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# Hydrogen Sulfide Attenuates Hyperhomocysteinemia-Induced Cardiomyocytic Endoplasmic Reticulum Stress in Rats

Hongling Wei,[1,*] Rongyuan Zhang,[1,*] Hongfang Jin,[1] Die Liu,[1] Xiuying Tang,[2] Chaoshu Tang,[3,4] and Junbao Du[1,4]

## Abstract

The mechanisms responsible for the cardioprotective effect of hydrogen sulfide ($H_2S$) are unclear. The present study was designed to examine whether $H_2S$ could regulate hyperhomocysteinemia (HHcy)-induced cardiomyocytic endoplasmic reticulum (ER) stress. A rat model of HHcy was produced, and H9c2 cells (rat embryonic heart–derived cell line) were cultured. The plasma homocysteine was measured by using HPLC. Plasma $H_2S$ concentration and myocardial $H_2S$ production were measured with a sulfide-sensitive electrode. Confocal immunofluorescent analysis for cardiomyocytic C/EBP homologous protein (CHOP) was performed. Glucose-regulated protein 78 (GRP78), CHOP, and caspase 12 expressions by myocardial tissues and cleaved caspase 12 and p-eIF2$\alpha$ expressions by H9c2 cells were detected with Western blotting. The results showed that methionine overload induced HHcy, resulting in a marked cardiomyocytic ER stress, whereas endogenous production of $H_2S$ was reduced in rats with HHcy. $H_2S$ supplementation, however, decreased expressions of ER stress–associated proteins, including GRP78, CHOP, and caspase 12, by myocardial tissues *in vivo*. The inhibition of endogenous $H_2S$ production further enhanced cardiomyocytic ER stress, but $H_2S$ supplementation effectively antagonized the H9c2 cell CHOP, cleaved caspase 12 and p-eIF2$\alpha$ expressions induced by Hcy, thapsigargin, or tunicamycin *in vitro*. The results suggest that $H_2S$ can attenuate cardiomyocytic ER stress in HHcy-induced cardiomyocytic injury. *Antioxid. Redox Signal.* 12, 1079–1091.

## Introduction

HOMOCYSTEINE (Hcy) is a sulfur-containing amino acid generated during methionine metabolism. Hyperhomocysteinemia (HHcy) is a pathologic condition in which plasma Hcy metabolism is disrupted (2, 30). Epidemiologic studies have indicated that HHcy is an independent risk factor for cardiovascular complications (3, 20). Severe HHcy is due to genetic defects, resulting in deficiencies in cystathionine-$\beta$-synthase (CBS) or methylenetetrahydrofolate reductase (48). Mild HHcy is due to mild impairment of the methylation pathway, such as deficiency in folate, vitamin $B_6$ or vitamin $B_{12}$ (24). HHcy is also related to several life-style factors (3). Patients with severe HHcy usually are first seen with neurologic abnormalities or premature arteriosclerosis, and cerebral thrombosis or myocardial infarction develops at around the age of 30 years (23). Patients with mild HHcy are more common in the general population (3). Mild HHcy can increase the risk of cardiovascular diseases in a concentration-dependent manner (29, 31).

The mechanisms by which HHcy induces cardiovascular injury are not defined. Studies of cellular and dietary models of HHcy have suggested several mechanisms to explain the cardiovascular changes associated with HHcy. Hcy can cause oxidative injury (26, 40), cellular hypomethylation (50), and inflammatory damage (13). Recently, endoplasmic reticulum (ER) stress was proposed to explain the pathogenic effects of HHcy (5, 25, 43), which may be a common pathway of injury of tissues and cells induced by HHcy. The ER lumen is a unique oxidative environment, critical for protein folding and formation of disulfide bonds (44). Disturbances such as cellular redox alteration, glucose deprivation, aberrant regulation of $Ca^{2+}$, and viral infection may cause accumulation of unfolded proteins in the ER (44). Three ER-associated pathways are

[1]Department of Pediatrics, [2]Department of Electron Microscopy, and [3]Institute of Cardiovascular Research, Peking University First Hospital, Beijing, P.R. China.
[4]Key Laboratory of Molecular Cardiovascular Diseases, Ministry of Education, Beijing, P.R. China.
*These authors contributed equally to this work.

subsequently activated, which leads to ER stress. Excessive and prolonged ER stress can trigger cell death (22). Glucose-regulated protein 78 (GRP78), C/EBP homologous protein (CHOP), and caspase 12 are molecular markers of ER stress. ER stress is considered to be an important mechanism involved in diabetes mellitus, HHcy, neurodegenerative diseases, and viral infections (18).

Hydrogen sulfide ($H_2S$) and Hcy are metabolites of methionine (35), but they exert entirely opposite effects. For example, Hcy stimulated proliferation of vascular smooth muscle cells (VSMCs), whereas $H_2S$ inhibited it (7, 39, 45); Hcy induced formation of reactive oxygen species (ROS), whereas $H_2S$ scavenged ROS (1, 32, 40, 49); Hcy impaired vascular relaxation, whereas $H_2S$ improved it (6). As a gasotransmitter with exclusive biologic effects, $H_2S$ can be endogenously generated by CBS and cystathionine-$\gamma$-lyase (CSE) in mammalian tissues (17, 38, 41). Studies have shown that HHcy-associated damage is due to reduced endogenous generation of $H_2S$. Supplementation of $H_2S$ or the $H_2S$-donor sodium hydrosulfide (NaHS) could ameliorate HHcy-induced injury (1, 6, 32, 40, 49). Lou *et al.* (19) recently reported that $H_2S$ exposure inhibited the elevated expression of ER stress markers in rat gastric ulcer cells. Yang *et al.* (47) showed that $H_2S$ induced the apoptosis of insulin-secreting $\beta$ cells by enhancing ER stress (47).

Given the importance of HHcy in the pathogenesis of cardiovascular disease and altered endogenous production of $H_2S$ in HHcy, it is imperative to understand whether the ER-stress status of cardiomyocytes is regulated by $H_2S$ and to investigate the underlying signaling cascade.

Therefore, the present study was undertaken to examine whether $H_2S$ could regulate hyperhomocysteinemia (HHcy)-induced cardiomyocytic ER stress in a rat model of HHcy *in vivo* and in an H9c2 cell line *in vitro* and investigate the possible mechanisms.

## Materials and Methods

### Animals and preparation of an HHcy model

Animal experimental protocols complied with the Animal Management Guidelines of the People's Republic of China. These protocols were approved by the Animal Research Committee of Peking University (Beijing, China).

Male Wistar rats (180–220 g) were purchased from Vital River (Beijing, China). Rats were housed in cages and fed a standard laboratory diet and fresh water. The cages were kept in a temperature-controlled room ($24 \pm 1^{\circ}$C), at a relative humidity of 65–70%, and a 12-h light/dark cycle.

The induction of HHcy was as described previously (33), with a minor modification. Thirty rats were randomly divided into three groups: (a) control group ($n = 10$), orally administered drinking water by gavage; (b) methionine group ($n = 10$), orally administered methionine solution by gavage (1 g/kg per day); and (c) methionine + NaHS group ($n = 10$), orally administered methionine solution by gavage (1 g/kg per day) plus NaHS by intraperitoneal injection (14 $\mu$mol/kg per day). Rats were maintained for 5 weeks.

### Cell lines and reagents

Rat embryonic heart–derived H9c2 cells were purchased from the American Tissue Culture Collection (Rockville, MD). Methionine, DL-homocysteine, L-cysteine, pyridoxal-5′-phosphate (PLP), DL-propargylglycine (PPG), thapsigargin (TG), and tunicamycin (TN) were from Sigma-Aldrich (St. Louis, MO). The antibody for GRP78 (3183), CHOP (2895), phospho-eukaryotic initiation factor 2 $\alpha$-subunit (eIF2$\alpha$) (3597), and eIF2$\alpha$ (2103) were from Cell Signaling Technology (Boston, MA). The antibody for $\beta$-actin (sc-47778) was from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody for caspase 12 (3282) was from Biovision (Mountain View, CA). Anti-rabbit [horseradish peroxidase (HRP)-labeled] antibody, anti-mouse (HRP-labeled) antibody, and electrochemiluminescence (ECL) Western blotting detection reagents were from Santa Cruz Biotechnology. The x-ray film for Western blotting was from Kodak (Rochester, NY). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), and L-glutamine were from Invitrogen (Carlsbad, CA).

### Cell culture

Cells were cultured in DMEM supplemented with 10% FCS, 2 m*M* L-glutamine, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin. They grew in a humidified atmosphere of 5% $CO_2$ at 37°C. Cells were made quiescent by incubation with serum-free DMEM for 24 h before use. At 70 to 80% confluence, cells were cultured with media supplemented with 0, 0.1, 0.5, 1.0, or 2.5 m*M* DL-homocysteine for 6 h. In experiments using donors or inhibitors of $H_2S$, cells were pretreated for 30 min with 0, 0.1, 0.5, and 1 m*M* NaHS or 0, 1, 2 and 5 m*M* PPG, respectively, and then supplemented with 2.5 m*M* DL-homocysteine for 6 h. Meanwhile, 10 $\mu M$ TG or 1.5 $\mu$g/ml TN was the positive control to induce ER stress.

### Measurement of the total concentration of Hcy in plasma

Blood was collected from the abdominal aortae of fasting rats after 5 weeks. The blood was promptly cooled on ice and centrifuged at 3,000 *g* for 20 min at 4°C. Serum was stored at −20°C until use. The total level of Hcy in plasma was measured with high-performance liquid chromatography (HPLC), as described (4). In brief, serum was reduced for 30 min at 4°C. Deproteinization was undertaken with 10% trichloroacetic acid. After centrifugation, the clear supernatant was mixed with NaOH, borate buffer (pH 9.5), and 7-fluorobenzo-2-oxa-1, 3-diazole-4-sulfonate. After derivatization at 60°C (1 h), the sample was analyzed with HPLC.

### Measurement of plasma $H_2S$ level

Plasma samples were obtained from abdominal aortae by using heparinized syringes and were immediately centrifuged. Plasma $H_2S$ concentration was determined with a sulfide-sensitive electrode (ELIT 8225; Electro Analytic Instruments Ltd., London, United Kingdom) and an ELIT Ion Analyzer (ELIT 9801; Electro Analytic Instruments), as previously described (11). In brief, 0.5 ml of antioxidant buffer (NaOH, 2.35 *M* and EDTA, 0.27 *M*) was added to standard $S^{2-}$ solution (10, 20, 30, 40, 50, 60, and 80 $\mu M$, respectively) or blood samples in the same volume and then well mixed. After being rinsed with distilled water and dried before each measurement, the electrode was immersed into mixtures in turn to record the electrode potential (voltage value, mV). Plasma $H_2S$ concentration was calculated

according to the standard-curve plotting by voltage value *versus* concentration on the basis of standard $S^{2-}$ solution.

### *Determination of $H_2S$ production rate in the heart*

$H_2S$ production rate was measured as described previously (11), with a slight modification. In brief, myocardium was homogenized in 50 m*M* ice-cold potassium phosphate buffer (pH 6.8). Then 0.5 ml of 10% (wt/vol) myocardium homogenates was added in the same volume reaction mixture containing 100 m*M* potassium phosphate buffer (pH 7.4), 10 m*M* L-cysteine, and 2 m*M* pyridoxal 5′-phosphate in a conical flask. A small center well contained 1 *M* NaOH to absorb $H_2S$. The flasks were then flushed with a constant stream of $N_2$ for 10 min before being sealed with a double layer. The catalytic reaction was initiated by transferring the flasks from an ice bath to a 37°C shaking water bath. After incubation at 37°C for 90 min, the reactions were stopped by adding 0.5 ml of 50% trichloroacetic acid to the mixture. Then the flasks were incubated in the shaking water bath for an additional 60 min at 37°C to ensure complete trapping of $H_2S$. The content from the center wells was with an antioxidant buffer in the test tubes. The $H_2S$ content was measured in a same way. The $H_2S$ production rate was expressed in nmol/(min/mg protein).

### *Ultrastructural detection by transmission electron microscopy*

Ultrastructural detection was carried out as described previously (27). In brief, myocardial tissues and H9c2 cells (final concentration, $1\times10^6$) were fixed in 2% glutaraldehyde overnight at 4°C. They were subsequently fixed with 1% glutaraldehyde and 1% osmic acid for 1 to 2 h. Specimens were dehydrated with acetone and embedded. They were cut into sections of 100 nm in thickness. Replicas were floated onto distilled water and picked up on 400-mesh copper grids. These were examined by using a JEM 1230 electron microscope (JEOL Ltd., Tokyo, Japan).

### *Western blot analysis*

Hearts were homogenized in lysis buffer (50 m*M* Tris-HCL, pH 7.5; 150 m*M* NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate), including a protease inhibitors and a phosphatase inhibitor. Cultured H9c2 cells were harvested and lysed in the same lysis buffer. After centrifugation at 13,000 *g*, supernatants were boiled in Laemmli loading buffer and separated with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). They were then transferred onto a nitrocellulose membrane, as described previously (10). The membrane was blocked with 5% skim milk in Tris-buffered saline (TBS) for 1 h at room temperature. The membrane was incubated with primary antibody at 4°C for 14 to 18 h. Appropriate HRP-labeled secondary antibodies were coupled with the blots for another 1 h at room temperature. The developed signal was visualized by using an enhanced chemiluminescence detection kit and quantified with AlphaImager (San Leandro, CA).

### *Immunfluorescence microscopy*

H9c2 cells were cultured on cover slides to 50% confluence, treated with Hcy (2.5 m*M*) for 6 h, and pretreated with NaHS or PPG for 30 min. TG and TN were the positive controls. Immunfluorescence was conducted as described previously (15). Cells were washed thrice with ice-cold phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for 20 min. They were blocked with 5% bovine serum albumin (BSA) in PBS containing 1% Triton X-100. CHOP antibody

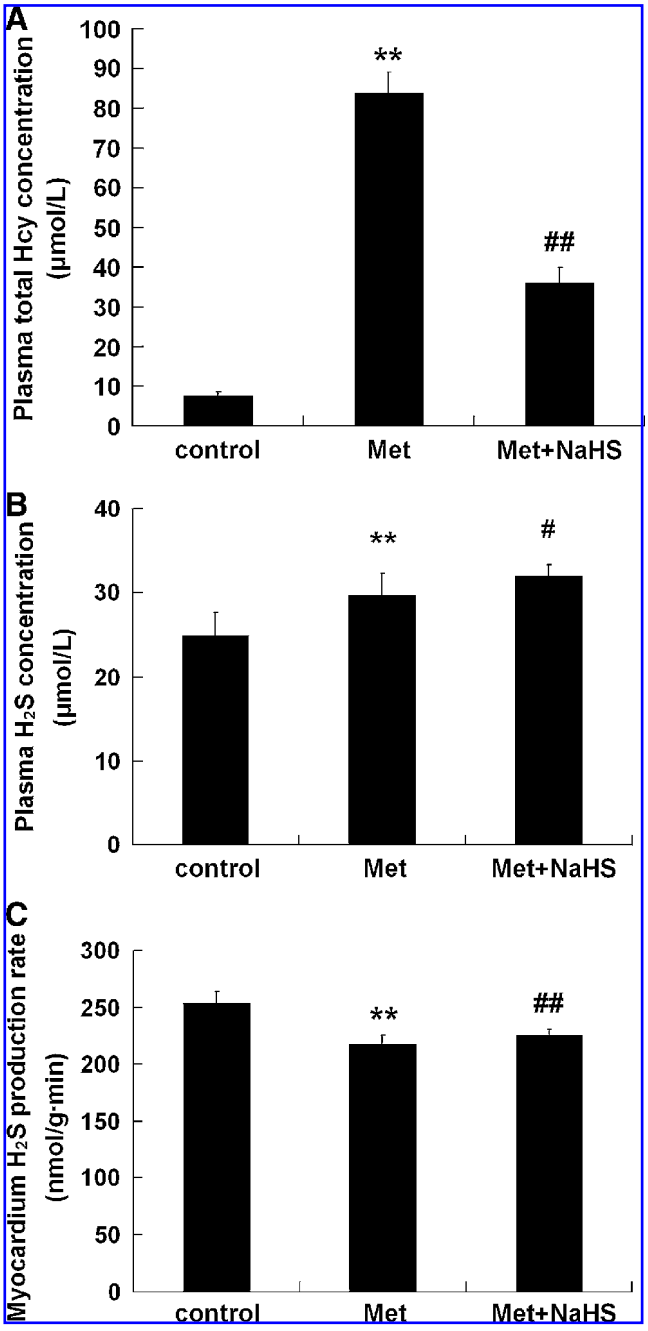


**FIG. 1. Excessive methionine supplementation induced HHcy and impaired the $H_2S$-production pathway.** (**A**) Plasma total Hcy level. (**B**) Plasma $H_2S$ concentration. (**C**) Myocardial $H_2S$ production rate. $H_2S$ = hydrogen sulfide; Met = methionine; NaHS = sodium hydrosulfide. $^{**}p < 0.01$ compared with control group, $^{\#}p < 0.05$; $^{\#\#}p < 0.01$ compared with methionine group.

was visualized by using fluorescein isothiocyanate (FITC)-conjugated anti-rabbit secondary antibody. Nuclei and actin filaments were marked with Hoechst 33324 and phalloidin, respectively.

### *Statistical analysis*

Data are expressed as mean ± SD. Statistical analysis was determined with one-way ANOVA followed by the least significant difference test or Dunnett's *post hoc* test. A value of $p < 0.05$ was considered significant. The analysis was performed by using SPSS 13.0 (Chicago, IL).

## Results

### *Effect of methionine supplementation on total Hcy concentration in the plasma of rats*

Plasma levels of Hcy increased by 10.3-fold ($p < 0.01$) for rats in the methionine group compared with rats in the control group (Fig. 1A). Plasma levels of Hcy decreased by 57% ($p < 0.01$) for rats in the methionine + NaHS group compared with rats in the methionine group (Fig. 1A).

### *Effect of HHcy on plasma $H_2S$ concentration and myocardial production rate in rats*

As compared with rats in the control group, plasma levels of $H_2S$ increased by 19% in rats of the methionine group ($p < 0.01$) (Fig. 1B), and the myocardial production rate of $H_2S$ decreased by 14% ($p < 0.01$) (Fig. 1C). Compared with that in HHcy rats, supplementation with the $H_2S$ donor NaHS increased plasma levels of $H_2S$ by 8% ($p < 0.05$) (Fig. 1B); and the myocardial production rate of $H_2S$ increased by 14% ($p < 0.01$) (Fig. 1C).

### *$H_2S$ supplementation ameliorated myocardial tissue injury in HHcy rats*

Ultrastructural examination of the myocardium in rats with HHcy demonstrated that, in the control group, cardiomyo-

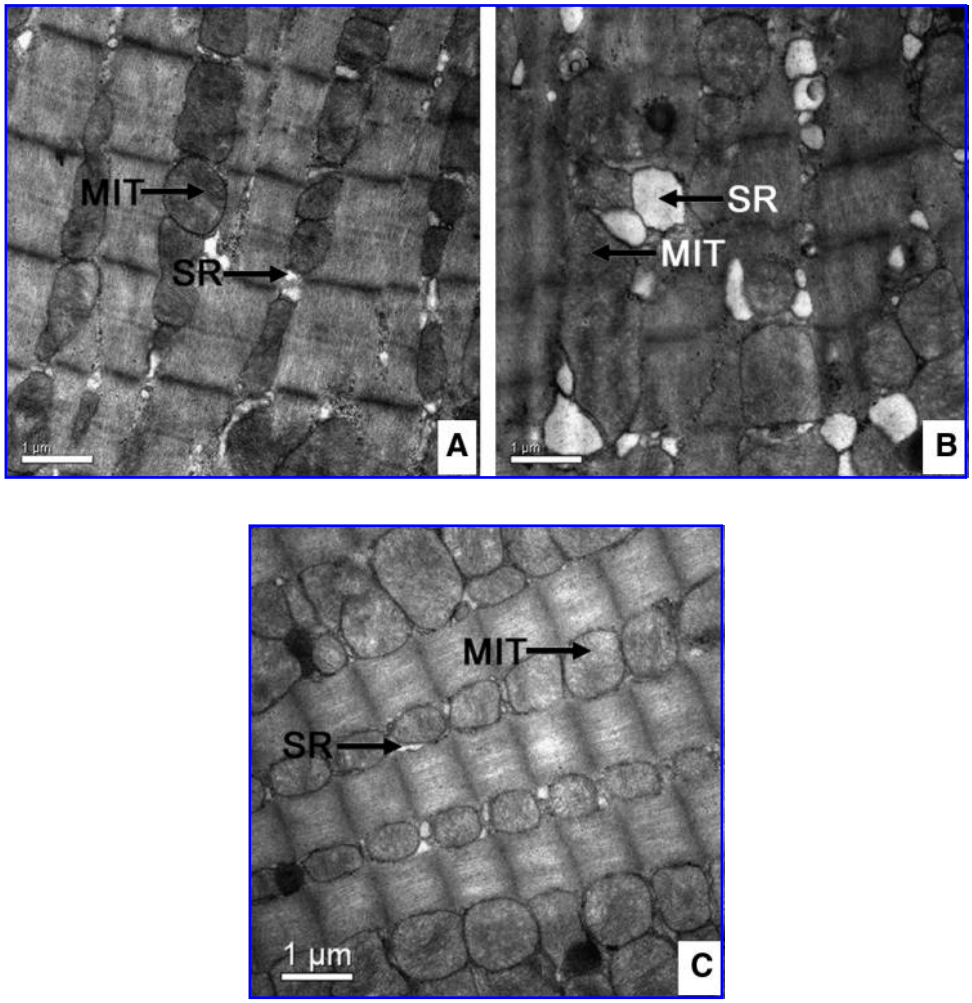


**FIG. 2. $H_2S$ supplementation ameliorated cardiomyocyte injury in HHcy rats.** (**A**) In the control group, the cardiomyocytes were lined up in an orderly manner; the structure of myocardial mitochondria was clear; and the SR was normal. (**B**) In the methionine group, the SR was obviously dilated; mitochondria, swollen; and mitochondria cristae, fractured and dissolved. (**C**) In the methionine + NaHS group, the SR was not dilated, and the structure of mitochondria was clear. MIT = mitochondria; SR = sarcoplasmic reticulum.

cytes were arranged in a line, the structure of myocardial mitochondria was clear, and the sarcoplasmic reticulum (SR) was normal (Fig. 2A). In the methionine group, the myocardial SR was clearly dilated, the mitochondria swollen, and mitochondria cristae fractured and dissolved (Fig. 2B). In the methionine + NaHS group, the myocardial SR was not dilated, and the structure of mitochondria was clear (Fig. 2C).

### *$H_2S$ supplementation decreased myocardial expression of GRP78, CHOP, and caspase 12 in rats with HHcy*

Compared with that of rats in the control group, the expression level of: myocardial GRP78 was increased by 56%; cleaved caspase 12 was increased by 94%; and CHOP was increased by 3.3-fold in rats of the methionine group (all $p < 0.01$). For rats in the methionine + NaHS group compared with rats in the methionine group, myocardial GRP78 expression was reduced by 22% ($p < 0.05$) (Fig. 3), cleaved caspase 12 expression was decreased by 42% ($p < 0.01$) (Fig. 4), and CHOP expression was decreased by 16% ($p < 0.01$) (Fig. 5).

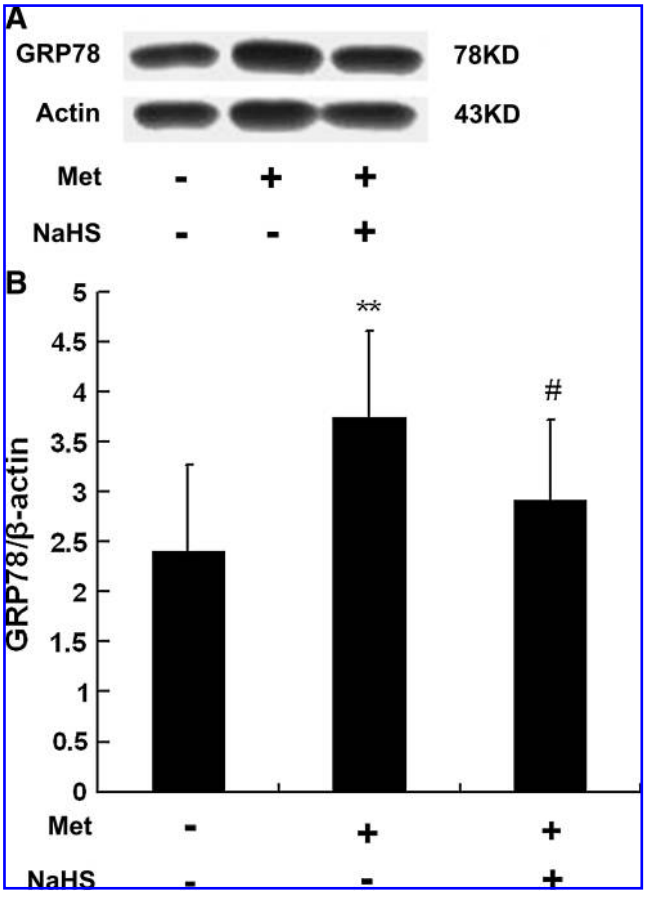


**FIG. 3. $H_2S$ supplementation ameliorated GRP78 expression in the myocardium in rats with HHcy.** (**A**) Myocardial tissue protein (80 $\mu$g) was separated on 8% SDS–PAGE and subjected to Western-blot analysis; relative GRP78 (78 kDa) expression was counted by comparing the gray intensity and the area of bands with $\beta$-actin (43 kDa). (**B**) The $H_2S$ donor NaHS ameliorated GRP78 expression. GRP78 = glucose-regulated protein 78. $^{**}p < 0.01$ compared with control group; $^{\#}p < 0.05$ compared with methionine group.

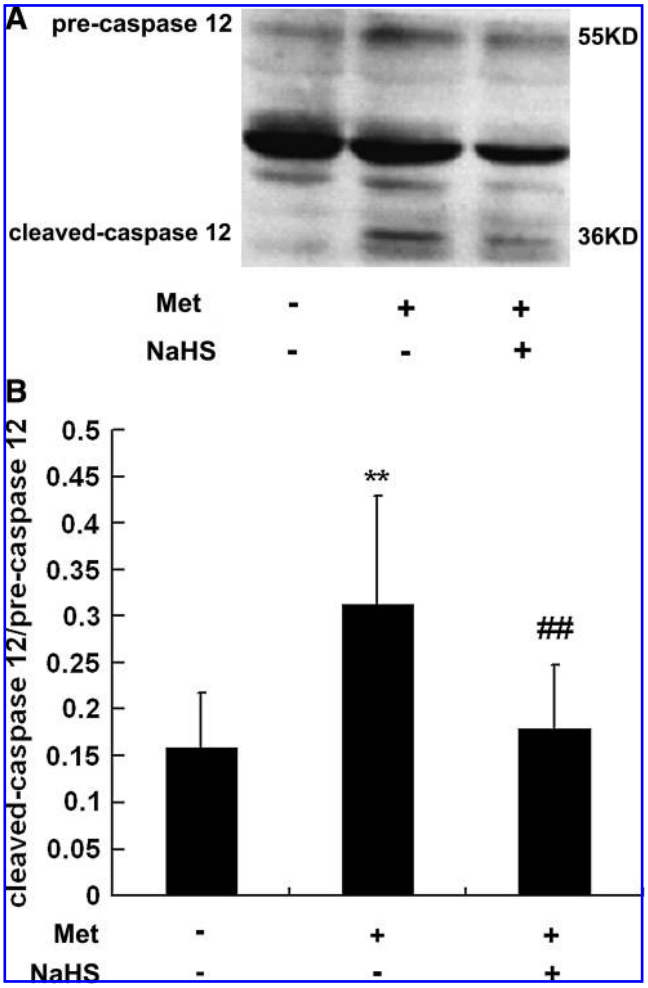


**FIG. 4. $H_2S$ supplementation ameliorated expression of cleaved caspase 12 in the myocardium of rats with HHcy.** (**A**) Myocardial tissue protein (80 $\mu$g) was separated on 10% SDS–PAGE and subjected to Western-blot analysis; cleaved caspase 12 (36 kDa) expression was counted by comparing gray intensity and area of bands with precaspase 12 (55 kDa). (**B**) The $H_2S$ donor NaHS ameliorated expression of cleaved caspase 12. $^{**}p < 0.01$ compared with control group; $^{\#\#}p < 0.01$ compared with methionine group.

### *$H_2S$ supplementation directly ameliorated injury to H9c2 cells induced by HHcy*

We carried out *in vitro* experiments to elucidate the direct effect of $H_2S$ on Hcy-induced cell injury. Myocardial ultrastructural examination showed that $H_2S$ donors directly ameliorated myocardial cell injury induced by Hcy.

In the control group, the nuclear contour was a regular ellipse, the nuclear envelope was clear and smooth, the karyoplasmic ratio was uniform, and chromatin was distributed uniformly. Organelles were abundant, the structure of mitochondria was clear, and the ER showed flat vesicles that were lined up in a regular manner (Fig. 6A).

In the HHcy group, cells were treated with 2.5 m*M* Hcy, and incubation was allowed to proceed for 6 h. The nuclear contour was deformed, the nuclear envelope fractured, and chromatin aggregated into a block structure. Organelles were

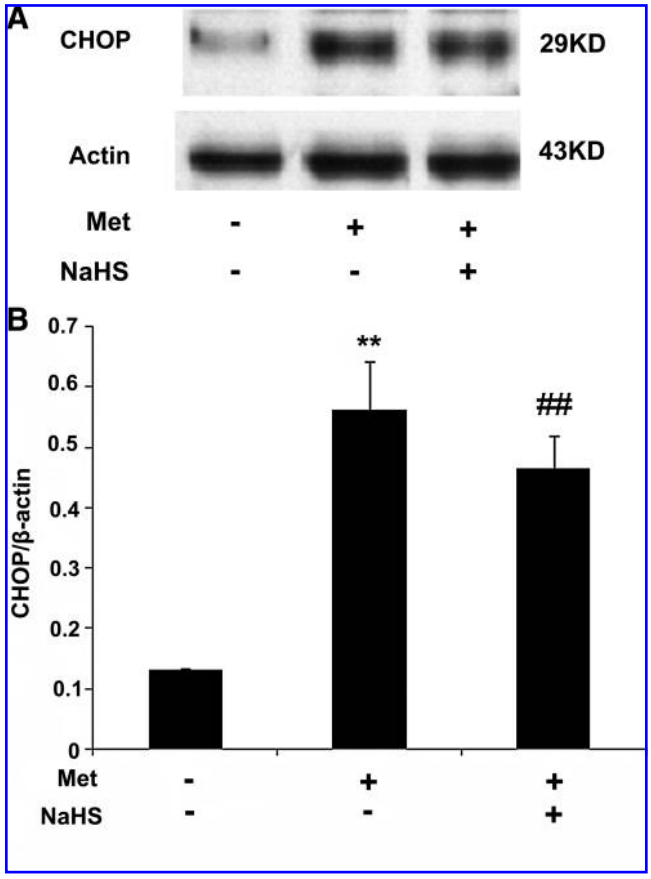


**FIG. 5. $H_2S$ supplementation ameliorated CHOP expression in the myocardium of rats with HHcy.** (**A**) Myocardial tissue protein (80 $\mu$g) was separated on 12% SDS–PAGE and subjected to Western-blot analysis; cleaved CHOP (29 kDa) expression was counted by comparing gray intensity and area of bands with $\beta$-actin (43 kDa). (**B**) The $H_2S$ donor NaHS ameliorated expression of cleaved CHOP. CHOP = C/EBP homologous protein. $^{**}p < 0.01$ compared with control group; $^{\#\#}p < 0.01$ compared with methionine group.

clearly deformed and arranged erratically, mitochondria were swollen, and mitochondria cristae fractured and dissolved. The ER was significantly dilated and showed round vesicles, and apoptotic bodies could be observed (Fig. 6B).

In the TN group, 1.5 $\mu$g/ml TN was added to cells, and the cells were incubated for 6 h to induce ER stress. The morphology of the nuclear contour was normal, the nuclear envelope was intact, and the chromatin was aggregated. Organelles were clearly deformed and arranged erratically, and vacuoles occupied the sites of internal structures in the organelles. Mitochondria were swollen, the ER significantly dilated and seen as round vesicles. Apoptotic bodies could be seen (Fig. 6C).

In the TG group, 10 $\mu M$ TG was added to cells, and cells were incubated for 6 h to induce ER stress. The nuclear contour was deformed, the nuclear envelope fractured, and chromatin aggregated into a block structure. Organelles were deformed, the mitochondria swollen, and the ER significantly dilated. Apoptotic bodies could be seen (Fig. 6D).

In the HHcy + NaHS group, cells were pretreated with the $H_2S$ donor NaHS for 30 min, followed by 2.5 m$M$ Hcy, and incubation was allowed to proceed for 6 h. The nuclear envelope was complete, and chromatin did not aggregate. Mitochondria were slightly swollen, and the ER was dilated (Fig. 6E).

In the HHcy + PPG group, cells were pretreated with the $H_2S$ production enzyme (CSE) inhibitor PPG for 30 min, followed by 2.5 m$M$ Hcy, and incubation allowed to proceed for 6 h. The nuclear contour was deformed, the nuclear envelope fractured, and chromatin aggregated into a block structure. Organelles were clearly deformed, mitochondria severely swollen, and the ER significantly dilated (Fig. 6F).

### *$H_2S$ supplementation directly decreased CHOP expression induced by Hcy in H9c2 cells*

Confocal immunofluorescent analysis of H9c2 cells showed that $H_2S$ donors directly decreased CHOP expression induced by Hcy. CHOP antibody was used to detect CHOP expression (green); Hoechst 33324 was used to detect nuclear expression (blue); and actin filaments were labeled with phalloidin (red).

In control cells, little CHOP expression was found in the nucleus (Fig. 7A). CHOP expression in cells treated with 2.5 m$M$ Hcy for 6 h was clearly increased (Fig. 7B). CHOP expression in cells given 1.5 $\mu$g/ml TN or 10 $\mu M$ TG for 6 h was increased (Fig. 7C and D). CHOP expression was reduced in cells pretreated with the $H_2S$ donor NaHS for 30 min and then administrated 2.5 m$M$ Hcy for 6 h (Fig. 7E). CHOP expression was enhanced in cells pretreated with CSE inhibitor PPG for 30 min and then administrated 2.5 m$M$ Hcy for 6 h (Fig. 7F).

### *$H_2S$ supplementation directly decreased expression of cleaved caspase 12 induced by Hcy in H9c2 cells*

Western blot analysis showed that administration of 1.0 m$M$ and 2.5 m$M$ Hcy increased the expression of cleaved caspase 12 by 30.2% and 55.9% (Hcy 1.0 m$M$, $p < 0.05$; Hcy 2.5 m$M$, $p < 0.01$) (Fig. 8B). Inhibition of endogenous production of $H_2S$ by PPG could increase the expression of cleaved caspase 12. PPG at concentrations of 1, 2, and 5 m$M$ increased the expression of cleaved caspase 12 by 9.9%, 19.8%, and 17.8% (for PPG at 1.0 m$M$, $p < 0.05$; for PPG at 2.5 m$M$, $p < 0.01$; and for PPG at 5 m$M$, $p < 0.01$) (Fig. 8C). NaHS at concentrations of 0.1 m$M$ and 0.5 m$M$ decreased the expression of cleaved caspase 12 by 25.8% and 45.5%, respectively (all $p < 0.01$) (Fig. 8D).

### *$H_2S$ supplementation directly decreased expression of p-eIF2$\alpha$ induced by Hcy in H9c2 cells*

Western blot analysis revealed that administration of 0.1 m$M$, 0.5 m$M$, 1.0 m$M$, and 2.5 m$M$ Hcy increased p-eIF2$\alpha$ expression by 13.6%, 17.6%, 21.7%, and 35.4%, respectively (all $p < 0.01$) (Fig. 9B). Inhibition of endogenous production of $H_2S$ by PPG could increase p-eIF2$\alpha$ expression. PPG at concentrations of 1 m$M$, 2 m$M$, and 5 m$M$ increased p-eIF2$\alpha$ expression by 8.3%, 10.5% and 19.7% (for PPG at 1.0 m$M$, $p < 0.05$, for PPG at 2.5 m$M$, $p < 0.05$, and for PPG at 5 m$M$, $p < 0.01$), respectively (Fig. 9C). NaHS at concentrations of 0.1 m$M$ and 0.5 m$M$ decreased p-eIF2$\alpha$ expression by 6.8% and 20.6% (for NaHS 0.1 m$M$, $p < 0.05$, and for NaHS 0.5m$M$, $p < 0.01$), respectively (Fig. 9D).

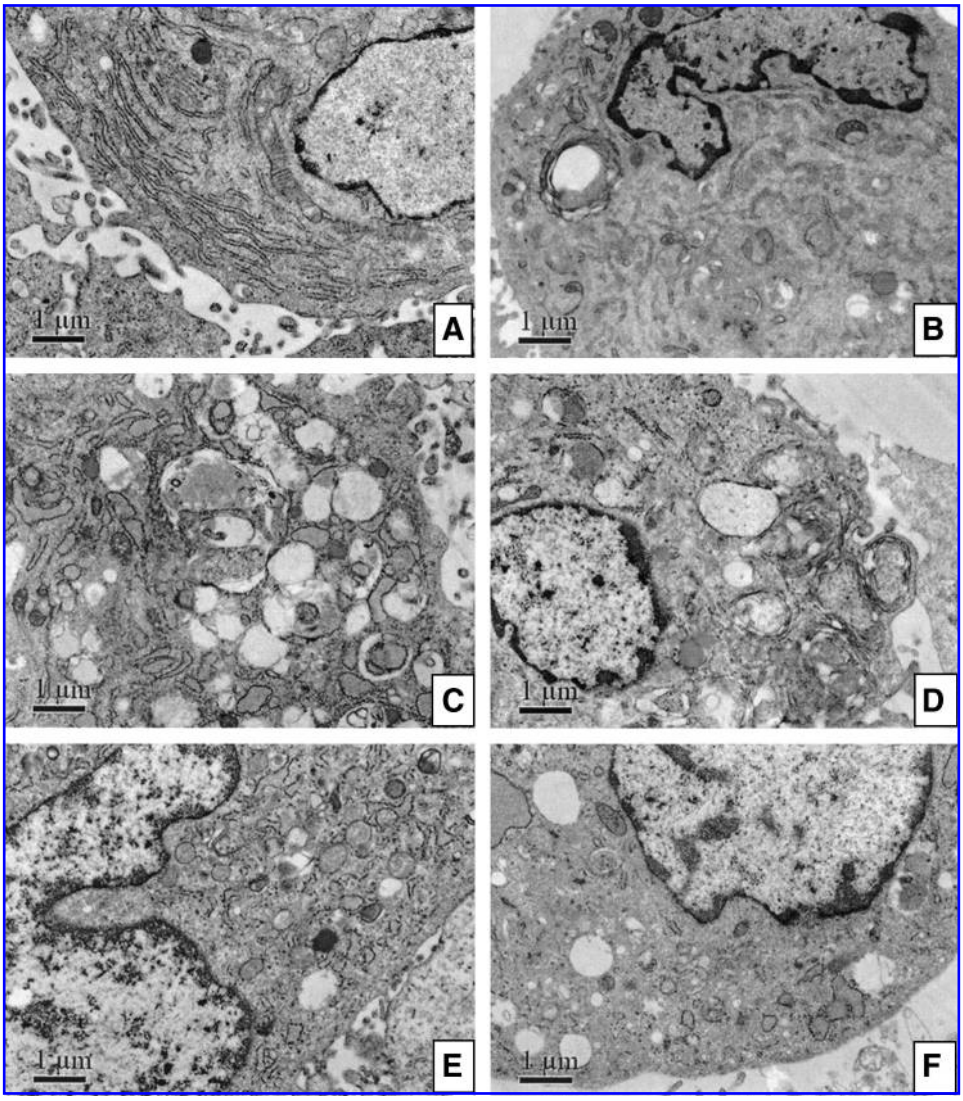


**FIG. 6. $H_2S$ supplementation directly ameliorated injury to myocardial cells induced by HHcy.** Ultrastructural detection was undertaken by using transmission electron microscopy. (**A**) In control cells, the nuclear contour was seen as a regular ellipse; the nuclear envelope was clear and smooth; the karyoplasmic ratio was uniform; and chromatin was uniformly distributed. Organelles were abundant; the structure of mitochondria was clear; and the ER demonstrated flat vesicles that were lined up in a regular array. (**B**) Cells were treated with 2.5 m*M* DL-homocysteine for 6 h. The nuclear contour was deformed; the nuclear envelope, fractured; and chromatin, aggregated into a block structure. Organelles were obviously deformed and arranged erratically; mitochondria were swollen; and mitochondria cristae fractured and dissolved. The ER was significantly dilated and with round vesicles; apoptotic bodies could be seen. (**C**) Cells were administered with 1.5 $\mu$g/ml TN for 6 h to induce ER stress. The morphology of the nuclear contour was normal; the nuclear envelope was intact; and chromatin aggregated. Organelles were obviously deformed and arranged erratically; vacuoles occupied the sites of internal structures in the organelles. Mitochondria were swollen; the ER was significantly dilated and demonstrated round vesicles; apoptotic bodies could be observed. (**D**) Cells were administered 10 $\mu M$ TG for 6 h to induce ER stress. The nuclear contour was deformed; the nuclear envelope, fractured; and chromatin, aggregated into a block structure. Organelles were obviously deformed; mitochondria, swollen; the ER, significantly dilated; and apoptotic bodies could be seen. (**E**) Cells were pretreated with the $H_2S$ donor NaHS for 30 min, followed by 2.5 m*M* DL-homocysteine for 6 h. The nuclear envelope was complete; chromatin did not aggregate; the mitochondria were slightly swollen; and the ER was dilated. (**F**) Cells were pretreated with the $H_2S$ inhibitor PPG for 30 min, followed by 2.5 m*M* DL-homocysteine for 6 h. The nuclear contour was deformed; the nuclear envelope, fractured; and chromatin, aggregated into a block structure. Organelles were obviously deformed; mitochondria were severely swollen; and the ER significantly dilated.

## Discussion

HHcy is an independent risk factor for cardiovascular complications (3, 18). ER stress has been proposed to explain the pathogenic effects of HHcy (5, 25, 43), which may be a common pathway of the injury of tissues and cells induced by Hcy. The signal-transduction events associated with Hcy-induced ER stress are described in Fig. 10. $H_2S$ is another metabolite of methionine (35), but it exerts entirely different effects. Chang *et al.* (1) recently showed that $H_2S$ could protect

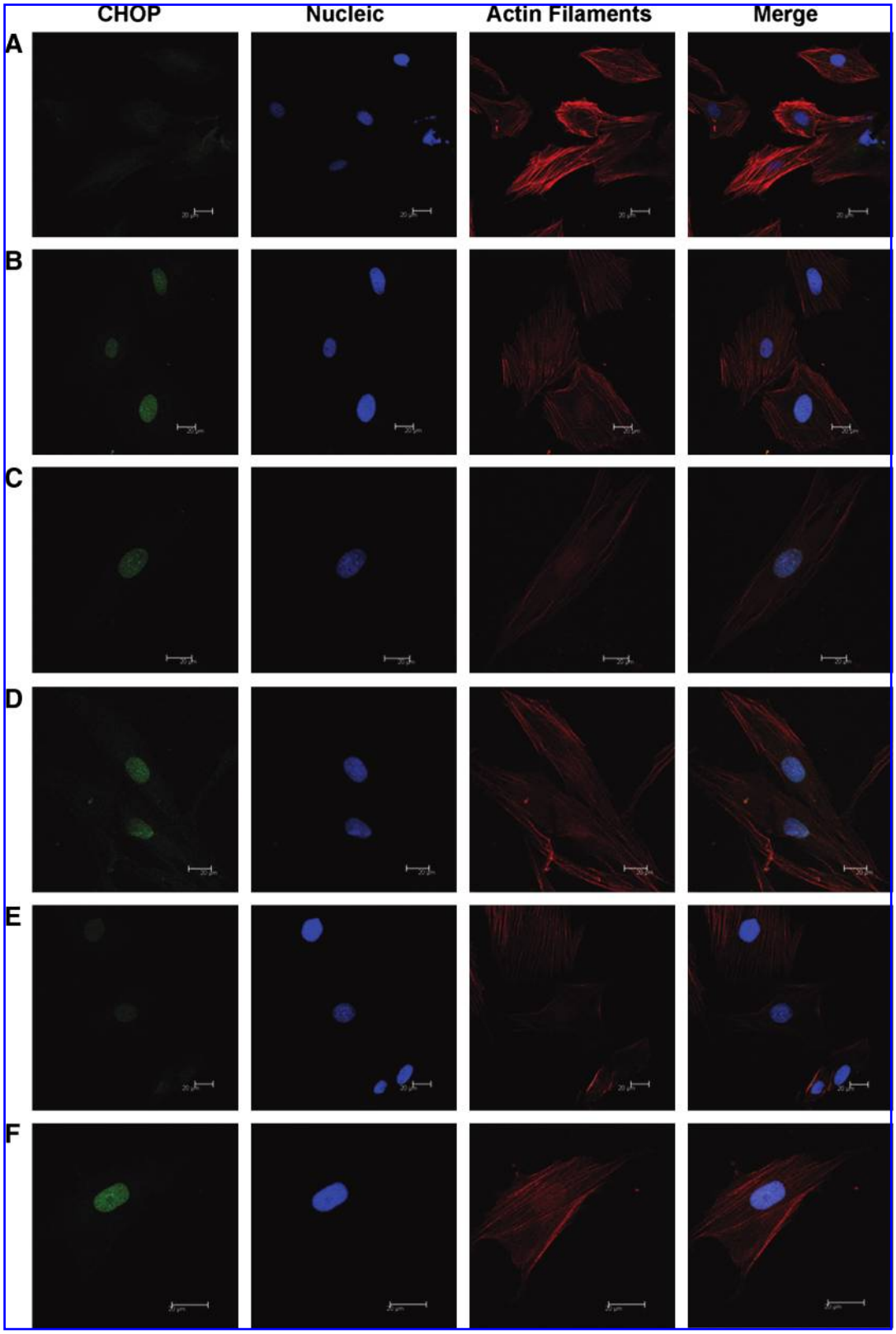


**FIG. 7. $H_2S$ supplementation directly decreased CHOP expression induced by Hcy.** Confocal immunofluorescent analysis of H9c2 cells showed that the $H_2S$ donor directly decreased CHOP expression induced by Hcy. CHOP antibody was used to detect CHOP expression (green); Hoechst 33324 was used to detect nuclear expression (blue); and actin filaments were labeled with phalloidin (red). (**A**) In control cells, CHOP expression in the nucleus was slight. (**B**) Cells were treated with 2.5 m*M* DL-homocysteine for 6 h. CHOP expression was increased. (**C**) Cells were treated with 1.5 $\mu$g/ml TN for 6 h to induce ER stress. CHOP expression was increased. (**D**) Cells were treated with 10 $\mu M$ TG for 6 h to induce ER stress. CHOP expression was increased. (**E**) Cells were pretreated with the $H_2S$ donor NaHS for 30 min followed with 2.5 m*M* DL-homocysteine for 6 h. CHOP expression was reduced. (**F**) Cells were pretreated with the CSE inhibitor PPG for 30 min, followed with 2.5 m*M* DL-homocysteine for 6 h. CHOP expression was enhanced.

the cardiovascular system against Hcy-induced damage. Whether the ER-stress status of cardiomyocytes and the underlying signaling cascade is regulated by $H_2S$ is not known. We conducted *in vivo* and *in vitro* experiments to determine whether $H_2S$ plays a profound role in regulating ER stress induced by HHcy.

In the present study, NaHS was used as an $H_2S$ donor. Under physiologic conditions, $H_2S$ exists at about one third as the undissociated form ($H_2S$) and about two thirds as $HS^-$ at equilibrium (9). NaHS can be spontaneously dissociated into $Na^+$ and $HS^-$ in solution, and then the latter associates with $H^+$ to produce $H_2S$. The influence of 1 m*M* sodium ion in the physiologic experiments is negligible in plasma containing 140 m*M* sodium ion (14). NaHS does not change the pH value of the medium. NaHS was widely used as an $H_2S$ donor to investigate the effect of $H_2S$ in many studies (36, 42, 46).

HHcy is a pathologic condition in which Hcy metabolism in the plasma is disrupted (2, 30). In our *in vivo* study, we successfully produced an HHcy model in the rat by administering methionine. Normal levels of Hcy are 5 to 15 m*M*. Mild HHcy is defined as 15 to 30 m*M*, intermediate HHcy, as 30 to 100 m*M*, and severe HHcy, as more than 100 m*M* (20). In our methionine group, plasma total Hcy levels indicated intermediate HHcy. Plasma $H_2S$ concentration and myocardial $H_2S$ production rate were reduced in HHcy rats, indicating that endogenous $H_2S$ exerted important effects on HHcy pathogenesis. Ultrastructural examination showed that in the methionine group, myocardial SR was clearly obviously dilated, the mitochondria swollen, and mitochondria cristae fractured and dissolved. SR is the smooth ER in cardiomyocytes. We chose GRP78, caspase 12, and CHOP as markers of ER stress. GRP78 is an ER-chaperon protein whose expression is increased during ER stress (21). CHOP and caspase 12 participate in ER stress–induced apoptosis (27). In the methionine group, accompanied by dilation of myocardial SR, expressions of GRP 78, caspase 12, and CHOP were all induced by HHcy. This indicated that severe ER stress was induced in the myocardium and that it may be the cause of HHcy injury.

$H_2S$ serves as a protective gaseous signaling molecule in cardiovascular systems. The physiologic concentration of $H_2S$ could exert effects on mitochondrial function and promote the survival of myocardial cells (8). ER is another important subcellular organelle critical for protein folding and the formation of disulfide bonds (44). Investigating whether $H_2S$ can exert effects on the ER is important. In the present study, supplementation of $H_2S$ not only reduced plasma total Hcy level, but

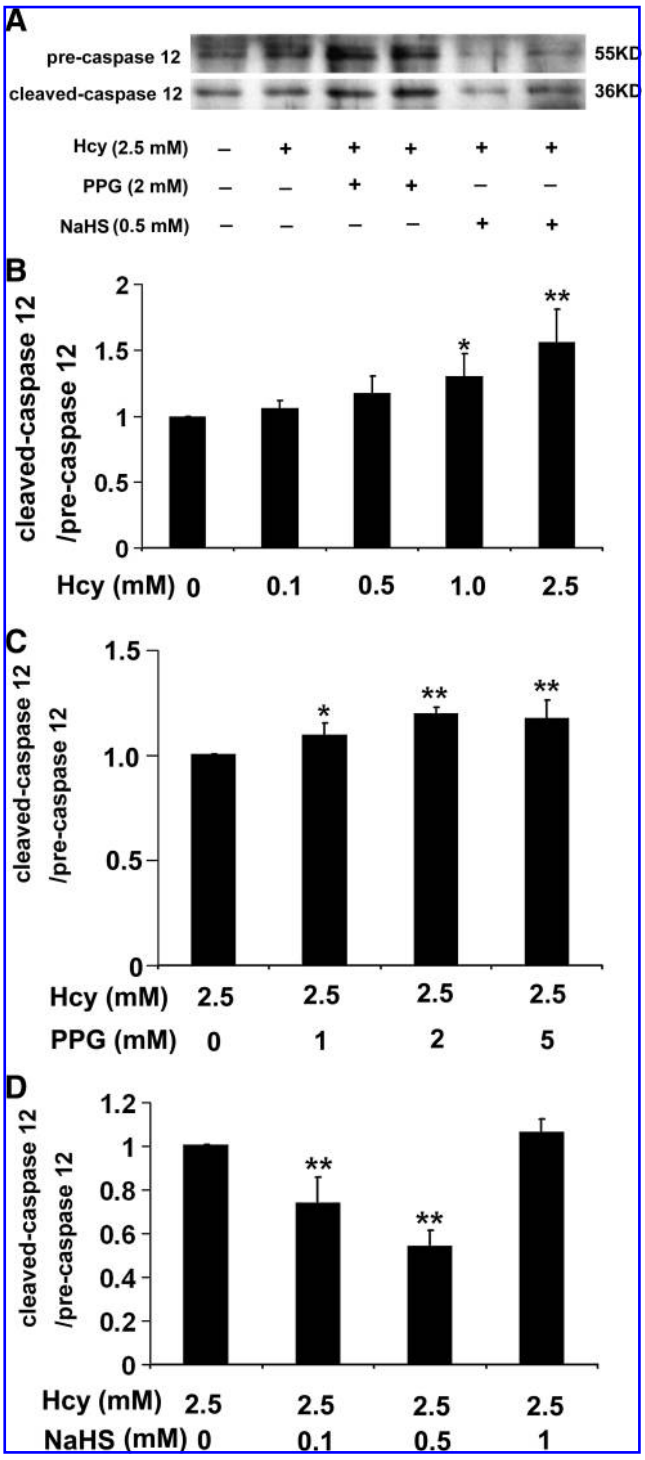


**FIG. 8. $H_2S$ supplementation directly decreased expression of cleaved caspase 12 induced by Hcy in H9c2 cells.** (**A**) H9c2 cell protein (30 $\mu$g) was separated on 10% SDS–PAGE and subjected to Western-blot analysis. Cleaved caspase 12 (36 kDa) expression was counted by comparing gray intensity and area of bands with pre-caspase 12 (55 kDa). (**B**) Cells were supplemented with 0, 0.1, 0.5, 1.0, or 2.5 m*M* Hcy for 6 h. DL-Homocysteine at concentrations of 1.0 and 2.5 m*M* increased expression of cleaved caspase 12. Cells treated with 0 m*M* DL-homocysteine were considered to be the control group, and the protein expression in this group was deemed to be 1. $^*p < 0.05$; $^{**}p < 0.01$ compared with control group. (**C**) Cells were pretreated for 30 min with 0, 1, 2 and 5 m*M* PPG, and then supplemented with 2.5 m*M* DL-homocysteine for 6 h. PPG at concentrations of 1, 2, and 5 m*M* increased expression of cleaved caspase 12. Cells treated with 2.5 m*M* Hcy were considered to be the control group, and the protein expression in this group was deemed to be 1. $^*p < 0.05$, $^{**}p < 0.01$ compared with control group. (**D**) Cells were pretreated for 30 min with 0, 0.1, 0.5, and 1 m*M* NaHS, and then supplemented with 2.5 m*M* DL-homocysteine for 6 h. NaHS at concentrations of 0.1 and 0.5 m*M* decreased expression of cleaved caspase 12. Cells treated with 2.5 m*M* Hcy were considered to be the control group, and the protein expression in this group was deemed to be 1. $^{**}p < 0.01$ compared with control group.

also alleviated myocardial injury. Ultrastructural examination showed that the structure of the myocardial SR was restored to its normal state, and that mitochondria recovered their shape. Further research demonstrated that $H_2S$ supplementation could decrease the expression of GRP78, caspase 12, and CHOP in the myocardium of rats with HHcy. The fact that $H_2S$ reduces expression of GRP78 and caspase 12 is in accord with $H_2S$ effects on stress-related gastric ulceration (19). In HHcy rats, $H_2S$ may regulate the expression of ER stress–associated markers to promote cardiomyocytic survival.

We carried out an *in vitro* study to explain further the direct effects of $H_2S$ on Hcy-induced ER stress. The H9c2 cell line is derived from rat embryonic heart and is used to study the exclusive molecular mechanisms of myocardial cells (12, 28, 34). We used four concentrations of Hcy (0.1, 0.5, 1.0, and 2.5 m*M*) to induce injury in accordance with previous studies (16, 34). Ultrastructural examination showed that 2.5 m*M* Hcy could damage H9c2 cells. The ER was significantly dilated and was seen as round vesicles; apoptotic bodies could be seen. These changes were consistent with the *in vivo* study. Cells treated with 2.5 m*M* Hcy showed increased expression of CHOP in the nucleus. The ER stress–inducers TN and TG exhibited the same morphologic changes, so we concluded that 2.5 m*M* Hcy could lead to ER stress. Caspase 12 is a specific proapoptotic ER stress marker. In our *in vitro* study,

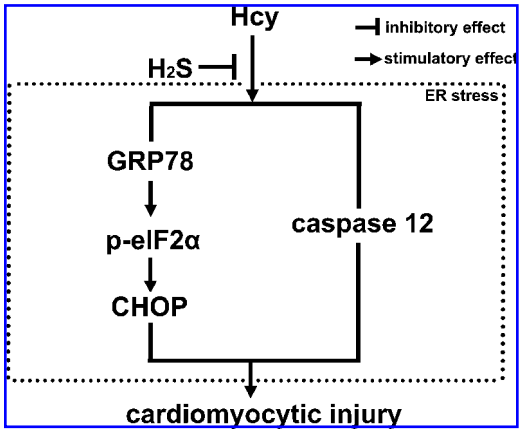


**FIG. 10. The diagram of the proposed pathway where hydrogen sulfide exerts its inhibitory effect on endoplasmic reticulum stress of the myocardium** The diagram shows signal-transduction events associated with homocysteine-induced endoplasmic reticulum (ER) stress. Hydrogen sulfide decreases expression of the ER stress–associated markers glucose-regulated protein 78 (GRP78), C/EBP homologous protein (CHOP), cleaved caspase 12, and p-eIF2α to lessen the cardiomyocytic injury.

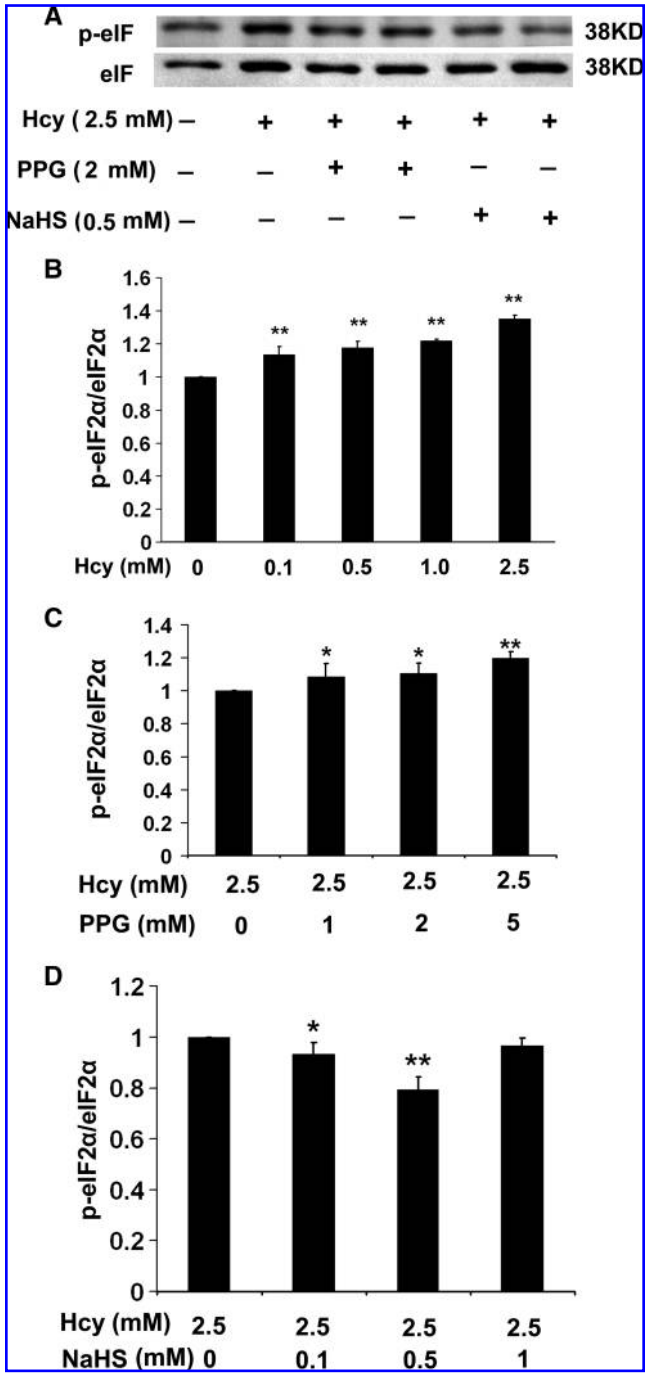


**FIG. 9. $H_2S$ supplementation directly decreased p-eIF2α expression induced by Hcy in H9c2 cells.** (**A**) H9c2 cell protein (30 μg) was separated on 10% SDS–PAGE and subjected to Western-blot analysis; p-eIF2α (36 kDa) expression was counted by comparing gray intensity and area of bands with eIF2α (36 kDa). (**B**) Cells were supplemented with 0, 0.1, 0.5, 1.0, or 2.5 m*M* DL-homocysteine for 6 h. DL-Homocysteine at concentrations of 1.0 m*M* and 2.5 m*M* increased p-eIF2α expression. Cells treated with 0 m*M* DL-homocysteine were considered to be the control group, and the protein expression in this group deemed to be 1. $^*p < 0.05$; $^{**}p < 0.01$ compared with control group. (**C**) Cells were pretreated for 30 min with 0, 1, 2, and 5 m*M* PPG, and then supplemented with 2.5 m*M* DL-homocysteine for 6 h. PPG at concentrations of 1, 2, and 5 m*M* increased p-eIF2α expression. Cells treated with 2.5 m*M* Hcy were considered to be the control group, and the protein expression in this group was deemed to be 1. $^*p < 0.05$; $^{**}p < 0.01$ compared with control group. (**D**) Cells were pretreated for 30 min with 0, 0.1, 0.5, and 1 m*M* NaHS, and then supplemented with 2.5 m*M* DL-homocysteine for 6 h. NaHS at concentrations of 0.1 and 0.5 m*M* decreased p-eIF2α expression. Cells treated with 2.5 m*M* Hcy were considered to be the control group, and the protein expression in this group was deemed to be 1. $^{**}p < 0.01$ compared with control group.

Western blot analysis showed that 1.0 m*M* and 2.5 m*M* Hcy could increase the expression of cleaved caspase 12, which further confirmed that ER stress was an important cause of HHcy.

eIF2α controls gene expression at the translational level and has critical roles in cell growth. Phosphorylation of eIF2α can block the initiation of gene expression (37). The ratio of p-eIF2α to eIF2α is an important factor in the ER-stress pathway. P-eIF2α can block the initiation of most gene expression at the translational level, but it may also enhance CHOP activity at a posttranscriptional level (44). Our data showed that all tested concentrations of Hcy (0.1, 0.5, 1.0, and 2.5 m*M*) increased the ratio of p-eIF2α and eIF2α, suggesting that P-eIF2α might be one of the targets of Hcy in ER stress.

In our *in vitro* study, cells not only were supplemented with $H_2S$, but also were pretreated with PPG. PPG is a CSE inhibitor that can inhibit endogenous production of $H_2S$ in the cardiovascular system. Ultrastructural examination showed that, in the HHcy + NaHS group, dilation of myocardial ER was reduced, but in the HHcy + PPG group, mitochondria and the ER were significantly dilated. These data suggested that $H_2S$ supplementation could alleviate Hcy-induced injury in H9c2 cells, whereas $H_2S$ inhibition could aggravate the damage. Further analysis showed that $H_2S$ supplementation directly decreased the expression of cleaved caspase 12 and p-eIF2α induced by Hcy in H9c2 cells, whereas $H_2S$ inhibition increased the expression of cleaved caspase 12 and p-eIF2α. The results indicated that endogenous $H_2S$ counteracted Hcy injury by regulating ER stress and might act as a novel therapeutic substance for HHcy.

In summary, our data showed that $H_2S$ could effectively ameliorate HHcy-induced cardiomyocytic injury along with decreased expressions of GRP78, CHOP, and caspase 12 in rats with HHcy. In H9c2 cells, Hcy and ER-stress inducers TN and TG successfully induced a marked cell injury and ER stress. The inhibition of endogenous $H_2S$ production further enhanced cellular ER stress, but supplementation of the $H_2S$ donor markedly antagonized cell injury in association with downregulation of CHOP, cleaved caspase 12, and p-eIF2α expressions. The results suggested that $H_2S$ could attenuate cardiomyocytic ER stress in HHcy-induced cardiomyocytic injury.

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## Author Disclosure Statement

No competing financial interests exist.

## References

1. Chang L, Geng B, Yu F, Zhao J, Jiang H, Du J, and Tang C. Hydrogen sulfide inhibits myocardial injury induced by homocysteine in rats. *Amino Acids* 34: 573–585, 2008.
2. Dayal S and Lentz SR. ADMA and hyperhomocysteinemia. *Vasc Med* 1: S27–S33, 2005.
3. de Bree A, Verschuren WM, Blom HJ, and Kromhout D. Lifestyle factors and plasma homocysteine concentrations in a general population sample. *Am J Epidemiol* 154: 150–154, 2001.
4. Dias VC, Bamforth FJ, Tesanovic M, Hyndman ME, Parsons HG, and Cembrowski GS. Evaluation and intermethod comparison of the Bio-Rad high-performance liquid chromatographic method for plasma total homocysteine. *Clin Chem* 44: 2199–2201, 1998.
5. Dickhout JG, Sood SK, and Austin RC. Role of endoplasmic reticulum calcium disequilibria in the mechanism of homocysteine-induced ER stress. *Antioxid Redox Signal* 9: 1863–1873, 2007.
6. Distrutti E, Mencarelli A, Santucci L, Renga B, Orlandi S, Donini A, Shah V, and Fiorucci S. The methionine connection: homocysteine and hydrogen sulfide exert opposite effects on hepatic microcirculation in rats. *Hepatology* 47: 659–667, 2008.
7. 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.
8. 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 U S A* 104: 15560–15565, 2007.
9. Eto K, Ogasawara M, Umemura K, Nagai Y, and Kimura H. Hydrogen sulfide is produced in response to neuronal excitation. *J Neurosci* 22: 3386–3391, 2002.
10. Fling SP and Gregerson DS. Peptide and protein molecular weight determination by electrophoresis using a high-molarity tris buffer system without urea. *Anal Biochem* 155: 83–88, 1986.
11. Geng B, Du JB, and Tang CS. Application of sensitive sulphur electrode assay to measure and analyze cystathionine-gamma-lyase/hydrogen sulfide pathway of cardiovascular tissues, cells and plasma in rats. *Beijing Da Xue Xue Bao* 37: 545–548, 2005.
12. Han XJ, Chae JK, Lee MJ, You KR, Lee BH, and Kim DG. Involvement of GADD153 and cardiac ankyrin repeat protein in hypoxia-induced apoptosis of H9c2 cells. *J Biol Chem* 280: 23122–23129, 2005.
13. Hofmann MA, Lalla E, Lu Y, Gleason MR, Wolf BM, Tanji N, Ferran LJ Jr, Kohl B, Rao V, Kisiel W, Stern DM, and Schmidt AM. Hyperhomocysteinemia enhances vascular inflammation and accelerates atherosclerosis in a murine model. *J Clin Invest* 107: 675–683, 2001.
14. 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.
15. Kondo S, Saito A, Hino S, Murakami T, Ogata M, Kanemoto S, Nara S, Yamashita A, Yoshinaga K, Hara H, and Imaizumi K. BBF2H7, a novel transmembrane bZIP transcription factor, is a new type of endoplasmic reticulum stress transducer. *Mol Cell Biol* 27: 1716–1729, 2007.
16. Levrand S, Pacher P, Pesse B, Rolli J, Feihl F, Waeber B, and Liaudet L. Homocysteine induces cell death in H9C2 cardiomyocytes through the generation of peroxynitrite. *Biochem Biophys Res Commun* 359: 445–450, 2007.
17. Li L and Moore PK. Putative biological roles of hydrogen sulfide in health and disease: a breath of not so fresh air. *Trends Pharmacol Sci* 29: 84–90, 2008.

18. Lin JH, Walter P, and Yen TS. Endoplasmic reticulum stress in disease pathogenesis. *Annu Rev Pathol* 3: 399–425, 2008.
19. Lou LX, Geng B, Du JB, and Tang CS. Hydrogen sulphide-induced hypothermia attenuates stress-related ulceration in rats. *Clin Exp Pharmacol Physiol* 35: 223–228, 2008.
20. Malinow MR, Bostom AG, and Krauss RM. Homocyst(e)ine, diet, and cardiovascular diseases: a statement for healthcare professionals from the Nutrition Committee, American Heart Association. *Circulation* 99: 178–182, 1999.
21. Mao C, Tai WC, Bai Y, Poizat C, and Lee AS. In vivo regulation of Grp78/BiP transcription in the embryonic heart: role of the endoplasmic reticulum stress response element and GATA-4. *J Biol Chem* 281: 8877–8887, 2006.
22. McCullough KD, Martindale JL, Klotz LO, Aw TY, and Holbrook NJ. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. *Mol Cell Biol* 21: 1249–1259, 2001.
23. McCully KS. Vascular pathology of homocysteinemia: implications for the pathogenesis of arteriosclerosis. *Am J Pathol* 56: 111–128, 1969.
24. McNulty H, Pentieva K, Hoey L, and Ward M. Homocysteine, B-vitamins and CVD. *Proc Nutr Soc* 67: 232–237, 2008.
25. Oka Y, Hirabayashi Y, Ishii T, Takahashi R, and Sasaki T. A monoclonal antibody against human homocysteine induced endoplasmic reticulum protein (Herp): a useful tool for evaluating endoplasmic reticulum stress. *Tohoku J Exp Med* 212: 431–437, 2007.
26. Rogers EJ, Chen S, and Chan A. Folate deficiency and plasma homocysteine during increased oxidative stress. *N Engl J Med* 357: 421–422, 2007.
27. Rutkowski DT, Arnold SM, Miller CN, Wu J, Li J, Gunnison KM, Mori K, Sadighi Akha AA, Raden D, and Kaufman RJ. Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs and proteins. *PLoS Biol* 4: 374, 2006.
28. Sardão VA, Oliveira PJ, Holy J, Oliveira CR, and Wallace KB. Vital imaging of H9c2 myoblasts exposed to *tert*-butylhydroperoxide: characterization of morphological features of cell death. *BMC Cell Biol* 8: 11–26, 2007.
29. Schnyder G, Roffi M, Flammer Y, Pin R, and Hess OM. Effect of homocysteine-lowering therapy with folic acid, vitamin B12, and vitamin B6 on clinical outcome after percutaneous coronary intervention: the Swiss Heart Study: a randomized controlled trial. *JAMA* 288: 973–979, 2002.
30. Selhub J. Homocysteine metabolism. *Annu Rev Nutr* 19: 217–246, 1999.
31. Selhub J, Jacques PF, Bostom AG, D'Agostino RB, Wilson PW, Belanger AJ, O'Leary DH, Wolf PA, Schaefer EJ, and Rosenberg IH. Association between plasma homocysteine concentrations and extracranial carotid-artery stenosis. *N Engl J Med* 332: 286–291, 1995.
32. Sen U, Basu P, Abe OA, Givvimani S, Tyagi N, Metreveli N, Shah KS, Passmore JC, and Tyagi SC. Hydrogen sulfide ameliorates hyperhomocysteinemia associated chronic renal failure. *Am J Physiol Renal Physiol* 2009. [Epub ahead of print]
33. Sharma M, Rai SK, Tiwari M, and Chandra R. Effect of hyperhomocysteinemia on cardiovascular risk factors and initiation of atherosclerosis in Wistar rats. *Eur J Pharmacol* 574: 49–60, 2007.
34. Sipkens JA, Krijnen PA, Meischl C, Cillessen SA, Smulders YM, Smith DE, Giroth CP, Spreeuwenberg MD, Musters RJ, Muller A, Jakobs C, Roos D, Stehouwer CD, Rauwerda JA, van Hinsbergh VW, and Niessen HW. Homocysteine affects cardiomyocyte viability: concentration-dependent effects on reversible flip-flop, apoptosis and necrosis. *Apoptosis* 12: 1407–1418, 2007.
35. Stipanuk MH. Sulfur amino acid metabolism: pathways for production and removal of homocysteine and cysteine. *Annu Rev Nutr* 24: 539–577, 2004.
36. Sun YG, Cao YX, Wang WW, Ma SF, Yao T, and Zhu YC. Hydrogen sulphide is an inhibitor of L-type calcium channels and mechanical contraction in rat cardiomyocytes. *Cardiovasc Res* 79: 632–641, 2008.
37. Su Q, Wang S, Gao HQ, Kazemi S, Harding HP, Ron D, and Koromilas AE. Modulation of the eukaryotic initiation factor 2 alpha-subunit kinase PERK by tyrosine phosphorylation. *J Biol Chem* 283: 469–475, 2008.
38. Tang C, Li X, and Du J. Hydrogen sulfide as a new endogenous gaseous transmitter in the cardiovascular system. *Curr Vasc Pharmacol* 4: 17–22, 2006.
39. Tsai JC, Perrella MA, Yoshizumi M, Hsieh CM, Haber E, Schlegel R, and Lee ME. Promotion of vascular smooth muscle cell growth by homocysteine: a link to atherosclerosis. *Proc Natl Acad Sci U S A* 91: 6369–6373, 1994.
40. Tyagi N, Moshal KS, Sen U, Vacek TP, Kumar M, Hughes WM Jr, Kundu S, and Tyagi SC. $H_2S$ protects against methionine-induced oxidative stress in brain endothelial cells. *Antioxid Redox Signal* 11: 25–33, 2009.
41. Wang R. The gasotransmitter role of hydrogen sulfide. *Antioxid Redox Signal* 5: 493–450, 2003.
42. Wang Y, Zhao X, Jin H, Wei H, Li W, Bu D, Tang X, Ren Y, Tang C, and Du J. Role of hydrogen sulfide in the development of atherosclerotic lesions in apolipoprotein E knockout mice. *Arterioscler Thromb Vasc Biol* 29: 173–179, 2009.
43. Werstuck GH, Lentz SR, Dayal S, Hossain GS, Sood SK, Shi YY, Zhou J, Maeda N, Krisans SK, Malinow MR, and Austin RC. Homocysteine-induced endoplasmic reticulum stress causes dysregulation of the cholesterol and triglyceride biosynthetic pathways. *J Clin Invest* 107: 1263–1273, 2001.
44. Xu C, Bailly-Maitre B, and Reed JC. Endoplasmic reticulum stress: cell life and death decisions. *J Clin Invest* 115: 2656–2664, 2005.
45. Yang G, Wu L, and Wang R. Pro-apoptotic effect of endogenous $H_2S$ on human aorta smooth muscle cells. *FASEB J* 20: 553–555, 2006.
46. Yang G, Wu L, Jiang B, Yang W, Qi J, Cao K, Meng Q, Mustafa AK, Mu W, Zhang S, Snyder SH, and Wang R. $H_2S$ as a physiologic vasorelaxant: hypertension in mice with deletion of cystathionine gamma-lyase. *Science*. 22: 587–590, 2008.
47. Yang G, Yang W, Wu L, and Wang R. $H_2S$, endoplasmic reticulum stress, and apoptosis of insulin-secreting beta cells. *J Biol Chem* 282: 16567–16576, 2007.
48. Yang QH, Botto LD, Gallagher M, Friedman JM, Sanders CL, Koontz D, Nikolova S, Erickson JD, and Steinberg K. Prevalence and effects of gene-gene and gene-nutrient interactions on serum folate and serum total homocysteine concentrations in the United States: findings from the third National Health and Nutrition Examination Survey DNA Bank. *Am J Clin Nutr* 88: 232–246, 2008.
49. 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* 351: 485–491, 2006.
50. Yideng J, Jianzhong Z, Ying H, Juan S, Jinge Z, Shenglan W, Xiaoqun H, and Shuren W. Homocysteine-mediated expression of SAHH, DNMTs, MBD2, and DNA hypomethylation potential pathogenic mechanism in VSMCs. *DNA Cell Biol* 26: 603–611, 2007.

Address correspondence to:
*Junbao Du, M.D., Ph.D.*
*The Department of Pediatrics*
*Peking University First Hospital*
*Xi-An Men Street No. 1*
*West District*
*Beijing 100034*
*P.R. China*

*E-mail:* junbaodu@ht.rol.cn.net

*Chaoshu Tang, Ph.D.*
*Institute of Cardiovascular Research*
*Peking University First Hospital*
*Xi-An Men Street No. 1*
*West District*
*Beijing 100034*
*P.R. China*

*E-mail:* tangchaoshu@263.net.cn

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**Abbreviations Used**

CBS = cystathionine-$\beta$-synthase
CHOP = C/EBP homologous protein
CSE = cystathionine-$\gamma$-lyase
DMEM = Dulbecco's modified Eagle's medium
eIF2$\alpha$ = eukaryotic initiation factor 2 $\alpha$-subunit
ER = endoplasmic reticulum
FCS = fetal calf serum
GRP78 = glucose regulated protein 78
Hcy = homocysteine
HHcy = hyperhomocysteinemia
HPLC = high-performance liquid chromatography
$H_2S$ = hydrogen sulfide
NaHS = sodium hydrosulfide
PAGE = polyacrylamide gel electrophoresis
PPG = propargylglycine
ROS = reactive oxygen species
SDS = sodium dodecyl sulfate
TBS = Tris-buffered saline
TG = thapsigargin
TN = tunicamycin
VSMC = vascular smooth muscle cell

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2. Yi-Hong Liu , Ming Lu , Li-Fang Hu , Peter T.-H. Wong , George D. Webb , Jin-Song Bian . 2012. Hydrogen Sulfide in the Mammalian Cardiovascular System. *Antioxidants & Redox Signaling* **17**:1, 141-185. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
3. Jiancheng Xu, Qi Zhou, Wei Xu, Lu Cai. 2012. Endoplasmic Reticulum Stress and Diabetic Cardiomyopathy. *Experimental Diabetes Research* **2012**, 1-12. [CrossRef]
4. Xiu-Yu Wang, Chun-Tao Yang, Dong-Dan Zheng, Li-Qiu Mo, Ai-Ping Lan, Zhan-Li Yang, Fen Hu, Pei-Xi Chen, Xin-Xue Liao, Jian-Qiang Feng. 2011. Hydrogen sulfide protects H9c2 cells against doxorubicin-induced cardiotoxicity through inhibition of endoplasmic reticulum stress. *Molecular and Cellular Biochemistry* . [CrossRef]
5. N. Krishnan, C. Fu, D. J. Pappin, N. K. Tonks. 2011. H2S-Induced Sulfhydration of the Phosphatase PTP1B and Its Role in the Endoplasmic Reticulum Stress Response. *Science Signaling* **4**:203, ra86-ra86. [CrossRef]
6. 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]
7. E. Dongó, I. Hornyák, Zs. Benk#, Levente Kiss. 2011. The cardioprotective potential of hydrogen sulfide in myocardial ischemia/reperfusion injury (Review). *Acta Physiologica Hungarica* **98**:4, 369-381. [CrossRef]
8. Yingmei Zhang , Zhi Xia , Karissa H. La Cour , Jun Ren . 2011. Activation of Akt Rescues Endoplasmic Reticulum Stress-Impaired Murine Cardiac Contractile Function via Glycogen Synthase Kinase-3#-Mediated Suppression of Mitochondrial Permeation Pore Opening. *Antioxidants & Redox Signaling* **15**:9, 2407-2424. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links] [Supplemental material]
9. Liman Luo, Stella Chen, Hongfang Jin, Chaoshu Tang, Junbao Du. 2011. Endogenous generation of sulfur dioxide in rat tissues. *Biochemical and Biophysical Research Communications* . [CrossRef]
10. Yingmei Zhang, Jun Ren. 2011. Thapsigargin triggers cardiac contractile dysfunction via NADPH oxidase-mediated mitochondrial dysfunction: Role of Akt dephosphorylation. *Free Radical Biology and Medicine* . [CrossRef]
11. Gábor Szabó, Gábor Veres, Tamás Radovits, Domokos Ger#, Katalin Módis, Christiane Miesel-Gröschel, Ferenc Horkay, Matthias Karck, Csaba Szabó. 2011. Cardioprotective effects of hydrogen sulfide. *Nitric Oxide* **25**:2, 201-210. [CrossRef]
12. Xin-Bao Wang, Xiao-Mei Huang, Todd Ochs, Xue-Ying Li, Hong-Fang Jin, Chao-Shu Tang, Jun-Bao Du. 2011. Effect of sulfur dioxide preconditioning on rat myocardial ischemia/reperfusion injury by inducing endoplasmic reticulum stress. *Basic Research in Cardiology* . [CrossRef]
13. Wei Li, Chaoshu Tang, Hongfang Jin, Junbao Du. 2011. Regulatory effects of sulfur dioxide on the development of atherosclerotic lesions and vascular hydrogen sulfide in atherosclerotic rats. *Atherosclerosis* **215**:2, 323-330. [CrossRef]
14. Li LI, Bang-chuan HU, Shi-jin GONG, Jing YAN. 2011. Homocysteine-Induced Caspase-3 Activation by Endoplasmic Reticulum Stress in Endothelial Progenitor Cells from Patients with Coronary Heart Disease and Healthy Donors. *Bioscience, Biotechnology, and Biochemistry* **75**:7, 1300-1305. [CrossRef]
15. Edward G Lynn, Richard C Austin. 2011. Hydrogen sulfide in the pathogenesis of atherosclerosis and its therapeutic potential. *Expert Review of Clinical Pharmacology* **4**:1, 97-108. [CrossRef]
16. Rui Wang . 2010. Hydrogen Sulfide: The Third Gasotransmitter in Biology and Medicine. *Antioxidants & Redox Signaling* **12**:9, 1061-1064. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]