Exogenous hydrogen sulfide restores cardiac function after trauma-hemorrhagic shock by inhibiting mitochondrial apoptosis

Lu Yang1*, Jiayan Lin2*, Xiaoyong Zhao1, Linong Yao1, Qian Ding1, Wei Chai1

1Department of Anesthesiology, Tangdu Hospital, Fourth Military Medical University, Xi’an, Shaanxi, China; 2Department of Anesthesiology, 113 Hospital of People’s Liberation Army, Ningbo, Zhejiang, China. *Equal contributors.

Received November 7, 2015; Accepted February 10, 2016; Epub March 15, 2016; Published March 30, 2016

Abstract: Cardiac dysfunction is a major complication of trauma-hemorrhagic shock (THS). Exogenous hydrogen sulfide (H2S) has been reported to attenuate THS-induced myocardial injury in our earlier study; however, the underlying mechanisms remain unclear. The aim of this study was to evaluate the anti-apoptotic effects of H2S in a rat model of THS-induced myocardial injury. Eighty rats were divided into 4 groups: Sham group, THS group, Vehicle group, and Sodium hydrosulfide (NaHS) group. THS was induced by midline laparotomy and hemorrhagic shock, followed by resuscitation. Vehicle or NaHS was administrated 10 min before resuscitation, and all animals were sacrificed at 2 h after resuscitation for subsequent analysis. The results showed that NaHS restored cardiac function; attenuated myocardial injury; and inhibited myocardial apoptosis. Reverse transcription-polymerase chain reaction (RT-PCR) and western blotting demonstrated that NaHS significantly up-regulated anti-apoptotic protein (Bcl-2) and down-regulated pro-apoptotic protein (Bax) and genes (caspase-9, bax). In addition, the activity of mitochondrial permeability transition pore (mPTP) and mitochondrial structure were protected by NaHS. Therefore, our results suggest that exogenous H2S attenuates THS-induced myocardial injury by inhibiting myocardial apoptosis, mPTP opening and mediating apoptosis-related gene and protein expressions after THS.

Keywords: Hydrogen sulfide, trauma-hemorrhagic shock, myocardial injury, mitochondrial apoptosis

Introduction

Hemorrhagic shock (HS) is a major complication of multiple trauma and commonly occurs in both civilian and military situations [1]. After trauma-hemorrhagic shock (THS), resuscitation leads to multiple organ dysfunctions, which is a significant contributor to the late mortality and intensive care [2]. Our previous studies found that exogenous hydrogen sulfide (H2S) attenuated THS-induced myocardial injury and improved cardiac function; however, the underlying mechanisms of these cardioprotective effects remain unclear [3, 4]. Earlier studies have reported the anti-apoptotic effect of H2S against myocardial ischemia/reperfusion (I/R) injury in some pathologic situations [5-11]. However, the studies exploring the anti-apoptotic effect of H2S during THS are sparse. It has been reported that extensive cardiac apoptosis was readily detected within 24 h after THS, which played an important role in the development of heart failure after resuscitation [12, 13]. Thus, in this study, we hypothesized that exogenous H2S exerted the cardioprotective effects by inhibiting myocardial apoptosis induced by THS.

Mitochondria are critical regulator of apoptotic cell death. The preservation of mitochondrial function and structure is linked to the inhibition of cell apoptosis [14-16]. Mitochondrial permeability transition pore (mPTP) opening in the inner mitochondrial membrane has been implicated in I/R [17, 18]. The opening of mPTP leads to the proton gradient and electrical potential disruption across the inner mitochondrial membrane which eventually induces the rupture of outer mitochondrial membrane and mitochondrial swelling [19]. Cytochrome c, apoptosis-
 Mechanisms of cardioprotective effects of exogenous H$_2$S

inducing factor (AIF) and Ca$^{2+}$ are then released after mitochondrial swelling, which activate the members of caspase family and lead to apoptosis [20]. In addition, Bcl-2 family is considered to have an important role in the mitochondrial apoptotic pathway as well [21]. The pro-apoptotic protein Bax forms oligomers in the mitochondrial outer membrane, which induces the release of pro-apoptotic factors like cytochrome c from mitochondria and promotes apoptosis. Oppositely, the anti-apoptotic protein Bcl-2 inhibits mitochondrial apoptosis via blocking the release and oligomerization of Bax [22-24]. Yao et al have found that sodium hydrosulfide (NaHS) dramatically inhibited mPTP opening and thus protected cardiomyocytes from hypoxia/reoxygenation-induced apoptosis [25]. Furthermore, Zhang et al reported that NaHS regulated Bax/Bcl-2 signaling after ischemia, which then prevented mitochondrial-related cell apoptosis in a rat model of myocardial I/R injury [7].

Thus, in this study, we investigated the effect of NaHS administration on cardiac apoptosis in a rat model of THS. We hypothesized that exogenous H$_2$S attenuated THS-induced cardiac injury by inhibiting myocardial apoptosis, preserving mitochondrial function, and regulating Bax/Bcl-2 signaling, thereby protecting the cardiac function.

**Materials and methods**

**Animals**

Eighty age-matched male Sprague-Dawley rats, weighing 250-300 g, were used in this study. All the research protocols were approved by the Animal Care and Use Committee at Fourth Military Medical University and in accord with the NIH Guide for the Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985). All animals were housed and maintained on a 12:12 light/dark cycle with free access to food and water in our laboratory. After 1 week of acclimatization, the rats were fasted overnight before the experiments but allowed free access to water.

**Drugs**

NaHS was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, U.S.A.). All chemicals and reagents were procured from local suppliers and were of analytical grade.

**Animal grouping and administration**

The rats were randomly divided into 4 groups: sham-operated group (Sham group; $n = 20$); trauma-hemorrhagic shock group (THS group; $n = 20$); THS with vehicle treatment group (Vehicle group; $n = 20$); and THS with NaHS treatment group (NaHS group; $n = 20$).

The rat model of THS was induced as described in our previous study [4]. Briefly, after anesthesia with 1% pentobarbital sodium (40 mg/kg), rats were laparotomized through a 5 cm midline abdominal incision to induced soft tissue injury. Polyethylene catheters (PE-50) were placed in both femoral arteries and the right femoral vein. Hemodynamic variables were measured via one of the arteries using a blood pressure analyzer (Powerlab System, AD Instruments, Bella Vista, NSW, Australia). Blood was then withdrawn rapidly to a mean arterial pressure (MAP) of 35-40 mmHg through the other artery until they could no longer maintain this MAP unless some Ringer lactate solution (RL) was administered. This procedure took about 10 min. The volume removed was recorded as maximal bleed out (MBO). After MBO had been achieved, hypotension was maintained between 35 and 40 mmHg by giving small volumes of RL until 40% of the MBO volume of RL had been returned (about 90 min from the beginning of bleeding). The animals were then resuscitated with RL in a volume four times of MBO over 60 min through the venous catheter.

All animals underwent trauma-hemorrhage and resuscitation except Sham group. Rats in Vehicle group and NaHS group were given the same volume of vehicle or NaHS solution (28 μmol/kg, i.p.) respectively at 10 min before the beginning of resuscitation. Two hours after the end of experimental or sham operations, ten animals from each group were re-anesthetized to determine cardiac function and collect blood samples. Then, the rats were sacrificed and the hearts were excised for mPTP activity, reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis. The other ten rats in each group were used for hematoxylin-eosin (HE) staining, electron microscopy examination and terminal deoxynucleotidyl transferase-mediated deoxyuridine
Mechanisms of cardioprotective effects of exogenous H₂S

**Table 1.** The primer sequences used for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax</td>
<td>5'-GTACAGGTTTGCAGCAGG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-CGTCCCAGCTGCAATG-3'</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>5'-GGCATCTTCCTCTCCAGC-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TCCAGCCTCGTTATCC-3'</td>
</tr>
<tr>
<td>Capsase-9</td>
<td>5'-CGTGGACATGGTCTTG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-ACGGTGTTGATGATGAGGC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-AGGCCGGTGTAGTGTG-3'</td>
</tr>
<tr>
<td></td>
<td>5'-TGCCGTTCCACCATCTC-3'</td>
</tr>
</tbody>
</table>

triphosphate (dUTP) nick-end labeling (TUNEL) assay.

**Determination of cardiac function**

The right carotid artery was cannulated with PE-50 tubing, and the catheter was advanced into the left ventricle to monitor and record the left ventricular pressure (LVP) and the positive and negative first derivatives of pressure (+dp/dtmax and -dp/dtmax).

**Myocardial enzyme assays**

Creatine kinase (CK) and lactate dehydrogenase (LDH) levels in the serum were measured using commercially available colorimetry kits (Jiancheng bioengineering Ins, Nanjing, China) according to the manufacturer's instructions. As described in our previous study, the blood samples were centrifuged at 4°C to collect serum [3]. The serum and regents were then added to the assay wells in order, and the optical density value of each well was detected with a spectrophotometer (PowerWave XS, BioTek Inc., Winooski, VT, U.S.A.) at 460 nm. The relative CK and LDH levels in serum were then calculated accordingly.

**Determination of mPTP activity**

Myocardial mitochondria were isolated using Mitochondria Isolation Kit (Genmed, China) according to the manufacturer's instruction. The mPTP function was reflected indirectly by calcium-induced mitochondrial swelling as described in our previous study [26]. Briefly, after myocardial mitochondria were isolated and identified by Janus green, calcium was administered in the mitochondrial suspension to induce the mitochondrial swelling. Swelling of mitochondria was measured as a change in light scattering of the mitochondrial suspension at 540 nm (A540) using a spectrometer (Molecular Devices, Sunnyvale, CA, USA) at 25°C. A decrease in A540 indicates an increase in mitochondrial swelling.

**Quantitation of mRNA expression**

Total RNA was extracted from the myocardial tissues with Trizol reagent (Takara, Japan) and reverse transcribed (RT) into cDNA with PrimeScript RT reagent Kit (Takara, Japan) according to the manufacturer's instructions. The cDNA was then used as a template for polymerase chain reaction (PCR) amplification of bcl-2, bax, caspase-9 and glyceraldehyde phosphate dehydrogenase (GAPDH). All the PCR reactions were started with heating 95°C for 3 min, followed by the repeated cycle of 95°C for 30 s, 55-58°C for 30 s and 72°C for 1 min, with additional 72°C for 7 min. After repeated thermal reactions for 30 cycles, the PCR products were electrophoresed on 1% agarose containing 5 μg/mL of ethidium bromide, and then scanned using an ultraviolet gel imaging system (BioRad, U.S.A.). Representative gels were photographed and densitometry was performed to assess quantities relative to GAPDH. The primer sequences were shown in Table 1.

**Western blot analysis**

Tissues were homogenized in cold RIPA buffer, and the concentration of protein was determined by BCA method (Beyotime Institute of Biotechnology, China). The protein samples were separated on 12% sodium dodecyl sulfate-polyacrylamide gel and then transferred to nitrocellulose membrane. After blocking with 5% non-fat milk, membranes were incubated with corresponding primary antibody: rabbit anti-Bax and anti-Bcl-2 antibodies (1:1000; Cell Signaling Technology, USA). After washing, the membranes were incubated with appropriate secondary antibody conjugated to the horse-radish peroxidase. The bands were then detected using enhanced chemiluminescence kit (Pierce, Rockford, IL, U.S.A.) and normalized to β-actin.

**Determination of myocardial apoptosis**

Formalin-fixed myocardial tissue was dehydrated, embedded in paraffin, and sliced into 5 μm-thick sections. Myocardial apoptosis was...
Mechanisms of cardioprotective effects of exogenous H$_2$S
detected by TUNEL assay with an in situ Cell
Death Detection Kit (Roche Applied Science,
Mannheim, Germany) following the manufac-
turer’s instructions. Briefly, the sections were
dewaxed, rehydrated and then permeabilized
with 10 µg/mL proteinase K for 30 min at room
temperature. The slides were then incubated
with TUNEL reaction mixture in a humidified
chamber for 60 min at 37°C in the dark. After
washing with PBS, the reaction was stopped by
immersing the sample in 50 µL converter-POD
buffer for 30 min at 37°C. Then the sections
were counterstained with hematoxylin. Finally,
the samples were developed using the diami-
nobenzidine substrate for 10 min. The samples
were then washed and examined under the
optical microscope (Olympus, Tokyo, Japan).
TUNEL-positive cells were counted in 10 ran-
domly chosen fields per section at a magnifica-
tion of 400-fold by an investigator blinded to
the experimental groups. The data were pre-

Morphological investigation
To investigate the morphologic changes
induced by THS in the hearts, HE staining was
performed according to the standard protocol.
5 µm-thick sections were used in this study and
the morphologic changes were evaluated by an
experienced pathologist who was blinded to
the experimental groups.

Electron microscopy examination
A segment of myocardial tissue (1 mm × 1 mm
× 1 mm) was obtained from the root of the ante-
rior papillary muscle of left ventricle in each
sample and fixed in 2.5% glutaraldehyde for
transmission electron microscopy examination.
Myocardial ultrathin sections were elaborated
according to the standard procedures. Changes
of the mitochondrial morphology were observed
under a JEM-2000EX transmission electron
microscope (JEOL Ltd., Tokyo, Japan). Five ran-
dom fields on each electron microscope sam-
Mechanisms of cardioprotective effects of exogenous H₂S

ple were selected to observe the mitochondrial damage.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) and analyzed by SPSS17.0 software (SPSS Inc., U.S.A). HR and MAP were analyzed using repeated measures analysis of variance (ANOVA) to test for the significant differences within and between groups. Further comparisons to evaluate mean differences at individual time points were made using an ANOVA with Student-Newman-Keuls (SNK-q) analysis. Other data were analyzed using one-way ANOVA followed by SNK-q test for intergroup comparisons after homogeneity test for variance. Differences were considered statistically significant when $P<0.05$.

Results

Exogenous H₂S attenuated THS-induced hemodynamic deterioration and cardiac dysfunction

The baseline MAP and HR were not significantly different among the four groups. The measured hemodynamic variables were significantly decreased during the shock period in THS group, Vehicle group, and NaHS group ($P<0.05$, Figure 1A, 1B), and began to rise up after resuscitation. MAP at each time point during resuscitation were significantly lower than Sham group, and NaHS administration increase the MAP values ($P<0.01$, Figure 1A). HR was higher than Sham group at 15 min and 30 min after resuscitation ($P<0.05$, Figure 1B), but thereafter, there was no statistical difference in HR among the four groups.

LVP, +dp/dtmax, and -dp/dtmax were significantly decreased at 2 h after resuscitation in THS group and Vehicle group, as compared with Sham group ($P<0.01$, Figure 1C, 1D). NaHS administration significantly restored these cardiac parameters ($P<0.01$, Figure 1C, 1D). No significant difference was observed in cardiac function between THS group and Vehicle group.

Exogenous H₂S decreased CK and LDH levels and attenuated myocardial injury

CK and LDH levels in the serum were significantly increased in THS group and Vehicle group compared to Sham group ($P<0.01$, Figure 2A, 2B) NaHS treatment significantly decreased CK and LDH levels in the rat serum ($P<0.05$, Figure 2A, 2B).
As shown in HE staining (Figure 2C), cardiomyocytes are well-arranged and uniformly-stained in Sham group. THS resulted in significant myocardial injury characterized by edema of myocardial cells, unevenly-stained cardiac muscle fibers, and abnormally-distributed cardiac muscle spaces. There was marked infiltration of inflammatory cells into the interstitial spaces with fragmentation of myocardium in the rat hearts of THS group and Vehicle group. NaHS administration significantly attenuated these changes induced by THS.

Exogenous H2S inhibited mPTP opening and preserved mitochondrial structure after THS

The optical density began to decline in each group after administration of calcium into the mitochondrial suspension (Figure 3A). Compared with Sham group, changes of A540 (A540_max-A540_min) were significantly more in THS group and Vehicle group (P<0.05, Figure 3B). And the changes were significantly decreased in NaHS group compared to THS group and Vehicle group respectively (P<0.05, Figure 3B). There was no significant difference in the changes of A540 between THS group and Vehicle group.

Mitochondrial structure was generally normal in Sham group and NaHS group, but serious damages were found in THS group and Vehicle group (Figure 3C). The mitochondria of Sham group were integrated and the cristae were neatly aligned. Comparatively, in THS group and Vehicle group, the mitochondria in the myocardial tissues were generally swelling and deformed with disarranged cristae and a lot of vacuoles. NaHS group displayed little changes in mitochondrial structure compared to Sham group (Figure 3C).

Exogenous H2S regulated Bax/Bcl-2 apoptotic signaling during THS

Compared with Sham group, bax and caspase-9 mRNA were significantly increased in THS group and Vehicle group, which was re-
Mechanisms of cardioprotective effects of exogenous H\textsubscript{2}S

versed by NaHS administration (P<0.05, Figure 4A, 4B). Meanwhile, bcl-2 mRNA was significantly decreased in the other three groups as compared with Sham group (P<0.05, Figure 4C), but there was no significant difference among the three groups. The expression of Bax protein was increased in THS group and Vehicle group compared with Sham group, whereas the expression of Bcl-2 protein was decreased (P<0.05, Figure 5). And NaHS treatment significantly reduced the expression of Bax and increased the expression of Bcl-2 (P<0.05, Figure 5).

Exogenous H\textsubscript{2}S Reduced THS-induced apoptosis

The number of apoptotic cells in the rat hearts after THS were obviously increased in THS group and Vehicle group compared to Sham group (P<0.01, Figure 6). In contrast, NaHS administration evidently alleviated the apoptosis compared to THS group and Vehicle group (P<0.01, Figure 6). And few apoptotic cells were detected in Sham group.

Discussion

In the present study, we demonstrated that exogenous H\textsubscript{2}S protected against THS-induced myocardial injury, inhibited myocardial apoptosis and improved the cardiac function. Specifically, administration of exogenous H\textsubscript{2}S decreased CK and LDH levels, reduced TUNEL-positive cells, and increased LVP, +dp/dtmax, -dp/dtmax levels in NaHS-treated animals. Additionally, exogenous H\textsubscript{2}S inhibited mPTP opening, protected against mitochondrial damage, and regulated the imbalance of Bax/Bcl-2 apoptotic signaling induced by THS.

Our previous study has found that exogenous H\textsubscript{2}S attenuated cardiac injury caused by THS; however, the exact mechanisms of this protec-
Mechanisms of cardioprotective effects of exogenous H$_2$S

tive effect have not been fully elucidated [4]. The anti-apoptotic effects of H$_2$S have been widely reported in different models of I/R injury both in vivo and in vitro [5-11, 25]. For example, NaHS significantly attenuates myocardial apoptosis induced by hyperhomocysteinemia or ligation of left anterior descending coronary artery in rats [6, 7]. In addition, NaHS pretreatment reduces apoptosis in cardiomyocytes following hypoxia/reoxygenation [9, 25]. But few studies have investigated the anti-apoptotic effect of H$_2$S on myocardial injury in THS rats, and even

![Figure 6. Effects of exogenous H$_2$S on myocardial apoptosis. A. Representative images of TUNEL analysis for apoptosis. Original magnification × 400; scale bars = 20 μm. B. THS caused a marked increase in the number of apoptotic cells in myocardial tissues, which was reversed by NaHS administration. **P<0.01 vs. Sham group; ###P<0.01 vs. THS group and Vehicle group.](image)
Mechanisms of cardioprotective effects of exogenous H$_2$S

fewer have addressed the underlying mechanisms. To the best of our knowledge, this work is the first evidence to demonstrate the cardioprotective effects of exogenous H$_2$S via inhibiting mitochondrial apoptosis in THS rats.

The mPTP plays a prominent role in determining the possibility of cells to survive or undergo apoptosis after I/R injury and a variety of other stresses [18]. Elrod and colleagues found that H$_2$S attenuated myocardial I/R injury by preservation of mitochondrial structure and function [5]. NaHS significantly attenuated rat myocardial apoptosis and preserved left ventricular function through cardiac mitochondrial protection [6, 11]. Similarly, Yao et al. reported that H$_2$S protected against hypoxia/reoxygenation-induced cardiomyocytes apoptosis via prevention of mPTP opening [25]. Consistent with these previous studies, we found that exogenous H$_2$S prevented mPTP opening and preserved mitochondrial structure at the time of myocardial reperfusion in the THS rats.

In addition, the Bcl-2 family is also a critical mediator of cardiac I/R injury through activation of apoptotic signaling [22, 27]. It has been reported that NaHS corrected the imbalance of Bax/Bcl-2 induced by I/R injury in a rat model of left coronary artery ligation [7, 28]. Zhou et al. have reported that NaHS increased Bcl-2 expression after isolated heart reperfusion [9]. Besides, NaHS administration up-regulated Bcl-2 expression and down-regulated Bax expression in alcohol intake-induced left ventricular remodeling in rats [29]. Similarly, in this study, we found that exogenous H$_2$S up-regulated Bcl-2 protein expression and down-regulated the protein and gene expression of Bax after resuscitation. However, the expression of bcl-2 mRNA was not changed by NaHS administration. Wang et al. reported that NaHS promoted Bcl-2 protein and mRNA expression in a rat model of heart failure after 6 weeks of NaHS administration, which suggested that the discrepancy in the results may be attributed to the earlier observation time point or the different NaHS administration methods in our study [11].

Although we demonstrated that exogenous H$_2$S reduced THS-induced myocardial apoptosis by inhibiting mPTP opening and regulating Bax/Bcl-2 signaling in this study, the exact mechanisms of how H$_2$S acting on mPTP and Bax/Bcl-2 signal have not been clarified. It has been reported that Ca$^{2+}$ overloaded is one of the main factors resulting in I/R injury after reperfusion [30]. The increase of intracellular Ca$^{2+}$ triggers the opening of mPTP, leading to the release of pro-apoptotic factors, which initiate the apoptotic cascade [31]. Some studies indicated that H$_2$S can prevent intracellular Ca$^{2+}$ overload by inducing intracellular acidosis via suppression of Na$^+$/H$^+$ exchanger-1 (NHE-1) on the cardiomyocytes, thereby inhibiting the opening of mPTP [32]. Besides, H$_2$S can also activate some pro-survival kinases, such as phosphoinositide 3-kinase (PI3K), protein kinase B (Akt) and glycogen synthase kinase-3β (GSK-3β) after I/R injury [9, 19, 25]. GSK-3β has been proposed as a viable target in the ischemic injury of the heart. Zhang et al. demonstrated that NaHS increased phosphorylation of GSK-3β and thus inhibited the opening of mPTP [19]. In addition, Yao et al. demonstrated that NaHS increased phosphorylation of GSK-3β at Ser9 by inducing the phosphorylation of Akt, which then inhibited the translocation of Bax to mitochondria and ultimately inhibited apoptosis [25]. However, as H$_2$S may also affect the mPTP activity and Bax/Bcl-2 signaling through other mechanisms, such as microRNA-1 (miR-1) and mammalian target of rapamycin complex 2 (mTORC2), more research is required to explore this question [8, 9].

In conclusion, our results confirmed that exogenous H$_2$S inhibited THS-induced myocardial injury and improved the cardiac function after resuscitation via inhibiting myocardial apoptosis, preventing mPTP opening and regulating the expression of apoptosis-related genes and proteins after THS. These data may provide a novel insight into the cardioprotective effects of H$_2$S and improve our understanding for the treatment of THS in the clinical setting.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (8127-2133, 81201024).

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Qian Ding and Wei Chai, Department of Anesthesiology, Tangdu Hospital, Fourth Military Medical University, Xi’an, Shaan-
Mechanisms of cardioprotective effects of exogenous H₂S

xi, China. Tel: 86-29-84777489; Fax: 86-29-84777439; E-mail: 250848637@qq.com (QD); Tel: 86-29-84777439; Fax: 86-29-84777439; E-mail: tdmzka@fmmu.edu.cn (WC)

References

[19] Lindsay J, Esposti MD and Gilmore AP. Bcl-2 proteins and mitochondria - specificity in mem-
Mechanisms of cardioprotective effects of exogenous H₂S


