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

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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.

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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