ANTIOXIDANTS & REDOX SIGNALING
Volume 11, Number 1, 2009

DOI: 10.1089/ars.2008.2073

Original Research Communication

H_2S Protects Against Methionine–Induced Oxidative Stress in Brain Endothelial Cells

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Abstract

Homocysteine (Hcy) causes cerebrovascular dysfunction by inducing oxidative stress. However, to date, there are no strategies to prevent Hcy-induced oxidative damage. Hcy is an H_2S precursor formed from methionine (Met) metabolism. We aimed to investigate whether H_2S ameliorated Met-induced oxidative stress in mouse brain endothelial cells (bEnd3). The bEnd3 cells were exposed to Met treatment in the presence or absence of NaHS (donor of H_2S). Met-induced cell toxicity increased the levels of free radicals in a concentration-dependent manner. Met increased NADPH-oxidase-4 (NOX-4) expression and mitigated thioredxion-1(Trx-1) expression. Pretreatment of bEnd3 with NaHS (0.05 m*M*) attenuated the production of free radicals in the presence of Met and protected the cells from oxidative damage. Furthermore, NaHS enhanced inhibitory effects of apocynin, *N*-acetyl-l-cysteine (NAC), reduced glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), N^{ω}-nitro-l-arginine methyl ester (L-NAME) on ROS production and redox enzymes levels induced by Met. In conclusion, the administration of H_2S protected the cells from oxidative stress induced by hyperhomocysteinemia (HHcy), which suggested that NaHS/H_2S may have therapeutic potential against Met-induced oxidative stress. *Antioxid. Redox Signal.* 11, 25–33.

Introduction

Methionine (Met) is an essential amino acid and is metabolized to homocysteine (Hcy), a sulfhydryl-containing nonprotein amino acid (32). It has been suggested that hyperhomocysteinemia (HHcy) is an independent risk factor for neurodegenerative diseases such as dementia, Alzheimer's disease (AD) and stroke (5, 16, 22, 28, 29, 33). Previous studies have shown that Hcy is critically involved in the pathogenesis of neurodegenerative disorders (11, 12). These unfavorable vascular effects of Hcy are believed to be due to one or both of the following: generation of reactive oxygen species (ROS) [including hydrogen peroxide (H_2O_2) and superoxide anion (O_2-) (10, 20, 47)], and a decrease in endothelial nitric oxide (NO) bioavailability (27, 31, 37) that play a critical role in endothelial cell damage and dysfunction.

Although hydrogen sulfide (H_2S) has been recognized as a toxic gas, recent H_2S research has been focused on its protective role in cardiovascular disease conditions. Like nitric oxide (NO) and carbon monoxide (CO) (40, 41), which are considered two gaseous transmitters, H_2S has been shown to be the third gaseous transmitter (39); moreover, H_2S plays important roles in several diseases. NO, CO, and H_2S share distinct properties which qualify them as gasotransmitters: a) they are small molecules of gas; b) they are freely permeable across membrane, do not act via specific membrane receptors; c) they are produced enzymatically (17). H_2S has been demonstrated to stimulate heme oxygenase expression and CO production and has bidirectional effects on the inducible NO synthase (23). However, much less is known about the physiological role of H_2S.

H_2S is endogenously generated in various mammalian tissues from Met–Hcy–Cys metabolism through the action of cystathione-β-synthetase (CBS) and/or cystathionine γ-lyase (CSE) enzymes (21). H_2S is a toxic gas and may act as a functional regulator in the nervous and cardiovascular systems

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(14). Although its neuromodulatory role has been demonstrated, little is known about its protective role in oxidative stress. Interestingly, recent studies have shown that H_2S is neuroprotective (4, 5, 18, 19). Physiological concentrations of H_2S in plasma have been reported to be between 45 μM and 300 μM (48, 50). At physiological concentrations, H_2S inhibits smooth muscle cell proliferation via the mitogen-activated protein kinase pathway and protects the following tissues/cells against oxidative stress: neurons, cardiomyocytes, pancreatic β-cells, and vascular smooth muscle cells (19.3). H_2S induces apoptosis by activating ERK and pro-caspase-3 (44). It has also been established that H_2S directly opens the K_{ATP} channel and causes reduction of vasorelaxation and transient blood pressure (49). However, the role of H_2S in the regulation of oxidative stress in endothelial cells is still unclear. In addition, H_2S acts as an endogenous scavenger for reactive oxygen species and reactive nitrogen species (RNS) (8, 9, 42). Therefore, the purpose of the present study was to determine a potential role of H_2S in preventing Met-induced oxidative damage in bEnd3 endothelial cells by modulating the production of ROS/RNS.

Materials and Methods

Materials

DCFH-DA(2′,7′-dichlorodihydrofluoresceindiacetate), DL-methionine (Met), Dulbecco's modified Eagle's medium (DMEM), dimethyl sulfoxide (DMSO), *N*-acetyl-l-cysteine (NAC), sodium hydrosulfide (NaHS), reduced glutathione (GSH), catalase (CAT), apocynin, superoxide dismutase (SOD), N^{ω}-nitro-l-arginine methyl ester (L-NAME), and DL-propargylglycine (PAG) were purchased from Sigma Chemical Company (St. Louis, MO). Specific antibodies against NOX-4 and Trx-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fetal bovine serum (FBS), phosphate-buffered saline (PBS), penicillin, and streptomycin were obtained from Gibco (Grand Island, NY). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), superoxide dismutase (SOD) assay kit were obtained from Cayman Chemical (Ann Arbor, MI).

Methods

Cell culture. bEnd3, an immortalized mouse brain endothelial cell line originally generated in 1990 (26), now commercially available at American Type Culture Collection, Manassas (ATCC, VA), was grown according to the supplier's instructions in DMEM supplemented with 4.5 g/l glucose, 3.7 g/l sodium bicarbonate, 4 m*M* glutamine, 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, pH 7.4. Cells were maintained in a humid chamber at 37°C in an atmosphere of 95% air and 5% CO_2 in 25 cm^2 tissue culture flasks (38). Confluent 25 cm^2 flasks were trypsinized and seeded at a density of 0.5–1.0 $\times$ 10^4 cells/cm^2 on to 6–12-well cell culture plate, and allowed to grow to ~70%–80% confluence.

Cell viability assay. Cell viability was determined by an MTT assay as originally described by Mosmann (27). In brief, bEnd3 cells were plated at a density of 10^5 cells/well on to 96-well tissue culture plates and incubated with Met (0.114 –2.3 m*M*), NaHS (0.05 –0.5 m*M*), and 500 μM PAG (inhibitor of CSE) in serum-free DMEM/F12 at 37°C for 24 h. Then, 10 μl of MTT reagent (5 mg/ml) was added to each well, and the plates were incubated for another 4 h. The medium was removed and wells were rinsed twice with PBS. To each well, 100 μl of crystal dissolving solution was added at room temperature to dissolve the formazan crystals for 5 min. The absorbance was measured at 570 nm with a spectramax3000 plate reader (Molecular devices, Sunnyvale, CA).

Measurements of homocysteine. Homocysteine was measured by using high pressure liquid chromatography (HPLC) as described earlier (33). HPLC analyses were performed using Class-VP 5.0 chromatograph (Shimadzu, Tokyo, Japan) containing a LC-10ADvp pump, a SIL-10ADvp auto-injector, a CTO-10Avp column oven, and a SPD-10Avp detector. The temperature inside the column was maintained at 37°C during analyses.

Sample preparation. Culture supernatants were collected and centrifuged to remove cell debris. To determine the Hcy level in the supernatants, 200 μl of supernatant was diluted with 100 μl of water and then 300 μl of 9 *M* urea (pH 9.0) was added. Fifty microliters of *n*-amyl alcohol was added to the solution as an antifoaming agent. Reduction of disulfides and cleavage of the protein-bound sulfur-containing amino acids was performed by the addition of 50 μl of $NaBH_4$ solution (10%, wt/vol) in 0.1 *N* NaOH. To perform the reaction, samples were incubated in a water bath at 50°C for 30 min. Samples were cooled at room temperature, and the reaction was stopped by the addition of 500 μl of 20% trichloroacetic acid. The proteins were separated by centrifugation for 4 min at 12,000 *g*, and supernatants were filtered using a 0.45 μm Millipore filter (32).

Measurement of H_2S production. bEnd3 (10^5 cells/well) were grown briefly in a 10 cm^2 dish. Cells became confluent following 24 h of treatment with Met at different concentrations. H_2S concentration in bEnd3 cells was measured as described previously (7, 48, 50).

Intracellular fluorescence measurement of reactive oxygen species. In order to measure the oxidized DCF levels in cells, we used the probe, 2′-7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA), as described previously (36). This membrane-permeable probe enters the cells and produces a fluorescent signal after intracellular oxidation by ROS. bEnd3 (10^5 cells/well) were grown briefly in 96-well plates. Cells were grown to confluence and treated for 24 h in serum-free DMEM/F12 media with or without the following: Met, NaHS, other various agents at different concentrations. The cells were washed with PBS, loaded with probe, DCFH-DA (10 μM), and incubated in dark for 2 h at 37°C in PBS. Thereafter, the cells were washed three times with PBS to remove the excess probe. Oxidized DCF was quantified by monitoring the DCF fluorescence intensity with excitation at 485 nm and emission at 530 nm with a spectra-max3000 plate reader (Molecular devices). Values were expressed in arbitrary units.

In situ labeling of ROS. bEnd3 cells were grown on 8-well cover glass plates and serum starved before the treatments with the following: 1.14 m*M* Met, 0.05 m*M* NaHS, or 1.14 m*M* Met^+ 0.05 m*M* NaHS for 24 h. Ros formation was visualized as described previously (36, 37).

Measurement of intracellular superoxide levels. The intracellular superoxide anion radicals were detected using superoxide dismutase (SOD) assay, using a kit from Cayman Chemicals. Briefly, bEnd3 were plated and treated with different agents in serum free DMEM/F12 media at 37°C for 24 h. Then cells were scraped with a rubber policeman and sonicated in cold 20 m*M* HEPES buffer, pH 7.2, containing 1 m*M* EGTA, 210 m*M* mannitol, and 70 m*M* sucrose, according to the manufacturer's recommendation. SOD activity was determined by spectrophotometry as the ability to inhibit the reduction of nitroblue tetrazolium (NBT) induced by xanthine-xanthine oxidase (1).

Immunoblot analysis. Cells were lysed in ice-cold-modified RIPA lysis buffer (Tris-HCl, 50 m*M*, pH 7.4; NP-40, 1%; 0.25% Na-deoxycholate, 150 m*M* NaCl; 1 m*M* EDTA; 1 m*M* PMSF; 1 μg/ml each of aprotinin, leupeptin, pepstatin; 1 m*M* Na_3VO_4; 1 m*M* NaF). Protein content of the lysate was determined using BCA protein assay (Pierce, Rockford, IL) kit. Protein samples were mixed with 1:1 vol/vol ratio with 2X sample loading buffer [800 μl glycerol, 1 ml 0.5 m*M* Tris-HCl (pH 6.8), 1.6 ml 10% (wt/vol) SDS, 400 μl 2-mercaptoethanol, 400 μl 0.05% (wt/vol) Bromophenol blue], boiled at 95–100°C for 5 min. Samples were cooled to room temperature and centrifuged to precipitate cell debris. Equal amounts of protein (20 μg) for each group were resolved by 10–15% SDS-PAGE. Protein was then electrophoretically transferred to a nitrocellulose membrane (BioRad, Hercules, CA). Transferred protein was blocked with 5% nonfat dry milk in TBS-T (50 m*M* Tris-HCl, 150 m*M* NaCl, 0.1% Tween-20, pH 7.4) for 1 h at room temperature. The blot was then incubated with appropriate primary antibody in blocking solution according to the supplier's specific instructions. Next, the blot was washed with TBS-T three times for 10 min each. The blots were incubated with appropriate horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature. Four more 10 min washes were performed, and ECL Plus substrate (Amersham Biosciences, Pittsburgh, PA) was applied to the blot for 5 min. The blot was developed using X-ray film (RPI Corp, Inc., Mount Prospect, IL) with a Kodak 2000A developer (Eastman Kodak, Rochester,NY). Image analysis was performed using UMAX PowerLock II (Taiwan, Republic of China).

Data analysis and statistics. Results were expressed as means ± SEM from at least seven independent experiments. Both paired and unpaired Student's *t* tests were used, where appropriate, for comparing the mean values between control and tested groups. The difference between mean values of multiple groups was analyzed by one-way analysis of variance (ANOVA), followed by a Scheffe's post-hoc analysis. Statistical significance was considered at $p < 0.05$. The arbitrary densitometry units (AU) were represented as percentage relative to control.

Results

Effect of NaHS on Met-induced cytotoxicity

To determine the protected role of hydrogen sulfide on Met-induced cytotoxicity, bEnd3 were cultured with Met with or without NaHS (donor of H_2S) at different concentrations for 24 h. The cell death was significantly increased in Met (0.114–2.3 m*M*) and NaHS (0.25–0.5 m*M*)-treated cells after 24 h compared to untreated cells. This effect was not observed when cells were treated with lower concentrations of Met or NaHS (Fig. 1A and B). The increase in cell death by high Met (1.14 m*M*) was attenuated by the pre-treatment with NaHS (0.05 and 0.1 m*M*), respectively (Fig. 1C). But this effect was reduced significantly by PAG (inhibitor of CSE) pretreatment (Fig. 1D).

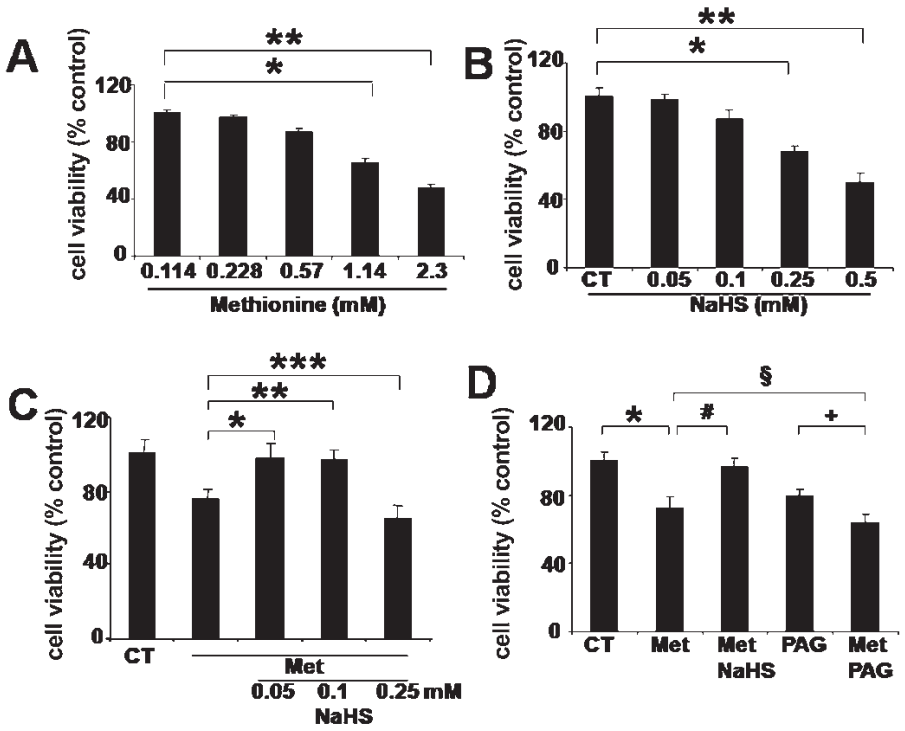

FIG. 1. Effect of hydrogen sulfide on Met–induced cytotoxicity. (**A**) Viability of bEnd3 cells treated with different concentrations of Met for 24 h; $n = 7$; $^{*}p < 0.05$; $^{**}p < 0.01$. (**B**) Cell viability measured after treatment with different concentrations of NaHS for 24 h; $n = 7$; $^{*}p < 0.05$; $^{**}p < 0.01$. (**C** and **D**) Cells were incubated with Met (1.14 m*M*) for 24 h in the presence or absence of NaHS or PAG; in (**C**) $n = 7$ in each group; $^{*}p < 0.05$; $^{**}p < 0.05$; $^{***}p < 0.05$. In (**D**), $n = 7$ in each group, $^{*}p < 0.05$; $^{\#}p < 0.05$; $^{\$}p < 0.01$; $^{+}p < 0.01$.

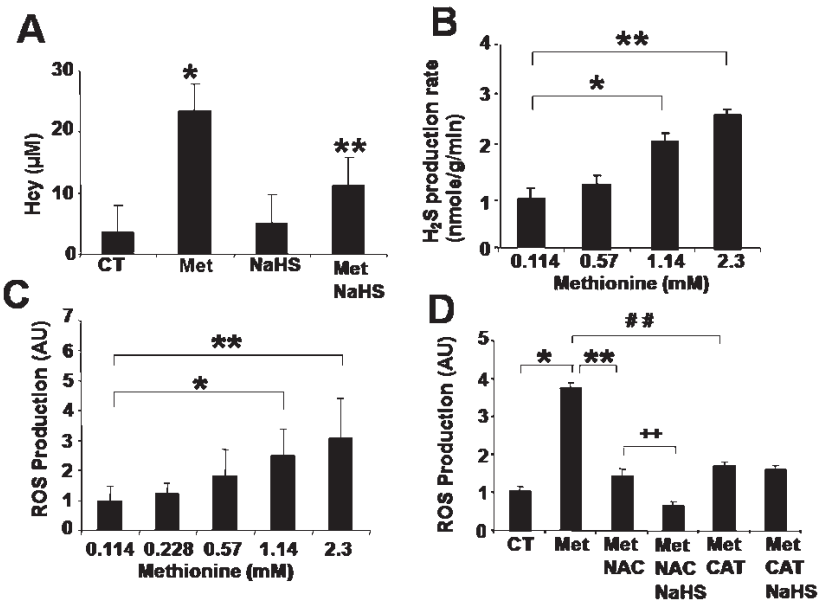

FIG. 2. Effect of hydrosulfide on homocysteine accumulation. (**A**) Data from chromatography of homocysteine from cell culture medium. The effect of the addition of Met (1.14 m*M*) and NaHS (0.05 m*M*) on Hcy levels; $^{*}p < 0.05$ compared to control (CT), $^{**}p < 0.01$compared to Met. (**B**) The measurement of H_2S in culture media after treatment with different concentrations of Met for 24 h. The cells were collected and homogenized to measure the H_2S production rate. $N = 7$ for each group, $^{*}p < 0.05$; $^{**}p < 0.01$. (**C**) ROS production, detected by 5-(6)-chloromethyl-2′,7′-dichlorodihydroflurorescein diacetate (DCFH-DA) staining after incubation of cells with Met at various concentrations for 24 h, $^{*}p < 0.05$; $^{**}p < 0.01$. (**D**) Effect of hydrogen sulfide on Met-induced reactive oxygen species (ROS) production in bEnd3 cells, ROS production was detected after treatment with Met (1.14 m*M*) in the presence or absence of NAC (50 μM), CAT (5 μ/ml) with or without NaHS, respectively; $n = 7$ for each group, $^{*}p < 0.05$; $^{**}p < .01$; $^{\#\#}p < 0.01$; $^{++}p < 0.01$.

Role of NaHS in Hcy accumulation

Cell culture medium was collected after 24 h from control (0.114 m*M* Met), high (1.14 m*M* Met), NaHS (0.05 m*M*), and 1.14 m*M* Met + NaHS (0.05 m*M*) treated cells. The medium was analyzed by HPLC. There was increased accumulation of Hcy in the high Met (1.14 m*M* Met)-treated group compared to that in control (Fig. 2A). Hcy levels were 3.6 ± 0.5, 23.38 ± 3.4, 5.1 ± 0.6, and 11.3 ± 1.2 μM in control Met, high Met, NaHS, and high Met + NaHS treated cells, respectively. These levels were similar and largely comparable with *in vivo* studies (4). The increase in Hcy accumulation in the high Met group was attenuated by NaHS treatment, which suggested that H_2S was a potent inhibitor of Hcy formation.

H_2S levels

To determine the effect of endogenously generated H_2S, bEnd3 cells were treated with different concentrations (0.114–2.3 mM) of Met for 24 h. H_2S production rate was markedly increased in a concentration-dependent manner (Fig. 2B).

NaHS attenuated Met-induced increases in intracellular ROS

Because the cytotoxicity of Met is known to be mediated mainly by oxidative stress, we investigated whether NaHS affects ROS formation by high Met using DCFH-DA fluorescence. Incubation of cells with different concentrations (0.114–2.3 m*M*) of Met for 24 h resulted in significant increases in ROS production in comparison with untreated cells (Fig. 2C). When the cells were treated with Met (1.14 m*M*) for different time periods (6, 12, 24 h), the 24 h treatment showed significant increases in ROS production (data not shown). When cells were incubated with antioxidant (NAC) or H_2O_2 scavenger (CAT), there were marked decreases in ROS production induced by the treatment with Met (1.14 m*M*, Fig. 2D). Furthermore, addition of NaHS (0.05 m*M*) significantly increased the inhibitory effects of NAC on Met-induced ROS production, while the effect could not be found in the CAT-treated group.

Interestingly, *in-situ* labeling (Fig. 3A–D) showed that the levels of intracellular ROS were increased in cells treated

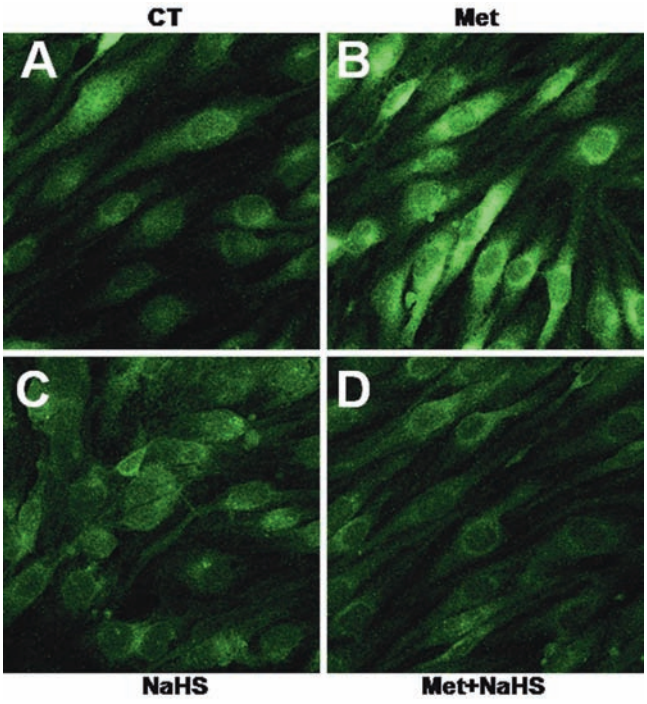

FIG. 3. *In situ* labeling of ROS. bEnd3 cells were grown on glass 8-well chambers and exposed to different treatments for 24 h. ROS production was evaluated by staining the cells with DCFH-DA. Images were acquired by laser confocal microscope (FluoView 1000) at an excitation of 488 nm and emission of 525 nm representative micrograph. (**A**) Control; (**B**) 1.14 m*M* Met; (**C**) 0.05 m*M* NaHS, (**D**) 1.14 m*M* Met + 0.05 m*M* NaHS; magnification 100×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

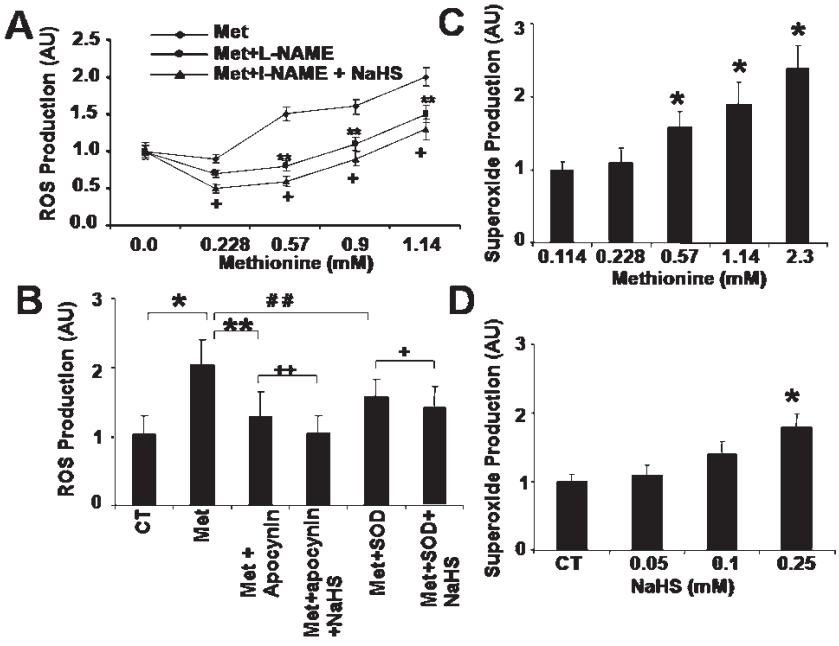

FIG. 4. Effect of H_2S on Met-induced generation of peroxynitrite ($ONOO^-$). (**A**) The bEnd3 cells were treated with Met 1.14 m*M*, Met 1.14 m*M* + L-NAME (100 μM) or Met (1.14 m*M*), + L-NAME (100 μM) + NaHS (0.05 m*M*) for 24 h and ROS were detected by using DCFH-DA probe; $^{**}p < 0.05$ compared to corresponding Met treatment. $^{+}p < 0.05$ compared to Met and L-NAME (100 μM) treatment. (**B**) ROS production was detected after bEnd3 cells were incubated for 24 h with Met (1.14 m*M*) in the presence or absence of apocynin (100 μM) or SOD (200 U/ml), with or without NaHS (0.05 m*M*), respectively. $N = 7$ in each group; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{\#\#}p < 0.01$; $^{++}p < 0.05$; $^{+}p < 0.05$. (**C**) Met-induced superoxide ($O_2^{\cdot -}$) production and effect of H_2S in bEnd3: $O_2^{\cdot -}$ production was measured after bEnd3 cells were treated with different concentrations of Met for 24 h. (**D**) $O_2^{\cdot -}$ production was determined in the treated cells after 24 h incubation with NaHS at different concentrations. $N = 7$ for each group. $^{*}p < 0.05$ compared to control.

with Met (1.14 m*M*, Fig. 3B), indicated by the increases in DCFH-DA fluorescence. However, when cells were treated with both NaHS (0.05 m*M*) and Met (1.14 m*M*), DCFH-DA fluorescence was decreased (Fig. 3D). This suggested that Met-induced intracellular ROS accumulation was attenuated by hydrogen sulfide. Cells treated with NaHS (0.05 m*M*) alone showed weak DCFH-DA fluorescence (Fig. 3C), similar to that of untreated cells (Fig. 3A).

NaHS attenuated Met-induced peroxynitrite formation

The generation of $ONOO^-$ requires rapid interaction of NO and $O_2^{\cdot -}$. To determine the effects of the interaction of Met and NaHS on $ONOO^-$ formation, cells were treated with different concentrations (0.114–2.3 m*M*) of Met for 24 h in the presence or absence of the NOS inhibitor, L-NAME (100 μM), NADPH oxidase inhibitor apocynin (100 μM), $O_2^{\cdot -}$ scavenger SOD (200 U/ml), with or without NaHS (0.05 m*M*). Figure 4A shows a concentration-dependent increase in Met-induced $ONOO^-$ formation. However, when bEnd3 cells were co-treated with NaHS, Met (1.14 m*M*), apocynin, or SOD, it ameliorated the inhibitory effect of apocynin, SOD, and L-NAME, respectively (Fig. 4B).

NaHS attenuated Met-induced superoxide anion production

To determine the protected role of hydrogen sulfide on Met-induced superoxide anion, we examined the release of superoxide anion by chemiluminescence assay. Figure 4C

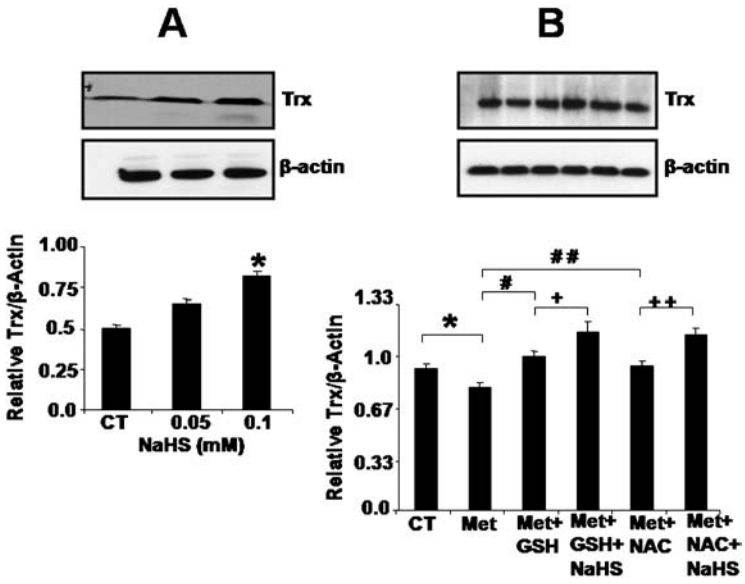

FIG. 5. Effect of hydrogen sulfide on Met-suppressed thioredoxin (Trx) expression. (**A**) bEnd3 cells were cultured and treated with different concentrations (0.05–0.1 m*M*) of NaHS alone for 24h; $^{*}p < 0.05$ compared to control. (**B**) bEnd3 cells were treated with Met (1.14 m*M*) in the presence or absence of the antioxidant NAC (50 μM), GSH (1 m*M*), with or without NaHS, respectively, for 24 h. Trx protein was measured in cell lysates by Western blot analysis, and membranes were stripped and reprobed with β-actin for equal loading. *Bottom*: graphical presentation of Trx (fold change over control). $N = 7$ for each group. $^{*}p < 0.05$ *vs.* untreated cells, $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$ vs. cells treated with Met in. $^{+}p < 0.05$ *vs.* cells treated with Met + NAC, $^{++}p < 0.05$ *vs.* cells treated with Met + GSH.

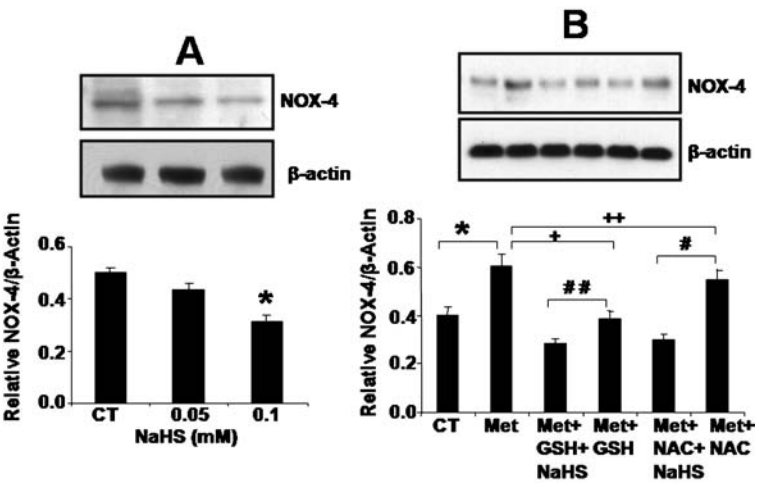

FIG. 6. Effect of hydrogen sulfide on Met-Induced NOX-4 expression. (**A**) Serum-starved bEnd3 cells were treated for 24 h with different concentration (0.05–0.1 m*M*) of NaHS alone for 24 h; $^{*}p < 0.05$ compared to control. (**B**) Serum-starved bEnd3 cells were either left untreated or were treated with Met (1.14 m*M*) in the presence or absence of the antioxidant NAC (50 μM), GSH (1 m*M*) with or without NaHS, respectively, for 24 h. After treatment, cell lysates were analyzed with 10–15% SDS-PAGE and subsequently by Western blot analysis; membranes were stripped and reprobed with β-actin for equal loading. *Bottom*: graphical presentation of NOX-4 (fold change over control). $N = 7$ for each group, $^{*}p < 0.05$ *vs.* untreated cells. $^{+}p < 0.05$; $^{++}p < 0.05$ *vs.* cells treated with Met group. $^{\#}p < 0.05$ *vs.* cells treated with Met + NAC, $^{\#\#}p < 0.01$ *vs.* cells treated with Met + GSH.

shows that 24 h of incubation with Met (1.14 m*M*) significantly increased $O_2^{\cdot -}$ levels in a concentration-dependent manner. The bEnd3 cells were incubated with different concentrations (0.05–0.25 m*M*) of NaHS for 24 h. The $O_2^{\cdot -}$ production was significantly increased only in 0.25 m*M* NaHS-treated cells, as compared to untreated cells (Fig. 4D).

Effect of NaHS on redox enzymes levels

To determine the protective role of hydrogen sulfide on Met-induced imbalance between the redox enzymes, we examined the Trx-1 and NOX-4 protein levels by Western blot analysis. Incubation of cells with 0.05–0.1 m*M* NaHS for 24 h resulted in a statistically significant increase in Trx-1 protein expression level (Fig. 5A) in comparison with untreated cells. Met (1.14 m*M*) alone resulted in a significant decrease in Trx-1 protein expression levels in comparison to the untreated control (Fig. 5B). Simultaneous incubation of Met (1.14 m*M*) with either NAC (50 μM) or GSH (1 m*M*) resulted in a significant increase of the Met downregulated Trx-1 protein expression compared with Met alone. Furthermore, when bEnd3 cells were treated with a combination [Met (1.14 m*M*); Met + NAC; Met + NAC + NaHS; Met + GSH; or Met + GSH + NaHS], the treatment reduced the effect of Met on Trx-1 protein expression as shown in Fig. 5B.

There was a concentration-dependent decrease in NOX-4 protein expression (Fig. 6A) after cells were incubated with NaHS (0.05 or 0.1 m*M*) for 24 h. Met (1.14 m*M*) alone significantly induced NOX-4 protein expression (Fig. 6A). Met-induced NOX-4 protein expression was markedly decreased in cells when they were pretreated with either NAC (50 μM) or GSH (1 m*M*). Furthermore, addition of NaHS (0.05 m*M*) significantly increased the inhibitory effects of NAC and GSH on Met-induced NOX-4 protein production in comparison with that in the absence of NaHS (Fig. 6).

Discussion

Our present study attempted to examine a novel link between the protective role of H_2S towards oxidative stress caused by HHcy in brain endothelial cells. Methionine, an essential amino acid, is converted to Hcy that then promotes neurodegenerative diseases through endothelial dysfunction (24, 25, 34). It is important to note that there are other respondents to increases in ROS, such as HO-1. Homocysteine generates ROS via the auto-oxidation of the thiol group (36, 44) or by decreasing the endothelial heme oxygenase-1 (HO-1) activity (30). The transcriptional upregulation of the HO-1 gene downregulated intracellular ROS (29). H_2S is an endogenous metabolic product of Met by the trans-sulfuration pathway that is dependent on two important enzymes: CBS and CSE (39). It has been demonstrated that H_2S reacts with at least four different ROS, superoxide radical anion, hydrogen peroxide, peroxynitrite, and hypochorite (8, 9, 41). All these compounds are highly reactive and their interaction with H_2S resulted in the protection of proteins and lipids from ROS/RNS-mediated damage (41, 42). Therefore, we hypothesize that H_2S may protect the cerebro-vasculature against Met-induced endothelial damage.

Our present study results show that high Met (1.14 m*M*) significantly decreased bEnd3 cells viability. Expectedly, the addition of NaHS (0.05 or 0.1 m*M*) significantly increased the cell viability as compared to the cells treated with high Met. Although H_2S (<0.1 m*M*) reduced cell viability, this was merely the effect of chemical cytotoxicity rather than physiological effect. But this effect was reduced significantly by PAG pretreatment (Fig. 1D), a result consistent with pre-

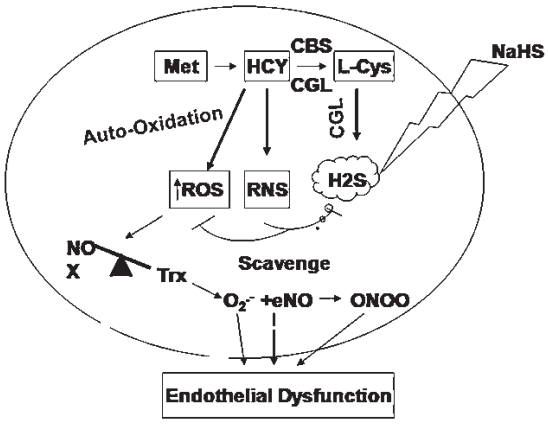

FIG. 7. Schematic presentation of proposed mechanism for the protective role of H_2S towards Met-induced oxidative stress in bEnd3 cells.

vious studies (32, 36, 43). PAG is an inhibitor of CSE, an enzyme responsible for endogenous H_2S formation. We found that, in the presence of PAG, cell viability was decreased in the setting of high Met.

The rapid interaction of superoxide with nitric oxide generated peroxynitrite, a potent mediator of oxidant-induced cellular injury (15, 34). In the present study, we demonstrated that ROS production was increased in a concentration-dependent manner after bEnd3 cells were treated with different doses of Met for 24 h. Met-induced ROS production was effectively blocked by NAC and H_2O_2 scavenger, CAT, and concentration-dependently inhibited by the NOS inhibitor, L-NAME. This provides evidence that Met can induce not only H_2O_2, but also $ONOO^-$ generation in mouse brain endothelial cells (Fig. 2). This finding is supported by the observed inhibitory effect of SOD and the effect of NADPH oxidase inhibitor, apocynin, on ROS formation (Fig. 3). According to previous studies, H_2S worked as a scavenger of oxygen-derived free radicals (9), which could contribute to the protective role of NaHS against the toxicity of H_2O_2 *in vitro* and *in vivo* model (45). We observed that Met-induced O_2^- production was markedly reduced by the O_2^- scavengers (SOD) as well as by NaHS (Fig. 4).

In addition, H_2S enhanced NO production via ERK1/2 activation, which suggested that H_2S may cooperate with NO in modulating their biological effects (13). Our results showed that high levels of H_2S can induce ROS and RNS formation, but low levels of H_2S can decrease H_2O_2, $ONOO^-$, and O_2^- generation induced by Met in bEnd3 cells. Furthermore, our results indicated that low concentrations of H_2S combined with certain agents, such as NAC, apocynin, SOD, or L-NAME, may synergistically increase their antioxidant effects, scavenge ROS, and protect vascular endothelium from Met-induced oxidant stress and cytotoxicity (Fig. 4). This is in contrast to functioning directly as an antioxidant. This protective role of H_2S may be similar to the protection of neurons from oxidative stress (18). The failure of NaHS combined with CAT to reduce Met-induced ROS formation might be explained by the proposed low efficiency of CAT compared with NAC in removing H_2O_2 at low concentrations (46). H_2S protects the cell damage by decreasing the ROS production by increasing SOD. The mechanism of Met-induced oxidative stress is not yet well known. Some studies suggested that Met-induced oxidative stress is possible through an NADPH oxidase-mediated pathway (36). Our results showed that Met (1.14 m*M*) significantly increased NOX-4 expression and, consequently, decreased Trx expression. bEnd3 cells treated with a combination of high Met, antioxidant, and NaHS may synergistically increase Trx expression and decrease NOX-4 expression, in comparison with the group treated with Met alone.

In this study, we employed different approaches to determine the protective role of H_2S on Met–induced oxidative damage. We presented evidence that H_2S is an important modulator of cellular cytotoxicity via redox cell pathways in the pathogenic conditions associated with HHcy. In conclusion, the data presented here provides a new mechanism by which H_2S reduces oxidative damage induced by Met. Thus, H_2S protected bEnd3 cells from oxidative damage. When considering previous studies along with our results with respect to antioxidant activity of H_2S and its effect on Met, we suggest that administration of H_2S might be an interesting potentially preventive strategy for reducing cerebrovascular complications in hyperhomocysteinemia (Fig. 7). However, the molecular mechanisms of a putative protective role of H_2S in neurogenerative pathogenesis should be further investigated.

Limitations

In "mild" human hyperhomocysteinemia (which is associated with an increased neurodegenerative diseases), plasma Hcy levels range from ~15 to 30 μmol/l. However, only a fraction of total plasma Hcy is in the reduced form *in vivo*. The concentrations used in the present study represent a ~100-fold dose. Three ranges of hyperhomocysteinemia are defined as follows: moderate (16–30 μM), intermediate (31–100 μM), and severe (>100 μM) (2, 6). Extracellular thiols are oxidized. Only a fraction of total plasma Hcy is in the reduced form *in vivo* and *in vitro*. NaHS, a donor of H_2S, was used at physiologically relevant concentrations (41).

Abbreviations

bEnd3, mouse brain microvascular endothelial cells; CAT, catalase; CBS, cystathione-β-synthetase; CSE, cystathionine γ-lyase; GSH, reduced glutathione; Hcy, homocysteine; HHcy, hyperhomocysteinemia; H_2S, hydrogen sulfide; L-NAME, N^{ω}-nitro-l-arginine methyl ester; Met, methionine; NAC, *N*-acetyl-l-cysteine; NaHS, sodium hydrogen sulfide; NOX4, NADPH-oxidase-4; PAG, DL-propargylglycine. ROS, reactive oxygen species; SOD, superoxide dismutase; Trx-1 ,thioredxion-1.

Acknowledgments

Supported in part by the National Institutes of Health grants HL-75185, HL-71010, and NS-51568.

References

1. Beauchamp C and Fridovich I. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Anal Biochem* 44: 276–287, 1971.
2. Bonaventura D, Tirapelli CR, Haddad R, Höehr NF, Eberlin MN, and de Oliveira AM. Chronic methionine load-induced hyperhomocysteinemia enhances rat carotid responsiveness for angiotensin II. *Pharmacology* 70: 91–99, 2004.
3. Elrod JW, Calvert JW, Morrison J, Doeller JE, Kraus DW, Tao L, Jiao X, Scalia R, Kiss L, Szabo C, Kimura H, Chow CW, and Lefer DJ. Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. *Proc Natl Acad Sci USA*. 104: 15560–15565, 2007.
4. Dayal S, Wilson KM, Leo L, Arning E, Bottiglieri T, and Lentz SR. Enhanced susceptibility to arterial thrombosis in a murine model of hyperhomocysteinemia. *Blood* 108: 2237–2243, 2006.
5. Faraci FM and Lentz SR. Hyperhomocysteinemia oxidative stress and cerebral vascular dysfunction. *Stroke* 35: 345–347, 2004.
6. Garlick PJ. Toxicity of methionine in humans. *J Nutr.* 136: 1722S–1725S, 2006.
7. Geng B, Cui Y, Zhao J, Fang Yu, Zhu Y, Xu G, Zhang Z, Tang C, and Du J. Hydrogen sulfide downregulates the aortic L-arginine/nitric oxide pathway in rats. *Am J Physiol Regul Integr Comp Physiol* 293: R1608-R1618, 2006.
8. Geng B, Yang J, Qi Y, Zhao J, Pang Y, Du J, and Tang C. H_2S

generated by heart in rat and its effects on cardiac function. *Biochem. Biophys. Res. Commun* 313: 362–368, 2004.
9. Geng B, Chang L, Pan C, Qi Y, Zhao J, Pang Y, Du Y, and Tang C. Endogenous hydrogen sulfide regulation of myocardial injury induced by isoproterenol. *Biochem Biophys Res Commun* 318: 756–763, 2004.
10. Heinecke JW, Rosen H, Suzuki LA, and Chait A. The role of sulfur-containing amino acids in superoxide production and modification of low density lipoprotein by arterial smooth muscle cells. *J Biol Chem* 262: 10098–10103, 1987.
11. Ho PI, Collins SC, Dhitavat S, Ortiz D, Ashline D, Rogers E, and Shea TB. Homocysteine potentiates beta-amyloid neurotoxicity: Role of oxidative stress. *J. Neurochem* 78: 249–253, 2001.
12. James SJ, CutlerP, Melnyk S, Jernigan S, Janak l, Gaylor DW, and Neubrander JA. Metabolic biomarkers of increased oxidative stress and impaired methylation capacity in children with autism. *Am J Clin Nutr* 80: 1611–1617, 2004.
13. Jeong SO, Pae HO, Oh GS, Jeong GS, Lee BS, Lee S, Kim YD, Rhew HY, Lee KM, and Chung HT. Hydrogen sulfide potentiates interleukin-1β-induced nitric oxide production via enhancement of extracellular signal-regulated kinase activation in rat vascular smooth muscle cells. *Biochem Biophys Res Commun* 345: 938–944, 2006.
14. Kamoun P. Endogenous production of hydrogen sulfide in mammals. *Amino acids* 26: 243–254, 2004.
15. Kanani PM, Sinkey CA, Browning RL, Allaman M, Knapp HR, and Haynes WG. Role of oxidant stress in endothelial dysfunction produced by experimental hyperhomocyst(e)inemia in humans. *Circulation* 100: 1161–1168, 1999.
16. Kang SS, Wong PWK, and Malinow MR. Hyperhomocyst(e)inemia as a risk factor for occlusive vascular disease. *Ann Rev Nutr* 12: 279–298, 1992.
17. Kasparek MS, Linden DR, Kreis ME, and Sarr MG. Gasotransmitters in the gastrointestinal tract. *Surgery* 143: 455–459. 2008.
18. Kimura Y and Kimura H. Hydrogen sulfide protects neurons from oxidative stress. *FASEB J* 18: 1165–1167, 2004.
19. Kimura Y, Dargusch R, Schubert D, and Kimura H. Hydrogen sulfide protects HT22 neuronal cells from oxidative stress. *Antioxid Redox Signal* 8: 661–670, 2006.
20. Kotamraju S, Konorev EA, Joseph J, and Kalyanaraman B. Doxorubicin-induced apoptosis in endothelial cells and cardiomyocytes is ameliorated by nitrone spin traps and ebselen. Role of reactive oxygen and nitrogen species. *J Biol Chem* 275: 33585-92, 2000.
21. Levonen AL, Lapatto R, Saksela M, and Raivio KO. Human cystathionine gamma-lyase: Developmental and *in vitro* expression of two isoforms. *Biochem J* 347: 1–5, 2000.
22. Loscalzo J. The oxidant stress of hyperhomocyst(e)inemia. *J Clin Invest* 98: 5–7, 2000.
23. úowicka E and Be towski J. Hydrogen sulfide (H2S)—the third gas of interest for pharmacologists. *Pharmacol Rep* 59: 24, 2007.
24. Mattson MP and Duan W. Apoptotic biochemical cascades in synaptic compartments: roles in adaptive plasticity and neurodegenerative disorders. *J. Neurosci Res* 58:152–166, 1999.
25. Mattson MP, Pedersen WA, Duan W, Culmsee C, and Camandola S. Cellular and molecular mechanisms underlying perturbed energy metabolism and neuronal degeneration in Alzheimer's and Parkinson's diseases. *Ann NY Acad Sci* 893:154–175, 1999.
26. Montesano R, Pepper MS, Möhle–Steinle U, Risau W, Wagner EF, and Orci L. Increased proteolytic activity is responsible for the aberrant morphogenetic behavior of endothelial cells expressing the middle T oncogene. *Cell* 10: 62: 435–45, 1990.
27. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55–63, 1983.
28. Muijsers RB, van Den Worm E, Folkerts G, Beukelman CJ, Koster AS, Postma DS, and Nijkamp FP. Apocynin inhibits peroxynitrite formation by murine macrophages. *Br J Pharmacol* 130: 932–936, 2000.
29. Obeid R and Herrmann W. Mechanisms of homocysteine neurotoxicity in neurodegenerative diseases with special reference to dementia. *FEBS Lett* 580: 2994–3005, 2006.
30. Rodrigo R, Passalacqua W, Araya J, Orellana M, and Rivera G . Homocysteine and essential hypertension. *J Clin Pharmacol* 43: 1299–1306, 2003.
31. Ryter SW and Choi AM. Heme oxygenase-1: Molecular mechanisms of gene expression in oxygen-related stress. *Antioxid Redox Signal* 4: 625–632, 2002.
32. Sawle P, Foresti R, Green CJ, and Motterlini R. Homocysteine attenuates endothelial haem oxygenase-1 induction by nitric oxide (NO) and hypoxia. *FEBS Lett* 23: 508: 403–406. 2001.
33. Selhub J. Homocysteine metabolism. *Annu Rev Med* 19: 217–246, 1999.
34. Sen U, Tyagi N, Kumar M, Moshal KS, Rodriguez WE, and Tyagi SC. Cystathionine-beta-synthase gene transfer and 3-deazaadenosine ameliorate inflammatory response in endothelial cells. *Am J Physiol Cell Physiol* 293: C1779–87, 2007.
35. Stamler JS, Osborne JA, Jaraki O, Rabbani LE, Mullins M, Singel D, and Loscalzo J. Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. *J Clin Invest* 91: 308–318, 1999.
36. Stampfer MJ, Malinow MR, Willett WC, Newcomer LM, Upson B, Ullmann D, Tishler PV, and Hennekens CH. A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians. *J Am Med Assoc* 268: 877–881, 1992.
37. Su JH, Anderson AJ, Cummings BJ, and Cotman CW. Immunohistochemical evidence for apoptosis in Alzheimer's disease. *Neuroreport* 5: 2529–2533, 1994.
38. Tawakol A, Omland T, Gerhard M, Wu JT, and Creager MA. Hyperhomocyst(e)inemia is associated with impaired endothelium-dependent vasodilation in humans. *Circulation* 95:1119–1121, 1997.
39. Tyagi N, Ovechkin AV, Lominadze D, Moshal KS, and Tyagi SC. Mitochondrial mechanism of microvascular endothelial cells apoptosis in hyperhomocysteinemia. *J Cell Biochem* 98: 1150–1162, 2006.
40. Tyagi N, Sedoris KC, Steed M, Ovechkin AV, Moshal KS, and Tyagi SC. Mechanisms of homocysteine-induced oxidative stress. *Am J Physiol Heart Circ Physiol* 289: H2649–H2656, 2005.
41. Tyagi N, Moshal KS, Tyagi SC, and Lominadze D. Gamma aminobutyric acid A receptor mitigates homocysteine-induced endothelial cell permeability. *Endothelium* 14: 315–323, 2007.
42. Wang R. Two's company, three's a crowd: Can H_2S be the third endogenous gaseous transmitter? *FASEB J* 16: 1792–1798, 2002.
43. Wang R, Wang ZZ, and Wu L. Carbon monoxide-induced vasorelaxation and the underlying mechanisms. *Br J Pharmacol* 121: 927–934, 1997.

44. Wang R, Wu L, and Wang ZZ. The direct effect of carbon monoxide on K_{Ca} channels in vascular smooth muscle cells. *PflÅgers Arch* 434: 285–291, 1997.
45. Whiteman M, Cheung NS, Zhu YZ, Chu SH, Siau JL, Wong BS, Armstrong JS, and Moore PK. Hydrogen sulphide: A novel inhibitor of hypochlorous acid-mediated oxidative damage in the brain? *Biochem Biophys Res Commun* 28: 794–798, 2005.
46. Whiteman M, Armstrong JS, Chu SH, Jia–Ling S, Wong BS, Cheung NS, Halliwell B, and Moore PK. The novel neuromodulator hydrogen sulfide: an endogenous peroxynitrite 'scavenger'? *J. Neurochem* 90: 765–768, 2005.
47. Yan SK, Chang T, Wang H, Wu L, Wang R, and Meng QH. Effects of hydrogen sulfide on homocysteine-induced oxidative stress in vascular smooth muscle cells. *Biochem Biophys Res Commun* 15: 485–491, 2006.
48. Yang G, Cao K, Wu L, and Wang R. Cystathionine gamma-lyase overexpression inhibits cell proliferation via a H2S-dependent modulation of ERK1/2 phosphorylation and p21Cip/WAK-1. *J Biol Chem* 279: 49199–49205, 2004.
49. Yonezawa D, Sekiguchi F, Miyamoto M, Taniguchi E, Honjo M, Masuko T, Nishikawa H, and Kawabata A. A protective role of hydrogen sulfide against oxidative stress in rat gastric mucosal epithelium. *Toxicology* 20: 11–18, 2007.
50. Yu BP. Cellular defenses against damage from reactive oxygen species. *Physiol. Rev* 74: 139–162, 1994.
51. Zhang Q, Du J, Zhou W, Yan H, Tang C, and Zhang C. Impact of hydrogen sulfide on carbon monoxide/heme oxygenase pathway in the pathogenesis of hypoxic pulmonary hypertension. *Biochem.Biophys.Res.Commun* 317: 30–37, 2004.
52. Zhao W, Zhang J, Lu Y, and Wang R. The vasorelaxant effect of H_2S as a novel endogenous gaseous K_{ATP} channel opener. *EMBO J* 20: 6008–6016, 2001.
53. Zhong C, Du J, Bu D, Yan H, Tang D, and Tang C. The regulatory effect of hydrogen sulfide on hypoxic pulmonary hypertension in rats. *Biochem Biophys Res Commun* 302: 810–816, 2003.
54. Zhu YZ, Wang ZJ, Ho P, Loke YY, Zhu YC, Huang SH, Tan CS, Whiteman M, Lu J, and Moore PK. Hydrogen sulfide and its possible roles in myocardial ischemia in experimental rats. *J Appl Physiol* 102: 261–268. 2006.

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Date of first submission to ARS Central, March 8, 2008; date of final revised submission, July 16, 2008; date of acceptance, July 16, 2008.