Carotid body denervation prevents the development of insulin resistance and hypertension induced by hypercaloric diets

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Abstract

Increased sympathetic activity is a well-known pathophysiological mechanism in insulin resistance (IR) and hypertension (HT). The carotid bodies (CB) are peripheral chemoreceptors that classically respond to hypoxia by increasing chemosensory activity in the carotid sinus nerve (CSN), causing hyperventilation and activation of the sympathoadrenal system. Besides its role in the control of ventilation, the CB has been proposed as a glucose sensor being implicated in the control of energy homeostasis. However, to date no studies have anticipated its role in the development of IR. Herein we propose that CB overstimulation is involved in the aetiology of IR and HT, core metabolic and hemodynamic disturbances of highly prevalent diseases like the metabolic syndrome, type 2 diabetes and obstructive sleep apnoea. We demonstrated CB activity is increased in IR animal models and that CSN resection prevents CB-overactivation and diet-induced IR and HT. Moreover we showed that insulin triggers CB, highlighting a new role for hyperinsulinemia as a stimulus for CB-overactivation. We propose that CB is implicated in the pathogenesis of metabolic and hemodynamic disturbances through sympathoadrenal overactivation and may represent a novel therapeutic target in these diseases.
Insulin resistance (IR), arterial hypertension (HT), obesity and dyslipidemia are core features of widespread diseases in western societies such as the metabolic syndrome, type 2 diabetes mellitus and obstructive sleep apnoea. Visceral obesity has been proposed to play a fundamental role in the simultaneous development of IR and HT that characterize these diseases(1). Recent findings suggest that peripheral IR is also a common feature in lean obstructive sleep apnoea(2) as well as lean polycystic ovarian syndrome(3), despite its strong relationship with visceral obesity. Similarly, the association of HT with obstructive sleep apnoea is independent from obesity(4), as demonstrated by hypertensive lean sleep apnoea patients. Altogether, these findings point out to the existence of an obesity-independent etiological factor that simultaneously causes IR and HT: the activation of the carotid bodies (CB) has recently been suggested as a putative candidate(5).

The CBs are arterial chemoreceptors that sense changes in arterial blood O$_2$, CO$_2$ and pH levels. Hypoxia and acidosis/hypercapnia activate the CBs, which respond by increasing the action potential frequency in their sensory nerve, the carotid sinus nerve (CSN). CSN activity is integrated in the brain stem to induce a fan of respiratory reflexes aimed, primarily, to normalize the altered blood gases via hyperventilation(6) and to regulate blood pressure and cardiac performance via sympathetic nervous system activation(7). The CB directly activates the adrenals via increased sympathetic drive and also increases sympathetic vasoconstrictor outflow to muscle, splanchnic, and renal beds(7,8). Enhanced sympathetic nerve activity is know to contribute to skeletal muscle IR and to impaired glucose tolerance, mainly due to sympathetic mediated lipolysis(9, 10) and also to increased arterial pressure(9). Recently, the CB was proposed to be a glucose sensor(11) and implicated in energy homeostasis control(12).
The objective of this study was to investigate the role of the CB in the pathogenesis of metabolic and hemodynamic disturbances by testing the hypothesis that CB activity is increased in IR and HT animal models. Also, to clarify the role of obesity as an independent factor in CB activation, we compared CB function in both obese and lean models of insulin resistance.

The second hypothesis tested was that insulin is a trigger for CB activation. In vivo experiments have previously shown that intravenous infusion of insulin causes a CB-dependent increase in ventilation(13). The authors concluded that this effect was associated with the hypoglycemia caused by insulin administration, however others have shown that low glucose is not a direct stimulus for rat CB chemoreceptors(14,15). These discordant results point towards insulin as a good alternative candidate to activate the carotid bodies.

Finally, we performed chronic CSN bilateral resections to test the hypothesis that preventing the carotid bodies from being overactivated averts the development of IR and HT, and also the increase in sympathoadrenal activity, induced by hypercaloric diets in animals. The data presented herein clarify the role of the CB in the pathogenesis of diet-induced IR and HT and unveil a new promising target for intervention in type 2 diabetes, metabolic syndrome and obstructive sleep apnoea.

**Research design and methods**

**Animals and experimental procedures**

Experiments were performed in Wistar rats (200–420 g) of both sexes, aged 3 months, obtained from the vivarium of Faculty of Medical Sciences. Two diet-induced IR and HT animal models were used: the rat submitted to a high-fat (HF) diet, a model that combines obesity, IR and HT(16,17) and the rat submitted to a high-sucrose (HSu) diet,
a lean model of combined IR and HT(16,18). Briefly, the control group fed a sham diet (7.4% fat+75% carbohydrate (4% sugar)+17% protein, SDS diets RM1, Probiológica, Portugal); the HSu model was obtained by administration of 35% sucrose (Panlab, Portugal) in drinking water during 28 days. The HF model fed a lipid rich diet (45% fat+35% carbohydrate+20% protein, Mucedola, Italy) during 21 days. The HSu and HF animals are validated in the literature as animal models of the metabolic syndrome(19).

To demonstrate that CB activity was increased in hypercaloric fed animals we compared HF and HSu with control group.

To evaluate the contribution of CB to the genesis of IR and HT, bilateral resection of CSN was performed 5 days prior to submitting the animals to standard or hypercaloric diets. The carotid artery bifurcations were located bilaterally and CSN were identified and either sectioned bilaterally or left intact (sham). These procedures were performed in aseptic conditions under ketamine (30mg/kg)/xylazine (4mg/kg) anaesthesia and brupenorphine (10µg/kg) analgesia. Chronic resection of CSN was confirmed by absence of ischemic hypoxia-induced hyperventilation prior to experiments.

Rats fed with standard diet were used to investigate if insulin triggers CB activation.

All test groups included equal number of males and females. Whenever an odd experimental number is displayed, this refers to the death of experimental units during the experimental procedure. Also, food and liquid intake was monitored during the treatments, in all groups of animals. Body weight and animal behavioural changes were assessed twice per week.

All measurements were performed with animals under sodium pentobarbital (60 mg/kg i.p.) anaesthesia, since pentobarbital was shown not to alter the metabolic parameters tested herein (KITT, fasting glycemia, insulinemia and free fatty acids) in comparison to conscious animals(20) nor insulin responses to glucose(21). 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. 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). Experimental protocols were approved by the Ethics Committee of the Faculty of Medical Sciences.

**Evaluation of basal ventilation and ischemic ventilatory responses in animal models of insulin resistance and hypertension**

A detailed description of these methods was previously published(22). Shortly, respiratory frequency (fR) and tidal volume (VT) were obtained by pneumotachography (Hugo SACHS Elektronik, Harvard Apparatus, Madrid, Spain) in anaesthetized and tracheostomized control rats and in rats submitted to hypercaloric diets. These respiratory parameters and blood pressure were continuously recorded in anesthetized and vagotomized rats breathing spontaneously and submitted to either bilateral occlusions (5–15s) of common carotid artery. 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(23). Control experiments were performed in animals submitted to bilateral cut of the carotid sinus nerve (CSN) in order to distinguish central and peripherally mediated effects.

**Effect of insulin on spontaneous ventilation in control animals**

Insulin effect on ventilation was assessed in control rats anesthetized, tracheostomized and vagotomized. Briefly, an insulin bolus (1, 5, 10, 50,100 and 200 mU/kg) was administered in external carotid artery and reaches the carotid body by being pushed by
the blood flow of common carotid artery. Ventilatory parameters as described above were monitored. Euglycemic clamp was maintained through glucose (10 mg/kg/min) perfusion into the femoral vein. Confirmation of CB-insulin mediated effect was done by measurement of ventilation after CSN cut.

Measurement of insulin sensitivity and mean arterial pressure

Insulin tolerance test (ITT) was used to measure insulin sensitivity(16,24). Mean arterial pressure monitoring was measured(16). After insulin sensitivity and mean arterial pressure evaluation, blood was collected by heart puncture and treated for quantification of soluble biomarkers(16). Visceral fat and adrenal medulla were collected after an abdominal laparotomy and weighted. Adrenal medullas were frozen in liquid nitrogen and stored placed at -80°C.

Measurement of plasma insulin, circulating free fatty acids, corticosterone and catecholamines levels and adrenal medulla catecholamines content

Plasma and serum were collected after heart puncture to ethylenediamine tetraacetic acid (EDTA) precoated tubes and to eppendorfs, respectively. Insulin concentrations and free fatty acids were determined in plasma and corticosterone was determined in serum(16). Corticosterone determination was obtained with a DetectX corticosterone Immunoassay kit (Arbor Assays, Madrid, Spain). For catecholamines quantification in plasma, 400 µl of plasma samples were purified and catecholamines were extracted and quantified as previously described(16). For quantification of catecholamines content in adrenal medulla, adrenal medullas previously frozen were homogenized in PCA 0.6N and their endogenous catecholamine content was quantified as described (15).
Carotid body dopamine and ATP release in response to hypoxia and to insulin

CBs were cleaned free of CSN nearby connective tissues under dissection microscope and incubated in Tyrode solution (15,25). To evaluate CB activity in IR and HT animal models, we have measured CB dopamine (plus DOPAC, its major metabolite) release. CB ATP and dopamine release in response to insulin were monitored in control animals. In brief, CBs were incubated in 500 µl (250 µl for ATP and 250 µl for dopamine for insulin effects) of Tyrode bicarbonate solution and cofactors for tyrosine hydroxylase and dopamine-β-hydroxylase (20 µM tyrosine, 100 mM ascorbic acid and 500 nM 6-methyl-tetrahydroptine) or Tyrode bicarbonate plus insulin (0.01-100 nM). Solutions were kept at 37°C and continuously bubbled with normoxia (20%O₂/5%CO₂/75%N₂), except when hypoxic stimuli were applied. Protocols for dopamine release in overfeeding rats include two 10 min normoxic incubations, followed by 10 min incubation in hypoxia (5%O₂/5%CO₂/75%N₂) and 2 post-hypoxic incubations in normoxia. Protocols for insulin effect on dopamine and ATP release include two 10 min incubation in normoxia, followed by 3 incubations with different insulin concentrations and 2 post-insulin incubations in normoxia. The solutions were renewed at each fixed time and all fractions were collected and quantified as previously described (15).

Western Blot analysis of insulin receptor, insulin receptor phosphorylation (phosphor-Tyr 1322) and tyrosine hydroxylase expression

For evaluation of insulin receptor phosphorylation, CBs were isolated, cleaned and incubated at 37°C during 30 min in Tyrode-solution containing 1 and 100 nM of insulin and bubbled with 20%O₂/5%CO₂/75%N₂. After, CBs were immediately frozen in liquid
nitrogen and placed -80°C. For CB insulin receptor and tyrosine hydroxylase expression, CBs after cleaned were frozen in liquid nitrogen. CBs were homogenized in Zurich medium containing a cocktail of protease inhibitors(26). Proteins were separated in a 10 or 12% SDS PAGE gel electrophoresis and electroblotted on nitrocellulose membrane (0.2µM BioRad, Madrid, Spain). To enhance detection sensitivity we used a three step Western blot protocol(27). After blocking, membranes were incubated with primary antibodies against insulin receptor (1:100, Sta Cruz Biotech, Madrid, Spain), insulin receptor phosphorylated (phosphor-Tyr1322, 1:50, Assay Designs, Portugal) and tyrosine hydroxylase (1:10000, Sigma, Madrid, Spain). The membranes were incubated in TBST (0.1%) containing biotin-conjugated goat anti-mouse IgG (1:10000, Millipore, Madrid, Spain) for 1 h, washed in TBST (0.02%), and incubated for 30 min in TBST (0.1%) containing horseradish peroxidase (HRP)-conjugated streptavidin (1:10000, Pierce, Madrid, Spain). Membranes were then washed in TBST (0.02%) and developed with enhanced chemiluminescence reagents (Immobilon Western, Millipore, Spain). Intensity of the signals was detected in a Chemidoc Molecular Imager (Chemidoc BioRad, Spain) and quantified using the Quantity-One software (BioRad, USA). The membranes were re-probed and tested for β-actin immunoreactivity (bands in the 42kDa region) to compare and normalise the expression of proteins with the amount of protein loaded.

**Chemoreceptor cell culture and intracellular Ca²⁺ measurements**

Cleaned CBs were enzymatically dispersed, and dissociated cells were plated on poly-L-lysine-coated coverslips maintained in culture for up to 24 h as previously described (28). Coverslips were incubated with fura-2 AM, mounted in a perfusion chamber, and fura-2 fluorescence was measured as the ratio of the fluorescent emission at 340/380 nm.
of chemoreceptor cells (29). General protocol for Ca\textsuperscript{2+} measurements consisted in a sequential incubation hypoxia (N\textsubscript{2}; 1 min), 5 min normoxic incubation (20%O\textsubscript{2}), 3 min incubation with 1 nM insulin, combination of both hypoxia (N\textsubscript{2}) and insulin (1 nM), 5 min normoxia (20%O\textsubscript{2}), 1 min hypoxia (N\textsubscript{2}) and finally 30 seconds of high external KCl.

Results

Administration of hypercaloric diets to Wistar rats produced changes in body weight, sympathetic nervous activity, blood pressure and insulin sensitivity similar to the ones observed in humans (2,16,30). Liquid intake was similar in all animals tested (control group: 101.21±3.09 ml/kg/day; HF animals: 89.50±3.93 ml/kg/day; and HSu animals: 93.22±2.59 ml/kg/day). No significant differences were observed in food intake (Control: 57.78±2.05 mg/kg/day; HF: 62.56±1.99 mg/kg/day; HSu: 51.22±4.51 mg/kg/day). The daily caloric intake was 164.7±5.8 kcal/kg/day in control animals, 299.0±9.4 kcal/kg/day in HF animals (p<0.001 vs control) and 332.8±12.8 kcal/kg/day for HSu animals (p<0.001 vs control). After CSN cut, the daily caloric intake was: 179.6±10.1 in the control group, 289.2±6.5 in the HF group and 327.6±10.5 kcal/kg/day in the HSu group. The daily caloric intake was not changed by CSN cut and there were no significant differences among the HF and HSu rats. Insulin resistance and hypertension were confirmed by measurement of insulin sensitivity and blood pressure in HF and HSu animals. The HF diet caused a decrease in K\textsubscript{ITT} from 4.69±0.33 % glucose/min in Control animals to 2.98±0.34 % glucose/min (P<0.01). The HSu diet decreased K\textsubscript{ITT} to 2.68±0.32 % glucose/min (P<0.01). HF and HSu diets caused a significant increase in mean arterial pressure compared with controls (MAP control = 95.99±3.21 mmHg; MAP HF = 142.31±2.47 mmHg; MAP HSu = 136.71±4.51 mmHg).
Fasting glycaemia was not significantly different in control and HF groups, although the HSu diet significantly increased fasting glycaemia in comparison with the control group (P<0.001) (data not shown).

**Carotid body is overactivated in insulin resistant and hypertensive rats**

Figure 1 demonstrates that CB activity is increased in animal models of insulin resistance and hypertension. Spontaneous ventilatory parameters (respiratory frequency, tidal volume and the product of these two parameters, minute ventilation) were increased in both HF and HSu animals, with a more pronounced effect in HF animals (Fig. 1a,d). Surgical CSN cut completely abolished the increase in spontaneous ventilation induced by the diets (Fig. 1c,d), showing that this effect is mediated by the CB. In addition, ventilatory responses to ischemic hypoxia, assessed as the increase in ventilation produced by common carotid artery occlusions for periods of 5, 10 and 15 seconds were augmented in HF animals (Fig. 1b,e). This increase in ventilation, which was proportional to the duration of the stimulus and was mediated through the carotid bodies as it was abolished by CSN cut (Fig. 1c).

In HSu animals only the response to an ischemic hypoxia of 5 seconds was significantly increased, and as observed in the HF model this was also abolished by CSN cut. We concluded that both the HF and the HSu rat models of IR and HT present an overstimulated CB, however, the more pronounced increases in spontaneous ventilation and in ischemic hypoxia induced-hyperventilation observed in HF animals suggest that these animals hold a higher degree of CB activation. Catecholamines, namely dopamine, are the best well characterized neurotransmitters in the CB (6), and its release in all mammalian species depends on extracellular Ca\(^{2+}\), is proportional to stimulus intensity and to the increase in CSN activity and therefore to CB function.
Thus, to confirm CB overactivation in HF and HSu animals, we measured both basal and hypoxia evoked-release of dopamine (plus DOPAC), the main metabolite of dopamine in the CB). We observed that basal release of dopamine was not significantly modified by hypercaloric diets (Fig. 1f), however the release induced by hypoxia (5%O₂) was increased 3.15 fold in HF and 2.12 fold in HSu rat models (Fig. 1g). Also, CBs weight was significantly increased by 36.71% and 27.13% in HF and HSu models, respectively (Fig. 1h), which suggests that overactivation of CB is due to hyperplasia of the organ. In fact, western blot analysis confirmed that the tyrosine hydroxylase expression, the rating enzyme for catecholamine biosynthesis increased by 64.4% in HF (p<0.01) and 30.8% in HSu animals (p=0.12) (Fig. 1i), confirming CB overactivity in these pathological animal models.

**Chronic carotid sinus nerve resection prevents insulin resistance and hypertension**

To test the involvement of the CB in the development of IR and HT, we performed a chronic CSN bilateral resection prior to hypercaloric diet administration, therefore blocking CB activity during the induction of insulin resistance. Rats submitted to CSN bilateral resection were compared with animals submitted to the same surgical procedure but in which CSN was left intact (sham). CSN bilateral resection was confirmed by the lack of increase in the ventilatory responses to ischemic hypoxia, assessed as common carotid artery occlusion (Fig. 2a). Sham procedure did not modify any of parameters evaluated (insulin sensitivity, mean arterial pressure, glycemia, insulinemia, free fatty acids, corticosterone, visceral fat, plasma catecholamines) when compared with animals control, HF and HSu animals not submitted to any surgical procedure (first paragraph results section, 16). Also, CSN bilateral resection did not alter liquid and food intake in any of the groups tested (data not shown).
Figure 2b depicts a representative curve of a typical insulin tolerance test in a control rat. Insulin sensitivity was significantly decreased by 42.08% and 53.61% in HF and HSu rats respectively (Fig. 2c). IR produced by hypercaloric diets, was completely prevented by CSN resection (Fig. 2c), linking CB dysfunction with the development of IR. In addition, we observed that CSN resection in control animals decreased insulin sensitivity, suggesting that CB also contributes to maintain metabolic control in physiological conditions. Mean arterial pressure, as previously described (16), was increased by 38.79% and 35.70% in HF and HSu rats, respectively, and this effect was totally prevented by CSN chronic resection (Fig. 2d). Glucose homeostasis and insulin secretion became normalized since fasting hyperglycemia and hyperinsulinemia returned to control values after CSN chronic denervation (Table 1). The increase in serum free fatty acids observed in HSu rats was abolished by CSN resection (Table 1). Neither HF and HSu diets nor CSN resection modified corticosterone levels (Table 1).

Due to the strong association between obesity and visceral fat with IR and HT (1,11,12), we tested if CSN resection could alter weight gain and visceral fat. In figure 2e absolute weights before and after administration of hypercaloric diets and also before and after CSN resection are depicted. HF, but not control or HF, animals significantly gained weight during the experimental period (Fig. 2e,f). We found that CSN resection significantly decreases weight gain in HF animals (Fig. 2f) and avoids visceral fat deposition (Fig. 2g). Since IR, HT and obesity are associated with sympathetic nervous system overactivity (1,11,12), and CB controls sympathetic outflow and sympathetic nerve activity (7,8), we also analysed sympathoadrenal activity, measured both as circulating and adrenal medulla catecholamines in our animal models. Plasma norepinephrine significantly increased in both HF and HSu rats in relation to control animals (HF = 48.40±7.72 pmol/ml; HSu = 71.32±9.04 pmol/ml; Control = 22.23±2.98
pmol/ml) (Fig 3a). Also, as depicted in figure 3b, plasma epinephrine increased 151.52% and 178.31% in HF and HSu, respectively (Control= 30.80±4.25 pmol/ml). These results suggest an increased sympathoadrenal activity (Fig. 3a,b) that was confirmed by the augmented catecholamines content in adrenal medulla of these animals (Fig. 3c,d). HF and HSu rats exhibited significant increases of 29.72% and 44.52% in adrenal medulla norepinephrine, respectively, and of 34.27% and 69.50% adrenal medulla epinephrine content, compared with the controls (norepinephrine control = 11.75±0.58 nmol/mg tissue; epinephrine control = 24.28±2.62 nmol/mg tissue, Fig. 3c and d). Chronic CSN cut did not affect sympathoadrenal activity in control animals; however, sympathoadrenal overactivation induced by hypercaloric diets was abolished in rats with CSN bilateral resection (Fig. 3a-d). These results demonstrate that CB plays a role in the genesis of IR and HT in animal models of type 2 diabetes and metabolic syndrome.

**Insulin triggers carotid body activation**

In the present work we propose that the stimulus for CB overactivation responsible for IR and HT is increased plasma insulin and therefore we hypothesize that insulin is capable of triggering CB activation. We used a 3 step western blot approach (27) to examine the presence of the insulin receptor in the CB and its phosphorylation in response to insulin. Western blot analysis demonstrated that insulin receptors are present in the CB (Fig. 4a) and that their phosphorylation increases in the presence of 1 and 100 nM insulin (Fig. 4a,b). Incubation of the CBs with 1 and 100nM insulin significantly increased insulin receptor phosphorylation by 98.6% and by 47%, respectively (Fig. 4b). We also tested if insulin receptor activation in the CB elicits a neurosecretory response by measuring intracellular Ca\(^{2+}\) and the release of
catecholamines and ATP, two of the neurotransmitters released from CBs in response to hypoxia (6,14,25,31,32). Fig 5a depicts, a bright-field image of a 20-h-old cell culture of dissociated CB and typical recording of intracellular cell Ca$^{2+}$, measured as the ratio of the fluorescent emission at 340/380 nm of chemoreceptor cells in basal conditions, in response to hypoxia (N$_2$), to 1nM of insulin and to 35mM of K$^+$ in left and right panels, respectively. Hypoxia significantly increased [Ca$^{2+}$]$_i$ by 15.97%. Also, 1nM insulin significantly increased [Ca$^{2+}$]$_i$ by 6.53%. When applied simultaneously, insulin and hypoxia increased intracellular Ca$^{2+}$ concentration by 21.53% suggesting that the transduction mechanisms by which the two stimuli operate are different. To evoke a neurosecretory response, the increase in [Ca$^{2+}$]$_i$ produced by insulin must be transduced into the release of neurotransmitters from the CB. Figure 5c and 5e show that insulin (10 nM) produced an increase in the basal release (black bars) of ATP and dopamine (plus DOPAC) from the whole CB in incubating solutions, and the effect was reversed after drug washout. The dose–response curves for the effect of insulin in neurotransmitter release in the whole CB are depicted in Fig 5d and f. The curves fitted a sigmoid with EC$_{50}$ of 0.552 nM and 6.17 nM and maximal effects of 257.9% and 265.1% for CB ATP and dopamine release, respectively. Note that concentrations above 400-500 pM are already compatible with an hyperinsulinemic state (33,34) and that when insulin was applied above 10 nM concentrations it evoked the release of ATP and DA (plus DOPAC) from CB in a similar magnitude as produced by hypoxia (5%O$_2$) (Fig 45 d,f).

Knowing that stimuli-induced CB activation results in hyperventilation (6), we assessed the effects of insulin on ventilation. In vivo experiments have previously showed that intravenous infusion of insulin-caused a CB-dependent increase in ventilation (13), an effect that was not due to hypoglycaemia per se, since low glucose
is not a direct stimulus for rat CB chemoreceptors (14,15). Therefore, we tested the effect of an intracarotid bolus of insulin on ventilation during an euglycemic clamp, to avoid the confounding effects of systemic hypoglycaemia. Figure 6a depicts a typical recording of pulmonary flow and tidal volume before and after an intracarotid administration of an insulin (50mU/kg) bolus. Insulin increased respiratory rate, tidal volume (Fig 6a, left panel Fig 6d) and the product of both parameters, minute ventilation (VE) (Fig. 6c) in a dose-dependent manner. The increase in ventilation induced by insulin is not immediate, showing a significant latency period (time to the onset of the response) comprised within the 106.0±4.04 and 188.5±3.51 seconds range (Fig. 6d). This observation is in accordance with the time-scale necessary for the activation of tyrosine kinase receptors, namely insulin receptors (35). Full dose-response curve for the effect of insulin in minute ventilation is depicted in Fig.6c fitting a sigmoid with an EC\textsubscript{50} of 35 mU/kg and a maximal effect of 60.41%. Figure 6e depicts a typical euglycemic clamp following an intracarotid administration of an insulin bolus of 50mU/kg. As expected the amount of glucose infused to maintain euglycemia increased in a insulin-dose dependent manner (figure 6f). The effect of insulin on ventilation was totally mediated by the CB, since CSN cut completely abolished the increase in ventilation induced by insulin (right panel Fig. 6d).

Discussion

This study represents a new conceptual framework regarding the pathogenesis of IR. Using a combination of neurochemical, physiological and cellular biology techniques we showed that CB activity is increased in models of metabolic syndrome and type 2 diabetes and that CB dysfunction is involved in the development of IR and HT. In addition, we demonstrated for the first time that insulin triggers the peripheral
chemoreceptors located in the CBs, suggesting that hyperinsulinemia may trigger CB-induced sympathoadrenal overactivity associated with metabolic disturbances.

Hyperinsulinemia is a known early pathological feature caused by increased secretory stress on the beta cell associated with peripheral insulin resistance caused by hypercaloric diets. Increased insulin levels trigger the CBs to activate the sympathetic nervous system, initiating a vicious cycle that worsens peripheral insulin action, impairs beta cell function and causes systemic hypertension. In line with these results, the CB rises as a new therapeutic target for intervention in metabolic disturbances.

We show herein, and also for the first time that CB activity is increased in diet-induced animal models of IR and HT. CB-mediated basal ventilation and ventilation in response to ischemic hypoxia were increased in the pathological models tested, as well as the CB chemoreceptor cell function - assessed both as hypoxia induced-release of dopamine and as tyrosine hydroxilase expression. The increase in CB cell function, together with increased CB weight observed in our experimental setting, are in agreement with the previous observations of Clarke et al. (36) showing that CB volume is increased in spontaneous insulin-dependent diabetic rats (strain BB/s), an effect that could not be attributed to an increase in the vascular component of the organ. We have also observed that HF animals exhibited more pronounced increases in both spontaneous ventilation and ischemic-hypoxia-induced-hyperventilation than HSu animals, suggesting that the HF animal-model is characterized by a higher degree of CB activation. Our results strongly suggest that there is an obesity-related factor that contributes to CB stimulation.

Although some authors have suggested that obesity does not enhance peripheral chemoreflex sensitivity (37) this topic remains controversial. It was shown that chronic intermittent hypoxia increases expression of TNF-α and IL-1β within the CB (38) and
that these pro-inflammatory cytokines may contribute directly to CB-mediated cardio respiratory changes evoked by intermittent hypoxia. Obesity is also characterized by a sub-clinical pro-inflammatory condition with increased secretion of adipokines, including leptin, tumour necrosis factor alpha, IL-1beta and IL-6, the same cytokines proposed as having a role in chemoreceptor changes observed in sleep apnoea. On the other hand, obesity has been associated with increased sympathetic nervous system activity through a leptin-mediated mechanism that is still unclear. Recently it was described that glomus cells in the carotid body express leptin receptors and are activated by intermittent hypoxia and systemic leptin injections, which suggests that leptin may be also represent an independent factor in CB activation.

Besides demonstrating that CB overactivity is present in animal models of IR and HT we have also shown that CSN bilateral resection totally prevented diet-induced IR and HT, as well as increased fasting plasma glucose, fasting plasma insulin, free fatty acids and systemic sympathoadrenal overactivity. In accordance with our results, it was previously observed by other authors that CB stimulation by corconium, a nicotinomimetic agent, causes a rise in circulating insulin that is reversed by CSN resection. We have also found that CSN resection decreased insulin sensitivity in control animals, which suggests a role for CB in metabolic control, not only in pathological but also in physiological conditions. This kind of mechanism is not novel in CB physiology, since it was recently proposed that the CB is involved in the counterregulatory response to hypoglycemia and in baroreflex control of blood pressure in humans.

Regarding the contribution of the CB to the development and maintenance of hypertension, our work agrees with previous results obtained by other groups in which it was observed that carotid sinus denervation prevented arterial pressure increase and
decreased sympathetic activity in spontaneous hypertensive young rats (43). It is known that, apart from chemoreceptor activity, CSN carries information related with baroreceptor activity. However, we would like to emphasize that the results obtained herein, both in the common carotid occlusion experiments and the carotid sinus denervation experiments reflect a carotid body chemoreceptor mediated effect. If there was a significant baroreceptor-mediated effect the animals would have become hypotensive in response to acute ischemic hypoxia and hypertensive after CSN denervation (a review see 44), which was not observed.

Our results show, for the first time that insulin triggers CB activation and that high insulin doses are an effective stimulus for CB overactivation. It is generally accepted that insulin stimulates the sympathetic nervous system, being fasting hyperinsulinemia one of the components of the sympathetic overactivation present in diabetes and the metabolic syndrome (45,46). However, insulin-induced sympathetic activity has been attributed to a central nervous system effect, since the infusion of insulin into the third cerebral ventricle increased sympathetic outflow, without significantly increasing adrenal and renal sympathetic activity (47,48). Without contradicting with these results, we show that insulin can also act on the carotid bodies to increase sympathoadrenal outflow. We demonstrated that insulin receptors are present in the CB and that its phosphorylation increases in response to insulin. As depicted in figure 3b, 1nM produced a higher degree of insulin receptor phosphorylation than 100nM. We expected to find a concentration-dependent relationship in CB insulin receptor phosphorylation, which we did not observe at high insulin concentrations. At high insulin levels insulin receptors are possibly saturated inducing a functional desensitization either by decreasing tyrosine kinase activity or by promoting insulin receptor endocytosis and degradation as it happens in human HepG2 cell line (49) and
in rat Fao cells (50). Also, we showed that insulin was capable of initiating a neurosecretory response measured as the increase in intracellular \( \text{Ca}^{2+} \) and the release of the neurotransmitters, ATP and dopamine, that is transduced into an increase in ventilation. The increase of ventilation induced by insulin is not novel (14), however in Bin-Jaliah’s work insulin was administered intravenously aiming to study the effects of insulin induced-hypoglycaemia in ventilation. Herein we administered insulin intracarotidally, to guarantee that the first site of insulin action is the CB; also we performed the experiments in euglycemic conditions, to avoid the confounding effects of systemic hypoglycaemia. These results together with the finding that the effect of insulin on ventilation disappears after CSN cut suggests that insulin action on ventilation is mediated by the CB.

In conclusion, we propose that insulin-triggered CB activation is responsible for increased sympathoadrenal activity and outflow creating a vicious cycle that culminates in severe IR and arterial HT, the core features of the metabolic syndrome and type 2 diabetes.

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experiments related with carotid body denervation and insulin resistance and in experiments related with carotid body overactivation in pathological rat models. Catecholamine quantification was performed in the laboratory of C.G., and C.G. also reviewed the manuscript. M.P.G. performed some of the experiments related with ATP quantification and insulin effects on ventilation and helped with the manuscript preparation. E.M. helped with the discussion. S.V.C. planned all the experiments, performed some of them, supervised the project and wrote the manuscript.

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Figure legends

Figure 1 Carotid body activity is increased in rat models of insulin resistance and hypertension. a and b, typical recordings of respiratory rate (bpm), tidal volume (ml) and blood pressure in basal conditions and in response to ischemic hypoxia, induced by occlusions of common carotid artery (OCC), in a control rat and in a rat submitted to a high fat (HF) diet. c, typical recording of ventilatory parameters after carotid sinus nerve cut in an HF rat. d, mean minute ventilation (VE, product of respiratory frequency and tidal volume) in control, HA and Hsu rats. e, effect of common carotid occlusion of 5, 10 and 15 seconds on VE in control, HF and Hsu rats. f, effect of hypercaloric diets on CB catecholamines (dopamine+DOPAC) basal release (20%O₂ + 5%O₂ balanced N₂) (n=5). g, effect of hypercaloric diets on the release of catecholamines from CB evoked by hypoxia (5%O₂ + 5%CO₂ balanced N₂) (n=5). h, effect of HF and Hsu diets in carotid bodies (CB) weight; control n = 19, HF n=27, Hsu n=24. i, effect of HF and Hsu diets on the immunoreactivity for tyrosine hydroxylase (TH, 60 KDa) expressed as the ratio TH/β-actin (43 KDa) expression. Left panel shows representative immunoreactivity for TH and β-actin in the CB in of control, HF and Hsu, animals. Bars (d, e, f, g, h and i) represent mean ± s.e.m. One and Two-Way ANOVA with Dunnett’s and Bonferroni multicomparsion tests, respectively; *p<0.05, **p<0.01, ***p<0.001 vs control; #p<0.05 vs values within the same group.

Figure 2 Carotid sinus nerve bilateral resection prevents insulin resistance and hypertension in high fat and high sucrose animal models. a, typical recording of
respiratory rate (bpm) and tidal volume (ml) in response to ischemic hypoxia, induced by occlusion of common carotid artery (OCC), in a rat submitted to carotid sinus nerve bilateral resection. The absence of increment in the ventilatory responses confirms carotid sinus nerve resection. b, representative glucose excursion curve for insulin tolerance test in a control rat. Details on KITT calculation are described methods section.  a, c, effect of carotid sinus nerve (CSN) resection on insulin sensitivity determined by the insulin tolerance test, expressed as constant rate for glucose disappearance (KITT) in control, high-fat (HF) and high-sucrose (HSu) diet rats. d, effect of CSN resection on mean arterial pressure in control, HF and HSu rats. e, absolute weight before and after hypercaloric diets administration and chronic sinus nerve resection f, increment in body-weight, calculated as total weight variation during the experimental period, in control, HF and HSu rats with and without CSN resection. g, visceral fat, weighed post-mortem and corrected to body weight in control, HF and HSu rats with and without CSN resection. Bars represent mean ± s.e.m. One and Two-Way ANOVA with Dunnett’s and Bonferroni multicomparison tests, respectively; *p<0.05, **p<0.01, *** p<0.001 vs control; #p<0.05; ##p<0.01, ###p<0.001 comparing values with and without CSN resection.

Figure 3 Carotid sinus nerve bilateral resection prevents sympathoadrenal overactivation in high fat and high sucrose animal models. a and b, effect of CSN resection on circulating catecholamines, norepinephrine (NE) and epinephrine (Epi), respectively. c and d, effect of CSN resection on adrenal medulla NE and Epi content, respectively. Bars represent mean ± s.e.m. Two-Way ANOVA with Bonferroni multicomparison tests, respectively; *p<0.05, **p<0.01, *** p<0.001 vs control; #p<0.05; ##p<0.01, ###p<0.001 comparing values with and without CSN resection.
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**a** representative Western blot showing insulin receptor immunoreactivity in the CB and insulin receptor phosphorylation immunoreactivity in control CBs and in response to 1 and 100 nM insulin (30 minutes incubation), respectively, corresponding to the 97 KDa band. A re-probing of the membranes with an anti-β-actin antibody, corresponding to the 42 KDa band is shown below the gels.  

**b** average insulin receptor phosphorylation in control and in CBs incubated with 1 and 100 nM insulin in relation to β-actin immunoreactivity(n = 3-4). **p<0.01, * p<0.05; One –Way ANOVA with Dunnett’s multicomparsion test comparing the groups with the control. Data represent mean ± s.e.m.

Figure 5  Insulin increases the neurosecretory responses in the carotid bodies  

**Figure 5** Insulin increases the neurosecretory responses in the carotid bodies. 

**a**, microscope field of dissociated rat carotid body cell culture and the typical recording of intracellular cell Ca$^{2+}$, measured as the ratio of the fluorescent emission at 340/380 nm of chemoreceptor cells in basal conditions, in response to hypoxia (N$_2$), to 1nM of insulin and to 35mM of K$.  

**b**, effect of insulin on intracellular cell Ca$^{2+}$, measured as means of the ∆RI in 179 chemoreceptor cells. In every cell the fluorescence signal was integrated as a function of time (running integral; RI).  

**c** and **d**, time course for the release of ATP from CB in response to insulin (10nM) and dose-response curve for insulin action on ATP release and its comparison with the effect of hypoxia (5%O$_2$ + 5%CO$_2$ balanced N$_2$). Release protocol consisted in a 2 incubations of CBs in normoxic solutions (20% O$_2$ + 5%CO$_2$ balanced N$_2$, 10 min), followed by insulin application during 30 minutes in normoxia and two final normoxic incubations.  

**e** and **f**, identical group of experiments than **c** and **d** but measuring catecholamines (dopamine + DOPAC)
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**Figure 6 Insulin increases ventilation through a carotid body mediated effect.** a, respiratory frequency (RR) and tidal volume (TV) recordings before and after administration of an intracarotid insulin (100mg/kg) bolus. b, mean basal ventilatory parameters, respiratory frequency, tidal volume and minute ventilation (VE) before insulin administration. c, dose-response curve for the effect of insulin (1, 5, 10, 50, 100, 200 mU/kg) on minute ventilation. To avoid the effect of hypoglycaemia the study the study of insulin effect on ventilation was made performed in euglycaemic conditions. Insulin effects on ventilation are means of 5-7 data. d, typical respiratory frequency and tidal volume recordings due to the administration of an intracarotid insulin (100mg/kg) bolus before and after carotid sinus nerve (CSN) cut. e, graph depicting a typical glucose perfusion curve to maintain euglycemia after insulin bolus and the levels of glycemia throughout the experiment. f, show total glucose concentrations perfused to maintain euglycemic clamp in response to the insulin concentrations (1, 5, 10, 50, 100, 200 mU/kg) tested. Values represent means ± s.e.m. One-Way ANOVA with Dunnett’s multicomparison test; *p<0.05, **p<0.01 vs basal values.
Table 1 Effect of carotid sinus nerve chronic resection on fasting plasma glucose, plasma insulin, serum free fatty acids and corticosterone levels in control, HF and HSu diet rats. Data with and without carotid sinus resection are means of 7-9 and 10-13 jvalues, respectively.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Glycaemia (mg/dL)</th>
<th>Insulinemia (µg/L)</th>
<th>Free fatty acids (µM)</th>
<th>Corticosterone (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without CSN resection</td>
<td>100.4 ± 4.2</td>
<td>1.9 ± 0.5</td>
<td>389.1 ± 40.5</td>
<td>4.34 ± 0.3</td>
</tr>
<tr>
<td>With CSN resection</td>
<td>95.4 ± 3.5</td>
<td>2.2 ± 0.0</td>
<td>468.6 ± 42.3</td>
<td>4.89 ± 0.1</td>
</tr>
<tr>
<td><strong>High fat diet</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without CSN resection</td>
<td>106.3 ± 2.5</td>
<td>4.6 ± 0.6 ***</td>
<td>436.5 ± 36.2</td>
<td>4.51 ± 0.1</td>
</tr>
<tr>
<td>With CSN resection</td>
<td>112.7 ± 3.9</td>
<td>2.0 ± 0.1 ###</td>
<td>377.8 ± 37.5</td>
<td>5.1 ± 0.1</td>
</tr>
<tr>
<td><strong>High sucrose diet</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without CSN resection</td>
<td>145.8 ± 9.6 ***</td>
<td>5.27 ± 0.3 ***</td>
<td>891.1 ± 93.2***</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>With CSN resection</td>
<td>95.6 ± 5.8 ###</td>
<td>1.9 ± 0.2 ###</td>
<td>431.8 ± 76.5###</td>
<td>4.6 ± 0.1</td>
</tr>
</tbody>
</table>

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