Hydrogen (H$_2$) Inhibits Isoproterenol-Induced Cardiac Hypertrophy via Antioxidative Pathways

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Background and Purpose: Hydrogen (H$_2$) has been shown to have a strong antioxidant effect on preventing oxidative stress-related diseases. The goal of the present study is to determine the pharmacodynamics of H$_2$ in a model of isoproterenol (ISO)-induced cardiac hypertrophy.

Methods: Mice (C57BL/6J; 8–10 weeks of age) were randomly assigned to four groups: Control group ($n$ = 10), ISO group ($n$ = 12), ISO plus H$_2$ group ($n$ = 12), and H$_2$ group ($n$ = 12). Mice received H$_2$ (1 ml/100g/day, intraperitoneal injection) for 7 days before ISO (0.5 mg/100g/day, subcutaneous injection) infusion, and then received ISO with or without H$_2$ for another 7 days. Then, cardiac function was evaluated by echocardiography. Cardiac hypertrophy was reflected by heart weight/body weight, gross morphology of hearts, and heart sections stained with hematoxylin and eosin, and relative atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) mRNA levels. Cardiac reactive oxygen species (ROS), 3-nitrotyrosine and p67 (phox) levels were analyzed by dihydroethidium staining, immunohistochemistry and Western blotting, respectively. For in vitro study, H9c2 cardiomyocytes were pretreated with H$_2$-rich medium for 30 min, and then treated with ISO (10 $\mu$M) for the indicated time. The medium and ISO were re-changed every 24 h. Cardiomyocyte surface areas, relative ANP and BNP mRNA levels, the expression of 3-nitrotyrosine, and the dissipation of mitochondrial membrane potential (MMP) were analyzed by dihydroethidium staining, immunohistochemistry and Western blotting, respectively. For in vitro study, H9c2 cardiomyocytes were pretreated with H$_2$-rich medium for 30 min, and then treated with ISO (10 $\mu$M) for the indicated time. The medium and ISO were re-changed every 24 h. Cardiomyocyte surface areas, relative ANP and BNP mRNA levels, the expression of 3-nitrotyrosine, and the dissipation of mitochondrial membrane potential (MMP) were examined. Moreover, the expression of extracellular signal-regulated kinase1/2 (ERK1/2), p-ERK1/2, p38, p-p38, c-Jun NH2-terminal kinase (JNK), and p-JNK were measured by Western blotting both in vivo and in vitro.

Results: Intraperitoneal injection of H$_2$ prevented cardiac hypertrophy and improved cardiac function in ISO-infused mice. H$_2$-rich medium blocked ISO-mediated cardiomyocytes hypertrophy in vitro. H$_2$ blocked the excessive expression of NADPH oxidase and the accumulation of ROS, attenuated the decrease of MMP, and inhibited ROS-sensitive ERK1/2, p38, and JNK signaling pathways.
INTRODUCTION

Heart failure is a global pandemic affecting an estimated 26 million people worldwide, posing an enormous burden to both individuals and society (Ambrosy et al., 2014). Heart failure is often preceded by left ventricular hypertrophy, which is characterized by an increase in the size of individual cardiac myocytes and re-expression of fetal cardiac genes, such as atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP; Magga et al., 1998; Heineke and Molkentin, 2006). Although cardiac hypertrophy has traditionally been considered as an adaptive response required to sustain cardiac output in response to stresses, long-standing hypertrophy will eventually lead to congestive heart failure, arrhythmia, and sudden death (Frey and Olson, 2003).

Increasing evidence suggests that diverse pathophysiological stimuli, including neurohumoral activation [such as angiotensin II (ANG II) and β-adrenoceptor stimulation], hypertension, ischemic heart diseases, myocardiitis, and diabetic cardiomyopathy, will contribute to cardiac hypertrophy and heart failure partially via inducing the production of excessive reactive oxygen species (ROS; Li et al., 2002; Zhang et al., 2007b; Zhang et al., 2015). The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and mitochondria have been proposed as primary sites of ROS generation (Dai et al., 2011a). ROS generated by NADPH oxidase was shown to stimulate and amplify mitochondrial ROS production and induce mitochondrial dysfunction, which can be reflected by the depression of mitochondrial membrane potential (MMP; Zorov et al., 2000; Dai et al., 2011a). The excessive accumulation of ROS subsequently activates downstream ROS-sensitive signaling pathways implicated in pathological cardiac hypertrophy. Therefore, blocking ROS will improve mitochondrial function and block downstream hypertrophic signaling, thus preventing the development of cardiac hypertrophy and progression to heart failure. Consistent with this notion, recent studies revealed that strategies targeted ROS and downstream signaling pathways modulated by ROS could be a better approach to improve cardiac hypertrophy (Burgoyne et al., 2012).

Molecule hydrogen (H₂), which is a colorless, odorless, tasteless, and flammable gas, has attracted considerable attention for improving oxidative stress-related diseases (Ohta, 2015). We recently revealed that intraperitoneal injection of H₂ protects against vascular hypertrophy induced by abdominal aortic coarctation (AAC) in vivo, and H₂-rich medium attenuates proliferation and migration of vascular smooth muscle cells (VSMCs) stimulated by ANG II in vitro (Zhang et al., 2016). Moreover, H₂ also has important role in protecting against heart diseases. Inhalation of H₂ attenuates left ventricular remodeling induced by intermittent hypoxia (Hayashi et al., 2011; Kato et al., 2014), and improves cardiac hypertrophy after germina matrix hemorrhage in neonatal rats (Lekic et al., 2011). However, the effects of H₂ on cardiac hypertrophy induced by β-adrenoceptor stimulation and the related signaling mechanisms still remain unclear. The aims of this study are, therefore, to determine the effect of intraperitoneal injection of H₂ on isoproterenol (ISO)-induced cardiac hypertrophy in vivo, and the effect of H₂-rich medium on ISO-induced H9c2 cardiomyocytes hypertrophy in vitro, as well as to identify the molecular mechanisms that may be responsible for its putative effects.

MATERIALS AND METHODS

Drugs and Chemicals

H₂ (99.999%; Guang Zhou Guang Qi GAS Co., Ltd, Guangdong, China) was stored in the seamless steel gas cylinder, and it was injected into an aseptic soft plastic infusion bag (100 ml; CR Double-Crane Pharmaceuticals Co., Ltd, Anhui, China) under sterile conditions immediately before intraperitoneal injection. ISO (I5627, Sigma–Aldrich, St. Louis, MO, USA) was dissolved in normal saline (5 mg/10 ml) under sterile conditions immediately before subcutaneous injection, and dissolved in double distilled water as 10 mM stock solution 30 min before use. The antibodies against extracellular signal-regulated kinase 1/2 (ERK1/2), p-ERK1/2, p38, p-p38, c-Jun NH2-terminal kinase (JNK), and p-JNK, p67 (phox) were from Cell Signaling Technology (Danvers, MA, USA). The antibody against β-actin was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-α-actin antibody was from Sigma–Aldrich (St. Louis, MO, USA). The antibody against 3-nitrotyrosine was from Abcam (Cambridge, MA, USA). JC-1 was from Beyotime Biotechnology (C2006, Jiangsu, China).

Preparation of H₂-rich Medium and Measurement of H₂ Concentration

H₂-rich medium was prepared as previously described (Zhang et al., 2016). The concentration of H₂ was measured by MB-Pt reagent (generously provided by Ming Yan, Shanghai Nanobubble Technology Co., Ltd, Shanghai, China) as previously described (Zhang et al., 2016). The H₂ concentration in our H₂-rich medium was no less than 0.6 ppm (0.6–0.9 ppm).

Cell Culture and Treatment

H9c2 rat cardiac myoblasts (a cardiomyoblast cell line derived from embryonic rat heart tissue; generously provided by Prof.

Conclusion: H₂ inhibits ISO-induced cardiac/myocyte hypertrophy both in vivo and in vitro, and improves the impaired left ventricular function. H₂ exerts its protective effects partially through blocking ROS-sensitive ERK1/2, p38, and JNK signaling pathways.

Keywords: hydrogen, β-adrenoceptor, cardiac hypertrophy, NADPH oxidase, reactive oxygen species, mitochondrial damage, MAPK
Animal Model of Cardiac Hypertrophy and Treatment Protocol

The C57BL/6J mice (aged 8–10 weeks, male) were obtained from the Laboratory Animal Center of Sun Yat-sen University. The animals were housed with 12-h light–dark cycles and allowed to obtain food and water ad libitum. All experimental procedures and protocols were approved by 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/100g/day) for 7 days as previously revealed (Tshori et al., 2006). Mice were randomly assigned to four groups: Control (Con) group (n = 10), ISO group (n = 12), ISO plus H2 group (n = 12), and H2 group (n = 12). H2 was given at the dose of 1 ml/100g/day by intraperitoneal injection as previously described (Huang et al., 2013; Zhang et al., 2016). Mice in ISO plus H2 group and H2 group received ISO consecutively for 7 days before receiving ISO, and continued for another 7 days. On the 8th day, mice in ISO group, and ISO plus H2 group received ISO for 7 days until animals were sacrificed on the 15th day. After sacrifice, hearts were excised, rinsed with PBS, and blotted dry. Hearts were weighed; the heart weight/body weight (HW/BW) ratios were calculated and expressed as milligrams HW per gram BW. Then hearts were snap frozen in liquid nitrogen within minutes and stored at −80°C until analyzed.

Echocardiography

Transthoracic echocardiography was performed to assess left ventricular function before sacrificed on the 15th day in a blinded manner. Mice were anesthetized with 1.5–2% isoflurane, and cardiomyocytes hypertrophic response was examined after 48 h of ISO challenge (Jeong et al., 2009).

Histological Analysis

Hearts were excised, washed with ice-PBS, fixed in 10% buffered formalin, and cut transversely close to the apex cordis to visualize the left and right ventricles. Several sections of heart (4–5 μm thickness) were prepared and stained with hematoxylin and eosin (H&E) for histopathology and then visualized by light microscopy.

Immunohistochemistry

For immunostaining, anti-sarcomeric α-actin antibody was used to assess the cell surface area of H9c2 cardiomyocytes as described previously (Akimoto et al., 1996). To assess 3-nitrotyrosine levels in heart, which can reflect formation of ONOO–, primary antibody against 3-nitrotyrosine (1:50) was used as previously described (Zhang et al., 2011).

Measurement of MMP

Mitochondrial membrane potential was determined by the dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) as previously described with slight modification (Cossarizza et al., 1993). Briefly, the treated cells were washed with PBS, and then incubated with JC-1 staining dye (culture medium: JC-1 working dye = 1:1) at 37°C in the dark for 20 min and rinsed three times with cold PBS, and analyzed by fluorescence microscope (Axio Observer Z1, Carl Zeiss. Inc.). The JC-1 aggregates, which was accumulated in the inner membrane of mitochondria, emitted red fluorescence and represented the high MMP, while green fluorescence reflected JC-1 monomer which entered in the cytosol following mitochondrial membrane depolarization. When mitochondria is damaged, the red/green ratio decreases. The ratio of JC-1 aggregates to monomer (red/green) intensity for each region was calculated by Image-Pro Plus software (version 6.0).

qRT-PCR

Total mRNA was extracted from left ventricles and H9c2 cardiomyocytes using TRIZol reagent (Invitrogen) according to the manufacturer’s instruction, and cDNA was synthesized using oligo (dT) primers with the Transcriptor First Strand cDNA Synthesis Kit (PrimeScript™ RT Master Mix, Takara). Selected gene differences were confirmed by qRT-PCR using SYBR green (SYBR® Premix Ex Taq™, Takara). The target gene expression was normalized to GAPDH gene expression. The primers for qRT-PCR are shown in Table 1.

Western Blotting

Western blotting was performed as previously described (Zhang et al., 2016). The membranes were incubated with primary (1:2000) and secondary (1:2000) antibodies by standard techniques. Immunodetection was accomplished using enhanced chemiluminescence (ChemiDoc XRS+ System, Bio-Rad, Hercules, CA, USA).

| Name          | Forward primer sequence (5’–3’)          | Reverse primer sequence (5’–3’)       |
|---------------|------------------------------------------|----------------------------------------|
| M-GAPDH       | GGTGGTCTCTCTGCGGACCTCA                   | TGGTACGGGTTCTTTAATCC                  |
| R-GAPDH       | GACATGCCGCGCTTGAAGAAC                   | AGGCCAGATGGCCCTTTAGT                  |
| M-ANP         | GCTCTTCCTCTCCACTCTG                     | TGCCTTCTGCTGTCTGAAAT                  |
| R-ANP         | GGGAGACCGACGCGCTCTCA                    | GGCCTCAATCCTGATC                     |
| M-BNP         | TCTGGGACCACTTTGAAAT                    | ATGGTTGGGAAATTTTGAT                  |
| R-BNP         | CTCCAGAAACATCCACCAGT                    | AGACGCCAACGCTGACT                      |
FIGURE 1 | Effects of hydrogen (H₂) on cardiac hypertrophy induced by isoproterenol (ISO) in vivo. (A) Gross morphology of hearts (top) and heart sections stained with H&E (bottom) after 1 week of ISO infusion with or without H₂ at the dose of 1 ml/100g/day. (B) The relative mRNA expression of hypertrophic marker atrial natriuretic peptide (ANP) to GAPDH (n = 3). (C) The relative mRNA expression of hypertrophic marker B-type natriuretic peptide (BNP) to GAPDH (n = 3). ∗P < 0.05 vs. Control (Con) and #P < 0.05 vs. ISO. Scale bar: 20 µm.

Assessment of Cardiac ROS Levels
Cardiac total ROS was stained with dihydroethidium (DHE, D-23107; Invitrogen) on fresh frozen sections as previously described (Zhang et al., 2014). Images were immediately acquired using confocal microscopy (Leica Model SPE, Leica Imaging Systems Ltd) using λex 405 nm laser excitation.

Statistical Analysis
Data are expressed as mean ± SD. Differences among groups were tested by one-way ANOVA. Comparisons between two groups were performed by unpaired Student’s t-test. A value of P < 0.05 was considered to be significantly different.

RESULTS

H₂ inhibited Cardiac Hypertrophy In vivo
In order to investigate the effects of H₂ on cardiac hypertrophy, ISO was used to induce cardiac hypertrophy in mice. As expected, mice with chronic ISO infusion exhibited cardiac hypertrophy compared to the control group, as indicated by the gross morphology of hearts, heart sections stained with H&E (Figure 1A). The hypertrophic marker gene ANP and BNP mRNA levels (Figures 1B,C, P < 0.05 vs. Con), and HW/BW ratio (Table 2, P < 0.05 vs. Con) were also increased. Pretreatment with H₂ (intraperitoneal injection) at the dose of...
TABLE 2 | Effects of hydrogen on cardiac dysfunction induced by isoproterenol (ISO) in vivo.

| Parameter       | Con  | ISO       | ISO+H₂  | H₂  |
|-----------------|------|-----------|---------|-----|
| Number (n)      | 10   | 12        | 12      | 12  |
| HW/BW (mg/g)    | 4.72 ± 0.08 | 5.81 ± 0.07* | 5.09 ± 0.14* | 4.69 ± 0.06 |
| LVEDD (mm)      | 3.24 ± 0.10 | 3.71 ± 0.06* | 3.45 ± 0.01* | 3.26 ± 0.08 |
| LVESD (mm)      | 2.05 ± 0.05 | 2.52 ± 0.07* | 2.35 ± 0.04* | 2.12 ± 0.07 |
| FS (%)          | 35.99 ± 0.13 | 31.56 ± 0.19* | 33.30 ± 0.45* | 35.68 ± 0.26 |

Hydrogen was given at the dose of 1 ml/100g/day by intraperitoneal injection. Heart weight, HW; Body weight, BW; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter. *P < 0.05 vs. Con and #P < 0.05 vs. ISO.

FIGURE 2 | Effects of H₂-rich medium on cardiomyocytes hypertrophy induced by ISO in vitro. (A) Photomicrographs of morphological change induced by ISO with or without H₂-rich medium. (B) Bar graph shows the relative cell surface area of cardiomyocytes stimulated by ISO with or without H₂-rich medium. (C) The relative mRNA levels of hypertrophic marker ANP to GAPDH (n = 4). (D) The relative mRNA levels of hypertrophic marker BNP to GAPDH (n = 4). *P < 0.05 vs. Con and #P < 0.05 vs. ISO. Scale bar: 20 µm.

1 ml/100g/day reversed these hypertrophic responses (Figure 1; Table 2, P < 0.05 vs. ISO). Moreover, H₂ injection alleviated the impaired left ventricular function, as evidenced by decreasing left ventricular end-systolic diameter (LVESD), left ventricular end-diastolic diameter (LVEDD), and increasing fractional shortening (FS%); Table 2, P < 0.05 vs. ISO). However, there were no significant changes between control group and H₂ group. Collectively, these data suggested that H₂ injection prevented the development of ISO-induced cardiac hypertrophy and preserved cardiac function in vivo.
H$_2$ attenuated Cardiomyocyte Hypertrophy In vitro

As the heart primarily consists of cardiomyocyte and fibroblast, therefore, we investigated whether H$_2$ could target cardiomyocyte for hypertrophic inhibition. H$_2$-rich medium and H9c2 cardiomyocytes were used for in vitro studies. First, we used CCK8 to investigate the possible cytotoxicity of H$_2$-rich medium on H9c2 cardiomyocyte. H$_2$ was shown to be non-cytotoxic for cardiomyocyte treating with H$_2$-rich medium for 48 h (data not shown). After 48 h of ISO stimulation, cardiomyocyte surface areas, and the hypertrophic marker gene ANP and BNP mRNA levels were significantly increased in H9c2 cardiomyocyte (Figures 2A–D, $P < 0.05$ vs. Con). H$_2$-rich medium attenuated these hypertrophic responses of H9c2 cardiomyocyte (Figures 2A–D, $P < 0.05$ vs. ISO). These data indicated that H$_2$ could also inhibit ISO-induced cardiomyocyte hypertrophy in vitro.

H$_2$ Blocked the Excess ROS Accumulation and Mitochondrial Damage

ROS play a critical role in the development of cardiac hypertrophy and heart failure (Burgoyne et al., 2012). ROS levels were increased in the left ventricular of ISO-infused mice compared with control mice, and this increase was inhibited by pretreatment with H$_2$ at the dose of 1ml/100g/day (Figure 3A). Moreover, another oxidative stress marker, 3-nitrotyrosine (3-NT), which reflects the formation of ONOO$^-$, was also upregulated by ISO stimuli, and suppressed by H$_2$ (Figure 3B). To confirm these in vivo findings, we evaluated the effects of H$_2$-rich medium on the levels of 3-NT stimulated by ISO in vitro. The accumulation of 3-NT was increased after ISO stimulation, while H$_2$-rich medium attenuated this effects (Figure 3C, $P < 0.05$).

To further understand the mechanism of H$_2$ in blocking ROS accumulation, we tested the NADPH oxidase subunit p67.
FIGURE 4 | Effects of H₂-rich medium on ISO-induced depression of MMP in vitro. After stimulated by ISO for 24 h with or without H₂-rich medium for 30 min pretreatment, MMP was measured by JC-1 staining followed by photofluorography (A). The quantification of the fluorescence intensity (red/green ratio) for each treatment was calculated by Image-Pro Plus software (B) (*n = 4). *P < 0.05 vs. Con and #P < 0.05 vs. ISO. Scale bar: 5 µm.

(phox) expression. Immunoblotting revealed the expression of p67 (phox) was increased in left ventricular of ISO-infused mice, and this increase was alleviated by H₂ (Figure 3D, *P < 0.05). As we have mentioned above, NADPH oxidase-derived ROS can stimulate and amplify mitochondrial ROS production and induce mitochondrial dysfunction (Zorov et al., 2000; Dai et al., 2011a), and these can be reflected by the change of MMP. ISO induced the depression of MMP, as indicated by high levels of green fluorescence and low levels of red fluorescence. Interestingly, H₂-rich medium blocked the depression of MMP induced by ISO (Figures 4A,B, *P < 0.05). Therefore, these data indicated that H₂ inhibited the excess ROS accumulation following ISO
stimuli through attenuating NADPH oxidase expression and mitochondrial damage.

**H$_2$ suppressed Mitogen-Activated Protein Kinases (MAPKs) Signaling In vivo and In vitro**

Based on the inhibitory effect of H$_2$ on the ISO-induced excess accumulation of ROS in vitro and in vivo, we further investigated its effect on the downstream hypertrophic targets, such as mitogen-activated protein kinases (MAPKs) signaling pathways. Following ISO stimuli, the phosphorylation of ERK1/2, p38 MAPK (p38), and c-Jun NH2-terminal kinase (JNK) were increased to the high level at 5 min, and came to the base line at 30 min (Figure 5, $P < 0.05$ vs. 0 min). These enhanced activation of MAPKs could be blocked by H$_2$-rich medium in vitro (Figure 6, $P < 0.05$ vs. ISO). Similarly, the activation of MAPKs were enhanced in the hearts of ISO-infused mice compared with control group (Figure 7, $P < 0.05$ vs. Con). Such changes were inhibited by pretreatment with H$_2$ in vivo (Figure 7, $P < 0.05$ vs. ISO). Thus, H$_2$ suppressed the enhanced phosphorylation of ERK1/2, p38, and JNK to alleviate ISO-mediated cardiac hypertrophy in vivo and cardiomyocyte hypertrophy in vitro.

**DISCUSSION**

The present study demonstrates that intraperitoneal injection of H$_2$ protects against ISO-induced cardiac hypertrophy and dysfunction in vivo and H$_2$-rich medium attenuates ISO-mediated cardiomyocyte hypertrophy in vitro. The cardioprotection of H$_2$ is mediated by direct interruption of NADPH oxidase expression and alleviating mitochondrial damage, these lead to inhibit the accumulation of ROS, and subsequently block downstream ERK1/2, p38, and JNK signaling.

H$_2$ has been emerged as an important blocker of heart diseases by various given manners. H$_2$ inhalation attenuates intermittent hypoxia (Hayashi et al., 2011; Kato et al., 2014), or ischemia/reperfusion (Hayashida et al., 2008), or germinal matrix hemorrhage-induced left ventricular remodeling (Lekic et al., 2011). Drinking H$_2$-rich water blocks cardiac fibrosis induced by left kidney artery ischemia/reperfusion injury (Zhu et al., 2015).
**FIGURE 6** | Effects of H\textsubscript{2}-rich medium on ISO-mediated MAPKs signaling activation \textit{in vitro}. Representative Western blot and quantification of ERK1/2 phosphorylation (A), p38 phosphorylation (B), and JNK phosphorylation (C) to their total protein expressions, respectively, \( n = 4 \). *P < 0.05 vs. Con and #P < 0.05 vs. ISO.

H\textsubscript{2}-rich saline injection also inhibits ischemia/reperfusion (Sun et al., 2009), or hypertension-mediated cardiac remodeling (Wang et al., 2011; Yu and Zheng, 2012). However, the effects of intraperitoneal injection of H\textsubscript{2} on cardiac hypertrophy induced by \( \beta \)-adrenoceptor stimulation have not yet been clarified. In this study, we prepared H\textsubscript{2}-rich medium, and developed new methods for giving H\textsubscript{2} in vivo by intraperitoneal injection of H\textsubscript{2}, and we find that H\textsubscript{2} not only attenuates ISO-induced cardiomyocyte hypertrophy \textit{in vitro} and cardiac hypertrophy \textit{in vivo}, but also improves the impaired cardiac function. As we have mentioned above, diabetic cardiomyopathy is also a contributor to cardiac hypertrophy and heart failure. H\textsubscript{2}-rich saline has been reported to improve early neurovascular dysfunction (Feng et al., 2013) and erectile dysfunction (Fan et al., 2013) in a streptozotocin-induced diabetic rat model. However, the effect of H\textsubscript{2} on diabetic cardiomyopathy is still under investigation. It has been reported that the gasotransmitter hydrogen sulfide (H\textsubscript{2}S) protects against pressure overload-mediated (Kondo et al., 2013) or arteriovenous fistula (AVF)-induced heart failure (Mishra et al., 2010). A question raised here is that whether the reciprocal interaction between H\textsubscript{2} and H\textsubscript{2}S exists during their regulation of cardiac hypertrophy.

The excess activation of ROS has been shown to contribute to the development of cardiac hypertrophy (Li et al., 2002; Zhang et al., 2005, 2007b; Burgoyne et al., 2012). In this study, we reveal that H\textsubscript{2} blocks ROS accumulation induced by \( \beta \)-adrenoceptor stimulation both \textit{in vitro} and \textit{in vivo}. The inhibitory effects of H\textsubscript{2} on ROS also have been reported in various animal models, such as heart ischemia/reperfusion injury (Zhang et al., 2011; Noda et al., 2013; Shinbo et al., 2013), brain injury (Ohsawa et al., 2007; Liu et al., 2011; Wang et al., 2012), renal injury (Li et al., 2016), chemotherapy-induced ovarian injury (Meng et al., 2015), metabolic syndrome (Song et al., 2013), etc. NADPH oxidase and mitochondria have been proposed as primary sites of ROS generation (Dai et al., 2011a). ROS produced by NADPH oxidase has the ability to stimulate and amplify mitochondrial ROS generation and induce mitochondrial dysfunction (Zorov et al., 2000; Dai et al., 2011a). Therefore, tyrosine kinase FYN interacts...
with the C-terminal domain of NOX4, and phosphorylates the tyrosine 566 on NOX4, thereby inhibiting apoptosis in the heart and preventing cardiac remodeling after pressure overload (Matsushima et al., 2016). Overexpression of catalase targeted to mitochondria, but not the overexpression of wild-type peroxisomal catalase, protects against ANG II-induced cardiac hypertrophy, fibrosis and mitochondrial damage, as well as heart failure induced by overexpression of Goq (Dai et al., 2011b). We found that H2 inhibits ISO-induced NADPH oxidase subunit p67 expression, and suppresses the dissipation of MMP. The excessive accumulation of ROS subsequently transmits signals to downstream ROS-sensitive signaling pathways, such as ERK1/2 (Li et al., 2002; Dai et al., 2011b), p38 MAPK (Li et al., 2002; Dai et al., 2011a), and JNK (Li et al., 2002; Kimura et al., 2005; Zhang et al., 2007a), NF-κB (Hirotani et al., 2002), PI3K/Akt (Sundaresan et al., 2009; Wang et al., 2013), and autophagy related signaling (Dai et al., 2011b), to induce pathological cardiac hypertrophy. Our results indicate that H2 markedly blocks ISO-induced ERK1/2, p38 and JNK activation in vivo and in vitro. These findings confirm that the anti-hypertrophic effect of H2 is partially achieved through blocking ROS-dependent MAPKs signaling. Yu Yongsheng et al. has reported that H2-rich saline inhibits cardiac hypertrophy in spontaneous hypertensive rats (SHRs) via blocking NF-κB activity (Yu and Zheng, 2012). H2-rich saline reduces myocardial reperfusion injury and improves heart function through down-regulating the expression of Akt and GSK3β (Yue et al., 2015), and blocking autophagy in myocardial tissue (Pan et al., 2015). However, whether PI3K/Akt, and autophagy signaling are related to the protective effects of H2 injection on pathological cardiac hypertrophy still needs further investigation.

CONCLUSION

Our study demonstrated that intraperitoneal injection of H2 attenuated β-adrenoceptor agonist (ISO)-mediated cardiac hypertrophy and dysfunction in vivo, and H2-rich medium blocked ISO-induced cardiomyocyte hypertrophic responses.
in vitro. Our results suggested that H₂ exerted anti-hypertrophic activity, at least in part, via alleviating NADPH oxidase expression and inhibiting the depression of MMP, and thus blocked ROS-sensitive MAPK signaling pathways.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: YZ and TW. Performed the experiments: YZ, JX, ZL, and CW. Analyzed the data: YZ and JX. Contributed reagents/materials/analysis tools: LW, PS, and PL.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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