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Regulation of the Heme Oxygenase-1/carbon monoxide system by hydrogen sulfide in murine coxsackievirus B3-induced myocarditis

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Abstract

To explore the impact of hydrogen sulfide (H_2S) on the heme oxygenase-1 (HO-1)/carbon monoxide (CO) system in coxsackie virus B3 (CVB3)-induced myocarditis. A total of 80 Balb/c mice were divided randomly into four groups designated N, C, P and S. Group N served as the negative control while groups C, P, and S were infected with CVB3 to induce myocarditis. Group P was additionally treated with DL-propargylglycine (PAG) to inhibit the generation of H_2S while Group S was treated with NaHS, an H_2S donor. Ten days after infection, heart sections were scored for histopathology. We also measured carboxyhemoglobin (COHb) levels in the blood and HO-1 expression by immunohistochemistry. 1. Each CVB3-infected group (C, P, and S) exhibited increased pathology, COHb levels, and HO-1 expression compared to uninfected controls. 2. Regarding histopathology, the score of group P was worse, while that of group S was better, than that of group C. 3. The P group COHb level was lower than group C, while the S group COHb level was higher than group C. 4. Positive HO-1 expression was seen in group C with reduced expression in group P and increased expression in group S. 5. A positive correlation was observed between the COHb concentration and HO-1expression; alternatively, a negative correlation was found between the histopathologic scores and both the concentration of COHb and the expression level of HO-1. Modulation of H_2S can play a regulatory role in the pathogenesis of VMC by impacting the HO-1/CO pathway.

Key words: Viral myocarditis (VMC), coxsackie virus B3 (CVB3), hydrogen sulfide (H_2S), carboxyhemoglobin (COHb), heme oxygenase-1 (HO-1).

Introduction

Viral myocarditis (VMC), which consists of localized or diffused myocardial lesions caused by viral infection, is a commonly acquired cardiovascular disease in children. Most researchers believe that VMC is mainly caused by enteroviruses such as coxsackie virus B3 (CVB3), and this virus is also used in experimental animal and cell modeling of VMC. Murine models of CVB3 infection share numerous biological parameters with CVB3-induced myocardial disease in humans (1). Recent studies suggest that apart from nitric oxide (NO) and carbon monoxide (CO), hydrogen sulfide (H_2S) is another gaseous signaling molecule in cardiovascular disease that is generated endogenously and can exert extensive biological effects (2). Therefore, this study adopted a VMC animal model and used sodium hydrosulfide (NaHS) and DL-propargylglycine (PAG) in mice to modulate H_2S *in vivo* thus investigating the impact of H_2S on the HO-1/CO pathway and the effect of H_2S in the pathogenesis of VMC.

Materials and methods

Mice

A total of 80 inbred Balb/c mice (male, 4-6weeks, 19.5±1.02g) were obtained from the animal breeding facilities in ShangHai Slac Laboratory Animal Co., Ltd.. The experiments were performed under room conditions characterized by 12 complete air changes per hour, a temperature of 22 ± 2.8, relative humidity of 55% ± 10%, and a 12-hr light/dark cycle. All mice received food and water *ad libitum*. The body weights of all mice were determined daily. Non-responding mice (as determined by a lack of weight loss) were not included in the evaluation.

Virus and animal infection

The virus was propagated in HeLa cells. Stocks of this myocarditic CVB3 variant (6.3×10^8 pfu/mL) were stored at -75°C until use. Mice were randomly assigned to control (Group N; n=20) and CVB3/myocarditis groups (n=60). Control mice were sham-infected with 0.1 mL phosphate-buffered saline (PBS). The myocarditis groups were inoculated intraperitoneally (i.p.) with 0.1 mL 10-5.69TCID50/mL CVB3 on day 0 and divided randomly into three groups designated as C, P and S (n=20 for each). The groups were treated daily as follows: group C was injected i.p. with 0.1 mL PBS; group P was injected i.p. with PAG at a dose of 40 mg/kg/d; and group S was injected i.p. with NaHS at a dose of 50 μmol/kg/d. All mice were observed daily, and 10 mice in each group were randomly sacrificed at day 10 at which time we recorded body weight and heart weight for calculation of the heart weight to body weight ratio. The experimental design and approach for the CVB3/myocarditis mouse model have been previously established by others (3).

Histopathological examination

Heart tissue was fixed in 4% formalin and embedded in paraffin. For histological examinations, 4mm sections were stained with hematoxylin and eosin (H&E). At least five adjacent sections were examined microscopically for the presence of lesions. The histological evidence of myocarditis and inflammation were classified

in terms of the degree of cellular infiltration and myocardial necrosis (3). They were scored on a scale of 0 to 4 as follows: 0 = no lesions; 1 = lesions involving <25% of the myocardium; 2 = lesions involving 25% to 50% of the myocardium; 3 = lesions involving 50 to 75% of the myocardium; and 4 = lesions involving 75% to 100% of the myocardium. The scores from adjacent sections were then averaged.

Carboxyhemoglobin (COHb) detection

The level of CO was represented by the concentration of COHb. Blood specimens were collected to test for the concentration of COHb using spectrophotometry.

Immunohistochemistry

HO-1 was detected by immunohistochemistry (IHC). Briefly, myocardial tissue sections were prepared using standard protocols and incubated with normal goat serum for 20 min at room temperature. Samples were then incubated with anti-HO-1,4 antibody at a 1:400 dilution overnight at 4°C. After washing, biotinylated goat anti-rabbit IgG was added for 20 min at 37°C and washed once again. Finally, samples were incubated with SAB Complex and DAB and counterstained with hematoxylin. ImagePro Plus 6.0 software was used to determine the average mean optical density (MOD) from 5 randomly selected myocardial images from each sample.

Statistical analysis

Statistical anlysis was performed using Prism software (GraphPad; San Diego, CA). A one-way ANOVA, including a Tukey post-test, was used to compare multiple groups. P values are included in individual figure legends. Linear regression analysis was performed where appropriate using both Microsoft Excel and Prism.

Results

CVB3-induced changes in body weight

Mice infected with CVB3 exhibited symptoms that included an affected habitus with restricted mobility, closed eyes, a distinct loss of weight, and even death. Mice in the uninfected control group N exhibited an increase in body weight, and none were found deceased. Ten days after virus infection, all infected mice exhibited a reduction of weight, but there were no differences of the heart weights in the evaluated mice in any group (data not shown). We also calculated the heart weight to body weight ratio to measure the extent of myocardial edama and reflect the degree of myocardial inflammation. For all CVB3-infected groups (C, P, and S) the heart weight to body weight ratio was significantly higher than the control group N (Fig. 1). However, we found that the ratio in group P was significantly higher than group C while the ratio in group S was significantly lower than group C.

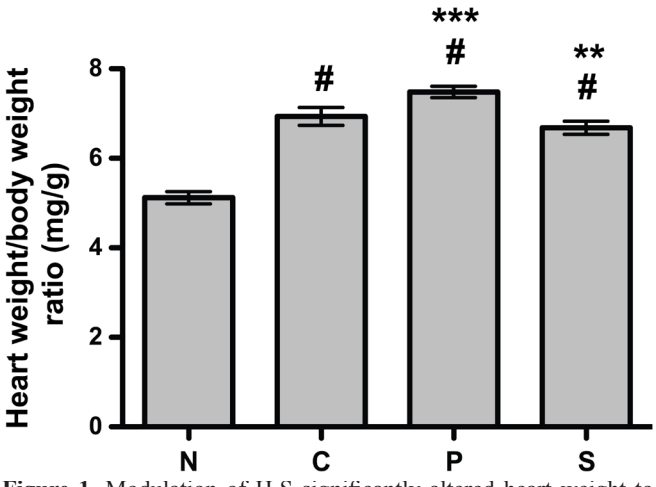

Figure 1. Modulation of H_2S significantly altered heart weight to body weight ratios. Graphical representation of heart weight to body weight ratios (mg/g) in all groups. # indicates $P < 0.001$ in comparison with group N. ** indicates $P < 0.01$ and *** indicates $P < 0.001$ in comparison with group C.

Effects of H_2S on histopathological changes characteristic of myocardial disease

Sections of heart tissue from control mice showed normal architecture of heart tissue and normal histopathology (group N; Fig. 2A). In the CVB3-infected group, myocardial inflammatory cell infiltration along with myocyte destruction was observed (group C; Fig. 2B). Severe injury to the myocardium including cellular infiltration, the disappearance of nuclear and cellular outlines, and necrotic areas characterized by calcification particles was observed in mice treated with PAG (group P; Fig 2C). However, the severity of cellular infiltration was significantly reduced in mice treated with NaHS (group S; Fig 2D). Heart sections were evaluated to determine histopathological scores using the Rezkalla measure. We found that mice treated with PAG had significantly more myocardial damage (as indicated by the histopathological scores) than the control CVB3-infected group C (Fig. 3). Alternatively, there was significantly less injury when infecetd mice were treated with NaHS. Thus, our data suggests that the level of H_2S can have a significant impact on the degree of myocardial damage in VMC; inhibiting H_2S increased damage while promoting H_2S reduced myocardial destruction.

Effects of H_2S on blood COHb levels

To determine the changes in carbon monoxide concentrations that occured following CVB3-induced myo-

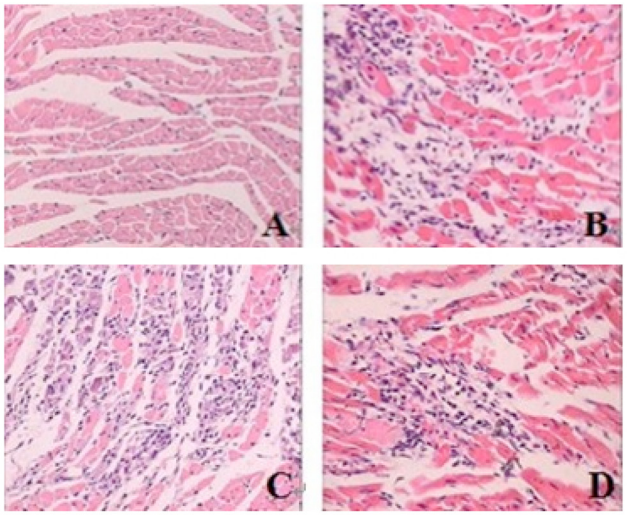

Figure 2. CVB3 induced myocardial damage. Heart sections were prepared as described in the Materials and Methods and stained with H&E. Representative images (x200) are shown from group N (A), group C (B), group P (C), and group S (D).

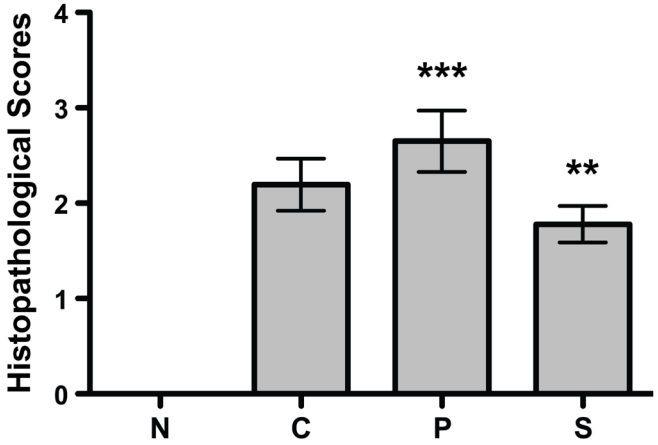

Figure 3. Modulation of H_2S significanlty altered myocardial damage. Graphical representation of the histopathological scores in all groups. ** indicates $P < 0.01$ and *** indicates $P < 0.001$ in comparison with group C.

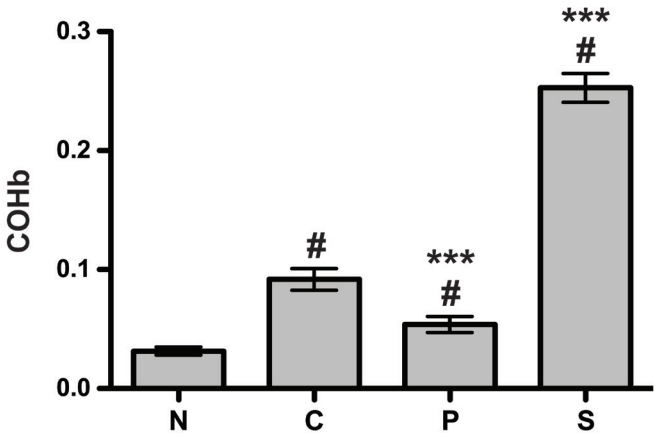

Figure 4. Modulation of H_2S significantly altered COHb concentrations. Graphical representation of COHb concentrations in all groups. # indicates $P < 0.001$ in comparison with group N, and *** indicates $P < 0.001$ in comparison with group C.

carditis, we monitored the levels of COHb in all groups of mice. Mice in all CVB3-infected groups (C, P and S) were significantly higher for COHb than those in the uninfected group N on day 10 after CVB3 inoculation. However, when compared with group C, the COHb levels were significantly lower in group P and higher in group S (Fig. 4). Thus, the reduction in H_2S following PAG treatment allowed for an increase in CO, but promoting H_2S reduced the presence of CO.

Effects of H_2S on HO-1 expression by immunohistochemistry

The expression of HO-1 protein can also be used as an indicator of H_2S activity during myocardial disease. Therefore we detected HO-1 by IHC using mean optical density (MOD). In the myocarditis groups (C, P, and S), myocardial cells were positive for HO-1 expression, but this was not seen in the control uninfected group N (Fig. 5A). In group C, positive HO-1 expression was detected in myocardial cells, myocardial interstitial cells and infiltrating inflammatory cells (Fig. 5B). However, HO-1 was only weakly expressed in group P (Fig. 5C). Finally, in group S, HO-1 expression was similar to that of group C, and strong positive staining granules are observed in areas of localized inflammation (Fig. 5D). The MOD of HO-1 IHC in groups C, P and S was significantly higher than those in group N after CVB3 inoculation (Fig. 6). Compared with group C, the MOD of HO-1 was significantly lower in group P and was significantly higher in group S. Thus, we found that the expression of HO-1 was significantly increased after virus inoculation. However, PAG treatment inhibited the activity of cystathionine-γ-lyase (CSE) which decreased the overall levels of HO-1, whereas exogenous H_2S increased the expression of HO-1 (Fig. 6).

Correlation analysis between myocardial disease and H_2S

To further correlate how the modulatation of H_2S activity could impact myocardial damage, we performed linear regression analysis using the 3 parameteres measured within: histopathogical scores, the concentration of COHb, and the level of HO-1 expression. Our data indicated a positive linear correlation between the concentration of COHb and the expression of HO-1 ($y=103.55x+9.2809$, $r=0.6561$, $p < 0.0001$, $n=39$) (Fig. 7A). Alternatively, the histopathological scores were negatively correlated with both the concentration of COHb ($y=-0.1514x+0.4675$, $r=-0.5672$, $p < 0.0001$, $n=29$) (Fig. 7B) and the expression of HO-1 ($y=-10.946x+50.198$, $r=-0.5005$, $p < 0.0001$, $n=29$) (Fig. 7C). Thus, in conclusion, our results suggest that using NaHS to increase H_2S *in vivo* subsequently increased CO and HO-1 which have a protective effect on the ex-

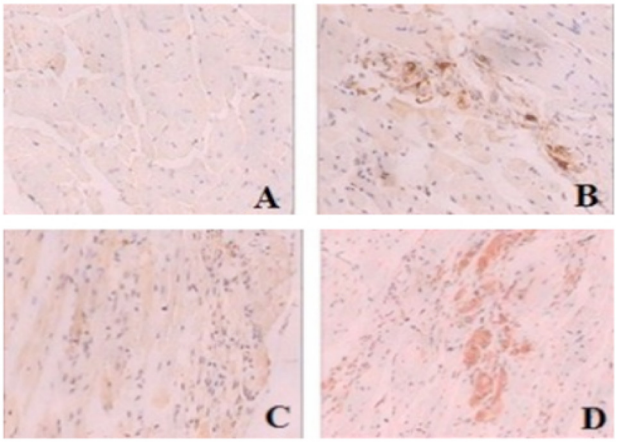

Figure 5. Upregulation of HO-1 in the heart following CVB3 infection. Representative images (x200) are shown from group N (A), group C (B), group P (C), and group S (D) following detection of HO-1 by IHC.

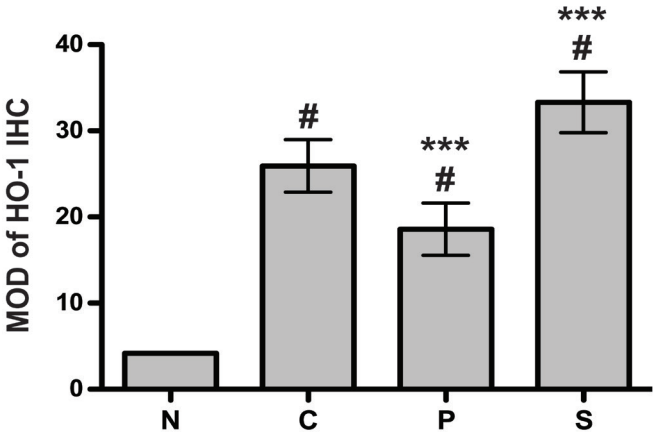

Figure 6. Modulation of H2S significantly altered HO-1 expression. Graphical representation of the MOD of HO-1 espression in all groups as determined by IHC. # indicates $P < 0.001$ in comparison with group N, and *** indicates $P < 0.001$ in comparison with group C.

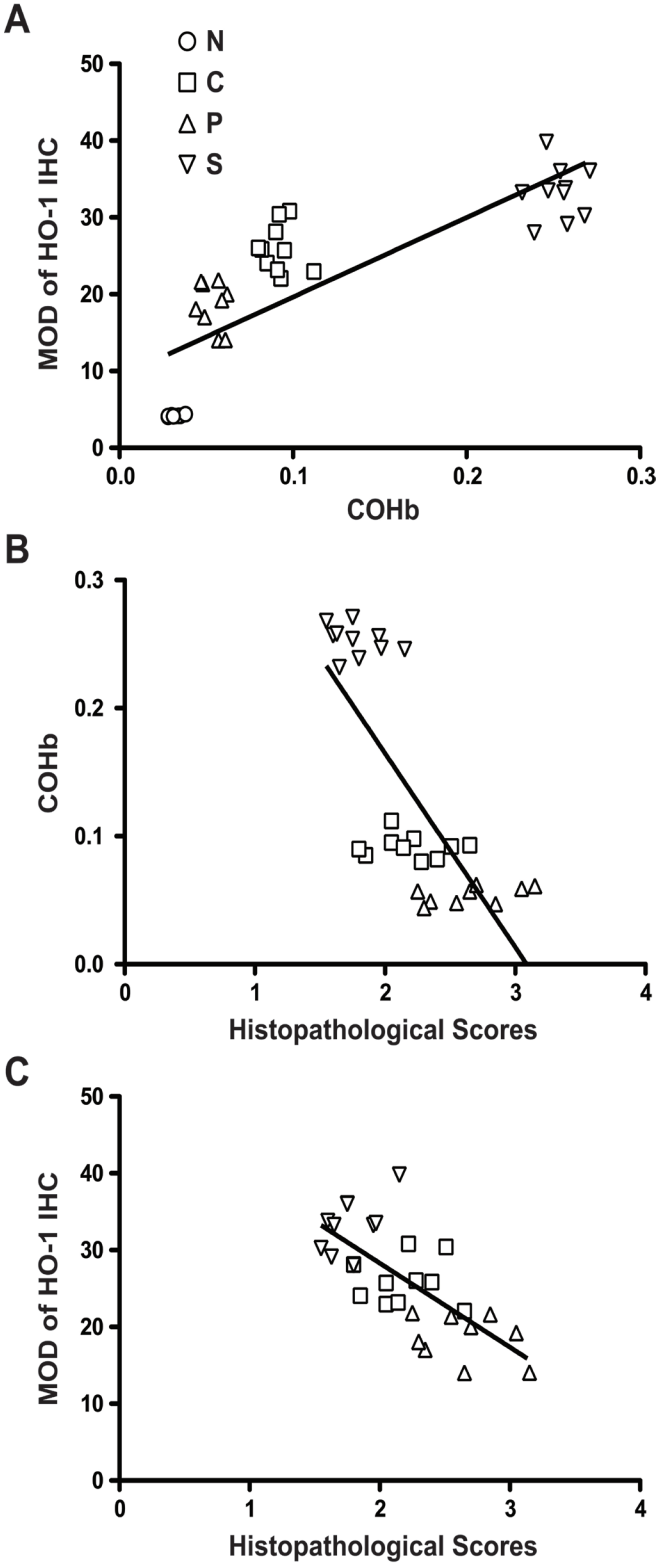

Figure 7. Myocardial disease is correlated with the modulation of the H_2S pathway. (A) Correlation analysis between the MOD of HO-1 by IHC and the COHb concentration. (B) Correlation analysis between histopathological scores and the COHb concentration. (C) Correlation analysis between histopathological scores and the MOD of HO-1 by IHC. Linear regression analysis can be found in the corresponding text.

tent of myocardial damage after CVB3 infection.

Discussion

VMC is one of the common cardiovascular diseases in children. Viral infections, particularly those caused by enteroviruses such as CVB3, can lead to heart tissue damage, infiltration of inflammatory cells, and foci of necrotic myocytes. Those histopathological changes in heart tissue are attributed to the virus itself and also to the immune response directed against CVB3 (4). Cystathionine-β-synthase (CBS) and CSE are two key enzymes involved in the synthesis of H_2S, both using pyridoxal 5'-phosphate as a cofactor (5-7). CBS is the main H_2S-producing enzyme in the brain, and CSE is involved in H_2S formation in the cardiovascular system (8, 9). The importance of CSE is recently demonstrated in a mouse lacking CSE, which showed the activity of H_2S was obviously decreased (10). As a new gaseous messenger molecule, H_2S forms specific pathways with its corresponding enzymes and plays important roles in physiological and pathophysiological conditions. H_2S, NO and CO may constitute a regulatory network in the vascular system (11). The interrelation and interaction of these gaseous transmitters in the pathogenesis of cardiovascular diseases has thus been acknowledged.

In this study, a VMC model was established by infection with CVB3 in mice. The results indicated that there was trace HO-1 expression in the normal control group, and HO-1 expression was significantly increased after CVB3 infection. The CSE inhibitor, PAG not only could suppress endogenous H_2S production but also could reduce expression of HO-1 and the concentrations of COHb. The severity of cellular infiltration was significantly increased in the PAG-treated group. Severe myocyte necrosis and the disappearance of nuclear and cellular outline were observed. On the contrary, an exogenous H_2S donor, NaHS, not only induced endogenous H_2S production but also increased the expression of HO-1 and the concentration of COHb. The myocardial inflammatory cell infiltration in the NaHS-treated group was significantly less than those in the infected, but untreated group. Thus, correlation analysis indicated the positive linear correlation between the concentrations of COHb and the immunohistochemical expression of HO-1; The histopathological scores presented negative linear correlation with both of the concentrations of COHb and the immunohistochemical expression of HO-1. This study revealed that H_2S could induce HO-1 expression, play a role in the upregulation of the HO-1/CO pathway, and contribute to cardioprotection in a mouse model of VMC.

There is reasonable speculation about the protection mechanism of H_2S: 1. H_2S relaxes vascular smooth muscles by the activation of K_{ATP} channels and the hyperpolarization of cell membranes (8). As a regulator of physiological cardiac function, H_2S could induce vasorelaxation, reduce central venous pressure, and reduce the load on the heart. 2. H_2S inhibits smooth muscle cell proliferation through the mitogen-activated protein kinase (MAPK) pathway (12); In addition, H_2S may enhance apoptosis via ERK-mediated pathways (13). These effects are important for maintaining the normal function of blood vessels and attenuating structural remodeling (14). 3. H_2S shows antiinflammatory effects by blocking the activation of NF-κB and inhibiting the expression of intercellular adhesion molecule-1 (ICAM-1) (15, 16). 4. H_2S effectively protects myocytes and contractile activity partly because of scavenging oxygen-free radicals and reducing the accumulation of lipid peroxidations (17). 5. It has been proven that the

endogenous CSE-H_2S pathway is regulated during the pathogenesis of myocardial ischemic injury (17).

Present reports show that H_2S, CO and NO may interact with each other and therefore play an important regulatory role in cardiovascular disease (11, 18). There is a negative feed back effect between the H_2S/CSE system and NO/NO synthase (NOS) system. Similarly, interaction between the NO/NOS system and HO-1/CO system may also involve negative feedback. H_2S may reduce the expression level of NOS and decrease the sensitivity of K_{Ca} channels to NO; however, NO may increase the expression of CSE and the cellular uptake of cystine (19). NO may also increase the transcriptional level of HO-1 mRNA and the expression level of CO, whereas CO may inhibit the transcriptional level of NOS mRNA and the expression level of NO (20).

H_2S induced the expression of HO-1 in myocardial tissue as well as promoted the formation of endogenous CO. H_2S also significantly decreased myocardial inflammation and prevented myocardial damage. Thus, our findings suggested that H_2S is a regulator of cardiovascular function that plays an important role in cardioprotective effects. Further studies modulating the H_2S pathway have the potential to remarkably promote the treatment of VMC.

Acknowledgements

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