Inhalation of hydrogen gas attenuates left ventricular remodeling induced by intermittent hypoxia in mice

Tetsuya Hayashi,1,2 Toshitaka Yoshioka,3 Kenichi Hasegawa,3 Masatoshi Miyamura,1 Tatsuhiro Mori,1 Akira Ukimura,1 Yasuo Matsumura,3 and Nobukazu Ishizaka1

1Faculty of Medicine, Department of Internal Medicine III, Takatsuki; Medical College, Takatsuki; 2Clinical Research Center, Osaka Medical College Hospital, Osaka; and 3Laboratory of Pathological and Molecular Pharmacology, Osaka University of Pharmaceutical Sciences, Takatsuki, Japan

Submitted 14 February 2011; accepted in final form 20 May 2011

Hydrogen (H2) gas is physiologically produced in the large intestine by intestinal bacteria during the fermentation of nondigestible carbohydrates (3). Recent studies have suggested that H2 gas may selectively scavenge hydroxyl radicals and thus exert an antioxidant effect (18). Ohsawa et al. (21) demonstrated that ingestion of H2-saturated water decreased oxidative stress and prevented atherosclerosis in apolipoprotein E knockout mice, whereas inhalation of H2 gas was reported to have a cardioprotective effect against ischemia-reperfusion injury in rats (9). However, the effects of H2 gas inhalation on progression of LV remodeling and alteration of the lipid profile have not been investigated in an experimental animal model of SAS. Therefore, we examined H2 gas inhalation using three different regimens to investigate its effect on lipid abnormalities and LV remodeling induced by intermittent hypoxia in mice.

MATERIALS AND METHODS

Animals. We used 8-wk-old male C57BL/6J mice (purchased from Clea Japan, Osaka, Japan) in this study. Animals were kept under a 12:12-h light-dark cycle and were allowed free access to standard chow and tap water. The study protocol and animal care methods were approved by the Osaka University of Pharmaceutical Sciences Experimental Animal Research Committee, and all experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Study protocol. Animals were placed in a chamber and were exposed to intermittent hypoxia (IH, repetitive cycles of 1 min each with 5 and 21% oxygen for 8 h during the daytime) with or without administration of H2 gas for seven consecutive days (n = 62). Repetitive administration of H2 gas (1.3 vol/100 vol) was done at the time of reoxygenation (H2-reoxy), during hypoxia (H2-hypo), or throughout the experimental period (H2-through). At 20 h after the last exposure to hypoxia, measurement of right ventricular and LV systolic pressures was performed by cardiac catheterization under anesthesia induced by intraperitoneal injection of pentobarbital sodium (50 mg/kg). After blood sampling, the heart was excised, and the upper half cut beneath the mitral valve level was subjected to light microscopic examination. The LV free wall myocardium was also excised for electron microscopic examination, immunohistochemistry, and real-time reverse transcription-polymerase chain reaction (RT-PCR).

Address for reprint requests and other correspondence: T. Hayashi, Dept. of Internal Medicine III, Osaka Medical College, 2-7 Daigakumachi, Takatsuki, Osaka 569-8686, Japan (e-mail: in3015@poh.osaka-med.ac.jp).
Measurement of lipid profiles. Plasma lipoproteins were analyzed using a high-performance liquid chromatography system at Skylight Biotech (LipoSEARCH, Akita, Japan), according to the protocol described elsewhere (32). Briefly, the cholesterol concentration in major lipoproteins and lipoprotein subclasses [from G1 (particle diameter >80 nm) to G13 (particle diameter >16.7 nm)] was determined by modified Gaussian curve fitting to resolve overlapping peaks (22).

Measurement of cross-sectional area and histological examination. Heart tissues were fixed in 10% formaldehyde, embedded in paraffin, and cut into 4-μm sections. Photographs sampled by a light microscope at 400 magnification were transformed to the binary images, and cardiac cross-sectional area was measured using ImageJ version 1.44 software [National Institutes of Health (NIH)]. To determine the mean cardiomyocyte diameter, the shortest diameters of 100 cardiomyocytes in each sample were measured using NIS-Elements version 3.07 software (Nikon), only in nucleated transverse sections stained with hematoxylin-eosin under a light microscope at 400 magnification. After staining with Sirius red, color images were taken from five randomly selected high-power fields (×200), and the percentage of collagen volume was calculated from the method previously reported (34). For electron microscopy, part of the LV myocardium was fixed in 4% paraformaldehyde containing 0.25% glutaraldehyde and 4.5% sucrose. Ultrathin sections obtained from the embedded blocks were stained with uranyl acetate and lead citrate and examined using an electron microscope (model H-7650; Hitachi, Tokyo, Japan) (7).

Immunohistochemistry for 4-hydroxy-2-nonenal protein expression. Immunohistochemical staining was performed to determine the amount of 4-hydroxy-2-nonenal (4-HNE)-modified protein adducts (8). Briefly, paraffin sections of the left ventricle were incubated with a monoclonal antibody directed against 4-HNE (product no. MNH-20; Japan Institute for the Control of Aging, Shizuoka, Japan) and a secondary antibody (biotinylated anti-mouse IgG), followed by the addition of Vectastatin Elite ABC reagent (Vector Laboratories, Burlingame, CA). The percent area of 4-HNE staining was measured by quantitative analysis (16). Results were compared with the mean percent area of control that was defined 1.0 and expressed as the 4-HNE expression ratio.

Detection of superoxide in the LV myocardium. To detect the production of superoxide in situ, freshly frozen unfixed LV specimens were incubated with 10 μmol/l of dihydroethidium (DHE; Molecular Probes, Eugene, OR) solution for 30 min in a light-protected humidified chamber at 37°C. Sections were then examined using a fluorescence microscope (BZ-8000; KEYENCE, Osaka, Japan). DHE fluorescence intensity was quantified using NIH Image 1.61 software and compared with the mean intensity of control (17).

Quantitative real-time RT-PCR. Total RNA was extracted from LV myocardial tissues using an RNeasy Mini Kit (Qiagen, Valencia, CA). Reverse transcription was performed with random hexamers and oligo(dT) primers. Quantitative real-time reverse transcription-polymerase chain reaction was performed on an ABI PRISM 7500 (Applied Biosystems, Foster City, CA). Detection of target gene expression was performed with the Melt Curve option, and the expression of each gene was normalized to the expression of the housekeeping genes 18S rRNA and the hypoxia-induced factor-1α (HIF-1α). The results were expressed as the fold change relative to normoxia.

Table 1. Effects of H2 gas inhalation on body weight, heart weight, and hemodynamics

<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Intermittent Hypoxia</th>
<th>Reoxygenation</th>
<th>Hypoxia</th>
<th>Throughout</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>13</td>
<td>12</td>
<td>18</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>23.7 ± 0.6</td>
<td>22.5 ± 0.3</td>
<td>22.1 ± 0.2</td>
<td>21.2 ± 0.2</td>
<td>22.0 ± 0.3</td>
</tr>
<tr>
<td>Heart wt, mg</td>
<td>107.2 ± 1.5</td>
<td>116.2 ± 3.1</td>
<td>107.3 ± 1.6</td>
<td>106.4 ± 2.4</td>
<td>105.2 ± 2.4*</td>
</tr>
<tr>
<td>Heart wt/body wt, mg/g</td>
<td>4.5 ± 0.1</td>
<td>5.1 ± 0.1*</td>
<td>4.8 ± 0.1</td>
<td>5.0 ± 0.1*</td>
<td>4.7 ± 0.1</td>
</tr>
</tbody>
</table>

Hemodynamic parameters

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>3</td>
<td>4</td>
<td>6</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>RVSP, mmHg</td>
<td>23.7 ± 3.2</td>
<td>33.5 ± 2.9</td>
<td>32.0 ± 1.3</td>
<td>30.4 ± 2.0</td>
<td>29.2 ± 2.0</td>
</tr>
<tr>
<td>LVSP, mmHg</td>
<td>106.0 ± 2.1</td>
<td>108.5 ± 1.8</td>
<td>100.7 ± 4.9</td>
<td>99.4 ± 2.4</td>
<td>110.5 ± 5.2</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals. Heart wt/body wt, heart weight-to-body weight ratio; RVSP, right ventricular systolic pressure; LVSP, left ventricular systolic pressure. P < 0.05 vs. normoxia (*) and vs. intermittent hypoxia (#).

AJP-Heart Circ Physiol• Vol. 301 • September 2011 • www.ajpheart.org
Superscript reverse transcriptase (Invitrogen, Carlsbad, CA). RT-PCR was performed using an ABI Step One sequence detector (PE Applied Biosystems, Foster City, CA). Taqman Probe and primers for the endogenous control mRNA (Ma99999915 for GAPDH) and the target mRNA [Ma00443258 for tumor necrosis factor-α (TNF-α), Ma00446191 for interleukin-6 (IL-6), and Ma00435304 for brain natriuretic peptide (BNP)] were purchased from Applied Biosystems. The reaction conditions were as follows: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. The level of the target mRNA was normalized to that of GAPDH mRNA (11).

**Statistical analysis.** Values are means ± SE. For statistical analyses, we used one-way ANOVA followed by Tukey-Kramer multiple-

Fig. 2. Plasma lipoproteins analyzed using high-performance liquid chromatography. Intermittent hypoxia (Hypoxia) elevated low- and very low-density lipoproteins (LDL and VLDL, respectively) of plasma cholesterol in mice (□). Inhalation of H2 gas throughout the experiment (▲) significantly suppressed the increases in LDL and VLDL. Marks and bars represent means ± SE. **P < 0.01 vs. normoxia (●); #P < 0.05 and ##P < 0.01 vs. hypoxia.

Fig. 3. Effects of H2 gas on plasma cholesterol subclasses in mice. Fractions from G3 through G10 are shown. Intermittent hypoxia (Hypoxia) significantly increased each plasma cholesterol fraction. In fractions G5, G6, G7, G8, G9, and G10, H2-reoxy as well as H2-throughout effectively suppressed the increase in plasma cholesterol. Values are means ± SE of the no. of animals in parentheses. **P < 0.01 vs. normoxia; #P < 0.05 and ##P < 0.01 vs. intermittent hypoxia.
comparison tests. A probability (P) value of <0.05 was taken as indicating statistical significance.

RESULTS

Body weight, heart weight, and hemodynamics. The heart-to-body weight ratio was increased significantly in IH and H2-hypo. H2-throughout significantly decreased the heart weight compared with IH (Table 1). No significant differences of right ventricular and LV systolic pressures were seen among the five groups.

Plasma cholesterol lipoprotein profile. IH significantly increased plasma levels of low- and very low-density cholesterol lipoproteins (LDL-C and VLDL-C, respectively) (Fig. 2). In fractions G5–G10, H2-reoxy and H2-throughout significantly suppressed the increase of LDL-C and VLDL-C caused by IH (Fig. 3). There were no significant changes of high-density lipoprotein cholesterol.

Histological findings. Light microscopy revealed that IH significantly increased the cardiac cross-sectional area, the cardiomyocyte diameter, and the percentage of perivascular fibrosis in the LV myocardium. These changes were suppressed significantly by inhalation of H2 gas. H2-throughout was more effective in reducing cardiac cross-sectional area than H2-reoxy and in preventing cardiomyocyte hypertrophy than H2-reoxy and H2-hypo (Fig. 4). Electron microscopy showed that IH increased nuclear invagination and the number of irregular mitochondria in the LV myocardium. Ballooning and loss of cristae in many mitochondria, and myelin-like figures, were observed occasionally. In the groups with H2 gas inhalation, especially in the H2-throughout group, the fine structure of the LV myocardium was well preserved (Fig. 5).

Superoxide production and 4-HNE expression in the LV myocardium. Superoxide production (detected by DHE labeling) and 4-HNE-modified protein adducts were increased significantly in the LV myocardium of IH mice. Inhalation of H2 gas significantly suppressed superoxide production and 4-HNE adducts in the LV myocardium (Fig. 6).

RT-PCR. IH significantly increased the expression of TNF-α, IL-6, and BNP mRNA in the LV myocardium. Inhalation of H2 gas, regardless of the timing of administration, significantly reduced the expression of TNF-α, IL-6, and BNP mRNA in the LV myocardium (Fig. 7).

DISCUSSION

We demonstrated in the present mouse model that inhalation of H2 gas at low concentrations (1.3 vol/100 vol) reduced IH-induced dyslipidemia when done at the time of reoxygenation and also prevented cardiomyocyte hypertrophy and perivascular fibrosis in the LV myocardium. Although H2 is reported to have no risk of igniting at concentrations of <4% (19), we only used 1.3 vol/100 vol H2 (the highest concentra-

![Fig. 4. Representative light micrographs and quantitative measurement of cardiac cross-sectional area, cardiomyocyte diameter, and percentage of collagen (%fibrosis) in the left ventricular (LV) myocardium. Intermittent hypoxia caused cardiac hypertrophy (D), and cardiomyocyte degeneration (E) and increased perivascular fibrosis (F) in the LV myocardium. Effects that were suppressed by H2 gas inhalation, especially in the H2-throughout group (G–I). Quantitatively, intermittent hypoxia significantly increased the cardiac cross-sectional area, the mean cardiomyocyte diameter, and perivascular fibrosis. Inhalation of H2 gas significantly suppressed these changes. Values are means ± SE of the no. of animals in parentheses. ***P < 0.001 vs. normoxia; #P < 0.05 and ###P < 0.001 vs. intermittent hypoxia.](http://ajpheart.physiology.org/)

AJP-Heart Circ Physiol • VOL 301 • SEPTEMBER 2011 • www.ajpheart.org
Fig. 5. Representative electron micrograph of the LV myocardium. Compared with the control group kept under normoxic conditions (A), intermittent hypoxia increased the no. of small and bizarre-shaped mitochondria (Mt) and deformity of nuclei (N) (white arrow) in the LV myocardium (B). Ballooning and loss of cristae of mitochondria and myelin-like figure (arrow) were observed in a myocardial cell judging from the basement membrane (arrowheads) and myofilaments (C). In the groups with H2 gas inhalation, especially in the H2-throughout group, the fine structure of the LV myocardium was well preserved, and lipofuscin and lysosomes were not increased (D). The scale bar indicates 1 μm.

Fig. 6. Dihydroethidium (DHE) labeling (A–C) and immunohistochemistry for 4-hydroxy-2-nonenal (4-HNE) (D–F) in the LV myocardium. Increased superoxide production (B) and oxidative stress (E) were observed in mice exposed to intermittent hypoxic stress, which effects were suppressed by H2 gas inhalation (C and F). Quantitatively, intermittent hypoxia significantly increased superoxide production and 4-HNE-modified protein adducts in the LV myocardium, whereas these changes were suppressed significantly by H2 gas. Suppression of superoxide production by H2 gas was greater in the H2-throughout group than the H2-hypo group. Values are means ± SE of the no. of animals in parentheses. ***P < 0.001 vs. normoxia; #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. hypoxia.
tion allowed for transportation of mixed oxygen/hydrogen gas under Japanese law.

IH relevant to SAS is known to cause dyslipidemia and increase oxidative stress, and SAS has been reported as a risk factor for cardiovascular disease (29, 27). There is increasing evidence that IH is independently associated with dyslipidemia (27, 4). However, the precise mechanisms by which IH induces dyslipidemia are not clear. Free radicals may be involved in the production of oxidized cholesterol (6). Li et al. (15) reported that IH, similar to that used in our experiment, for 5 days increased sterol regulatory element-binding protein-1 and stearoyl-CoA desaturase-1 in the liver of lean mice and also increased fasting serum total cholesterol and triglycerides, with a very small LDL-C peak detected by high-performance liquid chromatography. The increased levels of oxidized LDL in their study may have been related to IH-induced changes of hepatic lipid biosynthesis and cholesterol uptake (4). We also found that IH significantly increased VLDL-C and LDL-C, which was suppressed by H2 inhalation. Intriguingly, H2 gas given at the timing of reoxygenation (H2-reoxy) significantly suppressed increases in VLDL-C and LDL-C (cholesterol fractions G5–G10) induced by IH. These results suggest that radical scavenging by H2 gas is useful in normalizing lipid metabolism in the liver under hypoxic stress (30). Persistent dyslipidemia might exacerbate cardiovascular remodeling induced by hypoxic stress. Further studies, with longer observation periods, are required to clarify the effects of H2 inhalation on cholesterol uptake in the liver.

Oxidative stress plays an important role in the progression of cardiovascular disease (25). In this study, superoxide production and 4-HNE-modified protein adducts in the LV myocardium were increased by intermittent hypoxic stress, whereas these changes were inhibited by 1.3 vol/100 vol H2 gas. It may be that H2 gas reduces oxidative stress by scavenging free radicals. The present study suggested that inhalation of H2 gas suppresses oxidative stress and prevents IH-induced LV remodeling. Ohsawa et al. (20) showed that H2 protects cells and tissues against strong oxidative stress by scavenging the hydroxyl radical. It is also possible that H2 gas protects against oxidative stress by reducing other strong oxidant species in living cells. It was reported that H2 gas decreases the number of degenerated mitochondria and increases the number of healthy mitochondria (20). Our electron microscopy study demonstrated that inhalation of H2 gas increased the number of healthy mitochondria and decreased the number of degenerated mitochondria in the LV myocardium. Although the reason why giving H2 gas throughout tended to suppress TNF-α and BNP mRNA most effectively, no statistical differences were seen between the 3 H2 gas groups. Values are means ± SE of the no. of animals in parentheses. **P < 0.01 vs. normoxia; ###P < 0.01 vs. hypoxia.

**H2 GAS AND HYPOXIA-INDUCED LV REMODELING**

AJP-Heart Circ Physiol • VOL 301 • SEPTEMBER 2011 • www.ajpheart.org

---

Fig. 7. Quantitative RT-PCR. Representative blots of GAPDH show that GAPDH as a housekeeping gene was not affected by intermittent hypoxia. Intermittent hypoxia significantly increased expression of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and brain natriuretic peptide (BNP) mRNA in the LV myocardium. Inhalation of H2 gas significantly reduced cytokine expression in the LV myocardium. Although H2-throughout tended to suppress TNF-α and BNP mRNA most effectively, no statistical differences were seen between the 3 H2 gas groups. Values are means ± SE of the no. of animals in parentheses. **P < 0.01 vs. normoxia; ###P < 0.01 vs. hypoxia.
IH is known to increase C-reactive protein and activate various inflammatory cells, which have been implicated in the development of atherosclerotic lesions (13, 26, 24). We have previously reported that IH (30 s of 4.5–5.5% O2 followed by 30 s of 21% O2 for 8 h/day during the daytime for 10 days) increased TNF-α and IL-6 mRNA levels in the LV myocardium, changes that were possibly related to hypoxia-induced LV remodeling (8). We also reported that continuous hypoxia (10% O2 for 14 days) increased hypoxia-inducible factor-1α and vascular endothelial growth factor in the rat myocardium (10), although interstitial fibrosis was not increased, which had been reported by Sharma et al. (28). Transforming growth factor-β should be examined to evaluate the effect of hypoxia on interstitial fibrosis. Recently, Casals et al. (2) reported that hypoxia (5% O2) induces the synthesis and secretion of BNP in human cultured ventricular myocytes. In this study, IH (repetitive cycle of 1-min periods of 5 and 21% oxygen level for 8 h/day during the daytime for 7 days) increased TNF-α, IL-6, and BNP mRNA expression in the LV myocardium, which effects were significantly suppressed by inhalation of 1.3 vol/100 vol H2 gas, regardless of the timing of administration. Taken together with the histological findings, H2 gas given throughout the experimental period exhibited the greatest cardioprotective effects of the three different methods of H2 gas administration. These effects differed somewhat from the effects of H2 gas on dyslipidemia, so further studies are required.

In conclusion, H2 gas attenuated the development of IH-induced LV remodeling at least partly through the suppression of oxidative stress. Short-term inhalation of H2 gas was effective in reducing oxidative stress and in preventing LV remodeling induced by IH relevant to sleep apnea. Inhalation of H2 gas during hospitalization might be potentially useful for preventing cardiovascular events in patients with SAS.

ACKNOWLEDGMENTS

We express our gratitude to Y. Ogami, Y. Mizuoka, C. Ota, M. Ito, and M. Kobayashi for expert technical assistance. We also thank Skylight Biotech for technical assistance.

GRANTS

This study was supported by Kakenhi (23590267), a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology, and an Osaka Medical College Research Fund.

DISCLOSURES

None.

REFERENCES