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Carotid Body Function and Ventilatory Responses in Intermittent Hypoxia. Evidence for Anomalous Brainstem Integration of Arterial Chemoreceptor Input

M.C. GONZALEZ-MARTÍN,[1] M.V. VEGA-AGAPITO,[1] S.V. CONDE,[1,2] J. CASTAÑEDA,[1] R. BUSTAMANTE,[1] E. OLEA,[1] F. PEREZ-VIZCAINO,[3] C. GONZALEZ,[1]* AND A. OBESO[1]

[1] *Departamento de Bioquímica y Biología Molecular y Fisiología e Instituto de Biología y Genética Molecular, Facultad de Medicina, Universidad de Valladolid/CSIC, Valladolid, Spain*

[2] *CEDOC Departamento de Farmacología, Facudade de Ciencias Meedicas, Universidade Nova de Lisboa, Lisboa, Portugal*

[3] *Departamento de Farmacología, Facultad de Medicina, Universidad Complutense de Madrid, Madrid, Spain*

Obstructive sleep apnea is a frequent medical condition consisting in repetitive sleep-related episodes of upper airways obstruction and concurrent events of arterial blood hypoxia. There is a frequent association of cardiovascular diseases and other pathologies to this condition conforming the obstructive sleep apnea syndrome (OSAS). Laboratory models of OSAS consist in animals exposed to repetitive episodes of intermittent hypoxia (IH) which also develop cardiovascular pathologies, mostly hypertension. The overall OSAS pathophysiology appears to be linked to the repetitive hypoxia, which would cause a sensitization of carotid body (CB) chemoreflex and chemoreflex-driven hyperreactivity of the sympathetic nervous system. However, this proposal is uncertain because hyperventilation, reflecting the CB sensitization, and increased plasma CA levels, reflecting sympathetic hyperreactivity, are not constant findings in patients with OSAS and IH animals. Aiming to solve these uncertainties we have studied the entire CB chemoreflex arch in a rat model of IH, including activity of chemoreceptor cells and CB generated afferent activity to brainstem. The efferent activity was measured as ventilation in normoxia, hypoxia, and hypercapnia. Norepinephrine turnover in renal artery sympathetic endings was also assessed. Findings indicate a sensitization of the CB function to hypoxia evidenced by exaggerated chemoreceptor cell and CB afferent activity. Yet, IH rats exhibited marked hypoventilation in all studied conditions and increased turnover of norepinephrine in sympathetic endings. We conclude that IH produces a bias in the integration of the input arising from the CB with a diminished drive of ventilation and an exaggerated activation of brainstem sympathetic neurons.

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The carotid bodies (CB) are small arterial chemoreceptor organs formed by clusters of cells surrounded by a dense net of capillaries that facilitates the presentation of the blood-borne stimuli to chemoreceptor cells. Chemoreceptor cells are synaptically connected with the sensory nerve endings of the carotid sinus nerve (CSN), whose central projections terminate in the brainstem (Gonzalez et al., 1994). Current models of CB functioning consider that chemoreceptor express O_2-sensor(s) which are coupled to certain K^+ channels in such a manner that a decrease in PO_2 leads to inhibition of K^+ channels, cell depolarization, activation of voltage dependent Ca^{2+} channels, and augmentation of the release of neurotransmitters. These drive the chemoreceptor cell-nerve ending synapses to an increase in the action potential frequency in the CSN (see Gonzalez et al., 1992, 2009; Peers, 1997; Kemp, 2005). The end result of hypoxic (low PO_2) stimulation is the triggering of cardio respiratory reflexes, most significantly hyperventilation, aimed to normalize arterial blood gases. This fact gives a critical homeostatic significance to CB function (Gonzalez et al., 1994). It should be mentioned that CBs are responsible for the entire ventilatory response produced by hypoxic hypoxia, the only type of hypoxia occurring physiologically as in journeying or living at high altitude (Forster et al., 1976; Bisgard et al., 1976).

The CBs and CB-initiated reflexes play an adaptive function in response to chronic sustained hypoxia (Weil, 1986; Bisgard, 2000). On ascension to high altitude the CBs trigger a ventilatory response whose relationship to arterial PO_2 is comparable to that encountered at sea level in response to acute hypoxic tests. However, if the stay at high altitude is prolonged, a further increase in ventilation occurs. This extra gain of the chemoreflex, known as acclimatization, helps to minimize the impact of the environmental challenge by raising

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*Correspondence to: C. Gonzalez, Departamento de Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, Universidad de Valladolid, 47005 Valladolid, Spain.
E-mail: constanc@ibgm.uva.es

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arterial PO_2 and might constitute a compensatory mechanism in situations of hypoxemia produced by chronic respiratory diseases. Most of the extra gain of the CB chemoreflex seen in chronic hypoxia has its origin in the CB itself, although plastic changes in brain-stem integrative centers have also been described (Bisgard, 2000; Wilkinson et al., 2010).

Another type of chronic hypoxia that has a great clinical significance is the intermittent hypoxia (IH) encountered in patients with obstructive sleep apnea (OSA). These patients suffer repetitive episodes of obstruction of upper airways that occur during sleep (>30 episodes/sleep hour in some patients). Each obstruction produces a fall in arterial PO_2 that activates the CB generating a progressively increasing drive to brainstem respiratory nuclei that finally solves the obstruction and blood gases return to normality. Frequently, patients suffering OSA show a number of associated pathologies, mostly cardiovascular (hypertension, augmented acute vascular events) and neuropsychiatric (anxiety, depression, and cognitive impairment) (Sateia, 2003; Schröder and O'Hara, 2005), conforming what is known as obstructive sleep apnea syndrome (OSAS). It has been proposed that the CB chemoreflex plays a key pathogenic mechanism in the cardiovascular pathology in OSAS: repeated stimulation during the apneic episodes would cause a sensitization of the CB chemoreflex that would ultimately produce a sustained sympathetic drive and increase in circulating catecholamine (CA; Bao et al., 1997; Kumar et al., 2006).

Although this pathogenic schema has been proposed on the basis of studies in animal models of IH, there are several aspects of it that remain unsettled. For example, the ventilatory response to acute hypoxic tests that measures the gain of the CB chemoreflex has yielded conflicting results, both in OSAS patients and in IH animal models. Thus, several authors have found diminished hypoxic ventilatory responses in humans and several models of IH (Kimoff et al., 1997; Osanai et al., 1999; Waters and Tinworth, 2001; Reeves et al., 2006; O'Halloran et al., 2007). Other authors have found no change (Costes et al., 1995; Greenberg et al., 1999; Zoccal et al., 2008; Edge et al., 2009). While others have found that IH does indeed cause increased hypoxic ventilatory responses (Ling et al., 2001; Peng et al., 2006; del Rio et al., 2010). Similarly, measurements of adrenal medulla CA have yielded opposite results in the two available studies (Hui et al., 2003; Kumar et al., 2006) and measurements of circulating CA have yielded inconclusive results (Fletcher et al., 1992; Lesske et al., 1997; García-Río et al., 2000). In the present work we have studied the entire chemoreflex arch in rats subjected to IH. Specifically we have measured levels of catecholamines (CA) in CB chemoreceptor cells and their rate of synthesis and release with findings indicating a sensitization of chemoreceptor cells to hypoxia. We have measured electrical activity in the CSN to directly assess the final CB output to the brainstem with findings similarly indicating a hypoxic sensitization of the overall arterial chemoreception process. Ventilatory response to hypoxia was significantly attenuated indicating that IH causes central plastic reorganization of CB chemoreceptor input leading to a hyposensitivity of the ventilatory reflex. A facilitation of the vasopressor component of the chemoreflex arch is suggested by an increased turnover of CA in renal arteries (RA).

Materials and Methods

Animals and anesthesia

In this study we used 300–380 g of body weight adult Wistar male rats. When required, animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) dissolved in physiological saline. Animals were euthanized by an intracardiac overdose of sodium-pentobarbital. In handling the animals we followed the European Community Council directive of 24 November 1986 (86/609/EEC) for the Care and Use of Laboratory Animals. Protocols were approved by the Institutional Committee of the University of Valladolid for Animal Care and Use.

Exposure to intermittent hypoxia

Four rats were housed in hermetically shielding transparent methacrylate chambers (16 L), along with food and water. Each chamber has a gas inlet in front. Inside the chamber there is an incomplete wall that breaks the gas jet smoothing and slowing the flow of gas in the rats room situated 3 cm separated from the front wall. There are two outlets for the exit of gas from the chamber located at the back. In one of them there is an O_2 meter to continuously monitor the gas leaving the chamber. Gases flow into the chamber from tanks connected in cascade with stainless steel tubing and intercalated manometers to assure several days of supply. Flow of gases into the chamber is controlled by microprocessor driven electrovalves allowing predetermination of time and duration of entry of the desired gas. The electrovalve system is provided with a safety device for unwanted failure of the electrical power. In preliminary experiments the parameters of flow of gases were adjusted to achieve at the outlet of the chambers the desired gas mixture. Animals were exposed to IH for 8 and 15 days, from 8:00 to 16:00. The IH pattern was: 10% O_2 for 40 sec and 20% O_2 for 80 sec (i.e., 30 episodes/h).

In a selected group of spontaneously breathing anesthetized animals individually put into the IH exposure cages, anaerobic samples of blood (0.2 ml) were taken from the femoral artery. Blood withdrawal was slow and the removal of each blood sample took 20 sec. Multiple samples were taken from each animal at desired times. Blood gases tensions were immediately measured (Automatic Blood Gas System, ABL 5). Lowest values of PO_2 registered during the hypoxic episodes averaged 63.5 ± 2.5 mm Hg, although In all likelihood the real level of hypoxia attained was a little more intense because the nadir of PO_2 in the chamber lasted a few seconds and the withdrawal of blood took 20 sec. PaO_2 in blood samples withdrawn immediately after the hypoxic episodes recovered to control values. HbO_2 percentage prior to hypoxic episodes was above $\geq$96% and it reached lowest values of 91.7 ± 0.8%. Arterial PCO_2 dropped from pre-hypoxic levels of around 38 mm Hg to nadir values of around 27 mm Hg. In the 20-sec period immediately post-hypoxia it remained below the control PCO_2 of 38 mm Hg to recover before the onset of the next episode of hypoxia. Changes in PCO_2 clearly evidence the hyperventilation triggered by hypoxia.

Surgical procedures

The experiments were performed in the morning at around 9 a.m., that is, around 16 h after the completion of their last IH episode. Animals were tracheostomized and, after adequate dissections, bilateral blocks of tissue containing the carotid bifurcations were removed and placed in a lucite dissecting chamber filled with ice cold O_2-saturated Tyrode solution (in mM: NaCl, 140; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1.1; HEPES, 10; glucose, 5.5; pH 7.40). For the neurochemical experiments (measurement of content, synthesis, and release of CA), the CBs were identified, cleaned of surrounding tissues, and freed of the CSN with the aid of a dissecting microscope. Cleaned CB, were collected and saved differently according to experimental requirements. For CSN recording, the preparation CB-CSN was identified under a dissecting microscope in situ and then surgically removed as a tissue block which included the carotid bifurcation and the glossopharyngeal nerve. Once surgically cleaned of unwanted surrounding tissue, the preparation CB-CSN was digested during 3–5 min in collagenase type I (1 mg/ml) solution to loosen the perineurium. Thereafter the CB-CSN preparation was transferred to the recording chamber. In some animals, RA, from their emergence from the aorta to their arrival to the kidney hilum, were also removed, dissected free of surrounding

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connective tissue in the chilled saline, and stored until homogenization.

Basic hematology

Blood was obtained by direct cardiac puncture after an ample thoracotomy. Complete EDTA-blood was used for cellular blood counting in an Advia® Flow Cytometer (Bayer AG; Leverkusen, Germany).

Neurochemical experiments

Measurement of endogenous CA content in the CB and RA. For the analysis of endogenous unlabeled CA in the tissues, upon dissection CB and RA were placed individually in eppendorf tubes containing, respectively, 50 and 100 µl of ice-cold 0.3 N PCA. After weighing the tissues free of adherent PCA in an electrobalance (Supermicro, Sartorius), tissues were glass-to-glass homogenized, centrifuged, and aliquots (10–50 µl) of supernatants were directly injected into an HPLC system. The HPLC system was composed of a Milton Roy CM 400 pump, a Waters C18 (particle size 4 µm) column, a Waters U6K injector, a Bioanalytical Systems LC-4A electrochemical detector (set at a holding potential of 0.75 mV and a sensitivity of 1–5 nA). Mobile phase was, (in mM), Na_2HPO_4, 25, sodium octane sulfonate, 0.6 and EDTA, 0.1, with 6% MET-OH, pH adjusted to 3.2 with concentrated phosphoric acid. Identification and quantification of catecholamine was done against external standards and using Peak Sample Data Chromatography System software (Buck Scientific, East Norwalk, CT).

Measurement of the rate of CA synthesis in the CB and RA. General procedures for the synthesis experiments have been described in previous publications (Fidone and Gonzalez, 1982; Vicario et al., 2000). In brief, CB and RA were incubated during 2 h in a Tyrode solution containing tyrosine (30 µM), the natural precursor of CA, labeled with tritium (3,5-^{3}H-tyrosine, 6 Ci/mmol; Amersham, Barcelona, Spain); incubating solution also contained 100 µM 6-methyl-tetrahydropterine and 1 mM ascorbic acid, cofactors for tyrosine hydroxylase and dopamine-β-hydroxylase, respectively (Fidone and Gonzalez, 1982). Completed the labeling period, tissues were washed in ice-cold precursor-free Tyrode (5 min), homogenized and processed for HPLC as described above. Identification and ^{3}H-CA and labeled precursor in tissue samples were done against external standards and quantification was made by collection of the HPLC column effluents correspondent to the peaks of interest and scintillation counting of the collected effluents.

Measurement of 3H-CA release by the CB. The measurement of ^{3}H-CA release by the CB was performed in organs whose CA deposits have previously been labeled by their incubation as in the synthesis experiments but with high specific activity precursor (3,5-^{3}H-tyrosine, 40–50 Ci/mmol; Amersham). Following the labeling of ^{3}H-CA stores individual CB were transferred to a scintillation glass vial containing 4 ml of precursor-free Tyrode bicarbonate solution (composition as above except for the substitution of 24 mM NaCl by 24 mM $NaHCO_3$). Vials were kept in a shaker bath at 37°C for the entire experiment. Solutions were continuously bubbled with 20% O_2/5% CO_2, balance N_2 saturated with water vapor ($PO_2 \approx 136$–140 mm Hg), except during hypoxic stimulation and high K^+ stimulation (see below). During the first hour, the incubating solutions were renewed every 20 min and discarded. Thereafter, solutions were collected every 10 min for analysis of ^{3}H-CA content. Hypoxic and depolarizing stimuli consisted of 10 min incubations with low PO_2-equilibrated (7% O_2; $PO_2 \approx 46$ mm Hg) and high K^+-containing solutions (35 mM; equiosmolar Na^+ was removed). Collected solutions were acidified with glacial acetic acid to pH 3 and maintained at 4°C to prevent degradation of the ^{3}H-CA until analysis. The analysis included: adsorption to alumina (100 mg) at pH 8.6, washing of alumina with distilled water, bulk elution of all ^{3}H-catechols (1 ml of 1 N HCl), and scintillation counting. Raising of pH in the collected solutions from 3 to 8.6 was made by the addition to the vials under continuous shaking 3.6 ml of 2 M TRIS-buffer at a pH of 8.7. CBs from release experiments were analyzed as in the synthesis experiments.

Recording of the CSN activity

The CB-CSN preparation was transferred to a recording chamber mounted on a dissection microscope (Nikon) and superfused (37°C) with bicarbonate buffered saline (in mM: NaCl 120; $NaHCO_3$ 24; KCl 3; $CaCl_2$ 2; $MgCl_2$ 1.1; glucose 5; pH 7.40). Recordings of single or few fibers of CSN were made using a suction electrode. The pipette potential was amplified (Neurolog Digimiter, Hertfordshire, England), displayed on an oscilloscope, and stored in a PC computer (200 Hz acquisition rate, Axonscope, Axon Instruments, Foster City, CA). Chemoreceptor activity was identified (spontaneous generation of action potentials at irregular intervals) and confirmed by its increase in response to hypoxic superfusion (normoxia: solution equilibrated with 20% O_2 + 5% CO_2, balanced N_2; hypoxia: solution equilibrated with 0% or 5% O_2 + 5% CO_2, balanced N_2). CSN activity was digitalized, summed every second and converted into a voltage proportional to the sum. Hypercapnic stimulation consisted in the perfusion of the preparations with solutions equilibrated with 20% O_2 + 20% CO_2, balance N_2.

Whole body plethysmography

Ventilation was measured in conscious freely moving rats by whole body plethysmography. The system (Emka Technolgies, Paris, France) consists of 5 L metacrylate chambers continuously fluxed (2 L/min). Temperature was maintained in the chamber within the thermo-neutral range (22–24°C). Tidal volume (TV; ml/kg), respiratory frequency (Bf; breaths/min), and minute ventilation (MV; ml/min/kg) were measured. Briefly, the rats were placed in the plethysmographic chamber and breathed room air (control groups) for at least 30 min until adapted to the chamber ambient and they acquired a standard resting behavior. Thereafter we started recording ventilatory parameters during 20 min, followed by fluxing the chamber with a gas mixture containing 12%, 10%, or 7% O_2 (rest N_2; 2 L/min) and 5% CO_2 in air during 10 min. Each hypoxic or hypercapnic exposure was followed by a 20 min in air. The pressure changes within the chamber reflecting TV were measured with a high-gain differential pressure transducer. Ideally the frequency of pressure fluctuations is identical to breathing movements; spurious fluctuations of the pressure due to animal movements were electronically rejected. The amplitude of the pressure oscillations is proportionally related to TV; a calibration of the system by injections of 2–5 ml air into the chamber allowed a direct estimation of TV. Pressure signals were fed to a computer for visualization and storage for later analysis with EMKA software.

Presentation of data and statistics

Data are presented as means ± SEM. Statistical significance of differences was assessed using a two-tailed Student's *t*-test for unpaired data and for comparisons of more than two groups we have used a two-way ANOVA followed by Bonferroni multicomparison test.

Results

Animals

The protocol of IH applied in this study did not alter the gain in body weight, which was nearly identical to that observed in controls. Basic hematology, including red cell count, hematocrit, hemoglobin content, and other red cell related indexes were not different from control animals. White cell count was also not different from controls, but platelet count that in control rats was 970 ± 50 (×10^3/µl) was very significantly reduced to 630 ± 17 and 602 ± 12 (×10^3/µl; $P < 0.001$ in both cases) at 8 and 15 days of IH.

Carotid body CA content

Figure 1 shows the levels of endogenous CA in the CBs of control and 8 and 15 days IH animals. At 8 days the levels of both catecholamine, NE and DA, although showed a tendency to decrease were nearly normal. However at 15 days there were very significant decreases in both NE and DA content which

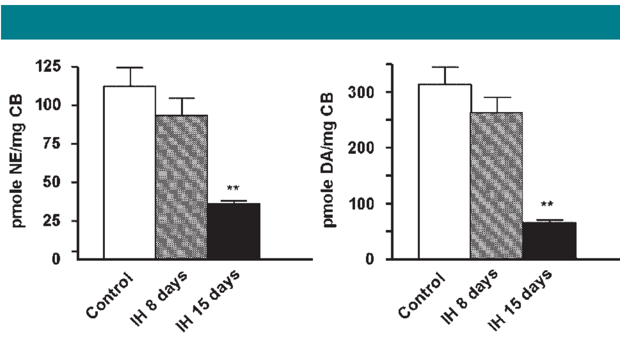

Fig. 1. Levels of catecholamines in the carotid bodies of control and 8 and 15 days intermittent hypoxic rats. Data are means ± SEM of 24 (control) and 9 individual values (8 and 5 days IH). **$P < 0.01$.

dropped from control levels of 112.3 ± 12.26 pmol/mg tissue (n = 24) for NE and 314.4 ± 29.87 pmol/mg tissue (n = 24) for DA to, respectively, 36.2 ± 2.29 and 65.4 ± 5.50 pmol/mg tissue (n = 9, $P < 0.01$ in both cases).

Carotid body and renal artery CA synthesis

The rates of CA synthesis from their natural precursor ^{3}H-tyrosine by the CBs of control and 8 and 15 days IH animals are shown in Figure 2. Measured NE and DA synthesis rates in control CBs were, respectively, 1.6 ± 0.14 and 52.0 ± 3.78 pmol/mg tissue/2 h (n = 24). The CBs obtained from animals exposed for 8 days to IH exhibited comparable rates of CA synthesis, but those obtained from 15 days IH animals exhibited a decreased rate of synthesis to levels 0.8 ± 0.2 pmol/mg tissue/2 h for NE (n = 9; $P < 0.05$) and to 19.1 ± 2.10 pmol/mg tissue/2 h for DA (n = 9; $P < 0.01$). Turnover times in the present experiments, that is, the ratios of CA content to rate of synthesis encountered in control CBs, were 141 and 12 h for NE and DA, respectively. At 8 days of IH turnover times were quite similar and at 15 days, while NE turnover time also remained nearly identical (144 h) that for DA decreased by nearly 50% to 6.88 h. These changes indicate that NE stores are reduced proportionally to the decreased rate of synthesis, but in the case of DA, its endogenous storage is reduced in a greater proportion than the rate of synthesis. The different behavior of both CA is due to the fact that most of NE in the CB is in sympathetic endings and not in chemoreceptor cells where DA is stored (see Discussion Section).

The marked effects of IH on the CA content and rate of synthesis in the CB prompted a comparison with the NE

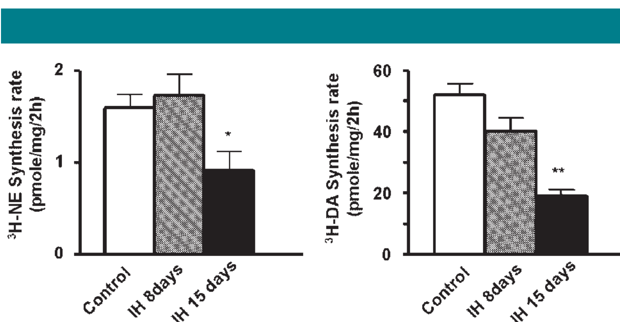

Fig. 2. Rates of 3H-catecolamines synthesis from their natural precursor 3H-tyrosine by carotid bodies of control and 8 and 15 days intermittent hypoxic rats. Data are means ± SEM of 24 (control) and 9 individual values (8 and 5 days IH). **$P < 0.01$.

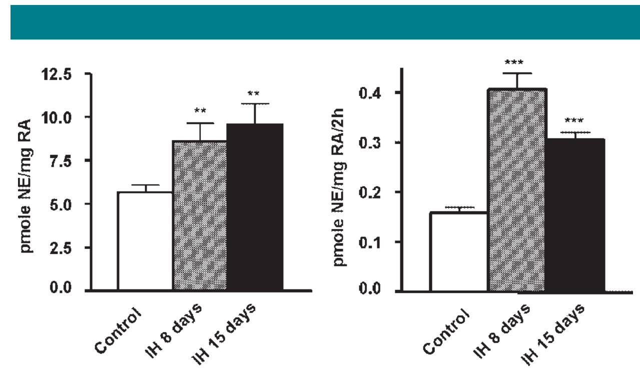

Fig. 3. Endogenous norepinephrine levels and rates of 3H-norepinephrine synthesis from its natural precursor 3H-tyrosine in the renal artery of control and 8 and 15 days intermittent hypoxic rats. Data are means ± SEM of 16 (control) and 9 individual values (8 and 5 days IH). **$P < 0.01$ and ***$P < 0.001$.

content and rate of synthesis in the sympathetic fibers/endings of the RA. As shown in Figure 3 the content of NE per milligram of renal artery in control rats was 5.7 ± 0.45 pmol (n = 16) and it increased at 8 and 15 days of IH exposure when it reached levels of 9.6 ± 1.20 pmol/mg renal artery tissue (n = 9; $P < 0.01$). The rate of ^{3}H-NE synthesis in control RA was 0.16 ± 0.01 pmol/mg tissue/2 h (n = 16) in control rats and increased to 0.3 ± 0.01 pmol/mg tissue/2 h in rats exposed to IH during 15 days (n = 10; $P < 0.001$). These data on renal artery contrast with findings in the CB. Thus, the renal CA storage capacity and rate of synthesis was increased by IH and turnover time remained nearly constant 71 h in control animals and 64 h in 15 days IH hypoxic animals.

Release of CA in the carotid body

Figure 4A,B shows the general protocol and mean time course of the ^{3}H-CA release experiments in CB of control and 15 days IH animals. CA release during normoxic periods was not significantly different between these two groups. In both cases it should be noted the slow decay of the released radioactivity with duration of the experiment while perfusing the CBs with normoxic solutions (20% O_2-equilibrated, basal release; dashed line across bars). Note also that normoxic incubating solutions in the periods following stimulation (10 min superfusion with 7% O_2-equilibrated solution and with a normoxic solution containing 35 mM K^+) contained higher amounts of radioactivity due to the slow washing of ^{3}H-CA and their labeled catabolites released during the stimulation. Finally, note the different scale in the *Y*-axis in control and 15 days IH. Computed stimulus-induced release (dpm above the dashed lines in Fig. 4A,B) shows that in response to hypoxia CBs of 8 days IH animals released ^{3}H-CA as control CBs, while CBs from 15 days IH animals released more than double control amount (3,565 ± 624 vs. 1,274 ± 383 dpm/CB; n = 16; $P < 0.001$; Fig. 4C). Contrary to that, CBs of control and 15 days IH animals released comparable amounts of ^{3}H-CA in response to the non-specific depolarizing high external K^+ (Fig. 4D).

Electrical activity in the carotid sinus nerve

The effects of IH on the electrical activity of the CSN were assessed on preparations isolated from control and 15 days exposed animals. Basal frequency measured in single-paucifiber preparations was near to 5 Hz (4.9 ± 0.91 Hz in control preparations) being less than half (2.2 ± 0.15 Hz; $P < 0.01$) in 15 days IH animals (Fig. 5B). Figure 5C shows mean responses to moderately intense (5% O_2-equilibrated solutions) and

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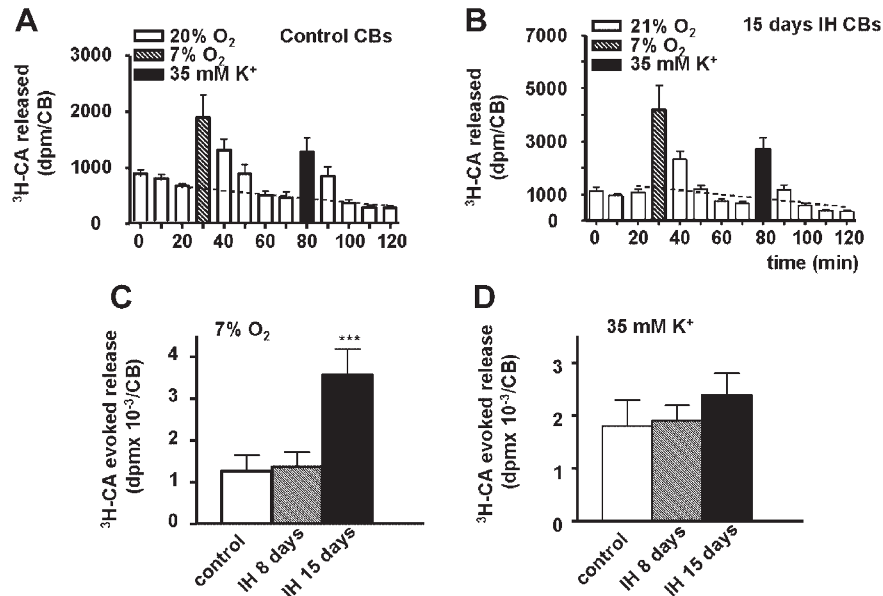

Fig. 4. Release of 3H-catecolamines by the carotid bodies of control and 8 and 15 days intermittent hypoxic rats. Effects of hypoxia and high external K+. A and B, respectively, the mean time course of the release by the carotid bodies of control and 15 days IH rats. Empty bars, superfusion with 20% O_2-saturated solutions, dashed bars superfusion with hypoxic solution (7% O_2-saturated) and black columns superfusion with solutions (20% O_2-equilibrated) but containing 35 mM K+. The dotted line crossing the histograms separates the basal release (below the line) from the stimulus-induced release (above the line). C and D: Mean evoked release by hypoxia and high external K+, respectively. Data are means of 16 (control) and 8 individual values (8 and 15 days IH). ***$P < 0.001$.

intense (N_2-equilibrated solutions) hypoxia and to hypercapnia (20% CO_2-equilibrated solutions) expressed as times basal normoxic activity. Data indicate that the CBs from IH animals generated much stronger acute hypoxic responses than controls, so even if basal activity was significantly lower absolute peak activity was nearly double in IH animals ($4.9\ Hz \times 5.63 = 27.7\ Hz$ vs. $2.2\ Hz \times 29.0 = 63.8\ Hz$ for 5% O_2, see sample recording in Fig. 5A, and $4.9\ Hz \times 8.7 = 42.63\ Hz$ vs. $2.2 \times 37.9 = 83.4\ Hz$ for N_2). However, the response to CO_2 (times basal) was only slightly higher in IH animals than in controls, making then the absolute hypercapnic peak frequency somehow smaller in IH than in control CBs. The pattern of the CSN response to hypoxia was also different. Thus, while in control preparations the response reached a peak promptly (40–60 sec) and exhibited a certain level of adaptation, in the CB-CSN preparations of IH animals the activity increased with nearly identical slope for longer period of time to reach the peakactivity at 90–120 s (Fig. 5D) with peak activity higher than in controls (see also Fig. 5A). The pattern of activity in control preparations is the common finding in the in control in vitro preparations (Fidone et al., 1982b; Conde et al., 2007). The latency of the hypoxic responses (not shown), which includes the renewal of the solutions in the dead space of the superfusion system, oscillated between 20 and 25 sec and there were not differences between control and IH animals. The response to hypercapnia was, as expected, faster, that is, latencies (11–14 sec, not shown) and time to peaks (26–33 sec; Fig. 5D) were smaller than for the hypoxic stimuli (Fidone and Gonzalez, 1986). There were not differences between control and IH animals in the hypercapnic responses.

Ventilation

Ventilation was studied in control and 8 and 15 days IH exposed animals. Figure 6A–C shows our main findings along with the protocol of the plethysmography. Breathing frequency (Bf; Fig. 6A) in control animals breathing room air atmosphere ranged between 77 and 88 breaths/min all along the experiments. Changing breathing atmosphere to 12%, 10%, and 7% O_2 significantly increased Bf to maximum of 151 ± 6 breaths/min in 10% O_2. Similarly, breathing in a 5% CO_2 containing air mixture increased Bf to 118 ± 3 breaths/min. In IH animals Bf did not show consistent variations at 8 days of exposure in any of the atmospheres studied but at 15 days of exposure it tended to be lower in all atmospheres, lowering being significant in 10% O_2 atmosphere to 131 ± 5 breaths/min ($P < 0.05$). TV/kg body weight (Fig. 6B) in control animals breathing normal air atmosphere in the different moments of the experiment ranged between 5.4 and 6.2 ml/kg. The 12% and 10% O_2 challenges caused modest increases in TV, but the most intense hypoxic atmosphere (7% O_2) very significantly increased TV to 8.8 ± 0.4 ml/kg. The 5% CO_2 also augmented significantly TV to 7.7 ± 0.3 L/kg. Exposure to IH for 8 days caused minor and variable modifications in TV that did not reach statistical significance in any condition. But after 15 days exposure in all conditions TV were smaller, being the decrease statistically significant in all atmospheres. Minute volume (MV)/kg body weight (Fig. 6C) in control animals breathing normal air atmosphere in the different moments of the experiment ranged between 404 and 526 ml/kg/min. Hypoxia produced an increase in MV directly related to its intensity, reaching levels of 781 ± 35 ml/kg/min in the 12% O_2 atmosphere, 898 ± 46 ml/kg/min in the 10% O_2 atmosphere, and 1048 ± 42 ml/kg/min in the 7% O_2 atmosphere. Similarly, breathing in air containing 5% CO_2 caused MV to increase to 892 ± 44 ml/kg/min. Exposure to IH for 8 days caused no change or a tendency to increase in MV at any given experimental time in normoxia, and in the normoxic period after breathing 10% O_2 the increase reached statistical significance (43% increase; $P < 0.01$). No differences were observed in hypoxic and hypercapnic atmospheres. Contrary to that, after 15 days exposure in all conditions MV were smaller than in unexposed animals, except

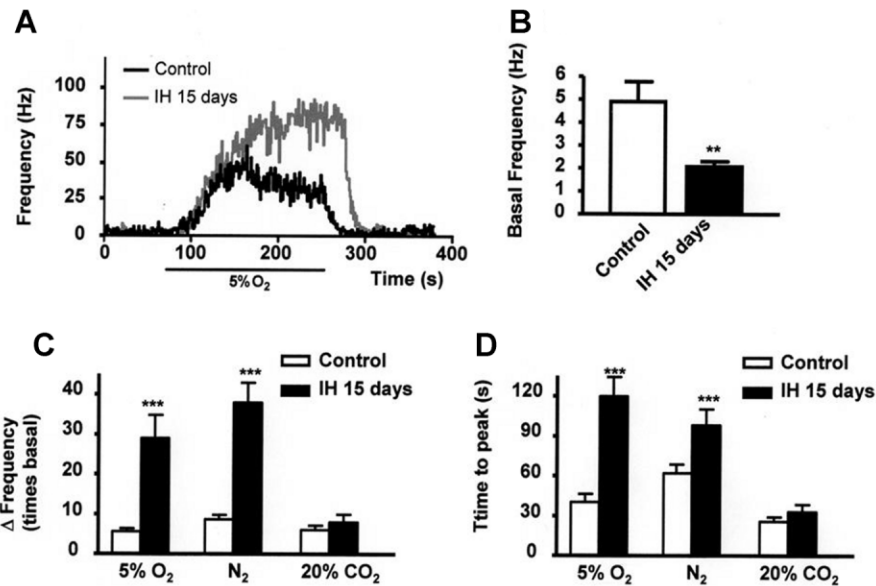

Fig. 5. Activity in paucifiber preparations of the carotid sinus nerve of control and 15 days ***$P < 0.001$. Effects of moderate and intense hypoxia and hypercapnia. A: Sample integrated response to moderate hypoxic stimulation of one control (black tracing) and one intermittent hypoxic animal (gray tracing). B: Mean basal normoxic (superfusion with 20% O_2-equilibrated solutions) in control (n = 10) and 15 days intermittent hypoxic (n = 9) animals **$P < 0.01$. C: Mean CSN responses in control and 15 days intermittent hypoxic animals to moderate and intense hypoxia and hypercapnia. Data (means ± SEM; n = 9) are expressed as times basal: empty bars, control; black bars, intermittent hypoxic animals. ***$P < 0.001$. D: Time required to reach maximal activity during stimulation. Data are means ± SEM (n = 9). Empty bars, control; black bars, intermittent hypoxic animals. ***$P < 0.001$.

while animals breathe 5% CO_2. In other words, after 15 days IH animals hypoventilated in air and the three hypoxic atmospheres tested.

Discussion

In the present work we have studied the effects of 8 and 15 days duration IH of moderate intensity as defined by nadir arterial PO_2 (63.5 ± 2.5 mm Hg) on the entire arterial CB chemoreceptor arch, from chemoreceptor cells to ventilation. We have also included a measurement of the utilization of CA by the sympathetic endings present in the renal artery. We have found that IH of 15 days duration produces marked changes in the levels and rate of CA synthesis in the CB chemoreceptor cells. It produces a marked decrease in the content and normoxic rate of synthesis of NE and DA. Yet, turnover time for DA, the main CA in chemoreceptor cells, decreased by nearly 50% indicating a relative higher rate of DA utilization in normoxia in chemoreceptor cells. During acute hypoxic stimulation chemoreceptor cells of the CBs of IH animals responded with an exaggerated release of ^{3}H-CA while the response elicited by a non-specific depolarizing stimulus (high external K^+) was not significantly altered. The output of the CB measured at the level of the CSN, that is, CB drive to brainstem, was diminished basally (normoxia) but exaggerated in response to hypoxia while response to high PCO_2/low pH remained nearly normal. Importantly enough, ventilatory responses were also altered in 15 days IH rats. They maintained Bf in all experimental conditions (atmospheres of 20%, 12%, 10%, and 7% O_2 and 5% CO_2 in air) but exhibited a significantly lower TV. Both factors made MV significantly lower in all conditions except in CO_2 atmosphere. As a whole, data demonstrate that IH disrupts the normal relationship encountered between CSN activity and ventilation. In control animals (and also in animals exposed to chronic sustained hypoxia) acute hypoxic tests increase the activity in the CSN and ventilation in parallel and in proportion to the intensity of the hypoxic tests (Fidone and Gonzalez, 1986; Gonzalez et al., 1994). In IH animals the exaggerated increase in CSN firing induced by acute hypoxia in comparison to controls failed to generate the proportionally exaggerated ventilatory response. Instead, IH animals hypoventilated in comparison to controls. This fact implies profound changes in the brainstem integrating respiratory centers (Kline, 2010) which are accompanied by marked changes in the dynamics of NE in vascular sympathetic endings, implying an exaggerated sympathetic activity. As a whole our results would indicate that IH causes an abnormal integration of the input from the CB to the nucleus tractus solitarius (nTS) to generate an exaggerated output to the rostral ventrolateral medulla. This area is a major source of excitation to the preganglionic sympathetic neurons in the intermediolateral column of the spinal cord (see below).

From Figure 1 it is evident that the rat CB is a catecholaminergic organ with DA being the dominant CA in relation to NE in a proportion of about 3:1. If we refer to chemoreceptor cells, this ratio would increase to 10 or more because most (≈80%) of the NE content in the rat CB is stored in the intraglomic sympathetic endings (Fidone and Gonzalez, 1982; Mir et al., 1982). In the CBs of 15 days IH animals there is a marked decrease in the content of both CA to around 25–30% of control level, but the proportion between both CA is roughly maintained. The rate of ^{3}HNE and ^{3}HDA from their natural precursor ^{3}HTyrosine (Fig. 2) indicates that in the CBs of control animals the ratio of their rate of synthesis (^{3}HDA/^{3}HNE) is around 30–35 exceeding markedly the ratio of content and indicating that DA is used, and therefore renewed, much more rapidly than NE. In fact, the rate of ^{3}HDA and ^{3}HNE synthesis, ≈26 pmol/mg of CB tissue/h and ≈0.85 pmol/mg of CB tissue/h,[1] respectively, indicates that the CB is using preferentially DA in comparison to NE as a signaling molecule.

[1]This rate of synthesis does not correspond with turnover rates. It is an underestimate because the synthesis is not lineal for two hours (see Vicario et al., 2000).

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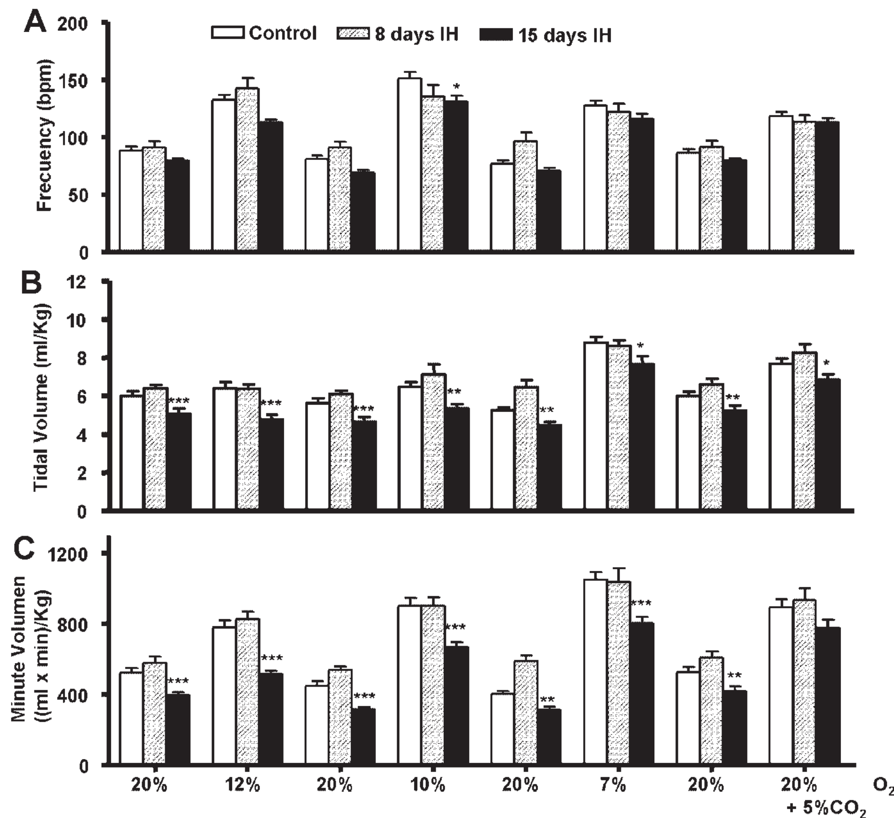

Fig. 6. Normoxic, hypoxic, and hypercapnic ventilation in control and 8 and 15 days intermittent hypoxic rats. A: Breathing frequency. B: Tidal volume by unit weight. C: Minute ventilation by unit weight. In all instances data are means ± SEM of 16–20 individual values for control animals, 8 individual values for 8 days, and 12 individual values for 15 days intermittent hypoxic rats. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

We have previously emphasized this fact (Fidone et al., 1982a; Vicario et al., 2000; Conde et al., 2006). In the CBs from the 15 days IH animals rates of synthesis drop to around 10 pmol/mg CB/h for DA and 0.5 pmol/mg CB/h for NE, evidencing that both CA, but particularly DA, is used at much lower rate in IH organs. However, the turnover times, that is, content/rate of synthesis for DA in IH animals drops from 12 to 6.88 h. Altogether data on CA content and rate of synthesis indicate that IH decreased drastically the normoxic storing capacity of the CB chemoreceptor cells for both CA, but reduced to a much lower extent their capacity to synthesize and use DA. These findings contrast with the well-known effects of chronic sustained hypoxia that increases both, the CA stores in chemoreceptor cells (Hanbauer et al., 1981; Pequignot et al., 1987) and the rate of CA synthesis (Caceres et al., 2007). These changes in CA metabolism in sustained hypoxia run in parallel to an increase in ventilation to hypoxia (acclimatization). In IH, animals did not show acclimatization but they rather showed tolerance to the acute hypoxic response. Moreover, the effects produced by IH appear to be rather specific of the hypoxic stimulation pattern and cannot be attributed to generalized alteration of the CA biosynthetic and storing machineries because in the sympathetic endings both the NE content and rate of synthesis is significantly increased (Fig. 3; see below).

The overall shape of the time course of ^{3}HCA release (mostly ^{3}HDA release) experiments is very similar in the CBs of control and 15 days IH animals (Fig. 4A,B). There are however important quantitative differences, being the most evident that the release of ^{3}HCA elicited by hypoxia is markedly increased (Fig. 4C). Once again, the effects of IH contrast with those of sustained hypoxia (Gonzalez-Martin et al., 2009) where a non specific and comparable sensitization leads to a parallel increase release in response to hypoxia and to high external K^+ which is probably due, at least in part, to an upregulation of L-type Ca^{2+} channels in the CB (Cáceres et al., 2009). However, in the case of IH data would indicate that sensitization occurs in the transduction cascade upstream of cell depolarization (Gonzalez et al., 1992, 2003). An upregulation of the O_2-sensing mechanisms and/or O_2-sensitive K^+ channels would satisfactorily explain findings. However, since cAMP stimulates the release of CA induced by hypoxia, and not the release induced by high K^+ (Perez-Garcia et al., 1991) by an EPAC mediated mechanism (Rocher et al., 2009) the upregulation of adenylyl cyclase or EPAC proteins can also account for the effects of IH. Additionally, as suggested by Peng et al. (2003), the possibility exists that increased reactive oxygen species of mitochondrial origin, probably due to a downregulation of mitochondrial superoxide dismutase (Nanduri et al. 2009) or an induction of some isoform of NADPH oxidase (Peng et al., 2009; He et al., 2010) is responsible for chemoreceptor cell sensitization to hypoxia. Yet, the target of ROS remains to be defined. In any case, future experiments are required to disclose among these possibilities.

All considerations made to the release of ^{3}HCA induced by hypoxia in the CBs of IH animals are directly applicable to the activity in the CSN elicited by hypoxia. However, we should make explicit that, even if during hypoxic stimulation in IH animals there is an increased DA concentration in the milieu

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surrounding the sensory nerve endings and a parallel increased level in neural discharge, we do not imply a cause-effect relationship between both parameters. In all likelihood, additional neurotransmitters co-released with DA (e.g., ATP that is co-stored with CA or acetylcholine; Nurse 2005), adenosine (Conde et al., 2009) or serotonin (Peng et al., 2009) are responsible for the augmentation of the hypoxic response. In sum, the genesis of the specific neural activity pattern (i.e., decreased basal activity, increased hypoxic activity and near normal hypercapnic response after 15 days of IH) must imply profound changes in the storing capacity and dynamics of possibly several neurotransmitter systems (exemplified by CA). Changes in the density and/or affinity of their receptors in the sensory nerve endings should also be considered.

The most striking finding was the marked decrease in ventilation in 15 days IH animals in all conditions except in hypercapnia. In a sense, the maintenance of hypercapnic ventilation constitutes in itself an excellent intra-experimental control. Three additional facts provide validation to the observed decrease in TV and MV in 15 days IH animals: (a) the data from 15 days IH animals were collected from two different experimental batches of animals exposed to IH and studied several weeks apart; (b) 15 days IH were studied concurrently with animals exposed to 8 days of IH which showed no change in ventilatory parameters, and (c) in another set of ongoing experiments with 15 days IH animals we have confirmed the ventilatory depression. In this context, we should ask the reasons for the discrepant findings on the modifications of the hypoxic ventilatory response produced by IH (see Introduction Section). We should agree with Edge et al. (2009) when they point out that there are several methodological reasons that can generate non-concordant findings. Among them we should mention the following: (a) differences in IH paradigm (i.e., frequency and intensity of hypoxic episodes), (b) time elapsing between last IH episode and measurements of ventilatory parameters, (c) duration of the IH (hours/day and days of IH), and (d) animal species, age, gender, and strain. Additionally, as recently discussed in the XVII Meeting of the International Society of Arterial Chemoreceptors (Valladolid, Spain, July 2008), it is possible that with some IH paradigms there is not a full recovery of blood PO_2 between hypoxic episodes and, therefore, instead of IH, animals are subjected to a sustained background of hypoxia with more intense hypoxic peaks.

The decreased MV in normoxic atmosphere and normal MV in hypercapnic atmospheres found in this study parallel the changes of activity recorded in the CSN. In hypoxic atmospheres a clear dissociation occurs between CSN activity and MV. The question emerges spontaneously: how is it possible that a more intense CB input to the nTS, particularly to the commissural and medial subnuclei of the caudal nTS (Katz et al., 1997), results in a decreased ventilatory response? To provide a tentative answer to this question we should also consider that the ventilatory patterns generated by IH are associated to an increased sympathetic tone as evidenced by many authors in their models of IH (e.g., Zoccal et al., 2007; Xing and Pilowsky, 2010). Moreover, the present study also shows the increased levels and turnover rate of NE in sympathetic endings, which should result in an increase in circulating NE because no less than 75% of the circulating NE comes form sympathetic endings (Goldstein et al., 2003; see Gonzalez-Martin et al., 2009). From a mechanistic point of view, our findings can be interpreted as the result of abnormal integration in the nTS which would generate abnormal outputs. For example, IH may be causing a deficient drive of the ventral respiratory column (that controls ventilation) and an exaggerated drive to the rostral ventrolateral medulla. This last area is a major source of excitation to the preganglionic sympathetic neurons in the intermediolateral column of the spinal cord normal coupling to respiration. Or, alternatively, the nTS can generate appropriate outputs, but postsynaptic mechanisms in the ventral respiratory column make it partially refractory to the CB chemoreceptor originated drive. The opposite can happen in brainstem sympathetic neurons. A combination of both mechanisms can be operative as current literature describes both abnormal integration at nTS and exaggerated activation of sympathetic neurons in IH (Zoccal et al., 2009; Kline 2010; Xing and Pilowsky, 2010). Our proposal of an anomalous integration of the CB input in the brainstem of IH animals emerges as an absolute necessity, because augmented sensitization of the CB function per se does not imply hyperactivation of the sympathetics. Thus, in sustained hypoxia many laboratories have evidenced a sensitization of the CB with an increased CB input into the brainstem (Gonzalez et al., 1994; Bisgard, 2000), yet there is not hyperreactivity of the sympathetics neither in high altitude residents nor in animal models of sustained hypoxia (Ward et al., 1995). In other words, increased CSN activity is not enough to trigger sympathetic sensitization, a particular "anomalous" integration of the activity is also required. The tendency to decrease of the ventilatory response to hypercapnia should be due to the fact that ventilation to hypercapnia is generated in only a small percentage ($\approx$30%) by the CB chemoreceptors being most of it generated at the central chemoreceptors located in the ventral medulla (Cherniack and Altose, 1997; Spyer and Gourine, 2009).

In sum, our study constitutes the first demonstration of the alteration of every element of the entire CB chemoreflex arch in IH animals demonstrating a sensitization of the afferent elements evidenced by exaggerated chemoreceptor cell and CSN responses to hypoxia and an alteration in the brainstem integration of the CB input leading to an altered function of the efferent arm of the reflex as evidenced by an exaggerated sympathetic neurotransmission and a reduced ventilation in normoxia and in response to hypoxia.

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