells, PC12 cells were exposed to 600 μM $CoCl_2$ for the indicated times (i.e., 3, 6, 12 and 24 h). Western blot analysis revealed that treatment with 600 μM $CoCl_2$ caused downregulation of GLT-1 expression in a time-dependent manner (Fig. 1). These data indicate that chemical hypoxia may reduce GLT-1 protein levels in PC12 cells.

H_2S reverses $CoCl_2$-induced downregulation of GLT-1 expression in PC12 cells. After PC12 cells were exposed to 600 μM $CoCl_2$ for 24 h, the levels of GLT-1 protein expression were markedly decreased (Fig. 2). However, pretreatment of PC12 cells with 400 μM NaHS for 30 min before exposure to $CoCl_2$ reversed this effect, suggesting that NaHS preconditioning may enhance GLT-1 protein expression level in $CoCl_2$-treated PC12 cells.

GLT-1 is involved in the cytoprotection of H_2S against $CoCl_2$-induced injury. To explore whether GLT-1 is involved in the cytoprotection of H_2S against $CoCl_2$-induced injuries, PC12 cells were pretreated with DHK (a inhibitor of GLT-1) at

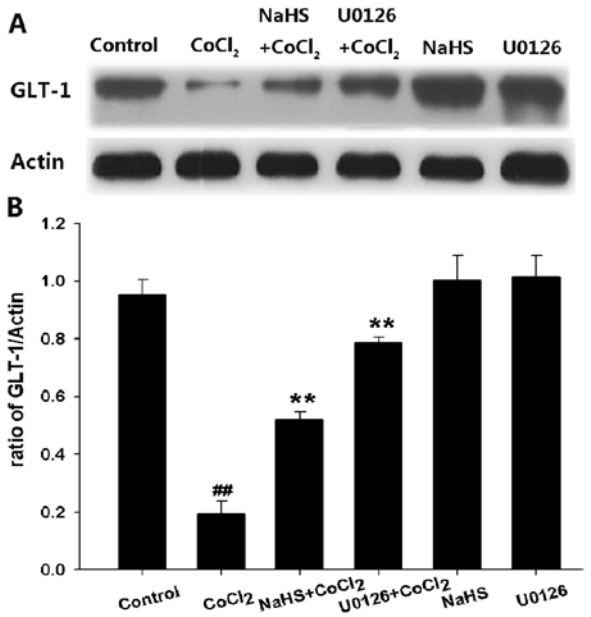

Figure 2. Effects of H_2S and U0126 on the downregulation of GLT-1 expression induced by $CoCl_2$ in PC12 cells. (A) PC12 cells were treated with 600 μM $CoCl_2$ for 24 h in the presence or absence of pretreatment with 400 μM NaHS for 30 min or 10 μM U0126, an inhibitor of MEK1/2, for 120 min. (B) Densitometric analysis for the results in (A). $^{\#\#}$P<0.01 vs. the control group; **P<0.01 vs. the $CoCl_2$ group.

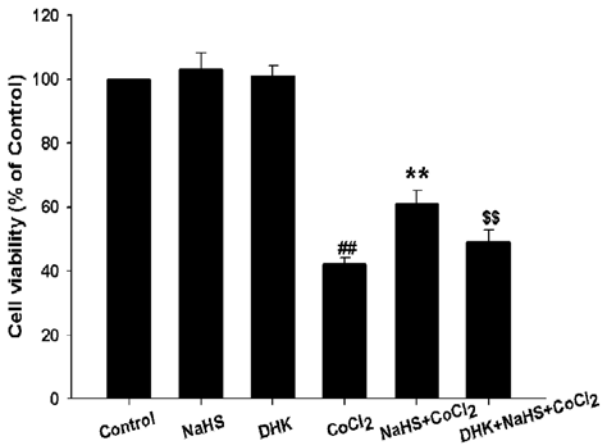

Figure 3. Role of GLT-1 in the cytoprotection of H_2S against the cytotoxicity induced by $CoCl_2$ in PC12 cells. PC12 cells were treated with 600 μM $CoCl_2$ for 24 h in the presence or absence of pretreatment with 400 μM NaHS for 30 min. To inhibit GLT-1, cells were pretreated with DHK for 30 min before pretreatment with 400 μM NaHS followed by exposure to 600 μM $CoCl_2$ for 24 h. The CCK-8 assay was performed to detect cell viability. $^{\#\#}$P<0.01 vs. the control group; **P<0.01 vs. the $CoCl_2$ group; $^{\$\$}$P<0.01 vs. the NaHS + $CoCl_2$ group.

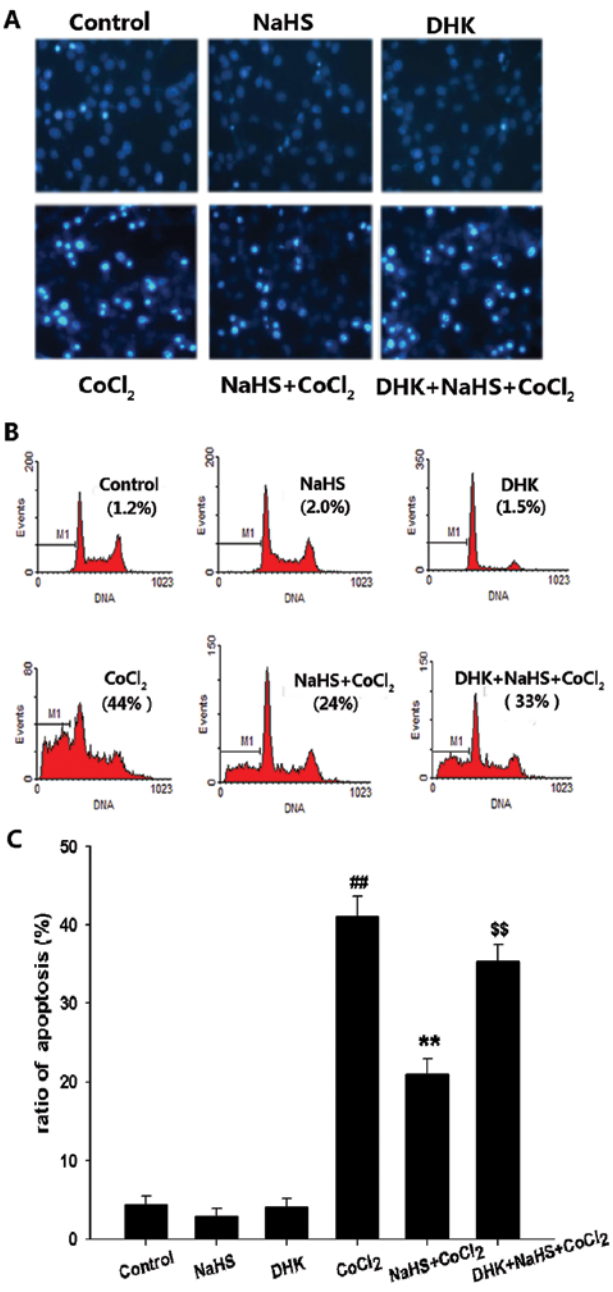

Figure 4. Role of GLT-1 in the cytoprotection of H_2S against apoptosis induced by $CoCl_2$ in PC12 cells. (A) Morphological changes in apoptotic PC12 cells assessed by Hoechst 33258 staining. (B) Apoptosis percentage of PC12 cells was determined by flow cytometry (FCM) as described in Materials and methods. (C) The statistical results of the FCM analysis for the apoptosis of the PC12 cells. Control group, normal PC12 cells; NaHS group, cells were treated with 400 μM NaHS for 30 min alone; DHK group, cells were treated with 400 μM DHK for 30 min alone; $CoCl_2$ group, cells were treated with 600 μM $CoCl_2$ for 48 h; NaHS + $CoCl_2$ group, cells were preconditioned with 400 μM NaHS for 30 min followed by exposure to 600 μM $CoCl_2$ for 48 h. DHK + NaHS + $CoCl_2$ group, cells were pretreated with 400 μM DHK for 30 min prior to NaHS preconditioning followed by the same protocols as in the NaHS + $CoCl_2$ group. $^{\#\#}$P<0.01 vs. the control group; **P<0.01 vs. the $CoCl_2$ group; $^{\$\$}$P<0.01 vs. the NaHS + $CoCl_2$ group.

400 μM for 30 min prior to NaHS preconditioning followed by exposure to 600 μM $CoCl_2$ for 24 h. As shown in Fig. 3, DHK pretreatment significantly blocked the protection of NaHS preconditioning against $CoCl_2$-induced cytotoxicity, the cell viability was considerably decreased, from 62±2.3% to 50±2.1% (P<0.01) (Fig. 3). Moreover, pretreatment with 400 μM DHK also markedly inhibited H_2S-induced anti-apoptotic effects, increasing the number of apoptotic cells with nuclear condensation and fragmentation (Fig. 4A) as well as the apoptotic percentage of PC12 cells compared with the NaHS pretreatment + $CoCl_2$ group (P<0.01) (Fig. 4B). Additionally, pretreatment of PC12 cells with DHK for 30 min before 400 μM NaHS preconditioning clearly inhibited H_2S-induced preservation of MMP (Fig. 5). These findings

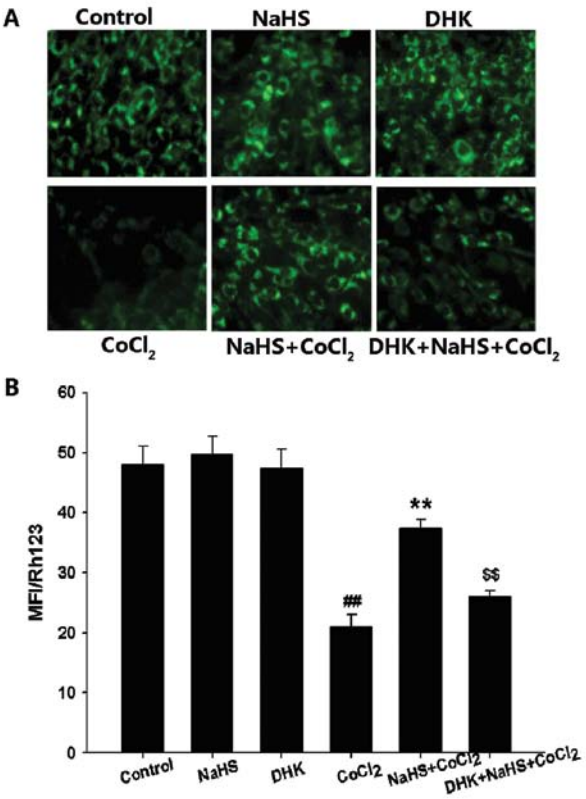

Figure 5. Role of GLT-1 in the cytoprotection of H_2S against mitochondrial damage induced by $CoCl_2$ in PC12 cells. MMP was measured by Rhodamine 123 (Rh123) staining followed by photofluorography. (A) Random micrographs of Rh123-derived fluorescence in PC12 cells. Control group, normal PC12 cells; NaHS group, cells were treated with 400 μM NaHS for 30 min alone; DHK group, cells were treated with 400 μM DHK for 30 min alone; $CoCl_2$ group, cells were treated with 600 μM $CoCl_2$ for 24 h; NaHS + $CoCl_2$, cells were pretreated with 400 μM NaHS for 30 min followed by exposure to 600 μM $CoCl_2$ for 24 h. DHK + NaHS + $CoCl_2$ group, cells were pretreated with 400 μM DHK for 30 min prior to NaHS preconditioning followed by the same protocols as in the NaHS + $CoCl_2$ group. (B) Quantitative analysis of the mean fluorescence intensity of Rh123 in each group, performed using ImageJ 1.410 software. $^{\#\#}$P<0.01 vs. the control group; **P<0.01 vs. the $CoCl_2$ group; $^{\$\$}$P<0.01 vs. the NaHS + $CoCl_2$ group.

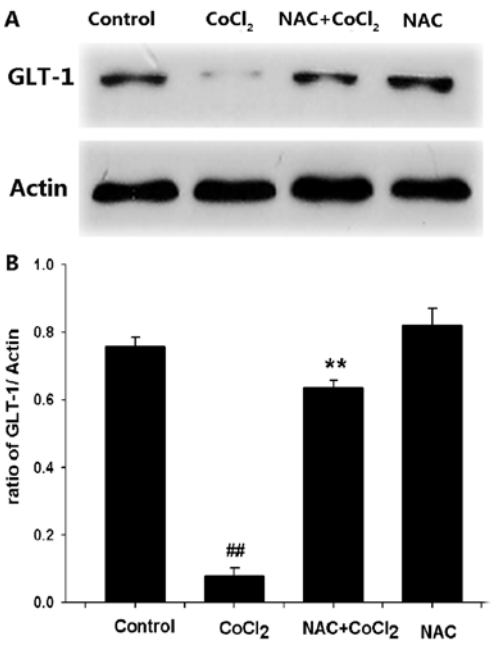

Figure 6. Effect of NAC on the downregulation of GLT-1 expression induced by $CoCl_2$ in PC12 cells. (A) PC12 cells were treated with 600 μM $CoCl_2$ for 24 h in the presence or absence of pretreatment with 500 μM NAC for 60 min. (B) Densitometric analysis for the results in (A). $^{\#\#}$P<0.01 vs. the control group; **P<0.01 vs. the $CoCl_2$ group.

suggest that GLT-1 contributes to the cytoprotection of H_2S against $CoCl_2$-induced injuries.

ROS are involved in the $CoCl_2$-induced downregulation of GLT-1 expression in PC12 cells. Since ROS generation inhibits glutamate uptake function (22), we examined whether ROS is involved in the $CoCl_2$-induced downregulation of GLT-1 protein expression in PC12 cells. Pretreatment of cells with 500 μM NAC (a ROS scavenger) for 60 min prior to exposure to 600 μM $CoCl_2$ for 24 h significantly blocked $CoCl_2$-induced downregulation of GLT-1 expression (Fig. 6). These data indicate that the inhibitory effect of $CoCl_2$ on GLT-1 expression may be associated with oxidative stress.

Activation of ERK1/2 contributes to the downregulation of GLT-1 expression induced by $CoCl_2$ in PC12 cells. Stimulation of ERK1/2MAPK also contributes to the inhibition of glutamate uptake (23). In order to investigate the effect of ERK1/2 activation on the downregulation of GLT-1 expression induced by $CoCl_2$, PC12 cells were pretreated with 10 μM U0126 (a MEK1/2 inhibitor) for 120 min prior to treatment with 600 μM $CoCl_2$ for 24 h. U0126 significantly reversed the inhibitory effect of $CoCl_2$ on the expression of GLT-1 in PC12 cells, suggesting that activation of ERK1/2 contributes to the downregulation of GLT-1 expression induced by $CoCl_2$ in PC12 cells (Fig. 2).

Discussion

GLT-1 has been classified as an astroglial transporter due to its predominant and widespread expression in astrocytes. In the present study, we found that PC12 cells expressed GLT-1, suggesting that GLT-1 may be involved in maintaining a normal level of glutamate in PC12 cells, which is consistent with a previous study (17). It is well known that GLT-1 plays a major role in glutamate re-uptake from the synaptic cleft after neuronal transmission (6,24,25). Lack of GLT-1 has indeed been shown to promote extracellular glutamate accumulation, excitotoxicity and, ultimately, cell death (26,27). GLT-1 has been estimated to represent up to 1% of total brain protein (6). The expression of GLT-1 is reduced in several animal models of neurodegenerative diseases, including traumatic brain injury (28) and hypoxic/ischemic insults (16,29). The levels of the GLT-1 and/or GLAST protein are also lower in the brain tissue from the patients with Alzheimer's disease (AD) (30) and Huntington's disease (31). The results of the present study showed that $CoCl_2$, a well-known hypoxia mimetic agent, attenuates expression of GLT-1 in a time-dependent manner. Our findings are comparable with a study showing that transient global ischemia reduces GLT-1 expression (16). Similarly, it was reported that GLT-1 protein levels are reduced in the brain in various models of central hypoxia/ischemia (16,29,32). Under hypoxic conditions (2.5 and 1% O_2 exposure for 24 h), glutamate uptake and GLT-1 protein levels

are significantly decreased in astrocytes (33). These studies all support our results. By contrast, Kobayashi and Millhorn (17) indicated that exposure of PC12 cells to hypoxia (1% O_2) for 6 to 24 h increases GLT-1 protein levels. Therefore, it is likely that the effects of hypoxia/ischemia on the expression of GLT-1 may be affected by many factors, including tissue or cell types, the level of hypoxia, manner of hypoxia induction and also the period of hypoxia/ischemia.

To clarify the mechanisms underlying the inhibitory effect of chemical hypoxia on GLT-1 expression, we tested the possible involvement of ROS. Several previous studies have shown that oxidative stress is implicated in glutamate clearance impairment and reduction of GLT-1 expression (18,21,34). Our recent studies have demonstrated the promotive effects of $CoCl_2$ on ROS production (19,20). In this study, we found that NAC, a ROS scavenger, can significantly block the inhibition of GLT-1 expression induced by $CoCl_2$, revealing that ROS partly contribute to the inhibitory effect of chemical hypoxia on the expression of GLT-1 in PC12 cells. We provide novel evidence for the role of ROS in $CoCl_2$-induced neuronal injury. Additionally, there is currently a lot of data demonstrating that oxidative stress may trigger and modulate the MAPK signaling pathways (21,35,36). We recently demonstrated that ROS can activate the MAPK pathway (20), linking to the possibility of an altered ERK1/2 activation that ultimately affects the expression of GLT-1. To confirm this possibility, we observed the effects of pretreatment of PC12 cells with UO126 (an inhibitor of MEK1/2) on the inhibition of GLT-1 expression by $CoCl_2$. Our results showed that U0126 clearly suppressed the $CoCl_2$-induced decrease in the expression of GLT-1, suggesting that the ERK1/2 pathway is involved in the inhibitory effect of $CoCl_2$ on GLT-1 expression. This is also a novel finding showing that the ROS-activated ERK1/2 pathway plays a role in the inhibition of GLT-1 expression by $CoCl_2$. Our findings are supported by previous studies (18,35). Lu *et al* (18) reported that PD98059, a specific ERK1/2 inhibitor, significantly reverses the reduction of trafficking of GLT-1 from cytoplasma to plasma membrane.

Although research on the regulatory mechanisms for GLT-1 expression has intensified, scarce data are available regarding the regulatory effect of gasotransmitter on the expression of GLT-1. H_2S, recently recognized as the third gasotransmitter alongside nitric oxide (NO) and carbon monoxide (CO) (39), has attracted extensive attention due to its multiple physiological and pathophysiological roles in various body systems (18-20,37-41). Kimura and Kimura (38) demonstrated the nueroprotective effect of H_2S against oxidative stress-induced injury in primary rat cortical neurons. H_2S also protects astrocytes from H_2O_2-induced neural injury (18). We recently found that H_2S protects PC12 cells against $CoCl_2$-induced damage by enhancing heat shock protein 90 (HSP90) (19), inhibiting the ROS-activated ERK1/2 and p38MAPK signaling pathways (20) and scavenging ROS (19,20). In the present study, we provide evidence for the first time that NaHS (a donor of H_2S) pretreatment prevents the $CoCl_2$-induced downregulation of GLT-1 expression in PC12 cells. Our results are in line with a recent study that H_2S protects astrocytes against oxidative stress-induced neural damage by increasing glutamate uptake (18). Based on our recent results (19,20,39-41) and other studies (18,21,35,36,38,42), there are several possible mechanisms responsible for the regulatory effect of H_2S on the expression of GLT-1: i) its antioxidation, by which H_2S can protect PC12 cells from $CoCl_2$-induced suppression of GLT-1 expression; ii) its inhibitory effect on the ERK1/2 pathway (20); and iii) H_2S functions as an ATP-sensitive potassium (K_{ATP}) channel opener (43). Hu *et al* (42) indicated that iptakalim, a K_{ATP} channel opener, can reverse the inhibition of glutamate uptake induced by N-methyl-4-4-phenylpyridinium (MPP^+) [used to stimulate Parkinson's disease (PD)-like conditions], revealing a role of the K_{ATP} channel opener in the functional regulation of glutamate transporter. Further research is required to confirm these findings.

We further explored the role of GLT-1 in the neuroprotection of H_2S against chemical hypoxia-induced injury. We found that pretreatment with DHK, a selective inhibitor of GLT-1, significantly reversed the protective effect of H_2S against $CoCl_2$-induced injuries, evidenced by a decrease in cell viability and an increase in apoptotic PC12 cells as well as MMP loss, suggesting that upregulation of GLT-1 expression may play an important role in the neuroprotective effects of H_2S.

In summary, in the present study, we have demonstrated for the first time that: i) both ROS and the ERK1/2 pathway contribute to the downregulation of GLT-1 expression induced by $CoCl_2$; ii) H_2S, a novel gaseous neuromodulator, reverses $CoCl_2$-induced downregulation of GLT-1 expression; and iii) upregulation of GLT-1 expression may play a crucial role in the neuroprotective effects of H_2S against chemical hypoxia-induced neuronal injury in PC12 cells. The findings of the present study may provide a potential neuroprotective therapeutic approach for treatment of hypoxia/ischemia-related neuronal injury. In addition, based on the notable findings that both levels of endogenous H_2S and GLT-1 are reduced in neurodegenerative diseases, such as AD and PD, we speculate that endogenous H_2S may be an important modulator of GLT-1. These findings remain to be confirmed in future studies.

Acknowledgements

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