

# 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  $\beta$ -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  $\mu$ M) for 5, 15 and 30 min, and then the protein expression levels of the autophagy marker microtubule-associated protein 1 light chain 3 $\beta$  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  $\beta$ -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

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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 3 $\beta$  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  $\beta$ -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. I5627; 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 under sterile conditions immediately prior to intraperitoneal injection. Primary antibodies against Atg7 (cat no. 2631) and LC3B (cat no. 3868) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The antibody against  $\beta$ -actin (cat no. sc-81178) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The anti-Beclin1 antibody (cat no. 3495; Cell Signaling Technology, Inc.) was kindly provided by Dr Zhi Zhao (Department of Gastrointestinal Surgery, The First Affiliated Hospital of Sun Yat-sen University). The goat anti-rabbit immunoglobulin (Ig)G horseradish peroxidase (HRP)-conjugated (cat no. 7074) and the horse anti-mouse IgG HRP-conjugated (cat no. 7076) secondary antibodies were purchased from Cell Signaling Technology, Inc.

**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 cardiac myoblasts (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% CO<sub>2</sub> 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  $\mu$ M ISO in DMEM/1% FBS for the indicated time (21). The 10  $\mu$ M 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  $\mu$ M) 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 $\pm$ 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+H<sub>2</sub>, n=6), and hydrogen group (H<sub>2</sub>, n=6). Mice in the ISO+H<sub>2</sub> 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 H<sub>2</sub> 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  $\mu$ g) were separated by SDS-PAGE (15% gel for LC3B and 10% gel for other proteins), and transferred

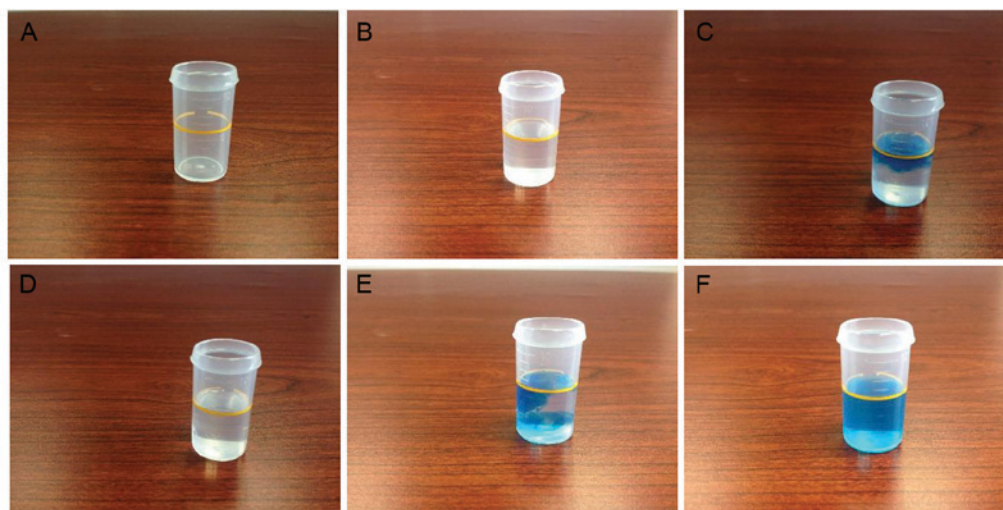


Figure 1. MB-Pt reagent was used to determine the hydrogen concentration in hydrogen-rich water. (A) Empty reference measuring cup. (B) Before titration, hydrogen-rich water was colorless. (C) A first drop of MB-Pt was added into 6 ml hydrogen-rich water, which was colored blue due to the MB-Pt. (D) Immediately, the blue color disappeared, then, the titration was repeated for N times. (E) At N+1 times, an additional drop of MB-Pt was added into the hydrogen-rich water, which turned blue. (F) The blue color at N+1 times remained and couldn't turn colorless. The hydrogen concentration was calculated as  $N \times 0.1$  ppm.

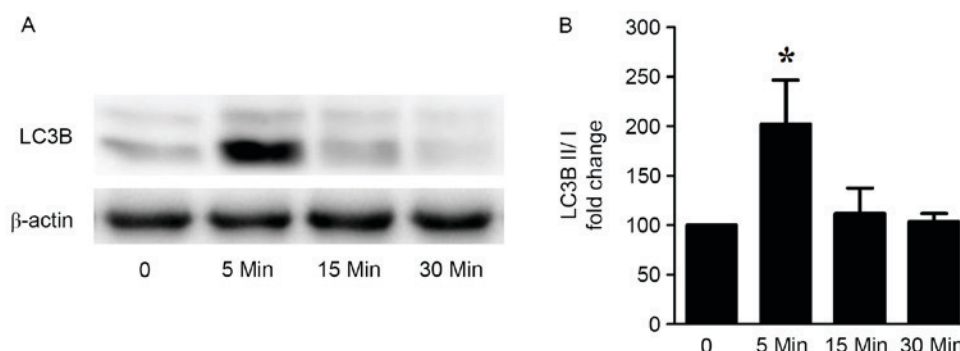


Figure 2. ISO stimulation induces LC3B activation in cardiomyocytes *in vitro*. (A) Representative western blot and (B) quantification of LC3B II to LC3B I ratio in H9c2 cardiomyocytes stimulated with ISO for 5, 15 and 30 min ( $n=4$ ). \* $P<0.05$  vs. 0 min. ISO, isoproterenol; LC3B, microtubule-associated protein 1 light chain 3 $\beta$ .

to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were incubated with Beclin1, Atg7, LC3B and  $\beta$ -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  $\beta$ -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  $\pm$  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  $\sim 0.6$ - $0.9$  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  $\mu$ M ISO was used to stimulate H9c2 cardiomyocytes for different durations (5, 15 and 30 min). The



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

Parameter	Control	ISO	ISO+H <sub>2</sub>	H <sub>2</sub>
Number	6	6	6	6
HW (mg)	0.12±0.01	0.15±0.01 <sup>a</sup>	0.13±0.01 <sup>b</sup>	0.12±0.01
BW (g)	25.17±1.17	25.33±1.51	25.67±1.97	24.92±1.24
HW/BW	4.78±0.21	5.74±0.13 <sup>a</sup>	5.16±0.15 <sup>b</sup>	4.73±0.14

Data are presented as mean ± standard deviation. <sup>a</sup>P<0.05 vs. Control; <sup>b</sup>P<0.05 vs. ISO. ISO, isoproterenol; H<sub>2</sub>, hydrogen; HW, heart weight; BW, body weight.

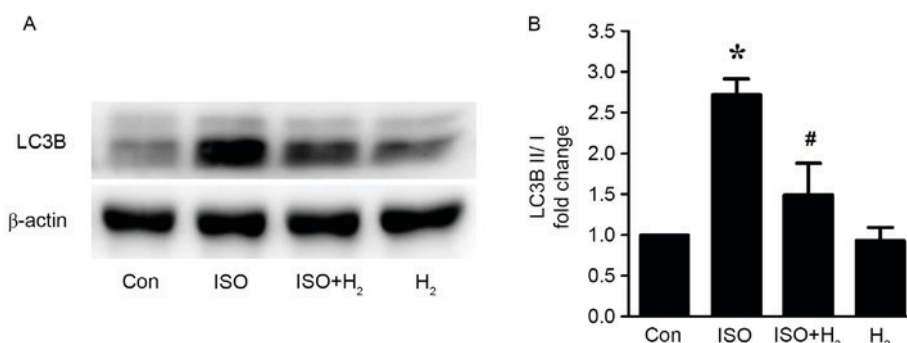


Figure 3. Hydrogen-rich medium pretreatment inhibits the ISO-induced LC3B activation *in vitro*. (A) Representative western blot and (B) quantification of LC3B II to LC3B I ratio in H9c2 cardiomyocytes following 5 min of ISO stimulation with or without hydrogen pretreatment (n=4). \*P<0.05 vs. Con; #P<0.05 vs. ISO. ISO, isoproterenol; LC3B, microtubule-associated protein 1 light chain 3β; Con, control, H<sub>2</sub>, hydrogen.

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

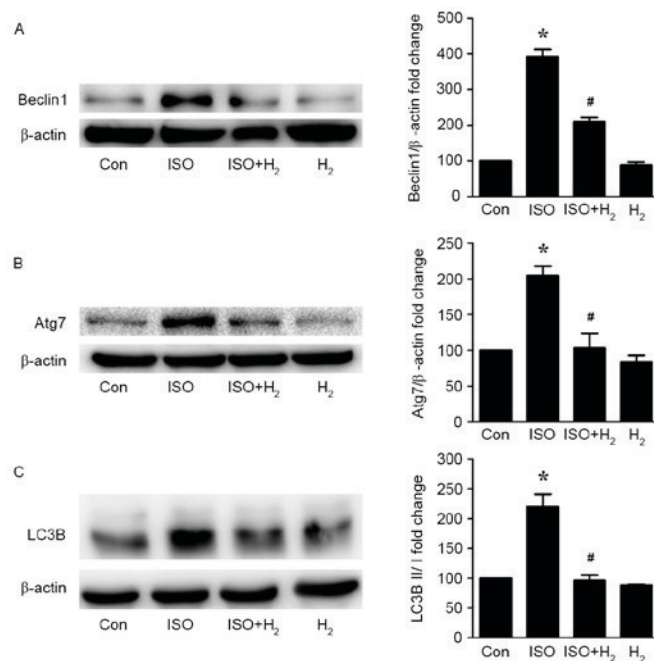


Figure 4. Intraperitoneal administration of hydrogen inhibits the ISO-mediated autophagy response *in vivo*. Representative western blots and quantification of relative expression levels of (A) Beclin1 to β-actin, (B) Atg7 to β-actin, and (C) LC3B II to LC3B I in the heart tissues of the experimental mice (n=4). \*P<0.05 vs. Con; #P<0.05 vs. ISO. ISO, isoproterenol; Atg7, autophagy-related protein 7; LC3B, microtubule-associated protein 1 light chain 3β; Con, control, H<sub>2</sub>, hydrogen.

intraperitoneal administration of hydrogen on cardiomyocyte autophagy activity was evaluated. The increased heart 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+H<sub>2</sub> 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  $\beta$ -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  $\beta$ -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

1. Hayashida K, Sano M, Kamimura N, Yokota T, Suzuki M, Maekawa Y, Kawamura A, Abe T, Ohta S, Fukuda K and Hori S: H(2) gas improves functional outcome after cardiac arrest to an extent comparable to therapeutic hypothermia in a rat model. *J Am Heart Assoc* 1: e003459, 2012.
2. Hayashi T, Yoshioka T, Hasegawa K, Miyamura M, Mori T, Ukimura A, Matsumura Y and Ishizaka N: Inhalation of hydrogen gas attenuates left ventricular remodeling induced by intermittent hypoxia in mice. *Am J Physiol Heart Circ Physiol* 301: H1062-H1069, 2011.
3. Kato R, Nomura A, Sakamoto A, Yasuda Y, Amatani K, Nagai S, Sen Y, Ijiri Y, Okada Y, Yamaguchi T, *et al*: Hydrogen gas attenuates embryonic gene expression and prevents left ventricular remodeling induced by intermittent hypoxia in cardiomyopathic hamsters. *Am J Physiol Heart Circ Physiol* 307: H1626-H1633, 2014.
4. Hayashida K, Sano M, Ohsawa I, Shinmura K, Tamaki K, Kimura K, Endo J, Katayama T, Kawamura A, Kohsaka S, *et al*: Inhalation of hydrogen gas reduces infarct size in the rat model of myocardial ischemia-reperfusion injury. *Biochem Biophys Res Commun* 373: 30-35, 2008.
5. Lekic T, Manaenko A, Rolland W, Fathali N, Peterson M, Tang J and Zhang JH: Protective effect of hydrogen gas therapy after germinal matrix hemorrhage in neonatal rats. *Acta Neurochir Suppl* 111: 237-241, 2011.
6. Sun Q, Kang Z, Cai J, Liu W, Liu Y, Zhang JH, Denoble PJ, Tao H and Sun X: Hydrogen-rich saline protects myocardium against ischemia/reperfusion injury in rats. *Exp Biol Med* (Maywood) 234: 1212-1219, 2009.
7. Zhang Y, Sun Q, He B, Xiao J, Wang Z and Sun X: Anti-inflammatory effect of hydrogen-rich saline in a rat model of regional myocardial ischemia and reperfusion. *Int J Cardiol* 148: 91-95, 2011.

8. Yu YS and Zheng H: Chronic hydrogen-rich saline treatment reduces oxidative stress and attenuates left ventricular hypertrophy in spontaneous hypertensive rats. *Mol Cell Biochem* 365: 233-242, 2012.
9. Zhang Y, Xu J, Long Z, Wang C, Wang L, Sun P, Li P and Wang T: Hydrogen (H<sub>2</sub>) Inhibits Isoproterenol-Induced Cardiac Hypertrophy via Antioxidative pathways. *Front Pharmacol* 7: 392, 2016.
10. Zhang YX, Xu JT, You XC, Wang C, Zhou KW, Li P, Sun P, Wang L and Wang TH: Inhibitory effects of hydrogen on proliferation and migration of vascular smooth muscle cells via Down-Regulation of mitogen/activated protein kinase and Ezrin-Radixin-Moesin signaling pathways. *Chin J Physiol* 59: 46-55, 2016.
11. Dai DF, Johnson SC, Villarin JJ, Chin MT, Nieves-Cintrón M, Chen T, Marcinek DJ, Dorn GW II, Kang YJ, Prolla TA, *et al*: Mitochondrial oxidative stress mediates angiotensin II-induced cardiac hypertrophy and Galphaq overexpression-induced heart failure. *Circ Res* 108: 837-846, 2011.
12. Porrello ER, D'Amore A, Curl CL, Allen AM, Harrap SB, Thomas WG and Delbridge LM: Angiotensin II type 2 receptor antagonizes angiotensin II type 1 receptor-mediated cardiomyocyte autophagy. *Hypertension* 53: 1032-1040, 2009.
13. Zhu H, Tannous P, Johnstone JL, Kong Y, Shelton JM, Richardson JA, Le V, Levine B, Rothermel BA and Hill JA: Cardiac autophagy is a maladaptive response to hemodynamic stress. *J Clin Invest* 117: 1782-1793, 2007.
14. Nakai A, Yamaguchi O, Takeda T, Higuchi Y, Hikoso S, Taniike M, Omiya S, Mizote I, Matsumura Y, Asahi M, *et al*: The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. *Nat Med* 13: 619-624, 2007.
15. Ohsumi Y: Historical landmarks of autophagy research. *Cell Res* 24: 9-23, 2014.
16. Mizushima N, Yoshimori T and Ohsumi Y: The role of Atg proteins in autophagosome formation. *Annu Rev Cell Dev Biol* 27: 107-132, 2011.
17. Levine B and Yuan J: Autophagy in cell death: An innocent convict? *J Clin Invest* 115: 2679-2688, 2005.
18. Levine B and Klionsky DJ: Development by self-digestion: Molecular mechanisms and biological functions of autophagy. *Dev Cell* 6: 463-477, 2004.
19. Levine B and Kroemer G: Autophagy in the pathogenesis of disease. *Cell* 132: 27-42, 2008.
20. Lavandro S, Chiong M, Rothermel BA and Hill JA: Autophagy in cardiovascular biology. *J Clin Invest* 125: 55-64, 2015.
21. Jeong K, Kwon H, Min C and Pak Y: Modulation of the caveolin-3 localization to caveolae and STAT3 to mitochondria by catecholamine-induced cardiac hypertrophy in H9c2 cardiomyoblasts. *Exp Mol Med* 41: 226-235, 2009.
22. Tshori S, Gilon D, Beeri R, Nechushtan H, Kaluzhny D, Pikarsky E and Razin E: Transcription factor MITF regulates cardiac growth and hypertrophy. *J Clin Invest* 116: 2673-2681, 2006.
23. Huang G, Zhou J, Zhan W, Xiong Y, Hu C, Li X, Li X, Li Y and Liao X: The neuroprotective effects of intraperitoneal injection of hydrogen in rabbits with cardiac arrest. *Resuscitation* 84: 690-695, 2013.
24. Seo T, Kurokawa R and Sato B: A convenient method for determining the concentration of hydrogen in water: Use of methylene blue with colloidal platinum. *Med Gas Res* 2: 1, 2012.
25. Nakatogawa H, Suzuki K, Kamada Y and Ohsumi Y: Dynamics and diversity in autophagy mechanisms: Lessons from yeast. *Nat Rev Mol Cell Biol* 10: 458-467, 2009.
26. Wang W, Tian L, Li Y, Wang X, Xia F, Li L, Li J and Zhang Z: Effects of hydrogen-rich saline on rats with acute carbon monoxide poisoning. *J Emerg Med* 44: 107-115, 2013.
27. Nagatani K, Wada K, Takeuchi S, Kobayashi H, Uozumi Y, Otani N, Fujita M, Tachibana S and Nawashiro H: Effect of hydrogen gas on the survival rate of mice following global cerebral ischemia. *Shock* 37: 645-652, 2012.