Role of gp91phox-containing NADPH oxidase in left ventricular remodeling induced by intermittent hypoxic stress

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SLEEP APNEA SYNDROME (SAS) is an important risk factor of cardiovascular diseases such as systemic and pulmonary hypertension, congestive heart failure, and stroke (18). Recent studies suggest that intermittent hypoxia due to SAS is associated with left ventricular (LV) dysfunction (1, 28). Plasma TNF-α and IL-6 levels are elevated in patients with SAS (24, 36), indicating the relationship between inflammatory pathways and intermittent hypoxia. In addition, oxidative stress has been reported to play an important role in the progression of LV remodeling, which is accompanied by hypertrophy of cardiomyocytes and interstitial fibrosis (17, 32). Thus intermittent hypoxia is clearly implicated in cardiovascular diseases, but the precise mechanisms have remained poorly understood.

NADPH oxidase has been identified as a major source of reactive oxygen species, which might aggravate hypertrophy of cardiomyocytes and interstitial fibrosis, and has been expressed in several kinds of cells, including endothelial cells (10), fibroblasts (34), and cardiomyocytes (19, 25). The typical NADPH oxidase is composed of membrane-bound gp91phox and p22phox subunits and cytotoxic p40phox, p47phox, and Rac-1 subunits (2). Recent studies have indicated that angiotensin II-induced cardiac hypertrophy was attenuated in gp91phox-deficient (gp91−/−) mice (4) and that gp91phox-containing NADPH oxidase activity increased in the failing human heart (14). However, the potential relevance to LV remodeling under intermittent hypoxia remains unclear. Therefore, the aim of the present study was to examine the role of NADPH oxidase, especially the gp91phox subunit, in the development of LV remodeling in mice exposed to intermittent hypoxic stress.

MATERIALS AND METHODS

Experimental protocol. Male gp91−/− mice (Jackson Laboratory, Bar Harbor, ME) and matched C57BL/6J wild-type mice at 7–12 wk of age were placed in a chamber (30 s of 4.5–5.5% O2 followed by 30 s of 21% O2) or exposed to normoxic conditions (Fig. 1). The animals were subjected to intermittent hypoxia for 8 h/day during the daytime for 10 consecutive days.

On the day after the last hypoxic exposure, a tail-cuff and a pneumatic pulse transducer (model BP-98A, Softron, Tokyo, Japan) were used to measure mean systemic blood pressure (MBP) in conscious mice. Then mice were randomly assigned from each group (n = 4–7 per group) and anesthetized with ketamine HCl (50 mg/kg ip) and xylazine (10 mg/kg ip) for echocardiography (SONOS 5500, Agilent Technology). The other mice in each group were adequately anesthetized with pentobarbitone sodium (40 mg/kg ip) for cardiac catheterization. A 1.4-Fr Micro-tip catheter (model SPR-671, Millar Instruments, Houston, TX) was introduced through the right jugular vein or the right carotid artery for measurement of right ventricular (RV) or LV pressure. After the hemodynamic measurement, blood samples were collected from the abdominal aorta for the measurement of lipid peroxide (LPO) in plasma. Then the heart was excised, and the upper half was used for light-microscopic examination. The free wall of the LV myocardium was excised for electron-microscopic examination, immunohistochemistry, RT-PCR, and EMSA.

All procedures were performed in accordance with our institutional guidelines for animal research; the protocol was approved by an independent committee of the Central Research Laboratory of the Osaka Medical College.

Light and electron microscopy. For light microscopy, the upper half of the heart was fixed in 10% formaldehyde, embedded in paraffin, and cut into 4-μm-thick sections. LV cardiomyocyte diameter was measured as previously described (12, 15, 26). Briefly, the...
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Fig. 1. A: intermittent hypoxic exposure system. Solenoid valve (SV)-N was opened to flush \( \text{N}_2 \) (500 l/min) into the chamber to reduce \( \text{O}_2 \) level. After SV-N was closed, SV-A was opened to flush compressed air. Valves were regulated via a personal computer with custom-made software. B: \( \text{O}_2 \) level in the chamber during each period. \( \text{O}_2 \) was reduced to 4.5–5.5% for 30 s and then returned to 21% in the following 30 s. Hatched bars indicate that SV-N or SV-A is open.

shortest diameters of cardiomyocytes were measured only in nucleated transverse sections stained with hematoxylin-eosin under a light microscope at \( \times 400 \) magnification. After Sirius red staining, color images were obtained from five randomly selected separate high-power fields (\( \times 200 \)) in five sections per mouse, and the collagen volume fraction was calculated by the method published previously (26, 35).

For electron microscopy, the specimens were 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 with an electron microscope (model H-7650, Hitachi) (13).

\( \text{NADPH oxidase activity.} \) NADPH-dependent superoxide production was measured by a lucigenin-enhanced chemiluminescence assay as described previously (35). The lucigenin concentration in the final reaction mixture was 5 \( \mu \text{mol/l.} \) NADPH-dependent superoxide production was expressed as relative light units per minute per milligram of protein.

**Immunohistochemistry for 4-hydroxy-2-nonenal protein.** The LV paraffin sections were deparaffinized, hydrated, and incubated with 3% \( \text{H}_2\text{O}_2 \) to reduce endogenous peroxidase activity. After nonspecific staining was blocked with a Vector M.O.M. immunodetection kit (Vector Laboratories, Burlingame, CA), the deparaffinized sections of LV myocardium were incubated with monoclonal antibody against 4-hydroxy-2-nonenal (4-HNE; no. MHN-20, Japan Institute for the Control of Aging, Shizuoka, Japan), and the percent area of 4-HNE staining was measured by a previously published method (35).

**Quantitative real-time RT-PCR.** Total RNA was extracted from heart samples with use of an RNaseasy mini kit (Qiagen, Valencia, CA). RT was performed with random hexamers and Superscript reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time quantitative PCR was performed using an ABI Step One sequence detector (PE Applied Biosystems, Foster City, CA). GAPDH mRNA was used as an endogenous control to enable relative mRNA quantification (Ma9999915). Tagman probe and primers for target mRNA (Ma00443258 for TNF-\( \alpha \), Ma00441724 for transforming growth factor (TGF)-\( \beta \), and Ma00446190 for IL-6) were purchased from Applied Biosystems. The cycling reaction conditions were as follows: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative quantity of target mRNA was evaluated by comparison with GAPDH mRNA expression.

**Assessment of NF-\( \kappa \text{B} \) activation by EMSA.** Nuclear extracts were prepared and EMSA were performed according to a previously described method (35). Double-stranded oligonucleotide containing the most common NF-\( \kappa \text{B} \) consensus binding site (\( 5^\prime \)-AGT TGA GGG GAC TTT CCC AGG C-3\(^\prime \); Promega, Madison, WI) was end-labeled with \([\gamma^32\text{P}]\)ATP using T4 polynucleotide kinase (Promega). After electrophoresis, gels were dried and exposed to imaging plates (Fuji Film, Tokyo, Japan). The protein-DNA complexes were visualized by autoradiography, and the relative intensities of bands were analyzed using NIH Image 1.61 software.

**Statistical analysis.** Values are means ± SE. For statistical analysis, we used one-way analysis of variance followed by Fisher’s protected least significant difference multiple comparison tests. Significance was recognized at \( P < 0.05 \).

**RESULTS**

**Heart and body weight.** Although body weight and heart weight were not changed by intermittent hypoxia in wild-type or \( \text{gp91}^{-/-} \) mice, the ratio of heart weight to body weight was significantly increased in wild-type, but not \( \text{gp91}^{-/-} \) mice (Table 1).

**Hemodynamic measurements.** Intermittent hypoxic stress did not affect MBP, LV systolic pressure, or LV end-diastolic pressure in wild-type or \( \text{gp91}^{-/-} \) mice. RV systolic pressure tended to increase in wild-type mice exposed to intermittent hypoxic stress. No increase in RV systolic pressure was seen in hypoxic \( \text{gp91}^{-/-} \) mice (Table 1). Echocardiograms showed no significant effect of intermittent hypoxic stress on the LV inner diameter of diastole or systole or deceleration time, although
ejection fraction was significantly increased in hypoxic gp91<sup>−/−</sup> mice compared with normoxic wild-type mice (Table 1).

**Histological findings.** Intermittent hypoxic stress significantly increased the mean diameter of cardiomyocytes and collagen volume fraction in wild-type mice (Figs. 2 and 3). Electron microscopy showed a variety of degenerative changes, including nuclear invagination, streaming of myofibers, and increased electron dense materials in mitochondria (Fig. 4). None of these degenerative changes induced by intermittent hypoxic stress were observed in gp91<sup>−/−</sup> mice.

**NADPH-dependent superoxide production.** NADPH-dependent superoxide production in LV myocardium was significantly increased by intermittent hypoxic stress in wild-type, but not gp91<sup>−/−</sup>, mice (Fig. 5A).

**Plasma LPO levels and 4-HNE expression in the LV myocardium.** Plasma LPO levels were significantly elevated in wild-type, but not gp91<sup>−/−</sup>, mice exposed to intermittent hypoxia (Fig. 5B). 4-HNE protein, a specific lipid peroxidation product and a marker of oxidative stress, was significantly increased by intermittent hypoxic stress in LV myocardium of wild-type, but not gp91<sup>−/−</sup>, mice (Fig. 5C).

**RT-PCR.** Intermittent hypoxic stress significantly increased the expression of TGF-β, TNF-α, and IL-6 mRNA in wild-type mice. In contrast to wild-type mice, all the increases induced by intermittent hypoxic stress were not seen in gp91<sup>−/−</sup> mice (Fig. 6).

**NF-κB binding activity.** NF-κB binding activity significantly increased in wild-type mice subjected to intermittent hypoxia

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### Table 1. Effect of hypoxia on hemodynamic and echocardiographic parameters

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<thead>
<tr>
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<th>Wild-Type Mice</th>
<th>gp91&lt;sup&gt;−/−&lt;/sup&gt; Mice</th>
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<tr>
<td></td>
<td>Normoxia</td>
<td>Hypoxia</td>
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<tr>
<td><strong>BW, g</strong></td>
<td>21.6±0.5 (8)</td>
<td>20.9±0.3 (13)</td>
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<tr>
<td><strong>HW, g</strong></td>
<td>0.092±0.004 (8)</td>
<td>0.103±0.004 (13)</td>
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<tr>
<td><strong>HW/BW, mg/g</strong></td>
<td>4.3±0.1 (8)</td>
<td>4.9±0.2† (13)</td>
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<tr>
<td><strong>Hemodynamic data</strong></td>
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<td></td>
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<tr>
<td><strong>MBP, mmHg</strong></td>
<td>79.8±1.7 (4)</td>
<td>81.3±1.5 (5)</td>
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<tr>
<td><strong>RVsyst, mmHg</strong></td>
<td>23.5±1.6 (6)</td>
<td>33.3±4.6 (12)</td>
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<tr>
<td><strong>LVsyst, mmHg</strong></td>
<td>93.3±3.8 (6)</td>
<td>94.0±3.5 (12)</td>
</tr>
<tr>
<td><strong>LVED, mmHg</strong></td>
<td>6.8±0.9 (6)</td>
<td>8.8±0.9 (12)</td>
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<tr>
<td><strong>Echocardiographic data</strong></td>
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<tr>
<td><strong>LVDd, mm</strong></td>
<td>2.5±0.1 (5)</td>
<td>2.8±0.2 (7)</td>
</tr>
<tr>
<td><strong>LVds, mm</strong></td>
<td>1.8±0.1 (5)</td>
<td>1.9±0.1 (7)</td>
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<tr>
<td><strong>EF, %</strong></td>
<td>59.9±1.9 (5)</td>
<td>64.5±3.1 (7)</td>
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<tr>
<td><strong>Dct, ms</strong></td>
<td>4.9±0.5 (5)</td>
<td>5.5±0.2 (7)</td>
</tr>
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</table>

Values are means ± SE of number of mice in parentheses. BW, body weight; HW, heart weight; MBP, mean blood pressure; RVsyst, right ventricular systolic pressure; LVsyst, left ventricular (LV) systolic pressure; LVED, LV end-diastolic pressure; LVDd, LV internal diameter of end diastole; LVds, LV internal diameter of end systole; EF, ejection fraction; Dct, deceleration time. *P < 0.05; †P < 0.01 vs. normoxic wild-type mice. ‡P < 0.01 vs. hypoxia-exposed wild-type mice.

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**Fig. 2.** Representative light micrographs of left ventricular (LV) myocardium. Hypertrophy of cardiomyocytes and disarray of myofibers are observed in wild-type, but not gp91<sup>−/−</sup> mice, exposed to intermittent hypoxia. Hematoxylin-eosin stain; original magnification ×100.
plays a crucial role in intermittent hypoxia-induced LV remodeling through an increase of oxidative stress might provide potential benefit, i.e., prevention of cardiovascular events in patients with SAS.

Intermittent hypoxia for 10 days tended to increase RV systolic pressure in wild-type mice, although there was no significant statistical difference. We previously reported that the effect of pulmonary hypertension on LV remodeling was slight in animal models exposed to continuous hypoxia for 3 wk (15, 35). Therefore, elevated RV pressure in this study was thought to have little effect on LV remodeling. On the other hand, RV systolic pressure in gp91\(^{-/-}\) mice was not affected by intermittent hypoxic stress. Liu et al. (21) demonstrated that chronic hypoxia did not increase RV systolic pressure in gp91\(^{-/-}\) mice and that gp91\(^{phox}\)-dependent superoxide production might play a pivotal role in the pathogenesis of pulmonary hypertension. Our similar observation might support the importance of gp91\(^{phox}\) in hypoxia-induced pulmonary hypertension.

No difference in MBP and LV systolic pressure among the four groups suggests that intermittent hypoxic stress might cause LV remodeling without changes in the afterload. Chen et al. (7) reported that 5 wk of intermittent hypoxic stress increased LV end-diastolic pressure and decreased cardiac output in rats. In the present study, however, LV systolic and diastolic function were not altered in wild-type or gp91\(^{-/-}\) mice, possibly because of our short experimental period. A longer period of intermittent hypoxia might cause LV dysfunction, as suggested by the degenerative myocardial cells in wild-type mice observed by electron microscopy. Another possibility is the interspecies difference in the sensitivity to hypoxic stress. At the least, an essential factor in the LV remodeling was hypoxia, rather than pressure overload, in the present study.

NADPH oxidase is a major source of superoxide production (11, 29), and NADPH oxidase-derived superoxide contributes to the progression of cardiovascular diseases, such as cardiac hypertrophy, heart failure, and hypertension (11, 27, 29, 31). Bendall et al. (4) reported attenuation of angiotensin II-induced cardiac hypertrophy through decreasing NADPH-dependent superoxide production in gp91\(^{-/-}\) mice. In the present study, intermittent hypoxic stress significantly increased NADPH-dependent superoxide production in LV myocardium of wild-type, but not gp91\(^{-/-}\), mice. Furthermore, hypertrophy of cardiomyocytes, increased interstitial fibrosis, and, consequently, LV remodeling were observed in wild-type mice subjected to intermittent hypoxia for 10 days. NADPH oxidase may be activated by several types of stimuli, including angiotensin II, TNF-\(\alpha\), and IL-6. We observed that the expression of TNF-\(\alpha\) and IL-6 mRNA in LV myocardium was enhanced in wild-type mice exposed to intermittent hypoxia. Although we could not examine circulating cytokine levels, plasma TNF-\(\alpha\) and IL-6 levels have been reported to be elevated in patients with SAS (24, 36). Thus TNF-\(\alpha\) and IL-6, which are involved in gp91\(^{phox}\)-containing NADPH oxidase induction in wild-type mice, might also play an important role in such induction under conditions of intermittent hypoxia. In addition, intermittent hypoxic stress significantly increased TGF-\(\beta\) mRNA in wild-type, but not gp91\(^{-/-}\), mice. TGF-\(\beta\) activates collagen gene expression and enhances the extracellular matrix protein synthesis (5, 8). Rosenkranz et al. (30) reported that overexpression of TGF-\(\beta\) in transgenic mice resulted in interstitial fibrosis
and hypertrophy of cardiomyocytes. We also observed that interstitial fibrosis was increased by intermittent hypoxia, and TGF-β might have played an important role in regulating LV remodeling through its direct and potent actions.

In the present study, intermittent hypoxic stress significantly increased TGF-β, TNF-α, and IL-6 mRNA expression in wild-type, but not gp91−/−, mice. The precise mechanisms for the failure of hypoxic stress to increase TGF-β, TNF-α, and IL-6 mRNA expression in gp91−/− mice is obscure. Perhaps there is another pathway that activates NADPH oxidase independent of TNF-α and IL-6. Angiotensin II activates NADPH oxidase mainly via angiotensin II type 1 receptors. Interestingly, hypoxic stress has been shown to increase the circulating levels of angiotensin II (37) and the expression of angiotensin

Fig. 4. Representative electron micrographs of LV myocardium. Fine structure of LV myocardium was normal in normoxic wild-type mice (A). Intermittent hypoxic stress caused a variety of degenerative changes (arrows), including nuclear invagination (B), streaming of myofibers (C), and myelin-figured inclusions (D). In gp91−/− mice, intermittent hypoxic stress had little effect on LV myocardium, although the number of mitochondria tended to increase (F) compared with normoxia (E). Scale bars, 1 μm.

Fig. 5. A: effect of intermittent hypoxic stress on NADPH-dependent superoxide production in homogenates from LV tissues of wild-type and gp91−/− mice. B: plasma lipid peroxide (LPO) levels in wild-type and gp91−/− mice exposed to normoxia or intermittent hypoxia. C: percent area of 4-hydroxy-2-nonenal (4-HNE) protein in LV myocardium from wild-type and gp91−/− mice exposed to normoxia or intermittent hypoxia. Values are means ± SE of number of animals in parentheses. RLU, relative light units.
II type 1 receptors (33). Evaluation of angiotensin II and cytokine expression in plasma and myocardium are needed to clarify the mechanisms in future studies.

Oxidative stress is involved in the pathophysiology of LV remodeling (17, 32). In the present study, NADPH-dependent superoxide production, plasma LPO levels, and 4-HNE expression were significantly increased by intermittent hypoxic stress in wild-type, but not gp91 \(^{-/-}\) mice. Therefore, oxidative stress might be enhanced under intermittent hypoxic conditions at least partly through gp91\(^{phox}\)-containing NADPH oxidase activation. Increased oxidative stress promotes NF-κB activation and the development of cardiac hypertrophy in vivo (3, 20). We observed that intermittent hypoxic stress increased NF-κB activity only in wild-type mice. Satoh et al. (31) reported that NF-κB might participate in some of the downstream effects of NADPH oxidase on cardiac hypertrophy. NF-κB also regulates the expression of inflammatory genes, including TNF-α and IL-6 (9, 22, 23). Taken together, these facts suggest that oxidative stress and NF-κB activation contribute to the development of LV remodeling.

In conclusion, 10 days of intermittent hypoxia in wild-type mice induced LV remodeling, which was accompanied by increased oxidative stress independent of systemic blood pressure. In contrast, LV remodeling did not occur in gp91\(^{-/-}\) mice exposed to intermittent hypoxia. Thus gp91\(^{phox}\)-containing NADPH oxidase plays a crucial role in the pathophysiology of intermittent hypoxia-induced LV remodeling.

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