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Hydrogen sulfide decreases the levels of ROS by inhibiting mitochondrial complex IV and increasing SOD activities in cardiomyocytes under ischemia/reperfusion

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ABSTRACT

Inhibition of oxidative stress has been reported to be involved in the cardioprotective effects of hydrogen sulfide (H_2S) during ischemia/reperfusion (I/R). However, the mechanism whereby H_2S regulates the level of cardiac reactive oxygen species (ROS) during I/R remains unclear. Therefore, we investigated the effects of H_2S on pathways that generate and scavenge ROS. Our results show that pretreating rat neonatal cardiomyocytes with NaHS, a H_2S donor, reduced the levels of ROS during the hypoxia/reoxygenation (H/R) condition. We found that H_2S inhibited mitochondrial complex IV activity and increased the activities of superoxide dismutases (SODs), including Mn-SOD and CuZn-SOD. Further studies indicated that H_2S up-regulated the expression of Mn-SOD but not CuZn-SOD. Using a cell-free system, we showed that H_2S activates CuZn-SOD. An isothermal titration calorimetry (ITC) analysis indicated that H_2S directly interacts with CuZn-SOD. Taken together, H_2S inhibits mitochondrial complex IV and activates SOD to decrease the levels of ROS in cardiomyocytes during I/R.

1. Introduction

Hydrogen sulfide (H_2S) is a gaseous messenger molecule that has recently been implicated in various physiological/pathological processes in mammals, including vascular relaxation [1], angiogenesis [2], ischemia/reperfusion (I/R) injury of the heart [3,4] and the function of ion channels [5]. Accumulating evidence demonstrates that H_2S exerts cardioprotective effects in animal models of I/R injury [3,4,6–8]. The mechanisms of this protection may be related to NO production [6], K_{ATP} channel activation [7], ERK and phosphatidylinositol 3-kinase (PI3K)/AKT pathways [3]. Because reactive oxygen species (ROS) is an important contributor to cardiac I/R injury, the effect of H_2S on ROS levels is worthy of our attention.

The mitochondrial respiratory chain is the main source of ROS during energy metabolism [9,10]. The production of ROS increases during pathological conditions, such as I/R injury to the heart, and excessive ROS have a pivotal role in the pathogenesis of myocardial I/R injury [11,12]. Therefore, therapies that protect the myocardium against I/R injury by inhibiting ROS have been explored [13–15]. In addition to pathways that generate ROS, scavenging pathways, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), have an important role in regulating the levels of ROS in cardiomyocytes.

Some studies have shown that H_2S can decrease the concentration of malondialdehyde (MDA) and increase the activity of SODs in rat hearts subjected to isoproterenol-induced injury [16], hyperhomocysteinemia [17] or traumatic hemorrhagic shock [18]; however, the underlying mechanisms behind these results were not elucidated. There is relatively little known about the effects of H_2S on cardiac ROS generation under the I/R condition. In the present study, to simulate an I/R model, we used isolated and cultured neonatal rat cardiomyocytes subjected to hypoxia/reoxygenation (H/R) [19,20]. Using this I/R model, we investigated the effect of H_2S on the regulation of ROS generating and scavenging pathways, as well as the underlying mechanism of this regulation.

2. Materials and methods

2.1. Reagents and antibodies

DMEM/F12 and fetal bovine serum (FBS) were from Invitrogen Gibco (Carlsbad, CA, USA). Sodium hydrosulfide (NaHS), which has been well established as a reliable H_2S donor [21,22], was from Sigma–Aldrich (St. Louis, MO, USA). Pure CuZn-SOD protein (from bovine erythrocytes) was purchased from Sigma–Aldrich (St. Louis, MO, USA). The antibodies used included rabbit anti-Mn-SOD polyclonal antibody (1:5000) (Millipore-Upstate, Billerica, MA, USA), rabbit anti-CuZn-SOD polyclonal antibody (1:5000) (Millipore-Upstate, Billerica, MA, USA), mouse anti-mitochondrial Complex IV monoclonal antibody (1:1000) (Abcam, Cambridge, UK) and rabbit anti-β-actin polyclonal antibody (1:4000) (Cell Signaling

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Technology, Danvers, MA, USA). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG were used as secondary antibodies (1:5000) (Cell Signaling Technology, Danvers, MA, USA).

2.2. Primary culture of neonatal rat cardiomyocytes

One-day-old Sprague–Dawley (SD) rat pups were purchased from Shanghai SLAC laboratory Animal Co., Ltd. (Shanghai, China). Primary cultures of rat neonatal cardiomyocytes were performed as previously published [23]. Neonatal rat hearts were quickly and aseptically excised from rat pups, cut into pieces, digested with 0.125% trypsin, and then collected and kept at 37 °C with 5% CO_2 for 1 h to exclude nonmyocardial cells. The cardiomyocytes in suspension were plated into 60-mm culture dishes at a density of 5×10^5 cells/ml in 10% FBS–DMEM/F12. The medium contained 100 mM 5-bromo-2′-deoxyuridine (BrdU) to prevent the proliferation of fibroblasts. After 3 days of culture, the cells were used in experiments. This study 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 Experimental Research, Shanghai Medical College, Fudan University.

2.3. H/R treatment protocol

The H/R treatment was performed as described by Woo et al. [24], with slight modifications. After being cultured for 3–4 days, the cardiomyocytes were washed with PBS three times and incubated with oxygen–glucose deprivation (OGD) medium, Hanks' balanced salt solution (HBSS: 125 mM NaCl, 4.9 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM $NaH_2PO_4 \cdot 2H_2O$, 1.8 mM $CaCl_2 \cdot 2H_2O$, 8.0 mM $NaHCO_3$, 20 mM HEPES, pH 6.4). The cells were incubated with 95% N_2 and 5% CO_2 at 37 °C for 4 h, and then in normoxic conditions (95% O_2 and 5% CO_2 at 37 °C) for 1 h for reoxygenation. The H/R and NaHS + H/R groups were subjected to the H/R treatment. The NaHS + H/R group was pretreated with NaHS for 30 min prior to the initiation of H/R. Cardiomyocytes cultured under normal conditions were used as the control group.

2.4. Measurement of ROS levels

The intracellular levels of ROS were measured using a cell-permeable non-fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Sigma–Aldrich, St. Louis, MO, USA). The dye loading was performed by incubating the cardiomyocytes with 10 μM DCFH-DA at 37 °C for 60 min. The production of ROS was examined using a spectrophotometer (Infinite M200, Tecan, Grödig, Austria) by measuring the fluorescence intensity of DCF at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

2.5. Measurement of mitochondrial respiratory activity

Mitochondria were isolated from cardiomyocytes using the Cell Mitochondria Isolation Kit (Beyotime Co., Nantong, China). Briefly, cardiomyocytes were collected, washed with PBS, and then suspended in ice-cold isolation buffer for 15 min. After the cells were homogenized, the homogenate was centrifuged at 600*g* for 10 min at 4 °C, and the supernatant was then centrifuged at 11,000*g* for 10 min at 4 °C. The mitochondria were collected in the sediments. The activities of the mitochondrial complexes were determined using the Mito Complex I, II, III, and IV Activity Assay Kits (GenMed Scientifics Inc., Wilmington, DE, USA).

2.6. Xanthine oxidase (XOD), CAT, GPx and SOD activity assays

The activities of XOD and antioxidants were measured using commercially available kits. The XOD and SOD activity assay kits were from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The CAT and GPx activity assay kits were from Beyotime Co. (Nantong, China). These experiments were performed according to the manufacturer's instructions.

2.7. Western blot analysis

Cardiomyocytes were collected and lysed, and the proteins were extracted and quantified using BCA reagent (Shen Neng Bo Cai Corp., Shanghai, China). Protein samples were separated on 12% SDS–PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore-upstate, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk for 1 h at room temperature and then incubated with primary antibodies overnight at 4 °C. After washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The specific bands were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific-Pierce, Waltham, MA, USA).

2.8. Interaction between H_2S and CuZn-SOD in a cell-free system

Using the SOD assay kit, a mixture of NaHS and purified CuZn-SOD was allowed to react in a cell-free system in the absence of cellular components at pH 7.8. The activity of SOD was measured using the SOD assay kit.

2.9. Isothermal titration calorimetry (ITC) assays to test the binding between H_2S and CuZn-SOD

A direct molecular interaction between H_2S and CuZn-SOD was examined using ITC (iTC_{200} system, MicroCal Inc., Northampton, MA, USA) at 25 °C. ITC is the gold standard for measuring biomolecular interactions [25] and can determine all binding parameters in a single experiment. When substances bind, heat is either generated or absorbed. ITC can directly measure the heat released or absorbed during a binding event. The measurement of this heat yields accurate information about the binding constants (K), reaction stoichiometry (N), enthalpy (ΔH) and entropy (ΔS). The assay was performed according to the manufacturer's instructions. Briefly, 25 consecutive injections of 2-μl aliquots of NaHS (500 μM) were added into the calorimeter cell containing 200 μl of 160 μM CuZn-SOD. The injections were made at 3-min intervals for all titrations. Origin 8.0 software was used to calculate ΔH, ΔS and K_d of the ITC experiments.

2.10. Statistical analysis

The data are presented as the mean ± SEM. Statistical analysis was performed with a one-way ANOVA using SPSS 11.5 for Windows. The results were considered significant when $P < 0.05$.

3. Results

3.1. H_2S reduced ROS levels in cardiomyocytes under the H/R condition

Using DCF fluorescence, we investigated the effect of NaHS on intracellular levels of ROS. At 5, 15, 30, 45 and 60 min after initiating reoxygenation, the intracellular ROS levels in the H/R group were higher than those in the control group. Compared to the

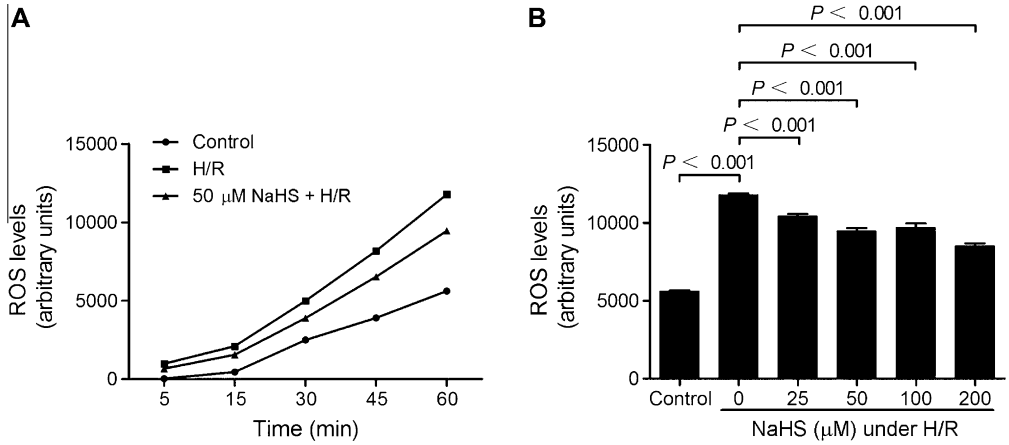

Fig. 1. The effects of NaHS on cellular ROS levels. (A) At 5, 15, 30, 45 and 60 min after the initiation of reoxygenation, NaHS (50 μM) pretreatment decreased ROS levels in H/R-treated cardiomyocytes ($n = 6$). (B) At 60 min after the initiation of reoxygenation, pretreating cardiomyocytes with different concentrations of NaHS (25, 50, 100 and 200 μM) reduced the increase in ROS levels induced by H/R treatment ($n = 6$).

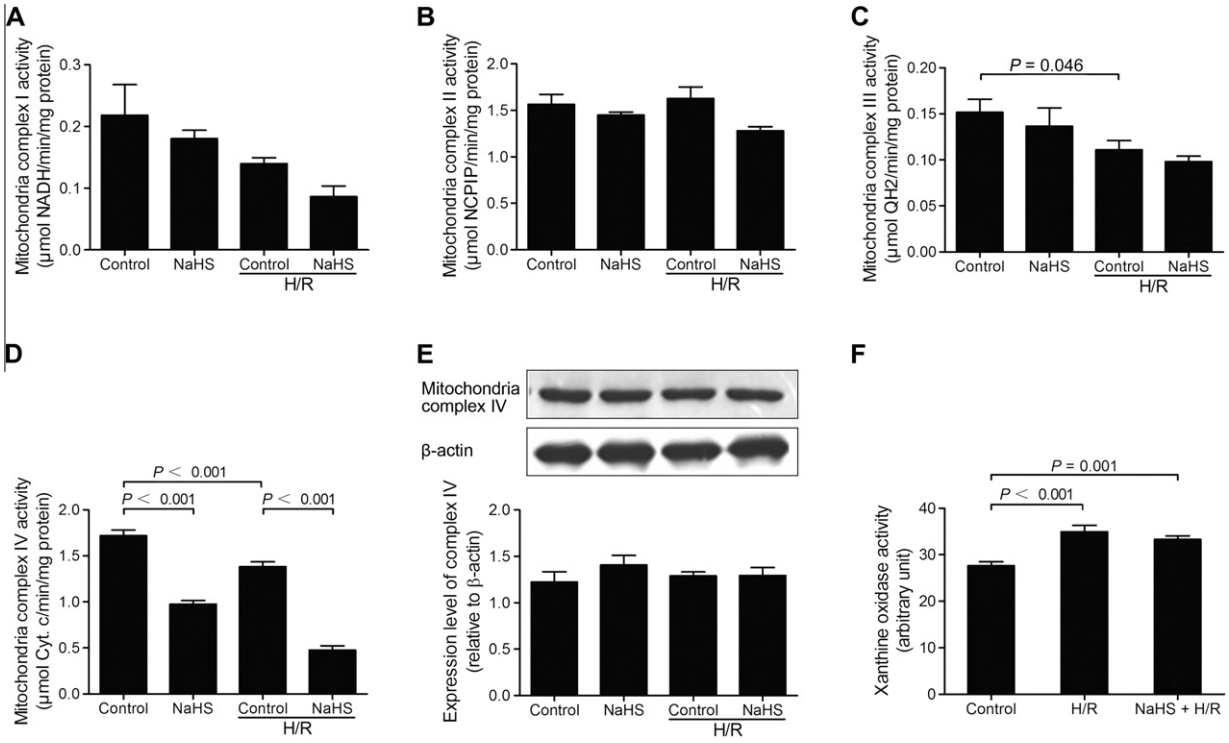

Fig. 2. The effects of NaHS on mitochondrial complexes and xanthine oxidase (XOD) activity. Cardiac mitochondrial complex I (A, $n = 6$), II (B, $n = 6$), III (C, $n = 6$) and IV (D, $n = 6$) activities were measured. The H/R treatment decreased the activities of mitochondrial complexes III and IV, and NaHS pretreatment further inhibited mitochondrial complex IV activity under the H/R condition. (E) The expression of mitochondrial complex IV was not changed under the H/R condition nor by NaHS pretreatment ($n = 4$). (F) The H/R treatment increased XOD activity, but NaHS (50 μM) did not affect XOD activity under the H/R condition ($n = 8$).

H/R group, the levels of DCF fluorescence in the NaHS + H/R group decreased when pretreated with 50 μM NaHS (Fig. 1A).

At 60 min after the initiation of reperfusion, the ROS levels in the H/R group increased significantly compared to the control group. The H/R-induced increase in DCF fluorescence was markedly inhibited by different concentrations of NaHS (25, 50, 100 and 200 μM) (Fig. 1B).

3.2. H_2S inhibited mitochondrial complex IV activity but not XOD activity in cardiomyocytes under the H/R condition

The activities of mitochondrial complexes III and IV, but not complexes I and II, were significantly reduced by the H/R treatment. The NaHS treatment alone had no effect on the activities of complexes I, II and III, but significantly decreased the activity of complex IV. Interestingly, in the cardiomyocytes subjected to H/R, the activity of complex IV, which was already decreased under the H/R condition, was further reduced by the NaHS pretreatment (Fig. 2A, B, C and D). To investigate whether the reduced activity of complex IV was caused by a decrease in protein content, the expression level of complex IV was measured. The results showed that the expression of mitochondrial complex IV was not changed under the H/R condition or by the NaHS pretreatment (Fig. 2E).

We also measured the activity of XOD, another enzyme that generates ROS under I/R conditions. The H/R treatment increased XOD activity. However, the NaHS (50 μM) pretreatment did not

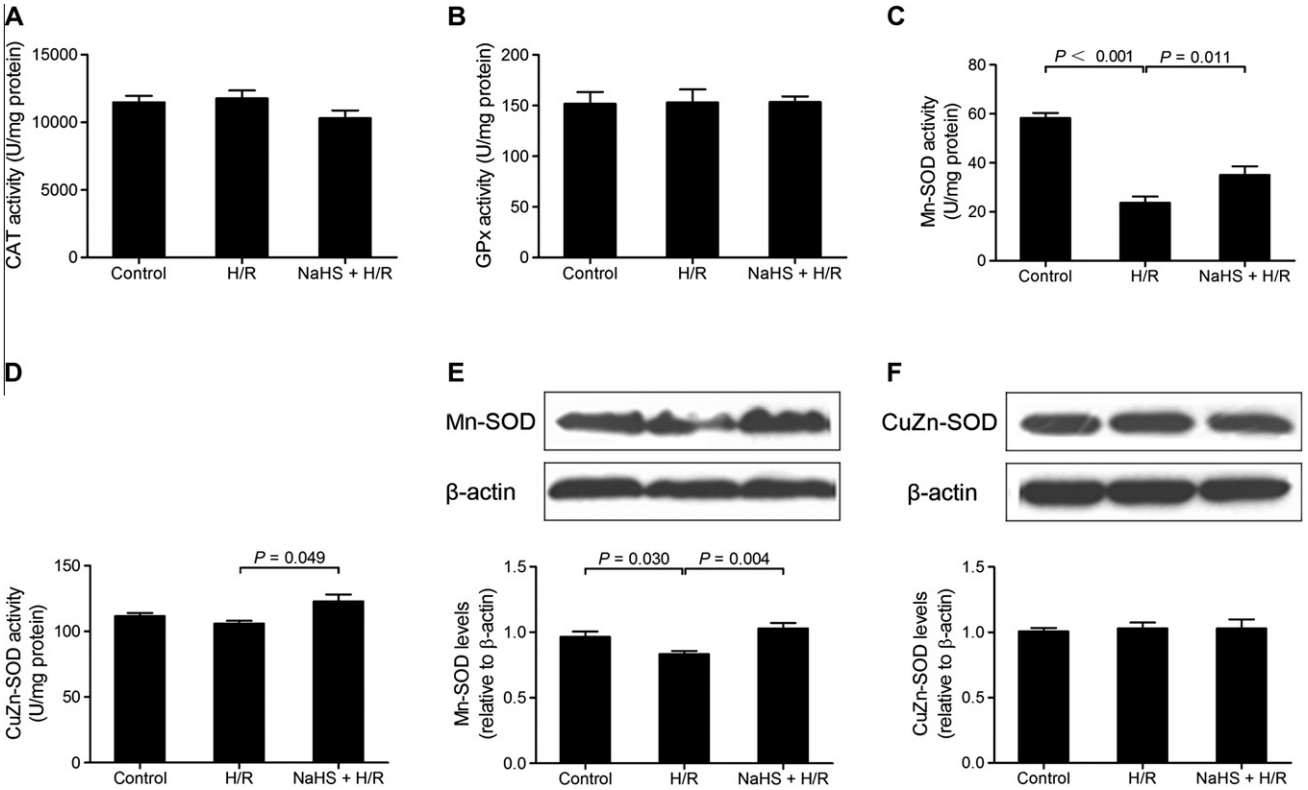

Fig. 3. The effects of NaHS on antioxidant activities. The intracellular activities of CAT (A, $n = 8$) and GPx (B, $n = 7$) were not changed by the H/R treatment alone or in combination with the NaHS pretreatment. (C) The activity of Mn-SOD decreased under the H/R condition and increased with NaHS pretreatment ($n = 6$). (D) NaHS pretreatment up-regulated the activity of CuZn-SOD under the H/R condition ($n = 6$). The expression of Mn-SOD (E, $n = 4$) but not CuZn-SOD (F, $n = 4$) was enhanced by NaHS pretreatment.

affect the regulation of XOD activity under the H/R condition (Fig. 2F).

3.3. H_2S activated SOD but not CAT and GPx in cardiomyocytes under the H/R condition

Compared to the control group, the intracellular activities of CAT and GPx under the H/R condition were not affected. Similarly, pretreating the cardiomyocytes with 50 μM NaHS did not affect the activities of CAT and GPx under the H/R condition (Fig. 3A and B).

In contrast, we observed a 60% reduction of Mn-SOD activity in cardiomyocytes subjected to the H/R treatment. NaHS significantly increased the activity of Mn-SOD in cardiomyocytes under the H/R condition. The activity of CuZn-SOD was not substantially affected under the H/R condition, but NaHS increased its activity under the H/R condition (Fig. 3C and D). To investigate whether the increase in SOD activities was due to increased expression, we measured the protein levels of Mn-SOD and CuZn-SOD. The results showed that the expression of Mn-SOD decreased under H/R but that the NaHS treatment increased the expression of Mn-SOD to control levels (Fig. 3E). Interestingly, the expression of CuZn-SOD was not affected under the H/R condition nor by the NaHS pretreatment (Fig. 3F).

3.4. H_2S increased CuZn-SOD activities through a direct interaction

The results described above show that NaHS increased CuZn-SOD activity without up-regulating its expression, which suggests that NaHS may directly activate CuZn-SOD. When NaHS was incubated with purified CuZn-SOD in a cell-free system without any other cellular components, NaHS activated CuZn-SOD (Fig. 4A). Because H_2S can be a reducing agent, we needed to rule out the possibility that H_2S can scavenge ROS to increase SOD activity in NaHS–CuZn-SOD system. We found that NaHS alone had little ROS scavenging activity, far less than that of CuZn-SOD (Fig. 4B).

We used ITC to determine if CuZn-SOD can directly interact with H_2S. The calorimetric profile showed that H_2S bound to CuZn-SOD in a molecular ratio of 0.141 ± 0.0301 with a K_d of $1.26 \times 10^5 \pm 7.75 \times 10^4\ M^{-1}$, a ΔH of $-5.733 \times 10^4 \pm 1.690 \times 10^4$ J/M, and a ΔS of -94.6 J/M/degree (Fig. 4C). The ITC results showed that heat is released when H_2S interacts with CuZn-SOD, suggesting that H_2S can bind to CuZn-SOD protein.

4. Discussion

Under physiological conditions, ROS is generated in cells, and increased ROS levels induce I/R damage in cardiomyocytes. The regulation of ROS levels is involved in the cardioprotection of H_2S [16–18]. However, to date, how H_2S regulates pathways involved in ROS homeostasis is not well understood.

In the present study, we show that H_2S reduced ROS levels in cardiomyocytes under H/R conditions *in vitro*. To explain how H_2S could reduce ROS levels, we examined the effects of H_2S on the intracellular pathways that generate and scavenge ROS. We found that H_2S inhibited the activity of mitochondrial complex IV in cardiomyocytes under the H/R condition. H_2S has a high affinity for mitochondria [26], and high doses of H_2S are toxic, which is attributed to its ability to directly inhibit mitochondrial complex IV [27,28]. Until now, this primary biochemical effect of H_2S has not been recognized to contribute to H_2S cardioprotection. Our results show that treating cardiomyocytes with 50 μM NaHS, which

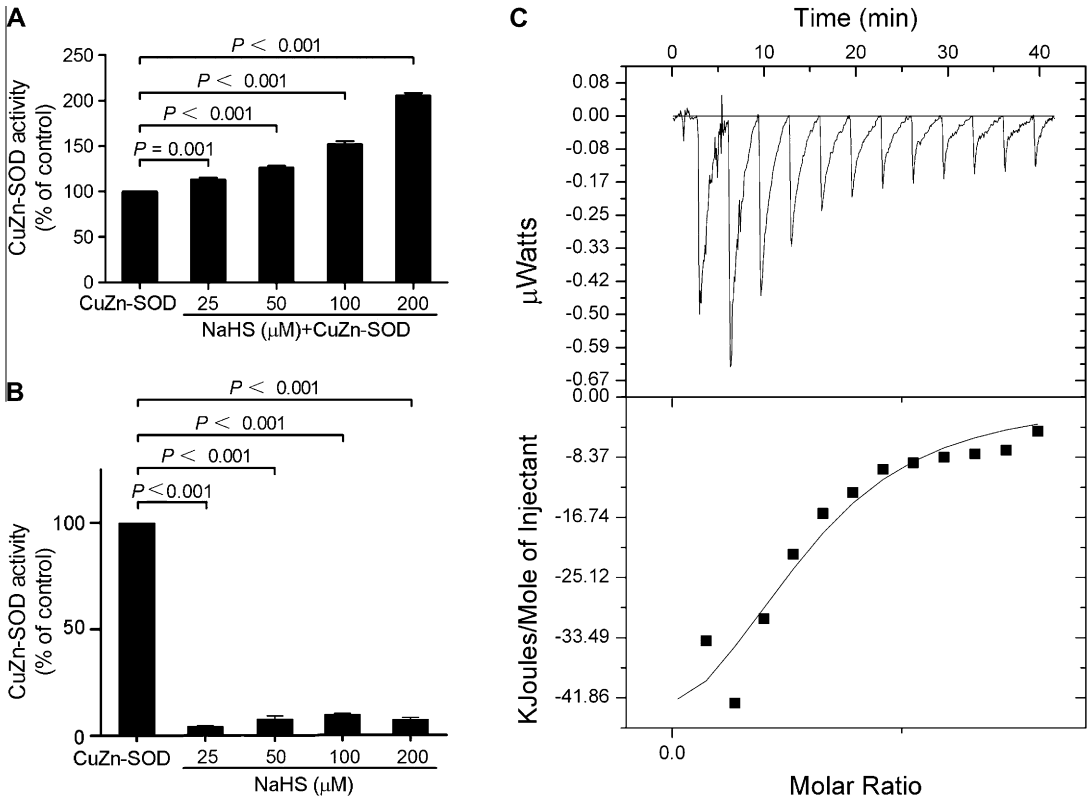

Fig. 4. The interaction between NaHS and CuZn-SOD. (A) NaHS activated CuZn-SOD in a cell-free system without any other cellular components ($n = 6$). (B) NaHS alone has little ROS scavenging activity ($n = 6$). (C) The ITC results indicate the heat released when H_2S interacts with CuZn-SOD. The upper panel shows the original data, and the lower panel shows the integrated heat for each injection with the curve fitted using a single-site binding model.

is close to the plasma level in rats [22], still inhibited complex IV activity under H/R conditions. There is increasing evidence that continual mitochondrial oxidative phosphorylation during I/R generates cytotoxic ROS [29]. Lesnefsky et al. [30] and Chen et al. [31] have reported that inhibiting mitochondrial respiration during I/R by inhibiting electron transport paradoxically decreases the release of ROS [29,30]. Consistent with these reports, our results show that H_2S can also inhibit electron transport, thus reducing harmful ROS generation under cardiac I/R.

In addition to a disordered electron transport cascade, the generation of excess ROS in hearts undergoing I/R involves an increase in XOD activity, a respiratory burst of leukocytes, and auto-oxidation of catecholamine. In our cultured cardiomyocytes, leukocytes and catecholamine do not exist, and H_2S did not affect the increase in XOD activity induced by H/R.

In the ROS scavenging pathways, O_2^- is converted to H_2O_2 by SOD, and H_2O_2 is subsequently reduced to H_2O and O_2 by CAT and GPx. In the present study, SOD was activated in the cardiomyocytes treated with H_2S. However, both CAT and GPx were not activated by H_2S. Similar to our results, Su et al. [32] reported that H_2S increases the activities of SOD and GPx during adriamycin-induced cardiomyopathy. Liu et al. [33] also showed that H_2S dramatically increase the levels of serum and intestinal SOD and GPx activities in intestinal I/R models. These studies suggest that H_2S exerts its protective role by increasing the activity of antioxidant enzymes.

However, little attention has been paid to the underlying mechanism of how H_2S activates SOD. In our study, the up-regulation in the expression of Mn-SOD can explain the H_2S-induced increase in Mn-SOD activity. Because the expression of CuZn-SOD was not affected by H_2S, it is unclear how H_2S enhances CuZn-SOD activity. The answer may involve the direct action of H_2S on CuZn-SOD. In a cell-free system, we found that H_2S increased the activity of CuZn-SOD and that H_2S alone possessed little ROS scavenging activity. We asked how H_2S could directly activate CuZn-SOD and used ITC to elucidate this event. ITC detected the heat that is released when H_2S interacts with CuZn-SOD and can be used to thermodynamically analyze the molecular binding between H_2S and CuZn-SOD. Using ITC, we confirmed that H_2S can bind to CuZn-SOD. As a small gaseous molecule, H_2S can freely diffuse through cell membranes without any specific transporters [8]; therefore, it is reasonable to infer that when encountering oxidative stress, H_2S may bind to a critical site on CuZn-SOD to directly and allosterically activate the enzyme.

In conclusion, H_2S inhibits mitochondrial complex IV in the ROS-generating pathways in cardiomyocytes. H_2S specifically activates both isoforms of SOD, CuZn-SOD and Mn-SOD. H_2S may activate CuZn-SOD through a direct interaction. This interaction would be a novel mechanism through which H_2S can exert its physiological and pathophysiological effects. By regulating pathways that generate and scavenge ROS, H_2S decreases ROS levels to protect cardiomyocytes during cardiac I/R.

Acknowledgments

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