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Biochemical and Biophysical Research Communications 361 (2007) 670–674
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Inhalation of hydrogen gas suppresses hepatic injury caused by ischemia/reperfusion through reducing oxidative stress

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Received 13 July 2007
Available online 25 July 2007

Abstract

We have recently showed that molecular hydrogen has great potential for selectively reducing cytotoxic reactive oxygen species, such as hydroxyl radicals, and that inhalation of hydrogen gas decreases cerebral infarction volume by reducing oxidative stress [I. Ohsawa, M. Ishikawa, K. Takahashi, M. Watanabe, K. Nishimaki, K. Yamagata, K.-I. Katsura, Y. Katayama, S. Asoh, S. Ohta, Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals, Nat. Med., 13 (2007) 688–694]. Here we show that the inhalation of hydrogen gas is applicable for hepatic injury caused by ischemia/reperfusion, using mice. The portal triad to the left lobe and the left middle lobe of the liver were completely occluded for 90 min, followed by reperfusion for 180 min. Inhalation of hydrogen gas (1–4%) during the last 190 min suppressed hepatic cell death, and reduced levels of serum alanine aminotransferase and hepatic malondialdehyde. In contrast, helium gas showed no protective effect, suggesting that the protective effect by hydrogen gas is specific. Thus, we propose that inhalation of hydrogen gas is a widely applicable method to reduce oxidative stress.

Keywords: ALT; Anti-oxidant; Hydrogen gas; Hydroxyl radical; Ischemia; Liver; MDA; Oxidative stress; Reperfusion

It is widely accepted that reactive oxygen species (ROS) are one of the causes of lifestyle-related diseases, cancer and the aging process. Moreover, when tissues are exposed to ischemia followed by reperfusion (I/R), ROS are extensively generated in the early stage of reperfusion to cause serious damage to tissues in various organs, including the liver [1], brain [2], heart [3], and kidney [4]. Prolonged hepatic warm ischemia aggravates oxidative stress after reperfusion, leading to severe reperfusion injury [1]; therefore, I/R injury caused by oxidative stress has been a major focus of basic and clinical research.

The most likely mechanisms underlying I/R-induced organ damage are multifactorial and interdependent, involving hypoxia, inflammatory responses, and free radical damage [5,6]. Although the etiology of I/R injury is poorly defined, oxygen free radicals appear to play important roles [7–11]. Thus, free radical scavengers are thought to be practical in the clinical setting of I/R damage. Actually, a number of agents, such as nicaraven [12], MCL-186 [13], MESNA [14], and α-tocopherol and $GdCl_3$ [15] have so far been tried as scavengers that are expected to prevent I/R injury.

We have recently demonstrated that molecular hydrogen selectively reduced the levels of hydroxyl radicals *in vitro* and that hydrogen molecule exerts a therapeutic antioxidant activity, using a rat middle cerebral artery occlusion model [16]; however, it remains unclear whether

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0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.07.088

a small molecule such as helium has a similar effect and whether the method could be applicable to other organs. Here, we show that inhalation of hydrogen gas is efficacious for hepatic injury caused by IR, whereas helium gas exhibits no effect.

Materials and methods

Animals. Male C57 BL/6N mice (4 to 5 weeks old, 15–18 g) were purchased from Seac Yoshitomi, Ltd. (Yoshitomi-cho, Fukuoka, Japan). The mice were maintained under the standard conditions with a 12-h light/dark cycle, and permitted ad libitum access to standard rodent chow and tap water. The experiments were conducted according to the Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Nippon Medical School.

Liver I/R model. Mice underwent an I/R procedure under general anesthesia with sevofrane (1.5%) in N_2O/O_2 (70:30) (flow rate of 1 L/min) gas. A model of partial hepatic ischemia was used in all experiments as described previously [17–20]. Briefly, after a midline incision was made and the portal triad (hepatic artery, portal vein, and bile duct) to the left lobe and the left middle lobe of the liver was occluded with a vascular micro clamp (FD562; Aesculap, South San Francisco, CA, USA) to produce 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 in a simple continuous manner. After 90-min ischemia, a second laparotomy was performed to remove the clamp. The abdomen was closed again in the same manner, followed by 180-min reperfusion.

Inhalation of H_2 gas. To administer H_2 gas to anesthetized mice, H_2 gas was supplied through a gas flowmeter, TF-1 (YUTAKA Engineering Corp., Tokyo, Japan) to the anesthetic gas (sevofrane (1.5%) in N_2O/O_2 (70:30) gas; flow rate of 1 L/min) 10 min before reperfusion until the end of reperfusion (total 190 min). The concentration of H_2 gas in the anesthetic gas was determined using a Breath Gas Analyzer™ Model TGA-2000 (TERAMECS, Kyoto, Japan).

Sample collections. At the end of reperfusion, the ischemic liver (left lobe) was taken for further experiments, and was cut into two pieces through the middle with a razor blade. One piece was immediately frozen in liquid nitrogen and stored at −80 °C until determination of the malondialdehyde (MDA) level. The other piece was further sliced into several pieces, and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH7.4) for histopathological study. Blood samples were also collected from the heart to measure a liver enzyme.

Hematoxylin–eosin staining. Fixed livers were dehydrated and embedded in paraffin. Tissues were sectioned (4-μm thickness) and stained with hematoxylin–eosin (HE).

Serum alanine aminotransferase activity. After clotting, a blood sample was centrifuged at 3000 rpm for 5 min at 4 °C. The top clear layer was centrifuged again under the same conditions to prepare serum. Activities of serum alanine aminotransferase (ALT) were examined using a Transaminase CII Testwako kit (Wako Pure Chemical Industries Ltd., Osaka, Japan). The result is shown in Karmen unit [21].

Hepatic malondialdehyde measurement. Hepatic MDA levels were determined using a BIOXYTHCH MDA-586 Assay kit (OxisResearch, Oregon, USA). Briefly, frozen ischemic liver tissues were homogenized in the presence of butylated hydroxytoluene. After centrifugation, free MDA in the supernatant was converted to a stable carbocyanin dye (maximum absorption at 586 nm) by the chemical reaction with *N*-methyl-2-phenylindole. Protein concentration was determined by the BCA Protein Assay (Pierce, Rockford, IL, USA) using BSA as a standard. MDA levels were normalized against protein (pmol/mg).

Results

Histopathological examination by HE staining

To investigate whether inhalation of hydrogen gas (H_2) protects the liver against hepatic I/R injury, we histopathologically analyzed liver sections prepared from mice subjected to I/R with or without H_2. When subjected to I/R insult without H_2, profound degeneration was observed in the whole section and zonal cytoplasmic vacuolization preferentially developed in the centrilobular region (white areas in Fig. 1). Quantitative analysis using NIH Image software revealed that the degenerated area occupied, on average, 65% of the whole section (Fig. 2). Inhalation of H_2 clearly attenuated the degeneration induced by I/R and the protective effect was in a concentration-dependent manner. Moreover, to exclude the possibility that this mitigation can be achieved by any gaseous molecule but is not specific to H_2, we treated mice with helium gas (He) instead of H_2. He gas did not show any protective effect against hepatic I/R injury (Fig. 1). These results indicate that H_2 gas reduces hepatic I/R injury at a concentration as low as 2–4%. The optimum concentration of H_2 was consistent with the previous results [16].

Serum alanine aminotransferase

To biochemically verify the results of the histological examination, we measured serum ALT levels in each experimental group. Inhalation of H_2 gas suppressed the release of ALT from the liver by half to one third compared with non-treatment of H_2 gas, while He gas could not decrease serum ALT levels (Fig. 3).

MDA in liver tissue

The MDA level is widely used as an indicator of free radical-mediated lipid peroxidation injury. We measured MDA levels in the liver because our previous study showed that hydrogen gas attenuates oxidative stress [16]. I/R insults increased hepatic MDA levels, as shown in Fig. 4 (0% H_2 gas). In contrast, inhalation of H_2 gas dramatically decreased MDA levels almost to the normal level. Inhalation of He gas instead of hydrogen gas slightly decreased MDA levels but not significant compared with the control (0% H_2 gas).

Discussion

It has been reported that hepatic warm I/R injury consists of two phases [22–25]. In the initial phase, Kupffer cells are activated by ischemia to produce reactive oxygen species (ROS) within 2 h after reperfusion, resulting in acute hepatocellular injury. The following late phase occurs 6 h after reperfusion, in which neutrophils, a well-known source of ROS [24,26–29], accumulate in the liver to more profoundly develop the hepatic damage. It is noted that

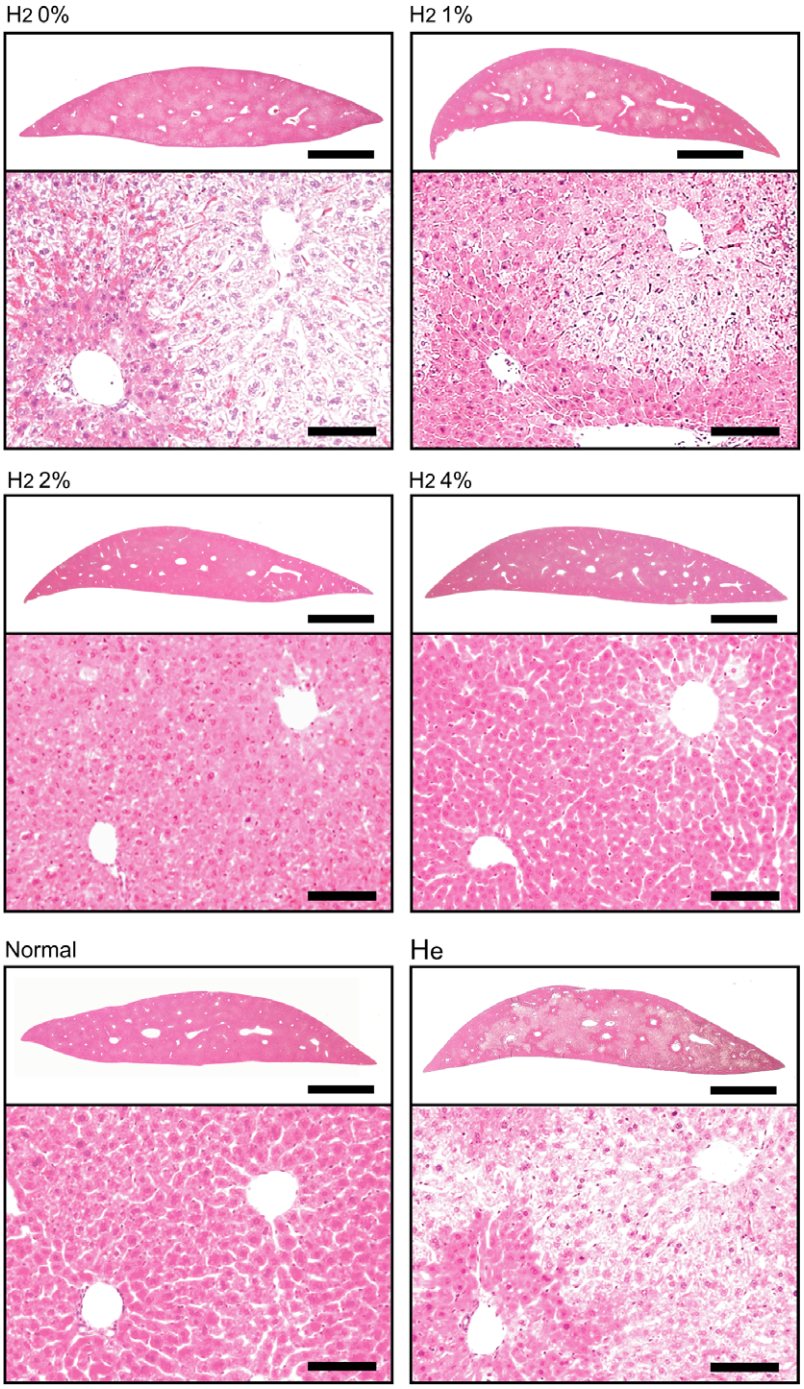

Fig. 1. Suppression of I/R injury by inhaling hydrogen gas. Under anesthetic gas, mice were subjected to a 90-min ischemic insult, followed by 180-min reperfusion. Ischemic livers were removed for fixing. Paraffin sections were prepared and subjected to HE staining. For mice to inhale hydrogen gas, hydrogen gas was supplied to the anesthetic gas 10 min before reperfusion and continued to be supplied until the end of reperfusion. Instead of hydrogen gas, helium gas (4%) was also inhaled by mice. Representative pictures are shown. Scale bar: 2 mm (upper panels) and 100 μm (lower panels).

reduced mitochondrial respiration activity in hepatocytes and sinusoidal endothelial cells can lead to the generation of ROS in the initial phase [30]. Thus, it is very important for mitigating hepatic I/R injury to inhibit or reduce ROS production/accumulation in the initial phase.

The hydroxyl radical is the most reactive product of ROS generated in cells. It is postulated that the hydroxyl radical is generated in biological systems from superoxide anion and hydrogen peroxide by the Haber–Weiss reaction or from hydrogen peroxide by the Fenton reaction [31,32]. It is biologically important to eliminate hydroxyl radicals, because superoxide anion and hydrogen peroxide are detoxified by antioxidant defense enzymes, superoxide dismutase, and peroxidase or glutathione-peroxidase, respectively; however, no enzyme detoxifies hydroxyl radicals. Various substances including glucose, mannitol, formate,

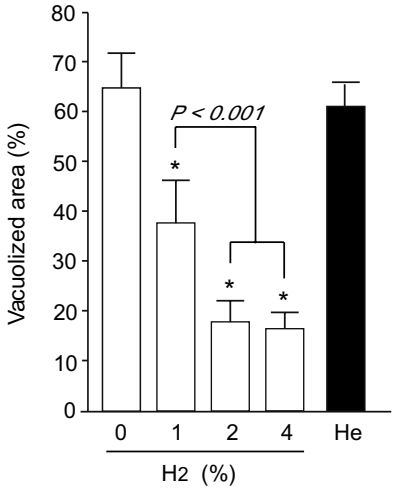

Fig. 2. Quantitative analysis of the vacuolized area induced by hepatic I/R. Whole images of HE-stained liver sections, as shown in Fig. 1 (upper panels), prepared from mice subjected to I/R with or without hydrogen gas ($n = 6$ each group) were analyzed to evaluate the relative area occupied by the vacuolized area (white area in Fig. 1) using NIH image software. Whole images of mice ($n = 6$) treated with helium gas (4%) instead of hydrogen gas were also analyzed. After statistical analysis by one-way ANOVA, the data are presented as the means with the standard deviation (vertical bars).

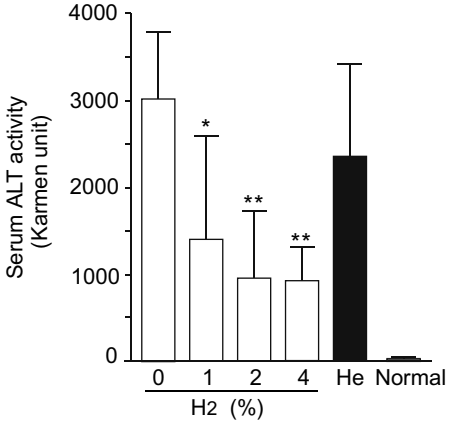

Fig. 3. Hydrogen gas decreased serum ALT levels. Mice ($n = 6$ each group) were subjected to I/R with or without hydrogen gas, and their serum ALT activities (expressed as Karmen units [21]) were examined. Mice ($n = 6$) treated with helium gas (4%) instead of hydrogen gas and normal mice (without any treatment; $n = 6$) were also analyzed. The mean is presented with the standard deviation. Statistical analysis was performed using one-way ANOVA. $^{*}P < 0.05$; $^{**}P < 0.005$, compared with 0% H_2 gas.

thiourea and dimetyl sulfoxide have been reported as a hydroxyl radical scavenger [33,34].

Since the hydrogen molecule is electronically neutral and is much smaller than the oxygen molecule, a hydrogen molecule is expected to easily penetrate the cellular and intracellular membranes, which prevent water-soluble antioxidants from entering cells and organelles such as mitochondria, a major source of ROS production.

In a recent study, we demonstrated that hydrogen molecules were detected in the blood after inhalation of hydrogen gas [16]. Hydrogen molecules reacted only with hydroxyl radicals, but could not react with less reactive oxidants, such as hydrogen peroxide [16]. Hydroxyl radicals easily react with cellular macromolecules, including DNA, proteins and lipids, to exert a strong cytotoxic effect. It is well known that the reaction of hydroxyl radicals and lipids results in lipid peroxidation. Peroxidation of arachidonic acid and linoleic acid produces MDA and hydroxynonenal (HNE). In this study, we showed that hydrogen gas significantly inhibited MDA production induced by hepatic I/R injury. We have also demonstrated that hydrogen gas inhibited HNE production induced by brain I/R injury and that hydrogen gas scavenged hydroxyl radicals produced in cultured cells [16]. These results suggest that hydrogen gas eliminates hydroxyl radicals induced by hepatic I/R injury.

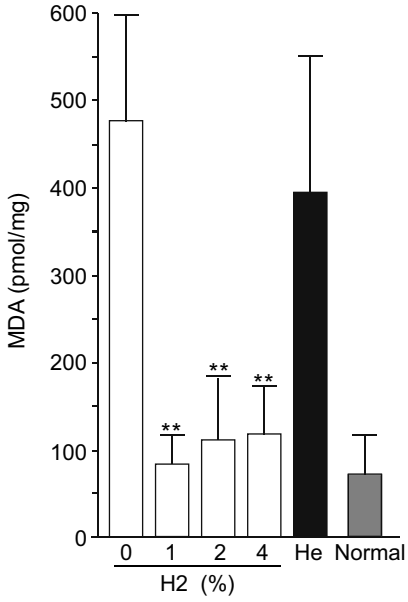

Fig. 4. Hydrogen gas significantly decreased levels of hepatic MDA, a marker of oxidative stress. Mice ($n = 6$ each group) were subjected to I/R with or without hydrogen gas. Ischemic livers were removed and homogenized in the presence of butylated hydroxytoluene. After centrifugation, free MDA in the supernatant was determined. Mice ($n = 6$) treated with helium gas (4%) instead of hydrogen gas and normal mice (without any treatment; $n = 6$) were also analyzed. Free MDA levels were normalized against protein contents in supernatants. The mean is presented with the standard deviation. Statistical analysis was performed using one-way ANOVA. $^{**}P < 0.0001$, compared with 0% H_2 gas.

Hydrogen gas has been used in medical applications to prevent decomposition sickness (DCS) in deep divers from safety profiles [35]. The development of intravascular bubbles is the main cause of DCS in deep divers. Nitrogen gas more easily develops bubbles than helium or hydrogen by changing with pressure conditions [36]. Thus, deep divers generally breathe binary mixtures of oxygen and helium or hydrogen to prevent DCS [37]. In this study, we showed that helium could not substitute hydrogen. Thus, in the prevention of DCS, H_2 and He do not function as antioxidants. Molecular hydrogen will be used in wide medical applications as an antioxidant.

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