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Intermittent hypoxia modulates nNOS expression and heart rate response to sympathetic nerve stimulation

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Mohan, R. M., S. Golding, and D. J. Paterson. Intermittent hypoxia modulates nNOS expression and heart rate response to sympathetic nerve stimulation. *Am J Physiol Heart Circ Physiol* 281: H132–H138, 2001.—Nitric oxide (NO) decreases norepinephrine (NE) release and the heart rate (HR) response to sympathetic nerve stimulation (SNS). We tested the hypothesis that the enhanced HR response to sympathetic activation following chronic intermittent hypoxia (IH) results from a peripheral modulation of pacemaking by NO. Isolated guinea pig double atrial/right stellate ganglion preparations were studied from animals that had been exposed to IH ($n = 20$) and control animals ($n = 22$). The HR response to SNS was significantly enhanced in the IH group compared with the controls. However, the increase in HR with cumulative doses (0.1–10 μM) of bath-applied NE was similar in both groups. Western blot analysis showed less neuronal NO synthase in the right atria from the IH group. In IH animals, the NO synthase inhibitor, N^{ω}-nitro-L-arginine (L-NNA; 100 μM) did not alter the increased HR response to SNS, whereas in control animals L-NNA significantly increased the HR response to SNS; an effect that was reversed with excess L-arginine. In conclusion, the enhanced HR response to SNS after IH may be related to a decreased inhibitory action of NO on presynaptic NE release.

hypoxic training; nitric oxide; sympathetic nervous system

THE ABILITY OF THE BODY to withstand hypoxic stress is determined by adaptive responses from the systemic circulation to redirect blood flow to essential organs (e.g., the brain and heart) (14) and improve oxygen extraction (3). Because these responses are linked to the responsiveness of the endothelium, a role for endothelium-derived nitric oxide (NO) has emerged in maintaining the balance between the metabolic demand and tissue oxygen supply during hypoxia. Chronic (3–4 wk) in vivo hypoxia (16, 17, 26, 31) as well as shorter hypoxic periods (12 h, 48 h, or 7 days) (31, 34) decreases endothelial NO synthase (NOS) protein levels and mRNA expression in the rat aorta along with reductions in levels of cGMP and nitrates (34). In vitro hypoxia (0–1% oxygen for 24 h) also decreases endothelial NOS protein and mRNA levels in bovine aortic cells (20, 25). Moreover, disruption of the NO-cGMP pathway by hypoxia may account for the impaired acetylcholine-induced relaxation in the rat aorta (34), suggesting an important role for oxygen tension in NO-regulated cardiovascular function.

Long-term exposure to intermittent hypoxia (IH) (e.g., obstructive sleep apnea) also elevates urinary and plasma catecholamines (7), muscle sympathetic nerve activity (37), systemic arterial blood pressure (6, 10), and sympathetic responsiveness to hypoxia/hypercapnia (8). Functionally, the enhanced sympathetic activity may be due to reduced NO-cGMP activation because this pathway decreases central sympathetic activity (40), the peripheral presynaptic release of norepinephrine (NE) (28), and the heart rate (HR) response to sympathetic nerve stimulation (SNS) (4). Therefore, we tested the hypothesis that chronic IH increases the HR response to SNS via a peripheral presynaptic modulation of pacemaking by the NO-cGMP pathway.

MATERIALS AND METHODS

Animal Care

All experiments were performed in accordance with Home Office license requirements (PPL 30/1133, Queen Anne's Gate, London, UK) and the Animals (Scientific Procedures) Act of 1986 (UK).

Forty-two male guinea pigs (Dunkin-Hartley) initially weighing between 125 and 150 g were housed in a temperature-controlled room (20 ± 1°C) with a 12:12-h light-dark cycle. Chow and water were provided ad libitum. At least 3 days after arrival from the breeder, they were randomly assigned into either control ($n = 22$) or IH ($n = 20$) groups.

IH and Control Protocols

Animals undergoing IH were subdivided into three cages, and each cage ($n = 4$) was placed inside a purpose-built hypoxic chamber. Chow and water were provided ad libitum within the chamber, and temperature was maintained between 20 and 22°C during the hypoxic exposure. The 12-h hypoxic training period commenced at 19:30 hours, corresponding to the dark phase of the 24-h cycle. Guinea pigs are not nocturnal, and therefore we chose to expose them to hypoxia overnight, when their activity and metabolism were reduced. In this way, maintenance of IH gas concentrations was easier because fluctuations in expired carbon dioxide were minimized. The fractional concentrations of oxygen ($F_{C_{O_2}}$) and carbon dioxide ($F_{C_{CO_2}}$) were continuously moni-

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tored by a O_2/CO_2 analyzer (Normocap 200, Datex), and manual adjustments to the flow of nitrogen and air maintained F_{CO_2} at 8–8.5% and F_{CCO_2} at 0.3–0.35%. Animals underwent IH for 21 consecutive days. Control animals remained in an ambient atmosphere (F_{CO_2} 21%, F_{CCO_2} 0.04%) for the same duration.

After the 21-day training period, control and IH animals were killed by cervical dislocation after exsanguination. The thorax and mediastinum were rapidly removed and placed in Tyrode solution aerated with 95% O_2-5% CO_2.

Guinea Pig Sinoatrial Node/Right Stellate Ganglion Preparation

Immediately after the mediastinum and thorax were removed, the ventricles were perfused with heparinized Tyrode solution (1,000 U/ml), excised, and weighed. The atria and right stellate ganglion were dissected free, and sutures (Ethicon 6/0 silk) were placed on the lateral edges of both atria. The preparation was then transferred to a preheated (37 ± 0.1°C) water-jacketed bath containing 100 ml of oxygenated Tyrode solution. The atria were vertically mounted, with the suture in the left atrium connected to a stainless steel hook and the suture in the right atrium attached to an isometric force transducer (model 60-2997, Harvard Apparatus). The right stellate ganglion was placed through a pair of platinum ring electrodes connected to a stimulator. Data were collected on a Power Macintosh 7500 computer (Apple Systems) using a Biopac MP100 acquisition system and Acknowledge software (Biopac Systems). HR (in beats/min) was triggered from contraction, and signals were displayed in real time. Data were stored on a CD ROM for offline analysis. Atrial samples were frozen at −70°C immediately after the in vitro protocols were completed .

Solutions and Drugs

Tyrode solution contained (in mM) 120 NaCl, 4 KCl, 2 $MgCl_2$, 0.1 NaH_2PO_4, 25 $NaHCO_3$, 2 $CaCl_2$, and 11 glucose. The solution was aerated with 95% O_2-5% CO_2 (pH= 7.4), and its temperature was maintained at 37 ± 0.1°C (Grant Instruments; Cambridge, UK). Solutions of N^{ω}-nitro-L-arginine (L-NNA) and L-arginine (L-Arg) were dissolved in Tyrode solution and prepared immediately before application. NE bitartrate was added from a 1 mM stock solution made up before the experiment using reagent-grade water from an Elga water purification system. All chemicals were purchased from Sigma (Dorset, UK).

Catecholamine Assay

HPLC analysis (St. Helier NHS Trust) for urinary catecholamines (NE and epinephrine) was used to provide indirect markers for any changes in sympathetic activity elicited by hypoxic exposure. Urine specimens (100 μl) in 2 μl of 3 mM hydrochloric acid were taken by bladder puncture after exsanguination and immediately frozen at −70°C. On the basis of the mean value of each measurement performed on the urine of seven IH and seven control animals, the total concentrations of NE and epinephrine were expressed in nanomoles per liter of urine.

Neuronal NOS Protein Measurement

Monoclonal mouse anti-neuronal NOS (nNOS) was used as the primary antibody, and horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG (NA931, Amersham) was used as the secondary antibody for Western blot analysis. Frozen tissue samples were thawed and lysed in buffer containing 50 mM HEPES (pH 7.4), 1 mM EDTA, 0.5% Triton X-100, and a cocktail of protease inhibitors (Complete Tablets, Roche Diagnostics). Protein concentrations were then measured using the Bio-Rad detergent-compatible protein assay kit, and total protein (50 μg) was separated on 7.5% SDS-PAGE gels at a constant 50 V for 12 h. Resolved proteins were transferred to a MSI polyvinylidene difluoride membrane (GRI) using a semidry transfer cell following the manufacturer's protocols (Bio-Rad). Membranes were blocked for 12 h in 3% dried milk in PBS-0.1% Tween 20 (PBST) at 4°C, washed twice in PBST, and incubated for 12 h in PBST containing 1% dried milk powder and primary antibody at 4°C. Blots were washed three times for 10 min each time in PBST, incubated with the secondary HRP-conjugated antibody for 30 min, and then washed as before. Unless specified, all incubations were done at room temperature. Antibody-bound proteins were detected using luminol-based chemiluminescence detection reagents (Renaissance, NEN Life Science) and exposure to chemiluminescence autoradiography film (Fugi MI-NP imaging film, GRI) for 10–60 min. Autoradiographs were digitized and relative band densities were determined using the Chemigenius gel documentation system and GeneTools gel analysis software (Syngene).

Experimental Protocols

The atria were equilibrated in Tyrode solution for 45–90 min until the HR did not alter by 10 beats/min over a 20-min period. After this equilibration period, the stellate ganglion was stimulated at 3 Hz at 10 V with a 1-ms pulse width for 30 s at 2- to 3-min intervals. Experimental protocols commenced after three consistent positive chronotropic responses to ganglion stimulation were achieved. The change in HR with SNS was calculated by the difference in 5-s averages in HR taken before the onset and cessation of a stimulation period.

Effect of IH on increase in HR with SNS. The right stellate ganglion was stimulated at 1, 3, 5, and 7 Hz (10 V, 1-ms pulse width, 30-s duration) to evaluate whether IH altered the HR response to SNS in the isolated double atrial preparations from control and IH animals. A minimum time of 120 s was left between consecutive stimulations.

Effect of IH on increase in HR with bath-applied NE. To test whether hypoxic training altered the HR response to sympathetic stimulation via a pre- or postsynaptic mechanism, the HR response to bath-applied NE (cumulative addition 0.1–10 μM) was measured in atria from IH and control animals and compared with the SNS response.

Role of NO on HR response to SNS in atria from IH and control animals. We examined the effects of the NOS inhibitor L-NNA (100 μM) on the increase in HR with SNS at 3 Hz. This single drug concentration (100 μM L-NNA) has been shown to enhance evoked NE release (28) and the HR response to SNS (4). The right stellate ganglion was stimulated alternately at 3 Hz (10 V, 1-ms pulse width) for three cycles. Fresh Tyrode solution was then placed in the organ bath, and L-NNA (100 μM) was equilibrated with the tissue for 20 min. A second period of three cycles of 3-Hz sympathetic stimulation was completed in the presence of L-NNA. After fresh Tyrode solution was added, L-NNA (100 μM) and L-Arg (1 mM) (the substrate for NOS) was applied to the preparation. After an equilibration period of 20–25 min, a final three cycles of 3-Hz sympathetic stimulation was completed. The increases in HR with SNS were averaged for control, L-NNA, and L-NNA + L-Arg treatment at 3-Hz stimulation.

Table 1. *Effect of IH on physical characteristics*

Groups	Body Weight, g	Growth Rate, g/day	Whole Heart Weight, mg	Ventricular Weight/ Body Weight, mg/g	Urinary Cathecholamines, nM	
					NE	Epinephrine
Control	398 ± 12	10.0 ± 0.3	1.3 ± 0.1	2.8 ± 0.1	13.9 ± 5.2	0.6 ± 0.4
IH	289 ± 8*	4.0 ± 0.3*	1.6 ± 0.1*	4.0 ± 0.2*	13.8 ± 5.8	1.3 ± 0.5

Values are expressed as means ± SE; n = 20–22 guinea pigs/group except for those measured for whole heart weights (n = 4/group) and urinary cathetcholamines (n = 7/group). IH, intermittent hypoxia; NE, norepinephrine. *$P \leq 0.05$, control vs. IH group.

Statistical Analysis

Data are presented as means ± SE. For comparison of physical characteristics after IH, unpaired t-tests were used to evaluate the difference between IH and control groups. For the double atrial/right stellate ganglion preparations, the difference in the positive chronotropic response to SNS between IH and control groups was compared using unpaired t-tests. A one-way repeated measures ANOVA followed by a Student-Newman-Keuls test for pairwise comparison was used to examine the effects of L-NNA and L-NNA + L-Arg on the increase in HR with sympathetic activation in both IH and control groups. Differences in HR with control, L-NNA, L-NNA + L-Arg treatments between IH and control groups were evaluated by using an unpaired t-test. Statistical significance was accepted at $P < 0.05$.

RESULTS

Animals in the control groups were allowed to breathe normoxic air daily, whereas those in the IH groups underwent daily 12-h hypoxic training periods, which commenced during the dark phase of their circadian cycle. During the 21-day experimental training period, F_{CO_2} and F_{CCO_2} were maintained at 8.22 ± 0.03 and 0.29 ± 0.01%, respectively. Relative to the control groups, the IH groups demonstrated typical adaptations to hypoxic exposure characterized by significantly ($P < 0.05$) lower body weights and growth rates (Table 1). Similarly, groups undergoing IH had significantly ($P < 0.05$) higher absolute heart weights as well as ventricular weight-to-body weight ratios (Table 1).

Urine Cathecholamine Measurements

Levels of urinary NE were similar in both IH and control groups. Epinephrine doubled in the IH group, but this trend did not reach statistical significance (Table 1).

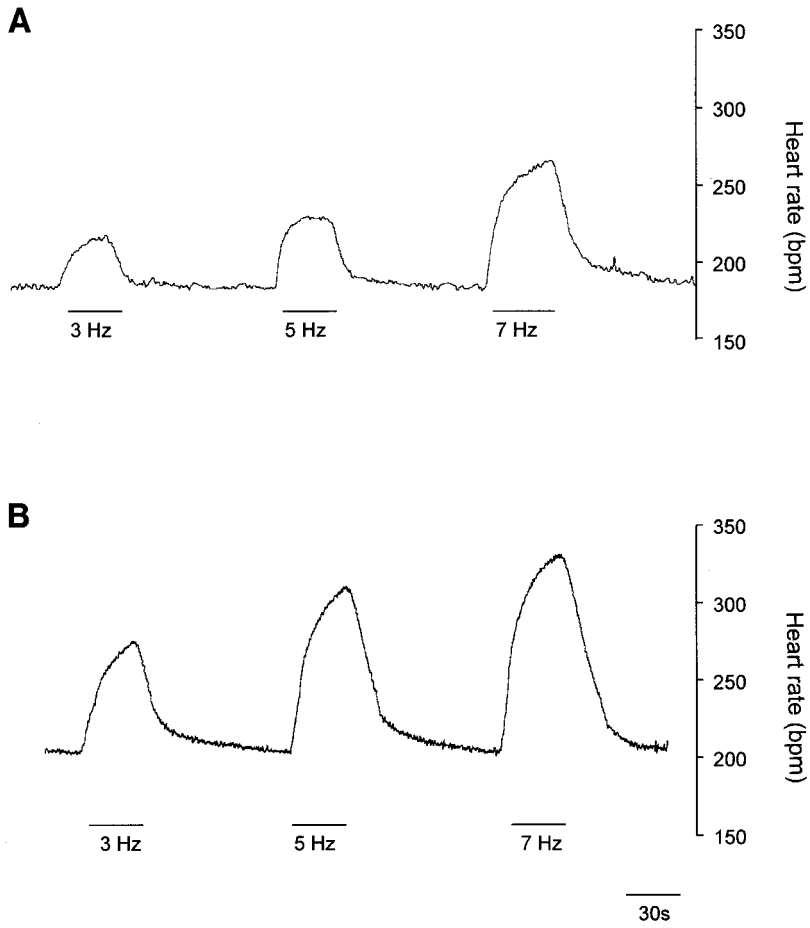

Fig. 1. Representative raw data traces showing the increase in heart rate [in beats/min (bpm)] during cardiac sympathetic nerve stimulation (SNS; at 3, 5, and 7 Hz, 10 V, 1-ms pulse width) in a double atrial/right stellate ganglion preparation from a control (*A*) and an intermittent hypoxia (IH) trained (*B*) animal. The right stellate ganglion was stimulated for 30 s, with 2–3 min between each stimulation period. The time calibration bar refers to both traces.

Effect of IH on Increase in HR with SNS and with Bath-Applied NE

Figure 1 shows representative raw data traces for the effect of sympathetic stimulation (3, 5, and 7 Hz) on HR (in beats/min) in isolated atrial preparations from an IH (Fig. 1*B*) and a control (Fig. 1*A*) animal. The increase in HR with sympathetic stimulation was significantly enhanced in atria from IH animals at 3 Hz ($P = 0.029$) and 5 Hz ($P = 0.018$). Figure 2*A* shows frequency-response curves for the mean positive chronotropic response to SNS (1, 3, 5, and 7 Hz) in IH ($n = 14$) and control ($n = 13$) groups.The positive chronotropic effect of the cumulative addition of NE (0.1–10 μM) was not significantly different between IH and control animals (Fig. 2*B*).

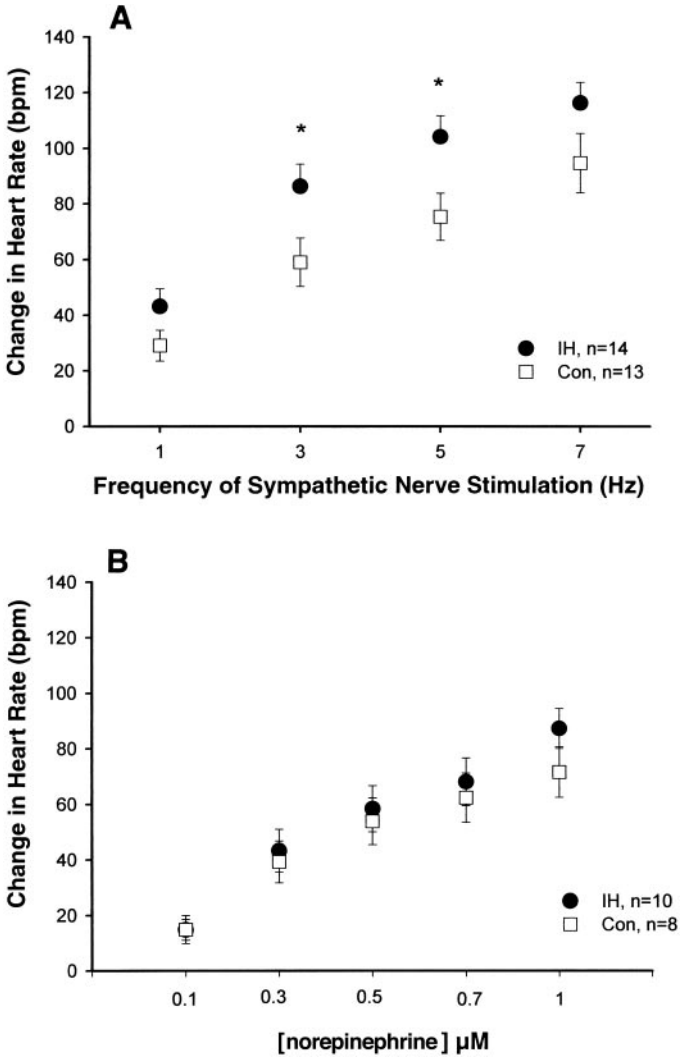

Fig. 2. Frequency-response curves for the increase in heart rate (in beats/min) with SNS in atria from control (Con; $n = 13$) and IH ($n = 14$) animals (*A*). The positive chronotropic response to sympathetic stimulation at 1, 3, 5, and 7 Hz was significantly attenuated in IH atria (unpaired *t*-test). *$P < 0.05$. *B*: dose-response curves for the increase in heart rate (in beats/min) with bath-applied norepinephrine (0.1–10 μM) in atria from control ($n = 7$) and IH ($n = 10$) animals.

Role of NO on Positive Chronotropic Response to SNS

In the IH group, L-NNA (100 μM) did not enhance the magnitude of the positive chronotropic response to sympathetic 3-Hz nerve stimulation (Fig. 3*B*). In the control group, L-NNA (100 μM) significantly increased the positive chronotropic response to sympathetic 3-Hz nerve stimulation, and this effect was significantly reversed with excess L-Arg (100 μM L-NNA + 1 mM L-Arg; Fig. 3*A*).

Changes in nNOS Expression After IH

Figure 4 shows results of Western blot analysis of nNOS protein expression using a specific monoclonal antibody in the atria from control and IH-trained animals. A 155-kDa protein band compatible with nNOS was absent in IH atrial samples but was identifiable in control samples. A 120-kDa protein band compatible with a nNOS variant (9) was identified in the total atrial protein samples from both control and IH groups. The level of this NOS protein was 52% lower in the IH group compared with the control group [control 5.4 ± 2.3 optical density (OD), $n = 4$; IH 2.6 ± 0.4 OD, $n = 4$]. Isolated guinea pig small intestines (negative control) and guinea pig forebrains (positive control) were used to verify nNOS antibody specficity.

DISCUSSION

We show that there is a significant peripheral component underlying the enhanced HR response to cardiac SNS after chronic IH and that this may involve a presynaptic modulation of NE release, because there was no difference in the HR response to bath-applied NE. In the control group, NOS inhibition significantly increased the HR response to SNS, an effect not seen in the IH group. In addition, the level of nNOS protein from the right atria was lower in the IH-exposed animals. When these data are taken together, they suggest that reduced bioavailability of NO may be responsible for the enhanced HR response to cardiac SNS after IH exposure because NO has been previously shown to inhibit NE release during cardiac SNS (28).

Efficacy of IH Exposure

The IH group demonstrated the typical adaptations of increased whole heart weight, higher ventricular weight-to-body weight ratios, and reduced body mass relative to the control group. Data from normobaric (13, 23) and hypobaric (24, 39) IH studies in rats report moderate degrees of cardiac hypertrophy defined by an increase in the heart weight-to-body weight ratio. The present study reports a 43% increase in the ventricular weight-to-body weight ratio in the IH group relative to the control group, which is within the range for IH-induced ventricular hypertrophy reported by others (13, 23, 39).

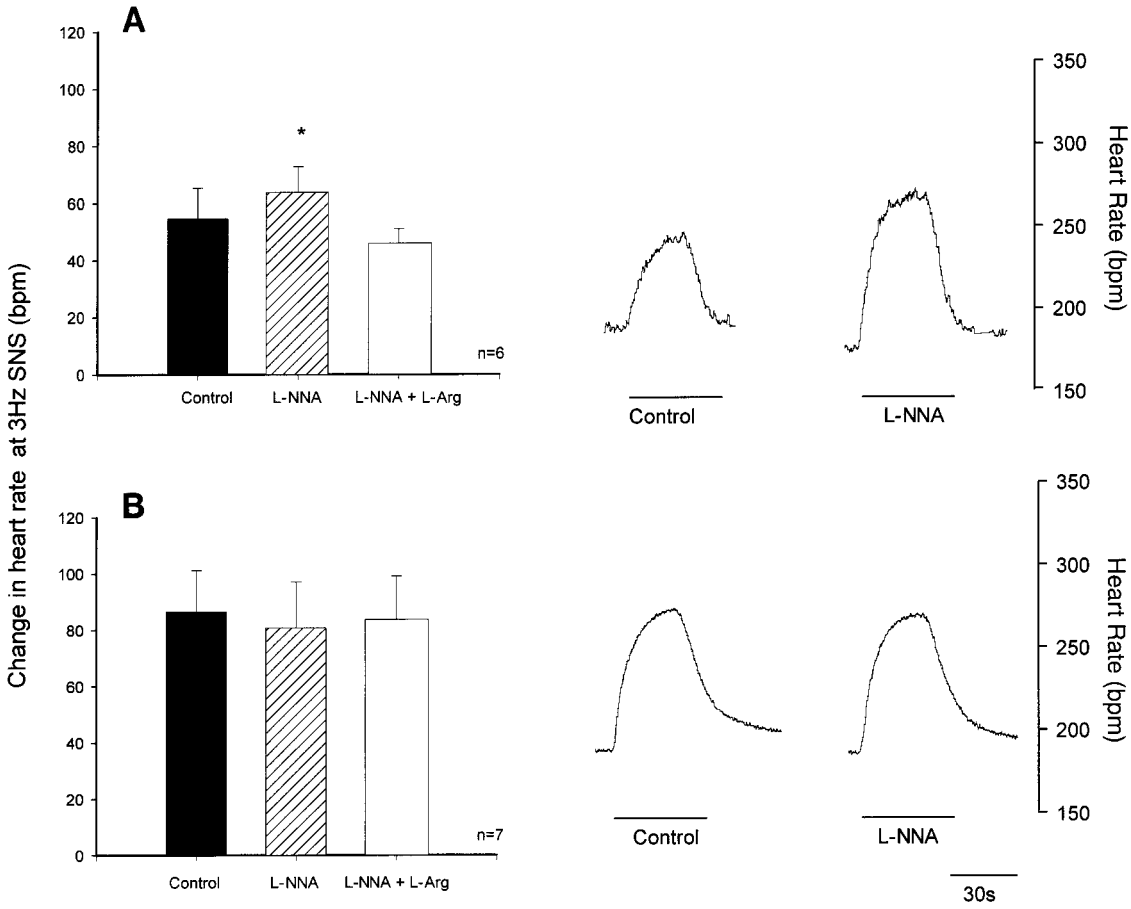

Fig. 3. *Right*: representative raw data traces showing the effect of nitric oxide synthase (NOS) inhibition with N^{ω}-nitro-L-arginine (L-NNA; 100 μM) on the increase in heart rate with right stellate ganglion stimulation at 3 Hz in control (*A*) and IH groups (*B*). *Left*: L-NNA significantly enhanced the magnitude of the positive chronotropic response to sympathetic activation in control atria at 3 Hz stimulation, and this effect was significantly attenuated with L-arginine (L-Arg). *$P < 0.05$ (ANOVA; $n = 6$). Note that the heart rate response to sympathetic stimulation remained significantly attenuated during NOS inhibition in the IH group (*left*, $n = 7$).

Hypoxia and Cardiac Autonomic Function

Hypoxia induces time-dependent changes in the cardiac autonomic nervous system (18). Acute hypoxic exposure decreases cardiac vagal tone (36) and increases both sympathetic activity and circulating catecholamines, resulting in an increase in HR and cardiac output (22, 36). After sustained exposure, the cardiovascular response is attenuated as HR and cardiac output fall to levels similar to those observed before hypoxic exposure (1). However, it is unclear whether the autonomic adaptations after hypoxic exposure result from changes in peripheral (e.g., presynaptic and/or postsynaptic) modulation of cardiac efferent activity or central sympathovagal outflow. Some evidence suggests that the initial increase in sympathetic activation in response to acute hypoxia may be antagonized by presynaptic activation of cholinergic muscarinic and α_2-receptors on the sympathetic nerve terminal (38). However, over longer periods of hypoxia, the blunting of sympathetic responsiveness has been attributed to downregulation of β-adrenoceptors (12), desensitization of β-adrenoceptors (35), and increased enzymatic activity of catechol-*O*-methyltransferase, leading to a more rapid breakdown of NE (21).

The enhanced HR response to peripheral SNS in the hypoxic group appears to be related to modulation of a presynaptic mechanism because the HR responses to bath-applied NE were similar between groups. Urinary NE levels were unchanged between both groups, whereas epinephrine was slightly elevated in the hypoxia group. These findings are consistent with previous reports (11, 27) showing that acute or prolonged hypoxia has limited effects on plasma or urinary catecholamines. The lack of an increase in global catecholaminergic activity may involve increased catecholamine clearance (19) as well as inhibition of the oxygen-sensitive tyrosine hydroxylase (2). However, we cannot exclude the proportion of urinary NE that is derived from renal noradrenergic nerves (15).

NO and Hypoxia

In the IH group, atrial nNOS protein expression at 155 kDa was absent, although the splice variant

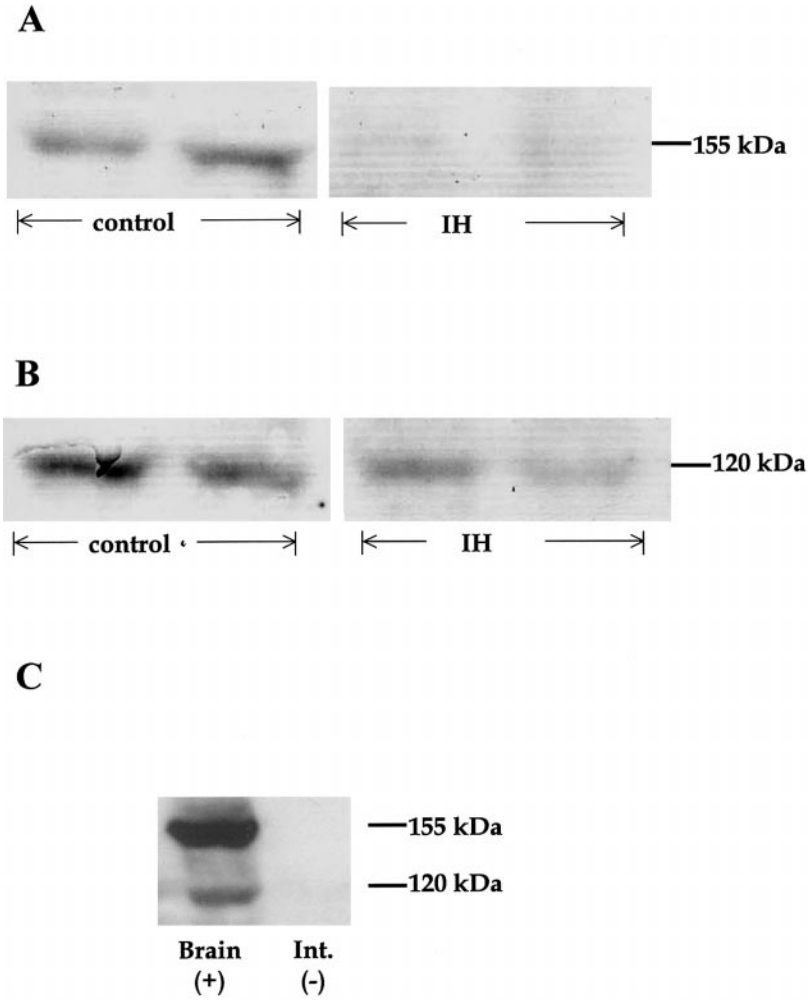

Fig. 4. Results of Western blot analysis of neuronal NOS protein expression using a specific monoclonal antibody in atria from control (n = 4) and IH (n = 4) animals. A 155-kDa protein band compatible with nNOS was absent in IH atrial samples but was identifiable in control samples (*A*). A 120-kDa protein band compatible with the nNOS variant was identified in the total protein sample from both control and IH groups (*B*). The level of this NOS protein was lower (by 52%) in the IH group compared with the control group (control 5.4 ± 2.3 OD, n = 4; IH 2.6 ± 0.4 OD, n = 4). Isolated guinea pig small intestine (Int; negative control, −) and guinea pig forebrain (positive control, +) were used to verify nNOS antibody specficity (*C*).

nNOS protein (120 kDa) was ~50% lower compared with the control group. Functionally, it is now well established that NO inhibits peripheral sympathetic activity in the heart. Inhibition of endogenous NO production with nonisoform-specific and nNOS inhibitors increases both NE release during cardiac SNS (28) and the HR response to cardiac SNS (5, 29). Conversely, NO donors or a cGMP analog attenuate the HR and inotropic responses to SNS in isolated guinea pig atria (4). In the current study, NOS inhibition significantly enhanced the effect of cardiac SNS on atrial beating rate in the control (but not the IH) group. When this result is viewed with the Western blot data, our results suggest that decreased NO bioavailability may be responsible for the increased sympathetic activation after 21 days of IH. However, we cannot exclude the possibility that reduced NO in vivo may increase central sympathetic activation after hypoxic training, because IH has been reported to activate central neural structures in the pathological development of diurnal systemic hypertension with obstructive sleep apnea (32).

The mechanism regulating NOS expression during hypoxia is poorly understood. At the molecular level, several transcriptional regulators that activate target genes in response to hypoxia may be important. One candidate is hypoxia-inducible factor (HIF)-1, which has been localized in catecholaminergic cells (33) and plays a general role in oxygen signaling to the nuclear transcriptional machinery (30). The inducible NOS isoform gene possesses a 5′ flanking hypoxia recognition sequence for HIF; however, it is not known whether the nNOS gene contains similar recognition sites that could regulate nNOS expression in cardiac sympathetic neurones during hypoxia.

Finally, high cardiac sympathetic activity is a negative prognostic indicator in the etiology of arrhythmia and sudden cardiac death. It remains to be established whether downregulation of cardiac sympathetic nNOS is an important functional marker in pathophysiological states promoting chronic IH.

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