Pediatric RESEARCH

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BASIC SCIENCE ARTICLE

Inhibition of endogenous hydrogen sulfide production improves viral elimination in CVB3-infected myocardium in mice

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BACKGROUND: To find whether administration of hydrogen sulfide has interaction with coxsackie virus B3 (CVB3) replication and spread.

METHODS: Six-week-old inbred male Balb/c mice were injected intraperitoneally with CVB3. Mice were randomized to four groups ($n = 10$ for each group): group N (sham infection + vehicle), group C (virus + vehicle), group P (virus + DL-proparglygylcine (PAG)), and group S (virus + sodium hydrogen sulfide (NaHS)). PAG and NaHS were administered intraperitoneally daily and mice were killed on day 4 after viral inoculation. Serum specimens were obtained to assay tumor necrosis factor-α (TNFα) level, and heart specimens were harvested for histological examination, 50% tissue culture infection dose (TCID50) assay, reverse transcription-polymerase chain reaction and Western blot analysis.

RESULTS: The ratio of heart-weight to body-weight and inflammatory scores showed no significant difference between infected groups. The circulatory and local concentrations of TNFα, nitric oxide synthase 2 messenger RNA, and protein were higher in group P, and were lower in group S compared to those in group C. Mice treated with PAG and NaHS had significantly lower and higher viral stocks than those inoculated with CVB3 only, respectively.

CONCLUSION: Inhibition of endogenous hydrogen sulfide production contributed to viral clearance in acute viremia of CVB3-induced myocarditis.

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INTRODUCTION

Previously, we have reported that cystathionine γ lyase/hydrogen sulfide (CSE/H_2S) pathway was upregulated in in vivo mouse model of viral myocarditis, and H_2S could alleviate the severity of myocardial injury and exert anti-inflammatory and cardioprotective effect.[1,2] Viral myocardits has two basic pathological processes, including viral attack in the early viremia and the following tissue inflammation caused by overproduction of proinflammatory cytokines. It is well known that both virus-induced direct injury and inflammation-mediated myocardial injury play key roles in the pathogenesis of viral myocarditis. However, to date, there was no effective strategy to eliminate coxsackie virus B3 (CVB3) in the early stage of myocarditis.

After systemic infection, the virus enters the myocyte, replicates in the cytoplasm, and may lead to myocardial lesions. Although the pathogenesis of CVB3 infection has been studied for decades, the exact factors to determine the initiation, occurrence, and progression of viral myocarditis remain unclear. Meanwhile, H_2S is an endogenously produced gaseous signaling molecule with diverse biological functions, and is capable of modulating many physiological processes. Recently, Ivanciuc et al.[3] reported that in a murine model of respiratory syncytial virus infection, H_2S donor played a protective role in virus-induced and inflammation-induced lesions in the lung. Naturally, a new question is raised that what is the impact of H_2S on the CVB3-mediated myocardial injury.

In the present study, we extend our study to find whether administration of H_2S has interaction with CVB3 replication and spread, to explore that the modulation of CSE/H_2S may provide a therapeutic option against virus-mediated damage.

METHODS

Murine model and treatment protocols

Six-week-old inbred male Balb/c mice purchased from the Shanghai Laboratory Animal Center (Shanghai, China) were inoculated intraperitoneally with $10^{-5.69}$ 50% tissue culture infection dose (TCID50)/ml of CVB3 diluted in phosphate-buffered saline (PBS) to a final volume of 100 µl. Group control was inoculated intraperitoneally with equal amounts of normal saline solution and was defined as group N ($n = 10$). Mice infected with CVB3 were administered intraperitoneally with DL-proparglygylcine (PAG) (40 mg/kg/day), sodium hydrogen sulfide (NaHS) (50 µmol/kg/day), and vehicle daily, named group P ($n = 10$), S ($n = 10$), and C ($n = 10$), respectively. All animal experiments in our studies were conformed to the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health (NIH publication no. 85-23, revised 1996) and were also approved by the Zhejiang University Committee for Animal Care and Use. Day 0

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was defined as the day of virus inoculation. After 4-day observation, all mice were killed to obtain blood specimens and heart preparations for further examination. The apical parts of the hearts were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin–eosin. The basal parts of the hearts were homogenized in the culture medium, and the supernatant virus titers were determined by TCID50 assay.

Virus and reagents

CVB3 Nancy strains were obtained from the Shangdong Academy of Medical Sciences (Shandong, China). Experiments were carried out in a biosafety level 2 laboratory. HeLa cells were form the Zhejiang Provincial Key Laboratory of Medical Genetics (Zhejiang, China). Fetal bovine serum and RPMI-1640 were purchased from Gibco. PAG and NaHS were used as an inhibitor of endogenous H_2S and H_2S donor, respectively, and were purchased from Sigma (St. Louis, MO, USA). Rabbit polyclonal anti-nitric oxide synthase 2 (NOS2) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SABC kit for immunohistochemistry was from Boster (Wubei, China). The primers for NOS2 and β-actin were synthesized by Genecore (Shanghai, China). The anti-tubulin antibody, horse radish peroxidase (HRP)-labeled secondary antibody, BeyoECL Plus, and ELISA kit were purchased from Beyotime (Jiangshu, China).

Histopathological examination

The ratio of heart-weight to body-weight (HW/BW) was calculated to indicate myocardial edema. Formalin-fixed heart specimens from each group were cleared and embedded in paraffin wax, cut into 4-μm-thick sections, mounted on glass slides, and stained with hematoxylin and eosin. Sections of the hearts were scored blindly by two observers according to the extent of cellular infiltration and myocardial necrosis, using a semiquantitative scale of 0 to 4. The scores were used as follows: 0, no lesion; 1+, lesion involving <25%; 2+, lesion involving 25 to 50%; 3+, lesion involving 50 to 75%; 4+, lesion involving 75 to 100%.

Immunohistochemistry

Well-prepared sections were also submitted for immunohistochemical staining. In brief, sections were dewaxed and rehydrated, followed by antigen unmasking by heating. After blocking, sections were incubated with rabbit polyclonal anti-NOS2 antibody at a dilution of 1:100 overnight at 4 °C and then secondary antibody for 30 min at room temperature, followed by incubation with streptavidin–biotin–peroxidase complex and 3′,3′-diaminobenzidine tetrahydrochloride, and counterstained with hematoxylin. Sections were then dehydrated and mounted with coverlips.

RT-PCR analysis

The messenger RNA (RNA) levels of heart NOS2 were estimated by a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) assay. Total RNA was isolated from hearts using TRIzol reagent according to the manufacturer's protocol. The sequence of sense and antisense primers are as follows: inducible nitric oxide synthase (iNOS/NOS2) (S-5′-ACGGACGA GACGGATAG-3′; A-5′-GGGCTTCAAGATAGGGA-3′); β-actin (S-5′-CCCATCTACGAGGGCTAT-3′; A-5′-TGTCACGCACGATTTCC-3′). Cycle sequencing was performed as follows: 30 cycles for iNOS and 20 cycles for β-actin of denaturation (94 °C for 30 s), annealing (53 °C for 30 s), and extension (72 °C for 60 s). The last cycle of 72 °C extended 5 min. Sterilized deionized water which replaced the DNA template severed as the blank control. The optical density of PCR products were measured to determine the relativel amount of iNOS/NOS2 messenger RNA (mRNA).

Western blot analysis

Heart specimens were homogenized on ice using tissue buffer containing phenylmethylsulfonyl fluoride. After centrifugation, the supernatant was collected for BCA assay. Protein lysates (50 μg) were boiled for 5 min under reducing conditions and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 120 V for 90 min in Tris-glycine-SDS running buffer. The gel was transferred for 1 h at 200 mA onto PVDF membranes. Membranes were then incubated for 1 h with blocking buffer (5% nonfat dry milk in Tris-buffered saline, 0.1% Tween-20 (TBST)), washed three times with TBST, and incubated overnight with the primary antibody (atni-iNOS/NOS2 antibody at a 1:400 dilution, anti-tubulin antibody at a 1:1000 dilution). After incubation, the membranes were washed three times for 5 min in TBST. Membranes were then incubated with the HRP-labeled secondary antibody at a 1:1000 dilution at room temperature for 1 h. Finally, the bands were visualized by BeyoECL plus.

Viral titer assay

The viral load in hearts was assayed by endpoint titration and measured by TCID50, as published elsewhere.[1] The serial dilutions of the supernatants from heart homogenates were coincubated with RPMI-1640 and 4% fetal bovine serum on HeLa cell monolayers in 96-well microtiter plates, which were examined daily for 5 days for the appearance of any cytopathic effect under an inverted microscope. The results were listed as $\log_{10}$ TCID50/mg tissue.

Tumor necrosis factor-α level

Level of tumor necrosis factor-α (TNFα) was determined in cardiac homogenates and plasma of mice using enzyme-linked immunosorbent assay kits. Assays were performed according to the manufacturer's instructions. The level of TNFα measured was listed as pg/ml.

Statistical analysis

Data were presented as mean ± SEM. Comparison of parameters among different groups was made with analysis of variance (one-way analysis of variance) followed by a post hoc multiple comparisons test (least significant difference), when required. $P < 0.05$ is indicative of statistical significant.

RESULTS

Myocardial histology

From days 0 to 4, no dead mice appeared. As shown in Fig. 1, a normal myocardial was seen in group N, and no obvious difference of inflammatory changes on heart sections was found between infected mice and infected mice treated with PAG and NaHS on day 4 after CVB3 inoculation, which was further

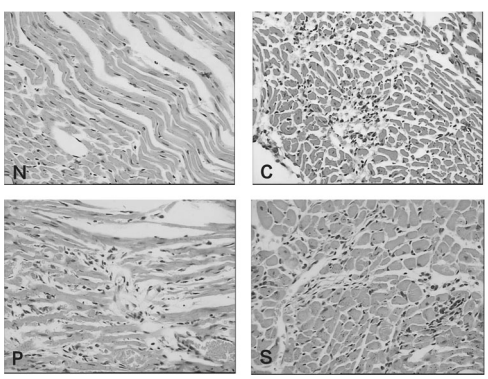

Fig. 1 Effect of sodium hydrogen sulfide (NaHS) and DL-propargylgylcine (PAG) on histopathology of the heart of mice with coxsackie virus B3 (CVB3). No inflammation was seen in group N. Groups C, P, and S showed a similar extent of myocardial lesion and cellular infiltration on postinfection day 4

demonstrated by the HW/BW ratio and inflammatory scores. These results suggested that CVB3 infection led to moderate inflammation on heart during early acute viremia, and both H2S and PAG treatments could not alleviate or exacerbate the inflammatory process.

Immunohistochemistry examination

As shown in Fig. 2, mice in group N had a negative expression of NOS2, and mice in groups C, P, and S had a mildly positive expression of NOS2. It was suggested that NOS2 might be a target that implicated in H_2S-treated mice with CVB3. To testify this hypothesis, NOS2 transcript and protein were further determined.

Cytopathic effects assay on HeLa cells

To evaluate the viral titer, the cytopathic effect (CPE) of CVB3 releasing from infected host cells was observed on HeLa cells. As shown in Fig. 3, HeLa cells adhered to the wall with well viability, and marked CPE was observed on HeLa cells coincubated with supernatant from infected-heart homogenates. Mice treated with PAG and NaHS had significantly lower and higher viral stocks than those inoculated with CVB3 only, respectively. In each infected group, supernatant of heart homogenates was coincubated with HeLa cells in the absence or presence with 10 and 50 μmol/l NaHS, and the viral loads showed no significant difference between these groups. It was suggested that CVB3 infection resulted in obvious CPE, and virus-mediated myocardial damage was predominant during early viremia in myocarditis. In vivo, inhibition of endogenous H_2S could limit viral replication and dissemination, but this effect did not appear again in vitro.

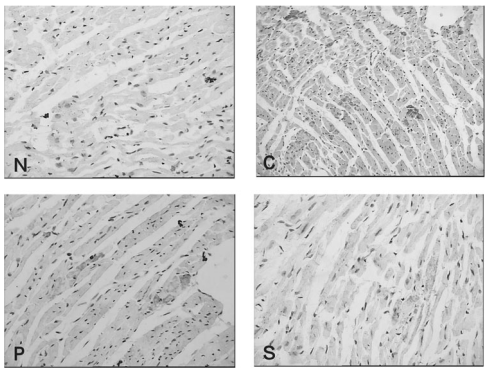

Fig. 2 Immunohistochemistry assay of nitric oxide synthase 2 (NOS2) on heart section in mice with coxsackie virus B3 (CVB3). No expression of NOS2 was detected in group N, and a mildly positive expression of NOS2 was detected in groups C, P, and S

Systemic and local TNFα levels

To explore whether tumor necrosis factor-α (TNFα) was involved in H_2S-treated mice with CVB3, we examined the concentrations of TNFα in the serum and heart. As shown in Fig. 4, compared to mice without CVB3 inoculation, infected mice had significant higher serum TNFα. Both circulatory and local concentrations of TNFα were higher in group P, and were lower in group S compared to those in group C.

Effect of H_2S on NOS2 mRNA and protein in mice with CVB3

As shown in Fig. 5, NOS2 mRNA and protein expressions were higher in group C than that in group N. Mice in groups P and S showed a significantly higher and lower expression levels, respectively, relative to those in group C.

DISCUSSION

The present data showed that there was no significant difference in myocardial inflammation as evidenced by the histological scores of myocardium and the ratio of HW/BW between infected groups with and without treatments. PAG treatment reduced viral titer in infected myocardium, while H_2S donor impaired viral clearance on day 4. Interestingly, no death mice were found during 4-day observation in this study, while occurred during 10-day observation in our previous experiment. It is suggested that viremia was more predominant than myocardial inflammation in

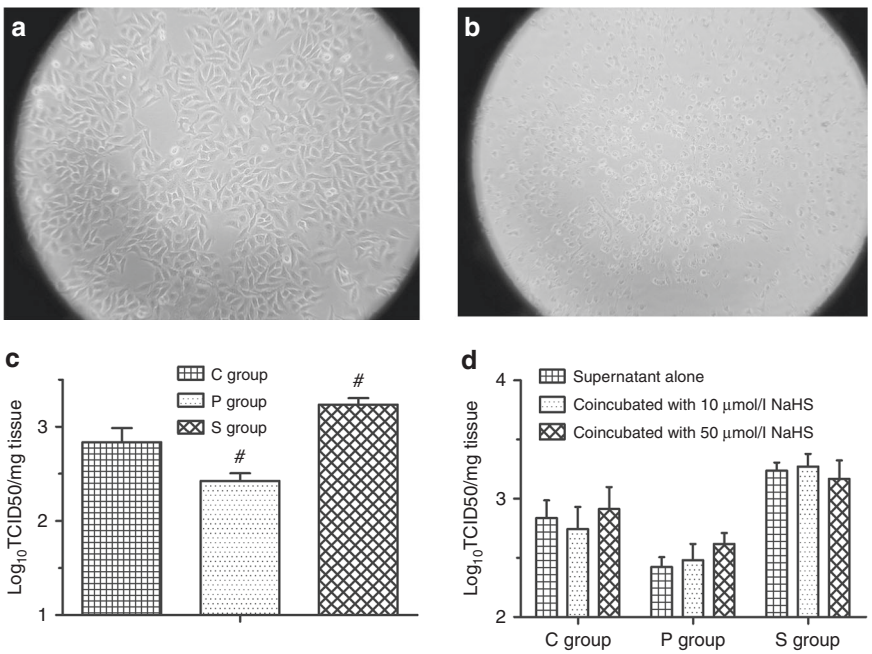

Fig. 3 CVB3 titer assay on HeLa cells. **a** HeLa cells adhered to the wall and grew well. **b** Cytopathic effects were notable on HeLa cells in the presence of supernatant from infected-heart homogenates. **c** DL-Proparglygylcine (PAG) treatment could alleviate cytopathic effects, while sodium hydrogen sulfide (NaHS) exacerbate. **d** Cytopathic effects showed no significant difference between HeLa cells coincubated with supernatant in the absence or presence with 10 and 50μmol/l NaHS. $^{\#}p < 0.05$ vs. group C

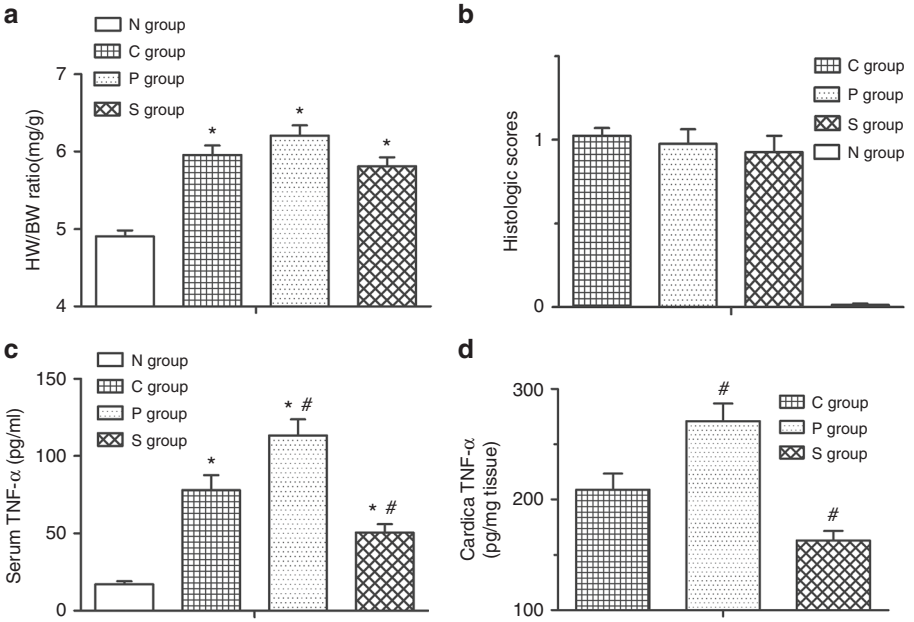

Fig. 4 Change of histology and tumor necrosis factor-α (TNFα) in hydrogen sulfide (H_2S)-treated mice with CVB3. **a** Each infected group had a higher heart-weight to body-weight (HW/BW) ratio than group N. The HW/BW ratio in group P and S was similar to that in group C. **b** The inflammatory scores showed no significant difference between group P, S, and C. **c** Serum TNFα in infected mice was higher than that in uninfected mice. Serum TNFα was higher in group P and lower in group S than that in group C. **d** Cardiac TNFα was higher in group P and lower in group S than that in group C. $^{*}p < 0.05$ vs. group N, $^{\#}p < 0.05$ vs. group C

the early stage of viral infection. These data provide evidence that H_2S was involved primarily in viral replication in the acute viremia of CVB3-induced myocarditis, and inhibition of H_2S had an antiviral activity. To determine the possibilities that may underlie this phenomenon, experiments were further carried out to analyze the NOS2 mRNA and protein expressions on infected heart, and the systemic and local levels of TNFα.

Our data displayed the level of TNFα in the serum, and the tissue was lower in the group treated with NaHS, but higher in the group treated with PAG, which suggested that PAG treatment resulted in an increase of both systemic and local TNFα secretion. H_2S treatment exert beneficial effect in a rat model of trinitrobenzene sulfonic acid induced colitis through decreasing TNFα expression in tissue.[4] H_2S administration showed protective role in a mode of myocardial ischemia/reperfusion (I/R) injury by inhibiting the surge of inflammatory cytokines such as TNFα.[5] Lee et al.[6] reported that H_2S could promote neuroprotection in the glial-mediated neuroinflammatory model by reducing the release of TNFα. These studies were consistent with our findings. However, it was also noted that the study reported by Kim et al.[7] showed that H_2S induced marked increase in the levels of TNFα in the RAW264.7 cells and in the forced swimming test mousel model. It seemed to be contradiction, and this difference could be explained by the different models. In fact, H_2S play different roles by activating different signaling pathways, such as through PI3K/Akt/Nrf2 pathway in the model of cerebral I/R injury,[8] by inactivation of P38 mitogen-activated protein kinase and nuclear factor-κB proteins.[6] A reduction of viral clearance was shown in the presence of H_2S as evident by elevated viral titers and viral replication in the heart in our study. It was noted that in in vitro study the pro-viral effect of H_2S did not appear again, which suggested that inhibition of H_2S alone could not block the life cycle of CVB3. Interestingly, several studies reported previously that TNFα was a potently antiviral cytokine. Blockage of endogenous TNFα signaling using soluble p75 TNFα receptor by electroporation was beneficial for the replication of viral on day 3 after infection.[9] The viral clearance was impaired in TNFα-knockout mice on day 7 after infection.[10] Recombinant human TNFα alleviated the surge of viral load induced by the over-expression of interleukin-6 in the viral myocarditis on day 3 after infection.[11] These studies suggested that during the acute viremia of viral myocarditis, TNFα possessed antiviral property. It could postulate that elevation of TNFα induced by inhibition of H_2S was beneficial to viral clearance.

Similar to the change of TNFα concentration, NOS2 expression on infected myocardial was also inhibited by H_2S while induced by PAG in our experiment. This result provided an evidence that inhibition of endogenous H_2S facilitated the induction of NOS2 expression. Oh et al.[12] have reported that administration of H_2S was able to inhibit NO production and iNOS expression in RAW264.7 macrophages stimulated with lipopolysaccharide. In IL-1β-mediated isolated human chondrocytes, H_2S showed anti-inflammatory properties with the occurrence of downregulation of NOS2 expression and reduction of NO production.[13] More recently, in the murine model of asthma, H_2S administration produced the same results as inhibition of NOS2 did by increasing glutathione/oxidized glutathione ratio and ameliorating airway inflammation.[14] These results were in line with our observations. Several studies have shown that NOS2 and its production NO had cardioprotection against viral attack. Induction of NOS2 expression blocked viral life cycle,[15] while inhibition of NOS2 activity or NOS2 gene knockout impaired viral elimination in vivo.[16] Moreover, nitric oxide donors can inactivate/blunt proteinases 2A and 3C of CVB3.[17] Additionally, peroxynitrite, formed by reaction of NO and superoxide, decreases viral replication through the formation of nitrotyrosine residues on VP1 (one of structural capsid proteins) and inhibits the entry of viral RNA into the host cell, while scavenging peroxynitrite/scavenger impaired viral clearance.[18] These suggested that NOS2 or its downstream effectors can inhibit viral RNA replication and protein synthesis by specific targets. Therefore, downregulation of NOS2 expression was one mechanism by which H_2S played a pro-viral role in CVB3-induced myocarditis. Interestingly, increases in the expression of TNFα and NOS2, induced by PAG, had little effect on myocardium 4 days

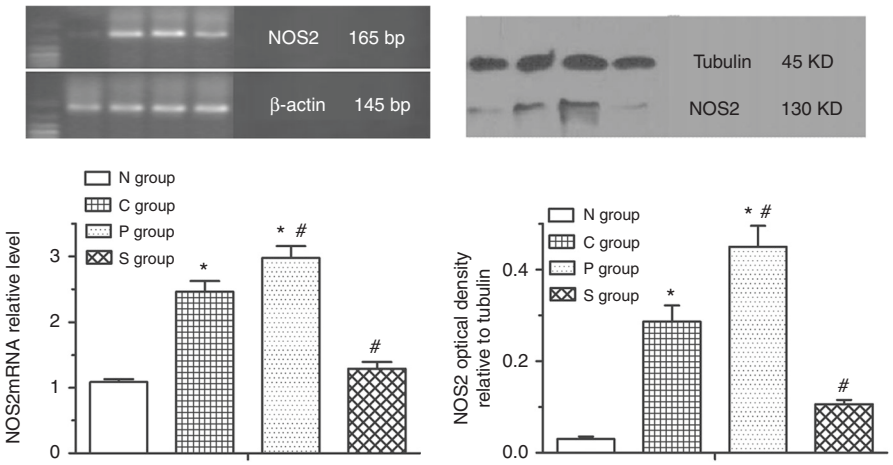

Fig. 5 Expression of nitric oxide synthase 2 (NOS2) messenger RNA (mRNA) and protein in mice with viral myocarditis. **a** Infected mice showed higher NOS2 mRNA expression than uninfected mice. NOS2 mRNA expression was higher in group P and lower in group S than that in group C. **b** Infected mice showed higher NOS2 protein expression than uninfected mice. NOS2 protein expression was higher in group P and lower in group S than that in group C. *$p < 0.05$ vs. group N, #$p < 0.05$ vs. group C

after CVB3 infection. These data suggested a possibility that H_2S may regulate immune response and redox state, by which PAG alleviated viremia in infected mice in the present study, but aggravated myocardial damages 10 days after inoculation in our previously study.[2]

There were additional possibilities for H_2S in the modulation of CVB3. H_2S treatment promoted the death of lymphocytes, including CD8+ T and in natural killer cells through decreasing mitochondrial membrane potential,[19] while CD8+ T lymphocytes normally protect against infection by killing infected cells via effector molecular such as perforins and granzymes. Furthermore, lymphocytes were also the main cells to produce and secrete NOS2 and TNFα. Therefore, the pro-viral effect of H_2S could be explained by the action of H_2S facilitating lymphocyte death. Another possible explanation is the direct role of H_2S and NO when forming nitrosothiol to clear away NO effect[20] and the reaction with unsaturated bonds of fatty acids to regulate NO release.[21] Interestingly, cardiac-specific overexpression of TNFα enhanced NOS2 expression and activity in myocardium,[22] which would further increase NOS2 level in PAG-treated virus-infected mice. The synergistic effect of elevation in NOS2 and TNFα, induced by PAG as observed in the present study, could strength significance of H_2S in modulating viral invasion, replication, and spread.

On the basis of these observations and our present data, it was reasonable to extrapolate that inhibition of endogenous H_2S production contributed to viral clearance in acute viremia of CVB3-induced myocarditis.

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AUTHOR CONTRIBUTIONS

W.H., F.Z.g: conceptualized and designed the study, acquired, analyzed and interpreted data, and drafted the initial manuscript. Y.W., Y.W., S.F., W.W., C.X., Y.Z.: acquired, analyzed, and interpreted data. F.G.: conceptualized and designed the study, coordinated and supervised data collection and analysis, and critically reviewed the manuscript. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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