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Age protects from harmful effects produced by chronic intermittent hypoxia

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Key points

- Episodes of intermittent hypoxia, as produced in obstructive sleep apnoea, cause a carotid-driven sympathetic hyperactivity and an oxidative status leading to cardiovascular and metabolic pathologies, which are less pronounced in patients >60–65 years old.
- In young rats (3–4 months) chronic intermittent hypoxia augments carotid body hypoxic responses, plasma catecholamine, renal catecholamine content and turnover, produces an oxidative status, and causes hypertension.
- In aged rats (22–24 months) chronic intermittent hypoxia does not alter carotid body responses, catecholamine-related parameters or redox status, and does not cause hypertension.
- It is concluded that age affords protection to harmful effects produced by chronic intermittent hypoxia.
- Possible mechanisms involved in age protection and the significance of our findings in the diagnosis and therapeutic approaches to obstructive sleep apneoa in the elderly are considered.

Abstract Obstructive sleep apnoea (OSA) affects an estimated 3–7% of the adult population, the frequency doubling at ages >60–65 years. As it evolves, OSA becomes frequently associated with cardiovascular, metabolic and neuropsychiatric pathologies defining OSA syndrome (OSAS). Exposing experimental animals to chronic intermittent hypoxia (CIH) can be used as a model of the recurrent hypoxic and O_2 desaturation patterns observed in OSA patients. CIH is an important OSA event triggering associated pathologies; CIH induces carotid body (CB)-driven exaggerated sympathetic tone and overproduction of reactive oxygen species, related to the pathogenic mechanisms of associated pathologies observed in OSAS. Aiming to discover why OSAS is clinically less conspicuous in aged patients, the present study compares CIH effects in young (3–4 months) and aged (22–24 months) rats. To define potential distinctive patterns of these pathogenic mechanisms, mean arterial blood pressure as the final CIH outcome was measured. In young rats, CIH augmented CB sensory responses to hypoxia, decreased hypoxic ventilation and augmented sympathetic activity (plasma catecholamine levels and renal artery content and synthesis rate). An increased brainstem integration of CB sensory input as a trigger of sympathetic activity is suggested. CIH also caused an oxidative status decreasing aconitase/fumarase ratio and superoxide dismutase activity. In aged animals, CIH minimally affected CB responses, ventilation and sympathetic-related parameters leaving redox status unaltered. In young animals, CIH caused hypertension and in aged animals, whose baseline blood pressure was augmented, CIH did not augment it further. Plausible mechanisms of the differences and potential significance of these findings for the diagnosis and therapy of OSAS are discussed.

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Abbreviations 24M, rats aged 22–24 months; 24MCIH, rats aged 22–24 months exposed to chronic intermittent hypoxia; 3M, rats aged 3–4 months; 3MCIH, rats aged 3–4 months exposed to chronic intermittent hypoxia; A, adrenaline; AP, arterial pressure; CA, catecholamine; CB, carotid body; CI, confidence interval; CIH, chronic intermittent hypoxia; CRP, C-reactive protein; CuZnSOD, cytoplasmic superoxide dismutase; DA, dopamine; E_{GSH}, glutathione redox potential; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; LPO, lipid peroxide; MnSOD, mitochondrial superoxide dismutase; MV, minute ventilation; NA, noradrenaline; NTS, nucleus of the tractus solitarius; OSA, obstructive sleep apnoea; OSAS, obstructive sleep apnoea syndrome; PaO_2, arterial oxygen pressure; RA, renal artery; ROS, reactive oxygen species; RVLM, rostroventrolateral medulla; SaO_2, arterial haemoglobin saturation; SOD, superoxide dismutase; SSA, 5-sulfosalicylic acid; TV, tidal volume; UA, upper airway.

Introduction

Obstructive sleep apnoea (OSA) consists of a repetitive obstruction of the upper airways (UAs) during sleep. Physiologically, in phases 3 and 4 of non-rapid eye movement sleep and mainly during the rapid eye movement periods of sleep, there is a decreased drive to inspiratory and UA opener muscles (Dempsey *et al.* 2010) resulting in a diminished air-pumping power and increased airways resistance. An abnormally high decrease in the drive to airways dilator muscles, or their abnormal response, leads to UA obstruction (Morrison *et al.* 1993). Typically, in OSA patients, a decrease in genioglossi activity causes the tongue to drop backwards and UAs close.

Each obstruction causes an episode of hypoxia leading to a picture of recurrent or chronic intermittent hypoxia (CIH) causing a fall in arterial oxygen pressure (PaO_2) and arterial haemoglobin saturation (SaO_2). Hypoxic episodes stimulate the carotid body (CB) chemoreceptors, triggering an increased motor output towards the inspiratory and UA dilator muscles and an arousal reaction, which together solve the obstruction (Dempsey *et al.* 2010, 2014). Once blood gases are restored, another cycle of UA obstruction is generated.

As OSA progresses, it frequently generates the obstructive sleep apnoea syndrome (OSAS), with associated pathologies: cardiovascular (hypertension and augmented acute vascular accidents; Dempsey *et al.* 2010; Gopalakrishnan & Tak, 2011), hepato-metabolic (insulin resistance, glucose intolerance, fatty liver disease; Morselli *et al.* 2012) and neuropsychiatric (anxiety, depression and cognitive-executive deficits; Sateia, 2003; Almendros *et al.* 2010; Gagnon *et al.* 2014). Epidemiological data show that OSAS-linked pathologies have a substantial worldwide economic impact on healthcare systems (Banno *et al.* 2009; Tarasiuk and Reuveni, 2013), and thus there is interest to understand their mechanisms with the aim to rationally address their therapies. Clinical (Adamson, 2009; Gami *et al.* 2013) and experimental studies (Fletcher *et al.* 1992) indicate that CIH is an important event in causing OSAS-associated pathologies. CIH causes CB sensitization (Peng *et al.* 2003; Del Rio *et al.* 2010), probably increasing CB chemoreceptor input to the brainstem (Zoccal *et al.* 2008; Kline, 2010; Gonzalez-Martín *et al.* 2011; Prabhakar *et al.* 2012; Ciriello and Moreau, 2013), leading to an exaggerated sympathetic tone, which generates hypertension and subsequent cardiovascular pathologies (Lesske *et al.* 1997; Kumar *et al.* 2006; Prabhakar *et al.* 2012; Olea *et al.* 2014). The swings in PaO_2 with each obstruction mimic ischaemia-reperfusion events, augmenting the production of reactive oxygen species (ROS). ROS generate a permanent status of vascular endothelium inflammation and dysfunction, lessening of its vasorelaxant and anti-adhesive phenotype, and facilitating cardiovascular pathologies (Lavie & Lavie, 2009, 2012; Jelic *et al.* 2010; Quintero *et al.* 2013).

Age is another factor to consider in OSAS. Several studies indicate that in aged subjects (>65 years) OSAS is more prevalent than in middle ages (30–65 years) but the clinical picture is less sharp and somehow paradoxical (Lavie and Lavie, 2009; Kobayashi *et al.* 2010; Martínez-García *et al.* 2010; Gagnon *et al.* 2014). Comparison of mortality in sleep laboratory cohorts of aged patients (>65 years) with matched general populations revealed that OSAS patients had a significantly lower mortality rate than expected (Lavie & Lavie, 2009), suggesting that oxidative stress, so closely associated with OSA, was implicated in the genesis of that protection by mechanisms similar to those responsible for heart and brain ischaemic preconditioning (Brzecka, 2005; Lavie and Lavie, 2009). Experimental studies of CIH effects in aged animals are scarce.

The present study aims to define potential distinctive patterns or intensities in alterations of the two main pathogenic mechanisms (sympathetic tone and oxidative status) involved in OSAS-linked pathologies by comparing the effects of CIH in young adult and aged rats. The findings indicate that CIH causes less intense modifications in CB function–sympathetic tone and oxidative stress in aged than in young adult animals.

Methods

Animals and anaesthesia

Young adult and old male Wistar rats (3–4 and 22–24 months old, respectively) were used, with free access to food and water and maintained under controlled conditions of temperature, humidity and a stationary light–dark cycle. Rats were distributed in four different groups: young 3–4 months old (3M), young chronic intermittent hypoxic (3MCIH), aged 22–24 months old (24M) and aged chronic intermittent hypoxic (24MCIH) rats. Except for CIH exposure and whole body plethysmography, experimental procedures were performed in animals anaesthetized with sodium pentobarbital (60 mg kg^{-1} body weight; I.P.) or ketamine plus diazepam (100 and 1.6 mg kg^{-1}, respectively; I.P.). Experiments were performed in the morning (from 08.30 to 13.00 h) of the day after the last intermittent hypoxic exposure, with animals fasting overnight. In handling the animals, we followed the European Community Council directive of 24 November 1986 (86/609/EEC) for the Care and Use of Laboratory Animals. The Institutional Committee of the University of Valladolid for Animal Care and Use approved the protocols. Animals were killed by a cardiac overdose of sodium pentobarbital.

Exposure to CIH

The specific protocol of CIH used in the present study consisted of cycles of 40 s, 5% O_2/80 s, air, equivalent to an apnoea–hypopnoea index of 30. The intermittent hypoxia was applied for 8 h day^{-1} (from 08.00 to 16.00 h) for 14 days. General design and functioning of our equipment to expose rats to intermittent hypoxia have been described in detail in previous publications (Gonzalez-Martin *et al.* 2011; Quintero *et al.* 2013). In recent experiments carried out on young rats and using the same protocol (Quintero *et al.* 2013), we have found that the lowest value of PaO_2 measured was 37.2 ± 0.9 mmHg, yielding mean lowest haemoglobin O_2 saturation of 73.5 ± 1.4%; haemoglobin O_2 saturation prior to hypoxic episodes was 96.5 ± 0.75%. The duration that SaO_2 was below 90% represented 25% of the 8 h duration of the hypoxic exposure.

Recording of carotid sinus nerve (CSN) activity

To record CSN activity, the CB–CSN preparation was exposed under a dissecting microscope and a block of tissue, including the carotid bifurcation and the glossopharyngeal nerve, was removed and placed in a Lucite chamber with ice-cold 100% O_2-equilibrated Tyrode solution (in mM: NaCl, 140; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1.1; Hepes, 10; glucose, 5; pH 7.40) for further dissection, freeing CB and CSN of surrounding connective tissue. The CB–CSN preparation was digested in collagenase type I (1 mg ml^{-1}; Warthington) solution to facilitate later isolation of single sensory units or paucifibre filaments of the CSN for neural activity recording (Rigual *et al.* 2002; Conde *et al.* 2006*a*). The experiments were performed using the times of digestion needed to obtain a maximal activity on the recording and therefore different times of collagenase digestion were used: 3–7 min in 3-month-old animals (3M and 3MCIH) and 20–30 min in 24-month-old animals (24M and 24MCIH), as we have observed that different times of digestion affect electrical activity of the nerve, mainly due to the increase in the epineurium and perineurium thickness of the CSN with age. These intervals represent also small changes produced by the different batches of the type 1 collagenase used. The CB–CSN preparation was maintained in ice-cold 100% O_2-equilibrated Tyrode solution until it was transferred to the recording chamber. Recording solution was Tyrode bicarbonate equilibrated with gas mixtures containing 5% CO_2 unless specified otherwise.

The CB–CSN preparation was transferred to a recording chamber mounted on a dissection microscope (Nikon) and perfused with normoxic Tyrode solution (equilibrated with 20% O_2–5% CO_2–balanced N_2 at 37°C). Extracellular recordings from single or multiple fibre filaments of CSN were made using a suction electrode. The pipette potential was amplified (NeurologDigimiter, Welwyn Garden City, 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 (perfusion with solutions equilibrated with 2% or 7% O_2 + 5% CO_2 + balanced N_2). The acidic hypercapnic stimulation consisted of perfusion of the preparation with solutions equilibrated with 20% O_2 + 20% CO_2, balanced N_2 (pH 6.8). Chemoreceptor activity was discriminated off-line for height and timing (Clampex 9.0, Molecular Devices). Oxygen tension in the recording chamber was measured with a needle electrode (no. 760, Diamond Micro Sensors, Ann Arbor, MI, USA) polarized to −0.8 V against an Ag/AgCl reference electrode also placed in the recording chamber.

Plethysmography

Ventilation was measured in conscious freely moving rats by whole body plethysmography as described in detail by Olea *et al.* (2011). The system (Emka Technologies, Paris, France; BUXCO Research Systems, Wilmington, NC, USA) consists of 5-litre metacrylate chambers continuously fluxed (2 l min^{-1}) with desired gas mixtures. Temperature was maintained in the chamber within the thermo-neutral range (22–24°C). Tidal volume (TV; ml kg^{-1}), respiratory frequency (breaths min^{-1}) and minute ventilation (MV; ml min^{-1} kg^{-1}) were measured.

Animals were placed in the plethysmographic chamber and breathed room air for at least 30 min until adapted to the ambient chamber conditions and they had acquired a standard resting behaviour. Thereafter recording of basal ventilatory parameters over 20 min started. Ventilatory parameters were measured in different atmospheres by fluxing the chamber during 10 min at 2 l min^{-1} with gas mixtures containing 12, 10 or 7% O_2 (rest N_2) and 5% CO_2 in air. Each recording in a special atmosphere was followed by a 10–20 min recovery period in air. The pressure changes within the chamber reflecting tidal volume were measured with a high-gain differential pressure transducer. Ideally the frequency of pressure fluctuations is identical to breathing movements; spurious fluctuations of the pressure due to animal movements were electronically rejected. The amplitude of the pressure oscillations is proportionally related to TV; a calibration of the system by injections of 5 ml of air into the chamber allowed direct estimation of TV. Pressure signals were fed to a computer for visualization, storage and offline analysis with Buxco software.

Measurement of endogenous catecholamine (CA) in plasma and renal artery (RA) tissue: measurement of the rate of ^{3}H-CA synthesis in renal arteries

Special care was taken in handling the animals used to measure plasma CA due to the lability of plasma CA levels. After anaesthesia rats were tracheostomized and pump ventilated with air for around 30 min. The chest was opened and blood was slowly withdrawn by direct puncture in the left ventricle. Citrated blood was centrifuged at 1000 *g* for 5 min at room temperature. Supernatant was transferred to tubes containing 60 mg ml^{-1} sodium metabisulfite and frozen at −80°C until the HPLC analysis of CA. For analysis of endogenous CA in RA, extra-renal segments of the arteries were removed and glass to glass homogenized in 0.1 N perchloric acid (PCA) containing 0.1 mM EDTA and centrifuged. Supernatants were stored at −80°C until HPLC analysis.

General procedures to study the rate of CA synthesis have been described in previous publications (Olea *et al.* 2014). In brief, RAs were incubated (37°C; 2 h) in Tyrode solution (in mM: NaCl, 140; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1.1; Hepes, 10; glucose, 5; pH 7.40) containing 30 μM of 3,5-^{3}H-tyrosine (the natural precursor of CA; 6 Ci $mmol^{-1}$; Perkin Elmer, Boston, MA, USA) and the cofactors for tyrosine hydroxylase and dopamine beta hydroxylase, 100 μM 6-methyl-tetrahydropterine and 1 mM ascorbic acid, respectively. Tissues were washed in precursor-free Tyrode solution (4°C; 5 min), homogenized and processed for HPLC analysis.

Plasma CAs were extracted from 500 μl of plasma using Waters Oasis-HLB cartridges after adequate conditioning following the instructions of the supplier (Waters Corporation, Milford, MA, USA). After convenient washings, CAs were eluted with 0.5 ml of citric acid (26.7 mM and 2.5% MeOH; pH 2.9) with a recovery of 98%. Eluates were either immediately injected in the HPLC system or refrozen at −80°C.

The HPLC system consisted of a Waters 600 controller pump, automatic injector Waters 717 plus Autosampler and BAS LC-4C Amperometric Detector. The mobile phase was: 25 mM Na_2HPO_4, 0.65 mM 1-octane sodium sulfonate acid, 0.1 mM EDTA, pH 3.46, and 6% MeOH. The sensitivity of the detector was modified as required. Amine identification was made against external standards and quantification was made with Peak Sample Data Chromatography System software (Buck Scientific, East Norwalk, CT, USA). Identification of ^{3}H-CA was done against internal standards and quantification by collecting of the HPLC column effluents and scintillation counting.

Plasma C-reactive protein (CRP) levels

CRP levels were measured in plasma of EDTA anticoagulated blood plasma using a commercial rat-specific ELISA (Cusabio Biotech; bioNovaCientifica, Madrid, Spain) following the instructions of the supplier.

Plasma adenosine levels

Adenosine quantification has been described in detail by Conde & Monteiro (2004). In brief, blood samples were collected from rat heart puncture to EDTA-precoated tubes and centrifuged during 10 min at 3000 *g*. Plasma was collected and adenosine was extracted and concentrated using Amicon Ultra-4 Centrifugal Filter Units (Millipore, Lisbon, Portugal). Adenosine was quantified by HPLC with UV detection. The HPLC system consisted of an LC 9-A pump, 7725i injector, SPD-6 AV UV-VIS wavelength detector and Class VP software to analyse the chromatograms (Shimadzu, Kyoto, Japan). The analytical column was a Lichrospher 100 RP-18 (125 4 mm, i.d., particle size 5 μm, Merck, Rahway, NJ, USA) protected by LichroCART 4–4 guard-columns (Merck). Columns and guard-columns were incorporated in the HPLC system through a ManuCART (Merck). The mobile phase was 100 mM KH_2PO_4 with 15% methanol, pH 6.5, run at a flux of 1.75 ml min^{-1}. External standards were prepared under the same conditions as the biological samples and adenosine identification and quantification was made against the standards.

Measurement of redox status parameters

a. Aconitase and fumarase activities. Activities of the two enzymes were determined as described by Quintero *et al.* (2013). Fresh tissues were minced in ice cold TES buffer (10 mM Tris, pH 7.4, 250 mM sucrose and 1 mM

EDTA) and mitochondria were isolated as previously described (Quintero *et al.* 2013). The final mitochondrial pellet was re-suspended in the same TES buffer and mitochondria were lysed by the addition of triton X-100 at a final concentration of 0.5% (w/v). Twenty, 40 and 10 μl of mitochondrial lysates from liver, lung and brain, respectively, were used for enzymatic measurements. The enzymatic activities were assessed following the increase in optical density at 240 nm for 8 min in the appropriate media: 30 mM sodium isocitrate, 50 mM Tris-HCl, pH 7.4, and 0.6 mM $MnCl_2$ for aconitase, and 50 mM sodium L-malate and 50 mM sodium phosphate buffer, pH 7.4, for fumarase. The linear rates during the last 3 min were used to evaluate enzymatic activities. The aconitase/fumarase ratio was expressed as the ratio of respective rates of optical density increase.

b. Reduced and oxidized glutathione (GSH and GSSG): glutathione redox potential. Tissues (liver, lung and brain) were quickly excised from the animals, washed in ice-cold saline, dry-blotted on filter paper, weighed and placed in Eppendorf tubes containing a solution of 5-sulfosalicylic acid (SSA) at 5% and 0.25 mM EDTA; SSA solution volume was adjusted to $\times 5$ tissue weight. Tissues were stored at -80°C until assay or immediately glass-to-glass homogenized at 0–4°C, centrifuged in a microfuge (4°C, 10 min) and the supernatant used to measure GSH and GSSG. Reduced and oxidized glutathione was determined by the Griffith method as described in detail by Gonzalez *et al.* (2004). From GSH and GSSG levels, the glutathione redox potential (E_{GSH}) was calculated using the Nernst equation $E_{GSH} = E^{o}_{GSH} - RT/zF \times \mathrm{Ln}\,[GSH]^2/[GSSG]$ and a value for E^{o}_{GSH} of -24 mV.

c. Measurement of lipid peroxides (LPOs). Tissues (brain, liver, lung) were homogenized in 20 mM phosphate buffer (pH 7.4; 0.2 w/v) and separated in three aliquots: one aliquot was used to determine LPO, the second to determine protein levels (Lowry) and the third to determine glutathione peroxidase (GPx) activity. The LPO aliquot was treated with 10 μl ml^{-1} of 0.5 M butylated hydroxytoluene in acetonitrile to prevent oxidation of the sample. LPO levels were determined immediately according to the assay kit supplier (Bioxytech LPO-586; OxisHealt Products, Portland, OR, USA). In the assay, malonaldehyde and 4-hydroxyalkenals present in the tissue sample react with a chromogen reagent at low temperature (45°C) and yield a stable chromophore with peak absorbance at 586 nm. This assay has been found to provide a convenient, sensitive and reliable index of lipid peroxidation. Low-temperature formation of the chromophore and prevention of tissue homogenate oxidation ensure the adequate stability of the analyses and the specificity of the assay.

d. Measurement of GPx activity. GPx activity was assayed as described by Quintero *et al.* (2013). The assay measures the disappearance of NADPH in the coupled reaction: GPx oxidizes GSH to GSSG in the presence of peroxide and GSSG is back-reduced to GSH by glutathione reductase using NADPH as a donor of reduction equivalents. The activity of GPx in the sample (Δabsorbance min^{-1}) was calculated on the basis of the molar extinction coefficient for NADPH (6220 UA mol^{-1}). One unit of activity is equal to one micromole NADPH oxidized min^{-1} mg^{-1} protein.

e. Catalase activity in tissues. Catalase activity was assayed using a modified version of the method of Aebi (see Quintero *et al.* 2013). Tissues were homogenized in cold 50 mM phosphate buffer (pH 7; 0.05–0.10 w/v) containing 1 mM EDTA and 0.1% Triton X-100, centrifuged (10,000 *g*; 15 min; 4°C) and the supernatant was immediately used to measure catalase activity or stored at -80°C until the assay. An aliquot of the supernatant (10–20 μl) was incubated for 1 min at 25°C with an excess of H_2O_2 (50 mM) generating water and oxygen. The reaction was stopped by addition of 900 μl of 15 mM sodium azide and 10 μl was assayed to evaluate remaining H_2O_2. Remaining H_2O_2 reacts with a colour reagent [150 mM potassium phosphate buffer (pH 7) containing 0.25 mM 4-aminoantipyrine, 2 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid and 0.7 U ml^{-1} peroxidase], generating a red quinoneimide dye that absorbs at 520 nm. Standard curves were constructed in parallel with concentrations of H_2O_2 between 1.25 and 7.5 mM. Samples without tissue homogenate, but with H_2O_2 (50 mM), were used to obtain the maximum rate of formation of red quinoneimide and by subtraction we calculated the H_2O_2 that was destroyed by catalase present in the tissue homogenates and transformed to enzymatic activity. One unit of catalase activity equals 1 μmol H_2O_2 degraded per minute per milligram of protein at pH 7 at 25°C at a substrate concentration of 50 mM H_2O_2.

f. Superoxide dismutase (SOD) activity in tissues. Tissues (liver, lung and brain) were homogenized in cold 0.25 M sucrose buffer (pH 7.4; 0.1 w/v) containing 10 mM Tris and 1 mM EDTA. The homogenates were centrifuged and the supernatant was used to measure SOD activity immediately or stored at -80°C. SOD activity was determined with an indirect method (SOD determination kit; Fluka, Madrid, Spain). Potassium cyanide (3 mM), which inhibits cytosolic SOD, allowed us to differentially determine mitochondrial and cytosolic SOD activities. SOD is expressed as activity units, one unit being the amount of enzyme capable of inhibiting by 50% the reduction of cytochrome *c* in a coupled system with xanthine oxidase at pH 7.8 and 25°C in a 3 ml reaction mixture.

Arterial pressure (AP) measurements

AP was measured in ketamine + diazepam anaesthetized rats. Animals were tracheostomized and pump ventilated with room air (CL Palmer, London, UK) (60 cycles min^{-1} and a positive expiratory pressure of 2 cmH_2O). The depressor effect of hypoxia was recorded by connecting the inlet of the respirator to a balloon filled with a 10% O_2 + 90% N_2 mixture. AP was recorded from the right common carotid artery with a calibrated (with a mercury manometer) pressure transducer (Transpac IV; ICU Medical, Inc., San Clemente, CA, USA) connected to an acquisition card (Power Lab 16SP; ADI Instruments, Castle Hill, Australia). Pressure signals were constantly monitored and data were stored for offline analysis. Mean arterial blood pressures are plotted.

Data presentation and statistical analysis

Along with some sample recording, data are presented as mean ± SD with their 95% confidence intervals (CIs). Comparison of the data obtained in each experimental group was made by one-way ANOVA, two-way ANOVA and two-way ANOVA for repeated measures as required. The data were considered to be significantly different when the statistical test yielded a P value <0.05.

Results

General characteristics of the animals

Young control animals weighed 366.2 ± 44.9 g and young animals exposed to CIH 369.8 ± 42.0 g. Aged 22–24 control rats had a mean body weight of 542.5 ± 73.0 g, and 24MCIH animals weighed 530.1 ± 75.6 g. Haematocrit values were measured in groups of eight animals. Young 3M and aged 24M rats had haematocrit values of 45.7 ± 4.7 and 42.9 ± 1.1%, respectively, and while in young rats CIH exposure did not alter this (46.1 ± 3.0%), in aged animals it produced a significant increase to 47.4 ± 3.2% ($P < 0.05$). Glycaemia was not different among groups, but there was a tendency to increase in both CIH groups (75.9 and 76.3 mg dl^{-1} in young and aged animals vs. 79.3 and 81.0 mg dl^{-1} in young and aged animals exposed to CIH).

Effects of CIH and age on CB chemoreceptor activity

Figure 1 depicts the effect of different times of digestion of the CB–CSN preparation on the chemosensory activity of the CSN in response to hypoxia in 3M and 24M rats. The responses of the CSN to hypoxia were influenced by the times of digestion, with the young animal preparations having maximal recording activity at 5–7 min and decreasing afterwards (Fig. 1*A* and *B*, left panel) and the old animals having its maximum between 25 and 30 min of digestion (Fig. 1*A* and *B*, right panel). Note also that young animals when submitted to lengthy digestion exhibit a decrease in the hypoxia-evoked chemosensory activity (Fig. 1*A* and *B*, left panel) and that in response to greater periods of digestion, such as 30 min, it is impossible to record (Fig. 1*A*, left panel), probably due to a loss of axon integrity. The low activity described herein when the CB–CSN preparation is submitted to low digestion times in aged animals (Fig. 1*A* and *B*, right panel) and its increase with the time of digestion correlates very well with the description that the epineurium and perineurium thickness increase with age as well as with perineurial thickness/fascicle size ratio (Kundalic *et al.* 2014; Campbell *et al.* 2013).

We highlight that we have performed the experiments to compare the effect of CIH on the CSN activity in 3M and 24M animals, in conditions that allow us to obtain maximal recording activity, and therefore 3–7 min in 3M animals and 20–30 min in 24M animals.

Figure 2 shows the output of the CB measured as chemosensory activity (action potential frequency) in the CSN in the four groups of rats. Figure 2*A* and *B* shows the time course of the CSN activity during application of an intense hypoxic stimulus in 3M *versus* 24M rats (left) and 3MCIH *versus* 24MCIH rats (right). The most salient difference is the great reduction in the response to hypoxia in the aged animals. The lower panels show mean values of the activity recorded in the CSN in different situations. Figure 2*C* shows that mean basal activity in 3M rats amounted to 3.7 ± 2.8 spikes s^{-1} [95% confidence interval)CI), 2.4–5.0; $n = 20$] being markedly diminished in 3MCIH rats, 1.4 ± 0.5 spikes s^{-1} (95% CI, 1.2–1.7; $n = 19$; $^{***}P < 0.001$). Comparable findings were obtained in 24M *versus* 24MCIH rats (3.5 ± 2.6 and 1.5 ± 0.9 spikes s^{-1}; $^{***}P < 0.001$; $n = 21$–23). Figure 2*D* shows the CSN responses elicited by intense and moderate hypoxia expressed as the value times basal. In 3M rats intense hypoxic stimulus caused neural activity to increase by a factor of 13.8 ± 6.6 (95% CI, 10.9–16.8) to a mean frequency of 51.5 Hz while in 3MCIH rats it increased by a factor of 44.1 ± 12.2 (95% CI, 34.0–53.3; $^{***}P < 0.001$) to 64.1 Hz. In 24M and 24MCIH rats the increases in activity elicited by intense hypoxia were also different and amounted, respectively, to 6.2 ± 5.5 (95% CI 3.5–8.9) and 22.3 ± 15.3 times basal (95% CI, 10.5–24.0; $^{***}P < 0.001$) or to 21.8 and 33.4 Hz. These increases were statistically smaller than those seen in young animals ($^{++}P < 0.001$, 3M *vs.* 24M and $^{+}P < 0.05$, 3MCIH *vs.* 24MCIH). As expected, the responses to less intense hypoxia (5% O_2) were smaller in all groups but the pattern of the response seen with intense hypoxia was maintained, i.e. aged rats respond to hypoxia less than young rats and at both ages the increment in discharges produced by hypoxia was higher in CIH rats. Figure 2*E* shows the responses

elicited by an acidic-hypercapnic stimulus, revealing a multiplication of basal activity by a comparable factor in all groups, albeit higher in CIH. Yet, considering that basal activity in 3MCIH and 24MCIH rats was about half that seen in corresponding 3M and 24M controls, the actual total activity elicited by the hypercapnic stimulus also was smaller in CIH than in control animals.

Effects of CIH and age on ventilation in different atmospheres

Figure 3 shows MV in the four groups of animals while breathing in atmospheres containing 20 (room air), 12, 10 and 7% O_2 balance N_2, and air with 5% CO_2. In room air (basal conditions) 3M rats breathed 554.8 ± 111.9 ml min^{-1} kg^{-1} (95% CI, 451.3–638.4; $n = 8$), 3MCIH animals 447.9 ± 121.8 ml min^{-1} kg^{-1} (95% CI, 296.7–599.2; $n = 8$), 24M rats 331.7 ± 53.5 ml min^{-1} kg^{-1} (95% CI, 282.2–381.3; $n = 8$; $P < 0.05$ *vs.* 3M) and 24MCIH 354.1 ± 51.9 ml min^{-1} kg^{-1} (95% CI, 306.0–402.1; $n = 8$). In low O_2 atmospheres MV increased in all groups and reached a maximum in 7% O_2 atmosphere in the 3M group of 1293.0 ± 87.6 ml min^{-1} kg^{-1} (95% CI, 1212.0–1374.0). In air/5% CO_2 atmosphere MV was also higher than in air atmosphere in all groups and again reached a maximum in the 3M group of 1213.0 ± 149.7 ml min^{-1} kg^{-1} (95% CI, 1088.1–1339.3). Exposure to CIH caused around a 20% decrease in the ventilation response at all atmospheres in young animals, making the comparison between 3M and

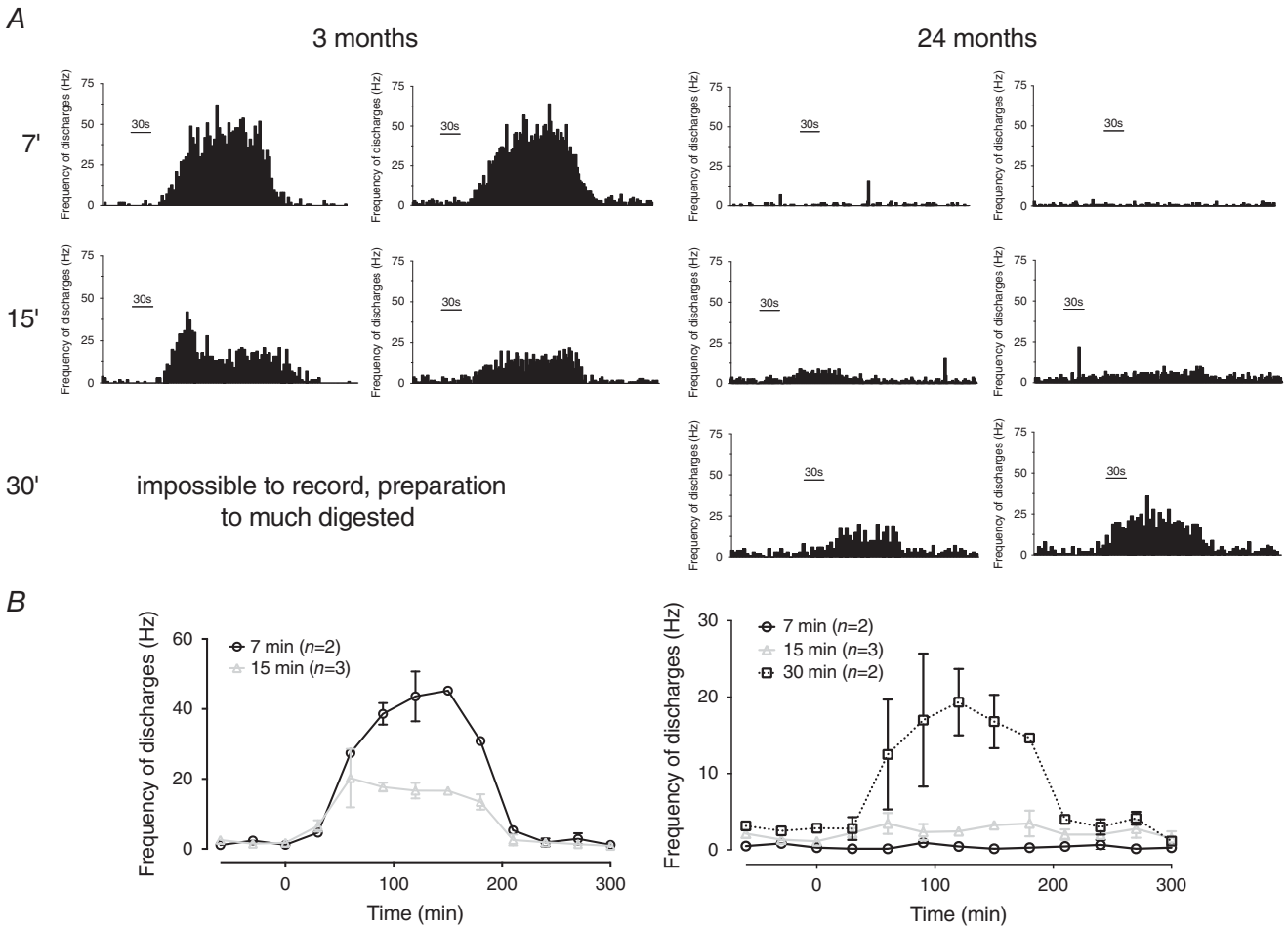

Figure 1. Effect of different times of digestion of the CB–CSN preparation on the electrical activity of the CSN evoked by hypoxia in 3M and 24M rats
Left and right panels represent, respectively, the recordings obtained in 3M and 24M animals. *A*, typical recordings of the chemosensory activity of the CSN evoked by hypoxia in CB–CSN preparation submitted to three different times of incubation: 5–7, 15 and 20–30 min in 3M and 24M animals. *B*, mean response of the CSN to hypoxia in CB–CSN preparation treated to different times of digestion, obtained in 2–3 different animals. Note that young animals submitted to lengthy digestion (15 min) exhibit a decrease in activity and in response to higher periods (30 min) of digestion it is impossible to obtain a good recording. The hypoxia used to increase the CSN response was 0% O_2 + 5% CO_2 (N_2) applied over 3 min. Data are means ± SD.

3MCIH rats statistically different ($^{*}P < 0.5$ and $^{**}P < 0.01$) in every case except breathing air. The control aged rats (24M group) ventilated less than control 3M rats, the difference in ventilation being highly significant in all atmospheres ($^{+++}P < 0.001$) except in air ($^{+}P < 0.05$). CIH did not cause any further decrease in ventilation in aged rats such that MV was statistically identical in 24M and 24MCIH rats in all atmospheres.

Table 1 shows breathing frequencies and tidal volumes (means ± SD) in the four groups of animals at different atmospheres ($n = 8$ in all cases). The effect of age is shown in Table 1*A*, indicating that in every atmosphere 24M animals breathed at lower frequencies and with lower tidal volumes, reaching statistical differences as indicated in the table ($^{+}P < 0.05$ and $^{++}P < 0.01$). To perceive the effect of intermittent hypoxia, the data in Table 1*A* and *B* should be compared. Note that in young animals (3M *vs.* 3MIH) intermittent hypoxia diminished by around 10% both breathing frequencies and tidal volumes in all atmospheres, leading to the nearly 20% decrease in MV shown in Fig. 3 (see Gonzalez-Martin *et al.* 2011; Olea *et al.* 2014). In aged rats the effect of CIH (24M *vs.* 24MCIH) was less obvious, breathing frequencies and tidal volumes fluctuating without a definite pattern.

Effects of CIH and age on plasma CA levels, RA CA content and rate of ^{3}H-CA synthesis

These three parameters were studied as indices of sympathetic activity. Figure 4 shows plasma noradrenaline (NA), adrenaline (A) and dopamine (DA) levels as well as NA/A ratios. Mean NA levels in 3M and 3MCIH rats were, respectively, 20.8 ± 10.4 (95% CI, 14.5–27.0; $n = 13$) and 39.9 ± 28.0 pmol ml^{-1} plasma (95% CI, 23.0–56.8; $n = 13$; $^{*}P < 0.05$). In 24M and 24MCIH rats, NA levels were, respectively, 17.3 ± 8.7 (95% CI, 9.3–25.3; $n = 8$) and

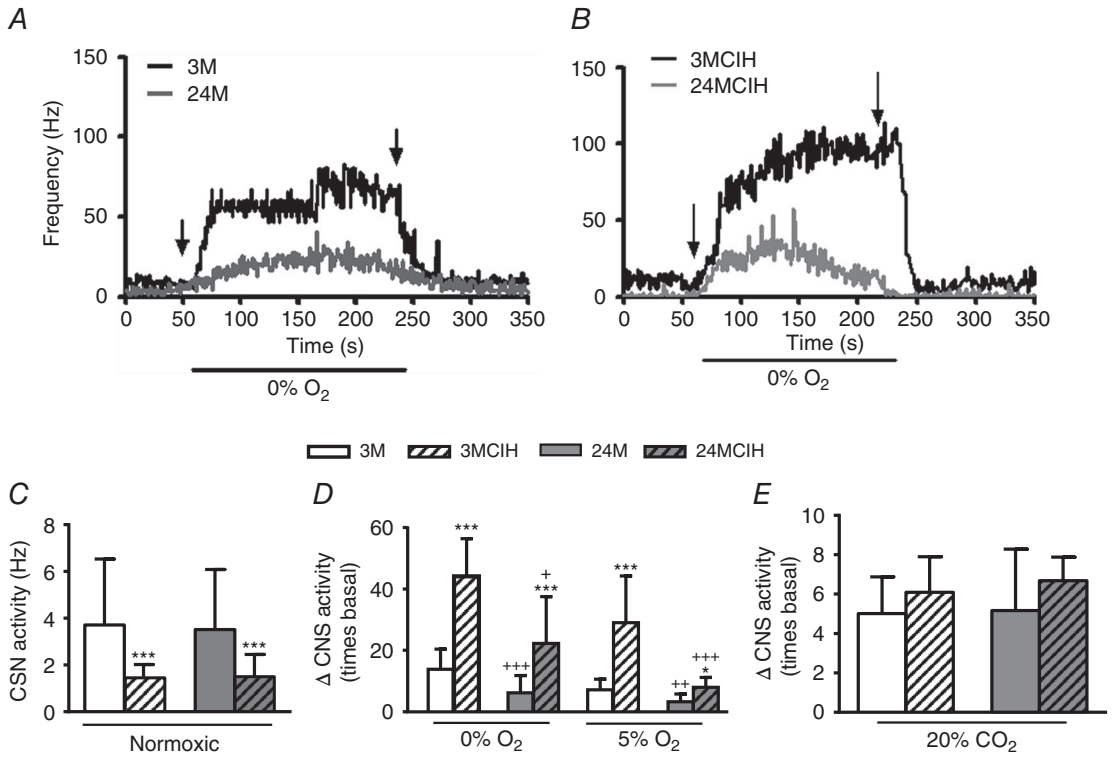

Figure 2. Effects of chronic intermittent hypoxia and age on carotid body chemoreceptor activity
A and *B*, time courses of the evolution of CSN activity in the four experimental groups on switching the superfusing solution from air to N_2-equilibrated (intense hypoxia). The most salient difference is the diminution of the response in 24M animals whether control or exposed to CIH; CIH causes an increase in the response (peak response) and differences in the time course of the response. *C*, mean basal activity obtained in the four groups of animals: young adult control, 3-month-old (3M); young experimental, exposed to CIH for 15 days (3MCIH); aged control, 24 months old (24M); and aged CIH-exposed animals (24MCIH). *D*, mean evoked activity elicited by mild and intense hypoxic stimuli expressed as times basal in the four experimental groups. *E* shows mean evoked activity elicited by a hypercapnic acidotic stimulus (superfusion with a solution equilibrated with 20% CO_2/pH 6.8). Bar charts represent mean ± SD. $^{*}P < 0.05$ and $^{***}P < 0.001$ refer to significant differences due to intermittent hypoxia exposure (i.e. 3M *vs.* 3MCIH and 24M *vs.* 24MCIH). $^{+}P < 0.05$, $^{++}P < 0.01$ and $^{+++}P < 0.001$ refer to age effects 3M *vs.* 24M and 3MCIH *vs.* 24MCIH. Statistical differences were assessed using a two-way ANOVA followed by Bonferroni's multiple comparisons test.

13.9 ± 6.8 pmol ml^{-1} plasma (95% CI, 8.3–19.6; $n = 8$), neither values being statistically different. Levels of A were similarly increased in 3MCIH *vs.* 3M rats ($^{**}P < 0.01$) and showed a tendency to increase, albeit non-significantly, in 24MCIH *vs.* 24M rats. Plasma NA/A ratios were highest in 24M animals with CIH causing a tendency to increase in young animals and to decrease in aged animals. Plasma DA levels were less than a 20th those of NA and A. In 3M rats DA levels amounted to 0.48 ± 0.15 pmol ml^{-1} plasma (95% CI, 0.40–0.57) and in 3MCIH rats were comparable and not statistically different. In 24M rats DA levels were higher, amounting to 0.75 ± 0.29 pmol ml^{-1} (95% CI, 0.44–1.05; $^{+}P < 0.05$) and CIH did not further increase them.

Plasma CA levels represent parameters intimately related to the activity of the sympathetic system: sympathetic endings spillover 90% of NA encountered in plasma and the adrenal medulla secretes all A and the remaining NA; the origin of plasma DA is multiple and not well defined (Goldstein *et al.* 1983, 2003). Yet, their rate of catabolism might distort the significance of plasma NA and A levels independently of their rate of spillover or secretion. To more precisely evaluate sympathetic activity, we have measured NA content and rate of synthesis in the sympathetic fibres/endings in the renal arteries. Figure 5*A* shows the levels of NA in renal arteries. In control 3M rats it amounted to 6.0 ± 1.8 pmol mg^{-1} tissue (95% CI, 5.2–6.8; $n = 21$) and exposure to CIH increased it to 9.4 ± 2.4 pmol mg^{-1} tissue (95% CI, 8.1–10.7; $n = 16$; $^{**}P < 0.01$). In aged rats, control and CIH animals have renal artery NA levels comparable to those seen in young rats. The rate of ^{3}H-NA synthesis in renal artery (Fig. 5*B*) was 0.50 ± 0.16 pmol mg^{-1} tissue h^{-1} (95% CI, 0.43–0.57) in 3M rats; in 3MCIH animals the rate of synthesis increased to 0.71 ± 0.08 pmol mg^{-1} tissue h^{-1} (95% CI, 0.64–0.78; $^{*}P < 0.05$). Control 24M rats exhibited a higher rate of ^{3}H-NA synthesis, 0.73 ± 0.21 pmol mg^{-1} tissue h^{-1} (95% CI, 0.57–0.88; $^{++}P < 0.01$ *vs.* 3M); contrary to the situation with young animals, in aged animals exposure to CIH tended to decrease the rate of ^{3}H-NA synthesis.

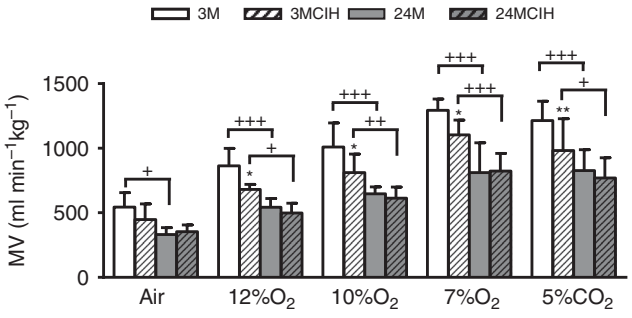

Figure 3. Effects of chronic intermittent hypoxia and age on ventilation at different atmospheres
Each group of four columns corresponds to minute ventilation (MV) in the indicated atmosphere. Data are means ± SD. Comparisons for statistical significance of the differences were made with a two-way ANOVA for repeated measures followed by Bonferroni's multiple comparisons test. $^{*}P < 0.05$ and $^{**}P < 0.01$ refer to significant differences due to CIH exposure (3M *vs.* 3MCIH). No statistical differences existed between 24M and 24MCIH. $^{+}P < 0.05$, $^{++}P < 0.01$ and $^{+++}P < 0.001$ refer to age effects 3M *vs.* 24M and 3MCIH *vs.* 24MCIH.

Effects of age and CIH on CRP and adenosine

CRP is an acute-phase reactant primarily synthesized in liver in response to inflammation mediators and released to plasma; in turn, CRP binding to endothelial cells may trigger generation of ROS, creating a vicious cycle that leads to the development of chronic inflammation and loss of the endothelial vase-relaxant and anti-adherent phenotype (Zhang *et al.* 2012). Figure 6*A* shows that levels of CRP in plasma of 3M rats amounted to 68.9 ± 20.2 μg ml^{-1} (95% CI, 56.1–81.7; $n = 12$) and in 3MCIH rats to 121.5 ± 12.5 μg ml^{-1} (95% CI, 111.0–131.9; $n = 8$; $^{***}P < 0.001$). In control aged rats (24M) CRP plasma levels were 55.2 ± 21.5 μg ml^{-1} (95% CI, 28.6–81.9) not different from those encountered in control young adult rats. CIH did not cause modifications in aged rats (57.7 ± 28.3 μg ml^{-1}; 95% CI, 28.0–87.4; $n = 6$).

Adenosine is a product of ATP metabolism, which can be recycled to resynthesize ATP itself (Conde *et al.* 2009). In addition to being a neurotransmitter–neuromodulator substance, adenosine has been postulated to substantially contribute to the ROS generation and inflammation encountered in OSAS patients, it being reported that the purine nucleoside is augmented in the plasma of these patients (see Lavie & Lavie, 2009). Recently it has been proposed that adenosine may promote an anti-inflammatory immunological status (Pettengill *et al.* 2013). Therefore, we felt it was important to ascertain whether CIH and/or age altered plasma adenosine levels. As shown in Fig. 6*B*, 3M rats had plasma adenosine levels of 599.5 ± 287.7 nmol l^{-1} (95% CI, 483.3–715.7; $n = 26$) and CIH exposure did not modify these levels. In 24M rats plasma adenosine levels amounted to 316.2 ± 116.4 nmol l^{-1} (95% CI, 261.7–370.7; $n = 20$; $^{+++}P < 0.001$ *vs.* 3M) and were not modified by exposure to CIH.

Effects of CIH and age on redox status parameters

Table 2 shows values for several indicators of the redox status monitored in brain, liver and lung tissues. The initial rows in the table correspond to GSH, GSSG and E_{GSH} in the tissues. Note that in young control 3M rats all tissues had GSH levels in the millimolar range with liver having the highest GSH levels approaching 10 mM. Note also that brain has the highest GSH/GSSG ratio so that in spite of

Table 1. Effects of chronic intermittent hypoxia and age on respiratory parameters

A

Animals	3M		24M	
Respiratory parameter	Breathing frequency (breaths min^{-1})	Tidal volume (ml kg^{-1})	Breathing frequency (breaths min^{-1})	Tidal volume (ml kg^{-1})
Atmosphere				
21% O_2 (air)	74.8 ± 8.5	7.1 ± 0.8	62.7 ± 9.3	5.9 ± 1.8
12% O_2	118.4 ± 12.9	7.5 ± 1.2	118.4 ± 17.3	6.1 ± 2.5
10% O_2	125.1 ± 19.6	8.3 ± 1.6	118.9 ± 27.02	6.3 ± 1.5^{+}
7% O_2	122.8 ± 26.7	11.3 ± 2.3	101.9 ± 23.84	8.4 ± 2.5^{++}
5% CO_2 in air	122.4 ± 10.1	10.5 ± 1.1	109.4 ± 20.1	7.9 ± 2.6^{++}

B

Animals	3MCIH		24MCIH	
Respiratory parameter	Breathing frequency (breaths min^{-1})	Tidal volume (ml kg^{-1})	Breathing frequency (breaths min^{-1})	Tidal volume (ml kg^{-1})
Atmosphere				
21% O_2 (air)	68.6 ± 10.8	6.5 ± 1.4	65.5 ± 11.6	5.5 ± 0.9
12% O_2	104.4 ± 16.4	7.0 ± 1.5	94.6 ± 15.7	5.4 ± 1.0
10% O_2	117 ± 10.1	7.0 ± 1.14	103 ± 20.8	6.2 ± 1.1
7% O_2	114.4 ± 15.5	9.9 ± 1.8	102.9 ± 24.9	8.3 ± 1.4
5% CO_2 in air	113.3 ± 14.4	9.3 ± 2.1	111 ± 20.2	7.0 ± 0.98

The table shows the breathing frequency and tidal volume from the four experimental groups of animals when breathing at different atmospheres. Data are means ± SD of eight individual values in the four groups of: young control (3M) and young CIH-exposed (3MCIH) rats and control aged (24M) and aged CIH-exposed animals (24MCIH). 3M and 3MCIH *vs.*, respectively, 24M and 24MCIH (effect of age) $^{+}P < 0.05$; $^{++}P < 0.01$ (3M *vs.* 24M tidal volume). Statistical differences while animals breathe in different atmospheres are not indicated. Two-way ANOVA for repeated measures as required followed by Bonferroni's multiple comparisons test.

having lower GSH levels it had an E_{GSH} comparable to that in the liver. Finally, lung had the lowest GSH/GSSG ratio and the lowest E_{GSH}. In young rats CIH caused no significant modifications in any glutathione-related parameter. In 24M animals the glutathione relationships among tissues just described were maintained, but in liver and lung the GSH/GSSG ratios were significantly lower than in 3M rats, and in lung also the E_{GSH} was less negative (−198.0 ± 3.0 *vs.* −213.0 ± 2.7; $^{+++}P < 0.001$). As in young animals, CIH did not alter glutathione-related

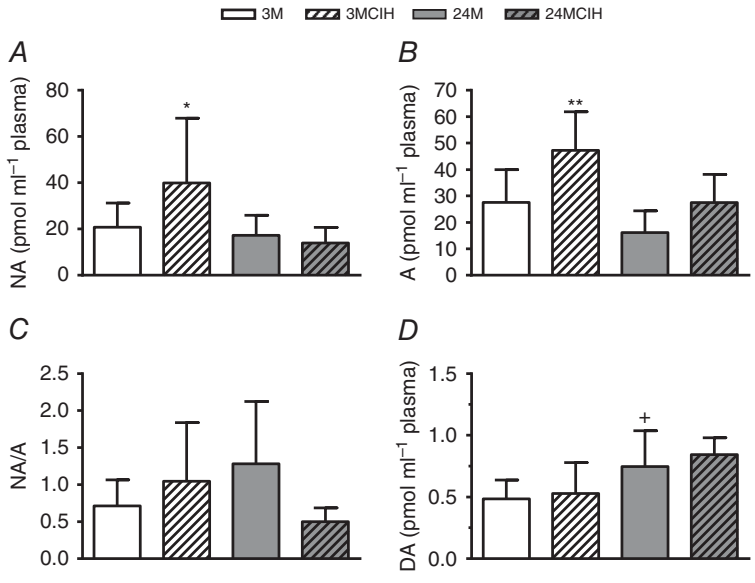

Figure 4. Effects of chronic intermittent hypoxia and age on plasma catecholamine levels
A and *B*, respectively, absolute noradrenaline (NA) and adrenaline (A) plasma levels in the four experimental groups as drawn. *C*, NA/A ratios. *D*, mean plasma dopamine (DA) levels. Data are means ± SD. One-way ANOVA, Tukey's multiple multicomparisons test. $^{*}P < 0.05$ and $^{**}P < 0.01$, statistical difference from correspondent controls (3M *vs.* 3MCIH); $^{+}P < 0.05$, statistical difference from young adult control animals (3M *vs.* 24M).

parameters in aged rats, except for a small increase in GSH in the lung ($^{*}P < 0.05$). Lipid peroxide levels (measured as malonaldehyde + 4-hydroxynonenals) were highest in lung tissue in 3M rats and although CIH tended to increase them in the three tissues, only in liver did the increase reach statistical significance. In 24M rats, LPOs were similar to those of 3M rats in brain and liver and surprisingly much lower in the lung. CIH did not alter LPOs in aged animals.

Cytoplasmic (CuZnSOD) and mitochondrial (MnSOD) superoxide dismutase activities were higher in liver than in brain and lung, and in the three tissues the ratio of cytoplasmic to mitochondrial dismutase activity was around 20–25. Intermittent hypoxia in young animals diminished the activity of the mitochondrial enzyme in brain and that of both enzymes in liver; no effect of CIH was observed in dismutating activity in lung. Age did not affect SOD activities except for a marked increase of the mitochondrial enzyme in lung tissue from 0.17 ± 0.12 units (95% CI, 0.027–0.32; $n = 5$) in young animals to 0.53 ± 0.11 units (95% CI, 0.44–0.62; $n = 8$; $^{++}P < 0.01$). Exposure of old rats to CIH did not affect SOD activities.

In every experimental condition GPx activity was maximal in liver and minimal in brain. In young 3M rats exposure to CIH increased GPx activity in lung from 90.7 ± 9.8 mU (95% CI, 81.7–99.8; $n = 8$) to 159.2 ± 10.4 mU (151.3–167.2; $n = 9$; $^{***}P < 0.001$) but did not affect activity in liver or brain. Ageing markedly augmented GPx activity in lung tissues ($^{+++}P < 0.001$) and CIH exposure to aged rats further increased lung GPx activity ($^{*}P < 0.05$). In liver, ageing also increased GPxs significantly ($^{+}P < 0.05$).

Catalase activity was also maximal in liver and in 3M young animals and reached 575 ± 97.7 units mg^{-1} protein (95% CI, 521.7–629.8; $n = 15$), being over 10 times lower in lung and over 100 times lower in brain. In 3MCIH rats, liver catalase activity diminished by nearly half ($^{***}P < 0.001$). Ageing caused catalase activity to increase in brain tissue ($^{+}P < 0.05$) and CIH showed a tendency to increase catalase activity in the three tissues in 24M rats.

Mammalian aconitase contains a $[4Fe–4S]^{2+}$ cluster which is susceptible to inactivation by oxidants, particularly $O_2^{\bullet-}$ and hydrogen peroxide, leading to the

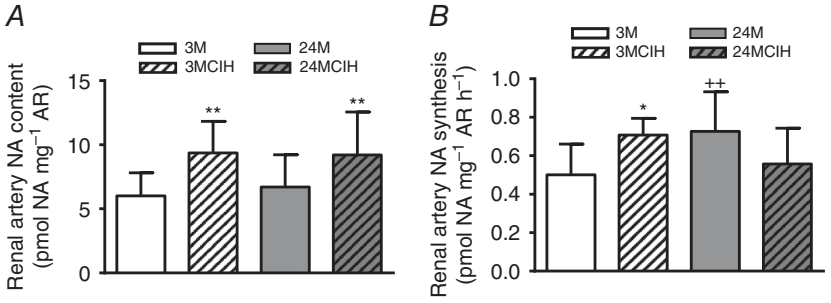

Figure 5. Effects of chronic intermittent hypoxia and age on renal artery catecholamine metabolism
A, renal artery catecholamine content in the four groups of animals. *B*, renal artery rate of ^{3}H-catecholamine synthesis. Data are means ± SD. Asterisks indicate significant differences imputable to CIH exposure, and pluses age-linked differences. One-way ANOVA, Tukey's multiple test. $^{*}P < 0.05$ and $^{**}P < 0.01$, statistical difference from correspondent controls unexposed to CIH. $^{++}P < 0.01$, statistical difference from young adult animals (3M *vs.* 24M).

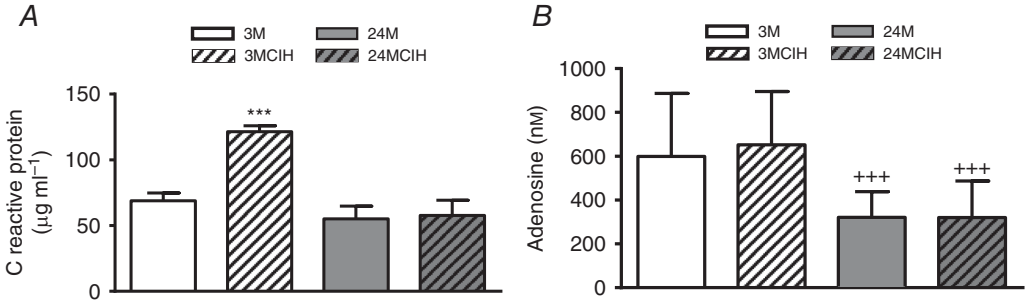

Figure 6. Effects of chronic intermittent hypoxia and age on plasma C-reactive protein (CRP) and adenosine levels
A, CRP plasma levels obtained from the four group of animals. *B*, plasma adenosine levels. Data are means ± SD. One-way ANOVA, Tukey's multiple multicomparisons test. $^{***}P < 0.001$ *vs.* 3M (CIH effect); $^{+++}P < 0.001$ *vs.* 3M and 3MCIH (age effect).

Table 2. Effects of chronic intermittent hypoxia and age on redox parameters in different tissues

Parameter	Tissue	Experimental group			
		3 months (3M)	3 months CIH (3MCIH)	24 months (24M)	24 months CIH (24MCIH)
GSH // GSSG (μmol g^{-1} tissue); GSH/GSSG ratio; E_{GSH} (mV)	Brain	1.6 ± 0.12 // 0.006 ± 0.002; 222 ± 32; −234 ± 9.2	1.6 ± 0.08 // 0.006 ± 0.002; 257 ± 16; −229 ± 4.7	1.65 ± 0.25 // 0.006± 0.001; 259 ± 17; −228 ± 3.9	1.79 ± 0.18 // 0.007 ± 0.001 253 ± 8.5; −229 ± 3.2
	Liver	8.3 ± 0.95 // 0.16 ± 0.08 101 ± 8.5; −234 ± 3.9	9.7± 0.8 //0.19 ± 0.05 91 ± 7.3; −235 ± 4.4	7.9 ± 0.93 // 0.12 ± 0.04 74 ± 7^{++}; −231 ± 5.4	8.05 ± 1.7 // 0.12 ± 0.03 68 ± 7.6; −231 ± 7
	Lung	2.1 ± 0.1 // 0.05 ± 0.009 59 ± 3.4; −213 ± 2.7	2.3 ± 0.17 // 0.06 ± 0.02 63 ± 7; −213 ± 5.7	1.61± 0.15^{+++} // 0.06 ± 0.007 27.3 ± 2.2^{+++}; −198 ± 3^{+++}	1.83 ± 0.08* // 0.06 ± 0.001 32.7 ± 2.5; −201 ± 1.9
LPO (nmol mg^{-1} protein)	Brain	0.31 ± 0.05	0.37 ± 0.13	0.32 ± 0.09	0.32 ± 0.10
	Liver	0.31 ± 0.15	0.46 ± 0.09*	0.31 ± 0.05	0.33 ± 0.09
	Lung	0.54 ± 0.11	0.59 ± 0.18	0.29 ± 0.09^{+++}	0.36 ± 0.10
CuZnSOD // MnSOD (units)	Brain	6.1 ± 1.73 //0.21 ± 0.04	5.3 ± 0.9 // 0.10 ± 0.02*	5.3 ± 1.8 // 0.36 ± 0.1	5.8 ± 1.3 // 0.35 ± 0.08
	Liver	36.8 ± 6.4 // 2.68 ± 2.21	20.7 ± 12.9** // 0.8 ± 0.6**	39.4 ± 4.7 // 3.2 ± 0.95	45.7 ± 7.8// 3.4 ± 0.5
	Lung	5.9 ± 1.5 // 0.17 ± 0.12	5.6 ± 0.3 // 0.23 ± 0.05	5.9 ± 0.8 // 0.53 ± 0.11^{++}	6.8 ± 1.5 // 0.6 ± 0.25
GPx (mU mg^{-1} protein)	Brain	27.6 ± 5.7	30.45 ± 5.6	27.7 ± 6.2	28.5 ± 8.6
	Liver	813.6 ± 165.6	736.2 ± 154.9	948.8 ± 69.26^{+}	899.3 ± 27.16
	Lung	90.74 ± 9.8	159.2 ± 10.3***	164 ± 27.2^{+++}	196.9 ± 32.3*
Catalase activity (U mg^{-1} protein)	Brain	4.95 ± 1.2	4.86 ± 0.71	6.43 ± 0.85^{+}	5.5 ± 0.93
	Liver	575.8 ± 97.7	353.9 ± 127.6***	604.9 ± 203.5	767.3 ± 68.55
	Lung	41.40 ± 7.2	40.75 ± 7.9	46.6 ± 6.1	51.3 ± 5.8

The table shows the effect of CIH and age in the four experimental groups: 3-month-old rats (3M), 3-month-old rats exposed to chronic intermittent hypoxia (3MCIH), 24-month-old rats (24M) and 24-month-old rats exposed to chronic intermittent hypoxia (24MCIH). Data are means ± SD of 5–10 individual values in the four groups of animals. 3M and 24M *vs.*, respectively, 3MCIH and 24MCIH (effect of CIH) ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. 3M *vs.* 3MCIH and 24M *vs.* 24MCIH); 3M and 3MCIH *vs.*, respectively, 24M and 24MCIH (effect of age) ($^{+}P < 0.05$; $^{++}P <$ 0.01; $^{+++}P < 0.001$. 3M *vs.* 24M). One-way ANOVA, Tukey's multiple multiple comparisons test.

release in ferrous form the Fe-α exposed to solvent. The net result is the formation of a $[3Fe–4S]^{1+}$ cluster and inactivation of the enzyme, making aconitase activity an adequate index of mitochondrial oxidative damage (Cantu *et al.* 2009). Fumarase, another tricarboxylic acid cycle enzyme, is resistant to oxidative damage (Cantu *et al.* 2009) such that the ratio of activities of aconitase to fumarase measured in the same tissue samples is a very reliable index of mitochondrial oxidative damage. Additionally, aconitase activity tends to decrease with age while fumarase does not (Yan *et al.* 1997). Figure 7 shows aconitase/fumarase ratios in liver, lung and brain. In liver of control 3M rats the aconitase/fumarase ratio was 0.13 ± 0.02 (95% CI, 0.11–0.14; $n = 8$) and diminished to 0.05 ± 0.01 (95% CI, 0.4–0.6; $n = 6$; $^{***}P < 0.001$) in 3MCIH rats. In eight aged control animals (24M) the ratio was nearly identical to that found in young rats and CIH did not cause a diminution of the enzyme ratio. In lung of 3M rats the ratio of aconitase/fumarse activities was higher than in the liver, 0.58 ± 0.04 (95% CI, 0.54–0.61; $n = 8$), and decreased to 0.26 ± 0.15 (95% CI, 0.12-0.39; $^{***}P < 0.001$) in 3MCIH rats. In 24M rats, the lung aconitase/fumarase ratio was about half that seen in control animals ($^{+++}P < 0.001$) and CIH did not alter it. In brain the aconitase/fumarase ratio in young control animals was highest, 0.84 ± 0.7 (95% CI, 0.78–0.90), and fell in 3MCIH rats to 0.36 ± 0.04 (95% CI, 0.33–0.39; $^{***}P < 0.001$). In 24M rats the ratio was 0.60 ± 0.5 (95%CI, 0.56–0.65; $^{+++}P < 0.001$ *vs.* 3M). Again CIH did not alter the ratio of activities in the brain of aged rats.

Effects of CIH and age on mean arterial blood pressure

Arterial blood pressure is the most easily and commonly explored cardio-circulatory parameter to ascertain the effects of CIH (see Fletcher *et al.* 1992), it being well known that age causes a slow and nearly steady increase in arterial blood pressure, both in humans and in experimental animals (Soltis, 1987; Franklin *et al.* 1997; Safar *et al.* 2011; Wills *et al.* 2011). Figure 8 shows that CIH augmented mean arterial blood pressure in young rats from 101.9 ± 7.2 mmHg (95% CI, 89.5–114.2; $n = 8$) to 150.9 ± 25.4 mmHg (95% CI, 127.5–174.5; $n = 7$; $^{***}P < 0.001$) in 3MCIH animals. By contrast, aged 24M animals exhibited, when compared with young animals, an elevated blood pressure of 128.1 ± 26.9 mmHg (95% CI, 110.0–146.2; $n = 11$) and CIH did not augment it, 132.9 ± 16.0 mmHg (95% CI, 122.6–143.1; $n = 11$). In all groups a 3 min episode of 10% O_2 breathing caused a 35–40% decrease in AP which recovered promptly after returning to air breathing. Heart frequency oscillated between 330 and 360 beats/min^{-1} in different groups without statistically significant differences among them. Similarly, the Fulton index of the heart oscillated between 0.23 and 0.25 with no statistical differences among them.

Discussion

We have used a comparative study of the effects of CIH in young adult and aged rats, along with their age-matched controls, to dissecting out the effects of CIH at both ages and the effects of age. The significance of basal activity recorded in a few fibres of a CSN preparation from different aged rats and subjected to specific experimental protocols should be interpreted cautiously. The observed differences may result from a different number of fibres included in the filaments split for recording due to variability of nerve structures in each experimental condition; this led to the expression of responses to different stimuli as values times basal activity (Fidone *et al.* 1982; Conde *et al.* 2006*a*). Experiments do not allow to discern whether differences in basal CSN activity are due to differences in the number of fibres. Comparing the results obtained in 3M and 3MCIH rats it is possible to say that CIH makes the CB hyper-reactive to hypoxia in young animals while maintaining or even lowering the reactivity to CO_2 (Gonzalez-Martín *et al.* 2011). Aging *per se*

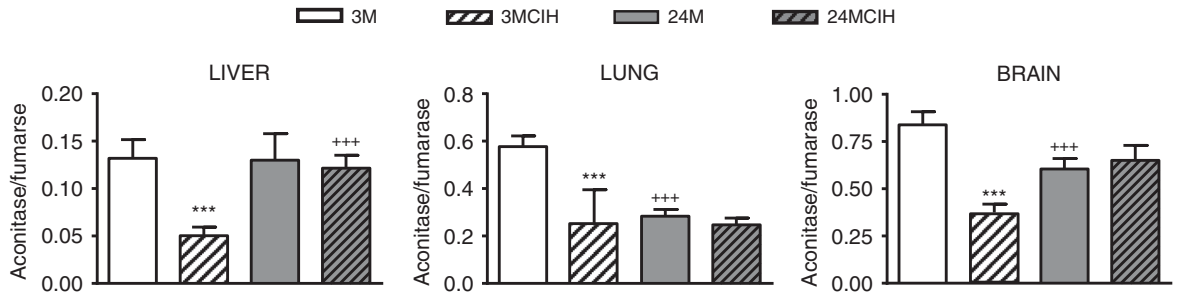

Figure 7. Effects of chronic intermittent hypoxia and age on and fumarase activity from liver, lung and brain mitochondria
Aconitase/fumarase ratio from liver, lung and brain tissue of the four groups of animals. Young adult control, 3-month-old (3M); young adult experimental, exposed to CIH for 15 days (3MCIH); aged control (24M); and aged CIH-exposed animals (24MCIH). Data are means ± SD. One-way ANOVA, Tukey's multiple multicomparisons test. $^{***}P < 0.001$ *vs.* 3M (CIH effect in young adult animals); $^{+++}P < 0.001$ *vs.* 3M and 3MCIH (age effect).

produces a marked CB hyporeactivity to hypoxia without affecting that to hypercapnia (Conde *et al.* 2006*a*); CIH effects on CB sensitiveness in old animals are comparable, but less intense than those in young animals. Coupling the activity in CSN to ventilation indicates that 3MCIH in comparison to 3M rats have augmented CB hypoxic input to brainstem and significantly lower ventilatory response, implying an augmented integration of the chemoreceptor input with a loss in gain of the chemoreceptor input to respiratory centres. This gain loss in the respiratory centre appears to be associated with a facilitation of the chemoreceptor information transference to the centre controlling sympathetic vasomotor areas. CIH would cause plastic changes, breaking the increased chemoafferent activity to stabilize breathing during CIH chemoreceptor activation and intensifying the sympathetic responses elicited by the hypoxic stimulation of the CB chemoreceptors (Kline, 2010; Moraes *et al.* 2012; Almado *et al.* 2014).

There is information supporting those notions and several plastic mechanisms have been proposed. For example, Greenberg *et al.* (1999) were pioneers in showing that intermittent hypoxia elicited a pattern of c-fos expression in well-defined regions of medulla oblongata involved in the tonic and reflex control of sympathetic activity: intermittent hypoxia increased expression of c-fos in nucleus of the tractus solitarius (NTS), which represents the first relay station of the CB chemoreflex, and in the rostroventrolateral medulla (RVLM), which represents the presympathetic neuronal origin of the sympathetic activity controlling cardiovascular functions (Guyenet, 2000; Almado *et al.* 2014).

The present findings show an increased sympathetoadrenal tone, including a very significant increase in renal sympathetic activity, with the final outcome being an augmented arterial blood pressure in young adult animals. The augmentation of plasma A levels (Fig. 4) indicates the increased activity of the splanchnic nerves which innervate the adrenal medulla (Xing & Pilowsky, 2010). The increase in plasma NA levels (Fig. 4) should reflect an augmentation of the sympathetic tone in many bodily territories. Indeed, the small percentage of NA coming from the AM, and even the lower percentage of NA spilling over to blood from the sympathetic endings, which recapture and recycle or metabolize most of the NA released, would favour an origin in wide sympathetically innervated areas (Goldstein *et al.* 1983, 2003). Several authors have found that CIH in animal models or in OSA patients augments sympathetic discharges in cervical, thoracic, splanchnic, renal and lumbar sympathetic nerves (see Prabhakar *et al.* 2005, 2012 for references). Data showing an increase in renal artery NA content and rate of synthesis (Fig. 5) indicate that renal sympathetic activity is augmented, suggesting that it plays a prime role in the genesis of the hypertension observed in young adult animals exposed to CIH (Fig. 8; see Fletcher *et al.* 1999; Linz *et al.* 2012).

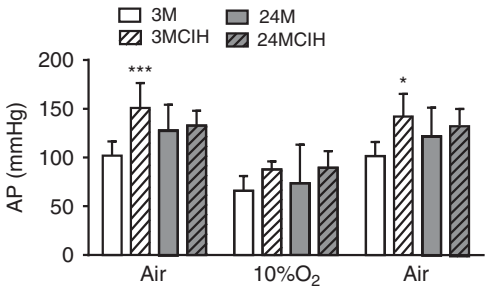

Figure 8. Effects of chronic intermittent hypoxia and age on mean arterial blood pressure (AP)
Mean arterial blood pressure from the four groups of animals breathing air or a 10% O_2 atmosphere. Data are means ± SD. ***$P < 0.001$ and *$P < 0.05$, statistically different from correspondent controls (3M).

The data for CB chemoreceptor activity and arterial blood pressure are different in old and young adult animals, both control and CIH-exposed. Pawar *et al.* (2008), comparing the CB responses to CIH of neonatal and adult (2–3 months old) rats, found a pronounced hypoxic sensitization of neonatal CB as well as morphological remodelling of the chemoreceptor tissue, indicating the existence of age-dependent changes of the CB response to CIH. It has also recently been shown (Peng *et al.* 2006; Nandury *et al.* 2015) that hypertension induced in CIH rodent models is linked to dysregulated hypoxia-inducible factor (HIF)-mediated transcription. CIH activates HIF-1 and suppresses HIF-2-mediated transcription. This dysregulation leads to an increased ROS generation in the chemosensory reflex, which is central to developing hypertension.

In previous studies we have discussed plausible mechanisms leading to diminished CB hypoxic responses in ageing, including a decrease in chemoreceptor cell mass, an increase in time of neurotransmitter turnover and as yet undefined changes in the O_2-sensing machinery (Conde *et al.* 2006*b*). The diminished CB chemoreceptor drive observed (Fig. 2) would produce the low ventilation in aged *vs.* young animals: CSN drive is an important contributor to set baseline ventilation, responsible for nearly the entire hypoxic drive and a significant contributor (60–70%) to the acidic hypercapnic ventilatory response (Fidone & Gonzalez, 1986; Gonzalez *et al.* 1994). However, the diminution of minute ventilation in aged animals herein and previously described (Monteiro *et al.* 2011) does not signify hypoventilation because it is well known that ageing is accompanied by a decrease in whole body O_2 consumption (compare fig. 6 of Gregerman, 1986 and Fig. 3 of the present study; see also Hawkins & Wiswell, 2003). A progressive and parallel variation in both parameters would maintain a constant alveolar minute ventilation to minute oxygen consumption ratio through the mammal's life span.

CIH causes a certain degree of CB sensitization, particularly to intense hypoxic stimuli (Fig. 2), although CIH-exposed aged rats show normal ventilation, suggesting that CIH does not elicit plastic modifications in brainstem nuclei capable of lessening the gain of the chemoreceptor input to respiratory centres (Fig. 3), and does not elicit plastic changes facilitating the transfer of chemoreceptor information to presympathetic neurons as no evidence for generalized sympathetic hyper-reactivity (Fig. 4) or hypertension in aged animals is observed (Fig. 8). Using whole body plethysmography in freely moving animals and avoiding spurious variables such as circadian variations and temperature effects we have consistently observed that CIH causes a diminution of ventilation in young adult rats (Gonzalez-Martin *et al.* 2011; Olea *et al.* 2014). The findings obtained in aged rats exposed to CIH indicate that plastic, adaptive or maladaptive processes (Prabhakar & Semenza, 2012) occurring in the brainstem of young adult animals and leading to hypertension do not take place in aged animals. The age-dependent loss of neural plasticity along with the synaptic and extrasynaptic mechanisms proposed to be involved are well known (Bergado & Almaguer, 2002; Guzman-Karlsson *et al.* 2014).

Oxidative stress seems to be a leading trigger for CIH-induced plastic changes, inducing a downregulation of HIF-2α activity and subsequent diminution of the expression of SOD, fundamental determinants of oxidative status (Penget *et al.* 2006; Prabhakar & Semenza, 2012). Contrary to the situation in young adult rats, CIH did not cause a decrease in the activity of any of the anti-oxidant enzymes assayed (SOD1, SOD2, GPx and catalase) in old animals. The increased AP baseline encountered in old rats was an expected finding. It is known that there is a multifactor age-dependent AP increase (Soltis, 1987; Franklin *et al.* 1997; Safar *et al.* 2011). Among those factors, in addition to augmented arterial stiffness, an increase in renal sympathetic activity seems to be important in the age-dependent AP rise (Hart & Charkoudian, 2014). A higher turnover rate of renal NA levels in aged rats (Fig. 5) is also present in our observations.

The effects of CIH on the genesis and significance of the alterations in redox parameters and their significance in young adult animals have been discussed in detail in recent publications (Quintero *et al.* 2013; Olea *et al.* 2014; see Prabhakar & Semenza, 2012). The CIH alteration of oxidative status affects tissue in a specific manner and liver is particularly sensitive (Table 2 and Fig. 7). Hepatic oxidative damage might help to explain the alterations in lipid metabolism encountered in OSA patients and animal models (Ahmed & Byrne, 2010). The significance of redox status alterations (pro-oxidative or oxidative status) has been linked to the cardiovascular pathologies encountered in OSA. Augmented ROS, as well as inflammatory status (Fig. 6), are direct mediators of endothelial damage resulting in loss of the anti-adherent, vasorelaxant and anti-proliferative phenotypes and drivers of the structural organization of vascular alterations (Lavie & Lavie, 2009; Jelic *et al.* 2010; Lavie and Lavie, 2012; Moraes *et al.* 2012). The lack of effects of CIH on plasma adenosine levels and the dual significance attributed to it (Lavie & Lavie, 2009; Pettengill *et al.* 2013) precludes a discussion of the meaning of this parameter. The lower levels found in aged animals would probably reflect a diminution of the overall metabolic rate with ageing (Conde *et al.* 2009).

The observed effects of age on redox parameters are a moderate decrease of the lung E_{GSH}, an increase of GPx activity, a diminution of LPO and an increase of mitochondrial SOD activity. There is also a decrease of the aconitase/fumarase ratio, mostly in the lung, which could be related to the age-dependent decrease in aconitase activity and mitochondrial oxidative capacity (Yan *et al.* 1997; Delaval *et al.* 2004). The increase in lung mitochondrial SOD suggests that mitochondrial oxidative damage is not a major determinant of the decrease in the aconitase/fumarase activity ratio. CIH did not alter any of the redox parameters measured in the pro-oxidative direction, indicating that aged animals tolerate CIH apparently without any of the ROS-triggered deleterious effects. There is a mild sensitization of the CB chemoreceptors, there is no augmented integration of chemoreceptor input causing hypoventilation and facilitation of sympathetic tone, and there is no sustained hypertension, all processes in which ROS participate (Prabhakar & Semenza, 2012). These findings do not show any sign of CIH causing oxidative status in aged animals. Each CIH episode in aged animals causes an P_{aO_2} swing that is considered to represent an ROS-generating event particularly in endothelial cells (Lavie & Lavie, 2009, 2012). Our findings indicate that there is no accumulating oxidative damage and the presumptive waves of ROS production do not seem to cause endothelial damage and permanent remodelling of blood vessels in old animals. It has been recently observed that transient and prolonged exposures to H_2O_2 are much better tolerated by endothelial cells from aged than from young adult rats (Socha *et al.* 2015).

The present study shows that ageing affords a protection against the deleterious effects produced by CIH in young adult animals and that this protection comes from two important pathogenic pathways in the genesis of CIH-linked co-morbidities: the absence of an augmented sympathetic tone and the absence of an oxidative status. Our findings contribute to the understanding and evaluation of the hazy clinical picture encountered in the late onset of OSAS patients. The treatment of hypertension in these patients could be more directed to reduce vessel stiffness and endothelial damage than to contractile properties of vascular smooth muscle. More in-depth studies on the cellular and molecular mechanisms

occurring during the normal ageing process and the resistance to the appearance of OSA co-morbidities are needed.

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Additional information

Competing interests

None of the authors has any conflict of interest.

Author contributions

The experiments were performed in the Department of Biochemistry and Molecular Biology and Physiology of the University of Valladolid. M.Q. and T.A. managed all redox experiments; E.O., S.Y., A.G.-N. and T.G.-M. carried on the plethysmography, blood pressure measurements, HPLC analyses of catecholamines and performed the statistical tests; S.C. performed the electrophysiological recordings in the carotid sinus nerve and analyses of adenosine. A.O. carried out most of the surgical procedures and designed the figures and C.G. and J.M.M. designed the experiments. C.G. wrote the first draft of the manuscript; all authors read and made suggestions to the manuscript. A.G.-N. and S.Y. reviewed the manuscript and incorporated the referees' suggested changes.

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