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The dual role of the cystathionine γ-lyase/hydrogen sulfide pathway in CVB3-induced myocarditis in mice

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ABSTRACT

The present study found that serum H_2S level, H_2S production rate, *CSE* mRNA and CSE protein levels were increased in CVB3-induced myocarditis. DL-proparglygylcine (PAG), an irreversible CSE inhibitor, decreased the infected myocardium titers on postinfection day 4, while NaHS, a H_2S donor, alleviated myocardial injury and necrosis, inflammatory cell infiltration and interstitial edema on postinfection day 10. These data reveal that the CSE/H_2S pathway is upregulated in the heart in a murine model of CVB3-induced myocarditis and that inhibition of endogenous H_2S is beneficial to treatment early in the disease while administration of exogenous H_2S is protective to infected myocardium during the later stage.

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Myocarditis is a leading cause of morbidity and mortality, especially in children. One of the most common pathogens resulting in viral myocarditis is coxsakievirus B3 (CVB3). To date, the therapeutic strategy for this infection is primarily supportive, and practical active therapy is still unavailable.

Meanwhile, hydrogen sulfide (H_2S) has recently become the third member of the gasotransmitter family, along with nitric oxide (NO) and carbon monoxide (CO) [1]. The mechanism of action for H_2S involves the reaction with the disulfide groups or metal ions in functional proteins [2] and the interaction with NO to form nitrosothiol coupled with no cGMP accumulation, which subsequently inhibits NO activity [3]. Previous studies have shown that H_2S plays key roles in a number of biological processes, including vasorelaxation, inflammation, apoptosis, ischemia/reperfusion and oxidative stress. For example, H_2S was demonstrated to be a vasodilator by opening K_{ATP} channels and hyperpolarizing membrane potential of vascular SMCs [4]. Cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS) are the two major H_2S-forming enzymes [1]. The CSE/H_2S pathway was reported to be predominant in myocardial tissue [5], which was further demonstrated using a CSE gene knock-out approach [6]. Interactions between gasotransmitters have been discussed extensively, including the H_2S downregulation of the nitric oxide synthase (NOS)/NO pathway by inducing the expression of heme oxygenase-1(HO-1)/CO [7]. Interestingly, the NOS/NO pathway has been shown to exert a direct antiviral effect, in part, by damaging viral RNA in CVB3-mediated myocarditis [8]. We have also previously reported that the HO-1/CO pathway was upregulated in CVB3-infected mice and that ZnPPIX, a HO-1 inhibitor, not only inhibited HO-1 overexpression, but also temporarily induced HO-1 expression, which protected the mice against myocardial injury during the early stage of the disease [9]. Given these findings, we then wanted to determine whether the CSE/H_2S pathway was involved in the pathogenesis of CVB3-related myocarditis.

Materials and methods

Materials. Experiments were carried out in a biosafety level 2 laboratory. All animal protocols were approved by the Animal Care and Use Committee of Wenzhou Medical College. Animals were purchased from the Shanghai Laboratory Animal Center (Shanghai, China) and kept under pathogen-free conditions at the animal center of Wenzhou Medical College. CVB3 Nancy strains were purchased from the Shandong Academy of Medical Sciences (Shandong, China). Hela cells were obtained from the Zhejiang Provincial Key Laboratory of Medical Genetics (Zhejiang, China). RPMI1640 and fetal bovine serum (FBS) were purchased from GIBCO®. DL-proparglygylcine (PAG), NaHS and *N*,*N*-dimethyl-*p*-phenylenediamine sulfate were purchased from Sigma (St. Louis, MO, USA). L-cysteine and pyridoxal 5′-phosphate were purchased from Solarbio (Beijing, China). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). The RevertAid™ First Strand DNA Synthesis Kit and PCR Master Mix were purchased from Fermentas (Vilnius, Lithuania). The primers for CSE (S-5′-CTGCCACCATTACG ATTACCC-3′; A-5′-TATCAGCACCCAGAGCCAAAG-3′) and β-actin (S-5′-CCCATCTACGAGGGCTAT-3′; A-5′-TGTCACGCACGATTTCC-3′) were synthesized by Genecore (Shanghai, China). The polyclonal

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anti-CSE antibody was purchased from PTGLAB (Chicago, IL, USA). The anti-tubulin antibody, HRP-labeled secondary antibody, SDS Lysis Buffer, phenylmethanesulfonyl fluoride (PMSF), BCA protein assay kit, PVDF membranes and BeyoECL Plus were all purchased from Beyotime (Jiangshu, China).

Cell culture, virus propagation. Hela cells were grown in RPMI1640 supplemented with 10% FBS. After reaching at least 90% confluence, CVB3 was added for 1 h, and the virus was then released from these cells by three freeze–thaw cycles. Samples were centrifuged at 7500 rpm at 4 °C for 10 min, and the supernatant was collected for further study.

Animal procedures. We randomly divided 115 six-week-old inbred male Balb/C mice into four groups, which were designated as N, C, P and S ($n = 25$ for N; $n = 30$ for C, P and S). Group N is non-infected mice, group C is infected control mice, and group P or S is infected mice treatment with PAG or NaHS. The mice in group N were inoculated intraperitoneally with phosphate-buffered saline (PBS) daily. The other three groups served as the myocarditis models and received $10^{-5.69}$$TCID_{50}$/ml of CVB3. From day 0, mice in group P or S received daily intraperitoneal injections of PAG (40 mg/kg/d) or NaHS (50 μmol/kg/d), respectively [10,11]. The mice in group C received daily injections of PBS. These mice were observed daily, and ten of the mice were randomly sacrificed on days 4 and 10 by bleeding from retroorbital plexus under intraperitoneal administration of Chloral hydrate. Blood specimens and hearts from the sacrificed mice were harvested for the following assays.

Measurement of serum H_2S. The basis and method of this assay are published elsewhere [12]. Briefly, H_2S interacts with zinc acetate to form zinc sulfide, which dissolves in a hydrochloride acid solution of *N*,*N*-dimethyl-*p*-phenylenediamine sulfate to yield, in the presence of ferric chloride, methylene blue. This solution can then be quantified by OD at a wavelength of 670 nm. In a 5 ml reaction system, 0.1 ml of serum was mixed with 2.5 ml distilled water, 0.5 ml zinc sulfide (1% w/v), 0.5 ml *N*,*N*-dimethyl-*p*-phenylenediamine sulfate (20 mmol/l) in 7.2 M HCl, 0.4 ml ferric chloride (30 mmol/l) in 1.2 M HCl and 1 ml trichloroacetic acid (10% w/v). This mixture was centrifuged for 15 min, and the optical density of the supernatant was determined. All samples were assayed twice, and a standard curve was made with the NaHS solution in order to calculate the H_2S concentration. The results are listed as μmol/l.

Assay of endogenous H_2S production in cardiac tissue. This assay was performed as previously described [5]. In brief, Heart (about 20 mg) was removed, weighted and homogenized in 50 mmol/l ice-cold potassium phosphate buffer (pH 6.8), and homogenates were regulated to a concentration of 10% (w/v). The protein concentration of homogenates was determined by BCA protein assay kit. All incubations were performed in 25 ml Erlenmeyer flasks with center wells made of cryovial test tubes (2 ml). Tissue homogenates were added to a reaction mixture containing 100 mmol/l potassium phosphate buffer (pH 7.4) and 10 mmol/l L-cysteine, 2 mmol/l pyridoxal 5′-phosphate. The center wells were filled with 1% zinc acetate and a 2.0×2.5 cm^2 piece filter paper to trap H_2S. Each flask was flushed with N_2 for 20 s to exclude O_2 and then sealed. After incubation for 90 min in a 37 °C shaking water bath, 0.5 ml of 50% trichloroacetic acid was added to the flasks to denature the protein. The flasks were incubated again for another 60 min to ensure a full trap. The contents of the center wells were then transferred to test tubes to detect H_2S concentration, which was calculated by the method mentioned above. The results are displayed as nmol/min/g protein.

RT-PCR analysis. RNA was extracted from the tissues using TRIzol reagent. After denaturing at 94 °C for 3 min, PCR was run 30 cycles (CSE) or 20 cycles (β-actin) of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 60 s, followed by one cycle of final extension at 72 °C for 5 min. A negative control without template cDNA was always included. The expected sizes of the PCR products were 339 bp for *CSE* or 145 bp for *β-actin*, and all PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining. The optical density of the expected bands was measured, and the OD ratio of *CSE* mRNA to *β-actin* mRNA was used to determine the relative amount of *CSE* mRNA.

Western blot analysis. The hearts were homogenized on ice using SDS Lysis Buffer with 1 mmol/l PMSF. A total of 50 μg of the protein lysates were separated on SDS–PAGE at 120 V for 90 min and then transferred onto PVDF membranes for 1 h at 200 mA. The membranes were incubated overnight with the primary antibody (rabbit polyclonal anti-CSE antibody at a 1:100 dilution, mouse monoclonal anti-tubulin antibody at a 1:1000 dilution), followed by incubation with the secondary antibody (goat anti-rabbit/mouse IgG antibody at a 1:1000 dilution) at room temperature for 1 h. Finally, the bands were visualized by BeyoECL Plus.

Assay of viral titer and myocardial virus concentration. Viral quantity was determined by 50% tissue culture infection dose (TCID50), as published elsewhere [8]. The supernatants of heart homogenates were serially diluted in PBS and planted onto Hela cell monolayers in 96-well flat-bottomed microtiter plates in the presence of RPMI1640 with 4% FBS and then incubated. The microtiter plates were examined daily for 5 days for the appearance of any cytopathic effect under an inverted microscope. The results were expressed as $\log_{10}$ TCID50/mg tissue. For the detection of virus titer, the assay was performed as described above, using virus dilutions instead of the tissue homogenate supernatants.

Histopathological study. Sections of the hearts were stained with hematoxylin and eosin. The percent area of cellular infiltration and myocardial necrosis was graded in a blinded manner and scored as follows: 0, no lesion; 1+, lesions involving <25%; 2+, lesions involving 25% to 50%; 3+, lesions involving 50% to 75%; 4+, lesions involving >75%.

Statistical analysis. Data were expressed as mean ± SEM, and statistical analysis was performed with a one-way ANOVA followed by a *post hoc* multiple comparisons test (*LSD*), when required. The Kaplan–Meier method and log-rank test were used to analyze survival. $P < 0.05$ was considered to be statistically significant.

Results and discussion

H_2S, which was formerly considered to be a colorless toxic gas with a rotten-egg odor, was recently reported to be a new gasotransmitter with multiple biological functions, including the regulation of cardiovascular homeostasis. Protective roles against atherosclerosis and ischemic-reperfusion injury have been shown for H_2S [13,14]. To date, however, the role of the CSE/H_2S pathway in viral myocarditis has remained unclear.

Our data suggested that CVB3-mediated myocarditis resulted in at least a twofold increase in serum H_2S and the production rate of H_2S in the myocardium. Intraperitoneal administration of PAG, which inhibits CSE activity by blocking substrates (such as cysteine) from binding the active site [15], reduced this effect (Fig. 1A and B). Incubating the myocardium homogenates of infected control with PAG (10 mmol/l [16]) showed an even more dramatic reduction of the H_2S production rate (Fig. 1C). The residual ability to produce small amounts of H_2S in the heart might result from CBS, and the discrepancy of the two inhibitory methods could be due to the metabolism of PAG *in vivo*. Furthermore, *CSE* mRNA and CSE protein expression were also enhanced in CVB3-infected mice compared to noninfected mice at both time points (Fig. 2A and B). Therefore, it is reasonable to extrapolate that CVB3 upregulates the CSE/H_2S pathway in cardiac tissues, which

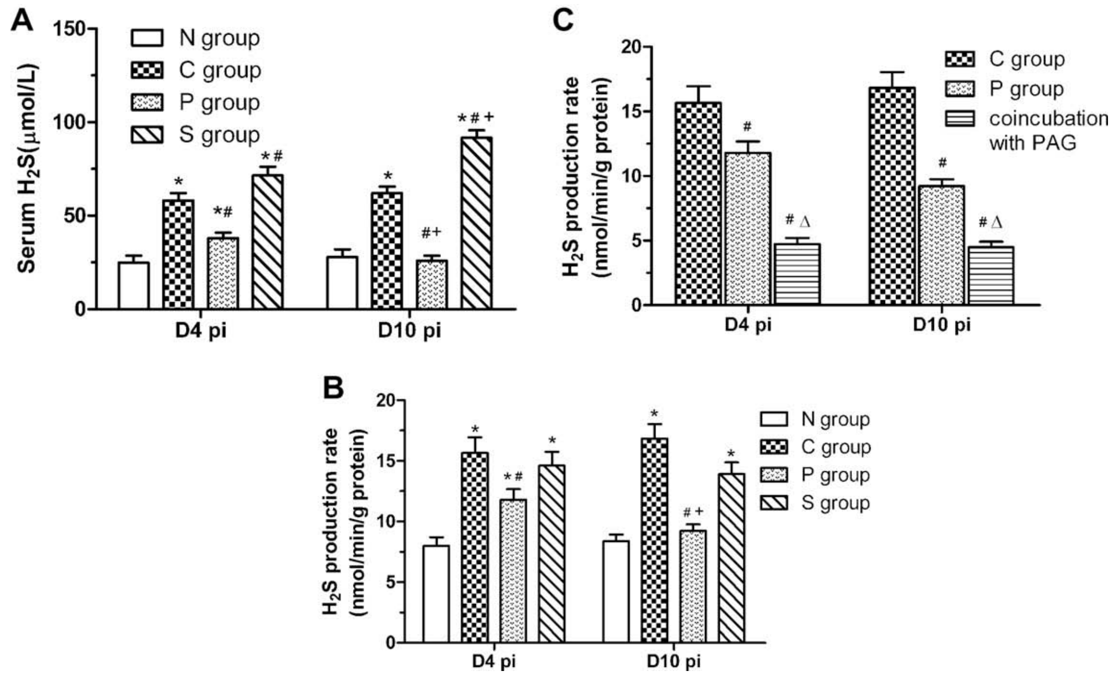

Fig. 1. (A) CVB3-induced more than a twofold increase in serum H_2S compared to basal levels. PAG or NaHS showed a significant decrease or increase compared to group C on days 4 and 10. (B) CVB3-induced a similar increase in the production rate of H_2S. Treatment with PAG for 4 and 10 days could reverse this effect, while treatment with NaHS had no impact on the rate. (C) The production rate of H_2S in infected myocardium by coincubation with PAG was more dramatic than the administration of PAG at two time points. $^{*}P < 0.05$ vs. group N, $^{\#}P < 0.05$ vs. group C, $^{+}P < 0.05$ vs. the same treatment group on day 4, $\Delta P < 0.05$ vs. group P.

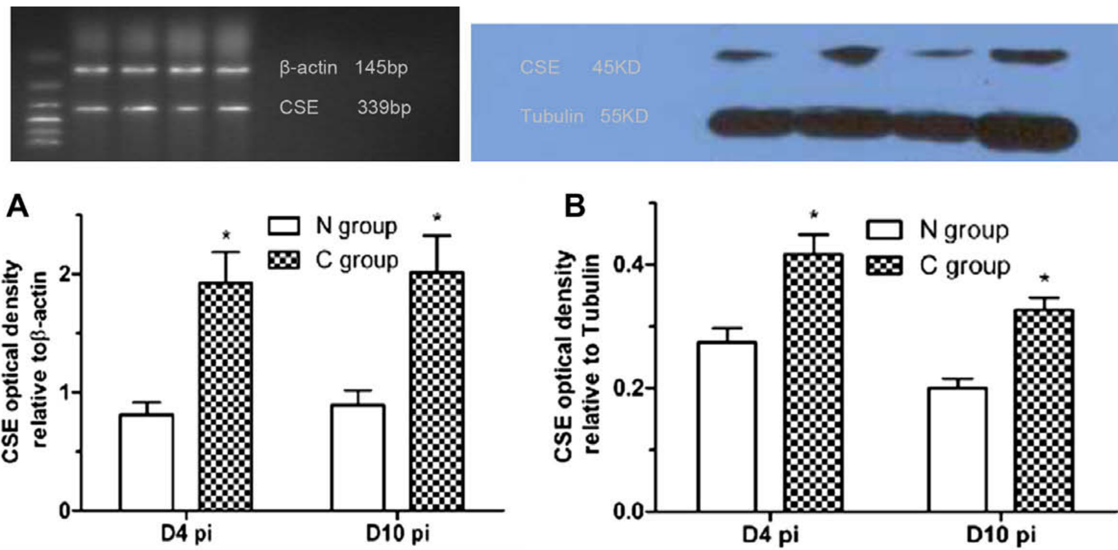

Fig. 2. (A) CVB3 increased CSE mRNA expression in group C compared to group N. (B) CVB3 increased CSE protein expression in group C compared to group N. $^{*}P < 0.05$ vs. group N.

causes the increased CSE expression and H_2S production that is observed in CVB3-induced myocarditis. This result raised questions about the exact role, if any, that the upregulated pathway played in the pathogenesis of viral myocarditis. One potential answer involved the exogenous administration of H_2S or the inhibition of endogenous H_2S.

Our data suggested that the myocardial viral titers of infected mice were higher on postinfection day 4 than on postinfection day 10 and that H_2S influenced viral load in the early stage (Fig. 3A). Using an endpoint titration assay, the administration of NaHS resulted in enhanced myocardial viral load, while the down-regulation of endogenous H_2S inhibited viral replication (Fig. 3A). On one hand, both two peaks of ERK1/2 activity and the activation of the PI3K/Akt pathway increased viral progeny release and viral protein expression *in vitro* [17,18]. On the other hand, H_2S also stimulated ERK1/2 and p38MAPK phosphorylation in RAW264.7 cells [7] and contributed to angiogenesis in the study of RF/6A endothelial cells by facilitating PI3K/Akt activation [11]. H_2S induction of the ERK1/2 and PI3K/Akt pathways is also related to its cardioprotective role against ischemia–reperfusion injury [14]. Whether H_2S promotes CVB3 dissemination via ERK1/2 or PI3K/AKt pathway merits further study. H_2S facilitates NK cell death

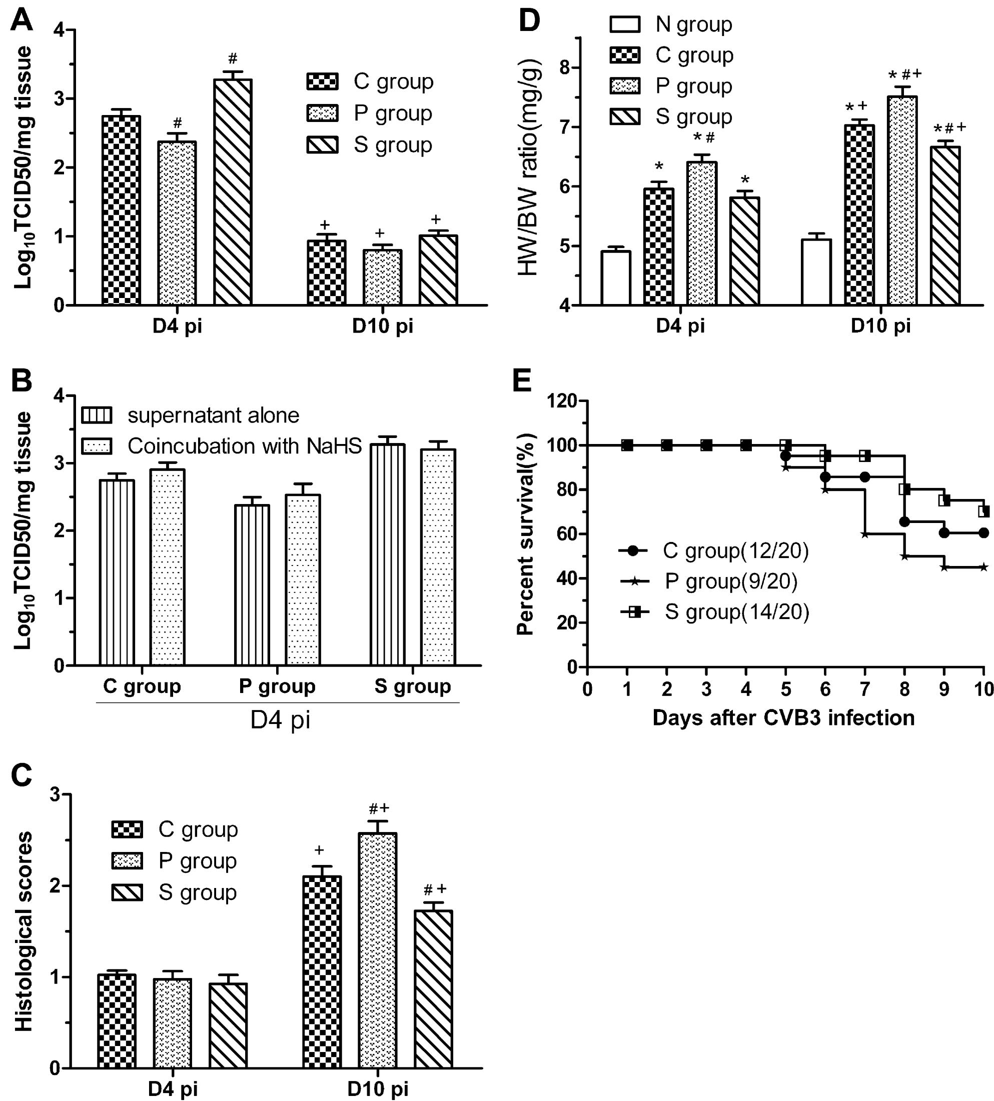

Fig. 3. (A) Endpoint titration was used to evaluate myocardial viral loads. The titers were higher in group S and lower in group P compared to the infected control group on day 4. No significant difference was found between the groups on day 10. (B) No significant difference was observed in the infected groups that were coincubated with NaHS compared to groups with no exposure to NaHS. (C) Scores were used to show the extent of myocardial infiltration and necrosis. No significant difference was observed between the groups on day 4. On day 10, however, the scores in group P or S were higher and lower, respectively, compared to those of group C. (D) The heart-weight to body-weight ratio was used to indicate myocardial edema. Treatment with PAG exacerbated myocardial edema on days 4 and 10, whereas treatment with NaHS attenuated myocardial edema on day 10. (E) The survival curve after CVB3 infection. The mice in groups C and P began to die on day 5 and on day 6 for those in group S. Using the log-rank test, no significant difference was shown in the survival rate of group P or S compared to group C. $^{*}P < 0.05$ vs. group N, $^{\#}P < 0.05$ vs. group C, $^{+}P < 0.05$ vs. the same treatment group on day 4.

[19], which may also be related to its early pro-viral effects. Further investigation into the exposure of supernatants of infected myocardium homogenates to NaHS (50 μmol/l [11]), however, failed to show a positive effect of H_2S on viral propagation (Fig. 3B), which suggested that H_2S is a synergistic effector of viral dissemination and that downregulation of H_2S alone is insufficient to prevent the spread of the CVB3 virus.

Our data also suggested that, on postinfection day 10, H_2S alleviated, while PAG exacerbated, CVB3-induced myocardial injury and necrosis, the infiltration of inflammatory cells and interstitial edema (Figs. 3C, D and 4). We speculated that H_2S possessed anti-inflammatory properties, which is consistent with the results of recent studies indicating that H_2S blocked NF-κB activation and led to a reduction of ICAM-1, which is an important inflammatory mediator [13,20], and that downregulation of the CSE/H_2S pathway facilitated leukocyte adherence and tissue swelling [21]. In contrast, a series of studies reported that H_2S had a pro-inflammatory role, including in a model of cecal ligation and puncture-induced sepsis [12,22,23], in a model of lipopolysaccharide (LPS)-induced endotoxemia [24] and in a model of caerulein-induced pancreatitis and associated lung injury [25]. The different results may be due to the animal model we studied was not physically and chemically but biologically caused inflammation. Another possible explanation of this discrepancy is that H_2S promotes lymphocyte death [19], which could contribute to its anti-inflammatory role in CVB3-induced myocarditis, while also preventing polymorphonuclear leukocyte apoptosis [26], which could lead to a pro-inflammatory function in neutrophil-mediated models. Overall, H_2S seems to have a bidirectional role in regulating inflammation.

Another important mechanism of H_2S cardioprotection may be its regulation of oxidative stress. Our colleagues have previously demonstrated that carvedilol protects against CVB3-induced myocarditis by decreasing malondialdehyde and increasing superoxide

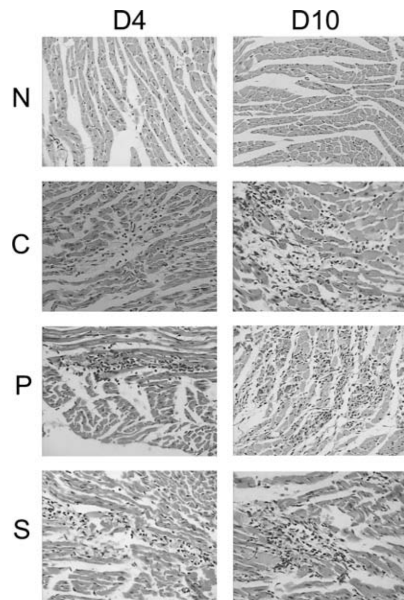

Fig. 4. Sections of infected hearts were stained with hematoxylin and eosin and observed under 200× magnification. No myocardial injury was found in group N. A mild accumulation of cellular infiltration in perivascular and interstitial regions was present in infected heart on day 4. A notable cellular infiltration and necrosis was seen in group C, which was more and less severe than group P or S, respectively, on day 10.

dismutase, thereby reducing oxidative stress injury [27]. In fact, considerable evidence suggests that H_2S is a potent antioxidant. Oxidative stress was attenuated by H_2S in high glucose-induced pancreatic beta-cells [28] and in methionine-induced brain endothelial cells [29]. Within these cells, H_2S acted as a scavenger for oxygen-free radicals, such as hydrogen peroxide, superoxide anions and peroxynitrite. In addition, H_2S facilitated γ-GCS/γ-glutamylcysteine (γ-GC) expression and increased the levels of glutathione, which is a major endogenous antioxidant [30]. Based upon our data, the 10-day inhibition of CSE activity with PAG, which leads to an underproduction of H_2S, significantly exacerbates the myocardial damage caused by CVB3 infection. This further supports the cardioprotective role of H_2S. H_2S can logically be extrapolated to play an antioxidant role in counteracting CVB3-induced myocarditis.

Both PAG and NaHS failed to improve the survival of the CVB3-infected mice (Fig. 3E), which suggested that H_2S exerted a pleiotropic effect and that a one-directional change of the pathway was unable to completely ameliorate viral myocarditis. Given the features of H_2S, one promising therapeutic strategy could involve the downregulation of the CSE/H_2S pathway during the early stages of CVB3 myocarditis and the upregulation of this pathway during the late stage of the disease.

In conclusion, the present data reveal that the CSE/H_2S pathway is upregulated in the cardiac tissue of a CVB3 myocarditis murine model. Inhibition of endogenous H_2S is beneficial during the early stage of this disease, while the administration of exogenous H_2S protects the infected myocardium during the later stage.

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