MOLECULAR HYDROGEN AMELIORATES LIPOPOLYSACCHARIDE-INDUCED ACUTE LUNG INJURY IN MICE THROUGH REDUCING INFLAMMATION AND APOPTOSIS

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ABSTRACT—Acute lung injury (ALI) is still a leading cause of morbidity and mortality in critically ill patients. Recently, our and other studies have found that hydrogen gas (H₂) treatment can ameliorate the lung injury induced by sepsis, ventilator, hyperoxia, and ischemia-reperfusion. However, the molecular mechanisms by which H₂ ameliorates lung injury remain unclear. In the current study, we investigated whether H₂ or hydrogen-rich saline (HS) could exert protective effects in a mouse model of ALI induced by intratracheal administration of lipopolysaccharide (LPS) via inhibiting the nuclear factor κB (NF-κB) signaling pathway-mediated inflammation and apoptosis. Two percent of H₂ was inhaled for 1 h beginning at 1 and 6 h after LPS administration, respectively. We found that HS-challenged mice exhibited significant lung injury characterized by the deterioration of histopathology and histologic scores, wet-to-dry weight ratio, and oxygenation index (PaO₂/FIO₂), as well as total protein in the bronchoalveolar lavage fluid (BALF), which was attenuated by H₂ treatment. Hydrogen gas treatment inhibited LPS-induced pulmonary early and late NF-κB activation. Moreover, H₂ treatment dramatically prevented the LPS-induced pulmonary cell apoptosis in LPS-challenged mice, as reflected by the decrease in TUNEL (deoxynucleotidyl transferase dUTP nick end labeling) staining-positive cells and caspase 3 activity. Furthermore, H₂ treatment markedly attenuated LPS-induced lung neutrophil recruitment and inflammation, as evidenced by down-regulation of lung myeloperoxidase activity, total cells, and polymorphonuclear neutrophils in BALF, as well as proinflammatory cytokines (tumor necrosis factor α, interleukin 1β, interleukin 6, and high-mobility group box 1) and chemokines (keratinocyte-derived chemokine, macrophage inflammatory protein [MIP] 1α, MIP-2, and monocyte chemotactic protein 1) in BALF. In addition, i.p. injection of 10 mL/kg hydrogen-rich saline also significantly attenuated the LPS-induced ALI. Collectively, these results demonstrate that molecular hydrogen treatment ameliorates LPS-induced ALI through reducing lung inflammation and apoptosis, which may be associated with the decreased NF-κB activity. Hydrogen gas may be useful as a novel therapy to treat ALI.

KEYWORDS—Acute lung injury, hydrogen gas, apoptosis, inflammation, chemokines

ABBREVIATIONS—ALI—acute lung injury; ARDS—acute respiratory distress syndrome; BALF—bronchoalveolar lavage fluid; ELISA—enzyme-linked immunosorbent assay; H₂—hydrogen gas; HMGB1—high-mobility group box 1; HS—hydrogen-rich saline; i.t.—intratracheal; KC—keratinocyte-derived chemokine; LPS—lipopolysaccharide; MCP-1—monocyte chemotactic protein 1; MIP-1α—macrophage inflammatory protein 1α; MIP-2—macrophage inflammatory protein 2; MPO—myeloperoxidase; PBS—phosphate-buffered saline; PMNs—polymorphonuclear neutrophils; TUNEL—deoxynucleotidyl transferase dUTP nick end labeling; W/D—wet-to-dry

INTRODUCTION

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), represent a spectrum of common syndromes in critically ill patients with a mortality rate of 30% to 50% (1, 2). Excessive cytokine-mediated inflammation plays a fundamental role in the pathogenesis of ALI (3). Moreover, nuclear factor κB (NF-κB) is a critical transcription factor required for maximal expression of many cytokines involved in the pathogenesis of ALI (4).

Recently, it is widely accepted that hydrogen gas (H₂) or hydrogen-rich saline (HS) exerts an effective therapeutic role in many disorders including stroke, ischemia-reperfusion injury, organ transplantation, sepsis, multiple organ dysfunction syndrome, type 2 diabetes, atherosclerosis, neurodegenerative diseases, oxygen toxicity via reducing oxidative stress, inflammation, and apoptosis (5–20). Furthermore, our recent studies have shown that H₂ inhalation significantly improves the survival rate and lung damage of septic mice in a concentration- and time-dependent manner (11, 12). Moreover, the beneficial...
effects of H₂ treatment on sepsis were associated with the decreased levels of oxidative stress and inflammatory cytokines in serum and tissues (11, 12). Besides, some reports show that H₂ treatment can improve the hyperoxia- or ventilator-induced lung injury through reducing inflammation and oxidation (21–23). Although the therapeutic efficacies of H₂ have been extensively studied, the detailed mechanism is unclear.

At low concentrations (<4.6% in air and 4.1% in pure oxygen), H₂ is neither inflammable nor explosive (19). Gram-negative bacteria are the leading cause of ALI/ARDS (3). Intratracheal administration of lipopolysaccharide (LPS), the major component of the outer membrane of gram-negative bacteria, is a well-established model of ALI (24). However, it remains unknown whether H₂ or HS treatment has any protective effects on LPS-induced ALI and, if so, whether downregulation of lung inflammation and apoptosis via inhibition of NF-κB plays any role in it. The present study was designed to test the hypothesis that molecular hydrogen attenuates LPS-induced ALI through downregulation of lung inflammation and apoptosis by suppressing the NF-κB activation.

MATERIALS AND METHODS

Animals

Adult male C57BL/6 mice weighing 20 to 25 g were provided by the Laboratory Animal Center of the Academy of Military Medical Sciences in Beijing, China. Animals were housed under specific pathogen-free conditions at 20°C to 22°C with a 12:12-h light-dark cycle. Standard animal chow and water were freely available. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Tianjin Medical University and performed in accordance with the National Institutes of Health (Bethesda, Md) guidelines for the use of experimental animals.

LPS-induced ALI

As previously described (24, 25), ALI was induced by intratracheal (i.t.) administration of LPS. Briefly, animals were anesthetized with an i.p. injection of pentobarbital sodium (50 mg/kg). They were orally intubated with a sterile plastic catheter and intratracheally given a single dose of aerosolized LPS (25 μg/mouse; Escherichia coli 0111:B4; Sigma, St. Louis, Mo). Control mice were intratracheally given 50 μL of sterile phosphate-buffered saline (PBS).

H₂ treatment

According to our previous studies (11, 12, 18), the animals were put in a sealed Plexiglas chamber with inflow and outflow outlets. Hydrogen gas was supplied through a gas flowmeter, TF-1 (YUTAKA Engineering Corp, Tokyo, Japan), and delivered by air into the chamber through a tube at a rate of 4 L/min. The concentration of oxygen in the chamber was maintained at 21% by using supplemental oxygen and continuously monitored with a gas analyzer (Medical Gas Analyzer LB-2, Model 40 M; Beckman Coulter, Inc, Fullerton, Calif). The concentration of H₂ in the chamber was continuously monitored with a commercially available detector (HY-ALERTA Handheld Detector Model 500; HY Scan, Valencia, Calif) and maintained at 2% during the treatment. Carbon dioxide was removed from the chamber gases with Baralyme. The animals without H₂ treatment were exposed to room air in the chamber.

Preparation of HS

The detailed information for the preparation of HS was described in previous reports (16, 20). Hydrogen gas was dissolved in normal saline for 6 h under high pressure (0.4 MPa) to a supersaturated level. The saturated HS was stored under atmospheric pressure at 4°C in an aluminum bag with no dead volume and was sterilized by γ-radiation. The hydrogen level in the saline was measured using gas chromatography as described by Ohsawa et al (7). The average hydrogen level was 0.85 mmol/L. Hydrogen-rich saline was freshly prepared every week to ensure that the concentration of hydrogen was more than 0.6 mmol/L.

Experimental design

Experiment 1: Effects of H₂ treatment on LPS-induced ALI in mice—Sixty-four animals were randomly divided into four groups (n = 16 per group): PBS, PBS + H₂, LPS, and LPS + H₂ groups. The animals in the PBS + H₂ and LPS + H₂ groups were exposed to 2% H₂ inhalation for 60 min starting at 1 and 6 h after i.t. administration of PBS or LPS, respectively. As a control, the animals from the PBS and LPS groups were given room air treatment at the same time points. The oxygenation index (PaO₂/FIO₂) was measured at 24 h after PBS or LPS administration. Moreover, the bronchoalveolar lavage fluid (BALF) was obtained for measuring the protein concentration as well as the number of total cells and polymorphonuclear neutrophils (PMNs) at 4 and 24 h after PBS or LPS administration (n = 6 per group at each time point). In addition, the lung samples were harvested for evaluating the histopathology (TUNEL) staining, caspase 3 activity, and NF-κB p65 nuclear translocation and DNA-binding activity.

Experiment 2: The underlying mechanisms of H₂ treatment in LPS-induced ALI—Additional 48 animals were used in this experiment and were randomly assigned to four groups (n = 12 per group). The grouping method and experimental protocols were the same as experiment 1. At 4 and 24 h after PBS or LPS administration, the inflammatory cytokines (tumor necrosis factor α [TNF-α], interleukin 1β [IL-1β], IL-6, high-mobility group box 1 [HMGB1], IL-10) and chemokines (keratinocyte-derived chemokine [KC], macrophage inflammatory protein 1α [MIP-1α], MIP-2, monocyte chemotactant protein 1 [MCP-1]) in the BALF were measured (n = 6 per group at each time point). In addition, the lung samples were harvested for evaluating the histopathology, W/D weight ratio, and MPO activity.

Oxygenation index analysis

To evaluate the oxygenation capability of lung, the ratio of oxygen tension to inspired oxygen fraction (PaO₂/FIO₂) was calculated. At 24 h of PBS or LPS administration, animals were anesthetized and given endotracheal intubation with a 20-gauge catheter. They were subjected to mechanical ventilation with pure oxygen at 7 mL/kg. The respiratory rate was 120 breaths/min. The animals were ventilated for 15 min before blood gas sampling. The arterial blood was obtained from carotid artery and measured with a GEM Premier 3000 gas analyzer (Instrumentation Laboratory, Milan, Italy).

Cell counts and protein concentration in BALF

Animals were subjected to bronchoalveolar lavage for collecting BALF by the methods described previously (26). Bronchoalveolar lavage fluid was obtained by cannulating the trachea with a 20-gauge catheter. Two volumes of 0.5 mL of PBS (pH 7.4) were instilled, gently aspirated, pooled, and resuspended. Lavage samples were centrifuged at 1,500 g for 10 min at 4°C. The supernatant was stored at −20°C. Furthermore, the cell pellet was resuspended in PBS, and subsequently, the number of total cells was determined using a hemocytometer (Beckman Coulter, Inc). The slides were visualized using Wright–Giensa staining (Beckman Coulter, Inc). PMNs were identified by a certified laboratory technologist in a blinded fashion. Total protein concentration in the BALF was determined using a standard commercial kit (Bio-Rad Laboratories, Hercules, Calif).

Histologic examination

Lungs were harvested for observing morphologic alterations at 24 h after PBS or LPS administration. The samples were fixed with 10% formalin for 6 h at room temperature, embedded in paraffin, and sectioned at 5-μm thickness. After deparaffinization and rehydration, the sections were stained with hema- toxylin and eosin. Histologic changes were evaluated by two pathologists who were blinded to the treatment regimen. A scoring system to grade the degree of lung injury was used, based on the following histologic features: edema, hyperemia and congestion, neutrophil margination and tissue infiltration, intraalveolar hemorrhage and debris, and cellular hyperplasia. Each feature was graded as absent, mild, moderate, or severe, with a score of 0 to 3. A total score was calculated for each animal (11).
**WD weight ratio**
To quantify the magnitude of pulmonary edema, we evaluated lung WD weight ratio. The harvested wet lung was weighed and then placed in an oven for 24 h at 80°C and weighed when it was dried. The ratio of wet lung to dry lung was calculated (11).

**MPO activity**
At 4 and 24 h after PBS or LPS administration, lungs were obtained and perfused with cold PBS to remove all blood, then weighed and stored at −80°C for more than 1 week before the MPO assay. The supernatant from lung homogenate was prepared for detecting MPO activity (11). Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol of peroxide per minute at 37°C and was expressed in unit per gram weight of wet tissue. The change in absorbance was measured spectrophotometrically at 590 nm by spectrophotometer (DU 640B; Beckman Coulter, Inc).

**TUNEL staining**
To detect DNA fragmentation in the nuclei of cells, we applied an In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) for quantification of apoptosis as described in our previous study (5). Apoptotic cells were manifested by brownish staining in the nuclei. Ten images were randomly selected from each section for counting at least 1,000 cells, and the apoptosis index was expressed as a percentage of TUNEL-positive cells (27). Two blinded pathologists performed the examination.

**Caspase 3 activity**
Lung homogenates were prepared, and the caspase 3 activity was measured with caspase 3/CPP32 Fluorometric Assay Kit (Biovision, Inc, Mountain View, Calif) in accordance with the manufacturer’s instructions (5). The assay was run in duplicate.

**Enzyme-linked immunosorbent assay**
The cytokines and chemokines in the BALF were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (mouse TNF-α, IL-1β, IL-6, IL-10, KC, MIP-1α, MIP-2, and MCP-1 ELISA kits are from R&D Systems, Minneapolis, Minn; HMGB1 ELISA kit is from IBL, Hamburg, Germany). All spectrophotometric readings were performed with a microplate reader (CA 94089; Molecular Devices, Sunnyvale, Calif). All experiments were performed according to the manufacturers’ instructions (11, 12).

**Western Blot Analysis**
To investigate the effects of H2 treatment on NF-κB p65 nuclear translocation, fresh lung tissues were harvested, and nuclear extracts were prepared with a nuclear extract kit (Active Motif, Carlsbad, Calif). Extracts were fractionated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblots were developed as described previously (21). The proteins were then electrotransferred to nitrocellulose membranes and blotted with anti-p65 antibody (Cell Signaling Technology, Beverly, Mass) and anti-β-TATA-binding protein antibody (Abcam, Cambridge, UK), and the protein expression was detected using an enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Piscataway, NJ).

**NF-κB activity**
The DNA-binding activity of NF-κB in lung tissues was quantified by ELISA, using the TransAM NF-κB p65 transcription factor assay kit (Active Motif). The nuclear extracts of lung tissues were prepared with a nuclear extract kit (Active Motif). According to the manufacturer’s instructions, all standards and samples were run in duplicate (28).

**Statistical analysis**
All values, except for histologic scores, are presented as mean ± SEM. The histologic scores were analyzed with Kruskal-Wallis test followed by the Mann-Whitney U test with Bonferroni correction. The intergroup differences of the rest data were tested by one-way analysis of variance followed by LSD-t test for multiple comparisons. The statistical analysis was performed using SPSS 16.0 software (SPSS Inc, Chicago, Ill). In all tests, P < 0.05 was considered statistically significant.

**RESULTS**

**H2 treatment attenuates LPS-induced lung injury in mice**
In the present study, we first investigated the effects of 2% H2 inhalation on lung histopathology and function in mice with either PBS or LPS challenge (Fig. 1). Lipopolysaccharide-challenged mice appeared to have significant lung injury characterized by alveolar wall thickening, infiltration of neutrophils into lung interstitium and alveolar space, consolidation, and alveolar hemorrhage (See Figure, Supplemental Digital Content 1, at http://links.lww.com/SHK/A132). Representative lung histology illustrate that hydrogen gas treatment resulted in a reduction of infiltrated inflammatory cells and a marked improvement in lung architecture in LPS-challenged mice. The animals were treated as described in Fig. 1. In addition, lung samples were harvested for hematoxylin and eosin (HE) staining. A, PBS group; B, LPS group; C, LPS+H2 group; original magnification, ×40.). Moreover, a scoring system to grade the degree of lung injury was used. Lipopolysaccharide-challenged mice showed the significant increase in lung histologic scores (P < 0.05 vs. PBS group, n = 10 per group; Fig. 1A), which was reduced by H2 treatment (P < 0.05, n = 10 per group; Fig. 1A). Meanwhile, LPS-challenged mice showed the significant increase in lung W/D ratio when compared with PBS group, which was also decreased by H2 treatment (P < 0.05, n = 6 per group; Fig. 1B). Interestingly, the PaO2/FiO2 was significantly decreased in LPS-challenged mice, which was improved by H2 treatment (P < 0.05, n = 6 per group; Fig. 1C). In addition, to exclude that H2 inhalation might cause hypoxia in mice with LPS or PBS administration, the arterial blood gas was measured in all groups during H2 treatment. There were no differences in the levels of arterial pH, PaO2, and PCO2 among all groups (data not shown). These results demonstrate that H2
treatment significantly improves the lung histopathology and lung function in LPS-challenged mice.

**H₂ treatment reduced the cells and protein in the BALF of LPS-challenged mice**

As shown in Figure 2, LPS-challenged mice showed the significant increase in total cells, PMNs, and total protein in the BALF at 4 and 24 h ($P < 0.05$ vs. PBS group, $n = 6$ per group), which were markedly reduced by H₂ treatment ($P < 0.05$, $n = 6$ per group). These data further indicate that H₂ treatment attenuates LPS-induced lung injury in mice.

**H₂ treatment decreased LPS-induced neutrophils recruitment into the lungs**

We also detected the lung MPO activity, an indicator of neutrophil infiltration, at 4 and 24 h after PBS or LPS administration (Fig. 2). The lung MPO activity of LPS-challenged mice dramatically increased ($P < 0.05$ vs. PBS group, $n = 6$ per group), which was inhibited by H₂ treatment ($P < 0.05$, $n = 6$ per group). These results suggest that H₂ treatment attenuates the lung inflammation in LPS-challenged mice.

**H₂ treatment prevented the lung cell apoptosis of LPS-challenged mice**

Moreover, we investigated the effects of H₂ treatment on pulmonary cell apoptosis in LPS-challenged mice by TUNEL staining and caspase 3 activity (Fig. 3). TUNEL staining identified few apoptotic cells in the lungs of PBS-challenged mice. In contrast, numerous lung cells were strongly positive for TUNEL staining in LPS-challenged mice. In the samples of H₂-treated mice, however, a few of the lung cells were positive for TUNEL staining (Fig. 3A). For quantitative measurement, the percentage of lung cells that were positive for TUNEL staining was recorded in each specimen (Fig. 3B). We found that LPS-challenged animals showed a significant increase in apoptotic cells ($P < 0.05$ vs. PBS group, $n = 6$ per group), which was reduced by H₂ treatment ($P < 0.05$, $n = 6$ per group). Similarly, we found that the caspase 3 activity was significantly increased in the lungs of LPS-challenged animals, which was prevented by H₂ treatment ($P < 0.05$, $n = 6$ per group; Fig. 3C). These results indicate that the i.t. administration of LPS increases the lung cell apoptosis, which can be significantly alleviated by H₂ treatment.

**H₂ treatment downregulated the cytokines and chemokines in the BALF of LPS-challenged mice**

As depicted in Figure 4, we found that the levels of both proinflammatory cytokines (TNF-α, IL-1β, IL-6, and HMGB1) and anti-inflammatory cytokine (IL-10) in the BALF were significantly increased at 4 and 24 h in LPS-challenged mice ($P < 0.05$ vs. PBS group, $n = 6$ per group). Hydrogen gas treatment markedly downregulated the levels of proinflammatory cytokines (TNF-α, IL-1β, IL-6, and HMGB1) in the BALF of LPS-challenged mice ($P < 0.05$, $n = 6$ per group), whereas H₂ treatment had no significant effects on anti-inflammatory cytokine IL-10 ($P > 0.05$, $n = 6$ per group). Furthermore, LPS-challenged mice exhibited the significantly increased levels of chemokines (KC, MIP-1α, MIP-2, and MCP-1) in the BALF at 4 and 24 h ($P < 0.05$ vs. PBS group, $n = 6$ per group), which were also reduced by H₂ treatment ($P < 0.05$, $n = 6$ per group). These results demonstrate that H₂ treatment downregulates the cytokines and chemokines in the BALF of LPS-challenged mice.

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**Fig. 2.** Hydrogen gas treatment reduced the cell counts and protein concentration in the BALF as well as the lung MPO activity of LPS-challenged mice at 4 and 24 h after LPS or PBS administration. A, Total cells in BALF. B, Polymorphonuclear neutrophils in BALF. C, Total protein concentration in BALF. D, Lung MPO activity. The animals were treated as described in Figure 1. The values are expressed as means ± SEM (n = 6 per group). *$P < 0.05$ vs. PBS group; †$P < 0.05$ vs. LPS group.

**Fig. 3.** Hydrogen gas treatment prevented the lung cell apoptosis in LPS-challenged mice. The animals were treated as described in Figure 1. The lung samples were harvested for measuring TUNEL staining and caspase 3 activity at 24 h after PBS or LPS administration. A, Representative lung TUNEL staining (original magnification ×40). B, Percentage of TUNEL-positive cells. C, Lung caspase 3 activity. The values are expressed as means ± SEM (n = 6 per group). *$P < 0.05$ vs. PBS group; †$P < 0.05$ vs. LPS group.
H₂ treatment inhibited LPS-induced pulmonary NF-κB p65 nuclear translocation and DNA-binding activity

In LPS-challenged mice, the lung NF-κB p65 nuclear translocation and DNA-binding activity were significantly elevated at 4 and 24 h (P < 0.05 vs. PBS group, n = 6 per group; Fig. 5). However, H₂ treatment significantly inhibited the lung NF-κB p65 nuclear translocation and DNA-binding activity of LPS-challenged mice (P < 0.05, n = 6 per group; Fig. 5). The lung NF-κB p65 nuclear translocation and DNA-binding activity had no significant differences between PBS and PBS + H₂ groups (P > 0.05, n = 6 per group; Fig. 5). The data demonstrate that H₂ treatment inhibits the lung NF-κB activation in LPS-challenged mice.

HS also attenuates LPS-induced ALI in mice

In addition, we investigated the effects of HS on LPS-induced lung injury. In this study, we found that i.p. administration of 10 mL/kg HS significantly improved the lung histologic scores, W/D ratio, PaO₂/FIO₂, and MPO activity, as well as total cells, PMNs, and total protein in the BALF of LPS-challenged mice (P < 0.05 vs. PBS group, n = 6 per group; Figs. 6 and 7). There is no statistically significant difference between H₂ treatment and HS treatment. These results indicate that HS also can ameliorate the LPS-induced ALI.

DISCUSSION

In the current study, we found that (1) the mice with i.t. administration of LPS exhibited significant lung injury, which was significantly improved by 2% H₂ treatment starting at 1 and 6 h after LPS administration; (2) H₂ treatment inhibited LPS-induced pulmonary early and late NF-κB activation; (3) H₂ treatment downregulated the pulmonary inflammation and cell apoptosis; and (4) HS had similar beneficial effects as hydrogen inhalation. In conclusion, these results demonstrate that molecular hydrogen ameliorates LPS-induced ALI by reducing lung inflammation and apoptosis, which may be associated with decreased NF-κB activity.
Gram-negative organisms account for approximately half of the infections predisposing to ALI, often in the setting of pneumonia or sepsis (1–3). Endotoxin (LPS) is the critical mediator of organ dysfunction and death associated with severe gram-negative infections. Some of the features of ALI/ARDS can be reproduced by administration of bacterial endotoxin (LPS), which acts via Toll-like receptor 4 to induce the expression of inflammatory cytokines and chemokines and upregulate leukocyte adhesion molecules, resulting in loss of microvascular and epithelial integrity and increased interstitial and alveolar edema (24). It is well established that i.t. administration of LPS can induce a model of ALI (24). In the present study, we successfully produced a mouse model of ALI by i.t. administration of aerosolized LPS (25 μg/mouse) according to previous reports (24, 25). We found that lung injuries, characterized by increased lung water content, disruption of lung architecture, extravasation of red blood cells, and accumulation of inflammatory cells, were present at 24 h after LPS administration in room air-treated animals, which is consistent with other studies (24, 25).

Recently, basic and clinical researches have shown that \( \text{H}_2 \) is an important physiological regulatory factor with antioxidant, anti-inflammatory, and antiapoptotic properties (19). Hydrogen gas inhalation can attenuate many kinds of lung injuries caused by ventilator, transplantation, hyperoxia, irradiation, and sepsis (11, 21, 22, 29, 30). In addition, HS or hydrogen-rich water reduces the burn-induced lung injury, paraquat-induced lung injury, and hyperoxic lung injury (3, 24). Inhaled LPS can cause neutrophilic inflammation and decrements in pulmonary function, which is dependent on the recruitment of neutrophils from the vascular space to the airspace (3, 24). In the present study, mice exposed to LPS exhibited a massive recruitment of inflammatory cells including neutrophils and macrophages in the airways. We found that the

![Image](https://example.com/image.png)

**Fig. 6.** Hydrogen-rich saline ameliorated the lung histologic scores (A), lung W/D weight ratio (B), and lung function (C) in LPS-challenged mice. The animals were i.p. injected 10 mL/kg HS or normal saline at 1 h after PBS or LPS administration, respectively. The oxygenation index (\( \text{Pa}_2/\text{F}_2 \)) was measured at 24 h after PBS or LPS administration. In addition, the lung samples were harvested for measuring the histopathology, W/D weight ratio. A, Lung histologic scores (the bar represents median, n = 10 per group). B, Lung W/D weight ratio. C, Oxygenation index (\( \text{Pa}_2/\text{F}_2 \)). The data of W/D ratio and \( \text{Pa}_2/\text{F}_2 \) are expressed as means ± SEM (n = 6 per group). *\( P < 0.05 \) vs. PBS group; †\( P < 0.05 \) vs. LPS group.

was given by 2% \( \text{H}_2 \) inhalation for 1 h beginning at 1 and 6 h after PBS or LPS administration, respectively. Intratracheal administration of LPS can induce lung injury characterized by the deterioration of lung histopathology and histologic scores and increase in lung W/D weight ratio as well as total protein in the BALF and lung function, which was improved by \( \text{H}_2 \) inhalation or HS treatment.

Pulmonary cell apoptosis is also considered to be important in the pathogenesis of ALI (33). In this study, apoptotic cells were first detected on the basis of positive TUNEL staining. As noted, numerous apoptotic cells were observed in the lungs of LPS-challenged animals. The number of TUNEL-positive lung cells was significantly reduced after \( \text{H}_2 \) treatment. On the other hand, at the molecular level, apoptosis is activated by the aspartate-specific cysteine protease (caspase) cascade, including caspases 3 and 12 (34). Caspase 3 is considered to be the most important of the executioner caspses and is activated by any of the initiator caspases (34). We also found that the caspase 3 activity was dramatically increased in the lungs of LPS-challenged mice, which was prevented by \( \text{H}_2 \) treatment. Previous studies also reported that \( \text{H}_2 \) inhalation or HS could have antiapoptotic roles reflected by the decrease in TUNEL staining–positive cells, caspase 3 activity, and caspase 12 activity (5, 16, 21). These results demonstrate that \( \text{H}_2 \) treatment prevents the lung cell apoptosis in LPS-challenged mice.

Neutrophilic inflammation is associated with ALI/ARDS (3, 24). Inhaled LPS can cause neutrophilic inflammation and decrements in pulmonary function, which is dependent on the recruitment of neutrophils from the vascular space to the airspace (3, 24). In the present study, mice exposed to LPS exhibited a massive recruitment of inflammatory cells including neutrophils and macrophages in the airways. We found that the

![Image](https://example.com/image.png)

**Fig. 7.** Hydrogen-rich saline reduced the cell counts and protein concentration in the BALF as well as the lung MPO activity of LPS-challenged mice. A, Total cells in BALF. B, Polymorphonuclear neutrophils in BALF. C, Total protein concentration in BALF. D, Lung MPO activity. The animals were treated as described in Figure 6. The lungs and BALF were harvested for measuring these indicators at 24 h after PBS or LPS administration. The values are expressed as means ± SEM (n = 6 per group). *\( P < 0.05 \) vs. PBS group; †\( P < 0.05 \) vs. LPS group.
total cells and PMNs in the BALF of LPS-challenged mice were significantly increased, which were attenuated by H2 treatment. Moreover, we investigated lung neutrophil infiltration by measuring the activity of lung MPO, a neutrophil-specific enzyme. Hydrogen gas treatment prevented the increase in lung MPO activity in LPS-challenged mice. On the other hand, we found that H2 treatment dramatically prevented the increase in proinflammatory cytokines (TNF-α, IL-1β, IL-6, and HMGB1) in the BALF of LPS-challenged mice, whereas it had no effect on anti-inflammatory cytokine (IL-10). Previous studies also reported that H2 inhalation or HS could reduce the release of proinflammatory cytokines (11, 12, 19). However, no research about its effects on anti-inflammatory cytokines was reported. These results demonstrate that H2 treatment ameliorates LPS-induced lung neutrophil infiltration and inflammation.

The increase in alveolar neutrophils is due, at least in part, to the enhanced chemokines. Some studies have shown that this cell infiltration was associated with an increase in BALF levels of chemoattractant cytokines such as KC, MCP-1α, MCP-1, and MIP-2 (3, 4, 24). Keratinocyte-derived chemokine and MIP-2 are major chemokines for neutrophils similar to human IL-8. Macrophage inflammatory protein 1α is a member of the CC chemokine family that is involved in the acute inflammatory state in the recruitment and activation of polymorphonuclear leukocytes. Similarly, MCP-1 is a chemokine important in the recruitment and adherence of monocytes and neutrophils to the endothelium. In this study, we found that the chemokines (KC, MIP-1α, MIP-2, and MCP-1) in the BALF are significantly elevated in mice subjected to LPS, which were prevented by H2 treatment. These results further indicate that H2 treatment reduces the LPS-induced lung inflammation through downregulation of neutrophils recruitment as well as proinflammatory cytokines and chemokines.

Nuclear factor κB is one critical transcription factor required for maximal expression of many cytokines and chemokines involved in the pathogenesis of ALI (4). It is well known that NF-κB regulates gene expression of cytokines, chemokines, and adhesion molecules. All of these factors play an important role in lung inflammatory injury. In addition, an in vivo animal study has shown an association between NF-κB activity and the expression of cytokines, chemokines, and vascular adhesion molecules (4). In this study, H2 treatment inhibited the lung NF-κB p65 nuclear translocation and DNA-binding activity in LPS-challenged mice. However, a previous study found that H2 inhalation reduced epithelial apoptosis in ventilator-induced lung injury via activating NF-κB (21). Chemical inhibition of NF-κB activation using SN50 reversed the protective effects of H2 in ventilator-induced lung injury (21). It is likely that H2 may have dual roles in regulation of NF-κB signaling pathway, depending on the experimental model, cell type, location and timing of stimuli, and a subunit composition of the NF-κB complex. But the beneficial effect of H2 treatment in LPS-induced ALI appears to be mediated through inhibition of NF-κB activation. Then we raise the question: how can H2 inhibit NF-κB activation? First, H2 can directly activate the NF-κB signaling through inhibiting the phosphorylation of IκB-α (35). Second, H2 can indirectly activate the NF-κB signaling through reducing oxygen free radical. Ohsawa et al. (7) found that H2 directly reacted with free radical species such as •OH, although the kinetic favorability of this direct reaction may be uncertain. Hydrogen gas, as a potential antioxidant, has certain unique properties: unlike most known antioxidants, H2 is permeable to cell membranes and can target organelles, including mitochondria and nuclei. Its rapid gaseous diffusion might make it highly effective for reducing cytotoxic radicals. Hydrogen gas specifically quenches exclusively detrimental reactive oxygen species (ROS), such as •OH and peroxynitrite (ONOO−), while maintaining the metabolic oxidation-reduction reaction and other less potent ROS, such as superoxide anion and H2O2. It is likely that H2 is mild enough not to disturb metabolic oxidation-reduction reactions or to disrupt ROS involved in cell signaling.

At low concentrations, H2 is neither explosive nor dangerous. Moreover, inhaled H2 at therapeutic dose has no adverse effects on the saturation level of arterial oxygen (SpO2) and hemodynamic parameters. In addition, HS is also a good choice in the clinic. Thus, molecular hydrogen may represent a promising future therapeutic option for ALI. Whether these and other protective effects of H2 application observed in animal models of lung injury can be extrapolated to the treatment of patients is the subject of ongoing clinical trials.

REFERENCES