Respiratory Network Enhances the Sympathoinhibitory Component of Baroreflex of Rats Submitted to Chronic Intermittent Hypoxia

Davi J.A. Moraes, Leni G.H. Bonagamba, Melina P. da Silva, André S. Mecawi, José Antunes-Rodrigues, Benedito H. Machado

Abstract—Chronic intermittent hypoxia (CIH) produces respiratory-related sympathetic overactivity and hypertension in rats. In this study, we tested the hypothesis that the enhanced central respiratory modulation of sympathetic activity after CIH also decreases the sympathoinhibitory component of baroreflex of rats, which may contribute to the development of hypertension. Wistar rats were exposed to CIH or normoxia (control group) for 10 days. Phrenic nerve, thoracic sympathetic nerve, and neurons in the rostral ventrolateral medulla and caudal ventrolateral medulla were recorded in in situ preparations of rats. Baroreflex regulation of thoracic sympathetic nerve, rostral ventrolateral medulla, and caudal ventrolateral medulla neurons activities were evaluated in different phases of respiration in response to either aortic depressor nerve stimulation or pressure stimuli. CIH rats presented higher respiratory-related thoracic sympathetic nerve inhibition during expiration, but not during inspiration, was enhanced in CIH when compared with control rats. In addition, CIH selectively enhanced the expiratory-related baroreceptor inputs, probably through caudal ventrolateral medulla neurons, to the respiratory-modulated bulbo-spinal rostral ventrolateral medulla presympathetic neurons. These findings support the concept that the onset of hypertension, mediated by sympathetic overactivity, after 10 days of CIH is not secondary to a reduction in sympathoinhibitory component of baroreflex. Instead, it was observed an increase in the gain of sympathoinhibitory component in in situ preparations of rats, suggesting that changes in the respiratory-related sympathetic network after CIH also play a key role in preventing greater increase in arterial pressure. (Hypertension. 2016;68:1021-1030. DOI: 10.1161/HYPERTENSIONAHA.116.07731.) ● Online Data Supplement

Key Words: arterial pressure ▪ baroreflex ▪ hypertension ▪ respiration ▪ sympathetic nervous system

Chronic intermittent hypoxia (CIH) is characterized by frequent activation of the peripheral chemoreceptors, which over time produces several cardiovascular dysfunctions, including mild hypertension.1-4 The CIH-induced hypertension in rats can be prevented by sympathetic nerve chemical denervation, renal sympathectomy, and adrenal medullectomy.5,6 Rats submitted to CIH also presented a larger fall in arterial pressure in response to ganglionic blockade and augmented power of oscillatory components at low and high frequencies in systolic arterial pressure.7 All these evidences indicate that hypertension in CIH rats is mediated through sympathetic overactivity. However, the mechanisms by which CIH produces sympathetic overactivity and hypertension are not yet characterized.

The central generator of respiration in the brain stem modulates the activity of presympathetic neurons in the rostral ventrolateral medulla (RVLM),S1-10 GABAergic RVLM-projecting neurons in the caudal ventrolateral medulla (CVLM),9 and the sympathetic nerves,1,2,12-14 affecting cardiovascular function. Therefore, changes in the respiratory-related sympathetic activity after CIH may contribute to mechanisms underlying the sympathetic overactivity and hypertension in rats. In this regard, we previously demonstrated that hypertensive CIH rats exhibited increased inspiratory-related firing frequency of barosensitive RVLM presympathetic neurons9 and sympathetic activity,3,4 which were generated by enhanced synaptic inputs from expiratory neurons.9 These data suggest that sympathetic overactivity after CIH is because of changes in the pattern of respiratory rhythm generator and its central modulation of sympathetic outflow.

Previous studies15-18 proposed that reduction in baroreflex gain also plays an important role in the development of sympathetic overactivity and hypertension after several days of CIH. However, studies by Lai et al16 demonstrated...
that rats submitted to CIH present increase in arterial pressure until the fifth day, while a reduction in spontaneous cardiac baroreflex gain was not observed until the 17th day. In addition, few days of CIH (7–10 days) did not attenuate the baroreflex control of renal sympathetic nerve activity of anesthetized, but it enhanced the evoked cardiac baroreflex gain of conscious CIH rats, suggesting that the onset of hypertension induced by CIH is not secondary to reductions in the baroreflex gain. In spite of some advances in our understanding of baroreflex function in rats after CIH, little is currently known about the functional changes in the sympathoinhibitory component of baroreflex induced by CIH and associated plasticity in the central pathways of the baroreflex. Considering that (1) respiratory modulation represents an important mechanism for control of RVLM presympathetic and CVLM GABAergic neurons and consequently the sympathetic outflow, (2) CIH increases the central respiratory modulation of RVLM presympathetic neurons and sympathetic outflow, and (3) respiration alters the sympathoinhibitory component of baroreflex; in this study, we hypothesized that changes in the respiratory network induced by CIH also reduce the sympathoinhibitory component of baroreflex and contribute to the development of hypertension. The data of this study performed in situ preparations of rats demonstrate that 10 days of CIH enhanced the respiratory-related sympathoinhibitory component of baroreflex by increasing the expiratory baroreceptor inputs, probably through CVLM GABAergic neurons, to the respiratory-modulated RVLM presympathetic neurons.

**Methods**

Detailed methods section is available in the online-only Data Supplement.

**Animals**

The experiments were performed on male Wistar rats (90–130 g) provided by the Animal Care Facility of the University of São Paulo, campus of Ribeirão Preto, Brazil. All experimental protocols were approved by the Ethics Committee on Animal Experimentation of the School of Medicine of Ribeirão Preto, University of São Paulo (protocol 064/2010).

**Chronic Intermittent Hypoxia**

Rats were divided into 2 experimental groups: rats exposed to CIH (6% of O, for 30–40 s, every 9 minutes and 8 hours a day) and rats maintained under normoxic condition (20.8% of O) during 10 days.

**Arterial Pressure Measurements in Conscious Animals**

At the end of the hypoxic or normoxic protocols, under anesthesia a catheter was inserted into the abdominal aorta through the femoral artery for arterial pressure measurements on the day after in conscious, freely moving rats under normoxic conditions.

**In Situ Perfused Preparations of Rats and Electrophysiological Recordings**

Control and CIH rats were prepared for in situ perfused preparations. Phrenic nerve (PN) and thoracic sympathetic nerve (tSN) were recorded as were single units using extracellular and whole-cell patch clamp recordings of RVLM and CVLM neurons.

**Baroreflex Activation**

The magnitude of the inhibitory responses of tSN activity and RVLM presympathetic neurons and excitatory responses of CVLM neurons in response to electric stimulation of the aortic depressor nerve (ADN; 0.2 ms at 400 Hz) was used as an index of their barosensitivity. The stimulus intensity was set as that required to elicit a fall in the perfusion pressure of >10 mm Hg in response to ADN stimulation at 50 Hz, 0.2-ms of duration for 2 s (threshold). Standard pulses obtained from the onset or offset of PN were delivered to the stimulator after a variable delay, allowing that the ADN could be stimulated in different phases of central respiratory cycle. ADN stimulation during postinspiration was triggered ≤1 s after offset of PN, ADN stimulation during second-half of expiration (E2 phase) was triggered after 2 s, whereas ADN stimulation during inspiration was triggered with no delay after the onset of PN. ADN stimulations that did not follow these criteria were not considered for data analysis.

In a subset of animals, baroreceptors were stimulated by briefly increasing the speed of the peristaltic pump, which increased the perfusion pressure by 50 mm Hg above the baseline of 70 to 80 mm Hg. These challenges in perfusion pressure were delivered in the 3 different respiratory phases.

**Histology**

Immunofluorescence was performed in brain stem sections from control and CIH rats to evaluate the location and phenotype of recorded RVLM neurons.

**Single-Cell Quantitative Real-Time Polymerase Chain Reaction**

Reactions for the single-cell quantitative real-time polymerase chain reaction were performed using the cytoplasm of the recorded CVLM neurons to confirm their inhibitory phenotype.

**Statistical Analyses**

Data were compared using 1-way ANOVA or 2-way ANOVA with Bonferroni post hoc testing or a Student unpaired t test (GraphPad Prism 4, La Jolla, CA) in accordance with the experimental protocol. Differences were considered significant at P<0.05.

**Results**

We first recorded arterial pressure in conscious, freely moving rats before evaluating the sympathetic activity in situ preparations and then the sympathoinhibitory baroreflex responses. CIH produced a significant increase in the mean arterial pressure of rats (109±1.3 mm Hg versus 90±1.5 mm Hg; P<0.0001; n=50). Using the same animals in in situ preparations, the tSN activity increased during inspiration with peak activity during postinspiration in control (n=27) and CIH (n=23) rats (Figure 1A). However, comparison between the groups indicated that hypertensive CIH rats showed a higher respiratory-related tSN activity at the end of E2 phase (late-expiratory activity; 59±1.5% versus 35±2.4%; P<0.0001; Figure 1A) in relation to control rats, as an index of respiratory-related sympathetic overactivity.

**Respiratory Modulation of Baroreflex Sympathoinhibition in Control and CIH Rats**

Baroreflex activation by either pressure stimuli (Figure S1A through S1C) or ADN stimulation produces sympathoinhibition, as well as a prolongation in the expiratory period in in situ preparations of rats, as described previously. The sympathoinhibitory response to ADN stimulation in control and CIH rats was respiratory-phase dependent. In control and CIH rats, the tSN activity was inhibited during ADN stimulation with
the greatest response occurring during postinspiration (control: $P<0.0001$; CIH: $P<0.0001$) and E2 phase (control: $P<0.0001$; CIH: $P<0.0001$) than inspiration (Figure 1A through 1F). In the sequence, we also evaluated whether CIH affects the respiratory modulation of ADN-evoked sympathoinhibition. Comparison between the groups indicated that the ADN-evoked sympathoinhibition during postinspiration (2-fold the threshold: 87.8±2.3% versus 70.2±2.1%; $P<0.001$; Figure 1C and 1D) and E2 phase (2-fold the threshold: 94.3±2.7% versus 72.5±3.1%; $P<0.001$; Figure 1E and 1F), but not during inspiration (2-fold the threshold: 57.3±2.8% versus 53.2±1.9%; Figure 1A and 1B), were enhanced in CIH than in control rats. Consistent with the observation above that the sympathoinhibition is more pronounced during expiration in CIH rats, the intensity required to produce 50% of inhibition of tSN during postinspiration (1±0.07 versus 1.52±0.07; $P<0.0001$; Figure 1D) and E2 phase (0.97±0.03 versus 1.47±0.08; $P<0.0001$; Figure 1F) was lower in CIH than in control rats. The voltage required to produce a fall of 10 mm Hg in perfusion pressure, to calculate the threshold (value 1), was also lower in CIH than in control rats (2.3±0.03 V versus 5.2±0.04 V; $P<0.0001$). In addition, the increase in the duration of expiration in response to baroreflex activation during postinspiration (4.4±0.12 s versus 5.7±0.17 s; $P<0.0001$; Figure 1C) or E2 phase (4.2±0.1 s versus 5.4±0.15 s; $P<0.0001$; Figure 1E) was smaller in CIH than in control rats.

Respiratory Modulation of Baseline Activity and Barosensitivity of RVLM Presympathetic Neurons From Control and CIH Rats

Sixty-one bulbospinal barosensitive presympathetic neurons (Figure S2) from control (n=33) and CIH (n=28) rats were extracellular recorded in the RVLM. Neurons were classified into 4 types according to their respiratory-related pattern, and the effect of ADN stimulation on their activities was then examined (Figures 2 through 4; Figure S3). Fourteen barosensitive RVLM presympathetic neurons from control (n=8) and CIH (n=6) rats exhibited a decrease in their firing frequency during PN discharge (inspiratory-inhibited neurons; Figure 2A). The magnitude of the respiratory modulation was 37±3.2% in neurons from control and 34.2±4% in neurons from CIH rats. There were no significant changes in their baseline firing frequency between control and CIH rats (7±1.1 Hz versus 6.7±2.2 Hz; Figure 2A). Their inhibitory responses to ADN stimulation were not dependent on respiratory cycle, in which the inhibition was similar during postinspiration and E2 phase (control: 51.3±2.7% versus 47±3.5%; CIH: 66.5±4.2% versus 64±4.3%; Figure 2C). CIH increased their inhibitory responses to ADN stimulation during both postinspiration (66.5±4.2% versus 51.3±2.7%; $P<0.05$; Figure 2B and 2C) and E2 phase (64±4.3% versus 47±3.5%; $P<0.01$; Figure 2C). Considering that the inspiratory-inhibited RVLM presympathetic neurons are inhibited during inspiration, it was not possible to evaluate their...
inhibitory response to ADN stimulation during this respiratory phase using extracellular recordings. Five RVLM presympathetic neurons from control (n=8) and CIH (n=7) rats exhibited a pattern consisting of an augmentation of their activity that reached a peak during postinspiration (postinspiratory-modulated neurons; Figure 3A). CIH produced an increase in their firing frequency only at the end of E2 phase (late-expiratory activity; 28±1.9 Hz versus 4±1.1 Hz; P<0.0001; Figure 3A), well correlated with the late-expiratory–related tSN overactivity (Figure 1A), as we described previously. The magnitude of the respiratory modulation was 44±4.1% in neurons from control and 47±3.1% in neurons from CIH rats. This type of presympathetic neuron from control and CIH rats was most effectively inhibited during postinspiration and E2 phase, despite their higher firing frequency (Figure 3C), and CIH enhanced the ADN-induced inhibition only during these respiratory phases (postinspiration: 94.3±3.8% versus 80.9±2.9%; P<0.05; Figure 3C and E2 phase: 95.4±3.3% versus 82.3±2.9%; P<0.05; Figure 3B and 3C).

Twenty-one RVLM presympathetic neurons exhibited an increase in their firing frequency during inspiration in control (n=12) and CIH (n=9) rats (inspiratory-modulated neurons; Figure 4A). There was no significant change in their baseline firing frequency between control and CIH rats (23±1.9 Hz versus 19±2.1 Hz; Figure 4A). This type of RVLM presympathetic neuron also exhibited the highest respiratory modulation among the 4 types of RVLM barosensitive neurons (control: 67±2.3%; CIH: 65±3.1%). ADN stimulation was most effective in the postinspiration and E2 phase in control and CIH rats, when their firing frequency was lowest (Figure 4C). In addition, CIH enhanced their inhibitory responses to ADN stimulation only during postinspiration (87.8±3.8% versus 68.7±3.3%; P<0.01, Figure 4B and 4C) and E2 phase (85.6±3.8% versus 70.3±4.3%; P<0.05; Figure 4C).

Respiratory Modulation of Baseline and Barosensitivity of Respiratory-Modulated CVLM GABAergic Neurons From Control and CIH Rats

Whole-cell patch clamp recordings were performed from CVLM neurons. Each neuron recorded was categorized by its most prominent pattern feature. Twenty-four neurons from control (n=11) and CIH (n=13) rats showed an increase in activity...
immediately after the PN burst (Figure 5A). The cytoplasm of these neurons were collected and found to express glutamic acid decarboxylase 67 mRNA (Figure 5C). Twenty-two neurons from control (n=10) and CIH (n=12) rats with inspiratory modulation were sharply activated at the PN activity (Figure 6A). The activity between PN bursts was fairly stable and considerably lower. The cytoplasm of these neurons were also collected and found to express glutamic acid decarboxylase 67 mRNA (Figure 6C). Both the population of respiratory-modulated CVLM neurons did not express either vesicular glutamate transporter 2, or glycine transporter 2 (Figures 5 and 6C).

There was no significant change in baseline firing frequency of either postinspiratory (13.4±1.3 Hz versus 12.7±2 Hz; Figure 5A) or inspiratory-modulated (24±0.7 Hz versus 22.1±1.5 Hz; Figure 6A) CVLM neurons between control and CIH rats. The inspiratory-modulated also exhibited the highest respiratory modulation of their baseline activity among the 2 types of CVLM barosensitive neurons (control versus CIH: inspiratory: 61±1.7% versus 62±2.5%; postinspiratory: 38.4±2.1% versus 43.1±2.4%). The respiratory modulation of postinspiratory-modulated CVLM neurons barosensitivity was similar to the activity pattern of these neurons. ADN stimulation was most effective in the postinspiration (control: P<0.0001; CIH: P<0.0001) and E2 phase (control: P<0.0001; CIH: P<0.0001), when their firing frequency was highest, than during inspiration. In addition, CIH enhanced their excitatory responses to ADN stimulation during postinspiration (68±3 versus 47±2.1 spikes; P<0.0001; Figure 5A) and E2 phase (65.1±2.4 versus 46.2±2 spikes; P<0.0001), but not during inspiration (27±2.3 versus 30.1±1.9 spikes; Figure 5B). On the other side, in the inspiratory-modulated CVLM neurons ADN stimulation was most effective during inspiration, when their firing frequency was highest, than during postinspiration (control: P=0.002; CIH: P<0.0001) or E2 phase (control: P=0.04; CIH: P=0.0003). Different from postinspiratory-modulated neurons, CIH did not affect their excitatory responses during inspiration (35.2±2.2 versus 31.2±2.1 spikes; Figure 6A), postinspiration (21.3±1.8 versus 22±1.9 spikes; Figure 6B) or E2 phase (20±3.2 versus 23±1.5 spikes).

To compare the intrinsic electrophysiological properties of respiratory-modulated CVLM neurons and to evaluate whether the effects of CIH on the sympathoinhibitory component of...
baroreflex function are dependent on changes in these intrinsic properties, we performed the blockade of their fast synaptic transmission. With respect to CIH rats, no differences were observed in (1) resting membrane potential (postinspiratory: −53.1±2.4 mV versus −53±2 mV; inspiratory: −52.1±2.5 mV versus −51.4±2.1 mV), (2) intrinsic firing frequency (postinspiratory: 15±1.4 Hz versus 16.1±2.3 Hz; inspiratory: 18.2±3.1 Hz versus 16±1.6 Hz), (3) input resistance [postinspiratory: 345±4.1 MΩ versus 335±4.2 MΩ; Figure 5D; inspiratory: 246±3.1 MΩ versus 257±4.9 MΩ; Figure 6D], and (4) number of spikes in response to positive current injection (Figures 5 and 6E) in relation to control rats. Postinspiratory and inspiratory-modulated CVLM neurons showed similar capacitance (41±1.7 pF versus 43±1.9 pF), resting membrane potential (−53±2 mV versus −51.4±2.1 mV), and intrinsic firing frequency (16.1±2.3 Hz versus 16±1 Hz). However, in relation to the inspiratory, postinspiratory CVLM neurons exhibited higher input resistance (335±4.2 MΩ versus 257±4.9 MΩ; P<0.0001; Figures 5 and 6D) and excitability (100 pA: 58.1±2.5 versus 41.2±3.4 spikes; P=0.0002; Figures 5 and 6E).

**Discussion**

This study demonstrates that 10 days of CIH selectively enhanced the expiratory-related sympathoinhibitory component of baroreflex in in situ preparations of rats by increasing the expiratory baroreceptor inputs, probably through CVLM GABAergic neurons, to the respiratory-modulated RVLM presympathetic neurons. These findings suggest that the early phase of hypertension, mediated by sympathetic overactivity, after 10 days of CIH is not secondary to a reduction in sympathoinhibitory component of baroreflex. Instead, an increase in the gain of sympathoinhibitory component was observed in in situ preparations of rats, a response that if observed in awake rats could effectively oppose mechanisms that promote an increase of arterial pressure in response to intermittent activation of peripheral chemoreceptors.

Several studies indicated that baroreflex gain is impaired in hypertension and resets to the high level of arterial pressure. In addition, the baroreflex activation has an inhibitory influence on the cardiovascular responses to chemoreflex activation. In previous studies, we demonstrated that 10 days...
of CIH increased both the peripheral chemoreflex sensitivity and the gain of cardiac component of baroreflex in rats. Thus, it is possible that the enhancement of the inhibitory components of cardiac and vascular sympathetic branches of the baroreflex evoked by 10 days of CIH plays a key role in trying to bring back arterial pressure to normal level after intermittent increases in response to each hypoxic episode, attenuating the excitatory influence of peripheral chemoreflex hypersensitivity and preventing greater increase in arterial pressure than that observed after CIH.

In this study, we are showing the central respiratory modulation of the baroreflex function, which is consistent with previous studies. Studies by Gilbey et al demonstrated that baroreflex bradycardia appears most sensitive when the afferents are activated in expiration, a time window in which the cardiac vagal motoneurons are more excitable. There is a parallel effect on the magnitude of the baroreflex-evoked decrease in sympathetic activity, with the larger effects also during expiration. Interestingly, CIH increased the expiratory-modulated, but not inspiratory-modulated, sympathoinhibitory component of baroreflex, suggesting that excitation of expiration plays a major role in increasing the baroreflex sensitivity after CIH. The classical central pathway involved in the baroreflex control of sympathetic activity operates via nucleus tractus solitary neurons, mediating the baroreceptor projections to the CVLM, which in turn inhibit the RVLM presympathetic neurons and hence lower sympathetic activity. The second proposed pathway is mediated by excitation of respiratory neurons, specifically by inhibitory postinspiratory (post-I) neurons of Bötzinger Complex in ventral medullary surface, which contribute for the greater inhibition of sympathetic activity and the increase in duration of expiration during baroreflex activation at expiration. Despite the greater baroreflex-induced sympathoinhibition during expiration in CIH rats, the increase in duration of expiration was smaller, suggesting different central pathways: one controlling the expiratory-related sympathoinhibition and another controlling the duration of expiration during baroreflex activation. We suggest that the excitation of inhibitory Bötzinger Complex post-I neurons during baroreflex activation is mainly involved in the reflex-induced increase of the duration of expiration because CIH reduced both the activity/excitability of inhibitory Bötzinger Complex post-I neurons and the increase in duration of expiration, but not the expiratory-related sympathoinhibitory component of baroreflex.

In this study, we verified that the central respiratory network modulates not only the ongoing activity of RVLM presympathetic neurons but also their responsiveness to baroreceptor inputs, as showed before in anesthetized rats. We suggest that the central respiratory modulation of RVLM presympathetic neurons excitability is responsible, at least in part, for their respiratory-dependent baroreflex response. This suggestion is consistent with the data showing that nonmodulated RVLM presympathetic neurons also do not present the respiratory modulation of their barosensitivity. We also observed greater baroreflex-mediated inhibition of RVLM presympathetic neurons during expiration in rats, showing that the sympathoinhibitory component of baroreflex is strongly dependent on the respiratory–sympathetic interactions. It is important to note that Miyawaki et al have
shown in vivo that a majority of the RVLM neurons recorded are more sensitive to baroreflex activation during inspiration, which are not in agreement with the tSN and RVLM presympathetic neurons recordings of this study in in situ preparations of rats, showing maximal baroreceptor inhibition during the expiratory phase. The presence of anesthesia in the experiments of Miyawaki et al. 32 and the different strains (Wistar versus Sprague–Dawley) and preparations used (in vivo versus in situ) might be critical in evoking different responses of RVLM presympathetic neurons.

It should be pointed out the absence of arterial pulsatility and the low level of perfusion pressure used in this study as the in situ preparation is perfused independently, using a peristaltic pump, which bypass the cardiac output. The pulsatility in in vivo preparations phasically activates the baroreceptors and the sympathetic activity shows a pulse-related modulation of activity that is a manifestation of this phasic baroreflex activation.33 This raises the issue of whether the pulselessness and the low level of perfusion pressure used in in situ preparation are leading to nonphysiological baroreflex responses, producing misleading results. In addressing this issue, it is important to emphasize that in our study we have obtained similar results using both pressure stimuli (static baroafferents activation) and ADN stimulation (intermittent baroafferents activation), and in both cases we have shown clear enhancement of respiratory modulation of sympathoinhibitory gain of the baroreflex after CIH in in situ preparation of rats. It is also worth noting that previous studies of the baroreflex in conscious freely moving rats have shown that the replacement of carotid pulsatile pressure to static pressure has little effect on resting systemic arterial pressure or heart rate,34 suggesting that our in situ observations, using static perfusion pressure, may be comparable with the respiratory modulation of the sympathoinhibitory baroreflex function of in vivo preparations from CIH, a matter that deserves additional experiments in the future. In our in situ preparation, the lower temperature used and the absence of pulmonary stretch receptor afferents may be responsible for the slow respiratory rate. Emerging evidence has suggested that slow respiratory rate can enhance the baroreflex sensitivity.35 However, this recording condition was identical in preparations from control and CIH rats. Therefore, we do not envisage that the slow respiratory rate accounts for the differences observed in the baroreflex function between control and CIH rats.

We previously demonstrated that sympathetic overactivity in hypertensive CIH rats is because of an increase of synaptic inputs to RVLM presympathetic neurons at expiration,9 but not because of changes in their intrinsic excitability.36 Surprisingly, it was observed that CIH also selectively increases the baroreflex inhibition of respiratory-modulated RVLM presympathetic neurons during expiration, suggesting that different and opposite mechanisms within the central expiratory generator control the level of sympathetic outflow after CIH. Although the central connections responsible for the selective enhancement of expiratory-modulated baroreflex sensitivity of RVLM presympathetic neurons were not fully characterized in this study, the following possibilities must be considered (1) an increase of excitatory synaptic transmission to the second-order barosensitive or pump nucleus tractus solitary cells37,38 gating mechanism for the larger sympathoinhibition during
expiration, (2) a phasic pontine influence of respiratory neurons on RVLM presympathetic neurons, or (3) an enhancement of sensitivity of respiratory-modulated sympathoinhibitory neurons of the CVLM. Studies by Mandel and Schreinhofer characterized different respiratory-modulated CVLM neurons with complementary firing patterns to sparsely projecting barosensitive RVLM presympathetic neurons described in this study. We found that CIH selectively enhanced the excitatory baroreflex responses of GABAergic postsynaptically-modulated CVLM neurons during expiration, but not during inspiration, suggesting that these CVLM neurons are the relay responsible for the enhanced expiratory modulation of sympathoinhibitory component of baroreflex after CIH. It is postulated that the pons plays an important role in modulating baroreflex function, and studies by Baekkey et al showed that pontine transection attenuated baroreflex sympathoinhibition, by eliminating the respiratory modulation. Many pontine neurons, including those located in the Parabrachial and Kölliker-Fuse nuclei, receive baroreceptive inputs and it is likely that the enhanced respiratory-related sympathoinhibition after CIH relays through the pons. Therefore, additional experiments are required to properly evaluate the electrophysiological properties and baroreflex responses of nucleus tractus solitary and respiratory pontine neurons after CIH.

Perspectives
Our data support the new concept that the early phase of sympathetic overactivity and hypertension developed in juvenile rats exposed to CIH for 10 days are not secondary to reduction in the sympathoinhibitory component of baroreflex. These data are similar to the phenomenon that we previously described in conscious CIH rats, in which 10 days of intermittent hypoxia also increased evoked cardiac baroreflex gain. However, the findings of this study do not imply that the baroreflex control of sympathetic activity may be reduced after a long-term exposure to CIH because different mechanisms might occur between the onset of hypertension and the long-term sustained increase in arterial pressure induced by CIH. Therefore, this study provides a foundation for more focused exploration of CIH-induced changes in central respiratory–sympathetic coupling as a potential mechanism underlying the onset of sympathetic overactivity and hypertension in rats. The cellular and molecular mechanisms underlying this changed coupling between sympathetic and respiratory activities observed after CIH exposure are currently under investigation in our laboratory.

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Disclosures
None.

References

Novelty and Significance

What Is New?

• Chronic intermittent hypoxia, a hallmark of obstructive sleep apnea, increases the baroreflex-evoked sympathoinhibition during expiration in in situ preparations of rats by selective enhancement of expiratory-related inhibitory baroreceptor inputs to respiratory-modulated bulbospinal presympathetic neurons.

What Is Relevant?

• The mechanisms underlying the sympathetic overactivity and hypertension in rats submitted to chronic intermittent hypoxia are unknown; our data support the contention that the onset of hypertension, mediated by sympathetic overactivity, after chronic intermittent hypoxia is not secondary to a reduction in sympathoinhibitory component of baroreflex.

Summary

Although counterintuitive, the enhanced respiratory-related sympathoinhibition by baroreceptor inputs to bulbospinal presympathetic neurons seems to counterbalance the sympathetic overactivity induced by intermittent activation of peripheral chemoreceptors in chronic intermittent hypoxic rats.
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METHODS

Chronic intermittent hypoxia

The chronic intermittent hypoxia (CIH) and control groups of rats were housed in collective cages and maintained in Plexiglas chambers (volume, 210 L) equipped with gas injectors of O2 and CO2 and sensors of O2, CO2, humidity and temperature. The CIH group was exposed to 5 min of normoxia (20.8% of O2) followed by 4 min of N2 injection to reduce the percentage of O2 from 20.8 to 6, remaining at this level for 30 - 40 s. After hypoxia, O2 was injected to return its percentage back to 20.8. This cycle with duration of 9 min was repeated over the time of 8 h a day (from 9:30 am to 5:30 pm) during 10 days. In the remaining 16 h of each day, the animals were exposed to 20.8% of O2. Control rats were exposed to 20.8% of O2 24 h a day during 10 days, as described in a previous study from our laboratory (1).

Arterial pressure measurements in conscious animals

At the end of the experimental protocol (CIH or normoxia), rats were anesthetized with tribromoethanol (250 mg.kg-1, i.p.; Sigma, St Louis, MO, USA) and a catheter was inserted into the abdominal aorta through the femoral artery for arterial pressure measurements. The catheter was tunneled subcutaneously and exteriorized through the skin in the back of the rat's neck. One day after, the arterial catheter was connected to a pressure transducer, and in turn, to an amplifier (Bridge Amp, ML221, ADInstruments; Bella Vista, NSW, Australia). The arterial pressure was recorded in conscious, freely moving rats under normoxic conditions during 60 min (PowerLab 4/25, ML845; ADInstruments).

In situ perfused preparations of rats

Rats were anesthetized with halothane (Astra Zeneca, Cotia, São Paulo, Brazil), in a level deep enough to abolish the withdrawal responses to noxious pinching of the tail and paw. The animals were then transected caudal to the diaphragm, exsanguinated, submerged in a cooled Ringer solution, decerebrated at the precollicular level to make insentient, skinned, and had descending aorta isolated. The heart was tied and removed to improve the stability of neuronal electrophysiological recordings. To expose the ventral medullary surface, the preparations were placed supine and the head was fixed on a silicon elastomer cushion using insect pins to ensure that the brain stem was orientated similarly in all preparations. The trachea and esophagus were removed. All muscles and connective tissues covering to the basilar surface of occipital bone were removed. The basilar portion of the atlantooccipital membrane was cut and the bone carefully removed using a micro-Rongeur (D.L. Micof, São Paulo, Brazil) to expose the ventral surface of the medulla in the anteroposterior extension from the vertebral arteries to the pontine nuclei (1, 2). Preparations were then transferred to a recording chamber, the descending aorta was cannulated and perfused retrogradely with Ringer solution (containing, in mM: 125 NaCl, 24 NaHCO3, 3 KCl, 2.5 CaCl2, 1.25 MgSO4, 1.25 KH2PO4 and 10 glucose) containing 1.25% poly(ethylene glycol) (an oncotic agent; Sigma) using a roller pump (Watson-Marlow 502s; Falmouth, Cornwall, UK) via a double-lumen cannula. The perfusion pressure was maintained in the range of 70-80 mmHg by adjusting the rate flow to 21–25 mL/min and by adding vasopressin to the perfusate (600–1.200 pM, Sigma). The perfusate was gassed continuously with 5% CO2-95% O2, warmed to 31–32°C, and filtered using a nylon mesh (pore size: 25 μm; Millipore, Billerica, MA, USA). A neuromuscular blocker (vecuronium bromide, 3–4 μg/mL; Cristália, Itapira, São Paulo, Brazil) was added to the perfusate to block contractions of the respiratory muscles and consequent movements that could prevent the fine electrophysiological recordings.
Simultaneous recordings of phrenic nerve (PN) and thoracic sympathetic nerve (tSN; T8–T12) activities were obtained using bipolar suction electrodes mounted on separate 3D micromanipulators (YOU-1; Narishige, Tokyo, Japan). The tSN activity was recorded in absolute units (microvolts). Based upon absolute values, we determined a percentage scale, in the range from 0 to 100, in order to compare the levels of baseline tSN during the different phases of respiratory cycle [inspiration, post-inspiration and second-half of expiration (E2 phase)] in control and CIH groups. This percentage scale was determined for each preparation, considering the maximal activity observed during the respiratory cycle as 100% and the electrical noise level as 0% obtained at the end of each experiment after the death of the preparation.

Single-unit recordings of respiratory-modulated barosensitive RVLM pre-sympathetic neurons (Duo 773 Electrometer; World Precision Instruments, Sarasota, FL, USA) were made using glass microelectrodes (30-50 MΩ) filled with 0.5 M of sodium acetate and 1.5% of biocytin (Molecular Probes, Grand Island, NY, USA) to juxtacellular labeling of recorded neurons (200 ms pulses of 1.0–4.0 nA at 2.5 Hz for 1–5 min). Microelectrodes were mounted in a 3D manipulator (MH3; Narishige) and positioned onto the ventral surface of medulla under visual control (binocular microscope; Carl Zeiss, Jena, Germany) using surface landmarks (trapezoid body and basilar artery) for orientation. A bipolar stimulating electrode was placed in the dorsolateral funiculus of spinal segments T2-T8 to allow antidromic activation of spinal axons. Standard criteria were used to verify the antidromic nature of evoked spikes (3), which requirements included: 1) invariant onset latency and; 2) the ability of spontaneous action potentials to cancel evoked spikes (i.e., the collision test).

Blind whole-cell patch-clamp recordings were performed from barosensitive CVLM neurons between 600-900 µm caudal to the caudal pole of the facial nucleus, 1.7-1.9 mm lateral to the midline and 50-700 µm dorsal to the ventral surface. The electrodes were filled with a solution containing the following (in mM): 130 K-gluconate, 4.5 MgCl2; 14 trisphosphocreatine, 10 HEPES; 5 EGTA; 4 Na-ATP; 0.3 Na-GTP; pH 7.3, 0.1% biocytin, ~300 mOsmol and had resistances of 5–8 MΩ when tested in bath solution. Current-clamp experiments were performed using an Axopatch-200B integrating amplifier (Molecular Devices, Sunnyvale, USA). Gigaseals (>1 GΩ) were formed and whole-cell configuration was obtained by suction. The intrinsic electrophysiological properties of CVLM neurons were evaluated in the presence of blockers of fast synaptic transmission in the perfusion solution [2.5–6.0 mM kynurenic acid, 20 μM bicuculline (free-base), and 1 μM strychnine; Sigma]. The input resistance was determined via linear regression applied to the linear portion of the voltage-current (V–I) relationship. Considering that CVLM neurons always presented spontaneous firing frequency after synaptic blockade, it was difficult to clearly determine a stable membrane potential that could unequivocally be defined as the resting potential. Therefore, we constructed an all-points histogram of 1 min of membrane potential value recorded in each experimental condition, and the resting membrane potential value was considered the value at which the cells spent most of the time, i.e., the peak of the distribution (1). A liquid junction potential of 15 mV was corrected off-line. The magnitude of respiratory modulation within PN-triggered histograms (bin width 10 ms) of RVLM pre-sympathetic and CVLM neurons was quantified as the percentage change in firing from the shortest to the largest bin during one complete cycle: percent modulation = [(largest bin count - shortest bin count)/shortest bin count] x 100.

All signals were amplified, band-pass filtered (0.05–5 kHz), and digitized (5–10 kHz; CED Micro1401; Cambridge Electronic Design, Cambridge, UK) to a computer using Spike2 software (version 5, Cambridge Electronic Design).
Barosensitivity of tSN, RVLM and CVLM neurons

To evaluate the pressure stimuli-evoked sympatho-inhibition, we calculated the tSN activity bounded by the points at which a pressure stimulus exceeded 75% of the difference between baseline and peak pressure. To evaluate the magnitude of the tSN barosensitivity, we compared tSN activity during the aortic depressor nerve (ADN) or pressure stimuli with those from 10 respiratory cycles immediately preceding the stimuli in order to compare the changes to an averaged series of cycles instead of just one. The ratio of the tSN activity between stimulation and the control condition was calculated as follows and used as an index of barosensitivity: percent of inhibition = [(baseline tSN activity - tSN activity during stimulus)/baseline tSN activity] x 100. We calculate the intensity required to produce 50% of inhibition of tSN to ADN stimulation by fitting the responses to different intensities (threshold, two-, three- and fourfold the threshold) using the following logistic equation (Prism4, Graphpad, La Jolla, CA; USA): Y=a1(a2−a1)/1+10(logx0−x)p, in which a1 is the minimal response; a2 is the maximum response; log(x0−x): correspond to 50% of inhibition and p is the slope.

ADN peri-stimulus time histograms of RVLM pre-sympathetic neurons were constructed in each of three phases of the respiratory cycle (bin width 10 ms). Control histograms were accumulated during the same phase, but without ADN stimulation. Control histograms were created from data acquired in the period immediately before ADN stimulation. The ratio of the activity between stimulation and control histograms was calculated as follows and used as an index of barosensitivity: percent inhibition = [(activity in control - activity in stimulation)/activity in control] x 100. To evaluate the barosensitivity of CVLM neurons, we compared their firing frequency responses to ADN stimulus with those from 10 respiratory cycles immediately preceding the stimulus in the same phase of respiratory cycle.

Histology

All preparations used for immunofluorescence were perfused first with PBS (0.1 M) and then with 4% paraformaldehyde (PFA) and brains removed and post-fixed in 4% PFA for 2–5 d. Transverse sections (50 μm thick) were cut through the medulla with a vibrating microtome (Leica, Wetzlar, Germany). The immunofluorescence was performed with free-floating sections. Sections were blocked and permeabilized in PBS containing 10% normal horse serum and 0.5% Triton X-100 for 1 h at room temperature. After three PBS washes, the slices were incubated in primary antibody mouse anti-tyrosine hydroxylase (TH; 1:1000; Millipore) for 24 h at 4°C. After three PBS washes, the sections were incubated in secondary antibodies: Alexa 488-conjugated streptavidin (1:500; Invitrogen, Grand Island, NY, USA) and Alexa 647-conjugated donkey anti-mouse (1:500; Jackson Laboratories, West Grove, PA, USA) for 1 h at room temperature. Slices were washed three times in PBS and mounted in Fluoromount (Sigma). Images were collected on a Leica TCS SP5 confocal microscope equipped with 488 and 633 nm laser lines and tunable emission wavelength detection.

Single-cell qRT-PCR

The cytoplasm of CVLM neuron was pulled into a patch pipette with a slight negative pressure. The material was then placed into a microtube containing High Capacity cDNA Reverse Transcription Kit reagents (Life Technologies, Carlsbad, CA, USA) and nuclease-free water for subsequent transcription in a thermocycler (The SimpliAmp, Life Technologies). A pre-amplification of the cDNA was performed using the TaqMan PreAmp Master Mix Kit (Life Technologies) with the following probes: Rn00690300_m1 (GAD67), Rn00584780_m1 (VGLUT2), Rn01475607_m1 (GLYT2) and NM_031144.2 (β-Actin; reference gene). The pre-
amplification protocol consisted of a hold temperature at 95°C during 10 min and 14 cycles of 95°C and 60°C during 15 s and 14 min, respectively. The reactions for the single-cell qRT-PCR were performed in singleplex and triplicate (StepOnePlus System, Life Technologies) using the same probes described above and the TaqMan Universal PCR Master Mix kit (Life Technologies) according to the manufacturer's recommendations.

SUPPLEMENTAL RESULTS

Respiratory modulation of pressure stimuli-evoked sympatho-inhibition in control and CIH rats

Transient increases in perfusion pressure (PP) were induced by increases in flow of perfusate. Delivering a transient pressure stimulus to activate ADN inputs at different phases of respiratory cycle showed that sympatho-inhibition in control and CIH rats was also respiratory-phase dependent. In control and CIH rats, the tSN activity was inhibited during pressure stimuli with greatest response occurring during post-inspiration (control: p<0.0001; CIH: p<0.0001) and E2 phase (control: p<0.0001; CIH: p<0.0001) than inspiration (Fig. S1, panels A-C). In the sequence, we also evaluated whether CIH affects the respiratory modulation of pressure stimuli-evoked sympatho-inhibition. Delivering a transient pressure stimulus showed that sympatho-inhibition was also enhanced in CIH rats during post-inspiration (89.3 ± 2.4 vs 76 ± 1.3 %; p<0.0001) and E2 phase (91 ± 1.9 vs 80 ± 2.3 %; p=0.0007), but not during inspiration (67 ± 2 vs 61 ± 3 %; n=37), in relation to control rats (Fig. S1, panels A-C). These data show that pressure stimuli mimic effects of ADN stimulation (Fig. 1) and are in agreement with our hypothesis that expiration enhances the sympatho-inhibitory component of baroreflex of rats submitted to CIH.

Location, spinal projection and phenotype of RVLM pre-sympathetic neurons

The RVLM pre-sympathetic neurons were recorded between 0-300 μm caudal to the caudal pole of the facial nucleus and 1.7-2.0 mm lateral to the midline. Typically, they were located 50-500 μm dorsal to the ventral surface. All RVLM pre-sympathetic neurons had spinal axons that send projections to T2-T8 (Fig. S2, panel A). The antidromic spikes exhibited invariant onset latency (Fig. S2, panel A). The average antidromic onset latency was not different between neurons from control (6 ± 1.2 ms) and CIH (5.8 ± 1.8 ms) rats. Neurons from control and CIH rats had axonal conduction velocities that averaged 2.8 ± 0.5 m.s⁻¹ and 2.5 ± 0.9 m.s⁻¹, respectively. Fig. S2, panel A, also shows that evoked spikes underwent time-controlled collision with spontaneous action potentials, thus confirming their antidromic nature. The same neurons were also considered to be barosensitive, since they were inhibited after stimulation of the ADN in different phases of respiratory cycle (Fig. S2, panel B). The adrenergic phenotype (C1 or non-C1) was determined for a subset of recorded neurons recovered after successful juxtacellular labeling. An example is shown in Fig. S2, panel C. Considering a pull of 42 recovered neurons, 7 from control (4 inspiratory-modulated and 3 non-modulated) and 8 from CIH rats (5 non-modulated and 3 inspiratory-modulated) were TH-ir and thus were designated C1 cells. The remaining 27 cells, 13 from control (8 post-inspiratory-modulated and 5 inspiratory-inhibited) and 14 from CIH rats (9 post-inspiratory-modulated and 5 inspiratory-inhibited), did not show detectable TH-ir and were classified as non-C1 cells. These data are in agreement with our previous study showing the phenotype distribution of respiratory-modulated RVLM pre-sympathetic neurons (1).
Non-modulated RVLM pre-sympathetic neurons present no respiratory modulation of their barosensitivity

Eleven RVLM pre-sympathetic neurons from control (n=6) and CIH (n=5) rats did not exhibit a clear respiratory-related discharge pattern in PN-triggered histograms (non-modulated; Fig. S3, panel A). This type of neuron exhibited no statistically significant respiratory-related modulation of its barosensitivity (Fig. S3, panel C). In addition, CIH did not affect their baseline firing frequency (13 ± 0.9 vs 12 ± 1.2 Hz; Fig. S3, panel A) or their barosensitivity [(inspiration: 40.9 ± 3.7 vs 47.9 ± 4.9%) (post-inspiration: 51.2 ± 5.7 vs 44.9 ± 5.4%) (E2 phase: 40.9 ± 4.2 vs 43.5 ± 4.2%), Fig. S3, panels B and C].

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Figure S1. Respiratory modulation of pressure stimuli-evoked sympatho-inhibition in control and CIH rats. (A) Thoracic sympathetic nerve (tSN) responses to transient increases in perfusion pressure (PP) during inspiration in control and CIH rats. (B) tSN responses to transient increases in PP during post-inspiration in control and CIH rats. (C) tSN responses to transient increases in PP during the second-half of expiration (E2 phase) in control and CIH rats. The pressure stimuli-evoked responses consisted of a decrease tSN as well as a prolongation of expiration when the stimulus was delivered during the post-inspiratory or E2 phase. Note the presence of late-expiratory activity (late-E; arrow) in tSN of CIH rats at the end of E2 phase. PN: Phrenic nerve.
Figure S2. Identification of RVLM pre-sympathetic neurons. (A) Representative RVLM pre-sympathetic neuron that was antidromically activated from the spinal cord. Asterisk indicates sweep when the antidromic spike was absent as the result of a collision with a spontaneous spike used to trigger the stimulus (stimulus artifact at arrow). (B) ADN stimulation (0.2 ms, 50 Hz, 2s; indicated by bar) inhibited the firing frequency of the same barosensitive RVLM pre-sympathetic neuron recorded in A. Duration of expiration was slightly increased during stimulation at post-inspiration (PI) and second-half of expiration (E2). (C) An inspiratory-modulated RVLM pre-sympathetic neuron labeled in situ with biocytin (Alexa 488, green) and tryrosine hydroxilase (TH) immunofluorescence revealed with Alexa 647 (red). Colocalization shown in yellow. Scale bars = 20 μm. I: inspiration. PN: phrenic nerve.
Figure S3. **Non-modulated barosensitive RVLM pre-sympathetic neurons from control and CIH rats.** (A) Phrenic nerve (PN)-triggered histogram of RVLM pre-sympathetic neurons from control and CIH rats, which did not exhibit clear respiratory modulation of their firing frequency. Note the absence of changes in their baseline firing frequency after CIH. (B) Average of PN-triggered histogram of all recorded non-modulated RVLM neurons from control and CIH rats before (black bars) and during ADN stimulation (red bars). Note their similar inhibitory responses to ADN stimulation during inspiration (I). (C) Average of the magnitude of inhibition of non-modulated RVLM pre-sympathetic neurons from control and CIH rats at post-inspiration (PI), I and second-half of expiration (E2). The magnitude of barosensitivity of non-modulated RVLM pre-sympathetic neurons was not statistically different throughout the respiratory cycle and CIH did not affect their barosensitivity.