High fat diet blunts the effects of leptin on ventilation and on carotid body activity

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Key points

- Leptin plays a role in the control of breathing, acting mainly on central nervous system; however, leptin receptors have been recently shown to be expressed in the carotid body (CB), and this finding suggests a physiological role for leptin in the regulation of CB function.
- Leptin increases minute ventilation in both basal and hypoxic conditions in rats. It increases the frequency of carotid sinus nerve discharge in basal conditions, as well as the release of adenosine from the CB. However, in a metabolic syndrome animal model, the effects of leptin in ventilatory control, carotid sinus nerve activity and adenosine release by the CB are blunted.
- Although leptin may be involved in triggering CB overactivation in initial stages of obesity and dysmetabolism, resistance to leptin signalling and blunting of responses develops in metabolic syndrome animal models.

Abstract Leptin plays a role in the control of breathing, acting mainly on central nervous system structures. Leptin receptors are expressed in the carotid body (CB) and this finding has been associated with a putative physiological role of leptin in the regulation of CB function. Since, the CBs are implicated in energy metabolism, here we tested the effects of different concentrations of leptin administration on ventilatory parameters and on carotid sinus nerve (CSN) activity in control and high-fat (HF) diet fed rats, in order to clarify the role of leptin in ventilation control in metabolic disease states. We also investigated the expression of leptin receptors and the neurotransmitters involved in leptin signalling in the CBs. We found that in non-disease

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M. J. Ribeiro and J. F. Sacramento contributed equally to this work

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conditions, leptin increases minute ventilation in both basal and hypoxic conditions. However, in the HF model, the effect of leptin in ventilatory control is blunted. We also observed that HF rats display an increased frequency of CSN discharge in basal conditions that is not altered by leptin, in contrast to what is observed in control animals. Leptin did not modify intracellular Ca^{2+} in CB chemoreceptor cells, but it produced an increase in the release of adenosine from the whole CB. We conclude that CBs represent an important target for leptin signalling, not only to coordinate peripheral ventilatory chemoreflexive drive, but probably also to modulate metabolic variables. We also concluded that leptin signalling is mediated by adenosine release and that HF diets blunt leptin responses in the CB, compromising ventilatory adaptation.

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Introduction

Leptin is an adipocyte-derived hormone with multiple effects in the physiology and pathophysiology of metabolism, energy homeostasis and endocrinology (Blüher & Mantzoros, 2015). It binds to leptin receptors, located throughout the central nervous system and in the peripheral tissues, to regulate satiety, food intake, energy expenditure, neuroendocrine systems and immune function (Blüher & Mantzoros, 2015). In humans leptin secretion is pulsatile and circadian, being significantly affected by disrupted sleep patterns that are often associated with obesity (Mullington *et al.* 2003). The scientific excitement related to the potential therapeutic impact of leptin faded when it was established that obesity and obesity-related cardiometabolic diseases were characterized by non-functional leptin, and subsequent hyperleptinaemia (Phillips *et al.* 2000, Sanner *et al.* 2004, Schmidt *et al.* 2006). Indeed, both human and animal studies demonstrated resistance to anorexic and weight-lowering effects of leptin in obese subjects while some of its actions on the sympathetic nervous system, namely renal and adrenal activation, remained preserved (Correia *et al.* 2002, Eikelis *et al.* 2003, Rahmouni *et al.* 2005), suggesting that leptin resistance, which characterizes obesity-related states, is tissue selective. In recent years, leptin has been shown to influence ventilation control, as the administration of the hormone reverses hypoxia and hypercapnia commonly encountered in leptin-deficient animal models (Tankersley *et al.* 1998, O'Donnel *et al.* 1999). Leptin is now known to play a role in the control of breathing, acting mainly on central nervous system structures; however, further actions on peripheral arterial chemoreceptors have also been suggested in the literature. Leptin receptors are expressed in type I cells of both rat and human carotid bodies (CBs), and this finding has been associated with a physiological role of circulating or locally produced leptin in the regulation of CB function (Porzionato *et al.* 2011). It has been observed that leptin administration induces the phosphorylation of signal transduction proteins within carotid body cells similar to the response produced by hypoxia (Messenger *et al.* 2012). These results promoted the idea that the ventilatory effects of leptin are, at least in part, mediated by the CB chemoreceptors; however, controversy regarding the role of leptin in the peripheral control of breathing function endures. Leptin microinjections into the nucleus tractus solitarii of rats increases pulmonary minute ventilation and tidal volume and enhances bioelectrical activity of the inspiratory muscles, suggesting a direct effect of leptin in ventilatory control through central respiratory control centres (Inyushkin *et al.* 2009). Results obtained in our lab also indicate that the stimulatory effect of leptin on ventilation is centrally mediated and not CB-controlled (Olea *et al.* 2015). The existence of functional leptin receptors in CB type I cells may be related to a distinct physiological action of the CB: besides its role in cardio-respiratory control, the CB has been proposed as a metabolic sensor, implicated in the control of whole body insulin sensitivity. Hypercaloric diets cause CB over-activation in rats, and this increased firing rate seems to be at the origin of insulin resistance and hyper-tension developed in these animals. Consistent with this notion, CB sensory denervation prevents metabolic and haemodynamic alterations in hypercalorically fed animals. Knowing that the CB expresses leptin and a functional leptin-B receptor (Porzionato *et al.* 2011), and that leptin and the CBs are implicated in energy metabolism, the objective of this work was to explore the influence of leptin on CB activity in a metabolic syndrome animal model.

We have previously reported that CB activity is increased in both lean and obese animal models of insulin resistance (Ribeiro *et al.* 2013). However, obese animals exhibited more pronounced increases in spontaneous ventilation, ischaemic hypoxia-induced hyperventilation, CB weight and tyrosine hydroxylase expression at the carotid bodies compared to lean animals (Ribeiro *et al.* 2013). We have also demonstrated that the increased ventilatory responses observed in both animal models were due to CB over-activation caused, at least partially, by hyperinsulinaemia

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(Ribeiro *et al.* 2013). However, when looking at plasma insulin levels, there were no significant differences between lean and obese animal models that could justify, *per se*, the differences found in CB-related functional parameters between the two animal models of insulin resistance. These results supported the hypothesis that there is an obesity-related factor that contributes to CB stimulation in a metabolic dysfunction setting. Knowing that hyperleptinaemia is a feature of obesity, we hypothesized that leptin could also contribute to the CB overactivation that is seen in metabolic models (Ribeiro *et al.* 2013) and that can aggravate insulin resistance and glucose intolerance. Herein we tested the effects of different concentrations of systemic and intracarotid leptin administration on ventilatory parameters and on carotid sinus nerve (CSN) activity in control and high-fat diet fed rats in order to clarify the role of leptin in ventilation control in metabolic disease states. We also investigated the expression of leptin receptors and assessed the neurotransmitters involved in leptin signalling in the CBs.

Methods

Ethical approval

All animal experimental and care procedures were approved by the Institutional Committee of the University of Valladolid and Faculdade de Ciências Médicas, NOVA Medical School for Animal Care. Principles of laboratory care were followed in accordance with the European Union Directive for Protection of Vertebrates Used for Experimental and Other Scientific Ends (2010/63/EU). Our work complies with the animal ethics guidelines as outlined in the editorial by Grundy (2015).

Animals and surgical procedures

Experiments were performed in adult male Wistar rats (250–400 g), aged 3 months, obtained from the vivarium of the Faculdade de Ciências Médicas, NOVA Medical School of Lisbon and from the vivarium of the Faculty of Medicine of the University of Valladolid. The rats were kept at a constant temperature (21°C) and a regular light (08.00–20.00 h) and dark (20.00–08.00 h) cycle, with food and water *ad libitum*. Two groups of rats were used: a control group that was fed a standard diet (14.5% protein, 10% fat, 55.1% carbohydrates; RM3, Special Diet Services, Witham, Essex, UK) and a high-fat (HF) diet group, a model that combines obesity, insulin resistance and hypertension, that was fed with 60% energy from fat (23.1% protein, 34.9% fat, 25.9% carbohydrates; 58Y1, TestDiet, St Louis, MO, USA) for 3 weeks (Ribeiro *et al.* 2013; Sacramento *et al.* 2017). Body weight and animal behavioural changes were assessed twice per week. At the end of 3 weeks on the diets, animals were tested for insulin sensitivity through an insulin tolerance test (Ribeiro *et al.* 2013; Sacramento *et al.* 2017). Rats were anaesthetized with sodium pentobarbital (60 mg kg^{-1} i.p.), since pentobarbital was shown not to alter glucose metabolism (Guarino *et al.* 2013), tracheostomized and the carotid arteries were dissected past the carotid bifurcation, excepted when *in vivo* experiments were performed to study effect of leptin on basal ventilation and on ventilatory responses to ischaemic hypoxia. For cell dissociation, adenosine release experiments and western blots, the CBs were cleaned free of CSN and nearby connective tissue. For the recording of CSN activity, the carotid artery bifurcations were placed in ice-cold/100% O_2-equilibrated Tyrode solution (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.1 mM $MgCl_2$, 10 mM Hepes and 5.5 mM glucose; pH 7.4). After dissection of surrounding tissue, the CB–CSN preparation was digested in collagenase type I (1 mg ml^{-1}; Worthington Biochemical Corp., Lakewood, NJ, USA) solution to loosen the perineurium. In each group of experiments, different animals were used.

At the end of the experiments, the rats were euthanized by an intracardiac overdose of pentobarbital, except when heart puncture was performed to collect blood. Death was confirmed by cervical dislocation.

Insulin tolerance test

Insulin sensitivity was evaluated through an insulin tolerance test (ITT) in conscious animals. The ITT consisted of the administration of an intravenous insulin (Humulin®, 100 IU ml^{-1}, Lilly, Lisboa, Portugal) bolus of 0.1 U (kg body weight)$^{-1}$ in the tail vein, after an overnight fast (approx. 16 h), followed by the measurement of the decline in plasma glucose concentration over a 15 min period. The constant rate for glucose disappearance (K_{ITT}) was calculated using the formula $0.693/t_{1/2}$. Glucose half-time ($t_{1/2}$) was calculated from the slope of the least square analysis of plasma glucose concentrations during the linear decay phase. Blood samples were collected by modified tail snip technique and glucose levels were measured with a glucometer (Precision Xtra Meter, Abbott Diabetes Care, Amadora, Portugal) and test strips (Abbott Diabetes Care).

Effect of leptin on basal ventilation and on ischaemic hypoxia ventilatory responses. A detailed description of these methods was published previously (Monteiro *et al.* 2011). In brief, control rats and rats submitted to the HF diet were anaesthetized with sodium pentobarbital (60 mg kg^{-1}, i.p.) and supplemented intravenously with 10% of the initial dose as necessary, to make them areflexic to a nociceptive stimulus (effects of corneal reflexes and pinch to the front paw on the rise in arterial blood pressure). The trachea was exposed in the neck, sectioned below the larynx and a differential air pressure transducer

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was connected to the distal end of the tracheostomy tubing to evaluate respiratory frequency, tidal and minute volume (emKa technologies, Paris, France) in anaesthetized and tracheostomized control rats and in rats submitted to the HF diet. Bilateral midcervical vagotomy was performed to abolish the role of vagal afferents innervating the lungs and the aortic chemoreceptors with a major influence on respiratory activity (Marek *et al.* 2008). The femoral artery and vein were cannulated under a dissection microscope to measure blood pressure and to administer leptin and anaesthetic supplements, respectively. Body temperature was maintained close to 37 ± 1°C using a heated underblanket controlled by a rectal thermistor probe. To study the effect of leptin on basal ventilation and on ischaemic hypoxia ventilatory responses, leptin (90, 180 and 270 ng ml^{-1}) was administered intravenously on the femoral vein and the respiratory parameters and blood pressure were continuously recorded in anaesthetized and vagotomized rats breathing spontaneously and submitted to bilateral occlusion (5–15 s) of common carotid artery. Additionally, to study the effect of leptin on CB-mediated respiratory responses in control and HF animals, a leptin bolus (90 and 270 ng ml^{-1}) was administered in external carotid artery reaching the CB by being pushed by the blood flow of common carotid artery. Ventilatory parameters as described above were monitored.

Effect of leptin on *ex vivo* carotid sinus nerve activity in basal and hypoxic conditions. The CB–CSN preparation was transferred to a recording chamber mounted on a dissection microscope (Nikon, Tokyo, Japan) and superfused with bicarbonate/CO_2 buffered saline (120 mm NaCl, 3 mM KCl, 2 mM $CaCl_2$, 1.1 mM $MgCl_2$, 5 mM glucose and 24 mM $NaHCO_3$, pH 7.4) equilibrated with 20% O_2 + 5% CO_2, balance N_2 at 37°C. Extracellular recordings from a single or a few fibres of CSN were performed using a suction electrode. The pipette potential was amplified (Neurolog, Digimiter, Welwyn Garden City, UK), filtered with low pass (5 kHz) and high pass filters (10 Hz), digitized at 5 kHz (Axonscope, 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 (0% O_2 + 5% CO_2, balance N_2). CSN unit activity was converted to logic pulses, which were summed every second and converted to a voltage proportional to the sum.

The effect of leptin (180 ng ml^{-1}; R&D Systems, Minneapolis, MN, USA) on the CSN activity has been investigated while superfusing the preparations with normoxic solution (20% O_2-equilibrated) for 5 and 30 min. Furthermore, the effect of 180 and 270 ng ml^{-1} of leptin on the CSN chemosensory activity was tested in normoxic solution (20% O_2-equilibrated) for 5 min and in hypoxic solution (0% O_2-equilibrated).

Effect of leptin on intracellular calcium in isolated chemoreceptor cells. Rats from the vivarium of the Faculty of Medicine of the University of Valladolid were anaesthetized with sodium pentobarbital (60 mg kg^{-1}, I.P), the bifurcations of the carotid arteries were removed and the CBs were cleaned free of nearby connective tissue. After this, the CBs were incubated (15 min) at 37°C in Ca^{2+}- and Mg^{2+}-free Tyrode solution (pH 7.2) containing collagenase (2.5 mg ml^{-1}, type IV, Sigma-Aldrich, Madrid, Spain) and bovine serum albumin (6 mg ml^{-1}, Fraction V, Sigma-Aldrich). After that, the solution was removed and the CBs were incubated for 10 min in a fresh Ca^{2+}- and Mg^{2+}-free Tyrode solution containing trypsin (1 mg ml^{-1}, type II, Sigma-Aldrich) and bovine serum albumin (6 mg ml^{-1}). Then, enzymatic activity was inhibited by adding 2.5 ml of Dulbecco's modified Eagle's medium (DMEM) at 4°C, supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin–fungizone and 2 mM L-glutamine. The cells were mechanically dispersed with a micropipette and centrifuged at 200 g for 5 min (Gallego-Martin *et al.* 2012). After centrifugation cells were placed in 100 μl of culture medium. Dispersed cells were plated as 25–30 μl drops on small poly-L-lysine-coated coverslips kept in 12-well plates and maintained in a humidified incubator (37°C; 5% CO_2 in air). Once the cells attached, 1 ml of supplemented DMEM was added to each well. In this medium 40 ng ml^{-1} leptin was added to the experimental cells and leptin-free medium to the control cells. At 24 h of incubation the medium was renewed with or without leptin. Control and experimental cells (with leptin) were maintained in the incubator for a total of 48 h until use for intracellular Ca^{2+} measurements.

Intracellular Ca^{2+} measurements in CB chemoreceptor dissociated cells have been described in previous publications (Gallego-Martin *et al.* 2012). Briefly, the dissociated cells were incubated with 2 μM Fura 2-AM (Thermo Fisher Scientific, Madrid, Spain) diluted in Tyrode solution at room temperature for 30–45 min under continuous orbital agitation. After that, coverslips were washed with Tyrode solution for 30 min and mounted in a perfusion chamber placed on the stage of a Nikon Diaphot 300 inverted microscope and connected to the perfusion system (maintained at 37°C). The perfusion of Tyrode–bicarbonate balanced solution (20% O_2, 5% CO_2, balance N_2), was performed by gravity, with a flow of 3 ml min^{-1}. During the experiment the cells were subjected to a hypoxic stimulus (Tyrode–HCO_3^- gassed with 95% N_2 and 5% CO_2) and depolarizing stimulus (Tyrode–HCO_3^- with 35 mM K^+). Dye wavelength excitation was alternated between 340 and 380 nm, and fluorescence emission at 540 nm was collected with a SensiCam digital Camera (PCO CCD imaging, PCO, Kelheim, Germany). The exposure time was 100 ms. The background was removed (MetaFluor program, Molecular

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Devices, Wokingham, UK) and the variations in the cytosolic [Ca^{2+}] were presented as the ratio between the fluorescence emitted after excitation at 340 nm and the fluorescence emitted after excitation at 380 nm (ratio 340 nm/380 nm). The illumination system and camera were driven by Axon Imaging Workbench 4.0 (Molecular Devices, Wokingham, UK) running on a Pentium computer.

CB *in vitro* adenosine release in response to hypoxia and to leptin. CBs were cleaned free of CSN and nearby connective tissues under a dissection microscope and incubated in Tyrode solution (Conde *et al.* 2012*a*). To evaluate the release of adenosine from CBs in response to hypoxia (2% O_2) and leptin (180 ng ml^{-1}) in control and HF animals, four CBs per experiment were incubated in 500 ml of Tyrode–bicarbonate solution with 2.5 μM of erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) (an inhibitor of adenosine deaminase) and 5 μM of *S*-(*p*-nitrobenzyl)-6-thioinosine (NBTI, an inhibitor of the equilibrative nucleoside transport system) in the presence or absence of leptin (180 ng ml^{-1}). Solutions were kept at 37°C and continuously bubbled with normoxic air (20% O_2 + 5% CO_2, balance N_2), except when hypoxic stimuli were applied (2% O_2 + 5% CO_2, balance N_2). The protocol for adenosine release included one 30 min normoxic incubation, followed by 30 min incubation in normoxia plus leptin and two post-leptin incubations in normoxia for 10 min, followed by 10 min of hypoxia (2% O_2) plus 10 min of normoxia. The solutions were renewed at each fixed time, and all fractions were collected and quantified by HPLC as previously described (Conde *et al.* 2012*a*). At the end of the experiment, the CBs were placed in 3 M Perchloric acid (PCA), weighed, homogenized and maintained at 0°C for 30 min. The samples were further centrifuged at 12,000 *g* for 5 min (4°C) and the adenosine content was quantified from the supernatant.

Western blot analysis of leptin receptor in the carotid body. For CB leptin receptor expression, CBs after cleaning were frozen in liquid nitrogen. The CBs were homogenized in Zurich medium containing a cocktail of protease inhibitors (Conde *et al.* 2012*b*). Proteins were separated in a 10 or 12% SDS-PAGE and electroblotted on nitrocellulose membrane (0.2 mmol l^{-1}; Bio-Rad, Madrid, Spain). To enhance detection sensitivity, we used a three-step western blot protocol (Johnson *et al.* 2009). After blocking, membranes were incubated with primary antibodies against leptin receptor (1:100; Santa Cruz Biotechnology, Madrid, Spain). The membranes were incubated in Tris-buffered saline with Tween (TBST) (0.1%) containing biotin-conjugated goat anti-mouse IgG (1:10,000; Millipore, Madrid, Spain) for 1 h, washed in TBST (0.02%), and incubated for 30 min in TBST (0.1%) containing horseradish peroxidase-conjugated streptavidin (1:10,000, Thermo Fisher Scientific, Porto Salvo, Portugal). Membranes were then washed in TBST (0.02%) and developed with enhanced chemiluminescence reagents (Immobilon Western; Millipore). Intensity of the signals was detected in a Chemidoc Molecular Imager (Chemidoc; Bio-Rad) and quantified using Quantity-One software (Bio-Rad). The membranes were reprobed and tested for β-actin immunoreactivity (bands in the 42 kDa region) to compare and normalize the expression of proteins with the amount of protein loaded.

Table 1. Effect of high-fat diet on insulin sensitivity (K_{ITT}), fasting plasma glucose and body weight

Treatments	Control	High-fat diet
K_{ITT} (% glucose min^{-1})	4.42 ± 0.12	1.91 ± 0.12****
Plasma glucose (mg dl^{-1})	87.10 ± 3.00	91.46 ± 3.16
Body weight (kg)	0.305 ± 0.018	0.440 ± 0.017****

Insulin sensitivity was determined by the insulin tolerance test and expressed as the constant rate for glucose disappearance (K_{ITT}). Data are means ± SEM of 10 animals. Student's *t* test: ****$P < 0.0001$ *vs.* control.

Statistical analysis

For statistical analyses data were evaluated using Prism version 6 (GraphPad Software Inc., La Jolla, CA, USA). The significance of the differences between the mean values was calculated by Student's *t* test and one- and two-way ANOVA with the Bonferroni multiple comparison test. Differences were considered significant at $P < 0.05$.

Results

Obesity is the major risk factor to develop insulin resistance, a metabolic abnormality associated with the development of type 2 diabetes. In accordance with that we observed that a HF diet induces insulin resistance, since it decreased by 56.79% the constant of the insulin tolerance test (K_{ITT}), an effect that occurs without changes in fasting glycaemia (Table 1). Moreover, and as expected, the HF diet increased body weight by 44.26%, compared to the control group (Table 1).

Effect of leptin on basal ventilation and on the ventilatory responses to ischaemic hypoxia

Figure 1 depicts the effect of leptin on basal ventilation and on the ventilatory responses to ischaemic hypoxia, assessed as occlusions of the common carotid artery, in control and high-fat diet animals. As previously described by Ribeiro *et al.* (2013), 3 weeks of high-fat diet induced

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an increase in basal respiratory parameters (Fig. 1*A*). Note the statistically significant increases in respiratory frequency (f_R) and minute ventilation ($\dot{V}_E$) in the obese model, but not in tidal volume (V_T). Figure 1*B* show the effect of intravenous administration of leptin (90, 180 and 270 ng ml^{-1}). Note, in the left panel, a typical increase in ventilation after an intravenous administration of leptin (180 ng ml^{-1}) in control and HF animals. Also, it is clear from the mean data presented in the right panel of the Fig. 1*B* that leptin increases significantly basal minute ventilation in control animals (90 ng ml^{-1}: 28.09 ± 6.48%; 180 ng ml^{-1}: 26.49 ± 6.28%; 270 ng ml^{-1}: 29.03 ± 8.79%). This effect was blunted in HF animals, as the high-fat diet decreased approximately by 60% the increase in ventilation induced by leptin (90 ng ml^{-1}: 11.71 ± 4.13%; 180 ng ml^{-1}: 11.83 ± 6.79%; 270 ng ml^{-1}: 10.41 ± 3.61%). The effect of leptin on ventilation was manifested mainly in f_R and not in tidal volume (data not shown). Figure 1*C* shows the effect of leptin administration on the ventilatory responses to ischaemic hypoxia. As previously described by several authors (Monteiro *et al.* 2011; Ribeiro *et al.* 2013), occlusion of common carotid artery increases ventilation. Herein we can see that leptin administration potentiates in a dose-dependent manner the ventilatory responses to ischaemic hypoxia in control animals. Note that the potentiation of the increase in ventilation induced by the 15 seconds occlusion of the common carotid artery produced by the highest dose of leptin (270 ng ml^{-1}) is lower than with the dose of 180 ng ml^{-1}, which may suggest a partial desensitization of leptin receptors. Also, note in Fig. 1*C* that in HF animals the effect of leptin in potentiating the response to ischaemic hypoxia is blunted. In order to confirm the results of the I.V. administration of leptin on ventilation, we decided to test two doses of leptin through an intracarotid administration (Fig. 1*D*). Intracarotid administration of leptin also increased minute ventilation in control animals, this effect being higher with the dose of 90 ng ml^{-1} (41.29 ± 9.85%) than with the dose

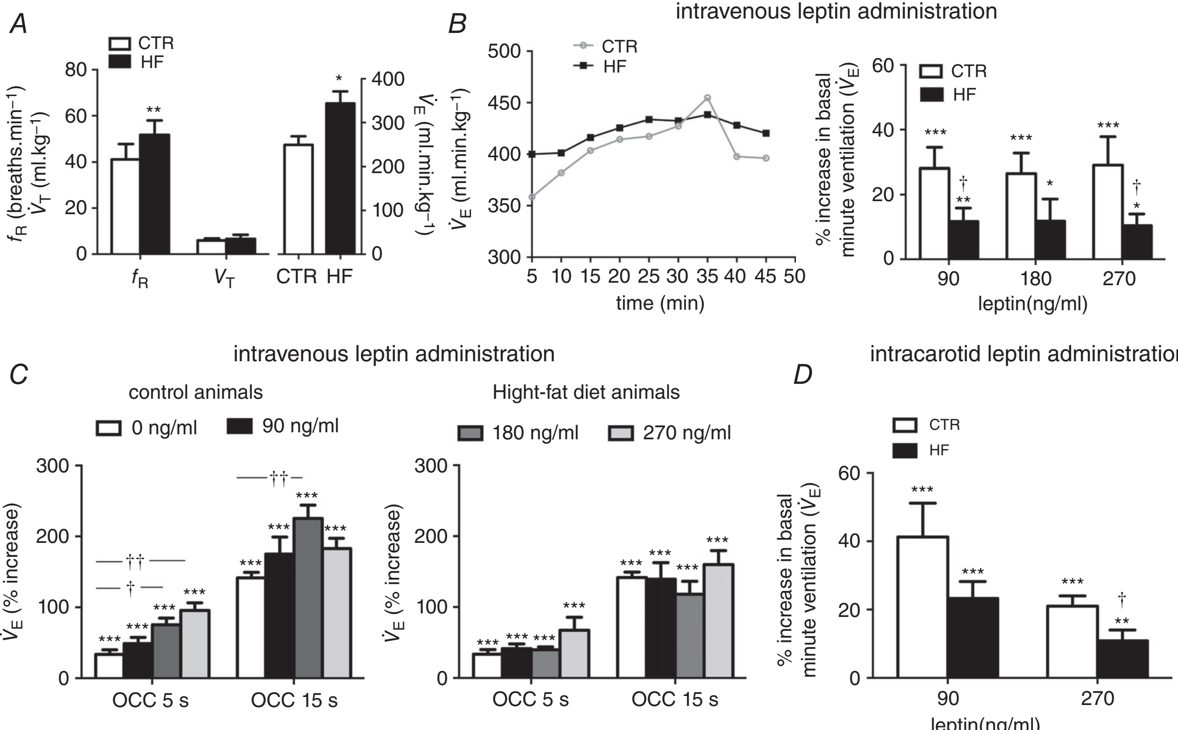

Figure 1. Effect of high-fat diet and leptin administration on ventilation
A, basal ventilatory parameters, respiratory frequency (f_R), tidal volume (V_T) and minute volume ($\dot{V}_E$), in control animals and in animals submitted to 3 weeks of high-fat diet. Student's *t* test in comparison with control animals: **P* < 0.05 and ***P* < 0.01. *B*, effect of intravenous leptin administration (90, 180 and 270 ng ml^{-1}) on basal minute volume. Left panel shows a typical increase in $\dot{V}_E$ after I.V. leptin administration (180 ng ml^{-1}). Right panel shows mean data for the effect of I.V. leptin administration on basal $\dot{V}_E$. *C*, effect of I.V. leptin administration on the ventilatory responses to ischaemic hypoxia, assessed as occlusion of the common carotid artery (OCC) for 5 and 15 s, in control and high-fat animals. *D*, effect of intracarotid administration of leptin (90 and 270 ng ml^{-1}) on basal minute ventilation in control and high-fat animals. Data represent the mean ± SEM of 8–10 control and 8–10 HF animals. One- and two-way ANOVA with Bonferroni multicomparison test, respectively: **P* < 0.05, ***P* < 0.01, ****P* < 0.001 *vs*. baseline; †*P* < 0.05, ††*P* < 0.01 comparing values with and without leptin. Baseline values are mean values of 10 min prior to leptin administration. Increases in response to leptin represent the mean of the highest values obtained.

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of 270 ng ml^{-1} (21.04 ± 2.99%). Also, here we showed that the increase in ventilation promoted by leptin is decreased in HF animals (90 ng ml^{-1}: 23.28 ± 4.92%; 270 ng ml^{-1}: 10.86 ± 3.13%).

Effect of leptin on carotid sinus nerve chemosensory activity

Figure 2 shows the effect of leptin on basal CSN chemosensory activity and on CSN response to hypoxia. Typical recordings of CSN chemosensory activity in response to 5 and 30 min of leptin (180 ng ml^{-1}) perfusion are displayed in Fig. 2*A*. Leptin (180 ng ml^{-1}) perfused during 5 and 30 min increased the basal CSN chemosensory activity by 50.51% (Fig. 2*B*) and 41.17% ($n = 4$, data not shown), respectively. Since the effect of leptin on CSN chemosensory activity is maintained over time, in the experiments that followed the preparations were exposed only to 5 min of leptin. Figure 2*B* presents the effect of leptin (180 and 270 ng ml^{-1}) on basal CSN chemosensory activity in control and HF animals. In control animals, 180 and 270 ng ml^{-1} of leptin increased mean basal activity by 111.50% and 43.36%, respectively. HF diet increased mean basal activity by 2.71 ± 0.40 impulses s^{-1}, compared with controls (1.13 ± 0.19 impulses s^{-1}), but leptin at both concentrations tested did not modify basal CSN chemosensory activity in HF animals. Leptin when tested in the control preparations did not delay the onset of the response (latency time, measured since the application of the stimulus) (Fig. 2*C*). HF diet decreased the latency time to 40.29 ± 4.58 s, compared to the control group (66.12 ± 6.47 s), and the application of leptin in this animal group increased latency time (HF 180 ng ml^{-1}: 62.85 ± 7.56 s; HF 270 ng ml^{-1}: 71.71 ± 9.54 s) (Fig. 2*C*). Also, the time needed to reach the maximal activity since the application of the hypoxia (time to peak) was not modified by leptin in the control group (Fig. 2*C*). The HF diet decreased the time to peak by 111.29 ± 10.54 s, compared with control group (151 ± 10.75 s). Leptin increased time to peak in the HF group (HF 180 ng ml^{-1}: 146.00 ± 12.26 s; HF 270 ng ml^{-1}: 159.71 ± 9.75 s) (Fig. 2*C*). Figure 2*D* displays typical recordings for the effect of leptin on the CSN chemosensory activity in response to hypoxia in control and HF animals. As can be clearly seen from the typical recordings as well as from the area under the curve for the CSN chemosensory response, leptin at both concentrations tested did not modify the response to hypoxia (Fig. 2*D*).

Leptin did not modify intracellular Ca^{2+} in CB chemoreceptor cells

Olea *et al.* (2015) have previously described that leptin did not modify the basal release of catecholamines from the rat CB nor potentiate the release evoked by hypoxia. To investigate if the effect of leptin on the CB is mediated by the release of a neurotransmitter through a Ca^{2+}-dependent exocytotic mechanism, we have evaluated the effect of leptin on intracellular calcium in isolated chemoreceptor cells. Intracellular Ca^{2+} measurements in dissociated chemoreceptor cells were performed for 48 h in the absence and in the presence of 40 ng ml^{-1} of leptin. Figure 3*A* and *B* shows sample recordings of the 340/380 fluorescence emission ratio and the running integral of the 340/380 ratio obtained in a control chemoreceptor cell loaded with fura-2 and in a leptin-treated cell loaded with the same fluorophore, in response to hypoxia (N_2) and 35 mM K^+. We can see clearly that chemoreceptor cells are present, as hypoxia induces the increase in intracellular Ca^{2+} in these cells. Figure 3*C* shows that intracellular Ca^{2+} responses to the different stimuli (N_2 and high K^+) increased in cells cultured for 48 h in the absence and in the presence of leptin. Note that the responses to hypoxia and high K^+ are of the same magnitude as those described previously by our laboratory (Gallego-Martin *et al.* 2012). Note also that leptin (about 4 times basal plasma levels) did not modify either the basal calcium transients in chemoreceptor cells or the increase in intracellular Ca^{2+} elicited by hypoxia or by high K^+. Data were expressed as the increase of the response integrated per minute.

Leptin induces the release of adenosine from the rat CB in control animals but not in animals submitted to a high-fat diet

Since, leptin did not change the release of catecholamines from the rat CB in basal conditions nor alter the release of these neurotransmitters elicited by hypoxia, and did not alter intracellular Ca^{2+} levels (Fig. 3), we tested the effect of leptin on the release of adenosine, a mediator that is known to be key in CB chemotransduction, and whose release is Ca^{2+} independent (Conde *et al.* 2012*a*). Figure 4 shows the effect of 180 ng ml^{-1} leptin on adenosine release from the CB. As can be seen in Fig. 4*A*, incubation of CBs with leptin increased significantly the release of adenosine by 186.9% (CTL 20% O_2: 0.37 ± 0.19 pmol (mg tissue)$^{-1}$ min^{-1}; CTL 20% O_2 + leptin = 1.06 ± 0.22 pmol (mg tissue)$^{-1}$ min^{-1}) in control animals. In contrast, in animals submitted to HF diet, leptin showed a tendency to decrease adenosine release from the CB (HF 20% O_2: 0.38 ± 0.10 pmol (mg tissue)$^{-1}$ min^{-1}; HF 20% O_2 + leptin: 0.16 ± 0.04 pmol (mg tissue)$^{-1}$ min^{-1}). Additionally, and since adenosine is one of the key mediators in CB hypoxic signalling, in the experimental protocol we tested the effect of hypoxia (2% O_2) on the release of adenosine. Figure 4*A* shows that in control animals, 2% O_2 elicits a significant adenosine release, increasing the release of this mediator by 262.2%, while in HF animals adenosine release seems to be compromised:

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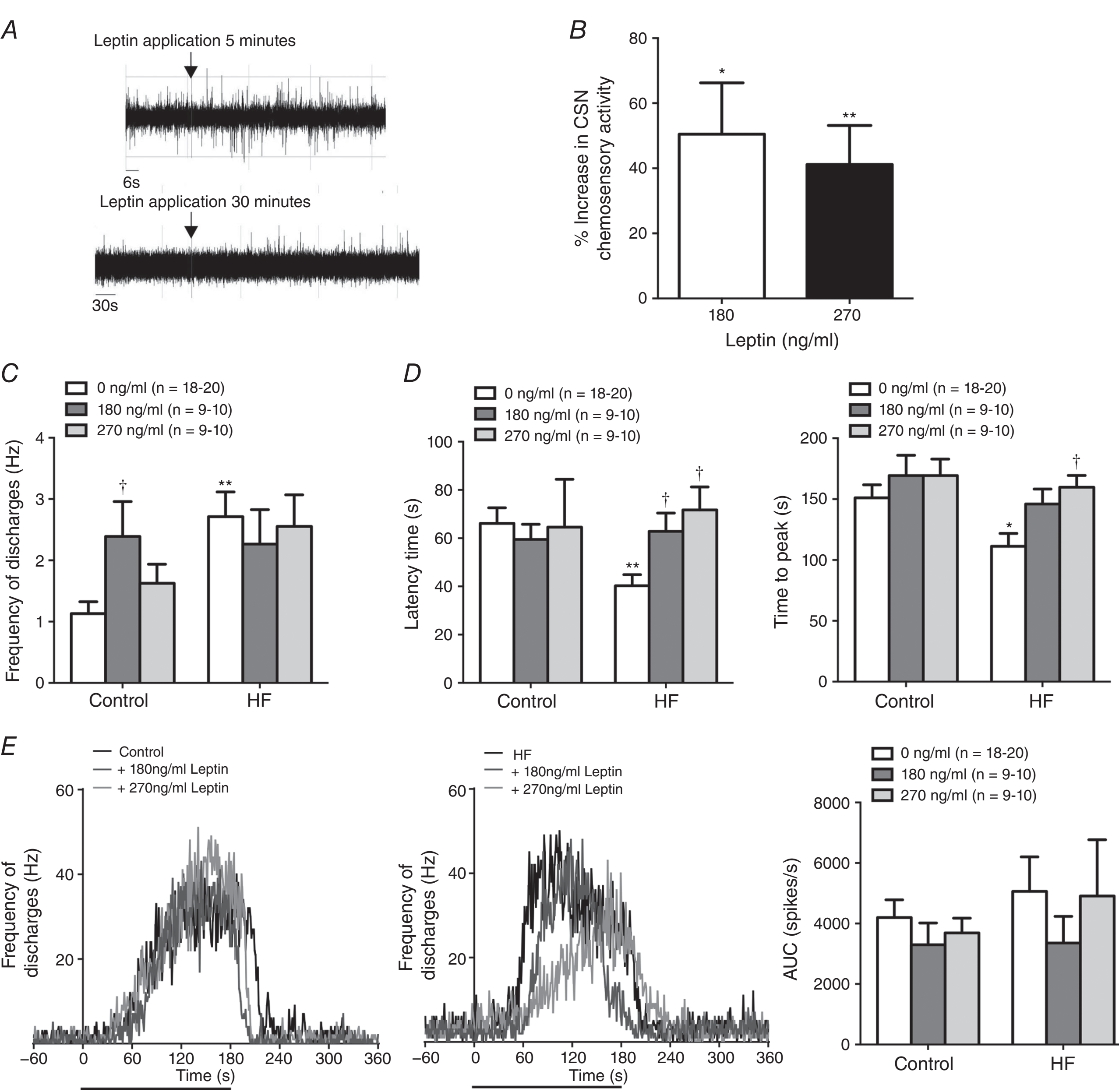

Figure 2. Effect of leptin on *ex vivo* carotid sinus nerve (CSN) basal activity and activity elicited by hypoxia (0% O_2) in control and high-fat (HF) animals
A, typical recordings of the effect of leptin (180 ng ml^{-1}) applied for 5 or 30 min on the frequency of action potentials of CSN in control animals. *B*, percentage increase of the effect of leptin (180 or 270 ng ml^{-1}) applied for 5 min on the frequency of action potentials of CSN in control animals ($n = 9 - 10$). *C*, mean basal frequencies of the CSN chemosensory activity. *D*, latency (the onset of the response) and peak (time to reach maximal activity) time for the effect of leptin on the hypoxic CSN chemosensory response. *E*, typical recordings and the area under the curve (AUC) for the effect of leptin (180 and 270 ng ml^{-1}) on the frequency of action potentials of CSN during superfusion with a solution equilibrated in response to 0% O_2 in control and HF animals. Data represent mean ± SEM. One- and two-way ANOVA with Bonferroni multicomparison test, respectively: $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs*. control or baseline (*B*); †$P < 0.05$ comparing values with and without leptin. Baseline values in the absence of leptin were obtained in the last 5 min prior to leptin administration. Leptin effects on baseline are mean of 5 ($n = 10$) or 30 min ($n = 4$) of CSN activity. Latency time was measured from the application of the stimulus until the moment when the nerve activity was higher than the baseline activity (defined as threshold). Time to peak was measured from the application of the stimulus until the moment the preparation reached maximal activity. AUC for the hypoxic response was measured from the application of the stimulus until the moment the activity of the nerve returned to baseline level.

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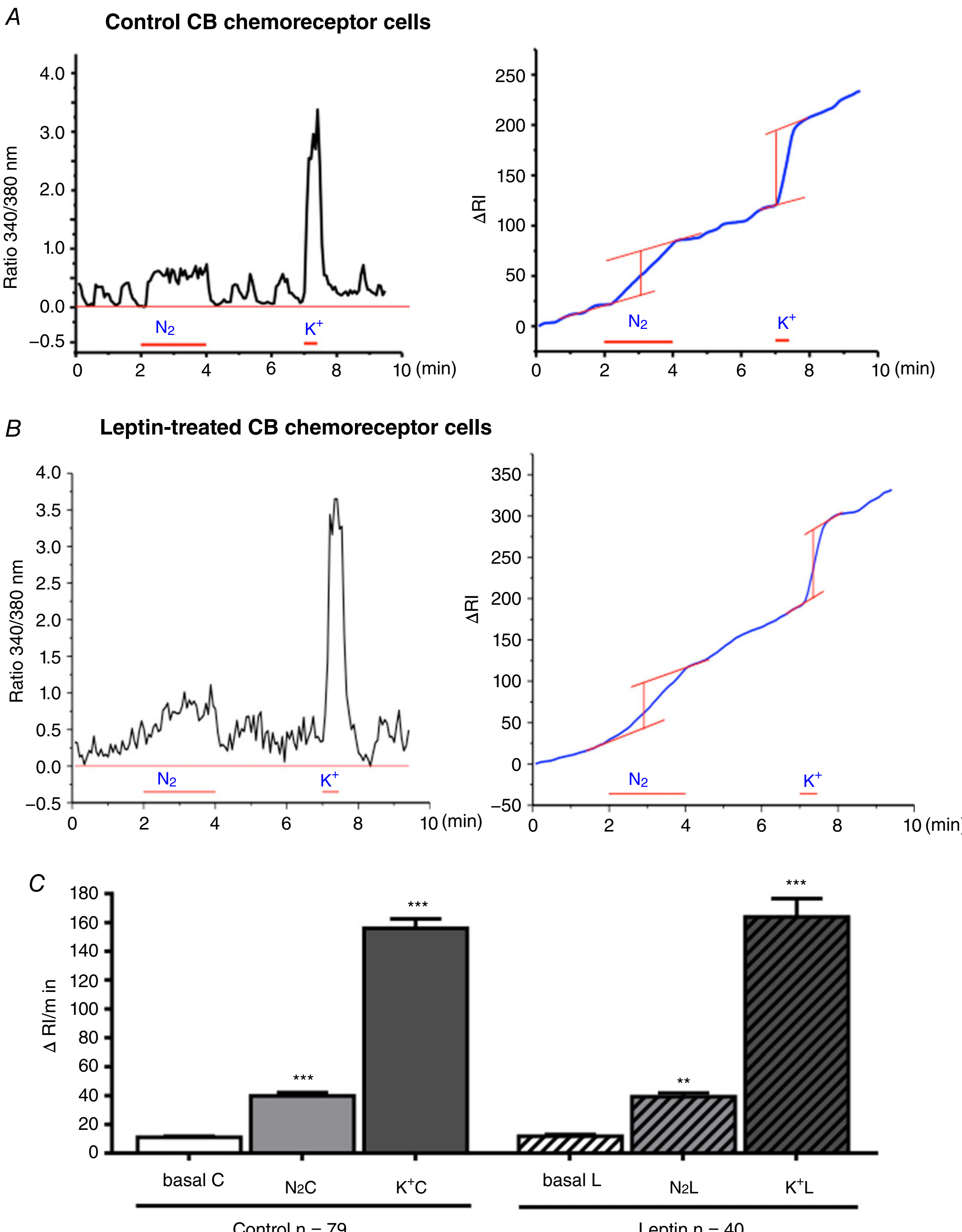

Figure 3. Effect of leptin on intracellular calcium in carotid body isolated chemoreceptor cells
A and *B* show, respectively, typical measurements of intracellular Ca^{2+} in chemoreceptor cells in the absence of leptin (control cells) and in leptin-treated cells. The left panel shows a sample recording of the 340/380 fluorescence emission ratio obtained in a control cell loaded with fura-2. The right panel shows the running integral of the 340/380 ratio. *C*, the effect of leptin (40 ng ml^{-1}) on mean intracellular calcium. Intracellular calcium levels were expressed as the increase in the response integrated per minute in control chemoreceptor

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cells (left columns) and in chemoreceptor cells cultured in a medium supplemented with leptin (40 ng ml^{-1}) (right columns) for 48 h under basal conditions and in response to hypoxia (0% O_2 + 5% CO_2 + 95% N_2) or 35 mM K^+. Data are means ± SEM of 79 control cells and of 40 cells cultured in the presence of leptin. One-way ANOVA with Bonferroni multicomparison test: $^{**}P < 0.01$ and $^{***}P < 0.01$ *vs.* control. [Colour figure can be viewed at wileyonlinelibrary.com]

2% O_2 in these animals increased the release of adenosine by only 39.8%. Note from Fig. 4*B* that the HF diet did not modify the adenosine content of the CBs, measured at the end of the experimental period.

Leptin receptors are overexpressed in high-fat diet CBs

Figure 5 shows the effect of high-fat diet on the expression of leptin receptor (Ob-R, short and long form – 100 and 125 kDa, respectively) in the CB. It can be seen that 3 weeks of high-fat diet induced a significant increase of 35% in the expression of leptin receptors.

Discussion

The results presented here support the previously described effect that in non-disease conditions leptin increases minute ventilation in both basal and hypoxic conditions. However, in a metabolic syndrome animal model, induced by hypercaloric diets, the effect of leptin in ventilatory control is blunted. We observed that HF rats display an increased frequency of CSN discharge in basal conditions that is not altered by the presence of leptin, in contrast to what is observed in control animals.

In basal conditions, HF animals exhibit higher frequency of CSN discharge, which is associated with increased CB adenosine release and a significant higher minute ventilation. The latter is associated with an increase in respiratory rate, but not in tidal volume. This animal model presents significant adipose tissue mass accumulation, which increases respiratory demands. As obesity becomes more pronounced it may cause a restriction to lung expansion that is compensated by an increase in ventilatory drive and in respiratory rate (O'Donnell *et al.* 1999).

Leptin does not modify the discharge frequency of the CSN in HF animals, at any dose tested, and this is probably due to a tonic overactivation of the CSN observed in resting conditions in these animals. In fact, we have showed that CB activity is increased, both in lean and in obese animal models of insulin resistance with obese animals exhibiting more pronounced resting CB activation compared to lean animals (Ribeiro *et al.* 2013). High-fat diet is known to induce weight gain, hyperinsulinaemia and hyperleptinaemia, associated with central leptin resistance (Dube *et al.* 2002). The increased activity of the CSN previously demonstrated in HF rats could be partially mediated by hyperleptinaemia, based on the hypothesis that leptin resistance is organ selective and does not develop in CB type I cells. The results obtained here support that leptin could stimulate CB and CSN activity in control animals, in a phase where leptin resistance is not yet established, contributing to an initial CB dysfunction in early obesity and prediabetes. However,

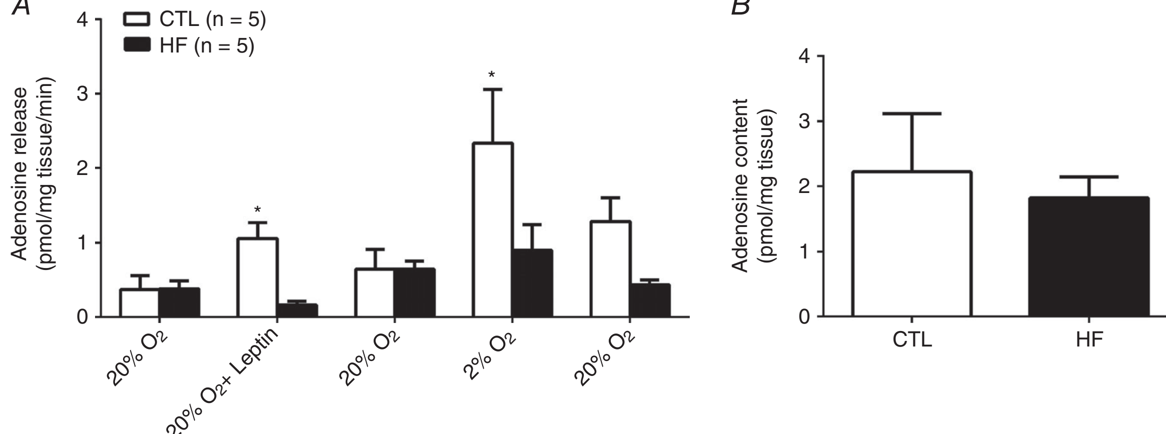

Figure 4. Effect of leptin and hypoxia (2% O_2) on the release of adenosine from the carotid body
A, effect of leptin (180 ng ml^{-1}) and hypoxia on the release of adenosine. *B*, the CB adenosine content at the end of the experimental protocol ($n = 5$, 4 CBs per experiment). Experimental protocol for adenosine release included CBs incubated for 30 min in normoxia (20% O_2) followed by 30 min of leptin in normoxia, followed by two periods of 10 min in normoxia, one period of 10 min in 2% O_2 and finally a 10 min period of normoxia. Values represent means ± SEM of 10 control and 10 HF animals. One-way ANOVA with Bonferroni multicomparison test: $^{*}P < 0.05$ adenosine release in CBs incubated with leptin or hypoxia *vs.* normoxic period.

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the absence of effect of exogenously administered leptin in HF animals does not support an effect of leptin in sustained CB overactivation. Blunting of leptin responses in these animals might be explained by saturated Ob-R leptin receptors in the CB, despite its 35% overexpression compared to controls, and by a pre-existing maximal effect of leptin-induced CB activation caused by diet-induced hyperleptinaemia, in parallel to what is observed in the central nervous system of these animals (Sainz *et al.* 2015).

Despite no observed changes in frequency discharges caused by leptin infusion in HF animals, both the latency time and time to peak in responses to hypoxia were affected by increasing doses of leptin in HF animals, which suggested that leptin changed the hypoxic responsiveness profile of the organ in a dose-dependent manner. The mechanism by which leptin modifies the time frame of hypoxic responses was not assessed in this experimental setting and remains to be clarified.

We have also shown that leptin does not modify intracellular calcium levels. Olea *et al.* have already reported that leptin does not affect catecholamine release in the CBs, suggesting that its signalling pathway at the CB does not involve calcium-mediated release of neurotransmitters (Olea *et al.* 2015). Adenosine is one of the key neurotransmitters in the CB whose release is calcium-independent (Gonzalez *et al.* 1994; Conde *et al.* 2012*a*) and we observed that leptin administration causes adenosine release in control animals but not in HF animals. Similar mechanisms have been described by Conde's group for the release of adenosine induced by hypoxia (Conde & Monteiro, 2004; Conde *et al.* 2006, 2012*a*). Therefore, leptin appears to signal adenosine release through a mechanism similar to the one evoked by hypoxia, and it still remains to be tested if the mechanism triggers the release of adenosine through an equilibrative nucleoside transporter (ENT) sensitive to NBTI, as described for low oxygen sensing (Conde & Monteiro, 2004).

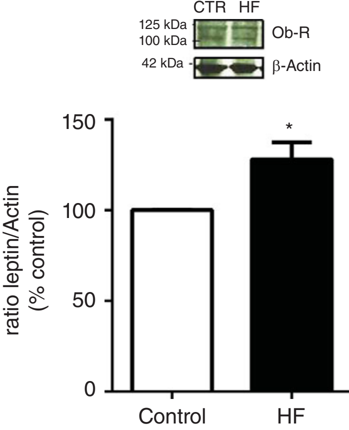

Figure 5. Effect of high-fat diet on the expression of leptin receptors on the rat carotid body (CB)
Top, a representative western blot for the expression of leptin receptor (Ob-R, short and long form – 100 and 125 kDa, respectively) and the loading protein, β-actin, at the CB. Bar graph represents the mean ratio leptin/actin of 5 CB samples. Bars represent means ± SEM. Student's unpaired t test: *$P < 0.05$ HF animals vs. controls. [Colour figure can be viewed at wileyonlinelibrary.com]

In HF animals, leptin-induced adenosine release by the CB did not occur, but the adenosine content of the HF CBs did not change in comparison with control animals. Prior changes in the activity of adenosine kinase with insulin resistance have been reported in the adipose tissue (Green *et al.* 1981) and therefore we can postulate that this reduced adenosine release could be due to altered activities of the enzymes involved in adenosine metabolism such as adenosine kinase, adenosine deaminase and purine nucleoside phosphorylase. Reduced hypoxic ventilatory responses have also been reported in obese Zucker rats and attributed to depressed adenosinergic peripheral excitatory mechanisms and to enhanced adenosinergic central depression mechanisms, both of which seemed to contribute to a blunted ventilatory response associated with obesity (Lee *et al.* 2005).

We conclude that the CBs represent an important target organ for leptin signalling in physiological conditions, not only to coordinate peripheral ventilatory chemoreflexive drive, but probably also to modulate metabolic variables. The results obtained in the present article do not deny an involvement of CBs in leptin-mediated ventilation effects since we have not performed the same experiments in CSN-denervated animals, but support that leptin could stimulate CB and CSN activity in control animals, in a phase where leptin resistance is not yet established. The relevance of CB leptin signalling is lost in a metabolic disease setting, since high-fat diets blunt leptin responses in the CB, probably due to development of CB leptin resistance, compromising ventilatory and also metabolic adaptation in these animals and contributing to the deleterious positive feedback loop observed in obesity.

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Additional information

Competing interests

The authors declare no conflicts of interest.

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Author contributions

The experiments described in the present paper were performed in the CEDOC, NOVA Medical School/Faculty of Medical Sciences, NOVA University and in the Department of Biochemistry, Molecular Biology and Physiology of the Faculty of Medicine of the University of Valladolid. The authors have contributed to the study as follows. Participated in research design: S.V.C. and A.O. Conducted experiments: M.J.R., J.F.S., T.G., E.O., S.Y., B.F.M. and S.V.C. Performed collection and data analysis: M.J.R., J.F.S., E.O., S.Y., B.F.M., M.G. and S.V.C. Wrote or contributed to the writing of the manuscript: S.V.C., M.G. and A.O. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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