Hydrogen Sulfide Is an Antiviral and Antiinflammatory Endogenous Gasotransmitter in the Airways

Role in Respiratory Syncytial Virus Infection

Teodora Ivanciuc¹, Elena Sbrana², Maria Ansar¹, Nikolay Bazhanov¹, Csaba Szabo³, Antonella Casola^{1,2,4,5*}, and Roberto P. Garofalo^{1,2,4,5*}

¹Departments of Pediatrics, ²Microbiology, and ³Anesthesiology, ⁴Sealy Center for Vaccine Development, and ⁵Sealy Center for Molecular Medicine, University of Texas Medical Branch, Galveston, Texas

Abstract

Hydrogen sulfide (H₂S) is an endogenous gaseous transmitter whose role in the pathophysiology of several lung diseases has been increasingly appreciated. Our recent studies *in vitro* have shown, we believe for the first time, that H₂S has an important antiviral and antiinflammatory activity in respiratory syncytial virus (RSV) infection, the leading cause of bronchiolitis and viral pneumonia in children. Our objective was to evaluate the therapeutic potential of GYY4137, a novel slow-releasing H₂S donor, for the prevention and treatment of RSV-induced lung disease, as well as to investigate the role of endogenous H₂S in a mouse model of RSV infection. Ten- to 12-week-old BALB/c mice treated with GYY4137, or C57BL/6J mice genetically deficient in the cystathionine γ -lyase enzyme, the major H₂S-generating enzyme in the lung, were infected with RSV and assessed for viral replication, clinical disease, airway hyperresponsiveness, and

inflammatory responses. Our results show that intranasal delivery of GYY4137 to RSV-infected mice significantly reduced viral replication and markedly improved clinical disease parameters and pulmonary dysfunction compared with the results in vehicle-treated control mice. The protective effect of the H₂S donor was associated with a significant reduction of viral-induced proinflammatory mediators and lung cellular infiltrates. Furthermore, cystathionine γ -lyase-deficient mice showed significantly enhanced RSV-induced lung disease and viral replication compared with wild-type animals. Overall, our results indicate that H₂S exerts a novel antiviral and antiinflammatory activity in the context of RSV infection and represent a potential novel pharmacological approach for ameliorating virus-induced lung disease.

Keywords: paramyxovirus; lung injury; airway hyperresponsiveness; cystathionine γ -lyase; antiviral

Hydrogen sulfide (H₂S) is an endogenous gaseous transmitter that participates in the regulation of physiological functions of the respiratory system, including smooth muscle contractility, pulmonary circulation, cell proliferation/apoptosis, oxidative

stress, and inflammation (1). Therefore, impaired H₂S production in human and experimental animal models has been implicated in the pathogenesis of chronic obstructive pulmonary disease (COPD), asthma, pulmonary fibrosis, and hypoxia-

induced pulmonary hypertension. H₂S is produced endogenously in mammals, including humans, by three enzymes: cystathionine γ -lyase (CSE), cystathionine- β -synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (2–4). Sulfide salts such

(Received in original form November 25, 2015; accepted in final form June 7, 2016)

*These authors contributed equally to this work.

This work was supported by National Institutes of Health grants R01 AI079246, R21 AI09088, R21 AI103565, and P01 AI062885; Department of Defense grant W81XWH1010146; and the University of Texas Medical Branch John Sealy Memorial Endowment Fund.

Author Contributions: T.I.: contributed to the conception of the manuscript and performed experiments, literature review, and drafting of the manuscript; E.S.: performed experiments and contributed to the revision of the manuscript; M.A.: performed experiments and literature review; N.B.: performed experiments and literature review; C.S.: contributed to the conception of the manuscript, literature review, and review of the manuscript; A.C.: contributed to the conception of the manuscript, drafting of the manuscript, literature review, and final manuscript review; and R.P.G.: contributed to the conception of the manuscript, literature review, drafting of the manuscript, and final manuscript review.

Correspondence and requests for reprints should be addressed to Roberto P. Garofalo, M.D., Department of Pediatrics, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-0369. E-mail: rpgarofa@utmb.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 55, Iss 5, pp 684–696, Nov 2016

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Originally Published in Press as DOI: 10.1165/rcmb.2015-0385OC on June 17, 2016

Internet address: www.atsjournals.org

Clinical Relevance

Hydrogen sulfide (H_2S) is an endogenous gasotransmitter that functions as a biologically relevant signaling molecule in mammals. H_2S is involved in various pathophysiological conditions of the respiratory system, including smooth muscle contractility. This study identifies H_2S as a novel molecule that can modulate viral replication and airway inflammatory responses, both important determinants of lung injury in respiratory syncytial virus infection, with the potential for rapid translation of such findings into novel therapeutic approaches for viral bronchiolitis and pneumonia.

as sodium hydrosulfide and sodium sulfide have been used widely to study the biological effects of H_2S in many cells, tissues, and animals. When used in cell culture, these salts generate a large burst of H_2S over a short time period. GYY4137 is a novel water-soluble H_2S donor that releases H_2S slowly over a period of hours (5). H_2S donors have been used to demonstrate how therapeutic H_2S administration exerts significant effects in various animal models of inflammation, reperfusion injury, and circulatory shock (6); however, their role in the context of viral infections is largely unknown.

We have shown recently, we believe for the first time, that the modulation of intracellular H_2S significantly affects cellular responses and viral replication in an *in vitro* model of respiratory viral infections caused by respiratory syncytial virus (RSV) and other paramyxoviruses (7). Treatment of both A549 cells and primary small alveolar epithelial cells with the H_2S donor GYY4137 significantly reduced the viral-induced release of proinflammatory mediators and significantly inhibited replication not only of RSV but of other paramyxoviruses, such as human metapneumovirus and Nipah virus, as well (7). On the basis of the observations from our *in vitro* studies, we used an *in vivo* model of RSV infection to address the role of H_2S in RSV-induced lung disease. In the current study, we found that GYY4137 administration

significantly attenuated RSV-induced body weight loss, clinical illness, and airway hyperresponsiveness (AHR). H_2S -donor treatment also significantly reduced pulmonary cytokine and chemokine production and neutrophil recruitment to the lung after RSV infection. To further explore the role of endogenous H_2S production in an experimental model of RSV infection, we used C57BL/6J mice genetically deficient in CSE enzyme ($CSE^{-/-}$), which exhibit a profound depletion of H_2S in peripheral tissues including the lungs (8). We found that endogenous H_2S modulates viral replication and disease severity in mice experimentally infected with RSV. These data suggest that endogenous H_2S plays a central role in protection against RSV infection, and that treatment with slow-releasing H_2S donors could provide a novel approach for the prevention and/or treatment of viral-induced pulmonary diseases.

Materials and Methods

RSV Preparation

The RSV long strain was grown in Hep-2 cells and purified by centrifugation on discontinuous sucrose gradients, as described (9, 10), and viral pools were titered in plaque-forming units (pfu) per milliliter using a methylcellulose plaque assay, as described (11). Ultraviolet (UV)-inactivated RSV was generated by exposing RSV to UV radiation (UVG-54; Entela, Upland, CA) for 30 minutes.

H_2S Donor

GYY4137 (morpholin-4-ium 4 methoxyphenyl [morpholino] phosphinodithioate) was purchased from Cayman Chemical (Ann Arbor, MI). GYY4137 was freshly prepared daily in PBS before mice delivery.

Ethics Statement

All procedures involving mice in this study were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston (Protocol: 9001002). The mice were killed by an intraperitoneal injection of ketamine and

xylazine and exsanguinated via the femoral vessels.

Mice and Infection Protocol

Ten- to 12-week-old female BALB/c mice were purchased from Harlan (Houston, TX). The 10- to 12-week-old male and female C57BL/6J mice (wild type [WT]) used in this work were purchased from the Jackson Laboratory (Bar Harbor, Maine). CSE KO mice on C57BL/6J background were generously provided by Dr. Solomon Snyder, Johns Hopkins University, Baltimore, MD. Under light anesthesia, mice were infected intranasally (i.n.) with 50 μ l of RSV diluted in PBS at a dose of 10^7 pfu or mock inoculated using the same volume of control buffer. In some experiments, BALB/c mice were inoculated with either RSV at a dose of 10^6 pfu for measurements of cytokines and chemokines, or with 10^5 pfu for pulmonary function testing. GYY4137 administration was performed i.n. at different doses and timing of RSV infection. CSE KO mice were used to examine the role of endogenous H_2S in the pathogenesis of RSV infection. Both sexes of CSE KO and WT age-matched mice were used. WT and CSE KO mice were inoculated i.n. with 10^7 pfu of RSV, in a total volume of 50 μ l, under light anesthesia. As mock treatment, all mice were inoculated with an equivalent volume of PBS. Daily determinations of body weight and illness score, bronchoalveolar lavage (BAL) differential cell counts, lung neutrophil counts by flow cytometry analysis, cytokines, chemokines, and type I IFNs measurements were made; in addition, lung viral titration, pulmonary histopathology, and pulmonary function testing were performed, as described previously (12, 13). CSE and CBS mRNA expression in lung tissue was analyzed by real-time polymerase chain reaction, as described previously (7, 8). Real-time visualization of H_2S generation in the lung was performed using the azide-based probe sulfidefluor-7 acetoxymethyl ester (7). Briefly, mice received one dose of GYY4137 or PBS and 1 hour later were injected intratracheally with sulfidefluor-7 acetoxymethyl ester (100 μ l; 10 μ M). Thirty minutes later, lungs were excised and imaged *ex vivo* in a UPV Small Animal Imaging System (Upland, CA) and were analyzed using Living Image software (Upland, CA).

Statistical Analysis

The data were evaluated using analysis of variance and two-tailed unpaired *t* tests for samples with unequal variances to determine significant difference between each set of two groups (GraphPad Prism 5.02; GraphPad Software, Inc., San Diego, CA). Results are expressed as mean $\pm$ SEM for each experimental group unless otherwise stated. $P < 0.05$ value was selected to indicate significance. All experiments were repeated at least three times; data in figures are shown from a representative experiment.

Results

GY4137 Treatment Ameliorates Viral-Induced Disease and Pulmonary Function in Response to RSV Infection

We have shown previously that RSV infection inhibits endogenous H₂S generation in airway epithelial cells, to a large extent by reducing the expression of the H₂S-generating enzyme CSE (7). In our initial experiments *in vivo*, we found that infection of mice also resulted in reduced expression of both CBS and CSE in the lung (see Figure E1A in the online supplement). Thus, to determine whether treatment with an H₂S donor could ameliorate RSV-induced disease, we initially assessed the effect of different GYY4137 treatment protocols on body weight loss. Release of H₂S can be measured directly in airway epithelial cells *in vitro* by the SF7-AM fluorescence probe (7), and we confirmed that this also occurs in the lung (Figure E1B). In the first protocol, animals were treated with various doses of GYY4137, ranging from 50 to 200 mg/kg, 1 hour before and 6 and 24 hours after infection. As shown in Figure 1A, mice inoculated with RSV alone lost weight progressively during the first 3 days of infection, with a peak of 15–20% loss by Day 3 postinfection (p.i.). Treatment with GYY4137 at any given dose consistently attenuated RSV-induced body weight loss, starting at Day 2 p.i. Treatment with additional doses of the GYY4137 during the following 2–3 days of infection did not result in further clinical benefit, with some signs of toxicity (ruffled fur) at the 200 mg/kg dose (data not shown).

We then determined whether the 50 mg/kg dose was effective if treatment was initiated after infection. The dose of

50 mg/kg GYY4137 was administered to mice as follows: (1) three doses, one at 2, 6, and 24 hours p.i., (2) two doses, one at 6 and the other at 24 hours p.i., and (3) one single dose 24 hours after infection. Mice treated with three or two doses of GYY4137 after RSV infection exhibited significantly attenuated body weight loss compared with vehicle-treated mice, although to a lesser extent than when the compound was administered before infection (Figure 1B). When GYY4137 was administered at 24 hours p.i., mice exhibited body weight loss similar to that of vehicle-treated infected mice (Figure 1B). Because the pretreatment protocol was the most effective in modulating RSV-induced disease, all subsequent experiments were performed using the dose of 50 mg/kg given 1 hour before and 6 and 24 hours after infection.

A positive effect of H₂S-donor administration was also observed on other clinical parameters of RSV infection that constitute the viral-induced illness score (see MATERIALS AND METHODS for details). Typically, the peak of illness severity coincides with the peak of RSV-induced body weight loss and occurs between Days 2 and 4 of infection (14). We observed a highly statistically significant difference in total illness score for GYY4137-treated versus vehicle-treated RSV-infected mice (Figure 1C), indicating that this treatment is effective in modulating RSV-induced clinical disease.

We and others have shown previously that RSV infection induces AHR in response to methacholine challenge (12, 15). To determine the effect of GYY4137 on pulmonary function, RSV-infected or mock-infected mice were assessed for AHR in response to methacholine challenge by whole-body plethysmography (Buxco Electronics, Inc., Sharon, CT), at Day 5 after infection. Aerosolized methacholine elicited significantly increased AHR in vehicle-treated mice infected with RSV, compared with all other groups. A significant difference was observed between vehicle- and GYY4137-treated RSV-infected animals, because GYY4137 strongly attenuated RSV-induced AHR at higher doses of methacholine (i.e., 25 and 50 mg/ml) (Figure 1D). Indeed, compared with the vehicle-treated RSV-infected group, GYY4137-treated RSV-infected mice showed an approximate twofold reduction in Penh values at a dose of 50 mg/ml. GYY4137 treatment did not alter baseline Penh values or AHR to methacholine in

mock-infected animals. The protective effect of GYY4137 on lung function was confirmed by analysis of airway resistance using the Flexivent system (Scireq, Montreal, PQ). As shown in Figure 1E, RSV-infected mice treated with GYY4137 exhibited lung resistance values similar to those measured in mock-infected animals. No differences in lung resistance were observed between vehicle- and GYY4137-treated mock-infected mice. Overall, these data indicate that slow-releasing H₂S donors significantly ameliorated clinical disease and lung function during RSV infection.

Treatment with GYY4137 Reduces Viral Replication

We have shown recently, we believe for the first time, that GYY4137 has an important antiviral activity *in vitro* against several members of the *Paramyxoviridae* family (7). To determine whether H₂S donor administration altered RSV replication in the lung, mice were treated with 50 mg/kg GYY4137 or control vehicle, starting 1 h before infection, as described above, and killed at Day 5 p.i., when peak viral titer occurs (14). A reduction ranging from 0.81 to 0.91 log of RSV peak titer was observed consistently across four independent experiments in GYY-treated animals (Figure 2A). Higher concentration of GYY4137 (i.e., 100 and 200 mg/kg body weight) did not show any greater antiviral effect (data not shown). The observed effect of H₂S donor on RSV replication in the lung appeared to be independent of the known antiviral activity of IFN- γ ; concentrations of this cytokine in BAL samples were comparable at Days 5 and 7 in infected mice treated with GYY4137 or vehicle control (data not shown). We then tested whether GYY4137 treatment could reduce viral replication when administered after RSV infection. For that, groups of mice were infected with RSV and treated with three doses (2, 6, and 24 hours after infection), two doses (6 and 24 hours after infection), or one dose (24 hours after infection) of GYY4137 (50 mg/kg) or vehicle. Results of these experiments showed that administration of GYY4137 up to 6 hours p.i. (in the first two protocols) was effective in reducing RSV viral titer in the lung, although to a lesser extent than observed in the pretreatment protocol, whereas administration at the later 24-hour time point was no longer effective in reducing viral replication (Figure 2B).

Figure 1. GYY4137 treatment attenuates respiratory syncytial virus (RSV)-induced disease and pulmonary lung function. (A) GYY4137 dose response *in vivo*. Mice were treated intranasally (i.n.) with different doses of GYY4137 (50 mg, 100 mg, and 200 mg/kg body weight) or an appropriate volume of vehicle (PBS) 1 hour before and 6 and 24 hours after infection. Mice were inoculated with either RSV or PBS. (A) Mice were monitored daily, and body weight was calculated on the basis of the original weight before the infection. Data are expressed as mean $\pm$ SEM ($n = 3-4$ mice/group). (B) Disease parameters—BALB/c mice were infected with 10^6 plaque-forming units (pfu) RSV and treated with GYY4137 or vehicle as follows: (1) three doses, at 2 hours and at 6 and 24 hours after infection, (2) two doses, one at 6 hours and one at 24 hours after infection, and (3) one dose, at 24 hours before infection. Data are expressed as mean $\pm$ SEM ($n = 4$ mice/group). * $P < 0.05$ compared with PBS/RSV at Days 1, 2, and 3 postinfection (p.i.). (C, D, and E) Mice were treated i.n. with GYY4137 (50 mg/kg body weight) or an appropriate volume of vehicle (PBS) 1 hour before and 6 and 24 hours after infection. Mice were inoculated with RSV or PBS. (C) Clinical illness scores of GYY4137/RSV (open squares) and RSV vehicle (solid squares) were measured from Day 1 to Day 7 p.i. (data shown for the 50 mg/kg dose). GYY4137/RSV-infected mice were assigned an illness score 0 starting with Day 3 p.i. (data shown as n.d. [not detected]). Sham (mock)-infected mice treated with either vehicle or GYY4137 received a healthy illness score 0 throughout the course of the experiment (data not shown). (D) Unrestrained, whole-body plethysmography (Buxco Electronics, Inc., Sharon, CT) was used to measure the enhanced pause (Penh) to evaluate airway hyperresponsiveness. Baseline and postmethacholine challenge Penh values were determined at Day 5 after infection. Penh values are presented as mean $\pm$ SEM ($n = 4-6$ mice/group). (E) Airway resistance (Day 5 p.i.) measured in mechanically ventilated mice by the Flexivent system. Data are expressed as mean $\pm$ SEM ($n = 3$ mice/group). * $P < 0.05$, ** $P < 0.001$, and *** $P < 0.0001$ compared with PBS/RSV group.

Figure 2. GYY4137 treatment reduces viral replication in RSV-infected mice. (A) Mice were treated with GYY4137 or vehicle and infected with either RSV or PBS and harvested at Day 5 postinfection to determine viral replication by plaque assay, expressed as pfu per gram of lung tissue. The bar graph represents mean $\pm$ SEM ($n = 4$ mice/group), ** $P < 0.001$ compared with the PBS/RSV group. (B) Mice were infected with 10^6 pfu RSV and treated with GYY4137 after infection as indicated. Data are expressed as mean $\pm$ SEM ($n = 4$ mice/group). At Day 5 postinfection, virus titer in the lung was determined by plaque assay. * $P < 0.05$ and ** $P < 0.001$ compared with the PBS/RSV group.

GYY4137 Treatment Decreases Pulmonary Inflammation in RSV-Infected Mice

Next, we investigated whether GYY4137 administration could modulate RSV-induced lung inflammation. GYY4137 or vehicle-treated mice (1 hour before and 6 and 24 hours p.i.) were infected with RSV or were mock inoculated and killed at Days 1, 3, 5, and 7 p.i. to collect BAL samples for total and differential cell count and at Day 7 p.i. for lung histopathology. A significant attenuation of total cell influx by GYY4137 treatment, compared with vehicle alone, was observed in RSV-infected mice at Days 1 and 3 p.i. (Figure 3A), but not at subsequent days (data not shown). Macrophages are the predominant cell type recovered from the BAL of uninfected mice, but after RSV infection, neutrophils become the predominant inflammatory cell in BAL and lung during the first few days of infection (16). GYY4137 administration significantly reduced neutrophil recruitment into the airways, both in BAL fluid (Figure 3A) and in lung tissue (Figure 3B), with a concomitant increase in the macrophage population. To further investigate histological changes in response

to H₂S donor, lung tissue from GYY4137- and vehicle-treated mock- and RSV-infected mice was harvested at Day 7 p.i. and subjected to hematoxylin and eosin staining. Lung histopathology analysis showed no airway inflammation in mock-infected GYY4137- or vehicle-treated animals (Figure 3C, upper panels). RSV-infected vehicle-treated mice had increased cellular infiltration in the perivascular and peribronchial spaces, which was greatly reduced by GYY4137 treatment (Figures 3C [lower panels] and 3D, expressed as pathology score).

GYY4137 Inhibits Production of Proinflammatory Mediators

RSV is a potent inducer of cytokines and chemokines, which have been shown to play an important role in viral-mediated lung inflammation and disease severity (17). In our mouse model, the peak of chemokine production occurs during the first 2 days of infection (18). Thus, to determine whether H₂S donor treatment was able to modulate RSV-induced proinflammatory mediator secretion, we measured cytokine, chemokine, and type I IFN levels in BAL samples collected at Day 1 p.i. In

RSV-infected mice, GYY4137 treatment significantly decreased the production of the proinflammatory cytokines IL-1 α , IL-1 β , IL-6, and tumor necrosis factor (TNF)- α , as well as other cytokines such as granulocyte-macrophage colony-stimulating factor and granulocyte-colony stimulating factor (Figure 4A). Similar results were observed with the release of the chemokines regulated upon activation, normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1 α , MIP-1 β , monocyte chemoattractant protein-1 (MCP)-1, and neutrophil chemokine (KC) (Figure 4B) and the secretion of both IFN- α and - β , which were all reduced by GYY4137 treatment (Figure 4C).

We have shown that GYY4137-mediated inhibition of RSV-induced cellular signaling and expression of proinflammatory genes in airway epithelial cells is a process distinct from its ability to inhibit viral replication (7). To determine whether this finding was true *in vivo*, mice were inoculated with UV-treated RSV (which was nonreplicating as assessed in HEp-2 cells by a plaque assay) and treated with GYY4137 or control vehicle, and BAL samples were collected to measure concentrations of cytokines and chemokines. As expected, UV-RSV induced secretion of cytokines known to also be released by cells such as alveolar macrophages that do not require viral replication, including IL-1 β , IL-6, TNF- α , MCP-1, MIP-1 β , and RANTES (13, 19). As shown in Figure 5, treatment of UV-RSV-inoculated mice with GYY4137 significantly reduced the levels of these cytokines in BAL, suggesting that H₂S exerted immunomodulatory and antiinflammatory activities in the lung, which are distinct from its inhibitory activity on viral replication.

CSE Deficiency Exacerbates Disease Severity, Airway Dysfunction, and Pulmonary Inflammation in RSV Infection

To further determine whether endogenous H₂S had a protective effect in RSV-induced lung disease, we investigated body weight loss, AHR, viral replication, cytokine/chemokine secretion, and lung histologic changes in RSV-infected mice lacking CSE, a key enzyme in the biosynthesis of H₂S in peripheral tissue, including the lung (8). CSE-deficient mice

Figure 3. GYY4137 reduces airway neutrophilia and lung inflammation after infection. Mice were treated intranasally with GYY4137 (50 mg/kg body weight) or an appropriate volume of vehicle (PBS) 1 hour before and 6 and 20 hours after infection. Mice were inoculated with either RSV or PBS as described in MATERIAL AND METHODS. Bronchoalveolar lavage (BAL) and lungs were collected at different time points after infection to determine differential cell counts by hematoxylin and eosin staining in (A) BAL and (B) neutrophil (CD11b⁺Gr1⁺) recruitment to the lung by flow cytometry analysis after staining with specific antibodies. (C) Lung samples were harvested at Day 7 postinfection, fixed for slide preparation, and stained with hematoxylin and eosin. Representative stained lung tissue sections from the indicated treatment. (D) Pathology score of prepared slides (scored as described in MATERIALS AND METHODS). The bar graph represents mean $\pm$ SEM ($n = 3-4$ mice/group). * $P < 0.05$ and ** $P < 0.001$ compared with PBS/RSV mice. Scale bars, 200 μ m. GR-1, granulocyte-differentiation antigen-1.

exhibit a profound depletion of H₂S in peripheral tissues (8). In our experiments, CSE KO mice exhibited enhanced body weight loss at the peak of the clinical disease and a delayed recovery to baseline weight compared with WT infected animals (Figure 6A). No differences in body weight loss were observed between WT and KO mock-infected mice. Although no

differences in baseline Penh values were observed between the WT and CSE KO animals, RSV-infected CSE KO mice exhibited a significantly enhanced sensitivity to methacholine challenge, demonstrated by greater Penh values at most of the doses tested, compared with WT infected mice (Figure 6B, left panel). Additional studies of lung mechanical

properties in artificially ventilated mice showed no differences in total lung respiratory resistance between the CSE KO and WT animals at low concentrations of methacholine; however, there was a significant increase in lung resistance in CSE KO RSV-infected mice at the higher concentrations (Figure 6B, right panel). Because we have shown recently that

Figure 4. GYY4137 inhibits proinflammatory mediator secretion in response to RSV infection. Mice were treated with GYY4137 or vehicle, RSV at the dose of 10^6 pfu, or sham infected and harvested at Day 1 postinfection to collect bronchoalveolar lavage samples to measure (A) cytokines and (B) chemokines by multi-Plex cytokine detection system, and (C) type I IFN by ELISA. The bar graph represents mean $\pm$ SEM ($n = 4-6$ mice/group). * $P < 0.05$, ** $P < 0.001$, and *** $P < 0.0001$ compared with the PBS/RSV group. G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; KC, neutrophil chemokine; MCP-1, monocyte chemoattractant protein-1; MIP-1, macrophage inflammatory protein-1; RANTES, regulated upon activation, normal T-cell expressed and secreted.

Figure 5. Effect of GYY4137 on cytokines/chemokine secretion in response to ultraviolet (UV)-inactivated RSV. Mice were treated intranasally with GYY4137 or vehicle and inoculated with either UV-inactivated 10^7 pfu RSV or mock infected. BAL was collected at Day 1 postinfection to measure cytokines and chemokines by a multi-Plex cytokine detection system. The bar graph represents mean $\pm$ SEM ($n = 4$ mice/group). ** $P < 0.001$, *** $P < 0.0001$ compared with the PBS/UV-RSV group.

CSE inhibition *in vitro* is associated with enhanced viral replication (7), we assessed viral titers in RSV-infected CSE KO and WT mice at Day 5 p.i. We observed a 50% increase in viral titer in CSE KO mice, compared with WT mice (Figure 6C), indicating that endogenous H_2S plays an important role in controlling RSV replication.

To determine whether treatment with an H_2S -releasing compound could rescue the exacerbated disease in CSE-deficient mice, GYY4137 was used at a dose of 50 mg/kg 1 hour before and 6 and 24 hours after infection. Similar to our observation in BALB/c mice, GYY4137 treatment of WT control mice (on C57BL/6 background) attenuated RSV-induced body weight loss at Days 1 and 2 p.i., with faster recovery at later time points. Moreover, GYY4137 treatment rescued body weight loss in RSV-infected CSE-deficient mice

when compared with vehicle-treated CSE-deficient littermates (Figure 6D). The protective effect of GYY4137 in CSE-deficient mice was also observed in studies of lung function because AHR in response to methacholine was significantly decreased in RSV-infected CSE-deficient mice treated with GYY4137 (Figure 6E). To investigate whether the lack of CSE affected RSV-induced proinflammatory response, BAL samples collected at Day 1 p.i. from CSE KO and WT mice were assessed for cytokines and chemokine levels by multi-Plex detection assay. We found that in the absence of CSE, RSV infection induced significantly higher levels of the cytokines TNF- α , IL-6, IL-13, and IL-12 (p40), compared with WT infected mice (Figure 7A). A similar effect was observed with the release of chemokines MIP-1 α and MIP-1 β (Figure 7A), with a trend toward increased

secretion for RANTES and MCP-1 (data not shown). Levels of IFN- γ and IL-4 measured in BAL samples at Day 5 p.i. by high sensitive ELISA were not statistically different in WT and CSE KO mice after infection with RSV (Figure 7B).

Finally, lung samples were harvested at Day 7 p.i., and lung sections were stained with hematoxylin and eosin. Lung histopathology analysis showed no airway inflammation in mock-infected WT and CSE KO mice; however, pulmonary perivascular and peribronchial inflammation, vasculitis, and alveolitis were significantly increased in the CSE KO mice in response to infection, compared with WT animals (Figure 7C). Overall, these data indicate a role of CSE and endogenous H_2S production in viral replication and inflammatory cellular responses in mice experimentally infected with RSV.

Figure 6. Cystathionine γ -lyase (CSE)-deficient mice have increased disease severity, viral replication, and lung inflammation. C57BL/6 (wild-type [WT]) and CSE^{-/-} (CSE KO) mice were infected intranasally with 10^7 pfu of RSV or PBS. (A) Mice were monitored daily, and body weight was calculated on the basis of the original weight before infection. Data are expressed as mean $\pm$ SEM ($n = 3$ –4 mice/group). (B) *Left panel*, unrestrained, whole-body plethysmography (Buxco Electronics, Inc., Sharon, CT) was used to measure the Penh to evaluate airway hyperresponsiveness. Baseline and postmethacholine challenge Penh values were determined at Day 1 p.i. Data are expressed as mean $\pm$ SEM ($n = 3$ –4 mice/group). *Right panel*, airway resistance (Day 1 p.i.) was measured in mechanically ventilated mice by the Flexivent system. Data are expressed as mean $\pm$ SEM ($n = 3$ –4 mice/group). (C) Viral replication. At Day 5 p.i., lungs were excised, and viral titers were determined by plaque assay. The bar graph represents mean $\pm$ SEM ($n = 3$ –4 mice/group). (D and E) C57BL/6 (WT) and CSE^{-/-} (CSE KO) mice were given GYY4137 or vehicle at a dose of 50 mg/kg 1 hour before and 6 and 24 hours after infection. Mice were infected intranasally with 10^7 pfu of RSV or PBS. (D) Mice were monitored daily, and body weight was calculated on the basis of the original weight before the infection. Data are expressed as mean $\pm$ SEM ($n = 2$ –3 mice/group). * $P < 0.05$ CSE KO/GYY4137/RSV versus CSE KO/PBS/RSV and WT/GYY4137/RSV versus WT/PBS/RSV at Day 1 postinfection; CSE KO/GYY4137/RSV versus CSE KO/PBS/RSV and CSE KO/PBS/RSV versus WT/PBS/RSV at Day 2 p.i., WT/GYY4137/RSV versus WT/PBS/RSV and CSE KO/PBS/RSV versus WT/PBS/RSV at Day 4 p.i. (E) Unrestrained, whole-body plethysmography (Buxco Electronics, Inc., Sharon, CT) was used to measure the Penh to evaluate airway hyperresponsiveness. Baseline and postmethacholine challenge Penh values were determined at Day 5 p.i. Data are expressed as mean $\pm$ SEM ($n = 2$ –3 mice/group). * $P < 0.05$, ** $P < 0.001$, and *** $P < 0.0001$ compared with the WT/RSV and the WT/GYY4137/RSV group and between CSE KO/RSV and CSE KO/GYY4137/RSV mice.

Discussion

Lung inflammation, which is initiated by the secretion of cytokines and chemokines from infected tissue-resident cells of the respiratory mucosa, plays an important role in the pathogenesis of RSV infection. Indeed, we and others have shown that modulation of the inflammatory response is associated with amelioration of clinical illness in animal models of RSV infection (12, 20–22). Recent studies have also pointed to an important correlation between the amount and kinetics of viral replication in the airways and the clinical outcome of RSV infections. Infants with greater RSV quantities in respiratory tract secretions have been shown to be at greater risk of prolonged hospitalization, intensive care unit stay, and mechanical ventilation (23, 24), whereas those with slower clearance of the virus have greater disease severity (25). This clinical and experimental evidence is important because new approaches to treat RSV infections are being developed, possibly with a combined spectrum of antiinflammatory and antiviral activities. In that regard, our discovery of H₂S as an endogenous biochemical pathway that can modulate inflammation and viral replication is of particular interest.

For several hundred years, H₂S has been known to exist as a noxious gas in animal tissues. Because H₂S is typically formed by commensal bacteria, it was not regarded as physiologically significant. However, recent studies have established that H₂S is indeed a biologically relevant signaling molecule in mammals (26). H₂S acts as a messenger molecule, and together with the volatile substances nitric oxide and carbon monoxide, it is defined as a gasotransmitter, playing physiological roles in a variety of functions such as synaptic transmission, vascular tone, angiogenesis, inflammation, and cellular signaling (1).

We have shown recently, we believe for the first time, that levels of intracellular H₂S modulates cellular responses and viral replication in an *in vitro* model of paramyxovirus infection (7), including RSV. Herein, we provide evidence that H₂S has a protective role in RSV infection *in vivo* as well, by modulating both inflammatory responses and viral replication. Indeed, our study shows that treatment of mice with an H₂S donor reduced RSV peak titer in the lung and

ameliorated clinical disease, including AHR. These effects were associated with a reduction in BAL and lung neutrophilia and overall lung pathology in RSV-infected H₂S-treated mice compared with RSV-infected untreated mice. The observed effect of the H₂S donor GYY4137 on RSV replication in the lung appeared to be independent of the known antiviral activity of IFN- γ (similar concentrations of this cytokine in mice treated or not treated with GYY4137) and IFN type I, the levels of which were in fact reduced in BAL samples of RSV-infected animals treated with GYY4137. This observation is not particularly surprising given the modest antiviral activity against RSV of endogenously produced IFN type I in mice (27), rather its contribution to the pathogenesis of airway inflammation (28). Moreover, our data show that administration of GYY4137 up to 6 hours after viral inoculation was effective in reducing RSV titer in the lung, a remarkable finding given the limitation of the mouse model in which the i.n. inoculation of virus results in its very rapid spread to the lower airways.

These findings were further supported by the evidence that (1) RSV infection causes a time-dependent reduction in the expression of H₂S-generating enzymes CSE and CBS, similar to our observations in epithelial cells; (2) CSE-deficient mice had increased RSV replication and greater disease and inflammatory mediator production compared with CSE-competent mice; and (3) antiviral activity and lung function (AHR) could be rescued in CSE-deficient mice by treatment with GYY4137. In humans, CSE expression and activity are regulated developmentally, as demonstrated by studies in premature infants, newborns, and infants in the first year of life, in which this enzyme has been measured and found to be delayed in maturation (29, 30). These findings are of particular relevance in relation to natural RSV infections, which cause the most severe disease during the first year of life, when the endogenous H₂S tone would likely be reduced, or in premature infants with smaller airways.

Although the mechanism(s) leading to increased AHR in the experimental mouse model of RSV infection are not fully understood, RSV-infected CSE-deficient mice showed increased AHR to methacholine challenge compared with WT infected mice. Moreover, the H₂S donor

GYY4137 significantly reduced AHR in RSV-infected BALB/c mice, further suggesting that the H₂S pathway in the lung is critical in relaxing airway smooth muscle and controlling viral-mediated airway reactivity. In this regard, some studies have shown that H₂S relaxes vascular smooth muscle by increasing ATP-sensitive K⁺ channel (K_{ATP}) channel currents and hyperpolarizing cell membrane (31), or by K_{ATP} channel-independent mechanisms, such as inhibition of Ca²⁺ release through the inositol-1,4,5-triphosphate receptor (32). In our mouse models of infection, treatment with the H₂S donor significantly decreased airway neutrophilia and secretion of inflammatory cytokines, which may contribute to RSV-induced AHR (12). Some limited studies have investigated the role of H₂S in the pathogenesis of asthma. In one study by Wu and colleagues (33), the serum level of H₂S was significantly lower in patients with asthma, compared with healthy control subjects, and significantly correlated with the severity of acute exacerbations and changes in FEV₁. In a mouse model of ovalbumin-mediated allergic inflammation, CSE deficiency was associated with increased Th2 cytokines and enhanced AHR after ovalbumin challenge, whereas exogenous H₂S supplementation was able to reduce it (34). In this regard, we did not observe any significant difference in Th1 or Th2 cytokines in CSE-deficient mice compared with WT control mice after RSV infection. In chronic diseases of the lung, data from the literature suggest a correlation of serum H₂S level with COPD severity, as defined by lung function and airway inflammation (35, 36). The serum H₂S level was significantly higher in patients with stable COPD than that in patients with acute exacerbation of COPD. In patients with stable COPD, serum H₂S levels were significantly lower in those with stage III obstruction, compared with those with stage I. This correlated positively with the percentage of predicted FEV₁ value.

Overall, our results support the notion that the antiviral activity of H₂S appears to be largely independent of its antiinflammatory properties. In airway epithelial cells, inhibition of RSV-induced cellular signaling and expression of proinflammatory genes by GYY4137 treatment occur separately from its ability to inhibit viral replication (7). Similarly,

Figure 7. Effect of CSE deficiency on cytokine and chemokine production and pulmonary inflammation after infection. C57BL/6 (WT) and CSE^{-/-} (CSE KO) mice were infected intranasally with 10^7 pfu of RSV or PBS. (A) Mice were infected with RSV or mock infected and killed at Day 1 postinfection to collect bronchoalveolar lavage samples. Cytokine/chemokine production was measured by a multi-Plex cytokine detection system. The bar graph represents mean $\pm$ SEM ($n = 3-4$ mice/group). (B) Bronchoalveolar lavage was collected at Day 5 after infection to measure IFN- γ and IL-4 by ELISA. Data are expressed as mean $\pm$ SEM ($n = 4$ mice/group). (C) Pathology score of lungs harvested at Day 7 postinfection, fixed, and hematoxylin and eosin stained. The bar graph represents mean $\pm$ SEM ($n = 3-4$ mice/group). * $P < 0.05$ compared with WT/RSV group.

treatment with GYY4137 significantly reduced cytokine and chemokine production in mice inoculated with UV-inactivated (i.e., nonreplicating) RSV. Endogenous H₂S production and exogenous H₂S administration have been associated with both proinflammatory and antiinflammatory effects in various models of disease (37). In the context of acute pancreatitis and in burn injury, for example, H₂S seems to play a proinflammatory role, whereas in other pathologies such as asthma, COPD, LPS-induced inflammation, and ischemia reperfusion, it displays antiinflammatory properties. In models of lung injury, administration of H₂S donors has often been associated with an antiinflammatory effect. For example, in a mouse model

of hyperoxia, treatment with sodium hydrosulfide was associated with reduced lung permeability and inflammation, because of decreased production of proinflammatory mediators such as IL-1 β , MCP-1, and MIP-2, and increased antiinflammatory cytokine expression (38). Similar results were obtained in other models of acute lung injury, such as the one associated with hemorrhagic shock or with bleomycin treatment (39, 40). In the only study investigating the antiinflammatory effect of H₂S administration in the course of a viral infection, GYY4137 suppressed Coxsackie B3-induced secretion of proinflammatory cytokines, an effect associated with suppression of activation of the nuclear factor- κ B signaling pathway (41).

Conclusions

In summary, our results establish, we believe for the first time, a critical protective role of the H₂S pathway in the development of disease, viral replication, and airway inflammation in an *in vivo* model of RSV infection, shedding insight into a new potential therapeutic approach for this important respiratory pathogen, and possibly other significant respiratory viral infections, by targeting the endogenous CSE/H₂S pathway. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Kimberly Palkowetz and Yinghong Ma for technical assistance and Cynthia Tribble for manuscript editing and submission.

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