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Original Article

Cardioprotection by H_2S postconditioning engages the inhibition of endoplasmic reticulum stress

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Abstract: Hydrogen sulfide (H_2S), 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_2S 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_2S 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_2S concentration. Furthermore, H_2S 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_2S 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 intensively 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_2S) has demonstrated that it in fact serves as an endogenous mediator in the context of myocardial protection [8, 9]. H_2S 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_2S 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_2S remain to be clarified, the general understanding on the cardioprotective effect of H_2S to date has led us to hypothesize that H_2S 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_2S 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_2S 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 reoxygenation. Cultured cardiomyocytes were randomly divided into three groups: 1) Control group (Con): normoxic condition for 16 h in DMSO 0.1%; 2) Hypoxia/reoxygenation group (H/R): hypoxia for 12 h and reoxygenation for 4 h; 3) NaHS postconditioning group (H/R + NaHS): after hypoxia for 12 h, 200 μmol/L NaHS added to DMEM before 4 h reoxygenation.

Myocardial ischemia/reperfusion model and experimental protocol

Male adult Sprague-Dawley (SD) rats weighing 200-250 g were obtained from the animal experimental center of Wuhan University School of Medicine. All animal experiments were performed in accordance with the guidelines for laboratory animal care of Medical College of Wuhan University, Hubei, China. The myocardial ischemia model was established by occlusion of the left anterior descending coronary artery (LAD) [22]. Briefly, all animals were anesthetized with urethane (1.0 g/kg, i.p.). A left lateral thoracotomy (3 cm incision between the third and fourth ribs) was performed to expose the heart. After pericardiotomy, the LAD was occluded with a 5-0 silk suture between the arterial cone and the left auricle, and the chest was closed immediately. At 0.5 h after the coronary occlusion, the chest was reopened and the suture was loosened for reperfusion for 2 h. Before and during the ischemia and reperfusion periods, heart rate (HR) and standard lead II electrocardiography (ECG) changes were recorded. The presence of myocardial ischemia was confirmed by significant ST segment elevation indicated by ECG. Successful reperfusion was defined as complete ST segment resolution.

The animals were allowed to acclimatize for one week before the experiments. A total of 60 rats were randomized into five experimental groups (n=12). 1) Sham group (Sham): Rats were subjected to the surgical procedures without the left anterior descending artery (LAD) occlusion; 2) Ischemia/reperfusion group (I/R): Rats were subjected to 0.5 h LAD occlusion, followed by 2 h of reperfusion; 3) NaHS postconditioning group (I/R + NaHS): After 0.5 h of LAD occlusion, NaHS (exogenous H_2S donor, 14 μmol/kg) was injected at the onset of reperfusion; 4) TUDCA inhibition group (I/R + TUDCA): TUDCA (25 mg/kg) was injected at the onset of reperfusion; 5) NaHS postconditioning and TUDCA inhibition group (I/R + TUDCA + NaHS): In addi-

tion to NaHS (14 μmol/kg) postconditioning, 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 G_1 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 ug/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 Modifit Flow Cytometry Software. The proportion of sub G_1 hypo-diploid cells was assessed by the histograms generated using the computer program. A total of 10^4 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'-GTGATGTTGTCATGGGCTTAGT-3' and reverse: 5'-TCGGCAGCAGAGGTAACAA-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a normalization control: forward: 5'-AAGCTGGTCATCAATGGCAAAC-3' and reverse 5'-GAAGACGCCAGTAGACTCCACG-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 H_2S concentration in plasma

H_2S 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-pphenylenediamine dihydrochloride in 7.2 mol/L HCl and 0.4 mL of 30 mmol/L $FeCl_3$ 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

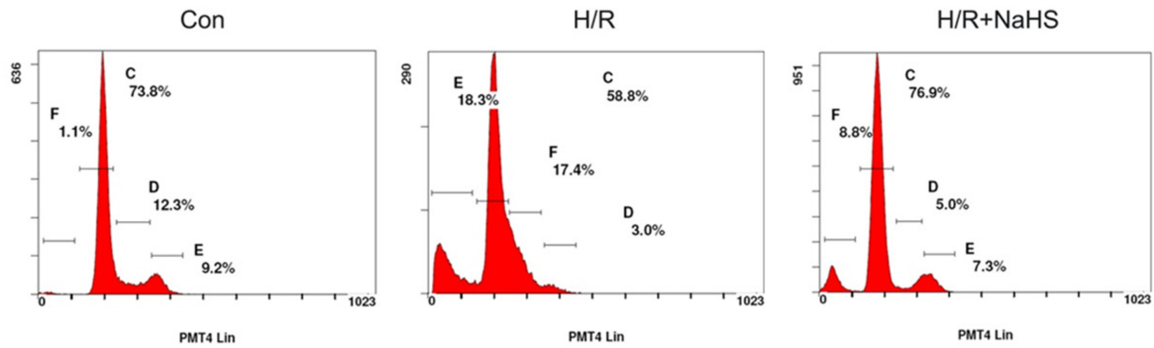

Figure 1. Effect of hydrogen sulfide (H_2S) postconditioning on H/R-induced cell death in rat H9c2 cardiac myocytes. H9c2 cells were exposed to hypoxia/reoxygenation (H/R) with or without NaHS (200 μmol/L NaHS) posttreatment. Representative histograms of flow cytometric analysis by propidium iodide (PI) staining were from 3 independent experiments. The peak of sub-G1 fraction is an indicator of the total number of dead cells.

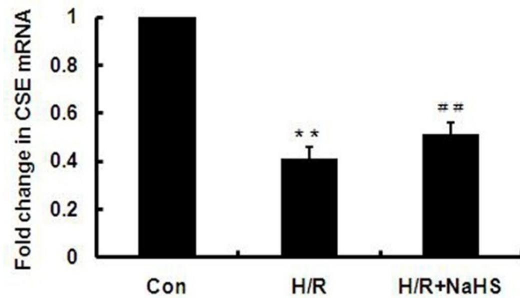

Figure 2. Effect of H_2S postconditioning on CSE mRNA expression in rat H9c2 cardiac myocytes. H9c2 cells were subjected to hypoxia 12 h followed by 4-h reoxygenation in the absence (H/R) or presence of NaHS (200 μmol/L NaHS) posttreatment. Cells cultured in a regular medium without being subjected to H/R were used as a control. The mRNA level of CSE was measured by a real-time PCR analysis. Data are presented as the mean ± SD (n=6). $^{**}P < 0.01$ versus the control group, $^{\#\#}P < 0.01$ versus the H/R group.

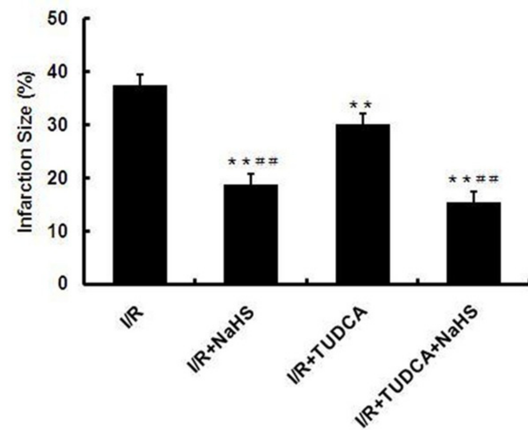

Figure 3. Effect of H_2S postconditioning on myocardial infarct size in rats. TTC-stained sections were obtained from rat hearts subjected to 0.5 h of ischemia followed by 2 h of reperfusion, with or without NaHS (14 μmol/kg) and/or TUDCA (25 mg/kg) postconditioning. Myocardial infarct size was expressed as a percentage of total left ventricle volume. Data are presented as the mean ± SD (n=6). $^{**}P < 0.01$ versus the I/R group, $^{\#\#}P < 0.01$ versus the I/R + TUDCA group.

significance of differences among groups was evaluated by a Student's t-test for unpaired data or Dunnett's t-test for multiple comparisons preceded by one-way analysis of variance (ANOVA). For all test, $P < 0.05$ was considered as statistically significant.

Results

H_2S postconditioning inhibited H/R-induced apoptosis and upregulated CSE mRNA level in H9c2 cells

To evaluate the effect of H_2S 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.

Real-Time PCR data showed that 200 μmol/L NaHS significantly elevated the level of CSE mRNA compared with the H/R group ($P < 0.01$) in H9c2, while the level of CSE mRNA decreased remarkably in H/R group than in the control group ($P < 0.01$) (**Figure 2**).

H_2S postconditioning decreased myocardial infarct size

Based on H_2S postconditioning preventing the H/R damage *in vitro*, we also monitored the effect of H_2S postconditioning on myocardial I/R injury *in vivo*. We measured the infarct size

Table 1. The incidence of ventricular arrhythmia in different groups

Group	Incidence of VPB (%)	Incidence of VT (%)	Incidence of VF (%)	Score (Mean ± SD)
Sham	0.0	0.0	0.0	0.000
I/R	62.5	100.0	75.0	4.250 ± 0.707**
I/R + NaHS	25.0	37.5	12.5	1.625 ± 0.517$^{\#\#,\Delta\Delta}$
I/R + TUDCA	37.5	50.0	25.0	3.125 ± 0.835$^{\#\#}$
I/R + TUDCA + NaHS	12.5	25.0	12.5	1.500 ± 0.535$^{\#\#,\Delta\Delta}$

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, $^{\Delta\Delta}P < 0.01$ versus the I/R + TUDCA group.

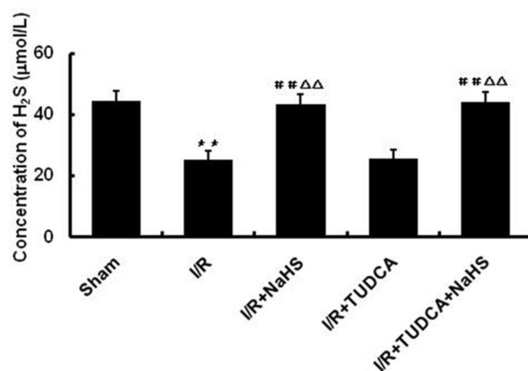

Figure 4. H_2S concentration in the plasma in rats. Rats were subjected to 0.5 h of left ventricle ischemia and reperfusion for 2 h with or without NaHS (14 μmol/kg) and/or TUDCA (25 mg/kg) posttreatment. The concentration of H_2S was measured from rat plasma as described in Materials and Methods. Data are presented as the mean ± SD (n=12). $^{**}P <$ 0.01 versus the Sham group, $^{\#\#}P < 0.01$ versus the I/R group, $^{\Delta\Delta}P < 0.01$ versus the I/R + TUDCA group.

in rats by TTC method. As shown in **Figure 3**, infarct size in I/R hearts was 37.41 ± 3.27%. Postconditioning with 14 μmol/kg NaHS and 25 mg/kg TUDCA respectively both reduced the infarct size significantly compared to the I/R group ($P < 0.01$). Compared with I/R + TUDCA group, the infarct size in I/R + NaHS group and I/R + TUDCA + NaHS group was further decreased ($P < 0.01$).

H_2S postconditioning reduced arrhythmia incidence

To further show H_2S postconditioning protective effect in I/R injury, we monitored the arrhythmia incidence during the 2 hour of reperfusion in rats. ECG from lead II showed that I/R-induced arrhythmia presented mainly as VPB, VT, and VF. The arrhythmia scores for the five experimental groups were shown in **Table 1**. Postconditioning with NaHS alone or together with TUDCA significantly reduced the arrhythmia score, compared with the I/R only or TUDCA only ($P < 0.01$).

H_2S postconditioning elevated the plasma concentration of H_2S

In addition, we also measured the plasma concentrations of H_2S in rats. H_2S concentration was significantly decreased after I/R compared with sham group ($P < 0.01$). Compared with the I/R group, H_2S 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 H_2S concentration compared with I/R group ($P > 0.05$).

H_2S postconditioning attenuated myocardial I/R-induced ER stress in rats

In order to investigate the mechanism underlying the protective effect of H_2S 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).

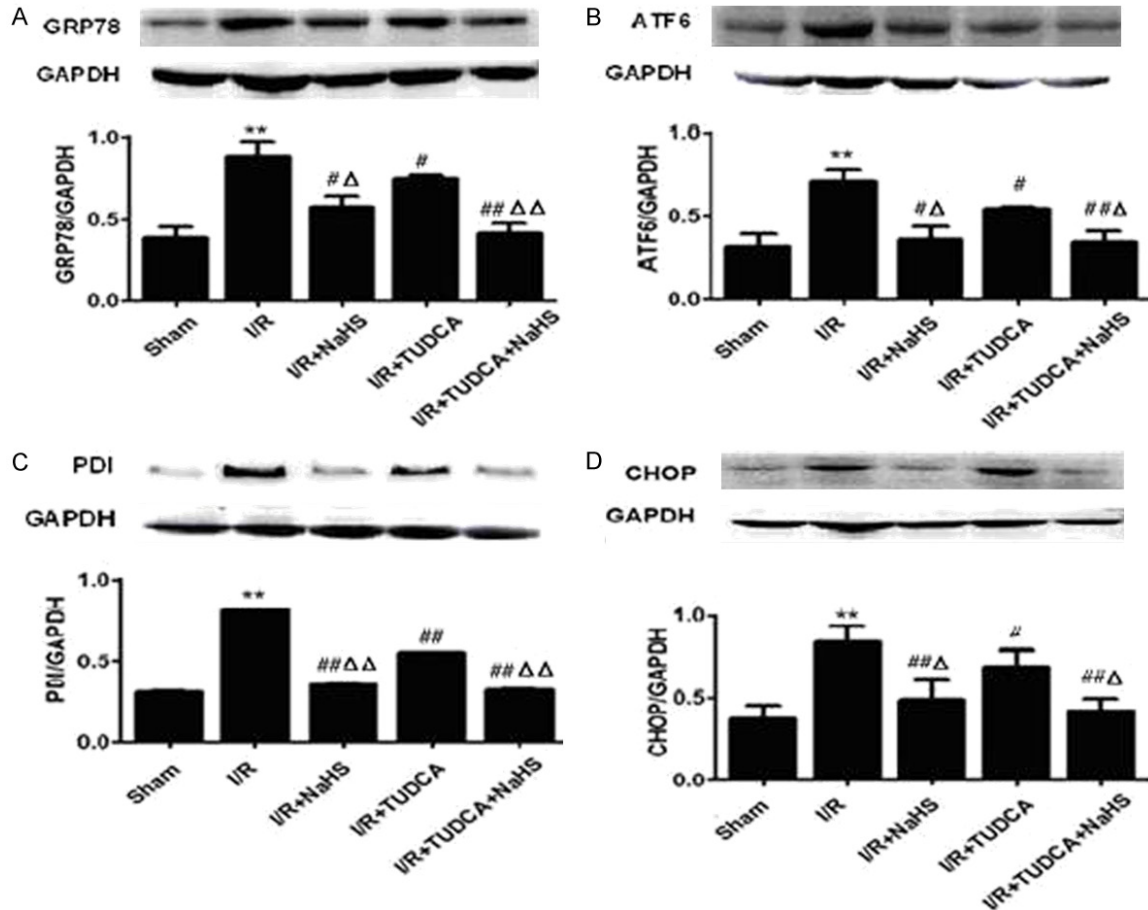

Figure 5. Effect of H_2S postconditioning on the expression of GRP78, ATF6, PDI and CHOP *in myocardium of rats.* Rats were subjected to 0.5 h of ischemia followed by 2 hour of reperfusion, with or without NaHS (14 μmol/kg) and/or TUDCA (25 mg/kg) postconditioning. The protein levels of GRP78 (A), ATF6 (B), PDI (C) and CHOP (D) in myocardium were measured by Western blot analysis. In all blots, staining for GAPDH was used as a loading control. Data are presented as the mean ± SD (n=6). $^{**}P < 0.01$ versus the sham group, $^{\#}P < 0.05$ versus the I/R group, $^{\#\#}P < 0.01$ versus the I/R group, $^{\Delta}P < 0.05$ versus the I/R + TUDCA group, $^{\Delta\Delta}P < 0.01$ versus the I/R + TUDCA group.

Discussion

During the last 10 years, pharmacological postconditioning for managing myocardial I/R injury was extensively studied [5]. H_2S 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_2S have not been fully elucidated. The results of the current study demonstrate that therapeutic administration of exogenous H_2S prior to the reperfusion period, provides significant myocardial protection in response to acute I/R injury. Our data found that: (1) H_2S postconditioning inhibits H/R-induced apoptosis and potentiates the CSE mRNA expression in rat H9c2 cardiac myocytes; *(2)* H_2S postconditioning significantly limits myocardial infarct size, reduces the number and duration of arrhythmias, as well as increases the plasma concentrations of H_2S *in vivo; (3)* H_2S postconditioning attenuates myocardial I/R-induced ER stress in rats. Taken together, these data indicate that H_2S 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-

strated that endogenously produced or exogenously supplied hydrogen sulfide (H_2S) 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 Bibli 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 H_2S 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, H_2S is predominantly generated by cystathionine-γ-lyase (CSE) [32]. It has been reported that treatment with exogenous H_2S or overexpression of CSE resulted in endogenous H_2S 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 H_2S concentration in rats plasma was significantly decreased after I/R, but increased obviously in the NaHS postconditioning group, and the levels of H_2S was related to the severity of heart injury. The results were consistent with previous study that endogenous H_2S was associated with the myocardial I/R injury and H_2S 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 H_2S 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 H_2S postconditioning to the protection against I/R injury. In the present work, we examined the influence of H_2S 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 H_2S) alleviated the upregulated expressions of GRP78, ATF6, PDI and CHOP in rats myocardium induced by I/R injury. H_2S is known to suppresse 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 H_2S exerts protective effect through inhibition of ER stress.

Furthermore, we compared the cardioprotection effect of H_2S postconditioning with tauroursodeoxycholic 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 arrhyth-

mias incidence in rats. In addition, TUDCA effectively inhibited I/R-induced ER stress. Compared with TUDCA, we were surprised to find that H_2S treatment showed a stronger protective effect. It suggested that the ERS pathway might not be the only pathway through which H_2S protected the heart from I/R-induced injury.

It should be noted that, growing evidence has shown that H_2S is capable to preserve mitochondrial function and ultimately promote cytoprotection. Elrod and his colleagues reported that H_2S limited the extent of myocardial infarction in mice and that the protection was associated with mitochondria protection [8]. In addition, H_2S inhibited the release of cytochrome c from mitochondria to preservate mitochondrial structure and function in order to attenuate myocardial injury [48]. H_2S 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 H_2S-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 H_2S postconditioning.

In summary, our present study revealed that administration of H_2S 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 H_2S 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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References

[1] Fordyce CB, Gersh BJ, Stone GW and Granger CB. Novel therapeutics in myocardial infarction: targeting microvascular dysfunction and reperfusion injury. Trends Pharmacol Sci 2015; 36: 605-616.

[2] Murry CE, Jennings RB and Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74: 1124-1136.

[3] Zaman J, Jeddi S, Daneshpour MS, Zarkesh M, Daneshian Z and Ghasemi A. Ischemic postconditioning provides cardioprotective and antiapoptotic effects against ischemia-reperfusion injury through iNOS inhibition in hyperthyroid rats. Gene 2015; 570: 185-190.

[4] Breivik L, Helgeland E, Aarnes EK, Mrdalj J and Jonassen AK. Remote postconditioning by humoral factors in effluent from ischemic preconditioned rat hearts is mediated via PI3K/Akt-dependent cell-survival signaling at reperfusion. Basic Res Cardiol 2011; 106: 135-145.

[5] Morel O, Perret T, Delarche N, Labeque JN, Jouve B, Elbaz M, Piot C and Ovize M. Pharmacological approaches to reperfusion therapy. Cardiovasc Res 2012; 94: 246-252.

[6] Hausenloy DJ. Cardioprotection techniques: preconditioning, postconditioning and remote conditioning (basic science). Curr Pharm Des 2013; 19: 4544-4563.

[7] Eltzschig HK and Eckle T. Ischemia and reperfusion--from mechanism to translation. Nat Med 2011; 17: 1391-1401.

[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 2007; 104: 15560-15565.

[9] Osipov RM, Robich MP, Feng J, Liu Y, Clements RT, Glazer HP, Sodha NR, Szabo C, Bianchi C and Sellke FW. Effect of hydrogen sulfide in a porcine model of myocardial ischemia-reperfusion: comparison of different administration regimens and characterization of the cellular mechanisms of protection. J Cardiovasc Pharmacol 2009; 54: 287-297.

[10] Sodha NR, Clements RT, Feng J, Liu Y, Bianchi C, Horvath EM, Szabo C, Stahl GL and Sellke FW. Hydrogen sulfide therapy attenuates the inflammatory response in a porcine model of myocardial ischemia/reperfusion injury. J Thorac Cardiovasc Surg 2009; 138: 977-984.

[11] Szabo G, Veres G, Radovits T, Gero D, Modis K, Miesel-Groschel C, Horkay F, Karck M and Szabo C. Cardioprotective effects of hydrogen sulfide. Nitric Oxide 2011; 25: 201-210.

[12] Ji Y, Pang QF, Xu G, Wang L, Wang JK and Zeng YM. Exogenous hydrogen sulfide postconditioning protects isolated rat hearts against ischemia-reperfusion injury. Eur J Pharmacol 2008; 587: 1-7.

[13] Li C, Hu M, Wang Y, Lu H, Deng J and Yan X. Hydrogen sulfide preconditioning protects against myocardial ischemia/reperfusion injury in rats through inhibition of endo/sarcoplasmic reticulum stress. Int J Clin Exp Pathol 2015; 8: 7740-7751.

[14] Bell RM and Yellon DM. There is more to life than revascularization: therapeutic targeting of myocardial ischemia/reperfusion injury. Cardiovasc Ther 2011; 29: e67-79.

[15] Rao RV, Poksay KS, Castro-Obregon S, Schilling B, Row RH, del Rio G, Gibson BW, Ellerby HM and Bredesen DE. Molecular components of a cell death pathway activated by endoplasmic reticulum stress. J Biol Chem 2004; 279: 177-187.

[16] Walter P and Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. Science 2011; 334: 1081-1086.

[17] Bassuk JA and Berg RA. Protein disulphide isomerase, a multifunctional endoplasmic reticulum protein. Matrix 1989; 9: 244-258.

[18] Xu C, Bailly-Maitre B and Reed JC. Endoplasmic reticulum stress: cell life and death decisions. J Clin Invest 2005; 115: 2656-2664.

[19] Martindale JJ, Fernandez R, Thuerauf D, Whittaker R, Gude N, Sussman MA and Glembotski CC. Endoplasmic reticulum stress gene induction and protection from ischemia/reperfusion injury in the hearts of transgenic mice with a tamoxifen-regulated form of ATF6. Circ Res 2006; 98: 1186-1193.

[20] Natarajan R, Salloum FN, Fisher BJ, Smithson L, Almenara J and Fowler AA 3rd. Prolyl hydroxylase inhibition attenuates post-ischemic cardiac injury via induction of endoplasmic reticulum stress genes. Vascul Pharmacol 2009; 51: 110-118.

[21] Khan M, Meduru S, Mostafa M, Khan S, Hideg K and Kuppusamy P. Trimetazidine, administered at the onset of reperfusion, ameliorates myocardial dysfunction and injury by activation of p38 mitogen-activated protein kinase and Akt signaling. J Pharmacol Exp Ther 2010; 333: 421-429.

[22] Maulik N, Engelman RM, Rousou JA, Flack JE 3rd, Deaton D and Das DK. Ischemic preconditioning reduces apoptosis by upregulating anti-death gene Bcl-2. Circulation 1999; 100: II369-375.

[23] Nicoletti I, Migliorati G, Pagliacci MC, Grignani F and Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 1991; 139: 271-279.

[24] Rao PR, Kumar VK, Viswanath RK and Subbaraju GV. Cardioprotective activity of alcoholic extract of Tinospora cordifolia in ischemia-reperfusion induced myocardial infarction in rats. Biol Pharm Bull 2005; 28: 2319-2322.

[25] Gao J, Guo J, Li H, Bai S, Li H, Wu B, Wang L, Xi Y, Tian Y, Yang G, Wang R, Wu L, Xu C and Li H. Involvement of dopamine D2 receptors activation in ischemic post-conditioning-induced cardioprotection through promoting PKC-epsilon particulate translocation in isolated rat hearts. Mol Cell Biochem 2013; 379: 267-276.

[26] Chunyu Z, Junbao D, Dingfang B, Hui Y, Xiuying T and Chaoshu T. The regulatory effect of hydrogen sulfide on hypoxic pulmonary hypertension in rats. Biochem Biophys Res Commun 2003; 302: 810-816.

[27] Xiao-Hong Y, Li L, Yan-Xia P, Hong L, Wei-Fang R, Yan L, An-Jing R, Chao-Shu T and Wen-Jun Y. Salusins protect neonatal rat cardiomyocytes from serum deprivation-induced cell death through upregulation of GRP78. J Cardiovasc Pharmacol 2006; 48: 41-46.

[28] Polhemus DJ and Lefer DJ. Emergence of hydrogen sulfide as an endogenous gaseous signaling molecule in cardiovascular disease. Circ Res 2014; 114: 730-737.

[29] Bibli SI, Andreadou I, Chatzianastasiou A, Tzimas C, Sanoudou D, Kranias E, Brouckaert P, Coletta C, Szabo C, Kremastinos DT, Iliodromitis EK and Papapetropoulos A. Cardioprotection by H2S engages a cGMP-dependent protein kinase G/phospholamban pathway. Cardiovasc Res 2015; 106: 432-442.

[30] Luan HF, Zhao ZB, Zhao QH, Zhu P, Xiu MY and Ji Y. Hydrogen sulfide postconditioning protects isolated rat hearts against ischemia and reperfusion injury mediated by the JAK2/STAT3 survival pathway. Braz J Med Biol Res 2012; 45: 898-905.

[31] Li H, Zhang C, Sun W, Li L, Wu B, Bai S, Li H, Zhong X, Wang R, Wu L and Xu C. Exogenous hydrogen sulfide restores cardioprotection of ischemic post-conditioning via inhibition of mPTP opening in the aging cardiomyocytes. Cell Biosci 2015; 5: 43.

[32] Pan LL, Liu XH, Gong QH, Yang HB and Zhu YZ. Role of cystathionine gamma-lyase/hydrogen sulfide pathway in cardiovascular disease: a novel therapeutic strategy? Antioxid Redox Signal 2012; 17: 106-118.

[33] Calvert JW, Elston M, Nicholson CK, Gundewar S, Jha S, Elrod JW, Ramachandran A and Lefer DJ. Genetic and pharmacologic hydrogen sulfide therapy attenuates ischemia-induced heart failure in mice. Circulation 2010; 122: 11-19.

[34] Wang XB, Huang XM, Ochs T, Li XY, Jin HF, Tang CS and Du JB. Effect of sulfur dioxide preconditioning on rat myocardial ischemia/reperfusion injury by inducing endoplasmic reticulum stress. Basic Res Cardiol 2011; 106: 865-878.
[35] Liu XH, Zhang ZY, Sun S and Wu XD. Ischemic postconditioning protects myocardium from ischemia/reperfusion injury through attenuating endoplasmic reticulum stress. Shock 2008; 30: 422-427.
[36] Minamino T and Kitakaze M. ER stress in cardiovascular disease. J Mol Cell Cardiol 2010; 48: 1105-1110.
[37] Bertolotti A, Zhang Y, Hendershot LM, Harding HP and Ron D. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nat Cell Biol 2000; 2: 326-332.
[38] Doyle KM, Kennedy D, Gorman AM, Gupta S, Healy SJ and Samali A. Unfolded proteins and endoplasmic reticulum stress in neurodegenerative disorders. J Cell Mol Med 2011; 15: 2025-2039.
[39] Chen JC, Wu ML, Huang KC and Lin WW. HMG-CoA reductase inhibitors activate the unfolded protein response and induce cytoprotective GRP78 expression. Cardiovasc Res 2008; 80: 138-150.
[40] Oyadomari S and Mori M. Roles of CHOP/GADD153 in endoplasmic reticulum stress. Cell Death Differ 2004; 11: 381-389.
[41] Wang XY, Yang CT, Zheng DD, Mo LQ, Lan AP, Yang ZL, Hu F, Chen PX, Liao XX and Feng JQ. Hydrogen sulfide protects H9c2 cells against doxorubicin-induced cardiotoxicity through inhibition of endoplasmic reticulum stress. Mol Cell Biochem 2012; 363: 419-426.
[42] Wei H, Zhang R, Jin H, Liu D, Tang X, Tang C and Du J. Hydrogen sulfide attenuates hyperhomocysteinemia-induced cardiomyocytic endoplasmic reticulum stress in rats. Antioxid Redox Signal 2010; 12: 1079-1091.
[43] Wei HJ, Xu JH, Li MH, Tang JP, Zou W, Zhang P, Wang L, Wang CY and Tang XQ. Hydrogen sulfide inhibits homocysteine-induced endoplasmic reticulum stress and neuronal apoptosis in rat hippocampus via upregulation of the BDNF-TrkB pathway. Acta Pharmacol Sin 2014; 35: 707-715.
[44] Li X, Zhang KY, Zhang P, Chen LX, Wang L, Xie M, Wang CY and Tang XQ. Hydrogen sulfide inhibits formaldehyde-induced endoplasmic reticulum stress in PC12 cells by upregulation of SIRT-1. PLoS One 2014; 9: e89856.
[45] Ikegami T and Matsuzaki Y. Ursodeoxycholic acid: mechanism of action and novel clinical applications. Hepatol Res 2008; 38: 123-131.
[46] Xie Q, Khaoustov VI, Chung CC, Sohn J, Krishnan B, Lewis DE and Yoffe B. Effect of tauroursodeoxycholic acid on endoplasmic reticulum stress-induced caspase-12 activation. Hepatology 2002; 36: 592-601.
[47] Azfer A, Niu J, Rogers LM, Adamski FM and Kolattukudy PE. Activation of endoplasmic reticulum stress response during the development of ischemic heart disease. Am J Physiol Heart Circ Physiol 2006; 291: H1411-1420.
[48] Wang X, Wang Q, Guo W and Zhu YZ. Hydrogen sulfide attenuates cardiac dysfunction in a rat model of heart failure: a mechanism through cardiac mitochondrial protection. Biosci Rep 2011; 31: 87-98.
[49] Alves MG, Soares AF, Carvalho RA and Oliveira PJ. Sodium hydrosulfide improves the protective potential of the cardioplegic histidine buffer solution. Eur J Pharmacol 2011; 654: 60-67.