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Thiosulfate Mediates Cytoprotective Effects of Hydrogen Sulfide Against Neuronal Ischemia

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Background—Hydrogen sulfide (H2S) exhibits protective effects in various disease models including cerebral ischemia–reperfusion (I/R) injury. Nonetheless, mechanisms and identity of molecules responsible for neuroprotective effects of H2S remain incompletely defined. In the current study, we observed that thiosulfate, an oxidation product of H2S, mediates protective effects of an H2S donor compound sodium sulfide (Na2S) against neuronal I/R injury.

Methods and Results—We observed that thiosulfate in cell culture medium is not only required but also sufficient to mediate cytoprotective effects of Na2S against oxygen glucose deprivation and reoxygenation of human neuroblastoma cell line (SH-SY5Y) and murine primary cortical neurons. Systemic administration of sodium thiosulfate (STS) improved survival and neurological function of mice subjected to global cerebral I/R injury. Beneficial effects of STS, as well as Na2S, were associated with marked increase of thiosulfate, but not H2S, in plasma and brain tissues. These results suggest that thiosulfate is a circulating “carrier” molecule of beneficial effects of H2S. Protective effects of thiosulfate were associated with inhibition of caspase-3 activity by persulfidation at Cys163 in caspase-3. We discovered that an SLC13 family protein, sodium sulfate cotransporter 2 (SLC13A4, NaS-2), facilitates transport of thiosulfate, but not sulfide, across the cell membrane, regulating intracellular concentrations and thus mediating cytoprotective effects of Na2S and STS.

Conclusions—The protective effects of H2S are mediated by thiosulfate that is transported across cell membrane by NaS-2 and exerts antiapoptotic effects via persulfidation of caspase-3. Given the established safety track record, thiosulfate may be therapeutic against ischemic brain injury. (J Am Heart Assoc. 2015;4:e002125 doi: 10.1161/JAHA.115.002125)

Key Words: apoptosis • cerebral ischemia • hydrogen sulfide • sulfidation • thiosulfate

Hydrogen sulfide (H2S) is produced by several enzymes in diverse tissues. H2S has a prominent role in vasorelaxation, neurotransmission, and inflammation. A number of studies suggest that H2S attenuates ischemia–reperfusion (I/R) injury in a variety of organs including brain, whether it is endogenously produced or exogenously administered as H2S gas or donor compounds.1–6 Along these lines, we reported that administration of Na2S or cardiomyocyte-specific overexpression of cystathionine gamma lyase, an enzyme that produces H2S, prevents neuronal death and improves long-term neurological outcomes and survival after cardiac arrest in mice.4,7 In a recent study, we also found that a novel H2S-releasing compound improves outcomes after global cerebral I/R.5 Nevertheless, mechanisms responsible for the cytoprotective effects of H2S remain incompletely defined. In particular, how biological effects of H2S are transported to the brain is largely unknown.

Levels of free H2S in circulating blood or cell culture medium at steady state are very low (nmol/L) and low μmol/L).8,9 Free H2S levels only transiently increase and quickly return to its baseline after administration of H2S donor compounds systemically or to cell culture medium.10 Therefore, for exogenously administered H2S to have pharmacological effects in remote organs or in cultured cells, H2S has to be converted to a metabolite that is stable in circulation or...
in culture medium, respectively. H$_2$S is serially oxidized to persulfide, sulfite (SO$_3^{2-}$), thiosulfate (S$_2$O$_3^{2-}$), and sulfate (SO$_4^{2-}$) in reactions catalyzed by several mitochondrial enzymes including sulfide quinone oxidoreductase, sulfur dioxygenase, thiosulfate sulfurtransferase (rhodanese), and sulfite oxidase (SO). H$_2$S or its metabolites can also be serially converted to sulfane sulfur-containing compounds. Sulfane sulfur is a sulfur atom with 6 valence electrons but no charge (represented as S$^0$). Biologically important sulfane sulfur-containing compounds include persulfides (R–S–SH), polysulfides (R–S–S–R), thiosulfate, and protein-bound elemental sulfur. H$_2$S and sulfane sulfur coexist, and recent reports suggest that sulfane sulfur-containing species may be the actual signaling molecules of the biological effects of H$_2$S.

In previous studies, we observed that beneficial effects of inhaled H$_2$S during murine endotoxic shock were associated with markedly increased plasma thiosulfate levels. Because thiosulfate is relatively stable in circulation, these observations indicate that thiosulfate may mediate protective effects of H$_2$S. However, thiosulfate is an anion that does not freely permeate across cell membranes. Therefore, for thiosulfate to exert any biological effects, it must be transported by some transport mechanisms into cells. Nonetheless, the role of thiosulfate transporter on the cytoprotective effects of H$_2$S has not been examined.

In the current study, we observed that thiosulfate converted from H$_2$S mediates the protective effects of Na$_2$S against cerebral I/R injury. Administration of sodium thiosulfate (STS) prevented in vitro and in vivo cerebral I/R injury by increasing intracellular concentrations of thiosulfate per se without increasing levels of H$_2$S and other H$_2$S metabolites. The protective effects of STS were associated with inhibition of c-jun N-terminal kinase (JNK) and caspase-3 and activation of extracellular signal-regulated kinase (Erk) 1/2. Furthermore, we revealed that the transport of thiosulfate across cell membrane and the protective effect of Na$_2$S and STS against oxygen glucose deprivation (OGD) and reoxygenation (OGD/R) were mediated by 1 of the SLC13 family proteins, sodium sulfate cotransporter 2 (SLC13A4, NaS-2), which is expressed in human and murine brain.

### Materials and Methods

#### Cell Culture

Human neuroblastoma cell line SH-SY5Y were cultured in Eagle’s medium/Ham’s F-12 50:50 mix (DMEM/F12; Cellgro by Mediatech, Inc) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin. Primary neuronal cultures were prepared from the cortex of embryonic day 15 C57BL/6J mice as previously described.

### Measurement of Sulfide and Thiosulfate by High-Performance Liquid Chromatography

Levels of sulfide and thiosulfate in the cell culture medium, SH-SY5Y cells, plasma, or brain were measured by using monobromobimane-based high-performance liquid chromatography (HPLC) analysis as previously described.

#### Cell Viability Assays

Cell viabilities of SH-SY5Y or primary cortical neurons were measured by using lactate dehydrogenase (LDH) assay or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously.

OGD/R

OGD for SH-SY5Y or for primary cortical neurons was performed by placing cells in a hypoxia chamber (STEMCELL Technologies Inc) for 15 or 2.5 hours followed by 24 or 21 hours of reoxygenation, respectively, as described previously.

### Measurement of Sulfide, Thiols, Thiosulfate, and Persulfides by Liquid Chromatography–Tandem Mass Spectrometry

Intracellular sulfide, homocysteine (Hcys), cysteine (Cys), glutathione (GSH), thiosulfate, CysSSH, or GSSH levels were measured, as described previously. In brief, 24 hours after the addition of STS at 0.25 mmol/L to the medium, SH-SY5Y cells were washed twice with PBS, scraped, collected, sonicated, and incubated in the methanol solution containing 5 mmol/L monobromobimane at 37°C for 30 minutes under dark conditions. After centrifugation, aliquots of the supernatants were diluted 10 to 100 times with distilled water containing known amounts of isotope-labeled internal standards and analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

### Western Blotting

Protein levels in SH-SY5Y were determined by standard immunoblot techniques. Primary antibodies were purchased from Cell Signaling Technology, Inc except the antibody against NaS-2 from Santa Cruz Biotechnology, Inc.

### Measurement of Caspase-3 Activity

Caspase-3 catalytic activity was determined based on measuring chromophore p-nitroaniline (pNA) after cleavage from the labeled substrate DEVD-pNA. Caspase-3 activity in the cell
lysate of SH-SY5Y was measured by using a colorimetric assay kit (EMD Millipore Corp) according to the manufacturer’s instructions. Inhibition of purified recombinant human caspase-3 activity by Na₂S or STS was examined by using the caspase-3 inhibitor screening assay kit (EMD Millipore) according to the manufacturer’s instructions with or without using (D,L)-dithiothreitol (DTT) solution.

**Persulfidation Assay (Modified Biotin Switch Assay)**

The assay was carried out as described previously. Briefly, SH-SY5Y cells were lysed, scraped, collected, sonicated, and centrifuged at 14 000 g for 5 minutes at 4°C to obtain supernatant of cell lysates. Supernatant was incubated with vehicle, Na₂S, or STS at 37°C for 30 minutes or 19 hours followed by the procedure of modified biotin switch assay and subjected to Western blotting analysis.

**Transfection of Plasmid DNA**

DH5α that was transformed with Myc-tagged pCDNA3-Casp3 (wild-type human caspase-3, Addgene) or Myc-tagged pCDNA3-Casp3 C163A (mutant human caspase-3, Addgene) was cultured in the LB medium containing 100 μg/mL ampicillin at 37°C for 2 days. Plasmids were purified by using QIAGEN plasmid kits. Transient transfections into SH-SY5Y cells were performed by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were used 24 hours after transfection.

**Gene Silencing by Transfection of siRNA**

Transfection of siRNA (scrambled sequence, Invitrogen, Stealth RNAi, cat# 12935-300, or NaS-2, Invitrogen, Stealth RNAi, cat# HSS119981) into SH-SY5Y cells were performed by using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer’s protocol. Cells were used 48 hours after transfection. Gene silencing was confirmed by using immunoblotting (Figure S4).

**Global Cerebral Ischemia and Reperfusion**

After approval by the Massachusetts General Hospital Subcommittee on Research Animal Care, all animal experiments were performed in accordance with the guidelines of the National Institutes of Health. Male mice (C57BL/6J, 8 to 9 weeks old) purchased from the Jackson Laboratory were anesthetized and subjected to 40 minutes of bilateral common carotid artery occlusion (BCAO) with microsurgical clips as previously described. To ascertain that BCAO induces global cerebral ischemia, we measured cerebral blood flow of mice at middle cerebral artery region by using a laser Doppler flowmetry (VMS-LDF; Moor Instrument Inc). STS at 10 mg/kg or vehicle was administered intraperitoneally (IP) 1 minute after the initiation of reperfusion. After reperfusion and recovery from anesthesia, mice were given IP 1 mL of 5% dextrose-enriched lactated Ringer’s solution with or without STS at 10 mg/kg daily for 1 week. Neurological function score (NFS) was evaluated as described previously.

**Statistical Analysis**

All data are presented as mean±SE. Data were analyzed by ANOVA by using Sigmastat 3.01a (Systat Software Inc) and the Prism 5 software package (GraphPad Software) unless otherwise described. Newman–Keuls multiple comparison post hoc test or Bonferroni post hoc test was performed for 1- or 2-way ANOVA, respectively, as required. P values <0.05 were considered significant.

**Results**

**Na₂S Protects Neurons From OGD/R**

We examined whether Na₂S improves viability of SH-SY5Y cells subjected to 15 hours of OGD followed by 24 hours of reoxygenation. Based on dose- (0.1, 0.2, or 0.5 mmol/L) and time-ranging (0.5, 3, 5, or 8 hours after the end of OGD) studies, we determined that 0.5 mmol/L and at 5 hours after the end of OGD were the most effective dose and time point to add Na₂S to improve viability of SH-SY5Y cells after OGD/R (Figure 1).

**Figure 1.** Effects of Na₂S on cell viabilities of SH-SY5Y after OGD/R. A, Protocol of OGD/R for SH-SY5Y. 8, Cell viabilities of SH-SY5Y after OGD/R. Na₂S at 0.1, 0.2, or 0.5 mmol/L was added at 0.5, 3, 5, or 8 h after the end of OGD. n=3 each; *P<0.05 vs control. LDH indicates lactate dehydrogenase; OGD/R, oxygen glucose deprivation and reoxygenation.
Thiosulfate Plays a Critical Role in the Protective Effect of Na$_2$S

We measured extracellular and intracellular levels of sulfide (sum of H$_2$S and HS$^-$; an index of unbound H$_2$S) and thiosulfate levels after the addition of Na$_2$S or STS via an HPLC method (Figure 2A through D). The result showed that extracellular and intracellular sulfide levels increased rapidly and returned to the baseline by 3 hours after the addition of Na$_2$S at 0.5 mmol/L to the cell culture medium. Extracellular thiosulfate levels increased to $\approx 0.25$ mmol/L, corresponding with the timing when extracellular and intracellular sulfide levels returned to their respective baseline levels, suggesting a stoichiometric conversion of sulfide to thiosulfate. The addition of STS at 0.25 mmol/L to the medium increased extracellular and intracellular thiosulfate levels to the similar levels with what was achieved after the addition of 0.5 mmol/L Na$_2$S. STS did not increase sulfide levels. We also observed that STS did not increase sulfide levels in medium and cells by using sulfide-specific fluorescent probes HSip-1 and HSip-1 DA, respectively (Figure S1). Taken together, these results suggest that most, if not all, sulfide is converted to thiosulfate in culture medium and cells. Based on these observations, we examined whether the addition of thiosulfate itself has neuroprotective effects against OGD/R (Figure 3A). STS added 5 hours after the end of OGD improved cell viabilities of SH-SY5Y in a dose-dependent manner. Further, STS added 30 minutes before starting OGD or 30 minutes after the end of OGD improved cell viabilities of murine primary cortical neurons (Figure 3B). To determine the role of extracellular thiosulfate converted from H$_2$S on the viability of SH-SY5Y cells after OGD, we changed culture medium with fresh medium to remove thiosulfate in the medium 3 hours after the addition of Na$_2$S at 0.5 mmol/L (Figure 3C and 3D). The time course for the medium-change experiment is shown in Figure S2. The protective effect of Na$_2$S was abolished by the medium change. On the other hand, replacing thiosulfate that was removed by the medium change with STS (0.25 mmol/L) restored neuroprotective effects. These results suggest that thiosulfate mediates the cytoprotective effects of Na$_2$S after I/R injury.

STS Increases Intracellular Levels of Thiosulfate but Not Sulfide and Persulfide

We measured levels of sulfide, Hcys, Cys, CysSSH, GSSH, thiosulfate, and GSH in the SH-SY5Y cells 24 hours after the addition of Na$_2$S or STS via an HPLC method (Figure 2A through D). The result showed that extracellular and intracellular sulfide levels increased rapidly and returned to the baseline by 3 hours after the addition of Na$_2$S at 0.5 mmol/L to the cell culture medium. Extracellular thiosulfate levels increased to $\approx 0.25$ mmol/L, corresponding with the timing when extracellular and intracellular sulfide levels returned to their respective baseline levels, suggesting a stoichiometric conversion of sulfide to thiosulfate. The addition of STS at 0.25 mmol/L to the medium increased extracellular and intracellular thiosulfate levels to the similar levels with what was achieved after the addition of 0.5 mmol/L Na$_2$S. STS did not increase sulfide levels. We also observed that STS did not increase sulfide levels in medium and cells by using sulfide-specific fluorescent probes HSip-1 and HSip-1 DA, respectively (Figure S1). Taken together, these results suggest that most, if not all, sulfide is converted to thiosulfate in culture medium and cells. Based on these observations, we examined whether the addition of thiosulfate itself has neuroprotective effects against OGD/R (Figure 3A). STS added 5 hours after the end of OGD improved cell viabilities of SH-SY5Y in a dose-dependent manner. Further, STS added 30 minutes before starting OGD or 30 minutes after the end of OGD improved cell viabilities of murine primary cortical neurons (Figure 3B). To determine the role of extracellular thiosulfate converted from H$_2$S on the viability of SH-SY5Y cells after OGD, we changed culture medium with fresh medium to remove thiosulfate in the medium 3 hours after the addition of Na$_2$S at 0.5 mmol/L (Figure 3C and 3D). The time course for the medium-change experiment is shown in Figure S2. The protective effect of Na$_2$S was abolished by the medium change. On the other hand, replacing thiosulfate that was removed by the medium change with STS (0.25 mmol/L) restored neuroprotective effects. These results suggest that thiosulfate mediates the cytoprotective effects of Na$_2$S after I/R injury.
addition of STS at 0.25 mmol/L to the medium by using the LC-MS/MS method. The result showed that the addition of STS to the medium did not alter the intracellular levels of sulfide, Hcys, Cys, and major antioxidative persulfides (CysSSH and GSSH), indicating that the protective effect of STS was not mediated via production of sulfide or antioxidative persulfides (Figure 4A through 4E). The addition of STS markedly increased intracellular levels of thiosulfate and modestly increased GSH in SH-SY5Y cells (Figure 4F and 4G).

**Na2S or STS Inhibits Activation of the Mitochondrial Pathway of Apoptosis**

To elucidate molecular mechanisms responsible for the improvement of cell viability by Na2S or STS, we examined protein levels of phosphorylated JNK, phosphorylated Erk1/2, phosphorylated Bad, Bcl-2, and cleaved caspase-3 in SH-SY5Y after OGD/R. Na2S at 0.5 mmol/L or STS at 0.25 mmol/L added 5 hours after the end of OGD attenuated phosphorylation of JNK and dephosphorylation of Erk1/2 and Bad (Figure 5A through 5C). The addition of Na2S or STS prevented the reduction of Bcl-2 and the increase in caspase-3 cleavage (Figure 5D and 5E). We also confirmed that Na2S or STS added 5 hours after the end of OGD inhibited caspase-3 activity in SH-SY5Y cells (Figure 5F). The medium change 3 hours after the addition of Na2S abolished the effects of Na2S on the changes of phosphorylation of JNK and Erk1/2. The addition of STS (0.25 mmol/L) after the medium change restored effects of Na2S on the phosphorylation of JNK and Erk1/2 (Figure 5G and 5H). These observations suggest that thiosulfate mediates the inhibitory effects of Na2S on the mitochondrial pathway of apoptosis.23–25

**Na2S or STS Inhibits Human Recombinant Caspase-3 Activity in Cell-Free System**

To examine whether Na2S or STS inhibits activated caspase-3 after cleavage, we measured proteolytic activity of human recombinant caspase-3, which was cleaved and preactivated in

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**Figure 3.** Effects of thiosulfate converted from H2S on cell viabilities after OGD/R. Cell viabilities of (A) SH-SY5Y or (B) primary cortical neurons of mice after OGD/R. STS was added 5 h after the end of OGD (A) or 5 min before starting OGD or 30 min after the end of OGD (B), n=3 each; Horizontal bar indicates *P<0.05 for control vs all other groups subjected to OGD/R. ** or ***P<0.05 or 0.01 vs vehicle. Cell viabilities of SH-SY5Y 24 h after OGD/R measured by (C) LDH or (D) MTT method. Na2S at 0.5 mmol/L was added 5 h after the end of OGD. Medium was replaced with fresh 1 MC with or without STS at 0.25 mmol/L 3 h after the addition of Na2S. n=5 or 6 each; **P<0.05 vs control (left most column), *P<0.05 vs OGD/R alone and #P<0.05. LDH indicates lactate dehydrogenase; MC, medium change; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OGD/R, oxygen glucose deprivation and reoxygenation; STS, sodium thiosulfate.

**Figure 4.** Intracellular levels of (A) sulfide, (B) Hcys, (C) Cys, (D) CysSSH, (E) GSSH, (F) thiosulfate, and (G) GSH in SH-SY5Y cells 24 h after the addition of vehicle (medium) or STS at 0.25 mmol/L to the medium measured by LC-MS/MS. Data were analyzed by using the Mann-Whitney test. n=3 each. CysSSH indicates cysteine persulfide; GSH, glutathione; GSSH, glutathione persulfide 2; LC-MS/MS, liquid chromatography–tandem mass spectrometry; n.s., not significant; STS, sodium thiosulfate.
a cell-free system. Na$_2$S or STS dose-dependently inhibited activity of recombinant caspase-3. Of note, the inhibitory effects of Na$_2$S and STS were abolished by DTT, suggesting that the inhibition of caspase-3 was mediated by the reversible modification of protein thiols including persulfidation (Figure 6A).

**Na$_2$S or STS Persulfidate Caspase-3 at Cysteine 163**

We found that incubating SH-SY5Y cell lysates with Na$_2$S at 0.5 mmol/L or STS at 0.25 mmol/L for 30 minutes persulfidated caspase-3. Levels of caspase-3 persulfidation were greater after incubation with Na$_2$S than with STS at 30 minutes after addition. However, levels of persulfidated caspase-3 were similar 19 hours after the addition of Na$_2$S or STS (Figure 6B).

To examine whether Na$_2$S or STS causes persulfidation at cysteine 163 (Cys163), which is the catalytically active site of caspase-3, we made SH-SY5Y cells transfected with wild-type human caspase-3 (WT caspase-3) or mutant human caspase-3 in which Cys163 was substituted with alanine (C163A). We examined whether persulfidation levels of mutant caspase-3$^{C163A}$ were lower than those of WT caspase-3 after the addition of Na$_2$S or STS to cell lysates. The successful transfection was confirmed by immunoblotting by using primary antibody against Myc-tag. As expected, cells transfected with mutant caspase-3$^{C163A}$ exhibited lower caspase-3 activity (Figure S3A and S3B). Protein levels of Myc-caspase-3 in SH-SY5Y transfected with WT caspase-3 were comparable with those in SH-SY5Y transfected with mutant caspase-3$^{C163A}$ (Figure S3C). The result showed that mutation at Cys163 markedly decreased persulfidation levels of caspase-3 at 30 minutes after the addition of Na$_2$S or STS to cell lysates (Figure 6C). This result indicates that Na$_2$S or STS causes persulfidation at Cys163 in caspase-3.

**Protective Effect of Thiosulfate Is Mediated by Sodium Sulfate Cotransporter 2 (NaS-2, SLC13A4)**

To elucidate how thiosulfate is transported across the cell membrane and accumulates in the cells, we compared...
thiosulfate levels in the SH-SY5Y cells transfected with siRNA of NaS-2 or scrambled sequence. The result showed that NaS-2 silencing markedly attenuated accumulation of thiosulfate, but not sulfide, 3 hours after the addition of Na2S or STS to the medium (Figure 7A and 7B). Moreover, NaS-2 silencing inhibited the improvement of cell viabilities of SH-SY5Y cells after OGD/R by the addition of Na2S or STS, indicating that the protective effects of Na2S or STS were directly mediated by thiosulfate, but not sulfide, transported into cells via NaS-2 (Figure 7C).

Thiosulfate Plays a Critical Role in the Protective Effect of H2S Against Global Cerebral I/R Injury

To determine the neuroprotective effects of NaS-2 and STS in vivo, we measured levels of sulfide and thiosulfate in mice plasma and brain tissues 90 minutes after IP administration of NaS-2 at 5 mmol/kg or STS at 10 mg/kg (40 μmol/kg). The administration of NaS-2 at 25 μmol/kg was reported to protect mice from brain I/R injury.27 The results showed that thiosulfate, but not sulfide, levels were increased 90 minutes after the administration of Na2S in plasma and brain (Figure 8A). Similarly, thiosulfate, but not sulfide, levels were increased 90 minutes after the administration of Na2S or STS. At these respective doses of Na2S and STS, plasma and brain thiosulfate levels were similar 90 minutes after the administration of Na2S or STS. Next, we examined protective effects of STS on outcomes after global cerebral I/R induced by BCAO (Figure 8B). Cerebral blood flow was decreased to <4% of the baseline within 5 minutes of BCAO (Figure S5). The single or daily administration of STS improved the 20-day survival rate of mice subjected to BCAO. The single administration of STS also improved NFS 24 hours after reperfusion and the 20-day survival rate of mice. These results suggest that STS exerts neuroprotective effects against cerebral I/R.

Discussion

The current study revealed that thiosulfate oxidized from H2S is essential and sufficient for the cytoprotective effects of NaS-2 in cultured neurons subjected to OGD/R. Incubation with NaS-2 stoichiometrically increased the levels of thiosulfate, but not H2S, in the cells, suggesting NaS-2 were almost completely converted to thiosulfate (Figure 2C). Administration of STS markedly increased cellular levels of thiosulfate,
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The protective effect of Na₂S or STS were associated with increased persulfide, Hcy, Cys, CysSSH, and GSSH in SH-SY5Y cells. Because GSH is an important intracellular reducing agent, it is possible that part of the beneficial effects of STS after OGD/R were mediated by increasing intracellular GSH. However, the percent increase of intracellular thiosulfate concentration was much greater than that of GSH after the addition of STS. Therefore, it is likely that thiosulfate directly exerted cytoprotective effects. The administration of Na₂S or STS markedly increased concentrations of thiosulfate, but not sulfide, in plasma and brain in mice. Taken together, our current observations suggest that thiosulfate is the “carrier” molecule responsible for the cytoprotective effects of H₂S.

It has been suggested that the phosphorylation of JNK and the dephosphorylation of Erk1/2 mediate cerebral I/R injury by promoting the dephosphorylation of Bad and the down-regulation of antiapoptotic Bcl-2, which lead to activation of the mitochondrial pathway of apoptosis. In the present study, we observed that the cytoprotective effect of Na₂S or STS in SH-SY5Y cells subjected to OGD/R was associated with the prevention of the phosphorylation of JNK and the dephosphorylation of Erk1/2. It has been reported that H₂S attenuates DNA damage in human endothelial cells and fibroblasts via persulfidation of mitogen-activated protein kinase-Erk kinase 1 at Cys341, which leads to Erk1/2 phosphorylation. Therefore, it is conceivable that Na₂S or STS prevents dephosphorylation of Erk1/2 via persulfidation of mitogen-activated protein kinase-Erk kinase 1.

It has been suggested that H₂S exerts its effects via protein persulfidation. Analogous to nitric oxide (NO)–dependent protein S–nitrosylation, persulfidation is another form of oxidative cysteine modification. It has been reported that nitrite, an oxidative metabolite of NO, causes S-nitrosylation of caspase-3 at Cys163 and inhibits caspase-3 activity in primary human umbilical vein endothelial cells. Similarly, in the current study, we observed that Na₂S and STS dose-dependently inhibited activity of cleaved caspase-3 in vitro. Interestingly, caspase-3 activity of SH-SY5Y cells subjected to OGD/R was completely inhibited by Na₂S although caspase-3 cleavage was incompletely prevented by Na₂S, suggesting that Na₂S directly inhibited caspase-3 activity after cleavage (Figure 5E and 5F). The inhibition of cleaved caspase-3 by Na₂S and STS were associated with persulfidation of caspase-3 at Cys163 (Figure 6A and 6B).

The protective effect of Na₂S or STS was facilitated by NaS–2–mediated transportation of thiosulfate, but not sulfide. Despite these results, we observed that thiosulfate inhibits the mitochondrial apoptosis cascade and caspase-3 activity. The cytoprotective effects of thiosulfate were responsible for the cytoprotective effects of H₂S.

Numerous studies reported cytoprotective effects of H₂S in vitro and in vivo. In the majority of studies, H₂S was administered by using simple sulfide salts, Na₂S or sodium hydrosulfide (NaHS). Once dissolved in water, both sulfide salts produce “hydrogen sulfide,” which exists as the equilibrium of H₂S and HS– (in 2:8 ratio) at physiological pH. However, because H₂S (and HS–) has a very short half-life in biological fluids including cell culture medium and blood, how H₂S reaches its presumed targets in the cells, and in the target tissues in the body when given in vivo, has been poorly understood. Our studies showed that H₂S was converted to thiosulfate in vitro and in vivo. While removal of thiosulfate from the medium abolished the protective effects of Na₂S, replacement of thiosulfate restored the protection. These results suggest that thiosulfate is not only required but also sufficient for the cytoprotective effects of Na₂S.

As shown in Figure 6, the inhibition of cleaved caspase-3 by Na₂S or STS in SH-SY5Y cells subjected to OGD/R was associated with the prevention of the phosphorylation of JNK and the dephosphorylation of Erk1/2/3. It has been reported that H₂S attenuates DNA damage in human endothelial cells and fibroblasts via persulfidation of mitogen-activated protein kinase-Erk kinase 1 at Cys341, which leads to Erk1/2 phosphorylation. Therefore, it is conceivable that Na₂S or STS prevents dephosphorylation of Erk1/2 via persulfidation of mitogen-activated protein kinase-Erk kinase 1.

It has been suggested that H₂S exerts its effects via protein persulfidation. Analogous to nitric oxide (NO)–dependent protein S–nitrosylation, persulfidation is another form of oxidative cysteine modification. It has been reported that nitrite, an oxidative metabolite of NO, causes S-nitrosylation of caspase-3 at Cys163 and inhibits caspase-3 activity in primary human umbilical vein endothelial cells. Similarly, in the current study, we observed that Na₂S and STS dose-dependently inhibited activity of cleaved caspase-3 in vitro. Interestingly, caspase-3 activity of SH-SY5Y cells subjected to OGD/R was completely inhibited by Na₂S although caspase-3 cleavage was incompletely prevented by Na₂S, suggesting that Na₂S directly inhibited caspase-3 activity after cleavage (Figure 5E and 5F). The inhibition of cleaved caspase-3 by Na₂S and STS were associated with persulfidation of caspase-3 at Cys163 (Figure 6A and 6B). Replacement of Cys163 of caspase-3 with alanine attenuated, but did not completely...
prevent, the persulfidation of caspase-3 induced by Na$_2$S at 0.5 mmol/L, indicating that Na$_2$S at 0.5 mmol/L might cause persulfidation at Cys163 and other cysteine residues (Figure 6C). We also observed that STS at 0.25 mmol/L caused persulfidation of WT caspase-3 but not mutant caspase-$3^{	ext{C163A}}$ (Figure 6C), suggesting that STS at 0.25 mmol/L persulfidated only Cys163 and no other cysteine residues of caspase-3. It has been suggested that sulfurtransferases such as thiosulfate sulfurtransferase mediate thiosulfate-induced protein persulfidation. These results suggest that Na$_2$S or STS inhibits caspase-3 activity via thiosulfate-mediated persulfidation of caspase-3 at Cys163.

It has been reported that STS increases thiosulfate levels in the brain, choroid plexus, and cerebrospinal fluid. We observed that the administration of Na$_2$S or STS increased the intracellular levels of thiosulfate in cultured neurons as well as in brain tissues. However, the mechanism responsible for the ability of Na$_2$S or STS to increase intracellular thiosulfate levels in the brain was unclear. Thiosulfate anion cannot easily pass across the cell membrane unless ion transporters or ion channels for thiosulfate exist on the cell membrane. Sodium/sulfate cotransporter 2 is a member of the SLC13 transporter family that mediates the intake of a wide variety of molecules including thiosulfate with the concomitant uptake of sodium ions. We observed that knockdown of NaS-2 markedly inhibited the accumulation of thiosulfate, but not sulfide, in SH-SY5Y cells incubated with Na$_2$S or STS and attenuated the cytoprotective effects of Na$_2$S and STS after OGD/R. These observations unambiguously indicate that thiosulfate mediates the cytoprotective effects of H$_2$S and thiosulfate. Furthermore, these results suggest that NaS-2 plays an important role in neuroprotective effects of H$_2$S or thiosulfate against cerebral I/R injury.

Although we observed neuroprotective effects of STS in a BCAO-induced global cerebral ischemia model of mice, effects of STS were studied only in young healthy male mice. Because a majority of patients who experience ischemic brain injury have multiple comorbidities, including vascular insufficiency, our observations may not readily translatable to the clinical situation. Further, we did not examine the impact of STS on histological brain injury in the current study, although the severity of brain injury correlates with the deterioration of neurological function score in this model. Neuroprotective effects of STS remains to be determined in more clinically relevant model of ischemic brain injury in future studies.

In summary, the current study uncovered, for the first time, that thiosulfate is necessary and sufficient to exert cytoprotective effects of H$_2$S in vitro and in vivo in the setting of I/R. We also identified a transport mechanism responsible for the uptake of thiosulfate into neurons, where it exerts antiapoptotic effects via persulfidation-mediated inhibition of caspase-3. The observed neuroprotective effects of thiosulfate against ischemic brain injury may be highly clinically relevant because STS has been clinically used for the detoxification of cyanide and other disorders in patients. In addition, toxicity of STS is very low compared with Na$_2$S or other sulfide donor compounds. Therapeutic potential of STS against ischemic neuronal injury warrants further studies.

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**Disclosures**

None.

**References**


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