Chronic intermittent hypoxia alters glutamatergic control of sympathetic and respiratory activities in the commissural NTS of rats

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Costa-Silva JH, Zoccal DB, Machado BH. Chronic intermittent hypoxia alters glutamatergic control of sympathetic and respiratory activities in the commissural NTS of rats. Am J Physiol Regul Integr Comp Physiol 302: R785–R793, 2012. First published December 28, 2011; doi:10.1152/ajpregu.00363.2011.—Sympathetic overactivity and altered respiratory control are commonly observed after chronic intermittent hypoxia (CIH) exposure. However, the central mechanisms underlying such neurovegetative dysfunctions remain unclear. Herein, we hypothesized that CIH (6% O₂ every 9 min, 8 h/day, 10 days) in juvenile rats alters glutamatergic transmission in the commissural nucleus tractus solitarius (cNTS), a pivotal site for integration of peripheral chemoreceptor inputs. Using an in situ working heart-brain stem preparation, we found that L-glutamate microinjections (1, 3, and 10 mM) into the cNTS of control rats (n = 8) evoked increases in thoracic sympathetic nerve (tSN) and central vagus nerve (cVN) activities combined with inhibition of phrenic nerve (PN) activity. Besides, the ionotropic glutamatergic receptor antagonism with kynurenic acid (KYN; 250 mM) in the cNTS of control group (n = 7) increased PN burst duration and frequency. In the CIH group (n = 10), the magnitude of L-glutamate-induced cVN excitation was smaller, and the PN inhibitory response was blunted (P < 0.05). In addition, KYN microinjections into the cNTS of CIH rats (n = 9) did not alter PN burst duration and produced smaller increases in its frequency compared with controls. Moreover, KYN microinjections into the cNTS attenuated the sympathoexcitatory response to peripheral chemoreflex activation in control but not in CIH rats (P < 0.05). These functional CIH-induced alterations were accompanied by a significant 10% increase of N-methyl-D-aspartate receptor 1 (NMDAR1) and glutamate receptor 2/3 (GluR2/3) receptor subunit density in the cNTS (n = 3–8, P < 0.05), evaluated by Western blot analysis. These data indicate that glutamatergic transmission is altered in the cNTS of CIH rats and may contribute to the sympathetic and respiratory changes observed in this experimental model.

nucleus tractus solitarius; glutamatergic neurotransmission; chemoreception

ACTIVATION OF PERIPHERAL CHEMORECEPTORS during acute hypoxia provides a powerful excitatory drive to respiratory and sympathetic networks resulting in coordinated respiratory and sympathetic reflex responses (17, 21, 39). Clinical and experimental studies reported that in conditions of chronic intermittent hypoxia (CIH), such as that observed in patients suffering from obstructive sleep apnea, the long-term and repetitive activation of peripheral chemoreceptors may evoke changes in the control of respiratory activity, excessive sympathetic outflow, and hypertension (24, 34, 40, 45, 56). Previously, we demonstrated that rats submitted to CIH exhibited reduction of central vagal postinspiratory activity and enhanced late-expiratory abdominal motor activity, indicating that the central control of expiratory activity is changed after CIH (60). The altered control of expiratory activity of CIH rats seems to contribute significantly to increase baseline sympathetic activity in these animals (58). This is supported by our findings obtained in in situ preparations showing that the higher levels of sympathetic activity of CIH-treated rats are, in part, entrained with the emergence of high-amplitude bursts in abdominal expiratory motor activity (43, 57, 60). Furthermore, it has been reported that CIH rats exhibited exaggerated sympathoexcitatory and phrenic responses to a new hypoxic challenge (14, 26, 38, 42), indicating a facilitation of the processing of hypoxic reflex responses. These findings suggest that CIH introduces plastic changes in the neurochemical mechanisms involved in the chemosensory control of respiratory and sympathetic activities.

The first synapses of peripheral chemoreceptors in the central nervous system occur at the nucleus tractus solitarius (NTS) (36, 41). This nucleus contains a complex neuronal circuitry responsible for integration and transmission of chemosensory information to other brain nuclei that control efferent respiratory and sympathetic reflex responses (18, 46). In addition, the NTS is involved in the regulation of baseline sympathetic and respiratory activities, as it receives the afferent information from arterial baroreceptors (7, 8) and pulmonary stretch receptors (10), and it hosts the respiratory neurons of dorsal respiratory column (19, 54). The majority of the peripheral chemoreceptor synapses are established in commissural NTS (cNTS) (41), in which L-glutamate plays an important role in the processing of peripheral chemoreflex responses (13, 17, 28). Moreover, the glutamatergic transmission within the NTS is required for the processing of baroreflexes (37) and Hering-Breuer (9) reflex responses, and microinjections of agonists (6) or antagonists (11, 17) of its receptors evoke dramatic changes in the respiratory pattern and its coupling with sympathetic activity.

Electrophysiological studies have reported an enhanced postsynaptic activity of NTS neurons from CIH rats due to an increased frequency of miniature excitatory postsynaptic currents (32). In addition, exogenous microinjections of AMPA evoked higher-amplitude currents in isolated NTS neurons from CIH rats (20), suggesting that glutamatergic signaling is altered in the NTS of rats submitted to CIH. However, these previous studies were performed in vitro, and there is no research on the implication of these glutamatergic changes in the NTS on the control of respiratory and sympathetic activities of CIH rats. Therefore, the goals of the present study were (1) to compare the sympathetic and respiratory responses elicited by microinjections of L-glutamate or its antagonist kynurenic acid into the cNTS of control and CIH-conditioned rats; and (2) to correlate response differences with alterations in the density of glutamatergic receptor subunits.

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MATERIALS AND METHODS

Animals. All experiments were performed on weaned juvenile male Wistar rats (P19–P21) obtained from the animal care facility of the University of São Paulo, Ribeirão Preto, Brazil. The animals were maintained under standard environmental conditions (23 ± 2°C; 12:12-h dark-light cycle) with water and chow available ad libitum. Experimental protocols were approved by the Ethical Committee on Animal Experimentation of the School of Medicine of Ribeirão Preto, University of São Paulo (protocols 019/2006 and 080/2007).

Chronic intermittent hypoxia. Chronic intermittent hypoxia (CIH) (n = 43) and control (n = 39) groups of rats were maintained inside Plexiglas chambers (volume 210 liters) equipped with gas injectors and O2, CO2, humidity, and temperature sensors. The CIH group was exposed to a protocol of 5 min of normoxia (fraction of inspired O2, FI02, of 20.8%) followed by 4 min of N2 injection to reduce FI02 from 20.8 to 6%. After 30–40 s of hypoxia, O2 was injected to return FI02 back to 20.8%. This 9-min cycle was repeated 8 h a day from (9:30 AM to 3:30 PM) for 10 days. During the remaining 16 h, thermal chambers were maintained at FI02 of 20.8%. N2 and O2 injections (White Martins, Sertãozinho, Brazil) were regulated by a solenoid valve system whose opening-closing control was operated by a computerized system (Oxycycler, Biospherrix, New York, NY). In the same room, in a side-by-side identical chamber, control rats were exposed to a FI02 of 20.8% 24 h a day for 10 days. The control rats were also exposed to a similar valve noise due to the frequent injection of O2 to maintain 20.8% FI02. To avoid stress due to direct jets of gas that could impact directly on the animals, gas injections were delivered through the upper part of the chambers.

In situ arterially perfused preparation. Working heart-brain stem preparations (45) from control (n = 15) and CIH rats (n = 19) were surgically prepared on the 11th day of the experimental protocol, as previously described (17, 47, 60). Briefly, rats were deeply anesthetized with halothane (AstraZeneca do Brazil, Cotia, SP, Brazil) until the loss of the paw withdrawal reflex, transected caudal to the diaphragm, and decerebrated at the precollicular level. This procedure rendered a system whose opening-closing control was operated by a computerized system (Oxycycler, Biospherrix, New York, NY). In the same room, in a side-by-side identical chamber, control rats were exposed to a FI02 of 20.8% 24 h a day for 10 days. The control rats were also exposed to a similar valve noise due to the frequent injection of O2 to maintain 20.8% FI02. To avoid stress due to direct jets of gas that could impact directly on the animals, gas injections were delivered through the upper part of the chambers.

Microinjections in the NTS. In separate groups of control and CIH animals, l-glutamate (25, 50, and 250 mM) and the antagonist of ionotropic glutamatergic receptors, kynurenic acid (KYN; 250 mM) were microinjected into the cNTS. The concentration of KYN 250 mM was considered the effective concentration to attenuate the sympathetic and respiratory responses elicited by microinjection of l-glutamate (EC50) into the cNTS of in situ preparations (10). The cNTS was approached using the following stereotaxic coordinates, in accordance with the atlas of Paxinos and Watson (49): 0.3 mm caudal to the atlas of Ronald (2000; 0.2 mm caudal to the atlas of Ronald); 0.3–0.4 mm ventral to the dorsal surface. Drugs (20–30 nl) were applied via three-barrel glass micropipettes coupled to a pico-pump system (Picospritzer II; Parker Instruments, Cleveland, OH). Microinjections of different concentrations of l-glutamate were performed unilaterally in a random sequence and with a time interval of 10–15 min between consecutive microinjections. KYN microinjections were performed bilaterally, and its effects on respiratory and sympathetic activities were monitored for 60 min.

Activation of peripheral chemoreceptors. Peripheral chemoreceptors were activated by intra-arterial injections of potassium cyanide (KCN 0.05%, 50 μl), as previously described (15), and respiratory and autonomic reflex responses were evaluated. This procedure was performed before and 2, 10, 30, 45, and 60 min after bilateral microinjections of KYN into the cNTS of control and CIH animals.

Histology. At the end of experiments the brain stem was rapidly removed and fixed by immersion for 5 days in 10% buffered formalin. Serial transverse sections of 18 μm were cut using a cryostat and stained with cresyl violet using the Nissl method. Histological site of microinjections in the cNTS were confirmed in the coronal brain stem sections of each rat preparation by optical microscopy, as illustrated in Fig. 1.

Western blot analysis. A large proportion of the cardiovascular and respiratory afferent inputs in the NTS establish their first central synapses on neurons expressing N-methyl-D-aspartate receptor 1 (NMDAR1) receptors (5). In addition, GluR 2/3 subunits were found in both cell body and processes of NTS neurons, with a pattern of distribution similar to NMDAR1 receptors (4). Thereby, in the present study, we evaluated the densities of NMDAR1 and GluR2/3 subunit receptors using Western blot analysis. For this, separate groups of CIH (n = 24) and control animals (n = 24) were deeply anesthetized with halothane; animals were decapitated, and the brain was rapidly removed. The brain stem was frozen in dry ice; using a cryostat, slices of 600-μm thickness were prepared to obtain 1-mm diameter bilateral punches of the cNTS (47). Punches from three animals were pooled; chopped on ice using a pestle with 1 ml of lysis buffer (50 mM NaCl, 2 mM MgCl2, 1.25 mM KH2PO4, 0.1 mM EGTA, 1% Triton; adjusted to pH 7.4) plus 52 mg/ml phenylmethylsulfonyl fluoride. The lysates of microinjections in the cNTS were confirmed in the coronal brain stem sections of each rat preparation by optical microscopy, as illustrated in Fig. 1.

Nerve recordings and data analyses. Sympathetic and respiratory nerves were isolated, and their activity was recorded using bipolar glass suction electrodes held in micromanipulators (Narishige, Tokyo, Japan). Left phrenic nerve (PN) discharges were recorded from its central end, and its rhythmic ramping activity was used as a continuous physiological index of respiration viability. The left cervical vagus nerve (cVN) was cut distally, and its central activity was recorded. The effluent activity of the left thoracic sympathetic nerve (tSN) was recorded from the sympathetic chain at T8–T12 level. All of the signals were amplified, band-pass filtered (0.05–5 kHz), and acquired in an A/D converter (CED micro 1401; Cambridge Electronic Design (CED), Cambridge, UK) to a computer using Spike 2 software (5 kHz; CED). The frequency of phrenic discharges was calculated from the time interval between consecutive integrated phrenic peak bursts, while the phrenic burst length (namely inspiratory time, T1) was determined as the time from the beginning to the peak of phrenic burst. The magnitude of changes in phrenic burst frequency elicited by l-glutamate microinjections (see below) was determined as the mean frequency during the 10-s epoch after microinjections. Thoracic sympathetic and central vagus activities were assessed by the area under the curve. The evoked alterations in all nerve activities were determined as the percentage of change in relation to the respective baseline activity prior to the stimulus. All of the analyses were carried out on rectified and integrated signals (time constant of 50 ms) and performed off-line using Spike 2 software with custom-written scripts (CED).
with rabbit anti-NMDAR1 (1:2,000), rabbit anti-GluR2/3 (1:1,000) or mouse anti-\(\alpha\)-tubulin (1:15,000) antibodies. The immunoreactive bands were visualized by incubation with the horseradish peroxidase-conjugated with anti-rabbit (1:5,000) or anti-mouse IgG (1:5,000), which were detected using an enhanced chemiluminescence (Super Signal, Thermo Scientific) and a radiographic film (Hyperfilm ECL, Amersham Biosciences). Immunoreactive bands were quantified by densitometry using the NIH Image J 1.40 software (National Institutes of Health, Bethesda, MD; http://rsbweb.nih.gov/ij/). Relative amounts of NMDAR1 and GluR2/3 detected were normalized with respect to \(\alpha\)-tubulin and expressed in arbitrary units.

**Statistical analyses.** The results were expressed as means ± SE and compared using Student’s unpaired and paired t-test or two-way ANOVA followed by Bonferroni post hoc test, depending on the experimental design. With respect to two-way ANOVA, this analysis was used to compare the magnitude of phrenic inhibitory responses to \(\text{L-glutamate} \) microinjections into the cNTS of control and the CIH group. The factors considered were time and CIH treatment: the former was used to evaluate the alterations in phrenic frequency along the timeline (irrespective to experimental group), and the latter was used to analyze the differences in phrenic responses between control and the CIH groups. The comparisons were carried out on GraphPad Prism software (GraphPad Software, ver. 4), and differences were considered significant at \(P < 0.05\).

**Drugs and providers.** \(\text{L-glutamate}, \text{kynurenic acid, and all salts used in Western blot analysis were purchased from Sigma Chemical (St. Louis, MO). Potassium cyanide (KCN) was purchased from Merck (Darmstadt, Germany). The drugs were diluted in NaCl 0.9% sterile solution (Samtec Biotechnology, Ribeirão Preto, Brazil), and their pHs were adjusted to 7.4 using sodium bicarbonate (Reagen, Rio de Janeiro, Brazil). Antibodies were obtained from Calbiochem (anti-NMDAR1; La Jolla, CA), Chemicon International (anti-GluR2/3; Temecula, CA) and Sigma (anti-\(\alpha\)-tubulin).**

**RESULTS**

Sympathetic and respiratory responses to microinjections of \(\text{L-glutamate} \) into the cNTS of CIH rats. Figure 2 shows the pattern of respiratory and sympathetic responses elicited by unilateral microinjections of different concentrations of \(\text{L-glutamate} \) into the cNTS of control and CIH rats. In the control group (\(n = 8\)), microinjections of \(\text{L-glutamate} \) into the cNTS produced a significant and concentration-dependent increase in the tSN (16 ± 6% at 25 mM; 25 ± 5% at 50 mM; and 27 ± 2% at 250 mM; time courses illustrated in Fig. 2B1–B3). These respiratory alterations elicited by \(\text{L-glutamate} \) microinjected into the cNTS were associated with significant increase in the tSN.
mM; Fig. 2D). In rats from the CIH group (n = 10), the increase in postinspiratory vagal activity in response to microinjections of L-glutamate in the cNTS was smaller (ΔcVN: 54 ± 17% at 25 mM; 85 ± 13% at 50 mM and 101 ± 11% at 250 mM; P < 0.05, Fig. 2C) and the inhibition of PN burst was blunted (ΔPN(mean of 0–10 s): 0.13 ± 0.07 Hz at 25 mM; 0.07 ± 0.07 Hz at 50 mM; and 0.03 ± 0.07 Hz at 250 mM; P < 0.05, time courses illustrated in Fig. 2B1–B3) compared with control group responses. Conversely, the sympathoexcitatory response to L-glutamate 250 mM (53 ± 9%), but not 25 (26 ± 6%) and 50 mM (44 ± 10%), was significantly higher in CIH than in control group (Fig. 2D, P < 0.05).

Effects of ionotropic glutamatergic receptor antagonism in the cNTS on baseline respiratory and sympathetic activities of CIH rats. The antagonism of ionotropic glutamatergic receptors in the cNTS of control and the CIH groups alter baseline PN but not tSN activity, as illustrated in Fig. 3A. As previously described (15), microinjections of KYN into the cNTS of control rats (n = 7) produced a large increase in frequency (0.34 ± 0.03 vs. 0.80 ± 0.06 Hz, P < 0.05, Fig. 3B) and time (0.67 ± 0.06 vs. 0.90 ± 0.12 s, P < 0.05, Fig. 3C) of PN bursts. In CIH animals (n = 9), the same concentration of KYN microinjected into the cNTS increased the frequency (0.29 ± 0.03 vs. 0.59 ± 0.05 Hz, P < 0.05, Fig. 3B) but not the time (0.63 ± 0.05 vs. 0.68 ± 0.05 s, Fig. 3C) of PN bursts. After KYN, the PN frequency was higher in control than in the CIH group (0.80 ± 0.06 vs. 0.59 ± 0.05 Hz, P < 0.05). Sixty minutes after microinjections, the time and frequency of PN bursts returned to control values (data not shown). With respect to baseline sympathetic activity, KYN microinjections into the cNTS produced negligible changes in the tSN mean values of control (ΔtSN: 1 ± 5%) and CIH rats (ΔtSN: −1 ± 3%).
ROLE OF GLUTAMATE IN NTS OF CIH RATS

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Sympathetic and respiratory responses to peripheral chemoreflex activation after ionotropic glutamatergic receptor antagonism in the cNTS of CIH rats. Activation of peripheral chemoreceptors of control rats (n = 6) by intra-arterial administration of KCN produced robust and coupled increases of tSN and PN frequency, as demonstrated in Fig. 4A. In the CIH group (n = 8), similar patterns of respiratory and sympathetic responses were noticed (Fig. 4A). Although the magnitude of KCN-induced increase in PN frequency was similar between CIH and control groups (ΔPN: 0.53 ± 0.06 vs. 0.49 ± 0.06 Hz, Fig. 4B), the sympathoexcitatory response was significantly higher in CIH than in control rats (120 ± 8 vs. 90 ± 11%, P < 0.05, Fig. 4C). In both groups, the antagonism of glutamatergic receptors in the cNTS did not affect the magnitude of increase in PN frequency (control: 0.49 ± 0.06 vs. 0.41 ± 0.05 Hz; CIH: 0.53 ± 0.06 vs. 0.47 ± 0.04 Hz before and after KYN, respectively; Fig. 4B). On the other hand, the same concentration of KYN microinjected into the cNTS produced a significant attenuation of sympathetic reflex response to peripheral chemoreceptor activation in control (90 ± 11 vs. 56 ± 6%, P < 0.05, Fig. 4C) but not in the CIH group (120 ± 9 vs. 106 ± 10%, Fig. 4C). Therefore, the magnitude of sympathetic chemoreflex response of CIH rats after KYN microinjections into the cNTS was greater than in control rats (Fig. 4C).

CIH exposure increased NMDAR1 and GluR2/3 immunoreactivity in the cNTS. Clear 110-kDa bands corresponding to glutamatergic receptor subunits are observed in cNTS samples from control and CIH rats. Representative blots were illustrated in Fig. 5. We observed that the intensities of NMDAR1 (1.11 ± 0.01 vs. 1.21 ± 0.02, P < 0.05, n = 3 each group) and GluR2/3 bands (0.99 ± 0.02 vs. 1.08 ± 0.03, P < 0.05, n = 7 each group) were significantly higher in CIH than in the control group (Fig. 5), indicating that the densities of these glutamatergic receptor subunits were increased in cNTS after CIH exposure.

DISCUSSION

Although accumulating clinical and experimental evidence supports the notion that exposure to CIH is an important risk factor for the development of sympathetic overactivity and hypertension (15, 25, 34, 59), the underlying mechanisms remain to be fully elucidated. There is evidence of plastic changes in peripheral (50) and central (16, 27, 32, 35, 60) components of chemosensory control of sympathetic and respiratory activities. The latter includes changes in the interaction between sympathetic and respiratory neuronal populations (1, 59) and alterations in neurochemical mechanisms in important brain stem areas (30, 31). Previous in vitro studies reported that glutamatergic signaling is altered in the NTS of rats submitted to CIH (20, 32). In the present study, we confirmed that CIH exposure alters NTS glutamatergic transmission, especially in its caudal aspect—a region that receives the

Fig. 4. Sympathoexcitatory response to chemoreflex activation after the antagonism of ionotropic glutamatergic receptors in the cNTS of control and CIH rats. A: response of raw and iSN and PN activities to peripheral chemoreceptor activation (bolus injection of KCN at arrows) before (baseline) and 2 min after KYN microinjections into the cNTS of control and CIH rats. Average values of magnitude of increase in PN frequency (ΔPN, B) and tSN (ΔtSN, C) in response to peripheral chemoreflex activation before (baseline) and 2 min after KYN in the cNTS of control (n = 7) and CIH rats (n = 9). *Significantly different from baseline, P < 0.05. #Significantly different from baseline control group response, P < 0.05.

Fig. 5. Immunoreactivity of ionotropic glutamatergic receptor subunits in the cNTS of CIH rats. Representative immunoreactive bands of NMDAR1 and GluR2/3 (GluR2/3) subunits in the cNTS of control and CIH rats (n = 3–8 each group) and their relation with α-tubulin band (bar graphs). *Significantly different from control group, P < 0.05.
majority of carotid-body peripheral chemoreceptor inputs (41). Moreover, in the present study, we also demonstrated that these changes in glutamatergic transmission in the cNTS of CIH rats may impose alterations in the normal functioning of respiratory and sympathetic networks. Thereby, our findings may represent a novel central mechanism contributing to sympathetic and respiratory dysfunctions observed after CIH exposure.

Several studies have pointed out that the glutamatergic neurotransmission within the NTS is essential for reflex control of respiratory and sympathetic activities since L-glutamate is required not only for the processing of afferent information from pulmonary stretch receptors (9), baroreflex receptors (37), and chemoreceptors (13) but also for the generation of eupneic respiratory pattern (15). Consonant with previous studies (6, 12), we observed that microinjections of L-glutamate into the cNTS of control rats elicited responses of increase in sympathetic activity and inhibition of phrenic inspiratory activity. According to Braga et al. (12), both respiratory and sympathetic responses to L-glutamate were attenuated after the antagonism of ionotropic glutamatergic receptors in the cNTS, indicating a critical role for these receptor subtypes in the mediation of respiratory and sympathetic responses to L-glutamate. The transient sympathoexcitatory response to microinjections of L-glutamate into the cNTS possibly involves the activation of direct pathways from cNTS neurons to presympathetic neurons of rostral ventrolateral medulla (RVLM), which are suggested to be involved in the processing of sympathetic response to peripheral chemoreflex activation (2, 3, 13). However, other indirect pathways, such as those involving the pons (29), should also be considered.

With respect to respiratory response, our results demonstrated that the response of phrenic burst inhibition to L-glutamate microinjections into the cNTS was associated with a robust increase in central vagal activity. The effenter activity of vagus during expiratory phase reflects the activity of postinhibitory (post-I) Bötzingner neurons (48, 53), which provide inhibitory inputs to inspiratory neurons of pre-Bötzingner complex and rostral ventral respiratory group, controlling inspiratory activity (22, 23, 44). In light of this, we suggest that microinjections of L-glutamate into the cNTS evoke a post-I neuronal activation that resulted in a long-lasting inhibition of inspiratory neuronal activity and, consequently, an apneic response. This hypothesis is in agreement with our previous studies showing that the antagonism of ionotropic glutamatergic receptors in the cNTS produced a significant reduction of baseline post-I vagal activity and increased phrenic burst length (17). Therefore, we suggest that L-glutamate at the level of cNTS plays a critical role in the control of post-I activity and inspiratory off-switch in in situ preparations. The neuronal sources underlying the ionotropic glutamatergic control of respiratory activity at the level of the cNTS require further studies to be fully elucidated, but it may involve the respiratory neurons of dorsal respiratory column (17) and connections with Bötzingner complex (BötC) (19, 55) and dorsolateral pons (33, 51, 53).

In CIH rats, both respiratory and sympathetic responses to microinjections of L-glutamate into the cNTS were altered. With respect to the former response, we observed that L-glutamate in the cNTS of CIH rats did not produce an inhibition of phrenic bursts as observed in controls. This reduced inhibitory responses of inspiratory activity of CIH rats were accompanied by attenuated responses of post-I vagal activation. These data indicate that the post-I neuronal activation in response to microinjections of L-glutamate in the cNTS is reduced in CIH rats, resulting in smaller depression of inspiratory neuronal activity and consequently of phrenic bursts. This concept seems to be in opposition to our Western blot analysis results showing that the density of NMDA and AMPA receptor subunits is enhanced in the cNTS of CIH rats. However, previous studies from our laboratory reported that baseline post-I vagal activity is reduced in CIH rats, suggesting that resting BötC post-I neuronal activity is depressed (60). This decreased activity of BötC post-I neurons may involve an increased inhibitory activity from BötC augmenting-expiratory neurons (60), which are reciprocally connected with post-I neurons by inhibitory pathways (23). In this scenario, we hypothesized that even with an increased density of glutamate receptors in the cNTS of CIH rats, the L-glutamate-induced excitatory inputs from cNTS to BötC resulted in a minor excitation of post-I activity because resting activity of BötC neurons was depressed. As a consequence, the magnitude of glutamate-induced apneic response in CIH rats was smaller compared with controls. However, additional experiments are required to test this hypothesis. Alternatively, it is possible that the depressed vagal and apneic responses of CIH rats may be related to a reduced glutamatergic signaling, specifically on respiratory neurons of cNTS recruited in these responses—a possibility that we cannot rule out in the present study and demands electrophysiological experiments to be elucidated.

In relation to sympathetic response, we observed that the sympathoexcitatory response induced by the highest concentration of L-glutamate microinjected were significantly greater in CIH rats, while the other L-glutamate concentrations presented a trend of larger amplitude. These data indicate that the sympathoexcitatory pathways from cNTS to presympathetic neurons of RVLM are facilitated in CIH rats. Considering our Western blot data, indicating an enhanced density of NMDA and AMPA receptors in the cNTS of CIH rats, we suggest that an augmented glutamatergic signaling in the cNTS contributes to this CIH-induced enhanced sympathetic response to L-glutamate microinjections in this region. At this step, we cannot exclude the involvement of altered neurotransmission in other important brain stem regions involved in the processing of this sympathoexcitatory response (52).

In addition to the experiments with agonist, we verified that the respiratory and sympathetic alterations induced by the antagonism of ionotropic glutamatergic receptors in the cNTS were attenuated in CIH animals. In agreement with our previous studies, microinjections of KYN into the cNTS of control rats elicited an increase in frequency and time of phrenic bursts—an effect associated with a KYN-induced depression of post-I activity (17). In CIH rats, the same concentration of KYN produced a small increase in respiratory frequency and no changes in duration of phrenic bursts. These attenuated effects of KYN in the cNTS of CIH rats may be related to a higher density of ionotropic glutamatergic receptors (as reported here) or to an enhanced spontaneous presynaptic L-glutamate release (32). In this context, we hypothesized that tonic glutamatergic neurotransmission involved in the control of inspiratory off-switch may be enhanced in the cNTS of CIH rats. However, we cannot exclude the possibility that the decreased effects of KYN in CIH rats might be associated with...
reduced release of endogenous L-glutamate at the cNTS level. These hypotheses require future experiments to be fully elucidated.

In the present study, we also verified that microinjections of KYN into the cNTS of in situ preparations of control and CIH rats produced no changes in baseline sympathetic activity. These findings contrast with those obtained in studies involving anesthetized and unanesthetized animals showing that the antagonism of ionotropic glutamatergic receptors in the NTS increases baseline sympathetic activity and arterial pressure, an effect associated with the antagonism of the neurotransmission on NTS neurons involved with the processing of sympatho-inhibitory component of baroreflex (8, 37, 46). Our data showing the lack of changes in baseline sympathetic activity after KYN in the NTS are probably related to the fact that the baroreceptors are unloaded in the in situ preparations due to the low perfusion pressure (50–70 mmHg). Therefore, the baroreflex sympathoinhibitory control of baseline TSN is reduced or even absent in this experimental condition, and the antagonism of glutamate receptors in the NTS produces no major changes in the baseline sympathetic activity.

In addition to changes in baseline respiratory activity, another important aspect related to experiments with antagonist relays on the sympathoexcitatory response to peripheral chemoreceptor stimulation. We observed that microinjections of KYN into the cNTS of control rats produced an attenuation of sympathetic reflex response, while in CIH rats, only a minor effect was observed. As far as the peripheral chemoreceptors are sensitized in CIH rats (50), an increased excitation from peripheral chemoreceptor may account for the reduced attenuation of chemoreflex-induced sympathetic response after KYN microinjections in the cNTS of CIH rats. However, our findings that are related to the increased density of ionotropic glutamate receptor and augmented L-glutamate-induced sympathetic response in the cNTS of CIH rats suggest that the enhanced glutamatergic transmission in the cNTS of CIH rats contribute to the augmented sympathoexcitatory response to peripheral chemoreceptor activation. In addition, the attenuation of chemoreflex sympathoexcitatory response after KYN microinjections into the cNTS could be also related to the attenuation of post-I activity and the consequent changes in the coupling between chemoreflex-induced respiratory and sympathetic responses (17). We suggest that the observed minor changes in sympathetic reflex response of CIH rats after KYN into the cNTS are associated with its attenuated effect on post-I activity. Therefore, these hypotheses pointed out that an enhanced glutamatergic transmission at the cNTS of CIH rats may contribute to the augmented sympathoexcitatory response to peripheral chemoreceptor stimulation. However, we cannot rule out that other neurotransmitter systems that are critical for the processing of the chemoreflex-induced sympathetic response, such as ATP (13), may also be altered and contribute to this phenomenon. All of these possibilities require further investigations.

Perspectives and Significance

Accumulating evidence suggests that the central mechanisms underpinning sympathetic overactivity associated with CIH exposure involve plastic changes in the neuronal circuits modulating both sympathetic and respiratory functions (43, 57, 60). The present study provides neurochemical evidence that glutamatergic neurotransmission is increased in the cNTS of rats submitted to CIH. Although we cannot point out the specific subpopulations of NTS neurons, in which glutamatergic transmission is altered, our data clearly demonstrated that these changes at the cNTS affect the normal control of respiratory and sympathetic activities. We hypothesize that these glutamatergic changes in the cNTS of CIH rats may be part of a complex adaptive process that take place during hypoxia to compensate the depressed BôC post-I neuronal activity (60) and maintain baseline control of inspiratory off-switch. Additionally, the enhanced cNTS glutamatergic transmission of CIH rats appears to significantly contribute to the exaggerated processing to chemoreflex-induced sympathoexcitatory response—a mechanism that may play a critical role in the development of hypertension in rats submitted to CIH or in patients facing the complex pathophysiological condition of obstructive sleep apnea (15, 45). It is also important to note that this impact of respiratory changes on the sympathetic overactivity due to neurochemical dysfunction may be considered the negative side of this complex network interaction. On the other hand, we should consider the potential positive side of understanding this complex neuronal mechanism, since it may imply that appropriated voluntary control of the respiratory function may contribute to lowering the sympathetic overactivity in hypertensive patients.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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