Original Article Exogenous H₂S reduces apoptosis and myocardial fibrosis in isoproterenol-induced rats by inhibiting activation of CaMKII/NF-κB signaling pathways

Shengquan Liu^{1*}, Maojun Liu^{1*}, Junrong Long¹, Wenting Tan¹, Fen Tang¹, Yan Li¹, Chun Chu², Jun Yang¹

¹Department of Cardiology, The First Affiliated Hospital of University of South China, Hengyang, Hunan, China; ²Department of Pharmacy, The Second Affiliated Hospital of University of South China, Hengyang, Hunan, China. *Equal contributors and co-first authors.

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Abstract: Purpose: This study aimed to figure out the effects of exogenous hydrogen sulfide (H_aS) on isoproterenol (ISO)-induced myocardial fibrosis and to verify potential mechanisms of cardiomyocyte apoptosis. Methods: Male Sprague-Dawley rats were randomly assigned into the control group, ISO group, ISO + H₂S group, and H₂S group. ISO was injected into the abdominal cavities of rats for 7 consecutive days to establish animal models of heart failure. Cardiac collagen deposition and collagen volume fraction (CVF) were measured by Masson's staining. Cardiomyocyte apoptosis was observed by TUNEL staining. Expression of related proteins in CaMKII/NF-KB signaling pathways, apoptosis, and MMPs/TIMPs was detected with the method of Western blotting. Expression of apoptosisrelated miRNAs was detected through RT-qPCR. Results: Compared to the control group, expression of CaMKII/ NF-kB, caspase2, and caspase3 proteins, as well as proapoptotic miRNAs, were significantly upregulated in the ISO group, while expression was downregulated in the ISO + H_aS group. Expression of Bcl-2 proteins was decreased in the ISO group but increased in the ISO + H₂S group. Masson's staining results showed that cardiac collagen deposition and CVF were significantly increased in the ISO group but decreased in the ISO + H_aS group. Findings obtained from TUNEL staining indicated that the ratio of TUNEL-positive cell nuclei in the ISO + $H_{a}S$ group was lower than that in the ISO group. Conclusion: The current study highlights that exogenous H₂S can inhibit myocardial fibrosis and cardiomyocyte apoptosis in ISO-induced rats. The mechanisms may be associated with downregulation of CaMKII/ NF-kB signaling pathways and expression of apoptosis-related miRNAs.

Keywords: Hydrogen sulfide, myocardial fibrosis, CaMKII/NF-κB signaling pathway, apoptosis, inflammatory reaction

Introduction

Heart failure is a common terminal manifestation and a leading cause of death in patients with cardiovascular disease. Myocardial remodeling is the most important pathophysiological change in heart failure. Myocardial fibrosis is a key part of it, whose occurrence and development are related to the myocardial toxicity of sympathy-catecholamines [1]. Myocardial fibrosis may lead to a decrease of myocardial contraction and relaxation. This is also closely related to the occurrence of arrhythmia and even sudden cardiac death. It has been reported that calcium circulation in cardiomyocytes is associated with myocardial contraction and relaxation [2]. Calcium regulation-associated proteins are important molecules in the regulation of calcium homeostasis in cardiomyocytes. Their expression and activity play an important role in regulating myocardial contraction and relaxation, which are involved in signal transduction mechanisms for myocardial remodeling and apoptosis in heart failure. In recent years, the roles of calcium/calmodulin kinase II in myocardial remodeling and heart failure have gained much attention from worldwide scholars [3]. CaMKII, an important calcium regulationassociated protein, belongs to the serine-threonine kinase family. A previous study has shown that CaMKII could regulate cardiomyocyte hypertrophy, apoptosis, and other pathophysiological processes through modulating expression of NF-κB [4]. NF-κB, an important transcription factor in eukaryotic cells, has a synergistic effect with STAT3, which is involved in the development of apoptosis and myocardial fibrosis [5]. It has been found that miRNAs, as transcriptional regulators, also play an important signal regulation role in apoptosis and myocardial fibrosis. Of these, miR-1 and miR-150, important members of small RNAs, have been shown to be implicated in the signal transduction mechanisms for apoptosis and myocardial fibrosis [6]. However, there have been few reports on whether CaMKII/NF-ĸB and miRNA are involved in the pathogenesis of ISO-induced myocardial fibrosis.

A new gas signaling molecule, H_2S has been found to have cardioprotective effects, able to improve interstitial collagen remodeling and myocardial fibrosis by relieving oxidative stress and inflammatory reactions [7]. However, whether H_2S can improve ISO-induced myocardial fibrosis and the nature of its internal mechanisms remains to be uncovered. Therefore, this study aimed to investigate the effects of exogenous H_2S on ISO-induced myocardial fibrosis by establishing rat models of ISO-induced myocardial fibrosis, exploring its internal mechanisms to regulate apoptosis and inflammatory reactions through modulating the CaMKII/NF- κ B signaling pathways and related miRNAs.

Materials and methods

Experimental animals

Forty adult male Sprague-Dawley (SD) rats (200 \pm 20 g) were provided by the Experimental Animal Center of University of South China. The rats were raised in well-ventilated and separate cages at 24 \pm 1°C indoors with a 12-h day/ night cycle. They were given free access to food and water. The rats were acclimatized for 1 week before the experiment. The experiment was carried out per the rules put forward in the Regulations for the Administration of Affairs Concerning Experimental Animals by the State Scientific and Technological Commission of the People's Republic of China.

Reagents and instruments

Isoproterenol hydrochloride (isoproterenol, ISO) was purchased from Meilun Bio (Dalian, China).

Sodium hydrosulfide (H₂S donor), diethyl pyrocarbonate (DEPC), and Tris were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal antibody TIMP-1 and glyceraldehyde phosphate dehydrogenase (GAPDH) were purchased from Wuhan Boster Biological Technology (Wuhan, China). CaMKK1 and AMPK were from Bioss (Beijing, China), diluted to 1:400. Rabbit polyclonal antibodies CaMKIIδ and Bcl-2 were obtained from ProteinTech (Chicago, IL, USA). Goat anti-rabbit antibody was from KPL (Clopper Road, Gaithersburg, Maryland, USA), diluted to 1:2000. TRIzol was obtained from Invitrogen (Carlsbad, CA, USA). The miRNA reverse transcription kit was from Cwbiotech Co. Ltd., (Beijing, China). Primer was from Genscript Biotechnology Company (Nanjing, China) and Taq, DL2000DNA Marker and dNTP were obtained from Beijing GenStar Biosolutions Co., Ltd., (Beijing, China). SYBGEEN PCR Master Mix was from Applied Biosystems, Foster City (California, USA). Hoefer miniVE, E-Blotter was from Bio-Rad (Hercules, California, USA). Ultraviolet spectrophotometer was purchased from Shimadzu (Kyoto, Japan). Realtime q-PCR and PCR plates were from Thermo Electron Corporation, Waltham (Massachusetts, USA).

Animal models and treatment

The rats were randomly classified into a control group, ISO group, ISO + H_2S group, and H_2S group, with 10 rats in each group. After feeding with a standard diet, rats in the ISO and ISO + H_2S groups were intraperitoneally injected with ISO (5 mg·kg⁻¹.d⁻¹) to establish the heart failure model, in accordance with modeling methods of Peter Krenek [8]. Rats in the ISO + H_2S and H_2S groups were treated with NaHS solution (56 µmol·kg⁻¹.d⁻¹) every day. Rats in the control and H_2S groups were intraperitoneally injected with equal doses of saline every day.

Echocardiographic detection

After 8 weeks of modeling, all rats were anesthetized with chloral hydrate. Cardiac functions were detected by an experienced physician using M ultrasound. Three cardiac cycle parameters were measured horizontally and continuously at the muscuil papillares of the macropinacoid. The mean value was adopted to calculate the left ventricular posterior wall thickness (LVPWs), left ventricular end-diastolic dimen-

Table 1. Primers	used	for	real-time PCR
analysis			

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miRNA	Primer sequence
miR-1	5'-TGGAATGTAAAGAAGTGTGTAT-3'
miR-150	5'-TCTCCCAACCCTTGTACCAGTG-3'
miR-29a	5'-ACTGATTTCTTTTGGTGTTCAG-3'
miR-214	5'-AGAGTTGTCATGTGTCT-3'
U6	5'-GCTTCGGCAGCACATATACTAAAT-3'

sion (LVDd), left ventricular end-systolic diameter (LVDs), and left ventricular fractional shortening (FS), along with other parameters.

Specimen collection and processing

The rats were weighed (body weight, BW) and killed. Their hearts were removed and weighed (heart weight, HW). Moreover, the heart mass index (HW/BW, mg/g) was also calculated. Ventricular muscles were fixed in 4% paraformaldehyde for Masson's staining. Some of the heart tissues were fixed in 2.5% glutaraldehyde solution for detection by a transmission electron microscope. The remaining heart tissues were placed in a -80°C freezer for cryopreservation. They were applied for the detection of Western blotting and RT-qPCR.

Masson's staining

Myocardium tissues were fixed in 4% paraformaldehyde, dehydrated with ethanol of concentration gradient, cleared with xylene, and embedded with paraffin. They were then made into 4 µm thick slices. The tissues were stained with the Masson's staining kit and observed under a light microscope (×400) for myocardial interstitial collagen accumulation. Three different perspectives were selected and processed with Image J. They were measured for collagen volume fraction (CVF).

Immunohistochemistry

The formalin-fixed and paraffin-embedded sections were dewaxed and dehydrated. Endogenous peroxidase was blocked with 3% hydrogen peroxide, rinsed with PBS three times, and blocked with 10% normal goat serum. Next, the tissues were incubated overnight at 4°C with primary antibody (rabbit polyclonal anti-collagen III and rabbit polyclonal anti-CD86; 1:50). The sections were then rinsed with PBS three times and incubated at 37°C with secondary antibody (polyclonal goat anti-rabbit horseradish peroxidase [HRP]-labeled immunoglobulin; 1:2000) for 30 minutes. Subsequently, the sections were washed with PBS three times again. The slides were developed with 3,3'-diaminobenzidine-hydrochloride (DAB) solution for 5 minutes and stained with hematoxylin for 5 minutes. They were dehydrated by alcohol and finally sealed with neutral resins.

TUNEL staining

Myocardial tissues were immersed in 10% formalin before being paraffin-embedded for sectioning. The tissues were stained by TUNEL solution, treated by H_2O_2 , and incubated with a reaction mixture including TdT and digoxigeninconjugated dUTP for 1 hour at 37°C. Next, labeled DNA was visualized by peroxidase-conjugated anti-digoxigenin antibody containing the chromogen of DAB. Apoptotic nuclei were then dyed with brown. The horizon was photographed and the ratio of apoptotic nuclei to myocardia tissue was counted.

Transmission electron microscope (TEM)

Tissues fixed in 2.5% glutaraldehyde were then fixed with 1% osmium acid for 2 hours. They were then rinsed with 0.1 M phosphoric acid 3 times, each time for about 10-15 minutes. The tissues were dehydrated in turn with 50%, 70%, and 90% acetone for 10-15 minutes, respectively, and 100% acetone for 15-20 minutes. Next, they were soaked at 37°C in the liquid mixture of pure acetone and embedding liquid (1:1) for 12 hours, then embedded with pure embedding liquid for 10-12 hour. They were put it in an oven at 37°C overnight and at 60°C for 12-24 hours. They were cut into slices of about 50-100 nm and re-stained with 3% uranyl acetate and lead nitrate. The transmission electron microscope was used to observe the arrangement of myocardial fibers and form of the mitochondria.

Western blotting

Myocardial tissue (0.1 g) was fully grinded on the ice. Cell lysis buffer 1:1 phenylmethyl sulfonylfluoride (PMSF) (700 μ L for each) was added to extract total protein. Protein concentrations were quantified by bicinchoninic acid (BCA) colorimetry. The protein was denatured by boiling

Table 2. Effects of $\rm H_2S$ on body weight, heart weight, and ratio of heart weight and body weight in each group. data are expressed as Mean \pm SD

Groups	Number	BW (g)	HW (mg)	HW/BW (mg/g)
Control	10	221.50 ± 23.67	591.70 ± 72.03	2.67 ± 0.17
IS0	9	243.89 ± 24.21	923.33 ± 92.87ª	3.79 ± 0.20^{a}
$ISO + H_2S$	9	233.89 ± 38.87	761.11 ± 154.30 ^{a,b}	3.24 ± 0.26 ^{a,b}
H_2S	10	220.50 ± 18.77	590.00 ± 63.94	2.67 ± 0.13

Abbreviations: A: Control group; B: ISO group; C: ISO + H_2S group; D: H_2S group. ^aP < 0.05 ISO group vs. Control group; ^{a,b}P < 0.05 ISO + H_2S group vs. ISO group.

Table 3. Effects of H_2S on the echocardiographic parameters in each group. Data are expressed as Mean $\pm SD$

Groups	Number	LVDd (mm)	LVDs (mm)	LVPW (mm)	FS (%)
Control	10	4.47 ± 0.15	2.64 ± 0.21	0.78 ± 0.04	40.89 ± 4.71
ISO	9	6.49 ± 0.34ª	5.04 ± 0.40ª	1.09 ± 0.16	22.20 ± 5.73ª
$ISO + H_2S$	9	$6.09 \pm 0.69^{a,b}$	$4.32 \pm 0.56^{a,b}$	1.08 ± 0.16	29.03 ± 4.41 ^{a,b}
H ₂ S	10	4.50 ± 0.19	2.66 ± 0.27	0.79 ± 0.06	40.85 ± 5.62

Abbreviations: Left ventricular end diastolic dimension (LVDd); left ventricular end systolic dimension (LVDs); left ventricular posterior wall (LVPW); fractional shortening (FS). ${}^{a}P < 0.01$ Control group vs. ISO group; ${}^{a,b}P < 0.05$ ISO + H_aS group vs. ISO group.

at 99°C for 10 minutes, separated through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with Tris-buffered saline with Tween20 (TBST) containing 5% skimmed milk for 1 hour at room temperature. Afterward, the membranes were incubated with blocking solution containing primary antibody (CaMKIIo, Bcl-2 and Caspase-3; 1:2000; CaMKIIδ, NF-κB and TIMP-1; 1:400) overnight at 4°C. Next, it was rinsed with TBST 3 times (10 minutes each time), incubated at room temperature for 1 hour with HRP-labeled antibody (1:2000), and rinsed again with TBST 3 times (10 minutes each time). PVDF membranes were detected by the chemiluminescence detection method. Image J software was used for optical density (OD) analysis of the target proteins, with corresponding gravness ratios representing relative levels of detected protein expression.

RT-qPCR

After all RNA-required equipment was sterilized, the TRIzol method was used to extract total RNA. RNA quality was measured by RNA agarose gel electrophoresis, with the absor-

bance ratio (1.8-2.0) at 260/280 nm. Concentrations were assessed using a spectrophotometer. cDNA was generated from 2 µg of total cellular RNA by reverse transcription (E. coli Poly (A) Polymerase 2.5U). PCR amplification reaction was carried out with SYBR based on cDNA. Primer sequences are shown in Table 1. The PCR procedure for realtime qPCR was pre-degeneration for 10 minutes (95°C), with 40 cycles of denaturation for 10 seconds (95°C), and annealing/extension 50 seconds (59°C). The melting curve was collected at 60-95°C. Relative expression levels of miRNAs, including miR-1, miR-29a, miR-150, and

miR-214, were analyzed using the $2^{\text{-}\Delta\Delta\text{C}t}$ method.

Statistical analysis

Experimental data was analyzed using SPSS 18.0 (Chicago, IL, USA). Results are expressed as mean \pm standard deviation (SD). LSD-t was used to analyze comparisons between groups and one-way analysis of variance (ANOVA) was used to analyze comparisons among multiple groups. P < 0.05 indicates statistical significance.

Results

Survival of rats

Two rats died during the experiment. One was in the ISO group and the other was in the H_2S + ISO group, of unknown causes.

BW, HW, and HW/BW of rats

HW/BW reflects cardiac hypertrophy and fibrosis, to some extent. Thus, the BW, HW, and HW/ BW of rats were all calculated. Compared with the control group, HW and HW/BW of rats in the ISO group were significantly increased. Relative to the ISO group, HW and HW/BW of rats in the



Figure 1. Masson's staining and Collagen volume fraction of myocardial tissues in each group (×400). Data are expressed as Mean \pm SD (n = 3). Abbreviations: A: Control group; B: ISO group; C: ISO + H₂S group; D: H₂S group. ^aP < 0.001 ISO group vs. Control group; ^{ab}P < 0.001 ISO + H₂S group vs. ISO group. Arrows indicate the deposition of collagen fibers in the myocardium.



Figure 2. Immunohistochemistry and the ratio of Collagen III to myocardial tissue in each group (×400). Data are expressed as Mean ± SD (n = 3). Abbreviations: A: Control group; B: ISO group; C: ISO + H₂S group; D: H₂S group. ^aP < 0.001 ISO group vs. Control group; ^{ab}P < 0.001 ISO + H₂S group vs. ISO group. Arrows indicate the area of Collagen III-positive.

 H_2S + ISO group were decreased, with statistical significance, while differences in BW were not statistically significant, as shown in **Table 2**. Results indicate that H_2S could relieve cardiac hypertrophy and fibrosis.

Echocardiography results

Parameters, such as LVDs, LVDd and FS, which evaluate left ventricular function, were detected in this present study. Compared with the control group, LVDs and LVDd of rats were significantly increased and FS was significantly decreased in the ISO group. In contrast to the ISO group, LVDs and LVDd of rats were significantly decreased and FS was significantly increased in the ISO + H₂S group. The above parameters were not significantly changed between the control group and H₂S group, as shown in **Table 3**. Present findings indicate that H₂S improves heart function of ISO-induced heart failure.

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Masson's staining results

To evaluate myocardial fibrosis, Masson's staining was carried out. Compared with the control group, for rats in the ISO group, their myocardial cells were arranged in disorder. Blue dye collagen fibers were significantly increased and CVFs were significantly increased. Compared to the ISO group, for rats in the ISO + H_oS group, blue dye collagen fibers were significantly decreased and CVFs measured of myocardial tissues were decreased. However, the control group and the H₂S group were not significantly different, as shown in Figure 1. Therefore, present findings demonstrate that myocardial fibrosis of heart failure rats was reduced after intervention with H₂S.

Immunohistochemistry results

To demonstrate myocardial fibrosis from levels of protein, collagen III was detected by immunohistochemistry. Compared with the control group, expression of collagen III was remarkably increased in the

ISO group. Expression of collagen III was remarkably decreased in the ISO + H_2S group, relative to the ISO group. The control group and H_2S group were not significantly different in expression of collagen III (**Figure 2**). Results show that H_2S could relieve expression of collagen III and attenuate myocardial fibrosis.

To observe whether inflammation response was involved in myocardial fibrosis, expression of CD86 was also detected by immunohistochemistry, aiming to clarify the inflammation reaction from a molecular level. Compared with the control group, the ratio of CD86-positive to myocardial tissue was remarkably increased in the ISO group. The ratio of CD86-positive to myocardial tissue was obviously decreased in the ISO + H_2S group, in contrast to the ISO group. The control group and the H_2S group were not significantly different in the ratio of CD86-positive to myocardial tissue to myocardial tissue (Figure 3). Present results indicate that H_2S could reduce

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Figure 3. Immunohistochemistry and the ratio of CD86-positive to myocardial tissue in each group (×400). Data are expressed as Mean \pm SD (n = 3). Abbreviations: A: Control group; B: ISO group; C: ISO + H₂S group; D: H₂S group. ^aP < 0.001 ISO group vs. Control group; ^{ab}P < 0.001 ISO + H₂S group vs. ISO group. Arrows indicate the area of CD86-positive.



Figure 4. TUNEL assay and the ratio of TUNEL-positive cell nuclei in each group (×400). Data are expressed as Mean ± SD (n = 3). Abbreviations: A: Control group; B: ISO group; C: ISO + H₂S group; D: H₂S group. ^aP < 0.001 ISO group vs. Control group; ^{ab}P < 0.001 ISO + H₂S group vs. ISO group. Arrows indicate the TUNEL-positive cell nuclei.

the inflammation response of myocardial fibrosis of heart failure rats.

TUNEL staining results

Apoptosis is related with myocardial fibrosis. TUNEL-assay in this study was used to detect cell apoptosis in the myocardium. Compared to the control group, the number of apoptotic nuclei of myocardial tissues was remarkably increased in the ISO group, while the number of apoptotic nuclei of myocardial tissues was remarkably decreased in the ISO + H_2S group, compared with the ISO group. The control group and the H_2S group were not significantly different in the number of apoptotic nuclei of myocardial tissue (**Figure 4**). Results reveal that H_2S could inhibit ISO-induced cell apoptosis in heart failure rats.

Transmission electron microscope results

In this experiment, transmission electron microscope was used to observe the ultrastructure, including myocardial fibers and mitochondria, aiming to explore the underlying mechanisms of deriving from H_aS against myocardial fibrosis. As shown in Figure 5, relative to the control group, for rats in the ISO group, myocardial fibers were arranged in disorder and with edema. Mitochondria showed significant swelling and was vacuolated and arranged in disorder. Cristae membrane was irregularly distributed, even fractured or missing. Compared with the ISO group, for rats in the H_sS + ISO group, myocardial fibers were arranged better, mitochondria showed slight swelling, and cristae membrane was slightly and irregularly distributed, as well as slightly fractured. Myocardial fibers and mitochondria of rats in the H₂S group and the control group were not significantly different. Present results demonstrate that H₂S could extenu-

ate injuries of the myocardial ultrastructure in heart failure rats.

Expression of MMPs/TIMPs and collagen II in myocardial tissues of rats in different groups

The imbalance of MMP_s/TIMP_s breaks down the balance of the ratio of collagen synthesis and degradation, possibly deteriorating myocardial fibrosis. In view of this, this study detected expression of relative proteins to reflect myocardial fibrosis. Compared to the control group, expression of TIMP1, TIMP3, MMP9, MMP2, and Collagen II in myocardial tissues was significantly increased, while that of MMP12 was significantly decreased for rats in the ISO group. Relative to the ISO group, expression of TIMP1, TIMP3, MMP9, MMP2, and Collagen II in myocardial tissues was significantly decreased, while that of MMP12 was



Figure 5. Transmission Electron Microscope to myocardial tissue in each group. Transmission election micrographs at scale bars of 1 μ m and 2 μ m. A: Control group; B: ISO group; C: ISO + H₂S group; D: H₂S group. The arrows indicate the myocardial fibers and mitochondria.

increased for rats in the ISO + H_2S group. However, expression of these proteins was not significantly different between the control group and H_2S group (**Figure 6**).

Expression of CaMKII δ and CaMKK1 in myocardial tissues of rats

To explore CaMkII/NF- κ B signaling pathways involvement in the development of myocardial fibrosis, this study detected expression of CaMKII δ and CaMKK1. As shown in **Figure 7**, compared with the control group, expression of CaMKII δ and CaMKK1 in myocardial tissues was significantly increased for rats in the ISO group. In contrast to the ISO group, expression of CaMKII δ and CaMKK1 in myocardial tissues was significantly decreased for rats in the ISO + H₂S group. Expression of CaMKII δ and CaMKK1 was not significantly different between the control group and H₂S group.

Expression of proteins related inflammation in myocardial tissues of rats in different groups

The current study also detected expression of NF- κ B and expression of proteins related to inflammation by Western blotting. Compared with the control group, expression of NF- κ B, STAT3, IL-6, and TNF- α in myocardial tissues was significantly increased for rats in the ISO

group. Compared with the ISO group, expression of NF- κ B, STAT3, IL-6, and TNF- α in myocardial tissues was significantly decreased for rats in the ISO + H₂S group. Expression of NF- κ B, STAT3, IL-6, and TNF- α was not significantly different between the control group and H₂S group (**Figure 8**). Present results indicate that H₂S could downregulate CaMkII/NF- κ B signaling pathways in ISO-induced heart failure rats.

Expression of Caspase3, Caspase2, and Bcl-2 in myocardial tissues of rats in different groups

To further investigate the mechanisms of apoptosis at the molecular level, expression of Caspase3, Caspase2, and Bcl-2 was examined. Compared to the control group, expression of Caspase3 and Caspase2 in myocardial tissues was significantly increased, while expression of Bcl-2 was decreased for rats in the ISO group. Compared with the ISO group, expression of Caspase3 and Caspase2 in myocardial tissues was significantly decreased and expression of Bcl-2 was increased for rats in the ISO + H₂S group. Expression of Caspase3 and Caspase2 was not significantly different between the control group and H₂S group (Figure 9). Present results reveal that H₂S could inhibit ISO-induced cell apoptosis in heart failure rats.





Expression of PPARG and AMPK in myocardial tissues of rats

The current study also measured expression of PPARG and AMPK. As shown in **Figure 10**, relative to the control group, expression of PPARG and AMPK in myocardial tissues was significantly decreased for rats in the ISO group. Compared with the ISO group, expression of PPARG and AMPK in myocardial tissues was significantly increased for rats in the ISO + H_2S group. Expression of PPARG and AMPK showed no significant differences between the control group and H_2S group.

Expression of miRNAs in myocardial tissues of rats in different groups

To expound the benefits of H₂S-elicited against apoptosis and myocardial fibrosis at the gene level, expression of miR-1, miR-29a, miR-214, and miR-150 were measured. Compared with the control group, expression of miR-1, miR-29a, and miR-150 in myocardial tissues was significantly increased, while that of miR-214 was decreased for rats in the ISO group. In contrast to the ISO group, expression of miR-1, miR-29a, and miR-150 in myocardial tissues was significantly decreased, while that of miR-214 was increased for rats in the ISO + H_aS group. Expression of miR-1, miR-29a, and miR-150 was not significantly different between the control group and H₂S group, as shown in Figure 11. Present results show that H₂S could down**Figure 7.** Expression of CaMKII δ and CaMKK1 protein in myocardial tissues from each group. Data are expressed as Mean \pm SD (n = 3). *P < 0.05 ISO group vs. Control group; **P < 0.05 ISO + H₂S group vs. ISO group.



regulate expression of miR-1, miR-29a, and miR-150, inhibiting cell apoptosis in ISO-induced heart failure.

Discussion

Heart failure, the terminal manifestation of various cardiovascular diseases, has a poor clinical prognosis. The mortality rate of patients with heart failure may be up to approximately 20%, annually. The ratio may be up to 50% in patients with severe heart failure. Myocardial remodeling is the key to determining clinical prognosis in the development of heart failure. Additionally, myocardial fibrosis is one of the main pathological changes of myocardial remodeling, mainly manifested by abnormal increasing and disorganization of cardiac collagen fibers, significant increasing of abnormal CVF, and imbalanced proportions of collagen [9]. The pathogenesis of myocardial fibrosis is not yet fully understood, but studies have elucidated that it is associated with sympathetic activation and catecholamine cardiotoxicity. During the development of heart failure, neuroendocrine activation can lead to increased catecholamine levels and persistent excitation of beta-adrenergic receptors. Additionally, catecholamine cardiotoxicity enables the promotion of myocardial apoptosis and release inflammatory mediators, thus further leading to the synthesis and deposition of extracellular matrix (ECM) [10]. MMPs is a major kind of enzyme that degrades extracellular matrix components.



TIMPs is an endogenous inhibitor of matrix metalloproteinases. It has been demonstrated that an imbalance between MMPs and TIMPs will lead to the accumulation of ECM collagens, causing myocardial fibrosis. This present study established myocardial fibrosis rat models through the injection of ISO. Results of echocardiography revealed that rates of FS in the ISO group were significantly lower than those in the control group. LVDd and LVDs of rats in the ISO group were significantly higher than those in the control group. Furthermore, HW and HW/ BW of rats in the ISO group were significantly increased. Masson's staining and CVF results showed that myocardial collagen deposition in myocardial tissues of rats in the ISO group was

Figure 8. Expression of inflammation related proteins in myocardial tissues from each group. Data are expressed as Mean \pm SD (n = 3). *P < 0.05 ISO group vs. Control group; **P < 0.05 ISO + H₂S group vs. ISO group.



significantly increased, in contrast to the control group, with myocardial fibers arranged in disorder. It was observed under the transmission electron microscope that, relative to the control group, rats in the ISO croup showed irregular arrangement of myocardial fibers. Moreover, expression of TIMP1 and TIMP3 in myocardial tissues was significantly increased. while expression of MMP12 was significantly decreased in the ISO croup, versus the control group. This indicates that expression of MMPs/ TIMPs was seriously disturbed. Present results suggest that ISO-induced myocardial fibrosis occurs in the rat myocardium, resulting in decreased myocardial remodeling and cardiac function, to some extent.



Figure 9. Expression of apoptosis-related proteins in myocardial tissues from each group. Data are expressed as Mean \pm SD (n = 3). *P < 0.05 ISO group vs. Control group; **P < 0.05 ISO + H₂S group vs. ISO group.

A newly discovered gas signal molecule involved in cell signal regulation, H₂S has attracted increasing attention from scholars. It has been reported that H₂S might play a role in myocardial protection in multiple cardiovascular disease models, such as viral myocarditis, acute coronary syndrome, and diabetic cardiomyopathy. Moreover, it has been demonstrated that H₂S has anti-oxidative stress abilities. It inhibits inflammatory reactions, improves myocardial remodeling, and inhibits myocardial fibrosis and other biological effects [11]. Mishra and others studies have found that H₂S is able to significantly reduce cardiac hypertrophy in rats with chronic heart failure [12]. GYY4137, the donor of H₂S, can also reverse myocardial fibrosis in rats with hypertension. It has also been reported that H₂S may participate in the regulation of calcium homeostasis and calcium channels, inhibiting the proliferation of human atrial fibroblasts and the transformation to myofibroblasts. Therefore, this study observed the effects of H₂S on myocardial fibrosis with NaHS

as an exogenous donor of H_2S , establishing rat models of ISO-induced myocardial fibrosis. Transmission electron microscope results suggest that the irregular arrangement of myocardial fibers was improved in the H_2S + ISO group. Additionally, the MMPs/TIMPs imbalance was significantly improved. Rats in the H_2S + ISO group had significantly decreased LVDs and LVDd, increased FS, and significantly decreased HW and HW/BW, compared to rats in the ISO group. Results suggest that H_2S can significantly improve the MMPs/TIMPs imbalance and much ISO-induced myocardial fibrosis, thus improving myocardial remodeling and left ventricular function.

The mechanisms of H_2S that inhibit myocardial fibrosis are still not very clear. Several studies have reported that it may be related to antioxidative stress, which inhibits inflammatory reactions, apoptosis, and other effects. It has been demonstrated that H_2S may be implicated in the regulation of calcium homeostasis and



calcium channels. CaMKII is a multifunctional serine-threonine kinase which plays a key role in regulating calcium homeostasis in cardiomyocytes. CaMKIIo is a major isozyme of CaMKII in the heart. It has been found that persistently excited β1-AR is able to activate CaMKII pathways, further leading to myocardial hypertrophy, myocardial fibrosis, and other pathological processes of myocardial remodeling [13]. Ca-MKII has been suggested to be involved in apoptosis signaling. Studies have shown that the activation of CaMKIIδ is an important medium in multiple inductive apoptosis signaling pathways [14]. Persistent excitation of β -AR can continuously activate CaMKII and induce myocardial apoptosis through mitochondrion apoptosis. Apoptosis can be inhibited by specific inhibiting CaMKII_δ [15]. Calcium-dependent protein kinase CaMKK, a calmodulin dependent protein kinase, may be involved in the activation and regulation of CaMK and AMPK, as well as the signaling regulation of multiple physiological and pathophysiological processes, including energy balance and glucose homeostasis. The present study revealed that ISOinduced myocardial fibrosis may be related to the upregulation of CaMKII and an increase of apoptosis. Results showed that, compared with the control group, rats in the ISO group had significantly increased expression of myocardial Caspase3 and Caspase2 and decreased ex-

Figure 10. Expression of AMPK and PPARG proteins in myocardial tissues from each group. Data are expressed as Mean \pm SD (n = 3). *P < 0.05 ISO group vs. Control group; **P < 0.05 ISO + H₂S group vs. ISO group.



pression of Bcl-2. TUNEL staining results also showed that apoptosis in myocardial tissues of rats in the ISO group was significantly increased and expression of CaMKIIo and CaMKK1 in myocardial tissues of rats in the ISO group was significantly increased. Results suggest that CaMKIIo pathways may be involved in the regulation mechanisms of ISO-included myocardial fibrosis and related to the development of myocardial fibrosis. Upon intervention, rats had decreased expression of Caspase3 in the myocardium and increased expression of Bcl-2. TUNEL staining detection also showed decreased apoptosis and significantly decreased expression of CaMKIIo and CaMKK1, suggesting that H₂S may inhibit apoptosis by downregulating expression of CaMKIIo, further improving ISO-induced myocardial fibrosis.

Inflammatory reactions are involved in the pathogenesis of myocardial fibrosis. Inhibited myocardial fibrosis mechanisms of H_2S are also related to anti-oxidative stress and inhibited inflammatory reactions. CaMKII δ is a nuclear transcription factor that plays a role in multidirectional regulation. Studies have shown that CaMKII may also be involved in the regulation mechanisms of inflammatory reactions. It has been reported that CaMKII delta gene deletion may reduce the inflammatory reactions of the heart and damage to cardiomyocytes due to



Figure 11. Expression of apoptosis-related miRNAs in myocardial tissues from each group. Data are expressed as Mean \pm SD (n = 3). *P < 0.05 ISO group vs. Control group; **P < 0.05 ISO + H₂S group vs. ISO group.

ischemic reperfusion. This may improve the negative remodeling and systolic dysfunction of infarcted myocardium. Additionally, several studies have found that CaMKII may promote the activation of NF-kB pathways in the ischemia reperfusion myocardium, playing a key role in myocardial inflammation reactions. Occurrence of inflammatory reactions in myocardial tissues may also be closely related to CaMKII/NF-KB [16-18]. NF-KB and STAT3, which are involved in the occurrence of apoptosis and myocardial fibrosis, also have synergistic effects. NF-KB may also regulate expression of multiple inflammatory cytokines, and activate MMPs, affecting the degradation balance of ECM and promoting occurrence of myocardial fibrosis. PPARy may interact with other transcription factors, such as NF-kB, to exert its gene regulating function. Collagen I, α -SMA, and TGF-B are not only negative regulators of fibrosis-related genes, but also play important roles in the regulation of myocardial energy metabolism [19]. This study found that, compared with the control group, rats in the ISO group had increased expression of myocardial NF- κ B and STAT3, with decreased expression of PPAR γ . However, decreased expression of NF- κ B and STAT3, increased expression of PPAR γ , and significantly decreased expression of inflammatory factors, such as IL-6 and TNF- α , was found upon H₂S intervention. Results suggest that H₂S may improve ISO-induced my-ocardial fibrosis by downregulating expression of CaMKII δ /NF- κ B and inhibiting the inflammatory reactions of myocardial tissues.

MicroRNAs, a highly evolutionarily conserved endogenous single stranded non-coding small RNA, are involved in multiple pathological and physiological processes, such as cell proliferation, differentiation, and apoptosis by regulating the expression of target genes with posttranscriptional mechanisms. Studies have indicated that miRNAs are involved in the regulation mechanisms of myocardial fibrosis, including miR-29, miR-1, miR-214, and miR-150. A study on H9c2 apoptosis that was induced by oxidative stress implied that the upregulated miR-1 can regulate expression of HSP60 to promote apoptosis. Moreover, some scholars have found that miR-214 may inhibit calcium overload and apoptosis by inhibiting CaMKII in ischemia-reperfusion injuries [20]. However, Eva van Rooij and Sadakatsu Ikeda found that upregulation of miR-214 may lead to occurrence of myocardial remodeling [21]. Additionally, the miR-29 family has also been shown to promote apoptosis and be involved in the regulation of myocardial fibrosis by regulating expression of extracellular matrix protein genes [22]. The present study also shows that miR-NAs may be involved in the regulation of myocardial fibrosis and apoptosis in rat models of ISO-induced myocardial fibrosis. Results revealed that rats in the ISO group had significantly increased expression of miR-1, miR-29a, and miR-150 in myocardial tissues, compared with the control group. Results also indicated that, relative to the ISO group, rats in the H₂S + ISO group had decreased expression of miR-1, miR-29a and miR-150, while no significant differences were found in terms of expression of miR-214 in myocardial tissues. Present results suggest that the mechanisms of H₂S improving myocardial fibrosis in rats may be involved with decreasing expression of miR-1, miR-29a, and miR-150, thus inhibiting apoptosis.

In conclusion, the current study has found that exogenous H_2S may inhibit ISO-induced myocardial fibrosis and apoptosis in rats through downregulation of CaMKII signaling pathways. It also found decreased expression of apoptosis-related miRNAs, including miR-1, miR-29a, and miR-150. However, whether there is a direct upstream and downstream relationship between miRNA and CaMKII δ , as well as the specific regulation mechanisms, requires further examination.

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Disclosure of conflict of interest

None.

Address correspondence to: Jun Yang, Department of Cardiology, The First Affiliated Hospital of University of South China, 69 Chuanshuan Road, Shigu District, Hengyang 421001, Hunan Province, China. Tel: 86-734-8578767, 86-18007340658; E-mail: yangjunincn@163.com; Chun Chu, Department of Pharmacy, The Second Affiliated Hospital of University of South China, 69 Chuanshuan Road, Shigu District, Hengyang 421001, Hunan Province, China. Tel: 86-13873409200; E-mail: yjchuchun@163.com

References

- [1] Wang B, Xu M, Li W, Li X, Zheng Q and Niu X. Aerobic exercise protects against pressure overload-induced cardiac dysfunction and hypertrophy via β3-AR-nNOS-NO activation. PLoS One 2017; 12: e0179648.
- [2] Hu ST, Liu GS, Shen YF, Wang YL, Tang Y and Yang YJ. Defective Ca(2+) handling proteins regulation during heart failure. Physiol Res 2011; 60: 27-37.
- Inesi G, Prasad AM and Pilankatta R. The Ca2
 + ATPase of cardiac sarcoplasmic reticulum: physiological role and relevance to diseases. Biochem Biophys Res Commun 2008; 369: 182-187.
- [4] Ling H, Gray CB, Zambon AC, Grimm M, Gu Y, Dalton N, Purcell NH, Peterson K and Brown JH. Ca²⁺/Calmodulin-dependent protein kinase II δ mediates myocardial ischemia/reperfusion injury through nuclear factor-κB. Circ Res 2013; 112: 935-944.
- [5] Dinasarapu AR, Gupta S, Ram MM, Fahy E, Min J, Sud M, Gersten MJ, Glass CK and Subramaniam S. A combined omics study on activated macrophages--enhanced role of STATs in apoptosis, immunity and lipid metabolism. Bioinformatics 2013; 29: 2735-2743.
- [6] Xu C, Lu Y, Pan Z, Chu W, Luo X, Lin H, Xiao J, Shan H, Wang Z and Yang B. The muscle-specific microRNAs miR-1 and miR-133 produce opposing effects on apoptosis by targeting HSP60, HSP70 and caspase-9 in cardiomyocytes. J Cell Sci 2007; 120: 3045-3052.
- [7] Meng G, Zhu J, Xiao Y, Huang Z, Zhang Y, Tang X, Xie L, Chen Y, Shao Y, Ferro A, Wang R, Moore PK and Ji Y. Hydrogen sulfide donor GYY4137 protects against myocardial fibrosis. Oxid Med Cell Longev 2015; 2015: 691070.
- [8] Krenek P, Kmecova J, Kucerova D, Bajuszova Z, Musil P, Gazova A, Ochodnicky P, Klimas J and Kyselovic J. Isoproterenol-induced heart failure in the rat is associated with nitric oxide-dependent functional alterations of cardiac function. Eur J Heart Fail 2009; 11: 140-146.
- [9] Zhang F, Dang Y, Li Y, Hao Q, Li R and Qi X. Cardiac contractility modulation attenuate

myocardial fibrosis by inhibiting tgf- β 1/smad3 signaling pathway in a rabbit model of chronic heart failure. Cell Physiol Biochem 2016; 39: 294-302.

- [10] Amin P, Singh M and Singh K. β-Adrenergic receptor-stimulated cardiac myocyte apoptosis: role of β1 integrins. J Signal Transduct 2011; 2011: 179057.
- [11] Wang R. Physiological implications of hydrogen sulfide: a whiff exploration that blossomed. Physiol Rev 2012; 92: 791-896.
- [12] Mishra PK, Tyagi N, Sen U, Givvimani S and Tyagi SC. H₂S ameliorates oxidative and proteolytic stresses and protects the heart against adverse remodeling in chronic heart failure. Am J Physiol Heart Circ Physiol 2010; 298: H451-456.
- [13] Xia P, Liu Y and Cheng Z. Signaling pathways in cardiac myocyte apoptosis. Biomed Res Int 2016; 2016: 9583268.
- [14] Anderson ME, Brown JH and Bers DM. CaMKII in myocardial hypertrophy and heart failure. J Mol Cell Cardiol 2011; 51: 468-473.
- [15] Zhu W, Woo AY, Yang D, Cheng H, Crow MT and Xiao RP. Activation of CaMKIIdeltaC is a common intermediate of diverse death stimuli-induced heart muscle cell apoptosis. J Biol Chem 2007; 282: 10833-10839.
- [16] Singh MV, Swaminathan PD, Luczak ED, Kutschke W, Weiss RM and Anderson ME. MyD88 mediated inflammatory signaling leads to CaMKII oxidation, cardiac hypertrophy and death after myocardial infarction. J Mol Cell Cardiol 2012; 52: 1135-1144.
- [17] Kashiwase K, Higuchi Y, Hirotani S, Yamaguchi O, Hikoso S, Takeda T, Watanabe T, Taniike M, Nakai A, Tsujimoto I, Matsumura Y, Ueno H, Nishida K, Hori M and Otsu K. CaMKII activates ASK1 and NF-kappaB to induce cardiomyocyte hypertrophy. Biochem Biophys Res Commun 2005; 327: 136-142.

- [18] Liu X, Yao M, Li N, Wang C, Zheng Y and Cao X. CaMKII promotes TLR-triggered proinflammatory cytokine and type I interferon production by directly binding and activating TAK1 and IRF3 in macrophages. Blood 2008; 112: 4961-4970.
- [19] Hazra S, Xiong S, Wang J, Rippe RA, Krishna V, Chatterjee K and Tsukamoto H. Peroxisome proliferator-activated receptor gamma induces a phenotypic switch from activated to quiescent hepatic stellate cells. J Biol Chem 2004; 279: 11392-11401.
- [20] Aurora AB, Mahmoud AI, Luo X, Johnson BA, van Rooij E, Matsuzaki S, Humphries KM, Hill JA, Bassel-Duby R, Sadek HA and Olson EN. MicroRNA-214 protects the mouse heart from ischemic injury by controlling Ca²⁺ overload and cell death. J Clin Invest 2012; 122: 1222-1232.
- [21] Ikeda S, Kong SW, Lu J, Bisping E, Zhang H, Allen PD, Golub TR, Pieske B and Pu WT. Altered microRNA expression in human heart disease. Physiol Genomics 2007; 31: 367-373.
- [22] van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, Hill JA and Olson EN. Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. Proc Natl Acad Sci U S A 2008; 105: 13027-13032.