Original Article
Cardioprotection by H$_2$S postconditioning engages the inhibition of endoplasmic reticulum stress

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Abstract: Hydrogen sulfide (H$_2$S), a novel signaling gasotransmitter in the system, plays a prominent role in modulating many pathophysiology processes. However, the pathways mediating its effects have not been well addressed. Here we investigated the impact of H$_2$S postconditioning on myocardium ischemia/reperfusion (I/R) injury and evaluated its potential inhibition of endoplasmic reticulum (ER) stress mechanism. In in vitro study H9c2, a kind of rat’s cardiomyocyte, was subjected to hypoxia/reoxygenation, and we found H$_2$S postconditioning reduced cell apoptosis and attenuated the expression level of cystathionine gamma-lyase (CSE) mRNA. In in vivo experiments of rats myocardial I/R injury, there were a decline in myocardial infarct size and cardiac arrhythmias, and a rise in plasma H$_2$S concentration. Furthermore, H$_2$S postconditioning significantly attenuated I/R-induced ER stress responses in myocardium, including the down-regulation of ER markers, GRP78, ATF6, PDI and CHOP. In summary, these results suggested that H$_2$S postconditioning exerted significant cardioprotective effects possibly through the inhibition of ER stress in I/R-induced myocardial injury.

Keywords: Hydrogen sulfide, ischemia/reperfusion, endoplasmic reticulum stress

Introduction
Acute myocardial infarction (AMI) is currently one of the most common causes of morbidity and mortality in the world. In the treatment of AMI, re-establishing coronary blood flow with the rapid use of reperfusion strategies such as thrombolysis, percutaneous coronary angioplasty and coronary artery bypass graft have been widely used in clinical settings. However, these processes may lead to severe injury associated with ischemia/reperfusion (I/R) [1]. In order to reduce myocardial I/R injury, therapeutic strategies such as pre- and post-conditioning, as well as pharmacological interventions have been extensively investigated [2-7]. And pharmacological postconditioning with better predictability, better control and convenient operation indicates better clinical prospect [5].

Recently, investigation in the gaseous signaling molecule hydrogen sulfide (H$_2$S) has demonstrated that it in fact serves as an endogenous mediator in the context of myocardial protection [8, 9]. H$_2$S was reported to protect the heart from myocardial ischemia-reperfusion in various studies [10-12]. The work from our laboratory had also shown that H$_2$S had cardiac protective effects not only in vivo model of rat I/R injury but also in vitro model of hypoxia/reoxygenation in rat H9c2 cardiomyocytes [13]. Although the complete signaling mechanism of H$_2$S remain to be clarified, the general understanding on the cardioprotective effect of H$_2$S to date has led us to hypothesize that H$_2$S postconditioning may contribute to the protective effect of I/R.

I/R injury is mediated primarily by oxidative stress, intracellular and mitochondrial calcium overload, and inflammatory cell accumulation in infarcted myocardial tissues [14]. These various factors could interfere with ER function and then cause endoplasmic reticulum stress (ERS) [15]. ERS is a condition in which unfolded proteins accumulate and aggregate during disruptions of ER homeostasis. When ERS occurs, dissociation of the glucose-regulated protein of 78
kDa (GRP78) serves as the monitor to activate and trigger the unfolded protein response (UPR) and cells develop a self-protective strategy to restore normal ER function. It has been documented that three sensors of ERS, termed inositol-requiring 1 (IRE1), PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6), are physiologically kept inactive by GRP78 [16]. In addition, protein disulfide isomerase (PDI) distributes mainly in the endoplasmic reticulum lumen to launch UPR [17]. However, if stress is too intense or persistent, C/EBP homologous protein (CHOP), caspase-12, and JNK are activated, and ER/SR stress-induced apoptosis can be initiated [18]. Some data suggest that attenuation of ERS-induced apoptosis can protect the heart against I/R injury [19-21]. Thus, therapeutic interventions targeting ERS represent promising strategies for the treatment of ischemic cardiovascular diseases. Based on these investigations, here we aimed to test whether administration of exogenous H\textsubscript{2}S would exert cardioprotective effects to acute myocardial I/R injury in vitro and in vivo. Furthermore, we used the TUDCA (tauroursodeoxycholic acid, a specific inhibitor of ERS) to investigate if the ERS-related protein participates in the cardioprotection of H\textsubscript{2}S postconditioning in rat myocardial I/R injury.

Materials and methods

Reagents

Sodium hydrosulfide (NaHS) was purchased from Sigma-Aldrich (St Louis, MO, USA). TUDCA was purchased from Calbiochem (La Jolla, CA, USA). GRP78, ATF6, CHOP and PDI antibodies were purchased from Santa Cruz Biotechnology (CA, USA). DMEM, fetal bovine serum (FBS) and TRIzol reagent were purchased from Gibico BRL (Calsbad, CA, USA). SYBR Green Real-time PCR Master Mix was purchased from TOYOBO Inc. (Japan).

Cell culture and hypoxia/reoxygenation

Rat H9c2 cardiac myocytes (Wuhan University Center for Animal Experiment, Wuhan, China) were cultured in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified incubator. Cells on culture plates were placed into the hypoxia chamber for 12 h to induce hypoxia, and then re-oxygenated with maintenance medium for 4 h to induce reoxy-
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Administration of the ERS inhibitor TUDCA (25 mg/kg) was performed at the onset of reperfusion.

**Determination of H9c2 cell apoptosis**

Flow cytometry was performed to determine the content of apoptotic sub G₁ hypo-diploid cells [23]. The cells were digested with trypsin, washed twice with ice-cold PBS, and fixed in 70% ethanol at 4°C overnight. Fixed cells were then washed with PBS and incubated with propidium iodide (50 μg/mL), RNase A (10 mg/mL), and 0.1% Triton X-100 for 10 min in the dark. Cardiomyocyte apoptosis was analyzed by flow cytometry (FACSCalibur, Rockville, Becton Dickinson, San Jose, CA) with ModFit Flow Cytometry Software. The proportion of sub G₁ hypo-diploid cells was assessed by the histograms generated using the computer program. A total of 10⁴ cells were detected in each of the samples.

**Real-time PCR analysis**

Total RNA was extracted from H9c2 cardiomyocytes by TRizol and used to synthesize cDNA. cDNA was then used as the template for PCR with the following primers: CSE forward: 5'-GT-GATGTTGCTATGGGTAGT-3' and reverse: 5'-TC-GGCAGCAGGTAACA-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a normalization control: forward: 5'-AAAGCT-GGTACATCGAGGCTCAGAAT-3' and reverse 5'-GAAG-GAGCAGTAGACTCCAGC-3'. The reaction conditions were as follows: 95°C for 1 min, followed by 40 repeated cycles of 95°C for 10 s, 60°C for 30 s, and a final extension at 72°C for 5 min.

**Myocardial infarct size determination**

The infarct size was determined by 1% 2, 3, 5-triphenyltetrazolium chloride (TTC) staining [24]. In brief, at the end of reperfusion, the hearts were rapidly excised from the thorax and washed by 4°C physiological saline. The left ventricle (LV) was separated from the heart and weighed, and then frozen for 3 h at -20°C. Then the LV was cut into 5 transverse slices (1-2 mm) and the slices were incubated in 1% TTC (pH 7.4) at 37°C for 10 min. This method has been shown to reliably identify necrotic myocardium (which appears pale) from viable myocardium that stains brick red. The pale necrotic myocardial tissue was separated from the stained portions and weighed. The total weight of the area of necrosis was calculated and expressed as a percentage of the total left ventricular weight [25].

**Arrhythmia score**

Ventricular arrhythmias were recorded by ECG during the 2 hour reperfusion period. Arrhythmia scores were reference to the following Lambeth Conference standard: 0 points: no arrhythmia; 1 point: occasional ventricular premature beats (VPBs); 2 points: frequent VPBs (bigeminal or trigeminal rhythm); 3 points: occasional ventricular tachycardia (VT); 4 points: sustained VT or occasional ventricular fibrillation; and 5 points: ventricular fibrillation or death.

**Measurement of H₂S concentration in plasma**

H₂S concentration in the plasma was determined by the method described previously [26]. In brief, immediately after ischemia/reperfusion, plasma was collected from heart of rats before sacrifice and centrifuged (4000 rpm, 10 min, Room Temperature). 0.5 mL of 1% zinc acetate and 2.5 mL of distilled water were mixed with 0.1 mL of plasma. Subsequently, 0.5 mL of 20 mmol/L N, N-dimethyl-p-phenylenediamine dihydrochloride in 7.2 mol/L HCl and 0.4 mL of 30 mmol/L FeCl₃ in 1.2 mol/L HCl were applied for 20 min at room temperature. The protein in the plasma was removed by adding 1 mL of 10% trichloroacetic acid to the reaction mixture and pelleted by centrifugation. The optical absorbance at 670 nm was measured with a spectrophotometer.

**Western blot analysis**

Western blot analysis of GRP78, ATF6, PDI and CHOP were performed with 10 μg of protein extract, obtained as described previously [27], using rat monoclonal antibodies (1:1000 dilution) as the primary antibodies and peroxidase-conjugated rabbit-anti-rat IgG antibody (1:5000 dilution; Santa Cruz Biotechnology, CA) as a secondary antibody. The developed signals were visualized using ECL detection kits and analyzed with PhotoShop software.

**Statistical analysis**

Statistical analysis involved the use of SPSS 13.0. Data were presented as mean ± SD. The
**Results**

**H$_2$S postconditioning inhibited H/R-induced apoptosis and upregulated CSE mRNA level in H9c2 cells**

To evaluate the effect of H$_2$S postconditioning on rat H9c2 cardiac myocytes in H/R model, we measured the apoptosis by flow cytometry. As shown in Figure 1, 200 μmol/L NaHS added at the time of reoxygenation significantly inhibited H/R-induced cell death.

**H$_2$S postconditioning decreased myocardial infarct size**

Based on H$_2$S postconditioning preventing the H/R damage in vitro, we also monitored the effect of H$_2$S postconditioning on myocardial I/R injury in vivo. We measured the infarct size...
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Table 1. The incidence of ventricular arrhythmia in different groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Incidence of VPB (%)</th>
<th>Incidence of VT (%)</th>
<th>Incidence of VF (%)</th>
<th>Score (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.00</td>
</tr>
<tr>
<td>I/R</td>
<td>62.5</td>
<td>100.0</td>
<td>75.0</td>
<td>4.250 ± 0.707**</td>
</tr>
<tr>
<td>I/R + NaHS</td>
<td>25.0</td>
<td>37.5</td>
<td>12.5</td>
<td>1.625 ± 0.517**ΔΔ</td>
</tr>
<tr>
<td>I/R + TUDCA</td>
<td>37.5</td>
<td>50.0</td>
<td>25.0</td>
<td>3.125 ± 0.835**</td>
</tr>
<tr>
<td>I/R + TUDCA + NaHS</td>
<td>12.5</td>
<td>25.0</td>
<td>12.5</td>
<td>1.500 ± 0.535**ΔΔ</td>
</tr>
</tbody>
</table>

Note: Rats were subjected to 0.5 h of left ventricle ischemia and reperfusion for 2 h with or without NaHS (14 μmol/kg) and/or TUDCA (25 mg/kg) posttreatment. Ventricular arrhythmia was monitored by ECG from lead II. Incidence of ventricular arrhythmia was expressed as a percentage of the total ventricular arrhythmias times. Statistical results of arrhythmic scores evaluating the cardiac arrhythmias recorded 2 hours during reperfusion in the different groups. Data are reported as mean ± SD (n=12). **P < 0.01 versus the Sham group, *P < 0.01 versus the I/R group, ΔΔP < 0.01 versus the I/R + TUDCA group.

Figure 4. H₂S concentration in the plasma in rats. Rats were subjected to 0.5 h of left ventricle ischemia and reperfusion for 2 h with or without NaHS (14 μmol/kg) and/or TUDCA (25 mg/kg) posttreatment. The concentration of H₂S was measured from rat plasma as described in Materials and Methods. Data are presented as the mean ± SD (n=12). **P < 0.01 versus the Sham group, *P < 0.01 versus the I/R group, ΔΔP < 0.01 versus the I/R + TUDCA group.

H₂S postconditioning elevated the plasma concentration of H₂S

In addition, we also measured the plasma concentrations of H₂S in rats. H₂S concentration was significantly decreased after I/R compared with sham group (P < 0.01). Compared with the I/R group, H₂S concentration were significantly elevated following postconditioning with NaHS or TUDCA + NaHS together (P < 0.01) (Figure 4). Postconditioning with TUDCA alone did not impact plasma H₂S concentration compared with I/R group (P > 0.05).

H₂S postconditioning attenuated myocardial I/R-induced ER stress in rats

In order to investigate the mechanism underlying the protective effect of H₂S postconditioning against myocardial I/R injury in rats, we measured the expression of ERS-related proteins in myocardium such as GRP78, ATF6, PDI and CHOP. As shown in Figure 5, expression levels of proteins GRP78, ATF6, PDI and CHOP were significantly elevated in the I/R group compared to the sham group (P < 0.01). When postconditioning with NaHS or TUDCA, the expression of these proteins decreased markedly compared with the I/R group (P < 0.05). Treatment with NaHS or TUDCA + NaHS inhibited the expression of ERS-related proteins more effectively than treatment with TUDCA (P < 0.05).
H$_2$S postconditioning attenuates myocardial I/R injury

Discussion

During the last 10 years, pharmacological post-conditioning for managing myocardial I/R injury was extensively studied [5]. H$_2$S has recently come to the fore due to some promising data demonstrating that this gasotransmitter confers cardioprotection in a variety of settings [28]. However, the underlying molecular mechanisms of the protective effect of H$_2$S have not been fully elucidated. The results of the current study demonstrate that therapeutic administration of exogenous H$_2$S prior to the reperfusion period, provides significant myocardial protection in response to acute I/R injury. Our data found that: (1) H$_2$S postconditioning inhibits H/R-induced apoptosis and potentiates the CSE mRNA expression in rat H9c2 cardiac myocytes; (2) H$_2$S postconditioning significantly limits myocardial infarct size, reduces the number and duration of arrhythmias, as well as increases the plasma concentrations of H$_2$S in vivo; (3) H$_2$S postconditioning attenuates myocardial I/R-induced ER stress in rats. Taken together, these data indicate that H$_2$S plays an important role in myocardial cytoprotection during the development of myocardial infarction through inhibition of ER stress.

Reperfusion is a critical treatment for minimizing myocardial damage following AMI (acute myocardial infarction). However, reperfusion can lead to new injury which is known as “reperfusion injury”. Previous studies have demon-
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...strated that endogenously produced or exogenously supplied hydrogen sulfide (H₂S) could contribute to cytoprotection during reperfusion injury in cardiomyocytes, in isolated ex vivo and in in vivo hearts [8, 9, 12]. Recently, Neel R. Sodha and his colleagues reported that therapeutic sulfide administrated at the time of reperfusion after sustained ischaemia provided protection in response to I/R injury in Yorkshire swine, such as, improving myocardial function, reducing infarct size and improving coronary microvascular reactivity [10]. Sofia-Iris Bibili also observed postconditioning of NaHS induced cardioprotection in rabbits and mice by reducing the infarct size [29]. Moreover, in isolated rat hearts and primary cultured neonatal cardiomyocytes, hydrogen sulfide postconditioning had been shown to play an important role in the cardioprotection against I/R-induced apoptosis [30, 31]. In agreement with the limited number of in vivo and in vitro studies [10, 29-31], our findings further supported that H₂S postconditioning was able to exert an effective protection against the injury subjected to I/R by reducing cardiomyocyte apoptosis in H9c2 cells, and decreasing infarct size and reducing arrhythmias incidence in rats model.

In the cardiovascular system, H₂S is predominantly generated by cystathionine-γ-lyase (CSE) [32]. It has been reported that treatment with exogenous H₂S or overexpression of CSE resulted in endogenous H₂S production, which was associated with profound protection against H/R-induced apoptosis [31], ischemia-induced heart failure and mortality in mice with myocardial ischemia-reperfusion injury [33]. Our findings did indeed demonstrate that H₂S concentration in rats plasma was significantly decreased after I/R, but increased obviously in the NaHS postconditioning group, and the levels of H₂S was related to the severity of heart injury. The results were consistent with previous study that endogenous H₂S was associated with the myocardial I/R injury and H₂S postconditioning could protect against reperfusion-induced injury.

Emerging data of myocardial I/R point to the role of ER stress (ERS) as one of the main events that can modulate cell tolerance to stress and survival after injury [19, 20, 34, 35]. A large number of ERS-associated proteins have been shown to be involved in the development of myocardial I/R [36]. In our previous study, excessive ER stress has been suppressed by H₂S preconditioning in both I/R model of the adult rats and hypoxia/reoxygenation (H/R) model of rat H9c2 cardiomyocytes [13]. Therefore, regulation of ER stress becomes critical in understanding the contribution of H₂S postconditioning to the protection against I/R injury. In the present work, we examined the influence of H₂S postconditioning on the expression of protein markers of ER stress, such as GRP78, ATF6, PDI and CHOP. GRP78, an ER-chaperon protein, plays a crucial role in regulation of the ER dynamic homeostasis [37-39]. As an initial step of ERS, GRP78 senses accumulation of misfolded/unfolded proteins and dissociates from the three ER transmembrane sensors, PERK, IRE1 and ATF6. Subsequently, many genes including GRP78, HERP, GRP94, and PDI were activated to initiate the UPR. CHOP (C/EBP homologous protein 10) is a transcription factor and a good biomarker of the presence of excessive ER stress [40]. The increase of CHOP plays a major role in inducing apoptosis. Within the current study, the increased level of GRP78, ATF6 and PDI expression in myocardium I/R injury (Figure 4) indicated the accumulation of misfolded/unfolded proteins in ER lumen, and the expression of CHOP, a major inducer of apoptosis, was increased. Moreover, our findings revealed that administration of NaHS (the donor of H₂S) alleviated the upregulated expressions of GRP78, ATF6, PDI and CHOP in rats myocardium induced by I/R injury. H₂S is known to suppress excessive ER stress to launch its protective effect in different models, such as doxorubicin-induced cardiotoxicity [41], hyperhomocysteinemia-induced cardiomyocytic injury [42], homocysteine-induced neuronal apoptosis [43], and formaldehyde-induced neurotoxicity [44]. Taken together, these findings support the notion that either exogenous or endogenous increasing of H₂S exerts protective effect through inhibition of ER stress.

Furthermore, we compared the cardioprotection effect of H₂S postconditioning with taurodeoxycholic acid (TUDCA), capable of inhibiting ER stress-induced apoptosis in vivo and in vitro [45-47], in myocardium I/R injury model. Our results revealed that treatment with TUDCA at the onset of reperfusion attenuated I/R injury by limiting infarct size and reducing arrhythm-
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memias incidence in rats. In addition, TUDCA effectively inhibited I/R-induced ER stress. Compared with TUDCA, we were surprised to find that H₂S treatment showed a stronger protective effect. It suggested that the ERS pathway might not be the only pathway through which H₂S protected the heart from I/R-induced injury.

It should be noted that, growing evidence has shown that H₂S is capable to preserve mitochondrial function and ultimately promote cytoprotection. Elrod and his colleagues reported that H₂S limited the extent of myocardial infarction in mice and that the protection was associated with mitochondria protection [8]. In addition, H₂S inhibited the release of cytochrome c from mitochondria to preserve mitochondrial structure and function in order to attenuate myocardial injury [48]. H₂S may protect mitochondrial function by downregulating mitochondrial respiration [49], limiting the generation of ROS and diminishing the degree of mitochondrial uncoupling [8]. These findings prompted us to investigate the possibility that mitochondria were also involved in H₂S-mediated cardioprotection in myocardial I/R injury in rats. Thus, further studies will be needed to investigate the association between ER stress and mitochondrial dysfunction underlying the cardioprotective effect of H₂S postconditioning.

In summary, our present study revealed that administration of H₂S prior to the onset of reperfusion markedly attenuates myocardial ischemia-reperfusion injury. The cardioprotective effect may be associated with the inhibition of ERS pathway. These findings should shed some lights on the therapeutic implication of H₂S in the clinical setting when administered concomitantly with the coronary revascularization process.

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Disclosure of conflict of interest

None.

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