ANTIOXIDANTS & REDOX SIGNALING
Volume 12, Number 1, 2010

DOI: 10.1089/ars.2008.2282

ORIGINAL RESEARCH COMMUNICATION

Hydrogen Sulfide Increases Glutathione Production and Suppresses Oxidative Stress in Mitochondria

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Abstract

Hydrogen sulfide (H_2S) is a synaptic modulator as well as a neuroprotectant in the brain. We recently showed that H_2S protects neurons from oxidative stress by increasing the levels of glutathione (GSH), a major cellular antioxidant, by more than twice that of a control through enhancing the cystine transport. Here we show that H_2S enhances the transport of cysteine to increase GSH production more than cystine transport and to redistribute the localization of GSH to mitochondria. The efficiency of GSH production enhanced by H_2S is even greater by fourfold under oxidative stress by glutamate. H_2S reinstated GSH levels in the fetal brain decreased by ischemia/reperfusion *in utero*. In addition, Neuro2a cells expressing a mitochondrial H_2S-producing enzyme, 3-mercaptopyruvate sulfurtransferase (3MST), along with cysteine aminotransferase (CAT), showed significant resistance to oxidative stress. The present study shows that H_2S protects cells from oxidative stress by two mechanisms. It enhances the production of GSH by enhancing cystine/cysteine transporters and redistributes GSH to mitochondria. H_2S produced in mitochondria also may directly suppress oxidative stress. It provides a new mechanism of neuroprotection from oxidative stress by H_2S. *Antioxid. Redox Signal.* 12, 1–13.

Introduction

Hydrogen sulfide (H_2S) is a synaptic modulator in the brain as well as a smooth-muscle relaxant (1, 23, 42, 59). Cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3MST) produce H_2S from cysteine (1, 23, 26, 48). H_2S selectively enhances the NMDA receptor–mediated responses and facilitates the induction of hippocampal long-term potentiation (LTP), a synaptic model of memory and learning (1). H_2S also modulates the synaptic responses of dorsal raphe serotonergic neurons (30) and regulates the release of corticotropin-releasing hormone from the hypothalamus (13). In astrocytes, H_2S increases the intracellular concentrations of Ca^{2+} that propagate into neighboring astrocytes as Ca^{2+} waves (42). These observations show that H_2S can act as a signal molecule in the brain.

H_2S dissociates to H^+ and HS^- in solution. In physiologic saline at 37°C and pH 7.4, less than one fifth of H_2S exists as the undissociated form (H_2S), and the remaining four fifths exist as HS^- plus a trace of S^{2-} at equilibrium with H_2S (14, 55). Most of H_2S is dissolved as HS^- and S^{2-} in alkaline conditions, whereas H_2S evaporated in acidic conditions. Because the pK_1 is 6.76 at 37°C, ~85% of H_2S exists as H_2S gas, and the remaining 15%, as HS^- at pH 6.0. Although it has not been possible to determine which form of H_2S (H_2S, HS^-, or S^{2-}, the mix of free inorganic sulfides) is active, the term "hydrogen sulfide" has been used. The term "hydrogen sulfide for total free sulfides" also is used here.

Two forms of glutamate toxicity exist; receptor-initiated excitotoxicity (9) and non–receptor-mediated oxidative glutamate toxicity (40). Oxidative glutamate toxicity has been observed in primary cultures of neuronal cells (41), neuronal cell lines (12, 38), and brain slices (54). Primary cultures of immature neurons, which do not express ionotropic receptors, have been used to study the effect of H_2S on oxidative glutamate toxicity (27, 28, 40) differentiated from excitotoxicity (9). We recently found that H_2S protects neurons from oxidative glutamate toxicity by increasing the levels of intracellular glutathione (GSH), a major endogenous antioxidant (27, 28). H_2S also protects cells from cytotoxicity caused by peroxynitrite, β-amyloid, and hypochlorous acid (31, 51, 57, 58). In oxidative glutamate toxicity, when extracellular concentrations of glutamate are increased, the import of cystine in exchange for glutamate by the cystine/glutamate antiporter is decreased. Because cystine is reduced to cysteine in cells for the synthesis of GSH, a decrease in the cystine import results in the decreased synthesis of GSH. H_2S reinstates the cystine import suppressed by glutamate (28).

It has been thought that cysteine normally exists as its oxidized form, cystine, in extracellular space (47, 53). However,

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significant amounts of cysteine (~20 μM) were measured in plasma and blood (46). Because H_2S readily passes through plasma membrane, it is possible that H_2S produced in the cells may be released into extracellular space and reduces cystine into cysteine. Because transporters of cysteine are widely distributed in various types of cells (5), once extracellular cystine is reduced into cysteine in the presence of H_2S, cysteine can readily be imported into cells and used for GSH production.

In addition to neurons, H_2S protects cardiac muscle from myocardial ischemia/reperfusion injury by preserving mitochondrial function (16, 50). Mitochondria play a key role in cell-death pathways. Although various types of proapoptotic signals start the cell-death cascade, they may all converge on the mitochondria (39). Perturbation of mitochondrial function causes loss of the mitochondrial transmembrane potential and the release of apoptogenic factors such as cytochrome *c* into the cytosol, ultimately resulting in cell death. For example, mitochondrial dysfunction caused by oxidative stress leads to numerous neurodegenerative and cardiovascular diseases (34, 39).

The present study shows that H_2S reduces cystine into cysteine in the extracellular space and makes cells efficiently transport cysteine into cells for GSH production. Cysteine transport enhanced by H_2S contributes to GSH production to a greater extent than does cystine transport. The production of GSH enhanced by H_2S is prominent under conditions of oxidative stress caused by glutamate. H_2S increases the production of GSH and its redistribution to mitochondria, and H_2S produced in mitochondria may contribute to suppressing oxidative stress.

Materials and Methods

Cell culture and toxicity assay

All animal procedures were approved by the National Institute of Neuroscience Animal Care and Use Committee. Primary cortical neurons were prepared from embryonic day 17 Sprague–Dawley rats as described (28). In brief, cells were dissociated from the cortex and maintained in modified Eagle's medium (MEM) supplemented with 30 mM glucose, 2 mM glutamine, 1 mM pyruvate, and 10% fetal calf serum (FBS). For toxicity studies, cells were seeded on poly-D-lysine–coated 96-well microtiter dishes at 50,000 cells/100 μl in each well and exposed to glutamate in the presence or absence of NaHS (10–150 μM) (Aldrich, Milwaukee, WI), β-mercaptoethanol (1–30 μM) (β-me;Wako, Osaka), *d,l*-dithiothreitol (10–100 μM) (DTT; Sigma, Saint Louis, MO), L-cysteine (7–70 μM) or L-cystine (500 μM) (Sigma) 24 h after the initial plating. The WST-8 {a tetrazolium salt, [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt]} viability assay was performed with the cell-counting kit-8 (Dojindo, Kumamoto, Japan) 24 h after the application of glutamate. Ten microliters of 10 mM WST-8 was added to each well, cells were incubated at 37°C, and absorption values at 450 nm were measured. Values of samples were normalized by subtracting values of direct reduction of WST-8 by NaHS, β-me, or DTT.

Mouse brain neuroblastoma, Neuro2a cells (44), and mouse hippocampal HT22 cells (27, 33) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 μ/ml) at 37°C in 10% CO_2.

Transfection of cells

Neuro2a cells were transiently transfected with expression plasmids by using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. In brief, cells (10^5 cells/well in 24-well plates) were transfected with 0.8 μg of expression plasmids. Twenty-four hours after transfection, cells were trypsinized and seeded at 5,000 cells/well in 96-well titer plates. The following day, cells were exposed to 10 mM glutamate. Twenty-four hours after the exposure, the viability of cells was assessed with WST assay.

Neuro2a cells expressing CBS, 3MST, CAT (cysteine aminotransferase), and their combination were generated by transfecting cells with expression plasmids, described previously (48).

Measurement of the amount of cysteine in the culture medium

The amount of cysteine in culture medium was measured by the method described previously (27). In brief, primary cultures of cortical neurons (3×10^6 cells/4 ml) plated in a 6-cm poly-D-lysine–coated dish, were treated with NaHS (100 μM) (Aldrich), β-me (10–100 μM), DTT (100 μM), or cysteine (100 μM). Two, 10, 20, 60, and 120 min after the addition of NaHS, β-me, DTT, or cysteine, 75 μl of culture medium was collected in a 1.5-ml Eppendorf tube containing 25 μl of 0.5 M CHES [2-(cyclohexylamino)-ethanesulfonic acid, pH 8.4]. The mixture was derivatized with 4 μl of 50 mM monobromobimane (mBBr) for 15 min in the dark. The reaction was terminated by adding 10 μl of 30% (vol/vol) acetic acid, and denatured protein was removed by centrifugation (15,000 g for 10 min). Samples were separated with a Waters Symmetry C18 (250×4.6-mm ID) column. The mBBr adduct was monitored with a scanning fluorescence detector (Waters 474) with an excitation wavelength at 370 nm and an emission wavelength at 485 nm.

Measurement of the levels of glutathione, γ-glutamyl-cysteine(γ-gc), and cysteine

The amount of GSH, γ-GC, and cysteine was measured with the method described previously (27). In brief, primary cultures of cortical neurons (3×10^6 cells/4 ml) plated in a 6-cm poly-D-lysine–coated dish, were treated with 1 mM glutamate or 5, 10, 30, and 50 μM BSO [L-buthionine-sulfoximine (Sigma) in the presence or absence of NaHS (10–150 μM), β-mercaptoethanol (1–30 μM), and *d,l*-dithiothreitol (100 μM), L-cysteine (7–70 μM) or L-cystine (500 μM)] for 4 h. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and harvested in phosphate buffer (0.1 M NaH_2PO_4, pH 5.8; 2 mM EDTA; with 0.05 mg/ml acivicin for the measurement of γ-GC; or without acivicin for cysteine and GSH). After sonication, cell lysates were centrifuged at 16,000 g for 10 min, and supernatants (75 μl) were derivatized with mBBr and analyzed with HPLC, as described earlier.

Measurement of activities of γ–glutamyl cysteine synthetase (γ-GCS) and glutathione synthase (GS)

Activities of γ-GCS and GS were measured as described previously (56). In brief, primary cultures of cells (10^7 cells/10 cm) were treated with NaHS (30 and 100 μM) or β-me (3 and 10 μM) for 4 h. Cells were gently scraped with ice-cold

PBS (pH 7.4) containing 20 m*M* boric acid and 1 m*M* serine (PBS-SB). Cell suspensions were sonicated and centrifuged at 15,000 *g* for 10 min at 4°C. Pellets were resuspended in PBS-SB. To measure enzymatic activity, cell suspensions (20 μl) were transferred to Eppendorf tubes containing 80 μl γ-GCS reaction mix (20 m*M* ATP, 100 m*M* L-glutamic acid, 1 m*M* EDTA, 20 m*M* $MgCl_2$, 200 m*M* Tris, pH 7.4) and preincubated at 37°C. The reaction with γ-GCS was initiated by adding 50 μl of 5 m*M* cysteine. Reactions were stopped by adding 50 μl ice-cold 200 m*M* 5-sulfosalicyclic acid (SSA) and vortexing. Samples were centrifuged at 15,000 *g* for 5 min. Supernatants were transferred to a new tube and mixed with 200 μl of 0.2*M* *N*-ethylmorpholine in 0.02 *M* KOH, and derivatized with 50 m*M* monobromobimane (5 μl) in the dark for 30 min, then mixed with 40 μl of 1 *M* SSA, vortexed, and centrifuged at 15,000 *g* for 10 min. Supernatants were applied to HPLC as described earlier.

To study the direct effect of NaHS and β-me on γ-GCS, NaHS or β-I plus 5 m*M* cysteine was added to cell suspensions. The reaction was stopped with SSA and analyzed for γ-gc with HPLC, as described earlier. GS activity also was measured. In brief, cell suspensions were preincubated in GS reaction mix (10 m*M* ATP, 100 m*M* L-glycine, 1 mM EDTA, 20 m*M* $MgCl_2$, 200 m*M* Tris, at pH7.4). The reaction was initiated by adding 50 μl of 5 m*M* γ-gc. The reaction was stopped with SSA. The amount of synthesized GSH was determined with HPLC analysis, as described earlier.

RT-PCR analysis

The expression of γ-GCS mRNA was measured with RT-PCR. In brief, rat primary cultures of cortical cells were incubated with NaHS (100 μ*M*) in the presence or absence of glutamate (1 m*M*) for 8 h. Total RNA was isolated from cells by using TRIzol reagent (Invitrogen, Carlsbad, CA) and cleaned with the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Single-stranded cDNA was synthesized with 1 μg RNA by using the EXscript RT reagent kit (Takara-bio, Ohtsu, Shiga, Japan) and used as a template for PCR analysis by using SYBR premix Ex Taq (Takara-bio) on a ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA). PCR was performed with denaturing at 95°C for 10 s, followed by 40 cycles of annealing and elongation at 95°C for 5 s and 60°C for 31 s. Taqman gene-expression assays (Applied Biosystems) specific to the catalytic subunit of γ-GCS (assay ID Rn00689049) were used as primers.

Isolation of mitochondrial and nuclear fractions from primary cultures of neurons or Neuro2a cells

Mitochondria and nucleus were isolated by modifying the method described previously (17). In brief, primary cortical cultures were plated at 10^7 cells in 10-cm poly-D-lysine–coated plates. Four hours after treatment with NaHS, β-me, or glutamate, cells were washed with PBS, harvested in PBS containing 2 m*M* EDTA, and centrifuged at 1,000 rpm for 5 min. Pellets were resuspended in ice-cold cell-homogenization medium (CHM; 150 m*M* $MgCl_2$, 10 m*M* KCl, 10 m*M* Tris Cl, at pH 6.7). Fifty microliters of the cell suspension was taken for measurement of total GSH. The suspension was left on ice for 2 min, and then homogenized in a Teflon homogenizer (1,500 rpm for 30 strokes) until most cells were ruptured. The homogenates were mixed with 330 μl of ice-cold CHM containing 1 *M* sucrose and centrifuged at 1,000 *g* for 5 min at 4°C. Pellets were resuspended in PBS and used as the nuclear fraction. The supernatants were filtered through Whatman No. 44 filter paper. Filtrates were centrifuged at 11,000 *g* for 10 min. The supernatants were used as the cytoplasmic fraction. Pellets were resuspended in 50 μl of mitochondria-suspension medium (10 m*M* Tris base, 2.5 *M* sucrose, at pH 7.0) and used as a mitochondrial fraction.

For isolation of mitochondria and nucleus from Neuro2a cells, cells were plated at 1.2×10^6 cells/15-cm culture dish. Because Neuro2a cells were less sensitive to glutamate toxicity than were primary cortical neurons, a higher dose of glutamate (20 m*M*) was used to induce oxidative glutamate toxicity. Higher doses of NaHS (100, 300 μ*M*) or β-me (10, 30 μ*M*) were also required to increase amounts of GSH in mitochondria and nucleus. Four hours after treatments, cells were harvested, and amounts of GSH in mitochondria and nucleus were measured according to the same method described for the primary cultures.

For isolation of mitochondria from rat brains, mitochondria were isolated by modifying the method described previously (17, 49). In brief, male Sprague–Dawley rats (8–10 weeks) were killed, and the brains were rapidly removed to ice-cold isolation buffer containing 0.32 *M* sucrose, 1 m*M* EDTA (potassium salt), and 10 m*M* Tris-HCl (pH 7.4). The tissue in isolation buffer (5×vol/wt) was homogenized with a Teflon homogenizer (1,500 rpm for 30 strokes). The homogenates were centrifuged at 1,000 *g* for 10 min at 4°C. Pellets were used as a nuclear fraction. The supernatant was transferred to a new tube and recentrifuged at 1,300 *g* for 10 min at 4°C. The supernatant was mixed with 50% Percoll to make a final concentration of 15%. The Percoll mixture (4 ml) was layered on 4 ml of 23% Percoll above 4 ml of 40% Percoll. Tubes were centrifuged at 31,000 *g* for 30 min at 4°C. The band (mitochondrial fraction) near the interface between the 23 and 40% Percoll layers was withdrawn with an 18-gauge needle, diluted with isolation buffer, and centrifuged at 14,000 *g* for 5 min at 4°C. The pellet was washed and then suspended in mitochondrial suspension buffer containing 75 m*M* mannitol, 25 m*M* sucrose, 100 m*M* KCl, 5 m*M* Tris-phosphate (pH 7.4), 0.05 m*M* EDTA (K^+ salt), and 10 m*M* Tris-HCl (pH 7.4). The upper portion of 23% Percoll was used as a cytoplasmic fraction.

Measurements of mitochondrial and nuclear GSHs

Mitochondrial, nuclear, cytoplasmic, and total GSHs were measured with HPLC, as described earlier, with some modifications. After sonication, these fractions were centrifuged at 15,000 *g* for 10 min. Supernatants were mixed with 0.125 *M* CHES and 0.1 m*M* DTT, and incubated for 1 h at 37°C to reduce GSSG into GSH. The reduced samples were mixed with 2 m*M* mBBr and left for 20 min at room temperature. Derivatization was stopped with 10 μl 30% acetic acid. The mixture was centrifuged at 15,000 *g* for 10 min. The supernatants were analyzed with HPLC, as described earlier.

For measurement of the GSH levels in isolated mitochondria to which GSH was exogenously applied, mitochondrial suspension aliquoted in Eppendorf tube was preincubated for 15 min at 37°C. Then NaHS (3, 10 μ*M*) or β-me (1, 5 μ*M*) was added to the suspension in the presence or absence of GSH (5 or 10 m*M*) and incubated for 1 h at 37°C. The reaction

mixture was centrifuged at 14,000 *g* for 2 min. Pellets were washed and resuspended in phosphate buffer. GSH levels in suspensions were determined as an adduct of monobromobimane with HPLC, as described earlier.

Western blot analysis

Fractionations of cytoplasm, mitochondria, and nucleus were confirmed with Western blot analysis with antibodies against specific markers. In brief, fractionated samples were diluted with 2× sample buffer containing 125 m*M* Tris-HCl, at pH 6.8, 4% SDS, 20% glycerol, and 10% β-mercaptoethanol. The protein contents of the samples were determined with the Bio-Rad protein assay, and the samples were separated with SDS-polyacrylamide electrophoresis. Western-blot analyses were performed with antibodies against Mn-SOD (as a mitochondrial marker; Stressgen, Victoria, BC, Canada), glucose-6-phosphate dehydrogenase (G6PD, as a cytosol marker; Bethyl Laboratories, Montgomery, TX), and histone H1(clone AE-4, as a nuclear marker; Upstate, Temecula, CA).

Intrauterine ischemia/reperfusion

The procedure was followed by (32) with modification. In brief, Institute of Cancer Research (ICR) pregnant mice on day 15 of pregnancy were randomly picked up and anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg, i.p.). NaHS (0.4375 μmol/kg) or vehicle was administered intraperitoneally to anesthetized mice 15 min before surgery. Abdomens of the mice were sterilized and incised in a longitudinal direction. The uterus was carefully pulled out without harming arteries, veins, and connecting tissues. Fetal ischemia was induced by clamping the bilateral uteroovarian arteries for 5 min by using micro-bulldog clamps. Reperfusion was achieved by removing the clamps from the arteries, and circulation was restored. The incision was sutured, and the sutured area was cleaned with 70% ethanol. Throughout the surgery and until 1 h after surgery, the body temperature was maintained at 37°C by using a heating pad and an overhead lamp. After that, the animals were individually housed in each cage and were allowed to recover. Twenty-four hours after reperfusion, mice were killed by cervical dislocation, and the whole brain of each embryo was removed. The pregnant mice treated with intrauterine ischemia/reperfusion did not die in 24 h. Two pregnant mice were used for each group.

Measurement of GSH levels in mouse fetal brains

Approximately 25 mg of brain was sonicated in 250 μl RIPA buffer (50 m*M* Tris-HCl at pH 7.6, 150 m*M* NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing a complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). An aliquot of lysates was saved for protein assay (DC protein assay; Bio-Rad, Hercules, CA). The remaining lysates were centrifuged at 1,600 *g* for 10 min. The amount of GSH in the supernatants was measured with HPLC, as described earlier, with some modifications. In brief, brain lysate (5 μl) was mixed with 0.125 *M* CHES, at pH 8.4, and 0.1 m*M* DTT, and incubated for 1 h at 37°C to reduce GSSG to GSH. The reduced samples were derivatized with mBBr and analyzed with HPLC, as described earlier.

Statistics

The data were analyzed by using StatView software (Abacus Concepts, Berkeley, CA) for one-way analysis of variance (ANOVA) with *post hoc* testing by using the Fisher PLSD multiple-comparison test.

Results

Reduction of cystine into cysteine by H_2S

We previously demonstrated that H_2S protects neurons from oxidative stress by enhancing the activity of cystine/glutamate antiporter that transports cystine into cells (27, 28). Because H_2S is a reducing substance and because approximately 20 μ*M* cysteine is contained in plasma and blood (46), it is possible that H_2S reduces cystine into cysteine in the extracellular space, where it can be transported into cells by the abundant cysteine transporter. To examine this possibility, NaHS, a donor of H_2S, was added to the medium initially containing 100 μ*M* cystine and 0.51 μ*M* cysteine, and we measured the levels of cysteine. The addition of NaHS (100 μ*M*) to the medium rapidly increased the concentrations of cysteine, which reached the maximal level 30 min after the addition of NaHS, and gradually decreased thereafter (Fig. 1). Approximately 10% of cystine is reduced by NaHS to yield 20 μ*M* cysteine. Other reducing agents, 100 μ*M* β-mercaptoethanol (β-me) and 100 μ*M* dithiothreitol (DTT), increased the concentrations of cysteine to a greater extent than did NaHS. Of the initial cysteine, 50% and 25% was reduced to obtain 100 and 50 μ*M* cysteine by DTT and β-me, respectively (Fig. 1). At 30 min, the reducing activity of 100 μ*M* NaHS is about equivalent to that of 30 μ*M* β-me.

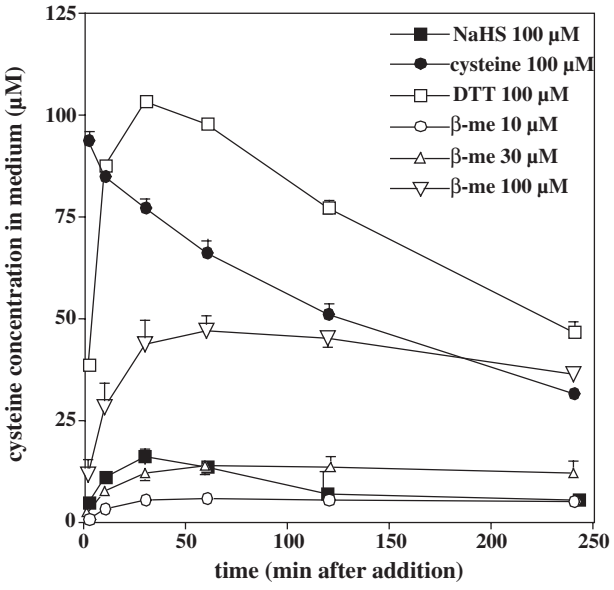

FIG. 1. Reduction of cystine to cysteine by NaHS, β-me, or DTT. Cysteine concentrations in the culture medium were measured with HPLC at 2, 10, 30, 60, and 120 min after the addition of NaHS (100 μ*M*), β-me (10, 30, and 100 μ*M*), or DTT (100 μ*M*). Oxidation of cysteine (100 μ*M*) after addition of culture medium also was monitored.

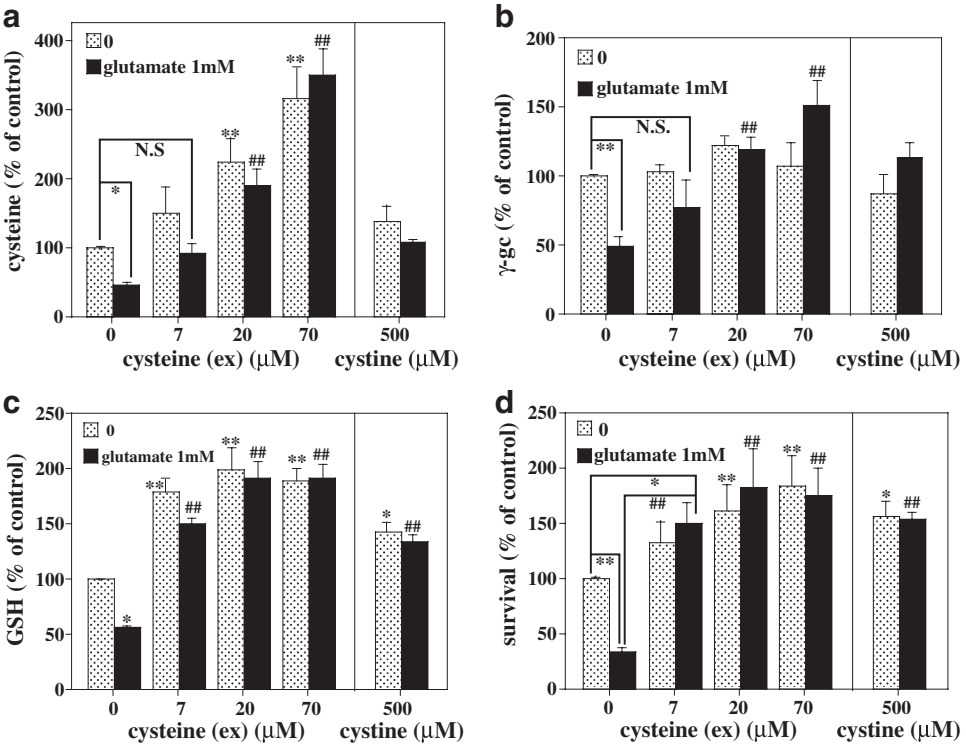

FIG. 2. Effects of extracellular cysteine or cystine on intracellular levels of cysteine, γ-gc, and GSH, and the survival of cells (a–c). The intracellular levels of cysteine (**a**), γ-gc (**b**) or GSH (**c**) were measured by HPLC 4 h after the addition of cysteine or cystine to culture medium in the presence or absence of glutamate. (**d**) Protection of neurons by extracellular cysteine or cystine from glutamate toxicity. Relative survival of neurons 24 h after the applications of cysteine or cystine in the presence or absence of glutamate (1 m*M*) was measured with the WST-8 assay. All the data expressed as percentage of control and the mean ± SEM of at least three experiments. Statistical differences were calculated with ANOVA. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* control, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs.* glutamate (1 m*M*).

Cysteine is efficiently transported into cells and used for the synthesis of GSH to protect cells from oxidative stress

Because the availability of intracellular cysteine [cysteine (in)] limits the production of GSH (35), we examined whether the increases of extracellular cysteine [cysteine (ex)] contributed to the synthesis of GSH by using cultures of rat primary cortical neurons. Cultures of 1- to 3-day-old neurons prepared from embryonic day 17, which do not express NMDA receptors (41), have widely been used to study oxidative glutamate toxicity (28, 41). Because the intracellular GSH levels reach the maximal level 4 h after the addition of H_2S (28), we measured the intracellular concentrations of cysteine, γ-glutamyl cysteine (γ-gc), and GSH at 4 h.

To examine whether cysteine (ex) is transported into cells more or less better than cystine, cysteine or cystine was added to culture medium, and concentrations of cysteine (in), γ-gc, and GSH were measured. Four hours after the addition of cysteine, the concentrations of cysteine (in) were greatly increased, in a dose-dependent manner, and reached ~300% of a control with 70 μ*M* cysteine (ex) (Fig. 2a). Although cystine is less soluble in aqueous solution than cysteine, cystine can still be dissolved to ~1.6 m*M* at 37°C and pH 7.3 in the human plasma (5). We therefore tested the effect of cystine on the intracellular cysteine. Even 500 μ*M* cystine did not significantly increase the concentrations of cysteine (in) (Fig. 2a). These observations suggest that the capacity for cysteine transport is large enough to transport cysteine (ex) readily into cells more efficiently than cystine.

It has been thought that extracellular glutamate (1 m*M*) decreases the concentrations of cysteine (in) mainly by inhibiting the cystine/glutamate antiporter (28, 40, 41). However, the concentrations of cysteine (in) decreased by glutamate were elevated by 7 μ*M* cysteine (ex) to approximately the initial levels (Fig. 2a). This observation confirms that glutamate mainly suppresses cystine import, but that the import of cysteine (ex) can compensate for cystine import that is suppressed by glutamate.

Although the availability of cysteine (in) became greater by increasing the concentrations of cysteine (ex), γ-gc production was only slightly increased (Fig. 2a and b). Because the intracellular concentration of γ-gc increases 2 h after the stimulation by NaHS but rapidly returns to a control level at 4 h (28, 43), it is likely that newly synthesized γ-gc is immediately used to synthesize GSH. This possibility is supported by the observation that the concentrations of GSH were increased to 180% of a control by 7 μM cysteine (ex) despite γ-gc concentrations remaining at a basal level (Fig. 2b and c). Although γ-gc levels suppressed by glutamate were recovered only to 77% of a control in the presence of 7 μ*M* cysteine (ex), the levels of GSH reached 150% of a control, even under the stress by glutamate in the presence of the same concentration of cysteine (ex) (Fig. 2b and c). In contrast to cysteine, even 500 μ*M* cystine was less effective than 7 μ*M* cysteine in increasing the concentrations of GSH, irrespective of the presence or absence of glutamate. These observations confirm that cysteine (ex) is transported more efficiently than cystine to generate GSH.

Because oxidative stress can be caused by decreasing the intracellular concentrations of GSH, which is restored by increasing the concentrations of cysteine (ex) (Fig. 2c), we examined whether the increase in the concentrations of cysteine (ex) protects cells from oxidative glutamate toxicity. Seventy percent of primary neurons died 20 h after the exposure to 1 m*M* glutamate (Fig. 2d), but most cells survived in the presence of 7 μ*M* cysteine (Fig. 2d). Cell viability increased with the increasing concentrations of cysteine (ex) and reached the maximal level (~180% of a control) with 70 μ*M* cysteine

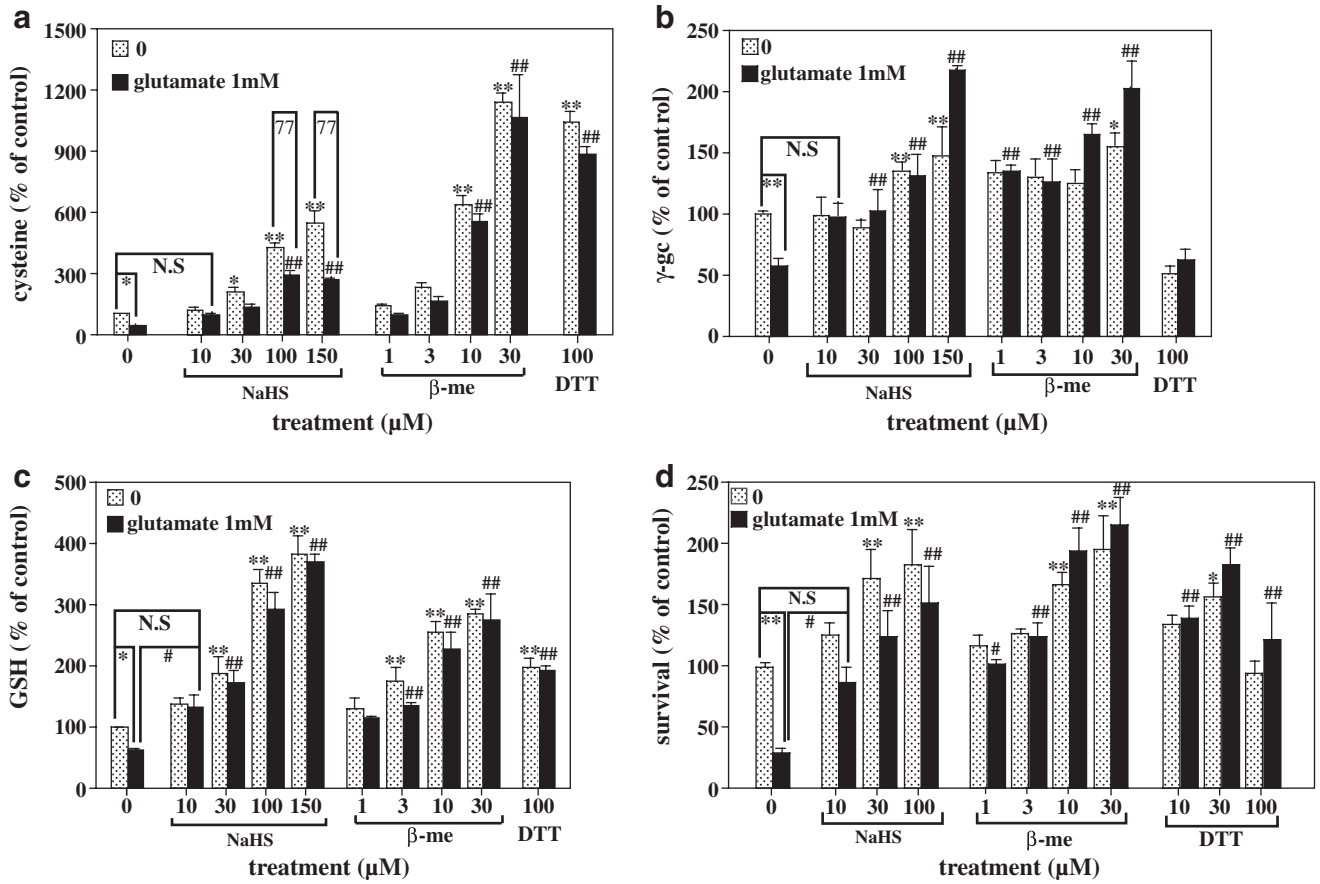

FIG. 3. Effects of H_2S, *β*-me, or DTT on intracellular levels of cysteine, *γ*-gc, and GSH, and survival of cells (a–c). The effect of H_2S, *β*-me, or DTT on the levels of cysteine, *γ*-gc, or GSH. The intracellular levels of cysteine (**a**), *γ*-gc (**b**), or GSH (**c**) were measured with HPLC 4 h after the addition of H_2S, *β*-me, or DTT to culture medium in the presence or absence of glutamate. (**d**) Protection of neurons from glutamate toxicity by H_2S, *β*-me, or DTT. Survival of neurons 24 h after the applications of H_2S, *β*-me, or DTT in the presence or absence of glutamate was measured with the WST assay. All the data represent the mean ± SEM of three to seven experiments. Statistical differences were calculated with ANOVA. $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* control, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs.* glutamate (1 m*M*), $^{\varsigma}p < 0.05$ and $^{\varsigma\varsigma}p < 0.01$ H_2S *vs.* H_2S in the presence of glutamate in (**a**).

(Fig. 2d). In contrast, to increase cell viability to ~150% of control, 500 *μM* cystine was required (Fig. 2c and d). Thus, cysteine (ex) improves cell viability and protects cells from oxidative stress more efficiently than does cystine.

Both cysteine and cystine increased survival to more than that of a control. Similar results were obtained for the effect of H_2S (Fig. 3d) (28), probably because these protect cells from the spontaneous cell death that occurs in primary cultures (2).

H_2S increases the concentrations of GSH more efficiently than β-me and DTT

Because H_2S, *β*-me, and DTT reduce cystine into cysteine (Fig. 1) (10, 24), we examined whether these reducing agents increase the concentrations of cysteine (in), and further tested whether the ability of increasing cysteine (in) simply causes the increase in *γ*-gc and GSH to enhance the survival of cells. The addition of these reducing agents to culture medium significantly increased levels of cysteine (in) (Fig. 3a). The levels of cysteine (in) reached the maximum with 150 *μM* NaHS, but twice as much with 30 *μM* *β*-me or 100 *μM* DTT as that achieved with NaHS (Fig. 3a). The H_2S-induced increase in cysteine (in) levels was significantly suppressed by glutamate, whereas the *β*-me– or DTT-induced increase was only slightly suppressed (Fig. 3a). These observations suggest that H_2S enhances cysteine/cystine transporters that are more sensitive to glutamate than are those enhanced by *β*-me or DTT.

Although cysteine (in) levels achieved in the presence of H_2S were less than those by *β*-me and DTT, H_2S produced greater amounts of GSH than did *β*-me and DTT (Fig.3a–c). The result suggests that cysteine (in) is more efficiently used to produce GSH in the presence of H_2S than are *β*-me and DTT. Cell survival suppressed by glutamate was completely reinstated by 30 *μM* NaHS (Fig. 3d).

To examine further whether the increase in cysteine (in) is not parallel to the increase in the production of GSH, changes in the concentrations of GSH were plotted against those of cysteine (in) in the presence or absence of H_2S or *β*-me (Fig. 4a). GSH levels were linearly increased when the concentrations of cysteine (in) were increased by H_2S (Fig. 4a). In contrast, GSH levels were increased up to approximately

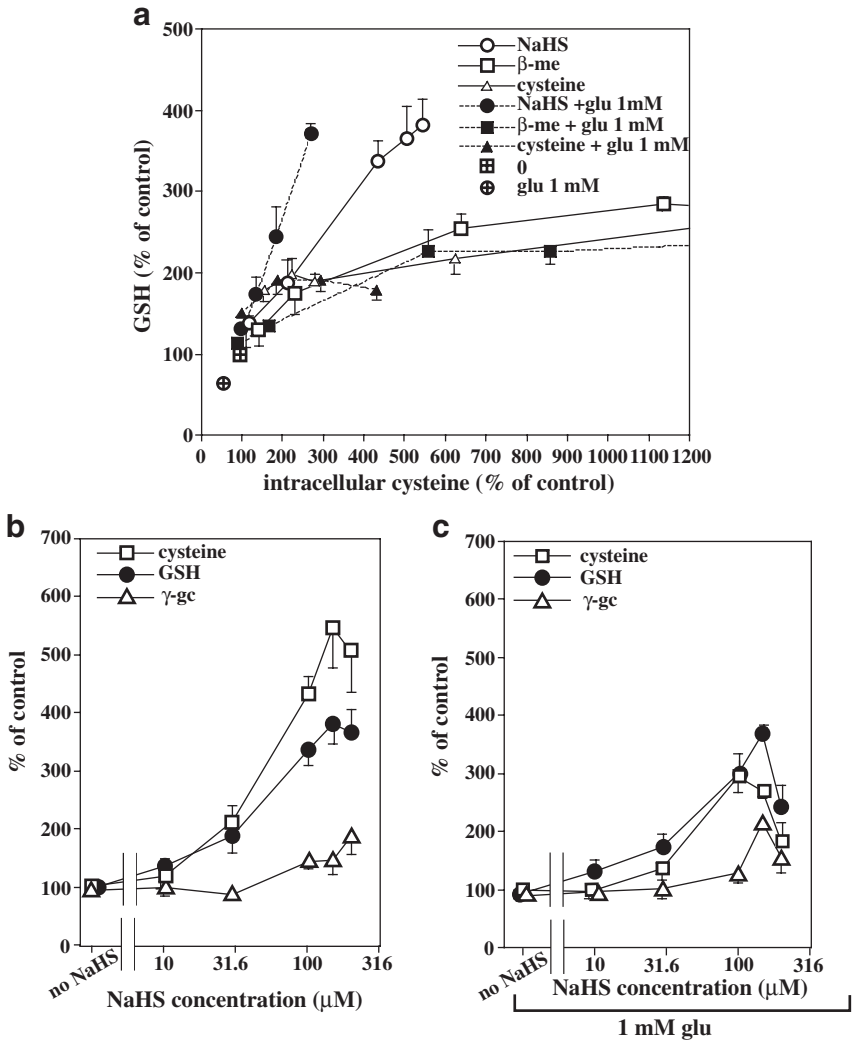

FIG. 4. The effect of H_2S, *β*-me, or extracellular cysteine on GSH production from intracellular cysteine. (**a**) Relation between GSH and intracellular cysteine in the presence of H_2S, *β*-me, or extracellular cysteine. The relative amounts of GSH were plotted against the corresponding amounts of cysteine (in) after the addition of H_2S, *β*-me, or cysteine (ex) in the presence or absence of glutamate. (**b**, **c**) The relative amounts of cysteine (in), *γ*-gc, and GSH after the addition of various doses of H_2S in the absence (**b**) or presence (**c**) of glutamate.

threefold and saturated even with higher concentrations of cysteine (in) by *β*-me or extracellularly applied cysteine (Fig. 4a). The slope of the GSH-against-cysteine (in) curve for H_2S was even steeper under oxidative stress caused by glutamate than it was without stress. This observation suggests that the efficiency of producing GSH from the same amounts of cysteine (in) is greater for H_2S under oxidative stress caused by glutamate than it is without stress. Another observation showed a similar result that the concentrations of cysteine (in) increased by H_2S were greatly decreased under oxidative stress by glutamate, but the production of GSH enhanced by H_2S was not affected under oxidative stress (Fig. 4b and c), indicating that H_2S is more efficiently produced from a smaller amount of cysteine (in) under oxidative stress. These observations confirm that the increase in the levels of cysteine (in) does not simply increase GSH levels and that the enhancement of the production of GSH by H_2S is not solely caused by its reducing activity.

H_2S enhances the activity of γ-GCS

GSH is synthesized by the consecutive catalysis of two enzymes, *γ*-glutamyl cysteine synthetase (*γ*-GCS) and glutathione synthetase (GS) (35). Because H_2S enhances the production of *γ*-gc and GSH, it is possible that H_2S modifies the activities of their producing enzymes. To examine this possibility, the effects of H_2S on GSH production suppressed by buthionine sulfoximine (BSO), a specific inhibitor for *γ*-GCS (20), was compared with those of *β*-me.

The efficiency of GSH synthesis, which is expressed as the GSH/cysteine (in) ratio, was plotted against the doses of BSO in Fig. 5. The GSH/cysteine ratio was suppressed by BSO in a dose-dependent manner. H_2S significantly reversed the reduction of the GSH/cysteine ratio caused by BSO and shifted the curve to the right, whereas *β*-me did not (Fig. 5). These observations suggest that H_2S may increase the activity of *γ*-GCS and confirmed our previous finding (28), and this enhancing activity of H_2S is one of critical differences from mere reducing substances.

Whether or not the increased activity of *γ*-GCS was due to the transcriptional regulation of this enzyme, the levels of *γ*-GCS mRNA were examined with quantitative PCR. Levels of *γ*-GCS mRNA 8 h after exposure to 100 μM NaHS were not significantly different ($105 \pm 5\%$ of a control) from those in the absence of NaHS (100%). Glutamate (1 mM) slightly suppressed the expression of *γ*-GCS mRNA ($82 \pm 3\%$), but the suppression was not recovered even in the presence of NaHS ($74 \pm 5\%$). The observations suggest that the increased total

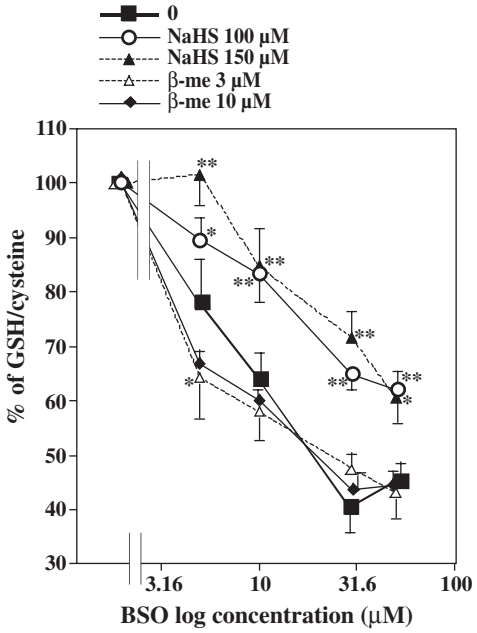

FIG. 5. H_2S reinstated GSH production suppressed by BSO. The ratio of GSH/cysteine (percentage of control) was plotted against doses of BSO in the presence or absence of H_2S or β-me. All the data represent the mean ± SEM of at least three experiments. Statistical differences were calculated between H_2S + BSO or β-me + BSO and BSO alone (*$p < 0.05$, **$p < 0.01$).

activity of γ-GCS was not due to the increase in the amount of γ-GCS.

The possibility that H_2S increases the activity of GS also was tested. Because no specific inhibitor for GS is known, the following experiments were performed. At least two possibilities exist for the activation of GS. One possibility is that H_2S activates a signal-transduction pathway to make GS in the active state. Another possibility is that H_2S enters into the cell and directly activates GS. To examine the first possibility, lysates were prepared from cells that had been exposed to H_2S or β-me for 4 h, and then enzymatic activity of GS in lysates was tested by measuring the amounts of newly synthesized GSH from γ-GC as a substrate. H_2S or β-me did not change the amount of GSH (data not shown).

To examine the second possibility, H_2S or β-me was added to lysates immediately before the initiation of the enzymatic reaction. Neither H_2S nor β-me changed the activity of GS (data not shown). These observations suggest that H_2S does not increase the activity of GS.

H_2S protects cells from oxidative stress caused by H_2O_2

To examine whether the protective effect of H_2S is effective, not only for glutamate toxicity but also the other oxidative stress, the effect of H_2S on H_2O_2-induced oxidative stress was examined. Oxidative stress on Neuro2a caused by 30 μM H_2O_2 was significantly suppressed by 300 μM NaHS (Fig. 6a). Similar results were obtained for HT22 cells (Fig. 6b). The effect of H_2S on GSH suppressed by H_2O_2 also was examined. H_2S recovered the levels of GSH suppressed by H_2O_2 (Fig. 6c). These observations suggest that H_2S protects cells from broader oxidative stress, including that caused by H_2O_2.

H_2S increases GSH levels in mitochondria as well as in the nucleus

Cellular GSH is localized mainly in the cytoplasm, and most of the remaining GSH is stored in mitochondria (21, 25). Because mitochondrial GSH is more resistant to the depletion caused by BSO than is cytoplasmic GSH (15), it is possible that H_2S increases the total cellular concentrations of GSH by increasing the pool in mitochondria. To examine this possibility, cellular compartments, including mitochondria, nucleus, and cytoplasm, were isolated from primary cortical cells, and the amounts of GSH contained in each fraction were measured.

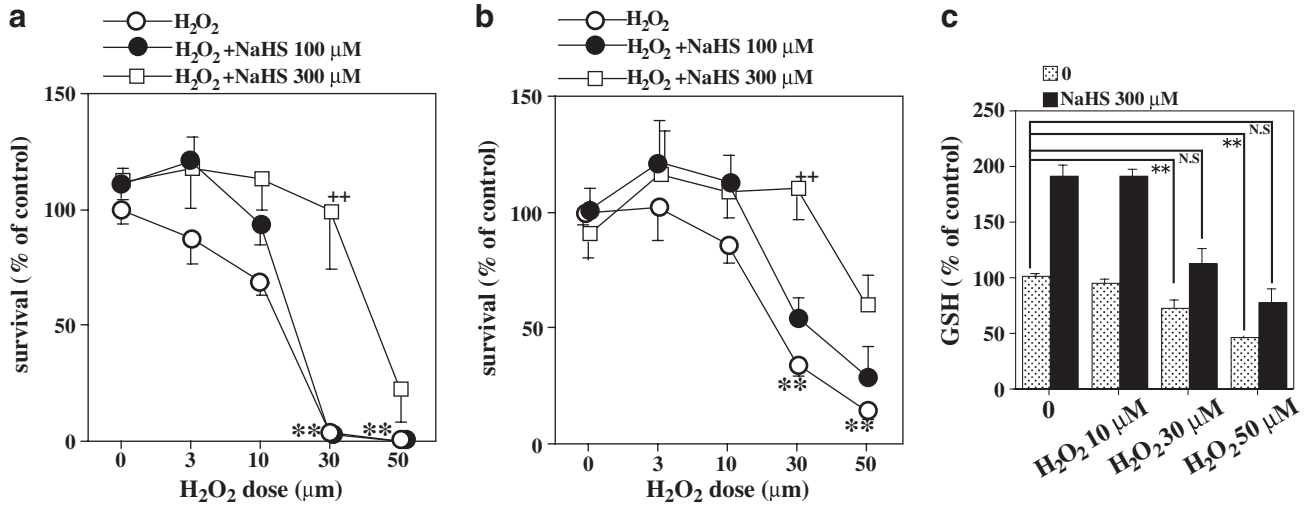

FIG. 6. H_2S protects neuronal cells from oxidative stress caused by H_2O_2 by increasing the levels of GSH (a, b). The survival rate of Neuro 2a (**a**) and HT22 (**b**) cells from H_2O_2 toxicity is increased in the presence of NaHS. H_2O_2 significantly decreased the rate of survival of cells (**$p < 0.01$), and NaHS significantly reinstated it ($^{++}p < 0.01$). (**c**) The levels of GSH in Neuro 2a cells decreased by H_2O_2 were recovered in the presence of NaHS (**$p < 0.01$).

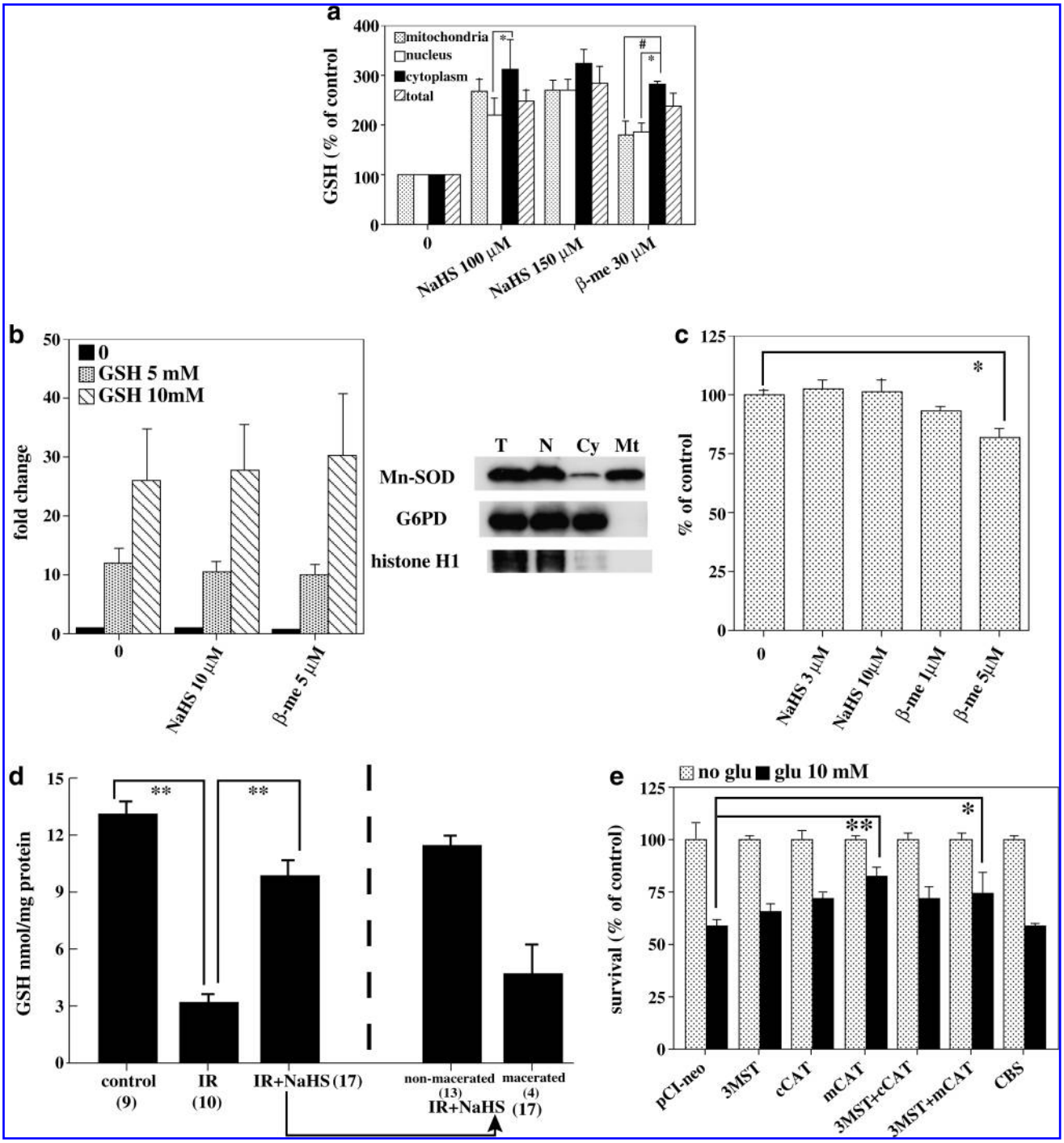

FIG. 7. H_2S increases mitochondrial and nuclear GSH. (**a**) The effect of H_2S or β-me on the subcellular distribution of GSH. H_2S or β-me was added to primary cortical neurons. (**b**) GSH levels increased in isolated mitochondria. Incubation of isolated mitochondria in the presence of GSH increased the levels of GSH in mitochondria. Note that NaHS and β-me did not have an effect on GSH levels in isolated mitochondria. The right column shows the Western blot analysis of total (T), nuclear (N), cytoplasmic (Cy), and mitochondrial (M) fractions with antibodies against specific markers (Mn-SOD; mitochondrial, G6PD; cytoplasmic, histone H1; nuclear markers). (**c**) β-me decreased endogenous levels of GSH in isolated mitochondria. Isolated mitochondria were incubated in the presence of NaHS or β-me for 1 h, and the levels of GSH were measured. $^{*}p < 0.05$; nuclear GSH *vs.* cytoplasmic GSH, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; mitochondrial GSH *vs.* cytoplasmic GSH. (**d**) GSH levels in the fetal brains decreased by ischemia/reperfusion (IR) *in utero* were reinstated by H_2S. All the brains 24 h after ischemia/reperfusion were macerated. In contrast, only four of 17 brains treated with NaHS (IR + NaHS) were macerated after ischemia/reperfusion. The GSH levels of the mice treated with IR + NaHS ($n = 17$) are separately shown, as those of the macerated ($n = 4$) and nonmacerated ($n = 13$) mice to the right. $^{**}p < 0.01$. (**e**) Cells expressing H_2S-producing enzymes are resistant to oxidative stress. Neuro2a cells were transfected with expression plasmids, and percentage survival after the exposure to 10 m*M* glutamate were assessed with WST assay. $^{*}p < 0.05$, $^{**}p < 0.01$.

H_2S increased GSH levels in each fraction to a similar extent, whereas β-me increased GSH levels of mitochondria and nucleus less than those of cytoplasm (Fig. 7a). These observations suggest that H_2S increases mitochondrial and nuclear GSH.

The direct effect of H_2S on the increase in mitochondrial GSH also was examined by applying NaHS or β-me to isolated mitochondria. When isolated mitochondria were incubated in the presence of 5 or 10 m*M* GSH for 1 h, the levels of GSH in mitochondria were greatly increased (Fig. 7b). The levels of GSH were not significantly changed in the presence of NaHS or β-me (Fig. 7b). The effect of NaHS and β-me on endogenous GSH in isolated mitochondria also was examined. The levels of GSH were not significantly changed by NaHS, whereas GSH levels were significantly decreased in the presence of β-me (Fig. 7c). These observations with isolated mitochondria suggest that GSH levels in mitochondria are increased by H_2S.

H_2S reinstates the GSH levels in the embryonic brain decreased by ischemia/reperfusion

Because H_2S protects cells by reinstating the GSH levels decreased by oxidative stress, the effect of H_2S on the GSH levels in the fetal brains after the ischemia/reperfusion injury caused by complete arrest of maternal–fetal blood supply was examined. All fetal brains ($n = 10$) 24 h after reperfusion were macerated, whereas, in the presence of NaHS, only four of 17 brains were macerated. No brains ($n = 9$) of vehicle mice were macerated. The GSH levels in the fetal brains 24 h after reperfusion were decreased to $\sim$24% ($n = 10$) of a control ($n = 9$) (Fig. 7d). Although the GSH levels of macerated brains with NaHS before ischemia/reperfusion ($n = 4$) were decreased to $\sim$36% of those of a control, those of nonmacerated brains ($n = 13$) were 87% of a control. The GSH levels of a total of macerated and nonmacerated brains with H_2S before ischemia/reperfusion ($n = 17$) were $\sim$75% of those of a control ($n = 9$). These observations indicate that H_2S protects fetal brains by reinstating the GSH levels of fetal brains decreased by *in utero* ischemia/reperfusion.

Expression of H_2S-producing enzymes attenuates oxidative glutamate toxicity

Because H_2S is produced from cysteine by 3MST with cysteine amino transferase (CAT) or by CBS in the brain (1, 48), it is possible that the expression of these enzymes may protect cells from oxidative glutamate toxicity. Because 3MST and CAT are localized mainly in mitochondria, which are the main sources of oxidative stress, H_2S produced by 3MST with CAT may directly suppress oxidative stress. To examine these possibilities, Neuro2a cells were transfected with expression plasmids encoding 3MST, mitochondrial (m) or cytoplasmic (c) CAT, and their combination, as well as CBS, and resistance of these cells to glutamate toxicity was examined with WST assay. The cells transfected with expression plasmids encoding mCAT alone and mCAT plus 3MST showed significant resistance to oxidative glutamate toxicity (Fig. 7e). Overexpression of mCAT alone and endogenous 3MST may be enough for protecting Neuro2a against oxidative glutamate toxicity. The expression of CBS did not show significant protection. It is probably because CBS is localized to the cytosol of astrocytes, and it may be less efficient to produce H_2S than 3MST with CAT in neurons or neural cells (48). These observations suggest that endogenously produced H_2S may directly suppress oxidative stress in mitochondria and may protect cells.

Discussion

Our previous study demonstrated that H_2S protects neurons from oxidative stress and improves the viability of cells by increasing the production of intracellular GSH, a major intracellular antioxidant (27, 28). We also showed that H_2S enhances the transport of cystine through the glutamate/cystine antiporter. The present study identifies a mechanism by which H_2S reduces cystine to cysteine in the extracellular space and thereby increases the transport of cysteine into cells by a transporter distinct from that of cystine. The contribution of cysteine transport to the production of GSH is much greater than that of cystine transport. H_2S redistributes GSH to mitochondria, which produce mainly reactive oxygen species. As an additional mechanism, H_2S produced by 3MST with CAT in mitochondria may directly suppress oxidative stress and protect cells (Fig. 7e).

GSH, which is a tripeptide consisting of cysteine, glutamate, and glycine, is a major antioxidant in the cellular defense against oxidative stress. Because the intracellular concentrations of cysteine are lower than those of glutamate and glycine, the availability of cysteine limits the *de novo* synthesis of GSH (19). In the extracellular space, most cysteine exists as its oxidized form, cystine (47). Our previous study showed that neurons import cystine *via* the cystine/glutamate antiporter (xc-) (11, 28, 40, 41, 45, 47) and reduce it to cysteine in the cell to use for the synthesis of GSH. The present study shows that H_2S reduces cystine to cysteine in the extracellular space and increases intracellular concentrations of cysteine to produce GSH, and that the cysteine transport in the presence of H_2S contributes to GSH synthesis to a greater extent than does cystine transport (Fig. 3a and c). It also is supported by our previous finding that the protecting effect of exogenously applied H_2S is attenuated by decreasing the extracellular concentrations of cystine (27).

Although both H_2S and β-me increased the concentrations of cysteine (in) and GSH (Fig. 3), the efficiency of increasing cysteine (in) and the sensitivity to the inhibition by glutamate were different between the two substances. Because the suppression of cysteine (in) by glutamate was completely reversed by β-me, but not by H_2S (Fig. 3a), it is possible that H_2S may enhance the activity of different transporters than those influenced by β-me to import cystine/cysteine. For example, cysteine is imported into cells by system ASC, which transports neutral α-amino acids including cysteine (29), or by $X_{A,G}^-$, excitatory amino acid transporters (7, 22). Because glutamate inhibits $X_{A,G}^-$ but not system ASC, and because our previous study showed that H_2S did not activate system ASC (28), the transporter enhanced by H_2S may be $X_{A,G}^-$. In contrast to H_2S, β-me reacts with cysteine to form a mixed disulfide of cysteine-β-me, which is taken up by cells mainly through the L system, which is not inhibited by glutamate (24). H_2S therefore has a different sensitivity to the inhibition by glutamate from β-me.

Because mitochondria do not synthesize GSH (21), cytoplasmic GSH is transported into mitochondria. The present study shows that the concentrations of GSH in isolated

mitochondria were increased, depending on the levels of extramitochondrial GSH (Fig. 7b), suggesting that the increased cytoplasmic GSH enhances its transport into mitochondria. β-me decreased the incorporation of GSH into isolated mitochondria, whereas H_2S did not (Fig. 7c). Because H_2S does not suppress the transport of GSH from cytoplasm into mitochondria, H_2S efficiently increases mitochondrial GSH. Because cell death is more closely related to the depletion of mitochondrial GSH rather than cytoplasmic GSH (18, 37), the increase of mitochondrial GSH by H_2S may contribute greatly to the protection of cells from oxidative stress.

In oxidative stress caused by ischemia/reperfusion, GSH levels are decreased. Total GSH was substantially decreased in mitochondria prepared from severely ischemic focal tissue in both the cerebral cortex and striatum (3). Mitochondrial GSH in hippocampus decreased by ischemia/reperfusion was restored by *S*-allyl L-cysteine, a water-soluble compound from garlic (4). Although the levels of mitochondrial GSH were not measured because of maceration of the fetal brains by ischemia/reperfusion in the present study, H_2S significantly recovered the GSH levels decreased by ischemia/reperfusion *in utero* (Fig. 7d). This observation *in vivo* supports the *in vitro* finding that H_2S protects brains by reinstating GSH levels decreased by oxidative stress.

Although less is known about nuclear GSH, it has a critical role in the synthesis of DNA (36, 52) and in the protection against oxidative damage or ionizing radiation (6). The increase in the levels of nuclear GSH by H_2S may also contribute to the protection of cells from oxidative damage.

H_2S facilitates the induction of hippocampal long-term potentiation (LTP) by enhancing currents mediated by NMDA receptors at the concentrations of 10–150 μM (1). Even at greater concentrations with a short application for 10 min, NaHS reversibly suppressed synaptic potentials without affecting the resting membrane potential or viability (1). In contrast, 8 h or longer exposure of matured primary cortical neurons, which express NMDA receptors, to 200 μM or greater concentrations of NaHS, caused cell death (8). Although the developmental changes in the release of H_2S have not well been understood, it is possible that H_2S release may be regulated at each stage of neuronal development so as not to cause cell death.

In conclusion, H_2S increases intracellular GSH concentrations by increasing the transport of cysteine to a greater extent than does that of cystine. In addition, H_2S enhances the redistribution of GSH into mitochondria. H_2S produced in mitochondria may also contribute to the protection of cells from oxidative stress.

Acknowledgments

This work was supported by a grant from the National Institute of Neuroscience to H.K.

Author Disclosure Statement

No competing financial interests exist.

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Date of first submission to ARS Central, September 5, 2008; date of final revised submission, August 12, 2009; date of acceptance, August 12, 2009.

Abbreviations Used

ANOVA = analysis of variance
BSO = l-buthionine-sulfoximine
CAT = cysteine aminotransferase
CBS = cystathionine β-synthase
CHES = 2-[cyclohexylamino]-ethanesulfonic acid
CHM = cell homogenization medium
CSE = cystathionine γ-lyase
DMEM = Dubach's modified Eagle's medium
DTT = *d,l*-dithiothreitol
FBS = fetal calf serum
γ-gc = γ-glutamyl-cysteine
γ-GCS = γ-glutamyl cysteine synthetase
GS = glutathione synthetase
GSH = glutathione
ICR = Institute of Cancer Research
LTP = long-term Potentiation
mBBr = monobromobimane
β-me = β-mercaptoethanol
MEM = modified Eagle's medium
3MST = 3-mercaptopyruvate sulfurtransferase
PBS = phosphate-buffered saline
SSA = 5-sulfosalicyclic acid
WST-8 = [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt]

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