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News & Views

Hydrogen Sulfide Enhances Reducing Activity in Neurons: Neurotrophic Role of H_2S in the Brain?

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ABSTRACT

Hydrogen sulfide (H_2S) can enzymatically be produced from cysteine in the brain. H_2S functions as a synaptic modulator as well as a neuroprotectant from oxidative stress in the brain. Here we show that H_2S specifically enhances the reducing activity in neurons and mouse neuroblastoma Neuro2a cells. An inhibitor of protein tyrosine kinase, genistein, suppresses the effect of H_2S, suggesting that tyrosine kinase may be involved in the enhancement of reducing activity by H_2S. The H_2S-specific enhancement of the reducing activity in neurons may lead to a neurotrophic role in the brain. *Antioxid. Redox Signal.* 9, 2035–2041.

H_2S ENHANCES THE REDUCTION OF TETRAZOLIUM IN NEURONS

It has been recognized that H_2S is a synaptic modulator in the brain (1, 17). It has also been demonstrated that H_2S protects neurons from oxidative stress by increasing the endogenous levels of glutathione (16). In a rat hippocampal neuronal cell line, HT22 cells, H_2S also enhances the activity of ATP-dependent K^+ channels and cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels to protect cells from glutamate toxicity (15). These observations suggest that H_2S may function as a neuroprotectant in the brain.

H_2S, which is well known as a toxic gas, inhibits cytochrome *c* oxidase (22), a component of the mitochondrial respiratory chain (7, 14) at high concentrations. High concentrations of H_2S also inhibit carbonic anhydrase (19, 23), monoamine oxidase (29), cholinesterase, and Na^+/K^+-ATPase (25), suggesting that the effect on several enzymes may be responsible for H_2S toxicity (2).

Tetrazolium salts, including MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), are reduced by cellular activity to form intensely colored formazans, and have often been used to quantitate the number of living cells. Because the formazan of MTT is water insoluble, it requires a solubilizing process with an organic solvent. A novel tetrazolium salt, 4-3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio.-1,3-benzene disulfonate sodium salt (WST-8) (13), whose formazan is water soluble, has recently been developed. WST-8 enables direct measurement of the formazan in the culture medium with high sensitivity and reproducibility using a membrane-permeable intermediate electron acceptor, 1-methoxy-5-methylphenazinium methosulfate (1-methoxy PMS).

The present study shows that H_2S specifically enhances the reducing activity in neurons. Other reducing agents do not exert such an effect in neurons. A tyrosine kinase inhibitor suppresses the effect of H_2S, suggesting that tyrosine kinase may be involved in enhancing the reducing activity in neurons caused by H_2S.

H_2S ENHANCES REDUCING ACTIVITY IN PRIMARY CULTURES OF NEURONS AND NEURO2A CELLS

Because H_2S protects neurons from oxidative stress, reducing activity in neurons in the presence of NaHS was examined by measuring the reduction of WST-8 in primary cultures of

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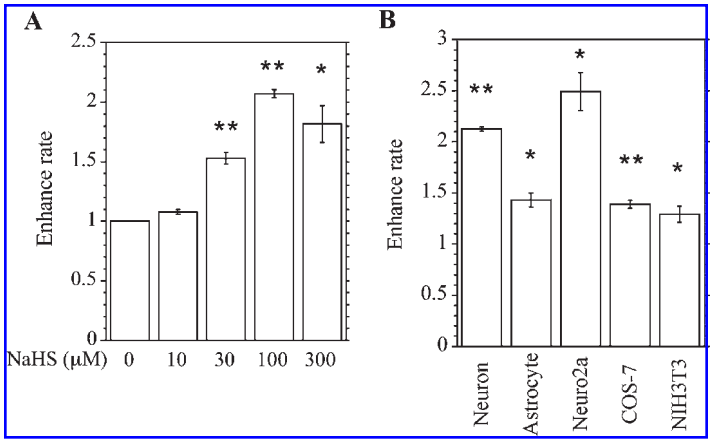

FIG. 1. NaHS enhances WST-8–reducing activities. (**A**) Dose-dependent enhancement of WST-8–reducing activity in primary cultures of neurons by NaHS. Cells were treated with NaHS for 1 h, and reduction of WST-8 was measured. (**B**) Enhancement of WST-8 reducing activity by NaHS in several cells. Primary cultures of rat cortical neurons (neuron) and astrocytes (astrocyte), Neuro2a cells, COS-7 cells, and NIH3T3 cells, were treated with 100 μM NaHS for 1 h, and reduction of WST-8 was measured. $^{*}p < 0.05$ and $^{**}p < 0.01$, compared with 0 μM by two-tailed one-sample t test. All the data represented as mean ± SEM from at least three independent experiments, each carried out at least in duplicate.

rat neurons. One hour after the application of NaHS, WST-8 was applied, and the amount of reduced WST-8 was measured. A treatment with NaHS increases the reduction of WST-8 in a dose-dependent manner, and maximal effect was obtained at 100 μM (Fig. 1A). A similar result was obtained for mouse neuroblastoma Neuro2a cells (see Fig. 1B). To test whether other cell types respond to NaHS to increase their reducing activity, the effect of 100 μM NaHS was examined for primary cultures of astrocytes, COS-7, and NIH3T3 fibroblast cell lines. The increase in the reduction of WST-8 in these cells was less than in primary cultures of neurons and Neuro2a cells (see Fig. 1B). These observations suggest that H_2S enhances the metabolic activity specifically in neurons and the neuronal cell line.

Because H_2S is a reducing agent, it is possible that the observed increase in the reduction of WST-8 is due to chemical reaction of WST-8 with H_2S. To examine this possibility, the reduction of WST-8 by NaHS in the absence of cells was tested. The increase of WST-8 reduction in the absence of cells, even without washing out of NaHS, was much less than that in the presence of cells with washing out of NaHS (Fig. 2A), suggesting that the observed increase of WST-8 reduction in cells in the presence of NaHS is not due to the chemical reaction between H_2S and WST-8 but is ascribed to the enhancement of the reducing activity of the cells induced by H_2S.

To examine whether the observed effect is specific for H_2S, several other reducing agents, GSH, DTT, α-tocopherol, and 2-mercaptoethanol, were tested. Each of these agents at 100 μM led to little enhancement of WST-8 reduction in neurons (see Fig. 2B). The result indicates that the increase in the WST-8–reducing activity in neuronal cells is specific for H_2S.

The time course of the effect of NaHS on enhancing the reducing activity was examined. When the exposure time was increased from 15 to 60 min, the enhancement on the reducing activity was increased in a time-dependent manner (Fig. 3A). The effect of longer exposure from 1 to 24 h was also examined. No significant change in enhancing activity of reduction

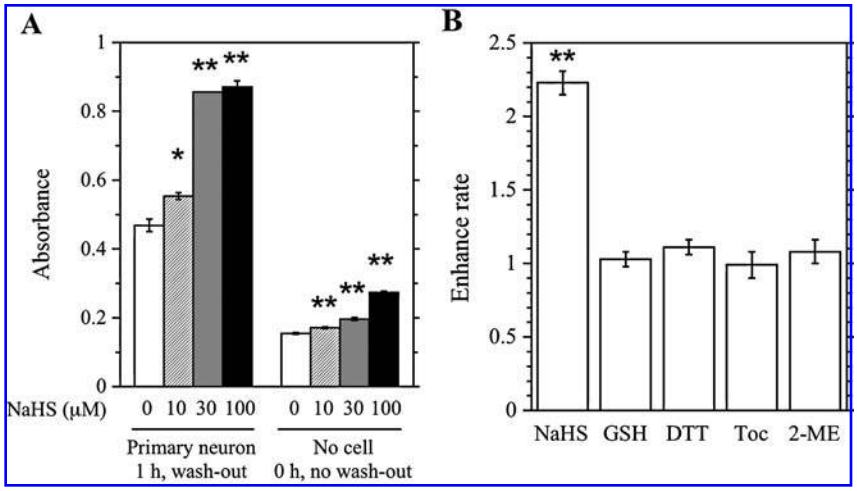

FIG. 2. WST-8 reduction by NaHS and a comparison with other reducing agents. (**A**) A comparison of WST-8 reduction by NaHS in the presence or absence of cells. Primary cultures of rat cortical neurons were treated with 0 μM (*open columns*), 10 μM (*shaded columns*), 30 μM (*grey columns*), or 100 μM (*solid columns*) of NaHS for 1 h. Wells were washed and replaced with medium containing WST-8. After incubation for 1.5 h, absorbance at 450 nm was measured. Conversely, the same concentrations of NaHS were added to the growth medium in the wells without cells. The reagent containing WST-8 was added to the wells immediately without washing, and absorbance at 450 nm was measured after 1.5 h of incubation. $^{*}p < 0.05$ and $^{**}p < 0.01$, compared with the relevant vehicle control by two-tailed Student's t test. All the data are represented as mean ± SEM of the experiments performed in triplicate. (**B**) Enhancement of WST-8 reduction by several reducing agents. The cells were treated for 1 h with 100 μM NaHS (NaHS), reduced glutathione (GSH), dithiothreitol (DTT), α-tocopherol (Toc), or 2-mercaptoethanol (2-ME), and reduction of WST-8 was measured. $^{**}p < 0.01$, compared with 0 μM by two-tailed one sample t test. The values are represented as mean ± SEM from three (Toc, 2-ME), four (GST, DTT), or six (NaHS) independent experiments, each carried out at least in duplicate.

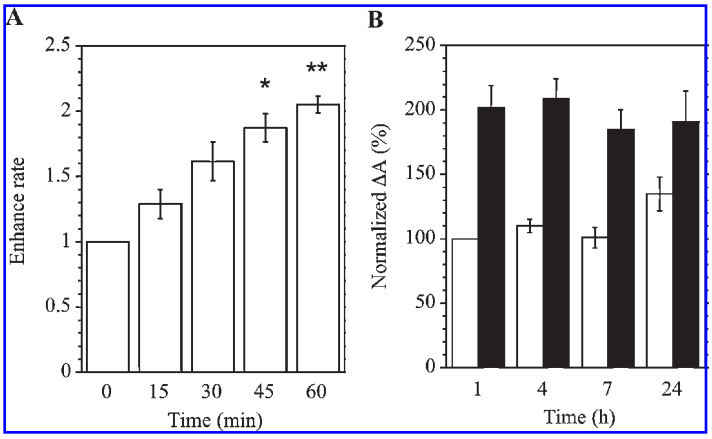

FIG. 3. Time course of the enhancement of WST-8 reduction by NaHS. (**A**) Short time course of the effect of NaHS. One hundred micromolar NaHS was applied to the cultures. After the indicated time, the wells were washed and replaced with medium containing WST-8. *$p < 0.05$ and **$p < 0.01$, compared with 0 min by two-tailed one-sample *t* test. (**B**) Longer time course of the effect of NaHS. The procedure was the same as for **A**. ΔA, the increment in the absorbance by treated cells (*solid columns*) or untreated cells (*open columns*), normalized to that for untreated cells at 1 h, is shown. All the data are represented as mean ± SEM of three independent experiments.

was found up to 7 h (see Fig. 3B). At 24 h, the enhancing activity was relatively decreased because of the background increase in the WST-8 reducing activity in cells in the absence of NaHS. These observations show that the enhancement of the reducing activity induced by NaHS was increased for 1 h, and the level was maintained at least for 7 h.

RESPIRATORY INHIBITION BY H_2S IS NOT RESPONSIBLE FOR THE INCREASE IN WST-8 REDUCTION

Because H_2S inhibits cytochrome *c* oxidase and suppresses the respiratory chain, it is possible that the reduction of WST-8 by H_2S is caused by the inhibition of cytochrome *c* oxidase. To examine this possibility, the effects of azide, another inhibitor of cytochrome *c* oxidase, were compared with those of H_2S. Neuro2a cells were treated with sodium azide (NaN_3) or NaHS in the presence of 2-deoxyglucose for 1 h, and the inhibition of cytochrome *c* oxidase was evaluated by measuring the amounts of intracellular ATP. NaN_3 greatly inhibited cytochrome *c* oxidase in concentrations >10 m*M* but did not enhance the reduction of WST-8 (Fig. 4A and B). In contrast, even 100 μM NaHS greatly enhanced the reduction of WST-8, though the same concentration of NaHS only slightly suppressed cytochrome *c* oxidase (see Fig. 4A and B). These observations suggest that inhibition of cytochrome *c* oxidase by H_2S is too weak to be responsible for the enhancement of the WST-8 reduction.

To examine the possibility that H_2S alters the cellular redox state through a mechanism other than the inhibition of cytochrome *c* oxidase, the effect of H_2S on the ratio of cellular NADH to total NAD (NADH plus NAD^+) was determined by applying NaHS to Neuro2a cells for 1 h. One millimolar NaN_3, which did not cause a substantial increase in WST-8 reduction (see Fig. 4B), greatly elevated the NADH/NADt ratio (Fig. 5A). In contrast, 100 μM NaHS, which greatly increased WST-8 reduction (see Fig. 4B), increased the (NADH/NADt) ratio less than did NaN_3 (see Fig. 5A). These observations suggest that the elevation of the NADH/NADt ratio by NaHS is not responsible for the increase in WST-8 reduction.

ATP-dependent K (K_{ATP}) channels (25, 30) are involved in the protection of neurons from oxidative stress by H_2S (15),

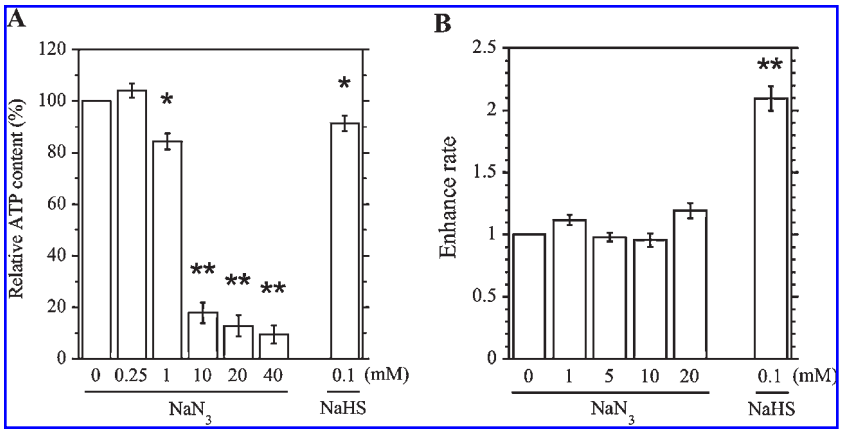

FIG. 4. Comparison of NaN_3 with NaHS in respiratory inhibition and enhancement of WST-8 reduction. (**A**) The levels of ATP in cells treated with NaN_3 or NaHS. Neuro2a cells were treated with 65 m*M* 2-deoxyglucose and indicated concentrations of NaN_3 or NaHS for 1 h. The cells were extracted with 2.5% trichloroacetic acid, and the ATP content of the extracts was determined by the luciferase assay. Relative ATP content of the cells normalized to that of cells treated with 2-deoxyglucose alone is shown. (**B**) NaHS but not NaN_3 induces WST-8 reduction. Neuro2a cells were treated with indicated concentrations of NaN_3 or NaHS for 1 h, and reduction of WST-8 was measured. *$p < 0.05$ and **$p < 0.01$, compared with a control by two-tailed one-sample *t* test. All the data are represented as mean ± SEM from three independent experiments.

and Ca^{2+} channels are activated by H_2S in astrocytes (21). Because it is possible that K_{ATP} and Ca^{2+} channels are involved in the enhancement of WST-8–reducing activity by H_2S, the effect of a K_{ATP} channel blocker, glibenclamide, and a broad-spectrum Ca^{2+} channel blocker, $LaCl_3$, was examined. Neither inhibitor affected the increase in the WST-8–reducing activity induced by H_2S (see Fig. 5B), indicating that neither K_{ATP} nor Ca^{2+} channels are involved in enhancing the WST-8–reducing activity caused by H_2S.

The involvement of mRNA and protein syntheses on the increase in the WST-8 reduction induced by H_2S was also examined by testing the effects of actinomycin D and cycloheximide. Neither actinomycin D nor cycloheximide inhibited the increase in the reduction (data not shown), suggesting that mRNA and protein syntheses are not involved in the enhancement of WST-8–reducing activity caused by H_2S.

INVOLVEMENT OF TYROSINE KINASES IN ENHANCING THE WST-8–REDUCING ACTIVITY OF H_2S

Previous studies showed that H_2S activated or inhibited extracellular signal-regulated kinases (ERK) or p38 mitogen-activated protein kinase (MAPK) in various types of the cell (6, 8, 26). It is therefore possible that signal transduction mediated by protein kinases is involved in the effect of H_2S. To examine this possibility, the effects of inhibitors of protein kinases were tested. Genistein, a broad inhibitor of tyrosine kinases, showed a significant inhibition of the effect of H_2S (Fig. 6A). In contrast, daidzein, an analog of genistein without the inhibiting activity of tyrosine kinases, showed no inhibition on the effect of H_2S.

We also tested the effect of another tyrosine kinase inhibitor, tyrphostin A23 (α-cyano-(3,4-dihydroxy)cinnamonitrile; also called AG18 or RG-50810). Although tyrphostin A23 alone inhibited the reduction of WST-8 itself, even without NaHS treatment to some extent, 50 and 100 μM tyrphostin A23 led to the inhibition on the enhancement induced by H_2S by 24% and 37%, respectively (see Fig. 6B). The inhibition is greater than that of 100 μM genistein (17%). An analogue of tyrphostin A23, tyrphostin A1 (α-cyano-(4-methoxy)cinnamonitrile; also called AG9), which lacks the inhibitory activity to epidermal growth factor (EGF)-receptor kinase, weakly inhibited both the enhancement of WST-8 reduction by H_2S and the reduction itself (see Fig. 6B). It is probably due to a less specific inhibitory activity of tyrphostin A1 on tyrosine kinases except for EGF-receptor kinase. Although further characterization of tyrphostin A1 is required, these observations suggest that tyrosine phosphorylation may be involved in enhancing the reducing activity induced by H_2S.

Staurosporine, a broad inhibitor of serine/threonine kinases, showed no effect on the enhancement, suggesting that staurosporine-sensitive serine/threonine kinases are not involved in the reducing activity of H_2S (see Fig. 6A).

CONCLUSIONS AND OPEN QUESTIONS

Tetrazolium salts, including WST-8 and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], are reduced by cellular activity to form intensely colored formazans and have often been used to quantitate living cells. Which activities are responsible for the reduction of tetrazolium salts is not fully understood. Although a decrease in MTT reduction has often been ascribed to a decrease in the cell viability due

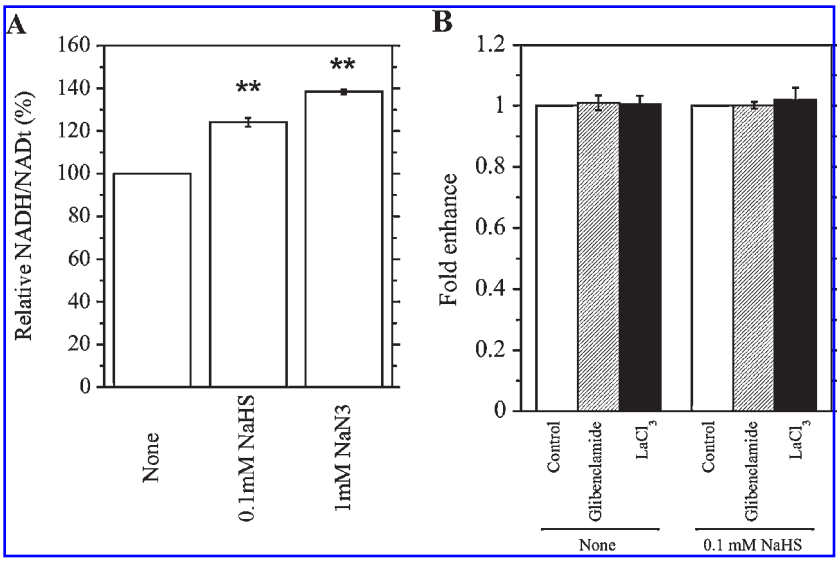

FIG. 5. Effect of NaHS on a ratio of NADH to total NAD, and effect of channel blockers on the enhancement of WST-8 reduction by NaHS. (**A**) A comparison of the effect of NaHS with NaN_3 on a ratio of NADH to total NAD. Neuro2a cells were treated with 0.1 mM NaHS or 1 mM NaN_3 for 1 h, and cellular NADH and NAD^+ were determined by enzymatic cycling assay. The NADH/NADt ratios (NADt:NADH plus NAD^+) normalized to that of nontreated cells (none). The NADH/NADt ratio of nontreated cells (0.195 ± 0.010) was represented as 100%. $^{**}p < 0.01$, compared with a control by two-tailed one-sample t test. (**B**) Glibenclamide and $LaCl_3$ have no effect on WST-8 reduction induced by NaHS. Primary cultured rat cortical neurons were pretreated for 30 min with either 10 μM glibenclamide (*shaded columns*), 10 μM $LaCl_3$ (*solid columns*), or none (*open columns*). One hundred micromolar NaHS was added to the cultures and incubated for 1 h, and reduction of WST-8 was measured. Relative enhancement rate normalized to the control (fold enhance) is shown. The enhancement rate by 0.1 mM NaHS without inhibitors was 2.06 ± 0.06. All the data are represented as mean ± SEM of three experiments.

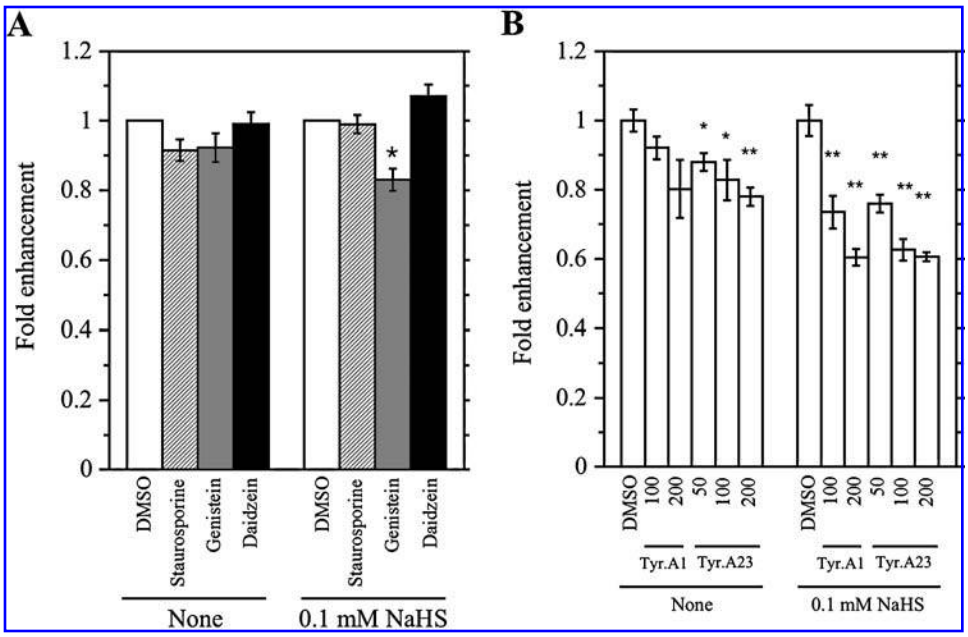

FIG. 6. Effect of protein kinase inhibitors on the enhancement of WST-8 reduction by NaHS. (**A**) Genistein decreases the enhancement of WST-8 reduction induced by NaHS. Neuro2a cells were pretreated for 30 min with either 50 n*M* staurosporine (*shaded columns*), 100 μM genistein (*grey columns*), 100 μM daidzein (*solid columns*), or 0.5% DMSO (*open columns*) as a vehicle control. One hundred micromolar NaHS was added to the cultures (0.1 m*M* NaHS) and incubated for 1 h. Reduction of WST-8 was measured. Relative enhancement rate normalized to the vehicle control (fold enhancement) is shown. The enhancement rate by 0.1 m*M* NaHS without protein kinase inhibitors was 2.87 ± 0.28. $^*p < 0.005$ compared with the relevant vehicle control (*i.e.*, unity) by two-tailed one-sample *t* test. The values are expressed as mean ± SEM of six independent experiments. (**B**) Tyrphostins decrease the enhancement of WST-8 reduction induced by NaHS. Neuro2a cells were pretreated for 30 min with different concentrations of tyrphostin A1, tyrphostin A23, or 0.5% DMSO as a vehicle control. Cells were then treated with 100 μM NaHS, and fold enhancement was determined as in (**A**). The enhancement rate by 0.1 m*M* NaHS without inhibitors was 2.47 ± 0.11. $^*p < 0.05$ and $^{**}p < 0.01$, compared with the relevant vehicle control by two-tailed Student's *t* test. The values are expressed as mean ± SEM of four wells in a quadruplicate experiment.

to mitochondrial dysfunction, the reduction can be catalyzed by enzymes located in postmitochondrial subcellular fractions (3, 11, 18) and plasma membrane (5, 9, 12, 20, 24). In the present study, because a plasma-membrane–permeable intermediate electron acceptor, 1-methoxy PMS, was present, the reduction of WST-8 (and its enhancement) could reflect enzymatic activities located in any sites in the cell, or reducing species such as superoxide radicals (27).

It was previously reported that genistein inhibits exocytosis of MTT formazan granules to the cell surface, whereas it does not inhibit the formation of intracellular formazan granules (18). In the present study, because plasma-membrane–permeable 1-methoxy PMS was present, exocytosis of the formazan should have little contribution to the reduction of WST-8. Genistein did not significantly inhibit WST-8 reduction in the absence of H_2S (see Fig. 6A). Therefore, the effect of genistein to inhibit the enhancement of the WST-8 reduction caused by H_2S is independent of the inhibition of exocytosis of the formazan.

H_2S in solution is volatile and chemically reactive. It therefore must be noted that the real working concentrations of H_2S could be lower than the one prepared and decreased in a time-dependent manner (15). When WST-8 reduction was compared between microwells without cells containing NaHS incubated at 37^0C for 1 h and microwells without incubation, WST-8 reduction was 20–60% less (depending on the H_2S dose) in incubated wells with cells than in wells without incubation in the absence of cells (data not shown).

Reduction of tetrazolium salts was used in a bioassay for lymphokines as an alternative for the ^{3}H-thymidine incorporation assay (4, 10). The rationale of this assay was the notion that the increase of the reduction of a tetrazolium salt by cells treated with cytokines reflects the maintenance of the mitochondrial activity by protection from cell death. However, in addition to this effect, it is also possible that the cytokine may enhance the reduction of tetrazolium salt on still-viable cells, similar to the effect of H_2S, in which the reducing activity of each cell is enhanced. For example, we observed an ~1.4-fold enhancement by 9% FBS in Neuro2a cells (data not shown). It is important to note that in our experiments, the interval between the beginning of FBS treatment and the application of WST-8 was 1 h. It excludes the possibility that the difference in cell numbers as a result of stimulating cell proliferation influences the result of assays. Therefore, the enhancement induced by FBS represents a qualitative change of living cells. With these similarities, however, the effect of H_2S is distinct from that of FBS, in which the enhancement of WST-8 reduction was not inhibited by genistein (data not shown).

In conclusion, H_2S enhances the reducing activity in neuronal cells, and this effect may be regulated by the signal trans-

duction in which phosphorylation of tyrosine is involved. H_2S may therefore have a trophic effect in the brain.

ABBREVIATIONS

DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; FBS, fetal bovine serum; GSH, reduced glutathione; K_{ATP} channels, ATP-sensitive K^+ channels; 1-methoxy-PMS, 1-methoxy-5-methylphenazinium methosulfate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NMDA, *N*-methyl-D-aspartate; tyrphostin A23, α-cyano-(3,4-dihydroxy)cinnamonitrile; tyrphostin A1, α-cyano-(4-methoxy)cinnamonitrile; WST-8, 4-3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate sodium salt.

APPENDIX

Notes

Chemicals. Sodium hydrogen sulfide (NaHS) hydrate, reduced glutathione (GSH), dithiothreitol (DTT), α-tocopherol, lanthanum chloride ($LaCl_3$), glibenclamide, 2-deoxyglucose, and staurosporine were purchased from Sigma-Aldrich (St. Louis, MO). 2-Mercaptoethanol, sodium azide (NaN_3), actinomycin D, cycloheximide, were purchased from Wako Pure Chemical Industry (Osaka, Japan), genistein from LKT Laboratories (St. Paul, MN), and daidzein from Extrasynthese (Genay Cedex, France).

Cell cultures. Primary cultures of neurons and astrocytes were prepared from the whole brains of the 17-day-old embryos of Sprague–Dawley rats by a modified method of a procedure previously reported (21). For cultures of neurons, the dispersed cells were initially seeded onto poly-D-lysine–coated 12-well culture plates (Becton-Dickinson, Franklin Lakes, NJ) with Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and 5% horse serum at a density of 4.6×10^5 cells per well. On the next day, cells were transferred into Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with 0.5 m*M* L-glutamine and B27 supplement minus AO (Invitrogen), and incubated at 37°C in 5% CO_2. The primary cultures of neurons were used 4 days after the initial seeding.

Cell lines, such as Neuro2a, COS-7, and NIH3T3, were maintained in DMEM supplemented with 10% FBS, and seeded at the day before assays.

Measurement of WST-8-reducing activity. For assays using Neuro2a, COS-7, and NIH3T3 cell lines, cells were seeded at the day before use into poly-D-lysine–coated 24-well culture plates with Neurobasal medium supplemented with 0.5 m*M* L-glutamine and B27 supplement minus AO at a density of 1.5×10^4 cells/cm^2. For application of various inhibitors such as glibenclamide, $LaCl_3$, actinomycin D, cycloheximide, staurosporine, genistein, and daidzein, medium was replaced with that supplemented with each inhibitor, and 0.1 volume of medium containing 1.1 m*M* NaHS was added 30 min after the application of each inhibitor. One hour after the application of NaHS, cells were washed twice with Neurobasal medium, and 1 ml for 12-well plates and 0.5 ml for 24-well plates of Neurobasal medium was supplemented with 0.5 m*M* L-glutamine and 0.1 volume of the reagent of the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) was added. This kit contains a tetrazolium salt, 4-3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate sodium salt (WST-8) (13), and an intermediate electron acceptor, 1-methoxy-5-methylphenazinium methosulfate (1-methoxy PMS). After 1.5 h of incubation, absorbance at 450 nm was measured with a microplate reader, Benchmark Plus (BIO RAD). The enhance rate R by H_2S was calculated by the following equation:

$$R = (A_t - A_0)/(A_u - A_0)$$

where A_t is the absorbance of the treated well, A_u is the untreated well, and A_0 is the well with no cells.

Determination of cellular ATP levels by luciferase assay. Neuro2a cells were seeded onto poly-D-lysine–coated 35-mm culture dishes (Becton-Dickinson) at a density of 1.5×10^4 cells/cm^2 with Neurobasal medium supplemented with 0.5 m*M* L-glutamine and B27 supplement minus AO. Cells were cultured overnight, and one-ninth volume of Neurobasal medium supplemented with 650 m*M* 2-deoxyglucose and 10 times concentrated NaHS or NaN_3 was added to the cultures and incubated for 1 h at 37°C. For evaluation of inhibition of cytochrome *c* oxidase, cells were washed with Dulbecco's phosphate-buffered saline (Nissui Pharmaceutical, Tokyo, Japan) and incubated with 1 ml per dish of 2.5% trichloroacetic acid at room temperature for 30 min. The resultant cell extracts were assayed using ENLITEN ATP assay system (Promega, Madison, WI) and a luminometer (Turner Designs TD-20/20, Sunnyvale, CA) as recommended by the manufacturer. The extracts were diluted immediately before the reaction with the buffer supplied by the kit (250 m*M* Tris-acetate, pH 7.75, 2 m*M* ethylenediaminetetraacetic acid), so that about 0.05 μl is used in a reaction of 120 μl.

Measurement of cellular NADH/NADt ratio. Neuro2a cells were seeded onto poly-D-lysine–coated 60-mm culture dishes and treated with NaHS or NaN_3 by a procedure similar to that for the measurement of WST-8–reducing activity described earlier. Cellular NADH and total NAD (NADH plus NAD^+) were determined by an enzymatic cycling assay, as reported previously (28).

Statistical analysis. Two-tailed Student's *t* test and two-tailed one-sample *t* test were performed, as shown in the figure legends.

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