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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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- Introduction – 792

- Discussion – 1675

Abbreviations used: CA, catecholamine; CB, carotid body; CH, chronic hypoxia; CSN, carotid sinus nerve; DA, dopamine; PG, petrosal ganglion; VE, minute volume;

Abstract

Sustained hypoxia produces a carotid body (CB) sensitization, known as acclimatization, which leads to an increase in the carotid sinus nerve (CSN) activity and ensuing hyperventilation greater than expected from the prevailing PO_2. Whether sustained hypoxia is physiological (high altitude) or pathological (lung disease), acclimatization has a homeostatic implication as it tends to minimize hypoxia. Caffeine, the most commonly ingested psychoactive drug and a non-selective adenosine receptor antagonist, alters CB function and ventilatory responses when acutely administered. Our aim is to investigate the effect of chronic caffeine intake on CB function and acclimatization using four groups of rats: normoxic, caffeine-treated normoxic, chronic hypoxic (12%O_2, 15 days) and caffeine-treated chronically hypoxic-rats. Caffeine was administered in drinking water (1mg/ml). Caffeine ameliorated ventilatory responses to acute hypoxia in normoxic animals without altering the output of the CB (CSN neural activity). Chronically hypoxic-caffeine-treated rats exhibited a decrease in the CSN response to acute hypoxic tests, but maintained ventilation when compared to chronic 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 indicate that caffeine abolishes CB acclimatization. The putative mechanisms involved in sensitization and its loss were investigated: expression of adenosine receptors in CB (A_{2B}) were downregulated and petrosal ganglion (PG; A_{2A}) were upregulated in caffeine treated-chronic hypoxic rats; both adenosine and dopamine release from CB chemoreceptor

cells were increased in chronic hypoxia and in caffeine-treated chronic hypoxia groups.

Introduction

The carotid bodies (CBs) chemoreceptors sense arterial blood gases and pH. Hypoxia and hypercapnia/acidosis activate CB chemoreceptor cells increasing their rate of neurotransmitters release (Gonzalez et al., 1994; Conde et al., 2012a), augmenting action potential frequency in the fibres 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 aimed to normalize blood gases and adequately distribute O_2 in the organism (Gonzalez et al., 1994).

Chronic hypoxia is a common situation which 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 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 hypoxic 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; Mkrtcian et al., 2012), causes hypertrophy of chemoreceptor cells a proliferation and widening of organ capillaries (Laidler and Kay, 1975) and it 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 while the A_{2A} effect is postsynaptic (Conde et al., 2006; Conde et al., 2008). The immatureness of the CB in neonates (Gonzalez et al., 1994) explains the prevalence of the central stimulatory effect in newborns animals while 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 it happens in hypoxia (Howell and Landrum, 1995).

The present study aims 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, chronic hypoxic and chronically

hypoxic-caffeine-treated. 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 alike those seen in normoxic animals and, at the same time, they presented increased VE, implying that caffeine centrally increases the reflex gain. Chronic hypoxic animals showed acclimatization manifested by an increase in CSN activity and VE. In comparison to chronic hypoxic animals, chronically hypoxic-caffeine-treated 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 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 were 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 hypoxic-induced release of adenosine, and did not change adenosine receptor expression. Chronically hypoxic-caffeine-treated rats showed DA and adenosine release responses comparable to those seen in chronic hypoxic animals, but exhibited decreased A_{2B} and increased A_{2A} receptor expression. These results are discussed in the frame of the current

literature aiming to understand the mechanisms of CB acclimatization and its loss in caffeine treated animals.

Material and Methods

Animals and anaesthesia. Chronic caffeine administration and exposure to chronic hypoxia. The experiments were performed in Wistar rats of both sexes aged 3-4 months (250-350g) obtained from the *vivarium* of the Faculty of Medicine of the University of Valladolid and from the *vivarium* of the Faculty of Medical Sciences of the Nova University of Lisbon. Animals were anaesthetized with sodium pentobarbital (60 mg/Kg, i.p.) and euthanized with an intracardiac overdose of the same anaesthetic. The Institutional Committee of the University of Valladolid and Nova University of Lisbon for Animal Care and Use approved the protocols. Control animals (N) were maintained in a normal room air atmosphere and chronic hypoxic rats (CH) were kept for 15 days in a chamber equilibrated with a gas mixture of 11-12% O_2 in 88-89 % N_2; $PO_2 \approx$ 80-84 mmHg and a constant a flow of 3L/min. The accumulation of CO_2 and water vapour 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 employed in order 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 23-26ºC. To study the effects of chronic caffeine intake we subdivided the N and CH groups into two new groups (with and without caffeine). Caffeine was administered in drinking water that

contained 1mg/mL of caffeine for 15 days (Gasior et al., 2002; Conde et al., 2012b). Therefore we defined four groups: normoxic (N), caffeine-treated normoxic (CafN, 15 days), chronic hypoxic (CH, 12%O_2, 15 days) and chronically hypoxic-caffeine-treated (CafH; caffeine ingestion + CH exposure). To study ventilator parameters we used an additional set of four groups in which rats were exposed to CH and ingested caffeine for only 8 days.

General surgical procedures. In the experiments where the release of endogenous adenosine and DA were analysed and due to the small size of the CB (≈50 µg) (Conde et al., 2006) it was necessary to make pools of 4 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 (in mM: NaCl 140; KCl 5; $CaCl_2$ 2; $MgCl_2$ 1.1; HEPES 10; glucose 5.5, pH 7.40) and CBs were cleaned (Vicario et al., 2000). To record CSN activity, the preparation CB-CSN was dissected of tissue surrounding the CB and the CSN. The CB-CSN preparation was digested during 3-5 min in collagenase type I (1mg/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 bifurcation-CSN-PG) were dissected free and placed in cold, oxygenated Tyrode 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 criovials 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 3000g for 10 minutes (4ºC) and supernatant plasmas were stored at -80ºC.

Quantification of plasma caffeine. After protein precipitation with PCA 30% and neutralization with KOH (4M)/Tris (0.4M), caffeine and its metabolites theobromine, theophyline and paraxanthine were quantified in the supernatant by reverse phase high performance liquid chromatography (HPLC) with low pressure gradient and UV detection at 274 nm as previously described 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-litre methacrylate chambers continuously fluxed (2 l/min) with controlled temperatures within the thermo-neutral range (22-24ºC). Tidal volume (V_T; ml) respiratory frequency (f; breaths/min; bpm) and minute ventilation (V_E; ml/min/Kg) were measured. Briefly, 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 behaviour. 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 in the Results section. All the gases were balanced with N_2 and applied at a flow of 2 l/min. The pressure change within the chamber reflecting tidal volume (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 air into the chamber allowed a direct estimation of V_T. Pressure signals were fed to a computer for visualisation 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 dis section microscope (Nikon) and superfused (37 ºC) with bicarbonate/CO_2 buffered saline (in mM: NaCl 120; $NaHCO_3$ 24; KCl 3; $CaCl_2$ 2; $MgCl_2$ 1.1; glucose 5; pH 7.40). Extracellular recordings from single or multiple fibre filaments of CSN were made using a suction electrode. The pipette potential was amplified (Neurolog Digimiter, Hertfordshire, England), filtered (1KHz), digitized at 6 K Hz 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 c onfirmed 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 ne edle electrode (no. 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 re sponse 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 receptors expression in the CB and PG. CBs and PGs were homogenized in Zurich medium (Tris-HCl 10 mM; EDTA 1 mM; NaCl 150 mM; Triton X-100 1%; Sodium cholate 1%; SDS 0.1%) and a cocktail of protease inhibitors. Regularly we used 4 PG and 6 CBs per electrophoresis lane (≈ 30 μg protein), so that in each electrophoresis we always had at least two lanes of control animals. Samples of the homogenates and the pre-stained molecular weight markers (Precision, BioRad, Madrid, Spain) were separated by SDS-Page (10% with a 5% concentrating gel) under reducing conditions and electro-transferred to nitrocellulose membranes (BioRad, Madrid, Spain). The membranes were blocked for 1 hour at room temperature with 0.5% I-Block (Tropix, Bedford, MA, USA) in TBS 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 carried out 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 (1:10000 dilution), respectively, for 1 h, washed again in TBST (0.02%), and incubated for 30 min in TBST (0.1%) containing horseradish peroxidase (HRP)-conjugated streptavidin (1:10000 dilution). Membranes were then washed in TBST (0.02%) and developed with enhanced

chemiluminescence reagents according to the manufacturer's instructions (Immobilon Western, Millipore, Spain). The intensity of the signals was detected in a Chemidoc Molecular Imager (Chemidoc BioRad, Spain) and quantified using the Quantity-One software (BioRad, USA). The membranes were then re-probed and tested for α-tubulin immunoreactivity (bands in the 65kDa region) in order to compare and normalise 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 in Tyrode containing EHNA (2.5 µM), an inhibitor of adenosine deaminase. The incubation media were kept at 37ºC and continuously bubbled with 20%O_2/5%CO_2 /75% N_2 saturated with water vapour, except when hypoxic stimuli were applied. Specific protocols for stimulus are provided in the Results section. 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 PCA 3M and 0.6M for adenosine and DA analyses, respectively. At the end of the experiment the CBs were immersed in 100 µl of PCA 0.6M and weighed. The collected fractions were kept for 10 min at 0 ºC and then centrifuged at 12000 g for 10 min (4 ºC). In 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 dopamine (DA) + DOPAC (its main catabolite) and therefore we would refer to the set as DA. For adenosine analyses, supernatants were recovered and adenosine was extracted from the medium as previously described by Conde

and Monteiro (2004). Endogenous adenosine release was quantified by HPLC with UV detection (Conde and Monteiro 2004).

Drugs and chemicals. Adenosine, caffeine, EHNA, PCA, KOH, Tris-HCl, EDTA, NaCl, Triton X-100, Sodium cholate, SDS, theophylline, theobromine and paraxanthine were obtained from Sigma (Sigma-Aldrich, Madrid, Spain). Adenosine was prepared as 5 mM stock solutions in water. Antibodies against A_{2A} and A_{2B} adenosine receptor as well as for α-tubulin were obtained from Sta Cruz Biotecnhology (USA).

Data analysis. The amount of adenosine endogenously released from CBs was expressed in pmol/mg tissue after the division of the absolute values obtained by four (four CBs used in each incubation) and corrected for the volume used and the medium weight of the 4 CBs. Data were evaluated using Graph Pad Prism Software, version 4 and were presented as mean ± SEM. 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* values of 0.05 or less were considered to represent significant differences.

Results

The ingestion of caffeine in the drinking water (1mg/ml) was stable throughout the experiments. Water consumption and body weight gain was comparable among groups. As expected we did not detect the presence of caffeine or its metabolites both in normoxic and in chronically hypoxic rats. 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 chronically hypoxic-caffeine-treated rats(4.01 ± 0.83 μg/ml). These values are within the range of 0.37 – 5.95 μg/ml previously described by Gasior *et al*. (2002) for doses of 0.25 – 1 mg/ml of caffeine in drinking water in rats. Caffeine plasma concentrations indicate that we are dealing with non-toxicological 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 15-35 μM, as a non-selective inhibitor of adenosine receptors, while IC_{50} values for phosphodiesterease inhibition and for Ca^{2+} release form 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 chronically hypoxic-caffeine-treated rats the word "basal" refers to 12% O_2 for both *in vivo* or *in vitro* experiments since the animals were maintained 8 or 15 days prior to 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 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 chronic hypoxic rats. Note first (Figure 1A), that in normoxic animals VE increased progressively with intensity of the acute hypoxic tests. Note also that chronically hypoxic animals (exposed to 12% O_2 during 8 and 15 days) exhibit higher VE than the normoxic group. The increase represented a 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 vs. chronically hypoxic animals reflects the acclimatization allowing to conclude that acclimatization to hypoxia was already expressed after 1 week of CH but was more accentuated after 2 weeks. Minute ventilation in response to an acute hypercapnic test (5% CO_2 in air; Figure 1B) was 46% ($p<0.05$) and 16% (n.s.) higher in8 and 15 days chronically hypoxic treated animals, respectively than in normoxic animals.

8 days 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 augmented in all hypoxic atmospheres; the increase (34.8%) reached statistical significance in response to 7%O_2 (Figure 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 apnoeic episodes (Steer et al., 2004). Chronically hypoxic-caffeine-treated groups showed ventilatory responses indistinguishable form those found in chronically hypoxic animals in all studied conditions (Figure 1D). Fifteen days chronically hypoxic-caffeine-treated animals showed a hypercapnic ventilatory response 57.9% higher than chronically hypoxic animals ($p<0.01$; Figure 1E). All together data indicate that the both acclimatization and the effect of caffeine were 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 Figures 2A to 2C, chronically hypoxic animals exhibited an augmented basal CSN activity (9.76 ± 0.89 impulses/s) in comparison to that observed in normoxic animals

(5.42 ± 0.81 impulses/s; p<0.001; Figure 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 chronically hypoxic and chronically hypoxic-caffeine-treated animals (Figure 2D).

CSN activity in response to all stimuli was normalized to correspondent basal activity so 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; n.s.) having no effect in the response to hypercapnia (compare N and CafN in Figure 2E). When 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 in Figure 2E). Since basal activity was nearly double in chronically hypoxic compared to normoxic animals, the data imply that absolute value of the evoked CSN activity for the hypoxic responses in chronically hypoxic animals was about double than in N 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 chrnonically hypoxic animals. In comparison to chronically hypoxic animals, chronically hypoxic-caffeine-treated exhibited about halved CSN peak frequency/basal frequency ratios to all stimuli (compare CH and CafH groups in Figure 2E). Summing 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 towards 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 chronic 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 previously reported 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 weigh of chronically hypoxic animals was 98.79 ± 4.55 μg (n = 14). Caffeine ingestion during CH exposure prevented the increase in CB weight, as in chronically hypoxic-caffeine-treated 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 HIF-1α and VEGF (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 chronic hypoxic rats. We limited our search to A_{2B} receptors in the CBs since 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 vs. Xu et al. 2006). Also, although rabbit chemoreceptor cells express A_1 receptors (Rocher et al. 1999) they have not been detected in rat cells (Gauda et al. 2000). Levels of expression of A_{2B} receptors in caffeine treated normoxic rats as well as in chronically hypoxic animals were not different from those seen in normoxic animals (Figure 4A and 4C), but in chronically hypoxic-caffeine-treated animals there was a downregulation 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 (Conde et al., 2009, Gauda et al., 2000) and A_{2B} mediated effects in the CB-CSN preparations of this species are presynaptically mediated. Figures 4B and 4D show that adenosine A_{2A} receptor expression were not different in normoxic, caffeine-treated normoxic and chronically hypoxic animals; however in chronically hypoxic-caffeine-treated rats there was an upregulation 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 in a sequential incubation of pools of 4 CBs in normoxic solutions (10 min), moderate hypoxia (7%O_2-saturated; 10 min), 2 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 chronically hypoxic-caffeine-treated rats ($p<0.01$ vs. correspondent normoxic group). Basal release of DA (left part of Figure 5A) was much lower than that of adenosine in all experimental groups (see units in 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 chronically hypoxic-caffeine-treated rats doubling those seen in corresponding normoxic groups ($p<0.001$).

The release of adenosine evoked by 7% and 2% O_2 is depicted in Figures 5B and 5C. In normoxic rats (Figure 5B) the release elicited by moderate hypoxia (7%O_2) equalled or overcame the elicited by intense hypoxia (2%O_2; see Conde et al., 2006 and 2012a); the hypoxia induced release of adenosine in caffeine treated normoxic rats was not different from that seen in normoxic animals. Comparing Figures 5B and 5C it can also be seen that: a) in the CB of chronic hypoxic rats acute hypoxic tests 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); b) 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 chronically hypoxic-caffeine-treated rats was not different (compare CH and CafH in Figure 5C); c) 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 prior to hypoxic stimulation is much higher for both hypoxic

stimuli in chronically hypoxic and chronically hypoxic-caffeine-treated rats than in corresponding normoxic groups; and d) basal release in all experimental groups did not show any time-dependent modification all along the experiments, evidencing the peculiarities of the metabolism and absence of cellular stores for this neurotransmitter.

Data on the release of DA are presented in Table 1. Notice that: a) 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 ≈40-50% smaller than in the initial normoxic period; b) When the release response was expressed as percentage of the correspondent control, the higher the stimulus (7% and 2% O_2, 10 min), the larger was the release induced by acute hypoxia; c) in chronically hypoxic and chronically hypoxic-caffeine-treated rats both hypoxic stimuli evoked a release response that percentagewise was comparable to those of correspondent normoxic groups; however in absolute amounts the stimuli were much larger; d) 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 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 that: 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 chronic 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 CH elicits acclimatization measured both as ventilatory responses and as CSN activity. Ventilatory sensitization to hypoxia was evident at day 8 and more pronounced at day 15 of CH. The acclimatization process was also evident at the level of CSN activity (15 days) since 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 vs 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 a nearly normal ventilation exhibit a markedly decreased acclimatization to CH (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: Since acute caffeine does not affect basal CSN frequency, while 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 the 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 the main responsible in generating the augmented CSN activity observed in chronic hypoxic animals during acute CB stimulation (Conde et al., 2006; 2012a).

Finally, although ATP is a potent excitatory neurotransmitter in the CB (Conde et al., 2012a; Nurse 2010), it has been proposed that CH does not change $P2X_2$ receptors 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 to these observations is that DA/ATP ratio in the dense-core neurotransmitter storage granules increases due to ATP decrease. This decrease would spare ATP in the unfavourable

situation of CH (Lindqvist et al., 2002), the net result being that in chronically hypoxic animals ATP release would be diminished. In fact, it was observed 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 (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 on 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 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 animal's 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 Langercrantz, 1999), being the treatment of election for apnoeas and other respiratory disrhythmias of the newborn (Mathew 2011). In addition, Pianosi et al. (1994) found that the slope of the line defined by the ratio VE/△mmHg of PCO_2 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 receptors 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, while acute caffeine inhibits the hypoxia-induced CSN activity without affecting normoxic activity (Conde et al., 2006; Howell and Landrum, 1995) and markedly inhibits basal and low intensity hypoxia induced release of DA (Conde et al., 2006), chronic caffeine tends to increase 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} 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 the CSN activity in response to hypoxia. Also, the trend to increase of A_{2B} receptor expression in the CB would explain the observed augmentation of the release of DA as 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 CH 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 CH. 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 evidence that the balanced dual actions of chronic caffeine in CH 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 (see also Riksen et al., 2009).

The marked decrease in the CSN response to hypoxia and hypercapnia in chronically hypoxic-caffeine-treated rats (vs. chronically hypoxic) 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 chronically hypoxic-caffeine-treated 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 state that the physiological implications of these

subtle changes are still unclear, however our data indicate that physiological significance is the loss of the sensitization of CB by chronic hypoxia. It can also be hypothesised 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 receptors expression in chemoreceptor cells and sensory nerve endings with and 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 CH 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 CH acclimatization in the ventilatory parameters. Extrapolating our findings in the rat to humans we postulate that normal caffeine ingestion ameliorates ventilation in response to acute hypoxia in normoxic subjects, would not harm hypoxic ventilation in human's submitted to chronic hypoxia, and would ameliorate their ventilatory responses to potential concurrent hypercapnia.

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Authorship Contributions

Participated in research design: Silvia V. Conde, Constancio Gonzalez

Conducted experiments: Silvia V. Conde, Maria J. Ribeiro

Performed data analysis: Silvia V. Conde, Maria J. Ribeiro, Ana Obeso, Ricardo Rigual

Wrote or contributed to the writing of the manuscript: Silvia V. Conde, Emilia C. Monteiro, Constancio Gonzalez

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Footnotes

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Legends of the figures

Figure 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). Ventilatory responses to acute hypoxia were assessed as minute volume (VE) (**A**). Effect of acute application of hypercapnia on minute ventilation in control rats and in rats exposed to 8 and 15 days of chronic hypoxia (**B**). Acute hypoxias were applied 3 times for 10 minutes intervallic 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 vs control values, One and Two-Way ANOVA with Bonferroni multicomparison test. Data represent means ± SEM.

Figure 2. Effects of chronic caffeine intake on the carotid sinus nerve (CSN) activity in normoxic and chronic hypoxic rats. **A**) Typical recording for the effect of chronic caffeine ingestion on the frequency of actions 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 chronic hypoxic rats. **C)** Typical recording for the effect of chronic caffeine ingestion on the frequency of actions potentials of CSN in response to N_2 in chronic hypoxic rats. Arrows indicate the period of application of acute hypoxic stimulus (N_2). Panels **D** and **E** represent, respectively, means of basal CSN activity and means of increases in peak frequencies, in control rats (N), in normoxic rats with chronic caffeine ingestion (CafN), in rats exposed to chronic hypoxia (CH) and in

chronic hypoxic rats after chronic caffeine ingestion (CafH). Data represent means ± SEM of n individual values given in the drawing. *P<0.05, **P<0.01, ***P<0.001 vs. normoxic values; #P<0.05, ##P<0.01 vs. chronic hypoxic values.

Figure 3. Effect of chronic caffeine intake, chronic hypoxia and both treatments applied together, on carotid bodies weight. Data represent means ± SEM. *P<0.05;***P<0.001; One-Way ANOVA with Bonferroni multi-comparison test. N, normoxic rats; CafN, normoxic submitted to chronic caffeine intake; CH, chronic hypoxic rats; CafH, chronic hypoxic rats submitted to chronic caffeine intake. *p<0.05; ***p<0.001.

Figure 4. A_{2B} and A_{2A} adenosine receptors immunoreactivity in t he carotid bodies (CB) and in PG, respectively in normoxic rats (N) and in rats submitted to different treatments: chronic caffeine intake (CafN), chronic hypoxia exposure (CH) and chronic caffeine + chronic hypoxia (CafH). **A** Western Blot comparing A_{2B} immunoreactivity, corresponding to 45 KD a band, when comparing CBs (controls and submitted to different paradigms). A re-probing of the membranes with an α-tubulin antibody, corresponding to the 65 KDa band is shown above the gel for A_{2B} receptors. **B** Western Blot comparing A_{2A} immunoreactivity, respectively, corresponding to 45 KDa band, when comparing PGs (controls and submitted to different paradigms). A re-probing of the membranes with α-tubulin antibody, corresponding to the 65 KDa band is shown above the gel for A_{2A}. **C** show the average relative A_{2B} immunoreactivity, respectively in the CB (n= 4-9) in the different paradigms expressed in relation to α-tubulin immunoreactivity. **D** show the average relative A_{2A} immunoreactivity,

respectively in the PG (n= 5-9) in the different paradigms expressed in relation to α-tubulin immunoreactivity. # P<0.05; Two-Way ANOVA with Bonferroni multi-comparison test, comparing values within the same group. Data represent means ± SEM.

Figure 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**) normoxia and chronically hypoxic rats (**C**). N, normoxic rats (n = 6); CafN, normoxic submitted to chronic caffeine intake (n = 7); CH, chronic hypoxic rats (n = 5); CafH, chronic hypoxic rats submitted to chronic caffeine intake (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 Bonferroni multi-comparison test.

Table I. Basal and hypoxia evoked release of endogenous dopamine in CBs of control normoxic, caffeine treated normoxic, chronic hypoxic and chronically hypoxic caffeine treated animals. In the columns referring to hypoxic stimuli (7 and 2% O_2) the upper values represent absolute amounts released in pmole/mg tissue/10 min and lower number represent the release during hypoxic stimulation expressed as percentage of preceding control (20% O_2 incubation)

Incubating Conditions / 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
Control, Normoxic, N	**10.4 ± 1.5**	**17.4 ± 1.7*** **167.30%**	**6.5 ± 1.5**	**14.2 ±2.1*** **218.46%**
Caffeine-treated normoxic, CafN	**11.1 ± 0.9**	**21.7 ± 2.0**** **195.50%**	**5.5 ± 1.2**	**13.3 ± 2.5**** **241.82%**
Chronic hypoxic (12%O_2, 15 days), CH	**21.2 ± 4.1**	**36.4± 2.9*** **171.69%**	**12.7 ± 2.2**	**36.0 ± 5.3**** **283.46%**
Chronically hypoxic-caffeine-treated , CafH	**25.9 ± 2.5**	**53.9 ± 5.4***** **208.10%**	**16.5 ± 1.5**	**38.0 ± 5.2**** **230.30%**

*P<0.05, **P<0.01, ***P<0.001 vs. preceding control values, Two-Way ANOVA with Bonferroni multicomparison test. Data represent means ± SEM

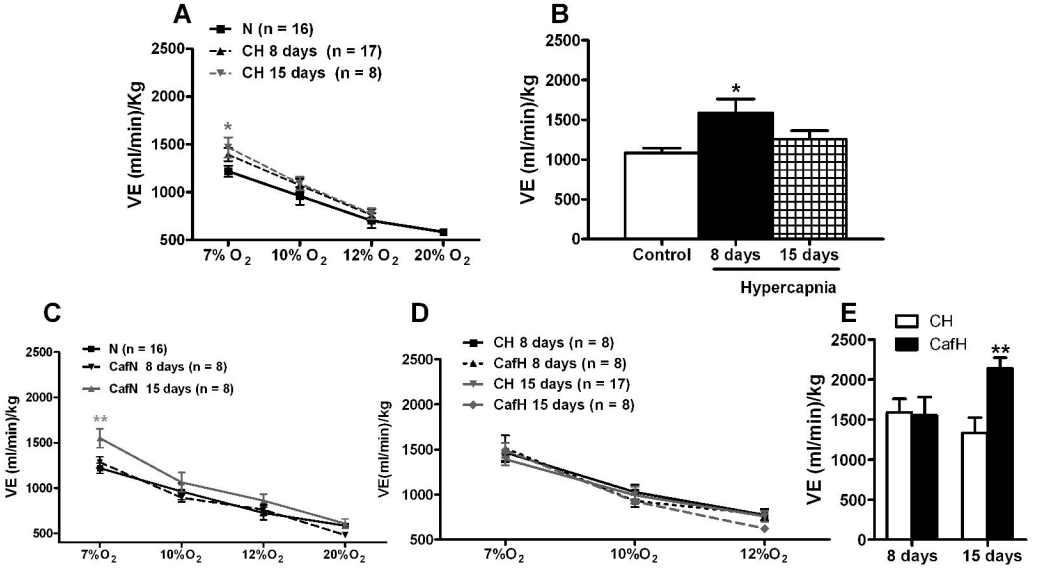
A
N (n = 16)
CH 8 days (n = 17)
CH 15 days (n = 8)
VE (ml/min)/Kg
2500
2000
1500
1000
500
*
7% O2
10% O2
12% O2
20% O2
B
VE (ml/min)/kg
2500
2000
1500
1000
500
0
*
Control
8 days
15 days
Hypercapnia
C
N (n = 16)
CafN 8 days (n = 8)
CafN 15 days (n = 8)
VE (ml/min)/kg
**
7%O2
10%O2
12%O2
20%O2
D
CH 8 days (n = 8)
CafH 8 days (n = 8)
CH 15 days (n = 17)
CafH 15 days (n = 8)
VE(ml/min)/kg
7%O2
10%O2
12%O2
E
CH
CafH
VE (ml/min)/kg
**
8 days
15 days

Figure 1

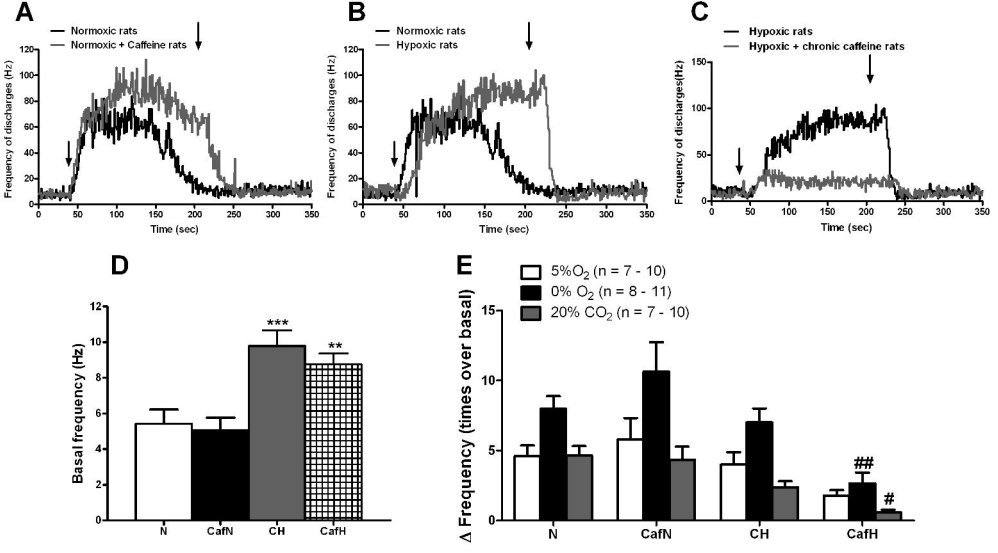
A
Normoxic rats
Normoxic + Caffeine rats
Frequency of discharges (Hz)
Time (sec)
B
Normoxic rats
Hypoxic rats
Frequency of discharges (Hz)
Time (sec)
C
Hypoxic rats
Hypoxic + chronic caffeine rats
Frequency of discharges(Hz)
Time (sec)
D
Basal frequency (Hz)
***
**
N
CafN
CH
CafH
E
5%O2 (n = 7 - 10)
0% O2 (n = 8 - 11)
20% CO2 (n = 7 - 10)
Δ Frequency (times over basal)
##
#
N
CafN
CH
CafH

Figure 2

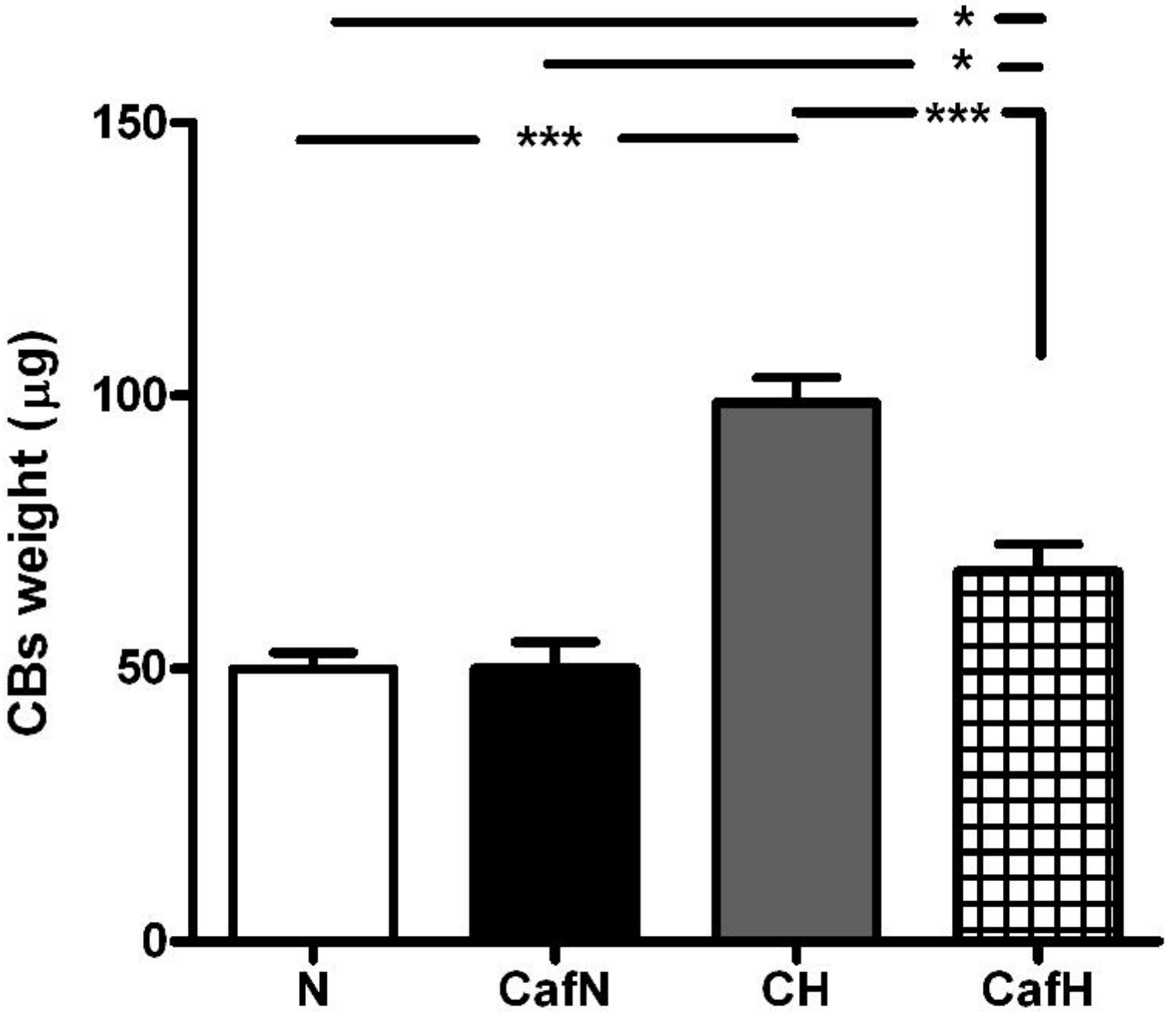

Figure 3

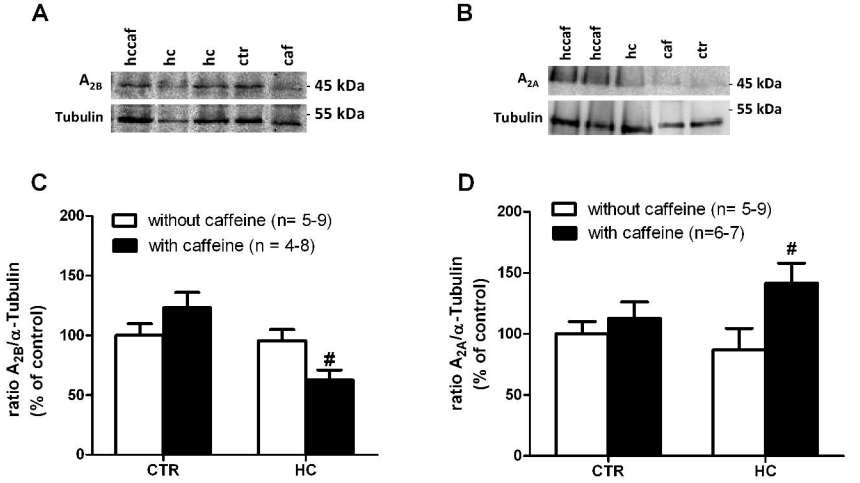
A
hccaf
hc
hc
ctr
caf
A2B
45 kDa
Tubulin
55 kDa
B
hccaf
hccaf
hc
caf
ctr
A2A
45 kDa
Tubulin
55 kDa
C
without caffeine (n= 5-9)
with caffeine (n = 4-8)
ratio A2B/α-Tubulin
(% of control)
200
150
100
50
0
#
CTR
HC
D
without caffeine (n= 5-9)
with caffeine (n=6-7)
ratio A2A/α-Tubulin
(% of control)
200
150
100
50
0
#
CTR
HC

Figure 4

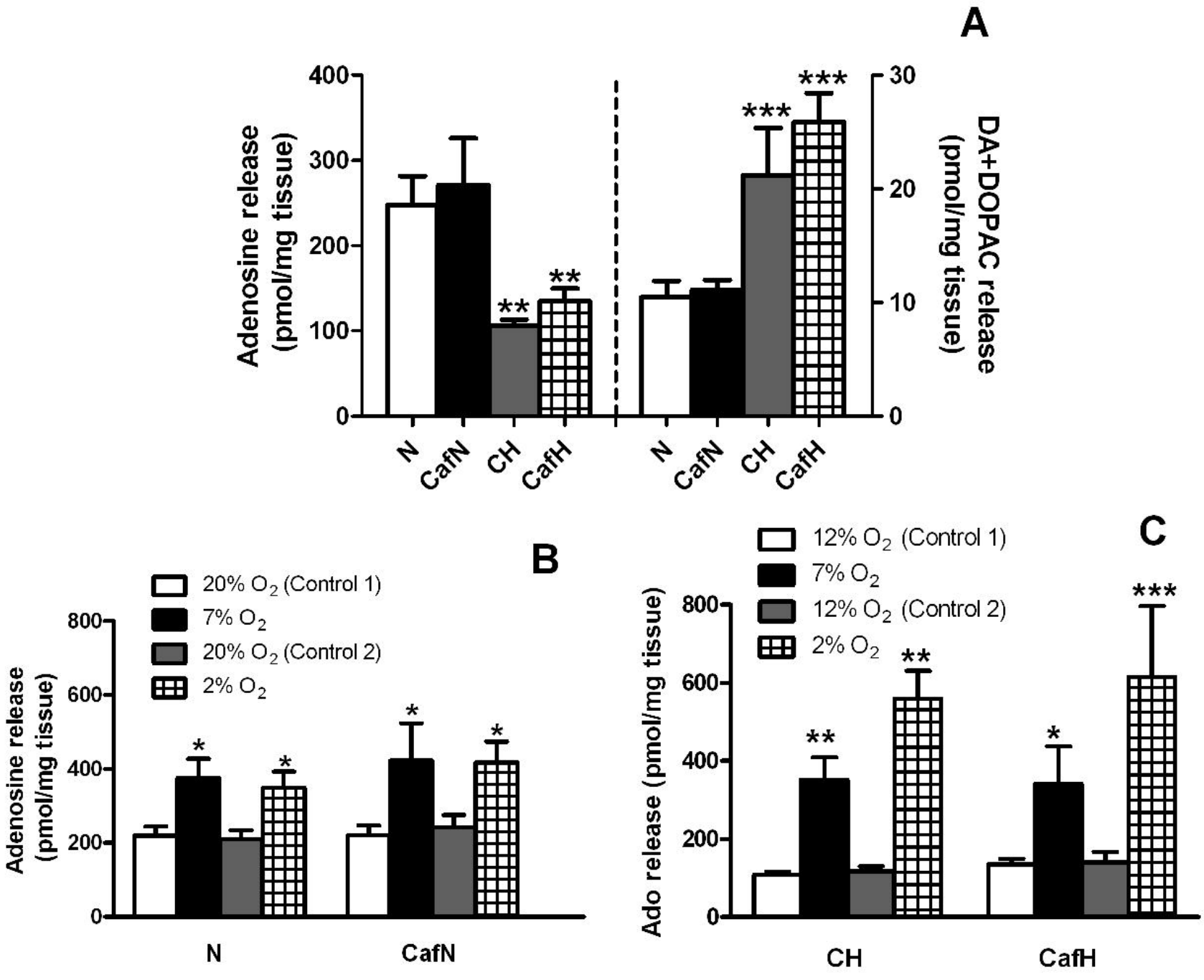
A
Adenosine release (pmol/mg tissue)
DA+DOPAC release (pmol/mg tissue)
400
300
200
100
0
30
20
10
0
**
**
***
***
N
CafN
CH
CafH
N
CafN
CH
CafH
B
20% O2 (Control 1)
7% O2
20% O2 (Control 2)
2% O2
Adenosine release (pmol/mg tissue)
800
600
400
200
0
*
*
*
*
N
CafN
C
12% O2 (Control 1)
7% O2
12% O2 (Control 2)
2% O2
Ado release (pmol/mg tissue)
800
600
400
200
0
**
**
*
***
CH
CafH

Figure 5