Published in final edited form as:

J Surg Res. 2012 November ; 178(1): 494–501. doi:10.1016/j.jss.2012.02.065.

Hydrogen Sulfide Decreases Reactive Oxygen in a Model of Lung Transplantation

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Abstract

Objectives—Ischemia-reperfusion injury (IRI) is a common complication following lung transplantation (LTx). IRI is thought to be mediated by reactive oxygen species (ROS). Hydrogen sulfide (H₂S) is a novel agent that has been previously shown to scavenge ROS and slow metabolism. We evaluated the impact of infused H₂S on the presence of ROS after reperfusion in an ex vivo model of LTx.

Methods—Heart-Lung blocks were recovered from New Zealand White rabbits (n=12) and cold stored in Perfadex solution for 18 hours. Following storage, the heart-lung blocks were reperfused ex vivo with donor rabbit blood. In the treatment group (n=7), a bolus of sodium hydrogen sulfide was added at the beginning of reperfusion (100 µg/kg) and continuously infused throughout the two hour experiment (1 mg/kg/hr). The vehicle group (n=5) received an equivalent volume of saline. Serial airway and pulmonary artery pressures were measured along with arterial and venous blood gases.

Results—Oxygenation and pulmonary artery pressures were similar between the two groups. However, treatment with H₂S resulted in a dramatic reduction in the presence of ROS after 2 hours of reperfusion (4851 ± 2139 vs. 235 ± 462 RFU/mg protein, p=0.003). There was a trend toward increased levels of cGMP in the H₂S treated group (3.08 ± 1.69 vs. 1.73 ± 1.41 fmol/mg tissue, p=0.23).

Conclusions—After prolonged ischemia, infusion of H₂S during reperfusion is associated with a significant decrease in the presence of ROS, a suspected mediator of IRI. To our knowledge, this study represents the first reported therapeutic use of H₂S in an experimental model of lung transplant.

Keywords

Lung transplantation; hydrogen sulfide; reactive oxygen species

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Conflicts: The authors have no relevant conflicts of interest.

INTRODUCTION

Although lung transplantation (LTx) is the gold standard of treatment for end-stage lung disease, 15–25% of LTx recipients experience severe post-transplant respiratory failure known as primary graft dysfunction (PGD).^{1–4} PGD is associated with progressive hypoxia, pulmonary edema, and increased pulmonary artery pressures.⁴ These sequelae result in an 8-fold increase in 30-day mortality, impaired long-term graft function, and an increased risk of bronchiolitis obliterans syndrome.⁴

The primary cause of PGD is ischemia-reperfusion injury (IRI).^{3, 4} During transplantation, the donor lungs are exposed to a period of cold ischemia. This ischemic phase leads to the production of reactive oxygen species (ROS) by endothelial cells, macrophages, and other immune cells. Additionally, multiple inflammatory mediators are generated, increasing pulmonary vascular permeability. After implantation, restoration of the oxygen supply results in a burst of ROS that can overwhelm the lung's anti-oxidant defenses resulting in additional damage, increased microvascular injury, and pulmonary edema. Although the cause of IRI is multifactorial, it is partly mediated by direct tissue damage caused by this generation of ROS.^{3, 4}

Hydrogen sulfide (H₂S), best known as a toxin, is a water soluble gas that is produced endogenously by cystathione γ -lyase and cystathionine β -synthase.^{5–7} While its mechanisms of action are still being explored, H₂S has been shown to be an avid free radical scavenger, binding ROS.^{5, 8, 9} Additionally, H₂S may slow metabolism by inhibiting cytochrome c oxidase and thus disrupting the mitochondrial electron transport chain resulting in the generation of less ROS.¹⁰ Exogenously administered H₂S has been shown to be protective in animal models of IRI including myocardial infarction,^{8, 11, 12} stroke,¹³ and liver,^{7, 14} kidney,¹⁵ and skin transplantation.¹⁶ However, the efficacy of H₂S in models of LTx has not been explored. Therefore, we undertook this study to investigate whether the administration of H₂S would decrease ROS and improve graft function in an experimental model of LTx.

METHODS

Model

These experiments were conducted using an ex vivo model of lung reperfusion whereby rabbit lungs were externally ventilated and perfused with donor rabbit blood. Our protocol has been described in detail previously and is represented schematically in Figure 1.¹ The experiment utilized 4 kg, male New Zealand White rabbits (Myrtle's Rabbitry Inc., Thompson Station, TN). This study was approved by the Animal Care and Use Committee at the Johns Hopkins University.

A total of 12 rabbits were divided into 2 groups and underwent en bloc heart-lung harvest followed by 18 hours of cold storage. These heart-lung blocks were subsequently reperused with donor rabbit blood for 120 minutes while physiologic data were recorded and tissue biopsies were taken.

Heart-Lung Block Harvest

All rabbits were anesthetized with intramuscular injection of ketamine (35 mg/kg) and xylazine (6.5 mg/kg). Additional sedation was given as needed with acepromazine (5 mg/kg). A tracheotomy was performed to facilitate endotracheal intubation and mechanical ventilation was initiated (Harvard ventilator apparatus, model 665; Harvard Apparatus Co., Holliston, MA). The experimental protocol ventilator settings were: Volume Control mode; rate 20 breaths/minute; tidal volume 10 mL/kg; Fractional inspired concentration of oxygen

(FiO₂) 100%. The chest was entered through a median sternotomy and the superior and inferior venae cavae, aorta, and pulmonary artery (PA) were isolated. Intravenous heparin (1000 U/kg) was given and 30 ug of prostaglandin E1 was injected directly into the PA. The PA was cannulated through a right ventriculotomy and the left atrium was cannulated directly. The lungs were flushed with 250 mL of cold (4 C) Perfadex solution (Vitrolife, Englewood, CO) via gravity drainage through the PA cannula. The superior and inferior venae cavae and the aorta were ligated. Topical cold ice slush was placed around the heart-lung block and the block was excised from the chest. The lungs were subsequently inflated and the block was preserved in Perfadex at 4 C for 18 hours.

Perfusion Pump System

Two donor rabbits were heparinized (1000 U/kg) and exsanguinated through the right ventricle to obtain 300 mL of whole blood for each reperfusion. Following cold storage, the heart-lung block was suspended by the trachea and ventilated at 10 mL/kg, 20 breaths/minute, with an FiO₂ of 100%. All animals were reperfused for 120 minutes with the donor rabbit blood using a Sarns 5000 roller head pump (Sarns Inc., Ann Arbor, MI). Blood removed via the left atrial cannula was collected in a reservoir and deoxygenated to achieve a PO₂ and PCO₂ of 60 mmHg (to simulate venous blood) before being returned to the heart-lung block through the PA cannula.

Experimental Groups

Twelve heart-lung blocks were procured, cold stored, and reperfused as described above. In the experimental group (n=7), a H₂S donor, sodium hydrogen sulfide (NaHS; Alfa Aesar, Ward Hill, MA) was dissolved in normal saline and given through the reperfusion circuit as a bolus dose (100 ug/kg) followed by a continuous infusion (1 mg/kg/hour) for the duration of reperfusion. In the control group (n=5), the same volume of normal saline was added to the circuit as a bolus and as a continuous infusion.

NaHS is known as a H₂S donor because it can form H₂S gas when dissolved in an aqueous solution. This occurs by the following mechanism: $\text{NaHS} \Rightarrow \text{Na}^+ + \text{HS}^-$ (1st reaction); $2\text{HS}^- \rightleftharpoons \text{H}_2\text{S} + \text{S}^{2-}$ (2nd reaction); $\text{HS}^- + \text{H}^+ \rightleftharpoons \text{H}_2\text{S}$ (3rd reaction).¹⁷

Hemodynamic Measurements

Physiologic measurements were recorded at baseline and then every 15 minutes for the duration of reperfusion. These measurements included airway, PA, and left atrial pressures. Every 15 minutes, PA and left atrial blood samples were analyzed for blood gas measurements (arterial blood gas analyzer, model 348, Chiron Diagnostics, Norwood, MA).

Lung Samples

Lung samples were taken from 10 of the 12 heart-lung blocks (2 were unable to be analyzed) at 4 time points during the experiment: immediately after procurement (prior to cold storage), immediately prior to reperfusion (after cold storage), after 1 hour of reperfusion, and after 2 hours of reperfusion. All lung samples were removed sharply after the application of hemoclips for hemostasis (large ligating clips; Weck, Research Triangle Park, NC). Samples were flash frozen in liquid nitrogen and stored at -80 C for biochemical analysis.

Reactive Oxygen Species Levels

ROS levels were examined in lung samples using a green fluorescence assay (OxiSelect *In Vitro* ROS Assay Kit, Cell Biolabs, San Diego, CA). The lung samples were homogenized on ice in phosphate buffered solution, centrifuged, and resuspended in assay buffer. The cell

permeable 2',7'-Dichlorodihydrofluorescein diacetate (DCFDA) fluorogenic probe was used to assess ROS levels. Fluorescence was read on a Spectramax M5 plate reader.

Cyclic GMP (cGMP) Levels

cGMP concentrations were determined by a commercially available enzyme immunoassay (Amersham cGMP Enzymeimmunoassay, GE Healthcare Life Sciences, Piscataway, NJ). Tissue samples were weighed and homogenized in 500 μ L of 6% trichloroacetic acid (TCA). Samples were centrifuged, and supernatants recovered and washed five times in 2 mL of water saturated ether. The aqueous layer was recovered and dried to recover a pellet. The pellet was re-suspended in cGMP kit assay buffer. The acetylation assay was performed according to the vendor's specifications. cGMP levels were calculated and reported as fmol/mg wet tissue weight.

Nitric Oxide Synthase (NOS) Activity

Lung tissue levels of both endothelial NOS (eNOS) and inducible NOS (iNOS) expression were evaluated by western blotting. For eNOS, 25 μ g of total protein was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), electrotransferred to a nitrocellulose membrane, and blotted using mouse monoclonal antibody to eNOS (BD Bioscience). For iNOS, 50 μ g of total protein was used and iNOS was determined using mouse monoclonal antibody to iNOS (BD Bioscience).

Statistical Analysis

Physiological data was evaluated by two independent statistical tests to assess the longitudinal differences between the treatment groups. First, repeated-measures analysis of variance (RM-ANOVA) was performed to evaluate the effects of NaHS over time. Post hoc comparisons at specific time points were evaluated with the Tukey-Honest significant difference tests. Multilevel random effects modeling was also performed to account for interactions both between and within each animal. The model used (generalized estimating equation, GEE) estimated the variance in the physiological parameters between animals and across time. Values for both the RM-ANOVA and the GEE analysis are presented when discussing physiologic parameters.

Results of biochemical activity assays were compared by the Student's t-test (cGMP, eNOS, iNOS) and by ANOVA for ROS. Data are presented as means $\pm$ standard deviation. P-values < 0.05 were considered statistically significant. All graphs are presented as mean values with error bars defining standard error. Statistical analysis was performed using Stata 11.2 (Stata Corporation LP, College Station, TX).

RESULTS

Physiologic Data

Prolonged cold storage produced a continuous deleterious effect on lung performance among all animals during reperfusion. In particular, the arterial partial pressure of oxygen (PO₂) declined throughout the 120 minute reperfusion while the mean pulmonary artery pressures (mPAP) increased (Figure 2). The arterial partial pressure of carbon dioxide (PCO₂) was fairly stable throughout reperfusion.

Animals treated with NaHS had similar oxygenation ($p=0.53$ by RM-ANOVA; $p=0.53$ by GEE) and ventilation ($p=0.99$ by RM-ANOVA; $p=0.57$ by GEE) as control animals. mPAPs were also similar between the two groups ($p=0.72$ by RM-ANOVA; $p=0.60$ by GEE).

Biochemistry

NaHS infusion was associated with significantly decreased levels of ROS compared to controls ($p=0.004$ by RM-ANOVA; $p=0.02$ by GEE; Figure 3). Post hoc testing revealed that ROS levels were significantly lower in animals treated with NaHS at 2 hours ($4,851 \pm 2,139$ vs. 235 ± 462 RFU/mg protein, $p=0.003$) but similar at baseline and at 1 hour. Moreover, in animals treated with NaHS, ROS levels at 2 hours were significantly lower than at baseline ($p=0.03$) and at one hour ($p=0.01$).

At the conclusion of reperfusion, there was a trend toward increased levels of cGMP in the NaHS treated rabbits compared to controls (3.08 ± 1.69 vs. 1.73 ± 1.41 fmol/mg tissue, $p=0.23$; Figure 4). The groups treated with NaHS tended to have higher levels of eNOS (1.77 ± 0.74 vs. 2.24 ± 0.70 , $p=0.54$) and lower levels of iNOS (1.96 ± 0.80 vs. 1.57 ± 0.64 , $p=0.37$) expression though these results were not significant (Figure 5).

DISCUSSION

In our experimental model of LTx, the infusion of the free radical scavenger, hydrogen sulfide, is associated with a significant decrease in the presence of ROS and a trend toward an increased level of cGMP. eNOS and iNOS expression were similar between the two groups. Additionally, physiological measures of lung performance including oxygenation, ventilation, and mPAP were similar.

LTx is often complicated by severe respiratory failure secondary to IRI. This injury is thought to be partially mediated by ROS. Thus, a therapy that can decrease the level of these damaging molecules has potential clinical utility in LTx. Although the levels of ROS declined in both groups over time, our experiment demonstrated a greater decline in ROS levels in the group treated with H_2S . Although ROS have been previously associated with IRI, in our experiment, reduction in ROS levels was not associated with improved clinical lung function.¹⁻⁴ Several explanations are possible.

First, it is possible that ROS are not a critical mediator of gas exchange or pulmonary vascular reactivity in the early reperfusion period. Other studies have also found free radical scavengers to be ineffective at improving oxygenation and reducing pulmonary pressures in models of LTx.¹⁸ Although ROS have been previously associated with IRI in LTx, it's possible that disruption of other biochemical pathways and modulation of inflammatory mediators are more clinically relevant in the early reperfusion period. Free radical damage is primarily mediated by direct tissue injury and the up-regulation of pro-apoptotic pathways through the modulation of intracellular calcium levels.³ While this damage may be important to long-term graft function, it may not affect early clinical performance. Further investigation with longer periods of reperfusion is warranted.

Second, it is possible that our model produces more severe lung injury than can be ameliorated by post-injury treatment. In our past experience with this model, we have found the rabbit lungs to be highly resistant to ischemic injury, leading us to use prolonged ischemia (18 hours) to ensure lung damage. However, while the lungs tend to be resistant to injury to a point, at approximately 18 hours, they appear to collapse, sustaining often irrecoverable injury. It is possible that our current model causes such severe injury that the additional oxidative stress caused by ROS is not clinically relevant. Thus mitigating ROS damage by decreasing ROS levels may not produce a clinically significant difference. Given these limitations, future investigation will make use of less severe injury as well as pre-ischemia H_2S treatment, aimed at preventing injury caused by prolonged ischemia.

Third, it is possible that our dose of H₂S was insufficient. In the literature, two different forms of intravenous H₂S donors have been utilized over a wide range of concentrations and in various different animal models. Initial bolus doses range from 100–3,000 ug/kg followed by maintenance infusion doses of 0.5–3 mg/kg/hour.^{9, 19–21} However, high doses of H₂S can cause acute lung injury.^{17, 22} Wary of causing iatrogenic lung injury, we chose a modest dose used by other investigators interested in cardiopulmonary function.^{8, 11} However, review of the temporal trends in ROS levels in our experiments suggests that the initial bolus dose was possibly too low as ROS levels do not differ between the groups until after 2 hours of continuous H₂S infusion. We suspect that a greater bolus dose would lead to an earlier differential in the presence of ROS, possibly leading to less free radical damage in the treated group and thus a greater clinical difference. This idea is supported by previous work that suggests there is a very narrow therapeutic window for effective administration of H₂S after IRI.²⁰

Previous research suggests that elevated levels of cGMP are associated with improved outcomes in LTx, mediated by decreased ROS levels, improved immunologic cell regulation, increased perfusion through vascular dilation, and the activation of cytoprotective mediators.^{1, 23, 24} In our experiment, infusion of H₂S was associated with increased levels of cGMP although this did not reach statistical significance. Nitric oxide synthases are an important pathway involved in the production of cGMP. However, in our experiment, levels of eNOS and iNOS were similar between the two groups. Previous research from our laboratory suggests that in LTx, eNOS may be protective and iNOS may be injurious.¹ H₂S has been previously associated with the upregulation of NOS 3 and the downregulation of iNOS through modulation of endogenous carbon monoxide and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).^{5, 20} In these experiments, eNOS and iNOS levels were not significantly affected by H₂S but cGMP levels were increased, suggesting either a mechanism of cGMP production outside the canonical nitric oxide (NO) pathway or a type II error. The relationship between NO and H₂S is not well understood, meriting further exploration.

Previous Literature

The administration of H₂S has been associated with cytoprotective effects in several models of IRI. In a model of myocardial infarction, Sodha et al.^{8, 11} demonstrated that H₂S was associated with decreased infarct size, preservation of cardiac function, decreased apoptosis, and decreased inflammatory mediators. Similar cytoprotective effects have been demonstrated in IRI models of liver, kidney, and skin transplantation.^{7, 14–16}

Previous research has also demonstrated H₂S to be protective of injury in several models of acute lung injury. In a model of ventilator-induced lung injury, Faller et al.²⁵ demonstrated that inhaled H₂S combined with mild hypothermia was associated with decreased pulmonary edema, neutrophil accumulation, and pro-inflammatory cytokine production. Similarly, in a model of smoke-induced lung injury, Esechie et al.²¹ found infused H₂S to be associated with decreased mortality and improved gas exchange. Several studies have verified that H₂S is associated with the decreased production of inflammatory mediators and a decreased immune response.^{26, 27} While we did not directly assay for immunologic or inflammatory molecules, the decreased levels of ROS exhibited in our experiment suggest a significantly blunted inflammatory response in the presence of H₂S.

Additionally, several investigators have reported that H₂S may cause pulmonary artery relaxation, reducing pulmonary artery pressure, particularly in models of hypoxic pulmonary hypertension.^{28, 29} Although we were not able to replicate this finding in our experiments, it is of particular importance in LTx where many of the recipients experience pulmonary

hypertension preoperatively secondary to their underlying disease and then postoperatively secondary to IRI.

Despite the potential advantages of H₂S in LTx, some caution is warranted. High levels of H₂S can be directly toxic to the lungs, causing acute lung injury and/or triggering apnea through regulation of CO₂ receptors.^{17, 22} Moreover, Perry et al.²⁶ have shown that H₂S can inhibit airway smooth muscle cells in human lung tissue in asthmatic patients. Although this may be beneficial in the setting of asthma, airway smooth muscle may be damaged during the LTx procedure, requiring proliferation and regeneration of these cells. Such a process may be inhibited by H₂S.

Finally, recent studies suggest that H₂S can be used to lower cellular metabolism, inducing a state of suspended-animation that allows animals to survive under hypoxic conditions.^{5, 30–32} In clinical LTx, hypothermic storage is used to decrease allograft metabolism and protect the lungs from ischemic injury. Interestingly, Blackstone et al.³¹ have shown that H₂S administration can reduce cellular metabolism and allow mice to survive severely hypoxic conditions. Though such research is in its early stages, the prospect of inducing a state of suspended-animation that protects organs from injury during ischemia and reperfusion by lowering their metabolic oxygen requirement is appealing and merits further investigation.

Limitations

In this study, we utilized an ex vivo model of lung transplantation. We did not induce brain death in the donors nor did we utilize immunosuppression. Therefore, we did not completely replicate the in vivo LTx procedure. Moreover, since we only conducted our experiment over 2 hours, we cannot evaluate long-term effects.

The ideal method of delivery, dosage, or duration of H₂S treatment, particularly in lung models, is unknown and wide ranges have been utilized in experimental models.^{9, 19–21} Furthermore, though a significant literature exists surrounding the use of hydrogen sulfide donors rather than H₂S gas, it is possible that the inhaled, gaseous form would produce better clinical results, particularly in LTx where a local effect would be possible, avoiding the potential complications of systemic administration. In future experiments, we plan to investigate the utilization of inhaled H₂S.

Moreover, our experiment may be limited by small sample size and thus susceptible to a type II error. Finally, we believe our experimental model is successful in producing severe lung injury; however, we speculate that this injury may be too severe, particularly for a model aimed at evaluating post-injury treatments. In the future, we plan to subject the lungs to shorter periods of ischemia and focus on therapies that prevent injury before it occurs.

Conclusion

In conclusion, after prolonged ischemia, infusion of the free radical scavenger hydrogen sulfide is associated with a significant decrease in the presence of ROS, a suspected mediator of IRI. To our knowledge, this preliminary study represents the first reported therapeutic use of H₂S in an experimental model of LTx. Further investigation is warranted to optimize the dosing and timing of H₂S administration.

Acknowledgments

We would like to thank Mr. Jeffrey Brawn, Mrs. Melissa Jones, and Mr. Chase Robinson for their outstanding technical assistance.

Funding

This research was supported by grant 90038390 from the Thoracic Surgery Foundation for Research and Education and by grant T32 2T32DK007713-12 from the National Institutes of Health. Dr. George is the Hugh R. Sharp Cardiac Surgery Research Fellow. Drs. Arnaoutakis and Beaty are the Irene Piccinini Investigators in Cardiac Surgery.

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Figure 1.

(A) Schematic diagram of the experimental model. The de-oxygenator is a membrane oxygenator through which a mixture of oxygen, carbon dioxide, and nitrogen are titrated to achieve a pulmonary artery pO_2 and PCO_2 of 40 mmHg (to simulate venous blood). (B) Photograph of a heart-lung block during reperfusion. The pump suction recovers any shed blood that is then returned to the venous reservoir. Abbreviations: PA, pulmonary artery; LA, left atrium.

A**Arterial Oxygen Levels**

B

Arterial Carbon Dioxide Levels

C

Pulmonary Artery Pressures

Figure 2.

Partial pressure of arterial oxygen (A), partial pressure of carbon dioxide (B), and (C) mean pulmonary artery pressures during reperfusion stratified by treatment. Control animals are depicted with a dashed line and animals treated with hydrogen sulfide are depicted with a solid line. P-values are based on repeated measures analysis of variance (RM-ANOVA) and modeling with the generalized estimating equation (GEE).

Figure 3.

Levels of reactive oxygen species (ROS) during reperfusion stratified by treatment. ROS is measured in related fluorescence units (RFU). P-values are based on repeated measures analysis of variance (RM-ANOVA), post hoc Tukey Honest test, and the generalized estimating equation (GEE).

Figure 4. Cyclic GMP levels stratified by treatment. cGMP levels are reported in fmol/mg of tissue. P-value is based on the Student's t-test.

B

eNOS
GAPDH

iNOS
GAPDH

Figure 5.
(A) eNOS and iNOS levels stratified by treatment. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a loading control for comparison. (B) Sample western blot demonstrating levels of NOS protein compared to the GAPDH loading control.