Chronic Intermittent Hypoxia Alters Rat Ophthalmic Artery Reactivity Through Oxidative Stress, Endothelin and Endothelium-Derived Hyperpolarizing Pathways

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Purpose. Obstructive sleep apnea recently has been associated with a higher frequency of ischemic optic neuropathies. Intermittent hypoxia (IH) has been proposed as a major component of obstructive sleep apnea cardiovascular consequences. However, there currently are no pathophysiologic data regarding the effect of IH on the ocular vascular system. Thus, we assessed the impact of chronic IH exposure on the morphology and vascular reactivity of the rat ophthalmic artery (OA).

Methods. Rats were exposed to 14 days of IH or normoxia (NX). Ophthalmic artery reactivity was studied using wire myography in rats treated or not with tempol (1 mM/day). Expression of endothelin-1 (ET-1) and its receptors, and of the three nitric oxide synthase (NOS) isoform genes was quantified using quantitative polymerase chain reaction (qPCR) in the retina and optic nerve. Structural alterations (optical and electron microscopy) and superoxide anion production were studied in OA sections.

Results. Superoxide ion expression in the OA wall was increased by 23% after IH exposure. Ophthalmic artery contractile response to 3.10^{-8} M ET-1 was increased by 18.6% and nitric oxide-mediated relaxation was significantly delayed in IH compared to NX rats. In the absence of nitric oxide, cytochrome P450 blockade increased relaxation to acetylcholine in IH rats and delayed it in NX rats. Tempol treatment abolished the IH-induced changes in OA reactivity.

Conclusions. These results strongly suggest that chronic IH induces oxidative stress in the rat OA, associated with endothelial dysfunction through alterations of nitric oxide and endothelium-derived hyperpolarising factors (EDHF) pathways.

Keywords: intermittent hypoxia, ophthalmic artery, endothelin, oxidative stress, nitric oxide

Obstructive sleep apnea is a common condition affecting 5% of the general population and 18% of those older than 50 years.[1] Obstructive sleep apnea is characterized by repetitive apneas during sleep, resulting in intermittent hypoxemia and sleep fragmentation. It is a well-known independent risk factor for hypertension, ischemic heart disease, atherosclerosis, coronary disease, autonomic dysfunction, and stroke.[2] The deleterious consequences of obstructive sleep apnea on the human vascular system include endothelial dysfunction,[3] elevated circulatory inflammatory mediator levels,[4] transient vasoconstriction after apneic episodes,[5] and increased vascular sensitivity to vasoconstrictors[6] associated with elevated circulating endothelin-1 (ET-1) concentrations during the night.[7] Moreover, decreased nitric oxide bioavailability has been linked to the hypoxemia/reoxygenation sequences[8] and to an enhanced release of superoxide anions by polymorphonuclear leukocytes in apneic patients. Animal studies reproducing the intermittent hypoxic stimulus of obstructive sleep apnea show that the vasoconstrictive response to ET-1 is increased[9,10] and the vasorelaxation in response to acetylcholine is decreased mainly in resistance arteries (gracilis and middle cerebral arteries),[11] but not in conductance arteries (carotid artery and aorta).[12]

Recent evidence in the literature suggests that obstructive sleep apnea may impair ocular vascular function, being mostly a risk factor for nonarteritic anterior ischemic optic neuropathy (for a review see Ref. 13). However, the relationship between obstructive sleep apnea and retinal or optic nerve alterations is not well understood, with the main hypotheses including a potential role of the endothelin system, oxidative stress, and inflammatory pathways.[14] Endothelin-1 mediates dose-dependent vasoconstriction in human ophthalmic and posterior ciliary arteries.[15] On the other hand, the nitric oxide system has a major vasodilatory role in the ocular vasculature, as shown in animals[16,17] and human ophthalmic artery (OA).[15] Thus, an imbalance in nitric oxide and ET-1 synthesis and/or bioavailability could be deleterious to the regulation of ocular blood perfusion.

We investigated the effects of 14 days of intermittent hypoxia (IH) exposure on the rat ocular vasculature by assessing the remodeling and vascular reactivity of the OA and involvement of nitric oxide, ET-1, and oxidative stress.

Materials and Methods

Ethical Approval

Male Wistar rats (7 weeks old, 275–299 g; Janvier Labs, Le Genest-Saint-Isle, France) were used for all experiments. Animal experiments were conducted in accordance with the guidelines from directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol was approved by the Committee on the Ethics of Animal Experiments of Grenoble Alpes University (authorization number 743-2015052818221958-v2). The rats were housed under diurnal lighting conditions and given free access to food and water. All surgical procedures were performed under general anesthesia, and all efforts were made to minimize suffering.

Chronic IH Exposure

Male Wistar rats (7 weeks old, 275–299 g; Janvier Labs) were exposed to either normoxia (NX) or IH for 14 days, as described previously.[12] Briefly, rats were exposed to IH cycling between ambient air (fraction of inspired oxygen [FiO_2] 21%, 30 seconds) and hypoxia (FiO_2 5%, 30 seconds), whereas normoxia cycling consisted of exposure to room air only. Rats were exposed to 1-minute cycles for 8 hours/day during the rat sleep period (daytime). Two subgroups of IH and NX rats ($n = 6$ in each) were treated with 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (tempol) at a dose of 1 mM in drinking water every day,[16] during the 14-day exposure to IH or NX. Arterial blood pressure was recorded at the end of IH or NX exposure in anesthetized animals, before blood and OA sampling for ex vivo experiments.

Isolated Ophthalmic Artery Preparation

A total of 101 animals were used for these experiments. After arterial blood pressure recording and blood collection, rats were killed by heart excision under deep anesthesia and decapitated. Ophthalmic artery dissection was performed as described previously.[18] The brain was removed rapidly, and the skull was placed in ice-cold oxygenated physiologic buffer (PSS, containing in mM: NaCl 130, $NaHCO_3$ 14.9, KCl 3.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.6, and glucose 11). Right and left OA from each rat were dissected carefully and mounted in a small-vessel myograph for isometric tension recording (Danish MyoTechnology, Aarhus, Denmark). The arteries were threaded onto two 40-µm tungsten wires. OA study using myography was previously validated in healthy rats (Supplementary Material SA).

Contractile responses of OA to cumulative doses of phenylephrine (10^{-9} to 10^{-4} M), serotonin (10^{-10} to 3.10^{-6} M), and ET-1 (10^{-10} to 10^{-6} M), and to a single dose of ET-1 (3.10^{-8} M) were assessed. To further explore endothelin receptor implication, the response to 3.10^{-8} M ET-1 was assessed in intact arteries in the presence of specific endothelin receptor A (ET_RA [BQ-123, 10^{-6} M]) or receptor B antagonists (ET_RB [BQ-788, 10^{-6} M]). Endothelial function was assessed by studying the response to cumulative doses of acetylcholine (10^{-9} to 10^{-4} M) in OA precontracted with serotonin (80% of maximal contractile response) in the absence and presence of several inhibitors: nitric oxide synthase (NOS) inhibitor N^G-nitro-L-arginine (L-NAME, 10^{-4} M), cyclooxygenase inhibitor indomethacin (10^{-4} M), prostacyclin synthase inhibitor *trans*-2-phenylcyclopropylamine hydrochloride (TPC, 10^{-4} M), and nonspecific cytochrome P450 inhibitor fluconazole (5.10^{-5} M). Endothelium-independent relaxation was assessed in response to the nitric oxide-donor sodium nitroprusside (10^{-9} to 10^{-4} M) in arteries precontracted with serotonin. The maximal effect (E_{max}) was the greatest response obtained with the agonist. The concentration of agonist producing 50% of the maximal effect (EC_{50}) was determined from each curve using a nonlinear curve fitting equation. The pEC_{50} value is the negative logarithm of the EC50. The doses of the various antagonists were based on data from the literature.

Quantitative RT-PCR (RT-qPCR)

Total RNA was extracted and isolated from retinas and optic nerves ($n = 9$ per group) with a miRNeasy micro kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. cDNA was synthetized from 0.5 µg total RNA by reverse transcripting the RNA with iScript Reverse Transcription Supermix (BioRad, Hercules, CA, USA). RT-qPCR was performed with a BioRad C1000 Thermal Cycler (BioRad), SsoAdvanced Universal SYBR Green Supermix (BioRad) and PCR primers (Sigma-Aldrich Corp., St. Louis, MO, USA) for the following rat genes: hypoxanthine guanine phosphoribosyltransferase 1; cyclophilin A; actin beta; *ET-1*; ET_RA; ET_RB; and endothelial, neuronal, and inducible nitric oxide synthase (*eNOS*, *nNOS*, and *iNOS*; (Supplementary Material SB). Reaction conditions were as follows: 95°C for 30 seconds, 95°C for 5 seconds, 60°C for 10 seconds, 72°C, for 30 seconds, then final extension (72°C, 4 minutes, followed by 25°C, 5 minutes). Every sample was tested in triplicate during three distinct runs, and milliQ water was used as negative control. RT-PCR products of all genes were separated on agarose gels and molecular weight was compared to predicted weight for each amplicon. Relative quantification of gene expression was performed using the comparative Ct method, with adjustment for amplification efficiency.[19] Relative expression of target genes was expressed as fold increase compared to NX rats.

Histology Procedures

Rats were anesthetized and killed as described in the previous section. Ocular globes with surrounding retrobulbar tissues were removed and either: (1) frozen in optimum cutting temperature compound (OCT TissueTek; Sakura Finetek, Torrance, CA, USA) and stored at –80°C until assayed (superoxide anions production); (2) fixed for 24 hours in 4% paraformaldehyde (PFA; in 0.1 M phosphate buffer, pH 7.4), dehydrated, and embedded in paraffin (morphometry); or (3) fixed overnight in 1% PFA/2.5% glutaraldehyde in PBS followed by 1 hour in buffered isotonic 2% osmium tetroxide solution, dehydration in ethanol, and subsequent embedding in epoxy resin for transmission electron microscopy ($n = 3$ eyes/ group). Ultrathin sections (50 nm thick) were cut on an ultramicrotome (UltraCutR, Leica Microsystems, Wetzlar, Germany), followed by contrasting with uranyl acetate/lead citrate before analysis on a transmission electron microscope.

Morphometry. Intima–media thickness and mean internal perimeter of OA were assessed in 5-µm thick paraffin-embedded cross-sections, as described previously.[20] Intima–media thickness and mean internal perimeter were evaluated using computer image analysis (MetaMorph6 software, Zeiss microscope; Carl Zeiss, Oberkochen, Germany). Ten sections of OA per rat were analyzed and averaged.

Measurement of Superoxide Anion Production. In situ production of superoxide anions was assessed using dihydroethidium staining in 12-µm thick frozen OA cross-sections as described previously.[21] In situ fluorescence was imaged using

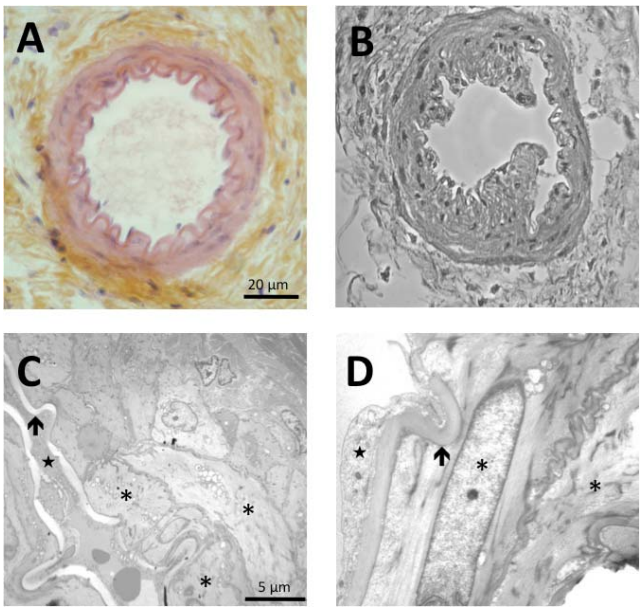

FIGURE 1. Effect of chronic IH on ophthalmic artery structure. (**A**, **B**) Representative optical microscopy images of hematoxylin-eosin–stained transverse sections (×40 magnification) used to evaluate intima–media thickness and mean inner perimeter (**A**) and location and morphology of intra-arterial cushions (**B**). (**C**, **D**) Representative electron microscopy images from NX (**C**) and IH (**D**) rats ($n = 3$ rats per group). *Smooth muscle cells from media. *Arrows* denote internal elastic lamina and stars denote endothelial cells.

confocal fluorescence microscopy (LSM 510 - Confocor 2; Carl Zeiss) and analyzed using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by National Institutes of Health [NIH], Bethesda, MD, USA).

Drugs

Acetylcholine, phenylephrine, serotonin, endothelin-1, BQ-123, BQ-788, L-NAME, indomethacin, *trans*-2-phenylcyclopropylamine hydrochloride and fluconazole were obtained from Sigma-Aldrich Corp.). Drugs were kept in accordance with manufacturer's recommendations until preparation of working-concentration solutions. All drugs were dissolved in distilled water, except BQ-123 and BQ-788, which were dissolved in dimethyl sulfoxide. Aliquots of working-concentration solutions were stored at −20°C and thawed once before use.

Statistical Analysis

All data are expressed as mean ± SD and the values of n refer to the number of animals in each group. Statistical analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Normality of data was assessed using Shapiro-Wilk normality tests. NX and IH groups were compared using Student's unpaired t-tests. For organ bath experiments, vasoconstrictor responses were expressed as a percentage of the maximum contraction induced by 60 mM potassium chloride. Relaxation to acetylcholine was expressed as a percentage of serotonin-induced contraction (80% of the maximal serotonin contraction). After normality testing, agonist responses were analyzed using ANOVA. Post hoc analysis was conducted with Bonferroni-corrected t-tests. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Chronic IH on Systemic Hemodynamic Parameters

Following 14 days of IH exposure, hematocrit was significantly increased by 15.6% (49.5% ± 3.1% vs. 42.8% ± 2.6% in IH compared to NX rats respectively; $P < 0.0001$). Mean, systolic and diastolic arterial blood pressures also were significantly increased by 12.1% (125.9 ± 12.9 vs. 112.0 ± 14.0 mm Hg; $P = 0.0001$), 9.2% (148.9 ± 15.9 vs. 136.4 ± 18.3 mm Hg; $P = 0.005$), and 14.1% (114.4 ± 13.0 vs. 100.2 ± 13.8 mm Hg; $P < 0.0001$), whereas the heart rate was not altered (376 ± 23 vs. 367 ± 40 beats/minute; $P = 0.3$) in IH compared to NX rats, respectively.

Effect of Chronic IH on Ophthalmic Artery Structure

IH did not induce vascular remodeling of OA, as shown by unaltered intima–media thickness (17.3 ± 2.1 vs.16.5 ± 2.7 µm in NX and IH groups, respectively; $P = 0.4$) and inner perimeter (342.2 ± 126.0 vs. 349.8 ± 45.9 µm in NX and IH groups, respectively; $P = 0.3$). V-shaped extensions of the OA wall into the lumen of the vessel, before branching of the central retinal artery and long posterior ciliary arteries, were identified as intra-arterial cushions (Fig. 1B). The location and macroscopic structure of these intra-arterial cushions were not altered by IH exposure.

Electron microscopy study of OA and optic nerve axons did not show ultrastructural changes of endothelial cells, vascular smooth muscle cells, and basal lamina of the vessel (Figs. 1C, 1D), nor in the myelin sheet and morphology of axons (data not shown).

Effect of Chronic IH on the Contractile Responses of Ophthalmic Artery

The mean contractile response to 60 mM potassium chloride was not significantly different in NX (7.7 ± 1.7 mN) and IH (7.9 ± 1.5 mN; Table) rats. Increasing doses of phenylephrine and serotonin induced a dose-dependent increase in vascular tone that was similar in both groups (Table; Supplementary Material SC).

In response to cumulative doses of ET-1, contraction was significantly higher in IH compared to NX rats at the dose of 3.10^{-8} M (Fig. 2A; $P = 0.01$). Administration of a single 3.10^{-8} M dose of ET-1 elicited an 18.6% greater contraction in IH compared to NX rats (Fig. 2B; $P = 0.04$). In both groups, contraction to 3.10^{-8} M ET-1 was almost completely abolished by the ET_RA antagonist BQ-123 (Fig. 2B; $P < 0.0001$). In contrast, the ET_RB antagonist BQ-788 significantly enhanced the contractile response to ET-1 in NX ($P < 0.0001$), but not in IH rats. Consequently, after ET_RB blockade, the contractile effect of ET-1 was greater in NX compared to IH rats (Fig. 2B; $P = 0.001$).

Effect of Chronic IH on the Relaxation Responses of OA

Acetylcholine caused a concentration-dependent relaxation of OA preconstricted with serotonin, and the response was delayed in IH compared to NX rats (Fig. 3A; $P < 0.0001$), as confirmed by pEC_{50} values (Table). The difference in acetylcholine response between NX and IH rats was maximal at 3.10^{-7} M (Fig. 3B). OA from NX and IH rats showed similar relaxation in response to sodium nitroprusside (Fig. 3C).

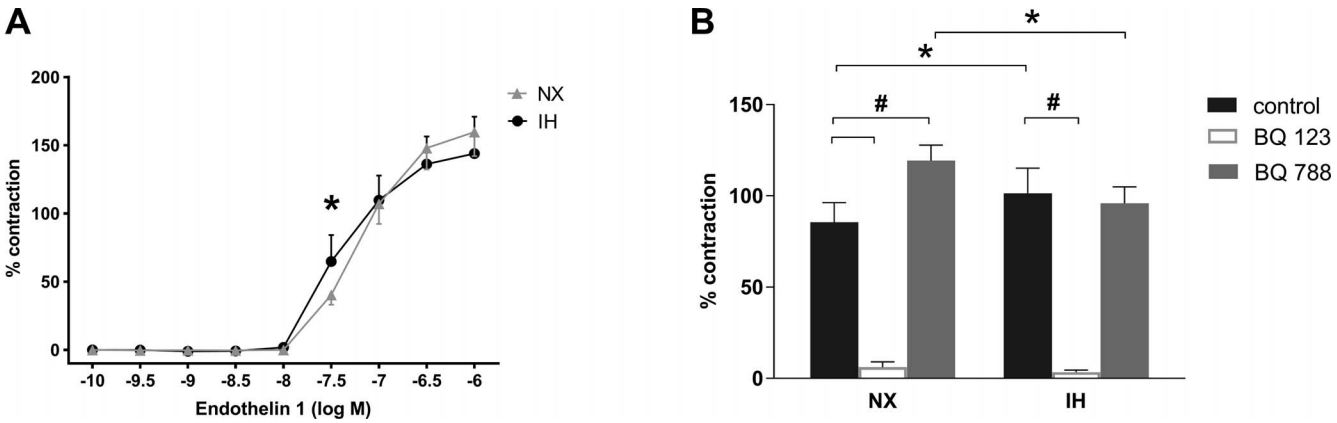

Figure 2. Effect of chronic IH on the contractile response of the rat ophthalmic artery to ET-1. (**A**) Cumulative dose-response curves to ET-1 (10^{-10} to 10^{-6} M) of ophthalmic arteries from NX and IH rats ($n = 6$ and 8, respectively). (**B**) Contraction in response to a single dose of ET-1 (3.10^{-8} M) alone (control, $n = 7$ NX and 6 IH) and in the presence of ET_RA (BQ 123, $n = 7$ per group) or ET_RB (BQ 788, $n = 7$ per group) receptor antagonists. Two-way ANOVA: $^*P < 0.05$, NX vs. IH; $\#P < 0.05$, control vs. ET_R antagonist.

The nonselective NOS inhibitor L-NAME significantly delayed the dose-dependent relaxation to acetylcholine between 10^{-7} M and 10^{-6} M in both groups (Fig. 4A; $P < 0.0001$). Nevertheless, the effect of L-NAME was greater in NX compared to IH rats, in particular at 3.10^{-7} M acetylcholine (Fig. 4B; $P < 0.001$).

The role of endothelium-derived hyperpolarizing factors (EDHF) was assessed using fluconazole, which inhibits EDHF production by cytochrome P450. In presence of L-NAME, fluconazole had opposing effects in OA of NX and IH rats, since it induced a significant left shift in the acetylcholine dose-response curve at 3.10^{-7} M in IH rats ($P < 0.0001$) and a nonsignificant right shift in NX rats (Fig. 4C). The difference in the response of OA from NX and IH rats in the presence of fluconazole was maximal at 3.10^{-7} M acetylcholine (Fig. 4D; $P < 0.0001$). Consequently, cytochrome P450 inhibition significantly enhanced vasorelaxation of OA in IH compared to NX rats at 3.10^{-7} and 10^{-6} M acetylcholine ($P < 0.0001$). In accordance, the pEC_{50} value was significantly higher in IH compared to NX rats (Table).

Finally, in the presence of L-NAME, the effects of nonselective cyclooxygenase inhibition (indomethacin) or specific prostacyclin inhibition (TPC) on the response to acetylcholine were similar in both groups (Supplementary Material SC).

Implication of Oxidative Stress in the Alterations of OA Reactivity Induced by Chronic IH

In situ production of superoxide anions in OA, evaluated by dihydroethidium fluorescence, was significantly enhanced by 23% in IH compared to NX rats ($n = 8$ and 7, respectively; $P = 0.03$; Fig. 5).

Treatment of rats with the superoxide dismutase mimetic tempol throughout IH or NX exposure prevented the IH-induced enhanced response to ET-1. The contractile response of OA to 3.10^{-8} M ET-1 was similar (77.5% ± 20% vs. 78.8% ± 29%) in tempol-treated NX ($n = 5$) and IH ($n = 5$) rats, respectively (Fig. 6A). Tempol treatment also normalized the OA relaxation to cumulative doses of acetylcholine, which did not significantly differ between tempol-treated NX ($n = 5$) and IH ($n = 6$) rats (Fig. 6B). The effect of L-NAME also was similar in both groups (Figs. 6C, 6D).

Finally, tempol treatment abolished the opposite effects of fluconazole in IH and NX rats. In the presence of L-NAME and fluconazole, the nitric oxide-independent relaxation of OA to acetylcholine was similar in tempol-treated NX ($n = 5$) and IH ($n = 6$) rats and did not differ from that observed in the presence of L-NAME alone (Figs. 6E, 6F).

OA from NX and IH rats treated with tempol showed a similar relaxation in response to sodium nitroprusside (data not shown).

Table. E_{max} and pD_2 Values of Ophthalmic Artery Dose-Response Curves

	NX	IH
Potassium chloride 60 mM		
E_{max} (mN)	7.7 ± 1.7 (51)	7.9 ± 1.5 (50)
Phenylephrine		
E_{max} (%)	127.8 ± 15.6 (12)	121.7 ± 9.4 (15)
pEC50	5.6 ± 0.2	5.8 ± 0.2
Serotonin		
E_{max} (%)	174.6 ± 22 (12)	175.2 ± 2.8 (15)
pEC50	7.1 ± 0.1	7.1 ± 0.2
Endothelin-1		
Emax (%)	161 ± 18 (6)	143.7 ± 29 (8)
pEC50	7.2 ± 0.1	7.4 ± 0.2
Acetylcholine		
E_{max} (%)	1.6 ± 3.6 (16)	3.0 ± 3.3 (16)
pEC50	6.8 ± 0.2	6.6 ± 0.2*
Sodium nitroprusside		
E_{max} (%)	20.8 ± 13.3 (6)	31.6 ± 7.7 (6)
pEC50	5.7 ± 0.7	5.3 ± 0.6
Acetylcholine + L-NAME		
E_{max} (%)	6.8 ± 7.5 (16)	4.4 ± 4.5 (16)
pEC50	6.3 ± 0.2	6.2 ± 0.2
Acetylcholine + L-NAME+ Indomethacin		
E_{max} (%)	1.3 ± 3.8 (8)	0 ± 5.0 (9)
pEC50	6.2 ± 0.2	6.2 ± 0.2
Acetylcholine + L-NAME + TPC		
E_{max} (%)	0 ± 2.5 (10)	1.7 ± 3.5 (8)
pEC50	5.9 ± 0.3	6.0 ± 0.4
Acetylcholine + L-NAME + Fluconazole		
E_{max} (%)	2.1 ± 8.7 (5)	0.4 ± 5.2 (6)
pEC50	5.9 ± 0.4	6.4 ± 0.3**

For phenylephrine, ET-1, and serotonin, E_{max} values are expressed as a percentage of contraction to 60 mM potassium chloride. For sodium nitroprusside and acetylcholine ± inhibitors, E_{max} values are expressed as a percentage of contraction to serotonin (80% of maximal contraction). Data are expressed as mean ± SD (n of ophthalmic arteries). Unpaired Student's t-tests: $^*P = 0.01$ and $^{**}P = 0.026$, NX vs. IH.

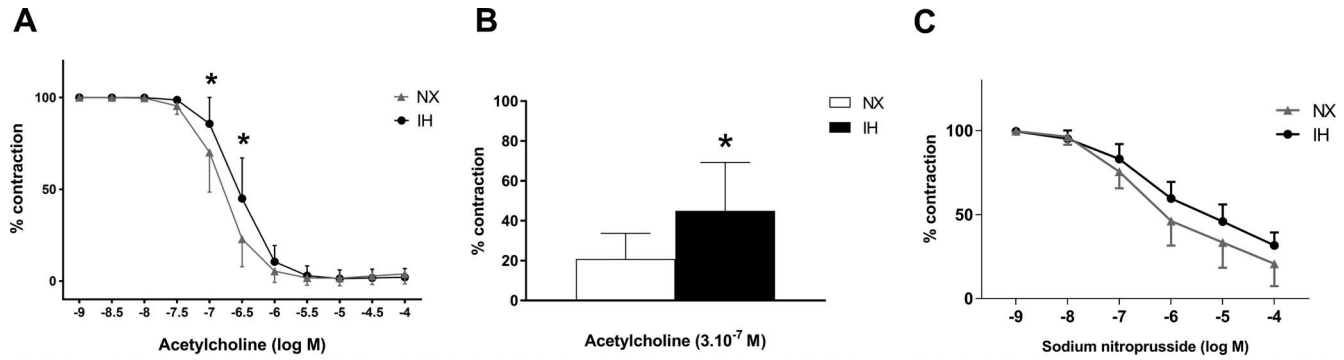

FIGURE 3. Effect of chronic IH on the relaxation of ophthalmic artery in response to acetylcholine and sodium nitroprusside. (**A**) Cumulative dose-response curves to acetylcholine (10^{-9} to 10^{-4} M) of ophthalmic arteries from NX and IH rats ($n = 16$ per group). Relaxation to acetylcholine was significantly delayed in IH compared to NX rats (two-way ANOVA, $^*P < 0.0001$). (**B**) The maximal difference between NX and IH rats was observed at 3.10^{-7} M acetylcholine (unpaired *t*-test, $^*P = 0.043$) (**C**) The cumulative dose-response curves to sodium nitroprusside (10^{-9} to 10^{-4} M) were not statistically different ($n = 6$ per group).

Effect of Chronic IH on Gene Expression in the Retina and Optic Nerve

We explored the impact of IH exposure on endothelin and nitric oxide system gene expression in the retina and optic nerve (Fig. 7). RT-qPCR analysis did not show any alteration in gene expression in the retina of rats exposed to 14 days of IH.

In the optic nerve head, IH exposure increased the expression of *eNOS* and *nNOS* genes (n = 9/group; P = 0.009) and of ET_RA and ET_RB genes ($n = 9$/group; $P = 0.01$).

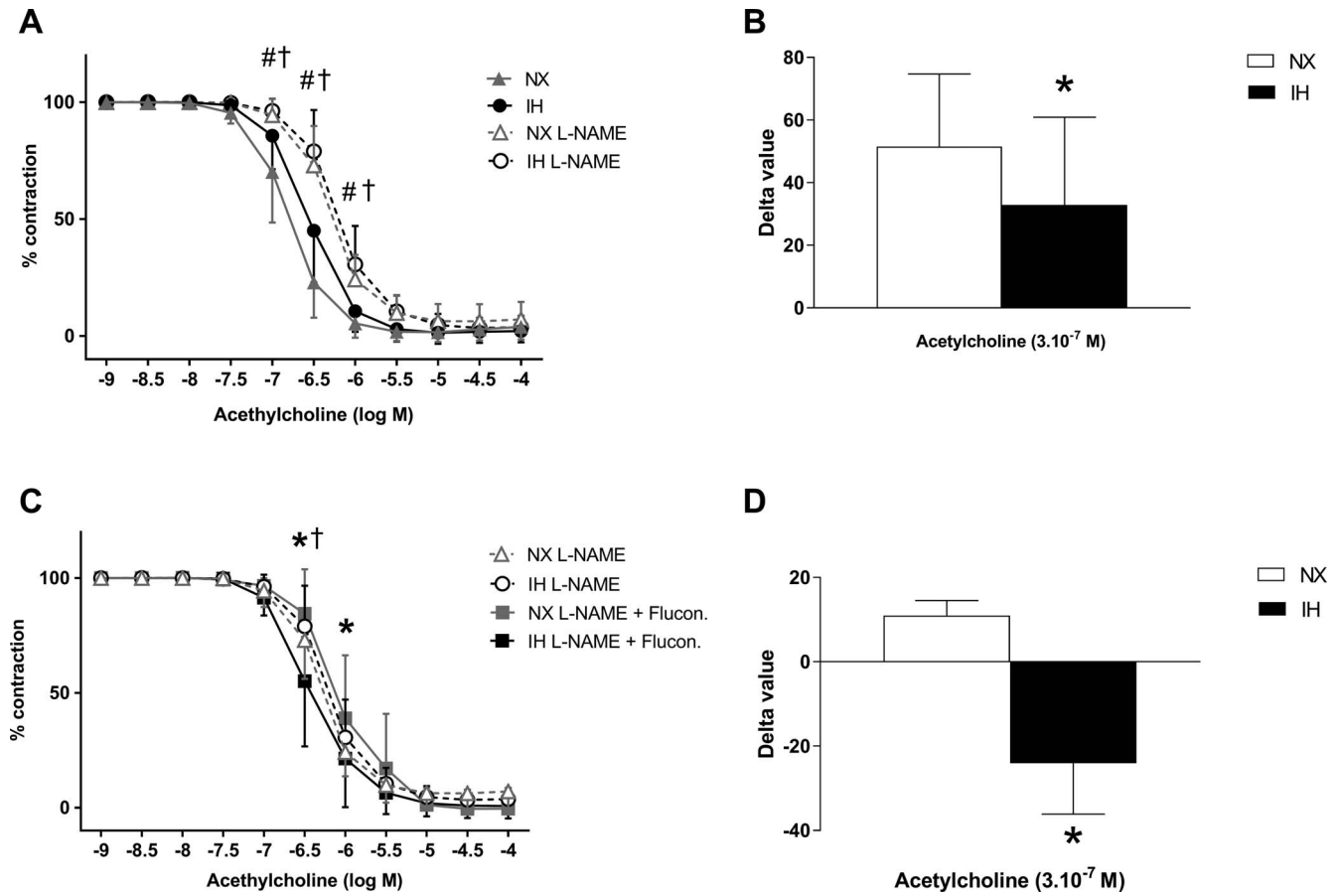

FIGURE 4. Effect of chronic IH on the response of ophthalmic artery to acetylcholine in the presence of NOS or EDHF inhibition. (**A**) Relaxation of ophthalmic arteries from NX and IH rats in response to cumulative doses of acetylcholine in presence of the nonspecific NOS inhibitor L-NAME (10^{-4} M, $n = 16$ per group). L-NAME induced a significant right shift of the dose-response curve in both groups ($\#P < 0.0001$ and $\dagger P < 0.0001$). (**B**) The inhibitory effect of L-NAME (delta values vs. acetylcholine alone) was more pronounced in arteries from NX compared to IH rats and the maximal difference between both groups was seen at 3.10^{-7} M acetylcholine ($^*P < 0.001$). (**C**) In the presence of L-NAME, EDHF inhibition by fluconazole (5.10^{-5} M) induced a significant left shift in the dose-response curve of IH rats only ($\dagger P < 0.0001$, $n = 6$ per group). This resulted in a significant difference between NX and IH rats ($^*P < 0.0001$). (**D**) The effect of fluconazole (delta values vs. + L-NAME) was, in fact, opposite in both groups and the maximal difference was seen at 3.10^{-7} M acetylcholine ($^*P < 0.0001$). Two-way ANOVA: *NX vs. IH; #inhibitors vs. acetylcholine alone, NX group; †inhibitors vs. acetylcholine alone, IH group.

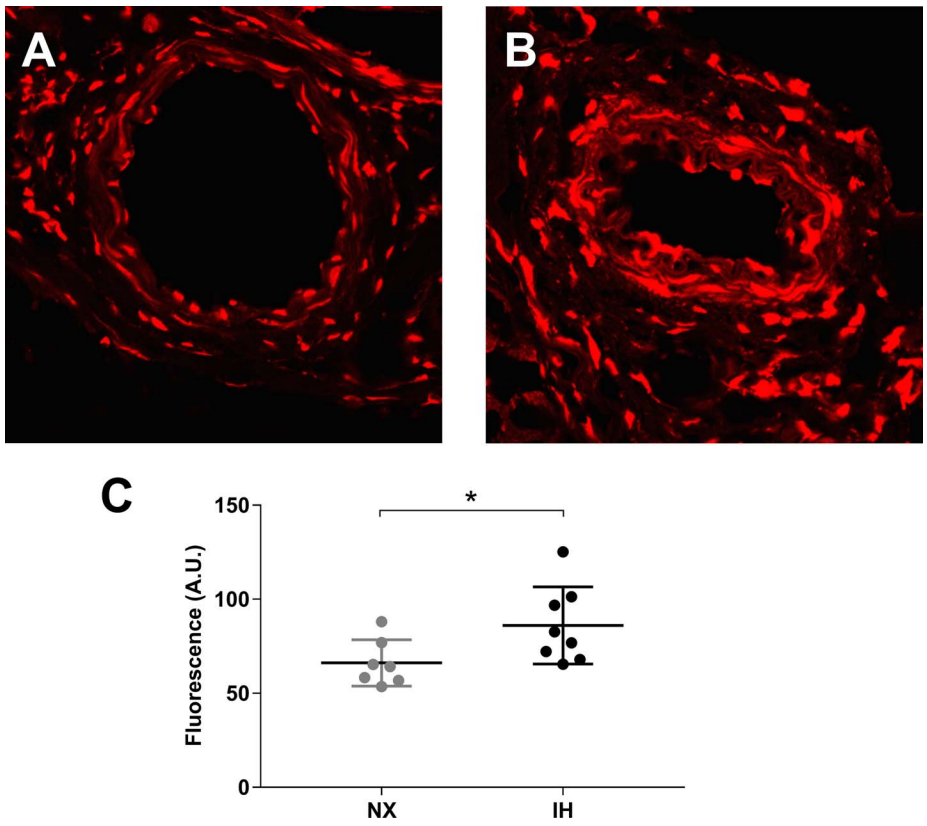

FIGURE 5. Superoxide ion production in ophthalmic artery. (**A**, **B**) Representative confocal images of dihydroethidium fluorescence in ophthalmic arteries from NX (**A**) and IH (**B**) rats (×40 magnification). (**C**) Scatter plot showing mean (*horizontal central bar*), SD (*vertical bars*), and individual values of dihydroethidium fluorescence (*A.U.*) in NX ($n = 7$) and IH ($n = 8$) rats. Unpaired Student's *t*-test: $^{*}P = 0.03$, NX vs. IH.

DISCUSSION

In the absence of structural modifications, the vasoreactivity of OA was affected by chronic IH, as shown previously in gracilis arteries.[22] We observed a specific and limited increase in the ET-1–mediated contractile response of OA from IH rats, without alteration in other vasoconstrictors response. ET-1 acts through activation of two types of receptors. ET_RA, predominantly expressed in vascular smooth muscle cells, mediate vasoconstriction, while ET_RB, found on endothelial muscle cells, mediate vasorelaxation mainly through the release of nitric oxide. However, at high ET-1 concentrations, vasoconstriction also can be produced by ET_RB present on vascular smooth muscle cells.[23]

Regarding the ocular circulation, our results are consistent with published data of ET-1 being the most potent vasoconstrictor of OA[18] and posterior ciliary arteries.[15,24] Although the increased reactivity to ET-1 of OA was limited to one dose after chronic IH exposure, this effect is consistent with the enhanced ET-1 sensitivity reported in other resistance vessels, such as fifth-order pulmonary[25] and fourth-order mesenteric arteries[9] from IH-exposed rodents. Variations in vascular bed response to IH are important to highlight, since they could be due to differences in IH models, but also to efficient mechanisms of vascular regulation specific to the ocular vasculature compared to other vascular beds. Indeed, these mechanisms, well known in human and animal models,[26] could result in a milder impact of IH stimulus on the ocular vascular function.

Endothelin-1 has an important role in IH-induced systemic hypertension[27] and vascular dysfunction. The increase in blood pressure associated with chronic IH is abolished by selective ET_RA antagonists[27] as well as by superoxide ion scavenging,[28] supporting the role of ET-1–mediated vasoconstriction and oxidative stress in vascular tone regulation in response to chronic IH. Our results suggested that the greater contraction in IH rats after ET-1 administration was due to a decrease in ET_RB-mediated relaxation. This suggests that ET_RB stimulation in rat OA produces a vasorelaxing effect and that this effect is impaired by chronic IH exposure. Assessment of OA response to an ET_RB agonist is needed to confirm the vasorelaxing effect of ET_RB. Furthermore, the impaired ET_RB-related vasorelaxation in IH group was observable only at the first dose of ET-1 eliciting a vasoconstrictive effect. The lack of difference at higher concentrations is most probably due to the potent vasoconstrictive activity of both receptors at high ET-1 doses. At the same time, potential differences in ET_RB-related vasorelaxing responses at low ET-1 concentrations could not be explored using wire myography, prompting us to continue the exploration of the ET-1 system at the dose 3.10^{-8} M.

Since activation of ET_RB leads to nitric oxide release,[23,29] the decrease in ET_RB-mediated relaxation of OA in response to IH could be linked to a reduction in nitric oxide bioavailability. This hypothesis was supported by the significant delay in nitric oxide-mediated vasorelaxation of OA observed in IH-exposed rats, and the unaltered vasorelaxation in response to exogenous nitric oxide (sodium nitroprusside). Nitric oxide-mediated vasorelaxation also has been shown to be impaired in other resistance arteries of animals submitted to chronic IH, such as middle cerebral arteries,[11] gracilis arteries,[11,22] and cremaster muscle arterioles.[30] In ocular vessels, nitric oxide is a major mediator of vascular relaxation in human OA,[15] porcine OA, and ciliary arteries,[16,17,23] as well as in canine OA.[31] These in

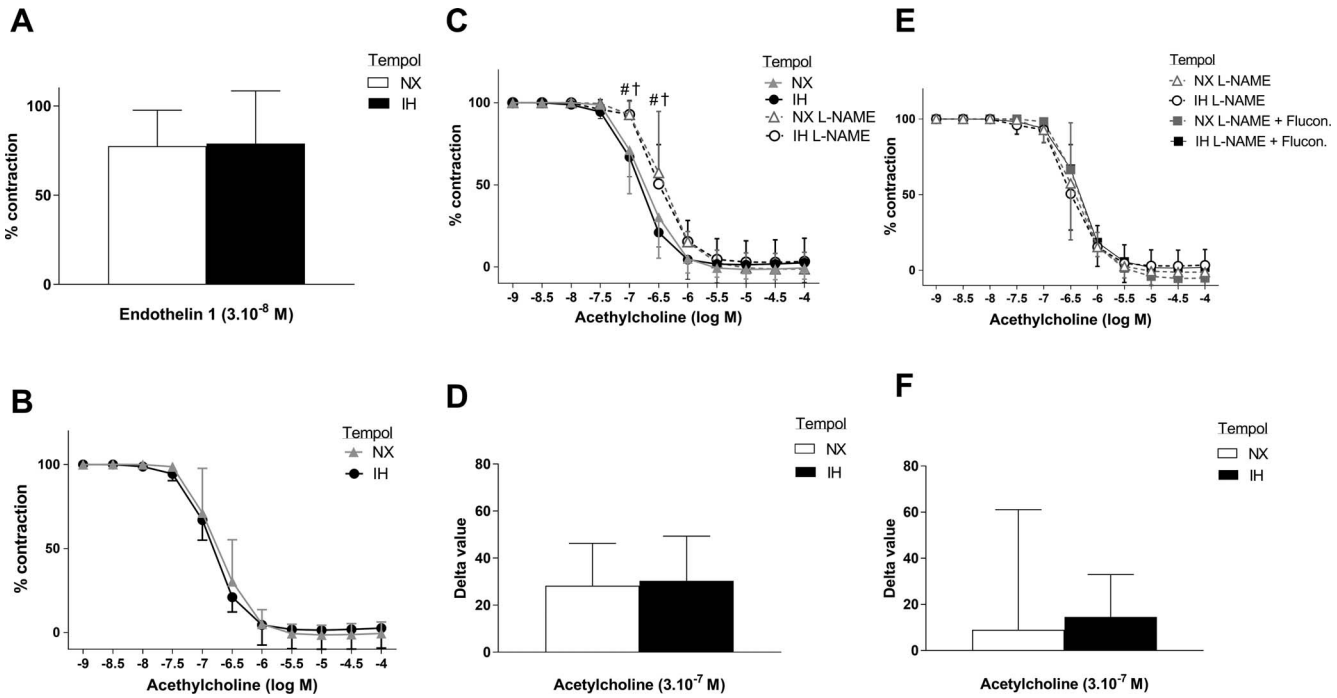

Figure 6. Ophthalmic artery reactivity in rats treated with tempol throughout exposure to NX or IH. Tempol administration during the 14-day exposure resulted in similar responses of ophthalmic arteries from NX and IH rats ($n = 6$ per group) in terms of: (**A**) Contraction in response to a single dose of ET-1 (3.10^{-8} M). (**B**) Response to cumulative doses of acetylcholine. (**C**) L-NAME–induced right shift in acetylcholine-induced relaxation curve. Two-way ANOVA: #$P < 0.05$ vs. acetylcholine alone in NX group; †$P < 0.05$ vs. acetylcholine alone IH group. (**D**) Delta value between the responses to 3.10^{-7} M acetylcholine in the presence of L-NAME vs. 3.10^{-7} M acetylcholine alone. (**E**) Response to cumulative doses of acetylcholine in presence of L-NAME and fluconazole. (**F**) Delta value between the responses to 3.10^{-7} M acetylcholine in the presence of L-NAME + fluconazole vs. 3.10^{-7} M acetylcholine in the presence of L-NAME only.

vitro data have been confirmed by experiments evaluating blood flow of the retina,[32] optic nerve head, and choroid.[33]

To our knowledge, our study is the first to investigate the involvement of nitric oxide and other vasorelaxing endothelium-derived molecules in rat OA. Reduced nitric oxide bioavailability in apneic patients,[34] associated with decreased endothelium-dependent vascular relaxation,[35] could potentially have detrimental effects on the ophthalmic circulation. We observed a potent vasorelaxation in response to acetylcholine that was only delayed by inhibition of nitric oxide release (L-NAME) and by combined inhibition of nitric oxide and prostacyclin release (L-NAME + TPC). Therefore, nitric oxide appears to have a smaller role in relaxation of OA in rats compared to humans.[15]

In our rat model, the IH-mediated OA dysfunction was associated with enhanced superoxide ion production in the OA wall and was abolished by the superoxide dismutase mimetic, tempol. This is consistent with the known increase in superoxide production in mesenteric,[27] pulmonary,[36] and cerebral[37] arteries of rats exposed to chronic IH. The normalization of OA endothelial function by tempol supports a direct role of reactive oxygen species in the decrease in nitric oxide bioavailability and the imbalance between vasodilating and vasoconstrictive cytochrome P450 products. This decrease

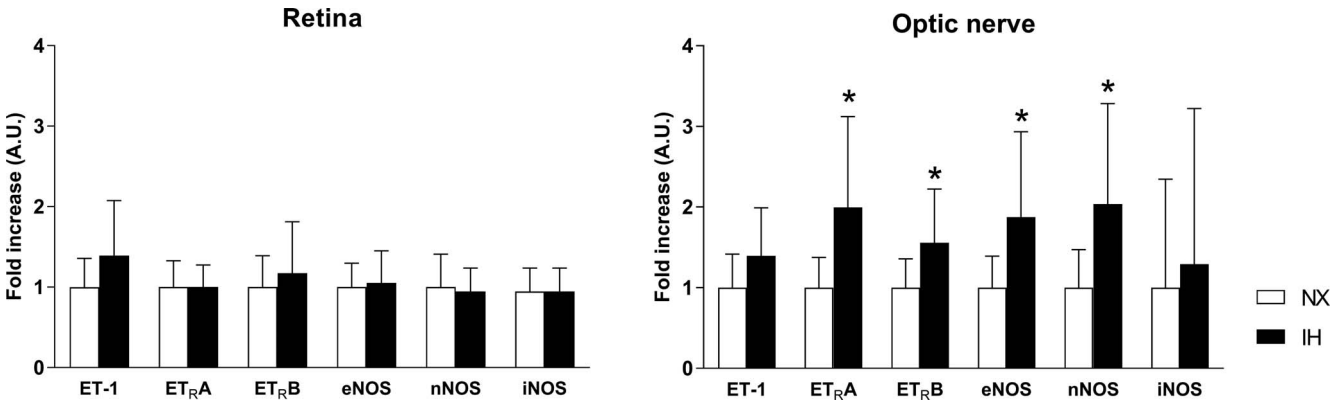

Figure 7. mRNA expression of *ET-1*, ET_RA, and ET_RB, *eNOS*, *nNOS*, and *iNOS* in the retina and optic nerve of rats exposed to 14 days of NX or IH. Relative gene expression is expressed as fold increase compared to that of NX rats ($n = 10$ rats per group). Unpaired Student's *t*-test: *$P < 0.05$, NX vs. IH.

in nitric oxide bioavailability could be secondary to eNOS uncoupling, leading to preferential superoxide ion formation[29] or to nitric oxide scavenging by superoxide ions, resulting in peroxynitrite formation.[38]

In the cardiovascular system, oxidative stress has a critical role in activating the ET-1 system to induce vascular remodelling,[27] but it also is enhanced by vascular ET-1 and ET_RA overexpression in response to chronic IH.[37] Therefore, we could hypothesize that the increase in oxidative stress associated with IH is involved in endothelin receptor and *NOS* gene overexpression observed in the optic nerve. Exploration of the expression of these genes after tempol treatment would be necessary to support this hypothesis.

Our results supported an important role of EDHF, particularly cytochrome P450 products, in the vasodilatory mechanisms of rat OA, as reported previously for the mouse OA (Manicam C. *IOVS* 2014;55:ARVO E-Abstract 4350). In our study, blockade of all cytochrome P450 products produced opposite effects in NX and IH rats, suggesting that chronic IH impairs the balance between vasorelaxing and vasoconstrictive cytochrome P450 products. In NX rats, fluconazole delayed acetylcholine-induced relaxation, revealing a dominant role of vasorelaxing epoxyeicosatrienoic acids in the response to acetylcholine. In contrast, fluconazole induced a left shift of this relaxation in IH-exposed rats, suggesting a primary contribution of vasoconstrictor 20-hydroxyeicosatetraenoic acid (20-HETE). This imbalance could be explained by various IH-induced alterations such as: (1) an increase in 20-HETE expression/activity, (2) a decrease in EET expression/activity, and (3) an increased expression/activity of soluble epoxide hydrolase. Cytochrome P450 requires oxygen to transform arachidonic acid into its vasoactive metabolites[39] and hypoxia reduces the synthesis of 20-HETE.[40] These observations suggest that chronic IH could alter 20-HETE synthesis, an interesting hypothesis in view of the involvement of 20-HETE in several models of ischemia-reperfusion brain damage.[41,42] To our knowledge, there are no data regarding the function of vascular cytochrome P450 enzymes in IH-exposed animals and obstructive sleep apnea patients. Therefore, the exploration of this pathway is mandatory to fully assess the mechanisms of IH/obstructive sleep apnea-induced vascular dysfunction. Moreover, additional experiments are necessary to fully explore the various EDHF pathways involved in the rat OA, since acetylcholine was able to induce complete vasorelaxation in the presence of combined NOS, PGI synthase, and cytochrome P450 blockade. In particular, the contribution of gap junctions and voltage-gated potassium channels should be explored in view of recent data in mouse OA.[43]

Finally, we have shown that *ET_RA*, *ET_RB*, *eNOS*, and *nNOS* genes were significantly upregulated in rat optic nerve after a 14-day exposure to IH. Interestingly, this was not observed in the retina, supporting the idea that regulation of these genes is tissue-dependent, as previously shown in carotid,[44] pulmonary,[45] and brain[46] arteries. Endothelin-1 is thought to be involved in glaucoma, through its vasoconstrictive effects on the optic nerve head[47] and by promoting reactive astrogliosis[48] and retinal ganglion cell death, either directly[49] or by disruption of axonal transport in the optic nerve through activation of ET_RA and ET_RB.[50] In cell culture, it induces the proliferation and hypertrophy of astrocytes from the human optic nerve head via ET_RA and ET_RB stimulation.[48,51] These observations support a potential effect of IH on the astroglial tissue[52] within the optic nerve. In physiologic conditions, nNOS is expressed in astrocytes, pericytes, and vascular nitrergic neurons in the optic nerve head, whereas eNOS is confined to endothelial cells. Nitric oxide is thought to play a signaling role between astrocytes and between astrocytes and axons.[53] The overexpression of eNOs and nNOS in optic nerve head of glaucoma patients[54] and the beneficial effect of reducing NOS activity on retinal ganglion cell death[55] underlines the potential detrimental impact of the IH-induced eNOs and nNOS upregulation in the rat optic nerve.

A limitation of our study is that we were able to perform only RT-qPCR on our samples due to insufficient material. Since changes in gene expression are not systematically observed at the protein level, it would be of interest to explore protein expression as well as posttranslational modifications of the genes investigated in this study, but also of other genes specific to the retina and optic nerve.

It also could be remarked that the study was performed on young animals while the prevalence of obstructive sleep apnea increases with age. Nevertheless, we and others have repeatedly demonstrated that IH per se is the main contributor to the deleterious consequences of obstructive sleep apnea, since it closely reproduces the cardiovascular alterations observed in apneic patients. Nevertheless, future studies investigating the ocular impact of IH in aged or obese rats would be of interest to observe the effect of combining various risk factors.

In conclusion, chronic IH exposure, such as that encountered in obstructive sleep apnea patients, alters the vascular function of the rat ophthalmic artery, characterized by a decrease in ET_RB-mediated vasorelaxation, delayed nitric oxide-mediated vasorelaxation, and an imbalance in the contribution of cytochrome P450 products to vascular tone. IH-mediated oxidative stress has a major role in OA dysfunction. Enhanced endothelin system, and *NOS* genes expression in the optic nerve during chronic IH suggests that the endothelin and oxidative system could be a potential target for the prevention and/or treatment of optic nerve diseases associated with obstructive sleep apnea such as nonarteritic anterior ischemic optic neuropathy.

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