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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

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Abstract

Molecular hydrogen (H_2) has recently attracted considerable attention for the prevention of oxidative stress-related vascular diseases. The purpose of this study is to evaluate the effects of hydrogen on proliferation and migration of vascular smooth muscle cells (VSMCs) stimulated by angiotensin II (Ang II) *in vitro*, and on vascular hypertrophy induced by abdominal aortic coarctation (AAC) *in vivo*. Hydrogen-rich medium (0.6~0.9 ppm) was added 30 min before 10^{-7} M Ang II administration, then the proliferation and migration index were determined 24 h after Ang II stimulation. Hydrogen gas (99.999%) was given by intraperitoneal injection at the dose of 1 ml/100 g/day consecutively for one week before AAC and lasted for 6 weeks *in vivo*. Hydrogen inhibited proliferation and migration of VSMCs with Ang II stimulation *in vitro,* and improved the vascular hypertrophy induced by AAC *in vivo*. Treatment with hydrogen reduced Ang II- or AAC-induced oxidative stress, which was reflected by diminishing the induction of reactive oxygen species (ROS) in Ang II-stimulated VSMCs, inhibiting the levels of 3-nitrotyrosine (3-NT) in vascular and serum malondialdehyde (MDA). Hydrogen treatment also blocked Ang II-induced phosphorylation of the extracellular signal-regulated kinase1/2 (ERK1/2), p38 MAPK, c-Jun NH_2-terminal kinase (JNK) and the ezrin/radixin/moesin (ERM) *in vitro*. Taken together, our studies indicate that hydrogen prevents AAC-induced vascular hypertrophy *in vivo,* and inhibits Ang II-induced proliferation and migration of VSMCs *in vitro* possibly by targeting ROS-dependent ERK1/2, p38 MAPK, JNK and ERM signaling. It provides the molecular basis of hydrogen on inhibiting the abnormal proliferation and migration of VSMCs and improving vascular remodeling diseases.

Key Words: ezrin/radixin/moesin, hydrogen, migration, mitogen-activated protein kinase, proliferation, vascular smooth muscle cells

Introduction

The vascular smooth muscle cells (VSMCs), as stromal cells of the vascular wall, play important roles in maintaining vessel tone and regulating blood pressure (17). Under physiological conditions, VSMCs are retained in a non-proliferative state, release some regulatory molecules for maintaining the normal functions of blood vessels (7), and regulate vascular tone mainly by NO-GC-cGMP-Ca^{2+} signaling (9). However, under pathological conditions, for example, hypertension, which is a potent stimulus for cell growth, migration, and proliferation, VSMCs re-enter the cell cycle for proliferation and migration in response to high levels

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of angiotensin II (Ang II), partly by the induction of excessive reactive oxygen species (ROS) (2, 30). Many migration and proliferation signaling events stimulated by ROS are mediated by members of the mitogen-activated protein kinase (MAPK) family, including the extracellular signal-regulated kinase1/2 (ERK1/2), p38 MAPK, and the c-Jun NH_2-terminal kinase (JNK) (2, 26), and by the ezrin-radixin-moesin (ERM) protein family (1, 12, 13). Consistent with this notion, recent studies revealed that treatment with antioxidants inhibited the abnormal proliferation and migration of VSMCs, and then improved vascular remodeling diseases (2, 19).

Hydrogen (H_2) has recently attracted considerable attention for the prevention of oxidative stress related vascular diseases (4, 21, 23, 27, 28, 32, 36). Hydrogen-rich saline inhibited endothelial dysfunction in spontaneous hypertensive rats (36), and reduced pulmonary artery medial smooth muscle layer hypertrophy and lumen stenosis on monocrotaline-induced pulmonary hypertension in a rat model (32). Hydrogen-rich saline also prevented neointima formation after carotid balloon injury *via* suppressing ROS production and inactivating the Ras-MEK1/2-ERK1/2 signaling pathway (4, 23). Hydrogen-rich medium inhibited VSMCs proliferation and migration induced by platelet-derived growth factor-BB (PDGF-BB) or fetal bovine serum (FBS) *in vitro* (4, 23). However, the effects of hydrogen on proliferation and migration of VSMCs stimulated by Ang II both *in vitro* and *in vivo*, and the related signaling mechanisms still remain unclear.

The aims of this study were, therefore, to determine whether hydrogen attenuates proliferation and migration of VSMCs stimulated by Ang II *in vitro*, and whether hydrogen protects against vascular hypertrophy induced by abdominal aortic coarctation (AAC) *in vivo*, as well as to identify the molecular mechanisms that may be responsible for its putative effects.

Materials and Methods

Drugs and Chemicals

Ang II (A9525, Sigma-Aldrich St. Louis, MO, USA) powders were dissolved as 10^{-4} M stock solution using the high pressure deionized water. Hydrogen gas (99.999%, Guang Zhou Guang Qi GAS Co., Ltd. Guangdong, PRC) was stored in the seamless steel gas cylinder, and it was injected into an aseptic soft plastic infusion bag under sterile conditions before intraperitoneal injection. The antibodies against ERK1/2, p-ERK1/2, p38 MAPK, p-p38 MAPK, JNK, p-JNK were from Cell Signaling Technology (Danvers, MA, USA). The antibodies against ERM and p-ERM were from ImmunoWay. The antibody against β-actin was from Santa Cruz Biotechnology. The antibody against 3-nitrotyrosine was from Abcam.

Preparation of Hydrogen-Rich Medium and Measurement of Hydrogen Concentration

The hydrogen-rich medium was prepared as previously described with a slight modification (8). Briefly, an aseptic soft plastic infusion bag with the volume of 100 ml was vacuumized, and then 20 ml Dulbecco's Modified Eagle Medium (DMEM; Gibco, New York City, NY, USA), which was supplemented with 100 U/ml penicillin/streptomycin (Hyclone), was injected into the bag. Hydrogen-rich medium was made by bubbling the medium with 99.999% hydrogen from the seamless steel gas cylinder until the bag was full of hydrogen. The medium was maintained at 4°C for more than 6 h before use. MB-Pt reagent (MiZ Company, Fujisawa-shi, Kanagawa-ken, Japan, a generous gift of Yan ming) was used to determine the concentration of hydrogen as previously described by Seo T. *et al.* (24). However, DMEM is a complicated mixture (containing amino acids, vitamins, inorganic salts and glucose), and DMEM itself can react with MB-Pt reagent. Thus, we used MB-Pt reagent to drop into hydrogen-rich water (prepared as hydrogen-rich medium) to indirectly reflect the concentrations of hydrogen in hydrogen-rich medium, and the hydrogen concentrations in hydrogen-rich water/medium was no less than 0.6 ppm (0.6~0.9 ppm).

Cell Culture and Treatment

The primary culture of rat aortic VSMCs was prepared as we have previously described (16). VSMCs were grown in DMEM (containing 5.5 mM glucose) supplemented with 100 U/ml penicillin/streptomycin and 10% FBS (Gibco) at 37°C under an atmosphere of 5% CO_2. Experiments were performed with cells from passages 4 to 8. VSMCs were grown to 60~70% confluence before being starved in FBS-free DMEM for 24 h. Quiescent VSMCs were pretreated with hydrogen-rich medium or control media for 30 min. After pre-treatment, cells were stimulated with 10^{-7} M Ang II for 24 h.

Wound Healing Assay

The relative cell migration ability was determined as previously described with a slight modification (27). Briefly, VSMCs was cultured to 100% confluence, and then cells were serum starved for 24 h in DMEM. Scratch 'wounds' were made with a 10 μl pipette tip and photographs were taken immediately after wounding. Before Ang II stimulation, the cultures were incubated with control medium or hydrogen-rich medium

for 30 min, then photographs were taken immediately again after treating cells with 10^{-7} M Ang II for 24 h. Cell migration to close the wounded area was measured. Results were expressed as a relative migration index according to the formula below:

Relative migration index = Distance of cell migration in treatment group/Distance of cell migration in control group × 100%.

Cell Proliferation Assay

The relative cell proliferation ability was determined as previously described with a slight modification (10). Briefly, after VSMCs were cultured in 96-well plates and received different treatments, the medium was changed and 10 μl CCK-8 solution (Dojindo, Japan) was added to each well at a 1/10 dilution, followed by a further 1.0 h incubation in the incubator. The mean optical density (OD) was measured at 450 nm with a microplate reader (Multiskan MK3 Microplate reader, Thermo Fisher Scientific Inc., Waltham, MA, USA). Results were expressed as relative cell proliferation ability according to the formula below:

Relative cell proliferation ability = OD treatment group/OD control group × 100%.

AAC-Induced Vascular Hypertrophy Model and Treatment Protocol

Male Wistar rats (weight about 200 to 225 g) were obtained from the Center for Animal Resources 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 the Institute for Animal Care and Use Committee at Sun Yat-sen University School of Medicine and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Rats were randomly assigned to four groups: Control group (n = 8), AAC group (n = 10), AAC plus hydrogen group (n = 10), hydrogen group (n = 8). AAC plus hydrogen group and hydrogen group were given hydrogen by intraperitoneal injection at the dose of 1 ml/100 g/day consecutively for one week before AAC and lasted for 6 weeks (14). Vascular hypertrophy was induced by AAC as previously described (18), which mainly displayed the increased in aortic medial smooth muscle cell number without a change in mean cellular volume or mass (22). Briefly, rats were anesthetized with chloral hydrate, and exposed their abdominal aortas. AAC was operated below the celiac trunk and above the superior mesentric artery using 1-0 silk and 21-gauge wire, which is removed to generate a defined construction. Five weeks after AAC, animals were sacrificed, blood samples were collected for measurement of malondialdehyde (MDA) and Ang II, and abdominal arteries with the coarctation site were excised, rinsed with ice-PBS. Then arteries were fixed in 10% buffered formalin until analyzed. Arteries were cut transversely above and close to the coarctation site. Several sections of arteries (4~5 μm thickness) were prepared and stained with hematoxylin and eosin (H&E) for histopathology and then visualized by light microscopy. After digitized images were obtained, the relative aortic medial thickness was measured by computer planimetry using cellSens Entry (Olympus, Shinjuku, Tokyo, Japan).

Western Blotting

Total proteins were extracted from VSMCs with the lysis buffer containing 100 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.3% phosphatase inhibitor cocktail (Sigma) and 1% protease inhibitor cocktail (Sigma). The protein concentration was determined using the Pierce BCA Protein Assay kit (Pierce, Waltham, MA, USA). Thirty micrograms of proteins were used for SDS-PAGE, and the proteins were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Primary and secondary antibodies were incubated with the membranes with the standard technique. Immunodetection was accomplished using enhanced chemiluminescence (ChemiDoc XRS+ System, Bio-Rad, Hercules, CA, USA).

Assessment of VSMCs ROS Levels

VSMCs ROS was examined using peroxide-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA, KeyGen BioTECH, Nanjing, Jiangsu, PRC) as previously described (5). Briefly, after VSMCs were cultured in 6-well plates and received different treatments, the medium was changed, cells were incubated with 10^{-5} M DCFH-DA in the dark for 60 min in the incubator. Treated cells were washed with DMEM for 3 times and harvested by trypsinization (without EDTA). Then, cells were plated in 96-well plate, and fluorescence of oxidized DCF was recorded with excitation/emission wavelength at 485/527 nm using fluorescence plate reader immediately.

Biochemical Analysis of MDA and Ang II

Blood was collected and subsequently was centrifuged for 10 min at 650 × *g*, and then serum was refrigerated at –80°C for subsequent experiments. Serum MDA was determined using a commercially available kit (Nanjing Jiancheng Bioengineering Institute, Nanjing,

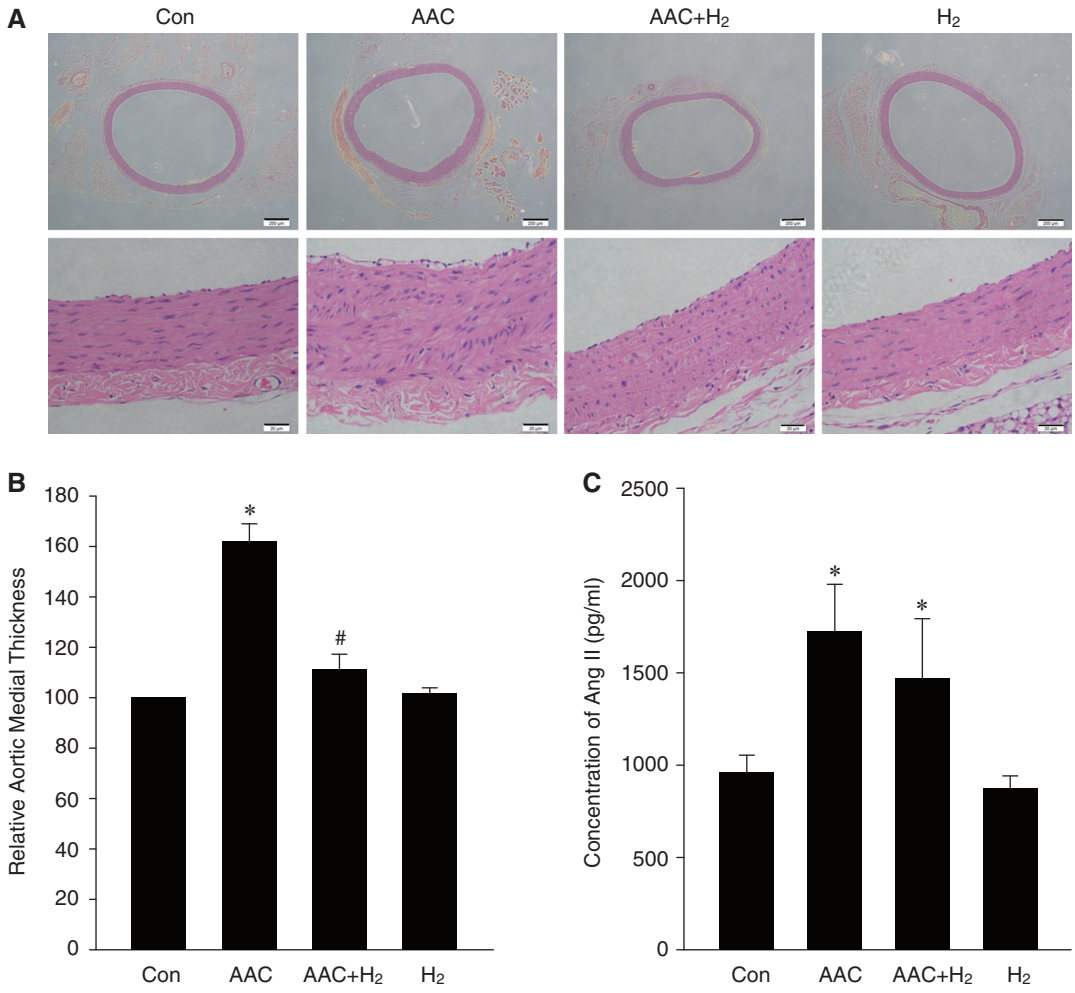

Fig. 1. The effect of hydrogen on vascular hypertrophy and serum Ang II levels induced by AAC. (A) H&E-stained sections show the abdominal aortic structures from normal group and AAC group with or without hydrogen. (B) The relative aortic medial thickness were quantified (n = 8~10). (C) Serum Ang II levels were monitored after 5 weeks of AAC (n = 8~10). $^{*}P < 0.05$ *vs*. Con, $^{\#}P < 0.05$ *vs*. AAC. Scale bar: 200 μm (upper panel), 20 μm (lower panel).

Jiangsu, PRC) according to the manufacturer's instructions. Serum Ang II was determined as previously described (34).

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

Hydrogen Attenuates AAC-Induced Vascular Remodeling In Vivo

In order to examine the inhibitory effect of hydrogen on vascular remodeling *in vivo*, we used AAC-induced vascular hypertrophy animal model. Five weeks after AAC, rats developed into vascular hypertrophy above the coarctation site and with higher serum Ang II level compared with control group (Fig. 1, A, B and C). Interestingly, hydrogen pretreatment inhibited vascular hypertrophy (Fig. 1, A and B). However, there were no significant changes in serum Ang II levels between AAC group and AAC plus hydrogen group (Fig. 1, C). Collectively, these data suggest that hydrogen prevents the development of AAC-induced vascular hypertrophy *in vivo*.

Hydrogen Reduces Ang II-Induced Migration and Proliferation of VSMCs In Vitro

To investigate the possible cytotoxity of hydrogen in VSMCs *in vitro*, we examined cell viability using CCK-8 assay. Hydrogen was determined to be non-cytotoxic for VSMCs after treating with hydrogen-rich

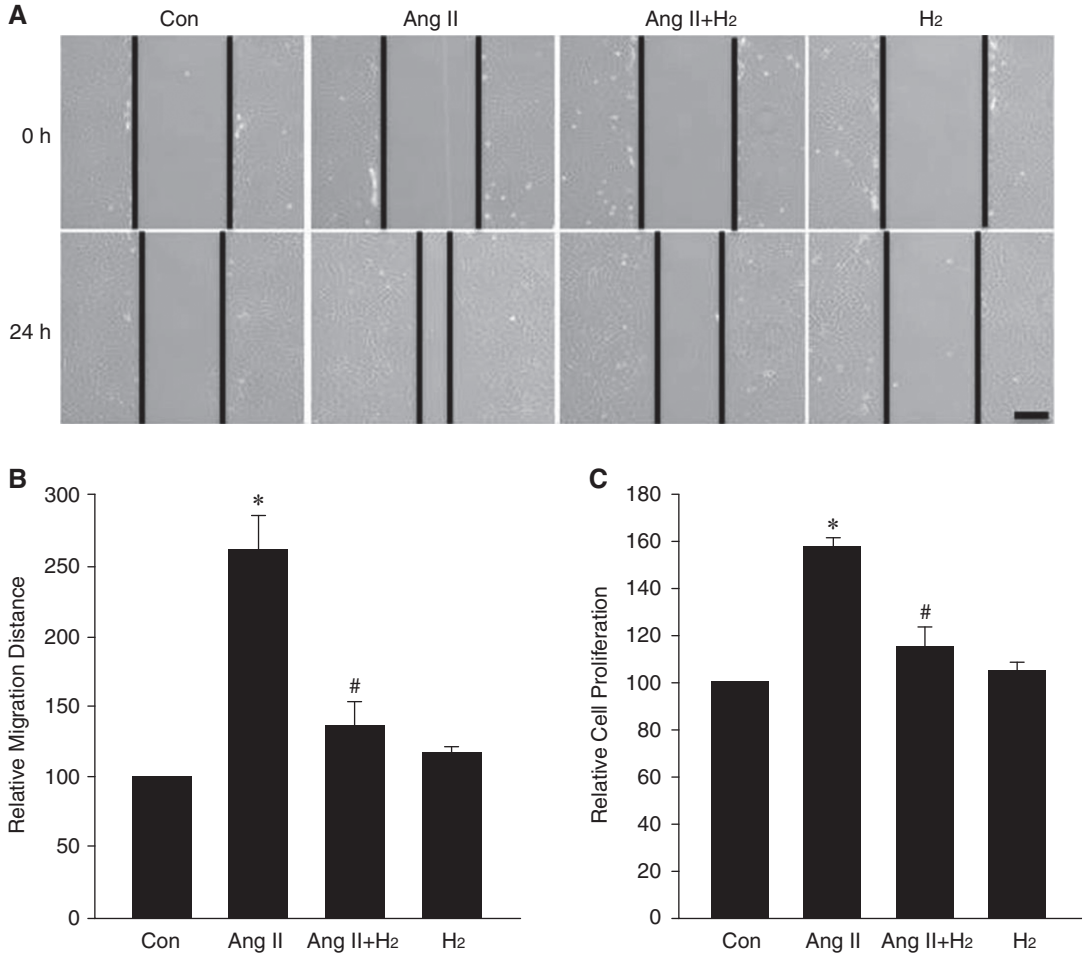

Fig. 2. Hydrogen inhibits VSMCs migration and proliferation stimulated by Ang II *in vitro*. (A) Migration assay was determined after treating with Ang II for 24 h with or without hydrogen. (B) The bar graph shows the migration index in VSMCs (n = 10). (C) Proliferation index was determined when treating with Ang II for 24 h with or without hydrogen (n = 6). $^{*}P < 0.05$ *vs.* Con, $^{\#}P < 0.05$ *vs.* Ang II. Scale bar: 100 μm.

medium for 24 h (data not shown). To examine the effects of hydrogen on Ang II-induced proliferation and migration of VSMCs *in vitro*, VSMCs were pre-incubated with hydrogen-rich medium for 30 min and then subsequently treated with Ang II (10^{-7} M) for 24 h. Hydrogen decreased VSMCs migration (Fig. 2, A and B) and proliferation (Fig. 2C) stimulated by Ang II. However, hydrogen alone had no effect on migration (Fig. 2, A and B) and proliferation (Fig. 2C) of VSMCs. Therefore, these data suggest that hydrogen inhibits VSMCs migration and proliferation stimulated by Ang II *in vitro*.

Hydrogen Blocks the Excess Production of ROS In Vitro *and* In Vivo

ROS play a critical role in the development of hypertension (including Ang II)-induced abnormal proliferation and migration of VSMCs *in vitro* and *in vivo* (2, 30, 31). In order to detect the levels of ROS, we used DCFH-DA to measure intracellular ROS. VSMCs were pretreated with hydrogen-rich medium for 30 min and subsequently incubated with 10^{-7} M Ang II for 24 h. The results showed that Ang II markedly increased the levels of ROS and this effect was blocked by hydrogen treatment (Fig. 3A). Recent studies have indicated that excess production of ROS could lead to lipid peroxidation, and lipid peroxidation has an important impact on vascular hypertrophy under pathological stimulation (3, 5, 23, 28, 32, 36). Therefore, we examined the effects of hydrogen on AAC-induced lipid peroxidation. Accordingly, hydrogen obviously suppressed serum lipid peroxidation induced by AAC (Fig. 3B). Moreover, another oxidative stress marker, 3-nitrotyrosine, which can reflect the formation of ONOO-, was also markedly inhibited in abdominal arteries above and close to the coarctation site by hydrogen treatment (Fig. 3, C and D). Thus, this is quite likely that hydrogen inhibits Ang II-induced oxidative stress of VSMCs *in vitro* or AAC-induced oxidative

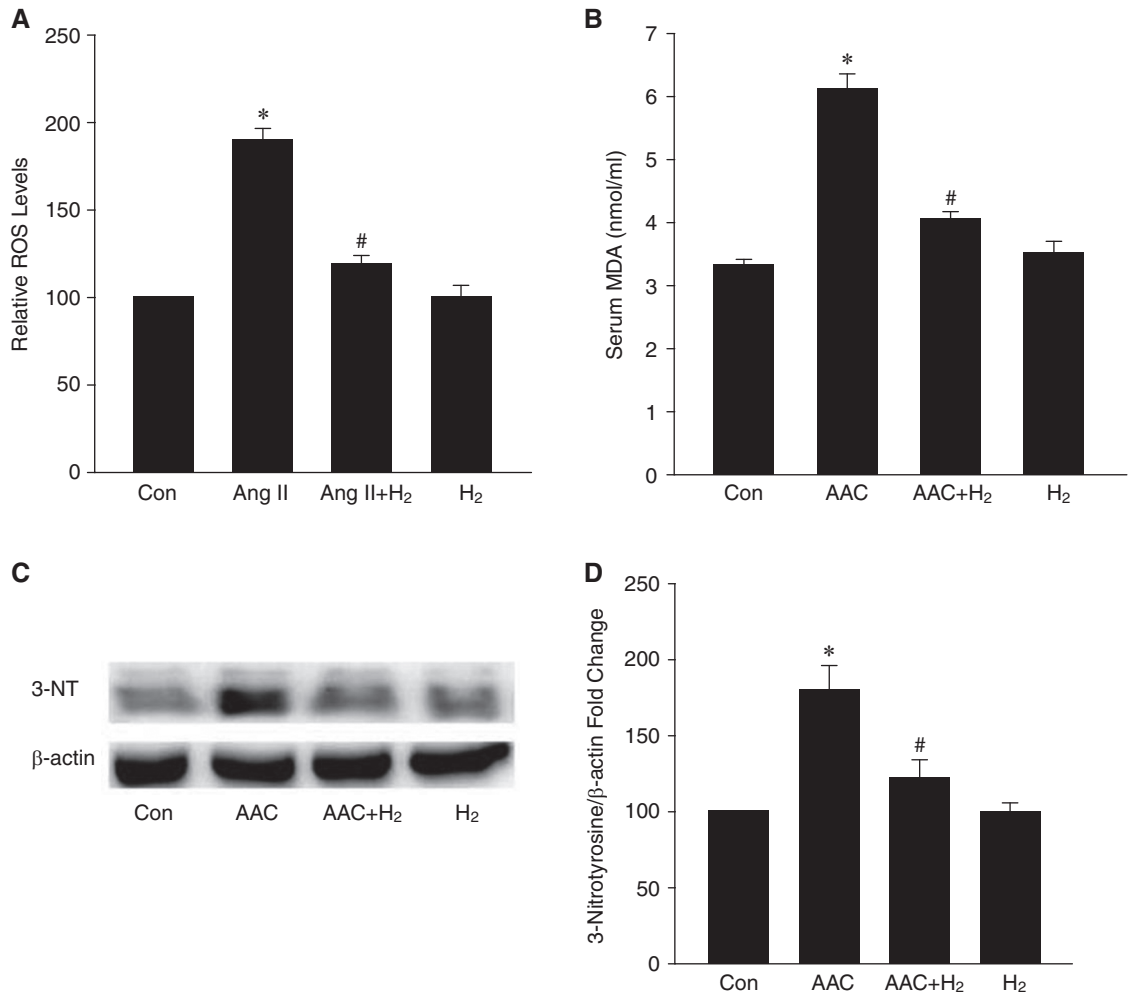

Fig. 3. Hydrogen blocks the excess production of ROS *in vitro* and *in vivo*. (A) 24 h after Ang II treatment with or without hydrogen, VSMCs were loaded with DCFH-DA and the cell-associated fluorescent intensities was determined (n = 6). (B) Serum MDA was measured 5 weeks after AAC with or without hydrogen (n = 8~10). (C) and (D) Representative western blot and quantification of 3-nitrotyrosine (3-NT) to β-actin in abdominal arteries 5 weeks after AAC with or without hydrogen (n = 3). $^{*}P < 0.05$ *vs.* Con, $^{\#}P < 0.05$ *vs.* Ang II or AAC.

stress *in vivo* through decreasing excess production of ROS.

Hydrogen Inactivates MAPK and ERM Signaling In Vitro

Based on the inhibitory effect of hydrogen on the excess production of ROS *in vitro* and *in vivo* in response to Ang II or AAC, we further investigated its effect on the downstream signaling targets, such as members of the MAPK family and ERM family *in vitro*. Our data suggested that ERK1/2, p38, JNK and ERM were significantly phosphorylated in Ang II-treated VSMCs compared with control group, and such changes were inhibited by pretreatment with hydrogen-rich medium (Fig. 4, A, B, C and D). Although hydrogen alone had no effect on the phosphorylation of ERK1/2, p38, JNK, it partially inhibited ERM phosphorylation (Fig. 4, A, B, C and D). Taken together, these findings suggest that ERK1/2, p38, JNK and ERM signaling play important roles in hydrogen-mediated inhibitory effects on Ang II-induced abnormal proliferation and migration of VSMCs *in vitro*.

Discussion

The present study demonstrates that intraperitoneal injection hydrogen protects against the development of vascular hypertrophy induced by AAC *in vivo,* and hydrogen-rich medium prevents the proliferation and migration of VSMCs stimulated by Ang II *in vitro*. The vascular protection of hydrogen is mediated by direct interruption of ROS-dependent ERK1/2, p38, JNK and ERM signaling, which leads to the inhibition of abnormal proliferation and migration of VCMCs.

Recently, mounting evidence uncovered that

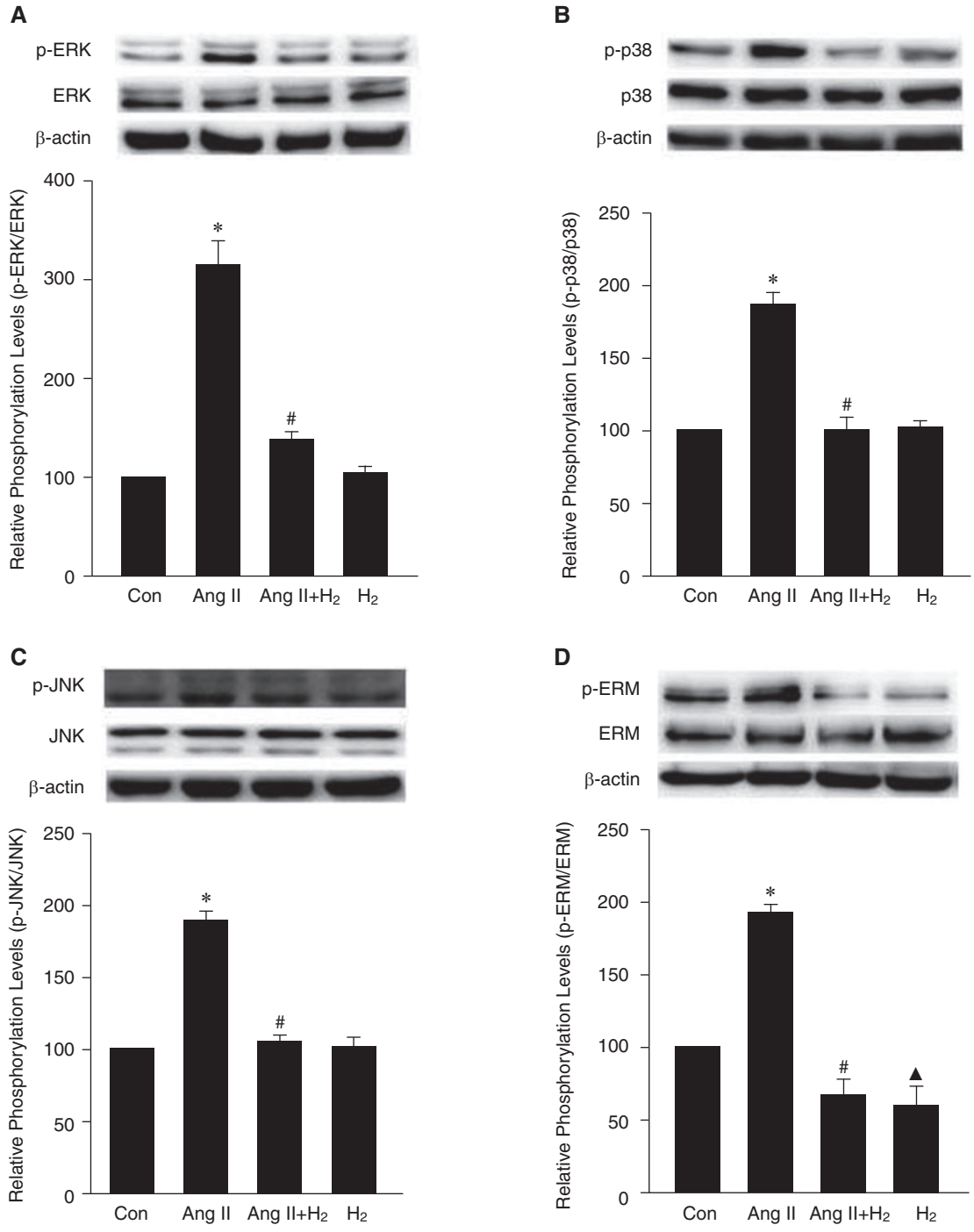

Fig. 4. Hydrogen inhibits MAPK and ERM signaling *in vitro*. (A) Representative western blot and quantification of ERK1/2 phosphorylation to its total protein expressions in the VSMCs stimulated by Ang II 24 h with or without hydrogen (n = 4). (B) Representative western blot and quantification of p38 phosphorylation to its total protein expressions (n = 4). (C) Representative western blot and quantification of JNK phosphorylation to its total protein expressions (n = 4). (D) Representative western blot and quantification of ERM phosphorylation to its total protein expressions (n = 4). $^{*}P < 0.05$ *vs.* Con, $^{\#}P < 0.05$ *vs.* Ang II, $^{\blacktriangle}P < 0.05$ *vs.* Con.

molecule hydrogen could prevent oxidative stress-related vascular diseases (4, 21, 23, 27, 28, 32, 36). Oral intake of hydrogen-rich water inhibited intimal hyperplasia in arterialized vein grafts in rats by preserving the endothelial integrity with less platelet and white blood cell aggregation (27). Hydrogen-rich saline prevented neointima formation after carotid balloon injury (4, 23), reduced pulmonary artery medial

smooth muscle layer hypertrophy and lumen stenosis on monocrotaline-induced pulmonary hypertension in a rat model (32), and also improved aortic hypertrophy and endothelial function in spontaneous hypertensive rats (36). Hydrogen-rich medium could inhibit VSMCs migration and proliferation induced by PDGF-BB or FBS *in vitro* (4, 23, 27). However, the effects of hydrogen on the development of vascular hypertrophy induced by AAC *in vivo,* and on the proliferation and migration of VSMCs stimulated by Ang II *in vitro* have not yet been clarified.

Unlike that the aortic medial hypertrophy in spontaneously hypertensive and Goldblatt hypertensive rats was due to the cellular hypertrophy, the AAC induced the increased in aortic medial smooth muscle cell number without a change in mean cellular volume or mass (22). Interestingly, we found that hydrogen attenuated vascular hypertrophy induced by AAC *in vivo.* However, hydrogen had no significant effect on circulating Ang II level, thereby the protective effect of hydrogen on vascular hypertrophy is possibly by blocking circulating Ang II actions on vessels (especially in VSMCs) rather than inhibiting its synthesis and secretion. Thus, we used Ang II-induced proliferation and migration of VSMCs *in vitro* to further explore the underlying molecule mechanisms of hydrogen.

The excess activation of ROS has been shown to contribute to the development of abnormal proliferation and migration of VSMCs both *in vitro* and *in vivo* (2, 30, 31). Importantly, previous studies showed that hydrogen could inhibit ROS excess activation in various animal models, such as malignant neoplasms (6, 35), polymicrobial sepsis (33), brain injury (20), cardiac injury (11) and so forth. Therefore, the inhibitory mechanisms of hydrogen on abnormal proliferation and migration of VSMCs were examined for its effect on ROS activation. We found that hydrogen blocked ROS activation induced by Ang II in cultured VSMCs *in vitro*, suppressed 3-nitrotyrosine formation in abdominal arteries above and close to the coarctation site, and reduced serum MDA levels induced by AAC *in vivo*. Taken together, these results indicate that the anti-proliferation and anti-migration effects of hydrogen can be in partly ascribed to blocking ROS activation.

It is well known that ROS transmits signals to prominent downstream pathways, such as MAPK and ERM pathways (13, 29). Among the MAPK, ERK1/2, p38 and JNK have been considered as the essential regulators of VSMCs proliferation and migration response (2, 25, 26, 30). Furthermore, ERM family also plays an important role in arteriosclerotic lesion formation *in vivo* induced by long-term continuous administration of Ang II (12). PDGF promotes VSMCs migration by activating ERM (1). Therefore, we further investigated the effect of hydrogen on the downstream signaling targets such as MAPK and ERM signaling pathways *in vitro.* Our data clearly demonstrated that hydrogen markedly blocked ERK1/2, p38, JNK and ERM activation *in vitro* in response to Ang II stimulus, without affecting the total protein of ERK1/2, p38, JNK and ERM. These findings confirmed ERK1/2, p38, JNK and ERM as pivotal regulators of Ang II-induced proliferation and migration of VSMCs, and revealed that the anti-proliferation and anti-migration effects of hydrogen was achieved through blocking ROS-dependent MAPK and ERM signaling. In accordance with our findings, Sun *et al*. (27) reported that drinking hydrogen-rich water effectively inhibited intimal hyperplasia in arterialized vein grafts in rats *via* inhibiting p38 activation. Chen *et al*. (4) also demonstrated that hydrogen-rich saline (or medium) inhibited neointimal hyperplasia induced by carotid balloon injury (or FBS-induced VSMCs proliferation and migration) through reducing ROS-induced activation of ERK1/2. However, hydrogen treatment inhibited LPS/IFNγ-induced phosphorylation of p38 and JNK, but did not affect that of ERK1/2 in RAW264 macrophage cells (15). The discrepancy on MAPK activation may be explained by different cell types, animal models and different stimuli.

In conclusion, our results demonstrate that hydrogen inhibits AAC-induced vascular hypertrophy *in vivo,* and suppresses proliferation and migration of VSMCs with Ang II stimulation *in vitro* by blocking excess activation of ROS, and we further demonstrate that ROS-dependent ERK1/2, p38, JNK and ERM signaling pathways are targets of the inhibitory actions of hydrogen *in vitro*. This study serves to elucidate the inhibitory effect of hydrogen on abnormal proliferation and migration of VSMCs and its related molecular mechanisms. More importantly, our results provide experimental evidence for the application of hydrogen in the treatment of vascular remodeling diseases.

Acknowledgments

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