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Original Research Communication

Hydrogen Sulfide Protects HT22 Neuronal Cells from Oxidative Stress

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ABSTRACT

Hydrogen sulfide (H_2S) is a neuromodulator in the brain and a relaxant for smooth muscle. H_2S protects primary cortical neurons from oxidative stress by increasing the intracellular concentrations of glutathione, the major antioxidant in cells. However, changes in glutathione alone are not sufficient to account for full protection in all types of nerve cells. H_2S is here shown to protect an immortalized mouse hippocampal cell line from oxidative glutamate toxicity by activating ATP-dependent K^+ (K_{ATP}) and Cl^- channels, in addition to increasing the levels of glutathione. The present study therefore identifies a novel pathway for H_2S protection from oxidative stress. *Antioxid. Redox Signal.* 8, 661–670.

INTRODUCTION

HYDROGEN SULFIDE (H_2S) can be produced from cysteine by cystathionine β–synthase (CBS), and the production rate in brain homogenates is 23 nmol H_2S/min per g-protein (1). H_2S facilitates hippocampal long-term potentiation (LTP) in neurons (1) and induces Ca^{2+} waves by activating Ca^{2+} channels in glia (31). H_2S also relaxes smooth muscle by activating ATP-dependent K^+ (K_{ATP}) channels (5, 16). In addition to functioning as a signal mediator, we recently demonstrated a neuroprotective effect of H_2S in a model of oxidative stress caused by glutamate (22). There are two forms of glutamate toxicity: receptor-initiated excitotoxicity (6) and nonreceptor-mediated oxidative glutamate toxicity, or oxytosis (30, 40). Oxytosis is a form of programmed cell death initiated by high concentrations of extracellular glutamate that prevents cystine uptake into cells, followed by the depletion of intracellular cysteine, the loss of glutathione, and severe oxidative stress. Oxidative stress is, at least in part, responsible for neuronal damage and degeneration in brain disorders, including stroke and epilepsy (7, 33). We recently showed that H_2S protects neurons from oxidative stress and enhances the activity of γ–glutamylcysteine synthetase and upregulates cystine transport, leading to increased levels of glutathione (22). Involvement of H_2S in cell proliferation and survival was also shown in blood vessels (9).

K_{ATP} channels, which regulate the release of neurotransmitters in the brain, are involved in protection against ischemia and glutamate excitotoxicity (11, 14, 24). K_{ATP} channels are also involved in the relaxation of smooth muscle induced by H_2S (5). The K_{ATP}-channel openers reduce abnormal excitatory synaptic activity and are neuroprotective (2). These protective effects are suppressed by K_{ATP}-channel blockers such as glibenclamide (24).

Glibenclamide also inhibits the activity of cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels (37). Glibenclamide induces apoptosis in hepatoblastoma cells by specifically inhibiting CFTR Cl^- channels (21). CFTR Cl^- channels and sulphonylurea receptors, a component of K_{ATP} channels, belong to the ATP-binding cassette superfamily, and are widely expressed in various organs including the brain (17, 28, 35).

Oxytosis has been observed in primary cultures of neuronal cells (22, 29), neuronal cell lines (27, 30), brain slices (41), and has recently been studied in the immortalized mouse hippocampal cell line, HT22 (8, 38). The present study

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demonstrates that H_2S protects HT22 cells from oxytosis by activating K_{ATP} and Cl^- channels in addition to increasing glutathione levels.

MATERIALS AND METHODS

Cell culture and toxicity assay

Mouse hippocampal HT22 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in 10% CO_2. For toxicity studies, HT22 cells were trypsinized (0.05% trypsin with 0.53 m*M* EDTA) and plated at 3×10^4 cells per ml in 96-well microtiter plates. After 24 h of incubation, cells were exposed to glutamate in the presence or absence of NaHS (Aldrich, Milwaukee, WI). Twenty hours after the addition of glutamate, cells were subjected to the WST-8 (a tetrazolium salt, 4-[3-(2-methoxy-4-nitro-phenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt) viability assay with kit-8 (Dojindo, Kumamoto, Japan). Ten μl of 10 m*M* WST-8 was added to each well, and cells were incubated for 1.5 h at 37°C, and the absorption values at 450 nm were measured. Percent survival was calculated as follows:

$$\text{Survival \%} = \frac{\text{Sample OD450-background control}}{\text{Untreated control-background control}} \times 100$$

where the untreated control is the OD450 of the medium with HT 22 cells in the absence of H_2S and background control is the OD450 of medium without cells. The results obtained by WST assay were confirmed by LDH assays and visual counting.

Lactate dehydrogenase (LDH) assay

HT22 cells were plated at 10^4 cells/ml in 96-well microtiter plates. After overnight incubation, the medium was changed to DMEM supplemented with 0.5% FBS. Cells were incubated in the low serum medium for 48 h, then exposed to glutamate in the presence or absence of NaHS. Since FBS contains LDH, which increases background absorbance, HT22 cells were incubated in low serum medium for the LDH assay. Sixteen h after the addition of glutamate, the microtiter plate was centrifuged at $250 \times g$ for 10 min, and the cell supernatant was used for the LDH assay (Cytotoxicity Detection kit (LDH), Roche Diagnostics, Basel, Switzerland). Cell supernatant (100 μl) was mixed with reaction mixture (100 μl) containing NAD^+, iodotetrazolium chloride, and sodium lactate and incubated in the dark for 30 min. The amount of formazan salt formed by LDH was measured at 490 nm. Percent cytotoxicity was calculated according to the instruction manual. Three controls were used. Background control is medium (DMEM supplemented with 0.5% FBS) only, high control is cells lysed with Triton X-100 (final 2%) to determine the maximum releasable LDH enzyme activity.

Low control is untreated HT22 cells used to determine the spontaneous LDH release. Percent toxicity was calculated by the equation,

$$\text{Cytotoxicity \%} = \frac{\text{Sample OD490-low control}}{\text{High control-low control}} \times 100$$

where Survival (% control) = 100 − cytotoxicity. A sister culture was used for the WST assay.

Measurement of H_2S

In the presence of a mild oxidizing agent, ferric ion, H_2S reacts with *N*,*N*-dimethyl-*p*-phenylenediamine to form methylene blue (MB). The amount of H_2S in the medium was determined by reversed-phase high-performance liquid chromatography (HPLC) as MB (12, 40). To assay the stability of NaHS, NaHS (300 μ*M*) was added to 10 cm culture dish containing 10 ml of DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). After 0, 15, 30, 60, and 120 min incubation at 37°C in 10% CO_2, 1 ml of medium was removed and transferred into an Eppendorf tube containing 0.1 ml each of 3 m*M* $FeCl_3$ and 2 m*M* *N*,*N*-dimethyl-*p*-phenylenediamine. After 20 min incubation at room temperature, the reaction mixture was loaded onto a Sep-Pak light tC_{18} (Waters) prewashed with 1 ml each of methanol and H_2O, then washed with 5 ml of H_2O. MB was eluted from Sep-Pak light tC_{18} with 0.5 ml methanol. Eluates were dried and dissolved in 50 μl of H_2O and analyzed with a Waters Symmetry C18 (5 μm, 4.6 × 250 mm ID). Absorbance at a wavelength of 668 nm was monitored with dual wavelength UV/Vis detector (Waters 2487) for the detection of MB.

Measurement of cysteine and glutathione levels

The amount of cysteine and glutathione was measured by the method described previously (22). Briefly, HT22 cells were treated with 5 m*M* glutamate, 300 μ*M* NaHS or both for 2, 4, 6, 8 h. Cells were washed twice with ice-cold PBS and harvested in phosphate buffer (0.1 *M* NaH_2PO_4 pH 5.8, 2 m*M* EDTA). After sonication, cell lysates were centrifuged at $16{,}000 \times g$ for 10 min, and supernatants were derivatized for HPLC. 75 μl of the supernatant was mixed with 0.5 *M* CHES (2-[cyclohexylamino]-ethanesulfonic acid), pH 8.4, then derivatized with 4 μl of 50 m*M* monobromobimane (mBBr) for 15 min in the dark. The reaction was terminated by adding 10 μl of 30% (v/v) acetic acid. Samples were analyzed with a Beckman Ultrasphere ODS (250 × 4.6-mm ID) column. The mBBr adduct was monitored by scanning fluorescence detector (Waters 474) with an excitation wavelength at 370 nm and an emission wavelength at 485 nm.

Statistics

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

RESULTS

It has been previously demonstrated that H_2S protects a mixed population of primary cultured neurons from oxytosis (22). To further investigate a neuroprotective role of H_2S against oxidative stress, the effect of H_2S was examined using a clonal hippocampal nerve cell line, HT22, a widely used

model for oxidative stress (39). HT22 cells exposed to 5 m*M* glutamate for 20 h die, while with the simultaneous application of NaHS most of the cells remain alive (Fig. 1A). NaHS alone did not have any effect on the cells. To determine the percentage of cells rescued by H_2S, a WST-8 assay was performed. WST is incorporated into live cells and reduced to a colored soluble formazan. A decrease in WST reduction represents a decreased number of viable cells (22). NaHS weakly protects cells from toxicity induced by glutamate at 10 μ*M* and its effect increases in a dose-dependent manner up to 100 μ*M* (Fig. 1A). NaHS alone did not show any toxic effect. Because 300 μ*M* NaHS showed greater protection than 100 μ*M*, 300 μ*M* was used in this study.

The protective effect of H_2S against oxidative stress was further examined using different concentrations of glutamate and both the WST-8 and LDH assays to measure cell death. Cell death caused by glutamate was efficiently suppressed by NaHS in both cases (Figs. 1B and C). Since the LDH assay requires low-serum medium and cell death in this model of glutamate toxicity is serum-dependent (8), fewer cells died in the presence of glutamate in low-serum medium than in complete medium. The results with the LDH assay support the observations with the WST-8 assay that H_2S protects HT22 cells from oxytosis.

NaHS dissociates to Na^+ and HS^- in solution, then HS^- associates with H^+ to produce H_2S. It does not matter whether the H_2S solution is prepared by bubbling H_2S gas or by dissolving NaHS. At physiological pH 7.4, approximately one-third of H_2S exists as undissociated form (H_2S), which can evaporate from the medium, and the remaining two-thirds as HS^- at equilibrium with H_2S (34). To estimate how much of H_2S + HS^- remains in the culture medium as a function of time, 300 μ*M* NaHS was added and the concentrations of H_2S + HS^- in the medium were measured over 2 h. Approximately one-third of H_2S + HS^- remains after 15 min of application and more than 90% is evaporated from the medium by 30 min (Fig. 2A). Therefore any effect of H_2S on cell physiology following as single application must be rapid and sustainable.

Since the oxytosis-induced cell death occurs 16 h after the application of glutamate, and since the effect of H_2S is rapid, the timing of the H_2S application to efficiently protect cells from oxytosis was examined. Cells started dying after 8 h of exposure to 5 m*M* glutamate and only 14% survived after 16 h (Fig. 2B). The simultaneous application of 300 μ*M* NaHS and glutamate delayed the onset of cell death to 12 h after the exposure to glutamate, but 63% of the cells survived even at 16 h (Fig. 2B). To assay whether H_2S protects early or late in the cell death cascade, 300 μ*M* NaHS was applied 2, 4, 6, 8, 10, and 12 h after the application of 5 m*M* glutamate, and cell survival was determined at 20 h. H_2S protection was only effective when added at early times after the exposure of cells to glutamate and decreased thereafter (Fig. 2C). The amount of time before the application of glutamate that NaHS can be added and still be protective was also examined. NaHS applied 12 h before glutamate is still effective in protecting cells from glutamate toxicity (Fig. 2D). Even though H_2S evaporates in 1 h (Fig. 2A), H_2S causes a commitment to survival that lasts for approximately 12 h. Therefore H_2S acts at an early stage following the application of glutamate and can delay the onset of toxicity when added well before the toxic insult.

Dependency of H_2S protection on extracellular cystine

Since cystine transport is enhanced in H_2S protection of neurons from oxidative stress (22), the dependency of H_2S protection on extracellular concentrations of cystine was examined. When the extracellular concentrations of cystine were decreased from 200 μ*M* to 0 μ*M*, toxicity measured 20 h later was increased in a dose-dependent manner (Fig. 3A). 100 μ*M* NaHS protects cells from toxicity induced by the

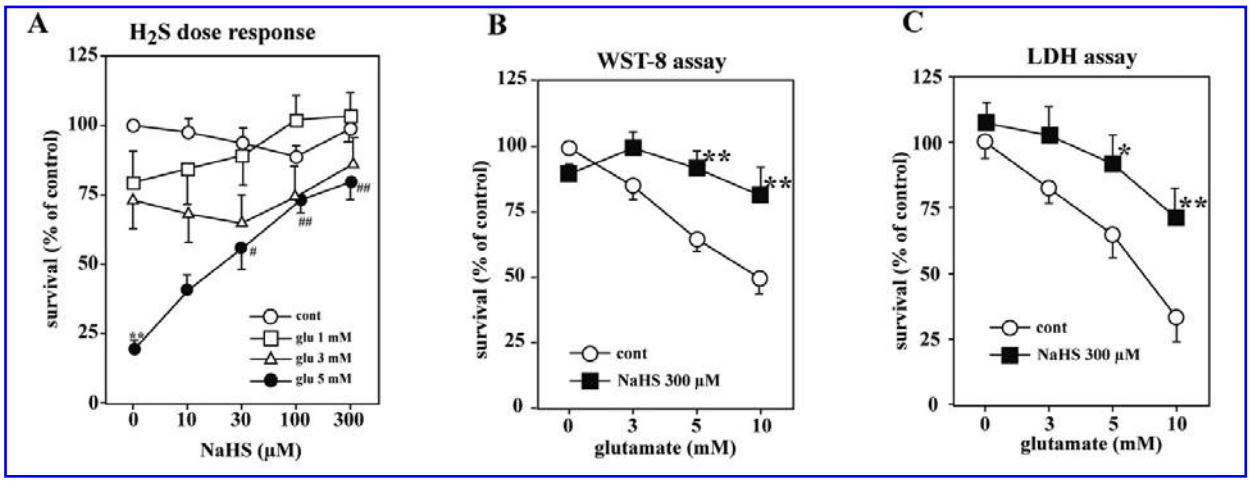

FIG. 1. H_2S protects HT22 cells from oxytosis. (**A**) The dose-dependent protection of cells by H_2S against glutamate toxicity. -◯-, control; -□- 1 m*M* glutamate; -Δ-, 3 m*M* glutamate; -●-, 5 m*M* glutamate. Relative survival of cells 20 h after the simultaneous application of glutamate and NaHS was measured with the WST assay and confirmed by visual counting. (**B**) WST-8 assay of H_2S protection. (**C**) LDH assay of H_2S protection. Both assays (**B**) and (**C**) were performed in low serum medium. All data in (**A–C**) represent the mean ± SEM of at least four experiments.

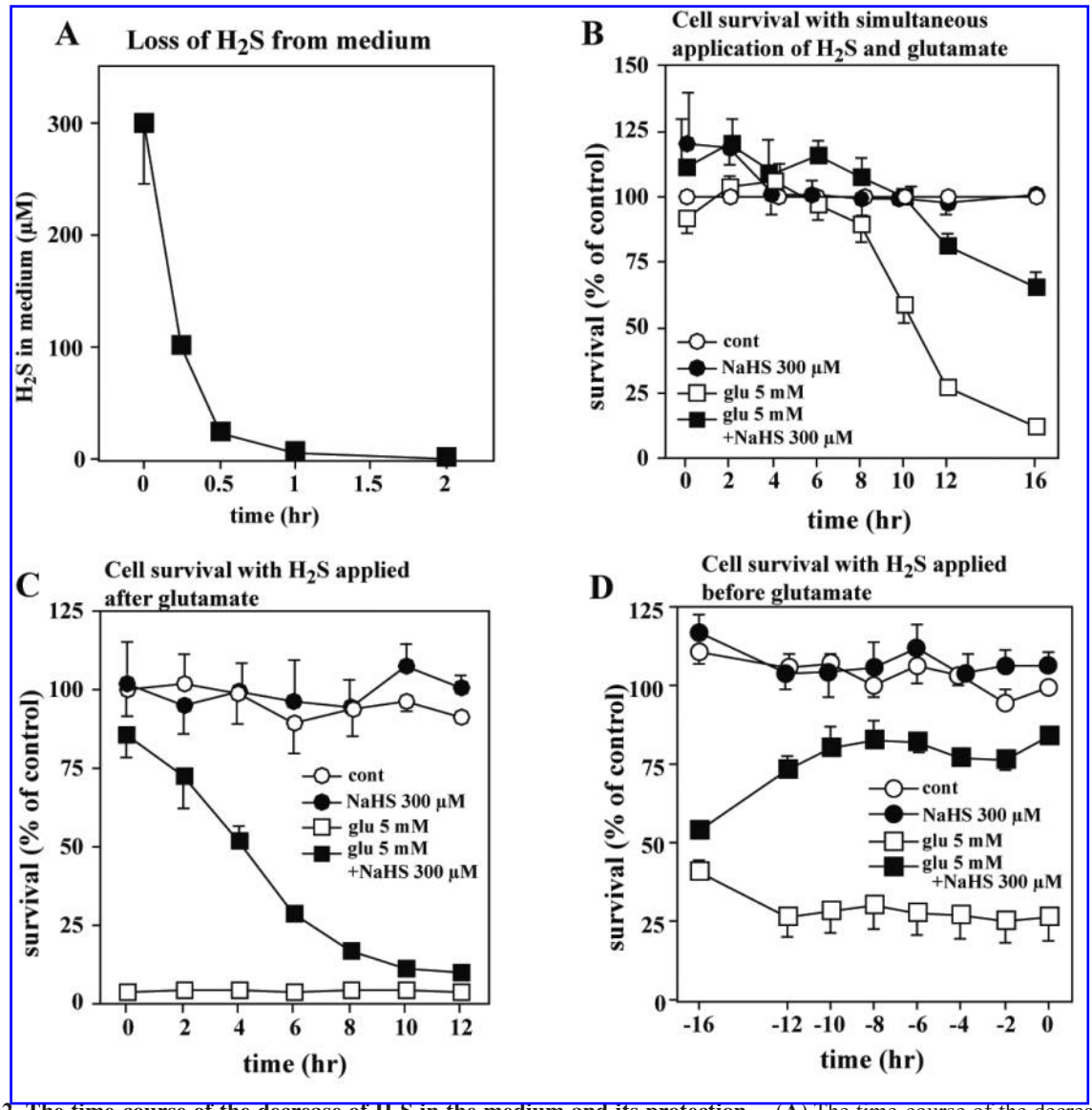

FIG. 2. The time-course of the decrease of H_2S in the medium and its protection. (**A**) The time-course of the decrease of H_2S in the medium. The levels of H_2S in the medium were measured after the application of 300 μ*M* NaHS. (**B**) The time-course of H_2S protection from glutamate toxicity. 300 μ*M* NaHS (-●-), 5 m*M* glutamate (-□-) or both (-■-) was applied to HT22 cells at 0 h and the WST-8 assay was performed at each time indicated. -◯-, control (medium alone). (**C**) and (**D**) The protection by H_2S applied at different times after (**C**) and before (**D**) the application of glutamate at 0 time. Glutamate was applied at 0 h and NaHS was applied to HT22 cells at 2, 4, 6, 8, 10, 12, and 16 h after (**C**) and before (**D**), the application of glutamate and the WST-8 assay was performed at 20 h after the application of glutamate. -◯-, control (medium alone); -l●- 300 μ*M* NaHS; -□-, 5 m*M* glutamate; -■-, both. All data in (**A**) to (**D**) represent the mean ± SEM of at least four experiments.

lower extracellular concentrations of cystine**.** Although toxicity induced by 10 μ*M* of cystine was partially protected by 100 μ*M* NaHS, toxicity induced by cystine-free media was not reversed (Fig. 3A). These observations show that H_2S requires at least a small amount extracellular cystine to protect cells from oxidative stress caused by direct cystine depletion.

The effect of H_2S on toxicity induced by a combination of low concentrations of glutamate and cystine was also examined. 1 m*M* glutamate with 100 μ*M* cystine elicited strong toxicity, but 300 μ*M* NaHS completely protected cells (Fig. 3B). These data again show that extracellular cystine is required for H_2S protection.

Changes in the levels of intracellular cysteine

Since cystine is transported inside the cells and reduced to cysteine, changes in the endogenous levels of cysteine in the presence of H_2S were examined. In the presence of H_2S, the levels of cysteine in cells are increased approximately 20% relative to those in cells in the absence of H_2S by 2 h after application, and they decreased thereafter (Fig. 4). At 2 h, even in the presence of glutamate, H_2S reinstates the levels of cysteine in cells to those in the absence of H_2S. These observations show that H_2S increases the levels of intracellular cysteine.

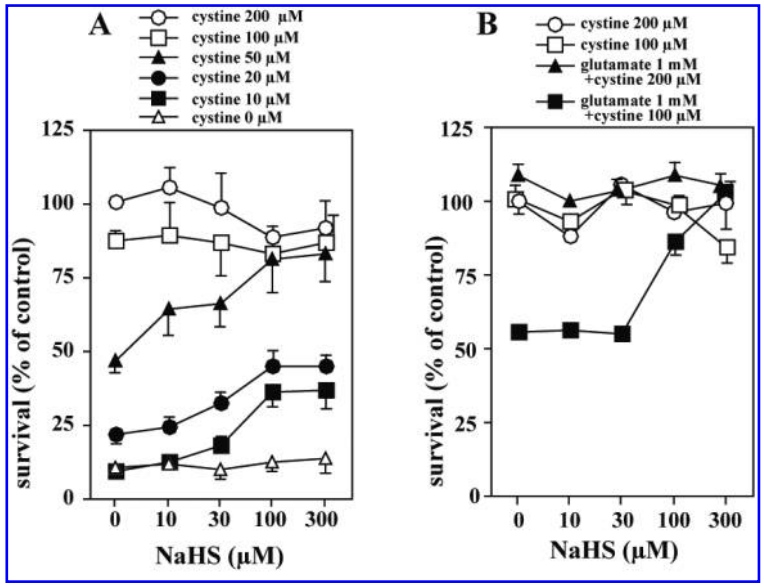

FIG. 3. H_2S protects HT22 cells from extracellular cystine depletion. The dose-dependent protection of cells by H_2S against cystine depletion (**A**) and both glutamate toxicity and cystine depletion (**B**). WST-8 assay was performed 20 h after the application of glutamate. (**A**) -◯-, control (200 μ*M* cystine); -□- 100 μ*M* cystine; -▲-, 50 μ*M*; -●-, 20 μ*M*; -■-, 10 μ*M*; -Δ-, cystine free. (**B**) -◯-, control (200 μ*M* cystine); -□- 100 μ*M* cystine; -▲-, 1 m*M* glutamate and 200 μ*M* cystine; -■-, 1 m*M* glutamate and 100 μ*M* cystine. All data in (**A**) and (**B**) represent the mean ± SEM of at least five experiments.

Changes in glutathione levels induced by H_2S

The exposure of HT22 cells, cortical neurons, and neuroblastoma cells to glutamate results in the depletion of glutathione (22, 30, 32, 38). Because H_2S protects primary cortical neurons from oxytosis by increasing the levels of glutathione (22), the effect of H_2S on glutathione levels in HT22 cells was also examined. Glutathione levels decreased by 5 m*M* glutamate were significantly recovered 2, 4, 6, and 8 h after the application of 300 μ*M* NaHS ($p < 0.05$ by the Student *t*-test) (Fig. 5). The levels of glutathione reinstated by H_2S are, however, less than those observed in cortical neurons (22), suggesting that an additional pathway may be involved in the protection of HT22 cells from oxytosis by H_2S.

Involvement of K_{ATP} and CFTR Cl^- channels in H_2S protection from glutamate toxicity

H_2S activates K_{ATP} channels in smooth muscle (16), and K_{ATP} channels may be involved in the protection of hippocampal neurons from glutamate excitotoxicity, ischemia, and oxidative injury (11, 14, 24). It is therefore possible that K_{ATP} channels are involved in the protection by H_2S against oxidative stress. To examine this possibility, the effect of glibenclamide and glipizide, K_{ATP} channel blockers, on H_2S protection was investigated. Glibenclamide suppressed the protection of cells by NaHS from glutamate toxicity in a dose-dependent manner (Fig. 6A). Glipizide showed the similar effects (Fig. 6B). In contrast, 5-hydroxydecanate (5-HD; 500 μ*M*), a selective inhibitor for mitochondrial K_{ATP} chan-

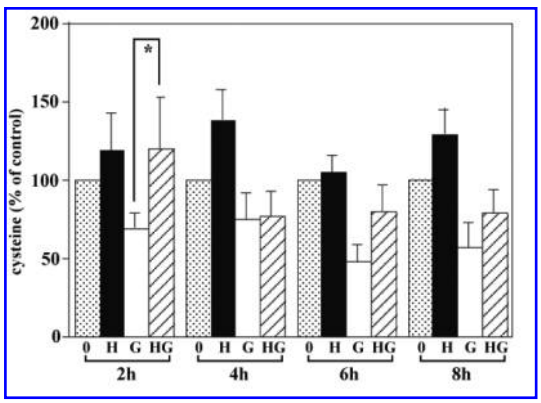

FIG. 4. H_2S increases the levels of cysteine decreased by glutamate. The endogenous levels of cysteine were measured by HPLC in the presence or absence of H_2S 2, 4, 6, and 8 h after the application of H_2S. H, 300 μ*M* NaHS; G, 5 m*M* glutamate; HG, both NaHS and glutamate. *$p < 0.05$ by Fisher's PLSD multiple comparison test. All data represent the mean ± SEM of at least four experiments.

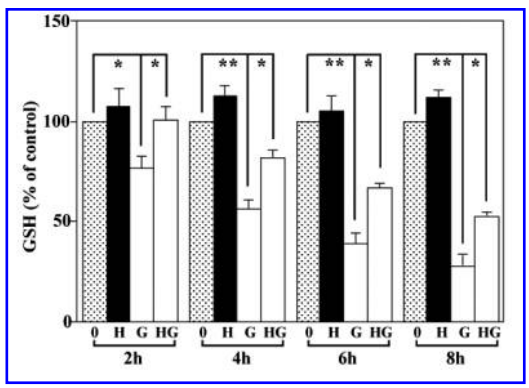

FIG. 5. Recovery of glutathione levels by H_2S. The effect of H_2S on glutathione levels. The effect of NaHS on the levels of glutathione was determined 2, 4, 6, and 8 h after the application of glutamate. H, 300 μ*M* NaHS; G, 5 m*M* glutamate; HG, both NaHS and glutamate. *$p < 0.05$ and **$p < 0.01$ by ANOVA. All data represent the mean ± SEM of at least four experiments.

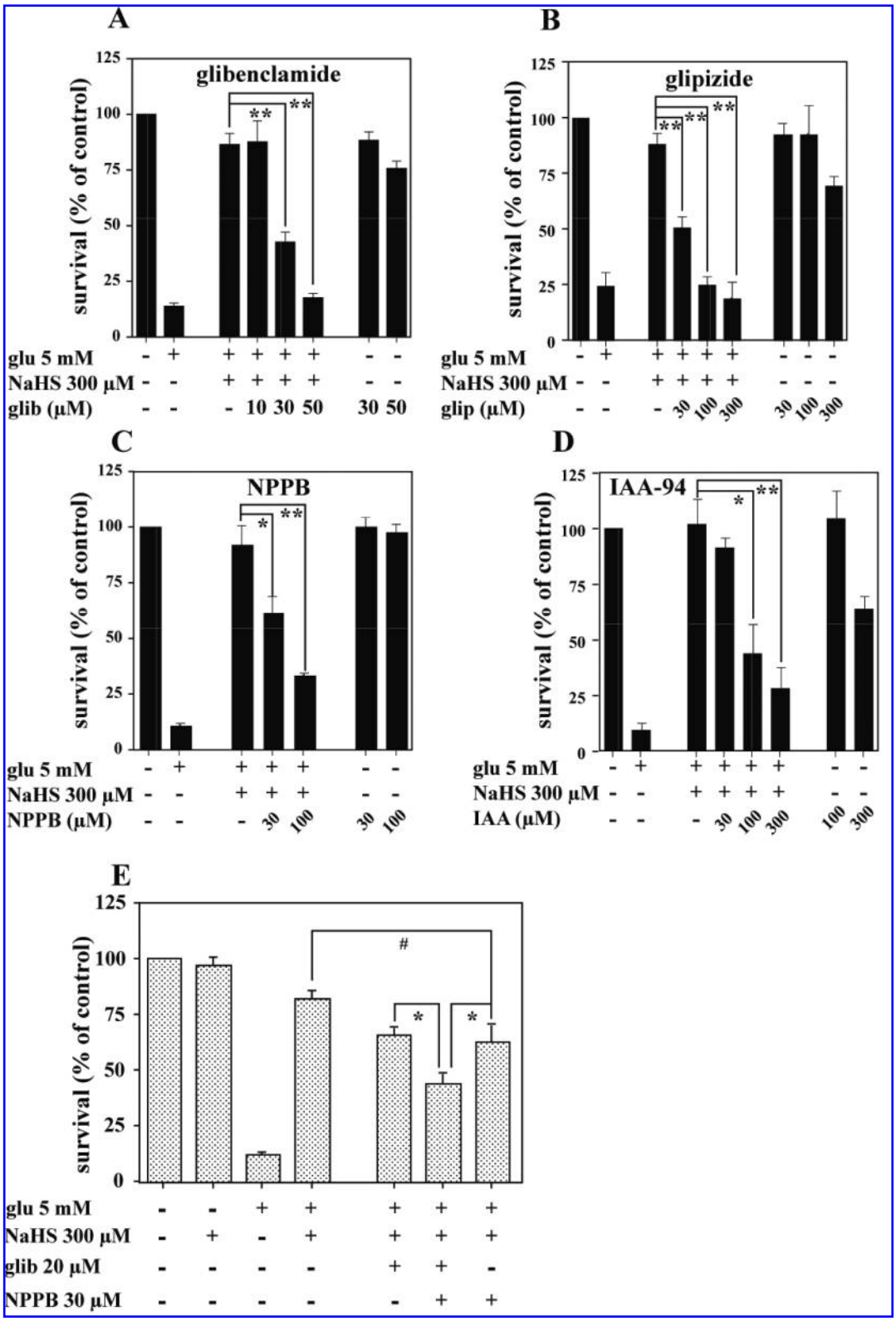

FIG. 6. Involvement of K_{ATP} and CFTR Cl^- channels in H_2S protection. (**A**, **B, C,** and **D**) The effects of K_{ATP} channel blockers, glibenclamide (**A**) and glipizide (**B**) and CFTR Cl^- channel blockers, NPPB (**C**) and IAA-94 (**D**) on H_2S protection. (**E**) The effect of simultaneously applied glibenclamide (20 µ*M*) with NPPB (30 µ*M*) on H_2S protection. Blockers were applied 30 min before the application of glutamate and NaHS, and the WST-8 assay was performed after 20 h. All data (**A**–**E**) represent the mean ± SEM of at least four experiments.

nels (3), did not suppress H_2S protection (data not shown). Although both glibenclamide and glipizide alone slightly reduce cell suvival at 50 and 300 μ*M*, respectively, both inhibitors more efficiently suppress H_2S protection from glutamate toxicity (Fig. 6). These observations suggest that K_{ATP} channels are involved in protecting HT22 cells by H_2S from oxytosis.

Glibenclamide also inhibits the activity of CFTR Cl^- channels (37) and induces apoptosis of hepatoblastoma cells (21). To examine whether CFTR Cl^- channels are involved in the protection of cells by H_2S from oxidative stress, the effects of other CFTR Cl^- channel blockers, 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and indyanyl oxyacetic acid (IAA-94) (19), were tested. NPPB and IAA-94 suppressed H_2S protection in a dose-dependent manner (Figs. 6C and 6D). In contrast, NPPB or IAA-94 alone only weakly suppresses survival of cells (Fig. 6). Therefore CFTR Cl^- channels may also be involved in the protection of HT22 cells by H_2S from oxytosis.

The effect of simultaneously applied K_{ATP} and Cl^- channel blockers on the protection by H_2S was also examined. In the presence of both glibenclamide and NPPB the protecting effect of H_2S on glutamate toxicity is further reduced, lowering cell survival (Fig. 6E). This observation supports above data that H_2S activates both K_{ATP} and CFTR Cl^- channels.

The involvement of K_{ATP} and CFTR Cl^- channels in the protecting effect of H_2S was also examined by using openers of these channels. Pinacidil, an opener of K_{ATP} channels that are localized to both plasma membrane and mitochondria (10), suppressed glutamate toxicity in a dose-dependent manner (Fig. 7A). In contrast, diazoxide (30–750 μ*M*), a specific opener of mitochondrial K_{ATP} channels (3), has no effect on oxytosis (data not shown). In the presence of levamisole, an opener of CFTR Cl^- channels (4), oxytosis is also efficiently suppressed in a dose-dependent manner (Fig. 7A). These observations suggest that the opening of K_{ATP} channels that are localized to the plasma membrane as well as CFTR Cl^- channels decreases glutamate toxicity.

To examine the effect of extracellular cystine on the protection by K^+ and Cl^- channels, the survival of cells induced by channel openers was tested in a culture medium with different concentrations of cystine. When extracellular concentrations of cystine were decreased from 200 to 0 μ*M*, the protection of cells by channel openers was greatly decreased (Fig. 7B). The dependency of protection by channel openers on extracellular cystine is similar to that caused by H_2S (Fig. 3A).

To further examine the involvement of K_{ATP} and CFTR Cl^- channels in H_2S-induced neuroprotection, H_2S was applied together with either pinacidil or levamisole and their effect on glutamate toxicity was studied. Although 30 μ*M* pinacidil or levamisole alone slightly suppresses glutamate toxicity, in the presence of low concentrations of H_2S, both openers significantly increased the protection of cells from glutamate toxicity (Fig. 7C). These observations support the present finding that H_2S activates K_{ATP} and CFTR Cl^- channels to prevent glutamate toxicity in HT22 cells.

To examine the possibility whether the activation of K_{ATP} and CFTR Cl^-channels changes the levels of glutathione and cysteine, their amounts were measured in the presence or absence of pinacidil or levamisole. The basal levels of glutathione or cysteine were not changed in the presence or absence of these channel openers (Figs. 7D and E). In addition, these openers only reinstated the cell viability (Fig. 7A) but not the levels of glutathione or cysteine decreased by glutamate. The activation of K_{ATP} and CFTR Cl^- channels by H_2S may therefore be independent of the increase in the levels of glutathione and cysteine or the activation of cystine transport.

DISCUSSION

Glutathione is the major cellular antioxidant and plays an important role in the protection of cortical neurons from oxidative stress by H_2S (22). H_2S protection of HT22 cells is dependent on the extracellular concentrations of cystine, and as with primary cortical neurons, H_2S increases the levels of intracellular cysteine and glutathione (Figs. 3, 4, and 5). However, the increases in the levels of glutathione in HT22 cells are less than those induced by H_2S in cortical neurons. H_2S increases glutathione levels, which are decreased by glutamate, by twofold of control in primary cultures of neurons (22), whereas H_2S only reinstates the levels of glutathione to a half of control in HT22 cells (Fig. 5). These observations suggest an additional pathway for H_2S protection to increasing glutathione levels.

In smooth muscle H_2S activates K_{ATP} channels (5). K_{ATP} channels are also involved in protection against ischemia and excitotoxicity (11, 14, 24). The K_{ATP} channel openers reduce abnormal excitatory synaptic activity and are protective against neuronal death (2). These protective effects are suppressed by K_{ATP} channel blockers, including glibenclamide (24). It is shown here that the activation of K_{ATP} channels localized to the plasma membrane is likely to mediate H_2S-induced neuroprotection. This conclusion is supported by the following experiments. (a) Glibenclamide and glipizide suppress the protection by H_2S from oxidative stress; (b) Pinacidil alone, a K_{ATP} channel opener, suppresses glutamate toxicity; (c) Neuroprotection is increased by the simultaneous application of H_2S and pinacidil. The observation that an opener and a blocker selective to mitochondrial K_{ATP} channels do not modulate protection by H_2S excludes the involvement of mitochondrial K_{ATP} channels (Figs. 6A, 6B, and 6E, and Figs. 7A and 7B).

CFTR Cl^- channels are found in the brain, including the cerebral cortex and hippocampus (20, 28). CFTR Cl^- channels and sulphonylurea receptors, a component of K_{ATP} channels, belong to the ATP-binding cassette superfamily, and both groups share significant sequence homologies (15). The Cl^- channel blockers NPPB and IAA-94 suppress protection by H_2S (Figs. 6C and 6D), and levamisole, an opener of Cl^- channels, efficiently suppresses glutamate toxicity (Fig. 7A). These observations suggest that Cl^- channels are also involved in protection by H_2S against oxytosis. The recent findings that a decrease in transmembrane Cl^- gradients causes cell death in hippocampal pyramidal neurons (36) and that the expression of CFTR gene is reduced in the hypothalamus of patients with Alzheimer's disease (23) suggest that homeo-

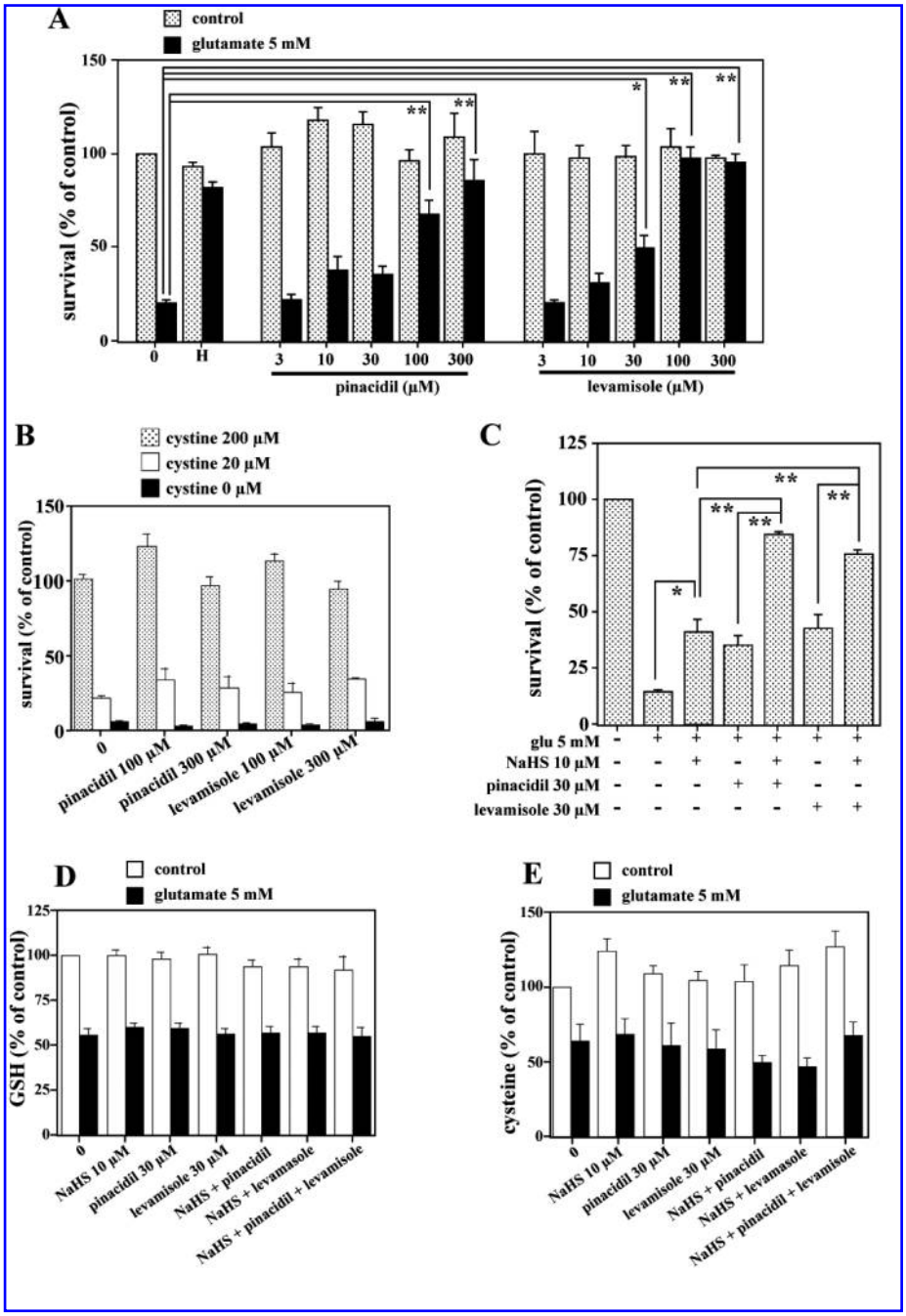

FIG. 7. The effect of K_{ATP} and CFTR Cl^- channel openers on oxytosis. (**A**) Effects of pinacidil, a K_{ATP} channel opener, and levamisole, a CFTR Cl^- channel opener, on glutamate toxicity. Pinacidil (3–300 μ*M*) or levamisole (3–300 μ*M*) was applied with glutamate and the WST-8 assay was performed after 20 h. $*p < 0.005$, $**p < 0.0001$ by Fisher's PLSD multiple comparison test. (**B**) Dependency of the survival effect of pinacidil or levamisole on extracellular cystine. The WST-8 viability assay was performed 20 h after the application of pinacidil or levamisole in the different concentrations of extracellular cystine. (**C**) Effects of simultaneously applied low concentrations of NaHS and pinacidil or levamisole on glutamate toxicity. $*p < 0.005$, $**p < 0.0005$ by Fisher's PLSD multiple comparison test. (**D** and **E**) The effect of channel openers on intracellular levels of glutathione and cysteine. The endogenous levels of GSH (**D**) and cysteine (**E**) were measured by HPLC 4 h after the application of substances. All data in (**A**–**E**) represent the mean ± SEM of at least four experiments.

stasis of transmembrane Cl^- gradients is required for normal cell survival.

The simultaneous application of blockers of K_{ATP} and Cl^- channels additively suppressed the protecting effect of H_2S on glutamate toxicity (Fig. 6E), and openers of K_{ATP} and Cl^- channels also additively reduced glutamate toxicity (Fig. 7C). Thus the activity of both channels may be enhanced by H_2S to protect HT22 cells from oxytosis.

In primary cultures of cortical neurons H_2S protects cells from oxytosis mainly by increasing glutathione levels in the cells (22). Because the concentrations of inhibitors of K_{ATP} channels or CFTR Cl^- channels, which were not toxic to HT22 cells, caused cell death in primary cultures, it was difficult to examine the effect of glibenclamide and NPPB on oxidative stress-induced cell death in primary cultures. Since the increase in glutathione levels induced by H_2S in HT22 cells is not as great as in primary cultures of neurons, we were prompted to examine other mechanisms and to show that H_2S activates K_{ATP} channels and CFTR Cl^- channels (Figs. 6 and 7). In primary cultures of neurons only the response from a mixture of cells

can be observed. However, K_{ATP} channels are highly expressed in neurons of hippocampus (13, 25), and expression of CFTR is greater in hippocampus than in cortex (28). Since HT22 is a cloned hippocampal neuronal cell line and primary cultures are a mixture of neurons, H_2S probably increases glutathione levels to protect all neurons but in addition may activate K_{ATP} and CFTR Cl^- channels in a subset group of neurons.

When the thiol group of K_{ATP} and CFTR Cl^- channels is oxidized, the activity of the channels is inhibited (18, 42). This suggests that the redox state of channels regulates their activity. H_2S can pass through cell membranes and may reduce oxidized thiol groups. It is also possible that the increased glutathione caused by H_2S may also enhance the activity of channels.

Although most of the H_2S in the culture medium evaporates within 30 min, H_2S protection as well as the recovery of glutathione levels decreased by glutamate lasts more than 8 h (Figs. 2 and 5). Even when added 12 h before glutamate, H_2S is effective in protecting cells from glutamate toxicity (Fig. 2D). In cortical neurons H_2S enhances the activity of γ–glutamylcysteine synthetase and cystine transporter activity to increase intracellular cysteine and glutathione (22). Since high concentrations of H_2S suppress respiration, it is possible that H_2S decreases the production of oxidants. This possibility was excluded, however, because there is no change in ATP levels in the presence of concentrations of H_2S used in the present study (data not shown). The present study shows that K_{ATP} and Cl^- channels are activated by H_2S to protect HT22 cells from oxidative stress (Figs. 4, 5, 6, and 7). Once cells are exposed to H_2S for a short period of time, H_2S activates pathways that increase the levels of glutathione and enhances the activity of the channels. Therefore H_2S protects cells from oxidative stress by mechanisms distinct from simply functioning as a reducing agent.

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ABBREVIATIONS

CFTR, cystic fibrosis transmembrane conductance regulator; CHES, 2-(cyclohexylamino)-ethanesulfonic acid; DMEM, Dulbecco's modified Eagle medium; FBS, fetal calf serum; 5-HD, 5-hydroxydecanate; HPLC, high-performance liquid chromatography; H_2S, hydrogen sulfide; IAA-94, indyanyl oxyacetic acid; K_{ATP} channel, ATP-dependent K^+ channel; LDH, lactate dehydrogenase; LTP, long-term potentiation; MB, methylene blue; mBBr, monobromobimane; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; PKC, protein kinase C; WST-8, 4-[3-(2-methoxy-4-nitro-phenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt).

REFERENCES

1. Abe K and Kimura H. The possible role of hydrogen sulfide as an endogenous neuromodulator. *J Neurosci* 16: 1066–1071, 1996.
2. Abele AE and Miller RJ. Potassium channel activators abolish excitotoxicity in cultured hippocampal pyramidal neurons. *Neurosci Lett* 115: 195–200, 1990.
3. Bajgar R, Seetharaman S, Kowaltowski AJ, Garlid KD, and Paucek P. Identification and properties of a novel intracellular (mitochondrial) ATP-sensitive potassium channel in brain. *J Biol Chem* 276: 33369–33374, 2001.
4. Becq F, Verrier B, Chang X-B, Riordan JR, and Hanrahan J. cAMP- and Ca^{2+}-independent activation of cystic fibrosis transmembrane conductance regulator channels by phenylimidazothiazole drugs. *J Biol Chem* 271: 16171–16179, 1996.
5. Cheng Y, Ndisang JF, Tang G, Cao K, and Wang R. Hydrogen sulfide-induced relaxation of resistance mesenteric artery beds of rats. *Am J Physiol Heart Circ Physiol* 287: H2316–H2323, 2004.
6. Choi DW. Glutamate neurotoxicity and diseases of the nervous system. *Neuron* 1: 623–634, 1988.
7. Coyle JT and Puttfarcken P. Oxidative stress, glutamate, and neurodegenerative disorders. *Science* 262: 689–695, 1993.
8. Davis JB and Maher P. Protein kinase C activation inhibits glutamate-induced cytotoxicity in a neuronal cell line. *Brain Res* 652: 169–173, 1994.
9. Du J, Hui Y, Cheung Y, Bin G, Jiang H, Chen X, and Tang C. The possible role of hydrogen sulfide as a smooth muscle cell proliferation inhibitor in rat cultured cells. *Heart Vessels* 19: 75–80, 2004.
10. Fischbach PS, White A, Barrett TD, and Lucchesi BR. Risk of ventricular proarryhythmia with selective opening of the myocardial sarcolemmal versus mitochondrial ATP-gated potassium channels. *J Pharmacol Exp Ther* 309: 554–559, 2004.
11. Goodman Y and Mattson MP. K^+ channel openers protect hippocampal neurons against oxidative injury and amyloid β-peptide toxicity. *Brain Res* 706: 328–332, 1996.
12. Haddad PR and Heckenberg AL. Trace determination of sulfide by reversed-phase ion-interaction chromatography using pre-column derivatization. *J Chromatogr* 447: 415–420, 1988.
13. Hernandez-Sanchez C, Wood TL, and Le Roith D. Developmental and tissue-specific sulfonylurea receptor gene expression. *Endocrinology* 138: 705–711, 1997.
14. Heurteaux C, Bertaina V, Widmann C, and Lazdunski M. K^+ channel openers prevent global ischemia-induced expression of *c-fos*, *c-jun*, heat shock protein, and amyloid β-protein precursor genes and neuronal death in rat hippocampus. *Proc Natl Acad Sci USA* 90: 9431–9435, 1993.
15. Higgins CF. The ABC of channel regulation. *Cell* 82: 693–696, 1995.
16. Hosoki R, Matsuki N, and Kimura H. The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem Biophys Res Commun* 237: 527–531, 1997.

17. Inagaki N, Gonoi T, Clement JP, IV, Namba N, Inazawa J, Gonzalez G, Aguilar-Bryan L, Seino S, and Bryan J. Reconstitution of I_{KATP}: an inward rectifier subunit plus the sulfonylurea receptor. *Science* 270:1166–1170, 1995.
18. Islam MS, Berggren P-O, and Larsson O. Sulfhydryl oxidation induces rapid and reversible closure of the ATP-regulated K^+ channel in the pancreatic beta-cell. *FEBS Lett* 319: 128–132, 1993.
19. Jentsch TJ, Stein V, Weinreich F, and Zdebik AA. Molecular structure and physiological function of chloride channels. *Physiol Rev* 82: 503–568, 2002.
20. Johannesson M, Bogdanovic N, Nordqvist AC, Hjelte L, and Schalling M. Cystic fibrosis mRNA expression in rat brain: cerebral cortex and medial preoptic area. *Neuroreport* 8: 535–539, 1997.
21. Kim J A, Kang YS, Lee SH, Lee EH, Yoo BH, and Lee YS. Glibenclamide induces apoptosis through inhibition of cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels and intracellular Ca^{2+} release in HepG2 human hepatoblastoma cells. *Biochem Biophys Res Commun* 261: 682–688, 1999.
22. Kimura Y and Kimura H. Hydrogen sulfide protects neurons from oxidative stress. *FASEB J* 18: 1165–1167, 2004.
23. Lahousse SA, Stopa EG, Mulberg AE, and de la Monte SM. Reduced expression of the cystic fibrosis transmembrane conductance regulator gene in the hypothalamus of patients with Alzheimer's disease. *J Alzheimers Dis* 5: 455–462, 2003.
24. Lauritzen I, De Weille JR, and Lazdunski, M. The potassium channel opener (−)-cromakalim prevents glutamate-induced cell death in hippocampal neurons. *J Neurochem* 69: 1570–1579, 1997.
25. Liss B, Bruns R, and Roeper J. Alternative sulfonylurea receptor expression defines metabolic sensitivity of K-ATP channels in dopaminergic midbrain neurons. *EMBO J* 18: 833–846, 1999.
26. Maher P. How protein kinase C activation protects nerve cells from oxidative stress-induced cell death. *J Neurosci* 2: 2929–2938, 2001.
27. Miyamoto M, Murphy TH, Schnaar RL, and Coyle JT. Antioxidants protect against glutamate-induced cytotoxicity in a neuronal cell line. *J Pharmacol Exp Ther* 250: 1132–1140, 1989.
28. Mulberg AE, Resta LP, Wiedner EB, Altschuler SM, Jefferson DM, and Broussard DL. Expression and localization of the cystic fibrosis transmembrane conductance regulator mRNA and its protein in rat brain. *J Clin Invest* 96: 646–652, 1995.
29. Murphy TH, Malouf AT, Sastre A, Schnaar RL, and Coyle JT. Calcium-dependent glutamate cytotoxicity in a neuronal cell line. *Brain Res* 444: 325–332, 1988.
30. Murphy TH, Miyamoto M, Sastre A, Schnaar RL, and Coyle JT. Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. *Neuron* 2: 1547–1558, 1989.
31. Nagai Y, Tsugane M, Oka J, and Kimura H. Hydrogen sulfide induced calcium waves in astrocytes. *FASEB J* 18: 557–559, 2004.
32. Oka A, Belliveau MJ, Rosenberg PA, and Volpe JJ. Vulnerability of oligodendroglia to glutamate: Pharmacology, mechanisms, and prevention. *J Neurosci* 13: 1441–1453, 1992.
33. Perry G, Cash AD, and Smith MA. Alzheimer disease and oxidative stress. *J Biomed Biotechnol* 2: 120–123, 2002.
34. Reiffenstein RJ. Toxicology of hydrogen sulfide. *Annu Rev Pharmacol Toxicol* 109–134, 1992.
35. Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL, Drumm ML, Iannuzzi MC, Collins FS, and Tsui L-C. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* 245: 1066–1073, 1989.
36. Sah R and Schwartz-Bloom RD. Optical imaging reveals elevated intracellular chloride in hippocampal pyramidal neurons after oxidative stress. *J Neurosci* 19: 9209–9217, 1999.
37. Sheppard D and Robinson KA. Mechanism of glibenclamide inhibition of cystic fibrosis transmembrane conductance regulator Cl^- channels expressed in a murine cell line. *J Physiol* 503: 333–346, 1997.
38. Tan S, Sagara Y, Liu Y, Maher P, and Schubert D. The regulation of reactive oxygen species production during programmed cell death. *J Cell Biol* 141: 1423–1432, 1998.
39. Tan S, Schubert D, and Maher P. Oxytosis: A novel form of programmed cell death. *Curr Top Med Chem* 1: 497–506, 2001.
40. Tang D and Santshi PH. Sensitive determination of dissolved sulfide in estuarine water by solid-phase extraction and high-performance liquid chromatography of methylene blue. *J Chromatogr A* 883: 305–309, 2000.
41. Vornov JJ and Coyle JT. Glutamate neurotoxicity and the inhibition of protein synthesis in the hippocampal slice. *J Neurochem* 56: 996–1006, 1991.
42. Wang W, Oliva C, Li G, Holmgren A, Lillig CH, and Kirk KL. Reversible silencing of CFTR chloride channels by glutathionylation. *J Gen Physiol* 125: 127–141, 2005.

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2. Ming Lu , Fang-Fang Zhao , Juan-Juan Tang , Cun-Jin Su , Yi Fan , Jian-Hua Ding , Jin-Song Bian , Gang Hu . 2012. The Neuroprotection of Hydrogen Sulfide Against MPTP-Induced Dopaminergic Neuron Degeneration Involves Uncoupling Protein 2 Rather Than ATP-Sensitive Potassium Channels. *Antioxidants & Redox Signaling* **17**:6, 849-859. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links] [Supplemental material]
3. Yan Pan, Shuang Ye, Dexiao Yuan, Jianghong Zhang, Yang Bai, Chunlin Shao. 2012. Radioprotective role of H2S/CSE pathway in Chang liver cells. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* . [CrossRef]
4. Jan Lewerenz , Sandra J. Hewett , Ying Huang , Maria Lambros , Peter W. Gout , Peter W. Kalivas , Ann Massie , Ilse Smolders , Axel Methner , Mathias Pergande , Sylvia B. Smith , Vadivel Ganapathy , Pamela Maher . The Cystine/Glutamate Antiporter System xc- in Health and Disease: From Molecular Mechanisms to Novel Therapeutic Opportunities. *Antioxidants & Redox Signaling*, ahead of print. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links] [Supplemental material]
5. Chris Peers , Claudia C. Bauer , John P. Boyle , Jason L. Scragg , Mark L. Dallas . 2012. Modulation of Ion Channels by Hydrogen Sulfide. *Antioxidants & Redox Signaling* **17**:1, 95-105. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
6. Hideo Kimura , Norihiro Shibuya , Yuka Kimura . 2012. Hydrogen Sulfide Is a Signaling Molecule and a Cytoprotectant. *Antioxidants & Redox Signaling* **17**:1, 45-57. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
7. Bridget Fox, Jan-Thorsten Schantz, Richard Haigh, Mark E. Wood, Phillip K. Moore, Nick Viner, Jeremy P. E. Spencer, Paul G. Winyard, Matthew Whiteman. 2012. Inducible hydrogen sulfide synthesis in chondrocytes and mesenchymal progenitor cells: is H2S a novel cytoprotective mediator in the inflamed joint?. *Journal of Cellular and Molecular Medicine* **16**:4, 896-910. [CrossRef]
8. Shinichi Kai , Tomoharu Tanaka , Hiroki Daijo , Hiroshi Harada , Shun Kishimoto , Kengo Suzuki , Satoshi Takabuchi , Keizo Takenaga , Kazuhiko Fukuda , Kiichi Hirota . 2012. Hydrogen Sulfide Inhibits Hypoxia- But Not Anoxia-Induced Hypoxia-Inducible Factor 1 Activation in a von Hippel-Lindau- and Mitochondria-Dependent Manner. *Antioxidants & Redox Signaling* **16**:3, 203-216. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
9. Bogusz Kulawiak, Adam Szewczyk. 2012. Glutamate-induced cell death in HT22 mouse hippocampal cells is attenuated by paxilline, a BK channel inhibitor. *Mitochondrion* . [CrossRef]
10. Neville N. Osborne, Dan Ji, Aman Shah Abdul Majid, Piero Del Sodato, Anna Sparatore. 2012. Glutamate oxidative injury to RGC-5 cells in culture is necrostatin sensitive and blunted by a hydrogen sulfide (H2S)-releasing derivative of aspirin (ACS14). *Neurochemistry International* . [CrossRef]
11. Patricia Horcajada, Ruxandra Gref, Tarek Baati, Phoebe K. Allan, Guillaume Maurin, Patrick Couvreur, Gérard Férey, Russell E. Morris, Christian Serre. 2011. Metal–Organic Frameworks in Biomedicine. *Chemical Reviews* 111214095646006. [CrossRef]
12. Matthew Whiteman, Sophie Le Trionnaire, Mohit Chopra, Bridget Fox, Jacqueline Whatmore. 2011. Emerging role of hydrogen sulfide in health and disease: critical appraisal of biomarkers and pharmacological tools. *Clinical Science* **121**:11, 459-488. [CrossRef]
13. Mitali Chattopadhyay, Ravinder Kodela, Niharika Nath, Cherease R. Street, Carlos A. Velázquez-Martínez, Daniel Boring, Khosrow Kashfi. 2011. Hydrogen sulfide-releasing aspirin modulates xenobiotic metabolizing enzymes in vitro and in vivo. *Biochemical Pharmacology* . [CrossRef]
14. J. Boisramé-Helms, P. Asfar, P. Radermacher, F. Meziani. 2011. Effets cardiovasculaires de l'hydrogène sulfuré. *Réanimation* . [CrossRef]
15. M. A. Aminzadeh, N. D. Vaziri. 2011. Downregulation of the renal and hepatic hydrogen sulfide (H2S)-producing enzymes and capacity in chronic kidney disease. *Nephrology Dialysis Transplantation* . [CrossRef]
16. Burkhard Kloesch, Melissa Liszt, Daniela Krehan, Johann Broell, Hans Kiener, Guenter Steiner. 2011. High concentrations of hydrogen sulphide elevate the expression of a series of pro-inflammatory genes in fibroblast-like synoviocytes derived from rheumatoid and osteoarthritis patients. *Immunology Letters* . [CrossRef]
17. Xiao-Qing Tang, Yuan-Yuan Zhuang, Li-Li Fan, Heng-Rong Fang, Cheng-Fang Zhou, Ping Zhang, Bi Hu. 2011. Involvement of KATP/PI3K/AKT/Bcl-2 Pathway in Hydrogen Sulfide-induced Neuroprotection Against the Toxicity of 1-methy-4-phenylpyridinium Ion. *Journal of Molecular Neuroscience* . [CrossRef]

18. A. A. Varaksin, E. V. Puschina. 2011. Hydrogen Sulfide as a Regulator of Systemic Functions in Vertebrates. *Neurophysiology* **43**:1, 62-72. [CrossRef]
19. W.N. Marsden. 2011. Stressor-induced NMDAR dysfunction as a unifying hypothesis for the aetiology, pathogenesis and comorbidity of clinical depression. *Medical Hypotheses* . [CrossRef]
20. Hideo Kimura. 2011. Hydrogen sulfide: its production, release and functions. *Amino Acids* **41**:1, 113-121. [CrossRef]
21. Zhanyong Li, Yiyi Wang, Yongling Xie, Zhuo Yang, Tao Zhang. 2011. Protective Effects of Exogenous Hydrogen Sulfide on Neurons of Hippocampus in a Rat Model of Brain Ischemia. *Neurochemical Research* . [CrossRef]
22. Minghui Jessica Chen, Zhao Feng Peng, Jayapal Manikandan, Alirio J. Melendez, Gek San Tan, Ching Ming Chung, Qiu-Tian Li, Theresa M. Tan, Lih Wen Deng, Matthew Whiteman, Philip M. Beart, Phillip K. Moore, Nam Sang Cheung. 2011. Gene profiling reveals hydrogen sulphide recruits death signaling via the N-methyl-D-aspartate receptor identifying commonalities with excitotoxicity. *Journal of Cellular Physiology* **226**:5, 1308-1322. [CrossRef]
23. Jian-Lin Shao, Xiao-Hong Wan, Yan Chen, Chun Bi, Hong-Mei Chen, Ying Zhong, Xin-Hua Heng, Jin-Qiao Qian. 2011. H2S Protects Hippocampal Neurons from Anoxia–Reoxygenation Through cAMP-Mediated PI3K/Akt/p70S6K Cell-Survival Signaling Pathways. *Journal of Molecular Neuroscience* **43**:3, 453-460. [CrossRef]
24. Ling Li, Peter Rose, Philip K. Moore. 2011. Hydrogen Sulfide and Cell Signaling. *Annual Review of Pharmacology and Toxicology* **51**:1, 169-187. [CrossRef]
25. Mohamed M. El-Seweidy, Nermin A.H. Sadik, Olfat G. Shaker. 2011. Role of sulfurous mineral water and sodium hydrosulfide as potent inhibitors of fibrosis in the heart of diabetic rats. *Archives of Biochemistry and Biophysics* **506**:1, 48-57. [CrossRef]
26. Yong-Peng Yu, Zhen-Guang Li, Dao-Zhen Wang, Xia Zhan, Jing-Hua Shao. 2011. Hydrogen sulfide as an effective and specific novel therapy for acute carbon monoxide poisoning. *Biochemical and Biophysical Research Communications* **404**:1, 6-9. [CrossRef]
27. Neal D. Mathew, David I. Schlipalius, Paul R. Ebert. 2011. Sulfurous Gases As Biological Messengers and Toxins: Comparative Genetics of Their Metabolism in Model Organisms. *Journal of Toxicology* **2011**, 1-14. [CrossRef]
28. Nermin A. H. Sadik, Mohamed M. El-Seweidy, Olfat G. Shaker. 2011. The Antiapoptotic Effects of Sulphurous Mineral Water and Sodium Hydrosulphide on Diabetic Rat Testes. *Cellular Physiology and Biochemistry* **28**:5, 887-898. [CrossRef]
29. Benjamin L Predmore, David J Lefer. 2011. Hydrogen sulfide-mediated myocardial pre- and post-conditioning. *Expert Review of Clinical Pharmacology* **4**:1, 83-96. [CrossRef]
30. Matthew Whiteman, Paul G Winyard. 2011. Hydrogen sulfide and inflammation: the good, the bad, the ugly and the promising. *Expert Review of Clinical Pharmacology* **4**:1, 13-32. [CrossRef]
31. Guangdong Yang. 2011. Hydrogen sulfide in cell survival: a double-edged sword. *Expert Review of Clinical Pharmacology* **4**:1, 33-47. [CrossRef]
32. B. Kloesch, M. Liszt, G. Steiner, J. Bröll. 2010. Inhibitors of p38 and ERK1/2 MAPkinase and hydrogen sulphide block constitutive and IL-1#-induced IL-6 and IL-8 expression in the human chondrocyte cell line C-28/I2. *Rheumatology International* . [CrossRef]
33. Peter W. Henderson, Sunil P. Singh, Andrew L. Weinstein, Vijay Nagineni, Daniel C. Rafii, Daniel Kadouch, David D. Krijgh, Jason A. Spector. 2010. Therapeutic Metabolic Inhibition: Hydrogen Sulfide Significantly Mitigates Skeletal Muscle Ischemia Reperfusion Injury In Vitro and In Vivo. *Plastic and Reconstructive Surgery* **126**:6, 1890-1898. [CrossRef]
34. Emilie Lagoutte, Sabria Mimoun, Mireille Andriamihaja, Catherine Chaumontet, François Blachier, Frédéric Bouillaud. 2010. Oxidation of hydrogen sulfide remains a priority in mammalian cells and causes reverse electron transfer in colonocytes. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1797**:8, 1500-1511. [CrossRef]
35. Matthias Derwall, Maren Westerkamp, Céline Löwer, Jan Deike-Glindemann, Nora Katharina Schnorrenberger, Mark Coburn, Kay Wilhelm Nolte, Nadine Gaisa, Joachim Weis, Katharina Siepmann, Martin Häusler, Rolf Rossaint, Michael Fries. 2010. HYDROGEN SULFIDE DOES NOT INCREASE RESUSCITABILITY IN A PORCINE MODEL OF PROLONGED CARDIAC ARREST. *Shock* **34**:2, 190-195. [CrossRef]
36. Serena Benedetti, Claudia Canino, Gaetana Tonti, Virginia Medda, Piergiorgio Calcaterra, Giuseppe Nappi, Fausto Salaffi, Franco Canestrari. 2010. Biomarkers of oxidation, inflammation and cartilage degradation in osteoarthritis patients undergoing sulfur-based spa therapies. *Clinical Biochemistry* **43**:12, 973-978. [CrossRef]
37. Vsevolod Telezhkin, Stephen P. Brazier, Sebastien H. Cayzac, William J. Wilkinson, Daniela Riccardi, Paul J. Kemp. 2010. Mechanism of inhibition by hydrogen sulfide of native and recombinant BKCa channels. *Respiratory Physiology & Neurobiology* **172**:3, 169-178. [CrossRef]

38. Guanghua Tang, Lingyun Wu, Rui Wang. 2010. Interaction of hydrogen sulfide with ion channels. *Clinical and Experimental Pharmacology and Physiology* **37**:7, 753-763. [CrossRef]
39. Sushil K. Jain , Rebeca Bull , Justin L. Rains , Pat F. Bass , Steven N. Levine , Sudha Reddy , Robert McVie , Joseph A. Bocchini , Jr. . 2010. Low Levels of Hydrogen Sulfide in the Blood of Diabetes Patients and Streptozotocin-Treated Rats Causes Vascular Inflammation?. *Antioxidants & Redox Signaling* **12**:11, 1333-1337. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
40. Peter W. Henderson, Andrew L. Weinstein, Josephine Sung, Sunil P. Singh, Vijay Nagineni, Jason A. Spector. 2010. Hydrogen Sulfide Attenuates Ischemia-Reperfusion Injury in In Vitro and In Vivo Models of Intestine Free Tissue Transfer. *Plastic and Reconstructive Surgery* **125**:6, 1670-1678. [CrossRef]
41. Ji-Gang Pan, Hai-Yan Hu, Jie Zhang, Hua Zhou, Li Chen, Yu-Hong Tang, Yu Zheng. 2010. Protective effect of hydrogen sulfide on hypoxic respiratory suppression in medullary slice of neonatal rats. *Respiratory Physiology & Neurobiology* **171**:3, 181-186. [CrossRef]
42. Hideo Kimura . 2010. Hydrogen Sulfide: From Brain to Gut. *Antioxidants & Redox Signaling* **12**:9, 1111-1123. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
43. A.S. Tay, L.F. Hu, M. Lu, P.T.H. Wong, J.S. Bian. 2010. Hydrogen sulfide protects neurons against hypoxic injury via stimulation of ATP-sensitive potassium channel/protein kinase C/extracellular signal-regulated kinase/heat shock protein90 pathway. *Neuroscience* **167**:2, 277-286. [CrossRef]
44. Peter W. Henderson, Sunil P. Singh, Daniel Belkin, Vamsi Nagineni, Andrew L. Weinstein, Jacob Weissich, Jason A. Spector. 2010. Hydrogen Sulfide Protects Against Ischemia-Reperfusion Injury in an In Vitro Model of Cutaneous Tissue Transplantation1. *Journal of Surgical Research* **159**:1, 451-455. [CrossRef]
45. Csaba SzaboMedicinal Chemistry and Therapeutic Applications of the Gasotransmitters NO, CO, and H 2 S and their Prodrugs . [CrossRef]
46. Yuka Kimura , Yu-Ichi Goto , Hideo Kimura . 2010. Hydrogen Sulfide Increases Glutathione Production and Suppresses Oxidative Stress in Mitochondria. *Antioxidants & Redox Signaling* **12**:1, 1-13. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
47. Boon Hian Tan, Peter T.-H. Wong, Jin-Song Bian. 2010. Hydrogen sulfide: A novel signaling molecule in the central nervous system. *Neurochemistry International* **56**:1, 3-10. [CrossRef]
48. Alma Martelli, Lara Testai, Maria Cristina Breschi, Corrado Blandizzi, Agostino Virdis, Stefano Taddei, Vincenzo Calderone. 2010. Hydrogen sulphide: novel opportunity for drug discovery. *Medicinal Research Reviews* n/a-n/a. [CrossRef]
49. G. F. Sitdikova, E. V. Gerasimova, N. N. Khaertdinov, A. L. Zefirov. 2009. Role of cyclic nucleotides in effects of hydrogen sulfide on the mediator release in frog neuromuscular junction. *Neurochemical Journal* **3**:4, 282-287. [CrossRef]
50. Alejandro K. Samhan-Arias, Miguel A. Garcia-Bereguiain, Carlos Gutierrez-Merino. 2009. Hydrogen sulfide is a reversible inhibitor of the NADH oxidase activity of synaptic plasma membranes. *Biochemical and Biophysical Research Communications* **388**:4, 718-722. [CrossRef]
51. Daniele Mancardi, Claudia Penna, Annalisa Merlino, Piero Del Soldato, David A. Wink, Pasquale Pagliaro. 2009. Physiological and pharmacological features of the novel gasotransmitter: Hydrogen sulfide. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1787**:7, 864-872. [CrossRef]
52. Norihiro Shibuya , Makiko Tanaka , Mikiharu Yoshida , Yuki Ogasawara , Tadayasu Togawa , Kazuyuki Ishii , Hideo Kimura . 2009. 3-Mercaptopyruvate Sulfurtransferase Produces Hydrogen Sulfide and Bound Sulfane Sulfur in the Brain. *Antioxidants & Redox Signaling* **11**:4, 703-714. [Abstract] [Full Text PDF] [Full Text PDF with Links]
53. Matthew Whiteman, Philip K. Moore. 2009. Hydrogen sulfide and the vasculature: a novel vasculoprotective entity and regulator of nitric oxide bioavailability?. *Journal of Cellular and Molecular Medicine* **13**:3, 488-507. [CrossRef]
54. Anna Sparatore, Elena Perrino, Valerio Tazzari, Daniela Giustarini, Ranieri Rossi, Giuseppe Rossoni, Kati Erdman, Henning Schröder, Piero Del Soldato. 2009. Pharmacological profile of a novel H2S-releasing aspirin. *Free Radical Biology and Medicine* **46**:5, 586-592. [CrossRef]
55. Mari Ishigami , Keiko Hiraki , Ken Umemura , Yuki Ogasawara , Kazuyuki Ishii , Hideo Kimura . 2009. A Source of Hydrogen Sulfide and a Mechanism of Its Release in the Brain. *Antioxidants & Redox Signaling* **11**:2, 205-214. [Abstract] [Full Text PDF] [Full Text PDF with Links]
56. Neetu Tyagi , Karni S. Moshal , Utpal Sen , Thomas P. Vacek , Munish Kumar , William M. Hughes Jr. , Soumi Kundu , Suresh C. Tyagi . 2009. H2S Protects Against Methionine–Induced Oxidative Stress in Brain Endothelial Cells. *Antioxidants & Redox Signaling* **11**:1, 25-33. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]

57. S Benedetti, F Benvenuti, G Nappi, N A Fortunati, L Marino, T Aureli, S De Luca, S Pagliarani, F Canestrari. 2009. Antioxidative effects of sulfurous mineral water: protection against lipid and protein oxidation. *European Journal of Clinical Nutrition* **63**:1, 106-112. [CrossRef]
58. D J Lefer. 2008. Potential importance of alterations in hydrogen sulphide (H2S) bioavailability in diabetes. *British Journal of Pharmacology* **155**:5, 617-619. [CrossRef]
59. Z FU, X LIU, B GENG, L FANG, C TANG. 2008. Hydrogen sulfide protects rat lung from ischemia–reperfusion injury. *Life Sciences* **82**:23-24, 1196-1202. [CrossRef]
60. L ROCHETTE, C VERGELY. 2008. Le sulfure d'hydrogène (H2S), un gaz endogène à l'odeur d'œuf pourri, pourrait être un régulateur des fonctions cardiovasculaires. *Annales de Cardiologie et d'Angéiologie* **57**:3, 136-138. [CrossRef]
61. Miguel Angel García-Bereguiaín , Alejandro Khalil Samhan-Arias , Francisco Javier Martín-Romero , Carlos Gutiérrez-Merino . 2008. Hydrogen Sulfide Raises Cytosolic Calcium in Neurons Through Activation of L-Type Ca2+ Channels. *Antioxidants & Redox Signaling* **10**:1, 31-42. [Abstract] [Full Text PDF] [Full Text PDF with Links]
62. K. Qu, S.W. Lee, J.S. Bian, C.-M. Low, P.T.-H. Wong. 2008. Hydrogen sulfide: Neurochemistry and neurobiology. *Neurochemistry International* **52**:1-2, 155-165. [CrossRef]
63. Winnie W. Pong, Renata Stouracova, Nina Frank, Jan P. Kraus, William D. Eldred. 2007. Comparative localization of cystathionine #-synthase and cystathionine #-lyase in retina: Differences between amphibians and mammals. *The Journal of Comparative Neurology* **505**:2, 158-165. [CrossRef]
64. Ken Umemura , Hideo Kimura . 2007. Hydrogen Sulfide Enhances Reducing Activity in Neurons: Neurotrophic Role of H2S in the Brain?. *Antioxidants & Redox Signaling* **9**:11, 2035-2042. [Abstract] [Full Text PDF] [Full Text PDF with Links]
65. Chang-qing CHEN, Hong XIN, Yi-zhun ZHU. 2007. Hydrogen sulfide: third gaseous transmitter, but with great pharmacological potential. *Acta Pharmacologica Sinica* **28**:11, 1709-1716. [CrossRef]
66. Csaba Szabó. 2007. Hydrogen sulphide and its therapeutic potential. *Nature Reviews Drug Discovery* **6**:11, 917-935. [CrossRef]