Hydrogen gas attenuates embryonic gene expression and prevents left ventricular remodeling induced by intermittent hypoxia in cardiomyopathic hamsters

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MATERIALS AND METHODS

SLEEP APNEA SYNDROME (SAS) is a breathing disorder characterized by recurrent episodes of apnea and/or hypopnea that increases the risk of cardiovascular morbidity and mortality (10, 19, 26). In persons with SAS, recurrent hypopnea/apnea leads to intermittent hypoxia (IH). The prevalence of SAS is much higher in patients with established cardiovascular disease, and central sleep apnea is associated with the more severe forms of heart failure (17), although the mechanisms underlying periodic breathing in patients with heart failure (HF) are complex and multifactorial.

Oxidative stress arises because of an imbalance between free radical production and endogenous antioxidant defenses and is increased in patients with HF (4). Free radicals have also been linked to endothelial dysfunction and increased sympathetic tone (2, 16), whereas intravenous infusion of antioxidants reduces free radical levels and attenuates sympathetic activity in animal models of HF (32). It has been suggested that hydrogen gas produced in the large intestine by intestinal bacteria might scavenge hydroxyl radicals (6), and it was recently reported that hydrogen gas selectively scavenges hydroxyl radicals and exerts an antioxidant effect (23). We previously reported that inhalation of hydrogen gas could prevent dyslipidemia and could also suppress oxidative stress in the left ventricular (LV) myocardium of mice exposed to IH (13). Accordingly, we considered that hydrogen gas might exert a cardioprotective effect in cardiomyopathic (CM) hamsters.

Because the effects of IH on cardiovascular disease have not been clarified, we performed the present study to examine the influence of IH on the heart in CM hamsters (an animal model of human hereditary cardiomyopathy) and to evaluate the antioxidant effect of hydrogen gas on the failing heart during exposure to IH.

MATERIALS AND METHODS

Animals. Normal Syrian hamsters (aged 24 wk, n = 22) were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan). By crossing BIO14.6 hamsters (Bio Breeders, Fitchburg, MA) and Syrian hamsters, we generated CM hamsters (aged 20–24 wk, n = 33) with homozygous deletion for the β-sarcoglycan gene (28). The hamsters were housed at a temperature of 24 ± 1°C and a humidity of 55 ± 10%, with lights on from 6:00 to 18:00 and free access to tap water and solid feed (NMF, Oriental Yeast, Tokyo, Japan). The Osaka University of Pharmaceutical Sciences Experimental Animal Research Committee approved the study protocol and the animal care methods. All experiments were conducted in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

Study protocol. Hamsters were placed in a chamber and exposed to IH (repeated cycles of 1.5 min of 5% oxygen and 5 min of 21%...
oxygen for 8 h during the daytime) for 2 wk with or without inhalation of hydrogen gas (3.05 vol/100 vol) during the hypoxic periods. Hamsters maintained under normoxic conditions in the same room served as controls. Thus we assessed Syrian hamsters housed under normoxic conditions (Syrian normoxia group), Syrian hamsters exposed to IH (Syrian hypoxia group), CM hamsters housed under normoxic conditions (CM normoxia group), CM hamsters exposed to IH (CM hypoxia group), and CM hamsters that inhaled a mixture of N₂/H₂ gas during IH (CM hypoxia + H₂ group). Cardiac function was evaluated by echocardiography under anesthesia induced by intraperitoneal injection of pentobarbital sodium (50 mg/kg) on the day after the last exposure to hypoxia.

**Echocardiographic analysis.** Transthoracic echocardiography was performed using a Vivid E9 instrument (GE Healthcare, Salt Lake City, UT). In brief, the LV ejection fraction (LVEF) was calculated from the LV end-diastolic volume (LVEDV) and LV end-systolic volume (LVESV) obtained using a modified Simpson's method (34). In addition, pulsed-wave Doppler spectra (early LV filling velocity (E wave) and late LV filling velocity due to atrial contraction (A wave)) of mitral inflow were recorded in the apical four-chamber view with the sample volume being placed near the tips of the mitral leaflets at the position where velocity was maximal and flow was laminar. The ratio of E wave velocity to A wave velocity (E/A ratio) was then calculated. Furthermore, the early velocity (e') and late velocity (a') of the mitral annulus were determined by tissue Doppler imaging, and the ratio of E to e' (E/e') ratio was calculated.

**Histological assessment.** Hearts (some arrested in diastole with saturated potassium chloride) were excised and transected below the mitral valve, after which the upper half was examined by light microscopy. The LV free wall myocardium was excised for assessment by electron microscopy, immunohistochemistry, and real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. For light microscopy, cardiac tissues were fixed in 10% formaldehyde, embedded in paraffin, and cut into 4-μm sections. Photographs were taken with an ECLIPSE 80i light microscope (Nikon, Tokyo, Japan) at a magnification of ×4 and were transformed into binary images, and the cardiac cross-sectional area was evaluated at a magnification of ×400 by the method previously reported (8) using ImageJ version 1.44 software (National Institutes of Health, Bethesda, MD). Briefly, in nucleated transverse sections stained with hematoxylin-eosin, cardiomyocytes with both a clear nucleus and intact cellular membrane were measured using NIS-Elements version 3.07 software (Nikon), and at least 30 cardiomyocytes were analyzed per heart. After staining with Sirius red, color images were randomly selected from five high-power fields (at a magnification of ×200), and the collagen volume ratio (%) was calculated as described elsewhere (33). The LV free wall myocardium was fixed in 4% paraformaldehyde containing 0.25% glutaraldehyde and 4.5% sucrose. Ultrathin sections were cut from the fixed blocks, stained with uranyl acetate and lead citrate, and examined using a Hitachi model H-7650 electron microscope (Hitachi, Tokyo, Japan) (12).

**Termal deoxyxynucleotid transferase-mediated dUTP-biotin end labeling.** LV myocardial tissue samples were assessed by terminal deoxyxynucleotid transferase-mediated dUTP-biotin nick end labeling (TUNEL) using Apop Tag peroxidase in situ apoptosis detection kits (Millipore Japan, Tokyo, Japan). Negative controls were processed with distilled water, and endomethrin was used as the positive control. After cells were counterstained with Mayer's hematoxylin, TUNEL-positive cells were counted as described elsewhere (15).

**Immunohistochemistry of 4-hydroxy-2-nonenal expression.** The amount of 4-hydroxy-2-nonenal (4-HNE)-modified protein adducts in paraffin sections of the left ventricle was determined by immunohistochemical staining. Sections were incubated with a monoclonal antibody for 4-HNE (Japan Institute for the Control of Aging, Shizuoka, Japan) and with a secondary antibody (biotinylated anti-mouse IgG), followed by incubation with Vectastain Elite ABC reagent (Vector, Burlingame, CA). The area of 4-HNE staining was then compared with the mean area on control sections (defined as 1.0).

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

**Quantitative real-time RT-PCR.** Total RNA was extracted from LV and RV myocardial tissues using TRI reagent (Molecular Research Center, Cincinnati, OH). Complementary DNA was synthesized from total RNA by reverse transcription using a Transcriptor Universal cDNA Master kit (Roche Diagnostics, Basel, Switzerland). The primers and probes were designed for the nucleotide sequence of hamster brain natriuretic peptide (BNP), β-myosin heavy chain (β-MHC), c-fos, c-jun, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using Probefinder v. 2.45 software (http://upcr.probefinder.com/roche3.html), which specifies a set of specific primers plus the TaqMan locked nucleic acid probe from the Roche Universal Probe Library collection. Universal Probes No. 38 for β-MHC, No. 46 for c-fos, No. 78 for c-jun, No. 80 for GAPDH, and No. 95 for BNP were purchased from Roche Diagnostics. The mRNA levels were

### Table 1. Body weight, heart weight, and heart weight/body weight ratio

<table>
<thead>
<tr>
<th>Group</th>
<th>BW, g</th>
<th>HW, mg</th>
<th>HW/BW ratio, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syrian normoxia</td>
<td>148 ± 4.50</td>
<td>0.418 ± 0.015</td>
<td>2.84 ± 0.114</td>
</tr>
<tr>
<td>Syrian hypoxia</td>
<td>149 ± 6.79</td>
<td>0.457 ± 0.018</td>
<td>3.08 ± 0.093</td>
</tr>
<tr>
<td>CM normoxia</td>
<td>134 ± 7.07</td>
<td>0.495 ± 0.023</td>
<td>3.74 ± 0.167*</td>
</tr>
<tr>
<td>CM hypoxia</td>
<td>131 ± 5.55</td>
<td>0.506 ± 0.009</td>
<td>4.29 ± 0.217** ,††</td>
</tr>
<tr>
<td>CM hypoxia + H₂</td>
<td>143 ± 7.04</td>
<td>0.504 ± 0.023</td>
<td>3.57 ± 0.185</td>
</tr>
</tbody>
</table>

CM, cardiomyopathy; BW, body weight; HW, heart weight. Data are shown as means ± SE (n = 5–13). *P < 0.05 compared with the Syrian hypoxia group; **P < 0.01 compared with the Syrian normoxia group; §P < 0.05 compared with the Syrian normoxia group; ††P < 0.005 compared with the Syrian hypoxia group; †‡P < 0.01 compared with the Syrian hypoxia group; †††P < 0.001 compared with the Syrian hypoxia group; §§P < 0.01 compared with the CM hypoxia group.

### Table 2. Echocardiographic findings

<table>
<thead>
<tr>
<th>Group</th>
<th>Dd, mm</th>
<th>Ds, mm</th>
<th>E, cm/s</th>
<th>A, cm/s</th>
<th>E/A</th>
<th>e', cm/s</th>
<th>E/e'</th>
<th>LVEF, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syrian normoxia</td>
<td>4.71 ± 0.17</td>
<td>3.15 ± 0.14</td>
<td>84.6 ± 3.13</td>
<td>60.9 ± 6.18</td>
<td>1.43 ± 0.08</td>
<td>5.23 ± 0.44</td>
<td>16.9 ± 1.80</td>
<td>60.2 ± 0.56</td>
</tr>
<tr>
<td>Syrian hypoxia</td>
<td>5.07 ± 0.17</td>
<td>3.59 ± 0.15</td>
<td>81.5 ± 5.92</td>
<td>54.8 ± 8.06</td>
<td>1.55 ± 0.13</td>
<td>4.23 ± 0.91</td>
<td>21.8 ± 3.93</td>
<td>61.5 ± 0.76</td>
</tr>
<tr>
<td>CM normoxia</td>
<td>5.89 ± 0.30</td>
<td>4.43 ± 0.44</td>
<td>79.5 ± 5.64</td>
<td>49.2 ± 7.42</td>
<td>1.69 ± 0.13</td>
<td>3.84 ± 0.43</td>
<td>21.5 ± 1.70</td>
<td>47.2 ± 3.41 ††</td>
</tr>
<tr>
<td>CM hypoxia</td>
<td>6.23 ± 0.36*</td>
<td>4.82 ± 0.50</td>
<td>77.9 ± 5.00</td>
<td>55.3 ± 4.34</td>
<td>1.43 ± 0.05</td>
<td>2.76 ± 0.24**</td>
<td>29.4 ± 2.29*††</td>
<td>37.2 ± 3.24*** ,†††</td>
</tr>
<tr>
<td>CM hypoxia + H₂</td>
<td>5.17 ± 0.28</td>
<td>3.61 ± 0.17</td>
<td>78.4 ± 4.42</td>
<td>56.7 ± 3.64</td>
<td>1.39 ± 0.03</td>
<td>4.04 ± 0.20</td>
<td>20.0 ± 1.59$</td>
<td>60.9 ± 0.92§§</td>
</tr>
</tbody>
</table>

CM, cardiomyopathy; Dd, left ventricular end-diastolic diameter; Ds, LV end-systolic diameter; E, early rapid filling wave of mitral inflow; A, atrial contraction wave of mitral inflow; e', early velocity of the mitral annulus; LVEF, left ventricular ejection fraction. Data are shown as means ± SE (n = 5–9). *P < 0.05 compared with the Syrian normoxia group; **P < 0.01 compared with the Syrian normoxia group; ††P < 0.005 compared with the Syrian hypoxia group; †‡P < 0.01 compared with the Syrian hypoxia group; †††P < 0.001 compared with the CM normoxia group; §P < 0.05 compared with the CM hypoxia group; §§P < 0.01 compared with the CM hypoxia group.
measured using a LightCycler (Roche Diagnostics) and were normalized by comparison with the level of GAPDH mRNA as the internal control.

Statistical analysis. Values are shown as means ± SE. Data were statistically analyzed by using one-way ANOVA followed by the Tukey-Kramer multiple comparison test, and \( P < 0.05 \) was considered to indicate a significant difference.

RESULTS

Heart weight and body weight. Heart weight was significantly increased in the CM hypoxia group compared with the Syrian normoxia group. The heart weight-to-body weight ratio was significantly increased in the CM hypoxia group compared with both the Syrian normoxia group and the Syrian hypoxia group (Table 1).

Echocardiographic findings. LVEF was significantly smaller in the CM normoxia group compared with the Syrian normoxia and Syrian hypoxia groups (Table 2). IH led to a significant increase of LV end-diastolic diameter, \( E/e' \), and LVEF in the CM hypoxia group, but not in the Syrian hypoxia group (Table 2 and Fig. 1). In contrast, these changes were less marked in the CM hypoxia + \( H_2 \) group, and LVEF showed significant improvement (Table 2 and Fig. 1).

Histological findings. In CM hamsters, exposure to IH induced cardiomegaly and a significant increase of cardiomyocyte cross-sectional area (Fig. 2). The CM hypoxia group showed an increase in degeneration of cardiomyocytes, including myofibrillar lysis, disarray of myofibers, and interstitial fibrosis (Fig. 2, \( H \) and \( I \)). Electron microscopy revealed mild myofiber disarray and variation of mitochondrial size in CM hamsters kept under normoxic conditions. Exposure to IH resulted in an increase of myofibrillar lysis, mitochondrial degeneration, dissociation of intercalated discs, and Z-line streaming (Fig. 3). All of these changes induced by IH in CM hamsters were suppressed by inhalation of hydrogen gas (Fig. 2, \( K \) and \( L \), and Fig. 3F).

TUNEL findings. The number of TUNEL-positive cells in the LV myocardium was significantly increased in the CM hypoxia group, whereas it was significantly reduced in the CM hypoxia + \( H_2 \) group (Fig. 4).

Superoxide production and 4-HNE expression in the LV myocardium. In the CM hypoxia group, superoxide production (detected by DHE labeling) and 4-HNE-modified protein adducts were significantly increased in the LV myocardium, whereas these changes were significantly suppressed in the CM hypoxia + \( H_2 \) group (Fig. 5).

Real-time RT-PCR. In the CM hypoxia group, expression of BNP and \( \beta \)-MHC mRNA was significantly increased in the LV myocardium compared with the Syrian normoxia group. Inha-
lation of hydrogen gas significantly reduced the expression of BNP and β-MHC mRNA in the LV myocardium (Fig. 6). Expression of c-fos and c-jun mRNA in the LV myocardium was also significantly increased in the CM hypoxia group, whereas inhalation of hydrogen gas significantly reduced myocardial expression of c-fos and c-jun mRNA (Fig. 6).

DISCUSSION

In the present study, we showed that the IH accelerated the degeneration of cardiomyocytes and aggravated systolic dysfunction in CM hamsters, whereas these deleterious effects of IH were not seen in Syrian hamsters. The CM hypoxia group displayed hypertrophy of cardiomyocytes, as well as increased expression of c-fos and c-jun mRNA. On the other hand, inhaling a low concentration (3.05 vol/100 vol) of hydrogen gas attenuated cardiomyocyte hypertrophy and perivascular fibrosis in the LV myocardium, resulting in the preservation of cardiac function.

LV diastolic function is frequently impaired in patients with SAS (1, 18). In the present study, exposure to IH for 2 wk mainly caused LV systolic dysfunction in CM hamsters and had a minimal effect on the heart in normal Syrian hamsters. We previously used dobutamine stress echocardiography to demonstrate that the myocardial contractile reserve is reduced in patients with severe SAS (25). Maeda et al. (20) recently reported that a longer duration of IH induces autophagy in the normal rat heart, which maintains contractile function and prevents necrosis. Their differing findings might be due to differences of the animal model and the duration of hypoxic stress. In addition, it should be remembered that the physiological conditions in our model are different from those in SAS patients, because we focused on IH and neglected barometric pressure and acid-base disturbance. Therefore, change of PaCO₂ should be taken into consideration in a future study.

CM hamsters, we found that IH increased cardiac hypertrophy, interstitial fibrosis, and cardiomyocyte degeneration (including mitochondrial destruction and myofibrillar lysis), but these changes did not occur in normal Syrian hamsters. Intriguingly, streaming of Z-lines was often observed in CM hamsters. It has been reported that Z-line degeneration is observed in the myocardium with genetic mutations of dystrophin-associated glycoproteins, including δ-sarcoglycan (31). The myocardium of CM hamsters would be expected to be fragile, and genomic deletion of δ-sarcoglycan might have
been the underlying cause of histological changes that were accelerated by IH in CM hamsters.

Although the precise mechanisms through which SAS aggravates heart failure are unknown, it has been suggested that oxidative stress might play an important role in the progression of cardiovascular disease (27). In the present study, superoxide production and 4-HNE-modified protein adducts were increased in the LV myocardium by IH, suggesting an increase of oxidative stress, and these changes were inhibited by inhaling a low concentration of hydrogen gas. Furthermore, the number of degenerated mitochondria was decreased, and the ultrastructural architecture of the LV myocardium was preserved in the CM hypoxia group. It has been reported that H2 gas selectively scavenges hydroxyl radicals (24). It has also been reported that hydrogen protects cells and tissues against oxidative stress by scavenging hydroxyl radicals (22) and might prevent a decrease of cellular ATP levels (23). Since we found that IH aggravated underlying abnormalities and reduced systolic function, one of the mechanisms through which adaptive servo-ventilation improves congestive HF might be alleviation of oxidative stress (11). We previously evaluated the effect of hydrogen gas inhalation on LV remodeling and dyslipidemia in mice when hydrogen was inhaled during reoxygenation, during hypoxia, or throughout the experimental period (13), and we showed that inhalation during hypoxia reduced oxidative stress and improved systolic dysfunction in the failing heart. Therefore, inhalation of hydrogen gas using adaptive servo-ventilation might be an effective treatment for patients with HF and periodic apnea. In the present study, oxidative stress was evaluated by immunohistochemistry. Our preliminary study demonstrated that malondialdehyde, a biomarker of lipid peroxidation, was significantly increased in LV myocardium after exposure to intermittent hypoxia (data not shown). Additional parameters of oxidative stress like malondialdehyde should be employed in future studies.

Myocardial remodeling is associated with upregulation of the expression of embryonic gene isoforms such as c-fos and c-jun (5, 21). BNP and β-MHC are well-established markers of cardiomyocyte hypertrophy. In the present study, changes of BNP and β-MHC mRNA expression showed that IH increased cardiac hypertrophy and that it was suppressed by inhaling hydrogen gas. We found that c-fos and c-jun mRNA expression was significantly increased in the LV myocardium of the CM hypoxia group. Increased c-fos and c-jun levels might suggest transcriptional activation of activator protein 1 (AP-1) (3), because AP-1 plays a pivotal role in the ERK/JNK signaling pathways and overexpression of c-jun and c-fos is a common feature of myocardial hypertrophy in rodents (3, 7). In turn, the upregulation of c-fos and c-jun might accelerate myocardial degeneration. Various stimuli can activate AP-1, including inflammatory cytokines, growth factors, and oxidative stress (9, 14, 30), whereas inhaled hydrogen gas scavenges free radicals and decreases oxidative stress, so that c-fos and c-jun mRNA expression was decreased by inhalation of hydrogen in the present study. AP-1 has been reported to induce aldose reductase-like gene, which is involved in the biosynthesis of PGF2α. It has been reported that PGF2α inhibits expression of the sarco(endo)plasmic reticulum Ca2+ -ATPase 2 (SERCA2)

Fig. 3. Electron micrographs of the LV myocardium. A normal Syrian hamster (A). Mt, mitochondria; N, nucleus. Mild myofiber disarray and variation of mitochondrial sizes are observed in CM hamster maintained under normoxic conditions (B). Intermittent hypoxia (IH) has increased myofibrillar lysis and Mt degeneration in a CM hamster (C). Partial dissociation (square) of intercalated disc (ID) (D) and Z-line (Z) streaming (square) (E) were often observed in the CM hypoxia group. All of the changes seen in CM hamsters due to IH were suppressed by inhaling hydrogen gas (F). Scale bar, 1 μm.
gene and thus causes diastolic dysfunction (29). Accordingly, further studies are needed with a focus on the molecular pathways involved in the pathogenesis of cardiomyopathy, including aldose reductase-like gene expression.

In conclusion, exposure of CM hamsters to IH promoted cardiomyocyte degeneration and systolic dysfunction, at least partly through increased oxidative stress, which might explain the poor prognosis of HF patients with SAS. Inhalation of hydrogen gas attenuated the deleterious changes in CM hamsters and might be a novel treatment strategy for HF, especially in patients who also have SAS.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

Fig. 5. Dihydroethidium (DHE) labeling (A–D) and immunohistochemistry for 4-hydroxy-2-nonenal (4-HNE; E–G) in LV myocardium of CM hamsters. Superoxide production (C) and oxidative stress (G) are increased in CM hamsters exposed to intermittent hypoxic stress, whereas these changes are suppressed by inhalation of hydrogen gas (D and H). Intermittent hypoxia significantly increased the levels of superoxide and 4-HNE-modified protein adducts in the LV myocardium, whereas inhalation of hydrogen significantly reversed these changes. Values are shown as means ± SE (n = 4–7).


