H₂S Attenuates LPS-Induced Acute Lung Injury by Reducing Oxidative/Nitrative Stress and Inflammation

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Key Words
Hydrogen sulfide • Endotoxemia • Lung • Oxidative stress • Nitrative stress • Inflammation

Abstract
Background: Hydrogen sulfide (H₂S), known as the third endogenous gaseous transmitter, has received increasing attention because of its diverse effects, including angiogenesis, vascular relaxation and myocardial protection. We aimed to investigate the role of H₂S in oxidative/nitrative stress and inflammation in acute lung injury (ALI) induced by endotoxemia. Methods: Male ICR mice were divided into six groups: (1) Control group; (2) GYY4137 treatment group; (3) L-NAME treatment group; (4) lipopolysaccharide (LPS) treatment group; (5) LPS with GYY4137 treatment group; and (6) LPS with L-NAME treatment group. The lungs were analysed by histology, NO production in the mouse lungs determined by modified Griess (Sigma-Aldrich) reaction, cytokine levels utilizing commercial kits, and protein abundance by Western blotting. Results: GYY4137, a slowly-releasing H₂S donor, improved the histopathological changes in the lungs of endotoxemic mice. Treatment with NG-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase (NOS) inhibitor, increased anti-oxidant biomarkers such as the total antioxidant capacity (T-AOC) and the activities of catalase (CAT) and superoxide dismutase (SOD) but decreased a marker of peroxynitrite (ONOO-) action and 3-nitrotyrosine (3-NT) in endotoxemic lung. L-NAME administration also suppressed inflammation in endotoxemic lung, as evidenced by the decreased pulmonary levels of interleukin (IL)-6, IL-8, and myeloperoxidase (MPO) and the increased level of anti-inflammatory cytokine IL-10. GYY4137 treatment reversed endotoxin-induced oxidative/nitrative stress, as evidenced by a decrease in malondialdehyde (MDA), hydrogen peroxide (H₂O₂) and 3-NT and an increase in the anti-oxidant biomarker ratio of reduced/oxidized glutathione (GSH/GSSG ratio) and T-AOC, CAT, and SOD activity. GYY4137 also attenuated endotoxin-induced lung inflammation. Moreover, H.-X. Zhang and S.-J. Liu contributed equally to this work and should be considered as co-first authors.

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treatment with GYY4137 inhibited inducible NOS (iNOS) expression and nitric oxide (NO) production in the endotoxemia lung. **Conclusions:** GYY4137 conferred protection against acute endotoxemia-associated lung injury, which may have been due to the anti-oxidant, anti-nitrative and anti-inflammatory properties of GYY4137. The present findings warrant further exploration of the clinical applicability of H₂S in the prevention and treatment of ALI.

**Introduction**

Acute lung injury (ALI) and its extreme manifestation, acute respiratory distress syndrome (ARDS), are acute complications resulting from stress situations such as trauma, burns, and sepsis [1-4]. ALI/ARDS is associated with an acute inflammatory process and is characterized by acute hypoxemic respiratory failure with severe alveolar and interstitial edema and neutrophil infiltration, resulting in a gradual decline of lung function [5]. Although there are many studies on ALI/ARDS, the current therapeutic strategies for ALI/ARDS are still very sparse, and the mortality rate remains high [6-8].

Recent evidence has demonstrated that oxidative stress plays an important role in the pathogenesis of ALI [9, 10]. As a result, the overproduction of reactive oxygen species (ROS) leads to oxidative protein modification and acute lung injury. Moreover, superoxide radicals can react with nitric oxide (NO) to synthesize peroxynitrite (ONOO⁻), leading to pathologic tissue injury by nitrative protein modification [11]. ROS and reactive nitrogen species (RNS) can lead to lung cell injury by many mechanisms, including (1) damage to DNA resulting in strand breaks and point mutations; (2) lipid peroxidation with the formation of vasoactive and pro-inflammatory molecules; (3) oxidation of proteins that alter protein activity; and (4) enhanced expression of pro-inflammatory genes by alteration of transcription factors such as nuclear factor (NF)-κB [12-15]. It has been reported that there are many potential sources of ROS, such as itinerant and resident leukocytes (neutrophils, monocytes, and macrophages), circulating oxidant-generating enzymes, and inhaled gases with high concentrations of oxygen [16, 17]. These studies provide more potential targets for the treatment of ALI.

Hydrogen sulfide (H₂S), which is produced by three enzymes, cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST), has recently been suggested to be “the third endogenous gaseous signaling transmitter” in mammalian tissues [18]. H₂S is reported to possess potent anti-oxidant, anti-inflammatory and other physiological regulatory functions and to act as a critical biological factor that is involved in the response to injury in the lung, liver, heart, adrenal gland, and other organs [19-24]. Notably, previous studies have suggested that H₂S plays an important role in the pathogenesis of ALI/ARDS. For example, it has been shown that inhalation of 80ppm H₂S decreases oxidative stress and cotton smoke-induced lung injury by reducing the production of iNOS and NO [25]. Further, the inhalation of 80ppm H₂S protects against lung injury caused by LPS by attenuating the pro-inflammatory responses. Furthermore, our previous studies have shown that the intraperitoneal injection of GYY4137 (a slow-releasing H₂S donor) at a concentration of 50 mg/kg alleviates lipopolysaccharide (LPS)-induced adrenal insufficiency by suppressing both oxidative and nitrative stress [26, 27]. However, it is not well known whether H₂S protects against acute lung injury by regulating ROS and RNS production in sepsis mice.

Given these pieces of evidence, this study aimed to define whether H₂S can improve LPS-induced pulmonary injury by modulating oxidative/nitrative stress and inflammation.
Materials and Methods

Animals

Male ICR mice (25-30 g), obtained from Shanghai SLAC Laboratory Animal Co. (Shanghai, China), were kept under standard laboratory conditions, with free access to food and water under a natural day/night cycle. This study was approved by Ethical Committee of Experimental Animals of Second Military Medical University.

Drug treatment

Animals were randomly divided into the following six groups: (1) Control group; (2) GYY4137 treatment group; (3) L-NAME treatment group; (4) lipopolysaccharide (LPS) treatment group; (5) LPS with GYY4137 treatment group; and (6) LPS with L-NAME treatment group. Mice underwent an intraperitoneal administration of LPS (5 mg/kg, Sigma-Aldrich, St. Louis, MO, USA), which was dissolved in sterile saline. GYY4137 (Cayman), dissolved in sterile saline, was injected i.p. immediately before the injection of LPS at a dose of 50 mg/kg. L-NAME (Sigma-Aldrich) was injected i.p. immediately before injection of LPS at a dose of 20 mg/kg. All chosen dosages were based on our previous study [28], and the control group was treated with an equal volume of saline. All mice were euthanized 24 h after the induction of endotoxemia. The left lower lung tissues were immersed in a 4% paraformaldehyde for histopathological analysis. Other parts of the lung tissue were stored at -80°C until use.

Histopathological analysis

For histopathological analysis, tissues were fixed in 4% paraformaldehyde overnight, embedded in paraffin and sectioned. Sections (4 µm thickness) were stained with hematoxylin and eosin (HE staining).

Measurement of the activity of CAT, SOD, T-AOC, MPO; the ratio of GSH/GSSG; the quantities of MDA, H₂O₂, IL-6, IL-8, IL-10, 3-NT

Lung tissues were collected as described above. The activities of CAT, SOD, T-AOC, MPO; the ratio of GSH/GSSG; and the quantities of MDA, H₂O₂, IL-6, IL-8, IL-10, and 3-NT were measured using commercial kits (Jiancheng Bioengineering Institute, China) according to the manufacturer’s instructions.

Western blot analysis

Protein extraction and western blot were performed as described previously [28]. Briefly, mouse lung tissues were homogenized in cold T-Per lysis buffer (Pierce). Equal amounts of protein samples (approximately 50 µg) were separated by 10% SDS-PAGE and subsequently transferred to nitrocellulose membranes. After blockage in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% dried milk powder (wt/vol) for 2 h at room temperature, membranes were immunostained using primary antibody (1:1000) for inducible nitric oxide synthase (iNOS) (Santa Cruz Biotechnology, Santa Cruz, CA) or β-actin (Santa Cruz) at 4 °C overnight. Then, membranes were incubated with a secondary horseradish peroxidase-conjugated antibody for 1 h at room temperature. Immunoreactive proteins were visualized using the enhanced chemiluminescence western blotting detection system (Santa Cruz). Staining intensity of the bands was measured using a densitometer (Syngene, Braintree, UK) together with the Genesnap and Genetools software (Syngene). To control for sampling errors, the ratio of band intensities to the β-actin was obtained to quantify the relative protein expression level.

NO production measurement

Total NO production in the mouse lungs were determined by measuring the concentration of nitrite, a stable metabolite of NO in vitro, with a modified Griess (Sigma-Aldrich) reaction method [29]. The absorbance was measured at 550 nm using a Bio-Rad (Hercules, CA) microplate reader, and the nitrite concentration was assessed with reference to the sodium nitrite standard curve.

Statistical analysis

Data were expressed as the means ± S.E.M. Statistical significance was estimated by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. All statistical analyses were done using SPSS 16.0 (SPSS, Inc.). A p-value less than 0.05 was considered significant.
Results

Histological changes in lung tissue sections
Sections of lung tissue were stained with H&E. Fig. 1A shows photomicrographs of the normal histology of lungs. The administration of LPS resulted in diffuse interstitial edema, alveolar thickening, extensive leukocyte infiltration into the interstitium and alveoli, and a marked decrease in alveolar air space (Fig. 1C). The administration of GYY4137, the slow-releasing H₂S donor, resulted in a significant attenuation of these pathological changes (Fig. 1D).

Effect of GYY4137 on MDA and H₂O₂ levels in lung tissues of endotoxemic mice
Wiesel et al. demonstrated that LPS treatment leads to oxidative stress in lung [30]. In the present study, the effect of GYY4137 and L-NAME on the oxidative status of endotoxemic lung tissues was investigated by determining MDA and H₂O₂. As shown in Fig. 2, compared with saline-treated control animals, a significant increase in lung MDA (Fig. 2A) and H₂O₂ (Fig. 2B) levels was detected in endotoxemic mice. The administration of GYY4137 significantly reversed the elevation of the MDA (Fig. 2A) and H₂O₂ (Fig. 2B) levels in the lung tissues of endotoxemic mice. However, the administration of L-NAME did not influence the MDA and H₂O₂ levels in endotoxemic mice lung tissues.

Fig. 1. GYY4137 treatment improves histopathological changes in the lung during endotoxemia. Mice were injected with LPS (5 mg/kg, ip). GYY4137 at a dose of 50 mg/kg was administered alone or immediately before injection of LPS. Twenty-four hours later, the left lower lung was removed for histopathologic examination using hematoxylin and eosin staining. A, Control; B, GYY4137 treatment; C, Endotoxemia; D, Endotoxemia with GYY4137 treatment. Original magnification, x200.

Fig. 2. Effect of GYY4137 and L-NAME on MDA and H₂O₂ levels in lung tissues of endotoxemic mice. Mice were injected with LPS (5 mg/kg, ip). GYY4137 at a dose of 50 mg/kg or L-NAME at a dose 20 mg/kg was administered immediately before injection of LPS. Twenty-four hours later, lung MDA (A) and H₂O₂ (B) levels were determined as described in the “Materials and methods”. Data are means±S.E.M. **p<0.01 vs Control. ##p<0.01 vs Endotoxemia.
Oxidative stress is often defined as an imbalance between pro-oxidants and anti-oxidants [31]. Thus, the present study further investigated the effect of GYY4137 and L-NAME on several anti-oxidant biomarkers, including the GSH/GSSG ratio, T-AOC activity and the activities of two important anti-oxidant enzymes CAT and SOD. Significant decreases in the GSH/GSSG ratio and the activity of T-AOC, CAT and SOD were observed in endotoxemic mice compared with control mice (Fig. 3A, B, C, D.). In addition, treatment with GYY4137 significantly attenuated the LPS-induced decreases of all of these anti-oxidative biomarkers. The administration of L-NAME significantly reduced the activity of T-AOC (Fig. 3B), CAT (Fig. 3C) and SOD (Fig. 3D) in lung tissues of endotoxemic mice but not the GSH/GSSG ratio (Fig. 3A).

Effect of GYY4137 on pro-inflammatory and anti-inflammatory factors in tissues of endotoxemic mice

LPS administration can increase the number of inflammatory cells in lung tissues [32]. As shown in Fig. 4, both GYY4137 and L-NAME attenuated the elevated IL-6, IL-8 and MPO levels induced by LPS in lung. Further, they also reversed the LPS-induced inhibition of IL-10 in endotoxemic mice.

Effects of GYY4137 on iNOS expression and NO production in lung tissues of endotoxemic mice

It is well known that NO production during inflammation is mainly due to inducible NO synthase (iNOS) [33]. As shown in Fig. 5, both iNOS expression and NO production were significantly increased in endotoxemic mice lung tissues. In addition, treatment with GYY4137 significantly decreased the elevated iNOS expression and NO production caused by LPS.
Because 3-NT is considered a marker of ONOO- action, we measured the 3-NT level in lung tissues of endotoxemic mice. As shown in Fig. 6, both GYY4137 and L-NAME decreased the elevated 3-NT level that was induced by LPS.

**Discussion**

Evidence from experimental and clinical studies indicates that a complex network of inflammatory cytokines and chemokines play a major role in the pathogenesis of inflammatory-induced lung injury from sepsis, pneumonia, aspiration, and shock [34]. In our study, quantitative analysis revealed that pro-inflammatory cytokines, such as IL-6 and IL-
8, were increased in the LPS groups compared with control groups; however, the level of anti-inflammatory cytokine IL-10 was decreased in the mice treated with LPS. Further, we determined the concentration of the MPO, which is a marker of neutrophil activity. In the present study, treatment with LPS increased the MPO levels. These data suggest that the inflammation induced by LPS plays an important role in the process of lung injury in septic mice.

It is well known that diverse pro-inflammatory compounds are capable of activating neutrophils in lungs of patients with ALI/ARDS. Furthermore, activated neutrophils in the lungs of patients with ALI/ARDS were a major source of ROS. Additionally, oxidative injury to the lungs mediated by ROS is an important facet of ALI/ARDS. Biologically important ROS include superoxide anion radical (O$_2^-$), H$_2$O$_2$ and hydroxyl radical (OH$^-$) [12]. In the present study, we found that LPS treatment increased the levels of H$_2$O$_2$ and MDA (a biochemical marker of peroxidative damage) and decreased the anti-oxidative biomarkers T-AOC, CAT, SOD.

Accumulating evidence has indicated that nitric oxide, an endogenous gas transmitter, is involved in the pathophysiology of acute lung injury (ALI) [35-37]. Under normal conditions, NO is mainly produced by neuronal NO synthase [nNOS] and endothelial nitric oxide synthase [eNOS] in the lung. However, NO production is mainly due to iNOS during sepsis [33]. NO induced by iNOS reacts with O$_2^-$ to create the potent oxidants ONOO-, leading to an increase in the production of RNS, which further aggravates pulmonary injury in ALI [11, 38-40]. The most well-known effect of ONOO- on proteins is the formation of 3-NT [41]. Moreover, 3-NT is a putative marker of NO-induced oxidant stress and peroxynitrite action. Many studies have shown that iNOS-derived NO led to lung injury, including substantial oxidative/nitrative stress and inflammation via peroxynitrite formation [28, 36, 42]. Consistently, our present report indicated that LPS induced lung tissue injury, followed by NO overproduction and iNOS overexpression. Although, in this study, L-NAME treatment did not decrease the H$_2$O$_2$ and MDA levels, it increased anti-oxidative biomarkers (T-AOC, CAT, SOD). Further, an increased level of 3-NT caused by LPS was rescued by L-NAME. These data suggest that LPS treatment induces ROS and NO production, resulting in oxidative/nitrative stress in the process of acute lung injury.

Previous studies have indicated that H$_2$S is clearly involved in endotoxemia-associated lung injury. However, the role of H$_2$S in lung injury remains controversial. Zhen et al. suggested that an intraperitoneal injection of DL-propargylglycine (PAG, CSE inhibitor) could reduce the serum concentration of H$_2$S and alleviate the lung pathology of rats with acute pancreatitis (AP) [43]. Madhav et al. also found that the prophylactic/therapeutic administration of PAG protected mice from acute pancreatitis-associated lung injury, as evidenced by a significant attenuation of lung MPO activity and by histological evidence of diminished lung injury [44]. However, some animal experiments have confirmed that an infusion of NaHS or H$_2$S inhalation has anti-oxidative and anti-inflammatory effects in animal models of various types of lung injury. Simone et al. clearly indicated that inhaling low-dose hydrogen (80 ppm) for 6 h prevents the development of acute lung injury by attenuating pro-inflammatory responses [45]. Li et al. suggested that treatment with H$_2$S (intraperitoneal injection of 0.1
mg/kg/day of 0.56 mol/L NaHS, H₂S donor) attenuated hypoxia-induced acute lung injury by abating oxidative stress, suppressing inflammation, and reducing lung permeability in mice [46]. Consistent with most of the literature in this area, we indicated that GYY4137 significantly attenuated endotoxemia-associated lung tissue injury. In addition, we provided several lines of evidence that GYY4137 treatment improved LPS-induced lung inflammation. GYY4137 restored lungs that had been affected by LPS-induced lung injury and increased neutrophil activation, which was associated with a negative modulation of pro-inflammatory factors, including IL-6 and IL-8, and a positive modulation of the anti-inflammatory factor IL-10. We also found that the administration of GYY4137 significantly decreased the production of NO and iNOS and the level of oxidative/nitrative stress in the lungs of septic mice. We provided several lines of evidence that GYY4137 treatment reduced the H₂O₂, MDA and 3-NT levels and restored the T-AOC activity, the GSH/GSSG ratio and the activities of SOD and CAT in the lung tissues of endotoxemic mice. Taken together, these data indicate that GYY4137 protects mice against endotoxemia-associated lung tissue injury, partly due to its potent anti-oxidant/nitrative and anti-inflammation properties.

For many decades, H₂S has been well known as a toxic gas with smell of rotten eggs and an environmental hazard, but it has recently received attention as “the third endogenous gaseous signaling transmitter,” in addition to NO and carbon monoxide (CO) in mammals, including humans [47]. Recently, endogenous levels of H₂S in mammalian tissues, such as the liver, kidney, brain, lung, and adrenal gland, have been measured in rats, humans and bovine species. Multiple studies have demonstrated that H₂S can play cytoprotective effects at micromolar concentrations; however, higher H₂S exposure tends to be cytotoxic to the cells. Thus, the development of H₂S or H₂S-releasing drugs as pharmaceuticals would require a detailed understanding of the toxicology, distribution, and metabolism of H₂S. The release of endogenous of H₂S from cells is likely to occur in lesser amounts and at a much slower rate than sodium hydrosulfide (NaHS). Therefore, GYY4137, a H₂S slow-releasing donor, may mimic the biological effects of naturally produced H₂S. Meanwhile, Li et al. reported that the administration of GYY4137 (133μmol/kg or 50 mg/kg) to rats did not cause significant cytotoxic effects or apoptosis or alter the cell cycle profile [48]. In our present study, we also found that the administration of GYY4137 at a concentration of 50 mg/kg could protect the adrenal gland against injury induced by LPS in septic mice. Therefore, GYY4137 may be a useful tool to probe the biological significance of H₂S in mammalian systems and may have therapeutic effects in many diseases.

Conclusion
In conclusion, the present study indicated that GYY4137 conferred protection against acute endotoxemia-associated lung injury, which may be due to the anti-oxidant, anti-nitrative and anti-inflammatory properties of GYY4137. The present findings warrant further exploration of the clinical applicability of H₂S in the prevention and treatment of ALI.

Acknowledgements
This work was supported by National Natural Science Foundation of China (No.81672266, No. 31271270, No. 31371164, No.31500945, No.81100228, NO. 31671213, NO. 81672266), State Key Lab of Space Medicine Fundamentals and Application grants (SMFA12B06,SMFA14K06), The Advanced Space Medico-engineering Research Project of China (NO.060402).

Disclosure Statement
The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.
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