Protective effect of hydrogen sulfide in a murine model of acute lung injury induced by combined burn and smoke inhalation

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ABSTRACT

Acute lung injury results in a severe inflammatory response, which leads to priming and activation of leucocytes, release of reactive oxygen and reactive nitrogen species, destruction of pulmonary endothelium, extravasation of protein-rich fluid into the interstitium and formation of oedema. Recently, H2S (hydrogen sulfide) has been shown to decrease the synthesis of pro-inflammatory cytokines, reduce leucocyte adherence to the endothelium and subsequent diapedesis of these cells from the microvasculature in vivo studies, and to protect cells in culture from oxidative injury. In the present study, we hypothesized that a parenteral formulation of H2S would reduce the lung injury induced by burn and smoke inhalation in a novel murine model. H2S post-treatment significantly decreased mortality and increased median survival in mice. H2S also inhibited IL-1β levels and significantly increased the concentration of the anti-inflammatory cytokine IL-10 in lung tissue. Additionally, H2S administration attenuated protein oxidation following injury and improved the histological condition of the lung. In conclusion, these results suggest that H2S exerts protective effects in acute lung injury, at least in part through the activation of anti-inflammatory and antioxidant pathways.

INTRODUCTION

H2S (hydrogen sulfide) is produced endogenously via the catalytic conversion of l-cysteine by two enzymes: CBS (cystathionine β-synthase) and CSE (cystathionine γ-lyase) [1]. Although both enzymes are required for H2S synthesis in some tissues, CBS acts mainly in the hippocampal and cerebellar regions of the brain [2], whereas CSE is thought to be the primary enzymatic source of H2S in the ileum [3], liver [2], vasculature and heart [4]. Recent studies have demonstrated the anti-inflammatory and cytoprotective effects of H2S [5]. For instance, in a study using human neuroblastoma cells, Whiteman et al. [6] reported on the ONOO− (peroxynitrite)-scavenging capabilities of H2S, thereby decreasing intracellular tyrosine nitration as well as attenuating oxidative stress. Other groups have shown that H2S modulates leucocyte–vascular endothelium interactions in vivo [7,8], as inhibition of CSE resulted in enhanced leucocyte adhesion and infiltration.

Key words: acute lung injury, burn and smoke inhalation, hydrogen sulfide, inflammation, interleukin-10, protein carbonylation.

Abbreviations: ALI, acute lung injury; B+S, burn and smoke inhalation; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; DNPH, 2,4-dinitrophenylhydrazine; H&E, haematoxylin and eosin; H2S, hydrogen sulfide; IL, interleukin; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; ONOO−, peroxynitrite; ROS, reactive oxygen species; s.c., subcutaneously; TBSA, total body surface area.

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Findings also suggest that H$_2$S may modulate cell viability. For example, significant increases in cell viability were observed in murine macrophages pretreated with H$_2$S and subjected to ONOO$^-$-induced nitrosative stress [9]. Additionally, cytotoxicity was attenuated in LPS (lipopolysaccharide)-stimulated murine macrophages when they were pre-treated with H$_2$S. The cytoprotective effects in this case were linked to attenuated NF-$\kappa$B (nuclear factor $\kappa$B) activation and subsequent nuclear translocation, and decreased NF-$\kappa$B-dependent gene transcription, such as iNOS (inducible nitric oxide synthase) [10].

All (acute lung injury) stems from a severe inflammatory response to toxic compounds such as inhaled smoke. The accumulation of inflammatory cells, such as activated neutrophils, lymphocytes and macrophages, in the lung is one of the hallmarks of ALI and results in severe tissue damage, leading to impaired pulmonary function [11]. Oxidative stress resulting from the presence of inflammatory cells contributes largely to the overall pathophysiology of ALI, causing the synthesis and secretion of inflammatory mediators, such as IL (interleukin)-1$\alpha$, IL-8 [12,13] and OONO$^-$ [14,15], which, in turn, may activate other pro-inflammatory signalling pathways and increase synthesis of adhesion molecules.

Although the mechanisms by which H$_2$S functions in preventing inflammatory cell injury are incompletely understood, Zanardo et al. [8] have demonstrated that, in the presence of excess L-cysteine, the precursor of the enzymatic synthesis of H$_2$S, there was not only a decrease in leucocyte infiltration after an inflammatory stimulus, but there was also a decrease in paw oedema formation as a result of decreased vascular permeability.

The aim of the present study was to investigate the effect of parenteral administration of an H$_2$S donor on B + S (burn and smoke inhalation)-induced ALI. We demonstrate that H$_2$S markedly improves survival in this model, and that this effect is coupled with (i) a down-regulation in IL-1$\beta$ levels in the lung, (ii) an up-regulation of IL-10, a prototypical anti-inflammatory cytokine, (iii) attenuated protein oxidation, and (iv) improved lung histology.

**MATERIALS AND METHODS**

**H$_2$S donor**

Sodium sulfide was produced and formulated to pH neutrality and iso-osmolarity by Ikaria Inc. using H$_2$S gas (Matheson) as a starting material [16]. The H$_2$S formulation was diluted in normal saline to the desired concentration and used immediately thereafter. All doses were freshly made daily in a rapid fashion and sealed in sterile airtight tubes until use.

**Animal model**

The study was approved by the Animal Care and Use Committee of the University Texas Medical Branch, Galveston, TX, U.S.A. All the animals were handled according to the guidelines established by the American Physiology Society and the National Institutes of Health.

Female C57BL/6 mice, weighing 20–26 g (4–6 weeks of age), were purchased from The Jackson Laboratory. The animals were anaesthetized using 4% vaporized isoflurane in air in a chamber, and were intubated using a custom-made endotracheal tube (modified from a 20-gauge angiocatheter; Baxter), allowed to breathe spontaneously under 2% isoflurane in air. The backs and flanks of the animals were shaved and 1.0 ml of 0.9% saline was injected s.c. (subcutaneously) to prevent deep tissue injury and spinal cord injury by flame burn under anaesthesia. The mice were randomized to three groups: (i) a sham group that received no injury, (ii) a combined B + S injury group that received both the burn [40% TBSA (total body surface area)] third-degree burn] and the smoke inhalation injury (two 30 s exposures of cool cotton smoke), and (iii) a B + S injury group treated with a sodium sulfide solution (2 mg/kg of body weight, s.c.).

**B + S injury**

The anaesthetized mice were covered with a Zetex cloth containing a rectangular opening corresponding to 40% of the mouse TBSA. A full-thickness flame burn was achieved by a Bunsen burner applied to exposed skin for approx. 10 s. Full-thickness injury was confirmed by loss of colouration and lack of bleeding on incision. Smoke inhalation was induced with a hand-made smoker device designed and constructed in our laboratory. The smoker, connected to tubing that provided a constant flow of air, was filled with 20 g of burning cotton towelling and then attached to the custom-made endotracheal tube via a T connection. A copper condenser coil was placed between the smoker and the endotracheal tube to cool down the hot smoke flow. Two sets of 30 s exposure of the cool cotton smoke were delivered. The animals were allowed to awaken after they were extubated and anaesthesia was discontinued. The animals were either given the vehicle or sodium sulfide (2 mg/kg of body weight, s.c.) and were resuscitated with 0.9% saline s.c. (100 ml/kg of body weight) immediately after injury, followed by an additional injection of saline (50 ml/kg of body weight) every 24 h during the remainder of the study period. Buprenorphine (2 mg/kg of body weight, s.c.) was given for analgesia in all animals every 24 h. The animals were then returned to their cages and allowed free access to food and water.

**Mortality study**

Mice in all groups ($n = 12$) were observed every 12 h for 120 h after the injury. No other parameters were measured in these mice.
Hydrogen sulfide in acute lung injury

ELISA
In a separate set of experiments, 18 mice were studied for 12 h after injury. Lung samples from the groups (sham, B + S control and B + S + treatment; n = 6 in each group) were collected and frozen immediately in liquid nitrogen. The tissue was homogenized using Tissue-Tearor (Bio-Spec Products) in 30 s bursts on ice and the homogenate was centrifuged at 2655 g for 30 min at 4 °C. The supernatant was collected, and ELISA kits (R&D Systems) were used to quantify IL-10 and IL-1β following the manufacturer’s instructions. The Bradford assay (Bio-Rad Laboratories) was used to measure protein content in the homogenate.

OxyBlot™ detection of protein carbonylation
Lung samples were homogenized in 10 vols of homogenization buffer [50 mmol/l Tris/HCl (pH 7.4), 1 mmol/l EDTA, 1 % (v/v) Nonidet P40, 150 mmol/l NaCl, 0.25 % sodium deoxycholate and 1:100 dilution of Protease Inhibitor Cocktail (Sigma–Aldrich)]. The protein concentration in the samples was determined by the Bradford assay with BSA as a standard. For detection of protein oxidation, an OxyBlot™ kit (Millipore) was used. The carbonyl groups in proteins were first derivatized with DNPH (2,4-dinitrophenylhydrazine) in the presence of 6 % (w/v) SDS. The kit used is sensitive to detect as little as 10 fmols of dinitrophenyl residues. To determine the specificity, the oxidized proteins provided by the kit were included as a positive control. Treatment of samples with a control solution served as a negative control for the DNPH treatment. The reaction was stopped after incubation for 15 min at room temperature (21 °C).

Western blotting and quantification
Samples were loaded on to 4–12 % gradient NuPAGE Novex Bis–Tris SDS/PAGE gels (Invitrogen) and run for 60 min at 200 V. Following electroblotting of proteins on to 0.2 μm nitrocellulose for 1 h at 100 V, the membrane was blocked and stained using OxyBlot™ kit reagents, according to the manufacturer’s instructions. Bands were visualized with chemiluminescence and captured on film. The total protein carbonylated was measured by reviewing all of the bands in each sample by densitometry. Densitometry was performed on scanned films using an Alpha Image™3400 system with AlphaEaseFC™ software. To quantify the amount of oxidation and to allow the comparison between the various samples, the oxidative index was defined as the ratio between densitometric values of the total OxyBlot™ bands and those stained for actin.

Lung histological assessment
Lung samples were fixed by complete immersion in 10 % (v/v) formalin for 3–5 days. Following fixation, samples were paraffin-embedded, sectioned at a thickness of 5 μm and stained with H&E (haematoxylin and eosin). Masked slides were examined by two observers, and the patterns of morphological changes were determined by a pathologist who was blinded to the treatment protocol.

Statistical analysis
Results was analysed using GraphPad Prism version 4, and values are presented as means ± S.E.M. The survival curve was analysed using the Mantel–Haenszel test. Other results were compared using ANOVA with a post-hoc Tukey test. A value of P < 0.05 was considered significant.

RESULTS
Mortality Study
Figure 1 shows the results of experiments on the survival rate of mice after being subjected to B + S injury. All animals in the sham group survived for 120 h (results not shown). Animals in the control group received saline vehicle alone and the observed median survival was 22 h after injury. After the first 24 h, only 33.3 % of the control animals were alive. There was a significant increase in median survival (75 h) in the mice that received H2S treatment (2 mg/kg of body weight, s.c.) after injury (P < 0.05). In the same group, eight out of 12 of the animals were alive during the first 24 h.

H2S attenuates the concentration of the pro-inflammatory cytokine IL-1β in lung tissue
Table 1 shows the results from experiments using lung tissue to quantify IL-1β concentrations after B + S treatment.
Table 1  Changes in IL-1β and IL-10 concentrations in lung tissue following B+S injury
Results are means ± S.E.M for all groups of mice. The sham group did not receive any injury; the control group received B+S injury and were given saline vehicle; the H2S treatment group were given the H2S donor compound diluted in saline after being subjected to B+S injury. Experiments were repeated at least twice.* P < 0.05 compared with the control group.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Group</th>
<th>IL-1β (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>124 ± 27</td>
<td>232 ± 69</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>187 ± 35</td>
<td>238 ± 47</td>
</tr>
<tr>
<td></td>
<td>H2S treatment</td>
<td>88 ± 14*</td>
<td>480 ± 48*</td>
</tr>
</tbody>
</table>

At 12 h after injury, the H2S donor compound significantly decreased the level of the pro-inflammatory cytokine in the lung by 50% when compared with the control animals (P < 0.05).

**Treatment with H2S increases the concentration of IL-10 in lung tissue**
Following B+S injury and saline vehicle treatment, the IL-10 concentration was unaffected compared with the sham group (Table 1). However, there was a 2-fold increase in IL-10 concentration in the lung after injury and treatment with the H2S donor compound compared with both sham and B+S-injured animals (P < 0.05).

**H2S attenuates oxidative stress following B+S injury**
B+S injury significantly increased the presence of protein carbonyl formation in the lung, whereas administration of H2S reversed this effect and decreased protein carbonyl formation, indicative of an overall antioxidant effect (Figure 2).

**Lung histology**
Animals in the control group that received B+S injury and saline vehicle had a pattern of increased epithelial disorganization, increased presence of airway exudates and oedema, whereas treatment with the H2S donor significantly attenuated this lung injury (Figure 3), as shown by the histology score (1.0 ± 0.10 in controls compared with 0.71 ± 0.08 with H2S treatment; P < 0.05).

**DISCUSSION**
The present study has examined the effect of parenteral administration of an H2S donor in a murine model of B+S-induced ALI. As demonstrated in the present and previous studies [17,18], B+S resulted in increased mortality and synthesis of pro-inflammatory cytokines. However, treatment with H2S improved mortality and decreased the lung tissue concentration of IL-1β, while the anti-inflammatory cytokine IL-10 was up-regulated. Additionally, we have demonstrated that protein oxidation due to B+S injury is increased markedly and this effect is attenuated by H2S administration.

H2S has been shown previously to exert a cytoprotective effect in several animal models of inflammation and to trigger the production of anti-inflammatory molecules [8,19,20]. In the present study, we found that mice exposed to B+S injury had early and high mortality, whereas H2S treatment significantly improved mortality. Relevant to our findings may be a study where H2S was shown to ‘scavenge’ ONOO⁻, decrease cytotoxicity and improve cell viability in a manner comparable with GSH, a known inhibitor of ONOO⁻ [6]. H2S supplementation has also been shown to potentiate cellular GSH + GSSG levels [21]. Additionally, on exposure to H2S, LPS-stimulated murine macrophages have been shown...
to not only decrease cytotoxicity, but also inhibit transcriptional activity [10]. In light of this, it is possible that exogenous H₂S exerts its protective effects through *de novo* synthesis of cytoprotective proteins and/or through activation of anti-inflammatory pathways.

Understanding the mechanism by which H₂S regulates key cytokines in the presence of ALI is considerably important, as increased synthesis and secretion of inflammatory mediators is thought to contribute to the overall pathology of the injury. In large animal studies, it has been reported previously that B+S injury triggers the transcriptional activation of several inflammatory genes, which led to increased cellular levels of ROS (reactive oxygen species) and reactive nitrogen species, and a large numbers of inflammatory mediators such as cytokines, chemokines and enzymes [22,23]. In particular, extensive lung parenchymal damage was observed in animals subjected to smoke inhalation. In our present study, mice were insufflated with cooled cotton smoke in order to minimize upper airway injury, thus mimicking the injury observed in human victims of house fires. Impaired oxygenation, as evidenced by attenuated \( \frac{P_{Ao_2}}{FiO_2} \) (partial pressure of arterial oxygen/fraction of inspired oxygen) ratios, is characteristic of this injury [24].

A marked decrease in the pro-inflammatory cytokine IL-1β occurred in lung tissue of H₂S-treated mice after B+S injury, suggesting the efficacy of H₂S in regulating neutrophil diapedesis and gene expression of adhesion proteins. A recent study [20] has demonstrated similar findings in that slow release of H₂S from a donor compound decreased inflammatory damage to the lung associated with LPS stimulation. In addition, the same H₂S donor formulation used in the present study has recently been shown to decrease myocardial IL-1β levels in the heart after myocardial ischaemia and reperfusion in a murine model *in vivo* [16]. Although the mechanistic details of how H₂S attenuates IL-1β is currently unknown, it will be interesting to investigate in future studies how various concentrations of H₂S *in vivo* alter the proteolytic cleavage of IL-1β from its ‘pro-form’ and, therefore, activity.

The tissue concentration of IL-10 was dramatically increased in mice treated with H₂S compared with the untreated group. Although IL-10 signalling is less well understood it is, however, widely accepted that it can inhibit immune function by suppressing adhesion molecules, inflammatory cells, such as macrophages and neutrophils, as well as pro-inflammatory mediators that are transcriptionally controlled by NF-κB [25]. It is therefore plausible that H₂S controls a central ‘switch’ in the inflammatory pathways which are simultaneously responsible for both the down-regulation of IL-1β and the up-regulation of IL-10. A similar pattern of response has been reported previously with a number of anti-inflammatory agents, including glucocorticoids, β-receptor agonists [26] and anti-inflammatory adenosine receptor ligands [27].

The generation of ROS is a consistent observation following smoke inhalation injury [28,29]. Because of the potential for ROS, such as hydroxyl radicals, to induce significant damage *in vivo*, cells and tissues possess antioxidant systems to scavenge and eliminate ROS. However, in ALI, this system is significantly overwhelmed by ROS-generating cells to the extent that antioxidants, such as GSH, are decreased in the lung [30]. Oxidative stress ensues and can lead to a state of altered cellular metabolism [31] or disruption of relevant function and systems. The physiological consequences of oxidative modification is seen where the protease–antiprotease system may become imbalanced or lung function compromised by surfactant or mucous dysfunction. The extent of pulmonary protein oxidation in patients with ARDS (acute respiratory distress syndrome) has been shown to be five times the level of oxidized proteins in control patients [30]. In the present study, we demonstrated that the H₂S donor compound is able to rescue smoke-exposed lungs from oxidative stress by decreasing protein oxidation and, thereby, improving survival. Histological changes in mice that underwent smoke exposure without treatment had deterioration of airway epithelial organization. Previous studies by Abdi et al. [32] and Hubbard et al. [33] have demonstrated significant sloughing of airway epithelium as early as 15 min after smoke insufflation and increased mucus production by 12 h. In the sham animals, there was evidence of damage due to intubation; however, the treated groups had a markedly

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**Figure 3** Representative light micrographs of H&E-stained lung sections from the control (A) and H₂S treatment (B) groups

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H2S in trauma-induced ALI. The present study, in combination with an emerging body of results ([7,8,16,34,35]; and overviewed in [5]), indicates that H2S-releasing compounds may represent a novel pharmacological intervention in the treatment of critical illness.

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