Protective and Detrimental Effects of Sodium Sulfide and Hydrogen Sulfide in Murine Ventilator-induced Lung Injury

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ABSTRACT

Background: The antiinflammatory effects of hydrogen sulfide (H_2S) and sodium sulfide (Na_2S) treatment may prevent acute lung injury induced by high tidal volume (HV_T) ventilation. However, lung protection may be limited by direct pulmonary toxicity associated with H_2S inhalation. Therefore, the authors tested whether the inhalation of H_2S or intravascular Na_2S treatment can protect against ventilator-induced lung injury in mice.

Methods: Anesthetized mice continuously inhaled 0, 1, 5, or 60 ppm H_2S or received a single bolus infusion of Na_2S (0.55 mg/kg) or vehicle and were then subjected to HV_T (40 ml/kg) ventilation lasting 4 h (n = 4–8 per group).

Results: HV_T ventilation increased the concentrations of protein and interleukin-6 in bronchoalveolar lavage fluid, contributing to reduced respiratory compliance and impaired arterial oxygenation, and caused death from lung injury and pulmonary edema. Inhalation of 1 or 5 ppm H_2S during HV_T ventilation did not alter lung injury, but inhalation of 60 ppm H_2S accelerated the development of ventilator-induced lung injury and enhanced the pulmonary expression of the chemoattractant CXCL-2 and the leukocyte adhesion molecules CD11b and L-selectin. In contrast, pretreatment with Na_2S attenuated the expression of CXCL-2

What We Already Know about This Topic

- Sulfides such as sodium sulfide and hydrogen sulfide have both protective and detrimental effects on cells
- High tidal volume ventilation can produce acute lung injury

What This Article Tells Us That Is New

- Inhalation of hydrogen sulfide has no beneficial effects on ventilator-induced lung injury in mice
- Intravascular administration of sodium sulfide attenuates the development of ventilator-induced lung injury through antioxidative signaling pathways

◇ This article is featured in "This Month in Anesthesiology." Please see this issue of ANESTHESIOLOGY, page 9A.

◆ This article is accompanied by an Editorial View. Please see: Aslami H, Schultz MJ, Juffermans NP: Hydrogen sulfide: A hot molecule. ANESTHESIOLOGY 2011; 115:921–2.

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and CD11b during HV_T ventilation and reduced pulmonary edema. Moreover, Na_2S enhanced the pulmonary expression of Nrf2-dependent antioxidant genes (NQO1, GPX2, and GST-A4) and prevented oxidative stress-induced depletion of glutathione in lung tissue.

Conclusions: The data suggest that systemic intravascular treatment with Na_2S represents a novel therapeutic strategy to prevent both ventilator-induced lung injury and pulmonary glutathione depletion by activating Nrf2-dependent antioxidant gene transcription.

MECHANICAL ventilation can be life-saving in acute respiratory failure but may contribute to lung injury because of its side effects.[1,2] Cyclic lung stretch during mechanical ventilation induces tissue disruption and activates proinflammatory pathways, promoting the formation of pulmonary edema and neutrophil infiltration.[3] In addition, cyclic lung stretch is associated with generation of reactive oxygen species and production of redox imbalance in the lung.[4–6] Together, these events may initiate and perpetuate oxidative stress and local and systemic inflammatory responses, enhance lung injury, and lead to multiorgan failure and mortality.[7–9] Developing novel therapies targeted at oxidative stress and inflammation in ventilator-induced lung injury (VILI) could be useful clinically.

Hydrogen sulfide (H_2S) is an endogenous gaseous transmitter.[10] The antiinflammatory and antiapoptotic effects of H_2S and other sulfides, such as sodium hydrogen sulfide or disodium sulfide (Na_2S), have been studied in various animal models of inflammation (reviewed in Baumgart *et al.*[11]) and oxidative stress.[12,13] In particular, Calvert *et al.* reported that Na_2S protects against cardiac ischemia or reperfusion injury by up-regulating Nrf2-dependent antioxidant and detoxification proteins.[14] Nrf2 (*i.e.*, nuclear factor E2-related factor 2) is a key transcription factor that regulates antioxidant genes as an adaptive response to oxidative stress or pharmacologic stimuli. Downstream targets of Nrf2 include direct antioxidant proteins (such as glutathione peroxidase, NAD(P)H oxidoreductase, *etc.*), thiol-metabolism associated detoxifying enzymes (such as glutathione-S-transferase, glutamate–cysteine ligase, thioredoxin, *etc.*), stress-response genes (heme oxygenase, heat-shock proteins, ferritin, *etc.*), and others. In a murine model of VILI, it has been shown that Nrf2-dependent antioxidant genes play a key protective role in reducing oxidative stress.[5] Whether or not H_2S can protect against VILI by modulating the expression of Nrf2-dependent antioxidant genes has not been reported.

Inhalation of H_2S gas is known to cause airway mucosa irritation and cytotoxicity,[10,15] has been reported to exert proinflammatory effects in various models (reviewed in Szabó[10] and Baumgart *et al.*[11]), and traditionally has been considered a health hazard. On the other hand, Faller *et al.* recently showed that inhaled H_2S at 80 ppm prevents lung injury in mice ventilated with a tidal volume of 12 ml/kg (plateau pressure 10–13 cm H_2O, *i.e.*, moderate stretch).[16] Nonetheless, molecular mechanisms responsible for the protective effects of H_2S inhalation against VILI are not completely understood. In addition, it remains uncertain whether inhaled H_2S is protective or deleterious during mechanical ventilation that produces increased lung stretch, such as in patients with acute respiratory distress syndrome.

To elucidate the role of H_2S in VILI, we examined the effect of inhaled H_2S in a model of lung injury induced by high tidal volume (HV_T) ventilation. We found that H_2S gas promotes VILI and enhances the pulmonary expression of leukocyte adhesion and chemoattractant molecules. Subsequently, we hypothesized that intravascular administration of Na_2S, avoiding direct exposure of the lung to H_2S gas, could provide better protection against VILI than could inhaled H_2S. We report that intravascular Na_2S both attenuates the pulmonary expression of chemoattractant and leukocyte adhesion molecules and enhances Nrf2-dependent expression of antioxidant genes, thereby attenuating VILI from HV_T ventilation.

Materials and Methods

This study was approved by the Institutional Animal Care and Use Committee (Subcommittee on Research Animal Care, Massachusetts General Hospital, Boston, Massachusetts), and conforms to the revised Guide for the Care and Use of Laboratory Animals.

Mouse Model of VILI

Male C57BL/6 mice (23.3 ± 1.0 g, mean ± SD) were subjected to HV_T ventilation as described previously[6] with minor modifications of inspired oxygen fraction (F_{IO2}) positive end-expiratory pressure, and alveolar recruitment. Briefly, after anesthesia was induced and tracheostomy performed, mice were ventilated in a volume-controlled mode (Inspira; Harvard Apparatus, Boston, MA) at a tidal volume (V_T) of 10 ml/kg, respiratory rate of 90 breaths/min, positive end-expiratory pressure of 2 cm H_2O, and F_{IO_2} 0.4 for 1 h (baseline ventilation). Immediately after the tracheostomy and after the initiation of ventilation, a carotid catheter was inserted for blood pressure monitoring, continuous infusion of anesthetics, and blood sampling. After 1 h of baseline ventilation, the ventilator settings were switched to an HV_T of 40 ml/kg, positive end-expiratory pressure of 1 cm H_2O, and respiratory rate of 60 breaths/min, and mice were ventilated for a maximum duration of 240 min of HV_T ventilation. The F_{IO_2} (0.4) was not changed. Because a plateau pressure exceeding 30 cm H_2O is associated with increased mortality[1] and commonly is used as a trigger for initiating rescue therapies in patients with acute respiratory distress syndrome, we aimed for plateau pressures of more than 30 cm H_2O to induce VILI in mice. In this model using normal lungs, a tidal volume of 40 ml/kg was necessary to increase plateau pressure above 30 cm H_2O, resulting in a peak pressure of ~35 cm H_2O.

Alveolar recruitment maneuvers were performed every 30 min during the baseline period and every 60 min during HV_T ventilation. Body temperature was maintained at 37°C with a heating pad. See Materials and Methods in Supplemental

Digital Content 1, http://links.lww.com/ALN/A775, which provides the detailed methodology of the mouse model of VILI.

Experimental Groups

After 30 min of baseline ventilation (*i.e.*, 30 min *before* the onset of injurious HV_T ventilation), mice were treated with either inhaled H_2S or intravascular Na_2S as follows: H_2S gas (hydrogen sulfide 100 ppm, balance nitrogen, MedTech-Gases, Medford, MA) was diluted and added continuously to the inhaled gas mixture ($F_{IO_2} = 0.4$) at a concentration of 0, 1, 5, or 60 ppm (n = 6 each) until the end of the study. Alternatively, mice received a single intraarterial bolus injection of Na_2S (sodium sulfide nonahydrate, Sigma–Aldrich, St. Louis, MO; 0.55 mg/kg in 5.5 ml vehicle/kg body weight, n = 8) or vehicle alone (Dulbecco's phosphate buffered saline, Sigma–Aldrich; 5.5 ml/kg body weight, n = 8).

The animals were killed after 240 min of HV_T ventilation or when their mean arterial pressure decreased to less than 60 mmHg for more than 5 min. Immediately after the animals were killed, respiratory mechanics were assessed and arterial blood was collected. Bronchoalveolar lavage (BAL) and the collection of lung tissues were performed as described in Supplemental Digital Content 1, http://links.lww.com/ALN/A775.

Control mice not subjected to HV_T ventilation (n = 4) were killed after 5 min of baseline ventilation.

Evaluation of Respiratory Mechanics and Blood Gas Analysis

We measured V_T, peak inspiratory airway pressure (PIP), inspiratory capacity, compliance of the respiratory system, pressure-volume curves, and arterial blood gas tensions, as described in Supplemental Digital Content 1, http://links.lww.com/ALN/A775.

Evaluation of Pulmonary Edema, Inflammation, and Histologic Changes

Protein concentration in BAL fluid was measured (Bradford assay; Sigma–Aldrich) to assess pulmonary edema formation. Lung inflammation was determined by measuring interleukin-6 (IL-6 enzyme-linked immunosorbent assay; R&D Systems, Minneapolis, MN) and leukocyte concentrations in BAL fluid.

Paraffin-embedded 6-μm lung sections were stained with hematoxylin and eosin or reacted with an antimouse neutrophil antibody (Cedarlane Laboratories, Burlington, Ontario, Canada) to evaluate histopathologic changes and neutrophil infiltration (see Materials and Methods in Supplemental Digital Content 1, http://links.lww.com/ALN/A775, which provides the detailed methodology of staining tissue sections).

Evaluation of Oxidative Stress and Pulmonary Gene Expression

Total, reduced (GSH) and oxidized (GSSG), glutathione concentrations in lung tissue were measured using an enzymatic assay (Cayman Chemical, Ann Arbor, MI).

Quantitative real-time polymerase chain reaction was used to determine the pulmonary expression of the neutrophil chemoattractant cytokine CXCL-2 (*i.e.*, macrophage inflammatory protein 2), the leukocyte adhesion molecules CD11b (*i.e.*, integrin α M), and L-selectin, as well as the following Nrf2-dependent antioxidant genes: NAD(P)H: quinone oxidoreductase (NQO1), glutathione-S-transferase A4 (GST-A4), and glutathione peroxidase 2 (GPX2) (see Materials and Methods in Supplemental Digital Content 1, http://links.lww.com/ALN/A775, which provides the detailed methodology of polymerase chain reaction).

Statistical Analysis

Animals subjected to HV_T ventilation *without* H_2S (0 ppm) were compared with H_2S-treated animals (60 ppm) subjected to HV_T ventilation and with animals not subjected to HV_T ventilation (controls) using ANOVA and Bonferroni multiple comparison tests (two comparisons: controls *vs.* 0 ppm H_2S; 0 *vs.* 60 ppm H_2S). Na_2S-treated animals subjected to HV_T ventilation were compared with vehicle-injected animals using Student unpaired *t* test (between-group comparison). Survival curves were compared with the log-rank Mantel–Cox test. Compliance of the respiratory system and PIP values at the beginning (1 h of HV_T) and end of HV_T ventilation were compared with the Student paired *t* test (within-group comparison). All tests were two-tailed and were performed with GraphPad Prism® version 5.01 for Windows (GraphPad Software, San Diego, CA). Data are expressed as mean ± SEM, unless indicated otherwise.

Results

Duration of HV_T Ventilation in Mice Treated with and without H_2S or Na_2S

Mice breathing 40% oxygen without added H_2S died of acute lung injury or fulfilled the criteria for being killed after 224 min (median) of HV_T ventilation (fig. 1). That is similar to the median survival time of mice ventilated with 1 ppm (236 min) or 5 ppm (215 min) of inhaled H_2S. In contrast, the median survival time during HV_T ventilation was shorter in mice breathing 60 ppm H_2S (166 min; $P = 0.0016$ *vs.* 0 ppm H_2S). The median survival time in vehicle-treated animals was 231 min. Remarkably, all Na_2S-treated animals were alive at the end of the experiment (240 min of HV_T ventilation) and were then killed.

Effect of Inhaled H_2S on Respiratory Mechanics during HV_T Ventilation

Ventilation with a tidal volume of 40 ml/kg resulted in a PIP of 33–35 cm H_2O in all groups during the first hour of HV_T

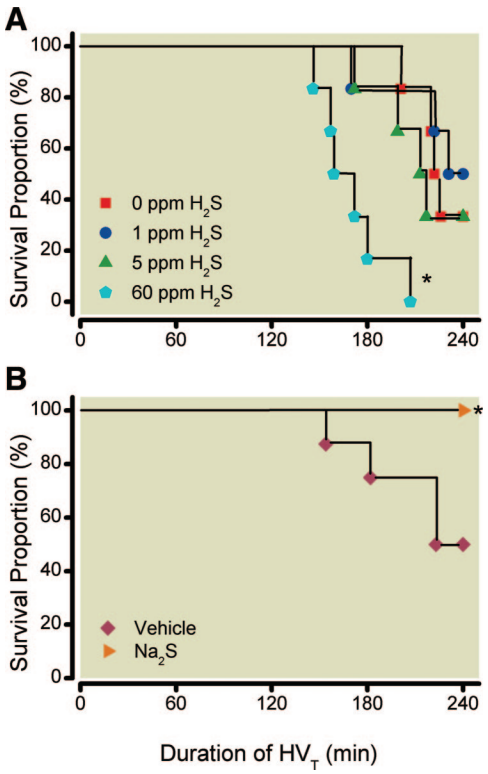

Fig. 1. Survival of mice subjected to high tidal volume (HV_T) ventilation (40 ml/kg) in the presence and absence of various concentrations of inhaled hydrogen sulfide (H_2S) (n = 6 for each concentration) (*A*) or after intravascular administration of sodium sulfide (Na_2S) (n = 8) (*B*). Mice were killed when their mean arterial pressure decreased to less than 60 mmHg (for more than 5 min) or after a maximum duration of 240 min of HV_T ventilation. * $P = 0.0016$ for 0 *versus* 60 ppm H_2S and $P = 0.0256$ for vehicle *versus* Na_2S.

ventilation. In mice breathing 40% oxygen without added H_2S, HV_T ventilation induced a gradual increase of PIP to 43 cm H_2O by the end of the experiment. A comparable increase in PIP was observed in mice ventilated with 1, 5, or 60 ppm H_2S (table 1). This increased PIP was associated

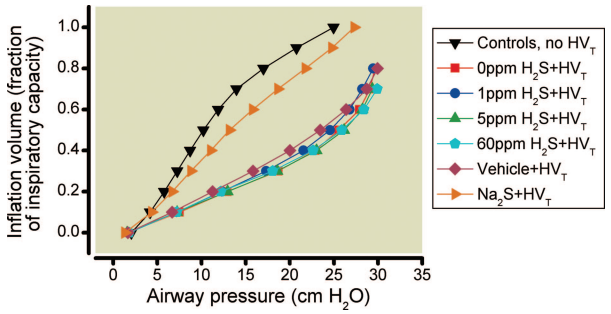

Fig. 2. Compliance of the respiratory system was assessed in mice subjected to high tidal volume (HV_T) ventilation in the presence and absence of various concentrations of inhaled hydrogen sulfide (H_2S) (n = 6 for each concentration) or after HV_T ventilation in mice pretreated with intravascular sodium sulfide (Na_2S) (n = 8) or vehicle (n = 8). Pressure-volume curves were plotted as the inflation volume (expressed as a fraction of inspiratory capacity) as a function of airway pressure and were compared with control mice not subjected to HV_T ventilation (n = 4). Deterioration of respiratory mechanics is indicated by a downward shift of the pressure-volume curve compared with controls. Na_2S treatment prevents the deterioration of respiratory system compliance induced by HV_T ventilation (40 ml/kg body weight). Pressure-volume curves are displayed as the mean of all curves in each experimental group. For clarity, only the mean values are displayed.

with a marked reduction of respiratory system compliance (table 1). Reduced respiratory system compliance was also reflected by a significant downward shift of the pressure-volume curves (fig. 2) measured in mice after HV_T ventilation with and without H_2S compared with those curves measured in mice not subjected to HV_T ventilation.

Effect of Inhaled H_2S on Arterial Blood Gas Tensions during HV_T Ventilation

In control mice, killed after 5 min of baseline ventilation (F_{IO_2} 0.4), the Pa_{CO_2} was 40 ± 4 mmHg, and the Pa_{O_2} was 239 ± 16 mmHg (fig. 3). After HV_T ventilation (0 ppm

Table 1. Respiratory Mechanics

	Peak Inspiratory Pressure (cm H_2O)		Respiratory Compliance (μl/cm H_2O)	
	1 h HV_T	End of HV_T	1 h HV_T	End of HV_T
Inhalation				
0 ppm H_2S	35 ± 0.3	43 ± 0.1*	38 ± 1.1	22 ± 1.6
1 ppm H_2S	34 ± 0.4	42 ± 0.7*	40 ± 1.3	20 ± 2.2*
5 ppm H_2S	35 ± 0.6	42 ± 0.1*	40 ± 0.6	18 ± 1.3*
60 ppm H_2S	33 ± 0.5	42 ± 0.2*	39 ± 0.9	18 ± 0.7*
Intravascular infusion				
Vehicle	34 ± 0.2	43 ± 0.3*	40 ± 0.4	24 ± 1.4*
Na_2S, 0.55 mg/kg	35 ± 0.2	39 ± 0.6†	39 ± 0.6	34 ± 2.3†

Respiratory mechanics were measured in mice subjected to high tidal volume (HV_T) ventilation. Values represent mean ± SEM after 60 min (1 h HV_T) and at the end of HV_T ventilation (maximum 240 min).

* $P < 0.05$ *vs.* 1 h HV_T. † $P < 0.05$ *vs.* vehicle.

H_2S = hydrogen sulfide; Na_2S = sodium sulfide.

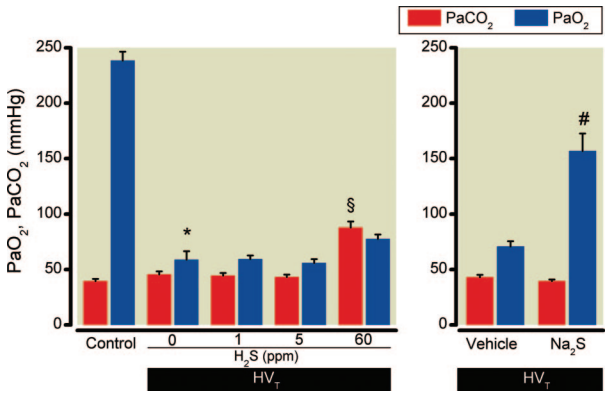

Fig. 3. Arterial oxygen (Pa_{O_2}) and carbon dioxide (Pa_{CO_2}) tensions of mice subjected to high tidal volume (HV_T) ventilation (F_{IO_2} 0.4) in the presence and absence of various concentrations of inhaled hydrogen sulfide (H_2S) (n = 6 for each concentration) or after intravascular administration of sodium sulfide (Na_2S) or vehicle (n = 8 in each group). Control mice were not subjected to HV_T ventilation but were briefly ventilated at tidal volume 10 ml/kg and F_{IO_2} 0.4 (n = 4). Pa_{O_2} and Pa_{CO_2} were measured in arterial blood obtained from the carotid artery. Arterial oxygenation was impaired by HV_T ventilation, but the decrease in Pa_{O_2} was attenuated by Na_2S pretreatment. * $P < 0.0001$ *versus* control, § $P = 0.0001$ *versus* 0 ppm H_2S, # $P = 0.0001$ *versus* vehicle. F_{IO2} = inspired oxygen fraction.

H_2S), Pa_{CO_2} did not change, but Pa_{O_2} decreased to 59 ± 17 mmHg at the end of the experiment. Similarly, in mice breathing 1 or 5 ppm H_2S, Pa_{CO_2} did not change but Pa_{O_2} decreased to 60 ± 6 and 56 ± 8 mmHg, respectively. In contrast, in mice breathing 60 ppm H_2S, Pa_{CO_2} increased to 88 ± 13 mmHg ($P = 0.0001$ *vs.* 0 ppm) and Pa_{O_2} decreased to 77 ± 10 mmHg (P = not significant *vs.* 0 ppm) in response to HV_T ventilation.

Effect of Inhaled H_2S on the Formation of Pulmonary Edema during HV_T Ventilation

To investigate whether alveolar-capillary barrier disruption with pulmonary edema formation contributes to the observed impairment of arterial oxygenation, the protein concentration in BAL fluid was measured (fig. 4). BAL fluid protein concentrations were consistently higher in all mice subjected to HV_T ventilation with and without inhaled H_2S than in controls.

Effects of Inhaled H_2S on BAL Fluid IL-6 and Leukocyte Concentrations during HV_T Ventilation

To evaluate pulmonary inflammation, we measured the concentrations of the proinflammatory cytokine IL-6 and the concentration of leukocytes in BAL fluid (fig. 5). BAL fluid IL-6 concentrations were greater in mice subjected to HV_T ventilation than in controls. In contrast, BAL fluid IL-6 concentrations were decreased in mice ventilated with 60 ppm H_2S than without H_2S (fig. 5A). BAL fluid leukocyte concentrations (fig. 5B) invariably were reduced by HV_T ventilation in mice compared with controls, independent of whether the mice were ventilated with or without H_2S.

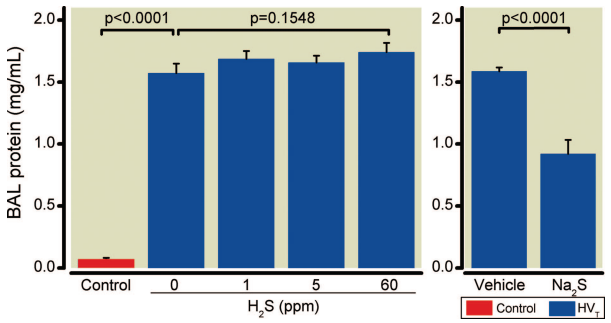

Fig. 4. Protein concentrations in bronchoalveolar lavage (BAL) fluid obtained from mice after high tidal volume (HV_T) ventilation. BAL fluid protein concentrations were measured subsequent to HV_T ventilation in the presence and absence of various concentrations of inhaled hydrogen sulfide (H_2S) (n = 6 for each concentration) or after intravascular administration of sodium sulfide (Na_2S) or vehicle (n = 8 in each group), and in control mice not subjected to HV_T ventilation (n = 4).

Effects of Inhaled H_2S on Pulmonary Expression of Leukocyte Chemoattractant and Adhesion Molecules during HV_T Ventilation

The pulmonary messenger RNA (mRNA) concentrations of CXCL-2, CD11b, and L-selectin in mice subjected to HV_T ventilation were approximately 7-, 3-, and 1.5-fold greater than in controls (fig. 6). Inhalation of 60 ppm H_2S during HV_T ventilation augmented the increase in CXCL-2, CD11b, and

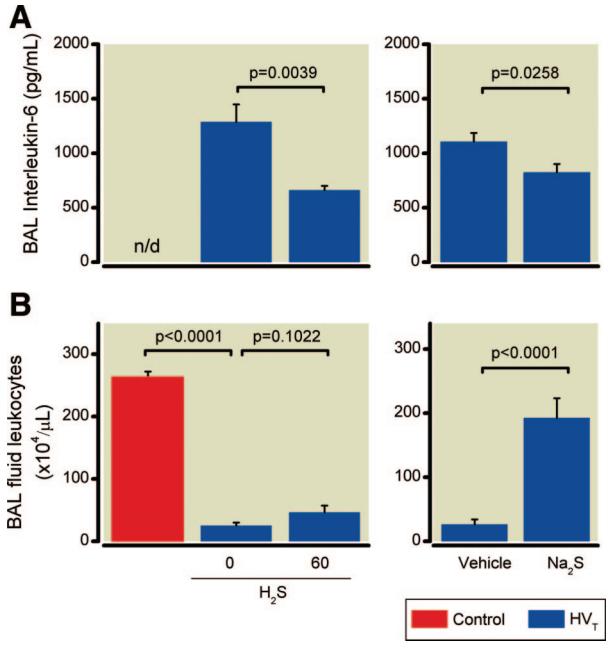

Fig. 5. Concentrations of interleukin-6 (IL-6) (*A*) and leukocytes in bronchoalveolar lavage (BAL) fluid (*B*) obtained from mice after high tidal volume (HV_T) ventilation. IL-6 and leukocyte concentrations were measured subsequent to HV_T ventilation in the presence and absence of inhaled hydrogen sulfide (H_2S) (n = 6 for each concentration) or after intravascular administration of sodium sulfide (Na_2S) or vehicle (n = 8 in each group), and in control mice not subjected to HV_T ventilation (n = 4). n/d = not detectable.

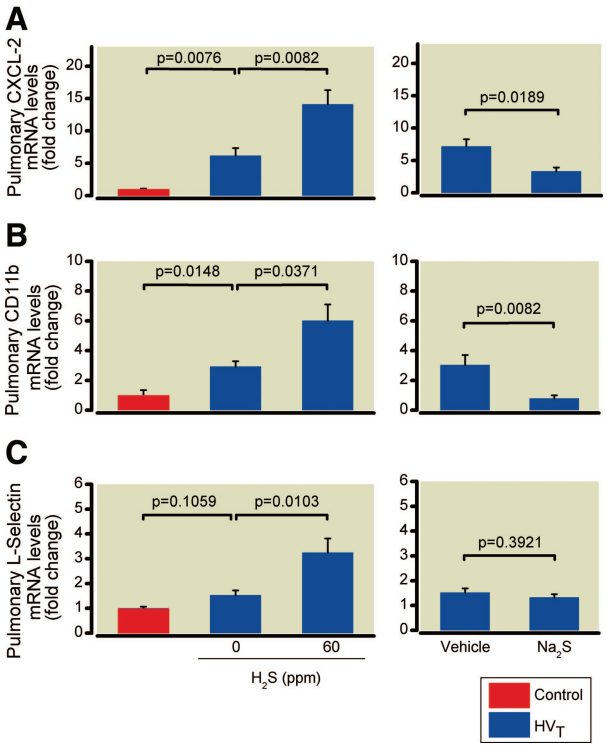

Fig. 6. Pulmonary messenger RNA (mRNA) concentrations of CXCL-2 (*A*), CD11b (*B*), and L-selectin (*C*) in mice subjected to high tidal volume (HV_T) ventilation in the presence and absence of hydrogen sulfide (H_2S) (n = 6 for each concentration) or after intravascular administration of sodium sulfide (Na_2S) or vehicle (n = 8 in each group). mRNA concentrations are expressed as fold change relative to the average expression values in control mice not subjected to HV_T ventilation (n = 4).

L-selectin mRNA concentrations (15-, 6-, and 3-increase *vs.* controls).

Effects of Inhaled H_2S on Oxidative Stress and Pulmonary Expression of Antioxidant Genes

The concentration of total glutathione and the ratio of reduced to oxidized glutathione (GSH/GSSG) were significantly lower in lungs subjected to HV_T ventilation than in controls (fig. 7). This decrease was independent of whether mice were ventilated with or without H_2S.

The pulmonary mRNA concentrations of NQO1 and GPX2 in mice subjected to HV_T ventilation were approximately 1.6- (not significant) and 2-fold higher than in controls (fig. 8). Inhalation of 60 ppm H_2S during HV_T ventilation attenuated the increase in NQO1 and GPX2 mRNA concentrations (1.1- and 1.3-fold increase *vs.* controls). HV_T ventilation with 0 or 60 ppm H_2S reduced the pulmonary mRNA concentrations of GST-A4 to 66% and 54%, respectively, of the concentrations measured in control mice.

Effect of Na_2S on Respiratory Mechanics during HV_T Ventilation

In vehicle-treated animals, PIP and respiratory system compliance deteriorated to the same extent as in mice receiving

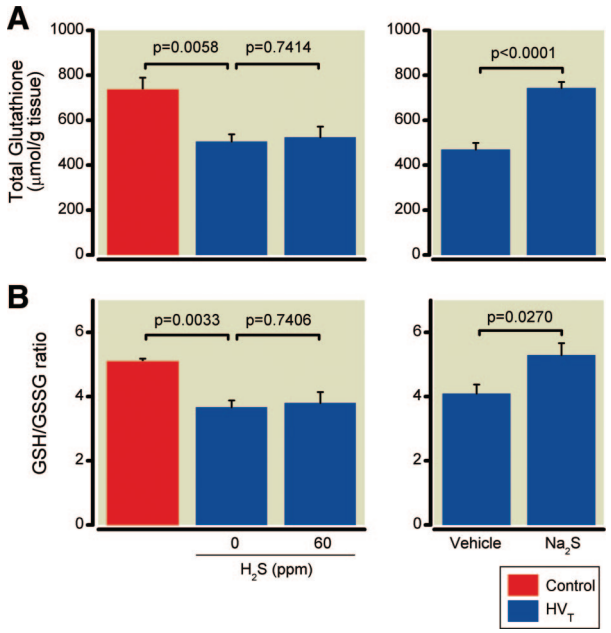

Fig. 7. Total glutathione concentrations (*A*) and the ratio of reduced to oxidized glutathione (GSH/GSSG) (*B*) in lung tissues of mice after high tidal volume (HV_T) ventilation in the presence and absence of inhaled hydrogen sulfide (H_2S) (n = 6 for each concentration) or after intravascular administration of sodium sulfide (Na_2S) or vehicle (n = 8 in each group) and in control mice not subjected to HV_T ventilation (n = 4).

HV_T ventilation alone (table 1). In contrast, treatment with Na_2S attenuated the PIP increase, preserved respiratory system compliance, and reduced the downward shift of the pressure-volume curve (fig. 2).

Effect of Na_2S on Arterial Blood Gas Tensions during HV_T Ventilation

In vehicle-treated animals, Pa_{CO_2} was stable and Pa_{O_2} decreased after HV_T ventilation (fig. 3). Compared with vehicle-treatment, Na_2S treatment attenuated the decrease in Pa_{O_2} in mice subjected to HV_T ventilation. At the end of the experiment, Pa_{O_2} was higher in Na_2S-treated animals than in vehicle-treated animals (157 ± 45 *vs.* 71 ± 14 mmHg, $P = 0.0001$).

Effect of Na_2S on the Formation of Pulmonary Edema during HV_T Ventilation

BAL fluid protein concentrations were greater in vehicle-treated mice subjected to HV_T ventilation than in controls (fig. 4). In contrast, BAL fluid protein concentrations were lower in Na_2S-treated mice than in vehicle-treated mice.

Effects of Na_2S on BAL Fluid IL-6 and Leukocyte Concentrations during HV_T Ventilation

BAL fluid IL-6 concentrations were greater in vehicle-treated animals subjected to HV_T ventilation than in controls. Na_2S

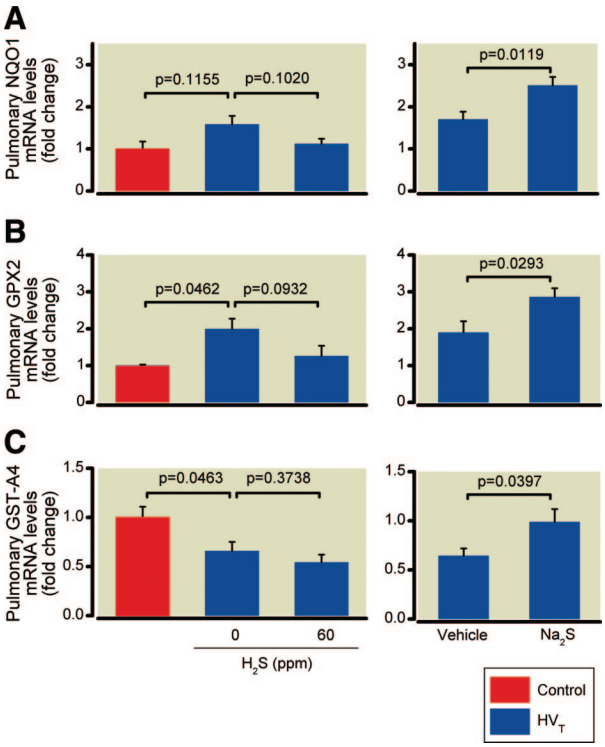

Fig. 8. Pulmonary messenger RNA (mRNA) concentrations of the Nrf2-dependent antioxidant genes NAD(P)H:quinone oxidoreductase, NQO1 (*A*), glutathione peroxidase 2, GPX2 (*B*), and glutathione-S-transferase A4, GST-A4 (*C*) in mice subjected to high tidal volume (HV_T) ventilation in the presence and absence of hydrogen sulfide (H_2S) (n = 6 for each concentration) or after intravascular administration of sodium sulfide (Na_2S) or vehicle (n = 8 in each group). mRNA concentrations are expressed as fold change relative to the average expression values in control mice not subjected to HV_T ventilation (n = 4).

treatment reduced BAL fluid IL-6 concentrations in mice subjected to HV_T ventilation (fig. 5A).

High tidal volume ventilation reduced the leukocyte concentration in BAL fluid obtained from vehicle-treated mice compared with that from controls. In contrast, Na_2S treatment restored the concentration of leukocytes in BAL fluid of mice subjected to HV_T ventilation (fig. 5B).

Effects of Na_2S on Pulmonary Expression of Leukocyte Chemoattractant and Adhesion Molecules during HV_T Ventilation

The CXCL-2 and CD11b mRNA concentrations measured in the lungs of vehicle-treated animals subjected to HV_T ventilation (7- and 3-fold increase *vs.* controls) were significantly attenuated by Na_2S treatment (3- and 0.8-fold increase *vs.* controls, fig. 6, A and B). In contrast, the pulmonary mRNA concentrations of L-selectin measured in vehicle-treated animals (1.5-fold increase *vs.* controls) did not differ from those measured in Na_2S-treated animals (1.3-fold increase *vs.* controls, fig. 6C).

Effects of Na_2S on Oxidative Stress and Pulmonary Expression of Antioxidant Genes

Total glutathione concentrations and the GSH/GSSG in lung tissues obtained from mice subjected to HV_T ventilation were significantly higher in Na_2S-treated animals than in vehicle-treated animals (fig. 7). The increase of antioxidant NQO1 and GPX2 mRNA concentrations measured in the lungs of vehicle-treated animals subjected to HV_T ventilation (1.7- and 1.9-fold increase *vs.* controls) was significantly enhanced by Na_2S treatment (2.5- and 2.9-fold increase *vs.* controls, fig. 8). Moreover, Na_2S treatment prevented the reduction of GST-A4 mRNA concentrations measured in vehicle-treated animals subjected to HV_T ventilation.

Pulmonary Histopathologic Changes from HV_T Ventilation

Histologic analysis of lung sections from mice treated with either 60 ppm inhaled H_2S or with injection of Na_2S revealed that HV_T ventilation induced signs of edema formation and airway epithelial disruption (fig. 9). These pathologic signs were similar in mice ventilated with or without 60 ppm H_2S. Fewer signs of edema and epithelial disruption were observed after Na_2S pretreatment. The number of neutrophils in lung tissue sections taken from three mice subjected to HV_T ventilation was found to be greater than the number of lung tissue neutrophils determined in two control animals not subjected to HV_T ventilation (fig. 10). In addition, the numbers of lung tissue neutrophils found after HV_T ventilation in three mice with and three mice without inhalation of 60 ppm H_2S were similar. In contrast, the number of neutrophils found in lung tissue sections taken from three mice subjected to HV_T ventilation after Na_2S pretreatment was smaller. Despite the small sample size of tissue sections used for neutrophil staining, the variance of lung tissue neutrophil concentrations is small. The interpretation of these data must be made with great caution.

Additional figures describing the effect of various concentrations of inhaled H_2S on BAL fluid leukocyte and IL-6 concentrations, pulmonary expression of leukocyte chemoattractant and adhesion molecules, and oxidative stress parameters and antioxidant gene expression are provided in Supplemental Digital Content 2, http://links.lww.com/ALN/A776, figures 1–4.

Discussion

This study was performed to evaluate whether inhalation of H_2S gas protects against lung injury from HV_T ventilation in mice. We found that inhalation of 1 or 5 ppm H_2S produces neither beneficial nor deleterious effects, whereas inhalation of 60 ppm H_2S accelerates the development of lung injury and death from HV_T ventilation. In contrast, treatment with a bolus infusion of Na_2S attenuates pulmonary edema formation, thereby limiting the deterioration of respiratory system compliance and arterial oxygenation. Our results suggest that these protective effects of Na_2S in VILI are linked to attenuated expression of chemoattractant and leukocyte ad-

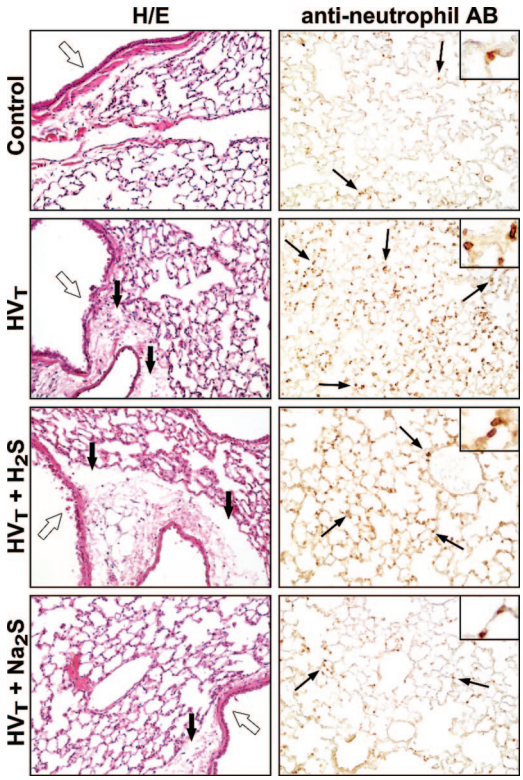

Fig. 9. Representative lung sections of mice subjected to high tidal volume (HV_T) ventilation in the presence and absence of 60 ppm hydrogen sulfide (H_2S) or after intravascular administration of sodium sulfide (Na_2S). These sections were stained with hematoxylin and eosin (H/E) or reacted with antibodies (AB) against neutrophils (*inset*: higher magnification) and were compared with sections from nonventilated control mice. In control mice, the airway epithelium (*open arrows*) is intact, no edema surrounding the airway is seen, and few neutrophils are visible (*thin black arrows*). HV_T ventilation induces airway disruption, edema formation between airways and adjacent vessels (*thick black arrows*), and neutrophil infiltration. Similar pathologic changes were present in lungs subjected to HV_T ventilation in the presence of 60 ppm H_2S. Less epithelial disruption and edema and fewer neutrophils are seen in lung tissue sections after Na_2S pretreatment.

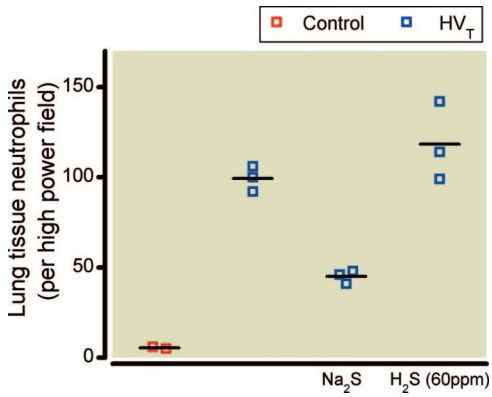

Fig. 10. Pulmonary infiltration with neutrophils in mice subjected to high tidal volume (HV_T) ventilation in the presence and absence of inhaled hydrogen sulfide (H_2S) (60 ppm) or after intravascular administration of sodium sulfide (Na_2S) (n = 3 in each group). Lung tissue sections were reacted with antibodies against neutrophils and were compared with lung sections obtained from control mice not subjected to HV_T ventilation (n = 2). Representative examples of lung sections are displayed in figure 9. Neutrophil concentrations are reported as the number of neutrophils per high power field (magnification ×40).

hesion molecules, enhanced expression of Nrf2-dependent antioxidant genes, and improved availability of reduced glutathione in the lung.

Our findings underscore the potential toxicity of H_2S gas inhalation in a rodent model of VILI. Lopez *et al.* reported cytotoxic effects and edema formation in respiratory tract tissues of rats breathing 10–400 ppm H_2S.[17] The primary biochemical mechanism responsible for the toxicity of H_2S gas is believed to involve the inhibition of cytochrome c oxidase.[18] Inhibition of cytochrome c oxidase blocks oxidative phosphorylation, a major source of cellular adenosine triphosphate synthesis. In turn, the abolishment of oxidative phosphorylation implies functional hypoxia (inability to use available oxygen for oxidative metabolism).[18] Both hypoxia and a lack of adenosine triphosphate are associated with pulmonary vasoconstriction, impaired alveolar fluid clearance, and pulmonary edema (reviewed in Bärtsch *et al.*[19]). Based on these considerations, the inhalation of 60 ppm H_2S is likely to have contributed to the formation of pulmonary edema in our murine model.

Faller *et al.* reported that inhalation of 80 ppm H_2S limited cytokine release in a model of lung injury in C57BL/6 mice caused by ventilation with a moderate tidal volume (12 ml/kg). Similarly, we found that BAL fluid IL-6 concentrations were lower when mice developed lung injury in the presence of 60 ppm H_2S (fig. 5). However, the current study demonstrates that under conditions of higher mechanical lung stretch (peak airway pressure 35 cm H_2O, as opposed to ~12 cm H_2O in the Faller *et al.* study), the potential antiinflammatory effects of inhaled H_2S are outweighed by the deleterious effects of inhaling 60 ppm H_2S, including enhanced pulmonary expression of chemoattractant and leukocyte adhesion molecules and earlier deaths (figs. 1 and 6). In addition, H_2S inhalation prevented the up-regulation of antioxidant gene expression in mice subjected to HV_T ventilation (fig. 8) and caused a decrease in both the GSH/GSSG and total glutathione concentrations in the lung (fig. 7).

Mechanical stretching of the lung may initiate intravascular pulmonary leukocyte sequestration in mice.[20] Various chemoattractant and adhesion molecules that are derived from epithelial and endothelial cells, as well as alveolar macrophages and fibroblasts, are responsible for inflammatory recruitment and migration of leukocytes from the circulation into injured lung tissue.[21] In particular, the neutrophil chemoattractant CXCL-2 and the adhesion molecules CD11b and L-selectin are reported to play a critical role in the pathogenesis of VILI and for control of leukocyte trafficking into the lung.[22,23] Ultimately, pulmonary neutrophil activation

and accumulation promotes microvascular permeability and edema formation.[3,22] Our results demonstrate that HV_T ventilation increases the pulmonary expression of CXCL-2, CD11b, and L-selectin. Moreover, the HV_T-induced increase in CXCL-2, CD11b, and L-selectin was enhanced markedly by concomitant inhalation of 60 ppm H_2S (fig. 7). These findings suggest that the pathophysiologic changes leading to an earlier death in mice ventilated with 60 ppm H_2S appear to be linked to enhanced expression of chemoattractant and leukocyte adhesion molecules in the lung.

As an alternative to the inhalation of potentially toxic H_2S gas, we examined whether intravascular administration of Na_2S can attenuate the development of VILI during HV_T ventilation. We found that systemic treatment with Na_2S protects against VILI and reduces GSH/GSSG imbalances (fig. 7) in mice subjected to HV_T ventilation. Our results also suggest that Na_2S attenuates the pulmonary expression of CXCL-2 and CD11b during VILI (fig. 6). This finding is supported by other reports demonstrating that both Na_2S and sodium hydrogen sulfide can impair leukocyte adherence to endothelium and subsequent diapedesis during inflammation *in vivo* by reducing the expression of endothelial and leukocyte adhesion molecules.[24,25] Increased pulmonary leukocyte adhesion in mice subjected to HV_T ventilation may also account for the low concentrations of BAL leukocytes reported here (fig. 5B) and elsewhere.[26] The protective effect of Na_2S in this model of lung injury produced by HV_T ventilation is consistent with the beneficial effect of sodium hydrogen sulfide in other rodent models of lung injury induced by oleic acid[27] or a skin burn and smoke inhalation.[28]

The current study demonstrates that Na_2S pretreatment, but not H_2S inhalation during HV_T ventilation, enhances the pulmonary expression of Nrf2-dependent antioxidant genes and stabilizes the concentration of reduced glutathione in the lung (fig. 6). A transcription factor for several antioxidant genes, Nrf2 is a master regulator of antioxidant response mechanisms (reviewed in Maher and Yamamoto[29]). Activation of Nrf2 results in the induction of many cytoprotective proteins. In the lung, Nrf2-dependent genes regulate the cellular redox status and have been reported to modulate pulmonary cellular responses and oxidative stress induced by mechanical ventilation.[5] The reasons inhaled H_2S and intravenous Na_2S have distinct effects in VILI in the current study are likely to be multifactorial. It is possible that pretreatment with Na_2S before injurious ventilation is required to induce the Nrf2-dependent antioxidant defense mechanisms that contribute to protecting against VILI. It is also conceivable that intravascular administration of Na_2S may preferentially target activation of Nrf2-dependent signaling in different cell types compared with inhaled H_2S (*e.g.*, activating vascular endothelial *vs.* airway epithelial cells). Analyzing the candidate cellular and molecular targets of H_2S and Na_2S in future studies may provide additional insights into their differential biologic effects.

Limitations

In patients, ventilator-associated lung injury usually occurs in lungs with reduced compliance caused by preexisting conditions (two-hit or multifactorial lung injury), including septic inflammation. The current study uses a one-hit model of VILI in healthy mouse lungs. Thus, the current study has limited clinical relevance. Although the current study demonstrates that Na_2S pretreatment protects against VILI, this study does not elucidate the potential dose-dependency of this protective effect and does not investigate the potential toxicity of Na_2S to central or peripheral organs. Lung tissue H_2S concentrations were not measured in this study. Comparing the lung tissue concentrations of sulfide species in future studies may help explain the differential biologic effects of inhaled H_2S and intravascular Na_2S and their dose-dependency.

Summary

Our study demonstrates that continuous inhalation of H_2S gas enhances the expression of leukocyte adhesion and chemoattractant molecules (CXCL-2, CD11b, and L-selectin), accelerates pulmonary edema formation, and promotes lung injury when it is inhaled at high concentrations (60 ppm) during mechanical ventilation with a high tidal volume. The deleterious effects of H_2S gas inhalation reemphasize that H_2S can cause pulmonary toxicity and do not suggest a substantial role for H_2S in the prevention and treatment of VILI in patients. In contrast, our data suggest that systemic intravascular treatment with Na_2S may represent a novel therapeutic strategy to prevent both VILI and GSH/GSSG imbalance by activating Nrf2-dependent antioxidant gene transcription.

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