Original Article

Inhaled hydrogen ameliorates endotoxin-induced bowel dysfunction

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Aim: Gastrointestinal dysmotility frequently occurs during sepsis and multiple organ failure, remaining a major cause of morbidity and mortality in critically ill patients. Previous studies have shown that hydrogen, a new therapeutic gas, can improve various organ damage associated with sepsis. In this study, we investigated the protective efficacies of inhaled hydrogen against lipopolysaccharide (LPS)-induced ileus.

Methods: Sepsis was induced in rats and mice by a single i.p. injection of LPS at 15 mg/kg for mice and 5 mg/kg for rats. Four groups of rats and mice including sham/air, sham/hydrogen, LPS/air, and LPS/hydrogen were analyzed. Hydrogen (1.3%) was inhaled for 25 h beginning at 1 h prior to LPS treatment. Gastrointestinal transit was quantified and cytokine levels, as well as neutrophil extravasation, in the intestinal muscularis propria were determined.

Results: Lipopolysaccharide challenge remarkably delayed gastrointestinal transit of non-absorbable dextran, associated with increased leukocyte recruitment and upregulation of pro-inflammatory cytokine mRNA expressions in the muscularis propria. Hydrogen significantly prevented LPS-induced bowel dysmotility and reduced leukocyte extravasation, as well as inhibition of inflammatory cytokine expression. *In vitro* analysis of cytokine levels after LPS treatment of cultured macrophages showed an increase of interleukin-10 by hydrogen regardless of the presence of nitric oxide.

Conclusions: This study showed the protective effects of hydrogen inhalation on LPS-induced septic ileus through inhibition of inflammation in the muscularis propria. These inhibitory effects on the pro-inflammatory response may be partially derived from anti-inflammatory cytokine interleukin-10 induction.

Key words: Hydrogen, ileus, inflammation, interleukin-10, sepsis

INTRODUCTION

GASTROINTESTINAL MOTILITY DISORDERS are common complications in the intensive care setting and are predictors of increased mortality and length of the stay in the intensive care unit (ICU). Several risk factors for developing gastrointestinal motility problems in the ICU setting have been identified and include sepsis, being on mechanical ventilation, and the use of vasopressors, opioids, or anticholinergic medications.[1]

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Sepsis accompanied by a systemic inflammatory response syndrome remains one of the leading causes of death and a major challenge in the ICU. In particular, sepsis is a severe clinical syndrome encountered and triggered in patients with infection, and can develop in the sequela of gastrointestinal motility disorders. Patients with sepsis experience a prolonged inhibition of coordinated bowel activity that causes accumulation of secretions and gas, resulting in nausea and vomiting, abdominal distension, and pain. During sepsis-induced ileus associated with luminal bacterial overgrowth, the intestinal inflammatory responses boost the systemic release of luminal pathogens including bacterial toxin, particularly endotoxin, and cytokines generated primarily by the gut. In addition, sepsis-induced ileus can lead to mucosal acidosis and gut ischemia, and eventually abdominal compartment syndrome causing ischemia in the intra-abdominal organs.[2,3]

Over the last decade, inhalation of hydrogen has been successfully shown as a potential therapeutic in a number of animal models, and the inhalation of hydrogen gas has now been tested in the field of intensive care medicine.[4–6] Hydrogen can be used at a safe density through a ventilation circuit. As hydrogen concentration monitors can be found for purchase at a low price, it is relatively easy to administer and manage the hydrogen. Therefore, we hypothesized that exogenously given inhaled hydrogen could ameliorate sepsis-induced ileus as a novel therapeutic strategy. We investigated the hypothesis using an established animal model.

MATERIAL AND METHODS

Animals

MALE C57BL/6J (wild-type) mice, aged 8 weeks, and Sprague–Dawley rats (both Clea Japan, Tokyo, Japan) were used in all experiments. All mice and rats were kept in individual stainless steel cages for 2–5 weeks before the experiments. All procedures involving rats were carried out in accordance with the guidelines of the Animal Care and Use Committees of the Hyogo College of Medicine (Nishinomiya, Japan) and complied with the National Research Council's Guide for the Humane Care and Use of Laboratory Animals.

Lipopolysaccharide-induced ileus

To create a sepsis-induced bowel dysfunction, a single i.p. injection of lipopolysaccharide (LPS, *Escherichia coli*) (O111:B4; Sigma, St. Louis, MO, USA) at 15 mg/kg in 0.5 mL was given to mice and 5 mg/kg in 0.5 mL was i.p. injected into rats. Sham control animals were given the same amount of saline instead of LPS.

Hydrogen treatment

For hydrogen gas treatment, cylinders with nitrogen-based, high-pressure, premixed gases were purchased (Japan Fine Products, Kanagawa, Japan). The manufacturer confirmed the concentrations of H_2 (1.3%), O_2 (21%), and N_2 (77.7%). In Japan, 1.3% is the highest concentration of H_2 that can be mixed and bottled under high pressure with 21% oxygen for clinical use. As a control, additional N_2 was given instead of H_2 (O_2, 21%; N_2, 79%). The premixed gases were delivered to the rats using a gas-exposure chamber (Natsume Seisakusho Co., Tokyo, Japan). Hydrogen or control gas (designated N_2) was given 1 h before and 24 h after i.p. injection of LPS or physiological saline. The sham control rats were placed under the air for 1 h before i.p. injection.

Experimental groups

Sepsis in mice was induced by i.p. injection of LPS. Four treatment groups were formulated for this study: (i) sham/H_2 (−), sham control animals treated with air; (ii) sham/H_2 (+), sham animals treated with 1.3% hydrogen in air; (iii) LPS/H_2 (−), animals received LPS plus treatment with air; and (iv) LPS/H_2 (+), animals received LPS plus treatment with 1.3% hydrogen in air. Animals were killed at different times, depending on the experimental protocols.

Determination of intestinal motility

Gastrointestinal transit was measured 24 h after LPS injection by evaluating the distribution of an enteral dose of fluorescein isothiocyanate (FITC)-labeled dextran (FD70; Sigma-Aldrich, St. Louis, MO, USA) using standard methods as previously described.[7,8] Briefly, mice were given FITC-dextran dissolved in distilled water (10 μL/g body weight) by gavage. Ninety minutes later, the animals were killed using cervical fracture under isoflurane anesthesia. The entire gastrointestinal tract from stomach to distal colon was excised and divided into 14 segments: stomach, small intestine (divided into 10 segments of equal length), cecum, and colon (divided into two segments of equal length). The luminal content of each segment was collected into a small tube. The supernatants were collected and fluorometrically assayed for FITC-dextran concentration. The transit of FITC-dextran along the gastrointestinal tract was summarized by calculating the geometric center (GC) for the distribution of the FITC-dextran using the following formula: GC = Σ (percentage of total fluorescent signal per segment × segment number)/100.

Neutrophil detection by myeloperoxidase

Whole-mount specimens of rat distal jejunum muscularis propria were prepared for histochemical analysis 24 h after operation. Freshly prepared whole mounts were stained with Hanker–Yates reagent (Polyscience, Eppelheim, Germany) for detection and quantitation of polymorphonuclear neutrophils showing myeloperoxidase activity.[9]

Real-time reverse transcription–polymerase chain reaction (RT-PCR)

The muscularis externa was stripped from the small bowel of control intestine and treated intestine 6 h following LPS injection, snap frozen in liquid nitrogen, and stored at −80°C. Mice mRNA were quantified in duplicate using SYBR Green two-step, real-time RT-PCR, as described

previously.[10] The following mRNAs were quantitated: interleukin (IL)-6, intercellular adhesion molecule (ICAM)-1, tumor necrosis factor (TNF)-α, inducible nitric oxide synthase (iNOS), IL-10, and Toll-like receptor (TLR)-4 and β-actin.

Cell culture experiments

RAW 264.7 mouse peritoneal macrophages were purchased from the American Tissue Cell Culture repository (Rockville, MD, USA) and grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 100 μg/mL gentamicin in a humidified atmosphere. Hydrogen gas treatment was carried out by culturing cells in a hypoxia chamber (Stemcell Technologies, Vancouver, Canada) infused with premixed gas with or without hydrogen (hydrogen, 3% H_2, 21% O_2, 5% CO_2, 71% N_2; control, 21% O_2, 5% CO_2, 74% N_2) (Praxair, Danbury, CT, USA). Cell culture dishes were placed in the chamber equipped with an airtight seal. Then, the chamber was flushed for ≥ 5 min with premixed gas (20 L/min) according to the manufacturer's instructions.[11] After 2 h of pretreatment with either H_2 or control gas, 1 μg/mL LPS (Sigma-Aldrich) or sterile saline was added to the culture media, and the culture plates were returned to the hypoxia chamber. Culture media and cells were collected 1 h (TNF-α) or 16 h (IL-10) after LPS treatment for cytokine determination by enzyme-linked immunosorbent assay. In some cases, RAW 264.7 macrophages were treated with 10 μM L-NAME (N-nitro-L-Arginine methyl ester, a selective inhibitor of nitric oxide synthase; Sigma-Aldrich) before exposing the cells to 1 μg/mL LPS in the presence or absence of H_2.

Statistical analysis

Results are expressed as mean ± standard error of the mean. Parametric data were analyzed with one-way ANOVA followed by post-hoc analysis with the Bonferroni correction. The lung injury score was analyzed with the Mann–Whitney *U*-test with the Bonferroni correction. The cell count was analyzed using ANOVA with Tukey–Kramer methods. JMP version 8 (SAS Institute, Cary, NC, USA) was used for all statistical analyses.

RESULTS

Inhaled hydrogen ameliorates bowel dysmotility

THE EFFECT OF LPS and hydrogen inhalation on gastrointestinal function was determined *in vivo* by measurement of gastrointestinal transit of non-absorbable liquid FITC-dextran for 90 min. Twenty four hours after anesthesia and saline treatment, there were no apparent alterations in intestinal transit over a period of 90 min in mice in air or hydrogen treatment. The LPS treatment in air resulted in significant delay of gastric transit, as shown in the transit distribution histograms, and the majority of the fluorescent markers stayed in the jejunum. In contrast, hydrogen inhalation significantly improved delay in intestinal transit (Fig. 1A).

Hydrogen inhalation reduced neutrophil recruitment

Lipopolysaccharide has proven to be a potent activator of resident intestinal macrophage and recruiter of circulating leukocytes. In this experiment, LPS caused a significant recruitment and extravasation of circulating leukocytes into the intestinal muscularis 24 h after the initiation of endotoxemia. Hydrogen inhalation of septic animals resulted in a significant reduction in the small bowel muscularis 24 h after LPS injection, compared to controls of air inhalation (Fig. 2).

Hydrogen inhalation inhibited pro-inflammatory cytokine mRNA upregulation

Inflammation in the muscularis propria was further evaluated by assessing the mRNA expression of several pro-inflammatory mediators. Intraperitoneal LPS injection caused increase of pro-inflammatory cytokine mRNA including IL-6, ICAM-1, TNF-α, and iNOS and TLR4, expression in the muscle layers. These upregulations were significantly inhibited by hydrogen inhalation (Fig. 3).

Hydrogen inhalation increase anti-inflammatory IL-10 mRNA expression

Lipopolysaccharide challenge led to an increase of anti-inflammatory IL-10 hydrogen inhalation, which can decrease the early pro-inflammatory cytokines in the intestinal muscularis propria. Hydrogen inhalation resulted in further significant elevation of IL-10 mRNA levels in intestinal muscularis (Fig. 3).

Hydrogen increased IL-10 levels *in vitro* independent of the presence of nitric oxide

To further study the mechanisms underlying the anti-inflammatory properties of hydrogen gas, as seen in the reduction of ileus after LPS challenge, we moved to an *in vitro* system.

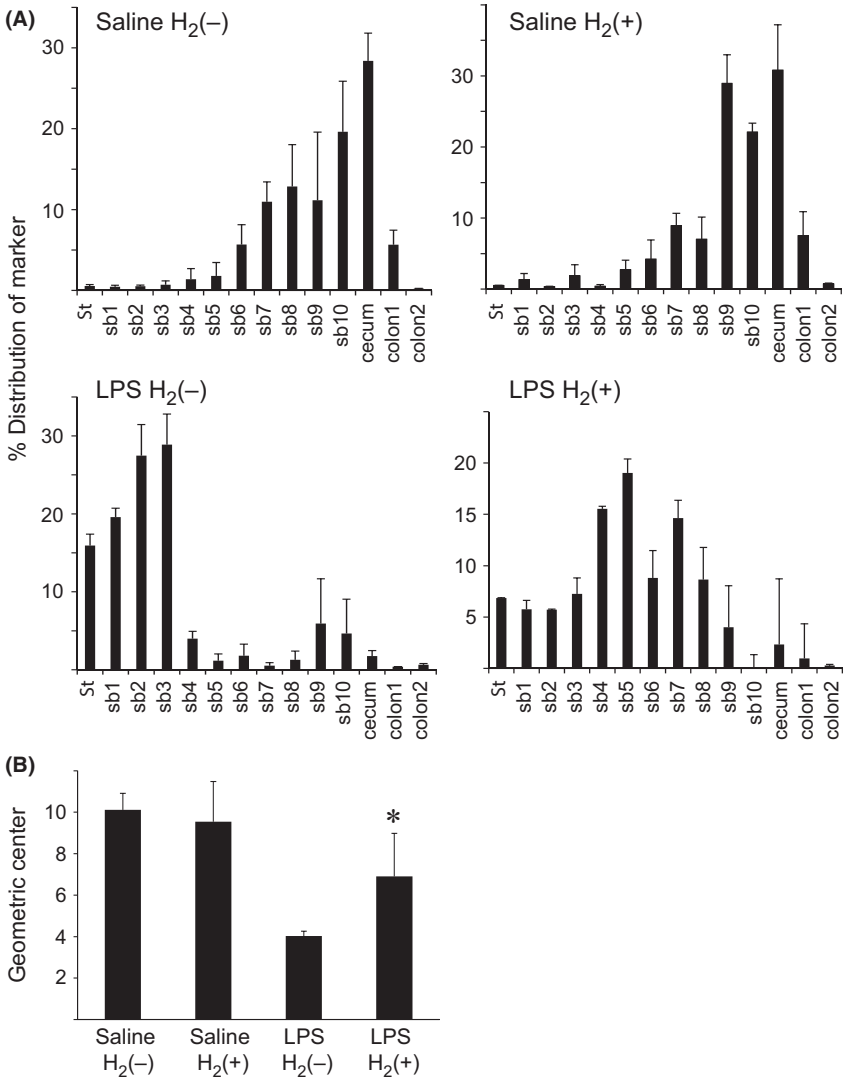

Fig. 1. A, Transit histograms for distribution of non-absorbable fluorescein isothiocyanate-labelled dextran along the gastrointestinal tract 90 min after oral administration in sham-operated animals and mice subjected to lipopolysaccharide (LPS) challenge with 1.3% hydrogen in air (H_2(+)) or without (H_2(−)). Gastrointestinal transit was significantly delayed after 24 h in the LPS H_2 group compared with the sham group, with many transit markers located in the middle of the jejunum. Hydrogen inhalation ameliorated gut dysmotility induced by LPS of the intestine. Data represent averaged percent distribution of fluorescence intensity from six animals for each group. Colon, colon divided into two segments of equal length; Sb, small intestine divided into 10 segments of equal length; St, stomach. B, Mean calculated geometric center from individual gastrointestinal transit distribution histograms. Animals showed an initial and significant decrease in the calculated geometric center, reflecting the suppression in gastrointestinal transit 24 h after LPS i.p. injection ($n = 6$; $^*P < 0.05$ vs LPS/H_2(−)).

RAW 264.7 mouse peritoneal macrophages were exposed to 3% H_2 or control gas and then treated with LPS to mimic an inflammatory stimulus. The LPS stimulation resulted in a marked elevation of TNF-α in the culture media within 1 h, and culture in the chamber containing 3% H_2 reduced TNF-α production. Interestingly, levels of IL-10, an anti-inflammatory cytokine, were significantly higher 16 h after LPS treatment in cells cultured in the presence of H_2 (Fig. 4). Next, we investigated whether nitric oxide (NO) mediated these anti-inflammatory effects by pretreating the cells with L-NAME, a nitric oxide synthase inhibitor. Inhibition of NO did not alter the decrease in TNF-α levels or

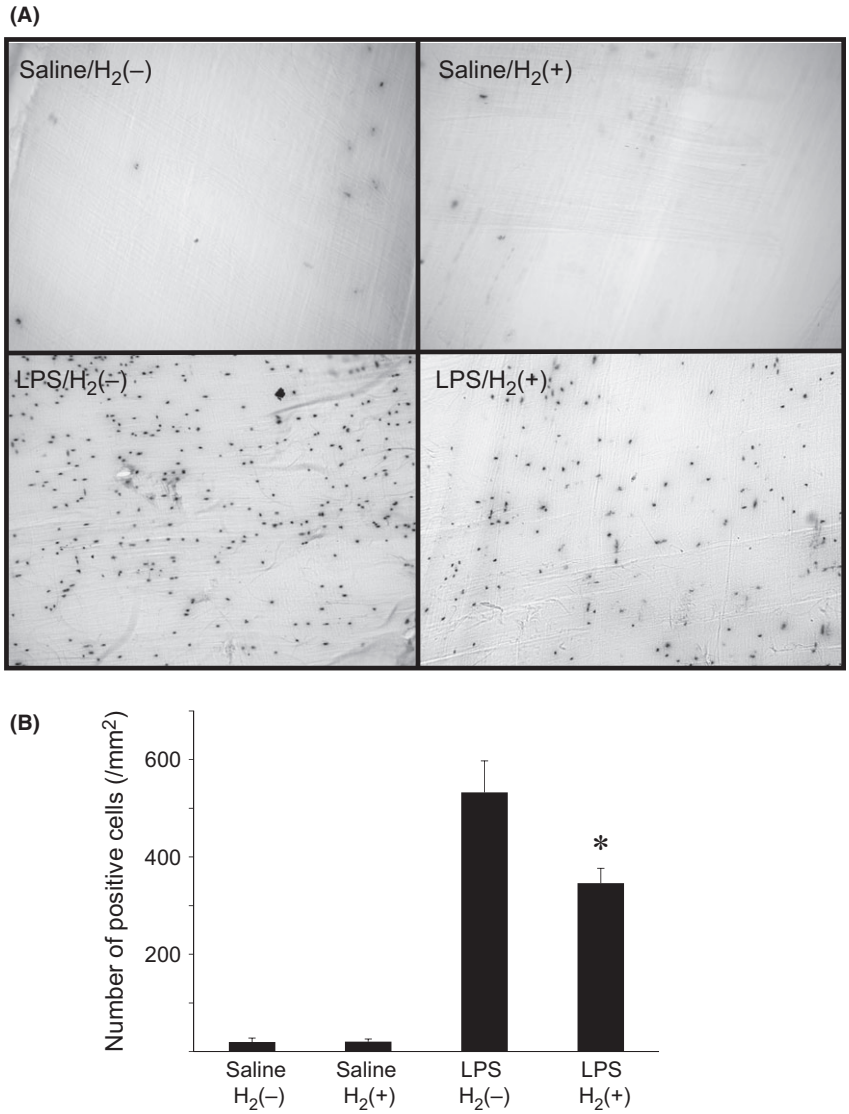

Fig. 2. A, Typical histochemically stained full thickness muscularis whole mounts for the presence of myeloperoxidase-positive polymorphonuclear neutrophils extravasated into muscularis propria. Images are representative of four individual experiments in which mice were received sham or lipopolysaccharide (LPS) challenge and were treated with (H_2(+)) or without (H_2(−)) 1.3% hydrogen in air. B, Histogram quantifying the number of extravasated neutrophils within the full thickness whole mount jejunal muscularis from each group (n = 4). As reflected in the histological analysis, LPS resulted in a significant cellular inflammatory response within the muscularis. Hydrogen inhalation decreased the number of myeloperoxidase-positive cells that extravasated into muscularis in response to LPS (n = 4; *P < 0.05 vs LPS/H_2(−)).

increase in IL-10 levels observed in the presence of H_2 and LPS, suggesting that NO does not play an important anti-inflammatory role in this setting (Fig. 4).

DISCUSSION

IN THE PRESENT study, we showed that exposure to hydrogen gas reduces the development of LPS-induced ileus in mice. Since the discovery of the anti-inflammatory and anti-oxidant effects of hydrogen, a number of experimental and clinical studies have indicated that hydrogen gas can be a promising new therapeutic method for various diseases, including sepsis. This study extended previous observations by showing that the efficacies of hydrogen associated with the regulation of leukocyte recruitment into the intestine and the sequent dysfunction of muscularis

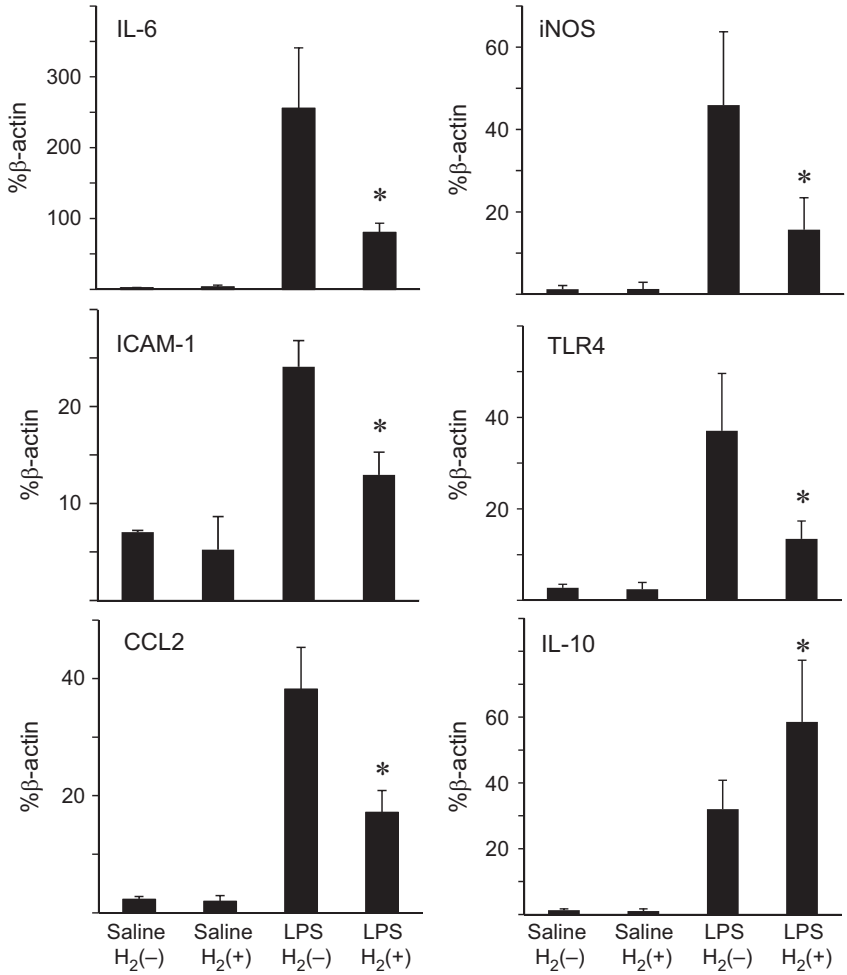

Fig. 3. Real-time reverse transcription-polymerase chain reaction analysis of mice or rats that received sham (saline) or lipopolysaccharide (LPS) challenge and treatment with (H_2(+)) or without (H_2(−)) 1.3% hydrogen in air. Analysis revealed increased expressions levels for interleukin (IL)-6, inducible nitric oxide synthase (iNOS), intercellular adhesion molecule (ICAM)-1, tumor necrosis factor (TNF)-α, and Toll-like receptor (TLR)4 in the muscularis propria compared to sham animals 6 h after LPS challenge. These upregulations were significantly inhibited by H_2 inhalation. Although IL-10 expression was significantly upregulated by LPS, hydrogen inhalation significantly increased IL-10 expression after 6 h compared to the LPS/H_2(−) group ($n = 4$, $^*P < 0.05$).

propria, at least in part, mediated through anti-inflammatory IL-10 induction.

The Surviving Sepsis Campaign, an international consortium of professional societies involved in critical care, treatment of infectious diseases, and emergency medicine, recently issued the third iteration of clinical guidelines for the management of severe sepsis and septic shock.[12] The pathogenesis and mechanisms of sepsis are complex, which include the excessive release of inflammatory cytokines, the action of oxidative stress, intestinal bacteria and endotoxin translocation, neutrophil dysfunction, microcirculatory impairment, mitochondrial dysfunction, and the imbalance between oxygen supply and oxygen consumption. Along with mucosal dysfunction, endotoxemia is known to be associated with alterations in gastrointestinal motility. Endotoxin exposure of the intestine results in the activation of the resident macrophages, which leads to the activation of transcription factors as well as the induction and liberation of various cytokines. The inflammatory mediators such as IL-6 and TNFα could result in diminished enteric neurotransmission. Mechanistically, the loss of inhibitory neuromuscular relaxation following LPS has been shown to be accompanied by a decrease in neuronal NOS expression, leading to bowel dysfunction.[13] Membrane TLR have been associated with sepsis-induced ileus, which in turn leads to pathogenic luminal bacterial overgrowth and translocation bacteria or

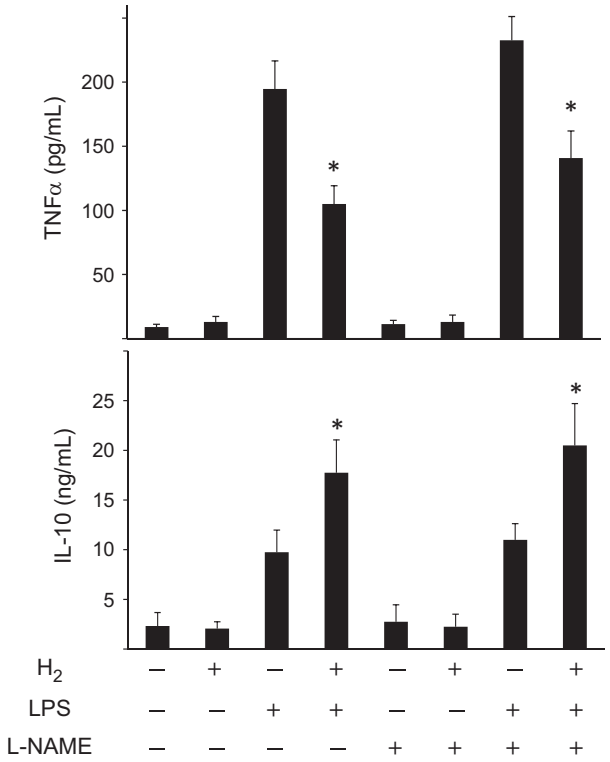

Fig. 4. *In vitro* analysis of cytokine levels after lipopolysaccharide (LPS) treatment of cultured macrophages. Enzyme-linked immunosorbent assay was carried out on the supernatants from cultures of RAW 264.7 cells. Each measurement was done in triplicate ($n = 3$; *$P < 0.05$ vs N_2). H_2, 1.3% hydrogen in air; IL-6, interleukin-6; L-NAME, N-nitro-L-Arginine methyl ester; TNF-α, tumor necrosis factor-α.

their toxins that cause ileus and mucosal dysfunction. TLR-4 activation by LPS demonstrated a time and dose-dependent response, leading to increasing dysmotility.[14]

Pro-inflammatory cytokines such as TNFα and IL-6 have been shown to be released early after an inflammatory stimulus of LPS, and play critical roles in inflammation and inflammatory bowel disease. The chemoattractant chemokine (C-C motif) ligand 2 (CCL2, previously referred to as monocyte chemoattractant protein-1), also plays a key role in the initiation of cellular inflammatory events within the intestinal muscularis during endotoxemia.[15] One finding of our study was that hydrogen treatment reduced the upregulation of pro-inflammatory mediator mRNA levels seen after LPS exposure.

Nitric oxide, produced by iNOS, has efficient inhibitory effects on smooth muscle contractility of the intestine.[16] As shown in this study, hydrogen inhalation for LPS insult significantly reduced intestinal muscularis iNOS mRNA expression in septic animals. Many studies have well established that NO is indeed a pro-inflammatory mediator, overproduction of NO plays a major role in the pathophysiology of septic shock, and induction of NOS with consequent excessive NO formation has been proposed as a major factor in pathologic vasodilation and tissue damage. Our study indicated that *in vitro* administration of hydrogen was able to inhibit the production of TNF-α levels in the culture media of macrophages.

Although the molecular mechanisms of protective effects of hydrogen in this study are not completely understood, our results indicated that humoral mechanisms that restrain or inhibit inflammation, including an upregulation of IL-10, by hydrogen inhalation also contributed to amelioration of sepsis-induced ileus. Despite the fact that NO is an important regulator of the inflammatory response after hemorrhagic shock, endogenous NO might not play a key role in the anti-inflammatory effects afforded by hydrogen.[17,18]

In conclusion, the current study showed that only 1.3% hydrogen gas, neither an inflammable nor explosive concentration, can have obvious protective effects on sepsis-induced ileus. Our results possibly resolve the clinical problem associated with sepsis and would have huge clinical impacts.

CONFLICT OF INTEREST

NONE.

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REFERENCES

1 Aderinto-Adike AO, Quigley EM. Gastrointestinal motility problems in critical care: a clinical perspective. J. Dig. Dis. 2014; 15: 335–44.
2 Hersch M, Gnidec AA, Bersten AD, Troster M, Rutledge FS, Sibbald WJ. Histologic and ultrastructural changes in nonpulmonary organs during early hyperdynamic sepsis. Surgery 1990; 107: 397–410.
3 Eskandari MK, Kalff JC, Billiar TR, Lee KK, Bauer AJ. LPS-induced muscularis macrophage nitric oxide suppresses rat jejunal circular muscle activity. Am. J. Physiol. 1999; 277: G478–86.
4 Kawamura T, Wakabayashi N, Shigemura N *et al.* Hydrogen gas reduces hyperoxic lung injury via the Nrf2 pathway

in vivo. Am. J. Physiol. Lung Cell. Mol. Physiol. 2013; 304: L646–56.
5 Kohama K, Yamashita H, Aoyama-Ishikawa M *et al.* Hydrogen inhalation protects against acute lung injury induced by hemorrhagic shock and resuscitation. Surgery 2015; 158: 399–407.
6 Huang CS, Kawamura T, Lee S *et al.* Hydrogen inhalation ameliorates ventilator-induced lung injury. Crit. Care 2010; 14: R234.
7 Nakao A, Schmidt J, Harada T *et al.* A single intraperitoneal dose of carbon monoxide-saturated ringer's lactate solution ameliorates postoperative ileus in mice. J. Pharmacol. Exp. Ther. 2006; 319: 1265–75.
8 Moore BA, Otterbein LE, Turler A, Choi AM, Bauer AJ. Inhaled carbon monoxide suppresses the development of postoperative ileus in the murine small intestine. Gastroenterology 2003; 124: 377–91.
9 Nakao A, Otterbein LE, Overhaus M *et al.* Biliverdin protects the functional integrity of a transplanted syngeneic small bowel. Gastroenterology 2004; 127: 595–606.
10 Nakao A, Kimizuka K, Stolz DB *et al.* Carbon monoxide inhalation protects rat intestinal grafts from ischemia/reperfusion injury. Am. J. Pathol. 2003; 163: 1587–98.
11 Kawasaki H, Guan J, Tamama K. Hydrogen gas treatment prolongs replicative lifespan of bone marrow multipotential stromal cells in vitro while preserving differentiation and paracrine potentials. Biochem. Biophys. Res. Commun. 2010; 397: 608–13.
12 Dellinger RP, Levy MM, Rhodes A *et al.* Surviving sepsis campaign: international guidelines for management of severe sepsis and septic shock: 2012. Crit. Care Med. 2013; 41: 580–637.
13 Buchholz BM, Bauer AJ. Membrane TLR signaling mechanisms in the gastrointestinal tract during sepsis. Neurogastroenterol. Motil. 2010; 22: 232–45.
14 Caddell KA, Martindale R, McClave SA, Miller K. Can the intestinal dysmotility of critical illness be differentiated from postoperative ileus? Curr. Gastroenterol. Rep. 2011; 13: 358–67.
15 Turler A, Schwarz NT, Turler E, Kalff JC, Bauer AJ. MCP-1 causes leukocyte recruitment and subsequently endotoxemic ileus in rat. Am. J. Physiol. Gastrointest. Liver Physiol. 2002; 282: G145–55.
16 Kalff JC, Schraut WH, Billiar TR, Simmons RL, Bauer AJ. Role of inducible nitric oxide synthase in postoperative intestinal smooth muscle dysfunction in rodents. Gastroenterology 2000; 118: 316–27.
17 Le Tulzo Y, Shenkar R, Kaneko D *et al.* Hemorrhage increases cytokine expression in lung mononuclear cells in mice: involvement of catecholamines in nuclear factor-kappaB regulation and cytokine expression. J. Clin. Invest. 1997; 99: 1516–24.
18 Hierholzer C, Harbrecht B, Menezes JM *et al.* Essential role of induced nitric oxide in the initiation of the inflammatory response after hemorrhagic shock. J. Exp. Med. 1998; 187: 917–28.