Hydrogen inhibits isoproterenol-induced autophagy in cardiomyocytes *in vitro* and *in vivo*

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**Abstract.** A previous study from our group has demonstrated that hydrogen administration can attenuate cardiovascular hypertrophy *in vivo* by targeting reactive oxygen species-dependent mitogen-activated protein kinase signaling. The aim of the present study is to determine the effect of hydrogen on cardiomyocyte autophagy during β-adrenoceptor activation *in vivo* and *in vitro*. We prepared hydrogen-rich medium, and the concentration of hydrogen was measured by using the MB-Pt reagent method. For the *in vitro* study, H9c2 cardiomyocytes were stimulated with isoproterenol (ISO; 10 µM) for 5, 15 and 30 min, and then the protein expression levels of the autophagy marker microtubule-associated protein 1 light chain 3β II (LC3B II) were examined by western blotting. The effect of hydrogen-rich medium was then tested by pretreating the H9c2 cardiomyocytes with hydrogen-rich medium for 30 min, then stimulating with ISO, and examining the protein expression levels of the autophagy marker LC3B II. For the *in vivo* study, mice received hydrogen (1 ml/100 g/day, by intraperitoneal injection) for 7 days prior to ISO administration (0.5 mg/100 g/day, by subcutaneous injection), and subsequently received hydrogen with or without ISO for another 7 days. Hypertrophic responses were examined by heart weight (HW) and heart weight/body weight (HW/BW) measurements. The protein expression of autophagy markers Beclin1, autophagy-related protein 7 (Atg7) and LC3B II were examined. The results demonstrated that excessive autophagy occurred following 5 min of ISO stimulation *in vitro*. This enhanced autophagy was blocked by pretreatment with hydrogen-rich medium. Furthermore, hydrogen improved the deteriorated hypertrophic responses and inhibited the enhanced autophagic activity mediated by ISO administration *in vivo*, as indicated by decreasing HW and HW/BW, and suppressing the protein expression levels of Beclin1, Atg7 and LC3B II. Therefore, the results of the present study demonstrated that hydrogen inhibited ISO-induced excessive autophagy in cardiomyocyte hypertrophy models *in vitro* and *in vivo*.

**Introduction**

Hydrogen has potential protective effects on cardiac remodeling. Hydrogen inhalation, commenced at the start of hyperoxic cardiopulmonary resuscitation (CPR), significantly improves brain and cardiac function in a rat model of cardiac arrest (CA) (1). Inhalation of hydrogen attenuates left ventricular remodeling induced by intermittent hypoxia (2,3), ischemia-reperfusion injury (4), and germinal matrix hemorrhage in neonatal rats (5). The ischemia-reperfusion injury of the heart in rats can also be attenuated by hydrogen-rich saline (6,7). Chronic hydrogen-rich saline treatment attenuates left ventricular hypertrophy in spontaneous hypertensive rats (8). Previous studies from our group also revealed that intraperitoneal injection of hydrogen prevents ISO-induced cardiac hypertrophy and dysfunction in mice (9), and suppresses abdominal aortic coarctation-induced vascular hypertrophy in rats (10). However, the molecular mechanisms by which hydrogen has a blocking effect on cardiac hypertrophy induced by β-adrenoceptor stimuli remain poorly understood.

Abnormal autophagic responses have been revealed as critical contributors of cardiac hypertrophy under various cardiovascular stresses (11-14). Autophagy is a mechanism whereby cytoplasmic components are sequestered in a double-membraned vesicle (autophagosome) towards delivery to the lysosome for breakdown in response to nutrient limitation, cellular stress, reactive oxygen species (ROS), or accumulation of protein aggregates or damaged organelles (15-18). The cellular events during autophagy follow distinct stages: Vesicle
nucleation (formation of the isolation membrane/phagophore), vesicle elongation and completion (growth and closure), fusion of the double-membraned autophagosome with the lysosome to form an autolysosome, and lysis of the autophagosome inner membrane and breakdown of its contents inside the autolysosome (19). These stages can be identified by a set of autophagy markers, including autophagy-related protein (Atg)1, Beclin1, Atg7 and microtubule-associated protein 1 light chain 3β II (LC3B II). A window of optimal autophagic activity appears to be critical to the maintenance of cardiovascular homeostasis and function; excessive or insufficient levels of autophagic flux can each contribute to heart disease pathogenesis (20). Whether hydrogen can influence cardiac autophagy during β-adrenoceptor stimulation remains unclear. The aim of the present study was, therefore, to determine the effects of hydrogen on autophagy in isoproterenol (ISO)-mediated cardiomyocyte hypertrophy in vivo and in vitro models.

Materials and methods

Drugs and antibodies. ISO (Cat no. 15627; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was dissolved using high pressure deionized water for cell culture study or dissolved in normal saline (5 mg/10 ml), under sterile conditions immediately prior subcutaneous injection, for the animal model study. Hydrogen (99.999%; Guang Zhou Guang Qi Gas Co., Ltd., Guangzhou, China) was stored in the seamless steel gas cylinder, and it was injected into an aseptic soft plastic infusion bag until the bag was full of gas with no dead volume. The bag with MB-Pt reagent (generously provided by Ming Yan, Shanghai Nanobubble Technology Co., Ltd., Shanghai, China.) with hydrogen-rich water, as previously described (10).

Preparation of hydrogen-rich medium and measurement of hydrogen concentration. Hydrogen-rich medium was prepared as previously described (9,10). Briefly, 20 ml Dulbecco’s Modified Eagle Medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 1% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin/streptomycin was injected into a vacuumed aseptic soft plastic infusion bag (100 ml; CR Double-Crane Pharmaceuticals Co., Ltd., Anhui, China). Then, 99.999% hydrogen from the seamless steel gas cylinder was bubbled into the aseptic soft plastic infusion bag until the bag was full of gas with no dead volume. The bag with hydrogen and medium was maintained at 4°C for >6 h prior to use. Hydrogen concentration was measured by the reaction of MB-Pt reagent (generously provided by Ming Yan, Shanghai Nanobubble Technology Co., Ltd., Shanghai, China.) with hydrogen-rich water, as previously described (10).

Cell culture and treatment. H9c2 rat cardiomyoblasts (a cardiomyoblast cell line derived from embryonic rat heart tissue, generously provided by Dr Runmin Guo, Guangdong Medical University, Zhanjiang, China) were grown in DMEM containing 5.5 mM glucose supplemented with 10% FBS and 100 U/ml penicillin/streptomycin under a humidified atmosphere of 95% air and 5% CO2 at 37°C. ISO powder was dissolved as 10 mM stock solution using high pressure deionized water 30 min prior to use. To investigate the effect of ISO on autophagy, cells were starved for 18 h in DMEM/1% FBS and then treated with 10 µM ISO in DMEM/1% FBS for the indicated time (21). The 10 µM ISO concentration was selected as it is routinely used for inducing cardiomyocyte hypertrophy (21). Cell Counting Kit-8 (Cat no. CK04; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to analyze the possible cytotoxicity of hydrogen-rich medium in cardiomyocytes as the product description. In order to investigate the effect of hydrogen on autophagy in response to ISO, the medium was replaced with hydrogen-rich medium for 30 min. Subsequently, the ISO (10 µM) treatment was continued in hydrogen-rich medium for the indicated time (21).

Animal model of cardiac hypertrophy and treatment protocol. C57BL/6J mice (aged 8-10 weeks; male; n=24; weight, 23.25±1.36 g) were obtained from the Laboratory Animal Center of Sun Yat-sen University (Guangzhou, China). The animals were housed with 12-h light/dark cycles and allowed access to food and water ad libitum. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee (Zhongshan School of Medicine, Sun Yat-sen University), and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication no. 85-23, revised 1996).

Cardiac hypertrophy was induced by subcutaneous injection of ISO (0.5 mg/100 g/day) for 7 days as previously described (9,22). Mice were randomly assigned to four groups: Control group (Con, n=6), ISO group (n=6), ISO plus hydrogen group (ISO+H2, n=6), and hydrogen group (H2, n=6). Mice in the ISO+H2 group received hydrogen (1 ml/100 g/day, intraperitoneal injection) for 7 days prior to ISO administration (0.5 mg/100 g/day, subcutaneous injection), and then received ISO with hydrogen for another 7 days (9,10,23). Mice in the ISO or H2 groups received only ISO or hydrogen administration alone, respectively. Control mice were untreated. Mice were sacrificed on the day 8 of ISO administration. Following sacrifice, hearts were excised, rinsed with ice-cold PBS, and blotted dry. Hearts were weighed, and the heart weight/body weight (HW/BW) ratios were calculated and expressed as mg HW per g BW. Then hearts were snap frozen in liquid nitrogen within min and stored at -80°C until further analysis.

Western blotting. Total protein was extracted from left ventricles and H9c2 cardiomyocytes, as previously described (9). The protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Protein samples (30 µg) were separated by SDS-PAGE (15% gel for LC3B and 10% gel for other proteins), and transferred
to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were incubated with Beclin1, Atg7, LC3B and β-actin primary antibodies (all, 1:2,000 incubated at 4˚C overnight) and their corresponding secondary antibodies [goat anti-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated for Beclin1, Atg7 and LC3B; and horse anti-mouse IgG HRP-conjugated for β-actin; all, 1:2,000; incubated at room temperature for 60 min] by standard techniques. Signals were detected using enhanced chemiluminescence (ChemiDoc XRS+ System; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein expression was quantified using the Image Lab 3.0.1 (Bate 1) (Bio-Rad Laboratories, Inc.).

Statistical analysis. Data are expressed as mean ± standard deviation. Statistical comparisons were performed with SPSS 20.0 software (IBM Corp., Armonk, NY, USA). Differences among groups were tested by one-way analysis of variance followed by Bonferroni’s method for post hoc analysis. Comparisons between two groups were performed by unpaired Student’s t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Hydrogen concentration analysis. DMEM is a complex mixture that contains reducing reagents, thus, it is not suitable for determining the hydrogen concentration by the MB-Pt reagent method directly. For this reason, hydrogen-rich water was prepared with the identical technique as the hydrogen-rich medium, and using the same volume of purified water instead of DMEM. The hydrogen concentration in the water was then determined in order to indirectly measure its concentration in hydrogen-rich medium. MB-Pt reagent was added into hydrogen-rich water until the solution was permanently stained blue (Fig. 1) (24). Using this method, the hydrogen concentration in normal purified water was determined to be <0.1 ppm, while in hydrogen-rich water it was ~0.6-0.9 ppm. These findings suggest that the medium infused by hydrogen in the present study was hydrogen-rich medium.

ISO stimulation induces cardiomyocyte excessive autophagy in vitro. To investigate the effects of ISO on autophagy in cardiomyocytes in vitro, 10 µM ISO was used to stimulate H9c2 cardiomyocytes for different durations (5, 15 and 30 min). The
autophagic marker LC3B was used to evaluate cardiomyocyte autophagy. The ratio of the protein expression levels of LC3B II/I was significantly increased following 5 min of ISO stimulation, and this increase was reduced back to basal levels at 30 min (Fig. 2). These results indicated that ISO induced an acute increased cardiomyocyte autophagy response.

Hydrogen-rich medium pretreatment inhibits the ISO-induced excessive autophagy in vitro. As discussed in our previous study, hydrogen-rich medium incubation can effectively inhibit cardiomyocyte hypertrophy in vitro (9). Therefore, in the present study, we investigated whether hydrogen may have an effect in regulating autophagy as a potential mechanism to inhibit cardiomyocyte hypertrophy. Firstly, the possible cytotoxicity of hydrogen-rich medium in cardiomyocytes was assessed by Cell Counting Kit-8 assay. Hydrogen-rich medium treatment for 48 h was not cytotoxic in cardiomyocytes (data not shown). When examining the protein expression levels of LC3B by western blotting, the results revealed that the ratio of LC3B II/I was significantly increased following 5 min of ISO stimulation in H9c2 cardiomyocytes (Fig. 3). The ISO-mediated increased autophagic activity, however, was significantly attenuated by pretreatment with hydrogen-rich medium for 30 min (Fig. 3). These data indicated that pretreatment with hydrogen-rich medium blocked the ISO-induced excessive autophagy in cardiomyocytes in vitro.

Hydrogen administration inhibits the ISO-induced excessive autophagy in a cardiac hypertrophy model in vivo. To further confirm the in vitro results, an ISO-induced cardiac hypertrophy in vivo model was generated in mice. Then, the effect of intraperitoneal administration of hydrogen on cardiomyocyte autophagy activity was evaluated. The increased heart weight

Table I. Heart weight and body weight measurements in the experimental mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>ISO</th>
<th>ISO+H₂</th>
<th>H₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>HW (mg)</td>
<td>0.12±0.01</td>
<td>0.15±0.01  a</td>
<td>0.13±0.01  b</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>BW (g)</td>
<td>25.17±1.77</td>
<td>25.33±1.51  a</td>
<td>25.67±1.97  b</td>
<td>24.92±1.24</td>
</tr>
<tr>
<td>HW/BW</td>
<td>4.78±0.21</td>
<td>5.74±0.13  a</td>
<td>5.16±0.15  b</td>
<td>4.73±0.14</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation. aP<0.05 vs. Control; bP<0.05 vs. ISO. ISO, isoproterenol; H₂, hydrogen; HW, heart weight; BW, body weight.
and HW/BW ratio that were observed in the ISO-treated mice indicated that the cardiac hypertrophy model was successfully established in vivo (Table I). Similar to the results from our previous study (9), intraperitoneal administration of hydrogen effectively inhibited the ISO-mediated cardiac hypertrophic responses (Table I). Western blot analysis revealed that ISO administration significantly induced the autophagy response in the left ventricles of the mice, as indicated by increased protein expression levels of the autophagic markers Beclin1, Atg7 and LC3B II in the ISO group compared with the control group (Fig. 4). Intraperitoneal administration of hydrogen significantly reversed the ISO-induced excessive autophagy in the heart, as measured by reduced autophagy marker expression in the ISO+H2 group compared with the ISO group (Fig. 4). Of note, hydrogen administration alone had no effect on autophagy activity in the heart. The present findings revealed that hydrogen administration inhibited the ISO-induced excessive autophagy in a cardiac hypertrophy model in vivo.

Discussion

The present study demonstrated that ISO induced an excessive and acute autophagy response in H9c2 cardiomyocytes in vitro and that hydrogen-rich medium pretreatment suppressed this ISO-induced excessive autophagy. In addition, using a mouse model of cardiac hypertrophy, the present study demonstrated that intraperitoneal administration of hydrogen significantly blocked the β-adrenoceptor agonist-mediated excessive autophagy in vivo.

Autophagy is an intracellular process that mediates protein degradation, organelle turnover, and recycling of cytoplasmic components in response to cellular stress or nutrient starvation (20,25). The process commences with formation of the autophagosome, a double-membrane structure of reticular origin that sequesters cytoplasmic components and ultimately fuses with a lysosome, where engulfed cargo is degraded by lysosome-derived acid hydrolases (20). Whereas basal levels of autophagy are required for cell survival, excessive levels or perhaps distinct forms of autophagic flux contribute to disease pathogenesis. In an angiotensin II-induced cardiomyopathy model, angiotensin II (1.1 mg/kg/d) infusion for 4 weeks induces cardiac mitochondrial damage, autophagy and biogenesis through mitochondrial ROS (11). Angiotensin II type 2 receptor has been reported to antagonize angiotensin II type 1 receptor-mediated cardiomyocyte autophagy (12). Zhu et al (13) have also revealed that excess autophagy contributes to pressure overload-induced cardiac hypertrophy and heart failure (13). In the present study, an ISO-induced cardiomyocyte hypertrophy model was established in vitro and in vivo. The results demonstrated that the autophagic marker LC3B was acutely activated in vitro following ISO stimulation. Similarly, activated LC3B and autophagy-related markers Beclin1 and Atg7 were significantly upregulated by ISO administration in vivo. These findings indicated that an excess autophagic response occurred during ISO-induced cardiomyocyte hypertrophy both in vitro and in vivo.

Hydrogen has been demonstrated to have anti-autophagy effects on neuronal protection (26,27). Treatment with hydrogen significantly attenuates neuronal injury and autophagy in the hippocampal cornu ammonis 1 sector, as well as reduces brain edema, following 24 h of reperfusion (27). Hydrogen-rich saline decreases the degree of autophagy in the later stage of acute carbon monoxide poisoning, thus maintaining homeostasis and enhancing neuronal survival (26). Consistent with these findings, the present results demonstrated that hydrogen-rich medium blocked ISO-induced cardiomyocyte excessive autophagy in vitro and in vivo, as measured by decreased expression of Beclin1, Atg7 and LC3B II. It is well known that ROS contributes to the increase in autophagy. We have recently revealed that hydrogen can block ISO-induced accumulation of ROS in the heart (9). Thus, the anti-autophagy activity of hydrogen in the heart may be related to its antioxidant effect. However, it should be noted that autophagy is regulated by a complicated signaling network, and the molecular mechanism by which hydrogen may block autophagy needs further investigation.

In summary, the present findings indicated that hydrogen-rich medium and intraperitoneal administration of hydrogen attenuated excessive autophagy in β-adrenoceptor agonist-induced cardiomyocyte hypertrophy models in vitro and in vivo, respectively. Therefore, hydrogen may be a useful natural agent for inhibiting stress-induced autophagy under certain conditions.

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References


