Hydrogen gas inhalation protects against liver ischemia/reperfusion injury by activating the NF-κB signaling pathway

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Abstract. Hydrogen has been demonstrated to function as a novel antioxidant and exert therapeutic antioxidant activity in a number of diseases. The present study was designed to investigate the effect of hydrogen inhalation on liver ischemia/reperfusion (I/R) injury in rats. The portal triad to the left lobe and the left middle lobe of the liver were completely occluded for 90 min. This was followed by reperfusion for 180 min. The rats subsequently underwent syngeneic orthotopic liver transplantation. Inhalation of various concentrations (1, 2 and 3%) of hydrogen gas and its administration for different durations (1, 3 and 6 h) immediately prior to the I/R injury allowed the optimal dose and duration of administration to be determined. Liver injury was evaluated through biochemical and histopathological examinations. The expression levels of proinflammatory cytokines, including tumor necrosis factor (TNF)-α and interleukin (IL)-6, were measured by enzyme-linked immunosorbent assay and quantitative polymerase chain reaction (qPCR). Liver nuclear factor κB (NF- κB) was detected by qPCR and western blot analysis. Inhalation of hydrogen gas at 2% concentration for 1 h significantly reduced the serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, the expression of cytokines, including IL-6, TNF- α , early growth response protein 1 (Egr-1) and IL-1β, and morphological damage. In addition, the mRNA and protein expression levels of NF-κB, heme oxygenase-1 (HO-1), B-cell lymphoma 2 (Bcl-2) and zinc finger protein A20 (A20) in rats where only the donors received hydrogen were significantly increased compared with those in rats where both the donor and recipient, or only the recipient received hydrogen. The results indicate that hydrogen

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inhalation at 2% concentration for 1 h prior to liver transplantation protected the rats from ischemia/reperfusion injury by activation of the NF- κ B signaling pathway.

Introduction

The application of medical gases in disease treatment remains a relatively unexplored field in medicine. However, accumulating evidence has demonstrated the attractive achievements of medical gases, especially hydrogen gas, in the treatment of various types of diseases including ischemic heart disease, stroke, sepsis, acute lung injury and inflammatory bowel disease (1-3). Gharib *et al* placed an animal model of chronic infectious hepatitis into a hydrogen hyperbaric chamber and found that the hydrogen significantly reduced liver injury and fibrosis, improved hemodynamics and increased nitric oxide synthase 2 and antioxidant enzyme activity (4).

Ischemia/reperfusion (I/R) injury is one of the key issues encountered during liver transplantation. It is closely associated with postoperative biliary complications, acute rejection and occasionally results in fatal injury (5-7). Therefore, decreasing I/R injury is critical for reducing complications following liver transplantation. Vascular endothelial cells are the main targets of I/R injury. Thus, in a preliminary experiment of the current study, liver endothelial cells were cultured in vitro to simulate liver transplantation I/R injury and hydrogen treatment. In addition, the levels of hydrogen and methane in blood samples from rats following hydrogen gas inhalation were measured using gas chromatography and it was successfully demonstrated that hydrogen was able to reach the target organ. Also, apoptosis of the vascular endothelial cells was detected using an Annexin V-PE apoptosis detection kit and it was observed that hydrogen treatment was able to significantly reduce the I/R injury-induced apoptosis of vascular endothelial cells. Hydrogen is highly diffusible and may reach subcellular structures such as nuclear and mitochondrial DNA and sites where reactive oxygen species are present. Hydrogen may also remove hydroxyl radicals, protect the mitochondrial membrane potential, maintain ATP synthesis and protect the DNA in the nucleus (8). Hydrogen is very convenient for patients to use and inhalation of hydrogen can be easily applied in clinical practice, particularly for mechanically ventilated patients in the perioperative period.

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hydrogen remain obscure and require elucidation. Therefore, in the current study, low concentrations of hydrogen were mixed with air to evaluate its protective effect against I/R injury associated with liver transplantation.

Materials and methods

Animals. Male Sprague-Dawley (SD) rats, weighing 250-300 g, were provided by the Experimental Animal Center of the Third Military Medical University (Chongqing, China). Rats were housed with free access to food and water under a natural day/night cycle. Rats were acclimated for seven days prior to any experimental procedures. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Third Military Medical University.

Inhalation of hydrogen gas. Different concentrations of hydrogen gas (1, 2 and 3%) were established by combining hydrogen with air, and subsequently compressed into oxygen bottles. During the process of hydrogen administration, rats were placed into a glass container which was connected to a pipeline. The hydrogen was administered to the conscious rats through the pipeline at a rate of 1.5 ml/h. The duration of hydrogen administration was 1, 3 or 6 h.

Model establishment. Immediately following the administration of hydrogen, the rats underwent an I/R injury procedure under general anesthesia with Sevofrane as described by Lord et al (9). A midline incision was created and the portal triad to the left lobe and the left middle lobe of the liver was occluded with a vascular microclamp to induce partial liver ischemia. Occlusion was verified visually by the color change of the left side of the liver to a paler shade. The abdominal muscles and peritoneum were closed with 5.0-nylon sutures. Following 90 min of ischemia, a second laparotomy was performed to remove the clamp. The abdomen was closed in the same manner and followed by 180 min of reperfusion. Then, immediately after I/R injury, syngeneic orthotopic liver transplants (OLTs) were performed using livers that were harvested from SD rats and stored for 18 h in University of Wisconsin (UW) solution, prior to being transplanted into syngeneic SD recipients with revascularization without hepatic artery reconstruction. There were three groups of rats that received liver transplants (each n=6), namely the hydrogen-treated donor and recipient group in which the donor and recipient rats both received hydrogen, the hydrogen-treated donor group in which only the donor rat received hydrogen, and the hydrogen-treated recipient group in which only the recipient rat received hydrogen. Right hepatic lobe tissue samples were taken at 1-h intervals following surgery and venous blood samples were collected after 3 h. Liver tissues were placed in 4% formaldehyde and stored in liquid nitrogen.

Hematoxylin and eosin (H&E) *staining*. Fixed livers were dehydrated and embedded in paraffin. Tissues were sectioned (4- μ m thickness) and stained with H&E.

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity. Following clotting, each blood sample was centrifuged at 1,100 x g for 5 min. The clear top layer was centrifuged again under the same conditions to prepare the serum. The activities of serum ALT and AST were examined using a Wako Transaminase CII-Test kit (Wako Pure Chemical Industries Ltd., Osaka, Japan).

Quantitative polymerase chain reaction (qPCR). The liver tissues were lysed with TRIzol[®] (Invitrogen Life Technologies, Carlsbad, CA, USA) and vigorously mixed with chloroform for 15 sec, then stored at room temperature for 3 min. Subsequently, they were centrifuged at 12,000 x g for 15 min at 4°C and the RNA was precipitated in the aqueous phase with isopropanol. The upper aqueous phase was transferred to a new microcentrifuge tube. The RNA was precipitated by adding 0.75% ethanol and centrifuged at 12,000 x g for ≤ 5 min at 4°C. The supernatant was removed and the RNA was dried at room temperature for 5-10 min. The mRNA expression levels of zinc finger protein A20 (A20), nuclear factor kB (NF-kB), heme oxygenase-1 (HO-1) and B-cell lymphoma 2 (Bcl-2) were determined using qPCR with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as a control. The expression levels of interleukin (IL)-6, tumor necrosis factor (TNF)- α , early growth response protein 1 (Egr-1) and IL-1β mRNA were also determined. The qPCR reactions were performed using an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) with the following conditions: 95°C, 10 min for one cycle; and then 95°C, 15 sec, 60°C, 1 min for 40 cycles. The expression levels of mRNA were quantified with $2^{-\Delta\Delta CT}$. The primer sequences are listed in Table I.

Enzyme-linked immunosorbent assay (ELISA). ELISA (R&D Systems, Minneapolis, MN, USA) was used to determine the expression levels of IL-6 and TNF- α in serum according to the manufacturers' instructions. The absorbance was measured at 450 nm using a microplate reader (Model 680; Bio-Rad, Hercules, CA, USA).

Transmission electron microscopy. Liver samples were fixed in 2.5% glutaraldehyde for 2 h and then rinsed in phosphate buffer. This was followed by a postfixation step for 2 h with 1% osmium tetroxide in phosphate buffer at 4°C. Afterwards, samples were dehydrated and embedded in resin. The samples were then trimmed and sectioned into slices (50~60 nm). Ultrastructural features were observed on a transmission electron microscope (TEM; Philips CM120 TEM; Philips, Amsterdam, The Netherlands) at 60 kV.

Western blot analysis. After treatment with different concentrations of hydrogen (1, 2 and 3%) for different durations (1, 3 and 6 h), total cell lysates were prepared and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For western blot analysis, the primary antibodies used included anti-A20, anti-NF- κ B, anti-HO-1, anti-Bcl-2 (Cell Signaling Technology, Inc., Beverly, MA, USA) and anti-GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). An anti-rabbit or anti-mouse secondary antibody conjugated with horseradish peroxidase was also used (Pierce Biotechnology, Inc., Rockford, IL, USA). Immunoreactive bands were detected with an enhanced chemiluminescence (ECL) kit for western blot detection using the ChemiGenius Bio Imaging System (Syngene, Frederick, MD, USA).

Gene	Forward primer	Reverse primer	
A20	ACCTGTTTCAAAAGGACTACGG	AAGGTAGCCAGAGGGGACG	
NF-κB	CTGCTTACGGTGGGATTGC	TGTTTCTTTCTCAGGGGGATTC	
HO-1	GCGAAACAAGCAGAACCCA	CCACCAGCAGCTCAGGATG	
Bcl-2	GTGAACTGGGGGGGGGGATTGT	GCATCCCAGCCTCCGTTA	
IL-6	AAGCCAGAGTCATTCAGAGCAA	TGGATGGTCTTGGTCCTTAGC	
TNF-α	CTTCTCATTCCTGCTCGTGG	ATCTGAGTGTGAGGGTCTGGG	
Egr-1	CAAGGGTGGTTTCCAGGTTC	GAAGGCTGCTGGGTACGGT	
IL-1β	GGGATGATGACGACCTGCTAG	CCACTTGTTGGCTTATGTTCTGT	
GAPDH	CCCATCTATGAGGGTTACGC	TTTAATGTCACGCACGATTTC	

Table I. Primers used in the quantitative polymerase chain reaction (qPCR).

A20, zinc finger protein A20; NF- κ B, nuclear factor κ B; HO-1, heme oxygenase-1; Bcl-2, B-cell lymphoma 2; IL-6, interleukin-6; TNF- α , tumor necrosis factor α ; Egr-1, early growth response protein 1; IL-1 β , interleukin-1 β ; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.



Figure 1. Effect of hydrogen gas inhalation on liver function. The activities of ALT and AST were measured by enzyme-linked immunosorbent assay following (A and B) hydrogen inhalation at different concentrations for 1 h and (C and D) 2% hydrogen inhalation for different durations. Data are expressed as mean \pm standard error of the mean. ALT, alanine aminotransferase; AST, aspartate aminotransferase. *P<0.05, **P<0.01 compared with the control group.

Statistical analysis. Data are presented as mean \pm standard error of the mean (SEM) and were statistically analyzed using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA). Comparisons between groups were made using analysis of variance (ANOVA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of hydrogen gas inhalation on liver function. The serum activities of ALT and AST were measured by ELISA. For rats that were administered different concentrations of hydrogen gas (1, 2 and 3%), the results revealed that gas inhalation at

all concentrations significantly reduced the ALT and AST activities compared with those in the control group (Fig. 1). In particular, a gas inhalation concentration of 2% achieved the optimal effect. Furthermore, for the rats treated with hydrogen at different time points, the results demonstrated that 1 h administration of gas prior to surgery led to a significant reduction in ALT and AST activities (Fig. 1).

Effect of hydrogen gas inhalation on cytokine expression. The mRNA expression of IL-6, TNF- α , Egr-1 and IL-1 β was measured using qPCR. Results revealed that hydrogen administration at 2% concentration significantly downregulated the mRNA levels of IL-6, TNF- α , Egr-1 and IL-1 β (Fig. 2).



Figure 2. Effect of hydrogen gas inhalation on cytokine expression. mRNA expression of IL-6, TNF- α , Egr-1 and IL-1 β was measured by quantitative PCR following (A-D) hydrogen inhalation at different concentrations for 1 h and (E-H) 2% hydrogen inhalation for different durations. Data are expressed as mean±standard error of mean. IL, interleukin; TNF, tumor necrosis factor; Egr-1, early growth response protein 1.*P<0.05, **P<0.01 compared with the control group.



Figure 3. Effect of hydrogen gas inhalation on IL-6 and TNF- α expression. Expression of IL-6 and TNF- α in serum was measured by enzyme-linked immunosorbent assay following (A and B) hydrogen inhalation at different concentrations for 1 h and (C and D) 2% hydrogen inhalation for different durations. Data are expressed as mean ± standard error of the mean. IL, interleukin; TNF, tumor necrosis factor.*P<0.05, **P<0.01 compared with the control group.

In addition, the expression levels of these cytokines were clearly decreased after hydrogen treatment for a period of 1 h (Fig. 2). The expression levels of IL-6 and TNF- α in serum were further examined using an ELISA. As demonstrated in Fig. 3, hydrogen gas inhalation at a concentration of 2% and

a duration of 1 h led to marked reductions in the levels of IL-6 and TNF- α expression.

Effect of hydrogen gas inhalation on liver morphology changes. The effect of hydrogen gas treatment on histopathological



Figure 4. Effect of hydrogen gas inhalation on liver morphology changes. The effect of 1-h hydrogen gas treatment on the histopathological changes in the livers of rats following ischemia/reperfusion (I/R) injury were determined by hematoxylin and eosin staining in (A) the control group and (B-D) the groups treated with hydrogen at concentrations of 1, 2 and 3%, respectively. Magnification, x400.



Figure 5. Effect of hydrogen gas inhalation on the subcellular morphological changes in liver tissues. Transmission electron microscopy was used to examine the subcellular morphological changes in liver tissues following exposure to hydrogen at different concentrations for various durations.

changes in the livers of rats with I/R is demonstrated in Fig. 4. Morphological examination revealed that the liver tissues of rats with I/R injury were severely damaged in the control group with severe edema, alveolar hemorrhage and extensive inflammatory cell infiltration. In the experimental groups, hydrogen treatment significantly alleviated liver edema, alveolar hemorrhage and inflammatory cell infiltration, suggesting that the liver injury induced by I/R was reduced by hydrogen treatment. In addition, following exposure to hydrogen, the morphological changes in subcellular structures in the liver tissues were further examined using a TEM. Gross morphological changes in the liver were clearly observed in



Figure 6. Hydrogen gas protects the liver against ischemia/reperfusion (I/R) injury by inhibiting the NF- κ B signaling pathway. The rats were randomly divided into three groups: hydrogen-treated donor and recipient (group A), hydrogen-treated donor (group B) and hydrogen-treated recipient (group C). The mRNA expression levels of (A) NF- κ B, (B) HO-1, (C) Bcl-2 and (D) A20 were measured by quantitative PCR and (E) the corresponding protein expression levels were determined by western blot analysis. Data are expressed as mean ± standard error of mean. NF- κ B, nuclear factor κ B; HO-1, heme oxygenase-1; Bcl-2, B-cell lymphoma 2; A20, zinc finger protein A20; GADPH, glyceraldehyde 3-phosphate dehydrogenase. *P<0.05, **P<0.01 compared with the control (Con) group.

the control group while hydrogen administration markedly reduced the I/R injury in the experimental groups (Fig. 5).

Hydrogen gas protects liver I/R injury by inhibiting the NF-κB signaling pathway. The current study then investigated whether the NF-κB signaling pathway was involved in initiating the protective effect of hydrogen on liver I/R injury. The rats were randomly divided into three groups: hydrogen-treated donor and recipient, hydrogen-treated donor, and hydrogen-treated recipient. The mRNA expression levels of NF-κB, HO-1, Bcl-2 and A20 were measured by qPCR. The results revealed that the mRNA expression levels of NF-κB, HO-1, Bcl-2 and A20 in the hydrogen-treated donor group were significantly increased compared with those in the other groups (Fig. 6). The expression levels of the corresponding proteins were then determined by western blot and demonstrated similar results (Fig. 6).

Discussion

In the current study, hydrogen gas inhalation by rats was used to investigate the protective effect of hydrogen on I/R injury during liver transplantation and identify the underlying mechanism. It was demonstrated that hydrogen inhalation was able to significantly suppress I/R injury in rats by downregulating ALT and AST activities, cytokine expression and morphological damage. In addition, the protective effect of hydrogen was identified to be mediated by the activation of the NF- κ B signaling pathway.

Compared with current drug therapy, the inhalation of hydrogen has several potential advantages. Hydrogen is physiologically safe for humans and is produced from undigested carbohydrates in the large intestine during fermentation (~150 ml/day). Colonic microflora continuously supply low doses of hydrogen into the blood circulation. Hydrogen is able to be metabolized by intestinal flora and may be discharged through the anus (10). It has been shown that <5% of hydrogen is not explosive and the successful application of hydrogen to prevent decompression sickness following deep diving has also been demonstrated to be safe (11). Oxygen produced by macrophages and neutrophils may kill bacteria, and this function is not affected by hydrogen. Therefore, hydrogen treatment does not affect cellular autophagy and other innate immune functions (12). In the present study, the activities of ALT and AST were markedly reduced following hydrogen treatment, indicating that hydrogen gas inhalation alleviated I/R injury in liver transplantation. Hydrogen gas inhaled at a concentration of 2% achieved the optimal effect. Furthermore, the rats were treated with hydrogen at different time points and it was revealed that 1 h administration of gas prior to surgery led to significant reductions in ALT and AST activities. Hydrogen treatment also led to marked reductions in IL-6, TNF-α, Egr-1 and IL-1ß expression. Analysis of changes to liver morphology following hydrogen inhalation using H&E staining and TEM revealed that hydrogen treatment at 2% concentration for 1 h prior to surgery significantly alleviated the tissue damage induced by I/R injury.

NF-κB is a nuclear transcription factor present in almost all animal cells and is involved in the cell response to stimulation by stress, cytokines and reactive oxygen species. NF-κB plays a critical role in mediating inflammatory and immune responses (13,14). Activation of NF-κB regulates an anti-apoptotic cascade and a number of anti-apoptotic genes including heme oxygenase, A20 and Bcl-2 (15,16). HO-1 is an inducible isoform produced in response to stress such as oxidative stress, hypoxia and cytokines (17). A20 has been identified to be a gene whose expression is rapidly induced by tumor necrosis factors (18). The protein encoded by this gene is a zinc finger protein and it has been shown to inhibit NF-κB activation as well as TNF-mediated apoptosis (19). The anti-apoptotic protein Bcl-2 has been demonstrated to prevent disruption of mitochondrial physiology and block cytochrome c release from mitochondria, which is a response gene of p53 and involved in p53-regulated apoptosis (20,21). The current study demonstrated that hydrogen gas inhalation resulted in a significant increase in NF-kB expression as well as in the expression levels of the anti-apoptotic genes HO-1, A20 and Bcl-2, particularly in the hydrogen-treated donor group compared with those in the hydrogen-treated donor and recipient group and the hydrogen-treated recipient group. The protein expression of these genes was determined by western blot analysis and revealed similar results. Together, these results demonstrated that the protective effect of hydrogen gas is dependent on the activation of the NF- κ B signaling pathway.

In summary, the present study demonstrated that hydrogen gas inhalation at 2% concentration for 1 h prior to liver transplantation protected rats from I/R injury by activating the NF- κ B signaling pathway.

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