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Intermittent Hypoxia-Induced Cardiovascular Remodeling Is Reversed by Normoxia in a Mouse Model of Sleep Apnea

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BACKGROUND: Intermittent hypoxia (IH) is the principal injurious factor involved in the cardiovascular morbidity and mortality associated with OSA. The gold standard for treatment is CPAP, which eliminates IH and appears to reduce cardiovascular risk. There is no experimental evidence on the reversibility of cardiovascular remodeling after IH withdrawal. The objective of the present study is to assess the reversibility of early cardiovascular structural remodeling induced by IH after resumption of normoxic breathing in a novel recovery animal model mimicking OSA treatment.

METHODS: We investigated cardiovascular remodeling in C57BL/6 mice exposed to IH for 6 weeks vs the normoxia group and its spontaneous recovery after 6 subsequent weeks under normoxia.

RESULTS: Aortic expansive remodeling was induced by IH, with intima-media thickening and without lumen perimeter changes. Elastic fiber network disorganization, fragmentation, and estrangement between the end points of disrupted fibers were increased by IH. Extracellular matrix turnover was altered, as visualized by collagen and mucoid interlaminar accumulation. Furthermore, left ventricular perivascular fibrosis was increased by IH, whereas cardiomyocytes size was unaffected. These cardiovascular remodeling events induced by IH were normalized after recovery in normoxia, mimicking CPAP treatment.

CONCLUSIONS: The early structural cardiovascular remodeling induced by IH was normalized after IH removal, revealing a novel recovery model for studying the effects of OSA treatment. Our findings suggest the clinical relevance of early detection and effective treatment of OSA in patients to prevent the natural course of cardiovascular diseases.

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KEY WORDS: atherosclerosis recovery; cardiovascular disease; continuous positive airway pressure; intermittent hypoxia; obstructive sleep apnea

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ABBREVIATIONS: ECM = extracellular matrix; H&E = hematoxylin and eosin; IH = intermittent hypoxia; IMT = intima-media thickness

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OSA is a highly prevalent disorder that affects 6% to 15% of the general population and is caused by repetitive upper airway occlusion during sleep.[1,2] OSA is an important public health problem because of its association with increased cardiovascular morbidity and mortality, including hypertension, coronary artery disease, congestive heart failure, heart attack, and stroke.[3,4] The major OSA components associated with cardiovascular consequences are large swings in intrathoracic pressure, postapneic arousals, and intermittent hypoxia (IH). IH is the main detrimental event leading to cardiovascular morbidity and mortality.[5,6]

Sympathetic overactivation, oxidative stress, and systemic inflammation are the main intermediary mechanisms associated with IH.[4,7] These abnormalities all contribute to the development of early and late cardiovascular remodeling, including increased blood pressure, endothelial dysfunction, carotid intima-media thickness (IMT), arterial stiffness, and accelerated progression of atherosclerosis, and induce cardiac rhythm and structural disturbances.[4,8]

Murine models have been used to study the adaptive and degenerative hemodynamic and structural alterations of the cardiovascular system induced by IH.[9] IH induces blood pressure elevation, endothelial dysfunction, enlargement of aortic IMT, cardiac hypertrophy, and extracellular matrix (ECM) alterations; increased systemic inflammation and activation of proinflammatory pathways in cardiovascular tissue; and increased risk of developing atherosclerotic plaques.[10]

CPAP, the gold standard therapy for patients with OSA, effectively improves daytime symptoms and quality of life, and might be an effective treatment for cardiovascular risk reduction.[11,12] Randomized controlled trials have demonstrated that CPAP therapy reduces blood pressure, sympathetic overactivity, and coagulation abnormalities and improves left ventricular ejection fraction.[13-16] CPAP has also been shown to improve endothelial function, IMT, and arterial stiffness in small studies.[5,17] However, there is no experimental evidence that elimination of IH reverses the cardiovascular remodeling induced by injurious hypoxic challenge.

To address this important issue, we established a murine model of recovery in which normal room air breathing is resumed after chronic IH challenge. We hypothesized that the resumption of normoxic conditions, which mimics CPAP treatment, could reverse the early cardiovascular morphological remodeling induced by IH. This recovery model will enable the study of the mechanisms involved in the therapeutic effects of OSA treatments in reversing injuries induced by IH in different organs.

Materials and Methods

Study Design

The study was approved by the Ethical Committee for Animal Research of the University of Barcelona and was performed on 6-week-old pathogen-free C57BL/6 male mice (Charles River Laboratories). The animals were housed in standard cages in a temperature- and light-controlled room (22°C-24°C; 14 hours of light, 10 hours of dark). A total of 40 mice were randomly assigned to IH exposure (n = 20 mice) or normoxia (n = 20 mice) for 6 weeks. After this IH phase, 10 mice from each group were anesthetized (urethane 20%, 1 g/kg) and euthanized by exsanguination, and aortas and hearts were excised. The remaining IH mice were subsequently subjected to a 6-week normoxic recovery phase to mimic CPAP treatment of patients with OSA and sacrificed, and tissue samples were excised as described below. The experimental design of the protocol is shown in Figure 1A. The groups were labeled N, normoxia; IH, intermittent hypoxia; N+R, normoxia with recovery phase; and IH+R, intermittent hypoxia with recovery phase.

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Intermittent Hypoxia

Chronic IH was applied as previously described.[18] For 6 weeks, mice in the IH group received 60 hypoxic events/h (20 s at 5% O_2 per min), during 6 h/d, corresponding to severe OSA. Control mice with normoxic breathing were placed in an identical system, but the hypoxic gas from the reservoir was replaced by room air. In the normoxic recovery phase, all mice were subjected to identical normoxic conditions.

Histomorphological Analyses

The mid thoracic aorta and left ventricle of the heart samples were perfused with phosphate-buffered saline, fixed with 4% paraformaldehyde, and embedded in paraffin for further histological analysis by an investigator blinded to the experimental group. The samples were stained with hematoxylin and eosin (H&E, Master Diagnostica), Gomori trichrome stain (Artisan Link Special Staining System; DAKO), or Alcian blue (Alcian blue 2.5; Bio-Optica). For measurements, images from four consecutive sections were processed using Image J (National Institutes of Health) and Adobe Photoshop CS6 (Adobe Systems Inc) software. All stained sections were captured with a digital microimaging network instrument (Leica-DMD-108; Leica Microsystems), and aortic autofluorescence was visualized using a fluorescence microscope (Olympus-BX51; Olympus).

Intima-media thickness: The cross-sectional IMT was quantified by morphometric analysis of the H&E stained sections (300 measurements for each animal).

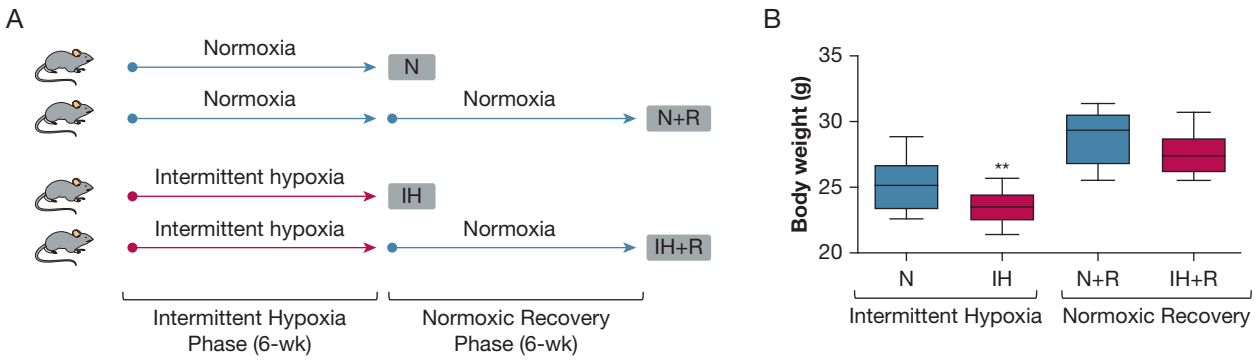

Figure 1 – *Mouse growth is altered by intermittent hypoxia and normalized after normoxic recovery. A, Experimental design of the study (n = 10, per group): male C57BL/6 mice exposed to room air (N) or to IH for 6 weeks, and mice exposed to N or IH and subsequently subjected to a period of normoxia (6 more weeks; N+R and IH+R). B, Box-plot representation of body weight in N and IH groups at 6 weeks (P = .005) and in N+R and IH+R groups at 12 weeks (P = .136). **P < .01 for intergroup comparisons. IH = intermittent hypoxia; IH+R = intermittent hypoxia plus normoxic recovery; N = normoxia; N+R = normoxia plus normoxic recovery.*

Alcian blue staining: The integrated density of the blue staining was quantified and adjusted to the corresponding aortic wall area to detect mucoid deposition.

Cardiac hypertrophy: The cross-sectional area of the cardiac myofibers with a circular running pattern was analyzed quantitatively using H&E stained sections (300 cardiomyocytes for each animal).

Cardiovascular fibrosis: Gomori trichrome stain was used to detect fibrosis in aortic and cardiac tissue. The fibrotic tissue was determined by measuring the positive collagen area adjusted to the total tissue area.

Elastic-network analysis: The aortic autofluorescence was used to perform elastic fiber analysis. The elastin disruption (ie, the complete fragmentation of one elastic fiber) and the distance between both ends of a fragmented fiber were quantified (adjusted by total aortic area and shown as percent space without fiber). In addition, we quantified the area with elastic fiber disorganization based on the inability to count the amount of organized elastic fiber.[19]

Data Analysis

Results were expressed as the mean ± SEM. Depending on normality and variance homogeneity, analysis of variance and Student *t* test or Mann-Whitney *U* test were performed. Statistical significance was set at a probability value of less than .05. Structural parameters were adjusted for body weight using a linear regression model.

Results

Body Weight

The body weight at baseline was similar in both groups. However, 6 weeks of IH decreased animal body weight ($P = .005$). After the normoxic recovery phase, the body weights of mice in the IH+R group were similar to those in the N+R group, suggesting a normalization of body weight after IH withdrawal (Fig 1B).

Morphological Vascular Remodeling

Intima-media thickness: The aortic IMT was increased by IH exposure vs that of the N group ($P = .03$). After normoxia, the IMT of mice in the IH+R group was normalized compared with its control, suggesting a recovery of aortic remodeling (Figs 2A, 2B). The aortic lumen perimeter did not exhibit significant changes, indicating expansive remodeling of the aortic wall induced by IH. Moreover, mice in the N+R and IH+R groups did not show statistically significant differences in lumen perimeter.

Elastin fiber disorganization and disruption: Six weeks of IH exposure induced elastin fiber disruption and increased the distance between both ends of the fragmented fibers (Figs 2C-E). These alterations were reduced compared with those of the N+R group, suggesting that the aortas of the IH+R group were subjected to a recovery remodeling process (Figs 2D, 2E). Furthermore, mice exposed to IH displayed an increase in zones of elastin fiber disorganization in the aortic wall, which was not observed in mice in the IH+R group compared with those in the N+R group (Fig 2F).

Aortic Mucoid deposition: Alcian blue staining revealed greater mucoid deposition in the vascular wall of the IH group between subintimal elastic fibers, specifically in regions neighboring the aortic lumen. Mucoid deposition in the aortic wall in the N+R and IH+R groups was similar to that in the normoxia group, suggesting normalization after normoxic recovery (Figs 3A, 3B).

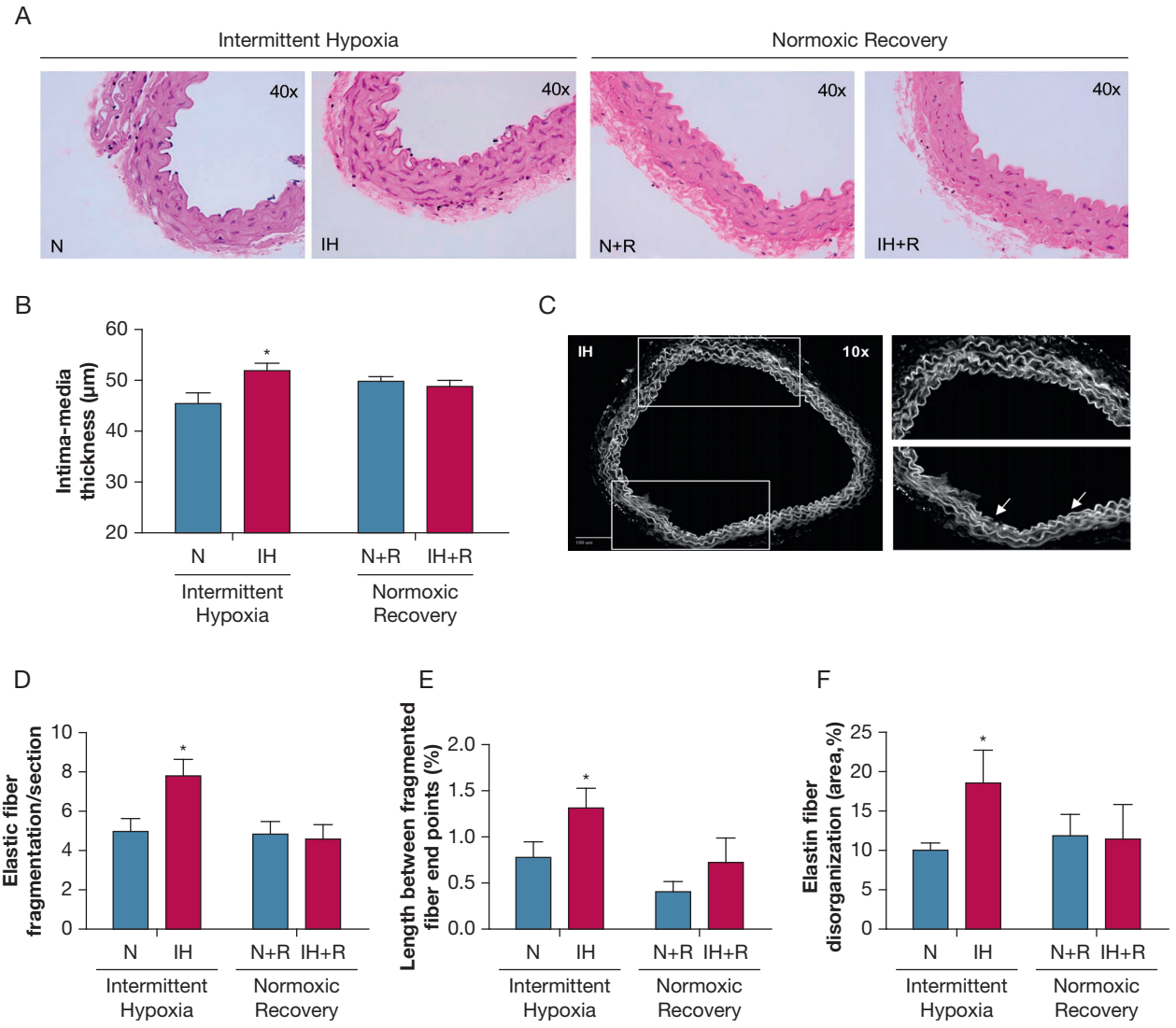

Figure 2 – *Aortic morphological remodeling associated with intermittent hypoxia and recovery after normoxic conditions. Morphological remodeling of mid thoracic aorta cross sections was assessed in C57BL/6 mice exposed to IH or room air (N) for 6 weeks and the recovery from exposure to IH or N after a period of normoxia (N+R, IH+R). A, Representative images of the aortic wall with H&E staining for each group (original magnification ×400). B, Histomorphometric analysis of intima-media thickness, IH vs N (P = .03) and IH+R vs N+R (P = .92). C, Representative pictures of the elastic network (original magnification ×100), revealed by autofluorescence, with magnification of zoom elastic fiber disorganization (top inset) and fragmentation (bottom inset, with arrows showing fragmented elastic fiber end points). D, E, Quantification of intima-media elastic fiber breaks (D), and the length between the ends of fragmented fibers adjusted by total aortic wall area (E) (shown as %). F, Elastin fiber disorganization area in the aortic wall adjusted by total area (shown as %). *P < .05; values are mean ± SEM. H&E = hematoxylin and eosin. See Figure 1 legend for expansion of other abbreviations.*

Aortic fibrosis: The collagen fiber content in the aortic wall was higher in mice exposed to IH for 6 weeks, suggesting the induction of collagen synthesis during IH exposure. Recovery under normoxic conditions of the IH+R group resulted in a decrease in aortic fibrosis, similar to the N+R group (Figs 3A, 3C).

Morphological Cardiac Remodeling

Mice exposed to IH for 6 weeks exhibited increased cardiac perivascular fibrosis compared with the normoxia group (Figs 4A, 4B). After the normoxic recovery phase, the extracellular collagen content of the IH+R group was no different from that of the N+R group (Figs 4A, 4B). The cross-sectional area of the left ventricular cardiomyocytes did not differ significantly between groups (Fig 4C).

Discussion

This study demonstrates that normoxic breathing after a period of chronic IH spontaneously reverts the early structural cardiovascular remodeling induced by this injurious challenge that characterizes sleep apnea. In the

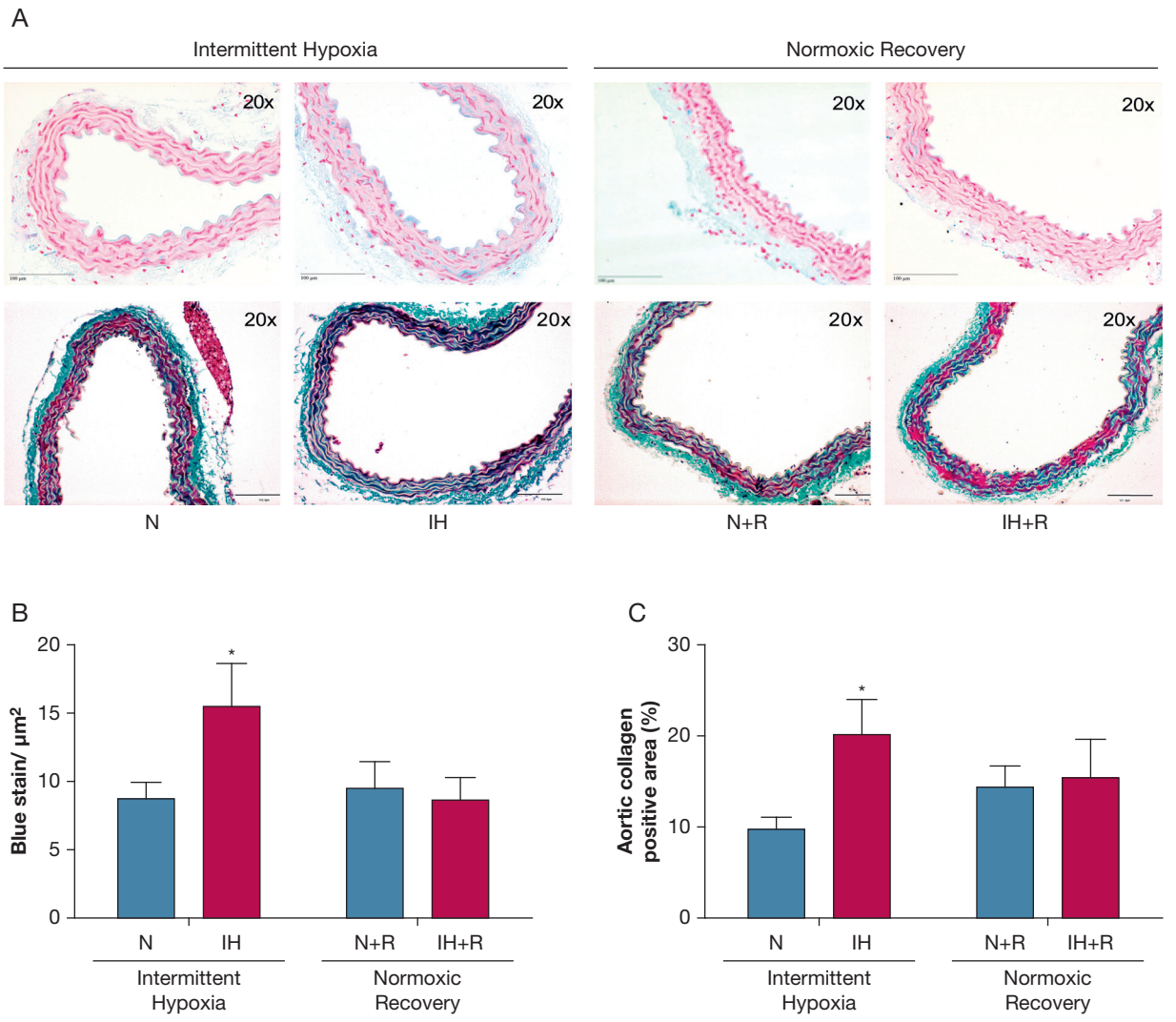

Figure 3 – *Aorta extracellular matrix remodeling induced by intermittent hypoxia and progression after recovery in normoxic conditions. Remodeling of extracellular components of the aortic wall was assessed in C57BL/6 mice exposed to IH or room air (N) at 6 weeks and in mice exposed to N or IH and subsequently subjected to normoxia (N+R, IH+R). A, top, Representative images of Alcian blue staining from the mid thoracic aorta to detect aortic wall mucoid deposition (original magnification ×200; mucins in blue); A, bottom, collagen-positive area of the intima-media (%). B, Representative images of Gomori trichrome stain to measure aortic wall fibrosis (original magnification ×200; collagen in green). C, Intima-media mucoid deposition shown as the ratio of the total blue density to the total aortic wall area. (%).*P < .05; values are mean ± SEM. See Figure 1 legend for expansion of abbreviations.*

field of experimental sleep apnea, considerable research has focused on analyzing the effects of IH, but few studies have analyzed the extent to which the deleterious effects of IH can be reversed by a period of post-IH normoxia, which mimics the clinical situation in which OSA therapy is applied to restore normal breathing. Our novel experimental setting strongly suggests that effective treatment could normalize early cardiovascular lesions induced by hypoxemia associated with OSA syndrome.

OSA integrates various pathophysiological triggers, but IH is the principal injurious factor that plays a pivotal role in the progression of cardiovascular diseases.[20] In addition to the evidence of adverse events caused by IH,[9] other studies have demonstrated beneficial effects of IH in both animal models and patients with OSA.[21] The opposing effects induced by IH depend mainly on the experimental time; long-term exposure (4-8 weeks) is required to cause detrimental effects.[22] In the present study, we assessed several morphological cardiovascular changes resulting from the direct effect of IH for 6 weeks, a common experimental paradigm to mimic severe OSA in patients. Our results confirmed the hypothesis that restoring normoxia by removing IH stress facilitates homeostatic cardiovascular restoration.

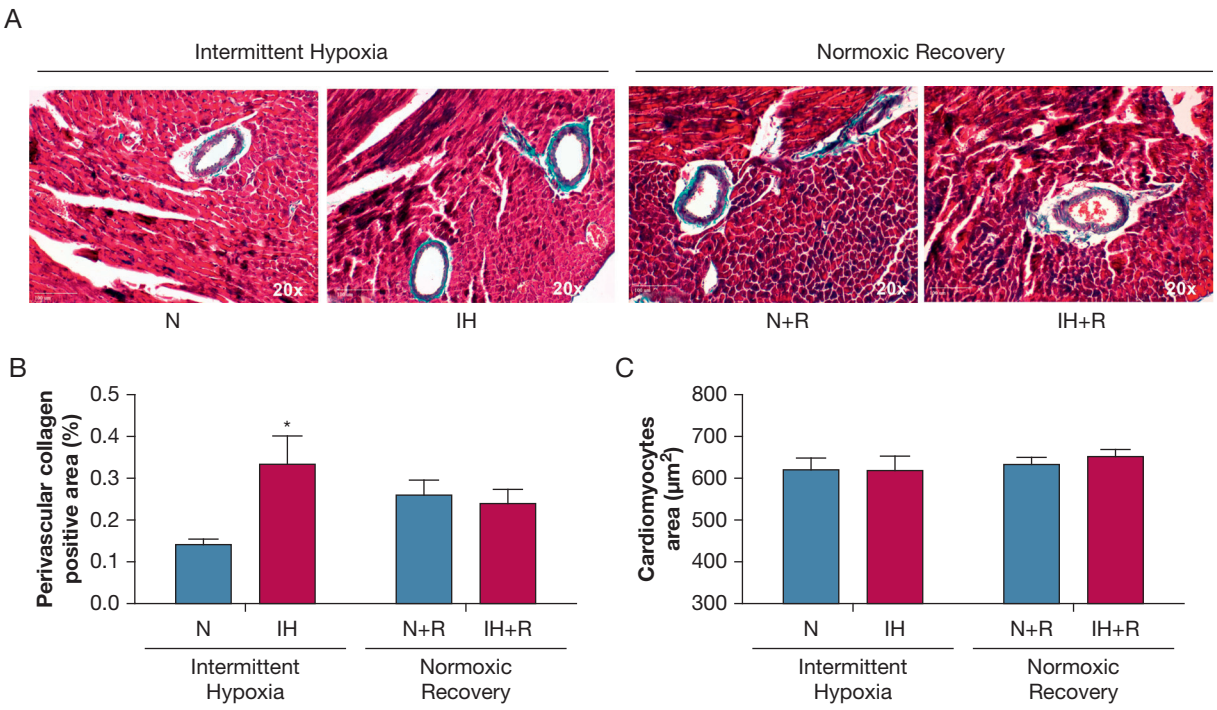

Figure 4 – *Cardiac morphological remodeling associated with intermittent hypoxia and the effect of recovery in normoxic conditions. Morphological remodeling of cardiac tissue was assessed in mice exposed to IH or room air (N) at 6 weeks and in mice exposed to IH or N that were subsequently subjected to normoxia (N+R, IH+R). A, Representative images of the left ventricle with Gomori trichrome stain to detect perivascular fibrosis (original magnification ×200; collagen in green). B, Analysis of perivascular fibrosis measured as collagen-positive area (%). C, Histomorphometric analysis of the left ventricular cardiomyocyte area of fibers with a circular pattern did not reveal statistically significant differences.* *P < .05; values are mean ± SEM. See Figure 1 legend for expansion of abbreviations.*

Vascular remodeling is dependent on dynamic interactions between local growth factors, vasoactive substances, and hemodynamic stimuli and is a response to long-standing changes in hemodynamic conditions.[23] IH[24] and sleep fragmentation[19] are independent factors that promote vascular remodeling in the aorta. IMT remodeling is an early predisposing event in atherosclerosis and plaque formation and is associated with increased cardiovascular risk.[25] Patients with OSA exhibit increased IMT in association with inflammatory markers and nocturnal oxygen desaturation.[26] Our findings confirm previous observations of expansive aortic remodeling with increased IMT without vascular dilatation as a result of IH exposure in mice.[27] Importantly, our novel experimental data on IMT normalization after normoxic recovery are in agreement with clinical data on patients with OSA who were treated with CPAP.[17]

We also observed that IH increased elastic fiber disorganization and disruption. The increase in the estrangement of the two end points of the disrupted lamina, reported in the present study, suggests a higher tensile stress in the aortic wall exposed to IH, leading to a stronger fiber break. Perturbations in the continuity of the elastic lamina have been implicated in early phases of atherosclerosis[28] and in vascular remodeling induced by sleep fragmentation.[19] Changes in elastin structure and distribution have been reported in a rat model of IH, but quantitative morphometric analysis was not performed.[29] However, we have quantitatively assessed elastic fiber organization and fragmentation of the aortic wall. Strikingly, our results demonstrate that the normoxic recovery in mice that had been previously exposed to IH enabled a normalization of the vascular elastic fiber network alterations.

Changes in the ECM have been implicated in the pathogenesis of atherosclerosis and play an important role in intercellular networking. These changes can lead to a fibroproliferative response, promoting lipid binding to the vascular wall and inducing foam cell formation.[30] We observed abnormal ECM turnover in the aortic wall in mice exposed to IH, which suggests that IH promotes collagen and mucopolysaccharide (proteoglycans and glycosaminoglycans) synthesis and deposition in interlaminar spaces. Importantly, we observed that this ECM remodeling could be normalized after a recovery period in normoxic conditions, which indicates the

possible activation of inhibitory and degradation pathways of collagen and mucopolysaccharide synthesis.

The ECM response to IH stress also includes morphological myocardial remodeling. We observed that IH induced perivascular fibrosis in the left ventricle, whereas interstitial fibrosis was not increased, in agreement with previous studies.[31] Perivascular fibrosis is substantially associated with the impairment of coronary blood flow and is involved in the progression of heart failure.[32] Because of significant independent associations between OSA and heart failure, many studies have evaluated CPAP as a treatment for patients with OSA who have heart failure.[33,34] In the present study, we observed a normalization of coronary perivascular fibrosis after recovery under normoxic conditions. Normoxia restoration was sufficient to reduce perivascular fibrosis, most likely because of the reduction of the fibroinflammatory response and oxidative stress production in myocardial tissue. This finding has clinical relevance and suggests that patients with OSA who have heart disease would benefit from effective breathing normalization, most likely because of the resulting improved coronary blood flow.

Cardiac remodeling includes hypertrophy that can exist in a state of compensation or progress to a decompensated state with time. We did not observe left ventricular hypertrophy, consistent with previous studies.[35] However, other studies have observed cardiac hypertrophy induced by IH.[31,36] The large disparity in results for left ventricular hypertrophy may reflect differences in species or strain or even the side of the heart,[22] which could explain our negative result for left ventricular hypertrophy.

Aortic wall and left ventricular remodeling induced by IH is the result of multiple interactions between intermediary mechanisms, including oxidative stress, systemic and tissue inflammation, metabolic deregulation, endothelial dysfunction, sympathetic overactivation, and blood pressure overload.[24,37] Our study did not focus on assessing changes in blood pressure; however, two similar studies found that C57BL/6 mice exhibit increments in blood pressure after 14 and 90 days of IH exposure.[22,38] Arterial blood pressure increases (10 to 20 mm Hg) in rodent models of IH are comparable with those of other experimental animal models of hypertension.[39] Thus, in mice that are exposed to IH, increases in blood pressure may induce functional, mechanical, and structural changes in the aortic wall in response to hemodynamic and biomechanical stress. Moreover, IMT, elastin fiber disruption, and interlaminar collagen accumulation induce arterial stiffness,[40] thereby contributing to systemic vascular resistance and arterial blood pressure elevation.

Reversibility of structural cardiovascular damage has been demonstrated in several animal models of hypertension through spontaneous reversion or through the use of several forms of antihypertensive treatment.[41-47] Celiprolol reduced cardiovascular alterations induced by hypoxic stress in mice exposed to IH.[48] The reversal of structural changes induced by elevated blood pressure suggests that several of our results could be explained by a reduction in blood pressure after the recovery phase in normoxic conditions.

The current study has several limitations. Recurrent apnea in patients results in IH, hypercapnia, sleep arousal, sleep fragmentation, and changes in intrathoracic pressure that may contribute to cardiovascular remodeling. However, our study focused exclusively on IH stress, which is a limitation because the mice model of hypoxemia associated with sleep apnea does not represent the totality of the complex disorder. However, IH is the most important pathophysiological component of sleep apnea that underlies cardiovascular complications, which was the principal outcome of our study. The most common index of cardiac hypertrophy is the measure of heart or ventricular weights related to body weight. We did not assess this parameter, but relating heart to body weight is not valid when the investigated groups do not exhibit similar body growth patterns, as we observed in this study.[49] The main strength of this work is that the use of a conventional mouse strain allowed us to assess the cardiovascular impact induced by IH per se and the subsequent recovery process under normoxic conditions, avoiding other confounding factors.

Conclusions

The current study demonstrates that IH induces preatherosclerotic remodeling characterized by IMT, elastin disruption and disorganization, accumulation of collagen fibers, and mucoid elements on the aortic wall. We also observed initial myocardial remodeling induced by IH exposure, specifically perivascular fibrosis. These cardiovascular remodeling events are virtually reversed when the IH stress was removed and mice were returned to normoxic conditions, mimicking the effective treatment of the hypoxic component of

OSA. The clinical relevance of our findings suggests that early detection of patients with OSA and the subsequent therapeutic intervention to normalize breathing may alter the natural course of cardiovascular diseases that are promoted by cyclic hypoxia and reoxygenation. Furthermore, we propose for the first time a murine model of IH followed by normoxia to study the potential benefits of IH resolution with CPAP treatment in patients with OSA, including restoring normal structure and function of the different organs challenged by this sleep breathing disorder. This recovery model may be a useful tool for future studies aimed at identifying possible cellular and molecular mechanisms and signaling pathways involved in the homeostatic and adaptive response to IH. Additionally, this model may be used in future studies to assess OSA treatments.

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