Hydrogen sulfide protects H9c2 cardiomyoblasts against H_2O_2-induced apoptosis

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Abstract

Reactive oxygen species (ROS) are highly reactive chemical species that may cause irreversible tissue damage, and play a critical role in cardiovascular diseases. Hydrogen sulfide (H_2S) is a gasotransmitter that acts as a ROS scavenger with cardioprotective effects. In this study, we investigated the cytoprotective effect of H_2S against H_2O_2-induced apoptosis in cardiomyocytes. H9c2 rat cardiomyoblasts were treated with H_2S (100 μM) 24 h before challenging with H_2O_2 (100 μM). Apoptosis was then assessed by annexin V and PI, and mitochondrial membrane potential was measured using a fluorescent probe, JC-1. Our results revealed that H_2S improved cell viability, reduced the apoptotic rate, and preserved mitochondrial membrane potential. An increased Bcl-2 to Bax ratio was also seen in myocytes treated with H_2S after H_2O_2-induced stress. Our findings indicated a therapeutic potential for H_2S in preventing myocyte death following ischemia/reperfusion.

Key words: Reactive oxygen species; ROS; Hydrogen sulfide; H_2O_2-induced apoptosis; Cardiomyocytes

Introduction

Reactive oxygen species (ROS) are highly reactive chemical species from cellular metabolism involving oxygen consumption (1). In normal tissues, approximately 5% of the consumed oxygen molecules are transformed into ROS (2). These ROS can initiate chain reactions in tissues, leading to irreversible damage in proteins, lipids, and nucleic acids (1). ROS play an essential role in regulating cell activities, such as gene expression, cell growth, and cell death (3). ROS can be detoxified by endogenous enzymes or free radical scavengers. However, the imbalance between antioxidants and oxidants leads to overproduction of ROS. This effect is associated with many multifactorial diseases, such as cardiovascular disorders (4,5). Global ischemia and reperfusion have been associated with the upregulation of ROS in cardiomyocytes (e.g., hydrogen peroxide [H_2O_2]), resulting in oxidative stress injuries (6). H_2O_2 may cause apoptosis in cardiomyocytes by activating the intrinsic apoptotic pathways. Therefore, H_2O_2 is often utilized to establish an in vitro model for ischemia and subsequent reperfusion (I/R) injury (4,5).

Hydrogen sulfide (H_2S) is predominantly synthesized from L-cysteine via cystathionine γ-lyase in the heart and vasculature. H_2S has drawn great scientific interest regarding myocardial protection. H_2S possesses almost all the beneficial cardiovascular effects similar to another well-characterized gasotransmitter, nitric oxide (NO) (7). Moreover, H_2S acts as a ROS scavenger without producing deleterious ROS, which is typically seen in NO. H_2S is rapidly emerging as a novel lipophilic cytoprotective signaling molecule with potent antioxidant, anti-inflammatory, and anti-apoptotic features (8,9). Our previous in vivo study revealed that the exogenous H_2S donor, sodium hydrosulfide (NaHS), has potent anti-inflammatory effects in the heart damaged by acute myocardial infarction, which may be partially due to the limited $CD11b^+Gr-1^+$ myeloid cells (10).

Although we have discovered the physiological and cardioprotective effects of H_2S, the signaling mechanisms that mediate these effects have not been thoroughly evaluated. This study aimed to elucidate the mechanisms by which H_2S prevents apoptosis of cardiomyocytes. In particular, we studied the mitochondrial pathway in response to H_2O_2-induced oxidative stress using an in vitro model that mimicked ischemia-reperfusion injuries.

Material and Methods

H$_2$S donor

H$_2$S was administered in the form of sodium hydrosulfide (NaHS) (Sigma-Aldrich, Cat. No.161527, USA). NaHS was freshly prepared in normal saline (0.9%) to the desired concentration before administration.

Cell culture

H9c2 cells (rat cardiomyoblasts) were obtained from the Cell Bank of the Chinese Academy of Sciences (China), and grown at a density of 10^5 cells/cm^2 as a monolayer. H9c2 cells were cultured in Dulbecco's modified Eagle medium (GIBCO, Cat. No. 11960-077, USA) supplemented with 10% v/v fetal bovine serum, 2 mM glutamine, 1% nonessential amino acids, 100 IU penicillin, and 100 µg/mL streptomycin under an atmosphere of 5% CO$_2$ saturated with water vapor at 37°C. The medium was replaced by fresh medium every two days. Subculture was done when the plates were more than 90% confluency.

Hydrogen peroxide treatment

To induce oxidative stress in H9c2 cells, the cells were cultured in serum-free medium containing 100 µM H$_2$O$_2$ for 24 h with or without a 30 min pre-treatment of 100 µM NaHS. H$_2$O$_2$ and NaHS were freshly prepared before each experiment. Control groups were treated with both H$_2$O$_2$ and NaHS simultaneously.

Apoptosis assay

An annexin V apoptosis detection kit (BD Biosciences, Cat. No. 556547, USA) was utilized to measure apoptosis of H2c9 cells following the manufacturer's instruction. After treatments, cells were washed twice with cold PBS, trypsinized, and then resuspended in the binding buffer at a concentration of 1×10^6 cells/mL. A 100 µL-aliquot of the cell suspension (1×10^5 cells) was then incubated with fluorescein isothiocyanate (FITC)-annexin V and propidium iodide for 15 min at room temperature in the dark. Apoptotic rate was analyzed using flow cytometry within 1 h.

Measurement of ROS production

The presence of intracellular ROS was detected using dihydroethidium (DHE, Sigma, Cat. No. D7008, USA), a sensitive fluorescent dye. According to the manufacturer's instructions, sub-confluent cells were pre-treated with or without 100 µM NaHS for 30 min, and then further incubated with 100 µM H$_2$O$_2$ for 24 h. Cells were then washed with PBS and incubated with 5 µM DHE at 37°C for 30 min. Fluorescence was captured with a fluorescent microscope, and the signal intensity was reported as a percentage of the control group.

Measurement of mitochondrial membrane potential (ψ_m)

Changes in the ψ_m were detected using a mitochondria-specific cationic dye, JC-1 (Life Technologies, Cat. No. T3168, USA). JC-1 is a lipophilic cation that can selectively enter into mitochondria. H9c2 cells with or without H$_2$O$_2$ treatment were incubated with 10 µg/mL JC-1 at 37°C in the dark for 10 min. The loading solution was then replaced with fresh medium, and the fluorescent signal was captured and analyzed using the fluorescence microscopy. Red emission indicates membrane potential-dependent JC-1 aggregates in mitochondria. Green fluorescence reflects the monomeric form of JC-1 appearing in the cytoplasm after mitochondrial membrane depolarization. Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio.

Western blot analysis

Western blot analysis was performed as previously described (11). Total protein lysates were collected for the standard immunoblot analysis. Protein concentrations were determined by a BCA protein assay. Aliquots of protein lysates (30 µg/lane) were loaded into sodium dodecyl sulfide-polyacrylamide gels (SDS-PAGE) and transferred to the PVDF membrane. The membrane was blocked and incubated with Bcl-2, Bax, and activated-caspase 3 p17 primary antibodies overnight at 4°C. Bcl-2 and Bax antibodies were purchased from Cell Signaling Technology (USA) (Bcl-2 Cat. No. 3498; Bax Cat. No. 14796), and activated-caspase 3 p17 antibody was from Bioworld (Cat. No. BS7004, USA). Blots were washed with TBST (Sigma-Aldrich), followed by incubation with corresponding horseradish peroxidase-conjugated secondary antibodies (Jackson Laboratory, USA). Lastly, the blots were visualized with enhanced chemiluminescence and were quantified by densitometry.

Statistical analysis

Data are reported as means ± SE. Different groups were compared using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls *post hoc* test. Comparison between two groups was assessed by *t*-test. P < 0.05 was considered statistically significant.

Results

H$_2$S reduced H$_2$O$_2$-induced apoptosis

To determine whether H$_2$S affected H$_2$O$_2$-induced apoptosis in H2c9 cells, we performed annexin V & PI assays. Flow cytometry was then utilized to evaluate the apoptotic rate (Figure 1A). Compared to the control group, H$_2$O$_2$ significantly induced cell apoptosis, but the presence of H$_2$S rescued H$_2$O$_2$ cytotoxicity, and increased cell viability (Figure 1B).

H$_2$S protected H9c2 cells from oxidative stress

Oxidative stress leads to detrimental changes in the cell signaling process. To determine whether H$_2$S has any effect on ROS activity under oxidative stress, we examined total ROS levels using DHE. Compared to the control group, H9c2 cells with H$_2$O$_2$ treatment showed an enhancement of fluorescence intensity by approximately
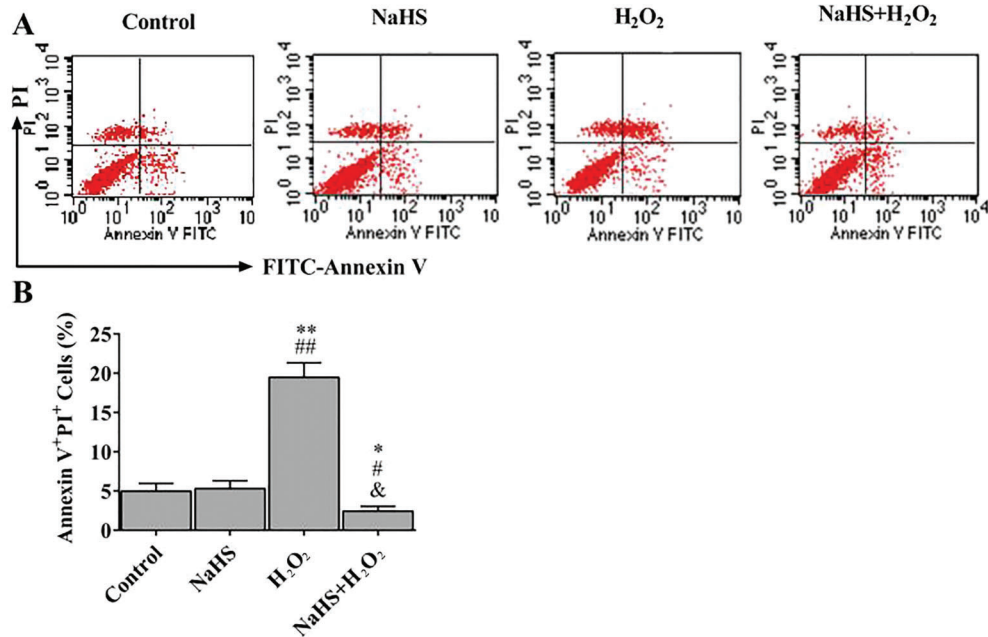
Figure 1. Hydrogen sulfide (H$_2$S) reduced H$_2$O$_2$-induced H9c2 apoptosis. **A**, Cell death analysis of treated cells was performed by flow cytometry with annexin V/PI double staining. Representative quantitative analysis is shown in **B**. Data are reported as means ± SE (n=6). *P < 0.05, **P < 0.01 *vs* control; #P < 0.05, ##P < 0.01 *vs* NaHS; &p < 0.01 *vs* H$_2$O$_2$ (ANOVA followed by Student-Newman-Keuls *post hoc* test).
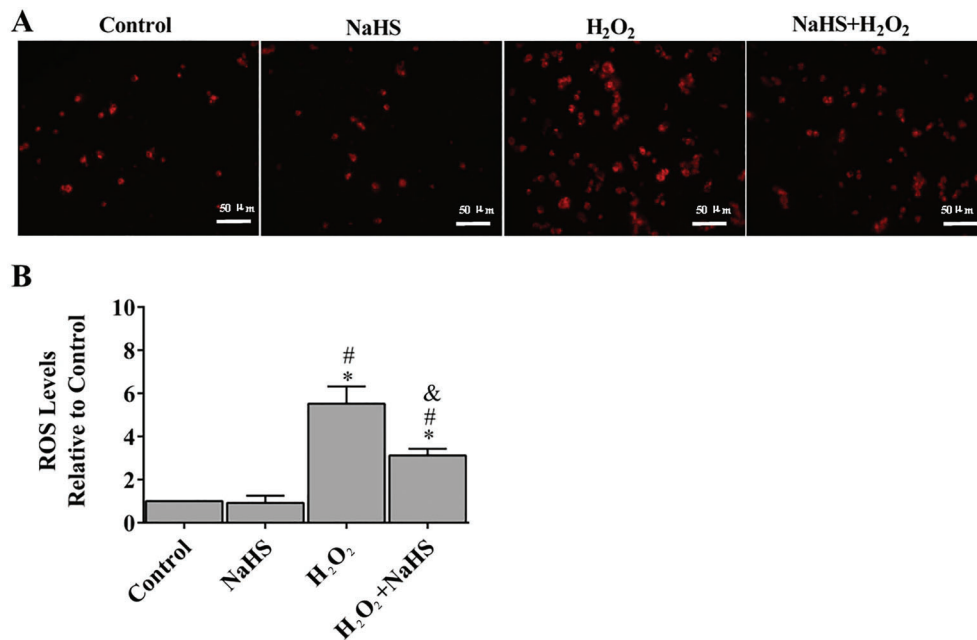

Figure 2. Hydrogen sulfide (H$_2$S) protected H9c2 cells against H$_2$O$_2$-induced oxidative stress (**A**). Intracellular superoxide anion production was detected with dihydroethidium and observed by fluorescent microscopy (**B**). The fluorescent signal was measured and quantified. Data are reported as means ± SE (n=6). *P < 0.01 *vs* control; #P < 0.01 *vs* NaHS; &P < 0.01 *vs* H$_2$O$_2$ (ANOVA followed by Student-Newman-Keuls *post hoc* test). ROS: reactive oxygen species.

six-fold. However, pre-treatment with H$_2$S demonstrated a significant rescue effect (50.2% reduction of the fluorescence intensity) (Figure 2).

H$_2$S prevented the loss of mitochondrial membrane potential (ψ_m)

Mitochondrial function is highly sensitive to oxidative damages. In this study, we applied the fluorescent dye JC-1 to investigate whether H$_2$S protected the physical functions of mitochondria from H$_2$O$_2$-induced cell stress in the cell model. In the control group, H9c2 cells exhibited

numerous brightly stained mitochondria that emitted orange fluorescence (Figure 3A). After treating with H$_2$O$_2$ for 24 h, H9c2 cells demonstrated fewer and less stained mitochondria (Figure 3B). H$_2$S pre-treatment, on the other hand, prevented H$_2$O$_2$-induced loss of mitochondrial membrane potential.

H$_2$S regulated the expression of apoptosis-related proteins

Compared to the expression levels of H$_2$O$_2$-treated H9c2 cells, the protein expression level of Bax was
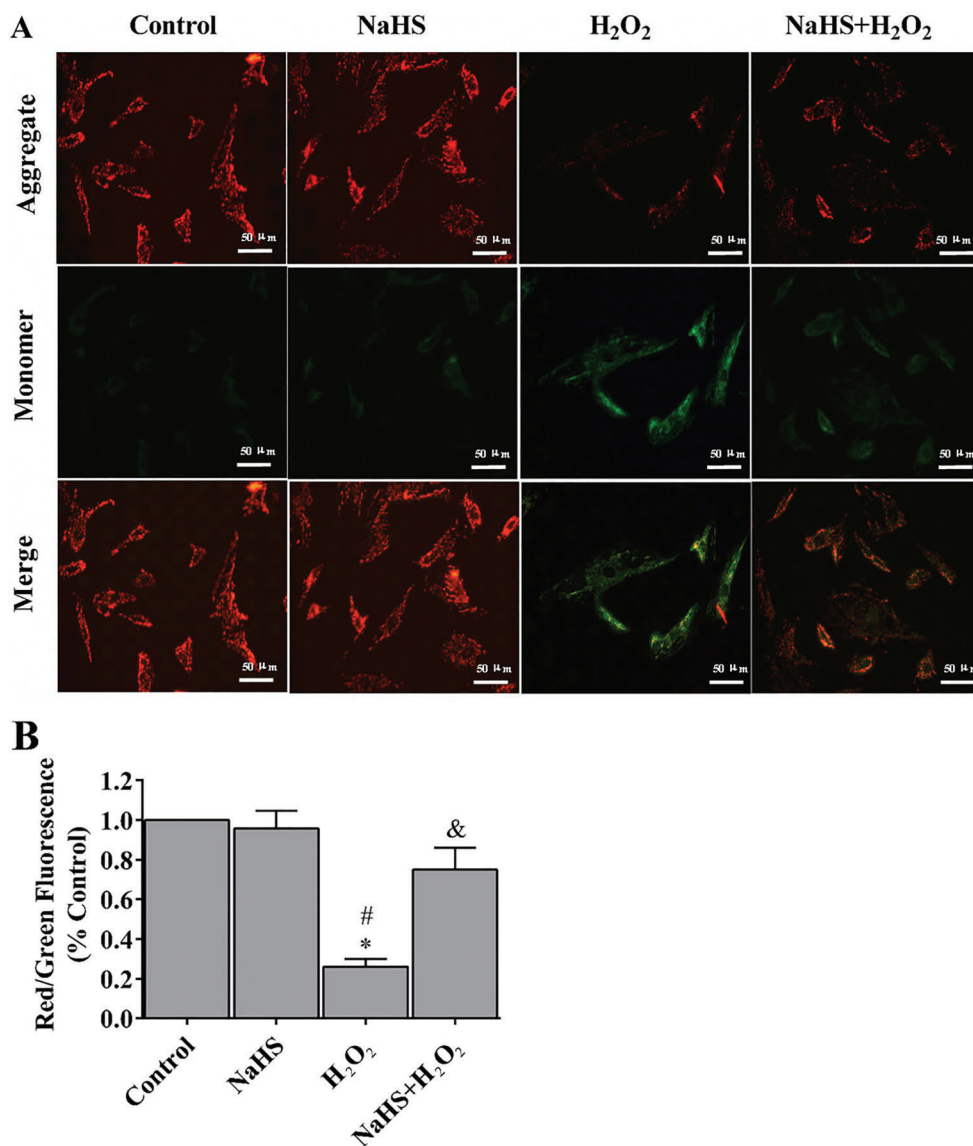

Figure 3. Hydrogen sulfide (H$_2$S) prevented the loss of mitochondrial membrane potential (ψ_m) (**A**). The ψ_m loss was determined by the lipophilic cationic probe JC-1. Red signal indicates JC-1 in mitochondria and green signal indicates cytosolic JC-1. Magnification $\times$400; bar: 50 μm. **B**, Quantitative analysis of membrane potential. Data are reported as means $\pm$ SE (n=6). *P$<$0.01 vs control; #P$<$0.01 vs NaHS; &P$<$0.05 vs H$_2$O$_2$ (ANOVA followed by the Student-Newman-Keuls post hoc test).
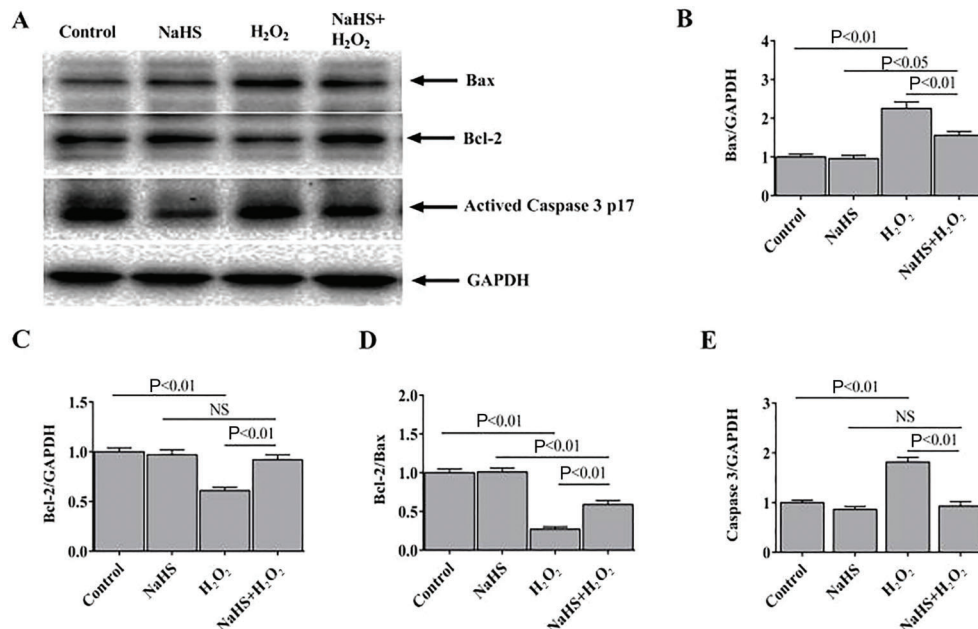
Figure 4. Hydrogen sulfide (H$_2$S) regulated the expression of apoptosis-related proteins. **A**, Representative immunoblots showing the expression of Bax, Bcl-2, and activated caspase 3 p17 in the H9c2 cells. Bax (**B**) and Bcl-2 (**C**) expression normalized to GAPDH (n=6). **D**, Densitometric analysis of the ratio of Bcl-2 to Bax. **E**, Activated caspase 3 p17 expression normalized to GAPDH. Data are reported as means ± SE (n=6) (ANOVA followed by Student-Newman-Keuls *post hoc* test). NS: not significant.

significantly reduced, while Bcl-2 expression was increased in the NaHS-treated cells (Figure 4A). Consequently, compared with the H$_2$O$_2$ group, the ratio of Bcl-2 to Bax was higher in the NaHS + H$_2$O$_2$ group (Figure 4B–D). The expression level of activated caspase 3, another apoptotic marker, was also examined (Figure 4E). The results suggested that H$_2$O$_2$ enhanced apoptosis in H9c2 cells, while the NaHS treatments decreased H$_2$O$_2$-induced apoptosis.

Discussion

As a gaseous signaling molecule, H$_2$S can freely diffuse across cell membranes and activate various cellular targets. This distinct feature makes H$_2$S an attractive pharmacological agent to treat cardiovascular diseases. Previous studies have demonstrated a compelling cardioprotective effect of H$_2$S in rat and mouse models (12–14). We have shown that H$_2$S pre-treatment efficiently protected human ventricular fibroblasts from H$_2$O$_2$-induced endoplasmic reticulum (ER) stress and prevented the activation of caspase cascade (15). In the current study, we showed that H$_2$S protected H9c2 cardioblasts from H$_2$O$_2$-induced oxidative stress. H$_2$S regulated the cell cycle, decreased apoptotic cells, and preserved mitochondrial membrane potential ψm that is essential for ATP production and homeostasis. H$_2$S also upregulated Bcl-2/Bax ratio, suggesting its critical role in the anti-apoptotic mechanisms.

Both studies indicated the holistic role of H$_2$S in protecting the cardiovascular system.

Oxidative stress is the imbalance between ROS production and detoxification. This imbalance impairs the capacity to repair the damages caused by reactive intermediates. Moreover, imbalance in oxidative states may lead to the generation of peroxides and free radicals, which in turn damage proteins, lipids, and DNA (1). Oxidative damage from H$_2$O$_2$ contributes to heart failure and tissue damages (16). The H$_2$O$_2$ molecule plays an essential role in the progression of oxidative stress and cardiac pathologies (17,18). In addition, excessive ROS damages mitochondria, opens its permeability transition pore (PTP), and thus induces mitochondrial permeability transition. These alterations cause mitochondrial depolarization and outer membrane rupture, leading to cell apoptosis or death (19,20). Apoptosis that occurs in the clinical setting (*e.g.*, open-heart surgery under cardiopulmonary bypass) is induced by various conditions and agents, including ROS, NO, calcium, and pressure overload, mechanical stress, tumor necrosis factor, and angiotensin II. Apoptosis has also been shown to play a pivotal role in the pathogenesis of ischemia/reperfusion (21).

Endogenous gaseous signaling mediators, such as H$_2$S, are formed in mammalian cells and tissues. H$_2$S may easily react with certain compounds, especially with reactive oxygen and nitrogen species, such as superoxide

radical anion (O_2-), H_2O_2, peroxynitrite (ONOO-), and hypochlorite (ClO-) (22–24). In the current study, we demonstrated that H_2O_2 induced ROS production and decreased mitochondrial membrane potential, suggesting an impairment of mitochondrial functions. H_2S treatment, however, attenuated the ROS production, restored mitochondrial membrane potential, and decreased cardiomyocyte apoptosis. H_2S may protect mitochondrial function via multiple pathways, such as activating AMPK in cardiomyocytes (25).

Previous studies showed that Bcl-2 family is upregulated during the opening of PTP (19,20). PEP-1-CAT, a fusion protein of anti-microbial peptide and antioxidant enzyme, protects cardiomyocyte from hypoxia/reoxygenation-induced injuries. PEP-1-CAT blocks Bcl-2/Bax/mitochondrial apoptotic pathway by inhibiting p38 MAPK while activating the PI3K/Akt and Erk1/2 signaling pathways (26). Here, we also found H_2S regulated the Bcl-2/Bax/caspase-3 signaling pathway in the rat cardiomyoblasts. The functions of Bcl-2 (anti-apoptosis) and Bax (pro-apoptosis) proteins are in opposition of the apoptotic pathway. When the apoptotic signal is present, caspase 3 (32 kD) will be cleaved and present as an active form (17 kD) to induce cell apoptosis (27). In our study, H_2O_2 treatment led to a shift in favor of the pro-apoptotic protein, Bax, as well as upregulated the downstream effector, caspase 3. On the other hand, H_2S rescued this apoptotic event and downregulated the level of caspase 3, demonstrating a cytoprotective effect in the rat cardiomyoblasts.

In conclusion, we demonstrated that NaHS has potent anti-apoptotic effects in cardiomyoblasts with H_2O_2-induced injuries. The anti-apoptotic function may be partially due to blocking ROS production in mitochondria, maintaining mitochondrial membrane integrity, and inhibiting activation of the Bcl-2/Bax apoptotic pathway. The current study suggested that H_2S may serve as an effective therapeutic option for treating ischemia-reperfusion injuries.

Acknowledgments

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