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Chronic Caffeine Intake in Adult Rat Inhibits Carotid Body Sensitization Produced by Chronic Sustained Hypoxia but Maintains Intact Chemoreflex Output

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ABSTRACT

Sustained hypoxia produces a carotid body (CB) sensitization, known as acclimatization, which leads to an increase in carotid sinus nerve (CSN) activity and ensuing hyperventilation greater than expected from the prevailing partial pressure of oxygen. Whether sustained hypoxia is physiological (high altitude) or pathological (lung disease), acclimatization has a homeostatic implication because it tends to minimize hypoxia. Caffeine, the most commonly ingested psychoactive drug and a nonselective adenosine receptor antagonist, alters CB function and ventilatory responses when administered acutely. Our aim was to investigate the effect of chronic caffeine intake on CB function and acclimatization using four groups of rats: normoxic, caffeine-treated normoxic, chronically hypoxic (12% O_2, 15 days), and caffeine-treated chronically hypoxic rats. Caffeine was administered in drinking water (1 mg/ml). Caffeine ameliorated ventilatory responses to acute hypoxia in normoxic animals without altering the output of the CB (CSN neural activity). Caffeine-treated chronically hypoxic rats exhibited a decrease in the CSN response to acute hypoxia tests but maintained ventilation compared with chronically hypoxic animals. The findings related to CSN neural activity combined with the ventilatory responses indicate that caffeine alters central integration of the CB input to increase the gain of the chemoreflex and that caffeine abolishes CB acclimatization. The putative mechanisms involved in sensitization and its loss were investigated: expression of adenosine receptors in CB (A_{2B}) was down-regulated and that in petrosal ganglion (A_{2A}) was up-regulated in caffeine-treated chronically hypoxic rats; both adenosine and dopamine release from CB chemoreceptor cells was increased in chronic hypoxia and in caffeine-treated chronic hypoxia groups.

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Introduction

The carotid body (CB) chemoreceptors sense arterial blood gases and pH. Hypoxia and hypercapnia/acidosis activate CB chemoreceptor cells, increasing their rate of neurotransmitter release (Gonzalez et al., 1994; Conde et al., 2012a), which augments action potential frequency in the fibers of the carotid sinus nerve (CSN), whose cell bodies are located in the petrosal ganglion (PG). Central integration of CSN activity elicits reflex hyperventilation and cardiocirculatory responses with the aim of normalizing blood gases and adequately distributing O_2 in the organism (Gonzalez et al., 1994).

Chronic hypoxia is a common situation that generates adaptive and compensatory responses meant to minimize hypoxia and its harmful effects. Physiologically, chronic hypoxia exists in animals and humans living or sojourning at high altitude, and pathologically it is encountered in many lung pathologies best exemplified by chronic obstructive pulmonary disease. Therefore, the study of chronic hypoxia has both basic and clinical interests. At the CB, chronic hypoxia

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ABBREVIATIONS: CB, carotid body; CSN, carotid sinus nerve; PG, petrosal ganglion; DA, dopamine; ACh, acetylcholine; VE, minute ventilation; PO_2, partial pressure of oxygen; N, normoxic; CafN, caffeine-treated normoxic; CH, chronically hypoxic; CafH, caffeine-treated chronically hypoxic; PCA, perchloric acid; HPLC, high-performance liquid chromatography; ANOVA, analysis of variance.

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generates a sensitization phenomenon, known as acclimatization, which results in amplified CSN activity and resultant hyperventilation for the existing level of hypoxia or acutely applied hypoxia tests (Bisgard and Neubauer, 1975; Bisgard, 2000). Thus, acclimatization is of prime importance to minimize hypoxia. Chronic hypoxia modifies CB gene expression (Huey and Powell, 2000; Ganfornina et al., 2005; Caceres et al., 2007; Mkrtchian et al., 2012), causes hypertrophy of chemoreceptor cells, a proliferation and widening of organ capillaries (Laidler and Kay, 1975), and also modifies neurotransmitter dynamics at the CBs [including dopamine (DA), acetylcholine (ACh), and ATP] (Powell, 2007). Yet, the mechanisms of acclimatization remain elusive.

Methylxanthines, particularly caffeine, stimulate ventilation and decrease frequency of apneic episodes in premature infants (Steer et al., 2004) via A_1 and A_{2A} adenosine receptor inhibition in central respiratory neurons (Herlenius and Lagercrantz, 1999), making caffeine the drug of choice to treat apneas of prematurity (Mathew, 2011). In the adult rat, acute caffeine administration acts on A_{2A} and A_{2B} adenosine receptors, inhibiting hypoxia-driven CSN activity by nearly 60%; A_{2B}-mediated inhibition is produced via interaction with DA metabolism in CB chemoreceptor cells, whereas the A_{2A} effect is postsynaptic (Conde et al., 2006, 2008). The immatureness of the CB in neonates (Gonzalez et al., 1994) explains the prevalence of the central stimulatory effect in newborns animals, whereas in adults, with CB function fully expressed, the acute peripheral inhibitory effect of caffeine takes over and inhibition of ventilation is noticeable only when the drive of ventilation depends mostly on CB chemoreceptors, as occurs in hypoxia (Howell and Landrum, 1995).

The aim of the present study was to define the effects of chronic caffeine intake on ventilatory responses in animals submitted to chronic hypoxia, in an attempt to clarify the pharmacodynamics of the most consumed psychoactive drug in the world (Fredholm et al., 1999; Chen et al., 2010) in a context of very high incidence of hypoxia-related pathologies in humans. Therefore, we measured the effects of caffeine on the afferent (CSN activity) and efferent (ventilation) arm of the CB chemoreflex in four groups of animals: normoxic, caffeine-treated normoxic, chronically hypoxic, and caffeine-treated chronically hypoxic. From the input-output relationships of the CB chemoreflexes [input = CSN activity; output = minute ventilation (VE)], reasonable inferences on the functioning of the reflex integrating respiratory centers can be made. Caffeine-treated normoxic animals exhibited levels of CSN activity like those seen in normoxic animals, and, at the same time, they presented increased VE, implying that caffeine centrally increases the reflex gain. Chronically hypoxic animals showed acclimatization manifested by an increase in CSN activity and VE. In comparison with chronically hypoxic animals, caffeine-treated chronically hypoxic animals showed a marked decrease in CSN responses to hypoxia, but nearly identical VE, i.e., chronic caffeine caused the loss of CB-sensitizing effects of chronic hypoxia together with a parallel increase in the centrally mediated gain of the reflex that maintained VE. Thus, chronic caffeine ameliorated ventilation in normoxic animals and caused no harm to ventilation in chronically hypoxic animals. In in vitro experiments, we measured the CB release of DA and adenosine and the expression of adenosine receptors in the CB (A_{2B}) and PG (A_{2A}) in the four groups of animals. Dopamine and adenosine release from CB was measured at rest and in low- and high-intensity hypoxic conditions. Caffeine ingestion in normoxic animals caused minor changes in DA and adenosine release and in the expression of adenosine receptors. Chronic hypoxia increased the resting and hypoxia-induced release of DA, diminished the resting and increased the hypoxia-induced release of adenosine, and did not change adenosine receptor expression. Caffeine-treated chronically hypoxic rats showed DA and adenosine release responses comparable to those seen in chronically hypoxic animals but exhibited decreased A_{2B} and increased A_{2A} receptor expression. These results are discussed in the frame of the current literature with the aim of understanding the mechanisms of CB acclimatization and its loss in caffeine-treated animals.

Materials and Methods

Animals and Anesthesia

Chronic Caffeine Administration and Exposure to Chronic Hypoxia. The experiments were performed in Wistar rats of both sexes aged 3 to 4 months (250–350 g), obtained from the vivarium of the Faculty of Medicine of the University of Valladolid (Valladolid, Spain) and from the vivarium of the Faculty of Medical Sciences of the Nova University of Lisbon (Lisbon, Portugal). Animals were anesthetized with sodium pentobarbital (60 mg/kg i.p.) and euthanized with an intracardiac overdose of the same anesthetic. The institutional committees for animal care and use of the University of Valladolid and Nova University of Lisbon approved the protocols. Control animals normoxic) were maintained in a normal room air atmosphere and chronically hypoxic rats were kept for 15 days in a chamber equilibrated with a gas mixture of 11 to 12% O_2 in 88 to 89% N_2 (PO_2 = ≈80 to 84 mm Hg) and a constant a flow of 3 l/min. The accumulation of CO_2 and water vapor inside the chamber was avoided by air renewal and by the presence on the ground of the chamber of a layer of soda lime granules. Every 4 days the chamber was opened to be cleaned and restocked with water and food. After each cleaning cycle, a high flow of gas was used to quickly drop the PO_2 in the chamber. The temperature and PO_2 in the chamber were monitored, and the animals were always kept with a seasonal day/light rhythm, with temperature controlled between 23 and 26°C. To study the effects of chronic caffeine intake we subdivided the normoxic and chronically hypoxic groups into two new groups (with and without caffeine). Caffeine was administered for 15 days in drinking water that contained 1 mg/ml caffeine (Gasior et al., 2002; Conde et al., 2012b). Therefore, we defined four groups: normoxic (N), caffeine-treated normoxic(CafN) (15 days), chronically hypoxic (CH) (12% O_2, 15 days), and caffeine-treated chronically hypoxic (CafH) (caffeine ingestion + chronic hypoxia exposure). To study ventilator parameters we used an additional set of four groups in which rats were exposed to chronic hypoxia and ingested caffeine for only 8 days.

General Surgical Procedures. In the experiments in which the release of endogenous adenosine and DA were analyzed and because of the small size of the CB (≈50 μg) (Conde et al., 2006), it was necessary to make pools of four CBs in each experiment to satisfactorily reach the limits of detection of the analytical techniques used. After tracheotomy, the carotid artery bifurcations were placed in ice-cold/100% O_2-equilibrated Tyrode's solution (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.1 mM $MgCl_2$, 10 mM HEPES, and 5.5 mM glucose; pH 7.40), and CBs were cleaned (Vicario et al., 2000). To record CSN activity, the CB-CSN preparation was dissected of tissue surrounding the CB and the CSN. The CB-CSN preparation was digested during 3 to 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.

To investigate A_{2A} and A_{2B} receptor expression in CBs and PGs, rats were decapitated, and the chemoreceptor complexes (carotid

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bifurcation-CSN-PG) were dissected free and placed in ice-cold, oxygenated Tyrode's saline solution, as described above. In brief, the vagus nerve was isolated and followed to its junction with the nodose-PG complex. CBs and PG were dissected and placed in cryovials in liquid nitrogen until further homogenization.

For plasma caffeine concentration determination, blood was collected from heart puncture in EDTA-precoated tubes and kept on ice. Blood samples were centrifuged at 3000*g* for 10 min (4°C), and supernatant plasmas were stored at −80°C.

Quantification of Plasma Caffeine. After protein precipitation with 30% PCA and neutralization with 4 M KOH-0.4 M Tris, caffeine, and its metabolites theobromine, theophylline, and paraxanthine were quantified in the supernatant by reverse-phase high-performance liquid chromatography (HPLC) with a low pressure gradient and UV detection at 274 nm as described previously by Conde et al. (2012b).

Whole-Body Plethysmographic Recordings of Ventilatory Responses in Response to Hypoxia and Hypercapnia. Ventilation was measured in conscious freely moving rats by whole-body plethysmography. The system (Emka Technologies, Paris, France) consisted of 5-liter methacrylate chambers continuously fluxed (2 l/min), with controlled temperatures within the thermoneutral range (22–24°C). Tidal volume (V_T) (milliliters). respiratory frequency (*f*) (breaths per minute), and VE) (milliliters per minute per kilogram) were measured. In brief, the rats were placed in the plethysmographic chamber and breathed room air for at least 30 min until they adapted to the chamber environment and acquired a standard resting behavior. Ventilatory parameters were recorded according to the protocols used with test gases applied for short periods (10 min). Specific protocols for normoxic and chronically hypoxic rats are provided under *Results*. All the gases were balanced with N_2 and applied at a flow of 2 l/min. The pressure change within the chamber reflecting V_T was 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 V_T; a calibration of the system by injections of 0.2 to 0.5 ml of air into the chamber allowed a direct estimation of V_T. Pressure signals were fed to a computer for visualization and storage for later analysis with Emka software.

Recording of Carotid Sinus Nerve Activity. The CB-CSN preparation was transferred to a recording chamber mounted on a dissection microscope (Nikon, Tokyo, Japan) and superfused (37°C) with bicarbonate-CO_2-buffered saline (120 mM NaCl, 24 mM $NaHCO_3$, 3 mM KCl, 2 mM $CaCl_2$, 1.1 mM $MgCl_2$, and 5 mM glucose; pH 7.40). Extracellular recordings from single or multiple fiber filaments of CSN were made using a suction electrode. The pipette potential was amplified (NeuroLog; Digimiter, Hertfordshire, England), filtered (1 kHz), digitized at 6 kHz (Axonscope; Axon Instruments, Molecular Devices, Wokingham, UK) and stored on a computer. Chemoreceptor activity was identified (spontaneous generation of action potentials at irregular intervals) and confirmed by its increase in response to hypoxia (normoxia: 20% O_2 + 5% O_2 + 75% N_2; hypoxia: 2% or 7% O_2 + 5% CO_2 + balanced N_2). CSN unit activity was converted to logic pulses, which were summed every second and converted in a voltage proportional to the sum. Chamber oxygen tension was measured with a needle electrode (760; Diamond Micro Sensors, Ann Arbor, MI) polarized to −0.8 V against a Ag/AgCl reference electrode placed in the recording chamber. The oxygen electrode current was also digitized in the same manner as above.

The CSN chemosensory activities in response to hypoxic and hypercapnic stimulation were achieved by perfusing the preparations with solutions equilibrated with gas mixtures containing 0% or 5% O_2 + 5% CO_2, balanced N_2, and 20% O_2 + 20% CO_2, balance of N_2, respectively.

Western Blot Analysis of A_{2A} and A_{2B} Adenosine Receptor Expression in the CB and PG. CBs and PGs were homogenized in Zurich medium (10 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium cholate, and 0.1% SDS) and a cocktail of protease inhibitors. We used 4 PG and 6 CBs per electrophoresis lane (≈30 μg of protein), so that in each electrophoresis we always had at least two lanes of control animals. Samples of the homogenates and the prestained molecular weight markers (Precision; Bio-Rad Laboratories, Madrid, Spain) were separated by SDS-polyacrylamide gel electrophoresis (10% with a 5% concentrating gel) under reducing conditions and electrotransferred to nitrocellulose membranes (Bio-Rad Laboratories). The membranes were blocked for 1 h at room temperature with 0.5% I-Block (Tropix, Bedford, MA) in Tris-buffered saline containing 0.02% Tween 20 (TBST). To enhance detection sensitivity we used a three-step Western blot protocol to detect A_{2A} and A_{2B} adenosine receptors in CBs and PGs performed as follows: A_{2A} and A_{2B} adenosine receptors were detected using mouse anti-A_{2A} adenosine receptor antibody (1:200 dilution) and rabbit anti-A_{2B} adenosine receptor antibody (1:200 dilution) incubated for 2 h in TBST (0.1%). Membranes for A_{2A} and A_{2B} adenosine receptor detection were washed in TBST (0.02%), incubated in TBST (0.1%) containing biotin-conjugated goat anti-mouse IgG and biotin-conjugated goat anti-rabbit IgG (1:10,000 dilution), respectively, for 1 h, washed again in TBST (0.02%), and incubated for 30 min in TBST (0.1%) containing horseradish peroxidase-conjugated streptavidin (1:10,000 dilution). Membranes were then washed in TBST (0.02%) and developed with enhanced chemiluminescence reagents according to the manufacturer's instructions (Immobilon Western; Millipore Iberica S.A.U., Madrid, Spain). The intensity of the signals was detected in a ChemiDoc molecular imager (Bio-Rad Laboratories) and quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA). The membranes were then reprobed and tested for α-tubulin immunoreactivity (bands in the 65-kDa region) to compare and normalize the expression of proteins with the amount of protein loaded.

Endogenous Release of Adenosine and DAs from Carotid Body. The CBs were incubated in 500 μl of Tyrode's solution containing *erythro*-9-(2-hydroxy-3-nonyl)adenine (2.5 μM), an inhibitor of adenosine deaminase. The incubation media were kept at 37°C and continuously bubbled with 20% O_2/5% CO_2/75% N_2 saturated with water vapor, except when hypoxic stimuli were applied. Specific protocols for stimulus are provided under *Results*. Stimulus included hypoxia of two intensities (7 and 2% O_2-equilibrated solutions). The collected fractions were divided in two equal aliquots and acidified with 3 and 0.6 M PCA for adenosine and DA analyses, respectively. At the end of the experiment, the CBs were immersed in 100 μl of 0.6 M PCA and weighed. The collected fractions were kept for 10 min at 0°C and then centrifuged at 12,000*g* for 10 min (4°C). For DA analyses, supernatants were frozen until further analysis by HPLC; DA was identified by its retention time and quantified against external standards (Vicario et al., 2000); data indicate that >90% of the released DA in all experimental groups is DA + 3,4-dihydroxyphenylacetic acid (its main catabolite), and, therefore, we refer to the set as DA. For adenosine analyses, supernatants were recovered, and adenosine was extracted from the medium as described previously by Conde and Monteiro (2004). Endogenous adenosine release was quantified by HPLC with UV detection (Conde and Monteiro, 2004).

Drugs and Chemicals

Adenosine, caffeine, *erythro*-9-(2-hydroxy-3-nonyl)adenine, PCA, KOH, Tris-HCl, EDTA, NaCl, Triton X-100, sodium cholate, SDS, theophylline, theobromine, and paraxanthine were obtained from Sigma-Aldrich (Madrid, Spain). Adenosine was prepared as 5 mM stock solutions in water. Antibodies against A_{2A} and A_{2B} adenosine receptors as well antibodies for α-tubulin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Data Analysis

The amount of adenosine endogenously released from CBs was expressed as picomoles per milligram of tissue after the division of

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the absolute values obtained by 4 (four CBs used in each incubation) and corrected for the volume used and the medium weight of the four CBs. Data were evaluated using Graph Pad Prism (version 4; GraphPad Software Inc., San Diego, CA) and are presented as means ± S.E.M. The significance of the differences between the means was calculated by one- and two-way analysis of variance (ANOVA) with Dunnett's and Bonferroni multiple comparison tests, respectively. $p \leq 0.05$ were considered to represent significant differences.

Results

The ingestion of caffeine in the drinking water (1 mg/ml) was stable throughout the experiments. Water consumption and body weight gain were comparable among groups. As expected, we did not detect the presence of caffeine or its metabolites in either normoxic or chronically hypoxic rats. The plasma caffeine concentration in caffeine-treated normoxic animals was 3.69 ± 1.02 μg/ml (or 19 μM), which was not significantly different from the concentration in caffeine-treated chronically hypoxic rats (4.01 ± 0.83 μg/ml). These values are within the range of 0.37 to 5.95 μg/ml described previously by Gasior et al. (2002) for doses of 0.25 to 1 mg/ml caffeine in drinking water in rats. Plasma caffeine concentrations indicate that we are dealing with nontoxicological caffeine concentrations with most (if not all) effects observed being mediated via inhibition of adenosine receptors. It is well known that caffeine has an IC_{50} in the range of 15 to 35 μM, as a nonselective inhibitor of adenosine receptors, whereas IC_{50} values for phosphodiesterase inhibition and for Ca^{2+} release from intracellular stores are, respectively, >10 and >100 times higher (Fredholm et al., 1999).

Throughout the *Results* and *Discussion* "basal" refers to data obtained either in an open air atmosphere or in solutions equilibrated with 20% O_2 in normoxic rats (normoxic and caffeine-treated normoxic),. For chronically hypoxic and caffeine-treated chronically hypoxic rats, the word "basal" refers to 12% O_2 for both in vivo or in vitro experiments because the animals were maintained for 8 or 15 days before the experiments in a 12% O_2 atmosphere. Gas mixtures contained 5% CO_2 if the experiments were performed in vitro. Data were normalized per unit of CB weight to account for the variations of the CB weights in the different experimental groups.

Effects of Chronic Caffeine Intake on the Ventilatory Responses Induced by Hypoxia and Hypercapnia in Control and Chronically Hypoxic Rats. Note first (Fig. 1A), that in normoxic animals VE increased progressively with the intensity of the acute hypoxia tests. Note also that chronically hypoxic animals (exposed to 12% O_2 during 8 and 15 days) exhibited higher VE than the normoxic group. The increase was 21% under 7% O_2 in animals exposed to chronic hypoxia during 15 days ($p < 0.05$). This divergence of the lines relating VE with breathing oxygen levels in normoxic versus chronically hypoxic animals reflects acclimatization, allowing the conclusion that acclimatization to hypoxia was already expressed after 1 week of chronic hypoxia but was more accentuated after 2 weeks. Minute ventilation

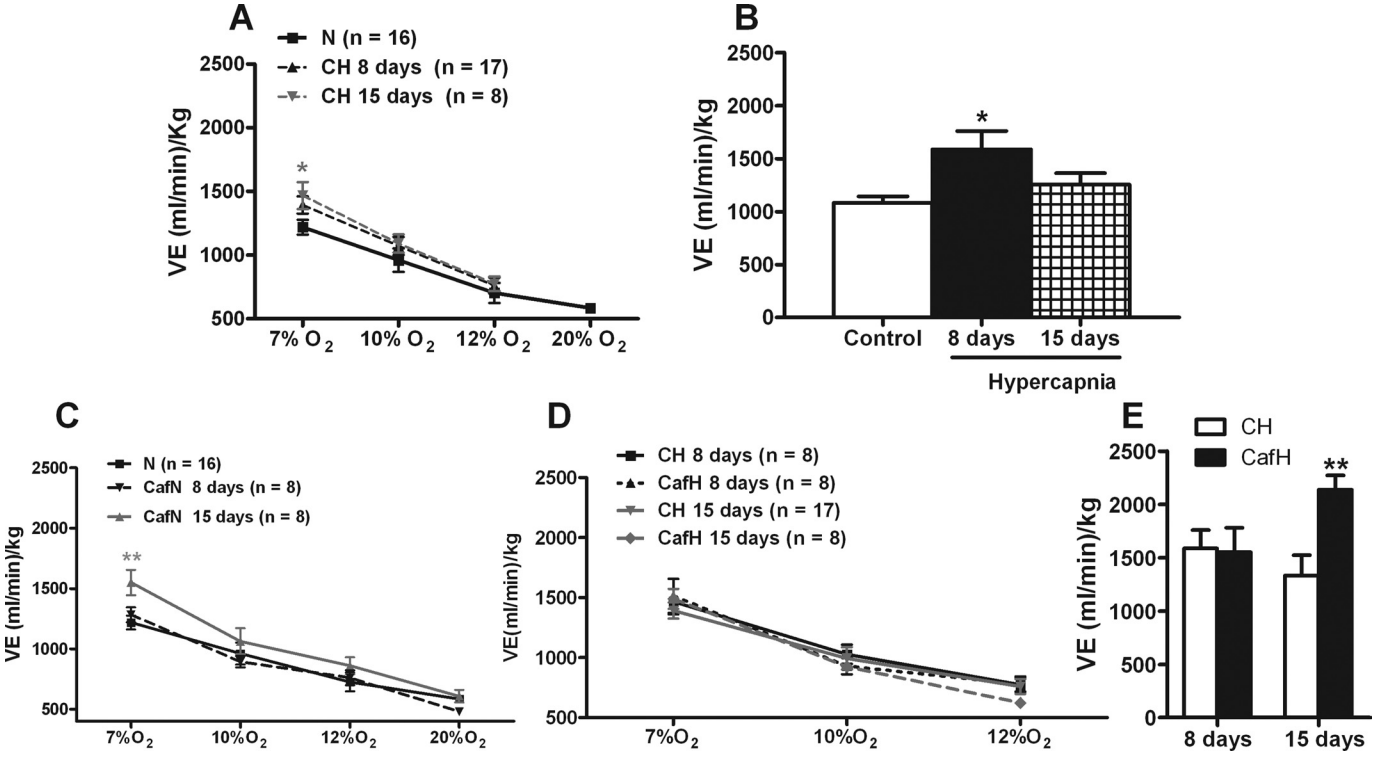

Fig. 1. Effect of 8 and 15 days exposure to hypoxia (12% O_2) on the ventilatory responses to acute hypoxia (10 and 7% O_2) and hypercapnia (5% CO_2). A, ventilatory responses to acute hypoxia were assessed as VE. B, effect of acute application of hypercapnia on minute ventilation in control rats and in rats exposed to 8 and 15 days of chronic hypoxia. C, effect of chronic caffeine exposure on VE values in response to acute hypoxias of several intensities (12, 10, and 7% O_2) in normoxic animals. D, VE values in response to acute hypoxic tests of several intensities (12, 10, and 7% O_2) in animals submitted to 8 and 15 days of chronic hypoxia. E, VE values in response to acute hypercapnia (5% CO_2) in animals exposed to 8 and 15 days of chronic hypoxia. Acute hypoxias were applied three times for 10-min intervals with periods of 10 min in normoxia. Hypercapnia was applied at the end of the experimental procedure between minutes 100 and 110. For each animal and for the entire population of animals, minute ventilation data were normalized to unit body weight. Minute volume values were corrected to the rat's weights. *, $p < 0.05$ versus control values, one and two-way ANOVA with a Bonferroni multicomparison test. Data represent means ± S.E.M.

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in response to an acute hypercapnic test (5% CO_2 in air) (Fig. 1B) was 46% ($p < 0.05$) and 16% (not significant) higher in animals exposed to chronic hypoxia for 8 and 15 days, respectively, than in normoxic animals.

Eight days of chronic caffeine intake did not produce significant alterations in VE in caffeine-treated normoxic animals in any of the atmospheres studied (20, 12, 10, and 7% O_2). However, after 15 days of caffeine ingestion, VE was augmented in all hypoxic atmospheres; the increase (34.8%) reached statistical significance in response to 7% O_2 (Fig. 1C). This long delay (>8 days in our experiments) for the manifestation of caffeine effects on ventilation is commonly seen in premature infants subjected to caffeine treatment for apneic episodes (Steer et al., 2004). Caffeine-treated chronically hypoxic groups showed ventilatory responses indistinguishable from those found in chronically hypoxic animals in all conditions studied (Fig. 1D). After 15 days, caffeine-treated chronically hypoxic animals showed a hypercapnic ventilatory response 57.9% higher than that of chronically hypoxic animals ($p < 0.01$) (Fig. 1E). Taken together, data indicate that both acclimatization and the effect of caffeine developed at day 15.

Effects of Chronic Caffeine Intake on Carotid Sinus Nerve Activity Evoked by Hypoxia and Hypercapnia in Normoxic and Chronically Hypoxic Rats. Although not clearly evident in the experiments depicted in Fig. 2, A to C, chronically hypoxic animals exhibited augmented basal CSN activity (9.76 ± 0.89 impulses/s) compared with that observed in normoxic animals (5.42 ± 0.81 impulses/s; $p < 0.001$) (Fig. 2D). Caffeine ingestion did not modify basal activity: there were no differences in the basal CSN action potential frequency between normoxic and caffeine-treated normoxic animals or between CH and CafH animals (Fig. 2D).

CSN activity in response to all stimuli was normalized to correspondent basal activity; thus, data are expressed as time over basal. Figure 2E shows that chronic caffeine intake in normoxia (caffeine-treated normoxic animals) tended to increase the CSN responses to moderate and intense hypoxia (25.5 and 33.6%, respectively; not significant), having no effect on the response to hypercapnia (compare N and CafN groups in Fig. 2E). Compared with normoxic animals, chronically hypoxic animals have nearly identical CSN peak frequency/basal frequency ratios in response to moderate (5% O_2) and intense hypoxia (0% O_2). The response to hypercapnic stimulus, however, decreased by 49% (compare N and CH groups in Fig. 2E). Because basal activity was nearly double in chronically hypoxic than in normoxic animals, the data imply that the absolute value of the evoked CSN activity for the hypoxic responses in chronically hypoxic animals was approximately double that in normoxic animals; i.e., chronic exposure to hypoxia has sensitized the CB to hypoxia. The absolute value of the response to hypercapnic stimulus was comparable in normoxic and chronically hypoxic animals. In comparison with chronically hypoxic animals, caffeine-treated chronically hypoxic animals exhibited approximately halved CSN peak frequency/basal frequency ratios to all stimuli (compare CH and CafH groups in Fig. 2E). To sum

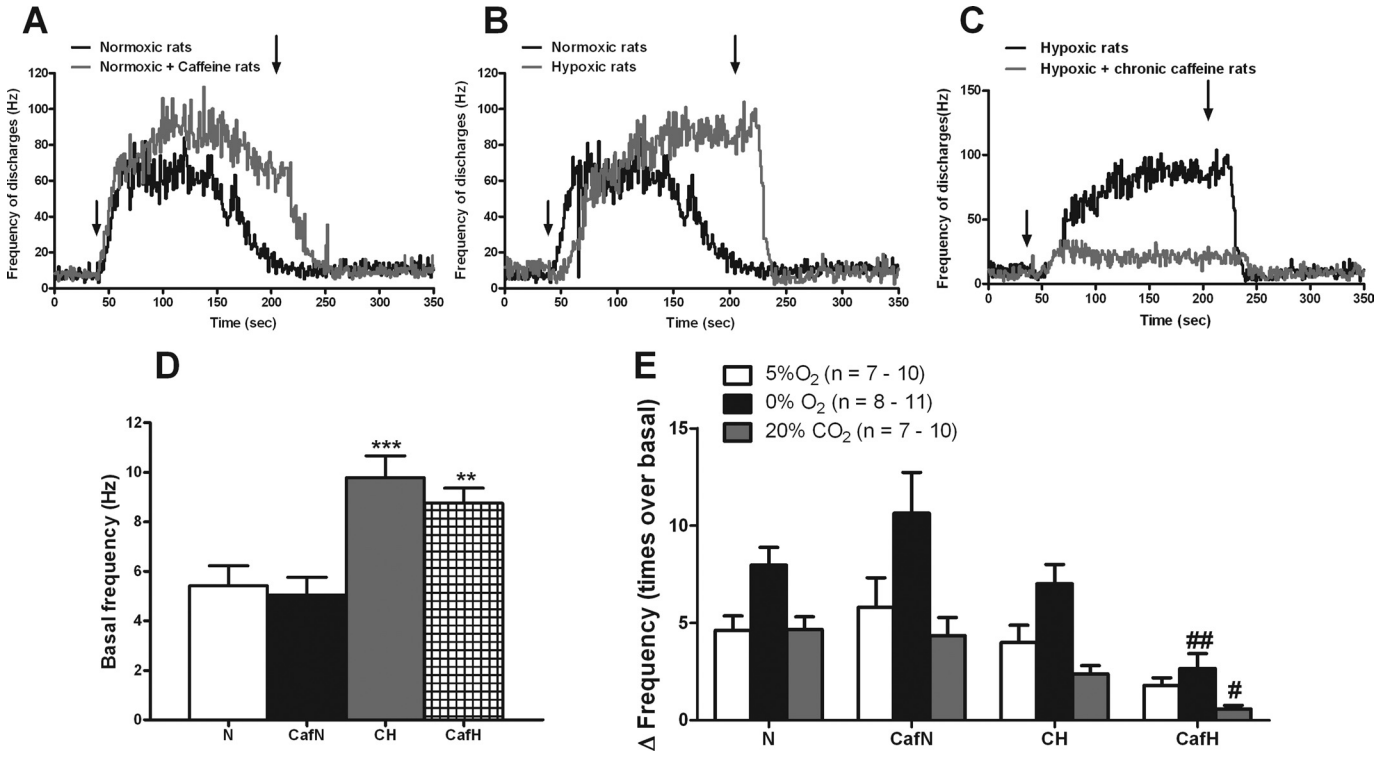

Fig. 2. Effects of chronic caffeine intake on CSN activity in normoxic and chronically hypoxic rats. A, typical recording for the effect of chronic caffeine ingestion on the frequency of action potentials of CSN in response to N_2 in normoxic rats. B, typical recording of the frequency of action potentials of CSN in response to N_2 (0% O_2) in normoxic and chronically hypoxic rats. C, typical recording for the effect of chronic caffeine ingestion on the frequency of action potentials of CSN in response to N_2 in chronically hypoxic rats. Arrows indicate the period of application of the acute hypoxic stimulus (N_2). D and E, means of basal CSN activity and means of increases in peak frequencies, respectively, in control (N), CafN, CH, and CafH groups. Data represent means ± S.E.M. of n individual values given in the figure. **, $p < 0.01$; ***, $p < 0.001$, versus normoxic values; #, $p < 0.05$; ##, $p < 0.01$, versus chronically hypoxic values.

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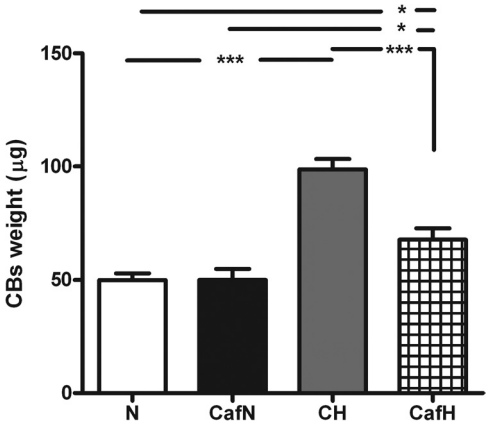

Fig. 3. Effect of chronic caffeine intake, chronic hypoxia, and both treatments applied together on carotid body weight. Data represent means ± S.E.M. *, $p < 0.05$; ***, $p < 0.001$, one-way ANOVA with Bonferroni multicomparison test.

up, our results uncover a dual effect of chronic ingestion of caffeine: a trend to augment acute hypoxic CSN responses in normoxic animals (opposite to the effect produced by acute caffeine) (Conde et al., 2006) and a clear inhibition of acute hypoxic (and hypercapnic) CSN responses in animals chronically exposed to hypoxia. In other words, chronic caffeine ingestion completely abolished the chronic hypoxia CB-mediated acclimatization to hypoxic stimulus. The experiments that follow were aimed to uncover the mechanism of CB-mediated acclimatization and the pathways through which caffeine eliminates it.

Effect of Chronic Caffeine Intake on Carotid Body Weight in Control and Chronically Hypoxic Rats. Figure 3 shows the weighs of the CB in the four experimental groups. In 16 control CBs (normoxic animals), the mean CB weight was 49.94 ± 2.95 μg, a value very similar to that reported previously by Conde et al. (2006) and not different from that of caffeine-treated normoxic rats (50.07 ± 4.81 μg; $n = 15$). The mean CB weight of chronically hypoxic animals was 98.79 ± 4.55 μg ($n = 14$). Caffeine ingestion during chronic hypoxia exposure prevented the increase in CB weight, because in caffeine-treated chronically hypoxic animals, the weight of the CB was 67.80 ± 5.01 μg ($n = 15$). The increase in CB size observed should be due to an increase in intraglomic blood vessels (Laidler and Kay, 1975) mediated by hypoxia-inducible factor-1α and vascular endothelial growth factor (Tipoe and Fung, 2003; Prabhakar et al., 2009) and by adenosine receptors (Auchampach, 2007).

Effects of Chronic Caffeine Intake on A_{2A} and A_{2B} Adenosine Receptor Expression in CB and PG in Normoxic and Chronically Hypoxic Rats. We limited our search to A_{2B} receptors in the CBs because adenosine effects on chemoreceptor cells (e.g., augmentation of DA release and increase in cAMP levels) are mediated by A_{2B} receptors (Conde et al., 2008) and also because functional data on the significance of A_{2A} receptors in chemoreceptor cells are controversial (see Kobayashi et al., 2000 versus Xu et al., 2006). In addition, although rabbit chemoreceptor cells express A_1 receptors (Rocher et al., 1999), they have not been detected in rat cells (Gauda, 2000). Levels of expression of A_{2B} receptors in caffeine-treated normoxic rats as well as those in chronically hypoxic animals were not different from those seen in normoxic animals (Fig. 4, A and C), but in caffeine-treated chronically hypoxic animals, there was a down-regulation in the expression of A_{2B} receptors (A_{2B}/tubulin ratios dropped to 61.71 ± 8.24% of that seen in chronically hypoxic animals; $p < 0.05$).

In PG, we limited our search to A_{2A} receptors because there is no evidence for the presence of A_1 receptors in the rat CB (Gauda, 2000; Conde et al., 2009) and A_{2B}-mediated

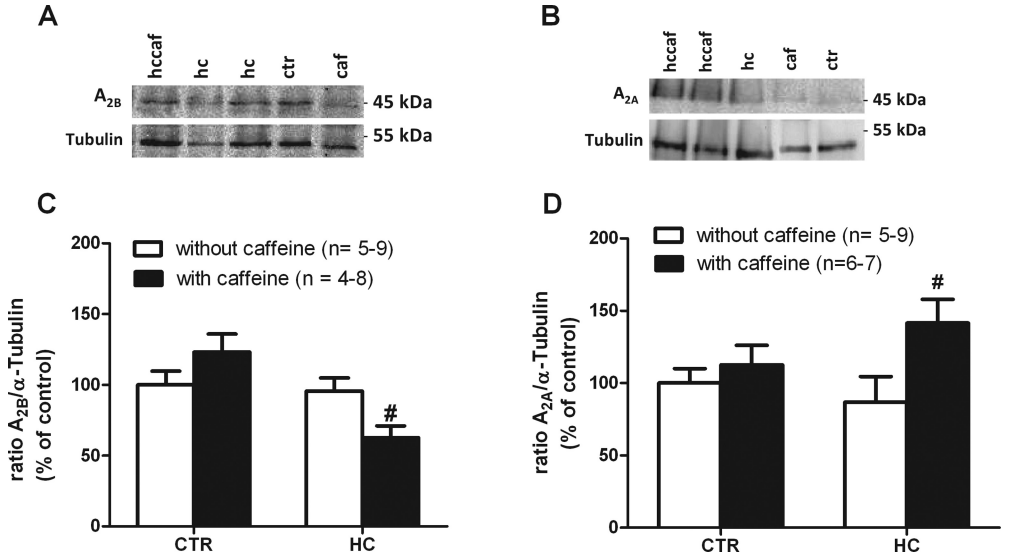

Fig. 4. A_{2B} and A_{2A} adenosine receptor immunoreactivity in the CB and PG, respectively, in normoxic rats (ctr) and in rats submitted to different treatments: chronic caffeine intake (caf), chronic hypoxia exposure (hc), and chronic caffeine + chronic hypoxia (hccaf). A, Western blot comparing A_{2B} immunoreactivity, corresponding to the 45-kDa band, in CBs (controls and submitted to different paradigms). A reprobing of the membranes with an α-tubulin antibody, corresponding to the 65-kDa band is shown below the gel for A_{2B} receptors. B, Western blot comparing A_{2A} immunoreactivity corresponding to the 45-kDa band, in PGs (controls and submitted to different paradigms). A reprobing of the membranes with an α-tubulin antibody, corresponding to the 65-kDa band is shown below the gel for A_{2A}. C, average relative A_{2B} immunoreactivity in the CB ($n = 4$–9) in the different paradigms expressed in relation to α-tubulin immunoreactivity. D, average relative A_{2A} immunoreactivity in the PG ($n = 5$–9) in the different paradigms expressed in relation to α-tubulin immunoreactivity. #, $p < 0.05$, two-way ANOVA with a Bonferroni multicomparison test, comparing values within the same group. Data represent means ± S.E.M.

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effects in the CB CSN preparations of this species are presynaptically mediated. Figure 4, B and D, shows that adenosine A_{2A} receptor expression was not different in normoxic, caffeine-treated normoxic, and chronically hypoxic animals; however, in caffeine-treated chronically hypoxic rats, there was an up-regulation of A_{2A} expression (A_{2A}/tubulin ratios were 141.57 ± 16.19% of that seen in chronically hypoxic animals; $p < 0.05$).

Effects of Chronic Hypoxia and Caffeine Ingestion on the Release of DA and Adenosine Release in Basal Conditions and in Response to Acute Hypoxia. The protocol for release experiments consisted of a sequential incubation of pools of four CBs in normoxic solutions (10 min), moderate hypoxia (7% O_2 saturated; 10 min), two additional normoxic 10-min incubations, a strong hypoxic incubation (2% O_2 saturated), and a final normoxic incubation. Figure 5A shows mean normoxic (basal) release of adenosine and DA in the four experimental groups measured in the initial 10-min normoxic incubation. Basal adenosine release by the CBs of normoxic and caffeine-treated normoxic animals was not different. Exposure of the animals to chronic hypoxia dramatically reduced basal release of adenosine to nearly half, both in chronically hypoxic and in caffeine-treated chronically hypoxic rats ($p < 0.01$ versus corresponding normoxic group). Basal release of DA (left part of Fig. 5A) was much lower than that of adenosine in all experimental groups (see units on both y-axes). Exposure to chronic hypoxia produced effects on the basal release of DA that mirrored those seen for adenosine, with basal DA release in chronically hypoxic and caffeine-treated chronically hypoxic rats double those seen in the corresponding normoxic groups ($p < 0.001$). The release of adenosine evoked by 7 and 2% O_2 is depicted in Fig. 5, B and C. In normoxic rats (Fig. 5B), the release elicited by moderate hypoxia (7% O_2) equaled or overcame that elicited by intense hypoxia (2% O_2) (Conde et al., 2006, 2012a); the hypoxia-induced release of adenosine in caffeine-treated normoxic rats was not different from that seen in normoxic animals. In a comparison of Fig. 5, B and C, it can also be seen that 1) in the CB of chronically hypoxic rats, acute hypoxia tests showed that the release of adenosine correlated with the intensity of tests (231% of basal release for 7% O_2 and 372% of basal release for 2% O_2), 2) chronic caffeine ingestion in animals exposed to chronic hypoxia did not alter basal or hypoxia-induced release of adenosine (i.e., the release in chronically hypoxic and caffeine-treated chronically hypoxic rats was not different) (compare CH and CafH groups in Fig. 5C), 3) the hypoxia-induced adenosine release (i.e., the release measured during the hypoxic stimulation subtracted from the basal normoxic release measured in the 10-min period immediately before hypoxic stimulation is much higher for hypoxic stimuli in both chronically hypoxic and caffeine-treated chronically hypoxic rats than in the corresponding normoxic groups, and 4) basal release in all experimental groups did not show any time-dependent modification during the experiments, indicating the peculiarities of the metabolism and absence of cellular stores for this neurotransmitter.

Data on the release of DA are presented in Table 1. Note the following findings. 1) In all groups, the basal release decay is time-dependent, so that in the second period of incubation with normoxic solutions after a period of hypoxic stimulation the release was ≈40 to 50% smaller than in the initial normoxic period. 2) When the release response was expressed as a percentage of the corresponding control, the higher the stimulus (7 and 2% O_2, 10 min), the larger was the release induced by acute hypoxia. 3) In chronically hypoxic

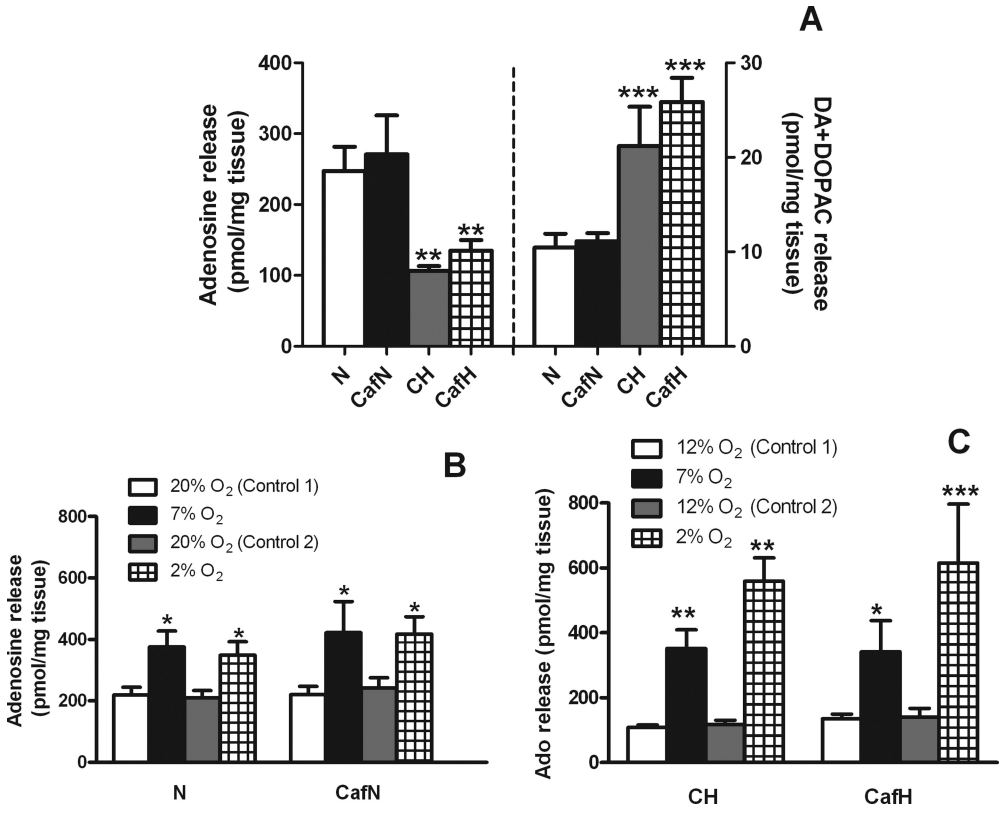

Fig. 5. Effects of chronic caffeine intake on the carotid body basal endogenous release of adenosine and dopamine (A) and on the release of adenosine elicited by acute hypoxia (7% and 2% O_2) in normoxic (B) and chronically hypoxic rats (C). N, $n = 6$; CafN, $n = 7$; CH, $n = 5$; and CafH, $n = 6$. Control 1 and Control 2 represent basal release before the application of the acute hypoxic stimulus. *, $p < 0.05$; **, $p < 0.01$, two-way ANOVA with a Bonferroni multicomparison test. DOPAC, 3,4-dihydroxyphenylacetic acid.

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TABLE 1

Basal and hypoxia-evoked release of endogenous dopamine in CBs of control normoxic, caffeine-treated normoxic, chronically hypoxic, and caffeine-treated chronically hypoxic animals

In the columns referring to hypoxic stimuli (7 and 2% O_2), values represent absolute amounts released and the values in parentheses represent release during hypoxic stimulation expressed as a percentage of the preceding control (20% O_2 incubation). Data represent means ± S.E.M.

Experimental Group	20 or 12% O_2, 10 min	7% O_2, 10 min	20 or 12% O_2, 10 min	2% O_2, 10 min
		pmol/mg tissue/10 min (%)		
Control, normoxic	10.4 ± 1.5	17.4 ± 1.7* (167.30)	6.5 ± 1.5	14.2 ± 2.1* (218.46)
Caffeine-treated normoxic	11.1 ± 0.9	21.7 ± 2.0** (195.50)	5.5 ± 1.2	13.3 ± 2.5** (241.82)
Chronically hypoxic (12% O_2, 15 days)	21.2 ± 4.1	36.4 ± 2.9* (171.69)	12.7 ± 2.2	36.0 ± 5.3** (283.46)
Caffeine-treated chronically hypoxic	25.9 ± 2.5	53.9 ± 5.4*** (208.10)	16.5 ± 1.5	38.0 ± 5.2** (230.30)

*$p < 0.05$, versus preceding control values, two-way ANOVA with Bonferroni multicomparison test.
**$p < 0.01$.
***$p < 0.001$.

and caffeine-treated chronically hypoxic rats, both hypoxic stimuli evoked a release response that was comparable percentage-wise to those of the corresponding normoxic groups; however, in absolute amounts the stimuli were much larger. 4) Chronic caffeine ingestion, both in normoxic and chronically hypoxic animals tended to augment the release response elicited by the mild hypoxic stimulus, but the response to an intense hypoxic stimulus was not consistently modified by caffeine ingestion; this finding indicates that adenosine modulates the release of DA elicited by moderate but not by intense hypoxia (Conde et al., 2006).

Discussion

The present work demonstrates the following. 1) Chronic hypoxia elicits acclimatization in the CB, as determined by both CB output (CSN activity) and ventilatory responses. 2) In normoxic animals, chronic caffeine ingestion affects neither basal nor hypoxia-elicited CSN activity but produces an increase in the ventilatory response to hypoxia, indicating that caffeine augments the gain of the chemoreflex through centrally mediated mechanisms. 3) In chronically hypoxic animals, caffeine ingestion abrogates acclimatization measured as CSN activity (afferent arm of the chemoreflex). However, acclimatization is still present in these animals if measured as the chemoreflex output (ventilatory response). Overall, our findings indicate that caffeine ingestion does not negatively affect the acclimatization phenomenon in adaptation to chronic hypoxia.

Carotid Body Sensitization by Chronic Hypoxia: The Acclimatization Process. We found that chronic hypoxia elicits acclimatization measured both as ventilatory responses and as CSN activity. Ventilatory sensitization to hypoxia was evident at day 8 and was more pronounced at day 15 of chronic hypoxia. The acclimatization process was also evident at the level of CSN activity (15 days) because both basal and hypoxic action potential frequency increased by day 15.

DA concentration in the milieu surrounding chemoreceptor cells and CSN sensory nerve endings is increased in CH animals (Table 1), suggesting that DA, whose function in normoxia is greatly discussed (Gonzalez et al., 1994 versus Iturriaga and Alcayaga, 2004; Iturriaga et al., 2009), definitively contributes to optimize CB sensitivity to hypoxia in CH animals. DA metabolic modifications comparable to those described in the CB have been reported to occur in the central projections of the CSN at the nucleus of the tractus solitarius, where it has been shown that DA facilitates acclimatization; in fact D_2-dopamine receptor-null mice, which in normoxia have nearly normal ventilation exhibit markedly decreased acclimatization to chronic hypoxia (Huey et al., 2003).

Chronic hypoxia does not alter the expression of adenosine receptors in CB (A_{2B}) and in PG (A_{2A}), but it profoundly affects the metabolism of adenosine. An explanation for the contribution of adenosine to augmented CSN activity that occurs in CH animals could be as follows: because acute caffeine does not affect basal CSN frequency, whereas it inhibits by nearly 60% the CSN elicited by hypoxia (Conde et al., 2006), it might be suggested that the significance of adenosine in setting basal CSN activity is minor, implying that the decrease in basal adenosine release observed in chronically hypoxic animals does not represent a brake for increased basal activity. Instead, the decrease in adenosine release appears to represent a major player in the genesis of the increased response to acute hypoxia. However, the generation of the increase in basal activity remains elusive. Although Chen et al. (2002, 2007) showed that endothelin contributes to it, available data indicate that endothelin receptors are expressed on chemoreceptor cells, and, therefore, its action must be mediated via modification of neurotransmitter release from the cells. We believe that increased adenosine release during CB stimulation is mainly responsible for generating the augmented CSN activity observed in chronically hypoxic animals during acute CB stimulation (Conde et al., 2006, 2012a).

Finally, although ATP is a potent excitatory neurotransmitter in the CB (Nurse 2010; Conde et al., 2012a), it has been proposed that chronic hypoxia does not change $P2X_2$ receptor density in PG and that contribution of endogenous ATP to the genesis of acute responses to hypoxia decreases in the CB of chronically hypoxic rats (He et al., 2006). An explanation for these observations is that the DA/ATP ratio in the dense-core neurotransmitter storage granules increases due to ATP decrease. This decrease would spare ATP in the unfavorable situation of chronic hypoxia (Lindqvist et al., 2002), the net result being that in chronically hypoxic animals ATP release would be diminished. In fact, in chronically hypoxic animals a change in the ratio of endothelin to ATP release from aortic endothelial cells due to a marked decrease in the release of ATP was observed (Bodin et al., 1992).

Another neurotransmitter, ACh, has classically been proposed to be involved in CB function (Gonzalez et al., 1994). We have not explored its significance in acclimatization because the capacity of rat chemoreceptor cells to synthesize ACh has been questioned (Gauda et al., 2004), and He et al. (2005) showed that blockers of cholinergic receptors were

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ineffective in inhibiting hypoxia-induced CSN activity in chronically hypoxic rats.

Chronic Caffeine Intake in Normoxic Animals. In comparison to normoxic rats, caffeine-treated normoxic animals exhibit CSN responses to hypoxia that tend to be higher and also significantly augmented ventilatory responses to hypoxia. The increase in VE implies that the excitatory effect of caffeine on ventilation results from a minor effect on peripheral chemoreceptors and a major effect on central respiratory neurons. This observation raises two questions: how does chronic caffeine increase the brainstem gain of the CSN input and how does it tend to increase CSN input itself?

The excitatory effect of chronic caffeine in response to hypoxia at the brainstem level is well known in infants (Herlenius and Lagercrantz, 1999), being the treatment of choice for apneas and other respiratory dysrhythmias of the newborn (Mathew 2011). In addition, Pianosi et al. (1994) found that the slope of the line defined by the ratio VE/partial pressure of carbon dioxide (millimeters of mercury) was duplicated by caffeine in adult humans. Several recent studies (Wang et al., 2005; Vandam et al., 2008) have shown that adenosine decreases respiratory rhythmogenesis by activating A_1 and A_2 receptors at different brainstem nuclei; therefore, the answer to the first question may reside in the A_1 and A_2 receptor antagonism by caffeine, which would result in the observed increase in VE.

The second question has a more complex answer. Chronic caffeine actions contrast with its acute effects (Jacobson et al., 1996; Riksen et al., 2009). Thus, whereas acute caffeine inhibits hypoxia-induced CSN activity without affecting normoxic activity (Howell and Landrum, 1995; Conde et al., 2006) and markedly inhibits basal and low-intensity hypoxia-induced release of DA (Conde et al., 2006), chronic caffeine tends to increase the CSN activity response to hypoxia, does not affect basal DA release, and increases the release induced by moderate hypoxia. Finally, although chronic caffeine did not modify the metabolism of adenosine in normoxic animals in our experimental setting, it showed a trend to increase both A_{2B} receptors in the CB and A_{2A} receptors in the PG. We hypothesize that the trend to increase A_{2A} receptors together with normal adenosine levels results in the observed increase in CSN activity in response to hypoxia. In addition, the trend to increase A_{2B} receptor expression in the CB would explain the observed augmentation of the release of DA because activation of A_{2B} receptors increases the release of DA via inhibition of dopaminergic D_2 chemoreceptor cell autoreceptors (Conde et al., 2008). Therefore, the observed propensity for CSN activity to increase in response to hypoxia in caffeine-treated normoxic animals is the additive result of the positive excitatory effect emerging from normal adenosine levels and augmented A_{2A} with the negative inhibitory effect caused by increased dopamine levels (Huey and Powell 2000; Iturriaga and Alcayaga, 2004).

Chronic Caffeine Intake in Rats Exposed to Chronic Hypoxia. Caffeine ingestion during chronic hypoxia did not alter the ventilatory response to hypoxia, but it increased ventilatory responses to hypercapnic stimulus. At the CSN level, caffeine abolished the sensitization induced by exposure to chronic hypoxia. These findings highlight a dual effect of caffeine in animals chronically exposed to hypoxia: the inhibition of the CB input to brainstem nuclei and a facilitatory effect at the central level, well exemplified by the hyperventilatory response to CO_2, which is mostly centrally mediated (Cherniak and Altose, 1997). Our results show that the balanced dual actions of chronic caffeine in chronic hypoxia represent an effective way of preventing an excessive hypoxic ventilatory response and resulting undesirable exaggerated hypocapnia and, more importantly, indicate that caffeine does not have deleterious effects in chronically hypoxic animals (Riksen et al., 2009).

The marked decrease in the CSN response to hypoxia and hypercapnia in caffeine-treated chronically hypoxic rats (versus chronically hypoxic rats) supports our previous conclusion that adenosine plays a major role in the genesis of the augmented CB responses to natural stimuli in acclimatized rats: this critical role is absent in caffeine-treated chronically hypoxic rats due to chronic adenosine receptor blockade. The mechanisms mediating these effects are not identified, but knowing that the excitatory action of adenosine is mediated nearly in the same proportion by A_{2A} and A_{2B} (Conde et al., 2008), up- and down-regulated, respectively, by caffeine ingestion in animals exposed to chronic hypoxia, we agree with the statement made by Bairam et al. (2012) that chronic caffeine treatment promotes subtle changes in the CB dopaminergic and adenosinergic pathways. These authors also stated that the physiological implications of these subtle changes are still unclear; however our data indicate that the physiological significance is the loss of the sensitization of CB by chronic hypoxia. It can also be hypothesized that the central facilitatory effect of caffeine is also mediated, at least partially, by interactions between the dopaminergic (i.e., central projections of CSN fibers) (Katz et al., 1997) and adenosinergic pathways (Gourine et al., 2002) in the nucleus of the tractus solitarius (Huey et al., 2003).

In conclusion, acclimatization, which is in great part CB-mediated, is a multifactorial process that involves modifications in DA and adenosine receptor expression in chemoreceptor cells and sensory nerve endings with an overall increase in the CB output. At the central brainstem level, a faithful reading of the augmented CB input leads to acclimatization translated into changes in ventilation. Chronic caffeine ingestion alters many of the CB effects of chronic hypoxia, leading to a loss of acclimatization sensed as CB output, but, at the same time, causes at the brainstem level an increase in the gain of the CB input reading leading to maintenance of chronic hypoxia acclimatization in the ventilatory parameters. Extrapolating our findings in the rat to humans, we postulate that normal caffeine ingestion would ameliorate ventilation in response to acute hypoxia in normoxic subjects, would not harm hypoxic ventilation in humans subjected to chronic hypoxia, and would ameliorate their ventilatory responses to potential concurrent hypercapnia.

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Authorship Contributions

Participated in research design: Conde and Gonzalez.
Conducted experiments: Conde and Ribeiro.
Performed data analysis: Conde, Ribeiro, Obeso, and Rigual.
Wrote or contributed to the writing of the manuscript: Conde, Monteiro, and Gonzalez.

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References

Auchampach JA (2007) Adenosine receptors and angiogenesis. *Circ Res* **101:**1075–1077.

Bairam A, Niane LM, and Joseph V (2012) Role of ATP and adenosine on carotid body function during development. *Respir Physiol Neurobiol*. http://dx.doi.org/j.resp.2012.06.016.

Bisgard GE (2000) Carotid body mechanisms in acclimatization to hypoxia. *Respir Physiol* **121:**237–246.

Bisgard G and Neubauer J (1975) Peripheral and central effects of hypoxia in the control of breathing, in *Regulation of Breathing* (Dempsey JA, Pack AI eds) vol. 79, pp 617–668, Marcel Dekker, New York.

Bodin P, Milner P, Winter R, and Burnstock G (1992) Chronic hypoxia changes the ratio of endothelin to ATP release from rat aortic endothelial cells exposed to high flow. *Proc Biol Sci* **247:**131–135.

Caceres AI, Obeso A, Gonzalez C, and Rocher A (2007) Molecular identification and functional role of voltage-gated sodium channels in rat carotid body chemoreceptor cells. Regulation of expression by chronic hypoxia in vivo. *J Neurochem* **102:**231–245.

Chen J, He L, Dinger B, Stensaas L, and Fidone S (2002) Role of endothelin and endothelin A-type receptor in adaptation of the carotid body to chronic hypoxia. *Am J Physiol Lung Cell Mol Physiol* **282:**L1314–L1323.

Chen J, He L, Liu X, Dinger B, Stensaas L, and Fidone S (2007) Effect of the endothelin receptor antagonist bosentan on chronic hypoxia-induced morphological and physiological changes in rat carotid body. *Am J Physiol Lung Cell Mol Physiol* **292:**L1257–L1262.

Chen X, Ghribi O, and Geiger JD (2010) Caffeine protects against disruptions of the blood-brain barrier in animal models of Alzheimer's and Parkinson's diseases. *J Alzheimers Dis* **20:**S127–S141.

Cherniak N and Altose M (1997) Central chemoreceptors, in *The Lung: Scientific Foundations* (Crystal R, West J, Weibel E, and Barnes P eds) pp 1767–1776, Lippincott-Raven, Philadelphia.

Conde SV, Gonzalez C, Batuca JR, Monteiro EC, and Obeso A (2008) An antagonistic interaction between A2B adenosine and D2 dopamine receptors modulates the function of rat carotid body chemoreceptor cells. *J Neurochem* **107:**1369–1381.

Conde SV and Monteiro EC (2004) Hypoxia induces adenosine release from the rat carotid body. *J Neurochem* **89:**1148–1156.

Conde SV, Monteiro EC, Obeso A, and Gonzalez C (2009) Adenosine in peripheral chemoreception: new insights into a historically overlooked molecule, in *The Arterial Chemoreceptors* (Gonzalez C, Nurse CA, Peers C eds) pp 145–159, Springer, Dordrecht, The Netherlands.

Conde SV, Monteiro EC, Rigual R, Obeso A, and Gonzalez C (2012a) Hypoxic intensity: a determinant for the contribution of ATP and adenosine to the genesis of carotid body chemosensory activity. *J Appl Physiol* **112:**2002–2010.

Conde SV, Nunes da Silva T, Gonzalez C, Mota Carmo M, Monteiro EC, and Guarino MP (2012b) Chronic caffeine intake decreases circulating catecholamines and prevents diet-induced insulin resistance and hypertension in rats. *Br J Nutr* **107:**86–95.

Conde SV, Obeso A, Vicario I, Rigual R, Rocher A, and Gonzalez C. (2006) Caffeine inhibition of rat carotid body chemoreceptors is mediated by A2A and A2B adenosine receptors. *J Neurochem* **98:**616–628.

Fredholm BB, Bättig K, Holmén J, Nehlig A, and Zvartau EE (1999) Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol Rev* **51:**83–133.

Ganfornina MD, Pérez-García MT, Gutiérrez G, Miguel-Velado E, López-López JR, Marín A, Sánchez D, and González C (2005) Comparative gene expression profile of mouse carotid body and adrenal medulla under physiological hypoxia. *J Physiol* **566:**491–503.

Gasior M, Jaszyna M, Munzar P, Witkin JM, and Goldberg SR (2002) Caffeine potentiates the discriminative-stimulus effects of nicotine in rats. *Psychopharmacology* **162:**385–395.

Gauda EB (2000) Expression and localization of A2a and A1-adenosine receptor genes in the rat carotid body and petrosal ganglia. A2a and A1-adenosine receptor mRNAs in the rat carotid body. *Adv Exp Med Biol* **475:**549–558.

Gauda EB, Cooper R, Johnson SM, McLemore GL, and Marshall C (2004) Autonomic microganglion cells: a source of acetylcholine in the rat carotid body. *J Appl Physiol* **96:**384–391.

Gonzalez C, Almaraz L, Obeso A, and Rigual R (1994) Carotid body chemoreceptors: from natural stimuli to sensory discharges. *Physiol Rev* **74:**829–898.

Gourine AV, Llaudet E, Thomas T, Dale N, and Spyer KM (2002) Adenosine release in nucleus tractus solitarii does not appear to mediate hypoxia-induced respiratory depression in rats. *J Physiol* **544:**161–170.

He L, Chen J, Dinger B, Stensaas L, and Fidone S (2006) Effect of chronic hypoxia on purinergic synaptic transmission in rat carotid body. *J Appl Physiol* **100:**157–162.

He L, Dinger B, and Fidone S (2005) Effect of chronic hypoxia on cholinergic chemotransmission in rat carotid body. *J Appl Physiol* **98:**614–619.

Herlenius E and Lagercrantz H (1999) Adenosinergic modulation of respiratory neurones in the neonatal rat brainstem in vitro. *J Physiol* **518:**159–172.

Howell LL and Landrum AM (1995) Attenuation of hypoxia-induced increases in ventilation by adenosine antagonists in rhesus monkeys. *Life Sci* **57:**773–783.

Huey KA and Powell FL (2000) Time-dependent changes in dopamine D_2-receptor mRNA in the arterial chemoreflex pathway with chronic hypoxia. *Mol Brain Res* **75:**264–270.

Huey KA, Szewczak JM, and Powell FL (2003) Dopaminergic mechanisms of neural plasticity in respiratory control: transgenic approaches. *Respir Physiol Neurobiol* **135:**133–144.

Iturriaga R and Alcayaga J (2004) Neurotransmission in the carotid body: transmitters and modulators between glomus cells and petrosal ganglion nerve terminals. *Brain Res Brain Res Rev* **47:**46–53.

Iturriaga R, Alcayaga J, and Gonzalez C (2009) Neurotransmitters in carotid body function: the case of dopamine—invited article. *Adv Exp Med Biol* **648:**137–143.

Jacobson KA, von Lubitz DK, Daly JW, and Fredholm BB (1996) Adenosine receptor ligands: differences with acute versus chronic treatment. *Trends Pharmacol Sci* **17:**108–113.

Katz DM, Finley JCW, Erickson JT and Brosenitsch TA (1997) Organization and development of chemoafferent input to the brainstem, in *The Carotid Body Chemoreceptors* (Gonzalez C ed) pp 150–170, Springer-Verlag, Berlin.

Kobayashi S, Conforti L, and Millhorn DE (2000) Gene expression and function of adenosine A_{2A} receptor in the rat carotid body. *Am J Physiol Lung Cell Mol Physiol* **279:**L273–L282.

Laidler P and Kay JM (1975) A quantitative morphological study of the carotid bodies of rats living at a simulated altitude of 4300 metres. *J Pathol* **117:**183–191.

Lindqvist A, Dreja K, Swärd K, and Hellstrand P (2002) Effects of oxygen tension on energetics of cultured vascular smooth muscle. *Am J Physiol Heart Circ Physiol* **283:**H110–H117.

Mathew OP (2011) Apnea of prematurity: pathogenesis and management strategies. *J Perinatol* **31:**302–310.

Mkrtchian S, Kåhlin J, Ebberyd A, Gonzalez C, Sanchez D, Balbir A, Kostuk EW, Shirahata M, Fagerlund MJ, and Eriksson LI (2012) The human carotid body transcriptome with focus on oxygen sensing and inflammation—a comparative analysis. *J Physiol* **590:**3807–3819.

Nurse CA (2010) Neurotransmitter and neuromodulatory mechanisms at peripheral arterial chemoreceptors. *Exp Physiol* **95:**657–667.

Pianosi P, Grondin D, Desmond K, Coates AL, and Aranda JV (1994) Effect of caffeine on the ventilatory response to inhaled carbon dioxide. *Respir Physiol* **95:**311–320.

Powell FL (2007) The influence of chronic hypoxia upon chemoreception. *Respir Physiol Neurobiol* **157:**154–161.

Prabhakar NR, Peng YJ, Kumar GK, Nanduri J, Di Giulio C, and Lahiri S (2009) Long-term regulation of carotid body function: acclimatization and adaptation—invited article. *Adv Exp Med Biol* **648:**307–317.

Riksen NP, Rongen GA, and Smits P (2009) Acute and long-term cardiovascular effects of coffee: implications for coronary heart disease. *Pharmacol Ther* **121:**185–191.

Rocher A, Gonzalez C, and Almaraz L (1999) Adenosine inhibits L-type Ca^{2+} current and catecholamine release in the rabbit carotid body chemoreceptor cells. *Eur J Neurosci* **11:**673–681.

Steer P, Flenady V, Shearman A, Charles B, Gray PH, Henderson-Smart D, Bury G, Fraser S, Hegarty J, Rogers Y, et al. (2004) High dose caffeine citrate for extubation of preterm infants: a randomised controlled trial. *Arch Dis Child Fetal Neonatal Ed* **89:**F499–F503.

Tipoe GL and Fung ML (2003) Expression of HIF-1α, VEGF and VEGF receptors in the carotid body of chronically hypoxic rat. *Respir Physiol Neurobiol* **138:**143–154.

Vandam RJ, Shields EJ, and Kelty JD (2008) Rhythm generation by the pre-Bötzinger complex in medullary slice and island preparations: effects of adenosine A_1 receptor activation. *BMC Neurosci* **9:**95.

Vicario I, Rigual R, Obeso A, and Gonzalez C (2000) Characterization of the synthesis and release of catecholamine in the rat carotid body in vitro. *Am J Physiol Cell Physiol* **278:**C490–C499.

Wang JL, Wu ZH, Pan BX, and Li J (2005) Adenosine A1 receptors modulate the discharge activities of inspiratory and biphasic expiratory neurons in the medial region of nucleus retrofacialis of neonatal rat in vitro. *Neurosci Lett* **379:**27–31.

Xu F, Xu J, Tse FW, and Tse A (2006) Adenosine stimulates depolarization and rise in cytoplasmic $[Ca^{2+}]$ in type I cells of rat carotid bodies. *Am J Physiol Cell Physiol* **290:**C1592–C1598.

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