Inhalation of hydrogen gas reduces infarct size in the rat model of myocardial ischemia–reperfusion injury


Department of Regenerative Medicine and Advanced Cardiac Therapeutics, Keio University School of Medicine, 35 Shinnomachi Shinjuku-ku, Tokyo 160-8582, Japan
Division of Cardiology, Keio University School of Medicine, Tokyo 160-8582, Japan
Division of Geriatric Medicine, Department of Internal Medicine, Keio University School of Medicine, Tokyo 160-8582, Japan
Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency, Saitama 332-0012, Japan
Department of Biochemistry and Cell Biology, Institute of Development and Aging Science, Graduate School of Medicine, Nippon Medical School, Kawasaki city 211-8533, Japan
Advanced Cardiac Therapeutics, Keio University School of Medicine, 35 Shinanomachi Shinjuku-ku, Tokyo 160-8582, Japan

Abstract

Inhalation of hydrogen (H2) gas has been demonstrated to limit the infarct volume of brain and liver by reducing ischemia–reperfusion injury in rodents. When translated into clinical practice, this therapy must be most frequently applied in the treatment of patients with acute myocardial infarction, since angioplastic recanalization of infarct-related occluded coronary artery is routinely performed. Therefore, we investigate whether H2 gas confers cardioprotection against ischemia–reperfusion injury in rats. In isolated perfused hearts, H2 gas enhances the recovery of left ventricular function following anoxia–reoxygenation. Inhaled H2 gas is rapidly transported and can reach ‘at risk’ ischemic myocardium before coronary blood flow of the occluded infarct-related artery is reestablished. Inhalation of H2 gas at incombustible levels during ischemia and reperfusion reduces infarct size without altering hemodynamic parameters, thereby preventing deleterious left ventricular remodeling. Thus, inhalation of H2 gas is promising strategy to alleviate ischemia–reperfusion injury coincident with recanalization of coronary artery.

© 2008 Elsevier Inc. All rights reserved.

Keywords:
Ischemia–reperfusion injury
Anti-oxidant
Myocardial infarction
H2

Acute myocardial infarction is a leading cause of death worldwide. Reduction of infarct size is an important therapeutic goal, since the size of the infarct is directly linked to short-term and long-term morbidity and mortality [1]. The prognosis of acute myocardial infarction has been improved dramatically with the development of highly successful approaches to restore blood flow by primary percutaneous coronary intervention (PCI) to the ischemic tissue [2]. Paradoxically, while coronary reperfusion improves the prognosis of acute myocardial infarction, it also leads to myocardial reperfusion injury by extending myocardial damage within the ischemic period [3]. Studies in animal models of acute myocardial infarction show that reperfusion injury accounts for up to 50% of the final size of a myocardial infarct [4]. Therefore, intervention to alleviate reperfusion injury at the time of coronary recanalization has been considered to be the promising strategy to further decrease infarct size and improve the prognosis after myocardial infarction.

The accelerated generation of reactive oxygen species (ROS) by reperfusion of the ischemic myocardium is a potential mediator of reperfusion injury [5–7]. Many attempts have been made to inhibit ROS production to limit the extent of reperfusion injury. However, the administration of ROS scavengers at the time of reperfusion has produced conflicting results [8,9]. That can be partially explained by the dual role of ROS in ischemia-reperfused hearts. The majority of detrimental effects associated with lethal reperfusion injury are attributed to hydroxyl radical (·OH), the most highly reactive oxygen species. By comparison, superoxide anion radical (O2·−) and hydrogen peroxide (H2O2) have less oxidative energy and, paradoxically, are implicated as crucial signaling components in the establishment of favorable tolerance to oxidative stress upon ischemia–reperfusion [10,11]. Consequently, the inhibition of both pathways can be deleterious.

Recently, Ohsawa et al. demonstrated that molecular hydrogen (H2) is a novel anti-oxidant with certain unique properties. (1) H2 is permeable to cell membranes and can target organelles,
including mitochondria and nuclei; (2) H2 specifically quenches exclusively detrimental ROS, such as ·OH and peroxynitrite (ONOO·), while maintaining the metabolic oxidation-reduction reaction and other less potent ROS, such as O2·−, H2O2, and nitric oxide (NO·); (3) inhalation of H2 gas limits the infarct volume of brain and liver if given at the appropriate time during reperfusion [12,13]. However, clinical application of reperfusion therapy for these organs is limited. When translated into the clinical practice, H2 gas inhalation therapy must be most frequently applied in the treatment of patients with acute myocardial infarction, since angioplastic recanalization of occluded infarct-related coronary artery is routinely performed.

The aim of this study was to investigate whether inhalation of H2 gas exerts cardioprotective effects during myocardial ischemia–reperfusion. We showed the inhaled H2 gas is rapidly transported and can reach even ‘at risk’ ischemic myocardium before coronary blood flow of the occluded infarct-related artery is reestablished. Inhalation of H2 gas during ischemia and reperfusion significantly reduces infarct size without altering hemodynamic parameters, thereby preventing deleterious left ventricular (LV) remodeling.

Materials and methods

Animals. All experimental procedures and protocols were approved by the Animal Care and Use Committees of the Keio University and conformed to the NIH Guide for the Care and Use of Laboratory Animals. Eight-week-old male Wistar rats were artificially ventilated under anesthesia with ketamine (60 mg/kg) and xylazine (15 mg/kg) given intraperitoneally. Temperature was maintained at 37.5 ± 0.5 °C using a thermostatically controlled heating blanket connected to a thermometer-probe placed in the rectum. H2 gas was administered through a ventilator and the flow volume was controlled by a gas flowmeter TF-1 (YUTAKA Engineering Corporation, Tokyo, Japan). The concentration of H2 gas in the gas mixture was determined using the Breath Gas Analyzer Model TGA-2000 (TERA-MECS, Kyoto, Japan). Saturation of arterial oxygen level (SaO2) was monitored by Clip sensor (PDR-43C) connected to Stand MECS, Kyoto, Japan). The concentration of H2 in the gas mixture was determined using the Breath Gas Analyzer Model TGA-2000 (TERA-MECS, Kyoto, Japan). The concentration of H2 in the gas mixture was determined using the Breath Gas Analyzer Model TGA-2000 (TERA-MECS, Kyoto, Japan).

Myocardial ischemia–reperfusion model. Regional myocardial ischemia was induced by transient occlusion of the left anterior descending coronary artery. After 30 min of ischemia, we removed the tube for myocardial reperfusion and closed the thorax with the suture intact. The suture around the coronary artery was retied 24 h after reperfusion and 2% Evans blue dye was injected into the LV cavity to retrospectively delineate the area at risk of myocardial infarction. The heart was removed, washed in phosphate buffered saline, and then sliced into sequential 1 mm thick sections was measured by planimetry using ImageJ software from the National Institutes of Health (Bethesda, MD, USA).

Statistical analyses. Values are presented as means ± SEM. Statistical significance was evaluated using the unpaired Student’s t-tests for comparisons between two mean values. Multiple comparisons between more than three groups were performed using ANOVA. A value of P < 0.05 was considered statistically significant.

Results

H2 gas improves the recovery of left ventricular function during reoxygenation after anoxia in isolated perfused hearts

We first studied the effect of H2 gas on the functional recovery after anoxia-reoxygenation in Langendorff-perfused rat hearts. Hearts were subjected to 40 min of anoxic perfusion with buffer equilibrated with either 100% N2 (Control group) or 100% H2 (H2 group) followed by 40 min of aerobic reperfusion with buffer equilibrated with 95% O2 and 5% CO2 (Fig. 1A). H2 gas significantly improved the recovery of LV developed pressure (LVPD), positive dP/dt, and negative dP/dt 40 min after reoxygenation (n = 10, P < 0.05, compared to control group, Fig. 1B).

Inhalation of H2 gas immediately increases the intramyocardial H2 gas concentration

Before we determined whether inhalation of hydrogen (H2) gas confers cardioprotection against ischemia–reperfusion injury, the regional delivery of inhaled H2 gas was investigated by monitoring the time-course of changes in H2 levels using a needle-shaped
hydrogen sensor electrode inserted directly into the tissues. When
2% H₂ gas was inhaled, the arterial H₂ levels started to increase
2 min after inhalation of H₂ gas and reached a maximum level after
5 min [1.82 ± 0.02% (n = 5)]. The incremental rate of H₂ saturation
for the non-ischemic myocardium was similar to that observed
in arterial blood with attaining a maximum of 1.73 ± 0.02%
(n = 5) (Fig. 2A). By contrast, the rate of increase in the H₂ satura-
tion was slower in the center of the thigh muscle with attaining
a maximum level of 0.50 ± 0.03% (n = 5) after 30 min (Fig. 2B and
Supplementary Fig.).

Of note, H₂ gas levels were increased even in the ischemic myo-
cardium (Fig. 2C). Although the incremental rate of H₂ saturation
was slower in the ischemic myocardium than in the non-ischemic
myocardium, the peak level of H₂ in the ischemic myocardium was
reached at approximately two thirds of the value observed in the
non-ischemic myocardium (Fig. 2D). After restoration of coronary
artery blood flow, the level of H₂ in the ischemic myocardium
immediately increased to the level observed in the non-ischemic
myocardium.

Inhalation of H₂ gas protects the heart from ischemia–reperfusion
injury

To investigate whether inhalation of H₂ gas protects the heart
from ischemia–reperfusion injury, rats were subjected to coro-
nary artery occlusion for 30 min followed by reperfusion for
24 h. H₂ gas was administered at the onset of ischemia and con-
tinued for 60 min after reperfusion. H₂ gas has no adverse effect
on heart rate and arterial oxygenation (Fig. 3A). There was no
significant difference in the temporal profile of LV end-systolic
function.
pressure. LV peak positive and negative LV dP/dt, between the control group and the 2% H2 gas inhalation group. Notably, LV-end-diastolic pressure after reperfusion was significantly lower in H2 gas inhalation group compared to control group (n = 5, 0.05).

In the absence of H2 gas inhalation, infarct size following ischemia–reperfusion was 41.6 ± 2.5% of the area at risk (n = 9). By comparison, inhalation of 0.5–2% H2 gas significantly reduced infarct size, with 2% H2 gas providing the most prominent effects (21.2 ± 1.6% of area at risk, n = 4, Fig. 3B and C). There was no significant difference in area at risk/LV among control group and H2 gas inhalation groups (data not shown). Consistent with those observations, the quantitative determination of 8-hydroxydeoxyguanosine (8-OHdG) immunoreactive area, a biomarker of oxidative stress, revealed that the level of oxidative injury elicited in the ‘at risk’ area was significantly smaller in the group receiving 2% H2 gas inhalation than that of control group (n = 5, 0.05, Fig. 3D).

**Inhalation of H2 gas reduces LV remodeling after ischemia–reperfusion injury**

To determine the impact of H2 inhalation at the time of ischemia–reperfusion on pathological LV remodeling, LV morphology and function were monitored by echocardiography 30 days after myocardial ischemia–reperfusion injury. Control rats showed maladaptive pathological remodeling after myocardial infarction, including dilatation of LV cavity, reduced LV systolic function. Notably, inhalation of H2 gas during myocardial ischemia–reperfusion reduced pathological remodeling after myocardial infarction (Fig. 4).

**Discussion**

This is the first study to demonstrate that inhalation of H2 gas, at an incombustible level, limit the extent of myocardial infarction.
resulting from myocardial ischemia–reperfusion injury, and thereby preserve LV function in vivo. The cardioprotective effect of H2 gas was also confirmed ex vivo Langendorff-perfused hearts subjected to anoxia-reoxygenation injury. The anti-oxidant properties of H2 were confirmed by the demonstration that (1) H2 improves the recovery of LV function during reoxygenation after anoxia, one of the oxidative stress model, in isolated perfused hearts; (2) inhalation of H2 gas ameliorates the level of 8-OHdG immunoreactivity in the ‘at risk’ area for infarction. The anti-oxidant action of molecular H2 may be explained, at least partially, by direct ROS scavenging effect. However, it remains unclear if the anti-oxidant action of H2 is also ascribed to the activation of the reperfusion injury salvage kinase pathways or a direct effect on mitochondrial energetics.

Gas inhalation as disease therapy has received recent interest. There are three endogenous gas signaling molecules, known as gas-transmitters, include nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H2S). The increased production of these gases under stress conditions may reflect the active involvement of these gases in the protective response. In pre-clinical experimental models of disease, including ischemia–reperfusion injury, the inhalation of exogenous CO or H2S has produced a favorable outcome for most vital organs [19–22]. However, the inherent toxicity of these gases must be investigated for gas inhalation to be considered an effective therapeutic strategy. It is unknown if the therapeutically effective threshold for CO or H2S can be attained locally in target organs without delivering a potentially toxic level of the gases via the lungs.

H2 is not produced endogenously in mammalian cells since the hydrogenase activity responsible for the formation of H2 gas has not been identified [23]. The spontaneous production of H2 gas in the human body occurs via fermentation of undigested carbohydrates by resident enterobacterial flora. H2 is transferred to the portal circulation and excreted through the breath in significant amounts. We demonstrated that H2 gas clearance method was employed to measure local blood flow in various tissues [24]. Since the heart is one of the most highly perfused tissues, the intramyocardial H2 concentration increases immediately following inhalation of H2, and attaining to almost compatible levels of that observed in arterial blood within 10 min. Of note, the regional H2 concentration in the ischemic myocardium reaches at two thirds of the value observed in the non-ischemic myocardium. This may occur through gaseous diffusion from the blood in the ventricular cavity and/or adjacent non-ischemic myocardium. These findings indicate that administration of H2 gas by inhalation, in patients with totally coronary artery occlusion, can efficiently increase the regional concentration of H2 in the ‘at risk’ area for myocardial infarction before reestablishing coronary blood flow within the occluded infarct-related artery.

We demonstrated that inhalation of H2 gas is promising strategies to alleviate ischemia–reperfusion injury at the time of reanamalsation of coronary artery. When translated into the clinical practice, inhalation of H2 gas must be most frequently applied in the treatment of patients with acute myocardial infarction in conjunction with routinely performed PCI procedures. Further understanding of the mechanisms underlying the signaling pathways involved in H2-mediated anti-oxidant activity, and the capacity of H2 to influence cellular metabolism, is required to fully exploit inhalation of H2 gas as a therapeutic strategy.

**Acknowledgments**

We thank M. Okada (NIHON KODEN), S. Kotouda (LMS laboratory and Medical Supplies), C. Ogawa, K. Nishimaki, M. Kamimura, S. Abe, K. Miyake, H. Kawaguchi, H. Shiozawa, and M. Ono for their technical assistance. M. Sano is a core member of the Global Center-of-Excellence (GCOE) for Human Metabolomics Systems Biology from MEXT. This work was supported by a PRESTO (Metabolism and Cellular Function) grant from the Japanese Science and Technology Agency awarded to M. Sano.

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.05.165.

**References**


