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Hydrogen gas protects against serum and glucose deprivation-induced myocardial injury in H9c2 cells through activation of the NF-E2-related factor 2/heme oxygenase 1 signaling pathway

QIANG XIE[1], XUE-XIANG LI[2], PENG ZHANG[1], JIN-CAO LI[1], YING CHENG[1], YAN-LING FENG[1], BING-SHENG HUANG[1], YU-FENG ZHUO[1] and GUO-HUA XU[1]

[1]Department of Cardiology, Hexian Memorial Hospital, Panyu, Guangzhou, Guangdong 511400; [2]Department of Cardiology, Laizhou People's Hospital, Laizhou, Shandong 261400, P.R. China

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Abstract. Ischemia or hypoxia-induced myocardial injury is closely associated with oxidative stress. Scavenging free radicals and/or enhancing endogenous antioxidative defense systems may be beneficial for the impediment of myocardial ischemic injury. Hydrogen (H_2) gas, as a water- and lipid-soluble small molecule, is not only able to selectively eliminate hydroxyl (·OH) free radicals, but also to enhance endogenous antioxidative defense systems in rat lungs and arabidopsis plants. However, thus far, it has remained elusive whether H_2 gas protects cardiomyocytes through enhancement of endogenous antioxidative defense systems. In the present study, the cardioprotective effect of H_2 gas against ischemic or hypoxic injury was investigated, along with the underlying molecular mechanisms. H9c2 cardiomyoblasts (H9c2 cells) were treated *in vitro* with a chemical hypoxia inducer, cobalt chloride ($CoCl_2$), to imitate hypoxia, or by serum and glucose deprivation (SGD) to imitate ischemia. Cell viability and intracellular ·OH free radicals were assessed. The role of an endogenous antioxidative defense system, the NF-E2-related factor 2 (Nrf2)/heme oxygenase 1 (HO-1) signaling pathway, was evaluated. The findings revealed that treatment with $CoCl_2$ or SGD markedly reduced cell viability in H9c2 cells. H_2 gas-rich medium protected against cell injury induced by SGD, but not that induced by $CoCl_2$. When the cells were exposed to SGD, levels of intracellular ·OH free radicals were markedly increased; this was mitigated by H_2 gas-rich medium. Exposure of the cells to SGD also resulted in significant increases in HO-1 expression and nuclear Nrf2 levels, and the HO-1 inhibitor ZnPP IX and the Nrf2 inhibitor brusatol aggravated SGD-induced cellular injury. H_2 gas-rich medium enhanced SGD-induced upregulation of HO-1 and Nrf2, and the HO-1 or Nrf2 inhibition partially suppressed H_2 gas-induced cardioprotection. Furthermore, following genetic silencing of Nrf2 by RNA interference, the effects of H_2 gas on the induction of HO-1 and cardioprotection were markedly reduced. In conclusion, H_2 gas protected cardiomyocytes from ischemia-induced myocardial injury through elimination of ·OH free radicals and also through activation of the Nrf2/HO-1 signaling pathway.

Correspondence to: Professor Guo-Hua Xu, Department of Cardiology, Hexian Memorial Hospital, 2 Eastern Qinghe Road, Panyu, Guangzhou, Guangdong 511400, P.R. China
E-mail: ghxu2008@163.com

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Introduction

Myocardial ischemic injury is a common pathological process in patients suffering from cardiac conditions, including atherosclerotic coronary artery disease, acute myocardial infarction and cardiac transplantation (1). However, the treatment costs for these diseases are high; therefore, it is important to investigate low-cost therapies that impede ischemia-induced myocardial injury.

The mechanisms underlying ischemia-induced cell damage are complicated and remain elusive. Increasing evidence suggested that oxidative stress is important in myocardial ischemic injury (2). Oxidative stress is characterized by marked increases in the production of reactive oxygen species (ROS), including the superoxide anion ($O_2^{\bullet-}$) and the hydroxyl radical (·OH), as well as non-radical molecules, including hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (3,4). Environmental stress factors, including ultraviolet rays, heat exposure, as well as ischemia and/or hypoxia, are major causes of oxidative stress, which may damage proteins, lipids and DNA, and eventually result in cellular death or the development of cancer (5,6). Under normal conditions, intracellular ROS levels are controlled by balancing ROS generation with ROS elimination. Once the balance is disrupted, for instance, through increased ROS generation and/or reduced elimination, ROS may aggregate and oxidative stress arises. Eliminating excessive ROS and enhancing endogenous antioxidation ability

have been applied clinically (7), and in myocardial ischemic injury, oxidant scavengers, antioxidant extracts, vitamin E and vitamin C have all demonstrated to have a potential therapeutic value (8). However, water-soluble vitamin C has a low transmembrane diffusion ability and it is difficult to accumulate vitamin C up to an effective level to eliminate ROS (9). Conversely, the lipid-soluble vitamin E is difficult to dissolve in the cytoplasm in order to neutralize ROS (10). These shortcomings have limited the wide clinical use of the two vitamins. Therefore, a small antioxidant with water- and lipid-solubility is expected to have greater application value.

Hydrogen (H_2) gas is an inexpensive medical gas generated by electrolysis of water. Similar to other gaseous molecules, including nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H_2S), H_2 gas has been demonstrated to exhibit numerous important cytoprotective effects in the nervous, cardiovascular and digestive systems (11-14). Unlike vitamin C and vitamin E, H_2 gas dissolves in water and lipids. In addition, the simple molecular structure and small molecular weight render H_2 gas a good antioxidant candidate in cells. A number of studies have suggested that H_2 gas may selectively scavenge ·OH free radicals (15,16). Therefore, H_2 gas may have broad clinical applications in the future. However, in cardiomyocytes, it has not been reported whether H_2 gas protects against ischemia-induced injury *in vitro*, which was the focus of the present study.

Abundant evidence indicated that enhancement of endogenous antioxidation activity exerts cardioprotective effects. For instance, upregulation of superoxide dismutase (SOD) or heme oxygenase-1 (HO-1) protects cardiomyocytes against ischemia and/or reperfusion-induced damage (17-19). Notably, in addition to scavenging ·OH free radicals, H_2 gas protection has been associated with induction of HO-1 controlled by NF-E2-related factor 2 (Nrf2) in rat lung transplant-induced injury and paraquat-induced oxidative damage in plants (20,21). H_2 gas protection of cardiomyocytes may therefore be associated with scavenging ·OH free radicals and upregulation of the Nrf2/HO-1 signaling pathway.

H9c2 cardiomyoblasts (H9c2 cells), originating from rat heart ventricular tissue, have widely served as an *in vitro* model for cardiac muscle in virtue of their morphological features and biochemical properties (22). In the present study, H9c2 cells were subjected to serum and glucose deprivation (SGD) or exposed to hypoxia provoked by a chemical hypoxia-mimicking agent, cobalt chloride ($CoCl_2$), to establish an ischemia/hypoxia-induced myocardial injury model. H_2 gas-rich medium was applied to investigate the cytoprotection, antioxidation and activation of the Nrf2/HO-1 signaling pathway as well as the involvement of scavenging ·OH free radicals and upregulation of the Nrf2/HO-1 signaling pathway in the cardioprotection by H_2 gas.

Materials and methods

Materials. $CoCl_2$ and protoporphyrin IX zinc (II) (ZnPP IX) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Brusatol (BR), an inhibitor of Nrf2, was provided by BOC Sciences (Shirley, NY, USA). A cell counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kyushu, Japan). Specific monoclonal primary antibodies against rat HO-1 and Nrf2 proteins were obtained from EPITOMICS of Abcam Company (Burlingame, CA, USA). High glucose and glucose-free Dulbecco's modified Eagle's medium and fetal bovine serum (FBS) were supplied by Gibco-BRL (Grand Island, NY, USA). A bicinchoninic acid (BCA) protein assay kit was purchased from Kangchen Bio-tech Inc. (Shanghai, China). An enhanced chemiluminescence (ECL) kit was obtained from Applygen Technologies Inc. (Beijing, China). An enzyme-linked immunosorbent assay (ELISA) kit for detection of 8-hydroxy-2'-deoxyguanosine (8-OHdG) was provided by Abnova Corporation (Taipei, Taiwan).

Cell culture. H9c2 cells were supplied by the Cell Bank at the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in high glucose DMEM supplemented with 15% FBS at 37˚C in an atmosphere of 5% CO_2 and 95% air. The cells were passaged approximately every two days after digestion with 0.2% trypsin.

Hypoxia or ischemia treatment. $CoCl_2$, a chemical hypoxia inducer, was co-incubated with H9c2 cells to induce hypoxia. An ischemia-induced myocardial injury model generated through SGD was prepared *in vitro* in the cell medium. The cell viability was used to indicate the extent of hypoxic or ischemic injury in the H9c2 cells.

Preparation of H_2 gas-rich medium. Pure H_2 gas (99.999% purity) was produced via electrolysis of water with a M177021 H_2 gas generator, supplied by Beijing Midwest Yuanda Technology Co., Ltd. (Beijing, China; 23). H_2 gas-rich medium was prepared freshly prior to saturating the medium with the generated H_2 gas for at least 30 min.

Determination of cell viability. Cell viability was analyzed by a CCK-8 assay following the manufacturer's instructions. H9c2 cells were plated in 96-well plates at a density of 5,000 cells/well. When the cells were grown to ~70% confluence, the indicated treatments were administered. At the end of the treatment, the CCK-8 solution (10 μl) at 1:10 dilution with FBS-free DMEM high glucose medium (100 μl) was added to each well followed by a further 3 h incubation at 37˚C. Absorbance (A) was measured at 450 nm with a microplate reader produced by Molecular Devices, LLC (Sunnyvale, CA, USA). The mean A was used to calculate the percentage of cell viability according to the following equation: Percentage of viable cells = (A treatment group - A Blank group)/(A Control group-A Blank group) x 100%. Experiments were performed six times (24).

Western blot analysis of protein expression. H9c2 cells were plated in 60-mm diameter petri dishes. Following the indicated treatments, the cells were harvested and total proteins or nuclear proteins were extracted and quantified with the BCA kit and used to measure HO-1 or Nrf2 expression levels, respectively. Subsequent to denaturation by heating at 100˚C for 5 min, equal quantities of proteins of the indicated groups were loaded and separated by 12% SDS-PAGE. The proteins in the gel were then transferred to a polyvinylidene fluoride membrane. Following blocking with 5% fat-free milk in Tris-buffered saline with Tween 20 (TBS-T), the membranes were incubated with rat monoclonal primary antibodies against HO-1 or Nrf2 overnight with gentle agitation at 4˚C. β-actin and Lamin B served as

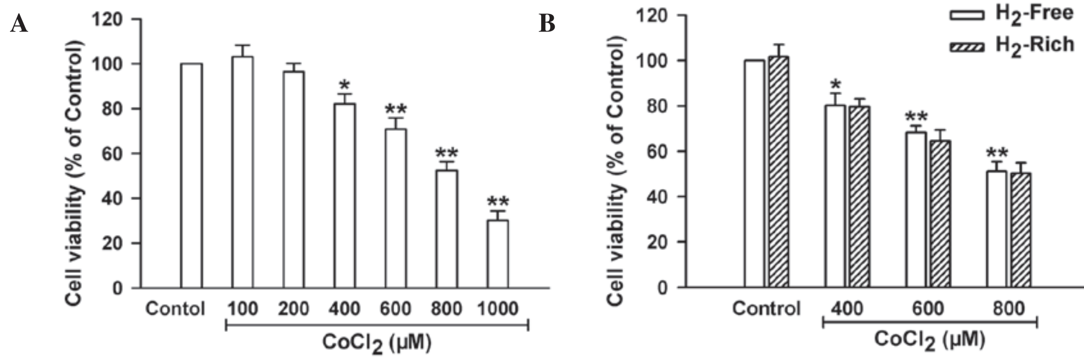

Figure 1. Effects of H_2 gas treatment on $CoCl_2$-induced injury in H9c2 cells. Following the indicated treatments, cell viability was measured by a cell counting kit-8 assay. (A) H9c2 cells were treated with increasing concentrations of $CoCl_2$ at 100-1,000 μM for 24 h. (B) H9c2 cells were treated with $CoCl_2$ at 400-800 μM for 24 h in the absence (H_2-Free) or presence (H_2-Rich) of H_2 gas application. Data are presented as mean ± standard deviation, n=6. *P<0.05, **P<0.01, compared with the control group.

loading controls. Subsequent to three washes with TBS-T, the membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. The membranes were washed again and developed with an ECL system. The membranes were then exposed to X-ray films (Kodac Company, Beijing, China). The integrated optical density of the protein bands was calculated by Image J 1.47 Software (National Institutes of Health, Bethesda, MD, USA).

Competitive ELISA for measurement of 8-OHdG. Intracellular ·OH free radicals cause oxidative damage to DNA to form 8-OHdG. Therefore, by measuring 8-OHdG levels in the cells, ·OH free radical content may be analyzed indirectly. H9c2 cells were plated in six-well plates and treated as indicated. At the end of the treatment, the cells were harvested and lysed. 8-OHdG levels in the lysate were determined according to the manufacturer's instructions (Abnova Corporation). The experiment was performed at least six times with similar outcomes.

Gene knockdown. Small interfering RNA (Si-RNA) against rat Nrf2 mRNA (GenBank accession no. AF037350; https://www.ncbi.nlm.nih.gov/genbank/) was synthesized by GenePharma Co., Ltd (Shanghai, China). The Si-RNA of Nrf2 (Si-Nrf2) and random non-coding RNA (Si-NC) were transfected into H9c2 cells using Lipofectamine 2000, according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). Si-Nrf2 and Si-NC (20 nmol/l) were incubated with the cells for 6 h followed by further incubation for 24 h in order to transfect the cells.

Statistical analysis. All data were analyzed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) and the results are expressed as the mean ± standard deviation. The significance of intergroup differences was evaluated by one-way analyses of variance. Differences were considered to be significant if the two-sided probability (P) was <0.05.

Results

H_2 gas-rich medium does not affect chemical hypoxia-induced myocardial injury. Hypoxemia can be imitated *in vitro* by chemical hypoxia or SGD treatment. H_2 gas protection in chemical hypoxia-induced myocardial injury was firstly assessed by treatment of H9c2 cells with $CoCl_2$ in H_2 gas-rich medium. As shown in Fig. 1A, treatment of H9c2 cells with increasing concentrations of $CoCl_2$ significantly reduced cell viability at concentrations of 400-1,000 μM. In order to observe H_2 gas effectiveness, the cell culture medium was saturated with H_2 gas generated by electrolysis of water. No difference in cell viability between the cells cultivated in H_2 gas-rich medium and those cultivated in H_2 gas-free medium was identified under the conditions of $CoCl_2$ exposure or rest (Fig. 1B). This result indicated that H_2 gas treatment did not influence chemical hypoxia-induced myocardial injury.

H_2 gas-rich medium alleviates SGD-induced cell injury. SGD treatment acts as another *in vitro* hypoxemia model by withdrawing FBS and glucose to mimic acute infarction-induced myocardial injury *in vivo*. When the cells were exposed to SGD for different time periods followed by further culture for 30 h, the cell viability of H9c2 cells was significantly reduced (P<0.01; Fig. 2A). Following exposure to SGD for 6-18 h, the H9c2 cells were cultured in H_2 gas-rich medium for 30 h. The findings demonstrated that, compared with H_2 gas-free culture, H_2 gas-rich culture significantly increased the viability of the cells subjected to SGD for 6 or 12 h (P<0.05), but not 18 h. The results suggested that when the cell injury induced by SGD was not severe, H_2 gas was able to promote cell survival.

H_2 gas-rich medium prevents SGD-induced ·OH free radical generation. One mechanism underlying H_2 protection is ROS scavenging, particularly of ·OH free radicals. To investigate whether H_2 gas-mediated myocardial protection was associated with scavenging ·OH free radicals, the ·OH levels in H9c2 cells were measured using ELISA. As shown in Fig. 3, exposure of the cells to SGD significantly enhanced intracellular ·OH levels (P<0.01). However, this effectiveness was significantly inhibited by incubation in H_2 gas-rich medium (P<0.01). This indicated that the elimination of ·OH free radicals may be an important mechanism of myocardial protection by H_2 gas.

Adaptive HO-1 induction contributes to H_2 gas inhibition of SGD-induced injury in H9c2 cells. HO-1 is endogenously

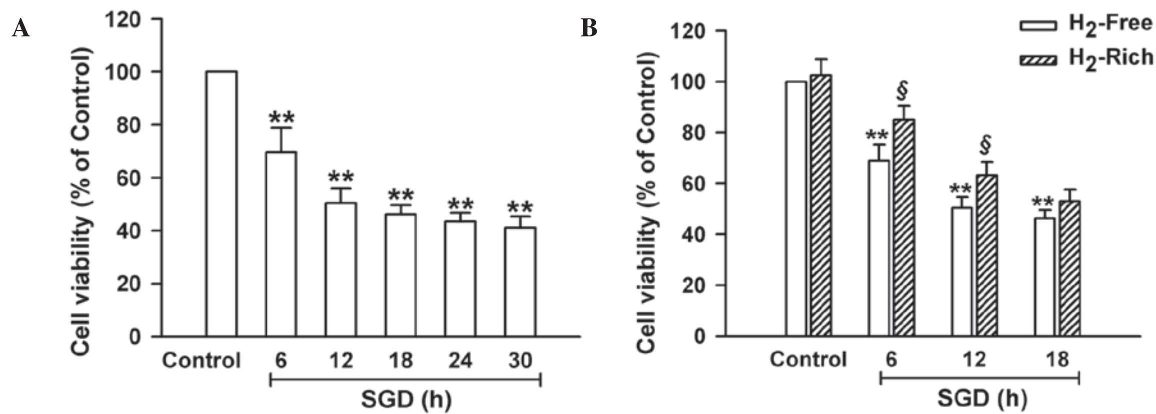

Figure 2. Effects of H_2 gas on SGD-induced injury in H9c2 cells. Following the indicated treatments, cell viability was measured with a cell counting kit-8 assay. (A) H9c2 cells were exposed to SGD between 6 and 30 h, followed by a further 30 h in normal culture. (B) H9c2 cells were exposed to SGD for 6, 12 or 18 h, respectively, in the absence (H_2-Free) or presence (H_2-Rich) of H_2 gas treatment. Data are presented as the mean ± standard deviation, n=6. *P<0.05, **P<0.01, compared with the control group. §P<0.05, compared with the SGD group. SGD, serum and glucose deprivation.

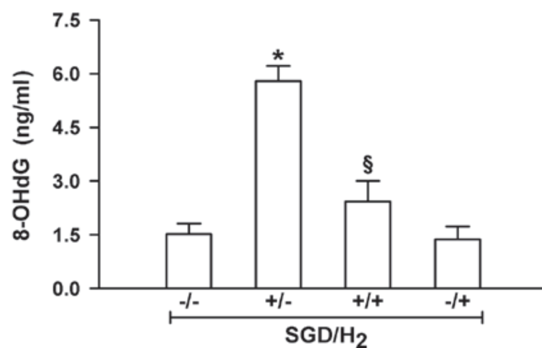

Figure 3. Effects of H_2 gas on SGD-induced hydroxyl free radical generation in H9c2 cells. The cells were exposed to SGD for 6 h in the absence or presence of H_2 gas application. Intracellular ·OH free radical content was indirectly measured by ELISA of 8-OHdG levels. Data are presented as the mean ± standard deviation, n=6. *P<0.01, compared with the control group. §P<0.01, compared with the SGD group. SGD, serum and glucose deprivation; 8-OHdG, 8-hydroxy-2'-deoxyguanosine.

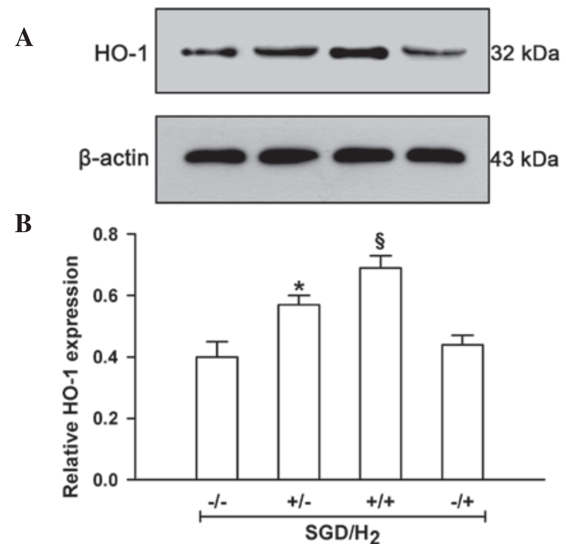

Figure 4. Levels of HO-1 under different conditions. H9c2 cells were exposed to SGD for 6 h followed by a further 30 h culture in H_2 gas-free/-rich medium. At the end of the treatments, HO-1 expression in the cells was detected by (A) Western blot assay and (B) quantitative analysis with ImageJ 1.47 software. Data are presented as the mean ± standard deviation, n=6. *P<0.01 compared with the control group. §P<0.05 compared with the SGD group. SGD, serum and glucose deprivation; HO-1, heme oxygenase 1.

produced, functioning as an antioxidation enzyme. In order to examine the role of HO-1 in SGD-induced myocardial injury, experiments to detect HO-1 levels were performed. The data in Fig. 4 show that exposure of H9c2 cells to SGD significantly upregulated HO-1 levels when compared with the cells under normal conditions (P<0.01). When the HO-1 inhibitor ZnPP IX was administered, SGD-induced cellular injury was found to be significantly aggravated (P<0.05; Fig. 5). These results suggested that HO-1 was beneficial in SGD-induced injury in H9c2 cells.

Upregulation of HO-1 is involved in H_2 gas-induced myocardial protection. H_2 gas-induced protection is not only associated with simple elimination of ·OH free radicals, but also with induction of endogenous genes, for instance, HO-1 (20,21). To clarify whether HO-1 is involved in H_2 gas-induced myocardial protection, experiments were conducted to observe the effect of H_2 gas on SGD-triggered HO-1 upregulation and the effect of HO-1 inhibition on H_2 gas-induced protection of H9c2 cells. The data in Fig. 4 reveal that exogenously applied H_2 gas significantly enhanced the upregulation elicited by SGD in H9c2 cells (P<0.05). Notably, inhibition of HO-1 with 10 μM ZnPP IX significantly reduced the H_2 gas-induced increase in cellular viability following SGD treatment (P<0.05; Fig. 6). The results suggested that HO-1 at least partially mediated the protection from ischemia provided by H_2 gas in H9c2 cells.

H_2 gas facilitates nuclear Nrf2 expression induced by SGD in H9c2 cells. Nrf2 is a transcription factor responsible for HO-1 expression. To address the role of Nrf2 in H_2 gas-induced myocardial protection, the effect of H_2 gas-rich medium on the changes of Nrf2 levels induced by SGD was investigated. As shown in Fig. 7, the SGD challenge resulted in a significant increase in nuclear Nrf2 expression levels, indicating its activation under SGD conditions in H9c2 cells (P<0.05). Notably, H_2 gas application following SGD exposure induced a significant increase in nuclear Nrf2 expression levels (P<0.05).

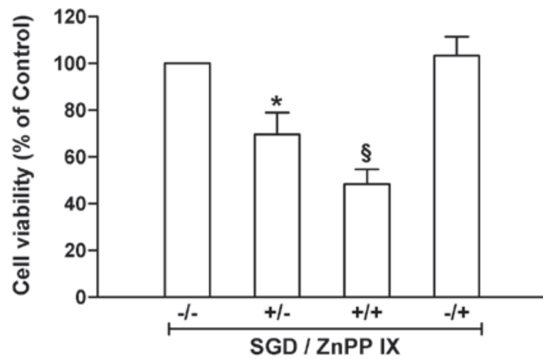

Figure 5. Effects of ZnPP IX on SGD-induced injury in H9c2 cells. The cells were exposed to SGD for 6 h followed by further culture with or without an additional 10 μM ZnPP IX treatment for 30 h. Following treatment, the cell viability was measured by the cell counting kit-8 assay. Data are presented as the mean ± standard deviation, n=6. *P<0.01 compared with the control group. §P<0.05 compared with the SGD group. SGD, serum and glucose deprivation; ZnPP IX, protoporphyrin IX zinc (II).

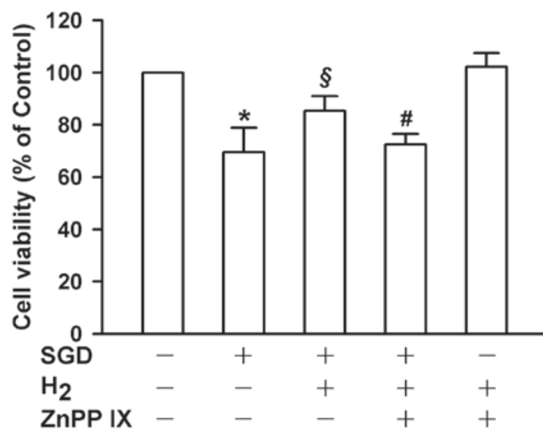

Figure 6. Effect of the indicated treatments on myocardial viability. H9c2 cells were exposed to SGD for 6 h followed by a further culture combined with H_2 gas with or without 10 μM ZnPP IX for 30 h. Following treatment, the cell viability was measured by the cell counting kit-8 assay. Data are presented as the mean ± standard deviation, n=6. *P<0.01 compared with the control group. §P<0.05 compared with the SGD group. #P<0.05 compared with SGD combined with the H_2 gas group. SGD, serum and glucose deprivation; ZnPP IX, protoporphyrin IX zinc (II).

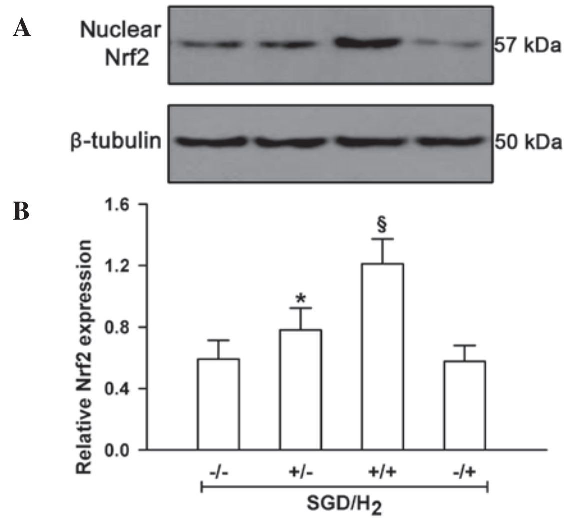

Figure 7. Effects of SGD and H_2 gas on expression of nuclear Nrf2 in H9c2 cells. The cells were exposed to SGD for 6 h followed by a further 30 h culture in H_2 gas-free/-rich medium. Following treatment, nuclear proteins were extracted and Nrf2 expression levels were detected by (A) western blot assay and (B) quantitative analysis with ImageJ 1.47 software. Data are presented as the mean ± standard deviation, n=6. *P<0.05 compared with the control group. §P<0.05 compared with the SGD group. SGD, serum and glucose deprivation; Nrf2, NF-E2-related factor 2.

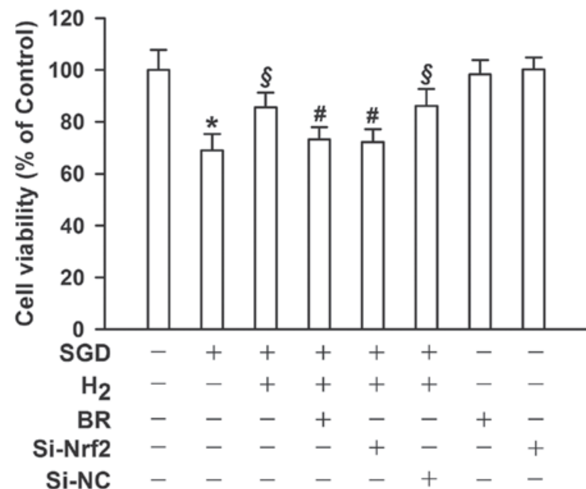

Figure 8. Effect of the different treatments on myocardial viability. Normal H9c2 cells were exposed to SGD for 6 h followed by a further 30 h culture combined with H_2 gas with or without 10 μM BR. Nrf2-deficient H9c2 cells caused by RNA interference assay were exposed to SGD for 6 h followed by a further 30 h culture combined with H_2 gas. Following treatment, the cell viability was measured by a cell counting kit-8 assay. Data are presented as the mean ± standard deviation, n=6. *P<0.01 compared with the control group. §P<0.05 compared with the SGD group. #P<0.05 compared with SGD combined with the H_2 gas group. SGD, serum and glucose deprivation; NC, negative control; BR, brusatol; Nrf2, NF-E2-related factor 2; Si, small interfering RNA.

Inhibition of Nrf2 with 10 μM BR significantly reduced myocardial protection by H_2 gas (P<0.05, Fig. 8).

In addition, genetic silencing of Nrf2 by RNAi (Si-Nrf2) also significantly inhibited H_2 gas-elicited HO-1 induction (P<0.05, Fig. 9) and myocardial protective action (P<0.05, Fig. 8). These results indicated that the H_2 gas protection from SGD-induced injury in H9c2 cells was at least in part mediated through the Nrf2/HO-1 signaling pathway.

Discussion

The results of the present study suggested that H_2 gas exhibited myocardial protection against ischemia-induced injury in H9c2 cells *in vitro* through elimination of ·OH free radicals and activation of the Nrf2/HO-1 signaling pathway. These findings provide further evidence of H_2 gas protection and deepen the understanding of the molecular mechanisms involved.

H_2 gas is a gas with novel medical application, in addition to NO, CO and H_2S, whose cytoprotective effects have gradually gained attention (15). The cytoprotective effect of H_2 gas has been investigated in the nervous, cardiovascular and digestive systems (11-14). In the present study, myocardial protection by H_2 gas was investigated in two distinct models: Chemical hypoxia-induced injury and SGD-induced injury in H9c2 cells. Treatment with chemical hypoxia-mimicking

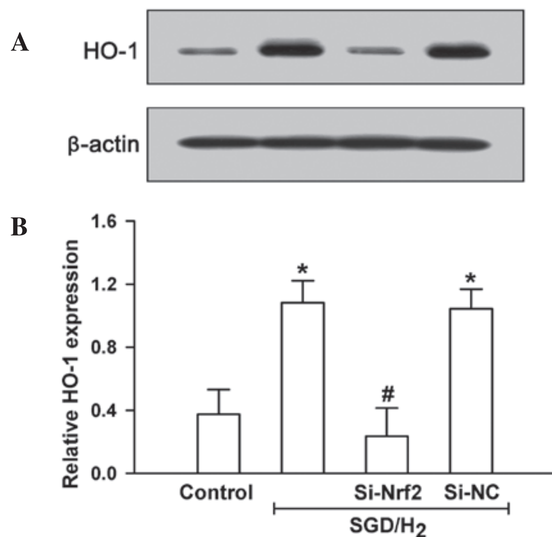

Figure 9. Effect of H_2 gas on the induction of HO-1 in Nrf2-deficient H9c2 cells. Following RNA interference with Si-Nrf2, which downregulated Nrf2 expression, the cells were exposed to SGD for 6 h followed by a further 30 h culture in the absence or presence of H_2 gas. Following treatment, HO-1 expression was observed by (A) western blot assay and (B) quantitative analysis with ImageJ 1.47 software. Data are presented as the mean ± standard deviation, n=6. *P<0.01 compared with the control group. #P<0.05 compared with SGD combined with H_2 gas group. SGD, serum and glucose deprivation; Nrf2, NF-E2-related factor 2; Si, small interfering RNA; HO-1, heme oxygenase 1.

agent $CoCl_2$, at 400-1,000 μM for 24 h, reduced cell viability in a concentration-dependent manner, although no effect of H_2 gas on $CoCl_2$-induced injury was observed. The effect of H_2 gas on SGD-induced injury was then analyzed. Notably, H_2 gas exerted marked myocardial protection, since its application impeded SGD-induced injury in H9c2 cells. These findings were in accordance with a study by Sun *et al* (29) on cardiac ischemia/reperfusion injury in a rat model. However, the findings of the present study also indicated that although $CoCl_2$ (25,26) and SGD (27) are frequently used in hypoxia/ischemia *in vitro* models, they may possess markedly different underlying injury mechanisms. In addition, as H_2 gas is a water- and lipid-soluble and simple molecule, H_2 gas may exhibit a greater clinical antioxidative value than the water-soluble vitamin C and lipid-soluble vitamin E.

Oxidative stress is critical in myocardial ischemic damage through overproduction of ROS (2). ROS in mammals include $O_2^{\bullet -}$, H_2O_2 and ·OH. $O_2^{\bullet -}$, H_2O_2 ROS are eliminated by corresponding enzymes. For example, $O_2^{\bullet -}$ may be catalyzed into O_2 and H_2O_2 via dismutation (28), and H_2O_2, one of the most powerful oxidizers, may be converted into H_2O by catalase or guaiacol peroxidase, but may also be converted into ·OH. Although ·OH free radicals are toxic to cells, enzymes responsible for ·OH elimination remain to be identified. Therefore, it is important for antioxidants to eliminate ·OH free radicals and/or inhibit the production of ·OH. In the present study, exposure of H9c2 cells to SGD was found to significantly increase intracellular ·OH free radical levels as identified through assessment of 8-OHdG. The ·OH levels after SGD stimulation were significantly reduced during cell cultivation in H_2 gas-rich medium, in concurrence with previous reports (15,16,29).

Increasing evidence suggested that the cytoprotective effect of H_2 gas is not only associated with simple elimination of ·OH free radicals, but also with numerous signaling molecules (20,21). A study on plants demonstrated that pretreatment with H_2 gas enhanced the salt tolerance of arabidopsis through zinc-finger transcription factor ZAT10/12 (30). Additionally, the HO-1 signaling pathway has been observed to be involved in H_2 gas protection against paraquat-induced oxidative injury (21). In animals, Cai *et al* (31) revealed that H_2 gas treatment alleviated tumor necrosis factor-alpha-induced rat osteoblast inflammatory injury via upregulation of SOD activity. Furthermore, evidence has indicated that HO-1 induction mediated H_2 gas mitigation of rat lung injury resulting from transplantation (20). In the present study, SGD exposure was found to markedly increase HO-1 expression. The mechanism of HO-1 in SGD-induced myocardial insult was further elucidated using a selective HO-1 inhibitor, ZnPP IX. Since the data indicated that the addition of ZnPP IX markedly aggravated the SGD-induced insult, HO-1 may exhibit a protective action against ischemia, a hypothesis which is supported by the results of a study by Hwa *et al* (32). Notably, exogenously applied H_2 gas was found to result in a further increase in HO-1 expression; application of ZnPP IX partially abolished this H_2 gas-triggered myocardial protection. Therefore, H_2 gas protection may be partially mediated by HO-1 induction. One study suggested that inhalation of H_2 gas combined with CO, a product of HO-1, enhanced its therapeutic efficacy in ischemia/reperfusion-induced myocardial injury (33). These findings, alongside those of the present study, support the hypothesis that the mechanisms underlying H_2 gas-induced myocardial protection are not limited to the elimination of the ·OH free radical and may also include upregulation of protective genes.

Nrf2 belongs to the NF-E2 superfamily of nuclear basic leucine zipper transcription factors. Under conditions of oxidative stress or pharmacological stimuli, Nrf2, as an adaptive response, regulates phase II gene expression of numerous enzymes that serve to detoxify pro-oxidative stressors (34). In the promoter region of certain genes, such as HO-1 and SOD, Nrf2 binds to the cis-acting regulatory element or enhancer sequence and induces gene expression (35). In the present study, SGD exposure markedly induced nuclear Nrf2 expression, which was further enhanced by H_2 gas administration. When the action of Nrf2 was inhibited by BR, H_2 gas-induced myocardial protection was significantly attenuated. Studies have indicated that BR may inhibit the Nrf2 signaling pathway (36,37). In addition, genetic silencing of Nrf2 may also impede H_2-induced HO-1 expression and increases in cell viability. These data suggested that the activation of Nrf2 is involved in H_2 gas protection and HO-1 induction. One study observed that another medical gas, H_2S, protected cardiomyocytes against ischemia-induced injury by activation of the Nrf2 signaling pathway (38). Nrf2-mediated myocardial protection has also been reported regarding a number of other compounds (39,40). For instance, in the nervous system, curcumin protected rat brains against focal ischemia via upregulation of the Nrf2/HO-1 signaling pathway (41). Therefore, the upregulation of the Nrf2/HO-1 signaling pathway is considered to be a general antioxidative mechanism of numerous drugs and compounds, and this signaling pathway may represent a novel molecular target of drug design.

In conclusion, the present study suggested that H_2 gas application not only directly scavenged ·OH free radicals but also enhanced the expression of proteins of the Nrf2/HO-1 signaling pathway in SGD-stimulated H9c2 cells. These findings provided basic information for the development of novel treatments of ischemic myocardial injury with H_2 gas.

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