Chronological Change of Right Ventricle by Chronic Intermittent Hypoxia in Mice

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**Study Objective:** No studies have investigated sequential changes in the heart on magnetic resonance imaging (MRI), along with observation of functional lung phenotypes and genetics, over the duration of chronic intermittent hypoxia (CIH). We investigated chronological changes in heart and lung phenotypes after CIH using a mouse model to provide new insights into the pathophysiology of sleep apnea-induced cardiovascular disease.

**Methods:** C57BL/6J adult male mice were randomized to 4 or 8 weeks of CIH. Cardiac cine-MRI images were analyzed to assess functional parameters of right ventricle (RV). Histopathological features of myocytes and pulmonary vessels, as well as genes involved in the endothelin (ET) system, were investigated.

**Results:** Function of the RV reduced significantly at 4 weeks and continuously decreased following another 4 weeks of CIH, although the rate of decrease was attenuated. Notably, persistence of reduced ejection fraction and end-systole RV wall thickness (WT) and increases in the ET system of the lungs and blood strongly implied the development of pulmonary hypertension after 8 weeks of CIH.

**Conclusions:** RV dysfunction with reduced end-systole RV WT could be a late phenotype in long-standing CIH and possibly also in obstructive sleep apnea.

**Keywords:** sleep apnea, cine-MRI, right ventricle, pulmonary hypertension, mouse, endothelin.

**Statement of Significance**
This study provides insight into changes in heart and lung phenotypes upon chronic intermittent hypoxia (CIH), as well as the pathophysiology of sleep apnea-induced cardiovascular diseases, via a mouse model. Cine-magnetic resonance imaging (cine-MRI) was applied to measure the right ventricle changes in CIH mouse model. Chronological changes in right ventricle function with CIH that suggest pulmonary hypertension were elaborated. Changes in the endothelin system from the lungs that might be associated with the mechanism of cardiovascular phenotypes in CIH mouse model were identified.

**INTRODUCTION**
Obstructive sleep apnea (OSA) is a sleep breathing disorder characterized by recurrent obstruction of the upper airway during sleep. Patients with OSA commonly have comorbidities of systemic or pulmonary hypertension, diabetes, cerebral stroke, or obesity.1 OSA is an independent risk factor for the development of cardiovascular diseases, such as arterial hypertension, heart failure, stroke, and pulmonary hypertension; its significance has been confirmed by several large epidemiologic studies.2–4 Sympathetic overactivation, oxidative stress, and systemic or local inflammatory reactions are the most contributing pathophysiology for OSA. The development of cardiovascular disease commonly requires several years of exposure to sleep apnea. However, even healthy OSA patients demonstrate subtle changes in vascular remodeling, such as atherosclerotic change.5,6 Therefore, investigating the exact pathophysiology of OSA-induced cardiovascular disease and its related genes or molecules in OSA patients is critical. However, OSA patients have various confounding factors that cause bias or misinterpretation of results. These factors limit the exploration of causal associations between OSA and cardiovascular diseases.

To overcome this obstacle, OSA animal models using chronic intermittent hypoxia (CIH) have been developed7 and used to study the essential pathogenesis of OSA-related complications.8 The common result of these studies is that increased oxidative stress seems to be critical in the progression of OSA-related cardiovascular diseases.9 Arterial hypertension, the most common disease of OSA, can develop through augmentation of the carotid chemoreflex via activation of HIF-1α following NOX2 and ROS production10,11 and enhancement of the sympathetic nervous12 or endothelin (ET) systems.13 OSA itself is generally agreed upon as leading to pulmonary hypertension (PH).14–16 However, consistent evidence of OSA-induced cardiac ventricular dysfunction or functional and morphological changes in the right ventricle (RV) is lacking. To our knowledge, no study has investigated sequential changes in the right heart on magnetic resonance imaging (MRI), along with observation of functional lung phenotypes and genetics, according to CIH duration. In this study, we used a CIH mouse model mimicking OSA to investigate chronological changes in heart function by cine-MRI and possible association with ROS system. We also attempted to describe histopathologic and associated genetic changes after 4 and 8 weeks of CIH exposure.

**MATERIALS AND METHODS**

**Animals and Exposure to CIH**
C57BL/6J adult male mice (about 8 weeks old) were randomized to 4 or 8 weeks of CIH (12 hours/day during daylight) or a Sham group exposed to air in identical chambers (4 weeks, sham: six mice, CIH: six mice, 8 weeks, sham: six mice, CIH six mice). All animals were provided standard mouse chow and water, ad libitum, at all times. All experimental protocols in this study were approved by the Institutional Animal Research Ethics Committee at the Yonsei Medical Center (IACUC Approval No. 2013-0299). All experimental
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Protocol methods were carried out in accordance with approved guidelines. Animals were housed in standard breeding cages. During CIH exposure, mice were transferred to a customized CIH chamber connected to a gas-control delivery system. Each 120-second cycle in the chamber had a 5% nadir of O₂ followed by restoration of O₂ to 21%. Gas-control delivery equipment (Live Cell Instrument, Korea) was designed to regulate nitrogen and oxygen flow into a customized chamber for intermittent hypoxia. The equipment was composed of programmable solenoids and flow regulators to control the inspired oxygen fraction, with timing and magnitude of arterial oxygen desaturation changes, as previously reported.15 Alterations in SpO₂ of mice in the CIH chamber were monitored with a pulse oximeter specifically designed for mice (Physiosuite, Kent Scientific©, Connecticut, USA). Mouse heart rates were stably maintained in the normoxic Sham chamber, and oxyhemoglobin levels were stably maintained above 90% (Figure 1). Mice in the CIH chamber showed sinusoidal fluctuations in oxyhemoglobin measured from peripheral blood according to cyclic change in FiO₂ from 5% to 21% (Figure 1). Control and CIH mice were weighed before hypoxia exposure and on the last day of each week of exposure. Summary of the characteristic of Sham and CIH animal is described in Supplementary Table 1.

Cardiac Magnetic Resonance Imaging

Cardiac MRI was performed with four Sham mice and four CIH mice for 4 weeks and five Sham mice and five CIH mice for 8 weeks at a 9.4 T magnet facility (Bruker, animal imaging facility, Avison Research Institute of Yonsei, Seoul, Korea) using an electrocardiogram (ECG)-triggered Cine-FLASH-sequence. Mice were anesthetized with 1.5% isoflurane in O₂ and positioned supine in a cradle. For optimal MR images for heart which can create strong motion artifact, an ECG electrode (SA Instruments, Inc., Stony Brook, New York, USA) was inserted into mouse forepaws and a respiration loop taped across the chest to R-waves detection. After positioning mice in the magnet (coil inner diameter 38 mm) with the heart in the center and scouting for long- and short-axis heart orientation with a double-gated, segmented gradient-echo sequence, shimming and pulse calibration were performed by Para Vision 5.0 operating software automatically before experiments. Using an ECG-triggered and respiratory-gated multiframe sequence (FLASH-cine protocol) with steady-state maintenance during respiration, 0.5-mm-thick slices were acquired in the short-axis orientation with 30 frames of images for one cardiac cycle. Imaging parameters comprised a field of view of 30.0 × 30.0 mm², matrix size of 256 × 256, echo time/repetition time = 2.1/6.0 milliseconds, a 20° sinc excitation pulse. 16 The measurements were averaged six times to increase signal-to-noise ratio. To define RV endocardial boundaries in short-axis views, we identified the RV cavity enclosed by the ventricular wall and mitral annulus and traced the endocardial border manually. Acquired cine-MRI data were captured as individual images by RadiAnt DICOM Viewer 3.4.2, then analyzed using region-of-interest measurements in Metamorph software: ejection fraction (EF), which was end-systole dimension divided by end-diastole dimension, and RV free wall thickness (WT) and ventricular diameter, which were measured in a mid-ventricular slice. RV diameters were given as endocardial diameters and evaluated in a septolateral direction, crossing the interventricular septum orthogonally. Analysis was performed using same images by two persons independently (Figure 2).

Hematoxylin and Eosin Staining

The heart of MRI measured mice (Sham four and CIH five for 4 weeks and five Sham mice and five CIH mice for 8 weeks) were used for histological analysis. Heart specimens were fixed and cleared of blood by perfusion with 3.8% paraformaldehyde and embedded in paraffin. For whole-heart morphology, hearts were cut longitudinally, and one half was embedded in paraffin. The other half was stored at −80°C for real-time polymerase chain reaction (PCR) analysis. Tissue sections (4 μm) were stained

![Figure 1](https://academic.oup.com/sleep/article-abstract/40/8/zsx103/3872269/fig1)
with hematoxylin and eosin (H&E) for routine histological analysis. From each mouse, one half of the lung tissue sample was stored at −80°C for real-time PCR analysis, and the other half was inflated with optimal cutting temperature compound (OCT; Sakura Tissue-Tek) before freezing. H&E staining of lungs and pulmonary arteries of about 50 μm in diameter was used for WT calculations. For calculating the thickness of PA walls, we used line measurement tools of MetaMorph. The values obtained by software were converted based on the bar (50 μm) in each image of H&E staining. More than 10 positions of walls from 10 arteries were used to measure and averages were summarized.

**Wheat-Germ-Agglutinin Staining for Myocyte Size Measurement**

To myocyte size analysis with wheat-germ-agglutinin (WGA) staining, fresh cryo tissue must be used, so we applied 8 weeks of CIH condition for four mice for each condition. To visualize myocyte boundaries for calculating myocyte size, Alexa Fluor 569-labeled WGA was used. Hearts were cut transversely, then embedded and frozen in OCT on dry ice. Frozen heart tissues were sliced into 4-μm sections at −20°C using a cryostat. After
staining with Alexa Fluor 569-labeled WGA, slides were visualized using an inverted fluorescence microscope (IX73-F22PH, Olympus). Cross-sectional cell areas were quantified by the region measurement software in Metamorph.

Masson’s Trichrome Staining for Muscularization of PA Wall
To visualize the fibrotic muscularized region around the PA wall, the slides with H&E staining were also stained with Masson’s trichrome. After staining, PA walls were pictured using an inverted light microscope (IX73-F22PH, Olympus). The dimensions of PA wall in images were calculated via two-dimension region tool of MetaMorph. The PA wall regions were drawn manually, then obtained the value of dimension. Muscularized area was selected blue color level after setting threshold, then the area was calculated. The value was converted to real μm² based on the square area using bar (50 μm).

Reverse-Transcriptase PCR and Quantitative Real-Time PCR Analyses
One half of the lung tissue sample from mice (Sham four and CIH five for 4 weeks and five Sham mice and five CIH mice for 8 weeks) stored at −80°C were used for mRNA analysis. Total RNA from lungs was extracted with TRIzol reagent (Invitrogen, Life Technologies™, Carlsbad, California, USA) according to the manufacturer’s instructions. cDNA was synthesized using AccuScript High Fidelity 1st Strand cDNA Synthesis kit (200436; Agilent Technologies, Santa Clara, California, USA) according to manufacturer’s instructions. Quantitative real-time PCR was performed in triplicate using TOPreal qPCR 2× PreMix (SYBR Green with high ROX) (RT501; Enzynomics, Daejeon, Korea). Reactions contained 25 ng of cDNA. Total RNA from heart halves or lung lobes was extracted for quantitative real-time PCR using the primer sets listed in Supplementary Table 2.

ELISA Analysis
Mouse serums after 8 weeks of sham (n = 6) or CIH (n = 6) was obtained from the tail vein before sacrifice. Mice used for cine-MRI measurement (8 weeks Sham [n = 4] and CIH [n = 4]) and mice of 8 weeks each conditions (n = 2) used for WGA staining were used for serum collection and ELIZA analysis. About 700 μL of serum were collected, from which plasma was obtained via centrifugation. To detect ET in mouse serum, an ET 1 ELISA kit (Enzo, cat. No #ADI-900-020A, New York, USA) was used. The plasma was diluted into a dilution solution in a 1:4 ratio, as recommended by the manufacturer. Absorbance at 450 was used to quantify the amount of ET, and serum amounts were calculated via the standard curve with supplied ET peptide.

Statistical Analysis
The results of multiple experiments are presented as means with standard error of the mean. Statistical analysis was performed with Student’s t-test or analysis of variance followed by Tukey’s multiple comparison test, as appropriate. p < .05 was considered statistically significant (*p < .05, **p < .01, ***p < .001).

RESULTS
Assessment of Cardiac Morphology and Function
Mouse heart function was assessed using cine-MRI in an axial or longitudinal axis according to the parameters described in the Materials and Methods section, and they were compared between the Sham and CIH groups (Figure 2). Analysis of Ry showed that EFs at 4 and 8 weeks were statistically lower in the CIH group than the Sham group (Figure 2G). We measured RV WT in the end-diastolic (ED) and end-systolic (ES) phases. RV ED-WT at 4 weeks was significantly lower in the CIH group than the Sham group. However, no difference in ED-WT was noted at 8 weeks (Figure 2H). In contrast, RV ES-WT at 4 weeks was not different between the CIH group than the Sham group, although it was significantly lower in the CIH group at 8 weeks (Figure 2I). These data indicated that RV morphology and function had already changed after 4 weeks of CIH and that the abnormalities had progressed following another 4 weeks of CIH. Especially, decreased RV ES-WT implied more impairment of pulmonary arteries and dilated RV after 8 weeks of CIH.

Histological Features of Cardiomyocytes and Hypertrophic Markers
Hearts were longitudinally sectioned and stained with H&E to observe gross morphological changes. Enlargement of the RV and slightly decreased RV WT were noticed at 4 weeks in the CIH group (Figure 3A). The enlarged RV cavity and decreased WT was severely enhanced after 8 weeks of CIH (Figure 3A). To compare the size of myocytes, heart sections were stained with WGA conjugated with Alexa Fluor 569 (red) (Figure 3B). Myocyte sizes of cross-sectional areas were calculated by measuring WGA-enclosed areas. The mean size of RV myocytes was significantly greater in the CIH group than the Sham group at 8 weeks (p < .001, Figure 3C).

Pulmonary Artery Histological Features
Lung tissue fixed with 4% paraformaldehyde was stained with H&E, and pulmonary arteries were observed. The accumulation of lymphocytes around the pulmonary artery (PA) was also more prominent in the CIH group at 4 and 8 weeks (Figure 4A). The endothelial wall of the pulmonary arteries was thicker in the CIH than the Sham group (Figure 4A). PA WTs were compared; the CIH group had significantly thicker PA walls than the Sham group (Figure 4B). PA muscularization was determined with Masson’s trichrome staining. Muscularized areas were measured using Metamorph software, and the CIH group showed a significant increase therein, compared to the Sham group (Figure 4, C and D).

ET System Changes in Hearts and Lungs
ET-1 is proposed to be an important mediator of carotid body chemosensory excitation and is present in the endothelium, blood vessels, and carotid bodies. Therefore, we investigated the ET system in blood and lung tissues. The mRNA levels for ET-1, ET-2, ET-3, and ET receptors (ETαA and ETβR) from lungs were all significantly elevated in the CIH group at 8 weeks (Figures 5A). Although the amount of ET-1 mRNA was smaller than ET-2 or ET-3, it was also significantly greater in the CIH group than the Sham group. To determine secreted ET protein levels, ELISA was performed to measure blood concentrations of only ET-1 because no good detection antibodies for ET-2 and ET-3 have been developed. ET-1 was also significantly higher in the CIH group than the Sham group at 8 weeks (Figure 5B).
mRNA levels for HIF-1 from lung tissue were also significantly elevated in the CIH group at 8 weeks (Figure 5C).

**Discussion**

In our study, we described chronologic changes in RV using three-dimensional cine-MRI, which was developed especially for small rodent experiments, with confirmation of changes in ETs in a murine CIH model.

Despite a large body of cardiovascular research on OSA, the influence of CIH on the right heart has not been clearly elucidated. Since the position of the RV is in a limited acoustic window, attempts to evaluate RV function by echocardiography are difficult. In addition, the complex geometry, contraction mode, and the unpredictability of changes in dimensions under pathophysiological conditions make assessment of morphology and function more difficult for the RV than the LV. Therefore, cine-MRI has advantages over two-dimensional echocardiography for measuring heart function because it requires fewer geometric assumptions and is more accurate and reproducible.
RV changes in OSA patients seem to vary, as the RV has a complex crescent shape and is surrounded by the LV, leading to difficulty in determining RV function with conventional two-dimensional echocardiography. Animal studies using dogs, rats, and mice support the hypothesis that OSA negatively affects ventricular function. RV dilation and dysfunction is important due to their close association with PH, which we confirmed as muscularization of pulmonary vessels that constantly increased over 8 weeks of CIH (Figure 4). The effect of OSA on right heart structure and function has been disputed, although many studies find they are associated in patients. RV systolic dysfunction is observed in OSA patients and is associated with disease severity. Impairment of RV function in OSA can be explained by stress that comprises a combination of increased pulmonary vascular resistance and RV EDV, leading to a reduction in RV EF. Despite controversial results, early investigations of RV function in OSA patients are essential because it might progress to PH, even in the absence of emerging cardiovascular disease. In our data, RV parameters showed significant morphological and functional changes after 4 weeks of CIH. RV EF and ED-WT were significantly decreased by 4 weeks of CIH. Among these parameters, RV ED-WT showed no difference between Sham and CIH group at 8 weeks; however, RV EF remained significantly lower and RV ES-WT became significantly lower in the CIH group at 8 weeks. At present, we cannot explain why RV-ED-WT is

Figure 4—Morphological analysis of the pulmonary artery and Masson's trichrome staining for muscularized area around pulmonary artery. (A) Representative H&E staining of PA (about 50-μm diameter). Scale bar, 50 μm. Wall thickness of PA in CIH group was increased compared to Sham group. (B) Summary of PA diameter measurement. PA-WTs in CIH group was significantly increased at both 4 and 8 weeks. PA walls at 8 weeks CIH had an increased diameter compared to 4 weeks of CIH (n = 6 per group; 10 positions in arteries and about 10 arteries per mouse were measured). (C) Muscularization of pulmonary arteries was determined with Masson's trichrome staining. (D) Muscularized area was measured using Metamorph software and the CIH group showed significantly increased muscularized area compared to the Sham group (**p < .01, ***p < .001). Br = bronchiole; PA = pulmonary artery; WT = wall thickness.
thinner at 4 weeks of CIH and RV-ES-WT is thinner at 8 weeks of CIH. However, these data clearly indicated that RV morphology and function had already changed to an abnormal state by 4 weeks of CIH; these abnormal changes persisted following another 4 weeks of CIH. Decreased RV ES-WT at 8 weeks of CIH implied more severe impairment in pulmonary arteries, and these results were also confirmed by histological analysis of heart and pulmonary arteries.

Microscopically, we found that intermittent hypoxia increased the size of RV myocytes (Figure 3, B and C). Such myocardial remodeling is associated with RV dysfunction in animal models with hypoxia or pressure overload\(^5\,\text{---}\,^6\,\text{---}\,^3\,\text{---}\,^4\) and might also be mediated by a ROS-signaling mechanism.\(^8\) RV remodeling may lead to PH characterized by pulmonary vasoconstriction, endothelial dysfunction, or vascular remodeling,\(^15\) for which HIF-1α is suggested as an important mechanism.\(^36\) In our results, thickening of PA-WT was enhanced at 4 weeks of CIH, and it was more evident at 8 weeks of CIH (Figure 4B). Similar results were reported by Fagan et al.\(^15\) who observed PH in mice after 4 weeks of CIH. This was an important finding because insidious PH may exist before overt severe cardiovascular diseases from CIH.

In our study, we investigated the ET system because it can contribute to elevation of blood pressure through sympathetic vasoconstriction in OSA.\(^13\,\text{---}\,^37\,\text{---}\,^38\) The association of plasma levels of ET-1 and hypertension under CIH was first reported by Kanagy et al. Also, an ET-1 antagonist is suggested as a therapeutic target for hypertension in OSA.\(^33\) ET-1 is expressed in the endothelium, blood vessels, and carotid body.\(^17\) Pawar et al.\(^39\) found enhanced basal production of ET-1 and upregulation of the ETrA receptor with CIH-induced ROS in rat neonatal carotid bodies. Our study found that the mRNA expressions of ET-1, ET-2, ET-3, ETrA, and ETrB were significantly increased after 8 weeks of CIH (Figure 5B). Compared to ET-1, ET-2 and ET-3 have not been extensively studied, and their functions are not well known. However, a significant role for ET-2 in CIH conditions can be speculated from some studies.\(^40\,\text{---}\,^42\) Since ET mRNA levels are considered to reflect protein levels thereof,\(^40\) the marked increases in ET2 and ET3 mRNA might imply a tremendous increase in ET-2 and ET3, greater than that in ET-1, after 8 weeks of CIH. Although no good detection antibodies for ET-2 and ET-3 have been developed, we carefully speculate that, if we can measure their amounts in blood samples, ET-2 and/or ET-3 could be more sensitive indicators of severe OSA than ET-1. Taken together, increases in ET system including its receptors, might play a critical role in long-term CIH.

Our study adds several new findings to the field. First, we applied a novel technique for measuring heart function using cine-MRI in a CIH mouse model. Second, we elaborated on chronological changes in RV function with CIH that suggest PH. Finally, we identified changes in the ET system from the lungs that might be associated with cardiovascular phenotypes in OSA. However, this study has some limitations that should be taken into consideration. First, the model system in this study was not coupled with EEG measuring sensors which shows sleep stage and mice were exposed to CIH during daylight time. This could be not enough to mirror real OSA condition. Second, the frequency of CIH cycle was slower than the CIH model in other studies. This could affect the time frame or severity in changing phenotype. Third, our CIH model is substantially different from OSA in human because of the lack in hypercapnic stimulus. It has been reported in previous literature that hypercapnic but not eucapnic IH can lead to increased RV mass compared to hypocapnic IH in rat.\(^43\) Therefore, this aspect

**Figure 5**—Endothelin system and HIF-1 changes during CIH in lung or serum. (A) ET-1, ET-2, ET-3, ETrA, and ETrB mRNA expression from lung tissue. ET-2, ET-3, ETrA, and ETrB expression was significantly higher at 8 weeks in CIH group. (B) ET-1 concentration in blood serum at 8 weeks for each condition. ELISA measured blood concentration of ET-1. (C) HIF-1 mRNA expression from lung tissue. HIF-1 was significantly higher in 8 weeks of CIH group. (*p < .05, **p < .01). CIH = chronic intermittent hypoxia; ET = endothelin.

**Figure 6**—Schematic tracing of sequential changes in phenotypes related to right ventricle or pulmonary hypertension with CIH in murine model. CIH = chronic intermittent hypoxia; EF = ejection fraction; ED-WT = end-diastole wall thickness; ES-WT = end-systole wall thickness; PA-WT = pulmonary artery wall thickness.
should be also considered when delineating result. Fourth, we did not determine PA pressure via a direct measurement. We assumed the development of PH based on phenotypic changes in the heart and lung including pulmonary vessels. Finally, we did not yet provide a signaling mechanism using a specific gene-engineered mouse in this study, although this is currently under investigation.

**CONCLUSIONS**

By integrated analysis, we finely documented the persistence of reduced RV EF and ES-WT using cine-MRI, which could be associated with the change of ROS system. In addition, we found the elevation of the ET system in the lungs and blood according to the change of RV phenotype. Therefore, we postulated that RV function could be retarded with the progression of PH upon CIH. ETs from the blood could be utilized as possible biomarkers to predict pathologic changes in the RV with PH.

**REFERENCES**


SUPPLEMENTARY MATERIAL
Supplementary material is available at SLEEP online.

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