Original Paper

Hydrogen Gas Attenuates Myocardial Ischemia Reperfusion Injury Independent of Postconditioning in Rats by Attenuating Endoplasmic Reticulum Stress-Induced Autophagy

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Key Words

Ischemic/reperfusion injury • Hydrogen • Ischemic postconditioning • Endoplasmic reticulum stress • Autophagy

Abstract

Background/Aims: To study the effect of inhaling hydrogen gas on myocardial ischemic/reperfusion(I/R) injury in rats. **Methods:** Seventy male Wistar albino rats were divided into five groups at random as the sham group (Sham). The I/R group (I/R), The ischemic postconditioning group (IPo), The I/R plus hydrogen group (IH₂) and the ischemic postconditioning plus hydrogen group (IPoH₂). The Sham group was without coronary occlusion. In I/R group, Ischemic/reperfusion injury was induced by coronary occlusion for 1 hour. Followed by 2 hours of reperfusion. In the IPo and IPoH₂ group, four cycles of 1 min reperfusion/1 min ischemia was given at the end of 1 hour coronary occlusion. While 2% hydrogen was administered by inhalation 5 min before reperfusion till 2 hours after reperfusion in both the IPoH₂ and IH₂ group. The heart and blood samples were harvested at the end of the surgical protocol. Then the myocardium cell endoplasmic reticulum(ER) stress and autophagy was observed by electron microscope. In addition, the cardiac ER stress and autophagy related proteins expression were detected by Western blotting analysis. **Results:** Both inhaling 2% hydrogen and ischemic postconditioning treatment reduced the ischemic size and serum troponin I level in rats with I/R injury, and inhaling hydrogen showed a more curative effect compared with ischemic postconditioning treatment. Meanwhile inhaling hydrogen showed a better protective effect in attenuating tissue reactive oxygen species. Malondialdehyde levels and immunoreactivities against 8-hydroxy-2'-deoxyguanosine and inhibiting cardiac endoplasmic reticulum stress and down-regulating autophagy as compared with ischemic postconditioning treatment. **Conclusion:** These results revealed a better protective effect of hydrogen on myocardial ischemic/reperfusion injury in rats by attenuating endoplasmic reticulum stress and down-regulating autophagy compared with ischemic postconditioning treatment.

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Introduction

Nowadays, acute myocardial infarction (AMI) is still a major cause of heart failure and cardiac death. Early management of AMI focuses on achieving immediate restoration of blood flow of the ischemic risk zone in order to minimise irreversible tissue damage. However, reperfusion, although is necessary to reestablish delivery of oxygen and substrate-rich blood to support cell metabolism and remove potentially damaging by-products of cellular metabolism, can elicit pathogenetic processes that paradoxically exacerbates heart function [1-4]. As reported, reperfusion causes additional damage to the reperfused myocardium, accounting for up to 40% of the final size [5]. What's more, recent studies indicate that post-ischemic 30 day mortality (about 8.5% after angioplasty and about 14% with thrombolysis) remains high [6]. Lines of studies have demonstrated that myocardial ischemic/reperfusion (I/R) might contribute to reperfusion arrhythmia, myocardial diastolic dysfunction, and irreversibly disordered metabolism and function [7].

The mechanisms underlying I/R injury are complex, but the majority of them involves generation of reactive oxygen species (ROS), which is fueled by reintroduction of molecular oxygen when the blood flow is reestablished while finally lead to myocardial cell death [5, 8]. In general, myocyte injury can progress along two distinct cell death pathways: necrosis and apoptosis [9]. Nonetheless, I/R injury which results in a higher proportion of necrotic myocytes will lead to a larger lesion than one in which apoptosis is predominant due to the associated inflammatory response [10]. There is exhaustive data available to support the up-regulation of autophagy following I/R in cardiomyocytes [11, 12]. Given the fact that depletion of high energy phosphate stores, autophagy can be further activated in response to act as a mode of cell death [13]. Thus autophagic cell death is defined as a modality of non-apoptotic or necrotic programmed cell death (PCD) in which autophagy serves as a cell death mechanism [14]. Diversity of researches indicate that autophagy can be activated by Endoplasmic reticulum (ER) stress. As the abundance of misfolded or unfolded proteins, promoted by oxidative stress from I/R injury, exceed the ER capacity, autophagy can be induced as a secondary response to degrade those proteins [15].

Other than ischemic preconditioning, ischemic postconditioning (IPoC) is a clinically practicable cardioprotective strategy against the I/R injury for it is defined as a short series of repetitive cycles of brief reperfusion and re-occlusion applied at the onset of reperfusion after a prolonged ischemic insult [16]. Previous observations showed beneficial effects on both animal and human studies, like limitation of infarct size, preservation of left ventricular function [17, 18], reduction of myocardial edema [19] and prevention of coronary microembolization [20]. These cardioprotective phenotypes from IPoC are attributed to secondary to its effect on various cellular mechanisms: the kinase pathway, survival activating factor enhancement pathway, Janus signal transducer and activator system, reduction in formation of reactive oxygen or nitrogen species, and attenuation of calcium overload [21, 22].

Hydrogen (H_2), considering its small size and electrically neutral properties, has advantageous distribution characteristics for its capability to penetrate biomembranes and diffuse into the organelle and nucleus [23]. In 2007, Ohsawa et al. [24], found that hydrogen act as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals, especially hydroxyl radical ($OH\cdot$), the most cytotoxic reactive oxygen species (ROS). In recent years, hydrogen gas (H_2) or hydrogen-rich saline are widely accepted to exert protective effects in many diseases including myocardial ischemia-reperfusion injury, neuropathic Pain, brain injury via regulating oxidative stress, inflammatory response and apoptosis [25-28]. Except in rat model, the protective effects of inhaling H_2 in myocardial ischemia-reperfusion injury also have been reported in dog model and patients experiencing ST-elevated myocardial infarction [29, 30]. Besides, our previous researches have indicated that hydrogen-rich saline dramatically attenuates cardiac and hepatic injury in doxorubicin rat model by inhibiting inflammation and apoptosis [31].

Based on these previous studies, this study was aimed to investigate the potential protective effects of H_2 and IPo on myocardial I/R injury in rat and whether the beneficial

effects was associated with the attenuation of endoplasmic reticulum stress and down-regulation of autophagy.

Materials and Methods

Animals

Seventy male Wistar albino rats provided by Experimental Animal Center of the First Affiliated Hospital of Harbin Medical University weighting an average of 200 g were used in this study in accordance with the Guidelines of Laboratory Animals of the First Affiliated Hospital of Harbin Medical University's protocol for care and use. They were housed with free access to food and water in a rodent facility under 12h light-dark cycle and the temperature of 20-25°C conditions. All rats were acclimated for seven days prior to any experimental procedures. All experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the First Affiliated Hospital of Harbin Medical University.

Preparation and estimation of hydrogen gas

As a kind of flammable gas, the concentration of 2% H₂ proves safe and therapeutic [32]. Hydrogen, produced from a hydrogen generator (HA300, Dura Safer Technology, Ltd Beijing, China) was premixed with air into an airtight bag of a vacuum till 2% to keep the concentration of hydrogen in the heart above 10ppm/g [33]. This inhaled gas bag was prepared freshly and measured with an H₂ sensor (SDH-B101, Sundo Technology. Co. Ltd, Shenzhen, China), then connected with a polyethylene tube to the mechanical ventilation system including rodent ventilator (R407, RWD Biotech. Co. Ltd, Shenzhen, China), air pump (R510-25, RWD Biotech. Co. Ltd, Shenzhen, China), gas recovery tank (R510-31, RWD Biotech. Co. Ltd, Shenzhen, China), ventilation package (R510-27), and rodent operating table (68620, RWD Biotech. Co. Ltd, Shenzhen, China) (Fig. 1A).

Experimental protocol

Seventy rats were divided into five groups at random as the sham group (Sham, n=14), the ischemic reperfusion group (I/R, n=14), the ischemic postconditioning group (IPo, n=14), the ischemic reperfusion plus hydrogen gas group (IH₂, n=14) and the ischemic postconditioning plus hydrogen gas group (IPoH₂, n=14). All rats were anesthetized by intraperitoneal administration of 1% pentobarbital sodium (40 mg/kg) once and ventilated with air or mixed gas (Fig. 1B). Under anesthesia, the animals were placed in a

Fig. 1. Experimental procedure. Preparation and inhalation of hydrogen gas (Fig. 1A). Myocardial ischemia was induced by transient occlusion of the left anterior descending coronary artery. I/R injury was induced by inflating the balloon occluder for 1 hour, followed by 2 hours of reperfusion. In the IPo and IPoH₂ group, four cycles of 1 min reperfusion/1 min ischemia (total time, 8 min) was given at the end of 1 hour coronary occlusion. While 2% H₂ was administered by inhalation 5 min before reperfusion till 2 hours after reperfusion in both the IPoH₂ and IH₂ group. The heart and blood samples were harvested at the end of the surgical protocol (Fig. 1B).

supine position with surface leads were placed subcutaneously to record the electrocardiogram (ECG). A left lateral thoracotomy was performed on the fourth intercostal space and the pericardium opened to provide exposure of the left anterior descending coronary artery (LAD). A 6-0 nylon suture was placed around the LAD at 2 to 3 mm from the tip of the left auricle, and a nontraumatic balloon occluder was placed over the artery. Coronary occlusion was induced by inflating the balloon occlude and confirmed with an elevated ST-segment change on the ECG. The Sham group underwent the same procedure but the balloon occluder was empty. In I/R group, I/R injury was induced by inflating the balloon occluder for 1 hour, followed by 2 hours of reperfusion. In the IPo and IPoH₂ group, four cycles of 1 min reperfusion/1 min ischemia (total time, 8 min) was given at the end of 1 hour coronary occlusion [19]. While 2% H₂ was administered by inhalation 5 min before reperfusion till 2 hours after reperfusion in both the IPoH₂ and IH₂ group. The heart and blood samples were harvested at the end of the surgical protocol.

Serum cardiac troponin I (TnI) level

Blood samples of all survived rats from aorta were collected into heparin-containing tubes, centrifuged at 3000 g for 15 min at 4°C, and measured within 2 hours. The serum concentrations of TnI, a kind of cellular component released from ruptured cardiomyocytes, were detected using ELISA kits in accordance with the manufacturers' instructions (Nanjing Jiancheng BioEngineering Institute, Nanjing, China).

ROS and malondialdehyde (MDA) levels of tissue

After euthanasia, cardiac tissues of rats were respectively washed in icy phosphate buffer saline (PBS). ROS was quantified by ELISA kits (Lanpai Biotech. Co. Ltd, Shanghai, China). MDA concentration, a presumptive marker of oxidant-mediated lipid peroxidation, was measured using another commercial kit (KeyGEN Biotech. Co. Ltd, Nanjing, China).

Histological study

After blood sample collecting, the rats were sacrificed and their hearts were rapidly excised for histopathological and biochemical analyses. Tissues were fixed with 10% buffered formalin, embedded in paraffin, sectioned into 2-μm-thick sections and stained with Heidenhain staining kits (DH0013, leagene Biotech. Co. Ltd, Beijing, China) to show early myocardial ischemic damage with black staining [34, 35]. 4 random fields at 100× magnification in each specimen were observed and photographed with a light microscope (DP73, Olympus Co, Japan) by 2 pathologists of blinded method.

Immunohistochemical staining.

Immunohistochemical staining of the heart tissues for 8-hydroxy-2-deoxyguanosine (8-OHdG) staining in the heart at 2 hours after reperfusion were carried out. The heart tissues were sectioned, and immersion fixed in 10% buffered formalin. The paraffin was cut into 4 μm thick serial sections, then deparaffinized in xylene, rehydrated using various grades of ethanol. Heat antigen retrieval was achieved at 100°C for 10 minutes with citrate buffer (PH 6.0) in microwave. Endogenous peroxidase activity blocked in 3% peroxide (15 minutes), and washed well in PBS. Then, the sections were incubated with primary goat polyclonal anti-8-OHdG antibody (1:200 dilution, no.ab10802, Abcam) overnight at 4°C, and secondary rabbit anti-goat IgG-HRP (PV9003, ZSGB-BIO). The immunoreaction was visualized by the 3, 3'-diaminobenzidine (DAB) reaction for 8-OHdG (with brown staining). Finally, slides were examined by a light microscope (DP73, Olympus Co, Japan), and quantitative statistical analysis was performed with the KS400 Image Analysis System (DP73, Olympus Co, Japan).

Transmission electron microscopy (TEM)

Fresh LV free wall myocardium samples (about 1 mm³) were fixed in 2.5% glutaraldehyde at 4°C overnight. Ultrathin sections were cut from the fixed blocks, processed according to standard procedures [36], and finally examined using an electron microscope (JEM-1200, JEOL Ltd, Tokyo, Japan).

Western blotting analysis

Western blotting was performed according to the commercial instruction. Total proteins were extracted from the tissues with the lysis buffer containing protease and phosphatase inhibitors for protein immunoblotting. Protein concentrations were measured by BCA protein assay kit with bovine serum

albumin (BSA) as standard (Beyotime, China). Protein samples were separated in each well of 12.5% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to Polyvinylidene fluoride (PVDF) membranes (Roche Applied Science). The blots were blocked with 5% BSA (Roche Applied Science) for 1 h at room temperature, then probed with primary antibody including glucose-regulated protein 78 (GRP78) (1:1000 dilution, no.11587-1-AP, Proteintech), tumor-necrosis factor- α (TNF- α) receptor-associated factor 2 (TRAF2) (1:1000 dilution, no.#4712, CST), Bcl-2 (1:1000 dilution, no.ab59348, Abcam), phosphorylated Bcl-2 (p-Bcl-2) (1:1000 dilution, no.ab138406, Abcam), Bcl-1 (1:1000 dilution, no.11306-1-AP, Proteintech), light chain 3-phosphatidyl ethanolamine (LC3)A/B (1:1000 dilution, no.#12741, CST), GAPDH (1:1000 dilution, no.TA-08, ZSGB). They were incubated at 4°C overnight. The membranes were washed with TBS-T and then incubated with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution, ZB-2301, ZB-2305, ZSGB) for 1 hour at room temperature. Finally, the bands were collected by Imaging System (Bio-Rad, Hercules, CA, USA). GAPDH was used as the control for equal loading of the protein.

Data processing & statistical analysis

Quantitative data were expressed as mean $\pm$ standard deviation (SD). An analysis of variance (one-way ANOVA) was with Tukey's post hoc test was used for multiple-group comparisons for all groups. A two-way ANOVA with replication was also used for comparison among the I/R, IPo, IH₂, and IPoH₂ groups to determine the two-factor interaction between the effects of postconditioning and H₂. Statistical significance was considered at a p value of < 0.05 . Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, USA).

Results

Effect of hydrogen on myocardial I/R injury

Representative Heidenhain stainings are shown in Figures 2A–E. Obvious myocardial ischemic injury was found in the I/R group from the light micrographs when compared with the Sham group ($P < 0.05$; Fig. 2F), while they were significantly ameliorated in the IPo, IH₂ and IPoH₂ group ($P < 0.05$; Fig. 2F). In addition, the ischemic size in the IH₂ and IPoH₂ groups were obviously decreased as compared with the IPo group ($P < 0.05$; Fig. 2F), while that in the IPoH₂ group also showed a salient decline when compared with the IH₂ group ($P < 0.05$; Fig. 2F). According to these results, we further investigated the protective effect of H₂ on serum TnI level, and the results showed that serum TnI level of I/R group was significantly increased as compared with Sham group ($P < 0.05$; Fig. 2G). Ischemic postconditioning treatment, inhaling H₂, or ischemic postconditioning plus inhaling H₂ significantly decreased the serum TnI level ($P < 0.05$; Fig. 2G). Furthermore, the serum TnI level in the IH₂ and IPoH₂ groups were significantly lower than that in IPo group ($P < 0.05$; Fig. 2G), whereas the rats with ischemic postconditioning plus inhaling H₂ treatment did not show a significant decline in the serum TnI level ($P < 0.05$; Fig. 2G). These data suggests that both ischemic postconditioning treatment and inhaling hydrogen gas could effectively relieve myocardial I/R injury. Besides, breathing H₂ shows better protective effect than ischemic postconditioning treatment in this study. In addition, considering the proven antioxidation of hydrogen, we further investigated the changes of ROS and MDA levels in the cardiac tissue.

The antioxidation effect of hydrogen on myocardial I/R injury

8-OHdG staining results are shown in Figures 3A–E, the positive staining for 8-OHdG, calculated as a percentage of the total number of nuclei, is shown in figure 3F. And compared with the Sham group, 8-OHdG positive staining was significantly increased in the I/R group. The number of positively stained nuclei was dramatically decreased in IPo, IH₂ and IPoH₂ group, but there was no significant difference in the 8-OHdG positively stained nuclei between the IPoH₂ and IH₂ group ($P > 0.05$; Fig. 3F). ROS and MDA levels in cardiac tissue were measured, shown that both ROS and MDA levels were significantly increased in the I/R group when compared with the Sham group ($P < 0.05$; Fig. 3G, 3H). In addition, they were significantly reduced in the IPo, IH₂ and IPoH₂ group ($P < 0.05$; Fig. 3A, B). What's more,

Fig. 2. Effect of hydrogen on myocardial I/R injury. Myocardial ischemic damage with black staining were processed for Heidenhain staining (100x magnification; Fig. 2A-2E). Besides, the ischemic size of each group was calculated in Fig. 2F ($n \geq 3$), cardiac TnI levels of all survived rats were shown in Fig. 2G ($n = 70$). Data are shown by mean $\pm$ SD. * $P < 0.05$ vs. Sham group. # $P < 0.05$ vs. I/R group. £ $P < 0.05$ vs. IPo group.

Fig. 3. The antioxidation effect of hydrogen gas on myocardial I/R injury. Immunohistochemical staining with antibodies against 8-OHdG in transverse sections of each groups were shown in Fig. 3A-3E (200x magnification), and the percentage of 8-OHdG positive nucleus of each group was calculated as shown in Fig. 3F ($n \geq 3$). The ROS (Fig. 3G, $n = 70$) and MDA (Fig. 3H, $n = 70$) levels of cardiac tissues in each group were detected. Data are shown by mean $\pm$ SD. * $P < 0.05$ vs. Sham group. # $P < 0.05$ vs. I/R group. £ $P < 0.05$ vs. IPo group. \$ $P < 0.05$ vs. IH₂ group.

compared with IPo group, the cardiac ROS and MDA levels in the IH₂ and IPoH₂ groups were obviously decreased ($P < 0.05$; Fig. 3G, 3H). The cardiac ROS levels in the IPoH₂ group was

Fig. 4. Inhaling hydrogen gas attenuates myocardial I/R injury by alleviating ER stress. The electron microscopy results of each group was shown in Figs. 4A-4E, I/R injury caused cardiomyocyte dilation of ER (Fig. 4B), but it was suppressed in the IPo, IH₂ and IPoH₂ groups (Fig. 4C-4E). Scale bar, 2 μm. Representative expression of GRP78 (Fig. 4F, 4G) and TRAF2 (Fig. 4F, 4H) of cardiac tissue in each group was detected. Data are shown by mean ± SD, n ≥ 3. *P < 0.05 vs. Sham group. #P < 0.05 vs. I/R group. £P < 0.05 vs. IPo group.

lower than that in IH₂ group ($P < 0.05$; Fig. 3G), whereas the MDA level in the IPoH₂ group did not show a salient decline as compared with the IH₂ group ($P > 0.05$; Fig. 3H). These findings indicate that ischemic postconditioning and hydrogen gas may act as antioxidant to decrease the cardiac ROS and MDA levels. And according to these results, hydrogen has a better antioxidation than ischemic postconditioning. Because oxidative stress injury could induce ER stress and autophagy, we further investigated the changes of endoplasmic reticulum stress and autophagy relative protein levels by western blotting.

Inhaling hydrogen gas attenuates myocardial I/R injury by attenuating ER stress

Compared with the Sham group, electron microscopy revealed obvious dilation of ER in I/R group rats (Fig. 4B). Whereas the dilation of ER induced by I/R injury was ameliorated in the IPo, IH₂ and IPoH₂ groups in varying degree. (Fig. 4C-4E). Besides, the results of expressions of GRP 78 and TRAF2 proteins are presented in Figures 4F-4H. The expressions of GRP 78 and TRAF2 in cardiac tissue were markedly increased in I/R group as compared with that in the Sham group. However, the elevation in the I/R group was obviously reduced in the IPo, IH₂ and IPoH₂ groups ($P < 0.05$; Fig. 4). Besides, the GRP 78 and TRAF2 expressions in the IH₂ and IPoH₂ groups were significantly lower than that in IPo group ($P < 0.05$; Fig. 4), whereas these proteins expression in the IPoH₂ group did not show a remarkable decline as compared with that in the IH₂ group ($P > 0.05$; Fig. 4). These results demonstrate that the reduction of I/R injury by breathing hydrogen gas is associated with the attenuation of ER stress.

Fig. 5. Inhaling hydrogen gas attenuates myocardial I/R injury by activating JNK pathway. Representative expression of p-Bcl-2/Bcl-2 (Fig. 5A, 5B) of cardiac tissue in each group was detected. Data are shown by mean $\pm$ SD, $n \geq 3$. * $P < 0.05$ vs. Sham group. # $P < 0.05$ vs. I/R group. £ $P < 0.05$ vs. IPo group.

Fig. 6. Inhaling hydrogen gas attenuates myocardial I/R injury by down-regulating of autophagy. The electron microscopy results of each group was shown in Figs 6A-6E, I/R injury caused increased autophagosome (Fig. 6B), but the autophagosome was reduced in the IPo, IH₂ and IPoH₂ groups (Fig. 6C-6E). Scale bar, 2 μ m. Representative expression of Bcl-1 (Fig. 8A, 8B) and LC3II/I (Fig. 8A, 8C) of cardiac tissue in each group was detected. Data are shown by mean $\pm$ SD, $n \geq 3$. * $P < 0.05$ vs. Sham group. # $P < 0.05$ vs. I/R group. £ $P < 0.05$ vs. IPo group.

Inhaling hydrogen gas attenuates myocardial I/R injury by activating JNK pathway

The results of expressions of Bcl-2 and p-Bcl-2 proteins are presented in Figures 5A-5C. The ratio of p-Bcl-2/Bcl-2 in cardiac tissue was significantly increased in I/R group as compared with that in the Sham group. While the elevation in the I/R group was significantly decreased in the IPo, IH₂ and IPoH₂ groups ($P < 0.05$; Fig. 5B). However, there was no significant difference of the ratio of p-Bcl-2/Bcl-2 among the IPo, IH₂ and IPoH₂

group ($P > 0.05$; Fig. 5B). These results demonstrate that hydrogen gas attenuates I/R injury by activating JNK pathway.

Inhaling hydrogen gas attenuates myocardial I/R injury by down-regulating autophagy

Compared with the Sham group, electron microscopy revealed increased autophagosome in I/R group rats (Fig. 6B). Whereas the autophagosome induced by I/R injury was reduced in the IPo, IH₂ and IPoH₂ groups in varying degree. (Fig. 6C-6E). The results of expressions of Bcln1 and LC3II/I proteins are presented in Figures 6F-6H. The expressions of Bcln-1 and LC3II/I in cardiac tissue were markedly increased in I/R group as compared with that in the Sham group. While the elevation in the I/R group was significantly decreased in the IPo, IH₂ and IPoH₂ groups ($P < 0.05$; Fig. 6). Besides, the Bcln-1 and LC3II/I expressions in the IH₂ and IPoH₂ groups were obviously lower than that in IPo group, whereas these proteins expression in the IPoH₂ group did not show a remarkable decline as compared with that in the IH₂ group ($P > 0.05$; Fig. 6). These results demonstrate that the reduction of I/R injury by breathing hydrogen gas is associated with the attenuation of autophagy.

Discussion

Clinically identified features of I/R injury may be reversible like arrhythmias or myocardial stunning, or irreversible like myocardial infarction or microvascular obstruction [37]. Lines of researches have shown that reperfusion salvaged large numbers of damaged myocytes, reperfusion itself also injures myocytes, so be called stunning [38]. Also it has shown that stunning results from O₂-derived free radicals impacting some portion of the contractile apparatus [39]. Considering the antioxidation effect of hydrogen, we investigated the antioxidation effects of H₂ on I/R in rats, and the changes of cardiac ROS, MDA and 8-OHdG levels indicated that inhaling H₂ attenuated I/R injury by inhibiting oxidative stress. MDA, as a presumptive marker of oxidant-mediated lipid peroxidation, its increase suggested the structure of cytomembrane or organelle membrane was damaged. Further more, we discovered that inhaling H₂ was significantly better than ischemic postconditioning on I/R injury.

As reported, endogenous ROS production, acts as stress signal and alters ER homeostasis making it dysfunctional [40]. In response to this signal, ER elicits a protective or adaptive response called unfolded-protein response (UPR) with an aim to restore ER homeostasis. GRP78, as a main UPR-upregulated protein, is an important chaperone which is typically increased when ER stress is encountered. In fact, upregulation of GRP78 can serve as an indicator of ER stress [41]. ER stress-induced activation of IRE1 α kinase activity recruits adaptor protein TRAF2, which further recruits c-Jun N-terminal kinase (JNK) activating several transcription factors [42]. Therefore, we further investigated the effects of H₂ on ER stress in rats with I/R injury, and the changes of expression of GRP78 and TRAF2 suggested the protective effects of H₂ and IPo on myocardial I/R injury in rat were linked to the attenuation of endoplasmic reticulum stress. Besides, inhaling H₂ had better effect on ER stress injury in rats compared with ischemic postconditioning.

Autophagy is activated during myocardial ischemia/reperfusion and several specific proteins are involved in this process, like Beclin1 and LC3 [43]. Beclin1 can interact with the anti-apoptotic protein Bcl-2 at the ER, with Bcl-2 inhibiting starvation-induced autophagy [44]. Also, it can be activated through IRE1-JNK pathway [45]. Activation of IRE1 stimulates disruption of Beclin-1 inhibitory complexes through phosphorylation of Bcl-2, thereby triggering autophagy [46]. And the results in this study revealed both inhaling H₂ and ischemic postconditioning could reduce the expressions of Bcln-1 and -Bcl-2/ Bcl-2 induced by I/R injury, besides, inhaling H₂ had a better effect in reducing the expression of these proteins when compared with ischemic postconditioning. The "nucleation" complex involving Beclin1 is recruited to form the phagophore. Subsequent vesicle elongation allows engulfment and sequestration of organelles or bulk cytosolic material. The conjugation of phosphatidylethanolamine

Fig. 7. Potential cardioprotective mechanisms of H₂ and IPO on I/R. Hydrogen gas and postconditioning attenuates myocardial ischemia reperfusion injury by inhibiting endoplasmic reticulum stress-induced autophagy.

(PE) to the microtubule-associated protein LC3, known as LC3-II, decorates mature autophagosomes, finally fuse with the lysosome for degradation of the sequestered cargo [47, 48]. We further investigated the expressions of LC3II/I in each group, the results showed both inhaling H₂ and ischemic postconditioning could reduce the expressions of LC3II/I induced by I/R injury, besides, inhaling H₂ had a better effect in reducing the expression of this protein as compared with ischemic postconditioning. In addition, ischemic size and TnI level caused by I/R injury also decreased by inhaling H₂ and ischemic postconditioning.

Autophagy is often described as “double-edged sword” for its contradictory role in different researches. While in our study it might look more like an urgent but faulty contingency plan as the cardiomyocyte has to take action when received death threats from I/R injury. Thus the reduction of autophagy is a result of reduced demand of processing the abnormal proteins or organelles.

Conclusion

Our present study investigates the alleviation of I/R injury with inhalation of hydrogen gas, independent of postconditioning, and demonstrates that it could reduce induction of autophagy by ER stress as our hypothesis (Fig. 7). Due to its safety, efficacy and convenience, inhalation of hydrogen gas may be considered as a potential therapy for I/R injury.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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