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

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ABSTRACT

Inhalation of hydrogen (H_2) 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 H_2 gas confers cardioprotection against ischemia–reperfusion injury in rats. In isolated perfused hearts, H_2 gas enhances the recovery of left ventricular function following anoxia-reoxygenation. Inhaled H_2 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 H_2 gas at incombustible levels during ischemia and reperfusion reduces infarct size without altering hemodynamic parameters, thereby preventing deleterious left ventricular remodeling. Thus, inhalation of H_2 gas is promising strategy to alleviate ischemia–reperfusion injury coincident with recanalization of coronary artery.

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 hydroxy radical (·OH), the most highly reactive oxygen species. By comparison, superoxide anion radical ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2) 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 (H_2) is a novel anti-oxidant with certain unique properties. (1) H_2 is permeable to cell membranes and can target organelles,

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including mitochondria and nuclei; (2) H_2 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 $O_2^{-}\cdot$, H_2O_2, and nitric oxide ($NO\cdot$); (3) inhalation of H_2 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, H_2 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 H_2 gas exerts cardioprotective effects during myocardial ischemia–reperfusion. We showed the inhaled H_2 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 H_2 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. H_2 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 H_2 in the gas mixture was determined using the Breath Gas Analyzer Model TGA-2000 (TERAMECS, Kyoto, Japan). Saturation of arterial oxygen level (SaO_2) was monitored by Clip sensor (PDR-43C) connected to Stand Alone Pulseoxymeter (CANL425SV). A Millar transducer catheter (SPR-320) was placed in the LV cavity via the left internal artery to monitor LV pressure using Polygraph system (NIHON KODEN; PEG-1000).

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. We stained the sections with 2,3,5-triphenyltetrazolium chloride (TTC) (3%) then measured the infarct (white), non-infarct (red), non-ischemic, (blue), and at risk areas (AAR) (white and red).

Echocardiography. Rats were anesthetized by inhalation with 1.5% isoflurane. Animals were anchored to a positionable platform in a supine position. Short axis echocardiography was accomplished with a Vevo 660 system (VisualSonics) with the use of a 600 series real-time microvisualization scanhead probe.

Measurement of H_2 gas concentration. H_2 gas concentration was measured in tissues using a needle-type H_2 sensor (Unisense). The electrode current was measured with a picoammeter (Keithley) attached to a strip chart. The negative current obtained from the H_2 sensor was converted to regional H_2 concentration using a calibration curve generated from known levels of H_2 saturated saline.

Langendorff-perfusion of the heart. Hearts were excised quickly from heparinized Wistar male rats (350 g) and perfused with modified Krebs–Henseleit buffer (118 mmol/l NaCl, 25 mmol/l $NaHCO_3$, 4.7 mmol/l KCl, 1.2 mmol/l $MgSO_4$, 1.2 mmol/l KH_2PO_4, 1.75 mmol/l $CaCl_2$, 0.5 mmol/l EDTA, 11 mmol/l glucose, and 5 mmol/l pyruvate) equilibrated with a gas mixture comprised of 95% O_2/5% CO_2 at 37 °C. Coronary perfusion pressure was maintained at 70 mmHg. A plastic catheter with a latex balloon was inserted into the LV. Before the induction of anoxia, hearts were paced at 5 Hz, and the LV end-diastolic pressure was adjusted to 10 mmHg by filling the balloon with water. Pacing was turned off during anoxia and turn on 10, 20, 30, or 40 min after reoxygenation to measure the recovery of LV function. Indices of LV function [LV systolic pressure, LVSP; LV diastolic pressure, LVDP; LV developed pressure (LVDP = LVSP − LVDP); and LV peak positive and negative dP/dt] were recorded as described previously [14–17].

Immunohistochemical procedures. Sample fixation, embedding, sectioning, and blocking were performed as described previously [18]. Briefly, hearts were perfused from the apex with PBS, perfusion-fixed with 4% paraformaldehyde/PBS, dissected, subsequently cryoprotected in sucrose solutions at 4 °C, embedded in OCT compound (Miles Scientific, Naperville, IL), and quickly frozen in liquid nitrogen. The fixed hearts were sectioned (8 μm) using a CM3050S cryostat (Leica, Nussloch, Germany). For immunostaining, sections were blocked in 5% BSA for 30 min at room temperature and stained with anti-8OH-dG (MOG-020P; Japan Institute for the Control of Aging; 1:800) antibodies overnight at 4 °C. Secondary antibodies conjugated Alexa Fluor 546 (Molecular Probes, Eugene, OR, USA; 1:200) were applied for 1 h at 4 °C. Nuclei were stained with TO-PRO-3 (Molecular Probes) in a mounting medium. Slides were observed under Fluorescence Microscope (LYMPUS BX-60). The 8-OHdG positive area as percentage of total left ventricles at serial short axis sections was measured by planimetry using Image/J 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

H_2 gas improves the recovery of left ventricular function during reoxygenation after anoxia in isolated perfused hearts

We first studied the effect of H_2 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% N_2 (Control group) or 100% H_2 (H_2 group) followed by 40 min of aerobic reperfusion with buffer equilibrated with 95% O_2 and 5% CO_2 (Fig. 1A). H_2 gas significantly improved the recovery of LV developed pressure (LVDP), positive dP/dt, and negative dP/dt 40 min after reoxygenation ($n = 10$, $^*P < 0.05$, compared to control group, Fig. 1B).

Inhalation of H_2 gas immediately increases the intramyocardial H_2 gas concentration

Before we determined whether inhalation of hydrogen (H_2) gas confers cardioprotection against ischemia–reperfusion injury, the regional delivery of inhaled H_2 gas was investigated by monitoring the time-course of changes in H_2 levels using a needle-shaped

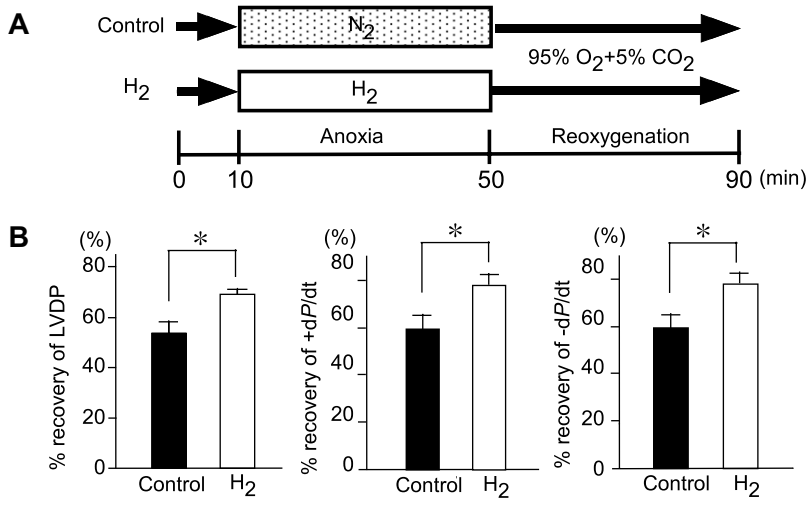

Fig. 1. H_2 gas improves the recovery of left ventricular function during reoxygenation after anoxia in isolated perfused hearts. (A) Experimental protocol of aoxia-reoxygenation. Isolated perfused rat hearts were subjected to 40 min of anoxia with buffer equilibrated with either 100% N_2 (control group) or 100% H_2 (H_2 group) followed by 40 min of aerobic reperfusion. (B) Comparison of percentage recovery of LVDP and peak positive and negative d*P*/d*t* 40 min after reoxygenation between control group and H_2 inhalation group ($n = 10$, $^*P < 0.05$, compared to control group).

hydrogen sensor electrode inserted directly into the tissues. When 2% H_2 gas was inhaled, the arterial H_2 levels started to increase 2 min after inhalation of H_2 gas and reached a maximum level after 5 min [1.82 ± 0.02% ($n = 5$)]. The incremental rate of H_2 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 H_2 saturation 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, H_2 gas levels were increased even in the ischemic myocardium (Fig. 2C). Although the incremental rate of H_2 saturation was slower in the ischemic myocardium than in the non-ischemic myocardium, the peak level of H_2 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 H_2 in the ischemic myocardium immediately increased to the level observed in the non-ischemic myocardium.

Inhalation of H_2 gas protects the heart from ischemia–reperfusion injury

To investigate whether inhalation of H_2 gas protects the heart from ischemia–reperfusion injury, rats were subjected to coronary artery occlusion for 30 min followed by reperfusion for 24 h. H_2 gas was administered at the onset of ischemia and continued for 60 min after reperfusion. H_2 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

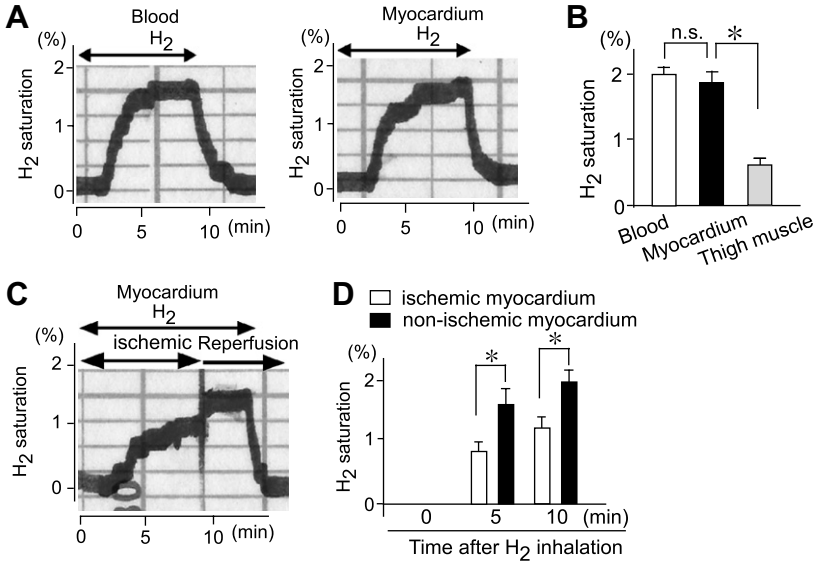

Fig. 2. Inhalation of H_2 gas increases the intramyocardial H_2 gas concentration. H_2 gas at 2% was administered by respiration to intubated rats receiving mechanical ventilation and the concentration of H_2 in tissue was recorded continuously. (A) A needle-type H_2 sensor was inserted in LV cavity (arterial blood) and non-ischemic LV myocardium. (B) Comparison of peak H_2 gas levels between arterial blood, non-ischemic LV myocardium, and thigh muscle ($n = 5$, $^*P < 0.05$, compared to the level of arterial blood). (C) The changes in the concentration of H_2 in 'at risk' area for infarction during ischemia and reperfusion. (D) Comparison of change in the H_2 concentration between non-ischemic and ischemic myocardium after H_2 inhalation ($n = 5$, $^*P < 0.05$, compared to the level of non-ischemic myocardium).

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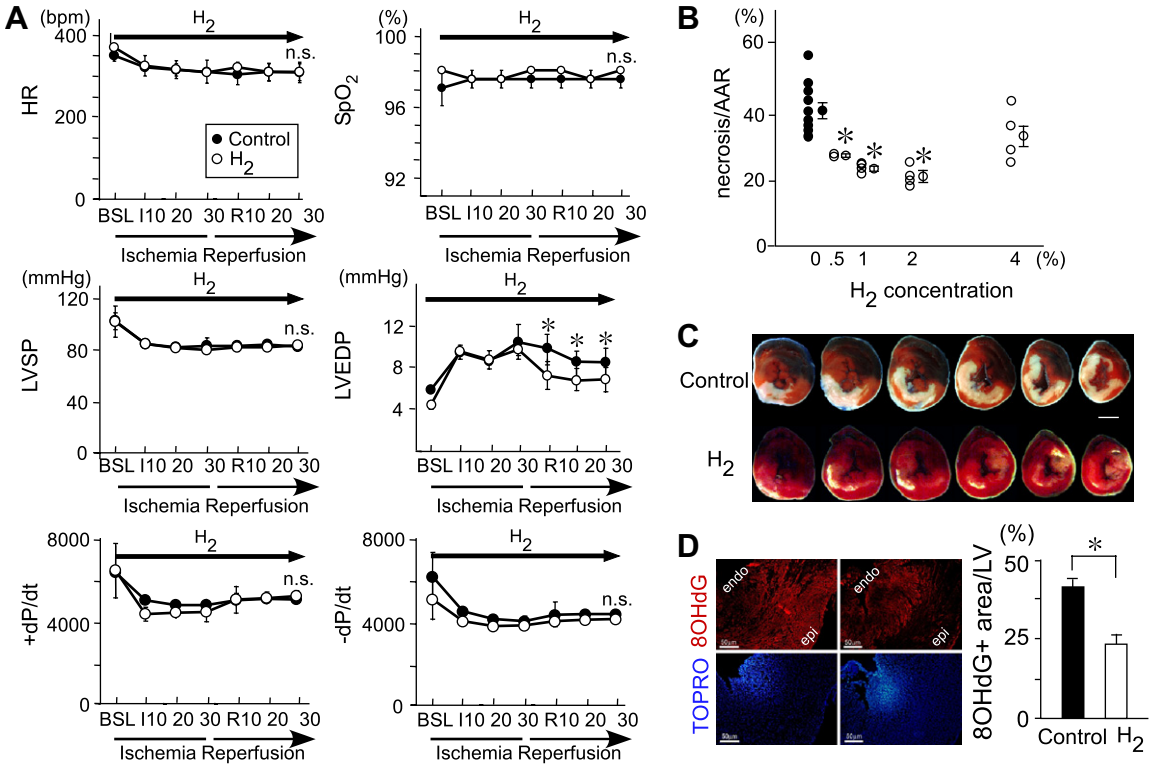

Fig. 3. Inhalation of H_2 reduces infarct size induced by ischemia–reperfusion injury. (A) Changes in heat rate (HR), oxygen saturation by pulse oximetry (SpO_2), and LV systolic pressure (LVSP), LV diastolic pressure (LVEDP) and LV peak positive and negative d*P*/d*t* were monitored during ischemia–reperfusion injury ($n = 5$ in each group). (B) H_2-dependent decrease in infarct size is expressed as the ratio of total infarct area/AAR ($^*P < 0.05$, compared to control group). (C) Representative photographs of serial heart sections obtained from rats subjected to myocardial ischemia–reperfusion injury in the presence or absence of H_2 inhalation. Bar = 2 mm. (D) Immunohistochemical staining with antibodies against 8-OHdG was performed 24 h after ischemia–reperfusion injury. Quantification of 8-OHdG immunoreactive area was expressed as percentage of total LV area at serial short axis sections ($n = 5$, $^*P < 0.05$, H_2 inhalation group compared to control group). endo, endocardium; epi, epicardium.

pressure, LV peak positive and negative LV d*P*/d*t*, between the control group and the 2% H_2 gas inhalation group. Notably, LV-end-diastolic pressure after reperfusion was significantly lower in H_2 gas inhalation group compared to control group ($n = 5$, $^*P < 0.05$).

In the absence of H_2 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% H_2 gas significantly reduced infarct size, with 2% H_2 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 H_2 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% H_2 gas inhalation than that of control group ($n = 5$, $^*P < 0.05$, Fig. 3D).

Inhalation of H_2 gas reduces LV remodeling after ischemia–reperfusion injury

To determine the impact of H_2 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 H_2 gas during myocardial ischemia–reperfusion reduced pathological remodeling after myocardial infarction (Fig. 4).

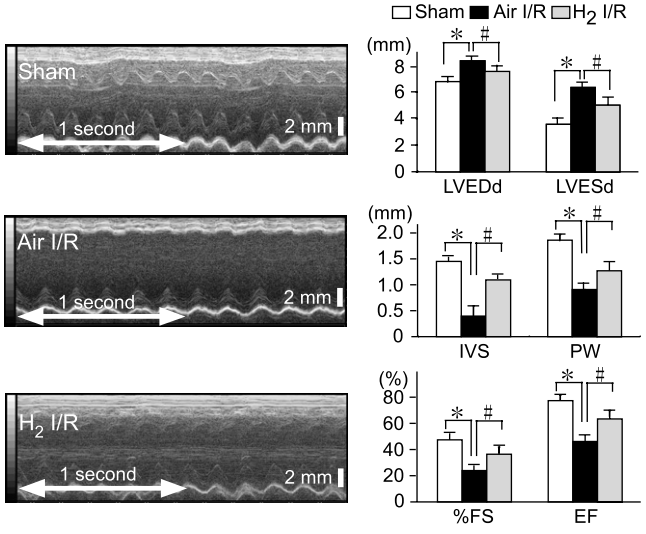

Fig. 4. Inhalation of H_2 gas reduces adverse LV remodeling. Representative M-mode echocardiographic images of sham-operated (sham), ischemia–reperfusion with air inhalation (Air_I/R), and ischemia–reperfusion with H_2 inhalation (H_2_I/R). Measurement of M-mode echocardiographic images in each group. LVEDd, LV endodiastolic diameter (μm); LVESd, LV endosystolic diameter (μm); IVC, intraventricular septum diameter (μm); PW, posterior wall thickness (μm); FS, fractional shortening (%); EF, ejection fraction (%) ($n = 5$, $^*P < 0.05$, compared to sham-operated group; $^\#P < 0.05$, compared to Air_I/R group).

Discussion

This is the first study to demonstrate that inhalation of H_2 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 H_2 gas was also confirmed *ex vivo* Langendorff-perfused hearts subjected to anoxia-reoxygenation injury. The anti-oxidant properties of H_2 were confirmed by the demonstration that (1) H_2 improves the recovery of LV function during reoxygenation after anoxia, one of the oxidative stress model, in isolated perfused hearts; (2) inhalation of H_2 gas ameliorates the level of 8-OHdG immunoreactivity in the 'at risk' area for infarction. The anti-oxidant action of molecular H_2 may be explained, at least partially, by direct ROS scavenging effect. However, it remains unclear if the anti-oxidant action of H_2 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 gasotransmitters, include nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfate (H_2S). 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 H_2S 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 H_2S can be attained locally in target organs without delivering a potentially toxic level of the gasses via the lungs.

H_2 is not produced endogenously in mammalian cells since the hydrogenase activity responsible for the formation of H_2 gas has not been identified [23]. The spontaneous production of H_2 gas in the human body occurs via fermentation of undigested carbohydrates by resident enterobacterial flora. H_2 is transferred to the portal circulation and excreted through the breath in significant amounts. We demonstrated that inhaled H_2 at therapeutic dose has no adverse effects on the saturation level of arterial oxygen (SpO_2) or hemodynamic parameters, including heart rate and LV pressure. H_2 dissolved in the blood is distributed to tissues proportional to regional blood flow, and is rapidly eliminated by the lungs. Accordingly, the H_2 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 H_2 concentration increases immediately following inhalation of H_2, and attaining to almost compatible levels of that observed in arterial blood within 10 min. Of note, the regional H_2 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 H_2 gas by inhalation, in patients with totally coronary artery occlusion, can efficiently increase the regional concentration of H_2 in the 'at risk' area for myocardial infarction before reestablishing coronary blood flow within the occluded infarct-related artery.

We demonstrated that inhalation of H_2 gas is promising strategies to alleviate ischemia–reperfusion injury at the time of recanalization of coronary artery. When translated into the clinical practice, inhalation of H_2 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 H_2-mediated anti-oxidant activity, and the capacity of H_2 to influence cellular metabolism, is required to fully exploit inhalation of H_2 gas as a therapeutic strategy.

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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

[1] A. Volpi, C. De Vita, M.G. Franzosi, E. Geraci, A.P. Maggioni, F. Mauri, E. Negri, E. Santoro, L. Tavazzi, G. Tognoni, Determinants of 6-month mortality in survivors of myocardial infarction after thrombolysis. Results of the GISSI-2 data base. The Ad hoc Working Group of the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI)-2 Data Base, Circulation 88 (1993) 416–429.
[2] E.C. Keeley, J.A. Boura, C.L. Grines, Primary angioplasty versus intravenous thrombolytic therapy for acute myocardial infarction: a quantitative review of 23 randomised trials, Lancet 361 (2003) 13–20.
[3] E. Braunwald, R.A. Kloner, Myocardial reperfusion: a double-edged sword?, J Clin. Invest. 76 (1985) 1713–1719.
[4] D.M. Yellon, D.J. Hausenloy, Myocardial reperfusion injury, N. Engl. J. Med. 357 (2007) 1121–1135.
[5] J.L. Zweier, Measurement of superoxide-derived free radicals in the reperfused heart. Evidence for a free radical mechanism of reperfusion injury, J. Biol. Chem. 263 (1988) 1353–1357.
[6] R. Bolli, B.S. Patel, M.O. Jeroudi, E.K. Lai, P.B. McCay, Demonstration of free radical generation in "stunned" myocardium of intact dogs with the use of the spin trap alpha-phenyl *N-tert*-butyl nitrone, J. Clin. Invest. 82 (1988) 476–485.
[7] T. Vanden Hoek, L.B. Becker, Z.H. Shao, C.Q. Li, P.T. Schumacker, Preconditioning in cardiomyocytes protects by attenuating oxidant stress at reperfusion, Circ. Res. 86 (2000) 541–548.
[8] J.T. Flaherty, B. Pitt, J.W. Gruber, R.R. Heuser, D.A. Rothbaum, L.R. Burwell, B.S. George, D.J. Kereiakes, D. Deitchman, N. Gustafson, et al., Recombinant human superoxide dismutase (h-SOD) fails to improve recovery of ventricular function in patients undergoing coronary angioplasty for acute myocardial infarction, Circulation 89 (1994) 1982–1991.
[9] V.J. Richard, C.E. Murry, R.B. Jennings, K.A. Reimer, Therapy to reduce free radicals during early reperfusion does not limit the size of myocardial infarcts caused by 90 minutes of ischemia in dogs, Circulation 78 (1988) 473–480.
[10] C. Penna, R. Rastaldo, D. Mancardi, S. Raimondo, S. Cappello, D. Gattullo, G. Losano, P. Pagliaro, Post-conditioning induced cardioprotection requires signaling through a redox-sensitive mechanism, mitochondrial ATP-sensitive K^+ channel and protein kinase C activation, Basic Res. Cardiol. 101 (2006) 180–189.
[11] J.M. Downey, M.V. Cohen, A really radical observation—a comment on Penna et al., Basic Res. Cardiol. 101 (2006) 190–191.
[12] I. Ohsawa, M. Ishikawa, K. Takahashi, M. Watanabe, K. Nishimaki, K. Yamagata, K. 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.
[13] K. Fukuda, S. Asoh, M. Ishikawa, Y. Yamamoto, I. Ohsawa, S. Ohta, Inhalation of hydrogen gas suppresses hepatic injury caused by ischemia/reperfusion through reducing oxidative stress, Biochem. Biophys. Res. Commun. 361 (2007) 670–674.
[14] M. Tani, Y. Suganuma, H. Hasegawa, K. Shinmura, Y. Hayashi, X. Guo, Y. Nakamura, Changes in ischemic tolerance and effects of ischemic preconditioning in middle-aged rat hearts, Circulation 95 (1997) 2559–2566.
[15] K. Shinmura, K. Tamaki, R. Bolli, Short-term caloric restriction improves ischemic tolerance independent of opening of ATP-sensitive K^+ channels in both young and aged hearts, J. Mol. Cell. Cardiol. 39 (2005) 285–296.
[16] K. Shinmura, K. Tamaki, K. Saito, Y. Nakano, T. Tobe, R. Bolli, Cardioprotective effects of short-term caloric restriction are mediated by adiponectin via activation of AMP-activated protein kinase, Circulation 116 (2007) 2809–2817.
[17] J.P. Headrick, Aging impairs functional, metabolic and ionic recovery from ischemia–reperfusion and hypoxia-reoxygenation, J. Mol. Cell. Cardiol. 30 (1998) 1415–1430.
[18] J. Endo, M. Sano, J. Fujita, K. Hayashida, S. Yuasa, N. Aoyama, Y. Takehara, O. Kato, S. Makino, S. Ogawa, K. Fukuda, Bone marrow derived cells are involved

in the pathogenesis of cardiac hypertrophy in response to pressure overload, Circulation 116 (2007) 1176–1184.
[19] C. Szabo, Hydrogen sulphide and its therapeutic potential, Nat. Rev. Drug Discov. 6 (2007) 917–935.
[20] J.W. Elrod, J.W. Calvert, J. Morrison, J.E. Doeller, D.W. Kraus, L. Tao, X. Jiao, R. Scalia, L. Kiss, C. Szabo, H. Kimura, C.W. Chow, D.J. Lefer, Hydrogen sulfide attenuates myocardial ischemia–reperfusion injury by preservation of mitochondrial function, Proc. Natl. Acad. Sci. USA 104 (2007) 15560–15565.
[21] R. Foresti, M.G. Bani-Hani, R. Motterlini, Use of carbon monoxide as a therapeutic agent: promises and challenges, Intensive Care Med. (2008).
[22] A. Kobayashi, K. Ishikawa, H. Matsumoto, S. Kimura, Y. Kamiyama, Y. Maruyama, Synergetic antioxidant and vasodilatory action of carbon monoxide in angiotensin II-induced cardiac hypertrophy, Hypertension 50 (2007) 1040–1048.
[23] M.W. Adams, L.E. Mortenson, J.S. Chen, Hydrogenase, Biochim. Biophys. Acta 594 (1980) 105–176.
[24] K. Aukland, B.F. Bower, R.W. Berliner, Measurement of local blood flow with hydrogen gas, Circ. Res. 14 (1964) 164–187.