Regulatory Effects of Hydrogen Sulfide on IL-6, IL-8 and IL-10 Levels in the Plasma and Pulmonary Tissue of Rats with Acute Lung Injury

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We examined the possible role of hydrogen sulfide (H2S) in the pathogenesis of oleic acid (OA)-induced acute lung injury (ALI) and its regulatory effects on the inflammatory response. Compared to control rats, the OA-treated rats had decreased partial pressure of oxygen in the arterial blood (PaO2) levels, an increased pulmonary wet/dry weight (W/D) ratio, increased index of quantitative assessment (IQA) score and increased frequency of polymorphonuclear (PMN) cells in the lung 2, 4 or 6 h after OA injection (0.1 ml/kg, intravenous injection). In addition, significantly increased IL-6, IL-8 and IL-10 levels together with decreased H2S levels were observed in the plasma and lung tissue of OA-treated rats compared to controls. Administration of the H2S donor sodium hydrosulfide (NaHS, 56 μmol/L, intraperitoneal injection) into OA-treated rats increased the PaO2 level, reduced the lung W/D ratio and infiltration of PMN cells, and alleviated the degree of ALI (measured by the IQA score). In addition, NaHS decreased IL-6 and IL-8 levels but increased IL-10 levels in the plasma and lung tissues, suggesting that H2S may regulate the inflammatory response during ALI via regulation of IL-6, IL-8 and IL-10. Thus, the down-regulation of endogenous H2S production might be involved in the pathogenesis of OA-induced ALI in rats. Exp Biol Med 233:1081–1087, 2008

Key words: hydrogen sulfide; acute lung injury; interleukin; polymorphonuclear

Introduction

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), are syndromes of acute respiratory failure. The mortality rate associated with ARDS has declined recently (1–3). However, ARDS is still one of the major causes of pulmonary and non-pulmonary morbidity in patients after hospital discharge (4), and the mechanism for ALI has not been fully established. It is known that inflammation is an important fundamental component of the pathogenesis. The imbalance between inflammatory and anti-inflammatory cytokines aggravates the inflammatory response and boosts the development of ALI/ARDS (5–8). IL-8 and IL-6 are all important inflammatory cytokines, while IL-10 is an anti-inflammatory cytokine in inflammatory response. The changes in ILs (IL-6, 8, 10) play critical roles in the inflammatory process of ALI.

Hydrogen sulfide (H2S) is a novel neurotransmitter in the cardiac and respiratory system (9). H2S has important pathophysiological effects in the progression of many diseases such as hypoxic pulmonary hypertension, myocardial injury, hypertension, and shock (10–14). However, the roles of H2S in the regulation of inflammation are still in dispute (15, 16). H2S donors reportedly inhibit aspirin-induced leukocyte adhesion in the endothelium of rat mesenteric venules, whereas inhibitors of H2S synthesis elicit leukocyte adhesion, which suggests that H2S probably plays an important part in the regulation of inflammatory

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response. Zhang (17) reported that H$_2$S acted as an inflammatory mediator in cecal ligation and puncture-induced sepsis in mice by upregulating the production of cytokines and chemokines via NF-kappa B. We hypothesized that H$_2$S is important in regulating the inflammatory response in the process of ALI. Therefore, in the present study, we examined the possible role of H$_2$S in the pathogenesis of oleic acid (OA)-induced ALI and its regulatory effects on the inflammatory cytokines interleukin (IL)-6, IL-8 and IL-10 to further our understanding of H$_2$S involvement in the development of ALI.

Materials and Methods

Materials. All animal care and experimental protocols complied with the Animal Management Rules of the Ministry of Health of the People’s Republic of China (documentation number 19890503) and the guide for the Care and Use of Laboratory Animals of the First Hospital, Peking University (China).

A total of 49 Sprague-Dawley male rats weighing 200–250 g were obtained from the Animal Center of Peking University First Hospital (China). The rats were housed at room temperature (25 ± 2 °C) in a controlled environment with a 12-h light/12-h dark cycle, lights on at 7:00 AM. All animals had free access to a standard laboratory chow, Xie-Li (SCXK2005–2007) from Beijing Ke-Ao Xie-Li Chow Company Ltd, containing protein (20 g/100 g), fat (4 g/100 g), calcium (1–1.8 g/100 g), fiber (<5 g/100 g), phosphorus (0.6–1.2 g/100 g) (Laboratory Animal Centre, AMMS, Beijing, China) and deionized water with 2 drinking bottles attached to each cage throughout the experimental period. Sodium hydrosulfide (NaHS) was purchased from Beijing Chemical Reagents Company (China). ELISA kits for detection of IL-6, IL-8 and IL-10 were purchased from Rapidbio (CA, USA). Other chemicals and reagents were of analytical grade.

Preparation of the Rat ALI Model (18–20). Rats were weighed and anesthetized with 0.25% pentobarbital sodium (40 mg/kg, intraperitoneal injection).

Analysis of Arterial Blood Gas. Blood samples of rats from OA group and OA+NaHS group were collected from the abdominal aorta at 2, 4, and 6 h after OA injection and blood samples of control group were drawn at 6 h after NS injection. All the blood samples were collected in self-filling, polypropylene syringes containing dry electrolyte-balanced heparin. Air pockets were expelled immediately after the sampling and the samples were thereafter mixed gently until testing PO$_2$, PCO$_2$ and pH on an automated blood gas analyzer (OMNITM-3, AVL, Switzerland) in 10 mins. These animals breathed air normally before the blood samples were collected.

Measurement of the Wet/Dry Weight (W/D) Ratio in the Lung Tissue. The upper lobe of the right lung was excised. Blood and water were removed from the lung surface and the wet weight was evaluated. The lobe was placed in an electric drying oven for 48 h at 80°C. Then the dry weight was measured and the W/D ratio calculated.

Leukocyte Differential Count in the Broncho-Alveolar Lavage Fluid (BALF). After orotracheal intubation and ligature of the hilum of the right lung, the left lung was lavaged twice with normal saline (2 ml), and the BALF was collected and centrifuged at 1500 rpm/min. The liquid supernatant was removed, and the sediment was spread on a slide and examined under a microscope: leukocytes were counted and the percentage of polymorphonuclear (PMN) cells (PMN%) was determined.

Morphology of Lung Tissues. The middle lobe of the right lung was fixed in 10% (wt/vol) formalin and routinely processed into paraffin sections (4–5 μm). Sections were used for hematoxylin and eosin staining (20). Six slices were selected from each group of rats, and 10 fields of each slice were visualized by microscopy (×400), Table 1 showed the scores used to assess the degree of pathological change. The average values were taken as a semi-quantitative histological index of quantitative assessment (IQA) of lung injury (21, 22).

Measurement of H$_2$S Content in the Plasma and Lung Tissue. The lung tissue homogenates for testing H$_2$S content were obtained from the lower lobe of the right lung which was homogenized in ice-cold 10 mmol/L phosphate buffered saline (pH 7.2). Plasma for testing H$_2$S content was obtained from the blood samples that were collected from abdominal aorta via a stainless steel needle into a heparinized syringe and immediately centrifuged.

<table>
<thead>
<tr>
<th>Score</th>
<th>Hyperaemia and edema</th>
<th>RBC and WBC infiltration</th>
<th>Hyaline membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No abnormalities</td>
<td>No RBC and WBC</td>
<td>No hyaline membrane</td>
</tr>
<tr>
<td>1</td>
<td>Mild</td>
<td>Very few</td>
<td>0–20% alveolus</td>
</tr>
<tr>
<td>2</td>
<td>Moderate</td>
<td>Part of alveolus</td>
<td>In 20–50% alveolus</td>
</tr>
<tr>
<td>3</td>
<td>Serious</td>
<td>Full of alveolus</td>
<td>In &gt;50% alveolus</td>
</tr>
</tbody>
</table>
Measurement of $H_2S$ content in plasma or homogenates involved use of an ELIT Ion Analyzer (ELIT 9801, Electro Analytic Instruments LTD, England) as mentioned in the previous study (23). In brief, 0.5 mL of sulfide antioxidant (SAOB, NaOH 2.35 mol/L and EDTA 0.27 mol/L) was added into 0.5 mL of $H_2S$ standard solutions (10, 20, 30, 40, 50, 60 and 80 $\mu$mol/L, respectively) and then stirred thoroughly. A sulfide-sensitive electrode (ELIT 8225, Electro Analytic Instruments LTD, England) and a reference electrode (ELIT 003n, Electro Analytic Instruments LTD, England) were rinsed in deionized water, blotted dry, immersed into mixture of SAOB and 10 $\mu$mol/L $H_2S$ standard solution. When a stable reading was displayed, the voltage value (mV) was recorded. This procedure was repeated for the other mixtures of SAOB and different concentration of $H_2S$ standard solutions in turn. When all the standards were measured, the standard curve of mV versus concentration was plotted. The electrodes were washed as before and the samples were measured in the same way as the measurement for standard solution. The sample data were plotted on the standard curve and the sample concentration was obtained.

**Measurement of IL-6, IL-8, and IL-10 Levels in the Plasma and Lung Tissue.** The lower lobes of the right lung were homogenized centrifuged as before. The liquid supernatants were obtained as homogenates for testing IL-6, IL-8 and IL-10 concentrations. Blood samples in each group were collected in heparinized tubes from the abdominal aorta and centrifuged at 3000 rpm. Cytokines levels were assayed using double-antibody sandwich Enzyme-Linked Immunosorbent Assay (ELISA), following the manufacturer’s instructions (Rapibio, CA, USA). Briefly, samples (100 $\mu$L) and IL-6 standards (1000, 500, 250, 125, 62.5 and 0 pg/ml) were added to the wells. Each was tested in duplicate. After 1 h of incubation at 37°C, samples were removed and the plates were washed with a washing buffer (consisting of PBS, 10 mmol/L pH 7.4 and Tween 20, 0.1%), soaked for a few minutes and washing was repeated for five times. Blot plates dry by tapping upside down on filter paper. Anti rat IL-6 biotin (100 $\mu$L) was added to each well of the plates, and left for 30 mins at 37°C. After five additional washing steps, 100 $\mu$L horse-radish peroxidase (HRP) was added to the wells, and left for 30 mins at 37°C. The plates were washed for five times again and 100 $\mu$L of tetramethylbenzidine (TMB) substrate was added to each well and shaken for 10 sec gently. The mixture was incubated in the dark for 30 mins at room temperature. Optical density (OD) value at 450 nm was measured by a Bio-Rad ELISA reader (Bio-Rad, Richmond, CA) after stopping the reaction by adding 100 $\mu$L of stop solution to each well. The standard curve of OD value versus concentration of IL-6 was obtained. The sample data was plotted on the standard curve and read off the sample IL-6 concentration. IL-8 and IL-10 contents in samples were run in the same assay.

**Data Analysis.** Results are expressed as means ± SD. ANOVA followed by a post hoc analysis (Bonferroni test) was used for comparison among groups. Analysis involved SPSS, version 11.5, for Windows. IQA scores were tested by the rank sum test. A value of $P < 0.05$ was considered to be significant.

**Results**

**Impacts of Hydrogen Sulfide Donor on PaO$_2$, Lung W/D Ratio, Leukocyte Differential Count in BALF, and IQA of Lung Injury in OA-Treated Rats.** In our rat model of ALI, the PaO$_2$ in OA-treated rats was significantly lower than in control rats at each time point examined (all $P < 0.01$, Table 2). In control rats few infiltrating PMN cells were observed and there was not evidence of hemorrhage or edema (Fig. 1a). In comparison, OA-treated rats showed diffuse edema and severe inflammatory cell infiltration in alveoli and interstitium of the lung, hemorrhage, and thickened interlobular septa (Fig. 1b). Significantly higher IQA scores were observed in the OA-treated rats compared to the controls at each time point (all $P < 0.01$, Table 3). The lung W/D ratio was also significantly higher in OA-treated rats than controls at each time point (all $P < 0.01$, Table 2). OA+NaHS-treated rats showed ameliorated OA-induced pulmonary injury, increased PaO$_2$ values (all $P < 0.05$, Table 2), lowered IQA scores (Table 3) and decreased W/D ratio in the lung tissue (all $P < 0.05$, Table 2) compared with the OA-alone group. $H_2S$ donor treatment attenuated PMN infiltration in the lung: the percentage of PMN cells in the BALF was significantly lower in the OA+NaHS group than the OA-alone group at each time point (all $P < 0.05$, Table 3). The degree of injury observed by histology was also ameliorated compared with that observed in the OA-alone group (Fig. 1c).

<table>
<thead>
<tr>
<th>Group</th>
<th>PaO$_2$ (mmHg)</th>
<th>Wet weight/dry weigh ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OA+NaHS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Comparison of PaO$_2$ and Wet/Dry Ratio Between the Untreated and Treated Rats

$^a$ $P < 0.01$ compared with control.
$^b$ $P < 0.05$ compared with OA.
H₂S Levels in the Plasma and Lung Tissue. H₂S levels in both the plasma and lung tissue of OA-treated rats were significantly lower than in controls at each time point (all \( P < 0.01 \), Table 4). NaHS significantly increased H₂S levels in the plasma and lung tissue compared with the OA-alone group (all \( P < 0.05 \), Table 4).

Levels of IL-6, IL-8 and IL-10 in the Plasma and Lung Tissue. We found significantly higher levels of IL-6, IL-8 and IL-10 in the plasma and lung tissue of OA rats than in controls (all \( P < 0.01 \), Tables 5, 6 and 7). Administration of the H₂S donor in the OA-treated rats significantly decreased the IL-6 levels in the plasma at each time point (2 h, \( P < 0.05 \); 4 and 6 h, \( P < 0.01 \)) and in the lungs at the 2 h (\( P < 0.05 \)) and 6 h (\( P < 0.01 \)) time points compared with the OA-alone group. Similarly, administration of the H₂S donor in OA-treated rats significantly decreased the IL-8 levels in the plasma (2 and 4 h, \( P < 0.05 \); 6 h, \( P < 0.01 \)) and lungs (2 h, \( P < 0.05 \); 4 and 6 h, \( P < 0.01 \)) compared with the OA-alone group. Interestingly, H₂S donor treatment in OA-treated rats significantly elevated IL-10 levels in the plasma by 34% at 4 h (\( P < 0.01 \)) and 35% at 6 h (\( P < 0.05 \)) and in the lungs by 15% at 6 h (\( P < 0.05 \)) compared with the OA-alone group.

Discussion

ALI is characterized by an acute inflammatory process in the airspace and lung parenchyma. In our rat model of ALI, OA-treated rats showed diffuse edema and severe inflammatory cell infiltration in alveoli and interstitium of the lung, hemorrhage, and thickened interlobular septa. Treatment with OA induced diffuse PMN cells infiltration into pulmonary alveolar spaces resulting in an increased percentage of PMN cells, lymphocytes and monocytes. Simultaneously, the PaO₂ in OA-treated rats was significantly lower than in control rats at each time point examined. Thus, intravenous injection of OA successfully induced ALI in our rats, as reported previously (18, 24, 25).

Recently, the gaseous messenger molecules nitric oxide (NO) and carbon monoxide (CO) were found to be involved in the mechanism of ALI/ARDS pathogenesis (26–28). H₂S, a newly characterized gaseous messenger molecule in the cardiovascular and respiratory systems, was found to be generated endogenously during cysteine metabolism in many types of mammalian cells in a reaction catalyzed by cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE). CSE and CBS expressions have been identified in human and other mammalian cells, including those from arteries, liver, kidney, brain, skin fibroblasts and blood lymphocytes (29). H₂S can protect nerve cells from oxidation injury (15) and Na₂S has been shown to inhibit chemotaxis and degranulation of PMN cells (30). In addition, H₂S donors reportedly inhibit aspirin-induced leukocyte adhesion in the endothelium of rat mesenteric venules, whereas inhibitors of H₂S synthesis elicit leukocyte adhesion (31). NaHS has been shown to inhibit apoptosis in isolated human neutrophils but had no effect on their bactericidal properties (32). Regardless, the role and significance of endogenous H₂S in the pathogenesis of ALI has not been clear.

To investigate the role of H₂S in the development of
ALI, the H\(_2\)S donor NaHS was administered to OA-treated rats (OA+NaHS group). In our study, we found that H\(_2\)S levels in both the plasma and lung tissue of OA-treated rats were significantly lower than in controls at each time point. At the same time, we found that NaHS significantly increased H\(_2\)S levels in the plasma and lung tissue compared with the OA-alone group. Interestingly, OA+NaHS group rats showed ameliorated OA-induced pulmonary injury, increased PaO\(_2\) values, lowered IQA scores and decreased W/D ratio in the lung tissue compared with the OA-alone

### Table 4. Comparison of H\(_2\)S Concentrations in Plasma and Lung Tissue Between the Untreated and Treated Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>H(_2)S concentration in plasma ((\mu)mol/L)</th>
<th>H(_2)S concentration in lung tissue (nmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 h</td>
<td>4 h</td>
</tr>
<tr>
<td>OA</td>
<td>21.30 ± 2.75(^a)</td>
<td>20.63 ± 1.26(^a)</td>
</tr>
<tr>
<td>OA+NaHS</td>
<td>22.61 ± 1.31(^a)</td>
<td>26.67 ± 3.44(^a,b)</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^a\) P < 0.01 compared with control.
\(^b\) P < 0.05 compared with OA.

### Table 5. Comparison of the Concentrations of IL-6 in Plasma and Lung Tissue Between the Untreated and Treated Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-6 concentration in plasma (pg/ml)</th>
<th>IL-6 concentration in lung tissue (pg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 h</td>
<td>4 h</td>
</tr>
<tr>
<td>OA</td>
<td>186.70 ± 23.85(^a)</td>
<td>238.50 ± 26.46(^a)</td>
</tr>
<tr>
<td>OA+NaHS</td>
<td>122.91 ± 20.80(^a,b)</td>
<td>185.37 ± 21.98(^a,c)</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^a\) P < 0.01 compared with control.
\(^b\) P < 0.05 compared with OA.
\(^c\) P < 0.01 compared with OA.

### Table 6. Comparison of the Concentrations of IL-8 in Plasma and Lung Tissue Between the Untreated and Treated Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-8 concentration in plasma (pg/ml)</th>
<th>IL-8 concentration in lung tissue (pg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 h</td>
<td>4 h</td>
</tr>
<tr>
<td>OA</td>
<td>184.11 ± 19.51(^a)</td>
<td>286.20 ± 53.34(^a)</td>
</tr>
<tr>
<td>OA+NaHS</td>
<td>134.88 ± 17.42(^a,b)</td>
<td>199.40 ± 34.56(^a,b)</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^a\) P < 0.01 compared with control.
\(^b\) P < 0.05 compared with OA.
\(^c\) P < 0.01 compared with OA.

### Table 7. Comparison of the Concentrations of IL-10 in Plasma and Lung Tissue Between the Untreated and Treated Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-10 concentration in plasma (pg/ml)</th>
<th>IL-10 concentration in lung tissue (pg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 h</td>
<td>4 h</td>
</tr>
<tr>
<td>OA</td>
<td>111.18 ± 11.46(^a)</td>
<td>115.60 ± 13.91(^a)</td>
</tr>
<tr>
<td>OA+NaHS</td>
<td>117.46 ± 24.28(^a)</td>
<td>154.48 ± 18.08(^a,c)</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

\(^a\) P < 0.01 compared with control.
\(^b\) P < 0.05 compared with OA.
\(^c\) P < 0.01 compared with OA.
group. H₂S donor treatment attenuated PMN cells infiltration in the lung. The degree of injury observed by histology was also ameliorated compared with that observed in the OA-alone group. Thus, administration of the H₂S donor significantly ameliorated the lung injury induced by OA, suggesting that the down-regulation of endogenous H₂S during ALI might be involved in the pathogenesis of ALI.

The mechanism by which H₂S regulates ALI pathogenesis remains unclear. Inflammatory cytokines have well-characterized effects on the pathogenesis of OA-induced lung injury (8). IL-6 is produced by many cell types including monocytes/macrophages, endothelial cells, fibroblasts, and smooth muscle cells, in response to stimulation by endotoxin. IL-1β, and tumor necrosis factor (TNF)-α (33–36). Circulating levels of IL-6 are good predictors of the severity of ARDS with different etiologies such as sepsis (37) and acute pancreatitis (38). The importance of IL-6 in the acute-phase response has been confirmed by its ability to stimulate synthesis of acute-phase proteins including C reactive protein from hepatocytes in vitro and in vivo (39–41). Patients with systemic inflammatory conditions such as sepsis/systemic inflammatory response syndrome also have increased circulating levels of IL-8 (42). In acute pancreatitis, increased IL-8 levels predict the severity of the disease (43, 44). The anti-inflammatory cytokine IL-10 (45–47) inhibits alveolar macrophage production of pro-inflammatory mediators involved in ARDS. Increased IL-10 plasma levels in animal models of endotoxaemia may inhibit the release of pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α from monocytes/macrophages, thereby preventing subsequent tissue damage (48–51).

We found that levels of IL-6, IL-8 and IL-10 in the plasma and lung tissue are at lower level in control rats. But OA injection made the levels of IL-6, IL-8 and IL-10 in the plasma and lung tissue increase significantly. Administration of the H₂S donor in the OA-treated rats significantly decreased the IL-6 and IL-8 levels in the plasma and in the lung. Interestingly, H₂S donor treatment in OA-treated rats significantly elevated IL-10 levels in plasma and in the lung. ALI in essence is an acute and uncontrollable inflammatory process. Inflammatory cytokines play crucial roles in the process. Simultaneous production of anti-inflammatory cytokines can potentially counteract the effects of pro-inflammatory cytokines and modify the intensity of the inflammation (52). So we conjectured that H₂S might play a protective role through changing the levels of inflammatory and anti-inflammatory cytokines in plasma and in the lung.

However, Huili Zhang (53) reported that injection of NaHS (10 mg/kg, i.p.) up-regulated leukocyte rolling and attachment significantly, as well as tissue levels of adhesion molecules in sepsis. They thought NaHS treatment enhanced the level of adhesion molecules and neutrophil infiltration in lung. Zhang (17) reported that injection of NaHS (10 mg/kg, i.p.) promoted inflammatory response in sepsis in mice by upregulating the production of cytokines and chemokines via NF-kappaB. In our study, the injection dose of NaHS was 56 μmol/L (3.1 mg/kg, i.p.). In this low dose we did not find the toxic effects of H₂S.

In summary, we have provided experimental evidence that demonstrates the importance of H₂S in both inflammatory and anti-inflammatory responses in OA-induced ALI. We further showed that H₂S donor NaHS could decrease the levels of inflammatory cytokines but increase that of anti-inflammatory cytokines. Our findings suggested that H₂S might play an important role in the acute inflammatory response. Because of the complicated functions of H₂S in inflammation process, further studies are needed to clarify the detailed mechanisms by which H₂S regulates inflammatory response.


