Respiratory Physiology & Neurobiology 170 (2010) 157–163

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

journal homepage: www.elsevier.com/locate/resphysiol

Time course of intermittent hypoxia-induced impairments in resistance artery structure and function

Nathan R. Philippi[a], Cynthia E. Bird[a], Noah J. Marcus[b,e], E. Burt Olson[c], Naomi C. Chesler[d], Barbara J. Morgan[a,e,*]

[a] John Rankin Laboratory of Pulmonary Medicine, Department of Orthopedics and Rehabilitation, University of Wisconsin, Madison, WI, United States
[b] John Rankin Laboratory of Pulmonary Medicine, Department of Kinesiology, University of Wisconsin, Madison, WI, United States
[c] John Rankin Laboratory of Pulmonary Medicine, Department of Population Health Sciences, University of Wisconsin, Madison, WI, United States
[d] Department of Biomedical Engineering, University of Wisconsin, Madison, WI, United States
[e] Respiratory Neurobiology Training Program, University of Wisconsin, Madison, WI, United States

ARTICLE INFO

Article history:
Accepted 2 December 2009

Keywords:
Vasodilation
Vascular endothelium
Fibrillar collagens
Isoprostanes

ABSTRACT

We previously demonstrated that chronic exposure to intermittent hypoxia (CIH) impairs endothelium-dependent vasodilation in rats. To determine the time course of this response, rats were exposed to CIH for 3, 14, 28, or 56 days. Then, we measured acetylcholine- and nitroprusside-induced vasodilation in isolated gracilis arteries. Also, we measured endothelial and inducible nitric oxide synthase, nitrotyrosine, and collagen in the arterial wall and urinary isoprostanes. Endothelium-dependent vasodilation was impaired after 2 weeks of CIH. Three days of CIH was not sufficient to produce this impairment and longer exposures (*i.e.* 4 and 8 weeks) did not exacerbate it. Impaired vasodilation was accompanied by increased collagen deposition. CIH elevated urinary isoprostane excretion, whereas there was no consistent effect on either isoform of nitric oxide synthase or nitrotyrosine. Exposure to CIH produces functional and structural deficits in skeletal muscle resistance arteries. These impairments develop within 2 weeks after initiation of exposure and they are accompanied by systemic evidence of oxidant stress.

1. Introduction

Exposure to chronic intermittent hypoxia (CIH) in rats causes vascular dysfunction. Specifically, we have documented that 14 days of CIH attenuates acetylcholine (ACh)- and hypoxia-induced vasodilation in the skeletal muscle and cerebral circulations (Phillips et al., 2004), increases stiffness of skeletal muscle resistance arteries (Phillips et al., 2006), and causes blood pressure and heart rate elevations that are apparent not only during the CIH exposures but also during the portion of the day when the rats are normoxic (Marcus et al., 2009a). These same impairments have been observed in patients with moderate to severe obstructive sleep apnea (OSA) (Carlson et al., 1996; Becker et al., 2003; Phillips et al., 2008). In such patients, the duration of OSA prior to diagnosis typically cannot be ascertained; therefore, the time course for development of impairments in vascular function is unknown. In addition, the mechanisms underlying these impairments are not completely understood. The purpose of this investigation was to perform *in vitro* assessment of resistance artery function and structure after 3, 14, 28, and 56 days of exposure to CIH in Sprague–Dawley rats, an established model of OSA. In addition, because oxidative stress and inflammation are putative contributors to OSA-related vascular dysfunction in humans (Lavie, 2009), we assessed the effects of CIH on urinary isoprostane excretion and expression of endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), and nitrotyrosine (NT) in the arterial wall.

2. Methods

2.1. Animals

Adult male Sprague–Dawley rats (Harlan, Madison, WI) were used for all experiments. They had *ad libitum* access to standard rat chow (Purina) and drinking water during exposure to either CIH or normoxia (see below). Room temperature and relative humidity were maintained at $24 \pm 1\,^{\circ}$C. and 20–70%, respectively. Rats were housed in accordance with recommendations set forth in the National Institutes of Health Guide for the Care of Laboratory Animals (NIH Pub. No. 85-23, Revised 1985). All protocols were approved by the School of Medicine and Public Health's Animal Care and Use Committee.

* Corresponding author at: 1300 University Avenue, 4245 Medical Sciences Center, Madison, WI 53706-1532, United States. Tel.: +1 608 265 2087; fax: +1 608 262 3285.
E-mail address: morgan@ortho.wisc.edu (B.J. Morgan).

1569-9048/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.resp.2009.12.003

2.2. Exposure to chronic intermittent hypoxia

Rats, in their home cages, were placed into a Plexiglas chamber and exposed to intermittent hypoxia for 12 h/day (from 18:00 to 06:00 h) for 3, 14, 28, or 56 days. Oxygen concentration in the chamber was monitored using a heated zirconium sensor (Fujikura America, Pittsburgh, PA). A microprocessor-controlled timer was used to operate solenoid valves that controlled the flow of oxygen and nitrogen into the chamber. The system was set to provide hypoxic exposures at 4-min intervals. During the first min of each cycle, nitrogen was flushed into the chamber at a rate sufficient to achieve a fraction of inspired oxygen (F_IO_2) of 0.10 within 60 s. This level of F_IO_2 was maintained for an additional 60 s. Oxygen was then introduced at a rate sufficient to achieve a F_IO_2 of 0.21 within 30 s and to maintain this oxygen level for the duration of the 4-min cycle.

2.3. Normoxic exposure

Control rats (NORM) were housed under normoxic conditions adjacent to the hypoxia chamber for 3, 14, 28, or 56 days. There, they were exposed to light, noise, and temperature stimuli similar to those experienced by the CIH rats.

2.4. Vessel harvesting procedures

On the day of study, each rat was anesthetized (50 mg/kg pentobarbital sodium, i.p.) and the small muscular branch of the femoral artery supplying the gracilis muscle was freed from surrounding tissue, covered with warmed physiological salt solution (PSS), and allowed to equilibrate *in situ* for 30 min. After equilibration, the artery was excised. Care was taken to minimize stretching, and the artery was handled only by the surrounding connective tissue. After excision, the artery was placed in warmed PSS composed of (in mmol/L): 119.0 NaCl, 4.7 KCl, 1.6 $CaCl_2$, 1.18 NaH_2PO_4, 1.17 $MgSO_4$, 24.0 $NaHCO_3$, 0.03 EDTA, and 5.5 dextrose and transferred to a superfusion-perfusion chamber (Living Systems Instrumentation, Burlington, VT).

2.5. Vessel reactivity studies

The artery was immersed in warmed PSS bubbled with oxygen (O_2), nitrogen (N_2) and carbon dioxide (CO_2) blended to achieve gas tensions of 145 mmHg O_2 and 40 mmHg CO_2 in the tissue bath. The proximal and distal ends of the artery were cannulated with glass micropipettes (120 μm, Living Systems Instrumentation, Burlington, VT) and secured to the pipettes using 10-0 nylon sutures. The vessel was stretched to the *in situ* length, and side branches were singly ligated with small strands teased from a 6-0 silk suture (Ethicon; Somerville, NJ) to ensure optimal pressurization. The inflow pipette was connected to a perfusion system that allowed control of intralumenal pressure and gas tensions. Vessel diameter was measured using television microscopy and a video micrometer (Living Systems Instrumentation, Burlington, VT). The level of baseline tone in the vessel was calculated as follows: $T = [(\Delta D \times D_{max}{}^{-1}) \times 100]$, where T is tone (in %), ΔD is the diameter increase from baseline to maximal relaxation, and D_{max} represents the maximum diameter of the vessel at baseline pressure (80 mmHg) under calcium-free conditions. Arteries exhibiting <20% baseline tone were excluded from analysis.

Responses of gracilis arteries to acetylcholine (10^{-6} mol/L, Sigma, St. Louis, MO) and sodium nitroprusside (SNP) (10^{-4} mol/L, Sigma, St. Louis, MO) were assessed by an investigator (NRP) blinded to group assignment. When these drugs were administered via the superfusate, flow was stopped by clamping the outflow pipette and the vessel was pressurized to 80 mmHg. Vessel diameter was monitored continuously and was measured at the point of its maximum value after the addition of the dilator agent. Responses to ACh and SNP were repeated after addition of 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (tempol) (100 μmol/L) to the tissue bath. After responses to vasodilator stimuli had been determined, maximum vessel diameter was measured in a relaxing solution containing (in mmol/L): 92.0 NaCl, 4.7 KCl, 1.17 $MgSO_4 \cdot 7H_2O$, 20.0 $MgCl \cdot 6H_2O$, 1.18 NaH_2PO_4, 24.0 $NaHCO_3$, 0.026 EDTA, 2.0 EGTA, and 5.5 dextrose.

2.6. Vessel morphometry and collagen measurements

After the gracilis artery was harvested for vessel reactivity studies, the animal was euthanized and the contralateral gracilis artery was perfused *in situ* with 4% paraformaldehyde at 80 mmHg for 45 min and then excised. The vessel was embedded in paraffin, cross-sectioned, and stained with picrosirius red to measure collagen content and hemotoxylin and eosin for vessel morphometry measurements (Histo-Scientific Research Laboratories, Mount Jackson, VA). The sections were visualized on an inverted microscope (TE-2000; Nikon, Melville, NY) and were captured using a Spot camera and software for image analysis (MetaVue; Optical Analysis Systems, Nashua, NH) by a single observer blinded to experimental condition (CEB).

In the hemotoxylin and eosin-stained tissue sections, intima-media thickness (IMT) was assessed with line measurement tools (after appropriate calibration) by averaging 12 equally spaced positions around the entire vessel circumference. To calculate the lumen diameter, circumference was determined by averaging the sizes of two circles—one drawn at the "peaks" of the endothelial folds and the other drawn at the "valleys". Wall to lumen ratio (W:L) was calculated by dividing IMT by lumen diameter.

In the sirius red-stained tissue sections, the area positive for collagen was identified under standard light by color thresholding and compared with the total tissue area in the field of view to produce a percent collagen in the artery wall (Junqueira et al., 1979). Collagen subtypes were identified using polarized light, under which the thicker Type I collagen fibers appear orange-red and the thinner Type III collagen fibers appear yellow-green (Rizzoni et al., 2006).

2.7. Immunohistochemistry of eNOS, iNOS, and nitrotyrosine (NT) in gracilis artery sections

Our goal was to determine whether CIH produces increases in these markers of oxidative stress and inflammation in the arterial wall. Paraffin-embedded, formalin-fixed tissue blocks were sectioned at 5 μm and mounted on slides. The sections were deparaffinized in xylene, and hydrated through graded ethanol to water. Antigen retrieval was performed in citrate buffer, pH 6.0 (10 mml/L citric acid, 0.05% Tween 20), at 95–100 °C for 20 min, then cooled to room temperature for 20 min. Non-specific binding was blocked with 10% goat serum in PBS for 1 h and endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 10 min. The slides were then incubated with the following primary antibodies in PBS with 1% goat serum and 0.1% Triton X-100 overnight at 4 °C: rabbit anti-eNOS (NeoMarkers, Freemont, CA) at 1:25, rabbit anti-iNOS (NeoMarkers, Freemont, CA) at 1:25, and mouse anti-nitrotyrosine (Zymed, S. San Francisco, CA) at 1:25. After washing with PBS, the sections were incubated with biotinylated goat anti-rabbit or anti-mouse secondary antibodies (Vector Laboratories, Burlingame, CA) at 1:200 in PBS for 1 h at room temperature. Slides were washed in PBS, and then incubated 30 min at room temperature with Vectastain ABC Elite (Vector Laboratories, Burlingame CA). Following three washes in PBS, slides were developed with AEC (Invitrogen, Carlsbad, CA), counterstained with Mayer's hematoxylin and coverslipped with crystal mount

Table 1
Body weight, age, hematocrit (Hct), and baseline gracilis artery tone of rats exposed to normoxia (NORM) or chronic intermittent hypoxia (CIH) for 3, 14, 28, or 56 days. Values are means ± SE.

	NORM (n = 6–8 per time point)				CIH (n = 6–8 per time point)				p values*	
	3 days	14 days	28 days	56 days	3 days	14 days	28 days	56 days	Group	Time
Mass, g	469 ± 2	393 ± 10	460 ± 14	466 ± 24	421 ± 13	397 ± 13	420 ± 7	437 ± 14	0.0040	<0.0001
Age, days	129 ± 3	95 ± 6	135 ± 11	169 ± 22	124 ± 12	113 ± 13	133 ± 10	156 ± 12	0.9838	0.0003
Hct	0.51 ± 0.03	0.52 ± 0.02	0.51 ± 0.02	0.53 ± 0.01	0.52 ± 0.03	0.52 ± 0.01	0.55 ± 0.01	0.57 ± 0.01	0.0500	0.2895
Tone	0.46 ± 0.09	0.56 ± 0.07	0.56 ± 0.05	0.28 ± 0.04	0.50 ± 0.08	0.54 ± 0.10	0.36 ± 0.05	0.49 ± 0.15	0.9385	0.2008

* All interactions were non-significant and between-group differences at all time points were non-significant.

(Biomeda, Foster City, CA). All reagents and chemicals are from Sigma, St. Louis, MO unless otherwise noted. Densitometry measurements were performed using NIH Scion Image software (Scion Corporation, Frederick, MD).

2.8. Urinary isoprostane excretion

The purpose of these measurements was to determine whether CIH affects this systemic indicator of oxidative stress. At the time of vessel harvest, urine was obtained from anesthetized rats via aspiration of the bladder. Samples were immediately frozen and stored at −80 °C until analyzed by enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor, MI). Values for urinary excretion of 8-isoprostane $PGF_{2\alpha}$, a systemic marker of oxidative stress, were expressed relative to urinary creatinine and to body weight.

2.9. Statistical analysis

Data were analyzed using 2-way ANOVA. Exposure group (CIH or NORM) and time (3, 14, 28, or 56 days) were the two factors. Bonferroni posttests were performed to compare NORM vs. CIH at each time point. p values <0.05 were considered statistically significant. In the text, tables, and figures, data are presented as means ± SE.

3. Results

3.1. Baseline characteristics at time of study (Table 1)

Two-way ANOVA revealed a main effect of CIH on body weight; however, posttests did not indicate statistically significant between-group differences at any point in time. CIH and NORM rats did not differ in age at any point in time; however, the rats exposed to both conditions for 14 days were somewhat younger than the rats studied at 3, 28, and 56 days of exposure. There was a small effect of CIH on hematocrit that was of borderline statistical significance (p = 0.05). Baseline tone in gracilis arteries did not differ as a function of group or time.

3.2. Vasodilator responses (Figs. 1 and 2)

We observed a main effect of CIH on ACh-induced vasodilation (p < 0.0001), with statistically significant between-group differences at 14 and 28 days of exposure. ACh-induced vasodilation in CIH vs. NORM remained attenuated after addition of tempol to the tissue bath (p < 0.0001). In contrast, there were no group (p = 0.4385) or time (p = 0.2841) differences in response to SNP. Gracilis artery maximal diameter, measured under Ca^{++}-free conditions, was significantly reduced in CIH vs. NORM (p = 0.0005), whereas there was no main effect of time on maximal diameter (p = 0.3137).

3.3. Vessel morphometry and collagen measurements (Table 2 and Fig. 3)

We observed no significant main effects of CIH on intima-media thickness (IMT) or wall to lumen ratio (W:L). There was, however, a significant main effect of time on IMT, with smaller values observed in 14-day rats of both groups. Both Type I (thicker, established) collagen fibers and Type III (thinner, newly laid-down) collagen fibers in the media were more abundant in CIH vs. NORM (p = 0.0432 and 0.0469, respectively).

3.4. eNOS, iNOS, and NT content in arterial wall (Table 3)

In the endothelium, there were no main effects of CIH on eNOS or iNOS expression; however, there were significant main effects of time on both variables. eNOS in endothelium was significantly more abundant in arteries from NORM vs. CIH rats after 14 days of exposure (p < 0.05). There was a significant effect of time, but not group, on endothelial expression of NT.

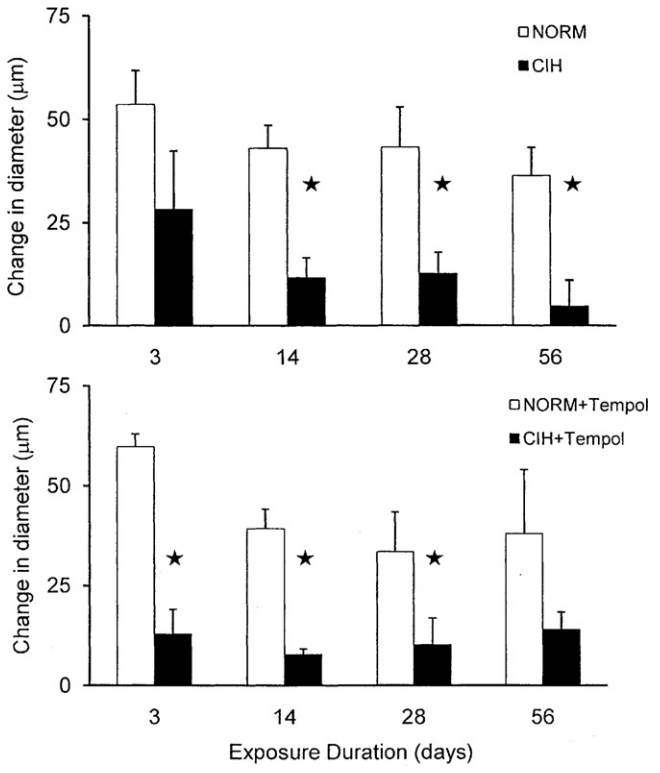

Fig. 1. Effect of CIH exposure on gracilis artery relaxation in response to acetylcholine (10^{-6} mol/L) before and after addition of tempol (100 μmol/L) to the superfusate. Upper panel: two-way ANOVA revealed a significant main effect of group but not time (p < 0.0001 and 0.1894, respectively). Lower panel: tempol had no effect on the group or time main effects (p < 0.0001 and 0.2657, respectively). No group-by-time interactions were observed. *p < 0.05 CIH vs. NORM at specified exposure duration.

Table 2
Intima-media thickness (IMT) and wall to lumen ratio (W:L) of gracilis arteries isolated from rats exposed to normoxia (NORM) or chronic intermittent hypoxia (CIH) for 3, 14, 28, or 56 days. Values are means ± SE.

	NORM				CIH				*p* values*	
	3 days	14 days	28 days	56 days	3 days	14 days	28 days	56 days	Group	Time
IMT, μm	34.1 ± 1.0	28.7 ± 1.5	30.5 ± 0.9	29.7 ± 2.4	34.4 ± 2.1	27.4 ± 0.8	33.9 ± 0.9	30.2 ± 3.0	0.5392	0.0015
W:L	0.62 ± 0.06	0.48 ± 0.06	0.57 ± 0.07	0.43 ± 0.05	0.51 ± 0.04	0.49 ± 0.06	0.71 ± 0.07	0.59 ± 0.13	0.3272	0.1553

* All interactions were non-significant and between-group differences at all time points were non-significant.

In the tunica media, NT varied as a function of time, but not group, with higher values observed in the 14-day rats. For iNOS expression, there was no main effect of group, whereas the main effect of time approached statistical significance.

3.5. Isoprostane excretion (Fig. 4)

There was a significant main effect of group on urinary excretion of 8-iso-$PGF_{2\alpha}$, with higher values in CIH-exposed rats ($p = 0.0224$). The main effect of time approached statistical significance ($p = 0.0705$).

4. Discussion

This study confirms our previous observations, made in rats exposed to CIH for 2 weeks, that CIH attenuates endothelium-dependent vasodilation in skeletal muscle resistance arteries and extends them by demonstrating that 3 days of CIH exposure is insufficient to consistently produce this impairment and that longer exposures (*i.e.* 4 weeks and 8 weeks) do not exacerbate it. The observed CIH-induced impairment in vasodilator function was accompanied by evidence of new collagen deposition in the tunica media and a decrease in maximal diameter of the artery. Urinary excretion of 8-isoprostane $PGF_{2\alpha}$ was elevated by CIH, suggesting increased oxidant stress at the systemic level, whereas there was no effect of CIH on NT content of the vascular wall. Expression of eNOS and iNOS in the arterial wall was likewise unaffected by CIH. The observed effects of CIH on vascular structure and function did not depend on the point in time that they were measured because no group-by-time interactions were noted. These findings indicate that: (1) exposure to CIH produces structural, as well as functional, deficits in skeletal muscle resistance arteries, (2) these impairments develop within 2 weeks of initiation of exposure, and (3) they are accompanied by systemic evidence of oxidant stress.

Our observations of impaired ACh-dependent vasodilation in isolated gracils arteries are consistent with previous findings in cremaster arterioles of anesthetized rats (Tahawi et al., 2001). These findings, combined with normal vasodilator responses to exogenous nitric oxide (NO) (Tahawi et al., 2001; Phillips et al., 2004) and reduced vasoconstriction with NOS inhibition (Tahawi et al.,

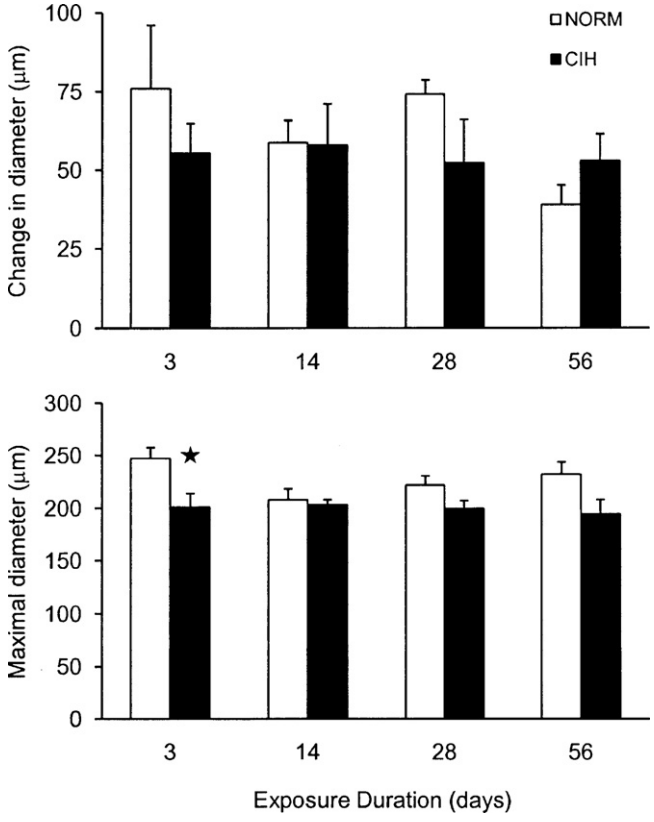

Fig. 2. Effects of CIH exposure on gracilis artery relaxation in response to sodium nitroprusside (10^{-6} mol/L) and maximal relaxation under Ca^{++}-free conditions. Upper panel: there were no significant main effects of group or time on the response to nitroprusside ($p = 0.4385$ and 0.2841, respectively). Lower panel: two-way ANOVA revealed a significant main effect of group but not time on maximal gracilis artery diameter ($p = 0.0005$ and 0.3137, respectively). No group-by-time interactions were observed. *$p < 0.05$ CIH vs. NORM at specified exposure duration.

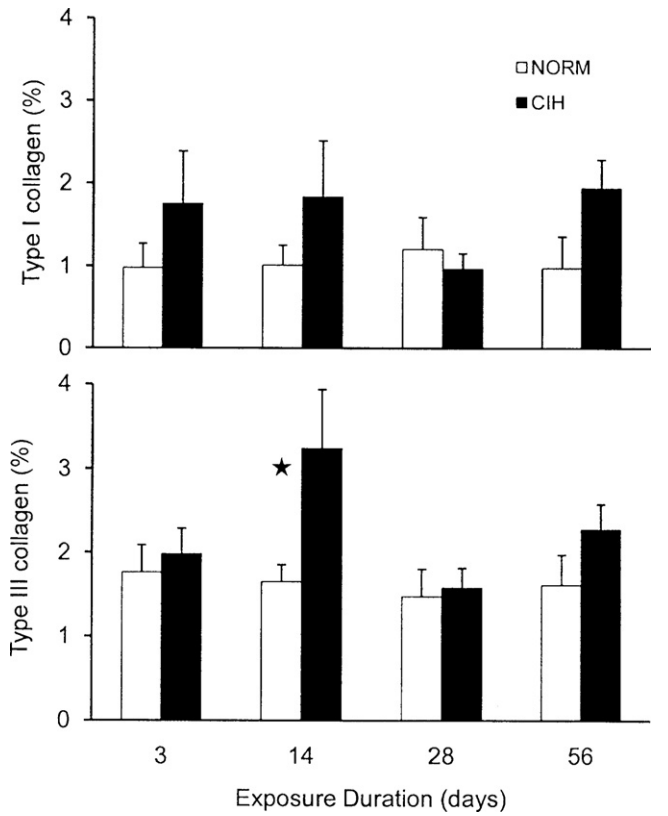

Fig. 3. Effects of CIH exposure on fibrillar collagen in gracilis artery medial layer. Two-way ANOVA revealed significant main effects of CIH on Type I (upper panel) and Type III (lower panel) collagen ($p = 0.0432$ and 0.0469, respectively). No group-by-time interactions ($p = 0.9469$ and 0.2382) and no main effects of time ($p = 0.5947$ and 0.1822) were observed. *$p < 0.05$ CIH vs. NORM at specified exposure duration.

Table 3
Expression of endothelial nitric oxide synthase (eNOS-e), inducible nitric oxide synthase (iNOS-e), and nitrotyrosine (NT-e) in endothelium and iNOS (iNOS-m) and NT (NT-m) in media of gracilis arteries isolated from rats exposed to normoxia (NORM) or chronic intermittent hypoxia (CIH) for 3, 14, 28, or 56 days. Values are means ± SE.

	NORM				CIH				*p* values*	
	3 days	14 days	28 days	56 days	3 days	14 days	28 days	56 days	Group	Time
eNOS-e	72.1 ± 6.8	86.1 ± 4.1	70.3 ± 2.9	61.2 ± 2.6	67.2 ± 2.6	72.2 ± 4.1†	76.0 ± 5.5	70.3 ± 6.2	0.7830	0.0443
iNOS-e	59.2 ± 3.7	69.8 ± 4.9	74.6 ± 4.4	67.2 ± 1.6	57.5 ± 3.9	65.4 ± 3.7	70.5 ± 5.4	63.4 ± 3.8	0.3040	0.0286
NT-e	52.0 ± 6.1	75.6 ± 5.3	44.8 ± 5.7	42.6 ± 6.8	45.9 ± 3.5	63.8 ± 5.8	42.7 ± 4.9	45.5 ± 5.2	0.3450	<0.0001
iNOS-m	58.0 ± 6.6	67.9 ± 5.0	69.9 ± 5.8	88.0 ± 9.9	65.9 ± 7.9	71.9 ± 4.9	66.3 ± 6.3	77.7 ± 7.2	0.9215	0.0596
NT-m	52.0 ± 5.8	83.7 ± 6.4	54.3 ± 8.0	43.2 ± 7.2	42.0 ± 3.8	78.9 ± 6.4	48.4 ± 5.8	50.5 ± 7.8	0.5242	<0.0001

* All interactions were non-significant.
† $p < 0.05$ vs. NORM at this time point.

2001), suggest that exposure to CIH decreases the bioavailability of NO. In the present study, CIH did not consistently affect eNOS expression; however, this finding does not preclude the possibility that CIH reduces NO bioavailability via alterations in eNOS activity or reduced availability of substrate or cofactors. Other investigators have shown that chronic exposure to continuous hypoxia attenuates ACh-induced vasodilation in rat hindlimb and that this attenuation is mediated, at least in part, by decreased availability of L-arginine (Reboul et al., 2009).

We also considered the possibility that the bioavailability of NO was reduced via scavenging by reactive oxygen species (*e.g.* superoxide ion). Our observation of increased urinary isoprostane excretion provides some evidence for CIH-induced oxidant stress at the systemic level; however, we found no evidence that CIH increases superoxide production in the arterial wall because CIH did not alter the NT content of the endothelium or tunica media. Furthermore, excess superoxide ion must not be a major contributor to the observed CIH-induced impairment in vascular function, because blunted endothelium-dependent vasodilation persisted in the presence of tempol, a superoxide dismutase mimetic. Thus, we consider it unlikely that excess superoxide in the vascular wall is responsible for decreasing the availability of NO. Nevertheless, we cannot discount the possibility that CIH-generated reactive oxygen species, including superoxide, acting at sites other than the vascular wall, contribute importantly to the detrimental effects of CIH on vascular regulation. Emerging evidence indicates that CIH enhances carotid chemoreflex sensitivity in a superoxide-dependent manner (Peng and Prabhakar, 2003). This adaptation, along with alterations in central nervous system regulation of sympathetic outflow, are thought to contribute to the sympathetic overactivity of CIH (Weiss et al., 2007; Marcus et al., 2009b). Oxidant stress can also derive from auto-oxidation of catecholamines (Kukreja and Hess, 1992); thus, enhanced sympathetic activation may be a cause, as well as a consequence, of oxidative stress in the setting of CIH.

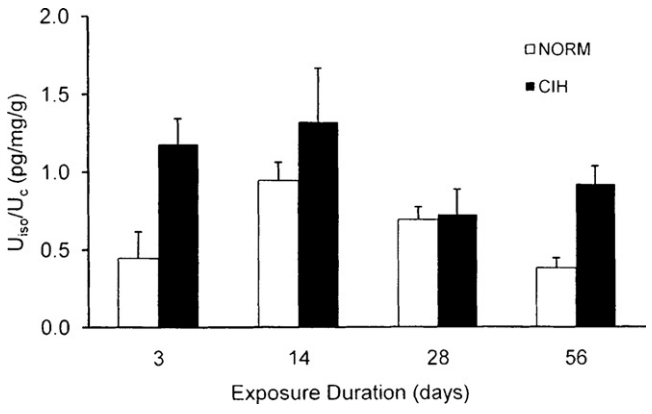

Fig. 4. Effect of CIH exposure on urinary isoprostane excretion. Two-way ANOVA revealed a significant main effect of CIH ($p = 0.0224$) and a non-significant main effect of time ($p = 0.0705$). U_{iso}, urine isoprostane; U_c, urine creatinine. No group-by-time interaction was observed ($p = 0.5018$) and there were no significant between-group differences at any time point.

Chronic sympathetic activation can produce vascular remodeling via trophic effects on vascular smooth muscle (Bleeke et al., 2004) and activation of adventitial fibroblasts (Stenmark et al., 2002). In this study, we did not make specific measurements of smooth muscle volume; however, we doubt that it was altered because there was no main effect of CIH on IMT. At 2 weeks of exposure, IMT was smaller in arteries from both groups of rats, an effect we attribute to the fact that they were somewhat younger in age. In contrast, we did observe an increase in collagen content and a decrease in maximal gracilis artery diameter, both of which are early indicators of remodeling. These structural changes are consistent with our previous finding that CIH causes a leftward shift of the stress–strain relationship in gracilis arteries, indicating an increase in vessel stiffness (Phillips et al., 2006). The mechanisms underlying CIH-induced structural alterations are not known; however, we speculate that surges in arterial pressure and flow during intermittent hypoxia cycles, along with elevations in baseline blood pressure (Marcus et al., 2009a), initiate vascular remodeling aimed at normalizing wall stress. Previous investigators demonstrated, in cultured human endothelial cells, that one episode of hypoxia/reoxygenation was sufficient to enhance production of metalloproteinases responsible for degradation of vascular basement membrane and extracellular matrix, an important initial step in the remodeling process (Ben-Yosef et al., 2002). In addition, hypoxia-induced inflammation (Nacher et al., 2007; Gonzalez et al., 2007) may contribute importantly to vascular remodeling via disruption of the balance between matrix metalloproteinases and their inhibitors (Jacob et al., 2001). In our study, CIH did not affect the amount of iNOS protein in the endothelium or tunica media; however, we did not measure activity of this enzyme.

We observed statistically significant effects of CIH on Type I (established) and Type III (newly laid-down) collagen. The effect of CIH on collagen deposition may be responsible for the increase in gracilis artery stiffness we observed in a previous study (Phillips et al., 2006) and for the decrease in gracilis artery maximal diameter we observed in the present study. In contrast, we do not believe that increased collagen deposition caused the observed decrement in endothelium-dependent vasodilation. If that had been the case, we would expect nitroprusside-induced relaxation to be diminished as well. However, it may be possible that impaired vasodilation and increased stiffness have a common cause, *i.e.* decreased availability of nitric oxide (NO). It has recently been appreciated that the endothelium participates in regulating arterial stiffness, and that NO is an important mediator of this effect (Wilkinson and McEniery, 2004).

A question that arises from this and previous (Tahawi et al., 2001; Phillips et al., 2004) studies is whether CIH-induced endothelial dysfunction causes, or is caused by, CIH-induced elevation in arterial pressure. Previously, we measured arterial pressure by telemetry in rats exposed to the identical CIH paradigm for 14 days (Marcus et al., 2009a). In that experiment, CIH increased mean

arterial pressure during the portion of the day when the intermittent hypoxia cycles occurred and also during the portion of the day when the animals were unperturbed. The increases reached statistically significant plateaus by day 3 and remained relatively stable for the duration of the exposure. In the present study, the decrease in endothelial function apparently occurred sometime between 3 and 14 days. Therefore, because the blood pressure response had a more rapid onset, it is unlikely that it was initiated by endothelial dysfunction. Our data do not rule out the possibility that endothelial dysfunction contributed to the maintenance of the pressure response.

4.1. Methodologic considerations

Our control animals were housed in cages adjacent to the hypoxia chamber but were not exposed to cyclic changes in airflow. Although both groups of rats were exposed to transient noises associated with solenoid valve operation and airflow change, we considered the possibility that the rats inside the chamber experienced stress induced by alterations in airflow and temperature. We consider it unlikely that our results were influenced by these factors because: (1) the inflow port of the hypoxia chamber contained baffles that minimized air jets, (2) the thermal environments were the same in the chamber and adjacent areas of the room where the NORM rats were housed (mean temperatures $23.9 \pm 0.1\,^{\circ}$C vs. $24.1 \pm 0.2\,^{\circ}$C.), and (3) we performed true sham exposures using normoxic gas flushing in a subset set of animals in a previous study (Phillips et al., 2004). The acetylcholine responses of arteries excised from these rats were no different from those from other normoxic control rats housed outside the chamber. In contrast, vasodilator responses were significantly greater in vessels from these sham animals than in those excised from rats exposed to CIH.

The terminal nature of our experiments necessitated the use of a between-subjects research design. For this reason, we could not make continuous measurements and pinpoint the exact time (between 3 and 14 days) required for CIH-induced impairments in vascular function and structure. Also, because our longest exposure was 8 weeks, we cannot, on the basis of our data, predict the effects of longer CIH exposures that more closely mimic human disease.

Our isolated vessel preparation allows limited inferences about the *in vivo* condition, *i.e.* the effects of CIH on vascular structure and function in the presence of vasomotor nerves, blood, and blood–borne vasoactive substances. Nevertheless, we did make morphometric measurements in stained sections of gracilis arteries that were fixed by *in situ* perfusion at constant pressure, thereby avoiding artifacts encountered *in vitro* (Schiffrin and Hayoz, 1997). Additionally, although the observed CIH-induced increases in urinary isoprostane excretion are reflective of oxidant stress at a systemic level (Rader and FitzGerald, 1998; Milne et al., 2005), we cannot on the basis of these data make inferences about oxidant stress in endothelium. The rats studied after 14 days of CIH or normoxia were somewhat younger than those studied after the other exposure durations. Although we do not know how this age effect may have influenced our results, we speculate that it may have been responsible for the variability in eNOS and nitrotyrosine expression observed in both experimental groups at the 2-week time point.

Finally, our semi-quantitative measures of collagen content and protein expression require confirmation with more precise methods and superoxide and NO production in the resistance vessels should be measured directly. Nevertheless, the present data strongly suggest that CIH produces endothelial dysfunction and elicits profibrotic changes in skeletal muscle resistance arteries.

4.2. Clinical correlates of the present findings

Several lines of evidence indicate that endothelial function is impaired in patients with OSA. Reductions in endothelium-dependent vasodilation in the forearm have been demonstrated by invasive and non-invasive means (Kato et al., 2000; Kraiczi et al., 2001; Nieto et al., 2004; Cross et al., 2008). A causal relationship between OSA and endothelial dysfunction was demonstrated by a study in which flow-mediated dilation in the forearm was improved by CPAP treatment (Ip et al., 2004). This beneficial effect was lost when CPAP was temporarily withheld. L-NMMA, a nitric oxide synthase inhibitor, caused greater reduction in forearm blood flow after vs. before CPAP treatment, which suggests that elimination of OSA augmented NO availability (Lattimore et al., 2006). Several other observations suggest that OSA reduces the bioavailability of NO. Decreased plasma levels of NO derivatives and normalization of these levels following CPAP treatment have been observed in patients with OSA (Ip et al., 2000; Alonso-Fernandez et al., 2009). Scavenging of NO by ROS is a potential explanation for the decrease in its bioavailability. In patients with OSA, increased production of superoxide by neutrophils (Schulz et al., 2000), increased biomarkers of lipid peroxidation (Lavie et al., 2004), and increased levels of 8-isoprostanes (Carpagnano et al., 2003; Alonso-Fernandez et al., 2009) have been observed.

OSA is also associated with alterations in arterial wall structure and biomechanics. Increased carotid intima-media thickness (Minoguchi et al., 2005; Tanriverdi et al., 2006) and increased arterial stiffness (Phillips et al., 2005; Baguet et al., 2005; Tanriverdi et al., 2006; Drager et al., 2007; Tsioufis et al., 2007; Kohler et al., 2008) have been observed in individuals with OSA.

In conclusion, the present findings support the notion that CIH in rats is a useful and appropriate model for studying the cardiovascular consequences of OSA in humans; however, the model fails to mimic all aspects of human sleep apnea. Specifically, hypoxic episodes are not accompanied by hypercapnia, sleep disruption is minor compared to that produced by OSA, and there are no dramatic swings in intrathoracic pressure. Nevertheless, the model produces many of the same detrimental effects on the cardiovascular system, including diurnal elevation in blood pressure (Fletcher et al., 1992; Becker et al., 2003; Marcus et al., 2009a), sympathetic activation (Carlson et al., 1993; Marcus et al., 2009b), impaired endothelium-dependent vasodilation (Carlson et al., 1996; Phillips et al., 2004), increased arterial stiffness (Phillips et al., 2006), and evidence of oxidant stress (Ip et al., 2000). In humans, these sequelae of OSA may predispose to cardiovascular disease. Furthermore, they may impair vascular regulation and oxygen delivery during episodes of apnea and other stresses (*e.g.* exercise). The present findings indicate that the negative cardiovascular consequences of CIH can be produced in relatively short periods of time. Therefore, we propose that this model is an efficient one for elucidating mechanisms and determining the frequency and severity of intermittent hypoxia required to produce vascular dysfunction. In addition, the model could be used to test putative pharmacological therapies for preventing or reversing the cardiovascular consequences of OSA.

Acknowledgements

The authors thank Joseph A. Hardin for performing the immunohistochemical staining and Dr. Yi-Hua Xu for assistance with the stain separation for NOS and NT measurements. We would also like to acknowledge the assistance of Dr. Julian H. Lombard for his thoughtful review of the manuscript. This work was supported by grants from the National Heart Lung and Blood Institute [grant numbers HL 074072 to BJM, T32 HL07654 to NJM].

References

Alonso-Fernandez, A., Garcia-Rio, F., Arias, M.A., Hernanz, A., de la Pena, M., Pierola, J., Barcelo, A., Lopez-Collazo, E., Agusti, A., 2009. Effects of CPAP on oxidative stress and nitrate efficiency in sleep apnoea: a randomised trial. Thorax 64, 581–586.

Baguet, J.P., Hammer, L., Levy, P., Pierre, H., Launois, S., Mallion, J.M., Pepin, J.L., 2005. The severity of oxygen desaturation is predictive of carotid wall thickening and plaque occurrence. Chest 128, 3407–3412.

Becker, H.F., Jerrentrup, A., Ploch, T., Grote, L., Penzel, T., Sullivan, C.E., Peter, J.H., 2003. Effect of nasal continuous positive airway pressure treatment on blood pressure in patients with obstructive sleep apnea. Circulation 107, 68–73.

Ben-Yosef, Y., Lahat, N., Shapiro, S., Bitterman, H., Miller, A., 2002. Regulation of endothelial matrix metalloproteinase-2 by hypoxia/reoxygenation. Circ. Res. 90, 784–791.

Bleeke, T., Zhang, H., Madamanchi, N., Patterson, C., Faber, J.E., 2004. Catecholamine-induced vascular wall growth is dependent on generation of reactive oxygen species. Circ. Res. 94, 37–45.

Carlson, J.T., Hedner, J., Elam, M., Ejnell, H., Sellgren, J., Wallin, B.G., 1993. Augmented resting sympathetic activity in awake patients with obstructive sleep apnea. Chest 103, 1763–1768.

Carlson, J.T., Rangemark, C., Hedner, J.A., 1996. Attenuated endothelium-dependent vascular relaxation in patients with sleep apnoea. J. Hypertens. 14, 577–584.

Carpagnano, G.E., Kharitonov, S.A., Resta, O., Foschino-Barbaro, M.P., Gramiccioni, E., Barnes, P.J., 2003. 8-Isoprostane, a marker of oxidative stress, is increased in exhaled breath condensate of patients with obstructive sleep apnea after night and is reduced by continuous positive airway pressure therapy. Chest 124, 1386–1392.

Cross, M.D., Mills, N.L., Al-Abri, M., Riha, R., Vennelle, M., Mackay, T.W., Newby, D.E., Douglas, N.J., 2008. Continuous positive airway pressure improves vascular function in obstructive sleep apnoea/hypopnoea syndrome: a randomised controlled trial. Thorax 63, 578–583.

Drager, L.F., Bortolotto, L.A., Figueiredo, A.C., Silva, B.C., Krieger, E.M., Lorenzi-Filho, G., 2007. Obstructive sleep apnea, hypertension, and their interaction on arterial stiffness and heart remodeling. Chest 131, 1379–1386.

Fletcher, E.C., Lesske, J., Qian, W., Miller III, C.C., Unger, T., 1992. Repetitive, episodic hypoxia causes diurnal elevation of blood pressure in rats. Hypertension 19, 555–561.

Gonzalez, N.C., Allen, J., Schmidt, E.J., Casillan, A.J., Orth, T., Wood, J.G., 2007. Role of the renin–angiotensin system in the systemic microvascular inflammation of alveolar hypoxia. Am. J. Physiol. Heart Circ. Physiol. 292, H2285–H2294.

Ip, M.S., Lam, B., Chan, L.Y., Zheng, L., Tsang, K.W., Fung, P.C., Lam, W.K., 2000. Circulating nitric oxide is suppressed in obstructive sleep apnea and is reversed by nasal continuous positive airway pressure. Am. J. Respir. Crit. Care Med. 162, 2166–2171.

Ip, M.S., Tse, H.F., Lam, B., Tsang, K.W., Lam, W.K., 2004. Endothelial function in obstructive sleep apnea and response to treatment. Am. J. Respir. Crit. Care Med. 169, 348–353.

Jacob, M.P., Badier-Commander, C., Fontaine, V., Benazzoug, Y., Feldman, L., Michel, J.B., 2001. Extracellular matrix remodeling in the vascular wall. Pathol. Biol. (Paris) 49, 326–332.

Junqueira, L.C., Bignolas, G., Brentani, R.R., 1979. Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. Histochem. J. 11, 447–455.

Kato, M., Roberts-Thomson, P., Phillips, B.G., Haynes, W.G., Winnicki, M., Accurso, V., Somers, V.K., 2000. Impairment of endothelium-dependent vasodilation of resistance vessels in patients with obstructive sleep apnea. Circulation 102, 2607–2610.

Kohler, M., Craig, S., Nicoll, D., Leeson, P., Davies, R.J., Stradling, J.R., 2008. Endothelial function and arterial stiffness in minimally symptomatic obstructive sleep apnea. Am. J. Respir. Crit. Care Med. 178, 984–988.

Kraiczi, H., Caidahl, K., Samuelsson, A., Peker, Y., Hedner, J., 2001. Impairment of vascular endothelial function and left ventricular filling: association with the severity of apnea-induced hypoxemia during sleep. Chest 119, 1085–1091.

Kukreja, R.C., Hess, M.L., 1992. The oxygen free radical system: from equations through membrane-protein interactions to cardiovascular injury and protection. Cardiovasc. Res. 26, 641–655.

Lattimore, J.L., Wilcox, I., Skilton, M., Langenfeld, M., Celermajer, D.S., 2006. Treatment of obstructive sleep apnoea leads to improved microvascular endothelial function in the systemic circulation. Thorax 61, 491–495.

Lavie, L., 2009. Oxidative stress—a unifying paradigm in obstructive sleep apnea and comorbidities. Prog. Cardiovasc. Dis. 51, 303–312.

Lavie, L., Vishnevsky, A., Lavie, P., 2004. Evidence for lipid peroxidation in obstructive sleep apnea. Sleep 27, 123–128.

Marcus, N.J., Olson Jr., E.B., Bird, C.E., Philippi, N.R., Morgan, B.J., 2009a. Time-dependent adaptation in the hemodynamic response to hypoxia. Respir. Physiol. Neurobiol. 165, 90–96.

Marcus, N.J., Li, Y.L., Bird, C.B., Olson, E.B., Smith, S.S., Sorenson, K.I., Schultz, H.D., Morgan, B.J., 2009b. Chronic intermittent hypoxia alters chemoreflex control of lumbar sympathetic nerve activity and carotid body protein expression. FASEB J. 23, 1008.1.

Milne, G.L., Musiek, E.S., Morrow, J.D., 2005. F2-isoprostanes as markers of oxidative stress in vivo: an overview. Biomarkers 10 (Suppl. 1), S10–S23.

Minoguchi, K., Yokoe, T., Tazaki, T., Minoguchi, H., Tanaka, A., Oda, N., Okada, S., Ohta, S., Naito, H., Adachi, M., 2005. Increased carotid intima-media thickness and serum inflammatory markers in obstructive sleep apnea. Am. J. Respir. Crit. Care Med. 172, 625–630.

Nacher, M., Serrano-Mollar, A., Farre, R., Panes, J., Segui, J., Montserrat, J.M., 2007. Recurrent obstructive apneas trigger early systemic inflammation in a rat model of sleep apnea. Respir. Physiol. Neurobiol. 155, 93–96.

Nieto, F.J., Herrington, D.M., Redline, S., Benjamin, E.J., Robbins, J.A., 2004. Sleep apnea and markers of vascular endothelial function in a large community sample of older adults. Am. J. Respir. Crit. Care Med. 169, 354–360.

Peng, Y.J., Prabhakar, N.R., 2003. Reactive oxygen species in the plasticity of respiratory behavior elicited by chronic intermittent hypoxia. J. Appl. Physiol. 94, 2342–2349.

Phillips, C., Hedner, J., Berend, N., Grunstein, R., 2005. Diurnal and obstructive sleep apnea influences on arterial stiffness and central blood pressure in men. Sleep 28, 604–609.

Phillips, C., Yee, B., Yang, Q., Villaneuva, A., Hedner, J., Berend, N., Grunstein, R., 2008. Effects of CPAP treatment and withdrawal in patients with obstructive sleep apnea on arterial stiffness and central blood pressure. Chest 134, 94–100.

Phillips, S.A., Olson, E.B., Lombard, J.H., Morgan, B.J., 2006. Chronic intermittent hypoxia alters NE reactivity and mechanics of skeletal muscle resistance arteries. J. Appl. Physiol. 100, 1117–1123.

Phillips, S.A., Olson, E.B., Morgan, B.J., Lombard, J.H., 2004. Chronic intermittent hypoxia impairs endothelium-dependent dilation in rat cerebral and skeletal muscle resistance arteries. Am. J. Physiol. Heart Circ. Physiol. 286, H388–H393.

Rader, D.J., FitzGerald, G.A., 1998. State of the art: atherosclerosis in a limited edition. Nat. Med. 4, 899–900.

Reboul, C., Gibault, A., Tanguy, S., Dauzat, M., Obert, P., 2009. Alteration of endothelium-mediated vasodilator response in the rat hindlimb vasculature consecutive to chronic hypoxic stress: NO and EDHF involvement. Vascul. Pharmacol. 51, 154–161.

Rizzoni, D., Paiardi, S., Rodella, L., Porteri, E., De, Ciuceis C., Rezzani, R., Boari, G.E., Zani, F., Miclini, M., Tiberio, G.A., Giulini, S.M., Rosei, C.A., Bianchi, R., Rosei, E.A., 2006. Changes in extracellular matrix in subcutaneous small resistance arteries of patients with primary aldosteronism. J. Clin. Endocrinol. Metab. 91, 2638–2642.

Schiffrin, E.L., Hayoz, D., 1997. How to assess vascular remodelling in small and medium-sized muscular arteries in humans. J. Hypertens. 15, 571–584.

Schulz, R., Mahmoudi, S., Hattar, K., Sibelius, U., Olschewski, H., Mayer, K., Seeger, W., Grimminger, F., 2000. Enhanced release of superoxide from polymorphonuclear neutrophils in obstructive sleep apnea. Impact of continuous positive airway pressure therapy. Am. J. Respir. Crit. Care Med. 162, 566–570.

Stenmark, K.R., Gerasimovskaya, E., Nemenoff, R.A., Das, M., 2002. Hypoxic activation of adventitial fibroblasts: role in vascular remodeling. Chest 122, 326S–334S.

Tahawi, Z., Orolinova, N., Joshua, I.G., Bader, M., Fletcher, E.C., 2001. Altered vascular reactivity in arterioles of chronic intermittent hypoxic rats. J. Appl. Physiol. 90, 2007–2013.

Tanriverdi, H., Evrengul, H., Kara, C.O., Kuru, O., Tanriverdi, S., Ozkurt, S., Kaftan, A., Kilic, M., 2006. Aortic stiffness, flow-mediated dilatation and carotid intima-media thickness in obstructive sleep apnea: non-invasive indicators of atherosclerosis. Respiration 73, 741–750.

Tsioufis, C., Thomopoulos, K., Dimitriadis, K., Amfilochiou, A., Tousoulis, D., Alchanatis, M., Stefanadis, C., Kallikazaros, I., 2007. The incremental effect of obstructive sleep apnoea syndrome on arterial stiffness in newly diagnosed essential hypertensive subjects. J. Hypertens. 25, 141–146.

Weiss, J.W., Liu, M.D., Huang, J., 2007. Sleep apnoea & hypertension: physiological bases for a causal relation: physiological basis for a causal relationship of obstructive sleep apnoea to hypertension. Exp. Physiol. 92, 21–26.

Wilkinson, I.B., McEniery, C.M., 2004. Arterial stiffness, endothelial function and novel pharmacological approaches. Clin. Exp. Pharmacol. Physiol. 31, 795–799.