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Original Article

Hydrogen sulfide improves cardiomyocytes electrical remodeling post ischemia/reperfusion injury in rats

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Abstract: Hydrogen sulfide (H_2S), produced by cystanthionine-γ-lysase (CSE) in the cardiovascular system, is an endogenous gaseous mediator exerting pronounced physiological effects as the third gasotransmitter in addition to nitric oxide (NO) and carbon monoxide (CO). Accumulating evidence indicated that H_2S could mediate the cardioprotective effects in myocardial ischemia model. Ventricular arrhythmia is the most important risk factor for cardiac mortality and sudden death after acute myocardial infarction (AMI). The potential impact of H_2S on cardiomyocytes electrical remodeling post ischemic insult is not fully explored now. Present study investigated the role of H_2S on cardiomyocytes electrical remodeling in rats with ischemia/reperfusion injury. H_2S concentration was reduced and arrhythmia score was increased in this model. CSE mRNA level was also upregulated in the ischemic myocardium. Exposure to exogenous NaHS reduced the action potential duration (APD), inhibited L-type Ca^{2+} channels and activated K_{ATP} channels in cardiomyocytes isolated from ischemic myocardium Exogenous H_2S application improves electrical remodeling in cardiomyocytes isolated from ischemic myocardium. These results indicated that reduced H_2S level might be linked to ischemia/reperfusion induced arrhythmias.

Keywords: Hydrogen sulfide, CSE, ischemia/reperfusion, action potential duration, L-type Ca^{2+} channels, K_{ATP} channels

Introduction

Hydrogen sulphide (H_2S), like nitric oxide and carbon monoxide, is an endogenously generated gaseous mediator exerting pronounced physiological effects on various organs including cardiovascular system [1]. Endogenous H_2S is produced enzymatically by the cysteine metabolic enzymes cystathionine b-synthase (CBS), cystathionine c-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase1 [2]. In the cardiovascular system, H_2S is predominantly generated by CSE, Previous studies demonstrated that endogenously synthesized H_2S could protect vascular tissues from atherogenic damage by reducing vessel intimal proliferation and inhibiting adhesion molecule expression [3]. The deficiency of CSE in mice leads to a decreased endogenous H_2S level, an age-dependent increase in blood pressure, and impaired endothelium-dependent vasorelaxation [4] and decreased endogenous H_2S production predisposes the animals to vascular remodeling and early development of atherosclerosis. Besides, reduced endogenous H_2S in plasma and myocardial tissue was shown in isoproterenol-induced myocardial injury on male Wistar rats [4]. Previous study showed that endogenous H_2S might mediate the cardioprotection effects exterted by ischemic postconditioning (IPO) in isolated rat hearts [5].

Additionally, exogenous administration of H_2S (in the form of NaHS) could decrease blood pressure in spontaneously hypertensive rats and in anaesthetized normotensive rats [6, 7]. Exogenous administration of H_2S also reduced infarct size in a rat model of coronary artery ligation [8]. Moreover, H_2S levels were reduced in heart failure mice and oral H_2S therapy prevented the transition from compensated to decompensated heart failure via upregulation of endothelial nitric oxide synthase and increase of nitric oxide bioavailability [9].

Myocardial ischemia is characterized by ionic and biochemical alterations, which could result in an unstable electrical substrate capable of initiating and sustaining arrhythmias [11]. At least 75% AMI patients suffered from arrhythmia in the peri-infarct period, which served as a major cause of mortality post AMI [10]. Previous study demonstrated that exogenous administration of H_2S could significantly decrease the duration and severity of ischemia/reperfusion-induced arrhythmias and increase the viability of cardiomyocytes isolated from isolated perfused rat hearts and cultured in simulated ischemia solution [11]. Above evidences suggest that there might be a close association between H_2S and arrhythmias induced by myocardial ischemia. Till now, the relationship between H_2S and ischemia/reperfusion-induced arrhythmia as well as cardiomyocytes electrical remodeling post ischemia is not fully explored. In this study, we investigated the relationship between H_2S and ischemia/reperfusion-induced arrhythmia and cardiomyocytes electrical remodeling post ischemia/reperfusion injury.

Material and methods

Animals

Wistar rats (20 ± 2 weeks old, weighing 200-250 g) were obtained from the Department of Experimental Animals, Chinese Academy of Sciences in Shanghai. The investigation conformed to the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH) of the United States and was approved by the Ethics Committee of the Xinhua Hospital. A total of 85 rats were used for the experiment, ischemia/reperfusion model mortality is about 15%.

Ischemia/reperfusion injury model in rats

Anaesthesia was induced with 10 g/L chloral hydrate by intraperitoneal injection. Rats were intubated and mechanically ventilated using a rodent ventilator (TKR-200 C Shanghai Xinman). A continuous electrocardiogram (ECG) telemetry transmitter was implanted in the abdominal cavity. A saline filled PE50 catheter, which was connected with a pressure transducer, was inserted through the carotid artery into the left ventricle to measure the left ventricular end-systolic pressure. A left thoracotomy and pericardiotomy were performed, and left anterior descending coronary artery was ligated with 5-0 silk thread. The artery was occluded for 30 min by a knot, and followed by reperfusion, the success was confirmed by observing the color changing of myocardium and monitoring with an electrocardiogram, left ventricular pressure quickly reduction and increasing of myocardial enzyme.

Arrhythmia score

Arrhythmias were recorded by ECG during the 30 minutes ischemia and 120 minutes 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.

Plasma H_2S level measurement

Immediately after ischemia/reperfusion, plasma was collected from femoral artery of rats before sacrifice and centrifuged (4000 rpm, 10 min, Room Temperature). Seventy-five microliters plasma was mixed with 250 ml 1% (w/v) zinc acetate and 425 ml distilled water in a tube to trap H_2S. Subsequently, N-dimethyl-p-phenylenediamine sulphate (20 μM; 133 μl) in 7.2 mM HCl was added followed by $FeCl_3$ (30 μM; 133 μl) in 1.2 M HCl. The protein in the plasma was removed by adding 250 ml of 10% tricholoacetic acid to the reaction mixture and pelleted by centrifugation at 12000 g (15 min). The absorbance of the resulting solution at 670 nm was measured with a spectrophotometer in a 96-well plate.

CSE expression assay

Immediately after blood collection, rats were sacrificed under deep anesthesia and chest opened, heart excised, washed with PBS buffer, Myocardium around ischemic area was obtained (about 100 mg) and quick frozen in liquid nitrogen and kept at -80°C till further examinations. RNA was extracted using TRIzol reagent as in the manufacturer's instructions (Invitrogen, CA, USA). The reverse transcription from mRNA to cDNA was performed with PrimeScript™ One Step RT-PCR Kit (TAKARA, JAP) with the manufacturer's instructions. Rat β-actin served as an internal control gene. The

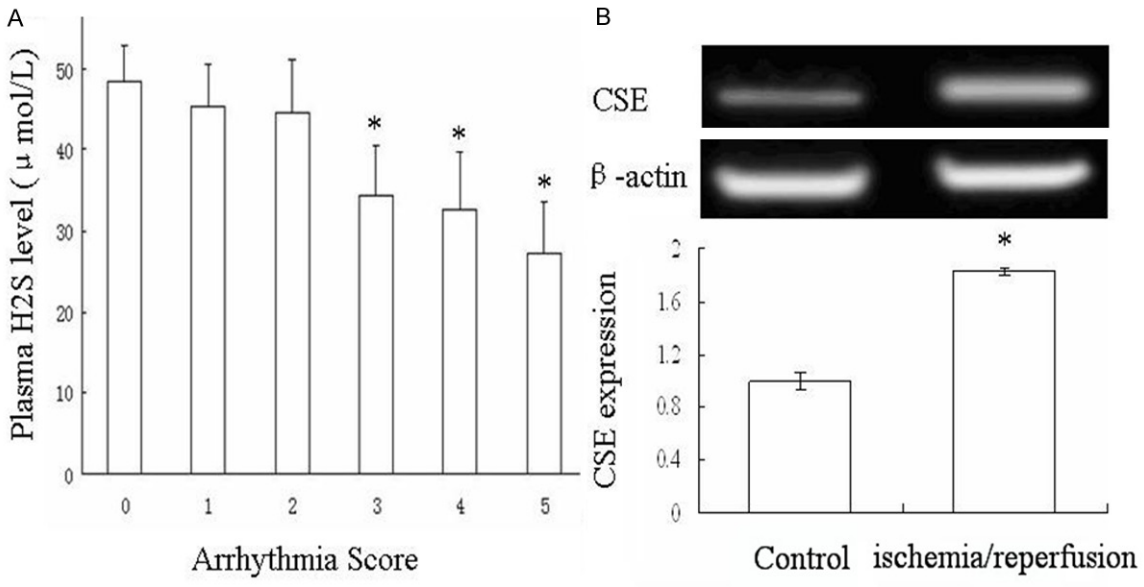

Figure 1. The plasma H_2S level and CSE expression of rats after ischemia/reperfusion. The ischemia/reperfusion rat model was set up and arrhythmia score was assessed according to Lambeth Conference standard. Plasma were collected to test the level of H_2S level in different group with different arrhythmia score. A. The tissue from ischemic area was collected to test the CSE expression by RT-PCR. B. Data are the mean ± SD of five mice per group. *P < 0.05 vs control group.

primer sequences of CSE and β-actin were as following: CSE (forward): 5'-GTG TCT GTT ACT TCC GAT GAC CTC-3'; CSE (reverse): 5'-CCT CGG CAG CAG AGG TAA CA-3'; β-actin (forward): 5'-CCG TAA AGA CCT CTA TGC CAA CA-3'; β-actin (reverse): 5'-CGG ACT CAT CGT ACT CCT GCT-3'.

Isolation of cardiomyocytes

Ventricular myocytes were obtained from hearts of male Wistar rats (200-250 g) by enzymatic dissociation according to the method described by Bian et al. [12] with some modifications. In brief, the heart was removed, mounted in a Langendorff apparatus, and perfused retrogradely through the aorta with a Krebs' solution containing 117 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.25 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11 mM glucose and bubbled with 95% O_2/5% CO_2 (pH 7.4, 34°C) at a constant flow rate of 13 ml/min. Cardiomyocytes were isolated from the myocardium around the ischemic area using a collagenase perfusion method as described previously. After separation of cardiomyocytes, cells were allowed to stabilize for 30 minutes before further experiment was commenced. Cardiomyocytes were treated with 50, 100, 200 μM NaHS or DMEM solution for 5 minutes, respectively.

Electrophysiological measurements

Cardiomyocytes were placed in a chamber on the stage of an inverted microscope (Leica, DMIL, Wetzlar, Germany) and continuously perfused at a constant rate (1.8 ml/min) with 35°C Tyrode's solution. The cells were patch-clamped in the whole-cell configuration using a patch-clamp amplifier (Axopatch 200B, Axon instruments, Burlingham, CA, USA). The signals were recorded and analyzed with pClamp 6.0 and Clampfit 9.0 softwares (Axon instruments). Briefly, patch electrodes were fabricated from borosilicate glass tubes and filled with a pipette solution containing (in mM) CsCl 120, $MgCl_2$ 2, $CaCl_2$ 1, Na_2ATP 5, EGTA 11, and HEPES 10 (pH adjusted to 7.4 with CsOH). Filled electrodes had a tip resistance of 3-5 MΩ. A period of 10-15 min was allowed after the whole-cell configuration was achieved. $I_{Ca,L}$, action potential and the K_{ATP} current were measured as described [13, 14].

Statistical analysis

All experimental data are presented as means ± SEM. Differences between groups were analyzed by one-way ANOVA followed by post hoc Tukey's test. A value of P < 0.05 was considered statistically significant.

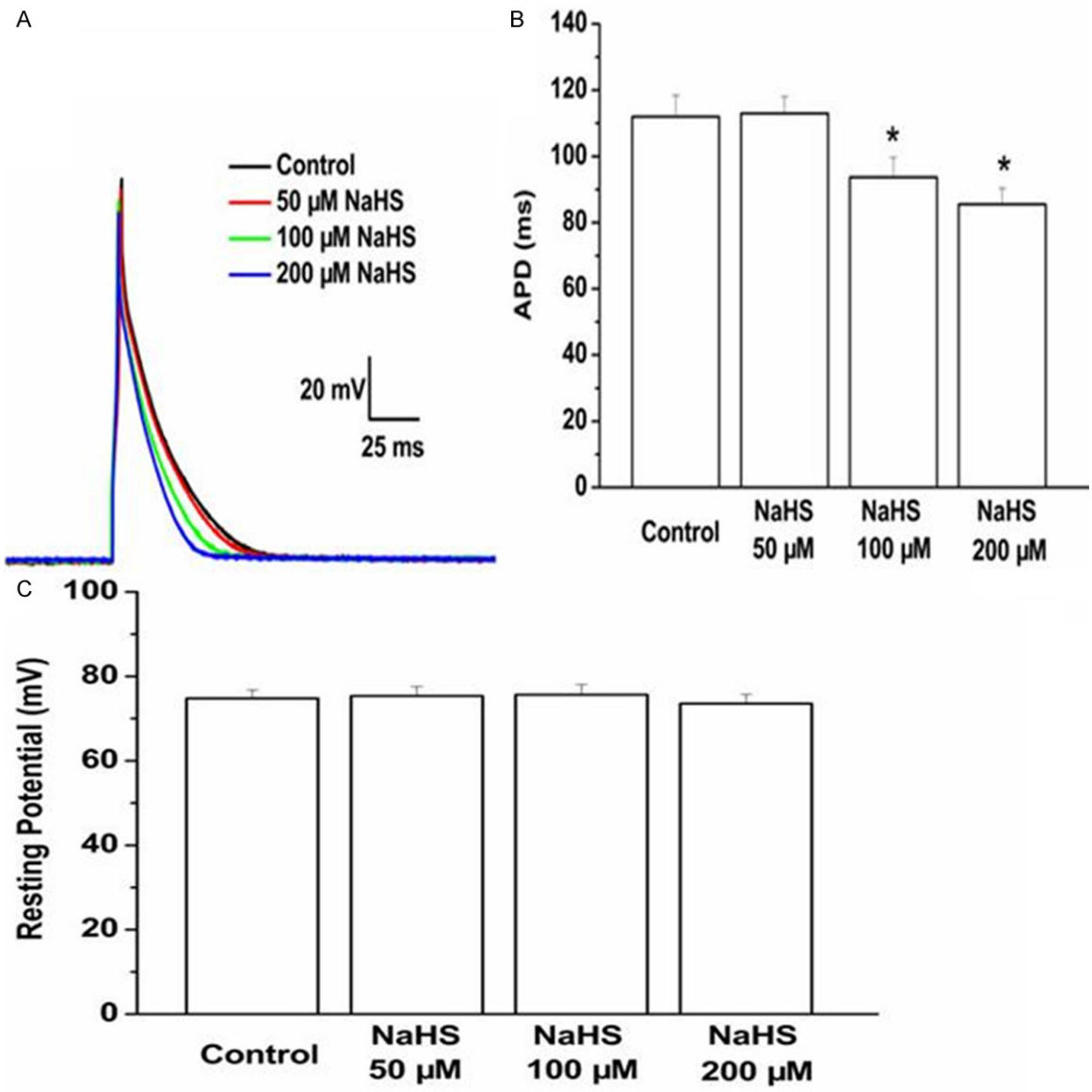

Figure 2. Effect of exogenous NaHS on the action potential duration and resting potential cardiomyocytes from myocardium around ischemic area were isolated and treated with NaHS. The NaHS levels were 50, 100, 200 μM. The action potential duration (A, B) and resting potential (C) were tested by patch-clamp amplifier. Data are the mean ± SD of six cells. *$P < 0.05$ *vs* control group.

Results

Plasma H_2S level was related with the development of post- ischemia/reperfusion arrhythmias

As results in **Figure 1A**, the plasma H_2S level was significantly lower in group with arrhythmia score ≥ 3 compared to control group with arrhythmia score = 0. CSE mRNA expression in infarct border zone was detected and the CSE mRNA expression was significantly upregulated in infarct border zone compared with tissue from normal control group (**Figure 1B**). These data indicated the potential relationship between plasma H_2S level and the development of post-ischemia/reperfusion arrhythmia.

Exogenous NaHS reduced the action potential duration in isolated ischemic cardiomyocytes

To test the role of exogenous H_2S on the electrical remodeling of cardiomyocytes, cardiomyocytes from infarct border zone were isolated. Resting potential (RP) and action potential duration (APD) of cardiomyocytes were mea-

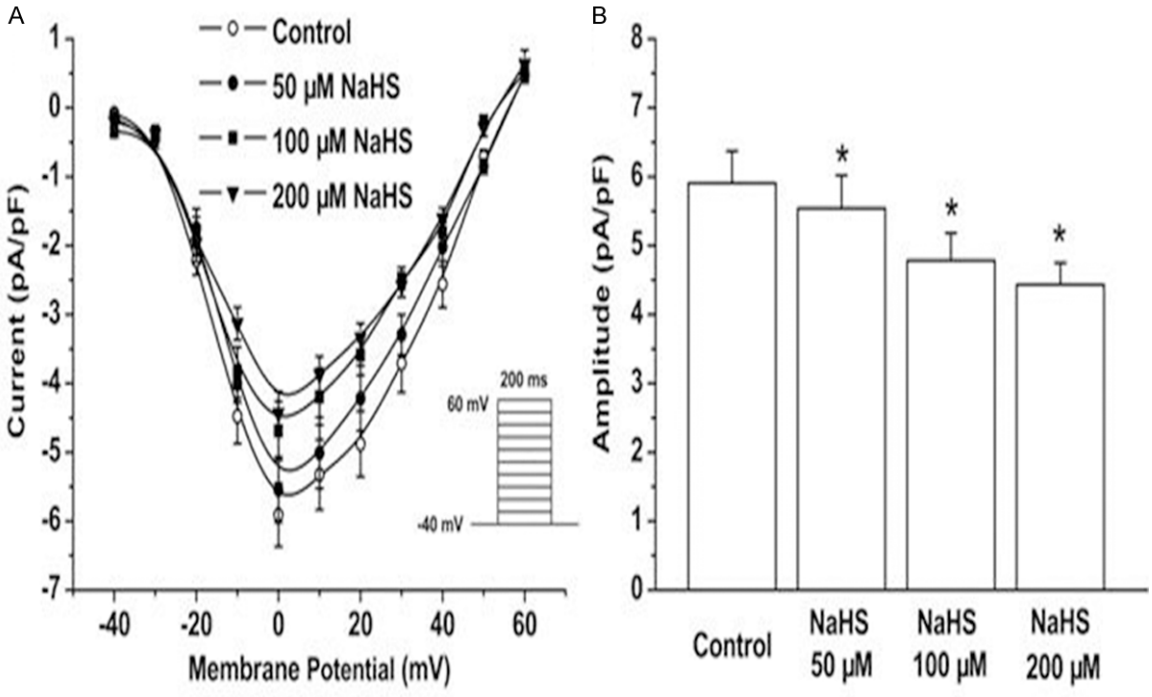

Figure 3. Effect of exogenous NaHS on L-type Ca^{2+} channels. Cardiomyocytes from myocardium around ischemic area were isolated and treated with 50, 100, 200 μM NaHS respectively. The L-type Ca^{2+} current was assessed by voltage clamp. The data were displayed with current pA/pF (A) and amplitude pA/pF (B). All data were calculated with the mean ± SD of six cells. *$P < 0.05$ *vs* control group.

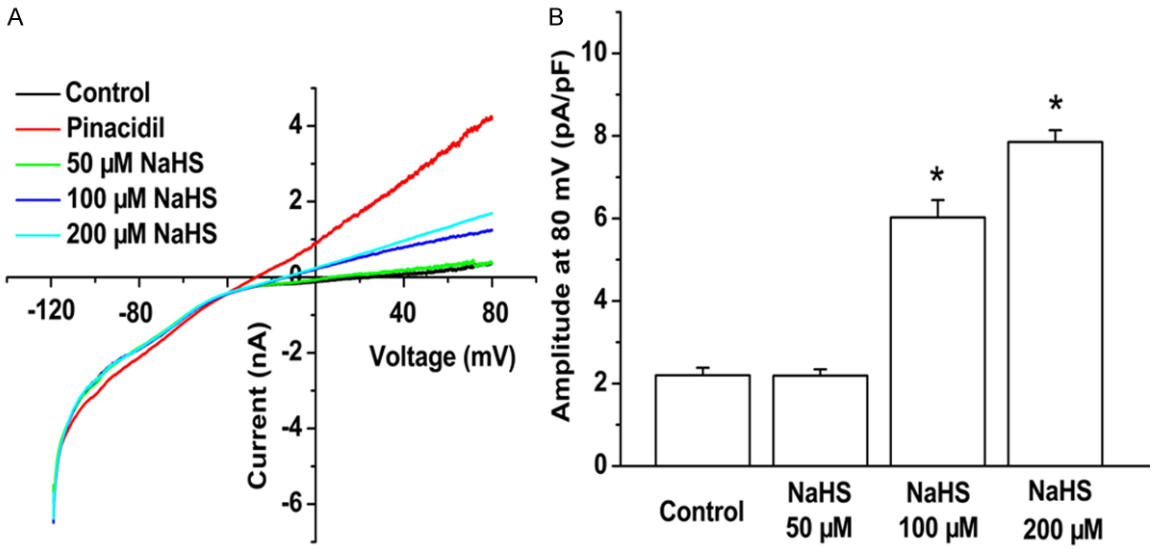

Figure 4. Effect of exogenous NaHS on ATP sensitive K^+ channels. Cardiomyocytes from myocardium around ischemic area were isolated and treated with 50, 100, 200 μM NaHS respectively. The ATP sensitive K^+ channels were assessed by voltage clamp. The data were displayed with current at different mV pA/pF (A) and amplitude at 80 mV pA/pF (B). All data were calculated with the mean ± SD of six cells. *$P < 0.05$ *vs.* control group.

sured post treatment for 5 minutes with various concentrations of NaHS. NaHS at concentration of 100 and 200 μM significantly reduced the APD compared with control cardiomyocytes

(**Figure 2A**, **2B**). However, the expose of NaHS had no impact on the resting potential (**Figure 2C**).

Exogenous NaHS inhibited the L-type Ca^{2+} channels

To indentify the role of H_2S on the activity of L-type Ca^{2+} channels, cardiomyocytes from infarct border zone were isolated *ex vivo*. The L-type Ca^{2+} current was assessed by voltage clamp. As showed in **Figure 3A**, **3B**, the L-type Ca^{2+} current was reduced in a dose-dependent manner post treatment with NaHS (50, 100, 200 μM NaHS) for 5 minutes.

Exogenous NaHS acitvated the ATP sensitive K^+ channels

The opening of K_{ATP} channels in the myocardium plays a pivotal role in cardioprotection during ischemia and reperfusion injury. To investigate the activity of H_2S on the K_{ATP} channels post-AMI, K_{ATP} current on cardiomyocytes isolated from infarct border zone was assessed by voltage clamp. The K_{ATP} current in isolated cardiomyocytes was significantly increased post 2 minutes treatment with NaHS (100, 200 μM) (**Figure 4A**, **4B**). Thus, exogenous NaSH could activate K_{ATP} current of post-AMI cardiomyocytes.

Discussion

Cardiac electrical and structural remodeling plays an important role in both cardiovascular health and disease [15]. Myocardial ischemia usually accompanies with cardiomyocytes electrophysiology alterations, resulting in arrhythmias and conduction abnormalities. Exogenous H_2S has been reported to be cardioprotective in various disease models. In this study, we tested serum H_2S concentration in rat with different arrhythmia score post ischemic insult. The results showed that the H_2S concentration was reduced with increasing arrhythmia score, which indicated that the reduction of endogenous H_2S might be linked with the development of arrhythmia post ischemia/reperfusion injury. In cardiomyocytes isolated from myocardium around ischemic area, exogenous NaHS reduced the action potential duration and L-type Ca^{2+} current while increased K_{ATP} current. Moreover, mRNA expression of CSE in myocardium around ischemic area was upregulated.

In the cardiovascular system, H_2S is predominantly generated by CSE [16]. Mice lacking the H_2S-producing enzyme cystathionine γ-lyase (CSE) exhibit elevated oxidative stress, and exacerbated myocardial and hepatic I/R injury [17]. In the heart, reducing the level of H_2S by inhibiting CSE increased myocardial infarct size [18]. Additionally, treatment with exogenous H_2S or genetic overexpression of CSE resulted in increased endogenous H_2S production, which was associated with profound protection against ischemia-induced heart failure and decreased mortality in mice with myocardial ischemia-reperfusion injury [19]. In this study, we demonstrated upregulated CSE mRNA expression in the myocardium around ischemic area. This phenomenon might reflect a compensatory local CSE expression upregulation to protection against ischemia-induced injury in this model.

In this study, cardiomyocytes from myocardium around ischemic area and were isolated and cultured. Exogenous H_2S was co-incubation with these cardiomyocytes to observe the role of H_2S on cardiomyocytes electrophysiology post ischmia/reperfusion injury. It is known that one of the hallmarks of secondary electrical remodeling post ischemic insult is repolarization abnormalities, specifically a prolongation of action potential duration (APD). The ionic mechanisms responsible for remodeling of the cardiac action potential involve a complex interplay between K^+, Ca^{2+} and Na^+ current [15]. These changes could markedly alter normal repolarization gradients in the heart and thus contribute to the development of abnormal heart rhythms (arrhythmogenesis) [20]. In this study, exogenous H_2S treatment of cardiomyocytes isolated from myocardium around ischemic area shortens the APD compared with control group, suggesting that H_2S treatment attenuated cardiomyocytes electrophysiology after ischemia-induced injury in this model.

The interplay among outward K^+ currents (Ik), inward Ca^{2+} currents (I_{Ca}) and the late component of the inward Na^+ current (I_{Na}) were changed after acute ischemic insult, which could also result in abnormal heart rhythms [15]. The opening of K_{ATP} channels in the myocardium plays a pivotal role in cardioprotection during ischemia and reperfusion injury and is a crucial component of the phenomenon termed cardiac ischemic preconditioning [21]. It was

shown that sarcolemmal K_{ATP} channels were important determinants in H2S-mediated cardioprotection in an isolated cardiac myocyte model [11]. It was shown that opening of sarcolemmal K_{ATP} could lead to potassium efflux, cell membrane depolarization and shortening of the action potential duration [22]. These events might reduce calcium influx, leading to a reduction of mechanical contraction and resulting in energy sparing in the myocardium during early reperfusion injury [23, 24]. Increasing evidence demonstrates that increased L-type Ca^{2+} density is an important mechanism for APD prolongation in mild to moderate hypertrophy [25]. Harbin Medical University, Harbin, Heilongjiang Hydrogen sulfide has been shown to inhibit L-type Ca^{2+} channels in the cardiomyocyte [26]. In line with above findings, H_2S administration also inhibited the L-type Ca^{2+} channels and activated the K_{ATP} channels in this ischemia/reperfusion model. These results indicated that exogenous H_2S could improve the cardiomyocytes electrical remodeling by activation of K_{ATP} channels post ischemic insult.

Understanding the cellular and molecular mechanisms of electrical remodeling might contribute to find out potential therapeutic targets for post-ischemic arrhythmias. This study suggested the relationship between endogenous H_2S and post-ischemic arrhythmia. Lower level of H_2S is negatively related to increased risk of arrhythmia after ischemia/reperfusion injury while exogenous H_2S treatment improved the post-ischemic cardiomyocytes electrical remodeling as shown by inhibited L-type Ca^{2+} channels and activated K_{ATP} channels.

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Disclosure of conflict of interest

None.

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