Inhaled Hydrogen Sulfide Protects against Ventilator-induced Lung Injury

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ABSTRACT

Background: Mechanical ventilation still causes an unacceptably high rate of morbidity and mortality because of ventilator-induced lung injury (VILI). Therefore, new therapeutic strategies are needed to treat VILI. Hydrogen sulfide can induce hypothermia and suspended animation-like states in mice. Hydrogen sulfide can also confer antiinflammatory and antiapoptotic effects. This study investigates the organ-protective effects of inhaled hydrogen sulfide during mechanical ventilation.

Methods: Mice were ventilated with a tidal volume of 12 ml/kg body weight for 6 h with synthetic air in the absence or presence of hydrogen sulfide (80 parts per million) and, in a second series, at either mild hypothermia or normothermia. Staining of lung sections determined the degree of lung damage by VILI score and apoptotic cells. Bronchoalveolar lavage fluid was analyzed for the cytokines interleukin-1β and macrophage inflammatory protein-1β and for neutrophil accumulation. Heme oxygenase-1 and heat shock protein 70 expression were assessed in the lung tissue by Western immunoblot analysis.

Results: Mechanical ventilation at both hypothermia and normothermia led to a profound development of VILI, characterized by pulmonary edema, increased apoptosis, cytokine release, neutrophil recruitment, and up-regulation of the stress proteins such as heme oxygenase-1 and heat shock protein 70. In contrast, the application of hydrogen sulfide during ventilation at either mild hypothermia or normothermia prevented edema formation, apoptosis, proinflammatory cytokine production, neutrophil accumulation, and inhibited heme oxygenase-1 expression.

Conclusions: Inhalation of hydrogen sulfide during mechanical ventilation protects against VILI by the inhibition of inflammatory and apoptotic responses. Hydrogen sulfide confers lung protection independently of its ability to induce mild hypothermia during ventilation.

What We Already Know about This Topic

- ❖ Mechanical ventilation can cause acute lung injury in critically ill patients
- ❖ Inhaled hydrogen sulfide can reduce inflammation and protect other organs, perhaps in part by inducing mild hypothermia

What This Article Tells Us That Is New

- ❖ In mice, inhaled hydrogen sulfide reduced inflammation and lung injury from mechanical ventilation that was independent of its effect on body temperature

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MECHANICAL ventilation is an indispensable tool for the clinical treatment of critically ill patients. Despite its life-saving properties, mechanical ventilation can induce lung injury in the healthy lung or exacerbate acute lung injury, referred to as ventilator-induced lung injury (VILI). VILI arises from cyclic stretch of the lung, leading to the tissue disruption, pulmonary edema, alveolar proteinosis, neutrophil influx, and release of inflammatory mediators.[1] The inflammatory response to mechanical ventilation is not restricted to the lung, but it may also spread to extrapulmonary organs, leading to a systemic inflammatory response and life-threatening multiple organ dysfunction.[2] Although

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◇ 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: Otulakowski G, Kavanagh BP: Hydrogen sulfide in lung injury: Therapeutic hope from a toxic gas. ANESTHESIOLOGY 2010; 113:4–6.

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modulation of ventilator settings such as applying low tidal volumes may improve lung injury in patients with acute respiratory distress syndrome,[3] VILI remains a major problem in the intensive care unit with an unacceptably high rate of morbidity and mortality.[2] Therefore, alternative therapeutic strategies must be developed to further minimize the risk of ventilator-associated lung injury. Among the possibilities, the induction of hibernation or suspended animation-like states in otherwise nonhibernating mammals may be of therapeutic interest. Several clinical trials have investigated the beneficial effects of suspended animation (*i.e.*, induced hypothermia) to prevent organ damage and improve clinical outcome.[4,5] Because suspended animation is known to mediate cytoprotection,[6] suspended animation-like states may conceivably alleviate VILI during mechanical ventilation.

Recently, hydrogen sulfide has been demonstrated to elicit a suspended animation-like state in mammals.[7,8] Hydrogen sulfide has long been exclusively regarded as a highly toxic gas with the smell of rotten eggs. In fact, high concentrations of inhaled hydrogen sulfide for extended exposure times might cause cytotoxicity, for example, within the respiratory tract,[9,10] and impair neurobehavioral function.[11] However, extensive research during the past few years has challenged this dogma with potential biomedical applications. Together with carbon monoxide and nitric oxide, hydrogen sulfide belongs to a class of endogenously synthesized gaseous molecules referred to as "gasotransmitters." In mammals, including humans, hydrogen sulfide is synthesized from L-cysteine by the cystathionine β-synthase or the cystathionine γ-lyase, both use pyridoxal $5'$-phosphate as a cofactor. Endogenous hydrogen sulfide exerts several biologic effects, including vasodilatation by opening smooth muscle adenosine triphosphate-sensitive potassium channels,[12] regulation of inflammatory responses,[13–15] or induction of the stress protein heme oxygenase-1 (HO-1).[16] Endogenously synthesized hydrogen sulfide has been shown to limit renal and myocardial ischemia–reperfusion injury.[17–19] Treatment with inhaled hydrogen sulfide or hydrogen sulfide donors can induce cardioprotective,[20,21] gastroprotective,[22] antinociceptive,[23,24] and antiinflammatory effects.[22,25] The latter effect of hydrogen sulfide involves the inhibited release of proinflammatory cytokines and neutrophil adhesion and activation.[22,25–27] These studies demonstrate that exogenously administered hydrogen sulfide might suppress inflammatory responses, a hallmark in the progression of lung injury in response to mechanical ventilation.

Based on these recent findings, the aim of the current study was to investigate the effects of inhaled hydrogen sulfide on the development of VILI in mice. We show that hydrogen sulfide substantially protects against VILI. This effect is mediated by the inhibition of neutrophil influx and inflammatory protein release. Although hydrogen sulfide decreases body temperature in animals, mild hypothermia does not seem to play a contributory role in the direct lung protective effect of hydrogen sulfide during mechanical ventilation.

Materials and Methods

Animals
Male C57BL/6N mice were obtained from Charles River Laboratories (Sulzburg, Germany) and used at a body weight of 25–27 g. All animal experiments were performed in accordance with guidelines of the local animal care commission (Ethics Committee University Freiburg, Freiburg, Germany, permission No. G-07/25). All mice were anesthetized with 90 mg/kg ketamine (intraperitoneal) and 1 mg/kg acepromazine (intraperitoneal) and placed on a heating pad.[28,29] A polyethylene catheter was inserted into the left carotid artery for direct blood pressure monitoring and for blood gas sampling, and a tracheotomy was established using a 20-gauge catheter. While nonventilated control mice were subjected to the instrumentation after 6-h spontaneous breathing and being killed immediately after, mice randomized to receive mechanical ventilation were connected to a rodent ventilator (Voltek enterprises, Toronto, Canada) *via* the tracheal canula. The ventilator was set to a tidal volume of 12 ml/kg body weight, frequency 80/min, positive end-expiratory pressure of 2 cm H_2O. Muscular relaxation was achieved by applying 2 mg/kg pancuronium (intraperitoneal). Anesthesia was maintained by continuous administration of ketamine, acepromazine, and pancuronium intraperitoneal as needed. A 0.7-ml saline bolus was injected intraperitoneally to compensate for evaporation during ventilation. Blood samples were withdrawn from ventilated animals after 30–45 min and 6 h and measured using an automated blood gas analyzer (ABL600/800, Radiometer, Copenhagen, Denmark) to ensure normal ventilation. Recruitment maneuvers (inspiratory hold on 30 cm H_2O for 5 s) were performed every 60 min during ventilation to prevent atelectasis. Body temperature (T_b [degrees Celsius, °C], measured with a rectal thermoelement), arterial blood pressure (millimeters mercury, mmHg), peak airway pressure, and plateau airway pressure (centimeters water) were continuously monitored (PowerLab 3/80, ADInstruments, Spechbach, Germany) and recorded every 30 min.

Experimental Groups
In the first set of experiments, nonventilated control mice were subjected to breathe synthetic air or synthetic air supplemented with 80 ppm H_2S spontaneously for 6 h (hydrogen sulfide, Air Liquide, Kornwestheim, Germany) in a sealed chamber. Mice receiving mechanical ventilation were randomized to be ventilated with either synthetic air or synthetic air supplemented with 80 ppm H_2S for 6 h. All ventilated mice were placed on a 36.5°C warm heating pad. In a second independent set of experiments, nonventilated control mice were subjected to breathe synthetic air. Mechanically ventilated mice were divided into four different groups: ventilation with synthetic air at mild hypothermia (mean T_b of 34°C), ventilation with synthetic air at normothermia (mean T_b of 36°C), ventilation with 80 ppm H_2S at mild hypothermia (mean T_b of 34°C), and ventilation with 80 ppm H_2S at normothermia (mean T_b of 36°C).

Tissue Sampling and Bronchoalveolar Lavage

At the end of each experiment, mice were killed. Tissue samples were snap frozen and stored at $-80°C$ for subsequent analysis. First, the left lung lobe was ligated, and a bronchoalveolar lavage (BAL) was performed *via* the tracheal catheter in the right lung lobes using 0.8 ml phosphate-buffered saline. The recovered volume was centrifuged, and the supernatant was snap frozen and stored at $-80°C$ until further use. The pellet was redissolved, and the relative amount of neutrophils was determined from methanol-fixed cytospin slides and stained with fast green, eosin, and thiazine (Diff-Quick, Medion Diagnostics AG, Düdingen, Switzerland). After the BAL, the right lobes were removed, snap frozen, and kept until further use at $-80°C$. Afterward, the left bronchus was reopened, and 2% paraformaldehyde was administered with a constant pressure of 20 cm H_2O *via* the tracheal catheter to unfold the lobe. Then, the lobe was extracted and kept in 2% paraformaldehyde for another 2 h followed by a 30% sucrose solution at 4°C overnight. The next day, the left lung lobe was embedded into optimal cutting temperature compound (Tissue-Tek®, Sikura Fintek GmbH, Staufen, Germany) and was frozen slowly on liquid nitrogen and stored at $-80°C$ until further use.

Cytokine and Chemokine Measurements

BAL aliquots were analyzed using interleukin-1β and macrophage inflammatory protein (MIP)-1β enzyme-linked immunosorbent assay kits (R&D Systems GmbH, Wiesbaden, Germany) according to the manufacturer's instructions.

Immunohistochemistry

Cryosections (6 μm) of the left lung were subjected to hematoxylin and eosin staining and analyzed in a blinded fashion. From each lung, four representative photographs were taken (magnification 400×). Five high-power fields were randomly assigned to each photograph, and alveolar wall thickness was analyzed by Axiovision software (AxioVS40LE, Zeiss, Jena, Germany). In each high-power field, the degree of lung damage was determined by a modified VILI score[30]: (1) thickness of the alveolar walls, (2) infiltration or aggregation of inflammatory cells, and (3) hemorrhage. Each item was graded according to the following five-point scale: 0, minimal damage; 1, mild damage; 2, moderate damage; 3, severe damage; 4, maximal damage. The degree of lung damage was assessed by the sum of scores ranging from 0 to 12 for each high-power field. The average of the sum of each field score per lung was compared among groups.

Detection of apoptotic cells was performed by terminal deoxynucleotide transferase-mediated dUTP-biotin nick-end labeling using the ApopTag Peroxidase Kit (S7100, Chemicon International, Millipore, Schwalbach, Germany) according to the manufacturer's instructions. From each lung, eight representative photographs (magnification 400×, 270 × 345 μm) were taken, the apoptotic cells were counted, and the number of apoptotic cells per mm^2 was calculated (corresponds to 6 μm^3).

Immunoblotting

The right upper lung lobe was homogenized in 30 mM Tris base including complete protease inhibitors (Roche Diagnostics, Mannheim, Germany). After determination of the protein content (Biorad Assay, Biorad, Germany), equal amounts were loaded onto a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane (Millipore). The membranes were incubated with antibodies against HO-1 (spa-895, Assay Designs, Biomol GmbH, Hamburg, Germany) or heat shock protein (HSP) 70 (ab-31010, Assay Designs) overnight at 4°C. After several washing steps, the membranes were incubated for 1.5 h with the appropriate secondary antibody, detected (ECL Western Blotting Analysis System, GE Healthcare, Buckinghamshire, United Kingdom), and ex-
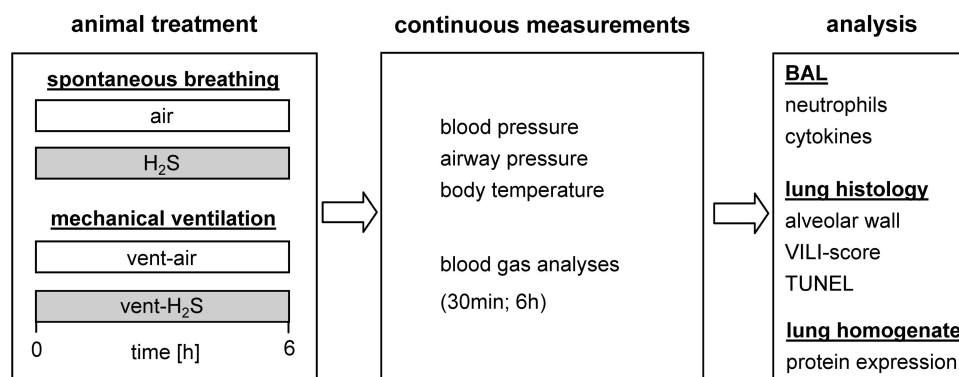
Fig. 1. Experimental design. Mice were subjected to breathe either air or 80 ppm hydrogen sulfide (H_2S) spontaneously for 6 h or they received mechanical ventilation (vent) with 12 ml/kg for 6 h with either synthetic air or synthetic air + 80 ppm H_2S. Airway pressure, blood pressure, and body temperature were continuously measured and noted every 30 min. Blood gas analyses were performed at the end of the experiment and in ventilated groups in addition to after the onset of ventilation. Bronchoalveolar lavage (BAL) and lung tissue were analyzed for the parameters depicted in the "analysis" box. TUNEL = terminal deoxynucleotide transferase-mediated dUTP-biotin nick-end labeling; VILI = ventilator-induced lung injury.

posed to radiographic films (GE Healthcare). Stripping and reblotting the membranes with glyceraldehyde-3-phosphate-dehydrogenase (CSA-335, Assay Designs) served as a control for equal loading and transfer. Densitometric analyses were performed using ImageJ‖ software (Bethesda, MD). Data represent fold induction of HO-1 or HSP70 with respect to glyceraldehyde-3-phosphate-dehydrogenase.

Statistical Analysis

Based on the assumed differences and variability in the data marking a biologic effect of treatment in each group, numbers of animals per group were estimated before the study. Therefore and as a votum of the local ethics committee, experiments were performed with six mice per group. Graphs represent means ± SEM. Data were further analyzed for normality before one-way ANOVA followed by the Student–Newman–Keuls *post hoc* test. A *P* value of less than 0.05 was considered significant. For two group comparisons, the unpaired Student *t* test was used (Sigmastat statistical software, Synstat Inc., Erkrath, Germany) followed by a Bonferroni correction. In that case, $P < 0.007$ was considered significant.

Results

Effect of Ventilation and Hydrogen Sulfide Treatment on Lung Injury

To examine the effects of hydrogen sulfide on VILI, mice were ventilated in the presence or absence of 80 ppm H_2S (fig. 1). First, we analyzed hematoxylin and eosin–stained lung sections (figs. 2A–D). Similar to control animals spontaneously breathing air for 6 h (fig. 2A), mice exposed to hydrogen sulfide for 6 h displayed no histologic signs of lung injury (fig. 2C). In contrast to the control animals, mechanical ventilation with air alone led to lung injury, which is expressed by substantial alveolar wall thickening and cellular infiltration (fig. 2B). Ventilation of mice with synthetic air supplemented with hydrogen sulfide reduced alveolar wall thickening and cellular infiltration (fig. 2D). The quantitative analysis of all experimental groups revealed that alveolar wall thickness was significantly increased by mechanical ventilation, whereas the application of hydrogen sulfide led to a decrease of wall thickness to control levels (fig. 2E). Next, we determined the degree of lung damage by a VILI score (fig. 2F). Although all nonventilated control animals exhibited baseline VILI scores, lung injury in air-ventilated mice was significantly increased. In sharp contrast and despite mechanical ventilation, additional hydrogen sulfide treatment abolished the increase in VILI score, suggesting that hydrogen sulfide treatment prevented the development of lung injury during mechanical ventilation.
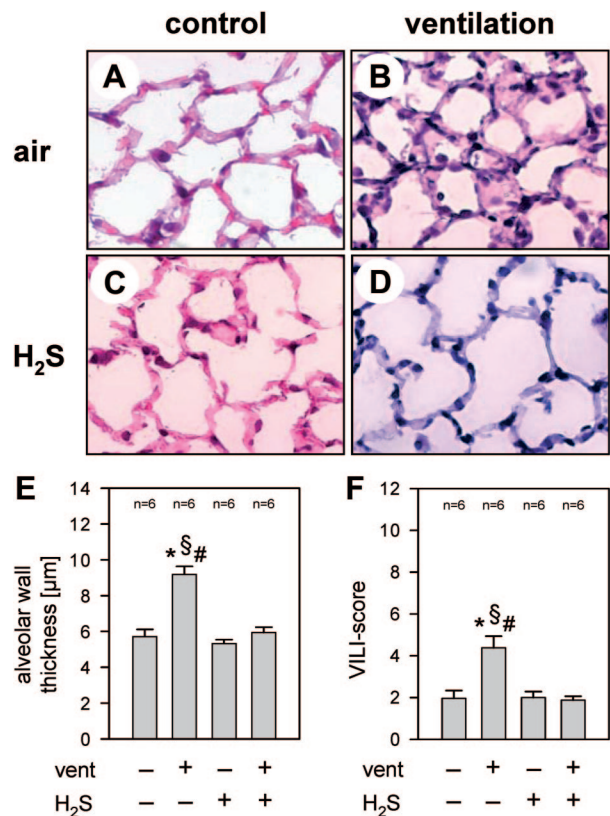
‖ National Institutes of Health. Available at: http://rsbweb.nih.gov. Accessed January 24, 2010.

Fig. 2. Effect of ventilation and hydrogen sulfide (H_2S) treatment on lung injury. As controls, mice were subjected to spontaneously breathe either air (*A*) or 80 ppm H_2S (*C*) or were ventilated (vent) with 12 ml/kg for 6 h with either synthetic air (*B*) or synthetic air + 80 ppm H_2S (*D*). The sections from the left lung lobe were hematoxylin and eosin stained. Representative pictures are shown for each experimental group (magnification ×400, *A–D*). High-power fields were randomly assigned per photograph to measure alveolar wall thickness (*E*) and ventilator-induced lung injury (VILI) score (*F*). Data represent means ± SEM for n = 6/group. Analysis of variance (Student–Newman–Keuls *post hoc* test), * $P < 0.05$ *versus* control; # $P < 0.05$ *versus* H_2S control; § $P < 0.05$ *versus* H_2S-ventilated group.

Effect of Ventilation and Hydrogen Sulfide Treatment on Lung Inflammation

The inflammatory response to mechanical stretch implies the release of inflammatory mediators such as cytokines and chemokines, leading to neutrophil infiltration into the lung.[1,31] Therefore, we determined the concentration of interleukin-1β and MIP-1β and the number of neutrophils in the BAL fluid. The measurements of interleukin-1β and MIP-1β by an enzyme-linked immunosorbent assay technique revealed that both proteins were nearly absent in BAL fluid of nonventilated controls and highly augmented in response to mechanical ventilation (figs. 3A and B). The production of cytokines and chemokines was completely inhibited when hydrogen sulfide was administered during ventilation and comparable with control levels (figs. 3A and B). The same observations were noted with respect to the migration of neutrophils. Compared with nonventilated
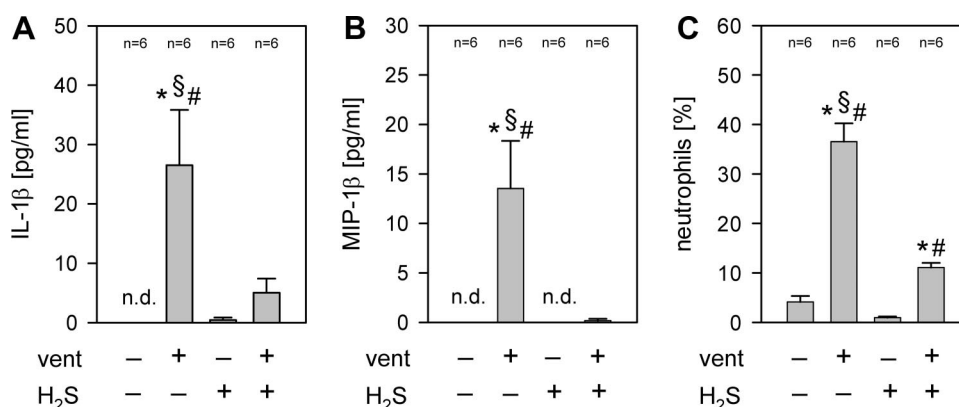
Fig. 3. Effect of ventilation and hydrogen sulfide (H$_2$S) treatment on lung inflammation. As controls, mice were subjected to spontaneously breathe air or 80 ppm hydrogen sulfide, or were ventilated (vent) with 12 ml/kg for 6 h with either synthetic air or synthetic air + 80 ppm H$_2$S as indicated. Bronchoalveolar lavage was performed in the right lung, and interleukin-1β (IL-1β, A), and macrophage inflammatory protein-1β (MIP-1β, B) contents were quantified by enzyme-linked immunosorbent assay. The relative amount of neutrophils (C) was determined by cytospin analysis. Graphs represent means ± SEM, n = 6/group. Analysis of variance (Student–Newman–Keuls *post hoc* test), * $P < 0.05$ *versus* control; # $P < 0.05$ *versus* H$_2$S control; § $P < 0.05$ *versus* H$_2$S-ventilated group. n.d. = not detected.

controls, neutrophil numbers were clearly increased in air-ventilated mice, whereas in hydrogen sulfide–ventilated mice, significantly less neutrophils were detectable (fig. 3C), indicating that the application of hydrogen sulfide prevents inflammation.

Effect of Ventilation and Hydrogen Sulfide Treatment on Apoptosis in the Lung

Next, we investigated the effect of hydrogen sulfide application on apoptosis in the lung during ventilation. Terminal deoxynucleotide transferase-mediated dUTP-biotin nick-end labeling staining of lung sections (figs. 4A–D) showed hardly any apoptotic cells in nonventilated untreated or hydrogen sulfide–treated controls (figs. 4A and C). In contrast, air-ventilated mice showed an increased number of apoptotic cells after mechanical ventilation for 6 h (fig. 4B). Hydrogen sulfide treatment significantly reduced the number of apoptotic cells (fig. 4D). These results were confirmed by quantification of apoptotic cells (fig. 4E), suggesting that hydrogen sulfide inhalation reduces apoptosis that is otherwise enhanced by mechanical ventilation.

Effect of Ventilation and Hydrogen Sulfide Treatment on Stress Protein Expression

It has been reported previously that hydrogen sulfide mediates antiinflammatory and antiapoptotic effects *via* the HO-1-signaling pathway.[16,32] To analyze its role during mechanical ventilation with respect to hydrogen sulfide-mediated effects, we assessed HO-1 protein expression in lung homogenates (fig. 4A, upper). Compared with both groups of control animals (fig. 5A, lanes 1 + 3), mechanical ventilation induced HO-1 expression that was diminished in the presence of hydrogen sulfide (fig. 5A, lane 4). Corresponding densitometric analysis of all experiments (fig. 5B) confirmed that the inhalation of hydrogen sulfide reduced ventilation-induced HO-1 protein expression. Be-

cause HO-1 is known to be a stress-inducible protein,[33] we sought to determine the expression of another stress-sensitive protein, that is, HSP70. Western blot analysis showed the up-regulation of HSP70 in response to mechanical ventilation (fig. 5A, middle). Here, a trend toward decreased HSP70 expression on hydrogen sulfide treatment was noted, but did not reach statistical significance (fig. 5C).

Effect of Ventilation and Hydrogen Sulfide Treatment on Airway Pressure, Blood Pressure, Blood Gas Analysis, and Body Temperature

Next, we asked whether hydrogen sulfide would exert an effect on airway pressure during mechanical ventilation. Figures 6A and B show the course of peak or plateau airway pressure, respectively, for air and hydrogen sulfide ventilation groups. Of note, insignificant decreases in airway pressure that occur once an hour are due to recruitment maneuvers. However, we could only detect minor differences between the two ventilation groups at the indicated time points (figs. 6A and B). Because hydrogen sulfide is described to mediate vasodilatation,[34,35] we investigated the influence of hydrogen sulfide treatment on blood pressure during mechanical ventilation. Inhalation of hydrogen sulfide did not influence systemic arterial blood pressure (fig. 6C) in our experimental system. Blood gas analysis of pH, P$_{CO_2}$, and P$_{O_2}$ within the first 30–45 min after the onset of ventilation and at the end of the experiment also did not differ between experimental groups (data not shown), indicating that normal ventilation was guaranteed. Previous findings demonstrated that hydrogen sulfide induces a suspended animation-like state in mice, which is characterized by a significant reduction in core body temperature (T$_b$) during hydrogen sulfide inhalation.[7,8] Even though all ventilated groups were treated equally according to the same fixed heating protocol, hydrogen sulfide supplementation significantly de-
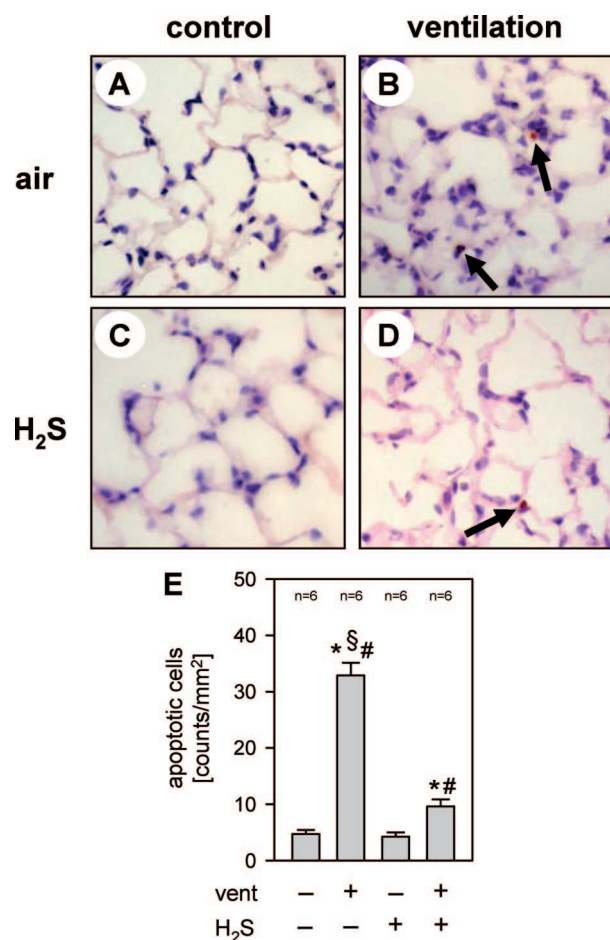
Fig. 4. Effect of ventilation and hydrogen sulfide (H_2S) treatment on apoptosis in the lung. As controls, mice were subjected to spontaneously breathe air (*A*) or 80 ppm H_2S (*C*), or were ventilated (vent) with 12 ml/kg for 6 h with either synthetic air (*B*) or synthetic air + 80 ppm H_2S (*D*). The sections from the left lung lobes were terminal deoxynucleotide transferase-mediated dUTP-biotin nick-end labeling stained. *Arrows* indicate apoptotic cells. Representative pictures are shown for each experimental group (magnification ×400). (*E*) Quantitative analysis of eight representative photos per lung, calculated in millimeter square. Graphs represent means ± SEM, n = 6/group. Analysis of variance (Student–Newman–Keuls *post hoc* test), * $P < 0.05$ *versus* control; # $P < 0.05$ *versus* H_2S control; § $P < 0.05$ *versus* H_2S-ventilated group.

Fig. 5. Effect of ventilation and hydrogen sulfide (H_2S) treatment on stress protein expression. As controls, mice were subjected to spontaneously breathe air or 80 ppm H_2S or were ventilated (vent) with 12 ml/kg for 6 h with either synthetic air or synthetic air + 80 ppm H_2S. Lung samples were taken from the upper right lobe for Western blot analysis. (*A*) Representative Western blots for heme oxygenase-1 (HO-1; *upper*), heat shock protein 70 (HSP70; *middle*), and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH; *lower*). (*B*, *C*) Densitometric analysis of all samples were normalized to GAPDH and expressed as fold induction for HO-1 (*B*) and HSP70 (*C*). Graphs represent means ± SEM, n = 5/group. Analysis of variance (Student–Newman–Keuls *post hoc* test), * $P < 0.05$ *versus* control; # $P < 0.05$ *versus* H_2S control; § $P < 0.05$ *versus* H_2S-ventilated group.

creased mean T_b from 36° to 34°C (group means over 6 h, fig. 6D), indicating that hydrogen sulfide inhalation causes mild hypothermia during mechanical ventilation.

Effect of Body Temperature on Lung Inflammation after Ventilation and Hydrogen Sulfide Treatment

Mild hypothermia alone has been described to reduce neutrophil accumulation in lung injury.[36] Therefore, we sought to analyze whether hydrogen sulfide mediates its effects through reduction of body temperature. In an independent set of experiments, T_b of air or hydrogen sulfide–ventilated mice was controlled to either reach normothermia or mild hypothermia (figs. 7A and B). Comparison of peak, plateau, and blood pressures
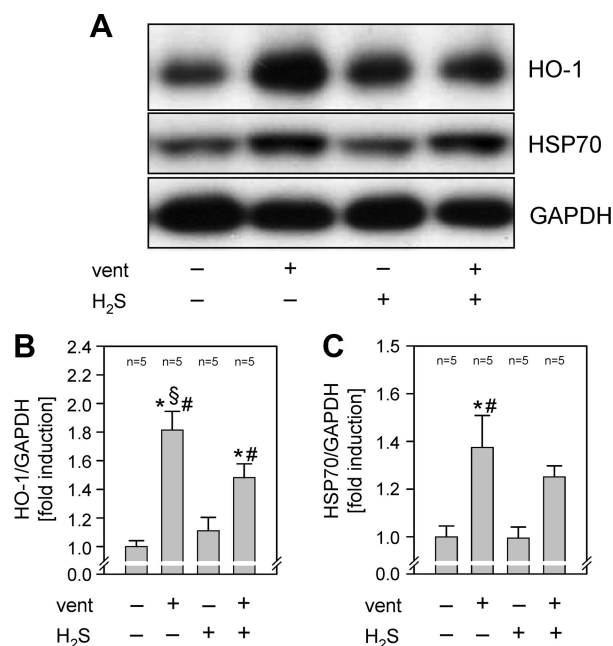
between all four groups revealed no differences (fig. 7C). Similar results were obtained by blood gas analysis (data not shown), indicating that mice in all groups had been ventilated equally.

Analysis of proinflammatory proteins by enzyme-linked immunosorbent assay showed that both interleukin-1β (fig. 8A) and MIP-1β (fig. 8B) were indeed significantly decreased in air-ventilated mice kept at mild hypothermia compared with air ventilation at normothermia. However, the relative number of neutrophils in both hypothermia and normothermia groups was identical and substantially increased when compared with the nonventilated control group (fig. 8C). Next, we tested whether the inhalation of hydrogen sulfide during ventilation at normothermia would abrogate the antiinflammatory effects observed during hypothermia. Most interestingly, ventilation with hydrogen sulfide at normothermia and hypothermia significantly decreased interleukin-1β, MIP-1β, and neutrophil infiltration to the same extent (figs. 8A–C). These results clearly demonstrate that the application of hydrogen sulfide during mechanical ventilation reduces inflammation independently of normothermia or hypothermia.
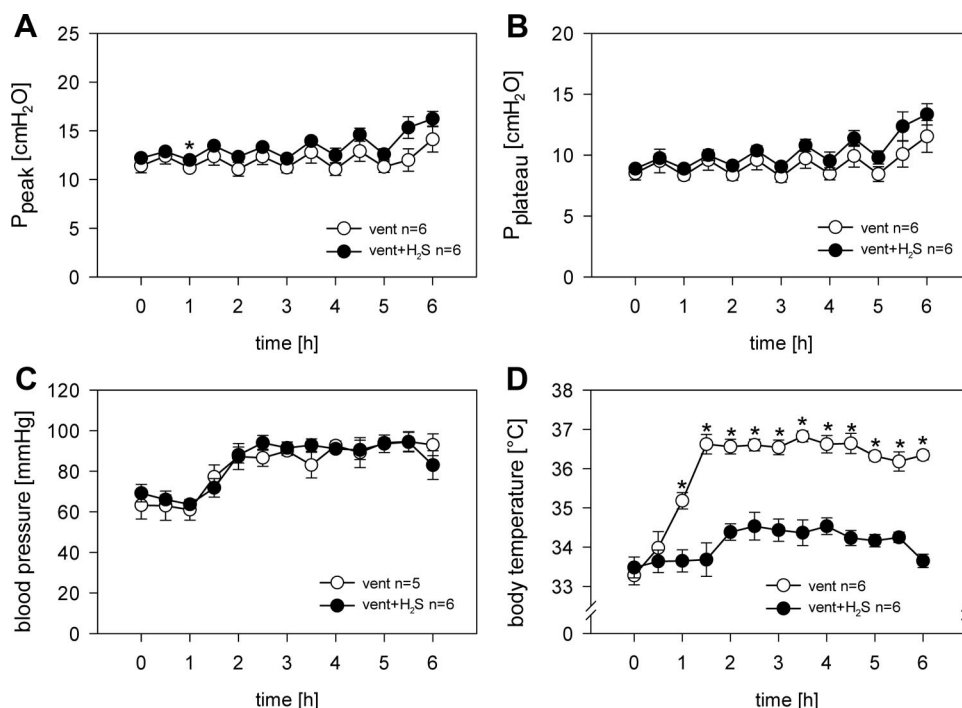
Fig. 6. Effect of ventilation and hydrogen sulfide (H_2S) treatment on airway pressure, blood pressure, and body temperature. Mice were ventilated with 12 ml/kg for 6 h either with synthetic air or synthetic air + 80 ppm H_2S. Peak (P_{peak}, *A*) and plateau airway pressure ($P_{plateau}$, *B*), arterial blood pressure (*C*), and body temperature (*D*) were recorded continuously and noted every 30 min. Graphs represent means ± SEM, n = 5–6/group. Student *t* test followed by Bonferroni correction. * $P < 0.007$.

Effect of Body Temperature on Lung Injury after Ventilation and Hydrogen Sulfide Treatment

To determine the effect of body temperature on lung injury after ventilation and hydrogen sulfide treatment, we stained the lung sections with hematoxylin and eosin (fig. 9). In contrast to nonventilated controls (fig. 9A), ventilation of mice with synthetic air led to a substantial increase in alveolar wall thickness, both in hypothermia (fig. 9B) and normothermia (fig. 9C). Nevertheless, quantitative analysis of wall thickness revealed that air ventilation at mild hypothermia slightly but significantly decreased alveolar wall thickening (fig. 9F). The degree of lung injury determined by the VILI score was substantially increased in both air-ventilated groups without statistical difference (fig. 9G). Finally, we analyzed lung sections of mice, ventilated with hydrogen sulfide at hypothermia and normothermia. Representative slides (figs. 9D and E) and quantitative analysis (fig. 9F) revealed that the thickening of alveolar walls was significantly reduced to the same level in hypothermia or normothermia. Similar results were obtained by applying the VILI score (fig. 9G). These data evidently argue for protective effects by hydrogen sulfide during the progression of VILI that are independent of its capacity to induce mild hypothermia.

Discussion

Mechanical ventilation causes lung injury characterized by edema and hyaline membrane formation, inflammatory cell infiltration, and hemorrhage.[1,37,38] In many studies, severe lung injury is provoked by ventilating rodents at high tidal volumes (20–40 ml/kg).[39–41] In our recent work[28,29] and in the current study, we have used an experimental protocol to ventilate mice with moderate tidal volumes (12 ml/kg), which are more relevant to those used in clinical practice. In this setting, mechanical ventilation results in a moderate degree of lung injury after 6 h, indicated by histology, quantitative analysis of alveolar wall thickening, and VILI score. In agreement with the recent publications demonstrating that the application of hydrogen sulfide donor compounds may significantly reduce lung injury in response to the instillation of oleic acid,[27] acute pancreatitis,[42] or combined burn and smoke inhalation[26] in animal models, our study provides the first evidence that hydrogen sulfide exerts the capacity to prevent VILI.

The development of VILI critically depends on the initiation of an inflammatory process, mainly characterized by cytokine release and neutrophil transmigration.[1,39,41] We and others have previously shown that among others, interleukin-1β and MIP-1β are released during mechanical ventilation.[28,29,39,43,44] Moreover, interleukin-1β receptor blockade has been demonstrated to protect against VILI.[39] We demonstrate here that the administration of hydrogen sulfide during mechanical ventilation prevents the release of proinflammatory cytokines and chemokines. Similar results have been obtained in other models of acute lung injury with the administration of NaHS, a hydrogen sulfide donor.[26,27] When attracted by cytokines, neutrophils play a key role in lung injury associated with mechanical ventilation.[1,39,41] In support of this notion, depletion of neutrophils prevents the

A animal treatment   continuous measurements   analysis

spontaneous breathing
| air |

mechanical ventilation
| vent-air   mean T_b 34°C |
| vent-air   mean T_b 36°C |
| vent-H$_2$S   mean T_b 34°C |
| vent-H$_2$S   mean T_b 36°C |

0   time [h]   6

continuous measurements

blood pressure
airway pressure
body temperature

blood gas analyses
(30min; 6h)

analysis

BAL
neutrophils
cytokines

lung histology
alveolar wall
VILI-score

B

C

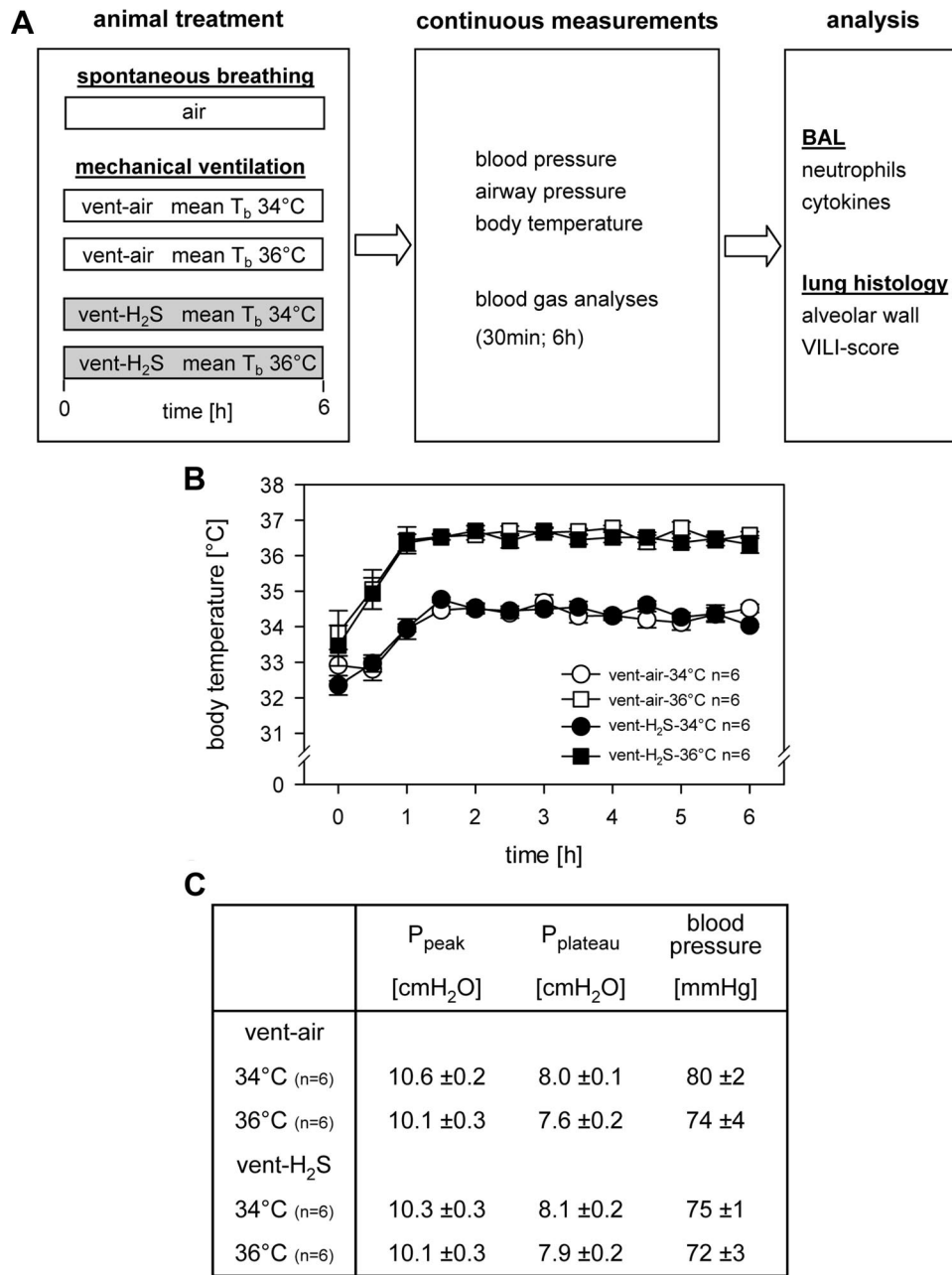|	P_{peak} [cmH$_2$O]	$P_{plateau}$ [cmH$_2$O]	blood pressure [mmHg]
vent-air			
34°C (n=6)	10.6 ±0.2	8.0 ±0.1	80 ±2
36°C (n=6)	10.1 ±0.3	7.6 ±0.2	74 ±4
vent-H$_2$S			
34°C (n=6)	10.3 ±0.3	8.1 ±0.2	75 ±1
36°C (n=6)	10.1 ±0.3	7.9 ±0.2	72 ±3

Fig. 7. Physiologic parameters for the second set of experimental groups. As controls, mice were subjected to spontaneously breathe air or were ventilated (vent) for 6 h either with synthetic air at a mean body temperature of 34°C or 36°C or with synthetic air + 80 ppm hydrogen sulfide (H$_2$S) at a body temperature of 34°C or 36°C (*A*). Body temperature (*B*), peak (P_{peak}) and plateau ($P_{plateau}$) airway pressure, and arterial blood pressure (*C*) were recorded continuously and noted every 30 min. Graph (*B*) represents means ± SEM, n = 6/group. Table in (*C*) shows mean group values over 6 h ± SEM, n = 6/group. BAL = bronchoalveolar lavage; VILI = ventilator-induced lung injury.

formation of hyaline membranes in the lungs of rabbits, despite high-tidal volume ventilation.[45] In our model, we could detect a high fraction of neutrophils in the BAL fluid from air-ventilated mice. In contrast, inhalation of hydrogen sulfide dramatically reduced neutrophil influx. Our findings are in agreement with previous reports, showing that hydrogen sulfide donors activate antiinflammatory pathways and efficiently abolish the accumulation of leukocytes in acute lung injury.[26,27,42] Taken together, these studies suggest that hy-

drogen sulfide prevents VILI primarily by blocking the inflammatory response.

During high tidal volume ventilation, VILI is characterized by the induction of apoptosis in lung tissue.[40,46,47] Assigned to our model, with the use of moderate tidal volumes, ventilation of mice with synthetic air led to a mild but significant increase of apoptotic cells in the lung. Nevertheless, inhalation of hydrogen sulfide during mechanical ventilation significantly reduced the number of apoptotic cells. Recent studies in various disease
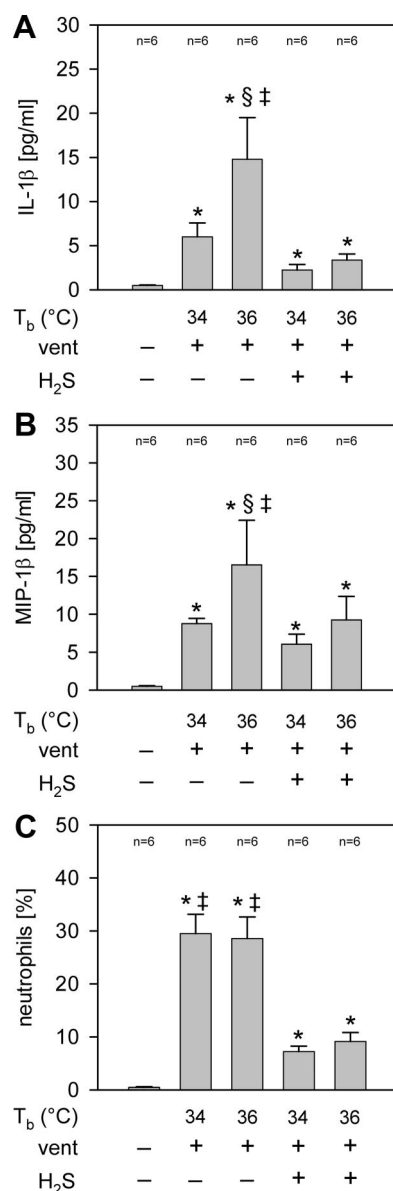
Fig. 9. Effect of body temperature on lung injury after ventilation and hydrogen sulfide (H_2S) treatment. Mice were ventilated (vent) for 6 h either with synthetic air at a mean body temperature (T_b) of 34°C (*B*) or 36°C (*C*) or with synthetic air + 80 ppm H_2S at a mean body temperature of 34°C (*D*) or 36°C (*E*). As controls, mice were subjected to spontaneously breathe air (*A*). Left lung lobes were harvested, fixed, cut, and hematoxylin and eosin stained. Representative pictures are shown for each experimental group (magnification ×400). High-power fields were randomly assigned per photograph, and alveolar wall thickness (*F*) and ventilator-induced lung injury (VILI) score (*G*) were determined. All graphs represent means ± SEM, n = 6/group. Analysis of variance (Student–Newman–Keuls *post hoc* test), * $P < 0.05$ *versus* nonventilated control; § $P < 0.05$ *versus* hypothermic air-ventilated groups; ‡ $P < 0.05$ *versus* H_2S-ventilated groups.

Fig. 8. Effect of body temperature on lung inflammation after ventilation and hydrogen sulfide (H_2S) treatment. Mice were ventilated (vent) for 6 h either with synthetic air at a mean body temperature (T_b) of 34°C or 36°C or with synthetic air + 80 ppm H_2S at a mean body temperature of 34°C or 36°C. As controls, mice were subjected to spontaneously breathe air. Bronchoalveolar lavage was performed in the right lung lobes and interleukin-1β (IL-1β, *A*), and macrophage inflammatory protein-1β (MIP-1β, *B*) contents were quantified by enzyme-linked immunosorbent assay. The relative amount of neutrophils (*C*) was determined by cytospin analysis. Graphs represent means ± SEM, n = 6/group. Analysis of variance (Student–Newman–Keuls *post hoc* test), * $P < 0.05$ *versus* nonventilated control; § $P < 0.05$ *versus* hypothermic air-ventilated group; ‡ $P < 0.05$ *versus* H_2S-ventilated groups.

models have clearly indicated the antiapoptotic effects of hydrogen sulfide application. For example, the hydrogen sulfide donor NaHS inhibits rotenone-induced cell apoptosis in an *in v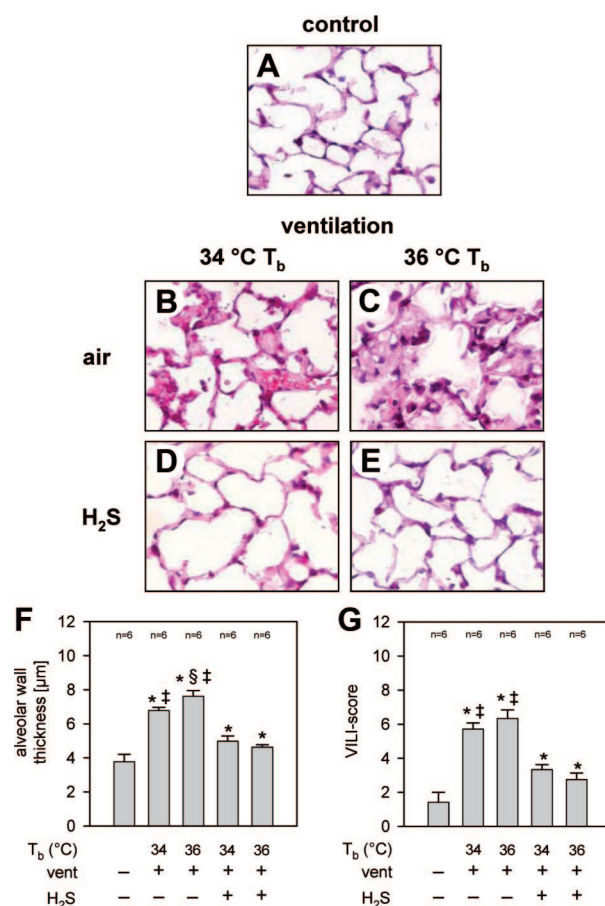itro* model[48] and apoptosis induced during cardiac ischemia–reperfusion.[18,49] Our data add to these findings by demonstrating that the inhalation of 80 ppm H_2S reduces apoptosis during mechanical ventilation.

The intracellular signaling pathways that are involved in hydrogen sulfide–mediated protection remain unclear. HO-1 is known for its antiinflammatory and antiapoptotic effects and, therefore, represents a candidate molecule in this protection (reviewed in Ref. 33). *In vitro* experiments showed that the application of hydrogen sulfide on lipopolysaccharide-stimulated macrophages led to the up-regulation of HO-1, subsequent inhibition of nitric oxide production, and nuclear factor-κB activation,[16] suggesting that

hydrogen sulfide may at least in part confer its protective effects through HO-1 regulation. However, in accordance with previous reports,[29,43,50] we found that cyclic stretching of the lung during mechanical ventilation greatly induced HO-1. Application of hydrogen sulfide did not further up-regulate HO-1 as we postulated, but it decreased its protein expression in the lung. Moreover, spontaneous breathing of hydrogen sulfide for 6 h failed to induce HO-1 in lung tissue. These findings suggest that in contrast to previous *in vitro* data, inhaled hydrogen sulfide does not prevent VILI by directly stimulating the HO-1 signaling pathway *in vivo*. Here, it seems more likely that HO-1 induction results as a stress response during mechanical ventilation and that hydrogen sulfide prevents its up-regulation by limiting the cellular stress associated with mechanical ventilation. Akin to HO-1, spontaneous hydrogen sulfide inhalation failed to modulate the expression of HSP70, another stress-inducible protein. As with HO-1, mechanical ventilation induced HSP70 protein[29,43,50] that showed a trend to decrease in the presence of hydrogen sulfide. On the basis of these data, we suggest that hydrogen sulfide does not protect against VILI *via* HO-1/HSP70 pathways, but it reduces cellular stress and thereby prevents stress-protein expression.

Because airway and perfusion pressure may affect the degree of VILI, these physiologic parameters were measured and analyzed. In this respect, we could not detect any differences in these parameters between hydrogen sulfide- and air-ventilated mice, suggesting no major impact of hydrogen sulfide on airway or vessel tone.[8] However, our data show a substantial difference between hydrogen sulfide- and air-ventilated mice regarding their body temperature during mechanical ventilation. Although both groups were subjected to the same fixed heating protocol, mean T_b of hydrogen sulfide–ventilated mice over 6 h did not exceed 34°C, whereas air-ventilated mice reached a mean T_b of 36°C. A decrease in T_b by inhalation of 80 ppm H_2S has been described recently[8,51] and was explained as the consequence of the induction of hypometabolism associated with induced suspended animation.[7,8] With respect to the current article, we hypothesized that decreased body temperature by itself might impact the development of VILI. Recent studies have implied protective effects of hypothermia either by increasing survival in hypoxia[52] or by inhibiting the migration of neutrophils in a mechanical ventilation and acid-induced lung injury model at a body temperature of 33°C.[36] Moreover, a body temperature of 27°C during injurious mechanical ventilation of lipopolysaccharide-challenged rats profoundly decreased lung injury parameters such as neutrophil influx, interleukin-1β levels, or histologic score in the lung.[53] Therefore, we examined whether the protective effects mediated by hydrogen sulfide inhalation were mainly due to the induction of hypothermia. In a separate experimental series, ventilating mice with synthetic air alone at 34°C T_b reduced alveolar wall thickening and interleukin-1β and MIP-1β release. However, VILI score and neutrophil influx were unaffected

in our model, suggesting that a slight decrease in mean T_b from 36°C to 34°C is not sufficient to completely prevent VILI. Moreover, hydrogen sulfide–mediated antiinflammatory and lung protective effects were present to the same extent irrespective of whether animals were kept under normothermia or mild hypothermia. At both temperatures, and without differences between groups (T_b 36°C and 34°C), hydrogen sulfide administration substantially prevented cytokine release, neutrophil influx, alveolar wall thickening, and lung injury measured by VILI score. These data clearly demonstrate that the application of hydrogen sulfide protects from ventilator-induced inflammation and lung injury independent of thermoregulatory processes. Moreover, a decrease of only 2°C seems to not significantly influence either lung injury or protection.

In the current study, we show for the first time that the inhalation of hydrogen sulfide during mechanical ventilation prevents VILI in mice. In this regard, hydrogen sulfide exerts antiinflammatory effects by limiting cytokine release and neutrophil transmigration. Hydrogen sulfide decreases body temperature to a mild hypothermia, yet the thermoregulation by hydrogen sulfide remains an independent factor for hydrogen sulfide–mediated lung protection.

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