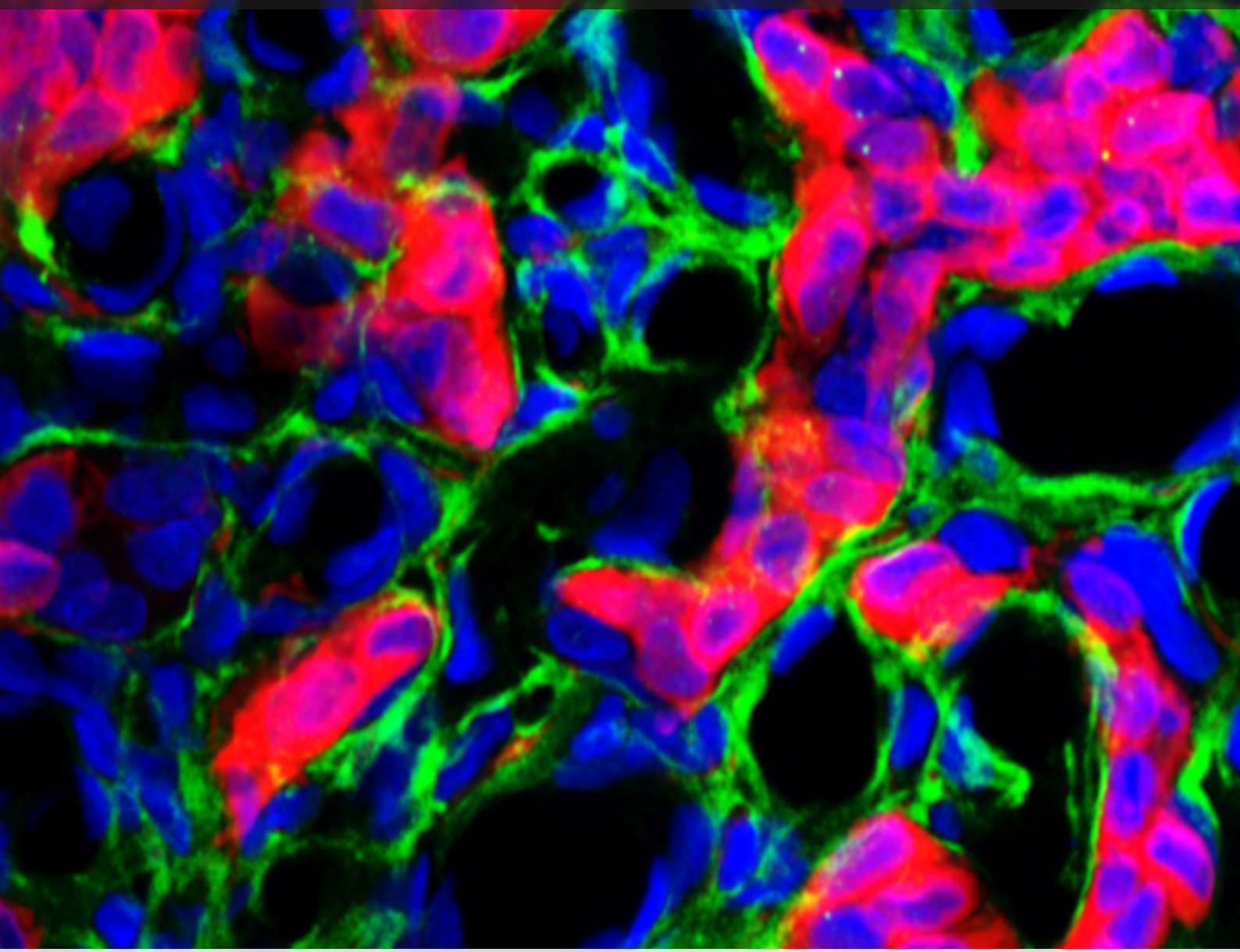
CAROTID BODY: A NEW TARGET FOR RESCUING NEURAL CONTROL OF CARDIORESPIRATORY BALANCE IN DISEASE

EDITED BY: Rodrigo Del Rio, Rodrigo Iturriaga and Harold D. Schultz
PUBLISHED IN: Frontiers in Physiology



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ISSN 1664-8714
ISBN 978-2-88919-943-3
DOI 10.3389/978-2-88919-943-3

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CAROTID BODY: A NEW TARGET FOR RESCUING NEURAL CONTROL OF CARDIORESPIRATORY BALANCE IN DISEASE

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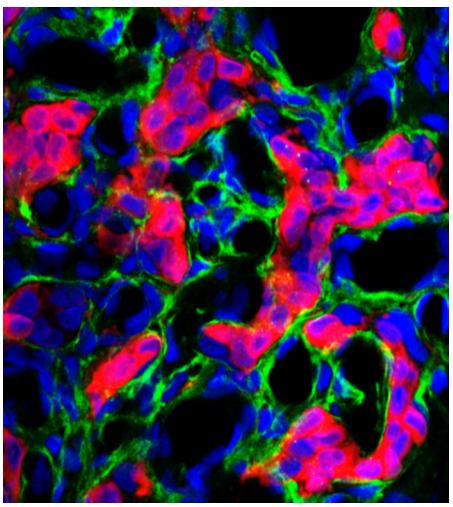

The Carotid Body. Type I cells stained with anti-tyrosine hydroxilase (red), Type II cells stained with anti-GFAP (green) and cell nuclei stained with Dapi (blue). Confocal microscopy image provided by Dr. Rodrigo Del Rio.

The carotid body (CB) is in charge of adjusting ventilatory and cardiovascular function during changes in arterial blood gases. Regardless this essential function, the CB has been implicated in the sensing of other physiological signals such as changes in blood flow and glucose levels. More important, malfunction of the CB chemoreceptors has been associated with the progression and deterioration of several disease states such as hypertension, heart failure, renal failure, insulin resistance, diabetes and sleep apnea. Although the mechanisms involved in the alterations of the CB function in pathophysiology are currently under intense research, the development of therapeutic approaches to restore normal CB chemoreflex function remains unsolved. Recent studies showing the effect of CB denervation in pathophysiology have unveiled a key role of these arterial chemoreceptors in the development of autonomic imbalance and respiratory disturbances, and suggest that targeting the CB could represent a novel strategy to improve disease outcome.

Unfortunately, classical pharmacotherapy intended to normalize CB function may be hard to establish since several cellular pathways are involved in the CB dysfunction. Augmented levels of angiotensin II, endothelin-1, cytokines and free radicals along with decreases in nitric oxide had all been related to the CB dysfunction. Moreover, changes in expression of angiotensin receptors, nitric oxide synthases and cytokines that take place within the CB tissue in pathological states also contribute to the enhanced CB chemoreflex drive. It has been shown in heart failure,

hypertension and obstructive sleep apnea that the CB becomes tonically hyper-reactive. During the progression of the disease this CB chemosensory facilitation process induces central nervous system plasticity. The altered autonomic-respiratory control leads to increased cardiorespiratory distress and the deterioration of the condition.

The focus of this e-book will be to cover the role of the CB in pathophysiology and to provide new evidence of the pathways involved in the maladaptive potentiation of the CB chemoreflex function. In memory of Professor Mashiko Shirahata and Professor Constancio Gonzalez.

Citation: Del Rio, R., Iturriaga, R., Schultz, H. D., eds. (2016). Carotid Body: A New Target for Rescuing Neural Control of Cardiorespiratory Balance in Disease. Lausanne: Frontiers Media. doi: 10.3389/978-2-88919-943-3

Table of Contents

frontiers in Physiology

EDITORIAL
published: 29 June 2015
doi: 10.3389/fphys.2015.00181

Editorial: Carotid body: a new target for rescuing neural control of cardiorespiratory balance in disease

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Keywords: carotid body, heart failure, sleep apnea, hypertension, autonomic function, sympathetic nervous system, insulin resistance

The carotid body (CB) is considered the main arterial chemoreceptor. Interestingly, the CB respond to a large repertoire of stimuli that can elicit a reflex cardiorespiratory response. For this reason, the pathophysiological role played by alterations in CB function during disease conditions is under intense research.

Since its discovery, the CB has been related almost exclusively to its pivotal role in adjusting ventilatory and cardiovascular function during acute or chronic alterations on blood gases (i.e., hypoxia and hypercapnia, sustained chronic hypoxia). However, recent advances has contribute to decipher the role played by the CB in the progression of highly prevalent diseases such as hypertension, heart failure and insulin resistance. Indeed, elegant studies showing the effect of CB neurotomy in pathophysiology have unveiled a key role of these arterial chemoreceptors in the development of autonomic imbalance, respiratory disturbances, systemic inflammation, and impairment of glucose metabolism (Fitzgerald, 2014). Therefore, targeting the CB rises as a novel therapeutic strategy to improve disease outcome. However, the mechanisms involved in the alterations of CB function in pathophysiology are not completely understood. Indeed, classical pharmacotherapy intended to normalize CB function may be hard to establish since several cellular pathways are involved in the CB dysfunction. Augmented levels of CB neuromodulators such as angiotensin II, endothelin-1, pro-inflammatory cytokines, cyclic nucleotides, hydrogen sulfide, carbon monoxide and free radicals had all been related to CB chemosensory facilitation (Iturriaga et al., 2014; Prabhakhar and Joyner, 2015). Indeed, it has been proposed that the enhanced CB chemoreflex drive induces central nervous system (CNS) plasticity.

In experimental chronic heart failure (CHF), the CB chemosensory activity is tonically elevated leading to sympatho-excitation and destabilization of breathing. Marcus et al. (2014) reviewed the contribution of the CB on the respiratory-sympathetic coupling in CHF and its role in the development of oscillatory breathing patterns and enhanced renal sympathetic nerve activity. The elimination of the CB afferents totally normalized breathing patterns and reduced sympathetic outflow suggesting that the CB plays a pivotal role in the progression of cardio-respiratory dysfunction during CHF.

It has been shown that exposure to chronic intermittent hypoxia (CIH), one of the main features of obstructive sleep apnea syndrome (OSA), induces a potentiation of CB chemosensory activity (Iturriaga et al., 2014). It has been proposed that episodic hypoxia during CIH induces CB sensory plasticity increasing neural afferent discharges to brainstem areas related to cardiorespiratory control (Fung, 2014; Iturriaga et al., 2014; Prabhakhar and Joyner, 2015).

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology

Received: *29 May 2015*
Accepted: *02 June 2015*
Published: *29 June 2015*

Citation:
Del Rio R, Iturriaga R and Schultz HD (2015) Editorial: Carotid body: a new target for rescuing neural control of cardiorespiratory balance in disease. Front. Physiol. 6:181. doi: 10.3389/fphys.2015.00181

The hyperactivation of the CB-mediated chemoreflex following CIH leads to hypertension. The cellular mechanisms underlying the CB sensitization during CIH are not completely known; however, it is well accepted that endothelin, angiotensin peptides, pro-inflammatory cytokines and oxidative stress are all involved in the sensory potentiation of the CB (Iturriaga et al., 2014). Fung (2014) reviewed the concept of a local renin-angiotensin system (RAS) within the CB, which could be promoting functional adjustments of the CB function during CIH. Interestingly, the RAS in the CB displayed a marked upregulation following CIH suggesting a plausible role during CB sensory plasticity. Therefore, specific blockers of the RAS should be of benefit in the control of CB-mediated hypertension during CIH. Accordingly, Diogo and Monteiro (2014) provide a comprehensive review of the different treatments and strategies to reduced blood pressure in both animal models and in humans with OSA. Intriguingly, there is a still lack of a unique treatment to control OSA-related hypertension. Therefore, future studies are needed.

Maintenance of glucose homeostasis is a key process for the proper metabolic functions of several tissues across the body. Accordingly, alterations on glucose sensing could severely impact the development of pathological conditions, especially those linked to sympatho-excitation (Conde et al., 2014; Gao et al., 2014). Nevertheless, the idea that CB chemoreceptor cells respond to changes in arterial glucose levels is still a matter of debate. However, Gao et al. (2014) provide compelling evidence showing that low glucose induced increases in intracellular Ca^{2+} levels and catecholamine release in isolated CB chemoreceptor cells. Furthermore, they described that glucose-sensing pathways in the CB are not shared by the oxygen sensing mechanisms by which the CB respond to low arterial oxygen levels (Gao et al., 2014). In addition, Conde et al. (2014) described the pivotal role of the CB in the development of insulin resistance and the consequent progression into type 2 diabetes. Furthermore, they proposed targeting the CB as a potential therapeutic strategy to improved glucose metabolism. In addition, Conde et al. (2014) proposed chronic caffeine intake as a plausible strategy intended to normalize CB function in insulin resistance and type 2 diabetes due to the well documented effects of caffeine on adenosine receptors within CB chemoreceptor cells. Unfortunately, there are no human studies showing a beneficial effect of caffeine intake during the transition from insulin resistance to diabetes. Future studies should be focused on the therapeutic effect of caffeine on the CB-mediated glucose sensing impairment during diabetes.

The immune system is the barrier of defense during pathogen infection. Recently, neuro-immunomodulation of the inflammatory process has been point out as a central step in the immune response. In this context, activation of peripheral sensory afferents by pro-inflammatory cytokines is of interest to understand the immunosensory modulation (Fernandez et al., 2014). Despite the evidence showing cytokine receptor expression on vagal paraganglia, the CB rises as a plausible sensor of inflammatory status since the CB displays cytokine receptors in chemoreceptor cells and that upon activation, elicit a reflex response intended to regulate the inflammatory response. Fernandez et al. (2014) reviewed the contribution of the CB on sepsis progression and the role of the CB on exacerbating the immune defense. Contrary to the above mentioned pathologies (heart failure, OSA, diabetes), during sepsis, activation of the CB appears to be helpful in the inflammatory process since it will induce corticoid release by the adrenal gland in a mechanism linked to an increase in sympathetic outflow. Thus, stimulation of the CB may be a suitable tool to improve the outcome during sepsis and infectious diseases.

Understanding the CB chemosensory process, regardless of it's involvement in pathophysiological events, is extremely relevant to improve knowledge in the field. The mechanisms that govern the CB chemoreceptor response to hypoxia are not fully understood. Nunes et al. (2014) reviewed the contribution of cyclic adenosine monophosphate (cAMP) to the CB chemosensory transduction process. Despite the fact that cAMP is considered mainly a metabolic product, its ability to modulate G-coupled receptors and change intracellular Ca^{2+} levels in CB chemoreceptor cells (or petrosal afferents) make this nucleotide an interesting candidate to be considered as a CB chemosensory modulator. In the same context of finding new pathways involved in the CB function, Mazzatenta et al. (2014) showed novel data regarding the role of galanin, a 30-aminoacid neuropeptide, in CB neurogenesis. Interestingly, the expression of this peptide in the CB seems to correlate with age. They showed that "old CBs" displayed less galanin compared to "young CBs." Therefore, it is plausible that galanin contributes to the loss of CB sensitivity during aging.

Also important in the CB-mediated chemoreflex is the mechanism by which the afferent information travels to the CNS. Retamal et al. (2014) reviewed the importance of the petrosal ganglion neurons in the CB chemotransduction process. Historically, this group of neurons was normally described as part of the "wiring" connection between the CB and the CNS. However, evidence from other sensory ganglia suggests that neuronal-to-glial cell communication can modulate sensory information. Thus, glial cells may modulate the excitatory or inhibitory status of petrosal ganglion neurons. The outcome would be an increase or decrease of the CB afferent input to the CNS. This exciting hypothesis deserves future investigations.

The CB undergoes structural and functional changes during development and in response to chronic sustained hypoxia. Taking into account the small size of the CB ($\approx$1 mm^3 in humans), novel techniques are always required to improve experimental approaches. Guidolin et al. (2014) provide an interesting new method to study structural changes in the CB. Using fractal analysis they study the extracellular matrix composition in fixed and stained sections from the CB. They found that fractal analysis of CB sections is a suitable tool to obtained quantitative data of the extracellular components present in the CB. This technique could be useful in the study of CB structural remodeling in disease conditions.

In summary, this collection of works provides a useful and timely update in the field of CB chemoreception and its contribution on the development and progression of several pathologies. Improving the knowledge on the role of the CB in health and disease will promote new avenues in the understanding of the CB-mediated chemoreflex.

Acknowledgments

RDR is supported by Fondecyt #1140275 from the Chilean Government, RI is supported by Fondecyt #1150040 from the Chilean Government and HDS is supported by a Program Project Grant from the Heart, Lung and Blood Institute of NIH (PO1-HL62222).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 14 January 2015
doi: 10.3389/fphys.2014.00524

Tasting arterial blood: what do the carotid chemoreceptors sense?

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The carotid bodies are sensory organs that detect the chemical composition of the arterial blood. The carotid body sensory activity increases in response to arterial hypoxemia and the ensuing chemoreflex regulates vital homeostatic functions. Recent studies suggest that the carotid bodies might also sense arterial blood glucose and circulating insulin levels. This review focuses on how the carotid bodies sense O_2, glucose, and insulin and some potential implications of these sensory functions on physiological regulation and in pathophysiological conditions. Emerging evidence suggests that carbon monoxide (CO)-regulated hydrogen sulfide (H_2S), stemming from hypoxia, depolarizes type I cells by inhibiting certain K^+ channels, facilitates voltage-gated Ca^{2+} influx leading to sensory excitation of the carotid body. Elevated CO and decreased H_2S renders the carotid bodies insensitive to hypoxia resulting in attenuated ventilatory adaptations to high altitude hypoxia, whereas reduced CO and high H_2S result in hypersensitivity of the carotid bodies to hypoxia and hypertension. Acute hypoglycemia augments the carotid body responses to hypoxia but that a prolonged lack of glucose in the carotid bodies can lead to a failure to sense hypoxia. Emerging evidence also indicates that carotid bodies might sense insulin directly independent of its effect on glucose, linking the carotid bodies to the pathophysiological consequences of the metabolic syndrome. How glucose and insulin interact with the CO-H_2S signaling is an area of ongoing study.

Keywords: glomus cells, K^+ channels, carbon monoxide, hydrogen sulfide, hypoglycemia, diabetes

INTRODUCTION

Based on the anatomical location and morphology, Frenando De Castro proposed that the function of the carotid body is to detect ".....chemical composition of the blood (chemical sensing) and the information is transmitted to the nerve terminals which by reflex action will influence on the functional activity of other organs" (De Castro, 1926). Independent studies by Jean-Francois Heymans and Corneille F. Heymans established that the carotid sinus region is divided into two different portions, the carotid body (glomus) which is stimulated by the chemical composition of the arterial blood, whereas the carotid sinus is the seat of the pressor receptors (Heymans and Heymans, 1927). Subsequently several investigators examined the effects of hypoxemia (i.e., reduced O_2 levels in arterial blood) on the carotid body. It is now established that hypoxemia increases carotid body sensory nerve activity and the ensuing reflex regulates cardiorespiratory functions (see Fidone and Gonzalez, 1986; Fitzgerald and Lahiri, 1986; Gonzalez et al., 1994; Kumar and Prabhakar, 2012 for references). Recent studies indicate that carotid bodies might also sense changes in arterial blood glucose and insulin levels (Koyama et al., 2000; Wehrwein et al., 2010; Ribeiro et al., 2013; Limberg et al., 2014). This brief review will focus on how the carotid bodies sense changes in arterial blood O_2, and emerging information about their role as sensors of glucose and insulin. The implications of these sensory functions of the carotid body on physiological regulation and in pathophysiological conditions will be highlighted.

ANATOMICAL LOCATION AND MORPHOLOGY OF THE CAROTID BODY

The carotid bodies are situated bilaterally at the bifurcation of the common carotid artery. The anatomical location of the carotid bodies favors detecting the changes in the arterial blood composition before the stimulus reaches the brain which is highly dependent on oxygen and glucose for sustained function. Sensory innervation to the carotid body is provided by a branch of the glossopharengeal nerve called the "carotid sinus nerve (CSN)." The cell bodies of the CSN reside in the petrosal ganglion. Autonomic innervation comes from the post-ganglionic fibers of the superior cervical ganglion. The carotid body receives the highest blood flow per tissue weight of any organ in the body, with a value of approximately 1000–2000 ml min^{-1} 100 g^{-1} (De Burgh Daly et al., 1954; Clarke et al., 1986; Barnett et al., 1988), which is two- to four-fold higher than the blood flow to the heart during heavy exercise (Duncker and Bache, 2008). The chemoreceptor tissue is composed of two major cell types: the type I (also called glomus) cells and type II cells. A substantial body of evidence suggests that type I cells are the initial sites of sensory transduction and they work in concert with the nearby afferent nerve ending as a "sensory unit;" whereas the type II cells are supporting

cells resembling glial cells of the nervous system (see Kumar and Prabhakar, 2012 for references).

CHARACTERISTICS OF THE CAROTID BODY SENSORY NERVE RESPONSE TO HYPOXIA

The sensory discharge of the carotid sinus nerve is low under normoxia (arterial PO_2 ~100 mmHg), which increases dramatically even with a modest drop in arterial PO_2 (e.g., 80–60 mmHg; Eyzaguirre and Lewin, 1961; Hornbein et al., 1961; Biscoe et al., 1970; Vidruk et al., 2001). The response is fast and occurs within seconds after the onset of hypoxia (Black et al., 1971; Ponte and Purves, 1974). Because of its high blood flow and exquisite sensitivity to hypoxia, the carotid body is uniquely suited to sense and respond to even a modest drop in PO_2.

ROLE OF GASEOUS MESSENGERS IN THE CAROTID BODY RESPONSE TO HYPOXIA

Emerging evidence suggests that the gaseous messengers carbon monoxide (CO) and hydrogen sulfide (H_2S) play a critical role in hypoxic sensing by the carotid body (Prabhakar, 2013). The following section describes how CO and H_2S contribute to the O_2 sensing by the carotid body.

CARBON MONOXIDE (CO)

Glomus cells express heme oxygenase-2 (HO-2), an enzyme that catalyzes the formation of CO (Prabhakar et al., 1995), and CO is a physiological inhibitor of the carotid body and glomus cell response to hypoxia (Prabhakar et al., 1995; Williams et al., 2004; Peng et al., 2014). CO levels are high during normoxia, and hypoxia decreases CO levels in a stimulus-dependent manner in the carotid body (Peng et al., 2014). These findings demonstrate that changes in O_2 levels are transduced to changes in CO production in the chemoreceptor tissue.

HYDROGEN SULFIDE (H_2S)

It is being increasingly recognized that H_2S is another gaseous messenger that participates in physiological functions (Yang et al., 2008). Whilst O_2 levels regulate CO, CO itself does not trigger the sensory excitation of the carotid body. Instead, CO contributes to the carotid body sensory excitation by regulating H_2S production from the enzyme, cystathionine-γ-lyase (CSE, Peng et al., 2010, 2014). CO suppresses H_2S levels by inhibiting CSE (Peng et al., 2010, 2014). As a consequence, during normoxia high CO levels are associated with low H_2S levels; whereas during hypoxia, low CO levels are accompanied with high H_2S levels, paralleling the sensory nerve excitation (Peng et al., 2010, 2014). Either genetic deletion or pharmacological blockade of CSE result in marked suppression of H_2S generation during hypoxia (Peng et al., 2010) leading to remarkable blunting of hypoxia-evoked sensory nerve excitation and ventilatory stimulation (Peng et al., 2010). These findings suggest that H_2S mediates the carotid body sensory nerve excitation by hypoxia.

How might H_2S contribute to carotid body excitation by hypoxia? The general consensus is that hypoxia depolarizes glomus cells by inhibiting certain K^+ channels leading to Ca^{2+}-dependent release of excitatory neurotransmitter(s), which stimulates the afferent nerve ending and increases the sensory nerve activity (Gonzalez et al., 1994; Kumar and Prabhakar, 2012; Moya et al., 2012; Nurse and Piskuric, 2012; Prabhakar and Peers, 2014). The following lines of evidence suggest that H_2S mediates hypoxia-induced glomus cell depolarization and voltage-gated Ca^{2+} influx: (a) like hypoxia, H_2S donor (NaHS) inhibits maxi-K^+ (Li et al., 2010; Telezhkin et al., 2010), TASK like K^+ channel activities and depolarizes type I cells (Buckler, 2012), (b) hypoxia-evoked Ca^{2+} influx is markedly reduced or absent in CSE null glomus cells or after pharmacological blockade of H_2S synthesis (Makarenko et al., 2012), (c) H_2S donor (NaHS) elevates $[Ca^{2+}]_i$ in glomus cells and this effect was absent in the absence of extracellular Ca^{2+} (Buckler, 2012; Makarenko et al., 2012) as well as by preventing the depolarization by voltage-clamping the cell at the resting membrane potential (Buckler, 2012), and (d) nifedipine, a blocker of L-type Ca^{2+} channel, prevents H_2S-as well as hypoxia-evoked $[Ca^{2+}]_i$ elevation in glomus cells (Makarenko et al., 2012). In addition, H_2S donor increases NADH auto fluorescence in glomus cells suggesting that H_2S might mediate its actions in part due to its effects on the mitochondrial electron transport chain (Buckler, 2012). These studies taken together suggest that CO-regulated H_2S, stemming from hypoxia, depolarizes type I cells by inhibiting certain K^+ channels, facilitates voltage-gated Ca^{2+} influx and thus produces sensory excitation of the carotid body.

IMPACT OF INHERENT VARIATIONS IN CO-H_2S SIGNALING ON THE CAROTID BODY O_2 SENSING

The chemosensory reflex is a critical regulator of breathing, sympathetic tone, and blood pressure (Fitzgerald and Lahiri, 1986; Kumar and Prabhakar, 2012). However, healthy human subjects exhibit substantial variations (about three-fold) in the chemosensory reflex as evidenced by variations in the ventilatory response to hypoxia (Weil, 2003). Such variations were also reported in rodents. For instance, in comparison to Sprague-Dawley (SD) rats, Brown-Norway (BN) rats display a markedly reduced ventilatory response to hypoxia (Strohl et al., 1997; Hodges et al., 2002), while Spontaneous Hypertensive (SH) rats exhibit an augmented one (Hayward et al., 2012). A recent study examined whether variations in the chemosensory reflex are due to differences in O_2 sensing by the carotid body in BN, SH, and SD rats (Peng et al., 2014). BN carotid bodies exhibited severely impaired glomus cell and sensory nerve responses to hypoxia, whereas SH rat carotid bodies showed augmented hypoxic response as compared with SD rats.

The low hypoxic sensitivity in the BN carotid body was associated with high CO and low H_2S levels; whereas, the augmented hypoxic sensitivity of SH rat carotid body was accompanied with low CO and high H_2S levels under both normoxia and hypoxia, respectively as compared with SD carotid bodies. The altered CO and H_2S levels in BN and SH rats was not associated with the changes in HO-2 and CSE proteins in glomus cells (Peng et al., 2014). Remarkably, treating BN carotid bodies with a heme oxygenase inhibitor decreased CO levels, increased basal and hypoxia-induced H_2S levels, and restored the magnitude of the hypoxic sensitivity, which was comparable to SD rats. Treating SH rat carotid bodies with a CO donor or a CSE inhibitor reduced H_2S levels and attenuated the hypoxic sensitivity (Peng et al., 2014). These findings suggest that high CO and low H_2S contribute to inherent hyposensitivity of the carotid body to hypoxia;

whereas, low CO and high H_2S leads to hypersensitivity of the carotid body to hypoxia, further supporting CO-regulated H_2S governs hypoxic sensing by the carotid body.

PHYSIOLOGICAL IMPLICATIONS OF CAROTID BODY O_2 SENSING

CONSEQUENCES OF HYPOSENSITIVITY OF THE CAROTID BODY TO HYPOXIA

BN rats exhibited reduced hypoxic ventilatory response (HVR) and near absence of hypoxia-evoked sympathetic nerve activity compared to SD rats (Peng et al., 2014). High-altitude hypoxia leads to a carotid body-mediated increase in breathing, or ventilatory adaptation to hypoxia (VAH) (Dempsey and Forster, 1982). A diminished HVR can result in attenuated VAH (Dempsey and Forster, 1982) and high-altitude pulmonary edema (Hackett et al., 1988; Matsuzawa et al., 1989; Hohenhaus et al., 1995). BN rats exposed to hypobaric hypoxia simulating 8500 m altitude for 16 h showed remarkable absence of VAH and profound pulmonary edema (Peng et al., 2014). Treating BN rats with a heme oxygenase inhibitor, improved ventilatory and sympathetic nerve responses to hypoxia, restored VAH and prevented hypobaric hypoxia-induced pulmonary edema (Peng et al., 2014).

CONSEQUENCES OF HYPERSENSITIVITY OF THE CAROTID BODY TO HYPOXIA

Spontaneous hypertensive (SH) rats (Przybylski, 1978) and human subjects with essential hypertension exhibit augmented ventilatory responses to hypoxia (Trezebski et al., 1983) and these effects were attributed to enhanced carotid body sensitivity to low O_2 (Przybylski, 1981; Trezebski et al., 1983). Later studies showed that the carotid body response to hypoxia is indeed augmented in SH rats (Fukuda et al., 1987) and carotid body chemoreflex mediates the heightened sympathetic nerve activity in SH rats (Tan et al., 2010). Based on these findings, Paton and co-workers (Abdala et al., 2012) tested whether ablation of the carotid bodies normalizes blood pressures in SH rats. They found that chronic bilateral sectioning of the carotid sinus nerves substantially lowered blood pressures in SH rats. Since L-propargylglycine (L-PAG), an inhibitor of H_2S synthesis, reduced the hypersensitivity of the carotid body in SH rats, Peng et al. (2014) examined whether L-PAG treatment affect the age-dependent development of hypertension in SH rats. Five-week-old SH rats were treated with either vehicle (saline) or L-PAG every day, with blood pressures measured every week for 5 weeks. Compared to vehicle-treated SH rats, L-PAG-treated SH rats presented a pronounced reduction in blood pressures. Ablation of the carotid bodies from 5-week-old SH rats also attenuated age-dependent hypertension to the same extent as L-PAG treatment. However, treating carotid body ablated rats with L-PAG caused no further decline in blood pressures, suggesting that the carotid bodies are the likely sites of action of L-PAG. These findings underscore a mechanism through which elevated H_2S signaling in the carotid body contributes to the hypoxic hypersensitivity and progression of hypertension in SH rats. The impact of inherent variations in CO-H_2S signaling on carotid body O_2 sensing and their consequences on physiological responses is schematically illustrated in **Figure 1**.

THE CAROTID BODIES: EXPANDING ROLE IN HUMAN PHYSIOLOGY AND PATHOPHYSIOLOGY?

In the above sections, we briefly reviewed the sensory transduction mechanisms that link carotid body function with hypoxia and also showed that stimulation of the carotid bodies by hypoxia is a key driver of ventilation and sympathetic nerve activity. Additionally, the data from the congestive heart failure (CHF) rats provide evidence that tonic or perhaps "overactive" stimulation of the carotid bodies in the absence of hypoxia might be an important driver of elevated sympathetic activity in a number of circumstances (Del Rio et al., 2013). In this context, there is evidence from animal models and also human studies suggesting that the carotid bodies are tonically active during normoxia and drive the increased sympathetic nerve activity in some patients with heart failure and also chronic kidney disease (Paton et al., 2013). In fact, excessive ventilation during exercise in heart failure

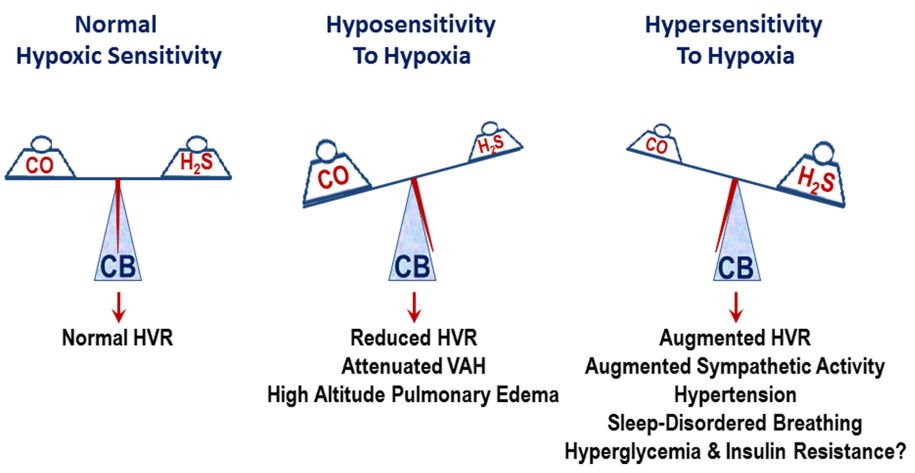

FIGURE 1 | Schematic presentation of the impact of inherent variations in carbon monoxide (CO) and hydrogen sulfide (H_2S) levels on the carotid body (CB) sensitivity to hypoxia and its influence on physiological responses. HVR, hypoxic ventilatory response; attenuated VAH, ventilatory adaptation to hypoxia.

patients is likely driven in part by hypersensitive carotid bodies and is associated with poor patient outcomes (Ponikowski et al., 2001). Hyperoxia or dopamine infusions to "turn off" the carotid body function in humans and in dogs can also blunt the sympathoexcitatory responses to a number of stressors like stimulation of metabosensitive skeletal muscle afferents during exercise (Ponikowski et al., 2001). Together these and other observations suggest that the role of the carotid body extends beyond O_2 sensing. These findings also suggest that carotid body sensory activity might be elevated in the normoxic state during hypertension, CHF and/or insulin resistance. A key questsion is whether this tonic activation is due to the enhanced CO-H_2S signaling in the carotid body under these conditions or some other mechanism(s).

WHAT ABOUT GLUCOSE AND HYPOXIA?

Over the last fifteen or so years evidence from a variety of models has suggested that the carotid bodies either directly sense arterial blood glucose concentrations or that prevailing glucose levels can influence the stimulus response curve of the carotid body to hypoxia. Pardal and López-Barneo (2002) reported that hypoglycemia stimulates glomus cells and enhances their responses to hypoxia. A recent study showed that glycogen depletion of the carotid body can lead to a brief period of hyperresponsiveness followed by hyporesponsiveness to hypoxia (Holmes et al., 2014). These findings are mirrored earlier observations in humans showing that maneuvers that cause whole body glycogen depletion initially stimulate ventilation, but that days of "semi-starvation" blunt the ventilatory responses to hypoxia but not to hypercapnia (Heigenhauser et al., 1983; Lindholm and Gennser, 2005). These studies indicate that acute hypoglycemia augments the carotid body responses to hypoxia, but a prolonged lack of glucose in the carotid bodies can lead to a failure to sense hypoxia.

WHAT ABOUT GLUCOSE *PER SE*?

The data presented above also raises questions whether carotid body can be sensors of blood glucose levels *per se*. As discussed earlier, this makes some teleological sense given the dependence of the brain on blood glucose and the location of the carotid bodies as sentinels in the arterial circulation just proximal to the brain. When the question is posed this way a number of interesting observations from *in vivo* studies are available. First, the counter regulatory hormonal responses to hypoglycemia induced by the insulin clamp technique in dogs are blunted in animals that have undergone carotid body resection (Koyama et al., 2000). Likewise, hyperoxia in humans (again to acutely "turn off" the carotid bodies) can also blunt the counter regulatory responses normally seen during hyperinsulinemic, hypoglycemic clamps (Wehrwein et al., 2010). There is also observational evidence in patients with COPD that correcting their arterial hypoxemia alters whole body glucose homeostasis in a manner consistent with the idea that the carotid body plays a role in the regulation of blood glucose (Jakobsson and Jorfeldt, 2006). Similar conclusions can also be drawn in diabetic patients on insulin who have received hyperbaric therapy for wound healing. In these patients, the risk of hypoglycemia seems increased during the hyperbaric treatment suggesting that it interferes with the ability of the carotid bodies to sense blood glucose (Al-Waili et al., 2006).

While the observations cited above are consistent with the idea that the carotid bodies also sense glucose, it should however, be noted that not all studies provide strong support for hypoglycemia as a stimulus for the carotid boides (Bin-Jaliah et al., 2004; Conde et al., 2007; Gallego-Martin et al., 2012). This could be due to technical or experimental design issues or complex interactions between glucose and insulin that we will discuss when we consider insuin as a potential stimulator of the carotid bodies.

How any sensing of blood glucose at the cellular level might differ or intersect with the hypoxic sensing via the CO-H_2S signaling or other mechanisms is currently unclear. Additionally, how carotid body regulation of blood glucose and sympathetic activty might be amplified in conditions like obstructive sleep apnea which is associated with both hypertension and diabetes are also unclear. This is especially important because tonically high levels of endogenous glucose production are a hallmark of type 2 diabetes and it seems reasonable to hypothesize that this might be driven in part by hyperresponsive carotid body (Basu et al., 2004).

A ROLE FOR INSULIN?

In a number of the *in vivo* and human studies mentioned above, hypoglycemia was generated using insulin infusions. Addtionally, in patients with type 2 diabetes insulin levels are generally higher for longer periods of time than in healthy subjects. In this context, insulin also has powerful sympathoexcitatory properties, and there is some evidence that it can stimulate ventilation (Ward et al., 2007). Furthermore, recent studies in rodents have identified insulin receptors on the glomus cells and linked the carotid body to a variety of the pathophysiological consequences of the metabolic syndrome (Ribeiro et al., 2013; Limberg et al., 2014). Together these observations suggest that insulin might stimulate the carotid body independently of changes in glucose. In fact, we have recently argued that periodic fluctuations in insulin in the context of the metabolic syndrome might be the "new" intermittent hypoxia (Limberg et al., 2014).

SUMMARY AND FUTURE DIRECTIONS

Great progress has been made in the last two decades on how glomus cells in the carotid body sense and transduce arterial oxygen levels. Understanding of the broad based phsyiological and pathophysiological repsonses evoked by the carotid body stimulation has also grown dramatically. All this new information has led to exciting new opportunities to investigate how insulin and glucose interact both acutely and chronically with the carotid body during both normoxia and hypoxia including intermittent hypoxia. This area is also ripe for translational research and team science linking the cellular mechanisms and adaptations, interogated *in vitro* with *in vivo* models including studies in humans. At a fundatmental level, understanding how various conditions associated with carotid body stimulation interact with the CO-H_2S pathway in the sensing of hypoxia will be of great interest. From a translational perspective it seems reasonable at this time to ask how much of the pathophysiology of sleep apnea/metabolic syndrome diad is being driven or amplified by the carotid body?

ACKNOWLEDGMENTS

The authors are grateful to Dr. Ying-Jie Peng for his help with the references and the figure. This research was supported by grants from National Institutes of Health, Heart, Lung and Blood Institute PO1-HL-90554 and UH2- HL-123610 (Nanduri R. Prabhakhar) and DK-090541 (Michael J. Joyner).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 20 October 2014; accepted: 20 December 2014; published online: 14 January 2015.

*Citation: Prabhakhar NR and Joyner MJ (2015) Tasting arterial blood: what do the carotid chemoreceptors sense? Front. Physiol. **5**:524. doi: 10.3389/fphys.2014.00524*

This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology.

frontiers in PHYSIOLOGY

REVIEW ARTICLE
published: 15 December 2014
doi: 10.3389/fphys.2014.00489

Neural reflex regulation of systemic inflammation: potential new targets for sepsis therapy

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Sepsis progresses to multiple organ dysfunction due to the uncontrolled release of inflammatory mediators, and a growing body of evidence shows that neural signals play a significant role in modulating the immune response. Thus, similar toall other physiological systems, the immune system is both connected to and regulated by the central nervous system. The efferent arc consists of the activation of the hypothalamic–pituitary–adrenal axis, sympathetic activation, the cholinergic anti-inflammatory reflex, and the local release of physiological neuromodulators. Immunosensory activity is centered on the production of pro-inflammatory cytokines, signals that are conveyed to the brain through different pathways. The activation of peripheral sensory nerves, i.e., vagal paraganglia by the vagus nerve, and carotid body (CB) chemoreceptors by the carotid/sinus nerve are broadly discussed here. Despite cytokine receptor expression in vagal afferent fibers, pro-inflammatory cytokines have no significant effect on vagus nerve activity. Thus, the CB may be the source of immunosensory inputs and incoming neural signals and, in fact, sense inflammatory mediators, playing a protective role during sepsis. Considering that CB stimulation increases sympathetic activity and adrenal glucocorticoids release, the electrical stimulation of arterial chemoreceptors may be suitable therapeutic approach for regulating systemic inflammation.

Keywords: systemic inflammation, sepsis, reflex control of inflammation, carotid body, vagus nerve

INFLAMMATORY RESPONSE AND ITS REGULATION

Inflammation can be defined as a "host defense in response to injury of vascularized tissues" (Majno and Joris, 1996). The inflammatory response –the first alert to the signals that address perturbation– involves an innate system of cellular and humoral responses that, following injury, will support the organism in attempts to restore tissue homeostasis (Chaplin, 2010). The inflammatory response involves a complex network of events involving several organizational levels: at the systemic level, the leakage of substances from the vascular compartment (e.g., water, salt, and proteins); at the cellular level, the activation of endothelial cells and macrophages, leukocyte-endothelium adhesive interactions, and the recruitment of leukocytes; at the subcellular level, the activation and aggregation of platelets, the release of proteases and oxidants from phagocytic cells, and the activation of the complement, clotting and fibrinolytic systems. All the above-mentioned events may assist in addressing a state of injury (Alvarez Perez Gil et al., 2012). During the inflammatory response, the number of inflammatory mediators found in the plasma and secreted by cells is broad and includes extracellular Danger-Associated Molecule Patterns (DAMPs), such as cellular debris, potassium, DNA, cytokines, histones and high-mobility group protein B1 (HMGB1), and Pathogen-Associated Molecular Patterns (PAMPs), such as bacterial lipopolysaccharide (LPS, endotoxin) or peptidoglycan, fungal zymosan or viral single stranded RNA (Deutschman and Tracey, 2014). DAMPs are sensed, leading to the assembly of the inflammasome, and DAMPs and PAMPs catalyze the formation of cell surface signalosomes. Both signalosomes and inflammasomes induce apoptosis, intracellular stress and other metabolic responses, such as the expression of pro-inflammatory cytokines (e.g., interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and HMGB1), cell proliferation and the amplification of the innate immune response (Lamkanfi et al., 2010).

Reversal or resolution of the inflammatory response implies that leukocytes are removed, either via lymphatics or apoptosis, and that the ongoing acute inflammatory response is terminated. As a consequence, during resolution, increased vascular permeability is reversed, and immune cell emigration from the blood compartment ceases (Rock et al., 2010).

Therefore, to maintain immunological homeostasis, inflammation resolution is essential for inhibiting the release and toxicity of potentially damaging inflammatory mediators (Nathan and Ding, 2010). Moreover, regulatory T cells, alternatively activated

macrophages and other cellular mechanisms also suppress excessive immune responses to prevent tissue damage. Numerous anti-inflammatory factors, such as cytokines (e.g., IL-4, IL-10, and transforming growth factor (TGF)-β), protease inhibitors, and antioxidant enzymes, which are present in plasma at very low concentrations, are induced by the immune challenge and are powerful anti-inflammatory factors comprising contain the acute inflammatory response. As a result, these regulatory factors decreased the production of pro-inflammatory mediators and reduce the number of immune cells accumulating in tissues (Buras et al., 2005). Nevertheless, there are several limitations of these humoral and cellular mechanisms: (i) they are slow compared to environmental changes; (ii) they are unable to integrate biological responses to numerous stimulating inputs across a network of immune tissues; and (iii) these mechanisms rely on the circulatory system and thus are anable to respond efficiently in a specific tissue or confined region (Andersson and Tracey, 2012).

An increasing amount of research shows that neural signals play a significant role in modulating the immune response (Glaser and Kiecolt-Glaser, 2005). In fact, both immune-suppression and immune-enhancement can be behaviorally modified in experimental animals (Cohen et al., 1994). Therefore, the central nervous system (CNS) communicates with the immune system and regulates it. Several pathways allow the CNS to regulate the transcription of immune response genes in peripheral tissues (**Figure 1**): (i) the hypothalamic–pituitary–adrenal (HPA) axis and the production of glucocorticoids, which suppress pro-inflammatory immune response genes (Herman et al., 2012); (ii) the sympathetic nervous system (SNS), which innervates primary and secondary lymphoid organs, the vasculature and peripheral organs and tissues, acting through norepinephrine to modify hematopoiesis and the interactions between antigen-presenting cells and lymphocytes (Nance and Sanders, 2007); (iii) SNS-induced epinephrine release by the adrenal gland, which upregulates the transcription of pro-inflammatory cytokines (Cole et al., 2007); (iv) the activation of the cholinergic anti-inflammatory system, whereby parasympathetic (vagal) outflow arrives at the celiac ganglion, and then releases acetylcholine (ACh) through cholinergic fibers from the splenic nerve, attenuating spleen cytokine production (Borovikova et al., 2000; Tracey, 2002; Rosas-Ballina and Tracey, 2009); (v) the local (peripheral tissues) release of physiological neuromodulators such as pain-related neuropeptides and enteric system-regulating neuropeptides, and circulating mediators, such as endogenous opioids, insulin-like growth factor, growth hormone, and other hormones (e.g., prolactin, Freeman et al., 2000) that can affect the innate and adaptive immune system (Irwin and Cole, 2011).

It is clear that in additionto neural reflexes maintaining homeostasis in other body systems, neural circuits also regulate immunity. Therefore, what is(are) the afferent pathway(s) by which the immune system relay signals to the central nervous system?

SYSTEMIC INFLAMMATION AND SEPSIS

Although inflammation is mostly a beneficial host response to foreign challenge or tissue injury, leading to the restoration of tissue structure and function, it can contribute to the pathogenesis of many diseases. Systemic inflammation results from the dysregulation of local inflammation when the host is unable to contain an insult, regardless of whether it is caused by bacteria, trauma, burn, or drug overdose. Sepsis is defined as "the systemic inflammatory response syndrome that occurs during infection" (Bone et al., 1992) and is mainly due to host cell stimulation (monocytes/macrophages, endothelial, and polymorphonuclear cells) to produce and release pro-inflammatory cytokines (Schletter et al., 1995). Sepsis involves the evidence of infection and may be associated with fever, tachycardia, tachypnea, altered white blood cell count, and decreased arterial oxygen partial pressure. Sepsis progression to severe sepsis and septic shock involves many pathological processes including hemodynamic abnormalities (e.g., hypoperfusion or hypotension) and also involves oliguria, lactic acidosis, acute alteration in mental state and multiple organ dysfunction (MOD) syndrome (Riedemann et al., 2003).

However, immunological mechanisms do not completely explain the basis of cellular dysfunction and MOD. Indeed, systemic inflammation affects several systems within the body, including metabolic, hormonal, and neural pathways (Singer et al., 2004; Carre and Singer, 2008). Thus, systemic inflammation initiates the disruption of communication between different organ systems, and subsequently, MOD reflects a progressive uncoupling that may become permanent. With an increasing projected incidence of 1.5% *per annum* in the United States, and an average cost per case of US$22,100 (Angus et al., 2001), sepsis syndromes and MOD are the main cause of death of critical care patients because despite many efforts and significant advances in maintaining therapies (Martin et al., 2003), there is no particularly effective therapy for these conditions (Riedemann et al., 2003). Thus, the knowledge of immunometabolic and neurophysiological mechanisms and the pathophysiology underlying sepsis progression to MOD and death could help to improve current therapies and identify new pharmacological therapeutic targets.

The pro-inflammatory cytokine TNF-α is an important mediator of the lethal effect of endotoxin (Tracey et al., 1986). In fact, reducing the activity or the expression of TNF-α significantly diminishes endotoxin-induced damage, and the degree of tissue damage can be correlated to the amount of TNF-α in serum (Yang et al., 2007). Damage may result in microvascular dysregulation and/or mitochondrial dysfunction (Crouser, 2004), which results in MOD and death. TNF-α is released during the first 30–90 min after exposure to LPS, triggering a second level of inflammatory cascades that involve other cytokines, reactive oxygen species, lipid mediators, and the up-regulation of cell adhesion molecules. Normally, the pro-inflammatory response is counter-balanced by a group of regulatory molecules, such as IL-10 (an anti-inflammatory cytokine), which attempt to restore immunological equilibrium (Scumpia and Moldawer, 2005). In fact, the main stimulus for IL-10 production is inflammation itself. Both TNF-α and IL-1β directly stimulate IL-10 production, suggesting the existence of a negative feedback loop, whereby the production of IL-10 is limited to the inflammatory process (Van Der Poll et al., 1994). Therefore, host damage can result directly by excessive inflammation, or indirectly through immune dysfunction, and host survival depends on the intensity of and the correct balance between pro- and anti-inflammatory responses.

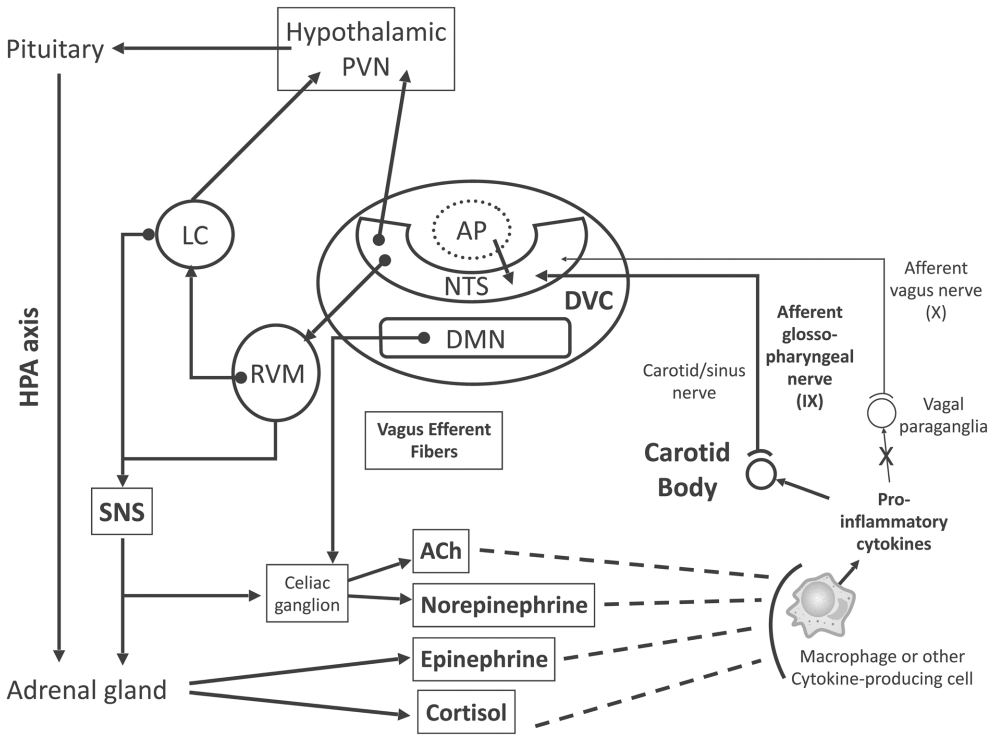

FIGURE 1 | Proposed model for inflammation-induced central nervous system (CNS) activation through carotid body (CB) chemoreceptors and subsequent efferent neuroimmunomodulator activation. Briefly, chemosensory transduction begins in immune cells, which release inflammatory mediators (i.e., TNF-α, IL-1β, and IL-6) to activate the CB. The CB is innervated by glossopharyngeal (cranial nerve IX) afferent neurons, the cell bodies of which are located in the petrosal ganglion, and their central projections end primarily within the dorsal vagal complex (DVC) of the *medulla* oblongata. The DVC consists of the *nucleus tractus solitarii* (NTS), the dorsal motor nucleus of vagus nerve (DMN), and the *area postrema* (AP). The DMN is the main site of origin of preganglionic vagus efferent fibers, which evoke acetylcholine (ACh) release from celiac ganglion neurons, inhibiting the pro-inflammatory response. The AP lacks a brain-blood barrier (dotted line, AP), projects into the NTS and is an important site for humoral immune-to-brain communication. The main portion of the CB chemosensory inputs is received by neurons in the NTS, which coordinate autonomic function and interaction with the endocrine system. Ascending projections from the NTS reach the hypothalamic paraventricular nucleus (PVN), an important structure involved in hypothalamus-pituitary-adrenal (HPA) axis activation, releasing cortisol, which, in fact, inhibits the pro-inflammatory response. Synaptic contacts also exist between neurons in the NTS and rostral ventrolateral *medulla* (RVM), which plays an important role in the control of cardiovascular and respiratory homeostasis. Neurons from the RVM project to the *locus coeruleus* (LC), which innervates higher brain sites, such as the PVN. Neuronal projections emanate from the RVM and LC to sympathetic preganglionic neurons (SNS) in the spinal cord, which innervates both the adrenal *medulla* and celiac ganglion for epinephrine and norepinephrine release, respectively, inhibiting the pro-inflammatory response. These ascending and descending connections provide a neuronal substrate for neuroimmunomodulatory mechanisms. Excitatory pathways, continuous lines; inhibitory pathways, dashed lines.

REFLEX REGULATION OF SYSTEMIC INFLAMMATION: IMMUNE-TO-BRAIN COMMUNICATION

Research into immunosensory activity has been focused on the origin of signaling, i.e., plasma pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6. In fact, direct injection of these cytokines into the brain causes fever, activation of the HPA axis and sickness-like symptoms, mimicking a real immune challenge (Quan, 2014). Immune system-derived signals are conveyed to the CNS through four different pathways. The circumventricular organs (CVOs) were among the first immune-to-brain pathways proposed (Blatteis et al., 1987; Stitt, 1990). These regions have a leaky brain-blood barrier (BBB), and several are situated near the CNS areas that are known to react against peripheral immune challenges, such as the *area postrema* (AP) and the *organum vasculosum*. The former contains neurons that project into the *nucleus tractus solitarii* (NTS) (Cai et al., 1996), a well-known target of neuroimmune activation, and the latter is involved in febrile responses. Another (second) afferent pathway occurs via the saturable transport of cytokines across the BBB (Banks and Erickson, 2010), contributing to an increase in neuroinflammation. A less direct pathway (third) is the binding of cytokines to brain endothelial cells, which evokes the release of paracrine mediators such as IL-1, IL6, and prostaglandins (Fabry et al., 1993; Cao et al., 1998; Quan, 2014). Finally, the fourth pathway occurs through the activation of peripheral sensory nerves, i.e., the vagus nerve (Goehler et al., 1997).

Wan et al. showed that subdiaphragmatic vagotomy blocks brain c-fos induction after the intraperitoneal (IP) administration of LPS (Wan et al., 1994), suggesting that neural, rather than humoral, pathways are capable of transmitting inflammatory signals to the brain. However, the involvement of peripheral sensory nerves in immunomodulation is controversial. Inflammatory

mediators released by immune cells are able to activate both vagal paraganglia (Goehler et al., 1997, 1999) and primary afferent neurons located in sensory ganglia, which, in turn, evokes host defense reflexes. Vagal paraganglia consist of two cell types: type I (glomus) cells and type II (sustentacular) cells (Berthoud et al., 1995). The cell bodies of vagal afferent neurons innervating vagal glomus cells (GC) are located in the nodose ganglion, and the central projections of these neurons arrive primarily at the dorsal vagal complex (DVC) of the *medulla oblongata*.

The DVC, which involves the AP, the dorsal motor nucleus of the vagus (DMN) and the NTS (Berthoud and Neuhuber, 2000), is the main site of origin of preganglionic vagus efferent fibers, whereas cardiovascular vagal efferents originate within the medullar *nucleus ambiguus* (NA). As previously mentioned, the AP is an important CVO and a putative site for humoral immune-to-brain communication. Neurons in the NTS receive the main portion of vagal sensory inputs. In turn, the NTS coordinates autonomic function and interaction with the endocrine system, and its ascending projections reach the hypothalamic paraventricular nucleus (PVN), an important structure involved in the HPA axis activation. Similarly, the NTS projects to the rostral ventrolateral medulla (RVM), which has an important function in the control of cardiovascular and respiratory homeostasis. Neurons from the RVM project to the *locus coeruleus* (LC), which innervates higher brain sites, such as the hypothalamus and PVN. Neuronal projections from the RVM and LC reach sympathetic preganglionic neurons in the spinal cord (Fernandez and Acuna-Castillo, 2012). Conversely, the PVN projects back to the RVM and NTS (Pavlov et al., 2003), completing the necessary connections for interaction between the HPA axis and autonomic (sympathetic and parasympathetic) cardiorespiratory reflex activation. Furthermore, these interactions provide a neuronal substrate for an immunomodulatory mechanism (**Figure 1**).

IMMUNOSENSORY SIGNALING THROUGH THE VAGUS NERVE

As previously mentioned, subdiaphragmatic vagotomy blocks brain c-fos induction after IP administration of LPS (Wan et al., 1994). Moreover, if the procedure is performed prior to the IP injection of IL-1β, the cytokine fails to evoke a hyperthermic response (Watkins et al., 1995). Thus, abdominal LPS or IL-1β activates sensory neurons in the vagus nerve, which, in turn, activates hyperthermia-related brainstem nuclei (Quan, 2014). In contrast, food-motivated behavior is suppressed by peripheral LPS or IL-1β; this depression is also blocked by vagotomy (Bret-Dibat et al., 1995).

Vagal primary afferent fibers are stimulated by IP administration of LPS (Goehler et al., 1999, 2000), and these fibers activate the CNS neurons responsible for the manifestations of systemic inflammatory response syndrome (Mascarucci et al., 1998; Borsody and Weiss, 2005). Bilateral subdiaphragmatic vagotomy prevents sickness manifestations and activation of the NTS, hypothalamus and LC in rats treated IP with LPS or IL-1β (Bluthe et al., 1994; Bret-Dibat et al., 1995; Gaykema et al., 1995; Hansen and Krueger, 1997; Borsody and Weiss, 2005).

TNF-α-induced vagal immunosensory activity increases (Emch et al., 2000) or decreases (Emch et al., 2002) vagal motor activity. The fever and hyperalgesia caused by IP LPS are suppressed by the abdominal transection of vagal trunks, though this has a little effect on the febrile response to intravenous (IV) or intramuscular LPS. As subdiaphragmatic vagotomy blocks the induction of c-Fos protein in the rat hypothalamus and brainstem following IP injection of LPS, visceral afferent innervations are involved in the response to LPS. Conversely, vagotomy has a minimal effect on c-Fos protein induced by the IV administration of LPS (Wan et al., 1994). Moreover, LPS induces c-Fos activation of NTS neurons, which persists after cervical bilateral vagotomy (Hermann et al., 2001). Nonetheless, these blockages of CNS c-Fos activation are controversial. Neurons from the abdominal region or vagus efferent fibers (perhaps those within celiac branches, which transport LPS from the peritoneal cavity to the blood) may mediate the response to LPS *per se*. Therefore, vagotomy may eliminate responsive neurons, or it may restrict the amount of LPS escaping into systemic circulation, diminishing the systemic responses to LPS (e.g., c-Fos protein induction in the CNS) (Lenczowski et al., 1997; Romanovsky et al., 2000).

Finally, IL-1β and TNF-α did not modify the frequency of discharge recorded from single fibers in a preparation of isolated superfused rat GC from vagal paraganglia (Mac Grory et al., 2010), despite the expression of cytokine receptor in vagal afferent fibers (Goehler et al., 1997). Furthermore, neither the basal nor hypoxia-induced discharge rate of vagal paraganglia are modulated by IL-1β, TNF-α or LPS, suggesting that these structures are not the afferent limb of an "immune reflex" (O'Connor et al., 2012) (**Figure 1**). Thus, the neural signals from immune chemosensory inputs should originate from other receptors, and the neural pathway of peripheral arterial chemoreceptors (i.e., the carotid body and its sensory ganglion) provides an interesting candidate.

THE STIMULATION OF EFFERENT VAGUS NERVE IN SEPSIS THERAPY

The role of the vagus nerve and its stimulation has been studied in systemic inflammation. Vagus nerve stimulation has an anti-inflammatory effect in endotoxemia and downregulates proinflammatory cytokine production in sepsis, decreasing the plasma levels of HMGB1 and improving survival in cecal ligation and puncture (CLP), a model of septic peritonitis. Unilateral cervical (Van Westerloo et al., 2005) or subdiaphragmatic vagotomy (Kessler et al., 2006) increases the plasma levels of pro-inflammatory cytokines, tissue damage and mortality in sepsis. Additionally, in septic rats, vagus nerve electrical stimulation attenuates and prevents hypotension (Song et al., 2008) and modulates coagulation activation and fibrinolysis (Van Westerloo et al., 2006), decreasing MOD. The therapeutic potential of vagal (cholinergic) efferent fibers to treat disorders characterized by cytokine dysregulation is reviewed elsewhere (Rosas-Ballina and Tracey, 2009; Tracey, 2009).

IMMUNOSENSORY SIGNALING THROUGH CAROTID BODY CHEMORECEPTORS

Anatomically, the carotid body (CB) is the largest paraganglion in the body (Mascorro and Yates, 1980), and its sensory innervation occurs through the carotid/sinus nerve, the nerve endings of

which establish abundant synapses with specialized GC (Verna, 1997). The cell bodies of sensory pseudo-monopolar neurons innervating the CB are mainly located in the petrosal ganglion (Kalia and Davies, 1978; Berger, 1980). Afferent carotid/sinus nerve fibers establish the first synapsis in the NTS, at the CNS, in the same way as vagal afferents (**Figure 1**) (Donoghue et al., 1984; Finley and Katz, 1992).

Due the rich vascularization and abundant chemosensory innervations, we recently proposed that the CB is a peripheral sensor for the presence of immunogenic agents in the blood. Although the canonical LPS receptor, Toll-like receptor (TLR)-4 (Fernandez et al., 2011), and TNF-α receptors are functional (Fernandez et al., 2008, 2011), TNF-α does not modify the chemosensory discharge recorded under normoxic conditions from the carotid nerves of *in vitro* perfused and superfused cat CB. Nevertheless, TNF-α reduces in a dose-dependent manner the hypoxia-induced enhanced frequency of chemosensory discharge (Fernandez et al., 2008) but enhances the $[Ca^{2+}]_i$ response to acute hypoxia of dissociated GC. The increase is significantly larger in cells from the CB of rats exposed to chronic hypoxia or chronic intermittent hypoxia (Lam et al., 2008, 2012). Finally, TNF-α receptor expression in human and mouse carotid bodies was observed using microarray analysis, though the technique did not detect TNF-α (Mkrtchian et al., 2012).

Glomus (type I) cells from rat CB express both IL-1 receptor type I (Wang et al., 2002) and IL-6 receptor α (Wang et al., 2006). *In vitro*-cultured GC respond to IL-1β with depolarization and a transient rise in $[Ca^{2+}]_i$. Furthermore, IL-1β significantly increases carotid/sinus nerve chemosensory discharge in anesthetized rats (Shu et al., 2007), though the extracellular administration of IL-6 induces a rise in $[Ca^{2+}]_i$ and catecholamine release from *in vitro*-cultured GC (Fan et al., 2009). In addition, the IP administration of IL-1β in rat GC up-regulates both IL-1 receptor type I and tyrosine hydroxylase (Zhang et al., 2007).

We have reported a significant and maintained increase in basal chemosensory discharge after IV infusion of LPS in cats (Fernandez et al., 2008). Additionally, LPS increases CB TNF-α expression in rats (Fernandez et al., 2011), though we have not assessed *in situ* TNF-α administration. Neither IL-1β nor IL-6 expression in the CB during sepsis has been reported, but systemic pro-inflammatory cytokines could reach the CB because of their extensive vascularization (Verna, 1979). Thus, increased basal CB chemosensory activity could be due to either IL-1β or IL-6 stimulation. IL-1β appears to mimic the responses of the CB to hypoxia (i.e., evokes GC $[Ca^{2+}]_i$ oscillations and induces the expression of hypoxia-inducible factor (HIF), a transcription factor essential for the maintenance of normal CB activity during hypoxia) and may, therefore, act in an autocrine manner to enhance the peripheral chemoreceptor drive during systemic inflammation.

In septic cats, CB sensitivity to both stimulant (hypoxia and nicotine) and depressant (hyperoxia) stimuli is decreased (Fernandez et al., 2008). *In vitro* experiments have shown that TNF-α reduces the hypoxia-induced enhanced frequency of chemosensory discharge in a dose-dependent manner. Thus, TNF-α modulates CB chemosensory activity, perhaps by inducing the GC to release an inhibitory transmitter, such as dopamine. This fact has not yet been tested.

Microarray analyses of human and mouse CBs have shown increased expression of many other genes involved in immune and inflammatory responses. In addition to the above-mentioned pro-inflammatory cytokines, the transcripts of nuclear factor (NF)-κB, IL-10R (but not IL-10), and HMGB-1 have also been found in human and mouse CBs (Mkrtchian et al., 2012). In addition, the IP administration of LPS in rats decreased the cytosolic fraction of IκBα in the CB, evoking subsequent NF-κB p65 translocation into the GC nucleus, which resulted in gene expression, i.e., TNF-α up-regulation (Fernandez et al., 2011).

The expression of pro-inflammatory cytokines and their receptors in the CB suggests that those cytokines may activate chemosensory neurons, even in the absence of sepsis syndrome, exerting a tonic control of cardiorespiratory, endocrine, autonomic, and/or immune functions. Consequently, pro-inflammatory cytokines, through GC membrane receptors, may modify chemosensory activity reaching the NTS, modulating specific components of the systemic inflammatory response (**Figure 1**).

Pentobarbitone-anesthetized cats treated IV with LPS showed tachypnea, tachycardia, and hypotension, symptoms comparable to patients with severe sepsis and septic shock. Of note, bilateral section of the carotid and aortic nerves prevented increased respiratory rates (Fernandez et al., 2008). Additionally, LPS enhances tonic CB chemosensory activity (i.e., the frequency of chemosensory discharges), whereas LPS reduces CB responsiveness to both transient excitatory (hypoxia and nicotine) and depressant ($F_iO_2 = 100\%$) stimuli (Fernandez et al., 2008). The reduced ventilatory responses to moderate and severe hypoxia observed in cats are similar to those observed in rats and in unanesthetized newborn piglets subjected to *E. coli* endotoxin infusion (McDeigan et al., 2003). This reduction in ventilatory responses is mediated –at least in part– by the inhibitory effect of endothelial nitric oxide (NO) on respiratory control mechanisms (Ladino et al., 2007).

Hyperoxia, which, in fact, reduces CB chemosensory activity (Fernandez et al., 2003), is associated with higher plasma levels of IL-6, IL-10 and TNF-α, a greater number of infected biological samples, and mortality in CLP-induced septic rats (Rodríguez-González et al., 2014). Consequently, the withdrawal of carotid chemo/baro-sensory function modifies the inflammatory response during sepsis syndromes through a network of neural, humoral and cytokine elements.

The activation of DVC neurons did not require intact vagal pathways, suggesting that peripherally generated TNF-α could act either directly on these neurons –because DVC displays the attributes of CVOs– (Hermann et al., 2001) or, more likely, through another neural afferent pathway. Bilateral vagotomy does affect c-Fos expression in the NTS (Hermann et al., 2001). However, we found that bilateral carotid/sinus neurotomy after IP administration of LPS suppresses both the LPS-induced increase in the number of c-Fos-positive neurons of the NTS –with no significant changes in AP c-Fos immunoreactivity– and the increased levels of plasma cortisol (Reyes et al., 2012). Accordingly, we suggest that the neural signals provided by peripheral receptors that are distinct from vagal paraganglia – such as arterial carotid chemoreceptors, the function of which is

intact after bilateral cervical vagotomy– produce prominent CNS manifestations of endotoxemia. These findings are particularly interesting because the CB induces –at least in part– an endocrine response to LPS by acting as an intermediate in the activation of the NTS by pro-inflammatory cytokines.

CAROTID BODY STIMULATION AS A TARGET FOR SEPSIS THERAPY: SYMPATHETIC ACTIVATION AND GLUCOCORTICOIDS RELEASE

The analysis of heart rate variability (HRV) gives a clear idea about the autonomic (sympathetic/parasympathetic) regulation of cardiorespiratory function. Decreased HRV is consistent with the pathogenesis of MOD; in fact, endotoxemic patients show decreased HRV (Godin et al., 1996; Rassias et al., 2005). Moreover, septic patients have an impaired sympatho-vagal balance that is characterized by a sustained sympatho-excitation accompanying hypotension (Barnaby et al., 2002), and chemo- and baro-denervation accelerates the drop in blood pressure (Vayssettes-Courchay et al., 2005). Finally, decreased parasympathetic activity is an excellent predictor of risk of death in patients with sepsis (Chen et al., 2008). Altogether, these data suggest that reflex arcs involved in maintaining the autonomic balance are altered during sepsis.

Carotid body stimulation provokes a wide array of cardiopulmonary and autonomic reflexes as well as endocrine responses (e.g., plasma release of catecholamines and cortisol) (Fitzgerald, 2014). In particular, chemoreflexes are important modulators of sympathetic activation (Abboud and Thames, 1983), and peripheral chemoreceptor activation elicits respiratory and cardiovascular effects and a sympatho-excitatory response (Alanis et al., 1968; Montarolo et al., 1976). Thus, tonic activation of carotid chemoreceptors during sepsis may also contribute to high levels of sympathetic activity (Kara et al., 2003). However, the administration of 100% O_2 decreases the heart rate, blood pressure and central sympathetic outflow (Kara et al., 2003), and hyperoxia-induced CB chemosensory activity withdrawal is associated with higher plasma levels of pro-inflammatory cytokines and mortality in septic rats (Rodríguez-González et al., 2014).

In addition, in anesthetized, paralyzed, ventilated and maintained normocapnic mongrel dogs, hypoxic hypoxia (the natural stimulus of CB chemoreceptors) increases the adrenal cortisol secretion rate, and surgical CB and/or aortic body deafferentation attenuates cortisol response (Raff et al., 1982). Thus, the CB exerts the main chemoreceptor influence on cortisol secretion during hypoxia. Interestingly, as mentioned above, bilateral carotid/sinus neurotomy attenuated the LPS-induced cortisol response in septic rats (Reyes et al., 2012).

Consequently, as a therapeutic target, the electrical stimulation of CB chemoreceptors could modify the inflammatory response during sepsis syndromes through a network consisting of neural (sympathetic activation), humoral (glucocorticoid secretion) and, as a consequence, cytokine elements.

CONCLUSIONS

The knowledge of immunometabolic and neurophysiological mechanisms and the pathophysiology of sepsis progression to produce organ dysfunction and death have helped in the improvement of current therapies and in identifying new pharmacological therapeutic targets.

Traditionally, the autonomic nervous system coordinates the fine-tuning of the cardiorespiratory relationship, maintaining appropriate metabolite and oxygen delivery to tissues. Several reflex arcs, such as arterial baroreflexes, central chemoreflexes, peripheral arterial chemoreflexes, and pulmonary stretch reflexes, maintain the autonomic (sympathetic-parasympathetic) equilibrium. Consequently, the interactions among those reflexes are clinically interesting because the pathophysiological over-reaction of a single reflex, which occurs in several disorders, may cause the suppression of the opposite reflex responses.

An increasing body of evidence obtained by us and other researchers shows that CB reflexes not only serves as a chemoreceptor for respiratory reflex responses but also as a sensor for immune status (Zapata et al., 2011) and as a modulator of autonomic balance, tending to coordinate the cardiorespiratory interplay devoted to maintaining oxygen homeostasis in different pathologies.

In summary, CB stimulation increases sympathetic activity and glucocorticoid release. Thus, increased basal CB chemosensory activity during sepsis could be responsible, at least in part, for the observed increase in plasma epinephrine and cortisol levels in septic patients. The resulting increased plasma anti-inflammatory mediators could modulate pro-inflammatory cytokine expression in cytokine-producing cells, thereby modifying systemic inflammation and sepsis resolution. The electrical stimulation of the carotid/sinus nerve is a potential therapeutic approach, though not yet assessed, for sepsis therapy.

ACKNOWLEDGMENTS

This work was supported by research grants from Fondo Nacional de Desarrollo Científico y Tecnológico—FONDECYT 1120976 (Ricardo Fernandez), 1110734 (Claudio Acuña-Castillo), and 3140414 (Gino Nardocci); and UNAB DI-354-13/R (Ricardo Fernandez). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 September 2014; accepted: 27 November 2014; published online: 15 December 2014.

Citation: Fernandez R, Nardocci G, Navarro C, Reyes EP, Acuña-Castillo C and Cortes PP (2014) Neural reflex regulation of systemic inflammation: potential new targets for sepsis therapy. Front. Physiol. ***5****:489. doi: 10.3389/fphys.2014.00489*

This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology.

frontiers in PHYSIOLOGY

MINI REVIEW ARTICLE
published: 04 December 2014
doi: 10.3389/fphys.2014.00474

Petrosal ganglion: a more complex role than originally imagined

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The petrosal ganglion (PG) is a peripheral sensory ganglion, composed of pseudomonopolar sensory neurons that innervate the posterior third of the tongue and the carotid sinus and body. According to their electrical properties PG neurons can be ascribed to one of two categories: (i) neurons with action potentials presenting an inflection (hump) on its repolarizing phase and (ii) neurons with fast and brisk action potentials. Although there is some correlation between the electrophysiological properties and the sensory modality of the neurons in some species, no general pattern can be easily recognized. On the other hand, petrosal neurons projecting to the carotid body are activated by several transmitters, with acetylcholine and ATP being the most conspicuous in most species. Petrosal neurons are completely surrounded by a multi-cellular sheet of glial (satellite) cells that prevents the formation of chemical or electrical synapses between neurons. Thus, PG neurons are regarded as mere wires that communicate the periphery (i.e., carotid body) and the central nervous system. However, it has been shown that in other sensory ganglia satellite glial cells and their neighboring neurons can interact, partly by the release of chemical neuro-glio transmitters. This intercellular communication can potentially modulate the excitatory status of sensory neurons and thus the afferent discharge. In this mini review, we will briefly summarize the general properties of PG neurons and the current knowledge about the glial-neuron communication in sensory neurons and how this phenomenon could be important in the chemical sensory processing generated in the carotid body.

Keywords: petrosal ganglia, sensory modality, chemosensory, mechanosensory, action potential

INTRODUCTION

The petrosal ganglion (PG) contains the soma of pseudomonopolar sensory neurons (Ramón y Cajal, 1909) that project to the posterior third of the tongue and the carotid sinus (CS) and body (CB) (Stensaas and Fidone, 1977). Although morphologically similar they constitute a heterogeneous population with regard to their sensory modality: both mechano–and chemosensory neurons can be recognized projecting to the periphery. Although there is no complete characterization of the population of neurons that project to the tongue, the neurons projecting to the carotid bifurcation appear to be segregated in terms of their electrophysiological (Belmonte and Gallego, 1983; Cummins et al., 2002; Varas et al., 2003) and morphological (Katz et al., 1983; Katz and Black, 1986; Kummer and Habeck, 1992) characteristics, the receptors that are expressed in their soma and the neurotransmitter that activate them (González et al., 1994; Iturriaga and Alcayaga, 2004; Nurse and Piskuric, 2013). As mentioned, PG neurons express receptors in their plasma membrane and respond to exogenous neurotransmitters application, characteristics that are present in other sensory ganglia in which there is intra-ganglionic information processing. Thus, we propose that PG have all the necessary components for intra-ganglionic information processing, and this may represent a future line of study in the carotid body-cardiorespiratory control.

ELECTROPHYSIOLOGICAL PROPERTIES OF PG NEURONS

Intracellular recordings show two major populations of neurons according to their action potential (AP) waveform: neurons with APs presenting an inflection (hump) on its repolarizing phase (**Figure 1A**) and neurons with fast and brisk APs (**Figure 1B**) (Belmonte and Gallego, 1983; Morales et al., 1987; Varas et al., 2003). Whole cell recordings from cultured PG neurons of the rat nodose-petrosal-jugular complex (NPJc) indicate that all neurons present Na^+ inward currents, although in 50% of these cells are tetrodotoxin (TTX)-resistant (Stea and Nurse, 1992). Ca^{2+} inward currents are also present in all neurons, mostly L-type. Outward K^+ currents are comprised by both the delayed rectifier and Ca^{2+}-dependent K^+ currents, the latter representing about 20% of the total outward current (Stea and Nurse, 1992). Most neurons (76%) respond with a single AP to long depolarizing pulses while the remaining ones responds with two or more APs to the same stimuli (Stea and Nurse, 1992). Similarly, intracellular recordings from cat PG neurons indicate that neurons with humped APs present both TTX-sensitive and insensitive

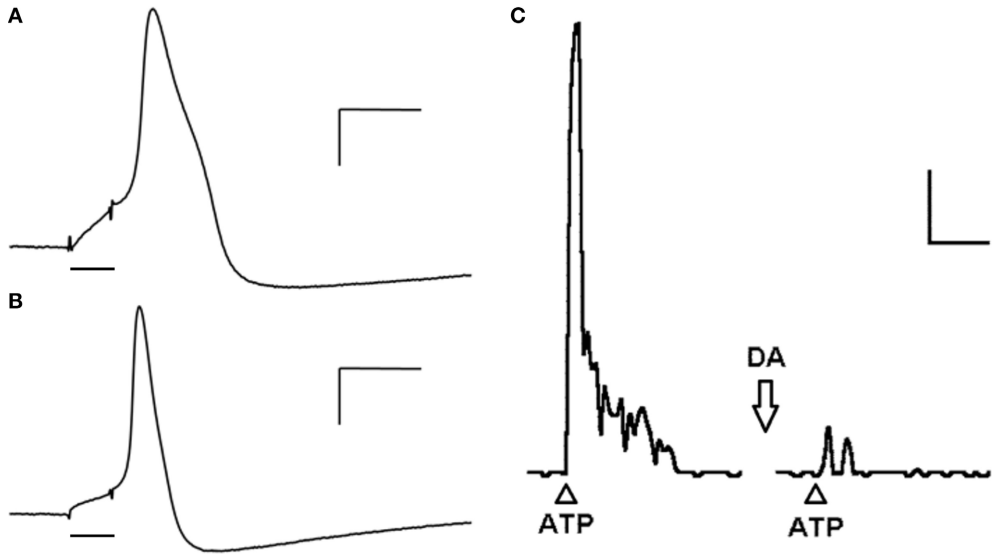

FIGURE 1 | Cat petrosal ganglion (PG) neurons in culture action potentials (APs), evoked by depolarizing pulses (bar), present an inflection (hump) in the repolarizing phase and long lasting hyperpolarization (A) or a smooth repolarizing phase and short hyperpolarization (B). Calibration: 10 mV, 5 ms. **(C)** A single dose of ATP (500 μg; arrowhead) applied to the PG increases transiently the frequency of discharge recorded from the carotid nerve. When the same dose of ATP is applied (arrowhead) after 30 s of a dose of dopamine (arrow; 1 mg) the response is significantly decreased. Calibration: 20 Hz, 15 s. Figure modified from Alcayaga et al. (2003a,b).

components (Gallego, 1983; Iturriaga et al., 2007), and that the depolarizing phase of the AP has an important Ca^{2+} component (Gallego, 1983). Reduction or blockade of Ca^{2+} currents reduces the AP amplitude and also the duration and amplitude of the AP hyperpolarization, suggesting the involvement of Ca^{2+}-dependent K^+ currents in the latter response (Gallego, 1983). On the other hand, the spikes of neurons with brisk APs are completely blocked by TTX (Belmonte and Gallego, 1983; Iturriaga et al., 2007). Because many of these recordings were performed on cultured or isolated PG neurons, there is no information on the sensory modality or peripheral target of the recorded neurons.

The central axotomy has no significant effect on PG neurons that present a hump in the AP, irrespective of their peripheral projection. However, peripheral axotomy decreases the conduction velocity, after hyperpolarization amplitude but increases AP duration without modifying resting membrane potential (Vm) or input resistance (Rin) (Belmonte et al., 1988). After axotomy spiking characteristics remained unchanged in neurons projecting through the glossopharyngeal branch (GPB), but the tonically discharging neurons projecting through the CSN increased by a factor of four (Gallego et al., 1987; Belmonte et al., 1988). Thus, electrical properties appear to be differentially modified according to the peripheral targets.

PETROSAL GANGLION NEURONS PROJECTING THROUGH THE CAROTID SINUS NERVE

ELECTROPHYSIOLOGICAL PROPERTIES

Cat PG neurons projecting through the CSN with myelinated axons can also be categorized according to their AP waveform. Thus, sensory neurons connected to the CB present humped APs with longer hyperpolarizations and phasic responses (Belmonte and Gallego, 1983; Varas et al., 2003). Conversely, barosensory neurons projecting to the CS present brisk APs with shorter hyperpolarizations and tonic discharges to long lasting depolarizations (Belmonte and Gallego, 1983; Varas et al., 2003), although humped APs have also been recorded in barosensory neurons (Belmonte and Gallego, 1983). In the cat, neurons with unmyelinated axons (C-fiber) have similar characteristics to myelinated ones (Varas et al., 2003), although tonic responses have also been reported in few of them (Belmonte and Gallego, 1983). Thus, cat PG neurons projecting through the CSN can be set apart by their AP waveform and spiking characteristics, which are highly correlated with the sensory modality they convey to the central nervous system (Varas et al., 2003). On the other hand, mouse and rat chemosensory PG neurons have unmyelinated axons (Donnelly, 1999; Donnelly and Rigual, 2000), with rat neurons presenting humped APs and tonic discharges (Donnelly, 1999).

Patch clamp recordings of rat isolated chemosensory neurons indicate that they present both transient and persistent TTX-sensitive Na^+ currents. Conversely, non-chemosensory PG neurons persistent Na^+ current is TTX-insensitive (Cummins et al., 2002). Molecular biology determinations indicate that both TTX-resistant ($Na_V1.8$, $Na_V1.9$) and TTX-sensitive ($Na_V1.1$, $Na_V1.6$, $Na_V1.7$) Na^+ channel isoforms are expressed in the rat PG (Cummins et al., 2002). Rat chemosensory neurons express several K^+ channels that underlie delayed rectifier currents ($K_V1.2$, $K_V1.5$, $K_V1.6$, $K_V2.1$), fast transient and inactivating currents ($K_V1.4$, $K_V4.3$), M-currents (KCNQ2, KCNQ3, KCNQ5), Ca^{2+}-dependent (KCa1.1) currents (Andrews and Kunze, 2001; Buniel et al., 2008), and also, hyperpolarization-activated cyclic nucleotide-gated (HCN2, HCN4) channels (Buniel et al., 2008).

However, a large variation in channel expression occurs within the chemosensory neurons reflected in different whole cell currents (Andrews and Kunze, 2001). Thus, although PG neurons appear as a homogeneous population, chemosensory neurons present subtle differences that could have different physiological and regulatory meaning.

Mechanosensory neurons appear to comprise a population of large, fast conducting neurons that present fast APs with short hyperpolarizations, generated by a TTX sensitive Na^+-current and a TEA-sensitive K^+-current, respectively. On the other hand, chemosensory neurons present APs of longer duration and long lasting hyperpolarization, resulting from the presence of Ca^{2+}-currents and a Ca^{2+}-dependent K^+-current, respectively, as well as other K^+-currents.

RESPONSES TO NEUROTRANSMITTERS

Many transmitter molecules have been indicated to participate in the generation and/or modulation of carotid chemosensory activity (González et al., 1994; Iturriaga and Alcayaga, 2004; Nurse and Piskuric, 2013). However, the postsynaptic effects have been slowly unraveling in the last decades (Iturriaga and Alcayaga, 2004; Nurse and Piskuric, 2013).

Cat and rabbit PG neurons projecting through the CSN increase their AP discharge frequency in response to acetylcholine (ACh), effect blocked by nicotinic ACh receptor (nAChR) antagonists (Alcayaga et al., 1998; Soto et al., 2010). In the cat, the activation of ACh muscarinic receptors (mAChRs) appears to be devoid of effects (Alcayaga et al., 1998) but in rabbits antagonizing mAChRs increased the magnitude of the ACh-induced responses, suggesting an inhibitory action for these receptors (Soto et al., 2010). Most (96%) identified cat chemosensory PG neurons depolarized and generated APs when ACh is applied to the soma while none of the barosensory ones responded (Varas et al., 2003), although activation of barosensory afferents by ACh and nicotine has been reported (Diamond, 1955). Nevertheless, activation of afferents with ACh and nicotine was obtained in the pressurized carotid bifurcation and vascular effects cannot be ruled out.

In cultured PG neurons ACh and nicotine induces depolarization (Zhong and Nurse, 1997; Varas et al., 2000) which is blocked by nAChR antagonists (Zhong and Nurse, 1997; Shirahata et al., 2000; Varas et al., 2006; Alcayaga et al., 2007). Agonist and antagonist sensibility indicate the presence of both α7 and α4β4 (Shirahata et al., 2000; Varas et al., 2006) or α4β2 nAChRs (Shirahata et al., 2000), in concordance with immunohistochemical evidence (Shirahata et al., 1998, 2000).

In a reconstituted system, containing rat NPJc neurons and CB cells, the basal neuronal activity as well as hypoxia induced increases in neuronal activity are partially blocked by nAChRs antagonists (Zhong et al., 1997; Nurse and Zhang, 1999, 2001; Zhang et al., 2000; Zhang and Nurse, 2004) and increased by an acetylcholinesterase inhibitor (Nurse and Zhang, 1999, 2001). These evidences indicate that in this preparation the basal and hypoxia–and hypercapnia-induced activity in NPJc neurons can be partly blocked by nAChRs antagonists.

The rabbit and cat PG neurons projecting through the CSN increase their spiking activity in response to ATP in a dose dependent manner (Alcayaga et al., 2000; Soto et al., 2010). These responses are blocked by P2X but not by P2Y antagonism (Alcayaga et al., 2000). Most (93%) identified chemosensory cat PG neurons respond to application of ATP to the perikarya with depolarization and spike trains while only 40% of barosensory neurons responded (Varas et al., 2003).

Whole cell recordings of cultured cat PG neurons show that ATP induces a dose dependent depolarization that increased the discharge frequency and a sustained inward current at a holding potential near the resting Vm (−60 mV) (Alcayaga et al., 2007). These responses are mimicked by α,β-methylene ATP and blocked by suramin, suggesting the involvement of P2X2,3 receptors in the generation of these responses (Alcayaga et al., 2007). In an *in vitro* preparation containing NPJc neurons and CB cells obtained from Sprague-Dawley rats, that respond as a reconstituted arterial chemoreceptor (Zhong et al., 1997), hypoxia- and hypercapnia-induced responses recorded from PG neurons are partially blocked by reduced extracellular Ca^{2+}/Mg^{2+} ratio and suramin, a nucleotide receptor blocker. These data indicate a synaptic, ATP-mediated activation of PG neurons (Zhang et al., 2000; Nurse and Zhang, 2001; Prasad et al., 2001; Zhang and Nurse, 2004). The presence of P2X2 and P2X3 subunits has been demonstrated in single cell RT-PCR of responding neurons in this preparation and by confocal immunofluorescence of rat NPJc neurons and neuronal terminals in the CB (Prasad et al., 2001). Moreover, mice lacking P2X2 and P2X3 subunits present reduced chemosensory afferent discharges and ventilatory responses (Rong et al., 2003). Nevertheless, the presence of P2X1 subunits has been reported in PG homogenates and other P2X subunits (i.e., P2X5, P2X7) may participate in ATP-induced intracellular Ca^{2+} increases in PG neurons (Nunes et al., 2012).

Recordings of identified cat chemosensory neurons (Varas et al., 2003) and neurons in a reconstituted system (Zhang et al., 2000; Nurse and Zhang, 2001) show that the responses induced by chemoreceptor stimuli are partially block by either nAChRs or nucleotide receptors blockade, and completely abolished by the joint administration of the blockers (Zhang et al., 2000; Nurse and Zhang, 2001; Varas et al., 2003). These results show that at least in these preparations chemosensory afferent activity depends almost exclusively on the synaptic communication mediated by ACh and ATP. Nevertheless, the afferent activity recorded from the CSN *in situ* and CB superfused *in vitro* indicates that responses induced by ACh and ATP are blocked by their respective blockers while ventilatory (Reyes et al., 2007b) and afferent activity remain largely unaffected (Reyes et al., 2007a,b) or only partially inhibited (Fitzgerald et al., 1997).

Dopamine is another neurotransmitter involved in the CB-petrosal neuron communication. The presence of D1- and D2-dopamine receptor mRNA in rat and cat PG (Schamel and Verna, 1993; Gauda et al., 1994; Bairam et al., 1998) and the enzyme tyrosine hydroxylase (TH) (Katz et al., 1983) have been demonstrated. The content of TH and the number of neurons expressing TH is increased after neuronal depolarization with KCl (40 mM) or veratridine (neurotoxin abolishing inactivation of

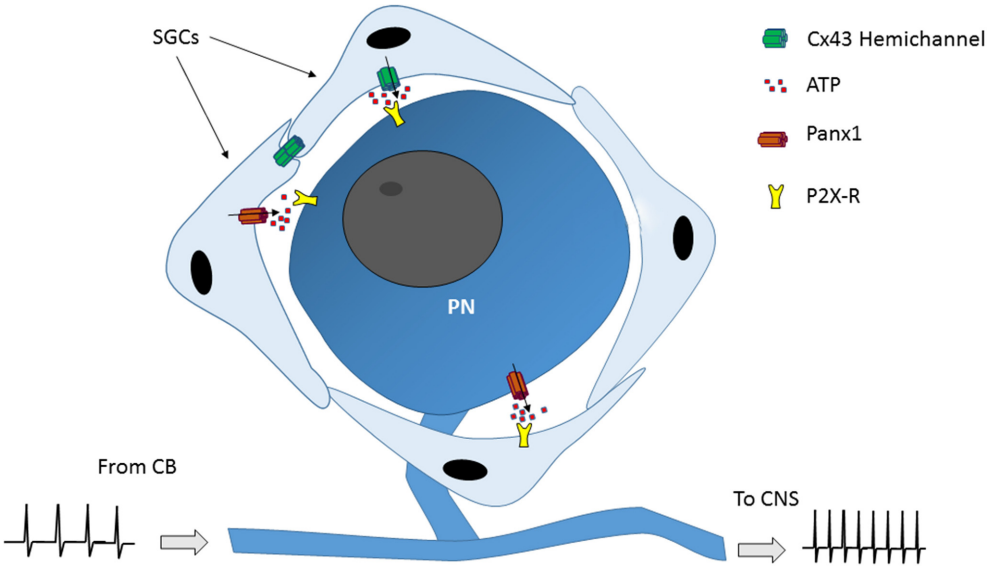

FIGURE 2 | Model of how satellite glial cells (SGCs) could modulate the petrosal neuronal activity. Connexin43 (Cx43) hemichannels and/or pannexin 1 (Panx1) channels present in SGCs when open allows the release of neurotransmitters to the extracellular space. Then, the neurotransmitters (i.e., ATP) activate receptors (i.e., P2X-R) in petrosal ganglion neurons (PN), which in turn induce their depolarization, which in turn may modulate the information from the carotid body (CB) to the CNS.

Na^+ channels) (Hertzberg et al., 1995), suggesting that the activity of petrosal neurons is a modulator of the TH expression in this neurons. Moreover, about 25% of petrosal neurons release dopamine in response to depolarizing signals *in vitro* (Iturriaga et al., 2003). Dopamine is released by CB cells in response to hypoxia (Hellström et al., 1976; Iturriaga et al., 1996, 2009; Iturriaga and Alcayaga, 1998) where it inhibits afferent activity (Zapata, 1975). However, when dopamine is applied in repetitive doses in short intervals the response becomes biphasic, observing an initial inhibition followed by an activation of the afferent discharge (Zapata, 1975). Similarly, low doses ($<10\,\mu g$) of dopamine applied directly to the cat PG *in vitro* enhance the responses to ACh (Alcayaga et al., 1999), while higher doses ($>200\,\mu g$) inhibit the responses to both ACh and ATP (Alcayaga et al., 1999, 2003a) (**Figure 2**). The inhibition induced by dopamine is blocked by spiroperone (Alcayaga et al., 1999), a D2 antagonist. Recently, it has been reported that sensory neurons projecting through the vagus nerve increase their activity in response to a media without Ca^{2+} and Mg^{2+}, increase that is blocked by dopamine (Retamal et al., 2014). All these data support the notion that dopamine is an inhibitory neurotransmitter for neurons in the PG and more specifically in those projecting to the CB. However, DA application to the rabbit PG produces a dose dependent increase in the neuronal discharge (Iturriaga et al., 2009), suggesting that the actions of a determined transmitter may be species specific.

The aforementioned data indicate that PG neurons that project to the CB are excited, at least, by ACh and ATP and those responses can also be modulated by, dopamine. However, the lack of complete elimination of responses to hypoxia *in situ* indicate that other transmitter molecules may also be involved in the generation of the afferent activity.

HAVE THE PETROSAL GANGLIA ALL THE NECESSARY ELEMENTS FOR INFORMATION PROCESSING?

As mentioned before, PG neurons express several different types of receptors -including ionotropic and metabotropic- in their somas. Rabbit's but not rat's petrosal neurons change their response to neurotransmitters as a consequence of normobaric chronic hypoxia (Iturriaga and Alcayaga, 2007; Icekson et al., 2013). However, in both preparations the CB chemosensory response to hypoxia was enhanced (Barnard et al., 1985; Iturriaga, 2013), suggesting that changes in the PG induced by changes in the oxygen level are not completely dependent on the activity of the CB. On the other hand, partial or complete elimination of the PG afferences have been used to treat some pathologies.

An increasing body of evidence shows that there is information processing into sensory ganglia related to the appearance and/or maintenance of chronic pain. Thus, satellite glial cells (SGCs) of trigeminal and dorsal root ganglion are activated in response to neuronal damage and/or to inflammatory process. This activation -correlated with an increased sensory neurons activity- (Blum et al., 2014; Song et al., 2014; Warwick et al., 2014) was importantly reduced by the intraganglionar application of connexin (Cx)-channel blockers (Dublin and Hanani, 2007; Huang et al., 2010; Hanani, 2012). It has been postulated that the cross-talk between neurons and SGCs is mediated by ATP release (through an undetermined pathway) from those cells (Suadicani et al., 2010). Cx- hemichannels and pannexin (Panx)-channels have been reported to be involved in autocrine and paracrine communication in several systems, including the central nervous system (Sáez et al., 2003; Orellana et al., 2013). Petrosal neurons and their surrounding SGCs express at least Cx43 (Chen et al., 1985; Retamal et al., 2014) and may also express Panx1, which was found in the whole NPJc (Retamal et al., 2014).

Thus, if this type of paracrine communication is present in petrosal ganglia, the opening of hemichannels in petrosal neurons and/or in SGCs could lead to an increase in the petrosal neurons activity (**Figure 2**), which in turn could induce for example diseases related to cardiovascular disorders. Thus, for example, CSN denervation partially corrected the sympathetic and respiratory variables in rabbits with experimental congestive heart failure (Marcus et al., 2014).

FUTURE DIRECTIONS

Since there are no works showing information processing in the PG, it is necessary to perform experiments in order to explore the possibility that SGCs and petrosal neurons communicate. This could be due to the release of neuro- and/or glio-transmitters through Cx hemichannels or Panx channels, as suggested by Retamal et al. (2014). Therefore, research on the communication of petrosal neurons and SGCs may answer questions concerning the oxygen or arterial pressure information processing such as; is there any effect of Cx or Panx channel blockers in the oxygen or arterial pressure information processing? Do Cx or Panx knockout animals present differences in that processing? Are SGCs important for the maintenance of normal neuronal activity? Since the expression of receptors and channels in petrosal neurons is modified by hypoxia and others stimulus, it could be interesting to assay if those modifications also modify the information from the CB to the CNS. As discussed, PG have all the necessary elements for information processing, hence it can modulate the information conveyed from the CB to the CNS, thus future study could focus in this putative modulation.

ACKNOWLEDGMENTS

This work was funded by grants FONDECYT #1120214 (Mauricio A. Retamal), 1090157 (Julio Alcayaga) and 1130177 (Julio Alcayaga), and Anillo #ACT1104 (Mauricio A. Retamal).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 03 October 2014; accepted: 17 November 2014; published online: 04 December 2014.
*Citation: Retamal MA, Reyes EP and Alcayaga J (2014) Petrosal ganglion: a more complex role than originally imagined. Front. Physiol. **5**:474. doi: 10.3389/fphys.2014.00474*

This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 02 December 2014
doi: 10.3389/fphys.2014.00468

Enhanced carotid body chemosensory activity and the cardiovascular alterations induced by intermittent hypoxia

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The carotid body (CB) plays a main role in the maintenance of the oxygen homeostasis. The hypoxic stimulation of the CB increases the chemosensory discharge, which in turn elicits reflex sympathetic, cardiovascular, and ventilatory adjustments. An exacerbate carotid chemosensory activity has been associated with human sympathetic-mediated diseases such as hypertension, insulin resistance, heart failure, and obstructive sleep apnea (OSA). Indeed, the CB chemosensory discharge becomes tonically hypereactive in experimental models of OSA and heart failure. Chronic intermittent hypoxia (CIH), a main feature of OSA, enhances CB chemosensory baseline discharges in normoxia and in response to hypoxia, inducing sympathetic overactivity and hypertension. Oxidative stress, increased levels of ET-1, Angiotensin II and pro-inflammatory cytokines, along with a reduced production of NO in the CB, have been associated with the enhanced carotid chemosensory activity. In this review, we will discuss new evidence supporting a main role for the CB chemoreceptor in the autonomic and cardiorespiratory alterations induced by intermittent hypoxia, as well as the molecular mechanisms involved in the CB chemosensory potentiation.

Keywords: autonomic dysfunction, carotid body, intermittent hypoxia, hypertension, oxidative stress

INTRODUCTION

The carotid body (CB) located in the bifurcation of the carotid arteries is the main peripheral chemoreceptor sensing arterial levels of PO_2, PCO_2, and pH. Also, changes in blood flow, temperature, osmolarity and glucose are able to elicit CB chemosensory excitation (Gonzalez et al., 1994; Pardal and López-Barneo, 2002; Iturriaga and Alcayaga, 2004; Iturriaga et al., 2007). The CB consists of clusters of chemoreceptor (glomus or type I) cells organized around the capillary network, synaptically connected to the nerve terminals of sensory neurons whose somata are in the petrosal ganglion, and surrounded by sustentacular glial (type II) cells. The most accepted model for chemoreception proposes that hypoxia closes K^+ channels, leading to glomus cell depolarization, entry of Ca^{2+} and the release of excitatory transmitters (ACh and ATP), which in turn increases the discharge in the nerve endings of the chemosensory neurons (Iturriaga and Alcayaga, 2004; Iturriaga et al., 2007). In the last years, new exciting evidences have shown that the CB plays a crucial role in the pathogenesis of several human sympathetic-mediated diseases, including obstructive sleep apnea (OSA), congestive heart failure, resistant hypertension and insulin resistance (Koyama et al., 2000; Prabhakar et al., 2005; Schultz et al., 2007; Iturriaga et al., 2009; Abdala et al., 2012; Del Rio et al., 2013; Paton et al., 2013; Porzionato et al., 2013; Ribeiro et al., 2013). Accordingly, targeting the CB in several pathological conditions has been proposed to be a future promising therapeutic tool for the treatment of sympathetic-mediated diseases. Indeed, the selective ablation of the CB markedly improve rat survival in experimental heart failure (Del Rio et al., 2013), prevent the development of insulin resistance and hypertension in rats fed with high sucrose diet (Ribeiro et al., 2013) and reduced high blood pressure in neurogenic and resistant hypertension (McBryde et al., 2013; Paton et al., 2013).

OBSTRUCTIVE SLEEP APNEA IS AN INDEPENDENT RISK FACTOR FOR SYSTEMIC HYPERTENSION

The OSA syndrome elicited by repeated upper airways occlusion, is usually associated with daytime sleepiness, fatigue, and deficits in attention and executive function (Beebe and Gozal, 2002; Idiaquez et al., 2014). Furthermore, OSA is recognized as an independent risk factor for systemic hypertension (~50% of OSA patients develop diurnal hypertension, Somers et al., 2008; Calhoun, 2010), and is associated with stroke, pulmonary hypertension, coronary artery disease and atrial fibrillation (Fletcher, 2000; Parati et al., 2007; Somers et al., 2008; Dempsey et al., 2010). Indeed, several epidemiological studies have shown that OSA is an independent risk factor for the progression of the hypertension, showing a positive relationship between the apnea/hypopnea index (AHI) and high blood pressures (Young et al., 1993; Peppard et al., 2000; Eckert and Malhotra, 2008; Marin et al., 2012). Moreover, results obtained from the Wisconsin Sleep Cohort (an ongoing 21-years longitudinal study performed on 1500 Wisconsin state employees) showed that untreated OSA patients have a high mortality risk associated with AHI (Nieto et al., 2000; Young et al., 2008). According to the "Recommendations for the management of

patients with obstructive sleep apnoea and hypertension" recently published by the European Union Cooperation in Scientific and Technological Research Action B26 on OSA, with the endorsement of the European Respiratory Society and the European Society of Hypertension (Parati et al., 2013) OSA is defined as "The combination of at least five obstructive breathing episodes per hour during sleep (apnoea, hypopnoea and respiratory effort related arousal events) and the following diagnostic criteria (A and/or B to be fulfilled). A: Excessive daytime sleepiness that is not better explained by other factors. B: Two or more of the following symptoms that are not better explained by other factors: Choking or gasping during sleep, recurrent awakenings from sleep, unrefreshing sleep, daytime fatigue and impaired concentration." According to this study, the AHI defines the severity of OSA: mild OSA: AHI 5–15 events/h; moderate OSA: AHI 15–30 events/h and severe OSA: AHI > 30 events/h (Parati et al., 2013).

PATHOPHYSIOLOGICAL MECHANISMS OF OSA-INDUCED HYPERTENSION

The cyclic obstruction of the upper airways during OSA leads to intermittent hypoxia and hypercapnia, negative intrathoraxic pressure, sleep fragmentation, and micro-arousals (Somers et al., 2008; Dempsey et al., 2010). During the airway occlusion, the resulting hypoxia and hypercapnia stimulates the CB chemoreceptor eliciting reflex acute sympathetic, hypertensive and hyperventilatory responses (Gozal and Kheirandish-Gozal, 2008; Somers et al., 2008; Garvey et al., 2009; Dempsey et al., 2010). Among these disturbances, the chronic intermittent hypoxia (CIH) is considered the main factor for the development of diurnal hypertension (Lavie, 2003; Gozal and Kheirandish-Gozal, 2008; Lévy et al., 2008; Somers et al., 2008; Arnardottir et al., 2009; Dempsey et al., 2010). Although the link between OSA and hypertension is well proved, the mechanisms underlying the pathogenesis of the hypertension are not entirely known. The most accepted proposal states that CIH elicits systemic oxidative stress, inflammation, and sympathetic hyperactivity, which led to endothelial dysfunction and the hypertension (Lavie, 2003; Somers et al., 2008; Garvey et al., 2009; Ryan et al., 2009; Dempsey et al., 2010). Nevertheless, conclusions from studies performed in OSA patients are controversial, because OSA patients often present concomitant morbidities (i.e., obesity and metabolic alterations), which are confounding factors that increase the cardiovascular risk. Thus, animal model of CIH, which simulates the hypoxic-reoxygenation episodes and reproduce several cardiovascular pathologic features of OSA including sympathetic hyperactivity and hypertension, are the gold-standard model to study mechanisms involved in OSA (Fletcher et al., 1992; Peng et al., 2003, 2011; Iturriaga et al., 2005, 2009; Prabhakar et al., 2005; Schulz et al., 2008; Dematteis et al., 2009; Del Rio et al., 2010, 2011a, 2012; Dumitrascu et al., 2013).

OSA produces sympathetic hyperactivity, demonstrated by an increased muscle sympathetic neural activity to blood vessels (Carlson et al., 1993) and excessive accumulation of urinary catecholamines (Dimsdale et al., 1995). Similarly, animals exposed to CIH show enhanced sympathetic responses to hypoxia, and develop systemic hypertension (Fletcher et al., 1992; Greenberg et al., 1999; Dick et al., 2007; Feng et al., 2008; Huang et al., 2009; Zoccal et al., 2009; Del Rio et al., 2010; Marcus et al., 2010). The autonomic dysfunction is characterized by enhanced sympathetic outflow, a reduction of the efficiency of the cardiac baroreflex sensitivity and alterations of heart rate variability (HRV). Indeed, non-invasive spectral analysis of HRV shows an increased ratio of low (LF) to high frequency (HF) band power, with a relative predominance of the LF band and a reduced contribution of the HF band, suggesting preponderance of the sympathetic drive in patients with OSA (Narkiewicz et al., 1998a; Shiomi et al., 1996) and animals exposed to CIH (Lai et al., 2006; Rey et al., 2008; Del Rio et al., 2010). Furthermore, it has been shown that CIH elicits vagal withdrawal, attributed in part to neuronal loss in ambiguous nucleus (Yan et al., 2008). Therefore, it is likely that the enhanced sympathetic to parasympathetic balance along with the reduction of the baroreflex could contribute to impair HRV and the regulation of vasomotor tone of blood vessels finally eliciting systemic hypertension.

In addition, OSA syndrome is also associated with endothelial dysfunction and vascular remodeling (Ip et al., 2004; Patt et al., 2010). OSA patients show an increased intima-media thickness (Minoguchi et al., 2005; Monneret et al., 2012) and a reduced nitric oxide-mediated vasodilatation (Kato et al., 2000). Similarly, some studies found that CIH reduced acetylcholine (ACh)-mediated vasodilation in rats (Tahawi et al., 2001; Dopp et al., 2011), but other reported a normal endothelial function in hypertensive CIH-treated rats (Julien et al., 2003; Lefebvre et al., 2006). Indeed, Lefebvre et al. (2006) found that CIH had no effect on the ACh-mediated vasodilatation of carotid, aortic and mesenteric beds, as well as on the contractile responses induced by noradrenaline and angiotensin II (Ang II) in arteries from CIH-rats compared to the arteries from control rats. However, they found that the contraction induced by endothelin-1 (ET-1) was higher in arteries from CIH-rats. More recently, Philippi et al. (2010) studied the time-course of the alteration of the endothelium dependent vasodilation in rats exposed to CIH. They found that CIH produces functional and structural changes in skeletal muscle arteries within the first 2 weeks of CIH, and those alterations were accompanied by systemic oxidative stress. Friedman et al. (2014) found that ROS generation during CIH activates NFATc3, which in turn increase the vascular response to ET-1. The administration of Tempol, a superoxide dismutase (SOD) mimetic, during CIH prevents the increased NFATc3 activity in the arteries from CIH-exposed mice, supporting that ROS is an important upstream signal in the CIH-induced NFATc3. Together, the available information suggest that vascular beds are affected by exposure to CIH, and that enhanced contractile responsiveness to vasoactive molecules such as ET-1 is critically dependent on ROS formation.

INTERMITTENT HYPOXIA ENHANCES CB CHEMOSENSORY DISCHARGES IN NORMOXIA AND HYPOXIA

Patients recently diagnosed with OSA, present potentiated pressor and ventilatory responses to hypoxia (Narkiewicz et al., 1998a,b, 1999), suggesting that the peripheral hypoxic chemoreflex were enhanced by CIH. Fletcher et al. (1992) were the first to obtain evidences that the CB is involved in the hypertension induced by CIH. They found that the bilateral CB

denervation prevented the development of hypertension in rats exposed to CIH for 35 days. Despite this seminal observation, the proposal that the CB contributes to the progression of the cardiovascular pathologies associated to OSA was not seriously considered. However, in the last decade a growing body of new evidences have support the proposal that the CB contributes to the progression of the CIH-induced hypertension (See for reviews: Prabhakar et al., 2005; Smith and Pacchia, 2007; Weiss et al., 2007; Somers et al., 2008; Garvey et al., 2009; Iturriaga et al., 2009; Dempsey et al., 2010). Recordings of rat and cat CB chemosensory discharges *in situ* and *in vitro* have demonstrate that CIH selectively increases basal chemosensory discharges in normoxia, and potentiates chemosensory and ventilatory responses to acute hypoxia (Peng et al., 2003, 2004; Rey et al., 2004, 2006; Prabhakar et al., 2005; Iturriaga et al., 2009; Del Rio et al., 2010, 2012). In addition, CIH induces plasticity of the CB chemosensory activity manifested as long-term facilitation. Indeed, Peng et al. (2003) found that chemosensory baseline discharges increased when the CB was excited by repetitive acute intermittent hypoxia in rats exposed to CIH. They reported that following 10 episodes of 12% O_2 lasting for 15 s, interspersed with 5 min of 95% O_2, the baseline chemosensory discharge increased with each episode of hypoxia, which persist for 60 min following the end of the hypoxic stimulus.

The mechanisms underlying the enhanced CB chemosensory reactivity to hypoxia induced by CIH are not entirely known (Iturriaga et al., 2009). Oxidative stress (Peng et al., 2003, 2009; Del Rio et al., 2010, 2012; Marcus et al., 2010), ET-1 (Rey et al., 2006, 2007; Pawar et al., 2009), Ang II (Lam et al., 2008, 2012; Fung, 2014), and pro-inflammatory cytokines (Iturriaga et al., 2009; Del Rio et al., 2011a, 2012; Lam et al., 2012) have been associated with the CB chemosensory potentiation. However, the primary molecular target responsible for the increased chemoreceptor discharge remains unknown. Recently, we studied the effects evoked by CIH on TASK K^+ channel activity and the depolarization induced by acute hypoxia in CB glomus cells from adult rats exposed to CIH (Ortiz et al., 2013). We measured membrane potential, single channel and macroscopic currents in the presence of TEA and 4-aminopyridine in CB chemoreceptor cells isolated from adult rats exposed to CIH for 7 days. CIH treatment did not change the resting membrane potential, but the hypoxic-evoked depolarization increased by 2-fold. Moreover, the hypoxic inhibition of the open probability of the TASK-K^+ channel was larger and faster in glomus cells from CIH-treated rats. This novel effect of CIH may contribute to explain the potentiation of CB oxygen chemoreception.

MOLECULAR MECHANISMS UNDERLYING ENHANCED CAROTID BODY CHEMOSENSORY ACTIVITY DURING INTERMITTENT HYPOXIA

OXIDATIVE STRESS CONTRIBUTES TO ENHANCE THE CAROTID CHEMOSENSORY ACTIVITY DURING INTERMITTENT HYPOXIA

ROS and reactive nitrogen species (RNS) have been proposed as mediators of the cardiovascular alterations in OSA patients (Christou et al., 2003; Lavie, 2003; Gozal and Kheirandish-Gozal, 2008; Jelic et al., 2008; Lévy et al., 2008) and animal exposed to CIH (Peng et al., 2003, 2009, 2011; Chen et al., 2005; Troncoso Brindeiro et al., 2007; Huang et al., 2009; Del Rio et al., 2010, 2012). Studies performed in OSA patients and animals exposed to CIH have shown that hypoxia-reoxygenation produces systemic oxidative stress due to the accumulation of ROS and RNS. Peng et al. (2003) proposed that superoxide radical participates in the potentiation of the rat CB chemosensory responses to hypoxia induced by CIH. They found that pre-treatment of rats for 10 days before and concomitant with the exposure to CIH with manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP), a SOD mimetic, prevents the CB chemosensory potentiation. In addition, they found that CIH decreases the activity of the aconitase enzyme in the CB and the activity of the complex I of the mitochondrial electron transport chain, suggesting that the mitochondria function is affected by CIH and represent a potential source of ROS production (Peng et al., 2003). In addition, Peng et al. (2009) found that acute hypoxia produced a larger increase in NOX activity in CBs from rats exposed to CIH for 10 days compared to the NOX activity found in control CBs, suggesting that NADPH oxidase contributes to generate ROS during CIH. Recently, Schulz et al. (2014) have shown that NADPH oxidase 2 (NOX2) knockout blocks the development of the hypertension induced by CIH. Indeed, they found that mice showed significant arterial blood pressure elevations after CIH. The hypertension was attenuated by 1 inhibition of NOX by apocynin, whereas NOX2 was not upregulated in the heart, aorta, and femoral and carotid arteries of CIH-mice. Therefore, they suggested that the CIH-induced arterial hypertension is mediated by ROS derived from an activation of NOX2 within cells located outside the cardiovascular system.

We studied the role of nitro-oxidative stress on the enhanced CB chemosensory function and hypertension in rats exposed to CIH for 21 days (Del Rio et al., 2010). We measured 3-nitrotyrosine (3-NT) formation in the CB as an index of oxidative stress. Superoxide reacts with NO to generate peroxynitrite, a powerful oxidizing agent that nitrates protein tyrosine-residues forming 3-NT. We found that CIH increased plasma lipid peroxidation and the formation of 3-NT in the CB. In addition, CIH enhanced the CB chemosensory and ventilatory responses to acute hypoxia, alters HRV and elicits hypertension. Concomitant administration of ascorbic acid reduced the increased systemic and local CB nitro-oxidative stress, the potentiation of CB chemosensory and ventilatory responses to hypoxia, as well as the hypertension in rats exposed to CIH (Del Rio et al., 2010). These results agree and extend previous observations that antioxidant treatment prevented the CB chemosensory potentiation (Peng et al., 2003) and the hypertension (Troncoso Brindeiro et al., 2007) in rats exposed to CIH.

The available evidence indicates that oxidative stress is involved in the CIH-induced CB potentiation, but it is matter of debate whether ROS are the primary signal, because ROS *per se* do not increase the CB chemosensory discharges. Indeed, H_2O_2 does not increase the carotid chemosensory discharge in rats (Peng et al., 2009) or cats CB (Osanai et al., 1997). In addition, modification of ROS production in rat glomus cells did not alter the catecholamine secretion, suggesting a lack of a causal link between ROS and glomus cells excitability (Gonzalez et al., 2007).

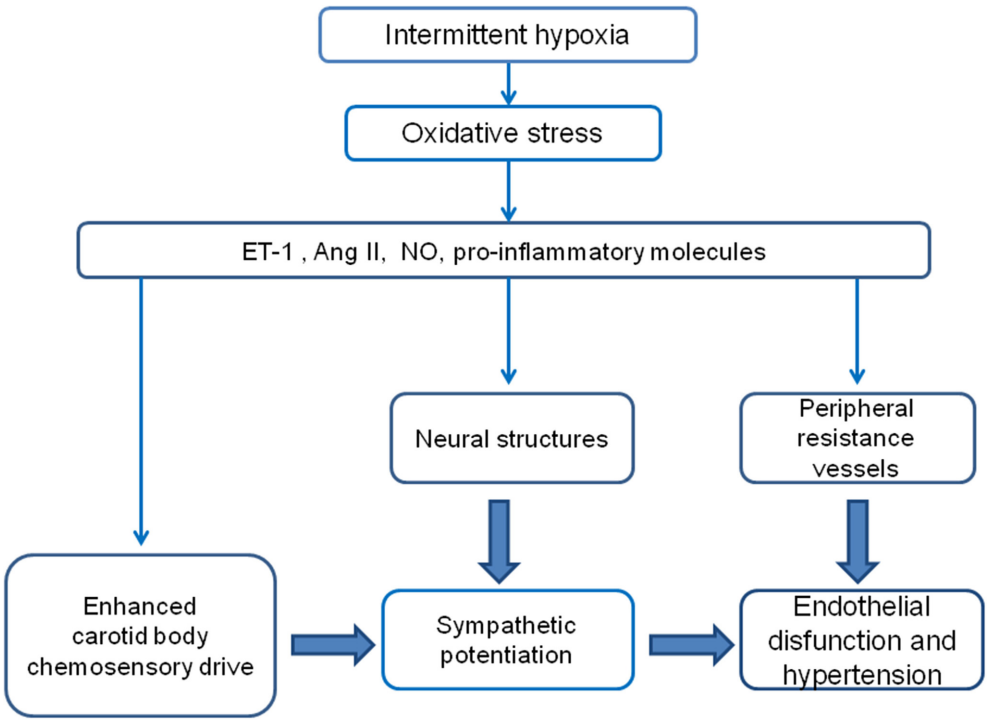

FIGURE 1 | Diagram of the proposed hypothetical mechanisms involved in the potentiation of the CB chemosensory response to hypoxia and the development of hypertension induced by CIH. It is likely that the hypoxic-reoxygenation cycles enhance the CB chemosensitivity to hypoxia, which in turn contributes to elicit a persistent augmented sympathetic neural drive.

Thus, it is possible that other molecules activated by the oxidative stress mediate the enhancing effects of CIH on CB oxygen chemoreception (See **Figure 1** and **Table 1**).

Table 1 | Possible mediator of the CIH effects on CB chemosensory potentiation.

Mediator	References
Endothelin 1	Rey et al., 2006, 2007; Iturriaga, 2013
Endothelin-1 (dependent on ROS)	Pawar et al., 2009; Peng et al., 2013
Reduced NO production (reduced nNOS and eNOS-ir levels).	Marcus et al., 2010; Del Rio et al., 2011a; Moya et al., 2012
Angiotensin II (dependent on O_2- production signaling through AT1 receptor)	Lam et al., 2008, 2012; Marcus et al., 2010; Peng et al., 2011; Fung, 2014
Pro-inflammatory cytokines	Iturriaga et al., 2009; Del Rio et al., 2011b, 2012; Lam et al., 2012

ENDOTHELIN-1

We and other have proposed that ET-1 is involved in the potentiation of the CB chemosensory discharge induced by CIH (Rey et al., 2006, 2007; Pawar et al., 2009; Iturriaga, 2013; Peng et al., 2013) and in the development of hypertension (Troncoso Brindeiro et al., 2007; Allahdadi et al., 2008). Rey et al. (2006) found that CIH increased 10-times the ET-1 immunoreactivity in endothelial, smooth muscle and glomus cells from CBs from cats exposed to CIH for 4 days, without changes in ET-1 plasma concentration. ET-1 elicits chemosensory excitation in both *in situ* and *in vitro* perfused cat CB preparation, but not in the superfused CB preparation, showing a predominant vascular effect. The CIH-induced potentiation of baseline discharges and hypoxic chemosensory responses in the perfused cat CB preparation was reduced by the unspecific ET-1 receptor blocker bosentan (Rey et al., 2006). These results suggest that a local increase of ET-1 in the CB may contribute to enhance the CB chemosensory tone induced by CIH, through a predominant vasomotor mechanism. Pawar et al. (2009) found that CIH enhanced the basal release of ET-1 and produces upregulation of the ET-A receptor, while the administration of MnTMPyP, which prevent the oxidative stress, reduced the increased release of ET-1 and the enhanced CB chemosensory responses to hypoxia. In the same way, the concurrent treatment with the ET-A receptor inhibitor BQ-123 prevented the development of the hypertension in rats exposed to CIH for 14 days (Allahdadi et al., 2008). Thus, ET-1 seems to be involved in the enhanced hypoxic CB chemosensory responses and in the progression of the hypertension following CIH. More recently, Peng et al. (2013) found that CIH increased the activity of the endothelin converting enzyme (ECE), which paralleled the raise of the ET-1 level in the neonatal rat CB. Since MnTMPyP prevented these effects, they proposed that oxidative stress was involved in the increased ET-1 expression. In addition, they found that hypoxia facilitates ET-1 release from CIH-treated CB, but not from control rat CB. These results support that a ROS-dependent release of ET-1, which activates the ET-A receptor is involved in the potentiation of the CB chemosensory responses

to hypoxia elicited by CIH in neonatal rats. However, it is worth to note that Del Rio et al. (2011a) and Lam et al. (2006) found that CIH transiently increases the levels of ET-1 in the adult rat CB during the first week of CIH, but later ET-1 levels returned to the control levels, suggesting that ET-1 may contribute to the enhanced CB responsiveness to hypoxia in the early phase of CIH.

NITRIC OXIDE

We studied the changes in the expression of eNOS in the CB, along with the progression of potentiated CB chemosensory responses to hypoxia in rats exposed to CIH for 7 to 21 days (Del Rio et al., 2011a). Exposure to CIH for 7 days enhanced CB chemosensory responses to hypoxia and produced a significant decrease in the eNOS immunoreactivity in the CB, which persisted for 21 days of CIH, suggesting that CIH may decrease the NO levels in the CB. Thus, we measured NO production—via nitrite generation in the incubation medium—from rat CBs exposed to CIH, and found a reduction in the NO production after 7 days of CIH that correlates with the reduced eNOS expression (Del Rio et al., 2011a; Moya et al., 2012). Since NO is an inhibitory modulator of CB chemosensory discharges, we hypothesized that a reduced NO level may contribute to enhance the basal CB discharges and the chemosensory responses to hypoxia (Moya et al., 2012). This interpretation is supported by the finding of Marcus et al. (2010), showing that CIH decreased the expression of the nNOS in the rat CB, suggesting that the removal of the normal inhibitory NO influence contributes to enhancing the CB chemosensory responses to hypoxia. We found a marked increase of 3-NT in the CB from rats exposed to CIH, which correlates with the enhanced chemosensory responses to hypoxia (Del Rio et al., 2011a), supporting the idea that oxidative-nitrosative stress plays a critical role in CB chemosensory potentiation induced by CIH (Iturriaga et al., 2009; Del Rio et al., 2010). Thus, the available data suggests that peroxynitrite formation due to the reaction of NO with the superoxide radical is a critical step in the CB chemosensory potentiation induced by CIH (Del Rio et al., 2010, 2011a).

ANGIOTENSIN II

The role of Angiotensin II on the enhanced CB chemosensory responses induced by CIH has been extensively reviewed by Fung (2014). The CB constitutively expresses the renin-angiotensin system (RAS), and responds to Ang II due to the functional AT-1 receptor expression in the CB glomus cells (Fung et al., 2001). Lam et al. (2014) found that CIH increased the expression of angiotensinogen and AT1 receptor in the rat CB glomus cells. They also found that the elevation of intracellular Ca^{2+} in response to exogenous Ang II was enhanced in glomus cells from CIH-rats. The pretreatment with losartan abolished the Ang II-induced Ca^{2+} response, suggesting an involvement of AT1 receptors, and attenuated the levels of gp91 (phox) and macrophage infiltration in the CB. Thus, the unregulated RAS expression may play a role in the enhanced CB chemosensory activity and local inflammation via AT1 receptor activation during CIH.

PRO-INFLAMMATORY CYTOKINES

Among the molecules up regulated in the CB by CIH, such as ET-1, Ang II, VEGF and iNOS (Rey et al., 2006, 2007; Lam et al., 2008, 2012, 2014; Del Rio et al., 2010, 2011a,b), pro-inflammatory cytokines have been proposed as mediators of the CB chemosensory potentiation induced by CIH (Lam et al., 2008; Iturriaga et al., 2009; Del Rio et al., 2011a, 2012) and cardiovascular pathologies in OSA patients (Vgontzas et al., 2004; Minoguchi et al., 2005; Biltagi et al., 2008; Ryan et al., 2009). Accordingly, we studied the time-course of the changes in the immunohistological levels of TNF-α, IL-1β, and IL-6 in the CB, along with the progression of the enhanced CB chemosensory responses to hypoxia in rats exposed to CIH for 7 to 21 days (Del Rio et al., 2011a). We found that CIH progressively increases the levels of TNF-α and IL-1β in the rat CB without modifying their plasma levels. On the contrary, Lam et al. (2012) reported that exposure of rats to intermittent hypoxia for 7 days increases the levels of IL-1β, TNF-α, and IL-6 in the CB, and found macrophage infiltration, which was reduced by daily treatment with the anti-inflammatory drugs dexamethasone or ibuprofen. Oxidative stress increases the synthesis of pro-inflammatory cytokines, mediated by the activation of the transcriptional factors NF-κB, activator protein 1 and HIF-1α (Prabhakar and Semenza, 2012). In response to oxidative stress, it is known that HIF-1α produced the translocation of NF-κB to the nucleus augmenting the expression of pro-inflammatory genes such as IL-1β, TNF-α, and ET-1 (Reuter et al., 2010). Accordingly, we found that CBs from rats exposed to CIH for 21 days showed higher levels of the p65 sub-unit of NF-κB suggesting a plausible role for this factor in the upregulation of the pro-inflammatory cytokines during CIH (Del Rio et al., 2012). We tested the hypothesis that CIH induced a ROS-dependent increased TNF-α and IL-1β levels in the CB, which may contribute to the CB chemosensory potentiation (Del Rio et al., 2012). Accordingly, we studied the effects of ibuprofen on TNF-α and IL-1β levels in the rat CB, the potentiation of the CB chemosensory and ventilatory hypoxic responses and the development of systemic hypertension (Del Rio et al., 2012). Ibuprofen prevented the overexpression of the cytokines, the enhanced hypoxic ventilatory response and the hypertension, but failed to block the enhanced CB chemosensory responses. Thus, our studies suggest that the upregulation of TNF-α and IL-1β in the CB induced by CIH is linked to oxidative stress, as well as the enhanced CB chemosensory responsiveness to hypoxia, but the chemosensory potentiation does not depend on the increased TNF-α and IL-1β levels in the CB. However, pro-inflammatory cytokines contribute to enhance the hypoxic ventilatory response and the hypertension induced by CIH, suggesting that multiple mechanisms may participate in the cardiorespiratory alterations induced by CIH.

CONTRIBUTION OF CENTRAL CARDIORESPIRATORY CENTERS AND ARTERIAL VESSELS TO THE HYPERTENSION INDUCED BY CIH

The sympathetic hyperactivity induced by CIH is likely to be the result of the enhanced CB chemosensory drive, but we cannot preclude excitatory effects of CIH on other structures of the chemorefelex pathway. Indeed, the same molecules that are

involved in the enhanced CB chemosensitivity (e.g., Ang II, ET-1, and NO) could act at multiple sites to contribute to CIH-induced arterial blood pressure rise (e.g., higher CNS centers, peripheral arteries vessels). The chemosensory petrosal neurons that innervate the CB glomus cells project to the NTS in the brainstem, which is the main integrative nucleus for visceral inputs. The NTS send projections to the RVLM that contain the pre-sympathetic neurons projecting to the pre-ganglionar neurons in the spinal cord. RVLM neurons participate in the control of BP, and in the CB-mediated activation of the sympathetic responses (Guyenet et al., 2010). It has been shown that CIH increased the expression of the neuronal activation markers c-Fos, and FosB/ΔFos in the NTS and RVLM. Indeed, Greenberg et al. (1999) found that CIH-exposure of rats for 30 days increased c-fos labeling in the NTS and the RVLM. More recently, several studies reported that CIH increases FosB/ΔFosB in the subfornical organ, the median preoptic nucleus, the paraventricular nucleus, the NTS and the RVLM (Knight et al., 2011; Cunningham et al., 2012; Bathina et al., 2013). Thus, other structures outside the brainstem might contribute to intermittent hypoxia-induced hypertension (e.g., paraventricular nucleus of the hypothalamus, as shown by Sharpe et al., 2013). The available evidences strongly suggests that oxidative stress is the key mediator of the enhanced CB chemosensory responses to hypoxia and the hypertension induced by CIH, but the actions of the oxidative stress on the BP regulation in rats exposed to CIH may occur in multiple sites of the chemoreflex pathway, including the NTS, RVLM, and/or the arterial blood vessels. Indeed, it has been proposed that superoxide anions in the brainstem contribute to elevate the arterial blood pressure in rat models of neurogenic hypertension such as the stroke-prone spontaneously hypertensive rat (Kishi et al., 2004) and Ang II induced hypertension (Chan and Chan, 2012). Although it is well known that oxidative stress, produced by Ang II and NADPH activation, in the brainstem elicits sympathetic activation, the role played by the oxidative stress induced by CIH in the progression of the hypertension is less known. In addition, Marcus et al. (2012) found that CIH impairs the vasodilatory responses in small arteries isolated from the skeletal muscle circulation in rats, an effect blocked by losartan, a Ang II type 1 receptor blocker. Intermittent hypoxia also caused an increase in the ratio of Ang II type 1 receptors (responsible for vasoconstriction and trophic effects) to Ang II type 2 receptors (responsible for vasodilation and anti-trophic properties) in peripheral arteries. On the other hand, oxidative stress has also been involved in the impaired vasodilatation in response to ACh in rats exposed to CIH. Indeed, the treatment of CIH-exposed rats with Tempol restores the normal vascular function (Phillips et al., 2006). Moreover, Dopp et al. (2011) reported that concomitant treatment with allopurinol, a xanthine oxidase inhibitor, attenuated the impairment of ACh induced vasodilatation in gracillis arteries of rats exposed to CIH for 14 days.

CONCLUSIONS AND FUTURE DIRECTIONS

The pathophysiological mechanisms involved in the development of hypertension in OSA are not fully understood. It is widely accepted that the CIH-induced oxidative stress contributes to enhance the CB chemosensory reactivity to oxygen and to the progression of the hypertension (**Figure 1**). Several studies have shown that concomitant administration of antioxidants, SOD mimetic, anti-inflammatory agents, ETA, and AT-1 receptor blockers, all of them reducing the levels of ROS formation and/or blocking the downstream signaling pathways induced by CIH, effectively prevents the enhanced CB chemosensory as well as the development of the hypertension. In addition, results showing that ablation of the CBs before the exposure to CIH significantly prevent the development of the hypertension strongly suggest a main role of the CB in the progression of the hypertension following CIH. However, the effect of the oxidative stress on the arterial blood pressure in rats exposed to CIH may also occur in multiple sites of the chemoreflex pathway, including the CB, the central cardiorespiratory centers and/or the arterial vessels. Thus, understanding how the oxidative stress and the molecules activated by CIH may interact at the CB and systemic levels would provide insights into the generation of the cardiovascular complications of OSA.

ACKNOWLEDGMENTS

This work was supported by grant 1100405 from the National Fund for Scientific and Technological Development of Chile (FONDECYT) and Project Puente/28 PUC.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 October 2014; accepted: 14 November 2014; published online: 02 December 2014.

Citation: Iturriaga R, Andrade DC and Del Rio R (2014) Enhanced carotid body chemosensory activity and the cardiovascular alterations induced by intermittent hypoxia. Front. Physiol. ***5****:468. doi: 10.3389/fphys.2014.00468*

This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

PERSPECTIVE ARTICLE
published: 24 November 2014
doi: 10.3389/fphys.2014.00438

Central role of carotid body chemoreceptors in disordered breathing and cardiorenal dysfunction in chronic heart failure

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Oscillatory breathing (OB) patterns are observed in pre-term infants, patients with cardio-renal impairment, and in otherwise healthy humans exposed to high altitude. Enhanced carotid body (CB) chemoreflex sensitivity is common to all of these populations and is thought to contribute to these abnormal patterns by destabilizing the respiratory control system. OB patterns in chronic heart failure (CHF) patients are associated with greater levels of tonic and chemoreflex-evoked sympathetic nerve activity (SNA), which is associated with greater morbidity and poor prognosis. Enhanced chemoreflex drive may contribute to tonic elevations in SNA by strengthening the relationship between respiratory and sympathetic neural outflow. Elimination of CB afferents in experimental models of CHF has been shown to reduce OB, respiratory-sympathetic coupling, and renal SNA, and to improve autonomic balance in the heart. The CB chemoreceptors may play an important role in progression of CHF by contributing to respiratory instability and OB, which in turn further exacerbates tonic and chemoreflex-evoked increases in SNA to the heart and kidney.

Keywords: carotid body chemoreceptors, Cheyne–Stokes respiration, sympathetic nervous system, heart failure, cardiorenal syndrome

INTRODUCTION

Abnormal oscillatory breathing (OB) patterns are frequently observed in diverse populations, including infants born prematurely (Copeman et al., 1964), patients with heart failure (Ponikowski et al., 1999), or end stage renal disease (Hanly and Pierrato, 2001), and in otherwise healthy humans who travel to high altitude (Lahiri et al., 1983). These abnormal breathing patterns most commonly occur during non-REM sleep when chemical control of breathing predominates; however, some heart failure patients exhibit OB during waking hours as well (Brack et al., 2007). OB is characterized by oscillations in tidal volume and/or respiratory frequency and is thought to occur as a result of physiological or environmental challenges that de-stabilize the respiratory control system. These challenges may include alterations in arterial blood gases and pH (decreased P_aO_2, decreased P_aCO_2, and increased pH), circulatory delay and reductions in systemic oxygen transport, and enhancement of respiratory chemoreflex function (Fanfulla et al., 1998). The etiology of OB is diverse; however a significant body of research indicates that enhanced chemoreflex sensitivity is a common element of most types of OB (Lahiri et al., 1983; Ponikowski et al., 1999; Al-Matary et al., 2004; Nock et al., 2004; Hering et al., 2007).

CHEMOREFLEX SENSITIVITY AND DISORDERED BREATHING IN HEART FAILURE

Cheyne–Stokes respiration (CSR), a form of OB in which oscillations in tidal volume are separated by apneic episodes, is highly prevalent in patients with chronic heart failure (CHF) (Mortara et al., 1997; Ponikowski et al., 1999; Giannoni et al., 2008). CSR is associated with increased morbidity and mortality, and decreased quality of life in this population (Hanly and Zuberi-Khokhar, 1996; Lanfranchi et al., 1999; Brack et al., 2007; Carmona-Bernal et al., 2008). Accumulating evidence suggests that enhanced central and/or peripheral chemoreflex sensitivity (Javaheri, 1999; Narkiewicz et al., 1999; Giannoni et al., 2008) as well as persistent hyperventilation/hypocapnia (Naughton et al., 1993; Fanfulla et al., 1998) contribute to the pathogenesis of CSR by causing instability of the respiratory control system (Naughton et al., 1993; Lorenzi-Filho et al., 1999, 2005; Pinna et al., 2000). The significance of the relationship between chemosensitivity and CSR is further underscored by the finding that high peripheral chemosensitivity is independently associated with poor prognosis and higher mortality risk in CHF patients but not in comparable CHF patients with low chemosensitivity (Ponikowski et al., 1999, 2001).

Numerous studies indicate that carotid body (CB) chemoreceptor-mediated responses to hypoxia and hypercapnia are augmented in CHF (Wilcox et al., 1993; Chua et al., 1996, 1997; Javaheri, 1999; Ponikowski and Banasiak, 2001; Ciarka et al., 2006; Giannoni et al., 2008). In a group of 60 CHF patients, approximately 60% had increased CB chemoreflex sensitivity (Giannoni et al., 2008). Most importantly, patients without augmented chemosensitivity did not exhibit CSR, and the incidence of CSR progressively

increased with enhancement of the CB chemoreflex. In other studies, deactivation of CB chemoreceptors with transient hyperoxia, or pharmacological attenuation of chemosensitivity with dihydrocodeine or acetazolamide significantly reduced central apnea incidence in CHF patients (Ponikowski et al., 1999; Fontana et al., 2011). These findings indicate an important relationship between CSR or cyclical breathing patterns and enhanced CB chemoreflex sensitivity.

Recent studies in animal models of CHF have further delineated the role of the CB chemoreceptors in OB. Studies from our laboratory have demonstrated enhanced ventilatory, sympathetic nerve, and carotid sinus nerve responses to isocapnic hypoxia as well as a tonic increase in resting afferent chemoreceptor discharge during normoxia in both rabbit and rat models of heart failure (Sun et al., 1999a,b; Li et al., 2005; Del Rio et al., 2013b; Haack et al., 2014; Marcus et al., 2014a). These increases in CB chemoreceptor activity coincide with an increase in measures of OB and the development of CHF (Marcus and Schultz, 2011). Denervation of the CB chemoreceptors (CBD) by CB ablation after the development of CHF results in abolition of chemoreflex responses, reduction of resting ventilation and sympathetic nerve activity (SNA), and reduction of apnea/hypopnea frequency and respiratory variability (Del Rio et al., 2013b; Marcus et al., 2014a). In other studies, pharmacologic attenuation of CB chemoreceptor activity with Simvastatin or an inhibitor of hydrogen sulfide production had similar efficacy in reducing apnea/hypopnea frequency and respiratory variability (Del Rio et al., 2013a; Haack et al., 2014).

Ablation of CB afferent activity in the aforementioned studies (Del Rio et al., 2013b; Marcus et al., 2014a) resulted in significant reductions in resting ventilation, which in turn would be expected to increase resting $PaCO_2$. CHF-CBD rabbits exhibited significant hypoventilation relative to normal animals for up to 9 days post CBD, the endpoint of the study (Marcus et al., 2014a). CHF-CBD rats exhibited hypoventilation compared to the ventilatory parameters obtained in normal animals when measured 2 days post denervation, but no hypoventilation was found at 14 weeks post CBD (Del Rio et al., 2013b). Thus, the salutary effect of CBD to stabilize the respiratory pattern in CHF could stem from an increase in P_aCO_2 above the apneic threshold, at least in the short-term, but abrogation of the elevated ventilatory loop gain mediated by the CB chemoreflex is likely to play an important role in reestablishing respiratory stability in CHF in the long-term.

Resting ventilation and sympathetic outflow are increased in CHF (Naughton et al., 1993; van de Borne et al., 1998). In our studies, CBD-reduced resting sympathetic outflow as well as ventilation, indicating that CB chemoreceptors play an important role in the tonic increases in both of these parameters in CHF. Central neural coupling between respiratory and sympathetic neural drive has been described in the literature (Haselton and Guyenet, 1989). It is possible that the elevated tonic input from CB chemoreceptors exacerbates respiratory-sympathetic coupling to account in part for their marked increase in CHF patients.

RESPIRATORY-SYMPATHETIC COUPLING IN HEART FAILURE

It is well-known that sympathetic discharge is actively modulated by respiration (Adrian et al., 1932; Haselton and Guyenet, 1989), and a growing body of evidence indicates that this modulatory influence may be altered in several different pathological states. Evidence of enhanced respiratory-sympathetic coupling has been found in three different animal models of hypertension (Zoccal et al., 2008; Simms et al., 2009; Toney et al., 2010) with differing etiologies (spontaneously hypertensive rat-SHR, Ang II/salt, and chronic intermittent hypoxia-CIH). Interestingly, in two of these models (SHR and CIH), enhanced CB chemoreflex sensitivity and tonic CB chemoreceptor afferent input to the brain stem have been shown to play a seminal role in mediating increased SNA and the development of hypertension (Fletcher et al., 1992; Peng et al., 2003; Del Rio et al., 2010; Marcus et al., 2010; Tan et al., 2010; Abdala et al., 2012). Furthermore, sympathetic drive increases in tandem with respiratory neural output after exposure to CIH (Zoccal et al., 2008). No studies have examined CB chemoreflex tone in the Ang II/salt model, however Ang II has been shown to play a role in enhancing CB chemosensitivity (Li et al., 2006), and thus it is plausible that tonic CB chemoreceptor input is elevated in this model as well. Evidence from these studies suggests that enhanced afferent activity arising from the CBs promotes respiratory-sympathetic coupling that in turn perpetuates sympathetic over activity.

Recent work from our lab (**Figure 1**) has shown that respiratory-sympathetic coupling is enhanced in CHF, and that the enhanced coupling coincides with sensitization of the CB chemoreflex (Marcus et al., 2014a). Furthermore, we demonstrated that respiratory-sympathetic coupling in CHF is critically dependent on the CB since it was markedly reduced or abolished after CBD (Marcus et al., 2014a). Taken together, these findings strongly suggest a central role for enhanced tonic CB chemoreceptor drive in the development of respiratory-sympathetic coupling in disease conditions characterized by autonomic imbalance and abnormal respiratory rhythms.

The mechanisms underpinning the relationship of CB chemoreflex drive to respiratory-sympathetic coupling in CHF are still unclear. A plausible hypothesis is that the entrainment between the respiratory and sympathetic neural drive may result from alterations in the neurons integrating CB afferents and initiating respiratory rhythm and sympathetic outflow in the brainstem. Indeed, there is evidence that CIH-induced sympatho-excitation results in an increase in the strength of the excitatory synapses at the level of the nucleus of the solitary tract, the paraventricular nucleus, and the rostral medulla (Kc et al., 2010; Kline, 2010; Silva and Schreihofer, 2011; Costa-Silva et al., 2012). Enhanced respiratory-sympathetic coupling is of major relevance in CHF patients in which hyperventilation is common, and in which frequent respiratory oscillations occur during CSR (**Figure 1**). Previous investigators have observed surges in SNA during the hyperpneic phase of CSR (Leung et al., 2006) which may be indicative of enhanced respiratory-sympathetic coupling, and which likely has important impact on downstream targets such as the heart and kidneys.

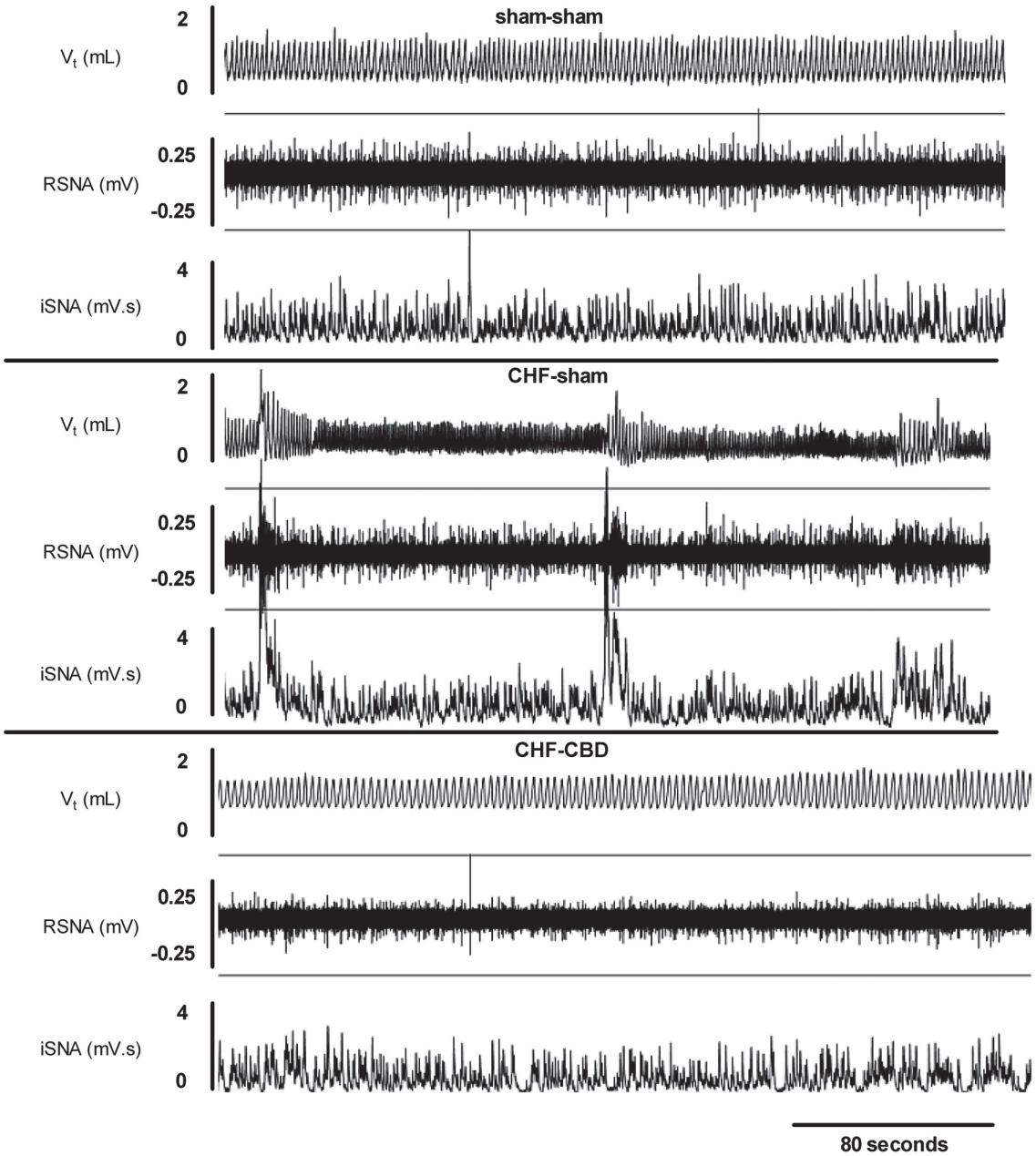

FIGURE 1 | Respiratory-sympathetic coupling in CHF. Oscillatory breathing patterns were apparent in CHF animals (middle panel) that were accompanied by concomitant oscillations in renal sympathetic nerve activity (RSNA). Respiratory and RSNA oscillations were not observed in CHF animals after carotid body denervation (bottom panel). CHF-chronic heart failure, CBD-carotid body denervation, V_t-tidal volume, RSNA-renal sympathetic nerve activity, iSNA-integrated renal sympathetic nerve activity. Reproduced with permission from Marcus et al. (2014a).

ROLE OF ENHANCED CHEMOREFLEX SENSITIVITY AND DISORDERED BREATHING IN CARDIAC AND RENAL DYSFUNCTION IN HEART FAILURE

In CHF patients, renal dysfunction is common and is associated with poor prognosis (Bock and Gottlieb, 2010). Development of renal dysfunction in CHF is particularly ominous because it can precipitate further decline in cardiac function, initiating a downward spiral of deteriorating cardiac and renal function, known as cardiorenal syndrome. While the etiology of cardiorenal syndrome is diverse, excessive sympathetic activation, volume retention and venous congestion, renal ischemia secondary to reductions in renal perfusion, and neuro-hormonal activation are thought to play central roles (Bock and Gottlieb, 2010). Tonic chemoreflex activation in CHF may contribute to cardiorenal syndrome by increasing sympathetic stimulation of the heart (Xing et al., 2014) and kidneys (Sun et al., 1999a) leading to increases

in peripheral vascular resistance and myocardial oxygen demand, increases in sodium and water retention, and activation of the renin-angiotensin system. In addition, the development of OB mediated by enhanced CB chemoreflex sensitivity may further exacerbate renal ischemia by eliciting additional chemoreflex-evoked renal vasoconstriction in addition to episodic hypoxemia (**Figure 1**).

Under normal circumstances, CB chemoreflex activation elicits a reduction in renal blood flow and glomerular filtration rate that is mediated by renal sympathetic nerves (Karim et al., 1987). In CHF, tonic elevations in renal SNA mediate sustained reductions in renal blood flow and alterations in angiotensin signaling (Clayton et al., 2011). Our preliminary findings indicate that the reduction in renal blood flow to CB chemoreflex activation is markedly accentuated in CHF animals. Further, CBD in CHF animals reduces renal SNA, increases renal blood flow, and decreases markers of renal injury and fibrosis (Marcus et al., 2014b), in addition to the reduction in disordered breathing and improvement in cardiac function mentioned previously (Marcus et al., 2014a). These findings suggest that tonic CB chemoreflex activation in CHF may contribute to renal pathology in part by its influence on sympathetic outflow (Hering et al., 2007) to the heart and kidneys (Sun et al., 1999b; Xing et al., 2014). In addition to the influence of tonic CB chemoreflex activation on resting renal SNA, additional surges in SNA may be superimposed by episodic hypoxemia associated with apneic episodes during sleep (van de Borne et al., 1998), augmented by an enhanced CB chemoreceptor sensitivity to hypoxia in CHF (Marcus et al., 2014a). This notion is supported by evidence from studies in clinical populations (Ryan et al., 2005).

Normalization of abnormal breathing patterns in CHF patients with continuous positive airway pressure (CPAP) or

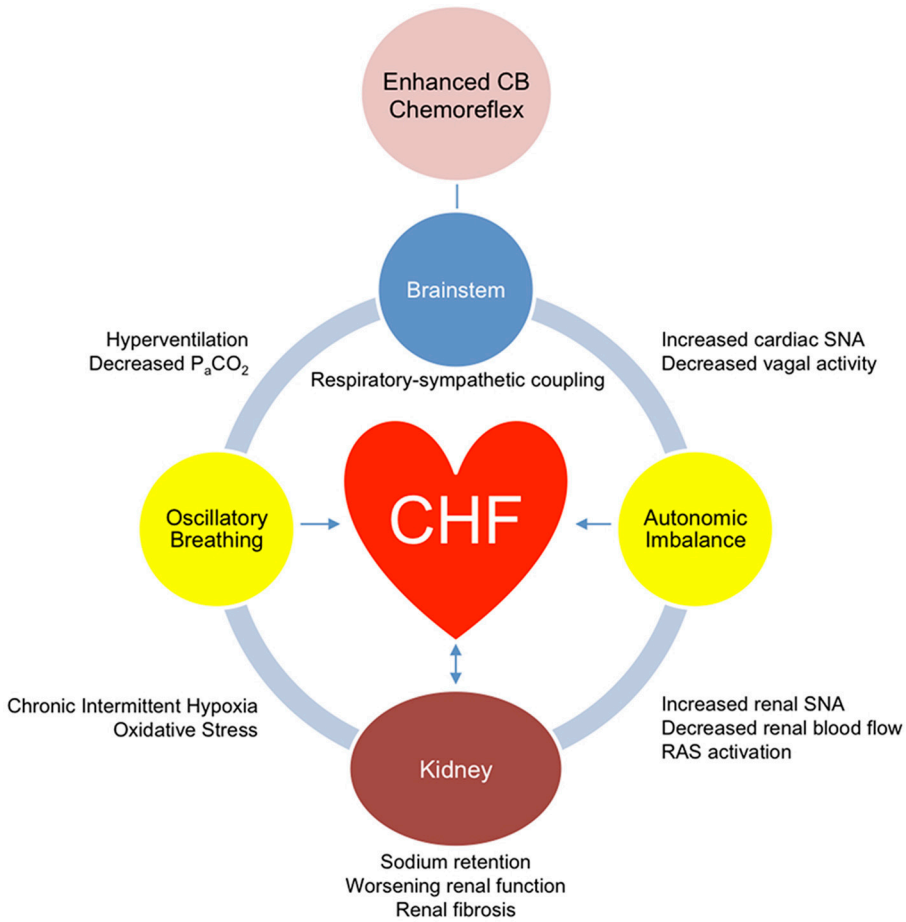

FIGURE 2 | Role of carotid body chemoreceptors in cardiac and renal dysfunction. Enhanced tonic afferent activity from carotid body (CB) chemoreceptors drives neuronal activity in brainstem centers that integrate peripheral afferents and control respiratory and sympathetic neural outflow. Hyperventilation due to the enhanced CB chemoreflex activation precipitates oscillatory breathing, which exacerbates sympathetic activation through respiratory-sympathetic coupling, in addition to exposing the heart and kidneys to intermittent hypoxia and oxidative stress. The CB-mediated enhanced respiratory-sympathetic coupling results in increased sympathetic and decreased vagal efferent outflow to the heart, which over time worsens cardiac function and development of fibrosis. Similarly, CB-mediated increases in renal SNA cause reductions in renal perfusion and activation of the renin-angiotensin system (RAS), which over time lead to worsening renal function and development of fibrosis. The combined deleterious effects of CB-mediated respiratory-sympathetic coupling on the heart and kidney advances the cardiorenal syndrome.

adaptive servo-ventilation (ASV) is associated with reduced tonic levels of sympathetic activation (Ryan et al., 2005), improved cardiac function, improved renal function, and improved prognosis (Koyama et al., 2011; Yoshihisa et al., 2011; Kasai et al., 2013; Owada et al., 2013). These improvements may be due to secondary effects of CPAP or ASV treatments to improve cardiac function via direct mechanical effects of pressure support ventilation on the heart (Takama and Kurabayashi, 2011), however they also likely reflect the reduction in CB chemoreflex sensitivity (Spicuzza et al., 2006), and consequent reduction in CB chemoreflex-mediated sleep disordered breathing and sympathoexcitation (Naughton et al., 1995; Despas et al., 2009). Our findings in an animal model of CHF support this notion of the functional consequences of enhanced respiratory-sympathetic coupling in CHF mediated by the CB. The reduction of disordered breathing patterns with CBD was sufficient to reduce renal SNA, increase renal blood flow, and improve cardiac function (Marcus et al., 2014a,b) and survival (Del Rio et al., 2013b), independent of any confounding effects of pressure support ventilation used in the aforementioned clinical studies.

CONCLUSION

Accumulating evidence suggests a critical role for the CB chemoreceptors in the etiology of several important pathophysiological aspects of CHF. CB chemoreceptors are a major driving force in the development of autonomic dysfunction and breathing abnormalities in CHF. Ablation of the CB chemoreceptors is sufficient to improve these parameters and leads to improved cardiac function (Marcus et al., 2014a) and survival (Del Rio et al., 2013b). The mechanisms by which the CB chemoreflex exacerbates cardiac deterioration and morbidity in CHF remain to be better elucidated, but disordered breathing, enhanced respiratory-sympathetic coupling, tonic and episodic increases in cardiac and renal SNA, and reductions in renal function likely play an important role (**Figure 2**). A case report published recently showed that unilateral CBD in a CHF patient resulted in modest improvements in autonomic function, cardiac function, and exercise tolerance, and reduced resting ventilation (Niewinski et al., 2013). This study supports findings from pre-clinical animal models and confirms the potential of CBD or other forms of CB modulation as a therapeutic option in CHF patients. Taken together, these findings suggest that CB-mediated disordered breathing and respiratory-sympathetic coupling in CHF plays an important role in the abnormalities of sympathetic outflow observed in CHF with negative clinical implications for cardiac and renal function (Marcus et al., 2014a,b).

ACKNOWLEDGMENTS

Noah J. Marcus is supported by a National Research Service Award fellowship from the Heart, Lung and Blood Institute of NIH (F32-HL108592-01A1). Rodrigo Del Rio is funded by Fondo Nacional de Desarrollo Científico y Tecnológico (Fondecyt #1140275) and Harold D. Schultz is funded by a Program Project Grant from the Heart, Lung and Blood Institute of NIH (PO1-HL62222).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 October 2014; accepted: 27 October 2014; published online: 24 November 2014.

*Citation: Marcus NJ, Del Rio R and Schultz HD (2014) Central role of carotid body chemoreceptors in disordered breathing and cardiorenal dysfunction in chronic heart failure. Front. Physiol. **5**:438. doi: 10.3389/fphys.2014.00438*

This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

METHODS ARTICLE
published: 05 November 2014
doi: 10.3389/fphys.2014.00432

Fractal analysis of the structural complexity of the connective tissue in human carotid bodies

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The carotid body (CB) may undergo different structural changes during perinatal development, aging, or in response to environmental stimuli. In the previous literature, morphometric approaches to evaluate these changes have considered quantitative first order parameters, such as volumes or densities, while changes in spatial disposition and/or complexity of structural components have not yet been considered. In the present study, different strategies for addressing morphological complexity of CB, apart from the overall amount of each tissue component, were evaluated and compared. In particular, we considered the spatial distribution of connective tissue in the carotid bodies of young control subjects, young opiate-related deaths and aged subjects, through analysis of dispersion (Morisita's index), gray level co-occurrence matrix (entropy, angular second moment, variance, correlation), and fractal analysis (fractal dimension, lacunarity). Opiate-related deaths and aged subjects showed a comparable increase in connective tissue with respect to young controls. However, the Morisita's index ($p < 0.05$), angular second moment ($p < 0.05$), fractal dimension ($p < 0.01$), and lacunarity ($p < 0.01$) permitted to identify significant differences in the disposition of the connective tissue between these two series. A receiver operating characteristic (ROC) curve was also calculated to evaluate the efficiency of each parameter. The fractal dimension and lacunarity, with areas under the ROC curve of 0.9651 (excellent accuracy) and 0.8835 (good accuracy), respectively, showed the highest discriminatory power. They evidenced higher level of structural complexity in the carotid bodies of opiate-related deaths than old controls, due to more complex branching of intralobular connective tissue. Further analyses will have to consider the suitability of these approaches to address other morphological features of the CB, such as different cell populations, vascularization, and innervation.

Keywords: morphometry, fractal parameters, co-occurrence matrix, Morisita's index, carotid body, drug-related death, heroin, aging

INTRODUCTION

The carotid body (CB) is the main peripheral arterial chemoreceptor, sensitive to reduction in pO_2 and pH and to increases in pCO_2. From a structural point of view, it is composed of lobules containing type I cells, positive for tyrosine hydroxylase, and type II cells, positive for glial fibrillary acidic protein. Type I cells are considered the true chemoreceptor elements. They are roundish and produce many different neurotransmitters and peptide neuromodulators. Type II cells are fusiform and envelop clusters of type I cells. They are usually considered supportive cells, although they may also be stem cell precursors for type I cells (Pardal et al., 2007; Platero-Luengo et al., 2014) and probably co-ordinate chemosensory transduction through interactions with the other cells of the CB (Tse et al., 2012). Connective tissue also characterizes the CB structure, mainly delimiting the glomic lobules (interlobular connective tissue) and partly branching in the lobular context (intralobular connective tissue). Neurotransmitters and neuromodulators released by type I cells mainly act on the afferent endings of the carotid sinus nerve, arising from the glossopharyngeal nerve. The CB also shows sensory innervation from jugular and nodose ganglia, post-ganglionic sympathetic nerve fibers from the superior cervical ganglion, and preganglionic parasympathetic and sympathetic fibers reaching local ganglion cells. Moreover, the CB is the structure in the body with the highest blood flow (Daly et al., 1954; Barnett et al., 1988) and local changes in blood flow have been considered to be involved in CB chemoreceptor discharge (Joels and Neil, 1963; Kirby and McQueen, 1984; Porzionato et al., 2006, 2011a,b).

The CB undergoes structural and functional changes during perinatal development (e.g., Porzionato et al., 2008a,b; De Caro et al., 2013), aging (e.g., Di Giulio et al., 2009, 2012; Zara et al., 2013a) and in response to a variety of environmental stimuli, such as chronic sustained hypoxia (e.g., Pardal et al., 2007; Platero-Luengo et al., 2014), chronic intermittent hypoxia (e.g., Iturriaga et al., 2009), chronic hyperoxia (e.g., Bavis et al., 2013), and exposure to nicotine (e.g., Stéphan-Blanchard et al., 2013). Several morphometrical approaches have been involved to address structural changes in the CB. Most morphometrical

parameters addressed in the literature are first order parameters, such as volumes or densities. Volume analyses may involve CB *in toto* or its different components (parenchyma, interlobular or intralobular connective tissue, vessels) (e.g., Dinsdale et al., 1977; Lack et al., 1986; Clarke et al., 2000; Porzionato et al., 2005). Innervation of the CB has mainly been evaluated in terms of density values (e.g., Kusakabe et al., 2003, 2004). The different cell types of the CB (type I and II cells, progenitors, macrophages, mast cells, and other immune cells) have been considered in the literature in terms of cell densities or total cell numbers (e.g., Pardal et al., 2007; Porzionato et al., 2013). Computer-assisted image analysis of protein expression in immunostained sections has also been performed through quantification of the immunoreactive area in order to estimate the percentage of tissue exhibiting positivity (e.g., Di Giulio et al., 2012; Zara et al., 2013b).

Size parameters alone, however, may be inadequate to fully characterize the microarchitecture generated by tissue components such as connective tissue, type II cells, vessels, and innervation, all characterized by a quite complex spatial arrangement. In fact, a pattern of this type, for each given size, can generate in the available space substantially different spatial textures characterized by different degrees of homogeneity and morphological complexity (Guidolin et al., 2004a,b). In the present study possible strategies to morphometrically estimate these morphological features of the CB tissue have been considered. In particular, they will be used for the analysis of the pattern of fibrosis induced in the CB by normal aging and opiate abuse in young people. Since data exist showing that a comparable increase in the amount of connective tissue in the CB occurs in both conditions, but with a likely different pattern of spatial distribution (Porzionato et al., 2005), this specific example can allow a test of the efficiency of the considered methods.

MATERIALS AND METHODS

TISSUE SAMPLES

Materials consisted of carotid bodies obtained at autopsy from 35 subjects who died of heroin/morphine intoxication (26 males, nine females; mean age ($\pm$ *SD*) 26 $\pm$ 3.5 years). In all cases, there was a clinical history of at least 3 years of heroin addiction. The other two groups for comparison consisted of 10 young (five males, five females; mean age 22 $\pm$ 3.4 years) and 10 aged subjects (five males, five females; mean age 66.5 $\pm$ 3.5 years) who died of trauma. All subjects were clinically without chronic pulmonary or cardiovascular disease. Cardiac hypertrophy or preceding myocardial infarction were excluded at autopsy. Autopsies were performed between 24 and 78 h after death. Specimens were taken of the right carotid bifurcation, including 20 mm of the common carotid and 20 mm of the internal and external carotid arteries.

Autopsies and all the procedures applied to process samples from human tissues have been performed according to the Italian Mortuary Police Legislation.

HISTOLOGICAL TECHNIQUES

Tissues were fixed in 10% phosphate-buffered formalin for 72 h, dehydrated through ascending alcohols and xylene, and paraffin embedded. Longitudinal serial sections, 5 μm thick, of the whole carotid bifurcation were then obtained, de-waxed (xylene and alcohol progressively at lower concentrations), and stained with Azan-Mallory (AM).

IMAGE ANALYSIS PROCEDURES

All the image analysis procedures were performed by using the ImageJ software (Schneider et al., 2012), freely available at http://rsb.info.nih.gov/ij/. They can be summarized as follows.

Image acquisition and pre-processing

Bright-field images of the AM-stained preparations were acquired by using a Leica DMR microscope (Leica Microsystems, Wetzlar, Germany) and a high resolution digital camera (DC 200, Leica Microsystems). At a primary magnification of ×20 one field per section, randomly chosen within the CB tissue, was selected and its image acquired in full colors (RGB, 24-bit), processed to correct shading, then filed TIFF (**Figure 1A**).

Since it showed the best contrast between the connective tissue and the CB parenchima, the red component of each acquired RGB image was selected for further processing (**Figure 1B**). Stromal structures and filaments can be easily segmented with conventional thresholding methods, and small remaining artifacts can be removed from the resulting binary image by applying a geometric filter to eliminate profiles within a specified range of area and/or shape (see Russ, 2011), leading to the generation of binary images (**Figure 1C**) of the connective component.

The amount of CB tissue it accounts for can be directly estimated from the corresponding binary image by evaluating the area fraction occupied by the binary pattern (Russ and Dehoff, 2000).

Morphological complexity of the pattern of the connective tissue

The obtained gray-level and binary images illustrated in **Figure 1**, however, can also represent the input data for procedures aimed at estimating indices able to capture more detailed morphological features, such as a characterization of the overall shape of the patterns generated by connective tissue, and of the way it arranges itself in the tissue. In these respect, three methods were considered in the present study. They are briefly detailed in the sections that follow.

Analysis of dispersion. To provide a quantitative evaluation of the dispersion in the tissue space of the binary pattern corresponding to connective tissue, Morisita's index (Morisita, 1962), one of the most robust distribution measures (Myers, 1978), was estimated. For this purpose, the binary image was divided into 12 sub-images and the number of pattern pixels in each sub-picture was evaluated. The index of dispersal (I_d) was then calculated using

$$I_d = n\left(\frac{\sum_{i=1}^{n} X_i^2 - N}{N(N-1)}\right)$$

where n is the number of sub-images, whereas N and X_i represent the number of pattern pixels in the image, and in each sub-image, respectively. The index value increases with increasing spatial dispersion of the pattern.

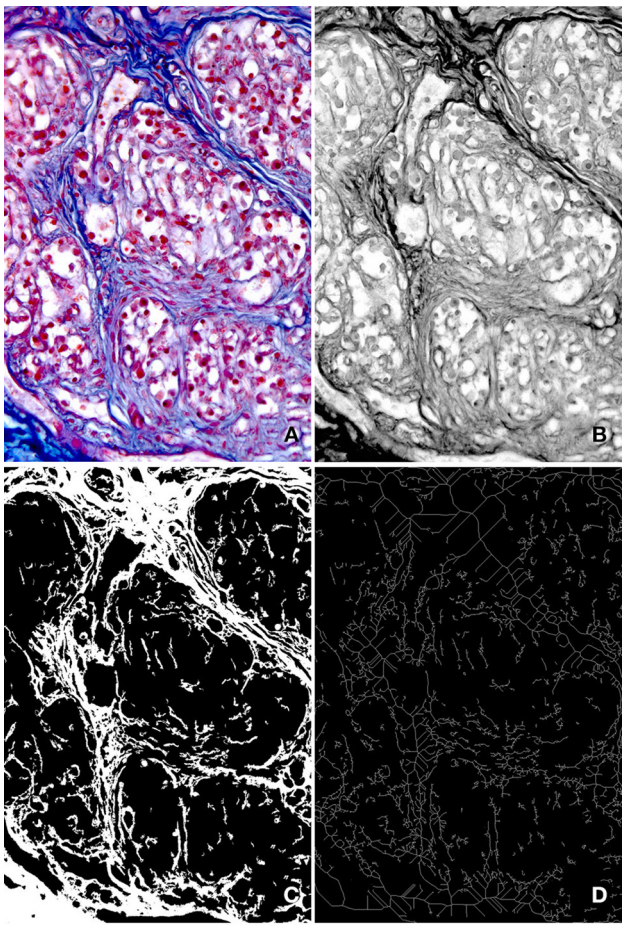

FIGURE 1 | Main steps of the image analysis procedure. (A) Full color (RGB, 24-bit) digital image of a microscope field stained with AM (primary magnification ×20). **(B)** Gray level image corresponding to the red component of the image in **(A)**. Due to the high contrast between connective tissue and parenchyma it exhibits, it was used to estimate the GLCM and to discriminate the connective tissue by proper thresholding. **(C)** Binary image of the connective tissue pattern, used to estimate the percent area it occupies and the Morisita's index. **(D)** Binary skeleton of the image in **(C)**, from which fractal parameters were estimated.

Gray level co-occurrence matrix analysis. Gray level co-occurrence matrix (GLCM) is a fast mathematical method for assessing image structural properties such as homogeneity, complexity, and level of disorder (see Pantic et al., 2012). It was first introduced by Haralick et al. (1973) and is based on a quantitation of the relationship between pixel brightness values in an image. This information can be extracted from a matrix $P_{\theta d}(i,j)$ (GLCM) describing how frequently two pixels with gray level i and j appear in the image separated by a distance d in the direction θ (Aggarwal and Agrawal, 2012). Haralick et al. (1973) described 14 parameters that can be calculated from the GLCM with the intent of describing the texture of an image. Today, however, those proven as the most useful in experimental and clinical medicine applications (see Losa and Castelli, 2005; Alvarenga et al., 2010; Pantic et al., 2012) are the following ones:

$$Entropy = -\sum_i \sum_j P(i,j)\log(P(i,j))$$

$$Angular\ second\ moment = \sum_i \sum_j \left[P(i,j)\right]^2$$

$$Variance = \sum_i \sum_j (1-\mu)^2 P(i,j)$$

$$Correlation = \frac{\sum_i \sum_j ijP(i,j) - \mu_x \mu_y}{\sigma_x \sigma_y}$$

where i and j are coordinates of the co-occurrence matrix, $\sigma's$ and $\mu's$ represent means and standard deviations along rows and columns of the matrix. They were computed with ImageJ by using the "texture analysis" plugin, developed by Julio E. Cabrera and Toby C. Cornish, and freely available at http://rsbweb.nih.gov/ij/plugins/texture.html.

Fractal analysis. To globally describe the complexity of form in quantitative terms the "Fractal dimension" (D) can be a valuable parameter (Guidolin et al., 2004b). It measures the rate of addition of structural detail with increasing magnification, scale, or resolution (Cutting and Garvin, 1987). D of the binary skeleton (**Figure 1D**) was estimated using the "box counting" method at multiple origins as indicated by Smith et al. (1996). Briefly, from grids of increasing size overlying the image, the number of boxes containing any pixel was counted. This number was recorded as a function of grid size and D was calculated, as −1 times the slope of the regression line, from a plot of the log of size on the x-axis and the log of box count on the y-axis. To minimize grid location effects, the algorithm started from a number (10 in our case) of locations, generating a set of values for D. The average value over all locations was considered as the final estimate of D. During the same analytical process "Lacunarity" was also calculated. This parameter is a measure of the nonuniformity (heterogeneity) of structure or the degree of structural variance within an object (Smith et al., 1996). It was estimated as the average of the coefficient of variation for pixel density over all grid sizes and locations (Bassinghtwaighte et al., 1994).

To perform the abovementioned analysis, the "FracLac for ImageJ" plugin by Audrey Karperien was used (freely available at http://rsb.info.nih.gov/ij/plugins/fraclac/fraclac.html).

Statistical analysis

Statistical analysis was done using GraphPad Prism software (GraphPad Inc., La Jolla, CA, USA) and SPSS statistical package (v. 13.0; IBM, Armonk, NY, USA). Data were analyzed by One-Way analysis of variance followed by Dunnet's test for multiple comparisons vs. the young control group. Bonferroni's test for comparisons between selected groups was used to determine possible statistically significant differences between opiate-addicted and aged cases. $p < 0.05$ was always used as the limit for statistical significance. In addition to the standard statistical difference tests, the ability of the various parameters to discriminate between aging and opiate addiction was estimated by calculating the ROC curves (Metz, 1978). ROC curve is constructed based on the fraction of true positives out of the positives (sensitivity) and the

fraction of false positives out of the negatives (specificity), taking into account different thresholds. The potential discriminatory performance of each parameter can be estimated by analyzing the area under its ROC curve (see Zweig and Campbell, 1993). A parameter with no discriminative value would have the area under the ROC curve approximately higher than 0.5 and lower than 0.6. Area between 0.6 and 0.7 indicates "poor" performance, area between 0.7 and 0.8 "fair" performance, and area between 0.8 and 0.9 "good" performance (Zweig and Campbell, 1993; Sandelowsky et al., 2011). Parameters belonging to the category "excellent" usually have the ROC areas higher than 0.9.

RESULTS

As shown in **Figure 2** a similar, statistically significant, increase in the total amount of connective tissue was observed in the CB from aged subjects and from young people who died of opiate intoxication when compared to normal young CB samples. It appeared mainly located between the lobes of parenchyma. From a qualitative point of view, however, some difference between the two groups can be observed in the spatial distribution of this tissue component. In particular a more complex branching of connective tissue within the CB parenchyma lobes seems to characterize the samples from subjects who died of opiate abuse when compared to normal aging (**Figure 3**).

As shown in **Table 1**, almost all of the morphometric parameters estimated to characterize the spatial organization of this tissue component indicated significant differences in this tissue feature between young controls and the other two groups of patients, with the only exception of D, that exhibited a similar value in young controls and aged subject and increased significantly only in opiate-addicted group. Interestingly, however, only some of these methods were able discriminate between aged subjects and young people who died of opiate abuse. As summarized in **Table 1**, "Angular second moment" of the GLCM, fractal parameters (D and lacunarity), and the Morisita's index assumed significantly different values in the two conditions.

For each of these parameters a ROC curve was calculated to illustrate the efficiency of the parameter as a binary classifier of CB fibrosis between aged and opiate addicted subjects. As presented in **Table 2**, the areas under the ROC curves indicated that the fractal parameters (D and lacunarity) were those exhibiting the highest discriminatory power.

DISCUSSION

A significant increase in connective tissue and a concomitant reduction in glomic parenchyma are well known characteristics of the CB tissue in both opiate-addicted and aged cases when compared to young controls. Consistently with previously reported data (Porzionato et al., 2005), also in the present study both these conditions led to an increase of the amount of connective tissue from about 30%, that can be observed in normal young people, to more than 40% of the CB. Such a change is in accordance with the findings of Hurst et al. (1985) who found progressive arteriosclerosis of the glomic arteries during aging, to which they ascribed the increase in connective tissue. Also in opiate addicts, the increase in connective tissue may be interpreted as a sign of early tissue aging, that can be ascribed to arteriosclerosis of the glomic arteries, to the recurrent episodes of hypoxia during reaction to opiate assumption, and/or to local inflammatory infiltrates (chronic carotid glomitis) (Porzionato et al., 2009).

The connective tissue, however, exhibits a complex spatial organization. It is mainly formed by large bundles of fibers located between the lobes of parenchyma, from which thin branches of intralobular connective tissue depart. When compared to young controls (see Porzionato et al., 2005), this intralobular component of the pattern appeared increased in both aged and opiate-addicted subjects. An analysis based on the simple estimate of a dimensional parameter (volume fraction) failed in detecting significant differences between the two conditions, although the branching pattern of connective tissue in opiate-addicted subjects appeared (at least qualitatively) more complex.

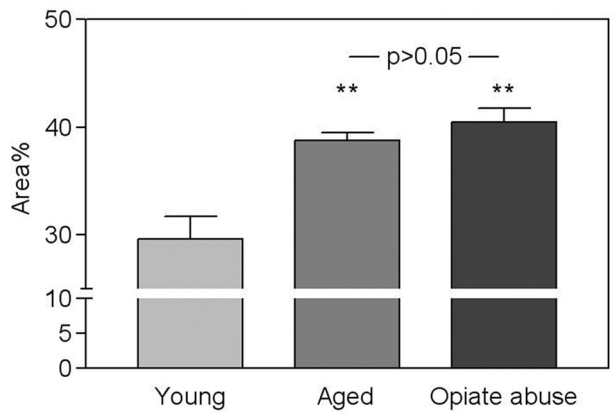

FIGURE 2 | Fraction of the CB corresponding to connective tissue in young controls, in aged subjects, and in young people who died of chronic opiate abuse. Values are average percent areas (± s.e.m.). As indicated both aging and opiate abuse induced a significant increase (**$p < 0.01$) of the total amount of connective tissue in the CB with respect to young control subjects. Between the two conditions however, no statistically significant differences can be detected.

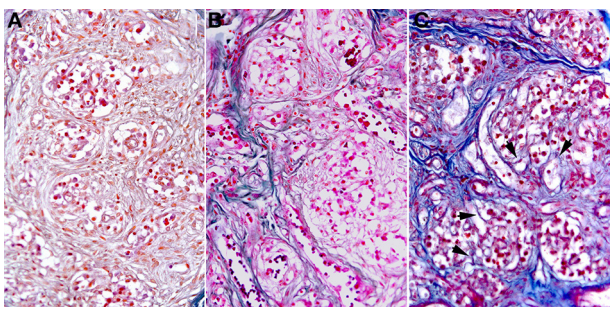

FIGURE 3 | Microscope fields of AM-stained samples from a young control (A), an aged subject (B), and from a subject who died of chronic opiate abuse (C). In the latter the spatial organization of the connective tissue appeared more complex, being characterized by a higher presence of thin branches of connective tissue within the parenchyma lobes. Some of them are highlighted by the arrow heads.

Table 1 | Mean values (± s.e.m.) of the parameters quantifying textural properties of the connective tissue in CB samples from normal young subjects, from aged subjects, and from young subjects who died of chronic opiate abuse.

Feature	Young controls	Aging	Drug-related deaths
Morisita's index	0.028 ± 0.002	0.060 ± 0.010(°)	0.083 ± 0.005(°°, *)
Angular second moment	0.0048 ± 0.0006	0.0020 ± 0.0007(°°)	0.0010 ± 0.0001(°°, *)
Variance	151.0 ± 12.9	97.2 ± 4.8(°°)	115.4 ± 5.2(°°)
Correlation	0.0010 ± 0.00017	0.0004 ± 0.00005(°°)	0.0004 ± 0.00003(°°)
Entropy	7.91 ± 0.150	8.36 ± 0.099(°)	8.44 ± 0.079(°°)
Fractal dimension	1.43 ± 0.028	1.447 ± 0.013	1.563 ± 0.006(°°, **)
Lacunarity	0.828 ± 0.017	0.500 ± 0.021(°°)	0.388 ± 0.009(°°, **)

°$p < 0.05$, °°$p < 0.01$ vs. the "Young controls" group; *$p < 0.05$, **$p < 0.01$ vs. the "Aging" group.

Table 2 | Area under the ROC curve for the parameters showing any discriminative power between drug-related deaths and aged subjects, and classification of their accuracy (see Zweig and Campbell, 1993; Sandelowsky et al., 2011).

Feature	Area under the ROC curve	Accuracy
Morisita's index	0.6529	Poor
Angular second moment	0.7895	Fair
Fractal dimension	0.9651	Excellent
Lacunarity	0.8835	Good

Thus, in the present study additional morphometric strategies were also explored, based on the analysis of the connective tissue pattern in terms of textural properties (such as homogeneity, complexity and level of disorder) more than overall amount. In particular, three different image analysis methods were tested: analysis of dispersion, analysis of the GLCM, and fractal analysis. Analysis of dispersion has been successfully used to study how a given population or morphological pattern fills the available space (see Goodenough and Goodenough, 2012). GLCM was successfully applied in nuclear magnetic resonance imaging (Li et al., 2009), computed tomography (Huber et al., 2011) and other clinical research areas. In fundamental medical and biology research, it was used to study tissue age-related structural degradation (Shamir et al., 2009), and chromatin structural changes during apoptosis (Losa and Castelli, 2005; Pantic et al., 2012). Fractal analysis proven very useful to characterize the complex morphology of vascular trees and the endothelial cells self-organization *in vitro* (Guidolin et al., 2004a,b). Although the interest was mainly focused on the comparison between opiate-addicted and aged cases (showing comparable amounts of connective tissue), the analysis was also extended to young cases for completeness.

The results indicated that GLCM, Morisita's index, and the fractal parameter "lacunarity" were able to discriminate normal CB tissue samples from those derived from aged or opiate-addicted people. However, since all the above mentioned parameters are measures of *homogeneity* (Morisita, 1962; Smith et al., 1996; Marrón, 2012; Pantic et al., 2013) describing how well a pattern fills the available space as a consequence of the triggering conditions, this finding could be partly related to the increase of connective tissue occurring following aging and opiate addiction.

More interesting findings emerge when conditions characterized by a similar total amount of CB connective tissue were compared. In this respect, the results of the present study indicate that some of the parameters provided by the tested methods (namely angular second moment, lacunarity, and Morisita's index) exhibited discriminatory power between CB normal aging and chronic opiate consumption, although in the presence of comparable amount of connective tissue. These parameters captured the qualitative observation (Porzionato et al., 2005) that following chronic opiate consumption the intralobular connective tissue branches in a more irregular way than in samples from aged subjects.

The parameter D deserves a more specific comment. Unlike the other parameters (estimating spatial homogeneity), it measures the rate of addition of structural detail with increasing magnification, scale, or resolution (Cutting and Garvin, 1987). Thus, it is particularly useful to describe in a compact form the "*complexity of shape*" of a structure. In this respect, the connective tissue pattern in the CB of opiate addicted subjects exhibited a statistically significant higher level of structural complexity when compared to the one observed in aged subjects. Also this finding is consistent with a higher degree of branching likely involving the intralobular regions of the CB. Interestingly, as far as this parameter is concerned, no significant differences were observed between aged and young subjects, showing that this parameter is not influenced (or only minimally) by the overall amount of connective tissue. Instead, the parameter D showed significantly different values between aged and opiate-addicted subjects, confirming the capability of this parameter to catch differences in the complexity of disposition of connective tissue, independently from quantitative aspects. Thus, we may state that beyond differences in the total amount, CB connective tissue is similarly organized in young and aged subjects whereas it is organized in a more complex irregular way in the opiate addicted subjects.

In addition to the standard statistical difference tests, the ability of the abovementioned parameters to discriminate between the two groups with comparable amount of connective tissue was here tested by ROC analysis, a technique essential in clinical sciences for evaluating the potential value of a diagnostic test (Pantic et al., 2013). According to this method, the potentially discriminatory performance of a parameter is evaluated by analyzing the area under the ROC curve. In this study the parameters provided by the fractal analysis (D and lacunarity) showed the highest accuracy and efficiency as binary classifiers. Thus, they appear as a

particular useful tool to complement dimensional parameters in order to describe complex tissue patterns (as the one exhibited by the connective tissue in the CB) and the changes they undergo under pathological conditions.

It can also be emphasized that the presented methods are not limited to the specific staining method used in the present study, but they can be applied to all the methods commonly used to visualize the connective tissue (as, for instance, Masson's trichromic and Sirius red), provided the image pre-processing includes a suitable segmentation step for this tissue component, such as color thresholding or color deconvolution (Ruifrok and Johnston, 2001; Rey et al., 2008), which must be considered preliminary to the use of the above methods. Furthermore, the analysis here presented could be easily adapted and extended to provide a morphometric description of spatial complexity of other CB tissue patterns, such as those generated by sustentacular type II cells, vessels, or innervation. In various experimental or clinical conditions, these structural components could have no changes in terms of volume fraction or density but changes in spatial disposition which could have a biological significance and which could be morphometrically described with the above methods, and particularly with fractal analysis.

AUTHOR CONTRIBUTIONS

All the authors (Diego Guidolin, Andrea Porzionato, Cinzia Tortorella, Veronica Macchi, and Raffaele De Caro) contributed substantially to the conception or design of the work, or to the acquisition, analysis, or interpretation of data of the work. All the authors drafted or revised the work critically for important intellectual content, and they approved the final version to be published. All the authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

ACKNOWLEDGMENT

The study was supported by a grant from the University of Padova (ex-60%) to Cinzia Tortorella.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 08 September 2014; accepted: 17 October 2014; published online: 05 November 2014.

*Citation: Guidolin D, Porzionato A, Tortorella C, Macchi V and De Caro R (2014) Fractal analysis of the structural complexity of the connective tissue in human carotid bodies. Front. Physiol. **5**:432. doi: 10.3389/fphys.2014.00432*

This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology.

frontiers in PHYSIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 31 October 2014
doi: 10.3389/fphys.2014.00427

In the carotid body, galanin is a signal for neurogenesis in young, and for neurodegeneration in the old and in drug-addicted subjects

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The carotid body is a highly specialized chemoreceptive structure for the detection of and reaction to hypoxia, through induction of an increase in hypoxia inducible factor. As tissue hypoxia increases with aging and can have dramatic effects in respiratory depression induced by drug addiction, we investigated the carotid body in young and old healthy subjects in comparison with drug-addicted subjects, including the expression of the neurotransmitter galanin. Galanin expression was recently reported for neuronal-like cells of the human carotid body, and it is implicated in several functions in neurons. In particular, this includes the regulation of differentiation of neural stem cells, and participation in the development and plasticity of the nervous system. Using immunohistochemistry detection, we demonstrate that galanin expression in the human carotid body in healthy older subjects and drug-addicted subjects is significantly reduced in comparison with healthy young subjects. This demonstrates not only the effects of normal aging and senescence, but also in the drug-addicted subjects, this appears to be due to a disorganization of the chemo-sensory region. With both aging and drug addiction, this results in a physiological reduction in neuronal-like cells, coupled with interlobular and intralobular increases in connective tissue fibers. Consequently, in both aging and drug addiction, this reduction of neuronal-like cells and the regeneration suggest that the carotid body is losing its sensory capabilities, with the transmission of chemoreceptive signals dramatically and vitally reduced. The level of galanin expression would thus provide a signal for neurogenesis in young subjects, and for neurodegeneration in older and drug-addicted subjects.

Keywords: galanin, human carotid body, neuronal-like cell, chemoreception, hypoxia, neurogenesis, drug addiction, aging

INTRODUCTION

In humans, the pleiotropic 30-amino-acid neuropeptide galanin is widely distributed in the central nervous system, where it is biologically active and participates in the modulation of several ascending neurotransmitter systems, including cholinergic, noradrenergic, and serotoninergic pathways (Tatemoto et al., 1983; Crawley et al., 2002). Three galanin receptors have been identified (GalR1-3), and these signal through G-protein-coupled mechanisms in tissue-specific and cell-specific manners, to modulate a wide array of homeostatic and pathological processes (Counts et al., 2003). Galanin acts as neurotrophic/neuroprotective factor for several neuronal populations, and it is involved in the plasticity of the nervous system. Furthermore, galanin administration results in up-regulation of genes involved in pro-survival/pro-neuronal signaling pathways, and increases the number of neurons arising from differentiation of olfactory sensory neuron progenitors (Cordeo-Llana et al., 2014). Treatment of wild-type and GAL knock-out neural stem cells with galanin and the GalR2-specific agonist Gal2-11 under differentiation conditions significantly promotes neuritogenesis, which is inhibited by the galanin antagonist M35 (Ma et al., 2008). Galanin thus regulates differentiating neural stem cells, and in this way it participates in the development and plasticity of the nervous system.

Recently, Di Giulio et al. (2014) reported on the selective expression of galanin in neuronal-like cells in the human carotid body. In previous studies, galanin has been described in the animal carotid body (Kameda, 1989; Ichikawa and Helke, 1993; Finley et al., 1995). Furthermore, in a study on the presence and localization of the three galanin receptor subtypes (at the mRNA and protein levels), GalR1 and GalR2 were identified in neuronal-like cells, but not in sustentacular cells, while GalR3 was negative for the whole of the carotid body (Porzionato et al., 2010).

The carotid body is a well-defined chemoreceptive anatomical structure with contiguity of function with the carotid arterial bifurcation. Its specialized physiological role is as an arterial chemoreceptor, to modulate the ventilatory volume and

frequency in response to hypoxia, hypercapnia, and acidosis. The carotid body has a lobular organization, with the lobes separated by thin connective septa. The cellular component includes neuronal-like, or Type I, cells, and sustentacular, or Type II, cells (Verna, 1979; Pallot, 1987). The neuronal-like cells are considered to be chemoreceptor units (Prabhakar, 2000, 2006; López-Barneo et al., 2008) that can release several neurotransmitters and neuromodulators in response to stimulating conditions (Iturriaga and Alcayaga, 2004; Nurse, 2005; Shirahata et al., 2007; Porzionato et al., 2008). This, in turn, elicits nervous impulses that are conveyed through glosso-pharyngeal afferent fibers that arise from the petrosal ganglion (Iturriaga et al., 2007). The sustentacular cells are glial-like cells that express astrocytic markers and have a supportive role (Pallot, 1987), although when exposed to prolonged hypoxia, they have been described as having a role in the production of stem-cell precursors for neuronal-like cells (Pardal et al., 2007).

In aging and, in particular, in a state of drug addiction, the carotid body undergoes several morphological, physiological, and biochemical modifications (Porzionato et al., 2005; Zara et al., 2013). Typically, there are changes in the cell populations, such as an increase in the connective tissue fiber compartment, which results in a reduction in the sensory tissue, and represents a sign of tissue aging. In drug-addicted subjects, early tissue aging has been coupled to episodes of respiratory depression and arteriosclerosis of the glomic arteries (Di Giulio et al., 2003; Porzionato et al., 2005; Zara et al., 2013).

In the present study, we investigated the expression of galanin in the human carotid body in young and old healthy subjects, in comparison with drug-addicted subjects. Our data indicate that galanin provides a signal for neurogenesis in young subjects, and for neurodegeneration in older subjects and in those under the pathological conditions of drug addiction.

MATERIALS AND METHODS

In the present study were selected human carotid bodies ($n = 15$) that were collected from 12- to 72-h postmortem subjects without chronic pulmonary or cardiovascular disease. Exclusion criteria as cardiac hypertrophy or previous myocardial infarction were also excluded at autopsy examination. Pathological carotid body specimens ($n = 8$; males; mean age, 27 ± 3.5 years) were collected from subjects with a history of drug addiction and with drug taking as the cause of death. Toxicological investigations for a group of drugs, including cocaine, methadone, amphetamines, benzodiazepines, cannabis, and alcohol, were performed on urine samples and venous blood samples, across concentration ranges of 0.5–103.2 mg/ml and 0.5–31.1 mg/ml, respectively. The control carotid body specimens were collected from young males ($n = 3$; mean age, 30 ± 3.5 years) and older males ($n = 4$; mean age, 70 ± 5.5 years) who had died by accidental trauma. The urine and venous blood samples of these controls were negative in the toxicological investigations. The study received ethical approval from the local Research Board.

The specimens were fixed in neutral 10% formalin, embedded in paraffin wax, and sectioned (5 μm), followed by histological staining with Mallory trichrome (Bio Optica; Milan, Italy). The mouse monoclonal anti-galanin antibody (H-11: sc166431; Santa Cruz Biotechnology; CA, USA) and anti-hypoxia-inducible factor (HIF) antibody (H1α 67, sc-53546; Santa Cruz Biotechnology; CA, USA) and developing kits (UltraVision LP Detection System HRP Polymer & DAB Plus Chromogen, Lab Vision Thermo Scientific; CA, USA) were used for the immunohistochemistry. For light microscopy and the data acquisition system, a Leica DM 4000 microscope was used, which was equipped with a Leica DFC 320 digital acquisition system (Leica Cambridge Ltd.; Cambridge, UK). QWin Plus 3.5 software (Leica Cambridge Ltd.; Cambridge, UK) was used to digitize the images and to compute the areas positive for the antibodies. Commercial software (SPSS and Origin) were used for the data and statistical analyses (One-Way ANOVA, with α level set at 0.001, or as specified).

Tissue degeneration and alteration detected by Mallory trichrome histological staining were considered as exclusion criteria. To detect any influence of the death-to-autopsy interval carotid body volume was assessed by Cavalieri's method. Further, the ratio between the areas occupied by connective tissue and parenchyma, such as intralobular connective tissue, and the area exhibited by the whole organ in five sections per subjects was taken as an estimate of the corresponding volume fractions, statistical analysis of the linear correlations was carried out under each condition, with an α level set at 0.05 (for detail in the method see Porzionato et al., 2005).

RESULTS

The Mallory trichrome staining of the human carotid body sections in the young and old healthy controls (**Figure 1**) showed tissue senescence related to aging. In the sections from the drug-addicted subjects, there was apparent pathological disorganization of the sensory areas and increases in both the interlobular and intralobular connective tissue fibers (**Figure 2**). The One-Way ANOVA defined a significant increase in connective tissue fibers in the older and drug-addicted subjects [$F_{(2, 33)} = 56.3$; $p < 0.001$], with *post-hoc* One-Way ANOVA showing significant increases in the connective tissue fiber with aging [young vs. old: $F_{(1, 24)} = 22.0$; $p < 0.001$], and between the young healthy subjects and those who were drug-addicted [$F_{(1, 22)} = 102.6$; $p < 0.001$].

The expression of galanin was revealed by immunohistochemistry in the sections from the healthy (young and old) and drug-addicted subjects. In the young healthy subjects, positive galanin labeling was restricted to the sensory areas at the level of the neuronal-like cells, while in the older healthy subjects, the labeling was decreased in this area; there was also a further dramatic reduction in galanin expression in the tissues from the drug-addicted subjects (**Figure 3**). One-Way ANOVA showed significant decreases in galanin labeling with aging [young vs. old: $F_{(1, 23)} = 12.5$; $p < 0.001$] and in the young healthy vs. drug-addicted subjects [$F_{(1, 23)} = 21$; $p < 0.001$], as also seen between all of the groups [$F_{(2, 37)} = 14.7$; $p < 0.001$].

The expression of HIF was also revealed by immunohistochemistry in the healthy (young and old) and drug-addicted subjects (**Figure 4**). One-Way ANOVA showed significant increases in HIF labeling with aging [young vs. old: $F_{(1, 9)} = 10.6$; $p < 0.001$], and in the young healthy vs. drug-addicted subjects

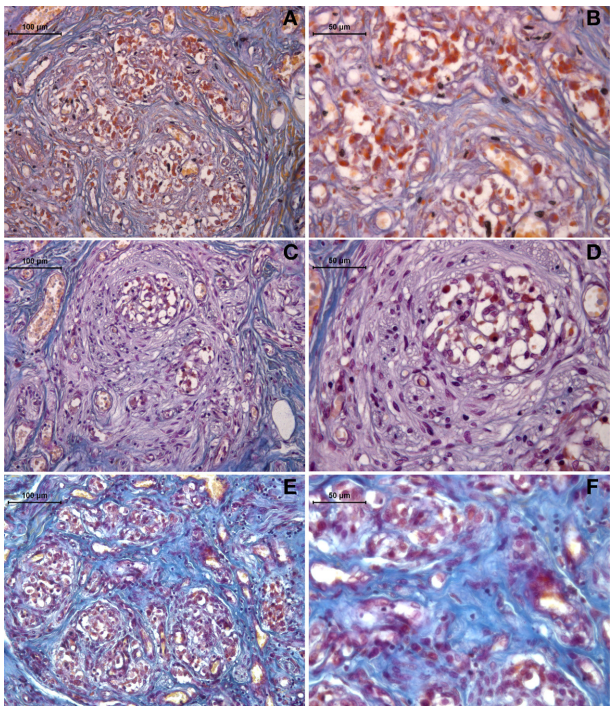

FIGURE 1 | Representative Mallory trichrome staining of human carotid body sections in young (A,B) and old (C,D) healthy subjects, compared to drug-addicted subjects (E,F). With the drug addiction, note the pathological disorganization of the sensory region and the increase in connective tissue fibers (blue), as interlobular (b) and intralobular (c).

FIGURE 2 | Morphometric analysis of the connective tissue fibers in human carotid body sections in healthy (young and old) and drug-addicted subjects, performed on Mallory trichrome staining. The One-Way ANOVA demonstrates a significant increase in the connective tissue fibers through the aging process and with drug addiction ($p < 0.001$).

$[F_{(1,\ 14)} = 4.9;\ p < 0.05]$, as also between all of the groups $[F_{(2,\ 21)} = 4.25; p < 0.05]$.

The effects of neurogenesis and hypoxia were also compared between the healthy (young and old) and drug-addicted subjects, as for galanin and HIF expression (**Figure 5**).

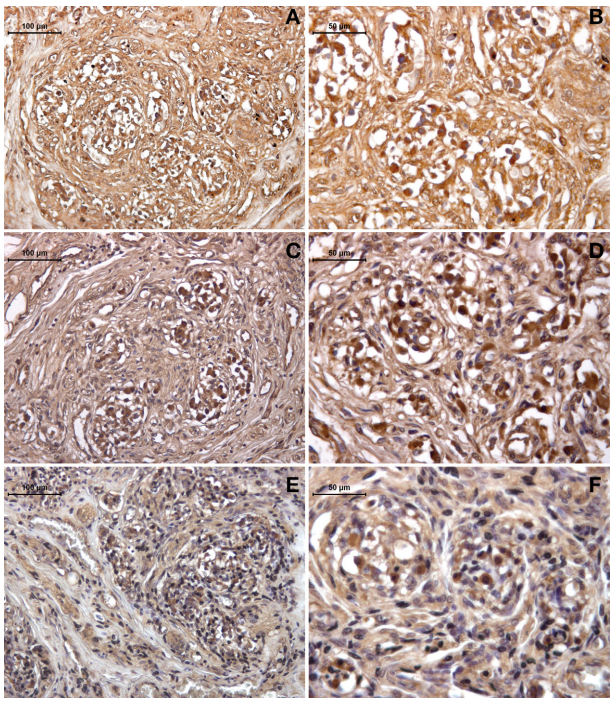

FIGURE 3 | Immunohistochemistry detection of galanin expression in human carotid body, in young (A,B) and old (C,D) healthy subjects, compared to drug-addicted subjects (E,F). With the drug addiction, note the scarce labeling that is higher than for the old healthy subjects, which shows a dramatic effect of the disorganization in the sensory region with the reduction of the neuronal-like cells and increases in the connective tissue fibers, seen as interlobular (left panel) and intralobular at higher magnification (right panel).

There were no statistically significant differences in the mean death-to-autopsy intervals between these healthy (young and old) and drug-addicted subjects. Furthermore, there were no statistically significant correlations seen between the death-to-autopsy interval on each parameter investigated ($p < 0.05$).

DISCUSSION

Although increases in connective tissue fibers are not exclusive to the aging process, its occurrence in this young population of drug-addicted subjects suggests activation of an early aging mechanism in the carotid body that would be due to the hypoxia effects of drug addiction (Porzionato et al., 2005; Zara et al., 2013). We have already shown that HIF expression increases with the aging process and in drug addiction (Zara et al., 2013), with the present data further confirming these previous data.

This early-aging phenomenon also appears to be also coupled with a selective reduction in the neuronal-like cells, which would also represent a specific character of this hypoxia effect. Furthermore, the most important evidence of the disorganization of the sensory region of the carotid body in these drug-addicted subjects was the significant reduction in galanin expression and the increase in HIF expression. The significance of these findings is in the line with a recent report where a loss of galanin led to a marked decrease in the rate of adult neurogenesis and a

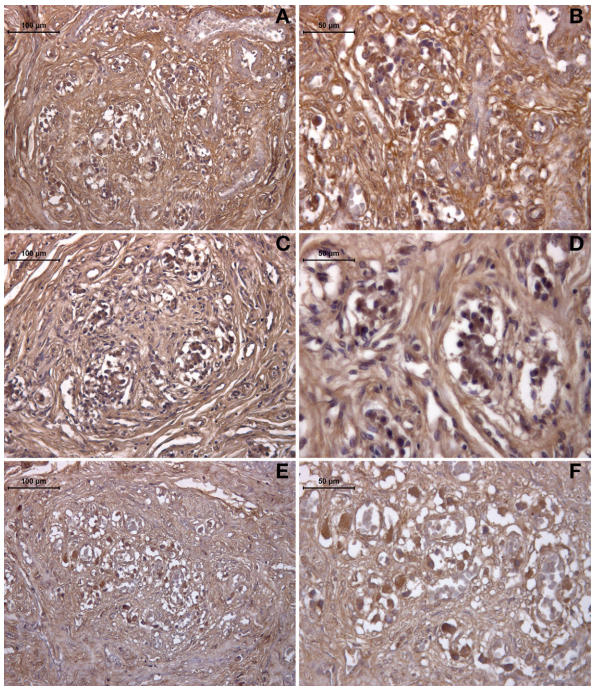

FIGURE 4 | Immunohistochemistry detection of HIF expression in the human carotid body, in young (A,B) and old (C,D) healthy subjects, compared to drug addicted subjects (E,F).

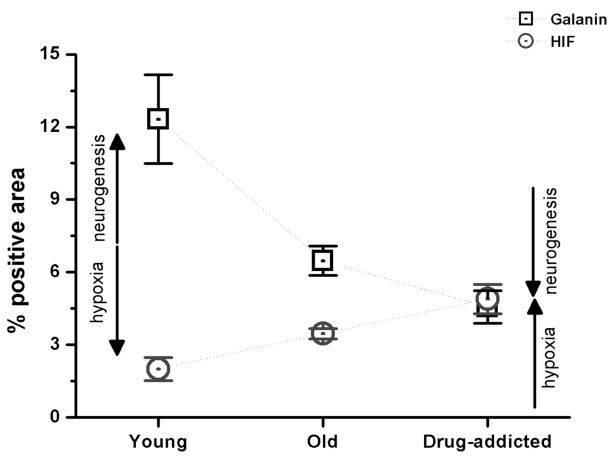

FIGURE 5 | Morphometric analysis of the positive areas for galanin and HIF in the human carotid body sections from the healthy young and old subjects, compared to the drug-addicted subjects, performed on anti-galanin immunohistochemistry labeling. One-Way ANOVA demonstrates a significant decrease in galanin expression on aging, and higher levels in drug-addicted subjects ($p < 0.001$); conversely, there is a significant increase in HIF expression upon aging, that is greater for the drug-addicted subjects.

reduction in the number of newly generated cells in the olfactory bulb (Cordeo-Llana et al., 2014).

The novelty of the present study is that it provides intriguing aspects related to the putative function of galanin in the carotid body. Galanin levels are potentially related to neuronal differentiation, such as was seen for neuroregeneration of olfactory sensory neurons (Cordeo-Llana et al., 2014). Galanin was expressed selectively in differentiating neuronal-like cells, and this is in line with what we have indirectly shown in the present study for the carotid body. This loss of galanin expression following aging and drug addiction, thus indicates here a reduction in regenerating neuronal-like cells, which in turn might suggest that the carotid body is losing its sensory capabilities. This is further supported by the increased levels of immunostaining for HIF, a known response to hypoxia (Semenza, 2000), and related to the aging process and to drug addiction (Zara et al., 2013; present study). As a consequence, the transmission of chemoreceptive signals appears to be dramatically and vitally reduced.

In conclusion, the consequences of aging and drug addiction seen here indicate severe cardio-respiratory impairment with an accelerated aging process. Our findings thus provide further evidence of the role of galanin as a modulator of neural stem cell function, and reinforce the importance of galanin for brain plasticity and repair.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 03 September 2014; accepted: 14 October 2014; published online: 31 October 2014.

Citation: Mazzatenta A, Marconi GD, Zara S, Cataldi A, Porzionato A and Di Giulio C (2014) In the carotid body, galanin is a signal for neurogenesis in young, and for neurodegeneration in the old and in drug-addicted subjects. Front. Physiol. ***5****:427. doi: 10.3389/fphys.2014.00427*

This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 29 October 2014
doi: 10.3389/fphys.2014.00418

Carotid body, insulin, and metabolic diseases: unraveling the links

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The carotid bodies (CB) are peripheral chemoreceptors that sense changes in arterial blood O_2, CO_2, and pH levels. Hypoxia, hypercapnia, and acidosis activate the CB, 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 panoply of cardiorespiratory reflexes aimed, primarily, to normalize the altered blood gases, via hyperventilation, and to regulate blood pressure and cardiac performance, via sympathetic nervous system (SNS) activation. Besides its role in the cardiorespiratory control the CB has been proposed as a metabolic sensor implicated in the control of energy homeostasis and, more recently, in the regulation of whole body insulin sensitivity. Hypercaloric diets cause CB overactivation in rats, which seems to be at the origin of the development of insulin resistance and hypertension, core features of metabolic syndrome and type 2 diabetes. Consistent with this notion, CB sensory denervation prevents metabolic and hemodynamic alterations in hypercaloric feed animal. Obstructive sleep apnea (OSA) is another chronic disorder characterized by increased CB activity and intimately related with several metabolic and cardiovascular abnormalities. In this manuscript we review in a concise manner the putative pathways linking CB chemoreceptors deregulation with the pathogenesis of insulin resistance and arterial hypertension. Also, the link between chronic intermittent hypoxia (CIH) and insulin resistance is discussed. Then, a final section is devoted to debate strategies to reduce CB activity and its use for prevention and therapeutics of metabolic diseases with an emphasis on new exciting research in the modulation of bioelectronic signals, likely to be central in the future.

Keywords: carotid body, chronic intermittent hypoxia, insulin resistance, metabolic dysfunction, obstructive sleep apnea

THE CAROTID BODIES

The carotid bodies (CB) are peripheral chemoreceptors located bilaterally in the bifurcation of the common carotid artery that classically sense changes in arterial blood such as low O_2 (hypoxia), high CO_2 (hypercapnia), and low pH (acidosis). Hypoxia and acidosis/hypercapnia activate the CB, inducing an increase in the frequency of discharge in the nerve endings of its sensorial nerve, the carotid sinus nerve (CSN). The CSN activity is integrated in the *nucleus solitary tract* to induce a myriad of respiratory reflexes aimed to normalize the altered blood gases, via hyperventilation (Gonzalez et al., 1994), and to regulate blood pressure and cardiac performance via an increase in the activity of the sympathetic branch of the autonomic nervous system (SNS) (Marshall, 1994) (see **Figure 1**). The chemoreceptor cells, also known as glomus or type I cells, are the main cellular constituent of the CB and are generally accepted as its chemosensory unit. These cells, which are derived of the neural crest, contain several classical neurotransmitters including, catecholamines [CA; dopamine (DA), and norepinephrine (NE)], serotonin, ACh, neuropeptides (substance P and enkephalins) and adenosine (Ado) and ATP (Gonzalez et al., 1994; Zhang et al., 2000; Rong et al., 2003; Buttigieg and Nurse, 2004; Conde and Monteiro, 2004; Conde et al., 2012a). All these substances, their agonists and antagonists are capable of modifying, inhibiting or stimulating CSN activity. In addition to chemoreceptor cells, the CB also possesses type II cells, or sustentacular cells and it has been proposed that they are adult neural stem cells sustaining neurogenesis *in vivo* in response to physiological stimuli, like chronic hypoxia, and acting in paracrine signaling during hypoxia (Pardal et al., 2007; Piskuric and Nurse, 2013).

ROLE OF CAROTID BODY IN METABOLISM

EVIDENCES FOR A ROLE OF CAROTID BODY IN GLUCOSE HOMEOSTASIS

The idea of a physiological role of the CB on the control of glucose metabolism was first suggested by Petropavlovskaya in the 50's. In this pioneer study it was shown that the stimulation of the CB induces a reflex hyperglycemia, an effect that is mediated

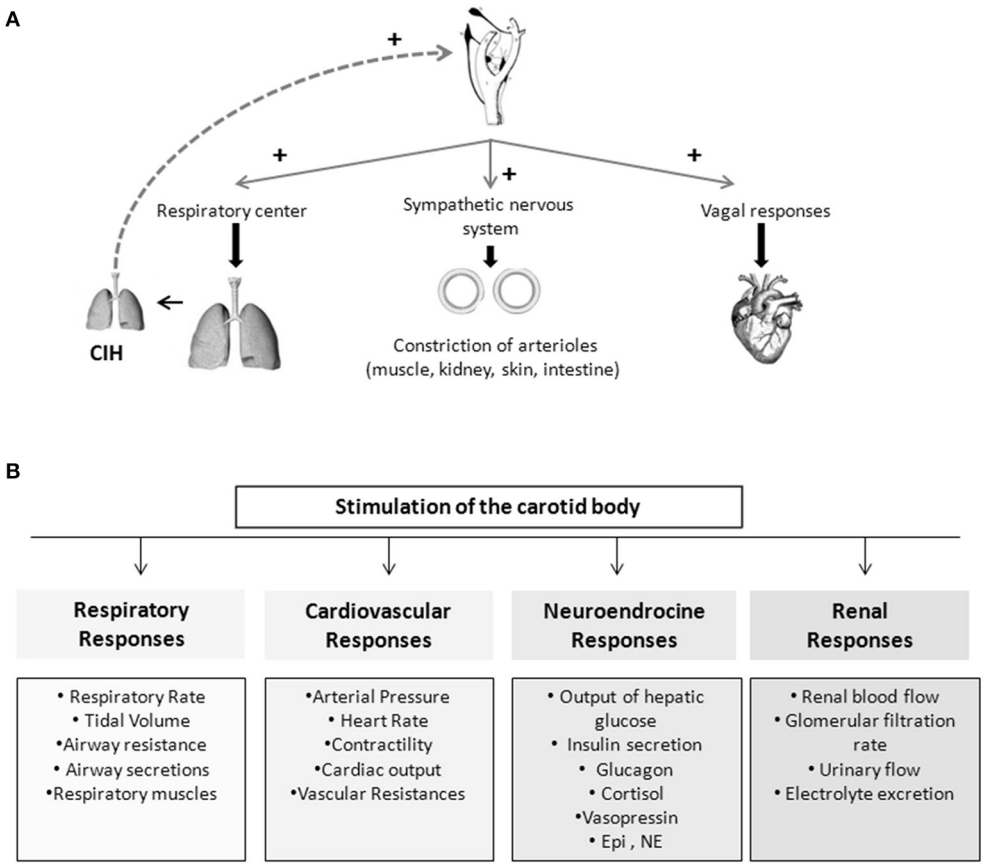

FIGURE 1 | Schematic representation of the chemoreflexes elicited by the carotid bodies. (A) Representation of important mechanism involved in the reflex-responses elicited by the carotid body. **(B)** Stimulation of the carotid body is capable of produce cardiovascular, respiratory, endocrine, and renal responses.

by the adrenal medulla, since it was not observed in adrenalectomized animals (Petropavlovskaya, 1953). Twenty five years later, Alvarez-Buylla and de Alvarez-Buylla (1988) confirmed those results by demonstrating that the pharmacological stimulation of the CB with cyanide (NaCN) produced an increase in hepatic glucose output in cats, this reflex response being eliminated by bilateral adrenalectomy or by surgical removal of the neurohypophysis (Alvarez-Buylla et al., 1997). Also, it was shown that changes in blood concentration in the CB-CSN, superfused *in vivo*, modify brain glucose retention, suggesting that chemosensory activity in the CSN controls brain glucose metabolism (Alvarez-Buylla and de Alvarez-Buylla, 1994). In parallel with the increase in hepatic glucose output, one would expect an increase in plasma insulin levels to ensure an adequate glucose utilization by the peripheral tissues and, in fact, stimulation of CBs by corconium, a nicotinomimetic agent, caused a rise in circulating insulin that was reversed by CSN resection (Anichkov and Tomilina, 1962). Later on, Koyama et al. (2000) demonstrated that CB plays an important role in glucose homeostasis *in vivo*, since dogs that have their CB resected presented lower arterial glucagon in basal conditions and reduced glucagon and cortisol levels during insulin-induced hypoglycemia, together with a marked decrease in endogenous hepatic glucose production in response to hypoglycemia, and with an increase in insulin sensitivity, independent of blood glucose level. These last results suggested for the first time that CB resection affects the response to moderate hyperinsulinemia and therefore, that the CB may play a role in glucose homeostasis that is not related with the hypoglycemic counterregulatory response.

The results obtained by Koyama et al. (2000) were supported by clinical studies where it was demonstrated that, the rate of glucose infusion necessary to maintain glucose levels in a hyperinsulinemic-hypoglycemic clamp was significantly higher during hyperoxia than in normoxia (Wehrwein et al., 2010). In the same study, the authors also observed that hyperoxia, which blunts CB activity, decreased the release of counter-regulatory hormones such as adrenaline, cortisol, glucagon and growth hormone, which seems to indicate that the CB play an important role in neuroendocrine responses during hypoglycemia (Wehrwein et al., 2010). However, the absence of adequate controls in hyperinsulinemic-euglycemic conditions in this study does not allow assigning the effects to the hyperinsulinemia *per se* or to hypoglycemia. In another clinical study designed to determine whether hypo- and hyperglycaemia modulate the ventilatory responses to hypoxia, it was shown that hypoglycemia, as well as hyperglycemia, produced an increase in ventilation and in the hypoxic ventilatory response, being the latter accompanied

by an increase in circulating counter-regulatory hormones (Ward et al., 2007). Interestingly, both hypo- and hyperglycemia were obtained under hyperinsulinemic conditions, and therefore it is possible that the effect in ventilation observed was due to hyperinsulinemia rather than to altered glucose concentrations. More recently, our laboratory has shown that CBs are overactivated in diet-induced animal models of insulin resistance and hypertension (Ribeiro et al., 2013). Also, we have demonstrated that insulin resistance and hypertension produced by hypercaloric diets are completely prevented by chronic bilateral CSN resection, and these results strengthen the link between CB dysfunction and the development of insulin resistance (Ribeiro et al., 2013). 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 (Ribeiro et al., 2013). Therefore, the research in the field performed since Petropavlovskaya work in the early 1950's strongly supports that the CB is a key organ in glucose homeostasis and that its dysfunction contributes to the pathogenesis of metabolic disturbances.

GLUCOSE SENSING IN THE CAROTID BODY

One of the hypotheses that came out to explain the role of the CB in glucose homeostasis was the potential of the CB as a glucosensor. Whereas some *in vivo* and *in vitro* studies, performed in cultured CB chemoreceptor cells or slices, had shown that CB could respond to blood glucose levels, (Koyama et al., 2000; Pardal and Lopez-Barneo, 2002; Zhang et al., 2007) others have completely denied a direct involvement of the CB in glucose sensing (Almaraz et al., 1984; Bin-Jaliah et al., 2004, 2005; Conde et al., 2007; Fitzgerald et al., 2009; Gallego-Martin et al., 2012). Due to these controversial results, the sensitivity of the CB to hypoglycaemia is still a hot topic in the CB field.

In cultured CB slices, perfusion with low or glucose-free solutions at a PO_2 ≈150 mmHg produced an increase in CAs release from chemoreceptor cells with a magnitude comparable to the response evoked by hypoxia and potentiated hypoxic responses (Pardal and Lopez-Barneo, 2002). Moreover it was found that low glucose inhibited K^+ currents (Pardal and Lopez-Barneo, 2002) in an extent similar to the observed by Peers during intense hypoxia (Peers, 1990); low glucose also promoted Ca^{2+} entry in chemoreceptor cells (Pardal and Lopez-Barneo, 2002). Lopez-Barneo's group published that sensitivity to low glucose and to hypoxia depends on different signal transduction mechanisms, although they converge on the final steps causing transmembrane Ca^{2+} influx and transmitter release (García-Fernández et al., 2007). Almost at the same time, but using an experimental model of co-culture of type I clusters and afferent petrosal neurons, Zhang et al. (2007) described that low glucose increased the spiking activity in the neurons, this increase being sensitive to purinergic and nicotinic blockers, implying that low glucose stimulates chemoreceptor cells and promotes the release of ATP and ACh. Contrasting with these results, CSN activity in freshly isolated cat and rat CB–CSN preparation was not modified by perfusion with glucose-free or low-glucose solutions (Almaraz et al., 1984; Bin-Jaliah et al., 2004, 2005). Also, Conde et al. (2007) demonstrated that low glucose concentrations neither activate the release of neurotransmitters, namely CAs and ATP, from the CB, nor altered basal and hypoxia (5% O_2)-induced CSN action potential frequency in freshly isolated whole CB preparations (Conde et al., 2007). In the same line, Fitzgerald et al. (2009) showed that the release of ATP from the cat CB was not modified in the presence of hypoglycemia but, surprisingly, they observed an increase in the release of ACh in the same conditions (Fitzgerald et al., 2009). Additionally, it was shown that withdrawal of glucose from the perfusion media did not activate the K_{ATP} channels, suggesting that this channel was insensitive to hypoglycemia (Kim et al., 2011). Altogether these results suggest that low glucose is not a direct stimulus for the CB chemoreceptors and do not support a significant physiological role of the CB as a glucose sensor.

Several differences can account for these discrepant results regarding glucose sensing in the CB, namely species differences, different dissociation protocols or culture conditions that lead to an altered cells phenotype, as suggested by Kumar (2007), or even the differences in the PO_2 levels used by some authors, as postulated by Zhang et al. (2007). However, Conde et al. (2007) have shown in the whole CB that low or absent glucose does not activate either chemoreceptor cells or the CB–CSN complex at different PO_2 tested in a very wide range (∼133, 66, 46, and 33 mmHg) and thus, differences in the PO_2 used in the experiments in intact preparations vs. slices or co-cultures is not the factor determining divergent findings, as suggested by Zhang et al. (2007). More recently, Gallego-Martin et al. (2012) demonstrated that in intact CBs cultured during 1 day, but not in freshly isolated organs, 0 mM glucose media potentiates the release of CAs elicited by hypoxia and that chemoreceptor cells in culture become transiently more dependent on glycolysis suggesting that the scarcity of glucose leads the cells to acquire the ability to increase their neurosecretory response to hypoxia. Another relevant issue in the discussion is the duration of glucose deprivation. While glucose reduction or deprivation did not have an effect when applied for short periods of time (<15 min), either in basal conditions or in response to hypoxia, when applied for longer periods of time (up to 120 min) it caused a spontaneous increase in basal release of CAs observable after 40 min of glucose deprivation. Concomitantly, bursts of CSN activity were observed with a comparable time course to the release of CAs, that culminated in a complete loss of the capacity of the CSN to respond to hypoxia (Conde et al., 2007). Consistent with these findings Holmes et al. (2014) have recently demonstrated that basal CSN activity was sustained during glucose deprivation approximately for 30 min before irreversible failure following a brief period of increased activity. Also, they showed that pharmacological inhibition of glycogenolysis and depletion of glycogen reduced the time to glycolytic run down, suggesting that glycogen metabolism in chemoreceptor cells allows glycogenolysis and the maintenance of CSN basal activity during hypoglycemia (Holmes et al., 2014). Therefore, glycogen metabolism may account for the differences reported in the capacity of the CB to sense glycemia and could contribute to CB responses in pathological conditions associated with an overstimulation of the organ.

IS INSULIN A STIMULUS FOR CB ACTIVATION?

A large body of literature supports a role for the central nervous system in insulin-induced sympathoexcitation, as the injection of insulin on *arcuate nucleus* and *paraventricular nucleus* has been shown to produce an increase in spinal sympathetic outflow, mediated by dorsal hypothalamus and rostral ventrolateral medulla (for a review see Dampney, 2011). However, this effect cannot be exclusively assigned to a centrally-mediated mechanism, since the injection of insulin into the carotid artery of anesthetized dogs produces an increase in blood pressure and sympathetic activity higher than the systemic insulin administration, being the effect abolished by ganglionic blockade (Pereda et al., 1962). These results were the first to suggest a role for the peripheral nervous system in insulin-mediated sympathetic activity. During the evaluation of a putative direct role of the CB in glucose sensing, Bin-Jaliah et al. (2004) observed that insulin infusion, used to produce hypoglycemia, increased minute ventilation and the rate of O_2 consumption (VO_2), an effect that was totally mediated by the CB, since CSN denervation blunted it. The same authors demonstrated afterwards that insulin-induced hypoglycemia was associated with a significantly increase in CO_2 chemosensitivity, an effect that was mediated by the CB, since the effect was lost in animals that had their CSN resected (Bin-Jaliah et al., 2005). Since *in vitro* hypoglycemia was incapable of modifying basal CSN activity (Bin-Jaliah et al., 2004; Conde et al., 2007) and blunted the response of CSN to hypercapnia (Bin-Jaliah et al., 2005) the elevation of ventilation observed *in vivo* by Bin-Jaliah's group was somehow surprising (Bin-Jaliah et al., 2004, 2005) and the hypothesis of being an indirect consequence of systemic hypoglycemia related to some other undetermined substance had to be considered. To pursue this hypothesis, our group has been dedicated to investigate whether insulin itself is capable of stimulating the CB and of eliciting a neurosecretory response. We have demonstrated the presence of insulin receptors in the rat CB by western-blot and its phosphorylation in response to insulin (Ribeiro et al., 2013). The presence of insulin receptors was also confirmed on finding that isolated whole CBs incubated with insulin accumulate more 2-deoxiglucose than the diaphragm muscle (Gallego Martin et al., 2014). Insulin is also capable to induce a rise in intracellular Ca^{2+} in chemoreceptor cells and to elicit the release of ATP and dopamine from the whole CB in a concentration-dependent manner (Ribeiro et al., 2013). As schematically represented in **Figure 2**, we have also shown that this neurosecretory response is transduced into an increase in ventilation in the whole animal, as insulin increased the spontaneous ventilation in a dose-dependent manner during an euglycemic clamp (Ribeiro et al., 2013). The increase in ventilation induced by insulin is mediated by the CB, since it is absent in animals that had their CSN resected (Ribeiro et al., 2013). Contrarily to our results, Bin-Jaliah et al. (2004) proposed that the ventilatory and metabolic effects observed *in vivo* were not due to insulin *per se*, since the increase in ventilation produced by insulin was absent during an euglycemic clamp. However, some differences in the methodology used can be in the basis of these discrepancies. In our study we have administrated a bolus of insulin intracarotidally to guarantee that the first site of insulin action is the CB, and not systemically as Bin-Jaliah et al. (2004, 2005). Also we performed a dose-response curve in which several concentrations of insulin were tested, making the results more robust in terms of concluding on a role of insulin in CB modulation. In fact, the neurosecretory response and the increase in ventilation elicited by insulin in our experimental setting support the idea that insulin is a very powerful stimulus for CB activation. Nevertheless, these findings do not exclude that the central nervous system is also involved in the sympathetic activation observed in response to circulating insulin and more studies are required to clarify the exact contribution of both the peripheral and the central nervous system in this process. It is undoubtedly however, that the overactivation of the SNS, measured as the increase in plasmatic CAs (norepinephrine + epinephrine) and in CAs (norepinephrine + epinephrine) content of the adrenal medulla (**Figure 3**) and the insulin resistance (**Figure 4**) seen in hypercaloric animal models are prevented by surgical resection of the CSN. These findings point toward a new role for the CB in the regulation of peripheral insulin sensitivity and in the pathogenesis of insulin resistance (Ribeiro et al., 2013).

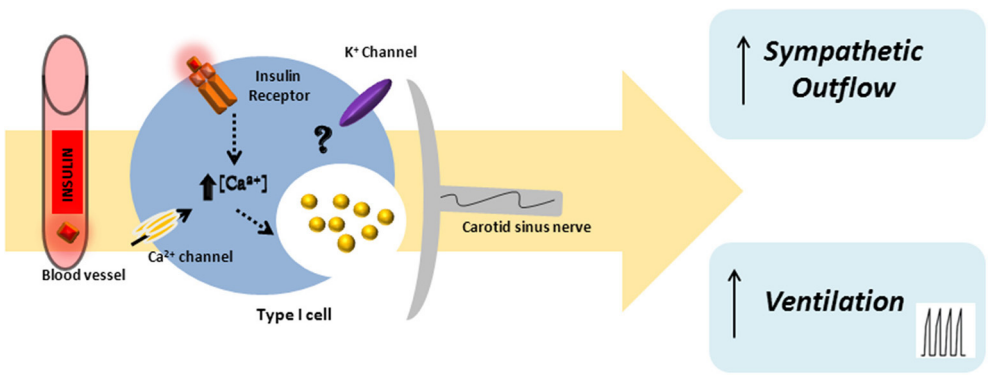

FIGURE 2 | Schematic representation of insulin action in the carotid body. Insulin will act on insulin receptors present in the carotid body chemoreceptor cells eliciting an increase in intracellular Ca^{2+} and the release of neurotransmitters, such as dopamine and ATP. The insulin-induced neurosecretory response in chemoreceptor cells is transduced in an increase in ventilation and in an augmented sympathetic outflow.

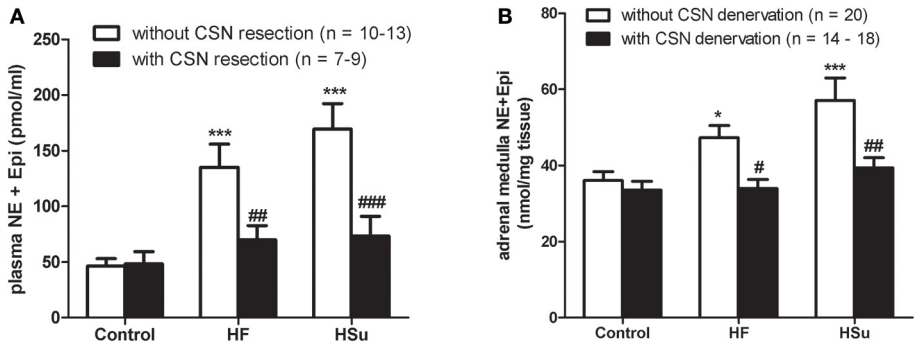

FIGURE 3 | Effect of carotid sinus nerve resection on sympathetic nervous system activity, measured as circulating catecholamines [norepinephrine (NE) + epinephrine (Epi)] (A) and adrenal medulla catecholamines (NE + Epi) content (B), in control, high fat (HF) and high sucrose (HSu) diet rats. Bars represent mean ± s.e.m. Two-Way ANOVA with Bonferroni multicomparison tests; $^{*}p < 0.05$, $^{***}p < 0.001$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. values within the same group (adapted from Ribeiro et al., 2013).

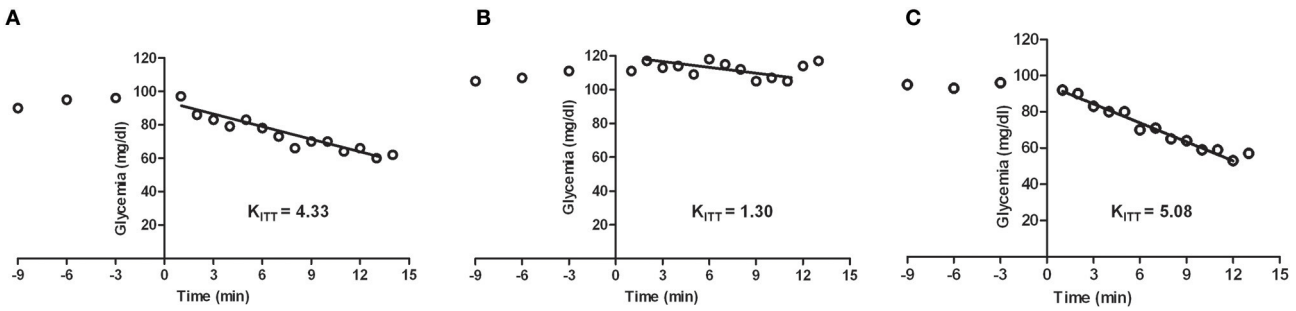

FIGURE 4 | Representative excursion curves for the insulin tolerance test in control (A), high fat (HF) (B), and high fat animals submitted to carotid sinus nerve resection (C) rats. Note that insulin sensitivity, expressed by the constant of the insulin tolerance test (KITT) decrease in the HF animals, this decrease being completely prevented by the bilateral resection of the carotid sinus nerve. HF animals were achieved by submitting the animals to a HF diet (45% lipid-rich diet) during 21 days. Bilateral resection of the carotid sinus nerve **(C)** was performed 5 days prior to submitting the animals to HF diet (adapted from Ribeiro et al., 2013).

LINKING INSULIN, SYMPATHETIC NERVOUS SYSTEM ACTIVATION AND METABOLIC DYSFUNCTION: THE ROLE OF THE CAROTID BODY

The sympathetic nervous system (SNS) is an important component of the autonomic nervous system playing a major role in the maintenance of homeostasis due to its involvement in the control of the cardiovascular system and of several metabolic processes. Sympathetic overactivity has been associated with several diseases, such as cardiovascular diseases (Graham et al., 2004), kidney disease (Converse et al., 1992), and metabolic disturbances, including type 2 diabetes (Huggett et al., 2003; Grassi et al., 2005, 2007; Kobayashi et al., 2010). In metabolic diseases the increase in sympathetic activation has been attentively associated with hyperinsulinemia, hyperleptinemia increased non-esterified free fatty acids, inflammation, and obesity among others, however the precise mechanisms remain to be unequivocally elucidated (Lambert et al., 2010).

INSULIN-INDUCED SYMPATHETIC OVERACTIVATION

It is known since the early 80's that insulin stimulates sympathetic nerve activity (Rowe et al., 1981) and, more recently, it has been shown that this stimulation occurs at blood insulin concentrations within the physiological range (Hausberg et al., 1995). In fact, the relationship between hyperinsulinemia and the increased sympathetic nerve activity lead Landsberg to propose in 1986 a causal relationship between metabolic disturbances, such as insulin resistance and dyslipidemia, and overactivation of the SNS (Landsberg, 1986).

In the last decades several reports were published, both in animals and in humans, supporting the hypothesis that insulin increases sympathetic nerve activity. In humans insulin has been shown to increase muscle sympathetic nerve activity (MSNA) (Anderson et al., 1991; Scherrer et al., 1993; Vollenweider et al., 1993) as well as norepinephrine levels (Anderson et al., 1991; Lambert et al., 2010) in euglycemic conditions. The MSNA response observed in response to insulin administration is both gradual (Anderson et al., 1991; Scherrer et al., 1993; Vollenweider et al., 1993, 1994; Banks, 2004) and sustained because MSNA remains increased even after plasma insulin levels return to baseline (Anderson et al., 1991; Scherrer et al., 1993; Vollenweider et al., 1993, 1994; Banks, 2004). In rats and dogs, insulin infusion also increases sympathetic nerve activity along with an increase in plasma norepinephrine levels (Liang et al., 1982; Tomiyama et al., 1992). However, the discovery that insulin infusion did

not increase sympathetic nerve activity in the skin in humans (Berne et al., 1992) and also that graded increases in plasma insulin failed to significantly increase renal or adrenal sympathetic activity in rats though leading to increased lumbar SNS activity, lead to the hypothesis that hyperinsulinemia produces regionally non-uniform increases in sympathetic nerve activity (Morgan et al., 1993). Also, while some authors claim that the relationship between insulin concentrations and sympathetic nerve activity is dose-dependent (Anderson et al., 1991; Berne et al., 1992), others have shown that this relationship is not apparent (Vollenweider et al., 1993, 1994) attributing this effect to a saturation of the receptors needed for insulin to cross the blood brain barrier (Banks et al., 1997; Dampney, 2011). The slow rise and fall in MSNA produced by hyperinsulinemia would be explained by the time insulin needs to cross the blood brain barrier (Banks, 2004).

As reviewed previously, our group demonstrated that insulin is capable of stimulating the CB eliciting a hyperventilatory response (Ribeiro et al., 2013) (**Figure 2**). These results are in accordance with the recent findings by Limberg et al. (2014) where hyperoxic silencing of carotid chemoreceptors reduced MSNA in hyperinsulinemic conditions, suggesting that the CB also mediates insulin-dependent sympathoexcitation in humans (Limberg et al., 2014).

THE ROLE OF CAROTID BODY IN METABOLIC DYSFUNCTION

SNS activation is implicated in the pathogenesis of metabolic diseases and in the specific components of the metabolic syndrome, such as insulin resistance, hypertension, dyslipidemia and obesity (Kahn and Flier, 2000; Esler et al., 2006; Tentolouris et al., 2006; Mancia et al., 2007). The idea that sympathetic hyperactivity contributes to the development of insulin resistance is not new (Defronzo, 1981), although the mechanisms involved in the association between sympathetic nerve activity and insulin resistance (Egan, 2003; Tentolouris et al., 2006; Tsioufis et al., 2007, 2011), are complex and not clearly understood, and several questions remain unanswered, including how is promoted the sustained activation of the SNS that characterizes metabolic diseases. Our group has recently proposed that the CB is the common link between sympathetic nerve activity, insulin resistance and hypertension (Ribeiro et al., 2013) (**Figure 5**). The CBs contribute to regulate blood pressure and cardiac performance via SNS activation (Marshall, 1994) and through an increased sympathetic drive, the CB directly activates the adrenals and increases the sympathetic vasoconstrictor outflow to muscle, splanchnic, and renal beds (Marshall, 1994; Cao and Morrison, 2001; Schultz et al., 2007). Therefore, we have hypothesized that an overactivation of the CB contributes to the genesis of insulin resistance, core pathological feature of metabolic disorders as type 2 diabetes or the metabolic syndrome. In fact, we have shown that animal models of diet-induced prediabetes develop an overactivation of the CB; measured as an increased spontaneous ventilation as well as increased respiratory responses to ischemic hypoxia; increased hypoxia-evoked release of dopamine and increased expression of tyrosine hydroxilase (Ribeiro et al., 2013). This overactivation of the CB results in an increase in SNS activity, measured as circulating CAs and the adrenal medulla CAs content (**Figure 3**), and in an reduction in insulin sensitivity (**Figure 4**) (Ribeiro et al., 2013). All these characteristic features of metabolic diseases were prevented by CSN resection (Ribeiro et al., 2013) meaning that the CB is primordial in controlling peripheral insulin sensitivity and that CB dysfunction is involved in the genesis of these disturbances.

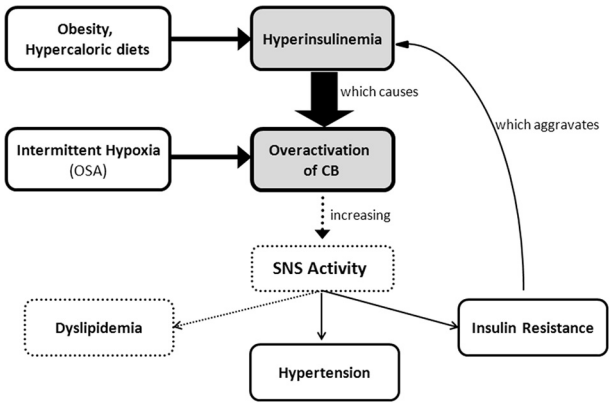

FIGURE 5 | Schematic representation of carotid body involvement in the development of insulin resistance through an increase in sympathetic nervous system activity. Overactivation of the carotid body caused by hyperinsulinemia and/or by chronic intermittent hypoxia originates an increase in sympathetic nervous system activity that promotes insulin resistance, hypertension, and probably dyslipidemia.

LINKING OBSTRUCTIVE SLEEP APNEA WITH METABOLIC DYSFUNCTION

OBSTRUCTIVE SLEEP APNEA

Obstructive sleep apnea (OSA) is the most common form of sleep disorder. It is characterized by repetitive collapse of the pharyngeal airway during sleep, which generally requires arousal to re-establish airway patency and resume breathing (Pillar and Shehadeh, 2008). Upper airway obstruction can result in either absent (apneas) or reduced (hypopneas) ventilation (Dempsey et al., 2010), despite persisting respiratory efforts, such that ventilatory requirements are not met. Consequently, hypoxemia and hypercapnia develop, which further stimulate respiratory effort. However, without spontaneous airway opening, the increased drive is ineffective to increase ventilation. Therefore, the apnea/hypopnea typically continues until the patient arouses from sleep and ends the obstruction. Following airway reopening, hyperventilation occurs to reverse the blood gas disturbances that developed during the respiratory event. The patient then returns to sleep and another obstruction develops (Eckert et al., 2009). The repetitive nature of these events results in the excessive daytime sleepiness (Punjabi et al., 1999), fatigue and neurocognitive dysfunction (Kim et al., 1997). Patients with OSA are classically characterized by the apnea-hypopnea index in mild OSA ($\geq$5 and $<$15 events/hour), moderate OSA ($\geq$15 and $<$30 events/hour), and severe OSA ($\geq$30 events/hour) (Kapur, 2010). OSA of at least mild severity (five or more events per hour of sleep) affects 5–20% of the general population (Young et al., 1993, 2002) with a prevalence of 17–24%

in men and 5–9% in women, and a tendency to even out after the menopause (Young et al., 1993; Bixler et al., 1998, 2001). The higher risk factors associated with OSA are age, male gender, and high body mass index. and this sleep disturbance is also linked to increased risk of hypertension, insulin resistance, glucose intolerance, type 2 diabetes, dyslipidemia, atherosclerosis and non-alcoholic fatty liver disease (Nieto et al., 2000; Newman et al., 2001; Punjabi et al., 2004; Drager et al., 2005; Reichmuth et al., 2005; Pulixi et al., 2014). The most effective and well-studied treatment for OSA is continuous positive airway pressure (CPAP) devices, which maintain upper airway patency during sleep, promote sleep continuity and significantly improve subjective and objective measures of daytime sleepiness (Patel et al., 2003).

The association between OSA and hypertension is well established (see Wolf et al., 2010 for a review). Bixler et al. (2000) demonstrated that OSA was independently associated with hypertension, both in men and women, being this relationship strongest in young subjects and proportional to the severity of the disease. The underlying mechanisms of OSA-induced hypertension are not completely understood, however it has been demonstrated that sympathetic activation plays a central role in the pathophysiological process. OSA patients, exhibit elevated blood pressure and elevated muscle sympathetic tone, as well as increased plasma CAs, an effect that diminishes with CPAP treatment (Somers et al., 1995; Kara et al., 2003). This high sympathetic drive is present even during daytime wakefulness when subjects are breathing normally and both arterial oxygen saturation and carbon dioxide levels are also normal (Kara et al., 2003; Narkiewicz and Somers, 2003). It was suggested that intermittent hypoxia resulting from apneas is the primary stimulus for evoking sympathetic excitation (Prabhakar et al., 2007, 2012) and that hypercapnia that occurs during apneas and even apnea, by itself, also contribute to sympathetic excitation (Prabhakar and Kumar, 2010; but see Lesske et al., 1997). Since the CB is the primary sensor for hypoxia and the ensuing reflex activates sympathetic nerve activity and elevates blood pressure (Lesske et al., 1997; Prabhakar and Kumar, 2010), it was suggested that CB overactivation by CIH produced by apneas would result in an increased sympathetic activity and hypertension. In fact, the surgical denervation of the CB prevented the increase in mean arterial blood pressure induced by CIH, as well as the adrenal demedullation and the chemical denervation of the peripheral SNS by 6-hydroxy dopamine (Lesske et al., 1997). The involvement of an increased sympatho-adrenal tone in CIH induced-hypertension was also suggested by the finding that acute hypoxia in CIH animals evoked the release of CAs from *ex vivo* adrenal medulla, an effect that is absent in controls, suggesting that direct activation adrenal medulla may account for the increase in blood pressure and plasma CAs seen in CIH animals (Kumar et al., 2006). In addition to the sympathetic tone, endothelial dysfunction, oxidative stress and inflammation have been proposed as potential mechanisms involved in the onset of the hypertension (see Gonzalez et al., 2012). However, evidence for a unique pathogenic mechanism has been difficult to establish in OSA patients because of concomitant co morbidities (Iturriaga et al., 2009; Del Rio et al., 2012).

CHRONIC INTERMITTENT HYPOXIA: LINKING CAROTID BODY AND OBSTRUCTIVE SLEEP APNEA

Chronic intermittent hypoxia (CIH), characterized by cyclic hypoxic episodes of short duration followed by normoxia, is a characteristic feature of OSA. The CB has been proposed to mediate the reflex increase in sympathetic activity and blood pressure associated with OSA due to CIH (Narkiewicz et al., 1999). In fact, several studies have demonstrated an increase in peripheral CB drive in OSA subjects. This increased CB peripheral drive was reflected by enhanced ventilatory and cardiovascular reflex responses induced by acute hypoxia (Somers et al., 1995; Narkiewicz et al., 1999) and also by an increase in basal tidal volume (Loredo et al., 2001). In a pioneer study, Fletcher et al. (1992a) demonstrated that 5 weeks of CIH induced an elevation of blood pressure in rats both during exposure to hypoxia and subsequently. In a succeeding publication, the same authors described that bilateral CB denervation prevented the development of hypertension in rats exposed to CIH for 35 days (Fletcher et al., 1992b), indicating that CB chemoreceptors are fundamental for the progression of CIH induced-hypertension. Consistent with these findings it was also demonstrated that CB denervation prevented the CIH-induced sympathetic activation (Prabhakar et al., 2005). In the last decade several reports have strengthened the idea that CIH resulting from sleep-disordered breathing leads to an overactivation of the CB, manifested by its increased sensitivity to hypoxia (Rey et al., 2004; Prabhakar et al., 2007; Peng et al., 2009). The recording of CSN discharge *in vitro* and *in situ* showed that exposure of animals to CIH increases the basal CSN discharge and enhances the chemosensory response to acute hypoxia (Peng et al., 2003; Rey et al., 2004; Gonzalez-Martín et al., 2011). Furthermore, Peng et al. (2003) demonstrated that CIH induces a CSN chemosensory long-term facilitation characterized by progressive increase in CSN activity with each hypoxic episode, remaining the baseline activity elevated approximately during 60 min after the last acute hypoxic stimuli. These authors have also suggested that, since the increase in CB sensory activity triggers sympathetic nerve discharge and an increase in blood pressure, sensory long-term facilitation contributes to the persistent increase in SNA and blood pressure that is observed in recurrent apnea patients (Peng et al., 2003). Peng et al. (2003) also found that when CIH-exposed rats were re-exposed to normoxia, the long-term facilitation and the augmented hypoxic ventilatory response was reversed. The reversible nature of the CB responses to CIH might explain why CPAP therapy reverses the adverse cardio-sympathetic effects in OSA patients (Kara et al., 2003). Also, CIH has no significant effect on CB weight (Obeso et al., 2012) nor morphology, as CIH did not produce significant differences in the total volume of the CB, number of glomus cells or glomus cell volume (Peng et al., 2003). The mechanisms underlying the CB overactivation induced by CIH are not well understood, with this effect being attributed to increased levels of endothelin-1 (Rey et al., 2006) and to reactive oxygen species (ROS) in the CB (Peng et al., 2003, 2009); however local expression of chemosensory modulators, like nitric oxide, and pro-inflammatory cytokines in the CB may have different temporal contribution to the CB chemosensory potentiation induced by CIH (Prabhakar et al., 2005; Del Rio et al., 2011).

Nevertheless, the possibility that alterations in the storing capacity and dynamics of possibly several neurotransmitter systems (e.g., CAs) (Gonzalez-Martín et al., 2011) cannot be excluded and changes in the density and/or affinity of their receptors in the sensory nerve endings could account for the overactivation of the CB seen in CIH.

OBSTRUCTIVE SLEEP APNEA, CHRONIC INTERMITTENT HYPOXIA, AND METABOLIC DYSFUNCTION

It is now consensual that OSA is independently associated with metabolic syndrome, which incorporates visceral obesity, hypertension, glucose intolerance, insulin resistance, and dyslipidemia (Bonsignore et al., 2013). Several studies have reported that metabolic syndrome is highly prevalent in OSA patients, with rates between 50 and >80% (Bonsignore et al., 2013). The indication of a relationship between OSA and the various pathological features of the metabolic syndrome, particularly insulin resistance, is recent when compared with the considerable body of evidence indicating that OSA can independently contribute to the development of sustained daytime hypertension. One of the earliest studies that showed that OSA is independently associated with insulin resistance was the performed by Ip et al. (2002), where the degree of insulin resistance was matched with body mass index and severity of OSA among 185 patients. Through a multiple linear regression, the authors found that obesity was the primary determinant of insulin resistance, but the patient's apnea-hypopnea index and minimal arterial O_2 saturation were also significantly contributors (Ip et al., 2002). In 2004 a large epidemiological study directly assessed OSA prevalence by polysomnography and measured glucose and insulin levels under fasting and after an oral glucose tolerance test in a subset of 2656 subjects from the Sleep Heart Health Study. The authors showed that subjects with mild or moderate to severe OSA had elevated fasting glucose and impaired oral glucose tolerance (Punjabi et al., 2004). Also, they demonstrated that the effect of OSA on glucose intolerance was independently associated with age, gender, body mass index and waist circumference (Punjabi et al., 2004). In another study, Punjabi and Beamer (2009), performed an intravenous glucose tolerance test in 118 non-diabetic subjects and found that the apnea-hypopnea index and the severity of nocturnal oxyhemoglobin desaturation were associated with decreased insulin sensitivity and pancreatic β-cell dysfunction, the effect being independent of age, sex and percent body fat (Punjabi and Beamer, 2009).

As expected by its association with insulin resistance, OSA may also be a risk factor for the development of type 2 diabetes, according to two large prospective studies. These two studies showed that regular snoring is associated with a 2- to 7-fold risk for type 2 diabetes over a period of 10 years (Elmasry et al., 2000; Al-Delaimy et al., 2002). Since snoring is not a clinical diagnostic for OSA, in a longitudinal study, Reichmuth et al. (2005) analyzed the data from 1387 subjects in the Wisconsin Sleep Cohort and examining the association between OSA, diagnosed by polysomnography, and the development of type 2 diabetes. Comparable to previous cross-sectional studies, a positive association between clinically diagnosed OSA and type 2 diabetes, after adjustment for age, sex, and waist girth was shown (Reichmuth et al., 2005). However, in a follow-up study of 978 subjects, the odds ratio for developing type 2 diabetes within a 4 years period for those with an apnea-hypopnea index of >15 events/hour did not reach statistical significance after adjustment for waist girth (Reichmuth et al., 2005). Since it is well described that insulin resistance precedes in approximately 10–15 years the development of type 2 diabetes (Nathan, 2002), the limitation of this work may be related with the duration of follow-up that was only 4 years. Therefore, further longitudinal studies would be necessary to fully examine the role of OSA in the development of type 2 diabetes.

The link between OSA and metabolic dysfunction was also sustained by the results obtained by Babu et al. (2005) showing that CPAP treatment for 3 months decreased postprandial glucose levels and glycated hemoglobin in type 2 diabetes patients with OSA, being the decrease higher when CPAP was used for more than 4 h per night (Babu et al., 2005). Also, Harsch et al. (2004a) observed an increase in insulin sensitivity, assessed through a hyperinsulinemic-euglycemic clamp, in type 2 diabetes patients after 3 months of effective CPAP treatment. In another study performed by Harsch et al. (2004b), in OSA patients without type 2 diabetes, it was observed that CPAP treatment increased insulin sensitivity within 2 days of therapy, with further improvements occurring at the 3 months follow-up. In contrast with the reported beneficial effects of CPAP on glucose metabolism and insulin resistance in OSA patients, some studies demonstrated that CPAP treatment for 3 or 6 months did not improve fasting glucose or insulin plasma levels (Ip et al., 2000). These differences among studies may be related with the treatment duration, lack of a control group, insufficient statistical power and absence of data on CPAP compliance.

The exact mechanism for the pathological changes that occur in glucose metabolism and insulin action in OSA patients is not completely understood. It is possible that multiple interrelated factors contribute to the complex interactions between OSA, obesity and glucose control. OSA is intrinsically associated with CIH and sleep loss due to sleep fragmentation, and both induce insulin resistance (Tasali et al., 2008). Recently, a lot of research has been published devoted to the study CIH and metabolic dysfunction in rodents however some of the data obtained is not consensual. It has been shown that mice exposed during 30 days to CIH exhibited elevated levels of fasting plasma insulin but comparable glucose levels and higher homeostasis model assessment (HOMA) index, indicating insulin resistance, an effect that was attributed to a pancreatic β-cell dysfunction (Wang et al., 2013). These results were sustained by the recent work of Gonzalez group where they observe that 15 days of CIH in rats induce insulin resistance, assessed by the HOMA index without affecting fasting glucose plasma levels and glucose tolerance (Olea et al., 2014). These findings obtained in mice and rats contrast with the recent publication by Shin and co-workers where they show that 4/6 weeks of CIH in mice increased fasting blood glucose, baseline hepatic glucose output but not insulin sensitivity measured through a hyperinsulinemic euglycemic clamp (Shin et al., 2014). These effects being mediated by the CB as CSN denervation prevented the CIH-induced hyperglycemia and the increase in hepatic glucose output (Shin et al., 2014). Whereas the

differences obtained in several metabolic parameters, like fasting glycemia, can be due to distinct species studied as well as to the different CIH paradigms, we must refer that HOMA index is a human index, an must not be used as the only index to assess insulin resistance in rodents.

Several intermediate mechanisms have been proposed to explain the pathological alterations in glucose metabolism in OSA: increased sympathetic activation, deregulation of the hypothalamus-pituitary axis and generation of ROS (Tasali et al., 2008). In addition, pancreatic β-cells are highly sensitive to hypoxia, and the subsequent shift to anaerobic glycolytic metabolism favors insulin resistance (Pallayova et al., 2011). Also, it was recently shown that mice exposed to 30 days CIH exhibited pancreatic β-cell dysfunction, manifested by impaired glucose-stimulated insulin secretion and increased mitochondrial ROS (Wang et al., 2013), which may contribute to the development of type 2 diabetes among sleep apnea patients. Finally, the oxidative status and activation of inflammatory pathways can also contribute to deregulation of metabolism (Tasali et al., 2008). It has been recently shown that 15 days to CIH in rats induce an oxidative status manifested by an increase in lipid peroxides and diminished activities of superoxide dismutases, an inflammatory status characterized by augmented C-reactive protein and nuclear factor kappa-B activation and a sympathetic hyperactivity assessed by plasma and renal artery CA levels and synthesis rate (Olea et al., 2014). Also, the same authors have shown that, as expected, the combination of CIH and obesity worsened the alterations observed (Olea et al., 2014).

Obesity is considered a major risk factor for the development and progression of OSA. It is estimated that 40% of obese individuals have OSA; consequently approximately 70% of individuals with OSA are obese (Vgontzas et al., 2000; Daltro et al., 2007). One possible mechanisms by which obesity may worsen OSA is due to fat deposition at specific sites of the body, namely in the upper airways. In fact, fat deposition in the tissues surrounding the upper airway appears to result in a smaller lumen and increased collapsibility of the upper airway, predisposing to apnea (Shelton et al., 1998; Schwab et al., 2003). This increase in fat deposition next to the upper airways can be found even in non-obese subjects with OSA (Mortimore et al., 1998). Fat deposits around the thorax (truncal obesity) also reduce chest compliance and functional residual capacity, and may increase oxygen demand (Naimark and Cherniack, 1960). Another fat depot that can contribute to OSA is visceral fat. Visceral obesity is common in subjects with OSA and is closely related with an increase in apnea index (Shinohara et al., 1997), Since obesity is positively correlated with OSA, weight loss and weight gain prevention offer a successful therapeutic approach to reduce the occurrence and the severity of OSA and its related mortality. In a longitudinal study, Peppard et al. (2000) showed that a 10% of weight loss predicted a 26% decrease in the apnea-hypopnea index, which suggest that even a modest weight loss may be effective in managing OSA and reducing new occurrence of OSA. Furthermore, CPAP treatment for 6 months led to visceral fat loss even if subjects did not lose weight (Chin et al., 1999). Short sleep fragmentation is associated with decreased levels of leptin, a hormone that lowers food intake, increases energy expenditure (Friedman and Halaas, 1998) and is secreted in proportion to body fat stores (Considine et al., 1996). In OSA subjects, several studies reported increased leptin levels compared to weight-matched control (Ip et al., 2000; Vgontzas et al., 2000), which correlated with OSA severity (Ip et al., 2000), and decreased after CPAP treatment (Chin et al., 1999).

Although obesity is the primary risk factor for OSA this disease also affects lean subjects, as Pamidi et al. (2012) demonstrated that young lean men, free of cardiometabolic disease, the presence of OSA is associated with IR and compensatory hyperinsulinemia to maintain normal glucose homeostasis (Pamidi et al., 2012). Therefore, from this study we can conclude that OSA may increase the risk of type 2 diabetes independently of traditional cardiometabolic risk factors. In the Sleep Heart Study (Seicean et al., 2008), a large community-based cohort of older individuals (>65 years of age), the presence of OSA was associated with a higher prevalence of prediabetes and occulted type 2 diabetes in the non-overweight group. Furthermore, the effect of CPAP treatment may be different between obese and non-obese subjects. Harsch et al. (2004b) showed that the improvement in insulin sensitivity was much smaller in obese subjects than in non-obese subjects, suggesting that in obese individual's insulin sensitivity is mainly determined by obesity and, to a smaller extent, by sleep apnea.

Obesity is known to be strongly associated with metabolic dysfunction, and that contributes to insulin resistance and glucose intolerance (Landsberg, 1996, 2001), nevertheless metabolic dysfunction can be present in lean OSA subjects (Pamidi et al., 2012). In CIH rodent models metabolic dysfunction is present without the obesity component (Carreras et al., 2012; Fenik et al., 2012; Wang et al., 2013; Shin et al., 2014), as it was described that animals submitted to CIH gain less weight (Carreras et al., 2012) or the similar weight (Olea et al., 2014) in comparison with controls. Also, the amounts of perirenal and epididymal fat found in CIH animals was similar to those found in controls (Olea et al., 2014). Taken together these results show that in OSA, obesity is not the only factor that contributes to metabolic dysfunction. The involvement of CB has been recently proposed as one of the links between CIH and sympathetic overactivity and metabolic dysfunction, since CB denervation prevents CIH-induced fasting hyperglycemia, although CB denervation was incapable of prevent insulin resistance (Shin et al., 2014), suggesting that other mechanisms can account for the CIH induced-insulin resistance. In fact, little is known regarding the molecular mechanisms behind this relationship, with the reduction of Glut4 metabolic fraction in skeletal muscle in CIH animals being the only mechanism described (Carreras et al., 2012). Therefore, detailed studies on the molecular mechanisms of insulin action in insulin-sensitive tissues will contribute enormously to better understand the paradigm of CIH-induced insulin resistance, and so the relationship between OSA and metabolic dysfunction.

FUTURE PERSPECTIVES

In the last couple of years, several reports of non-classical roles of the CB on glucose homeostasis and metabolic regulation have

been published, contributing to launch the CB as a putative therapeutic target for the treatment of endocrine diseases. Our group has been actively involved in the process and recently we described that chronic CB overstimulation is implicated in the etiology of diet-induced insulin resistance (Ribeiro et al., 2013). We have also described that surgical resection of the CSN prevents the development of dysmetabolic changes induced by hypercaloric treatments in rats (Ribeiro et al., 2013), an observation that contributed to strengthen that CB blockade/modulation represents a novel and unexploited therapeutic approach.

Besides the surgical resection of the CB, its overactivation can also be prevented pharmacologically with an old, well-studied and very safe drug: caffeine. Sustained caffeine administration prevents the development of hypertension, impaired glucose tolerance and insulin resistance in prediabetes animal models (Conde et al., 2012b; Panchal et al., 2012). The protective effect of chronic caffeine administration was accompanied by prevention of weight gain and decreased visceral fat in obese animals; however caffeine also exerted its positive metabolic effects in lean models of insulin resistance and hypertension independently of weight loss (Conde et al., 2012b). A putative mechanism related with blockade of adenosine receptors in the CBs and, therefore, with the inhibition of CB-mediated sympathetic overactivation by chronic caffeine administration has been proposed as a paradigm shift to explain the reduction of insulin resistance, blood pressure and type 2 diabetes risk induced by sustained consumption of this xanthine (Conde et al., 2012b,c; Ribeiro et al., 2013). The translation of these promising results into human medicine, namely through controlled clinical trials is still lacking—but the epidemiological data available strongly indicate that caffeine should integrate a normal healthy diet, and actually contribute to decrease the incidence of type 2 diabetes and obesity in high-risk populations (van Dam and Hu, 2005; Bhupathiraju et al., 2014).

Another way of modulating CB activity would be to directly target its effector, the SNS. The SNS may also represent a putative target to treat metabolic diseases related with insulin resistance, particularly if modulated regionally in classical insulin-target tissues like the skeletal muscle. This pinpoint modulation may be achieved through the use on Bioelectronic Medicines, electronic devices connected to individual peripheral nerve fibers, aiming to correct pathological electrical patterns and restore health (Famm et al., 2013). This new area of therapeutics is emerging right now, with the promise and ambitious goal of modulating specific peripheral nerves. Due to the important role the CBs seem to play in both the metabolic and hemodynamic control, they represent a natural candidate for Bioelectronic Medicines to be tested in a not so distant future.

ACKNOWLEDGMENTS

This work was supported by the Portuguese Foundation for Science and Technology [Grants EXPL/NEU-SCC/2183/2013] and by the L'Oreal/FCT/UNESCO Medals of Honor for Women in Science 2009 (to Sílvia V. Conde) and by Spanish Ministry of Economy and Competitiveness [Grant BFU2012-37459 to Constancio Gonzalez] and by Spanish Ministry of Health-Institute Carlos III [CIBERES to Constancio Gonzalez].

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 11 September 2014; accepted: 10 October 2014; published online: 29 October 2014.

Citation: Conde SV, Sacramento JF, Guarino MP, Gonzalez C, Obeso A, Diogo LN, Monteiro EC and Ribeiro MJ (2014) Carotid body, insulin, and metabolic diseases: unraveling the links. Front. Physiol. ***5****:418. doi: 10.3389/fphys.2014.00418*

This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 28 October 2014
doi: 10.3389/fphys.2014.00406

Revisiting cAMP signaling in the carotid body

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Chronic carotid body (CB) activation is now recognized as being essential in the development of hypertension and promoting insulin resistance; thus, it is imperative to characterize the chemotransduction mechanisms of this organ in order to modulate its activity and improve patient outcomes. For several years, and although controversial, cyclic adenosine monophosphate (cAMP) was considered an important player in initiating the activation of the CB. However, its relevance was partially displaced in the 90s by the emerging role of the mitochondria and molecules such as AMP-activated protein kinase and O_2-sensitive K^+ channels. Neurotransmitters/neuromodulators binding to metabotropic receptors are essential to chemotransmission in the CB, and cAMP is central to this process. cAMP also contributes to raise intracellular Ca^{2+} levels, and is intimately related to the cellular energetic status (AMP/ATP ratio). Furthermore, cAMP signaling is a target of multiple current pharmacological agents used in clinical practice. This review (1) provides an outline on the classical view of the cAMP-signaling pathway in the CB that originally supported its role in the O_2/CO_2 sensing mechanism, (2) presents recent evidence on CB cAMP neuromodulation and (3) discusses how CB activity is affected by current clinical therapies that modify cAMP-signaling, namely dopaminergic drugs, caffeine (modulation of A_{2A}/A_{2B} receptors) and roflumilast (PDE4 inhibitors). cAMP is key to any process that involves metabotropic receptors and the intracellular pathways involved in CB disease states are likely to involve this classical second messenger. Research examining the potential modification of cAMP levels and/or interactions with molecules associated with CB hyperactivity is currently in its beginning and this review will open doors for future explorations.

Keywords: cAMP signaling, carotid body, pharmacology, phosphodiesterase inhibitors, adenylyl cyclase, adenosine, dopamine, antipsychotics

INTRODUCTION

Adequate homeostatic regulation of arterial oxygen (P_aO_2), carbon dioxide (P_aCO_2), pH and blood glucose are important processes in physiology. Highly specialized chemosensory type I cells of the mammalian carotid bodies (CBs) sense acute changes in P_aO_2, P_aCO_2 and pH, and, upon stimulation, release neurotransmitters (NTs) that either inhibit or activate chemosensory fibers projecting into the central nervous system (CNS). The functional consequence of CB stimulation is the initiation of important cardiovascular, respiratory and metabolic reflexes. These reflexes include an increase in minute ventilation, a sympathetically mediated elevation in heart rate and peripheral vasoconstriction and an augmentation in adrenaline release from the adrenal medulla, with the latter leading to an increase in arterial blood glucose concentration.

Recently, interest in CB physiology has attracted considerable attention because of its emerging associations with chronic cardiovascular disease (McBryde et al., 2013). CB dysfunction and increases in chemoafferent discharge promote neurogenic hypertension in sleep disordered breathing (Prabhakar and Peng, 2004), chronic heart failure (Schultz et al., 2013) and essential hypertension (Abdala et al., 2012; McBryde et al., 2013). Moreover, the CB is a principal regulator in initiating insulin resistance in animal models of prediabetes and metabolic syndrome (Ribeiro et al., 2013). Therefore, the modulation of CB function may be necessary to prevent and treat some of these conditions. A good understanding on the modulation of the cellular processes occurring downstream of the CB transduction machinery, may not only promote drug development that modify CB chemodischarge to prevent or treat disease, but will also increase the awareness that CB chemodischarge can be an inadvertent side effect of drugs used to treat other diseases.

CB type I cells contain molecular sensors that, when activated, trigger transduction cascades that produce cellular depolarization, Ca^{2+} influx and NT and/or neuropeptide secretion. The list of characterized NTs/neuromodulators (NMs) and respective receptors in the CB has increased considerably over the last 20 years (**Table 1**). These NTs/NMs have the potential to activate metabotropic and ionotropic receptors located on type I cells (autoreceptors), on afferents of the carotid sinus nerve (CSN, post-synaptic receptors), or both, exerting either excitatory or inhibitory actions (**Table 1**). The activation of

Table 1 | Receptors in the carotid body.

NT/NM	Receptor			CSN activity	Species	Localization	References
	Subtype	Metabo-tropic	Iono-tropic				
DA[a]	D_1	G_s	–	(–)?	Rat, cat, rabbit	PG, SCG, whole CB, blood vessels	Almaraz et al., 1991; Bairam et al., 1998
	D_2	G_i/G_o	–	(–)[a]	Rat, rabbit, cat, mice, human	Whole CB, PG, and SCG, type I cells of CB, nerve endings	Dinger et al., 1981a; Mir et al., 1984; Czyzyk-Krzeska et al., 1992; Bairam and Khandjian, 1997; Fagerlund et al., 2010; Kåhlin et al., 2010
NE/E	α_{2A}	G_i/G_o	–	(–)	Rat, rabbit, cat	SCG, whole CB, type I cells, sympathetic innervation, blood vessels?	Kou et al., 1991; Almaraz et al., 1997; Gauda, 2002
	α_1	G_q/G_{11}	–	?	Mice (KO)	Carotid arteries	Deighan et al., 2005;
	ß$_2$	G_s	–	(+)	Rat	Whole CB	Mir et al., 1983
Ach[b]	M_2/M_4	Gi/Go	–	?	Cat, rabbit	Type I cells, CB, SCG, PG	Dinger et al., 1981b, 1991; Shirahata et al., 2004; Bairam et al., 2006
	M_1/M_3	G_q/G_{11}	–	(+)	Cat, rabbit	Type I cells, CB, SCG, PG	Dinger et al., 1981b, 1991; Shirahata et al., 2004; Bairam et al., 2006
	α4, α7, and ß2, α4ß2 hete	–	√	(+)	Rat	Type I cells, PG, CSN afferents	Obeso et al., 1997; Zhong and Nurse, 1997; Gauda, 2002; He et al., 2005; Conde and Monteiro, 2006a; Niane et al., 2009; Meza et al., 2012
	α3, α4, α7??, ß2, ß4				Cat	Whole CBs, SCG, PG, CSN afferents	Alcayaga et al., 1998, 2007; Shirahata et al., 1998; Bairam et al., 2007
	α3, α4, α5, α7??, ß2, ß4				Mice	CB tissue sections	Kåhlin et al., 2010
	α3, α7, ß2				Human	Whole CB	Fagerlund et al., 2010
ATP	$P2X_{2/3}$, P2X3, P2X2,	–	√	(+)	Rat, cat, humans, mice	PG afferents, whole CB, SCG	Prasad et al., 2001; Rong et al., 2003; Alcayaga et al., 2007; Bairam et al., 2007; Fagerlund et al., 2010
	$P2Y_1$	G_q/G_{11}, G_i/G_o	–	(–)?	Rat	Type I cells	Xu et al., 2005
	$P2Y_2$	G_q/G_{11}, G_i/G_o	–	(–)?	Rat	Type II cells	Xu et al., 2003
Ado	A_1	G_i/G_o	–	(–)?	Rat Rabbit	PG Type I cells	Gauda, 2002 Rocher et al., 1999
	A_{2A}	Gs	–	(+)	Rat, human	Whole CB, post synaptically on CSN	Kobayashi et al., 2000; Fagerlund et al., 2010
	A_{2B}	Gs	–	(+)	Rat	Whole CB, type I cells, PG	Kobayashi et al., 2000; Conde et al., 2006b, 2008

(Continued)

Table 1 | Continued

NT/NM	Receptor			CSN activity	Species	Localization	References
	Subtype	Metabo-tropic	Iono-tropic				
5-HT	5-HT_{2A}	G_q/G_{11}, G_i/G_o	–	(–)	Rat	type I cells, PG (just a few in PG)	Zhang et al., 2003
	5-HT_3	–	√	(+) Na^+, K^+, Ca^{2+}	Rat	PG	Wang et al., 2002b
	5-HT_{5A}	G_i/G_o	–	(–)	Rat	Type I cells, PG and SCG	Wang et al., 2000
Histamine	H_1	G_q/G_{11}	–	(+)	Cat, human	Type I cells,PG	Del Rio et al., 2008; Lazarov et al., 2009
	H_2	G_q/G_{11}	–	?	Cat	Whole CB	Del Rio et al., 2008
	H_3	G_i/G_o	–	(–)	Cat, human	Type II cells, PG	Del Rio et al., 2008; Lazarov et al., 2009
SP	NK_1	Gs	–	(+)	Rat, cat	SCG, PG, chemoreceptor efferents	Prabhakar et al., 1990; Gauda, 2002
ET	ET_A	G_q/G_{11}	–	?	Rat	Type I cells	Chen et al., 2002b
EPO	EPOR	–	–	?	Rat	CB clusters	Lam et al., 2009
TR	TRKB	–	–	?	?	Type I cells	Porzionato et al., 2008
Kiss	KissR	G_q/G_{11}	–	?	Rat and human	Type I cells, SCG	Porzionato et al., 2011
Cytokine	IL-1B, IL-6Rx, IL-1RI	–	–	(+)	Rat	CB, type I cells	Wang et al., 2002a; Lam et al., 2008
	TNF-R1, TNF-R2	–	–	(–)	Cat, rat	Type I cells	Fernández et al., 2011
	TLR4	–	–	(–)?	Rat	Type I cells ?	Fernández et al., 2011
Ang II	AT1	G_q/G_{11}	–	(+)	Rat	Type I cells	Fung et al., 2001
ENK	δ ??	G_i/G_o ??	–	(–)	Rat	Whole CB	Gauda, 2002
GABA	$GABA_A$	–	√	(–)	Rat	Sensory nerve (CSN) endings	Zhang et al., 2009
	$GABA_A$ (α2, α3, β3, γ2)	–	√	(–)	Cat	Type I cells, and cell bodies and nerves of PG	Igarashi et al., 2009
	$GABA_A$ (α2, β3, γ2)	–	√	(–)	Human	Whole CB	Fagerlund et al., 2010
	$GABA_B$	G_i/G_o	–	(–)	Rat, mice	Type I cells	Oomori et al., 1994; Fearon et al., 2003

[a] *Mainly inhibitory, but excitatory in rabbit (Iturriaga et al., 2009).* [b] *Although less characterized in rat, the nAchRα3,4,5,7, and ß2,4 are present in type I cell, α7 in the CNS afferents and α3,4,7, and ß2,4 in PG; the mAchR M1 and M2 are in type I cells, M1 in CSN afferents and M1 and M2 in PG neurons of cat and rabbit (for a revision, Shirahata et al., 2007). ?, suggested, but no direct evidences/not known; NT/NM, neurotransmitters/neuromodulators; DA, dopamine; NE/E, norepiniphrine/epiniphrine; NE, norepiniphrine; Ach, acetylcholine; ATP, adenosine triphosphate; Ado, adenosine; 5-HT, serotonine; GABA, gamma-aminobutyric acid; ENK, enkephalins; SP, substancia P; ET, endothelins; TR, trophin; AngII, Angiotensin; AC, adenylyl cyclase; +, excitatory; –, inhibitory; CB, carotid body; SCG, superior cervical ganglion; PG, petrosal ganglion.*

excitatory postsynaptic receptors is translated into an increase of CSN action potential frequency, and it is this signal that is conveyed to the CNS. Stimulation of excitatory autoreceptors induces an increase in $[Ca^{2+}]_i$ and subsequent further release of NTs/NMs.

Retrograde communication between petrosal ganglion (PG) neurons and CB type I and type II cells is another source of NTs/NMs release in the CB. PG neurons present catecholaminergic traits (Katz et al., 1983; Katz and Black, 1986) and catecholamines (CAs) are released from cultured PG neurons upon stimulation (Iturriaga et al., 2003). Moreover, nitridergic autonomic neurons located in the glossopharyngeal and carotid nerve may also modulate the CB function (Campanucci et al., 2012). The pannexin-1 channel opening have been recently shown to be important in reciprocal cross-talk pathways between type I and type II cells, particularly in purinergic transmission (ATP and Ado) (Nurse, 2014).

The specific NT profile, receptor expression and cellular effects changes with early postnatal development, and in some cases exhibits interspecies variability [e.g., dopamine (DA) exerts inhibitory effects on the CB in most species, except rabbit, (Iturriaga et al., 2009)].

Despite the numerous different NTs/NMs released from the type I cell, even under basal conditions, a convergence upon a common signaling pathway could confer the overall CB excitability and establish its sensitivity to physiological stimuli. Cyclic adenosine monophosphate (cAMP) is a common downstream signaling molecule of numerous receptors expressed in the type I cells, and is coupled to cellular energetic status (AMP/ATP ratio). This article therefore aims to summarize how changes in CB cAMP levels in physiology, pathology and following pharmacological intervention may be central to alterations in type I cell excitability leading to chemoafferent discharge and cardiorespiratory and metabolic reflex responses.

CLASSICAL UNDERSTANDING OF cAMP-SIGNALING PATHWAY IN THE CAROTID BODY

An involvement of cAMP in the CB chemotransduction (late 70s-early 80s) was originally prompted by the identification of secreted NTs and their receptor-mediated effects on CB chemoreceptor responses (**Table 1**). These secreted NTs included DA (Gonzalez and Fidone, 1977), acetylcholine (ACh) (Eyzaguirre et al., 1965), noradrenaline (NA), substance P, serotonin and prostaglandin E_2 (Pérez-García et al., 1993 for early references), and adenosine (Ado) (McQueen and Ribeiro, 1981; Monteiro and Ribeiro, 1987; Conde and Monteiro, 2004), acting through specific G-protein coupled receptors (**Table 1**). For those NTs that confer an excitatory response, cAMP levels were increased, while for those that confer an inhibitory response cAMP levels were decreased.

Fitzgerald and co-workers, were the first to identify cAMP in the CB cat homogenates (Fitzgerald et al., 1977). Subsequent studies showed that injection of isoprenaline increased cAMP accumulation in the rat CB (Mir et al., 1983) and elevated chemoafferent discharge frequency in cat and rabbit models via stimulation of beta-adrenoreceptors (Folgering et al., 1982). Moreover, administration of dibutyryl cyclic AMP (db-cAMP, cAMP analog) was found to mimic the excitatory effect of adenosine on chemosensory discharge (McQueen, 1983).

Following these findings, a new wealth of evidence emerged supporting a role for the cAMP in CB chemotransduction and/or chemotransmission (Wang et al., 1989, 1991a; Fidone et al., 1990; Pérez-García et al., 1990; Cachero et al., 1996; Summers et al., 2002). Multiple investigations reported rises in CB cAMP accumulation following hypoxia exposure (Fidone et al., 1990; Pérez-García et al., 1990), an effect that appeared to be specific for chemoreceptor tissue (Wang et al., 1989) and was dependent on NT release (Pérez-García et al., 1990). Activation of adenylyl cyclases (AC), by forskolin (FSK), potentiated CB CA secretion and CSN discharge frequency over a range of O_2 tensions from 30 to 0%, in the intact rabbit CB preparation (Almaraz et al., 1991; Wang et al., 1991a). In addition, FSK and db-cAMP both inhibited the type I cell O_2-sensitive K^+ current, emphasizing similarities between excitatory cAMP and hypoxic signaling cascades (López-López et al., 1993). Hypercapnia exposure also elevated cAMP content (Pérez-García et al., 1990) and FSK augmented the hypercapnic CA release (Pérez-García et al., 1991). In isolated rabbit CB type I cells, cAMP analogs potentiated inward Ca^{2+} current in a manner that was comparable with hypercapnia (Summers et al., 2002). Despite these data, it was not universally accepted that endogenous cAMP was physiologically relevant in the CB.

Delpiano et al. reported, using an *in vitro* preparation of the cat CB, that anoxia exposure induced only small increases in cAMP levels (Delpiano and Acker, 1991). Furthermore, severe whole body hypoxia exposure caused both increases and decreases in CB cAMP accumulation (Delpiano and Acker, 1991), and short periods of hypoxia (2.5–5 min) failed to alter the cAMP levels in rat CB (Mir et al., 1983). K^+ and Ca^{2+} currents, both important in hypoxic chemotransduction, were shown to be insensitive to an array of cAMP analogs in the rat CB type I cells (Hatton and Peers, 1996); inwardly rectifying Cl^- current is directly activated by cAMP (Carpenter and Peers, 1997). The inter-experiment variability, differences in species and age, in CB dissection methods, O_2 and CO_2 stimulus intensity, duration of incubation periods, CB preparations (*in vitro*, *in vivo*, whole CB vs. isolated cells or carotid sinus nerve (CSN) preparations) and cAMP detection methods (radioimmunoassay, enzyme-immunoassay, protein binding saturation assays) have all been credited for the discrepancies reported in the literature regarding the relevance of cAMP signaling in CB function (**Table 2**).

Thus, there was still a requirement to further characterize and better understand the physiological significance of cAMP in the CB. To consider cAMP signaling as a physiological modulator of the chemoreceptor activity, disruption of cAMP generation, metabolism, or its intracellular effectors would need to be synonymous with functional modification of basal CB activity and/or its responses to hypoxia/hypercapnia. $[cAMP]_i$ is tightly regulated by AC, by enzymes involved in its degradation (phosphodiesterases; PDE), and by the fluctuating activity of downstream effectors (Kamenetsky et al., 2006). The AC activities are highly integrated and determined by receptor-mediated changes in G-stimulatory (G_s) and G-inhibitory (G_i) proteins as well as by

Table 2 | Effects of different work conditions on cAMP levels in the carotid body.

[cAMP]	Species	Anesthesia	Preparation	Basal conditions				Stimulus				Technique	Units	References
				O_2 (%)	CO_2 (%)	Time (min)	Inc.media	O_2 (%)	CO_2 (%)	Time (min)	Inc. media			
=[a]	Cat 12 month	Na-pentob.	Whole CB, *In vivo*, *In vitro*	R.A. 30	R.A. 3	n/a 60/120	n/a Locke's	5 0	n/a 3	3/10/20 2	n/a Locke's+ [IBMX] 0.8 mM	RIA	pmol/CB	Delpiano et al., 1984
↑	Cat 12month	Na-pentob.	Whole CB, *In vitro*	35	4	180	Locke's modified	3	4	2	Locke's modified + [IBMX] 0.8 mM	RIA	pmol/CB	Delpiano and Acker, 1991
↑	Rabbit adult	Na-pentob.	Whole CB, *In vitro*	100	0	30	Tyrodes's	5	0	10	Tyrodes's	RIA	pmol/mg tissue	Wang et al., 1989
↑	Rat adult	Na-pentob.	CB slices, *In vitro*	100	0	30	Locke's + [theophyline] 10 mM	4	0	10	Locke's + [theophylline] 10 mM	Immune-reactivity	% positive cells	Wang et al., 1991b
↑	Rabbit adult	Na-pentob.	Whole CB, *In vitro*	100/95	0/5	30	Tyrodes's	0/5/7/10	5/20	10	Tyrodes's + [FSK] 0.01 mM, [IBMX] 0.5 mM and [Ca^{2+}] 2 mM	RIA	pmol/mg tissue	Pérez-García et al., 1990
↑	Rabbit adult	CO_2	Whole CB, *In vitro*	21	5	20	HCO_3^- enriched-medium	21	10	5	HCO_3^- enriched-medium	EIA	pmol/μg protein	Summers et al., 2002
↑	Rabbit adult	Na-pentob.	Whole CB, *In vitro*	20	5	30	Tyrodes's modified + [HCO_3^-] 24 mM	7	5	10	Tyrodes's modified + [HCO_3^- 24 mM + [IBMX] 0.5 mM	RIA	pmol/mg tissue	Cachero et al., 1996
↑	Rabbit adult	Na-pentob.	Whole CB, *In vitro*	100	0	30	Tyrode's modified	5	0	10	Tyrode's modified	RIA	pmol/mg tissue	Chen et al., 1997
=	Rat adult	Urethane	Whole CB, *In vivo*	R.A	R.A.	n/a	n/a	5	0	2/5	n/a	Protein binding	pmol/CB	Mir et al., 1983
=	Rat (3, 12, 24 months)	Na-pentob.	Whole CB, *In vitro*	95	5	15	Tyrodes's modified	95/20/10/5	5	30	Tyrodes's modified + [IBMX] 0.5 mM	EIA	pmol/mg tissue	Monteiro et al., 2011
=	Rat adult	Na-pentob.	Whole CB, *In vitro*	20/95	5	15	Tyrodes's modified	5	5	30	Tyrodes's modified + [IBMX] 0.5 mM	EIA	pmol/mg tissue	Nunes et al., 2010

[a]*Small increases in cAMP levels were observed in hypoxia only in the absence of IBMX. Inc., incubation; pentob., pentobarbital; R.A., room air; n/a, not applicable; RIA, radioimmunoassay; Locke's (in mM): NaCl 128, KCL 5.6, $CaCl_2$ 122.1, D-glucose 5.5, $NaHCO_3$ 10 and Hepes 7; Tyrode's (in mM):NaCl 112, KCl 4.7, $CaCl_2$ 2.2, $MgCl_2$ 1.1, Na-glutamate 42, Hepes 5, glucose 5.6, pH 7.4; Tyrode's modified solution (in mM):NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1.1, Hepes 10, glucose 5.5, pH 7.42; HCO_3^- medium (in mM): NaCl 117, KCl 4.5, $CaCl_2$ 2.5, $MgCl_2$ 1, sucrose 10, glucose 11, HCO_3^- 23, pH 7.42; CB, carotid body; EIA, Enzyme Immuno Assay; IBMX, Isobutyl-1-methylxanthine.*

CO_2 and HCO_3^- (depending on specific AC isoforms- see below). $[cAMP]_i$ can also be modified by direct diffusion from one cell to another through gap junctions (Bevans and Harris, 1999) or through transport to the extracellular milieu where it produces regulatory functions in multiple tissues (for a review Bankir et al., 2002; Hofer and Lefkimmiatis, 2007). Downstream effectors classically include protein kinase A (PKA) (Taylor et al., 1990), cyclic nucleic gated ion channels (Craven and Zagotta, 2006) and exchange proteins activated by cAMP (EPACs) (De Rooij et al., 1998).

Recently, better research tools have become available to more accurately detect intracellular cAMP and its regulation, thereby allowing us re-examine the enzymatic regulation of cAMP within the CB and its intracellular targets, during normoxia, hypoxia and hypercapnia conditions.

NOVEL FINDINGS CHARACTERIZING THE ENZYMATIC REGULATION OF cAMP ACCUMULATION IN THE CAROTID BODY

Over the last decade, the enzymes involved in the cAMP-pathway signaling in the CB have been identified, and their activity modulated by natural stimuli. Novel findings have been recently reported as to how O_2/CO_2 exposure affects the CB cAMP-signaling.

THE ROLE OF ADENYLYL CYCLASES IN THE CAROTID BODY ACTIVITY

The AC are enzymes that catalyze the synthesis of cAMP through the cyclization of ATP. There are two main classes: the classic NT-sensitive transmembrane (tmAC) and the more recently described soluble adenylyl cyclase (sAC) (for a review see Kamenetsky et al., 2006). The activity of the former is primarily influenced by extracellular signals (e.g., NTs, hormones, pharmacological agents) and is further subclassified in terms of G-protein associations, Ca^{2+} related signaling pathways (Halls and Cooper, 2011) and more recently by CO_2 interactions (Townsend et al., 2009; Cook et al., 2012). sAC is regulated directly by HCO_3^- and Ca^{2+}, in a pH independent manner, as being shown primarily in testis and further extended to other organs as described below (Chen et al., 2000; Jaiswal and Conti, 2003).

The presence of the different AC mRNA transcripts was only reported recently; intact rat CBs (16-17 postnatal days) express tmAC1, tmAC2, tmAC3, tmAC4, tmAC6 and tmAC9, with tmAC1, tmAC4 and tmAC6 exhibiting the highest fold expression level (Nunes et al., 2013). sAC mRNA has also been identified, and is expressed at greater levels in the CB than in non-chemosensitive neuronal tissues (Nunes et al., 2009). The studies on AC mRNA were performed in whole CB (containing type I and type II cells, vessels, nerve endings, etc.). Whether there is a specific and clearly distinguished physiological function for each AC isoform, in the CB is unknown. Many agents that target cAMP signaling pathways are also likely to non-selectively act on the respective G-protein coupled receptors or PDE, thus making individual AC targeting challenging. However, in other tissues, individual AC isoforms do demonstrate unique functionality and this has led to increased interest in identifying specific AC isoforms as potential drug targets (Pierre et al., 2009). Genetic association studies have been valuable in unraveling the importance of specific AC in physiology and disease. For instance, a polymorphism of AC6 have been associated with alterations in blood pressure and heart rate regulation in humans (Hodges et al., 2010). Point mutations of the AC3 gene are also associated with decreased insulin release in animal models of type 2 diabetes (Abdel-Halim et al., 1998). Correlating specific mutations in tmAC genes with CB dysfunction and hypertension across patient populations may help refine CB disease related research. This data is currently unavailable but could be invaluable given the emerging relevance of the CB in cardiovascular system pathology.

Although ATP binding to ionotropic receptors likely mediates excitatory chemodischarge to hypoxia, DA and Ado are two key participants in modifying type I cell and/or post-synaptic cAMP via their modification of tmAC activity. Hypoxia-induced raises in type I cell $[Ca^{2+}]$ and ^{3}H-DA neurosecretion are depressed in the presence of specific D_2 receptor agonists (Benot and López-Barneo, 1990; Carroll et al., 2005; Conde et al., 2008), an effect that is associated with a reduction in CB cAMP content in both conditions. Deficiency of D_2 receptors in adult mice blunts type I cell neurosecretion, but not CSN responses to hypoxia, possibly consistent with opposing pre-synaptic and post-synaptic neuromodulation (Prieto-Lloret et al., 2007). Systemic inhibition of Ado receptors decreases, but does not abolish, the CB mediated acute phase of the hypoxic ventilatory response (Lee et al., 2005). Using *in vitro* CB preparations, Conde et al. reported that blocking Ado receptors depresses hypoxic induced CA release and chemoafferent activity, an effect that is greater in milder rather than severe hypoxic conditions (Conde et al., 2006b, 2012a). D_2 receptors are negatively coupled to AC while Ado A_{2B} are positively coupled to AC. Blockage of Ado A_{2B} receptors counteract the decrease in cAMP elicited by D_2 receptor activation suggesting an A_{2B} and D_2 autoreceptor interaction accounting for overall $[cAMP]_i$ in the type I cell (Conde et al., 2008). In acutely dissociated type I cells, Ado A_{2A} receptor inhibition abolishes the $[Ca^{2+}]_i$ elevations evoked by Ado (Xu et al., 2006). Since both A_{2A} and A_{2B} receptors exert their actions through excitation of tmACs (reviewed in Ribeiro and Sebastião, 2010), it is the increase in $[cAMP]_i$, that is most likely to account for its overall chemostimulatory function. Accordingly, directly inhibiting tmACs with SQ22536, does indeed depress hypoxic induced CA-secretion (Rocher et al., 2009).

These findings do not, however, confine CB cAMP content to the regulation of DA and Ado. Essentially any NT/receptor system that is coupled to tmAC will alter cAMP levels in the CB, including histamine/H_1 and H_3 receptors (Del Rio et al., 2008, 2009; Thompson et al., 2010), adrenaline/β-adrenergic receptors (Mir et al., 1983; Hauton et al., 2013), pituitary adenylate cyclase-activating protein (PACAP)/PAC_1 receptor (Xu et al., 2007; Roy et al., 2013), among others (also see **Table 1**).

sAC activity has been described in numerous tissues where changes in HCO_3^-/CO_2 are essential to their function. For instance in the testis, where sAC is highly expressed, sAC mediates sperm maturation and acquisition of motility (Buck et al., 1999; Hess et al., 2005). In the kidneys it regulates recycling of V-ATPse (Pastor-Soler et al., 2003), in airway epithelial cells sAC regulates the ciliary beat frequency (Schmid et al., 2007), and in corneal

endothelium it plays a role in the activation of the cystic fibrosis transmembrane conductance regulator (Sun et al., 2004). sAC mRNA has now been identified in the whole CB, and although the sAC mRNA cellular localization has not been demonstrated, it is expressed at greater level in the intact organ than in other non-chemosensitive neuronal tissues (Nunes et al., 2009, 2013).

The physiological role of sAC in CO_2 sensing was only recently studied in the CB chemoreceptors and its function appears somewhat equivocal. Contrary to those that reported rises in cAMP content, and PKA dependent Ca^{2+} current during isohydric hypercapnia (Pérez-García et al., 1990; Summers et al., 2002), observations from our laboratory indicate that increasing the HCO_3^-/CO_2 ratio from 24mM /5% (normocapnia) to 44mM/10% (isohydric hypercapnia) does not alter cAMP content, PKA activity or CSN discharge frequency and, under these conditions, these assays were insensitive to the sAC inhibitor KH7 (Nunes et al., 2013). We did however show that KH7 decreased cAMP content under basal conditions and we speculated that sAC contributes more to normocapnic rather than hypercapnic $[cAMP]_i$, at least in the rat CB. Examining the extent of sAC HCO_3^- saturation in normocapnia/normoxia and whether this alters type I cell $[Ca^{2+}]_i$, chemoafferent frequency or responses to hypoxia/acidosis may thus be an important area for future investigation.

THE ROLE OF PHOSPHODIESTERASES IN THE CAROTID BODY ACTIVITY

PDE catalyze the hydrolysis of the 3-cAMP phosphate bonds of adenosine 3,5—cyclic monophosphate to AMP. According to the pharmacological principle that the regulation of the second messenger degradation can often make a more rapid and larger percentage change in concentration than comparable regulation of the rates of synthesis, PDEs are important modulators of cAMP levels, preventing uncontrolled diffusion of cAMP through the cell and consequently contributing to the formation of localized pools or gradients of cAMP within the cell (Lugnier, 2006). There are 11 known distinct PDE isoforms, each displaying unique substrate affinity and variable adjustment to endogenous co factors and pharmacological inhibitors (Bender and Beavo, 2006).

Uncharacterized PDE was first identified in the CB in 1977 by Hanbauer and Lovenberg, and these studies provided the first evidence for an O_2 dependent cAMP affinity (Hanbauer and Lovenberg, 1977). From then on, studies aiming to indirectly assay CB AC activity and manipulate cAMP levels in responses to different oxygen concentrations have been performed in the presence of the xanthine 3-isobutyl-1-methylxantine (IBMX), a non-selective PDE inhibitor (k_i = 1–10 mM, Dousa, 1999 and IC_{50} = 2–50 mM, Bender and Beavo, 2006) with potential to block Ado receptors (k_i = 7.28 mM, Daly et al., 1991). A particular limitation of IBMX is its inability to inhibit PDE7 and PDE8 (Lugnier, 2006).

The PDE4 isoform was recently proposed as a major regulator of cAMP-hydrolyzing activity in the rat CB (Nunes et al., 2010). PDE4 comprises four subtypes (PDE4A, PDE4B, PDE4C, and PDE4D) with at least 35 known splice variants (Bender and Beavo, 2006). Selective pharmacological inhibition of PDE4 increases CB cAMP content in normoxia and causes even greater rises during hypoxia. These increases are however considerably lower than those observed in the presence of IBMX, suggesting a physiological role of additional isoforms (Nunes et al., 2010). However, at the time of this review, no functional data relating PDE4 activity with CB responses to hypoxia or hypercapnia has been published; functional studies are necessary to further strengthen the position of PDE4 in the CB. Intriguingly, given that PDE4 activity increases with chronic hypoxia in O_2-sensitive pulmonary arteries and blood (Maclean et al., 1997; Spoto et al., 1998; Millen et al., 2006), a compensatory role for PDE4 to counter CB hyperactivity, although speculative, is plausible. That said, the consequences of chronic hypoxia or chronic intermittent hypoxia exposure on PDE activity in the CB remains to be explored.

THE ROLE OF cAMP EFFECTORS IN THE CAROTID BODY ACTIVITY: PROTEIN KINASE A, EXCHANGE PROTEIN ACTIVATED BY cAMP AND CYCLIC NUCLEOTIDE GATED CHANNELS

PKA is the classical downstream effector of cAMP. It is a holotetrameric serine/theonine kinase composed of two regulatory and two catalytic subunits. Four cAMP molecules bind to the regulatory subunits, each with two cAMP binding sites. The cAMP binding promotes the dissociation of the catalytic subunits that bind ATP to become catalytically active and phosphorylate serine and threonine residues in intracellular targets such as A-kinase anchoring proteins (AKAPS) and ion channels. AKAPs tether PKA to particular cellular organelles and to the plasma membrane confining the PKA signaling to a small pool within the cells (Beene and Scott, 2007). In the nucleus, PKA can phosphorylate transcription factors, such as cAMP response element binding protein (CREB), and thus regulate gene expression.

A physiological role for PKA in CB chemotransmission is at present controversial. In dissociated rabbit type I cells, PKA inhibition by PKA inhibitor (PKAi) diminishes the rise in L-type Ca^{2+} current in response to isohydric hypercapnia (Summers et al., 2002). However, we reported that PKA activation status, as measured by Fluorescent Resonance Energy Transfer (FRET) based reporters, is unaltered during isohydric hypercapnia in isolated rat type I cells (Nunes et al., 2013). Multiple blockers of PKA have no effect on hypoxic CA-secretion in the intact rat CB preparation (Rocher et al., 2009). In contrast, acute rises in type I cell $[Ca^{2+}]_i$ evoked by Ado and PACAP are essentially abolished by PKA inhibition with H89 (10 μM) (Xu et al., 2006, 2007). Sustained plateau CSN activity mediated by PACAP is inhibited by only 41% in the presence of H89, in a preparation including the carotid bifurcation- CB-CSN-superior cervical ganglion (Roy et al., 2013). Furthermore, type I cell activation by methylcholine is sensitive to tmAC inhibition but not H89 inhibition, which suggests that cAMP signaling cascades in the CB are independent of PKA activation (Thompson and Wyatt, 2011). Whether these multiple discrepancies reflect fundamental species differences, different preparations (whole CB vs. cultures or type I cells) or other unidentified experimental factors are unclear, but precaution must be taken when using H89 due its reported non-specific inhibitory effect (Lochner and Moolman, 2006). In cellular preparations, it is likely that transmitters released from type I cells are lost to the superfusate and so their potential excitatory

or inhibitory autoregulation of the type I cell chemosensitivity to hypoxia or hypercapnia is not apparent. Additionally, the contribution of retrograde communication (PG neurons to CB cells) should be also taken in account (Katz et al., 1983; Katz and Black, 1986; Iturriaga et al., 2003) as well as the new concept of tripartite sensory synapse between type I, type II and PG neurons (Nurse, 2014). Also, we now know that the contribution of the NTs to the hypoxic chemosensitivity in the CB depends on hypoxic intensity meaning that different hypoxic intensities will evoke the release of different NTs (Conde et al., 2012a) and therefore the differences observed in PKA activation can reflect distinct hypoxic intensities/mediators involved.

Different effects of PDE4 inhibitors on cAMP accumulation induced by hypoxia (Nunes et al., 2010), could suggest a different degree of PDE phosphorylation induced by differences in PKA activity mediated by hypoxia (Bender and Beavo, 2006).

Complex cAMP driven mechanisms through PKA and extracellular signal regulated kinase (ERK) mediated phosphorylation can modify PDE4 specific isoforms activity and subsequently alter the sensitivity to selective inhibitors (Bender and Beavo, 2006). Thus, determining whether acute hypoxia causes PDE4 activation by PKA or ERK mediated phosphorylation would be of interest.

EPAC is a guanine nucleotide exchange factor (GEF) for the RasGTPase homologs, Rap1 and Rap2. EPAC is composed of two regions: an N-terminal regulatory region containing a cAMP-binding site and a C-terminal catalytic region, with GEF activity (De Rooij et al., 1998). In the inactive conformation, EPAC is folded and the regulatory domain functions as an auto-inhibitory domain. cAMP binding unfolds the protein, allowing Rap to bind (for a review Gloerich and Bos, 2010). Rap GTPases cycle between an inactive GDP-bound and an active GTP-bound state, with GEFs mediating the exchange of GDP for GTP. GTPase-activating proteins then convert Rap to the inactive form. The activated Rap-GTP activates a variety of different mechanisms in the cell: promotes integrin-mediated cell adhesion, gap junction formation and $ERK_{1/2}$ MAPK-mediated protein phosphorylation, stimulates phospholipase C-ε which hydrolyzes PIP2 to generate diacylglycerol, and the Ca^{2+} mobilizing second messenger IP_3 (for a review Holz et al., 2006).

Rocher and co-workers initially proposed a physiological role for EPAC in the CB by examining the effects of the EPAC activator (8-pCPT-2′-O-Me-cAMP) and inhibitor (brefeldin) on the release of CAs (Rocher et al., 2009). Specifically, 8-pCPT-2′-O-Me-cAMP reversed the action of SQ22536 and brefeldin inhibited CA-secretion during hypoxia by approximately 50%. These authors suggested that the effectors of EPAC were likely to be the exocytotic machinery and K^+ channels (Rocher et al., 2009). More recently, this group has identified the expression of both EPAC1 and EPAC2 in the rat CB (Ramirez et al., 2012). In addition, EPAC activation by cAMP is proposed to cause downstream stimulation of the IP_3 receptor in the endoplasmic reticulum (Thompson and Wyatt, 2011) along with activation of PKC (Roy et al., 2013). Thus, crosstalk between the G_s/G_i (cAMP-related) and G_q signaling pathways within the type I cell likely occurs. Better characterization of this interaction could be particularly insightful given the known upregulation of G_q signaling associated with CB dysfunction in sleep disorder breathing (Peng et al., 2006) and CHF (Li et al., 2006).

cAMP can directly bind to cyclic nucleotide-gated (CNGC) and hyperpolarization-activated cyclic nucleotide-modulated (HCNC) ion channels. These channels belong to a superfamily of voltage-gated cation channels, and thus the binding of cAMP to these channels is translated into changes in membrane potential and influx of Ca^{2+} and Na^+. By conducting Ca^{2+}, they can stimulate Calmodulin (CaM) and CaM-dependent kinases and, in turn, modulate cAMP production by regulating activity of AC and PDE. Since CNGC and HCNC are also permeable to Na^+ and K^+, they can also alter the membrane potential in electrically active cells. The presence of these channels in the rat CB has been suggested by the work of Stea and co-workers (Stea et al., 1995); however, others have reported that cAMP analogs do not affect Ca^{2+} currents in type I cells (López-López et al., 1993). HCNC ion channels have not been characterized in the CB.

cAMP can be released from a variety of cell types and tissues (for a review see Bankir et al., 2002). Transport of cAMP moves against a concentration gradient, that is temperature dependent, unidirectional and requires energy (Rindler et al., 1978). One of the proposed functions of the extracellular cAMP is to regulate extracellular Ado levels. Extracellular cAMP can be metabolized by ecto-phosphodiesterases to adenosine monophosphate (5′-AMP), and then by ecto-5′-nucleotidases to Ado (Conde et al., 2009). Interestingly, extracellular cAMP can modulate phenotype, function and differentiation of human monocytes through A_{2A} and A_{2B} Ado receptors (Sciaraffia et al., 2014). The contribution of extracellular cAMP to Ado production/Ado receptors activation has never been investigated in the CB, and cAMP released from the CB has never been quantified.

Intracellular cAMP can diffuse intercellularly through well-characterized gap junctions (Bennett et al., 1991; Bevans et al., 1998; Bevans and Harris, 1999). In the rat CB, connexin 43 (Cx43) gap junctions are found between type I cells and carotid nerve terminals, and mediate intercellular communications and transport of small molecules and ions (Abudara and Eyzaguirre, 1996; Eyzaguirre, 2007). Electrical coupling, gap junction formation and connexin expression are regulated by cAMP (Abudara and Eyzaguirre, 1998; Abudara et al., 1999, 2000; Eyzaguirre, 2007) and chronic hypoxic exposure (Chen et al., 2002a).

All together, these findings suggest different regulations of cAMP signaling in the CB, mediated not only by the enzymes directly involved in the synthesis and degradation of this signaling molecule, but also by a variety of effectors that can modulate its accumulation, and consequently, trigger changes in the CB activity.

CURRENT UNDERSTANDING OF THE ROLE OF cAMP-SIGNALING PATHWAY ON THE OVERALL CAROTID BODY CHEMOSENSITIVITY

Observations from our laboratory show that cAMP levels are higher during normoxia than in hypoxic or hyperoxic superfusate in the whole CB from young and adult rats (Monteiro et al., 2011). These results support our view that cAMP-pathway may be involved in the maintenance of basal activity of the CB (translated in basal release of NTs or basal CSN electrical activity), suggesting a homeostatic and/or adaptative role for the

cAMP-pathway in the rat CB. Although, inter-species differences may exist (Delpiano and Acker, 1984, 1991; Wang et al., 1989; Pérez-García et al., 1990; Cachero et al., 1996; Chen et al., 1997). Findings from experiments studying NTs systems using CB from rabbits vs rat and cat often differ. For example, dopaminergic influence on CB function is excitatory in the rabbit while it is inhibitory in CB from other mammalian species (Fidone et al., 1982; Vicario et al., 2000a), with higher DA secretion in rats, combined with lower cAMP accumulation (through D_2 receptor activity).

Together with the other reported actions of cAMP signaling in the CB (**Table 3**), our results are consistent with the view that the cAMP-pathway is involved in the maintenance of basal CB excitability and thus the basal release of NTs and CSN sensory discharge frequency. The observed reduction in hypoxic sensitivity when cAMP signaling is targeted indicates that basal levels of cAMP act to prime the CB to subsequent hypoxic stimulation. This is likely achieved through the basal regulation of PKA and EPAC and possibility other, as yet unidentified downstream effectors. Given the synergy between hypoxia and hypercapnia, the basal type I cell $[cAMP]_i$ may also confer the excitability to high CO_2 or H^+ although further evidence is required to confirm this. It would be of considerable interest and relevant to humans to determine how chronic hyperoxia and/or hypercapnia modifies cAMP signaling in the CB.

NOVEL TECHNIQUES TO STUDY cAMP SIGNALING IN THE CAROTID BODY

With tmACs restricted to membranes, and sAC, PDEs and cAMP effectors widely distributed within the cells, cAMP accumulation is spatially and temporally controlled, generating cAMP microdomains. Thus, a comprehensive study of cAMP signaling should complement the cAMP quantifications made in CB homogenates using RIA (Pérez-García et al., 1991; Wang et al., 1991a; Cachero et al., 1996) or EIA (Batuca et al., 2003; Conde et al., 2008; Nunes et al., 2010, 2013; Monteiro et al., 2011), which are themselves static and terminal assays. Measurements in intact type I cells using reporter protein constructs may allow for a more detailed quantification of cAMP in distinct subcellular compartments. Nowadays, a variety of FRET-based biosensors are available to visualize signaling dynamics in living cells (Sample et al., 2014). The literature is void of data obtained using life imaging techniques to manipulate cAMP in the CB. Our laboratory in collaboration with the laboratory of Dr. Jin Zhang, have been successful in using FRET-based reporters in CB type I cells to interrogate cAMP-signaling pathways (Nunes et al., 2013).

Table 3 | Effects mediated by cAMP signaling in the carotid body.

Effects mediated by cAMP	Preparation	Technique	Agents that modulate [cAMP]	References
Increase of junctional conductance	Type I cells (young rats)	Dual-voltage clamping	dB-cAMP (1mM), 8-Br-cAMP (1 mM, 3 h)	Abudara and Eyzaguirre, 1998
Increase of the tyrosine hydroxylase gene expression elicited by hypoxia	Whole CB (adult rats)	Reverse-Transcriptase-polymerase chain reaction	FSK (0.01 mM, 3 h)	Chen et al., 1995
Activation of Cl^- currents	Type I cells (P10 rats)	Patch-clamp/whole-cell recording	cAMP (0.2 mM) 8-bromoadenosine-cAMP (2 mM)	Carpenter and Peers, 1997
Increase of Na^+ and Ca^{2+} inward currents and capacitance	Type I cells (P5-12 rats)	Patch-clamp/whole-cell recording	dB-cAMP (0.2–1 mM) and FSK (0.01 mM) up to 15 days	Stea et al., 1995
Induction of Na^+- channels and hypertrophy of type I cells	Type I cells (P5-12 rat)	Patch-clamp/whole-cell recording	Bt2-cAMP (1 mM, upto 14 days)	Stea et al., 1992
Potentiation of (30% O_2)-evoked CA release and CSN discharge	Whole CB, CSN (adult rabbit)	CSN activity recording; CA release	FSK (0.01 mM, 10 min)	Wang et al., 1991a
Increase of DA release elicited by hypoxia (5% O_2)	Whole CB (adult rabbit)	CA release	FSK (5–10μM), dB-cAMP (2mM) IBMX (0.5 mM), ISO (0.01–0.050 mM)	Pérez-García et al., 1990, 1991, 1993
Elevation of Ca^{2+}- currents (mimic the effect of hypercapnia)	Type I cells (adult rabbit)	Whole-cell recording	8-Br-cAMP (0.5 mM, 10 min)	Summers et al., 2002
Increase of GAP-43 and neurofilament (NF68 and NF160 kD) expression and neurite outgrowth	Type I cells (P5-7 rat)	Double-label immunofluore scence	dB-cAMP (1mM), FSK (0.01 mM), up to 2 weeks	Jackson and Nurse, 1995

dB-cAMP or Bt2-cAMP, Dibutyryl-cAMP; 8-Br-cAMP, 8-Bromoadenosine-cAMP; FSK, Forskolin; P, Postnatal day; CA, catecholamines; CSN, Carotid Sinus Nerve; DA, Dopamine; ISO, Isoprostane; IBMX, Isobutyl-1-methylxanthine; GAP-43, Growth-Associated Protein 43; CB, carotid body.

Image-based techniques can be used to understand interactions between cAMP and other mediators that raise $[Ca^{2+}]_i$ levels. Intensification of $[Ca^{2+}]_i$ signals have been linked with raised levels of angiotensin II, endothelin-1, cytokines, insulin and free radicals along with decreases in nitric oxide: levels of these substances are changed in disease states, associated with CB dysfunction (Chen et al., 2002b; Rey et al., 2006; Fung et al., 2007; Li et al., 2010; Schultz, 2011; Del Rio et al., 2012; Lam et al., 2012; Ribeiro et al., 2013). Intracellular Ca^{2+} levels can influence cAMP signaling directly through modulation of the activity of PDE and AC isoforms or indirectly through PKC activity, not only by allosteric regulation, but also by desensitization of GPCRs. In this sense, a compensatory cAMP mechanism may function to partially restore some sort of homeostatic control within the type I cell despite serious remodeling of the sensory transduction cascade in CB dysfunction.

As described above, our current view is that cAMP has an important role in the CB homeostasis, as suggested by the higher cAMP levels under normoxic conditions. Understanding how $[Ca^{2+}]_i$ and cAMP signaling is modified in conditions that lead to CB dysfunction, will be fundamental to understanding the role of these second messengers in the CB transduction/neurotransmission mechanism in health and disease.

IS CAROTID BODY IMPLICATED IN THE EFFECTS OF DRUGS THAT MODIFY cAMP FOR THERAPEUTIC PURPOSES?

The following section focuses on the CB mediated effects induced by currently clinical therapy that target cAMP-signaling. It is not aimed to extensively describe the putative drug effects that can be mediated by the CB but only to give insights that can stimulate future research in a field where the information is scarce. **Figure 1** summarizes molecular targets in type I cell and CSN endings that may be affected by drugs-induced changes in cAMP accumulation.

Exogenous DA is extensively used in human to improve cardiac output and peripheral perfusion in patients with cardiogenic and septic shock. Several years after using DA, its inhibitory effects on ventilation in man were described and attributed to an effect on chemoreceptor reflexes (Welsh et al., 1978). Twenty years later, Van de Borne et al. (1998) showed that repeated use of DA impairs the ventilatory response to hypoxemia due to an inhibitory effect on CSN activity, which explains why when administered in low doses to conscious patients, DA reduces the discomfort caused by

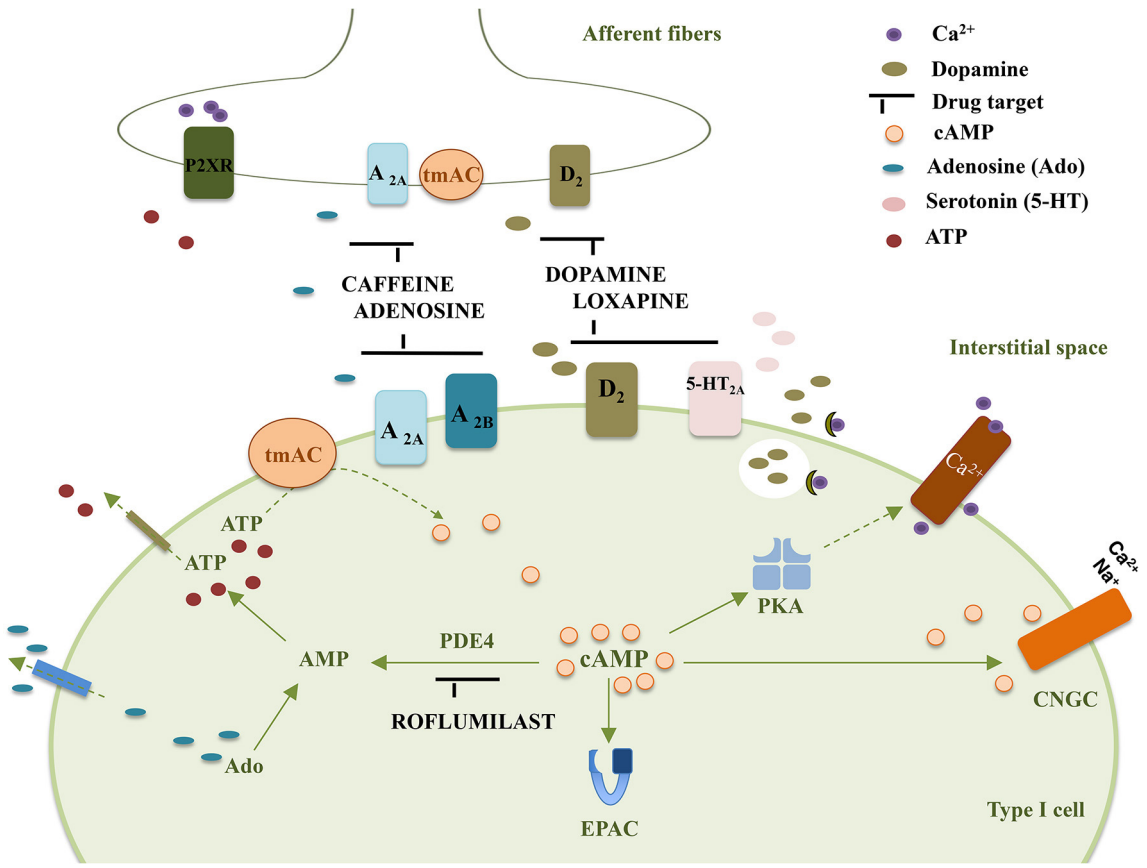

FIGURE 1 | Representation of some drug targets in type I cells and CSN endings that affect cAMP accumulation in the carotid body. tmAC, transmembrane Adenylyl Cyclase; PKA, Protein Kinase A; EPAC, Exchange Protein Activated cAMP; D_2, Dopamine receptor D_2; A_{2A}, Adenosine receptor A_{2A}; A_{2B}, Adenosine receptor A_{2B}; $5\text{-}HT_{2A}$, serotonin receptor $5\text{-}HT_{2A}$; P2XR, ATP ionotropic P2X receptor; Ado, adenosine; PDE4, Phosphodiesterase 4; CNGC, Cyclic Nucleotide Gated channel.

hypoxemia. Although its clinical use as vasopressor remains, both DA and NA have been used: DA decreases CB activity while NA does not appear to have an effect on CB activity (Zapata, 1975; Debaveye and Van den Berghe, 2004).

The impact of chronic use of *antipsychotics* (D_2 antagonists) on peripheral chemoreflexes is unknown but beneficial effects of loxapine on agitation and breathing patterns during weaning from mechanic ventilation have been described (Sztrymf et al., 2010). These findings open doors to a promising field to explore CB manipulation to improve adaptation to mechanical ventilation.

Acute administration of *Ado* (full agonist of A_{2A} and A_{2B} receptors) is clinically useful to revert paroxysmal supraventricular tachycardia and causes dyspnea and chest discomfort mediated by CB activation (Watt et al., 1987; Reid et al., 1991). *Caffeine* is a non-selective Ado antagonist that has been used to prevent and treat apnea of prematurity due, primarily, to the blockade of inhibitory Ado A_1 receptors in the CNS. Moreover, the effects of chronic coffee consumption have been extensively studied in the last years and it is now consensual that coffee, and probably caffeine, may reduce the risk of type 2 diabetes mellitus and hypertension, as well as other conditions associated with cardiovascular risk such as obesity and depression (O'Keefe et al., 2013). In fact, research in our laboratory have shown that chronic caffeine intake decreases circulating CAs, prevents diet-induced insulin resistance and hypertension (Conde et al., 2012b) and restores insulin sensitivity in aged rats (Guarino et al., 2013). Knowing that at the CB, caffeine blocks excitatory Ado A_{2A}/A_{2B} receptors (Conde et al., 2006b) and that CB denervation prevents the development of insulin resistance and hypertension induced by hypercaloric diets (Ribeiro et al., 2013) the CB modulation by caffeine can improve conditions associated to sympathetic mediated CB hyperactivity (e.g., hypertension). Other effects of caffeine have been described in the CB e.g., mobilization of Ca_i^{2+} stores in the CB cells by ryanodine receptor activation (Vicario et al., 2000b; Mokashi et al., 2001). However, they do not seem to be relevant in the clinical setting because their effects are achieved with toxic concentrations (Fredholm et al., 1999).

Roflumilast, an oral selective PDE4 inhibitor, was approved in 2011 by both the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of severe chronic obstructive pulmonary disease (COPD), due to its anti-inflammatory and bronchodilator effects. No evidences of roflumilast effects on CB activity have been reported and those are difficult to address essentially because of their CNS effects and the variability of the PDE inhibitors efficacy in hypoxic conditions (Nunes et al., 2010). Curiously, the roflumilast efficacy to reduce the risk of COPD exacerbations has only been shown in patients that experience reduced dyspnea (Rennard et al., 2014). Since CB resection relieves dyspnea in COPD patients and improves FEV_1 (Force Expiratory Volume) (Whipp and Ward, 1992) but exacerbates hypoxemia and hypercapnia and overall worsen the long term outcome (Stulbarg et al., 1989), the link between the mechanism of roflumilast action in COPD patients and CB activity merits further studies.

From the above evidence, one can conclude that the manipulation of cAMP signaling pathway is important to address O_2/CO_2 related diseases. However, manipulation of cAMP signaling may have consequences in the CB, that are clinically relevant and that have not yet been identified.

CONCLUSIONS

The importance of cAMP to CB physiology has moved from a discarded player in the O_2 chemotransduction to a central signaling molecule that is converged upon by multiple NTs/NMs, which collectively maintain an equilibrated CB activity. It remains to be seen whether modification of cAMP can improve patient outcomes in diseases associated with CB impairment or hyperactivity. We suggest that cAMP has an important role in the homeostasis of the CB since cAMP levels seem to be higher under normoxic conditions. Despite the increase in knowledge of CB physiology, the activity of tyrosine hydroxylase is still the hallmark of the CB and cAMP the classical second messenger of dopamine D_2 receptor signaling.

Understanding how calcium and cAMP cooperate in dysfunction CB, will be fundamental to understand the role of these second messengers in the CB transduction mechanism. Additionally, systemic pharmacological manipulation of cAMP signaling can have clinically relevant consequences mediated by the CB. This may proven to be an exciting field of research that is still currently unexplored.

ACKNOWLEDGMENT

This study was funded by an operating grant PTDC/SAU-TOX/112264/2009.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 August 2014; accepted: 01 October 2014; published online: 28 October 2014.

*Citation: Nunes AR, Holmes AP, Conde SV, Gauda EB and Monteiro EC (2014) Revisiting cAMP signaling in the carotid body. Front. Physiol. **5**:406. doi: 10.3389/fphys.2014.00406*

This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology.

frontiers in PHYSIOLOGY

REVIEW ARTICLE
published: 15 October 2014
doi: 10.3389/fphys.2014.00398

Glucose sensing by carotid body glomus cells: potential implications in disease

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The carotid body (CB) is a key chemoreceptor organ in which glomus cells sense changes in blood O_2, CO_2, and pH levels. CB glomus cells have also been found to detect hypoglycemia in both non-primate mammals and humans. O_2 and low-glucose responses share a common final pathway involving membrane depolarization, extracellular calcium influx, increase in cytosolic calcium concentration, and neurotransmitter secretion, which stimulates afferent sensory fibers to evoke sympathoadrenal activation. On the other hand, hypoxia and low glucose induce separate signal transduction pathways. Unlike O_2 sensing, the response of the CB to low glucose is not altered by rotenone, with the low glucose-activated background cationic current unaffected by hypoxia. Responses of the CB to hypoglycemia and hypoxia can be potentiated by each other. The counter-regulatory response to hypoglycemia by the CB is essential for the brain, an organ that is particularly sensitive to low glucose. CB glucose sensing could be altered in diabetic patients, particularly those under insulin treatment, as well as in other medical conditions such as sleep apnea or obstructive pulmonary diseases, where chronic hypoxemia presents with plastic modifications in CB structure and function. The current review will focus on the following main aspects: (1) the CB as a low glucose sensor in both *in vitro* and *in vivo* models; (2) molecular and ionic mechanisms of low glucose sensing by glomus cells, (3) the interplay between low glucose and O_2 sensing in CB, and (4) the role of CB low glucose sensing in the pathophysiology of cardiorespiratory and metabolic diseases, and how this may serve as a potential therapeutic target.

Keywords: carotid body, glucose sensing, O_2 sensing, hypoglycemia, intermittent hypoxia, sleep apnea, chronic hypoxia, diabetes

INTRODUCTION

Hypoglycemia, or a low blood glucose level, is a physiological condition that is detected by the body to trigger compensatory counter-regulatory responses, which are essential for maintaining glucose supply to organs, such as the brain, strictly dependent on this metabolite for survival. Alterations of glucose sensing might play an important pathogenic role in several diseases, especially those related to sympathoexcitation. The carotid body (CB) is a key chemoreceptor organ that may critically participate in glucose homeostasis. The first study linking the CB to glucose metabolism was reported more than 25 years ago (Alvarez-Buylla and de Alvarez-Buylla, 1988), and knowledge of the molecular mechanism underlying CB glucose sensing has advanced recently due in part to improvements in CB preparations that are suitable for *in vitro* recording of physiological parameters (Pardal and Lopez-Barneo, 2002a). The role of the CB in several cardiorespiratory and metabolic disorders has also been studied in the past few years (Paton et al., 2013; Ribeiro et al., 2013; Schultz et al., 2013) with the CB recently proposed as a potential therapeutic target for these diseases (McBryde et al., 2013).

CAROTID BODY AND O_2 SENSING

The CB, the main arterial chemoreceptor, is located at the carotid artery bifurcation. The CB is composed of clusters (glomeruli) of electrically excitable neuron-like glomus (type I) cells surrounded by glia-like sustentacular (type II) cells. Type II cells, or a subpopulation of them, have recently been identified as neural stem cells that contribute to the growth of the organ in conditions of chronic hypoxemia (Pardal et al., 2007; Platero-Luengo et al., 2014). Type I glomus cells have secretory vesicles containing dopamine and other neurotransmitters. CB glomus cells sense changes in the chemical composition of blood, including O_2 tension (PO_2), CO_2 tension, pH, and other stimuli (reviewed by Lopez-Barneo et al., 2008; Kumar and Prabhakar, 2012).

A major physiological function of the CB is to sense changes in blood PO_2, as this variable is not detected by central chemoreceptors. CB glomus cells behave as O_2-sensitive presynaptic-like elements. During hypoxia, O_2-sensitive K^+ channels are closed in the plasma membrane of glomus cells, which triggers membrane depolarization, Ca^{2+} influx, and neurotransmitter release. This signal is sent to the brainstem respiratory centers by afferent

fibers of the carotid-sinus nerve to mediate a compensatory acute hyperventilatory response in order to increase O_2 tension in the blood (Weir et al., 2005; Lopez-Barneo et al., 2008). Besides the CB glomus cells, O_2-sensitive ion channels have been described in numerous cell classes, such as chromaffin cells in the adrenal medulla, neuroepithelial bodies of the lung, pulmonary and systemic vascular smooth muscle, and heart myocytes among others (see for review Lopez-Barneo et al., 1999, 2001).

CAROTID BODY AND GLUCOSE SENSING

GLUCOSE SENSING IN DIFFERENT ORGANS

The brain is very sensitive to decreased glucose supply from the blood. Glucose-sensitive neurons have been found in different regions of the brain (Routh, 2002), including the hypothalamus (Biggers et al., 1989; Dunn-Meynell et al., 2002; Levin et al., 2004; Burdakov et al., 2006) and striatum (Calabresi et al., 1997) to mediate reflexes that counter-balance the changes of glucose level. Glucose-sensitive neurons have specific functional and molecular properties. Glut2, a low-affinity glucose transporter is expressed in some glucose-sensing cells (Schuit et al., 2001; Thorens, 2001). Glucokinase, a low-affinity hexokinase characteristic of pancreatic beta cells, seems to play an important role in both glucose-stimulated and inhibited neurons (Dunn-Meynell et al., 2002). In addition to the well-established role of central neurons in glucose control, numerous pieces of evidence indicate that glucose sensors also exist at the periphery and that they have an essential physiological role (Cane et al., 1986). In addition to α-cells of the pancreas, hypoglycemia-sensitive cells have also been suggested to exist in the liver (Hamilton-Wessler et al., 1994), near the portal vein (Hevener et al., 1997), and in the adrenal gland of the newborn (Livermore et al., 2012).

CAROTID BODY AS A SENSOR OF LOW GLUCOSE

The first evidence linking the CB with glucose metabolism was reported by Alvarez-Buylla and de Alvarez-Buylla (1988), Alvarez-Buylla and Roces de Alvarez-Buylla (1994). More recently, *in vivo* studies demonstrated that the counter-regulatory response to insulin-induced hypoglycemia is impaired in CB-resected dogs (Koyama et al., 2000). Moreover, these animals exhibit suppressed exercise-mediated induction of arterial plasma glucagon and norepinephrine and, therefore, cannot maintain blood glucose levels during exercise (Koyama et al., 2001).

Direct molecular proof of the CB as a glucose-sensing organ was first reported by Pardal and López-Barneo using the CB thin slice preparation and amperometry techniques (Pardal and Lopez-Barneo, 2002b). In this *in vitro* system, rat CB glomus cells secrete neurotransmitter when exposed to a glucose-free solution (**Figures 1A,B**) (Garcia-Fernandez et al., 2007). This secretory activity is reversible, depending on external Ca^{2+} influx (**Figure 1C**), and is proportional to the degree of glucopenia. Responses to hypoglycemia, including neurotransmitter release and sensory fiber discharge, have also been observed in other *in vitro* studies using rat CB slices (Garcia-Fernandez et al., 2007; Zhang et al., 2007), rat CB/petrosal ganglion co-culture (Zhang et al., 2007), and cat CB (Fitzgerald et al., 2009). Recently, the hypoglycemia-mediated secretory response has also been detected in human glomus cells dispersed from post mortem CBs (Ortega-Saenz et al., 2013) (see below). However, this topic is controversial as other groups have failed to detect glucose sensing by explanted CBs or dissociated rat CB cells (Bin-Jaliah et al., 2004; Gallego-Martin et al., 2012). Bin-Jaliah et al. (2004) reported CB stimulation in rats secondary to insulin-induced hypoglycemia. However, they proposed that sensing of hypoglycemia by the CB could be an indirect phenomenon dependent on other metabolically mediated blood borne factor. Systemic studies performed in humans have also reported opposing results regarding the role of the CB in hormonal counter-regulatory responses to hypoglycemia (Ward et al., 2009; Wehrwein et al., 2010). Although not fully understood, these discrepancies could possibly result from differences in CB sample preparation or limitations in experimental design. In any event, taken together the available experimental data suggests that low glucose sensing by CBs is likely to be a general phenomenon among mammals that has potential pathophysiological implications.

MOLECULAR AND IONIC MECHANISMS OF LOW GLUCOSE SENSING BY CAROTID BODY GLOMUS CELLS

The molecular mechanisms underlying CB glomus cell activation by hypoglycemia have been investigated in both lower mammals and human CB tissue samples (Pardal and Lopez-Barneo, 2002b; Garcia-Fernandez et al., 2007; Zhang et al., 2007; Fitzgerald et al., 2009; Ortega-Saenz et al., 2013). In our initial study we reported that, like O_2 sensing by the CB, macroscopic voltage-gated outward K^+ currents are inhibited in patch-clamped rat glomus cells exposed to glucose-free solutions (Pardal and Lopez-Barneo, 2002b). However, we soon realized that besides this phenomenon, low glucose elicits a membrane depolarization of ∼8 mV (**Figures 1D,E**) (Garcia-Fernandez et al., 2007), which is the main process leading to extracellular Ca^{2+} influx into glomus cells, as demonstrated by microfluorimetry experiments using Fura-2-AM labeled cells (**Figure 1F**) (Pardal and Lopez-Barneo, 2002b; Garcia-Fernandez et al., 2007; Ortega-Saenz et al., 2013). The increase in intracellular Ca^{2+}, which is demonstrated by the inhibition of the secretory activity by Cd^{2+}, a blocker of voltage-gated Ca^{2+} channels (Pardal and Lopez-Barneo, 2002b; Garcia-Fernandez et al., 2007), results in exocytotic neurotransmitter release (Pardal and Lopez-Barneo, 2002b; Garcia-Fernandez et al., 2007; Zhang et al., 2007; Ortega-Saenz et al., 2013). This neurotransmitter release triggers afferent discharge and activation of counter-regulatory autonomic pathways to increase the blood glucose level (Zhang et al., 2007; Fitzgerald et al., 2009). The depolarizing receptor potential triggered by low glucose has a reversal potential above 0 mV and is due to the increase of a standing inward cationic current (carried preferentially by Na^+ ions) present in glomus cells (**Figures 1G,H**) (Garcia-Fernandez et al., 2007). Indeed, in contrast with hypoxia, low glucose decreases the membrane resistance of glomus cells recorded with the perforated patch configuration of the patch clamp technique to ∼50% of control (González-Rodríguez and López-Barneo, unpublished results). As reported by others (Carpenter and Peers, 2001), the background Na^+ current plays a major role in chemotransduction by glomus cells as it sets the membrane potential to relatively depolarized levels, near the threshold for the opening of Ca^{2+} channels.

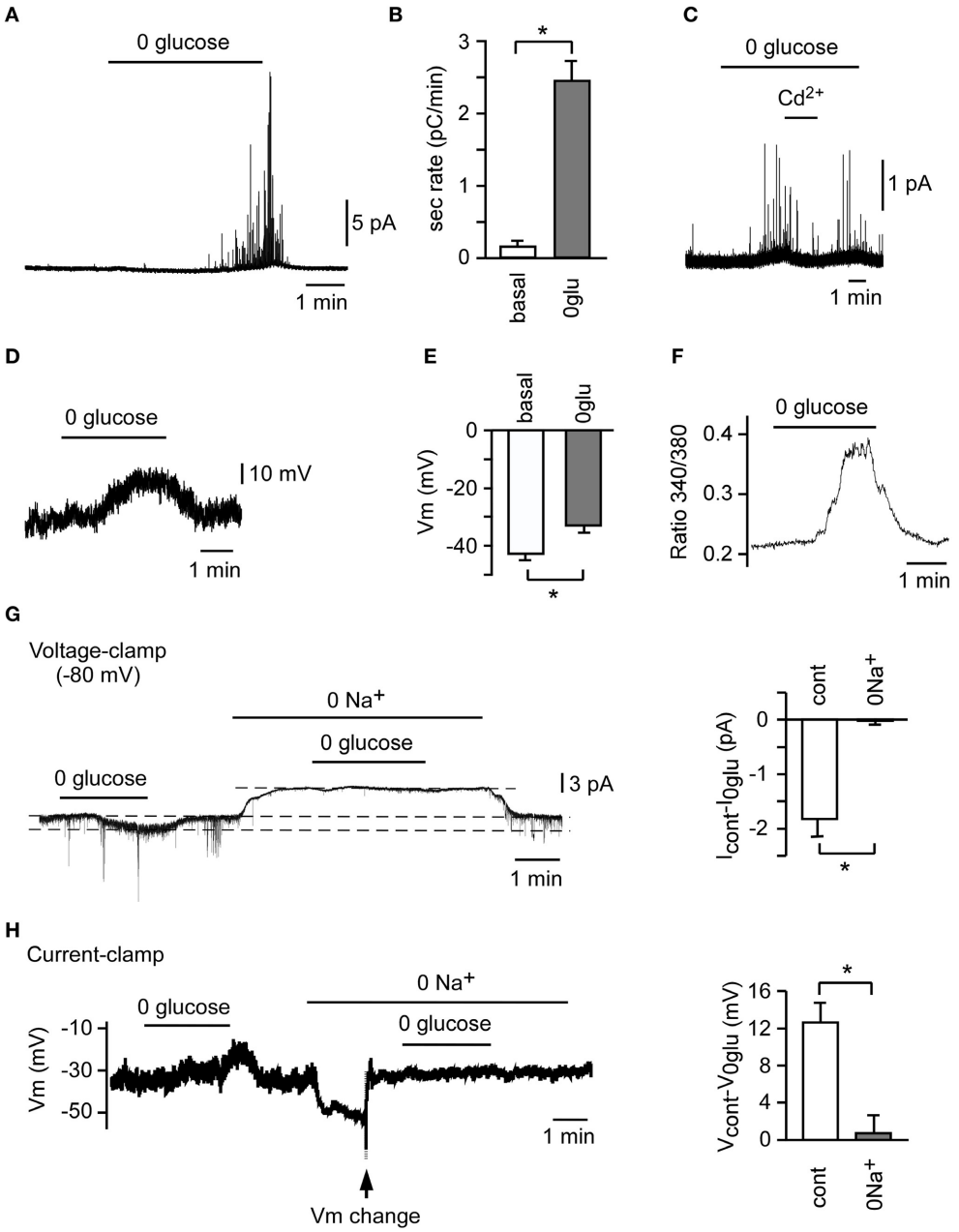

FIGURE 1 | Counter-regulatory response to hypoglycemia in rat carotid body (CB) slices and isolated glomus cells. A representative secretory response **(A)** and average secretion rate **(B)** induced by glucopenia in glomus cells from CB slices ($n = 3$). **(C)** Abolition of the secretory response to hypoglycemia by 100 μM Cd^{2+}. A representative depolarizing receptor potential **(D)** and average membrane potential **(E)** induced by 0 glucose in CB glomus cells ($n = 25$). **(F)** Reversible increase in cytosolic Ca^{2+} concentration in a Fura-2-loaded glomus cell in response to 0 glucose. **(G)** Abolition of 0 glucose-induced increase in current (Icontrol-I0glu) by replacement of extracellular Na^+ with N-methyl-D-glucamine (0 Na^+) in voltage-clamped glomus cells ($n = 3$). **(H)** Inhibition of 0 glucose-induced depolarization (Vcontrol-V0glu) by replacement of extracellular Na^+ with N-methyl-D-glucamine (0 Na^+) in current-clamped glomus cells ($n = 3$). To compensate for the hyperpolarization induced by 0 Na^+, Vm was changed manually to the previous resting value (arrow) $*p < 0.05$ (Modified from Garcia-Fernandez et al., 2007).

GLUCOSE TRANSPORT AND METABOLISM IN THE CAROTID BODY DURING LOW GLUCOSE SENSING

The mechanism of low glucose sensing by CB glomus cells does not seem to be the same as high glucose sensing by other glucose-sensing cells in terms of glucose transport and metabolism. Glut2 and glucokinase, molecules specifically expressed in high glucose-sensing cells (Schuit et al., 2001; Thorens, 2001), are not expressed in the CB (Garcia-Fernandez et al., 2007). However, glucose metabolism appears to be necessary for low glucose sensing by the CB, since non-metabolizable glucose fails to prevent the

glucose deficiency-induced catecholamine secretion by glomus cells (Garcia-Fernandez et al., 2007).

REGULATION OF CAROTID BODY LOW GLUCOSE SENSING

SIMILARITIES AND DIFFERENCES BETWEEN LOW GLUCOSE AND O_2 SENSING

O_2 and low-glucose sensing by the CB share many similarities. Both signaling pathways involve the inhibition of voltage-gated K^+ channels, plasma membrane depolarization, influx of extracellular Ca^{2+}, neurotransmitter release, and afferent nerve firing to transmit the signal to the brain, in order to trigger counter-regulatory responses to increase blood O_2 tension and glucose concentration. On the other hand, the initial steps of the signaling pathways are different for each. Low glucose triggers a depolarizing receptor potential, which is dependent on the activation of background cationic Na^+-permeable channels (Garcia-Fernandez et al., 2007), which do not seem to be regulated by hypoxia (Carpenter and Peers, 2001). Although voltage-gated K^+ channels are inhibited upon exposure of CB glomus cells to low glucose, this inhibition has a minimal effect regarding neurotransmitter secretion (Garcia-Fernandez et al., 2007). Indeed, as stated above, low glucose induces a decrease in the input resistance of cells, whereas the predominant effect of hypoxia is an increase in input resistance. Although glomus cells normally secrete neurotransmitters in response to glucose and hypoxia, there are cells that respond to only one of these two stimuli (**Figures 2A,B**). Moreover, rotenone, a specific mitochondrial complex I inhibitor, which blocks hypoxia-induced catecholamine secretion (Ortega-Saenz et al., 2003), shows no effect on the low glucose-induced secretory activity in CB cells (**Figures 2C,D**) (Garcia-Fernandez et al., 2007). Therefore, it appears that sensitivities to hypoglycemia and hypoxia depend on separate signal transduction mechanisms, although they share the same final steps leading to transmembrane Ca^{2+} influx and neurotransmitter release. The mechanism of CB O_2 sensing is as yet unknown; however a considerable body of knowledge including our rotenone data, suggests that mitochondria may play an important direct or indirect role (Ortega-Saenz

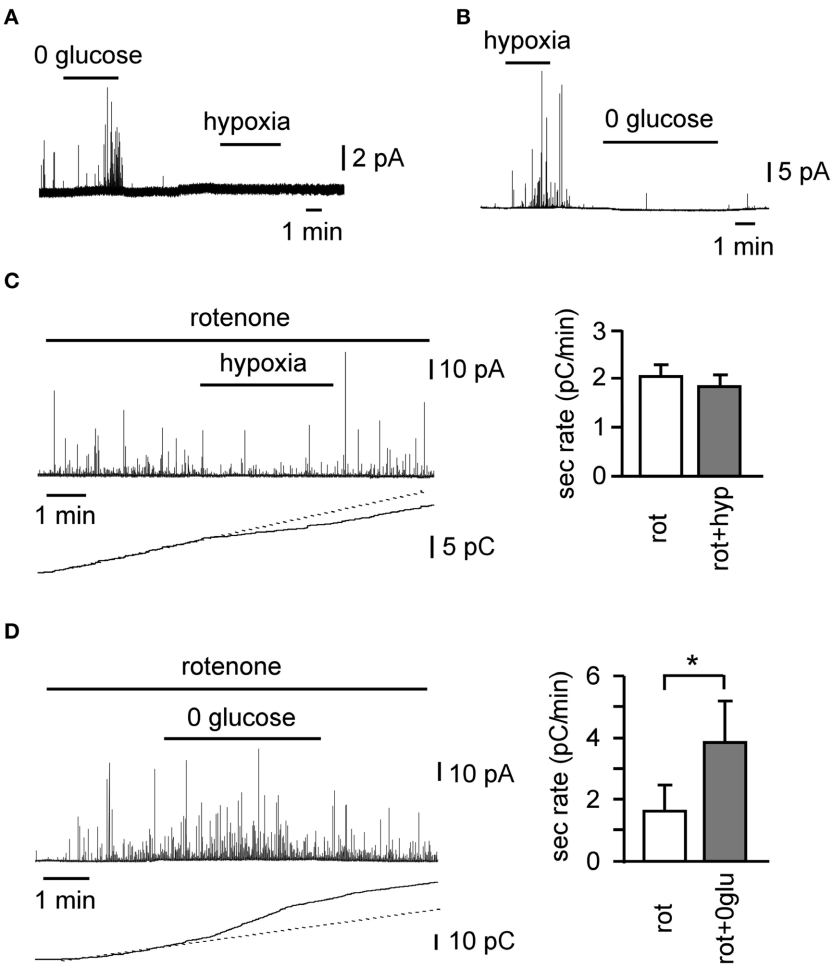

FIGURE 2 | Differential sensitivity of glomus cells to oxygen and low glucose in rat carotid body slices. (A,B) Examples of cells with differential secretory responses to hypoxia and low glucose. Differential effect of 100 nM rotenone on the secretory response induced by hypoxia **(C)** ($n = 14$) and hypoglycemia **(D)** ($n = 5$), as demonstrated by a representative amperometric recording, cumulative secretion signal, and average secretion rate. *$p < 0.05$ (Modified from Garcia-Fernandez et al., 2007).

et al., 2003; see Buckler and Turner, 2013 for an update and references). The fact that rotenone does not alter glomus cell responses to hypoglycemia indicates that low glucose sensing is not related to oxidative phosphorylation and could depend on metabolites of the glycolytic pathway (Garcia-Fernandez et al., 2007).

INTERPLAY BETWEEN LOW GLUCOSE AND O_2 SENSING

The brain is very sensitive to decreases both in arterial O_2 tension and glucose level. Being a polymodal sensor of O_2, glucose, pH, CO_2, etc., a coordinated response to hypoxia and hypoglycemia by CB chemoreceptors could prevent to a major extent the detrimental effects caused by both conditions. Although a small percentage of CB glomus cells respond specifically to only hypoxia or low glucose (Garcia-Fernandez et al., 2007), in a majority of glomus cells hypoxia and hypoglycemia can potentiate each other's response, such as is seen with neurotransmitter release and afferent discharge (Pardal and Lopez-Barneo, 2002b; Zhang et al., 2007; Fitzgerald et al., 2009). The secretory response to low glucose increases in the presence of low PO_2 in rat CB slices (Pardal and Lopez-Barneo, 2002b), and we have recently shown that glomus cells in the human CB are also glucose sensors and show the same responses (cell depolarization, increased cytosolic Ca^{2+} and neurotransmitter secretion), as described in lower mammals (**Figures 3A–D**). In this preparation, hypoxia ($6\%O_2$) potentiates low glucose-induced catecholamine secretion, whereas low glucose further induces Ca^{2+} influx during hypoxia (**Figures 3D,E**). The effect of hyperoxia on hypoglycemia and the effect of hyperglycemia on hypoxia are less well known. A recent human study suggested that hyperoxia could blunt the hypoglycemia effect (Wehrwein et al., 2010). Another study suggested that both hypo and hyperglycemia could increase the hypoxic response in human subjects (Ward et al., 2007).

INTERMITTENT HYPOXIA AND GLUCOSE SENSING

No direct evidence has been reported regarding the effect of intermittent hypoxia on glucose sensing by the CB. In rat CB glomus cells, intermittent hypoxia enhances acute hypoxia-induced membrane depolarization and the inhibition of TASK-like K^+ channels (Ortiz et al., 2013). Intermittent hypoxia has also been found to augment the CB sensory response to acute hypoxia and to enhance the hypoxic ventilatory chemoreflex in neonatal rats (Peng et al., 2004). However, a recent study reported an exaggerated activation of CB afferent activity accompanied by hypoventilation in a rat model of intermittent hypoxia when exposed to acute hypoxia (Gonzalez-Martin et al., 2011). It is logical to speculate that intermittent hypoxia could potentiate the carotid chemoreceptor response to hypoglycemia, as occurs with hypoxia. Indeed, intermittent hypoxia has been found to be associated with altered glucose metabolism in rodent models. Intermittent hypoxia results in an increase in fasting glucose and a decrease in insulin level in neonatal rats, which is associated with a disturbed glucose homeostasis (Pae et al., 2013). In mouse, intermittent hypoxia triggers increased fasting glucose and decreased sensitivity to insulin, with the former being reversed by discontinuation of exposure to hypoxia (Polak et al., 2013). Few human studies have been carried out to study the relationship between intermittent hypoxia and glucose homeostasis. Individuals exposed to intermittent hypoxia demonstrate an increased sympathetic nerve activity (Cutler et al., 2004), while male adults exposed to high altitude hypoxia have decreased insulin sensitivity (Larsen et al., 1997).

INSULIN AND CAROTID BODY GLUCOSE SENSING

In addition to hypoxia and intermittent hypoxia, insulin was found recently to be a regulator of the CB response to hypoglycemia. Indeed, insulin was proposed as a new intermittent hypoxia-like agent, and carotid chemoreceptors have been suggested to contribute to insulin-mediated sympathoexcitation (Limberg et al., 2014). Animal studies indicate that CB cells have insulin receptors and respond to increases in insulin levels by inducing sympathetic activation, as demonstrated by altered arterial blood pressure, breathing, and neurotransmitter release (Bin-Jaliah et al., 2004; Ribeiro et al., 2013). The combined activation of CB chemoreceptors by insulin and low glucose may serve as a counter-balance mechanism to limit the decrease of glucose levels in insulin-treated patients. In this regard, it would be interesting to explore whether long-lasting CB exposure to high glucose, as occurs in diabetic patients, alters the low glucose sensitivity of glomus cells.

CAROTID BODY DYSFUNCTION IN DISEASE STATES

CB acts as a combined oxygen and glucose sensor to facilitate activation of the counter-regulatory measures in response to small reductions of either variable. Such measures include, on one hand, hyperventilation and increased blood pressure to facilitate blood-borne O_2 supply to organs and, on the other hand liver glycogenolysis and insulin resistance of peripheral tissues to combat hypoglycemia. Diseases altering the structure and function of CB chemoreceptors could have detrimental effects, leading to dysregulation of glucose homeostasis.

OBSTRUCTIVE SLEEP APNEA

Obstructive sleep apnea (OSA) is a common clinical syndrome characterized by intermittent hypoxia and sleep fragmentation. OSA is a well-established significant risk factor for cardiovascular disease and mortality. As indicated above Intermittent Hypoxia and Glucose Sensing, chronic intermittent hypoxia results in CB chemoreceptor over-stimulation and augmentation of CB sensory responses in rats (Peng et al., 2003) and humans (Cutler et al., 2004). Intermittent hypoxia has been found to be associated with altered glucose metabolism and insulin resistance in rodent models (Pae et al., 2013; Polak et al., 2013), but its effects on glucose homeostasis in humans are as yet unstudied. It can be expected that CB overstimulation and growth seen in OSA patients (Nair et al., 2013; Abboud and Kumar, 2014) should lead to hyperglycemia and over-sensitivity to low glucose. Nevertheless, O_2 and glucose act on separate sensing mechanisms in glomus cells and, in addition, OSA can be accompanied by hypertension and diabetes. Therefore, the impact of OSA syndrome on CB-mediated glucose homeostasis requires future studies using human CB tissue samples (Ortega-Saenz et al., 2013).

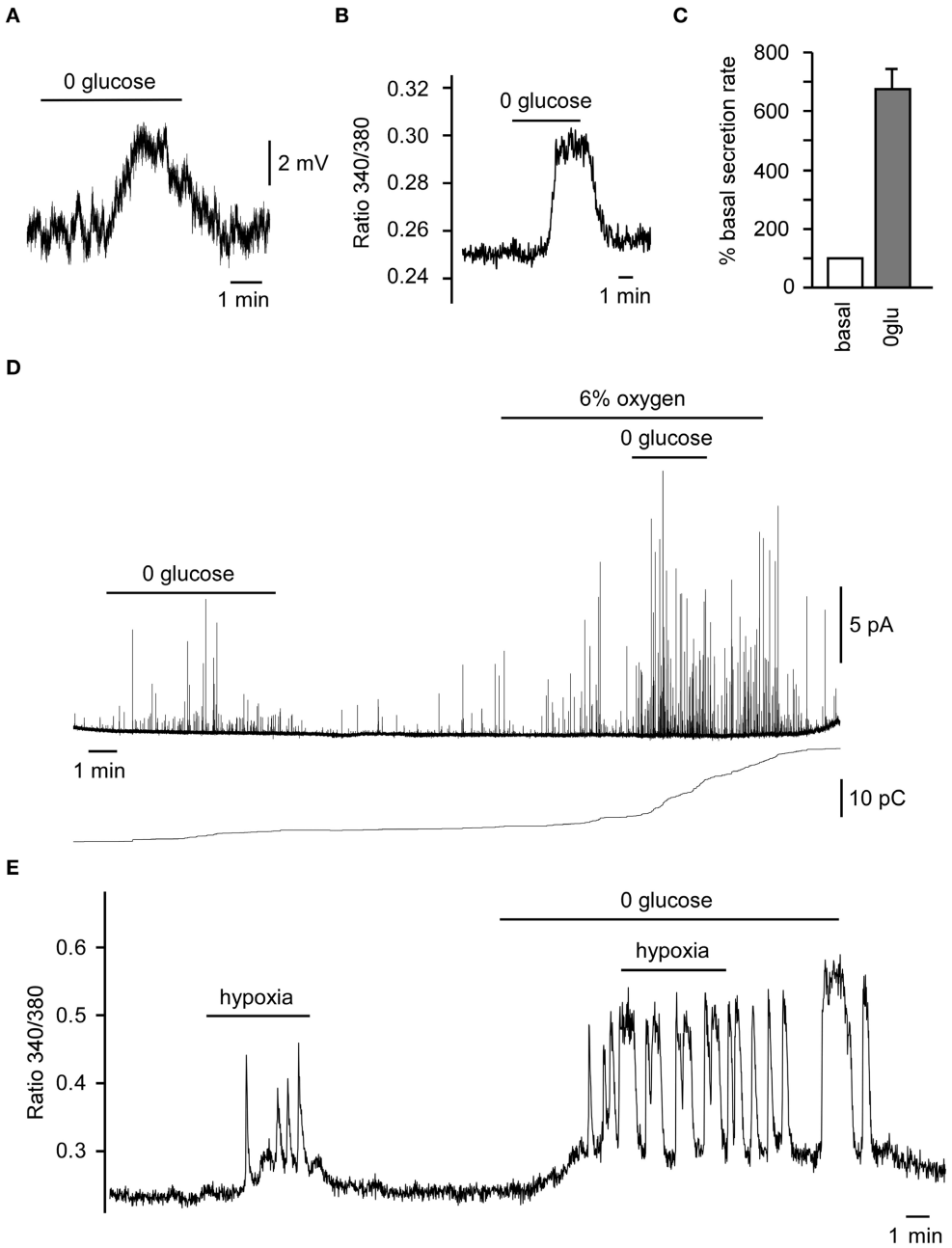

FIGURE 3 | Responses of human carotid body (CB) glomus cells to low glucose and hypoxia. (**A**) Depolarizing receptor potential recorded in a current-clamped human glomus cell in response to glucopenia. **(B)** Reversible increase in cytosolic Ca^{2+} in a Fura-2-loaded glomus cell exposed to 0 glucose. **(C)** Average secretion rate induced by hypoglycemia ($n = 2$). **(D)** Secretory response to 0 glucose of glomus cells in CB slices and the potentiation of the 0 glucose-induced secretory response by mild hypoxia (6% O_2) as demonstrated by a representative amperometric recording (top) and cumulative secretion signal (bottom). **(E)** Representative recording of a reversible increase of cytosolic Ca^{2+} in a Fura-2-loaded glomus cell, demonstrating the potentiation of the hypoxic-response by hypoglycemia. Modified from Ortega-Saenz et al. (2013).

DIABETES

Type 2 diabetes is a major chronic disease associated with high morbidity, mortality, and economic burden. Glucose sensing is essential for insulin-treated diabetic patients to counter-regulate insulin-induced hypoglycemia. It has been proposed that the CB dysfunction, increasing sympathetic tone and catecholamines in the blood, could possibly contribute to the pathogenesis of type 2 diabetes and essential hypertension (Nimbkar and Lateef, 2005). Using a computed tomographic angiography technique, enlargement of the CB is observed in patients with diabetes mellitus, hypertension, and congestive heart failure relative to controls, which supports the proposed functional relationship between

the CB and sympathetically mediated disease states (Cramer et al., 2014). In insulin-dependent diabetic rats, the CB volume is increased, due to an increase in the extravascular volume (Clarke et al., 1999). It is still unclear whether the CB enlargement is a cause of diseases or a consequence of disease progression. Whether CB glucose sensing is altered in diabetic patients is also unknown (see below).

RELATIONSHIP BETWEEN OBSTRUCTIVE SLEEP APNEA AND DIABETES

OSA syndrome and type 2 diabetes are also strongly linked to each other. Patients with OSA have an increased incidence of impaired glucose metabolism and are at an increased risk of developing type 2 diabetes (Tasali et al., 2008). On the other hand, the majority of patients with type 2 diabetes also have OSA (Tasali et al., 2008). Although the mechanism is most likely multifactorial, chronic intermittent hypoxia experienced by OSA patients could trigger CB chemoreceptor over-activity, leading to insulin resistance and abnormal glucose metabolism (Tasali et al., 2008). Indeed, insulin resistance is developed in both lean mice (Iiyori et al., 2007) and genetically obese mice (Polotsky et al., 2003) treated with intermittent hypoxia. The secretory activity of the CB is increased in the insulin-resistant rat model, whereas carotid sinus nerve resection prevents CB over-activation and diet-induced insulin resistance (Ribeiro et al., 2013). Therefore, sympathoexcitation due to CB over-stimulation could play an important role in the pathogenesis of both OSA and type 2 diabetes.

CONCLUSIONS

Carotid chemoreceptors work in coordination with other glucose sensing organs to counter-regulate hypoxia and hypoglycemia. The responses to hypoxia and hypoglycemia could be potentiated by each other. Failure to respond to these stresses could lead to malfunction of organs, such as the brain, which is highly sensitive to glucose and O_2 levels. Indeed, defects in CB function have been associated with several respiratory disturbances, particularly in the newborn (reviewed by Lopez-Barneo et al., 2008). CB over-stimulation could also exert detrimental effects, as has been demonstrated in OSA, hypertension and type 2 diabetes. However, whether the intrinsic glucose responsiveness of glomus cells is altered in these diseases is yet to be determined. Due to the essential role of the CB in sympathetic activation, this organ could serve as a potential therapeutic target for diseases with sustained hyperinsulinemia and sympathoexcitation, such as obesity, hypertension, sleep apnea, metabolic syndrome, cardiovascular disease, and diabetes (Paton et al., 2013). Evaluation of CB size in these conditions can be now studied with noninvasive computed tomography angiography (Nair et al., 2013; Cramer et al., 2014). However, bilateral surgical ablation of the CB performed in asthmatic patients or during neck tumor surgery causes permanent abolition of the ventilatory response to hypoxia. In addition, this condition causes a decrease in the CO_2 sensitivity of the respiratory center and, in some cases, long term resting hypoventilation and hypercapnia (reviewed by Timmers et al., 2003, see also Dahan et al., 2007). The counter-regulatory response to hypoglycemia could be also altered in patients who have had their CB removed, a status particularly critical in diabetic patients subjected to insulin treatment and therefore at high risk of hypoglycemia. Unilateral CB resection appears to be well tolerated (reviewed by Timmers et al., 2003, see also Minguez-Castellanos et al., 2007), thus making this likely to be a safer therapeutic option. Ideally, new reversible pharmacological tools should be developed to inhibit CB function. In this regard, selective inhibition of the O_2-sensing mechanisms or CB growth in chronic hypoxia (Platero-Luengo et al., 2014) could reduce CB over-activation while maintaining intact the counter-regulatory response to low glucose.

ACKNOWLEDGMENTS

This research was supported by the Botín Foundation and the Spanish Ministry of Economy and Innovation (SAF program).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 July 2014; accepted: 25 September 2014; published online: 15 October 2014.

*Citation: Gao L, Ortega-Sáenz P, García-Fernández M, González-Rodríguez P, Caballero-Eraso C and López-Barneo J (2014) Glucose sensing by carotid body glomus cells: potential implications in disease. Front. Physiol. **5**:398. doi: 10.3389/fphys.2014.00398*

This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 22 September 2014
doi: 10.3389/fphys.2014.00361

The efficacy of antihypertensive drugs in chronic intermittent hypoxia conditions

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Sleep apnea/hypopnea disorders include centrally originated diseases and obstructive sleep apnea (OSA). This last condition is renowned as a frequent secondary cause of hypertension (HT). The mechanisms involved in the pathogenesis of HT can be summarized in relation to two main pathways: sympathetic nervous system stimulation mediated mainly by activation of carotid body (CB) chemoreflexes and/or asphyxia, and, by no means the least important, the systemic effects of chronic intermittent hypoxia (CIH). The use of animal models has revealed that CIH is the critical stimulus underlying sympathetic activity and hypertension, and that this effect requires the presence of functional arterial chemoreceptors, which are hyperactive in CIH. These models of CIH mimic the HT observed in humans and allow the study of CIH independently without the mechanical obstruction component. The effect of continuous positive airway pressure (CPAP), the gold standard treatment for OSA patients, to reduce blood pressure seems to be modest and concomitant antihypertensive therapy is still required. We focus this review on the efficacy of pharmacological interventions to revert HT associated with CIH conditions in both animal models and humans. First, we explore the experimental animal models, developed to mimic HT related to CIH, which have been used to investigate the effect of antihypertensive drugs (AHDs). Second, we review what is known about drug efficacy to reverse HT induced by CIH in animals. Moreover, findings in humans with OSA are cited to demonstrate the lack of strong evidence for the establishment of a first-line antihypertensive regimen for these patients. Indeed, specific therapeutic guidelines for the pharmacological treatment of HT in these patients are still lacking. Finally, we discuss the future perspectives concerning the non-pharmacological and pharmacological management of this particular type of HT.

Keywords: antihypertensive drugs, blood pressure, chronic intermittent hypoxia, hypertension, obstructive sleep apnea

CHRONIC INTERMITTENT HYPOXIA-RELATED DISORDERS

Is is well established that intermittent hypoxia (IH) affects control of breathing, the autonomic nervous system and the cardiovascular system (Foster et al., 2007). Chronic intermittent hypoxia (CIH) is a feature that is present in interstitial lung disease (Fletcher et al., 1992a) and sleep-disordered breathing (SDB), and it has also been shown to occur in patients with hepatopulmonary syndrome (Tanné et al., 2005; Ogata et al., 2006; Palma et al., 2008). Since several years ago, there has been growing interest concerning CIH due to the high relevance of the part assumed to be played by sleep-related breathing disorders in chronic diseases.

Sleep apnea/hypopnea disorders include centrally originated diseases and obstructive sleep apnea (OSA). Central sleep apnea (CSA) is characterized by a lack of drive to breathe during sleep, resulting in insufficient or absent ventilation and compromised gas exchange (Eckert et al., 2007). In CSA, the cessation of respiration during sleep is not associated with ventilatory effort and there is sleep fragmentation due to arousals associated with reflexes activated by the ensuing hypoxemia (Paiva and Attarian, 2014). The major manifestations of CSA include high altitude-induced periodic breathing, idiopathic CSA, narcotic-induced central apnea, obesity hypoventilation syndrome, and Cheyne-Stokes breathing in heart failure (Eckert et al., 2007). While the precipitating mechanisms involved in the several types of CSA may diverge, unstable ventilatory drive during sleep is the principal underlying feature (Eckert et al., 2007). CSA is diagnosed in approximately 5% of the patients who undergo a polysomnographic study (Khan and Franco, 2014). On the other hand, OSA is briefly characterized by repetitive episodes of airflow cessation (apnea) or airflow reduction (hypopnea) caused by an obstructed or collapsed upper airway during sleep. Unlike CSA, obstruction occurs in OSA despite the central drive to breathe and inspiratory muscle activity (Levitzky, 2008). An appreciable number of factors are known to be linked to upper-airway collapse, namely reduced airway dilator muscle activity during sleep, upper-airway anatomy, obesity, decreased end-expiratory lung volume, ventilatory control instability, and rostral fluid shifts (Kapur, 2010). The repetitive episodes of apnea and hypopnea characteristic of OSA are closely associated with CIH, hypercapnia and an increase in intrathoracic pressure, leading to recurrent

arousals and significant changes in sleep architecture. OSA is affecting a growing proportion of the common population, and the estimated prevalence in the 1990s was 9% for women and 24% for men among middle-aged adults (Young et al., 1993). In addition, CSA can occur concomitantly with OSA. This last condition, recently labeled complex sleep syndrome, is observed in approximately 15% of the patients following treatment with continuous positive airway pressure (CPAP) (Paiva and Attarian, 2014). In a few words, complex sleep syndrome is a form of SDB in which CSA persists or emerges when obstructive events have disappeared using a positive pressure device (Khan and Franco, 2014). In clinical practice, when a few central apneas are observed in polysomnograms of patients with OSA, they are normally ignored because we do not presently understand their potencial clinical relevance.

Nowadays, it is well known that the outcomes of these sleep-related breathing disorders can lead to vascular diseases, contributing to a considerable increase in overall cardiovascular risk. The desaturation-reoxygenation sequence, a typical pattern coupled with the majority of respiratory events, is thought to be responsible for most of the associated cardiovascular morbidity (Lévy et al., 2012). Although OSA has been associated with several cardiovascular conditions, it has been more closely etiologically connected to systemic HT (Kapa et al., 2008), and the link between HT and OSA is now widely accepted and supported by different findings. Most episodes of OSA are coupled with sleep disruption, which *per se* increases sympathetic nerve activity and blood pressure (Morgan et al., 1996). In addition, the occurrence of arousals appears to enhance the pressor effects of asphyxia during OSA (Morgan et al., 1998), contributing synergistically to blood pressure increase. In any case, studies in both animals and humans underline the major role of hypoxia itself in promoting an increase in blood pressure (Brooks et al., 1997b; Tamisier et al., 2011).

Regarding CSA, this SDB, like OSA, is strongly linked to cardiac disease and cardiovascular outcomes (Brenner et al., 2008). Indeed, the majority of patients with CSA have underlying cardiovascular disease, primarily heart failure, which is considered the most common risk factor for CSA, followed by atrial fibrillation (Bradley and Phillipson, 1992). Moreover, like OSA, CSA has been implicated in heart failure pathophysiology (Mehra, 2014) and occurs in 30–50% of patients with left ventricular dysfunction and heart failure caused by HT, cardiomyopathy and ischemic heart disease (Bradley and Floras, 2003). Thus, CSA has significant co-morbidity with many cardiac conditions, which clearly contributes to an increase in the associated mortality and morbidity.

Besides systemic HT, chronic intermittent alveolar and systemic arterial hypoxia-hypercapnia can cause pulmonary HT (PH). SDB has also been found to be associated with PH, being considered one of the potential etiologies of PH (Galie et al., 2009). During episodes of OSA, the subsequent oscillations in PaO_2 lead to a cyclical pattern of vasoconstrictions and relaxations in the pulmonary circulation responsible for the marked fluctuations observed in pulmonary arterial pressure (Dempsey et al., 2010). The perpetuation of this pattern leads to fixed elevations in pulmonary pressure (Dempsey et al., 2010). Some data suggest that even slight changes in pulmonary function, in the absence of lung disease, are able to induce PH in patients with OSA. Furthermore, it is important to bear in mind that PH could also be a cause of abnormal arterial blood gases during wakefulness (Dempsey et al., 2010) and that OSA itself can lead to PH (Sajkov and McEvoy, 2009). The major consequence of the increased pulmonary artery pressure, together with increased blood viscosity (a consequence of the renal release of erythropoietin subsequent to hypoxemia), is the occurrence of right ventricle hypertrophy leading to *cor pulmonale* (Levitzky, 2008). The prevalence of this chronic cardiopulmonary condition among patients with SDB is estimated to range from 17 to 52% (Minic et al., 2014), and 20–30% of untreated OSA patients suffer from PH (Dumitrascu et al., 2013). Even if PH in this group of patients is typically not severe (Badesch et al., 2010), OSA patients with PH have a higher mortality rate than OSA patients without PH (Minai et al., 2009). A recent meta-analysis shows that CPAP is associated with a mild but statistically significant reduction in pulmonary artery pressure in OSA patients (Sun et al., 2014). This decrease might translate into a better outcome in patients with PH secondary to OSA. However, more studies are needed to confirm this assumption.

Taking into account its high prevalence and its associated adverse impact on cardiovascular, metabolic and other health outcomes, this review focuses on OSA and systemic HT.

OSA AND HT: HOW RELEVANT IS THIS LINKAGE?

Since 2003, OSA has formally been recognized as a frequent and important secondary cause of HT and is one of the first causes to be screened mainly in patients with a suggestive phenotype, refractory HT and a non-dipping profile (Chobanian et al., 2003; Mancia et al., 2007). More recently, OSA has been identified as an independent risk factor for HT (Lavie et al., 2000; Peppard et al., 2000; Marin et al., 2012), as one of the major clinical conditions that favors poorly controlled HT (Oliveras and Schmieder, 2013), and as the most common condition associated with resistant HT (Pedrosa et al., 2011). OSA and HT are two prevailing risk factors for several cardiovascular events (Wang and Vasan, 2005; Baguet et al., 2009). Due to their high prevalence and cardiovascular morbidity (Wolf et al., 2007; Malhotra and Loscalzo, 2009), OSA and HT are now acknowledged as public health problems. Epidemiological data show that the estimated overall prevalence of HT among patients with OSA is approximately 50% and an estimated 30–40% of hypertensive patients are diagnosed with OSA (Calhoun, 2010), confirming the bidirectional relationship between OSA and HT. Moreover, OSA and HT are chronic diseases mostly diagnosed in active adults and because of the associations between OSA and obesity and advancing age, the public health burden of OSA related to cardiovascular disease is expected to rise in the coming years (Dempsey et al., 2010). The use of both antihypertensive drugs (AHDs) and CPAP in these patients is for life and consequently treatment is associated with a high impact both in terms of costs and in patients' quality of life. Indeed, OSA generates an impressive economic burden, including medical costs, when compared to other equally relevant chronic diseases (Kapur, 2010; Badran et al., 2014).

OSA AND HT: WHAT IS THE PROBLEM?

CPAP is considered the gold standard treatment for mild, moderate and severe OSA due to its remarkable ability in providing pneumatic splitting of the upper airway and effectiveness in reducing the apnea-hypopnea index (AHI), symptoms, and cardiovascular morbidity and mortality (Hla et al., 2002; Pepperell et al., 2002; Wolf et al., 2007; Epstein et al., 2009; Mannarino et al., 2012). Besides preventing hypoxemia, sleep disturbance and apnea episodes, CPAP reduces sympathetic activity, systemic inflammation and oxidative stress (Yorgun et al., 2014). However, the results found for the effectiveness of CPAP on blood pressure (BP) control are still controversial. **Table 1** summarizes the results of original studies in which the effect of CPAP on BP has been analyzed. Whereas some studies and meta-analyses (Bakker et al., 2014; Varounis et al., 2014) have reported modest effects for CPAP in lowering BP, others tend to support the beneficial effect of CPAP treatment on BP reduction and attenuating the risk of developing HT. In any case, although the lowering effect of CPAP on BP is relevant in terms of overall cardiovascular risk reduction, this effect is very limited when compared to the performance of AHDs in patients with essential HT (Pépin et al., 2009). Thus, treating HT in patients with sleep apnea is proving to be a difficult task and there is consensus that the use of AHDs is mandatory. In spite of this, data on AHDs regimens in patients with OSA are scarce and there is a lack of specific therapeutic guidelines for the pharmacological treatment of HT in these patients. Furthermore, the effects of AH agents on OSA patients are not consistent (Parati et al., 2012) and there are no data on the efficacy of specific AHDs regimens when associated with CPAP.

A new treatment for OSA patients is the oral appliance/mandibular advancement device (Guralnick and Bakris, 2012). Oral appliance therapy is an important alternative to CPAP for some patients with mild to moderate OSA (Iftikhar et al., 2013). Despite a recent study (Andrén et al., 2013) and a recent meta-analysis (Iftikhar et al., 2013) which have shown some beneficial effects of this device in reducing blood pressure measurements, larger and longer randomized control trials are needed to confirm the effects of oral appliance therapy on BP control.

Clearly, more studies are required to identify first-line AHDs regimens for optimal BP control in this particular group of hypertensive patients (Tsioufis et al., 2010; Parati et al., 2013). Moreover, HT related to OSA needs to be managed as a specific entity and an earlier diagnosis of this type of HT seems to be as relevant as the selection of AHDs regimens. This work provides, for the first time, a systematic review on the efficacy of AHDs in HT related to OSA.

WHAT MODELS ARE AVAILABLE TO STUDY HT RELATED TO OSA?

Due to the high complexity and heterogeneity associated with OSA, considerable variability can be observed between reports addressed at the study of this disease. In addition, the scarcity of opportunities for patient investigation, in particular at the cellular level, has compromised progress in understanding the pathophysiology of OSA and the development of novel and specific treatments for this disorder. To overcome some of these limitations, several animal models and more recently, a model of OSA in healthy human volunteers (Tamisier et al., 2009, 2011) have been developed. Animal models, especially of IH, mimic OSA more easily than human models. The small size of rodents allows more rapid and intense changes in SaO_2 whereas humans require longer periods of hypoxia to induce arterial oxyhaemoglobin desaturation (Foster et al., 2007). The combination of these two approaches is certain to contribute to the consolidation of prevention strategies and the development of more suitable treatments for OSA patients.

ANIMAL MODELS

The major advantage of the use of animal models is that they allow single components of the disease to be evaluated, accurately controlling the triggering events in terms of both severity and duration, and providing homogeneous populations (Lévy et al., 2012). These models also provide an excellent opportunity to explore the underlying mechanistic pathways of HT related to OSA and their consequences under controlled conditions. Moreover, animal models have enabled the study of parameters that have proved difficult to assess in humans, particularly due to the need for organ harvesting to explore the mechanisms underlying the consequences of IH at the molecular level (Dematteis et al., 2009). Thus, studies with animal models are good tools for overcoming some confounding factors present in human studies (e.g., the presence of comorbidities, disease duration, and behavioral and environmental variables) (Badran et al., 2014), and for providing more specific information concerning the efficacy of drugs to be tested.

In 2009, Dematteis et al. used the terminology homologous (sharing the cause or pathophysiology of the human disease), predictive (responding to treatment similarly to the human disease) and isomorphic (displaying symptoms similar to those of the human disease although their cause and pathophysiology may differ) to categorize sleep apnea models (Dematteis et al., 2009). According to these categories, most sleep apnea models are only partially isomorphic, focusing on a specific aspect of the human disease. As a matter of fact, none of the currently available animal models reproduce all aspects of human sleep apnea and they present some important limitations. Nonetheless, the animal models of sleep apnea have brought out most of the available knowledge in this field and furthermore, almost all cardiovascular diseases known to be present in patients with OSA have been replicated in these models (Dumitrascu et al., 2013).

The effective use of animals to study sleep apnea implies recognition of the natural similarities and differences between animals and humans to ensure the reliability of the experimental results. For instance, as rodents are nocturnal animals, the stimulus must be applied during the sleep-dominant phase of the diurnal cycle. Moreover, in humans the circadian distribution of sleep tends to be consolidated and normally monophasic, with a daily sleep duration of 7–8 h, whereas it is polyphasic, relatively fragmented and with a duration of 12–15 h in rodents (Toth and Bhargava, 2013). Another issue is related to the fact that rodents sleep in the prone position (Golbidi et al., 2012); it is well known that supine OSA is the dominant phenotype of OSA syndrome and that the supine position favors upper airway collapse in humans (Joosten et al., 2014). Furthermore,

Table 1 | CPAP effect on blood pressure.

Study design	n	Study duration	HT patients (%)	AHDs (Y/N)	Mean CPAP use (h/night)	BP outcome	References
RCT; parallel group; blinded endpoint	194	12 weeks	100	Yes	5	↓ 3.1 mmHg MBP ↓ 3.2 mmHg DBP ↓ 3.1 mmHg SBP (NS)	Martínez-García et al., 2013
RCT; parallel group	118	4 weeks	10	Yes	4.9	↓ 3.3 mmHg 24 h MBP	Pepperell et al., 2002
Case -controlled study	48	4 weeks	79	Yes	5.1	↓ 5.2 mmHg DBP ↓ 3.8 mmHg SBP (NS)	Zhao et al., 2012
Prospective randomized trial	32	9 weeks	66	Yes	5.5	↓ ± 10 mmHg MBP ↓ ± 10 mmHg DBP ↓ ± 10 mmHg SBP (During both day and night-time)	Becker et al., 2003
RCT; multicenter; parallel group	723	4 years	51.5	Yes	5.0	NS on new-onset HT	Barbé et al., 2012
Prospective, single-center, long-term follow-up	91	5 years	100	Yes	*NA*	NS on 24 h BP, SBP and DBP	Kasiakogias et al., 2013
RCT; parallel group	40	6 months	100	Yes	6.01	↓ Awake SBP (6.5 mmHg) and DBP (4.5 mmHg) NS nocturnal SBP and DBP	Pedrosa et al., 2013
Retrospective chart review study	98	1 year	100	Yes	6.3	↓ 5.6 mmHg MBP (resistant HT group) ↓ 0.8 mmHg MBP (controlled BP group)	Dernaika et al., 2009
RCT; crossover	28	8 weeks	100	Yes	4.8	↓ 2.1 mmHg 24 h MBP (CPAP group) ↓ 9.1 mmHg 24 h MBP (valsartan group)	Pépin et al., 2009
Prospective cohort study	86	6 months	55	Yes	4.8	↓ 4.92 mmHg 24 h MBP	Robinson et al., 2008
Observational study	24	12 weeks	0	No	*NA*	↓ 5.3 mmHg 24 h MBP	Yorgun et al., 2014
Prospective cohort study	196	6 months	85	Yes	*NA*	↓ 2.7 mmHg DBP ↓ 2.1 mmHg SBP	Börgel et al., 2004
RCT; multicenter; double-blinded	340	12 weeks	100	No	4.5	↓ 1.5 mmHg MBP ↓ 1.3 mmHg mean DBP ↓ 2.1 mmHg mean SBP	Durán-Cantolla et al., 2010
RCT; multicenter; parallel group	44	6 weeks	*NA*	Yes	5.0	NS on 24 h SBP and DBP	Barbé et al., 2001
RCT; crossover study; sham placebo	35	10 weeks	100	Yes	5.2	NS on overall 24 h MBP	Robinson et al., 2006
Observational, monocentric; cohort study	495	3.4 years	40.4	Yes	*NA*	↓ Occurrence of systemic arterial HT	Bottini et al., 2012

(Continued)

Table 1 | Continued

Study design	n	Study duration	HT patients (%)	AHDs (Y/N)	Mean CPAP use (h/night)	BP outcome	References
RCT; single-blinded	44	13.2 weeks	100	Yes	5.1	Additional ↓ in office BP and ambulatory BP monitoring (CPAP+ 3 AHDs)	Litvin et al., 2013
RCT, multicenter	359	1 year	100	Yes	4.7	↓ 2.19 mmHg DBP NS ↓ 1.89 mmHg SBP NS	Barbé et al., 2009
RCT	36	3 months	*NA*	No	5.2	↓ 2 mmHg office DBP ↓ 5 mmHg office SBP ↓ 5 mmHg 24 h DBP ↓ 5 mmHg 24 h SBP	Drager et al., 2011
RCT; parallel group	64	3 months	100	Yes	>5.8	↓ 6.98 mmHg 24 h DBP ↓ 9.71 mmHg 24 h SBP	Lozano et al., 2010

AHDs, antihypertensive drugs; BP, blood pressure; CPAP, continuous positive airway pressure; DBP, diastolic blood pressure; HT (%), percentage of hypertensive patients; MBP, mean blood pressure; NA, information not available; NS, no significant effect; RCT, randomized controlled trials; SBP, systolic blood pressure; ↓, decrease.

additional care must be taken to minimize external factors (e.g., light exposure, photoperiod, noise, disruptions in the home environment, and post-surgical care in studies, for instance requiring implantation of telemetric devices) able to influence sleep in animals used in experimental research (reviewed in Toth and Bhargava, 2013).

The experimental animal models developed to mimic OSA have recently been reviewed (Dematteis et al., 2009; Golbidi et al., 2012; Davis and O'Donnell, 2013; Toth and Bhargava, 2013) and assembled taking into account the main injuries triggered by OSA. Despite attempts to use large animals (e.g., dogs, lambs, and pigs) to simulate upper airway obstruction, most research on the cardiovascular consequences of OSA has been performed in rodents. Alternative models (e.g., cell cultures incubated in specific devices that perform oxygen fluctuations mimicking sleep apnea-related IH), mainly relevant to signaling investigation (Kumar et al., 2003; Gozal et al., 2005; Ryan et al., 2005), represent a complementary approach to the most widely used sleep models. However, in spite of the recommendations to refine, reduce and replace (the 3Rs programme), these alternative models cannot replace animal models in the study of HT.

The natural models of sleep apnea include the English bulldog, the historic natural model of spontaneous obstruction (Hendricks et al., 1987), the sleep-related central apnea models [e.g., Sprague-Dawley rats (Carley et al., 2000), spontaneously hypertensive (SH) rats (Carley et al., 1998), C57BL/6J (Julien et al., 2003; Liu et al., 2010)], and the Zucker obese rat in which apnea is obesity-related (Ray et al., 2007; Lee et al., 2008; Iwasaki et al., 2012). The experimentally-induced models (e.g., the sleep deprivation model, induced airway obstruction and the CIH model) are the most widely used. Due to model limitations and lack of extensive study, we only briefly describe the induced airway obstruction model and the sleep deprivation model. Special focus will be given to the CIH model, based on the assumption that IH is the most effective paradigm to induce HT related to OSA and probably the most relevant stimulus regarding the cardiovascular sequelae of OSA.

Induced airway obstruction model

Briefly, the airway obstruction model involves surgical intervention (an endotracheal tube), which is an invasive procedure, or alternatively the use of a specific chamber with a latex neck collar that induces recurrent airway obstruction. This latter procedure, developed by Farré et al. (2007), is associated with high levels of stress due to the restriction of animal movement. In both approaches, the degree of obstruction is adjustable (Golbidi et al., 2012) and in the case of induction of obstruction through endotracheal tube, the $PaCO_2$ can be adjusted to mimic human sleep apnea (Golbidi et al., 2012). Many experiments using this method have not monitored the sleep state of the animals, but more recent studies have incorporated sophisticated apparatus that is able to detect sleep-awake states and allow close coordination between the initiation of airway obstruction and sleep onset (Schneider et al., 2000).

This model allows the study of the potential consequences of strenuous breathing against an obstructed airway and can be used to study the cardiovascular consequences and risk factors of OSA (e.g., systemic inflammation and coagulation), and to investigate the mechanisms that underlie OSA (Salejee et al., 1993; Nácher et al., 2007, 2009; Almendros et al., 2008, 2011; Othman et al., 2010). However, to the best of our knowledge, no study has yet shown that this obstruction model is able to mimic HT related to OSA. Furthermore, when testing AHDs, it became crucial to ensure the selection of a stress-free paradigm as it has been shown that any source of external stress on rodents can significantly increase heart rate and blood pressure (Brown et al., 2000; Kramer et al., 2000; Balcombe et al., 2004; Bonnichsen et al., 2005) and therefore contribute to confounding the experimental results. Finally, as the rat models of obstruction or asphyxia were developed in restrained or anesthetized rats, they are not good models for chronic administration of oral drugs, particularly AHDs.

Sleep deprivation model

In the last few years, several approaches have been used to trigger sleep deprivation in different animals, the rat being the animal of choice to date (Colavito et al., 2013). In the "multiple platform technique," the animal is aroused from sleep when the characteristic loss of muscle tone that accompanies paradoxical sleep causes it to fall off the platform (Suchecki and Tufik, 2000). The "gentle handling" procedure, by far the most popular method, is based on direct interaction with the experimenter, who actively keeps the animal awake through the use of external stimulation (e.g., mild noises, tapping or gentle shaking of the cage, or by direct contact with the animal either using a soft brush or by hand), or by the introduction of novel objects or nesting material in the cages, which typically leads to active exploratory behavior (Colavito et al., 2013).

These models are most often used to evaluate the neurophysiological aspects of OSA (Van Dongen et al., 2003; Haack and Mullington, 2005; McKenna et al., 2007; Ward et al., 2009; Nair et al., 2011) due to the high similarity between the structures of the nervous systems of rodents and humans (Badran et al., 2014), and to illustrate some mechanistic pathways induced by this trigger (McGuire et al., 2008; Tartar et al., 2010; Liu et al., 2011; Perry et al., 2011). Nevertheless, some studies have also aimed to evaluate the cardiovascular outcomes induced by this OSA feature and have suggested that sleep fragmentation may have a far more important role in cardiovascular changes observed in OSA patients (Golbidi et al., 2012). Even so, sleep deprivation studies have produced mixed results regarding BP outcomes.

In 1997, Brooks et al. suggested that sleep fragmentation, triggered by auditory stimulus, induced only acute changes in BP and did not affect daytime BP (Brooks et al., 1997a,b). In the same way, Bao et al.'s results showed that sleep fragmentation in rats, using acoustic stimuli for 35 days, did not elicit an increase in BP, probably due to some adaptation behavior (Bao et al., 1997). However, more recent studies have shown that sleep deprivation leads to increased plasma concentrations of epinephrine and norepinephrine (Andersen et al., 2005), ET-1/2 levels (Palma et al., 2002), and increased heart rate and systolic blood pressure (Andersen et al., 2004; Perry et al., 2007). In addition, sleep fragmentation enhances plasma inflammatory cytokines (e.g., TNF-α, IL-6, IL-1α, and IL-1β), leading to increased oxidative stress and inflammation (Yehuda et al., 2009). These results add further evidence demonstrating that sleep deprivation may lead to serious cardiovascular consequences and may aggravate hypertensive features. However, despite the potential of the sleep deprivation model to induce HT related to OSA, it does not exactly mimic sleep fragmentation and presents one major shortcoming regarding the evaluation of AHDs efficacy that should be taken into account. Sleep deprivation is a stressful method and it is still unclear whether the method is itself a stressful stimulus (Palma et al., 2002). Thus, in conclusion, sleep deprivation models are useful tools for unveiling various aspects of sleep function, studying the effects of sleep loss on subsequent brain function at the molecular, cellular and physiological levels, and evaluating cognitive impairment, but should be used with caution whenever stress can act as a confounding factor and compromise data interpretation.

CIH model

IH is now established as the dominant model of sleep apnea. Generally, this model makes use of specific ventilated chambers in which the animals are housed and cyclically exposed either to normoxia/hypoxia or room air to mimic the most relevant consequences of OSA. Hypoxic conditions can also be achieved by surgical intervention (an endotracheal tube) or by the use of a mask, which involves animal restraint and consequently high levels of stress (Golbidi et al., 2012). In either case, animals breathe nitrogen-enriched air alternating with oxygen or normal air (Dematteis et al., 2009). Thus, as with O_2, nitrogen plays an important role in this model as the flushing of the chambers with this gas allows the gradual lowering of O_2. The duration of the hypoxic and normoxic phases of the IH cycle, as well as the slopes of FiO_2, decrease and increase, and are dependent on cage/chamber size and the gas flows and mixtures (Dematteis et al., 2009).

The standard animal model of OSA was that described in the landmark study of Fletcher and Bao (1996). Despite the presence of some drawbacks, this model has successfully been employed to study the changes in systemic arterial pressure and the impact of IH on a wide range of cardiovascular outcomes. One of the major limitations pointed to in this model is the absence of recurrent upper airway obstruction, abolishing the acute hemodynamic changes due to the negative intrathoracic pressure (Badran et al., 2014). Marked negative intrathoracic pressure induces acute hemodynamic changes that are probably the starting point for chronic cardiovascular diseases (Bonsignore et al., 1994). Despite the absence of upper airway occlusion, some respiratory efforts (intermittent tachypnea) occur, corresponding to a fluctuating hyperventilation that follows the IH cycles (Dematteis et al., 2009). However, this disadvantage allows the evaluation of CIH effects, namely chronic blood gas exchanges, without the interference of the mechanical aspects of OSA.

This model also fails to reproduce the transient hypercapnia, or at least eucapnia, which occurs in humans determined by airway occlusion. The first question concerning this issue should be: is $PaCO_2$ relevant in humans? Hypercapnia is not a standard parameter analyzed in polysomnographic recordings in patients and therefore there is no consensus on the impact of $PaCO_2$ in arterial blood pressure in patients with OSA. In clinical studies of patients with moderate OSA, the changes in $PaCO_2$ have seemed to be irrelevant (Epstein et al., 2001) or have shown a slight increase (Tilkian et al., 1976) during the apneic events. However, a $PaCO_2$ increase may contribute to the severity of the cardiovascular consequences of OSA (Cooper et al., 2005). The results shown by Fletcher et al. in rats suggest that the exposure to hypercapnia during IH is not a critical factor as the effect of IH on diurnal BP is similar, independently of the lower or higher levels of CO_2 (Fletcher et al., 1995). Moreover, Bao et al. found that eucapnic IH in rats is a more powerful stimulus for inducing acute BP increase than hypocapnic IH (Bao et al., 1997). Similarly, Lesske et al. showed comparable changes in BP between two groups submitted to IH with or without hypercapnia (Lesske et al., 1997). On the other hand, based on the results of different CIH experimental protocols in rodents, Kanagy concludes that the level of $PaCO_2$ influences the magnitude of an increase

in BP (Kanagy, 2009). Concretely, eucapnic hypoxia induces a faster and greater increase than hypocapnic hypoxia (Kanagy, 2009), through mechanisms that presently remain unknown. Moreover, the greatest increases in BP have been observed in studies in which hypocapnia was prevented by CO_2 administration (Morgan, 2009). Likewise, Tamisier et al., in a study performed in humans, reported that hypercapnic hypoxia leads to greater sympathetic activation than hypocapnic hypoxia (Tamisier et al., 2009). In line with these findings, the presence of hypocapnic or eucapnic hypoxia conditions leads to an underestimated increase in BP that must be taken into account. In conclusion, although some data suggest that $PaCO_2$ may influence physiological responses to IH, further studies are needed to evaluate the combined effect of IH and hypercapnia. Another drawback that could be attributed to the IH paradigm is the fact that it is not accompanied by sleep fragmentation and does not incorporate monitoring of sleep.

Each group of researchers has applied its own specific paradigm and these discrepancies may compromise the straightforward comparison of the results. The several paradigms of CIH, which simulate the cyclical pattern of hypoxia experienced by patients with OSA, diverge in some respects, namely in the animal species involved, e.g., Sprague-Dawley rats (Fletcher et al., 1995; Kanagy et al., 2001; Tahawi et al., 2001; Allahdadi et al., 2005; Chen et al., 2005; Phillips et al., 2005; Lai et al., 2006), Wistar rats (Dunleavy et al., 2005; Lefebvre et al., 2006), C57BL/6J mice (Julien et al., 2003), and CF-1 mice (Rosa et al., 2011), the severity of hypoxia, the number of hypoxic episodes *per* hour of sleep, the number of days of hypoxic exposure (exposure duration), and CO_2 manipulation. **Table 2** summarizes the variability observed in the CIH models.

These models typically create moderate to severe oxygen desaturation, thereby mimicking severe forms of OSA and may therefore not be applicable to mild and moderate clinical OSA (Dematteis et al., 2009). CIH models with cycles of FiO_2 of 5% or less usually mimic severe forms of OSA in humans and produce maximal changes in BP and heart rate (Dematteis et al., 2008). However, higher FiO_2 (8–10%) has been used in rodent models of CIH (Soukhova-O'Hare et al., 2008; Knight et al., 2011; Perry et al., 2011; Bathina et al., 2013).

The duration and frequency of hypoxic/normoxic periods are adjustable; usually, the higher the frequency the shorter the IH cycles (Golbidi et al., 2012). There is a sizeable discrepancy regarding the duration of IH cycles, ranging from 120 cycles/h (30 s cycle; Fletcher et al., 1992a; Julien et al., 2003; Dematteis et al., 2008), 80 cycles/h (6 min cycle; Knight et al., 2011), 60 cycles/h (1 min cycle; Campen et al., 2005), and when the chambers are larger, longer cycles are often used, reducing the number of cycles/h (Zoccal et al., 2007, 2008; Silva and Schreihofer, 2011) of daytime exposure, from 4 h/day (Kalaria et al., 2004), 6 h/day (Lai et al., 2006), 7 h/day (Fletcher et al., 1992a), 8 h/day (Chen et al., 2005; Belaidi et al., 2009; Zoccal et al., 2008, 2009; Knight et al., 2011; Silva and Schreihofer, 2011; Dyavanapalli et al., 2014; Schulz et al., 2014), 10 h/day (Liu et al., 2013) to 12 h/day (Lin et al., 2007). The exposure duration of 8 h/day seems to be that on which there is the greatest consensus (see **Table 2**). The duration of exposure seems to affect the study outcomes more than the hypoxic nadir or the rate of hypoxic cycling (Davis and O'Donnell, 2013).

An advantage of CIH models is they allow exposures that can be extended over months, enabling the investigation of chronic consequences that might occur in humans (Toth and Bhargava, 2013). The number of days necessary to induce an increase in BP seems to be dependent on the CIH paradigm. Some authors suggest that the BP increase triggered by CIH represents a time-dependent effect (Prabhakar et al., 2001; Hui et al., 2003; Dematteis et al., 2008; Zoccal et al., 2009). Moreover, both the time and severity of hypoxia have been shown to play an important role in the cardiovascular response (Li et al., 2007; Perry et al., 2007). It has recently been shown that a period of 14 days is not long enough to induce structural changes in cardiovascular structures, but these are already apparent after 35 days of incubation (Dematteis et al., 2008). Moreover, Iturriaga et al. report that the exposure of rats to CIH for 14 days enhanced the ventilatory response to hypoxia and produced a significant shift in heart rate variability, but these cardiorespiratory alterations occurred without noticeable changes in mean arterial BP until 21 days of CIH exposure (Iturriaga et al., 2010). Whereas some short-term protocols (7–14 days) cause a significant increase in BP (Belaidi et al., 2009; Knight et al., 2011; Silva and Schreihofer, 2011; Bathina et al., 2013), others show an increase in BP that occurs only after long-term exposure (35 days) to CIH (Prabhakar et al., 2001, 2005; Chen et al., 2005; Zoccal et al., 2009) (see **Table 2**). Finally, most IH paradigms in rodents do not include CO_2 supplementation (Fletcher et al., 1999; Lin et al., 2007; Iturriaga et al., 2010; Perry et al., 2011; Bathina et al., 2013). In fact, only some authors have manipulated the CO_2 levels (Ooi et al., 2000; Kantores et al., 2006; Dyavanapalli et al., 2014) and fixed the values along the protocol (see **Table 2**).

Independently of the paradigm used to induce HT related to OSA, previous reviews are unanimous in reporting the development of mild HT, despite the divergent changes in arterial blood gases (Kanagy, 2009) (see **Table 2**). The exceptions found in this review (Kalaria et al., 2004; Belaidi et al., 2009; Iturriaga et al., 2010; Perry et al., 2011) are all related to the method used for BP measurement. It is apparent that arterial catheterization is not an accurate method of measuring BP in CIH models. The methods most often used for BP measurement (for a review, see Kurtz et al., 2005) in IH models (see **Table 2**) are the tail-cuff method (Allahdadi et al., 2005; Chen et al., 2005; Soukhova-O'Hare et al., 2008; Belaidi et al., 2009; Totoson et al., 2013), radiotelemetry (Fletcher, 2000; Tahawi et al., 2001; Lai et al., 2006; Knight et al., 2011; Bathina et al., 2013; Sharpe et al., 2013; Dyavanapalli et al., 2014; Schulz et al., 2014), and arterial catheterization (Kanagy et al., 2001; Kalaria et al., 2004; Campen et al., 2005; Lin et al., 2007; Belaidi et al., 2009; Zoccal et al., 2009; Iturriaga et al., 2010; Perry et al., 2011; Silva and Schreihofer, 2011; Totoson et al., 2013).

HUMANS

The variety of models of IH in healthy human subjects is much less impressive than that observed for animal models of sleep apnea. In terms of the exposure time, these models are usually divided into short-term and chronic (Foster et al., 2007). In

Table 2 | Reports on the effects of CIH on blood pressure.

Species	Hypoxia cycle, Nadir FiO_2, duration and CO_2 manipulation (Y/N)	BP measurement	Effect on BP	References
Sprague-Dawley rats	20 cycles (90 s each) of 21–5% O_2 and 0–5% CO_2/h; 7 h/day; 35 days; Yes	Tail-cuff method	↑ MAP (25–28 mmHg)	Allahdadi et al., 2005
Sprague-Dawley rats	80 cycles (6 min each) 21–10% O_2/day; 8 h/day; 7 days; No	Telemetry	↑ MAP (7–10 mmHg)	Knight et al., 2011
Sprague-Dawley rats	5% O_2 12 times/h; 8 h/day; 7–21 days; No	Arterial catheterization	No changes in MAP	Iturriaga et al., 2010
Wistar rats	10% O_2 for 4 h/day and 21% O_2 for 20 h/day; 56 days; Yes (PCO_2 < 0.02%)	Arterial catheterization	No differences in systemic pressure	Kalaria et al., 2004
Sprague-Dawley rats	2/3–20.9% O_2 (3–6 s +15–18 s; 2 cycles/min); 6–8 h/day; 35 days; No	Telemetry	↑ MAP (16 mmHg)	Tahawi et al., 2001
C57BL/6J mice	21–5% O_2 (60 s); 12 h/day; 5 weeks; No	Arterial catheterization	↑ Systemic BP (7.5 mmHg)	Campen et al., 2005
SHR + Wistar rats	21–10% O_2 (1 min cycles: 20 s + 40 s); 8 h/day; 14 days; No	Tail-cuff method + Arterial catheterization	Enhanced HT development in SHR + NS in Wistar rats	Belaidi et al., 2009
Sprague-Dawley rats	2/3–20.9% O_2 (3–6 s + 12 s; 2 cycles/min); 6–8 h/day; 35 days; No	Telemetry	↑ MAP (10 mmHg)	Fletcher, 2000
LCR and HCR	21–10% O_2 (3 min cycles); 8 h/day; 7 days; No	Telemetry	↑ MAP in both groups	Sharpe et al., 2013
Sprague-Dawley rats	20 cycles (90 s each) of 21–5% O_2 and 0–5% CO_2/h; 8 h/day; 11 days; Yes	Arterial catheterization	↑ MAP (30 mmHg)	Kanagy et al., 2001
Sprague-Dawley rats	48 cycles (45 + 30 s) 20.9–2/6% O_2/h; 6 h/day; 30 days; No	Telemetry	↑ MAP (19.3 mmHg)	Lai et al., 2006
C57BL/6J mice	21–5.7% O_2 (alternating every 6 min); 12 h/day; 90 days; No	Arterial catheterization	↑ MAP (19.8 mmHg)	Lin et al., 2007
Sprague-Dawley rats	21–10% O_2 for 5 s every 90 s; 10 h/day; 4 weeks; No	Tail BP telemeter	↑ MAP (37 mmHg)	Liu et al., 2013
SHR	21–10% O_2 (alternating every 90 s); 12 h/day; 30 days; No	Tail-cuff method	↑ SBP and DBP (NA mmHg)	Soukhova-O'Hare et al., 2008
Sprague-Dawley rats	21–4/6% O_2 (every 60 s); 8 h/day; 5 days/week; 5 weeks; No	Tail-cuff method	↑ MAP (12 mmHg)	Chen et al., 2005
Wistar rats	1 min cycles with 30 s of a 5% FiO_2; 8 h/day; 14–21 days; No	Tail-cuff method + Arterial catheterization	Rapidly ↑ MAP (NA mmHg)	Totoson et al., 2013
Sprague-Dawley rats	21–6% O_2 (9 min cycles); 8 h/day; 14 days; No	Arterial catheterization	↑ MAP (9 mmHg)	Silva and Schreihofer, 2011
Wistar rats	20.8–6% O_2 (9 min cycles: 5 min Nx); 8 h/day; 10 days; No	Arterial catheterization	↑ MAP (12 mmHg) ↑ SBP (9 mmHg) ↑ DBP (8 mmHg)	Zoccal et al., 2009
Wistar- Hannover rats	21–10% O_2 (2 min + 2 min); 1000–1600 h; Yes (PCO_2 < 0.01%)	Arterial catheterization	No differences in MAP	Perry et al., 2011

(Continued)

Table 2 | Continued

Species	Hypoxia cycle, Nadir FiO_2, duration and CO_2 manipulation (Y/N)	BP measurement	Effect on BP	References
Sprague-Dawley rats	10 cycles (6 min each) of 21–6% O_2 and 0–5% CO_2/h; 8 h/day; 28 days; Yes	Telemetry	↑ SBP (39 mmHg) ↑ DBP (33 mmHg)	Dyavanapalli et al., 2014
C57BL/6J mice	21–7% O_2 (120 s each cycle); 5 days/week; 8 h/day; 6 weeks; No	Telemetry	Significant ↑ MAP	Schulz et al., 2014
Sprague-Dawley rats	21–10% O_2 (cycle duration: NA); 8 h/day; 7 days; No	Telemetry	↑ MAP that persisted after CIH exposure	Bathina et al., 2013

BP, blood pressure; CIH, chronic intermittent hypoxia; DBP, diastolic blood pressure; h, hour; HCR, high aerobic capacity rats; HT, hypertension; LCR, low aerobic capacity rats; MAP, mean arterial pressure; NA, information not available; NS, no significant effect; Nx, normoxia; min, minutes; s, seconds; SBP, systolic blood pressure; SHR, spontaneously hypertensive rats; ↑, increase.

short-term IH models, generally the exposure time (20–60 min) and the duration of the hypoxia or voluntary apnea period (30 s) are very limited. The protocols of Cutler et al. and Tamisier et al. are good examples of short-term models (Cutler et al., 2004; Tamisier et al., 2009). In contrast, Foster et al. made use of a chronic model, exposing healthy human volunteers to an hour of IH (5 min hypoxia alternating with 5 min normoxia) daily for 2 weeks (Foster et al., 2005). As in the animal models of IH, only some studies have controlled the level of CO_2 (Foster et al., 2005), whereas others have not (Tamisier et al., 2009). Regardless of the protocol followed, exposing humans to CIH implies careful supervision.

In 2001, Xie et al. exposed nine healthy human subjects during wakefulness to 20 min of isocapnic hypoxia (arterial O_2 saturation, 77–87%) and 20 min of normoxic hypercapnia (end-tidal PCO_2, 15.3–8.6 Torr above eupnea) on two separate days. The subjects breathed through a leak-free nasal mask and the neurocirculatory and ventilatory responses to these two stimuli were further evaluated (Xie et al., 2001). These authors found that hypoxia induced a sympathetic activation that outlasted the chemical stimulus, whereas hypercapnia evoked a short-lived sympathetic activation (Xie et al., 2001). Years later, in a study performed with a larger sample ($n = 31$), Cutler et al. used a model of IH induced by voluntary apnea (30 s of hypoxic apnea every 1 min—simulating an AHI of 60/h—for 20 min) to determine if the cessation of breathing is important in prolonged sympathetic activation (Cutler et al., 2004). This study also included two other groups that were exposed to intermittent hypercapnic hypoxia and to intermittent isocapnic/hypoxia, respectively (Cutler et al., 2004). Their results support the hypothesis that short-term exposure to intermittent hypoxic apnea results in sustained elevation of post-ganglionic muscle sympathetic nerve activity and that hypoxia is the primary mediator of this response (Cutler et al., 2004). The data reported by Leuenberger et al. one year later were in line with these results (Leuenberger et al., 2005). They also found, in a study that enrolled 26 patients, a sustained sympathetic activation and also a transient elevation of BP following 30 min of voluntary end-expiratory apneas primed with a hypoxic gas mixture and lasting for 20 s in each minute (Leuenberger et al., 2005).

Foster et al. carried out three main studies in healthy human volunteers. The first aimed to determine the ventilatory, cardiovascular and cerebral tissue oxygen response to two protocols of IH (Foster et al., 2005). This study involved 18 patients randomly assigned to short-duration IH (1 h of 12% O_2 separated by 5 min of normoxia) or long-duration IH (30 min of 12% O_2). Both groups had 10 exposures over 12 days. Their findings show a rise in mean arterial blood pressure (MAP) that occurs throughout the daily exposure to short-duration IH but not during exposure to long-duration IH; moreover, they demonstrate that the vascular processes required to control blood flow and O_2 supply to cerebral tissue in a healthy human are delayed following exposure to 12 days of isocapnic IH (Foster et al., 2005). In 2009, the same group reinforced the enrollment of IH on the pathogenesis of cardiovascular and cerebrovascular disease in patients with OSA (Foster et al., 2009). They exposed 10 healthy subjects to IH (2 min of hypoxia: nadir $P_{ET,O2} = 45.0$ mmHg, alternating with 2 min of normoxia: peak $P_{ET,O2} = 88.0$ mmHg for 6 h) for 4 consecutive days and concluded that IH alters BP (MAP increased by 4 mmHg) and induces an increase in cerebral vascular resistance (Foster et al., 2009). More recently, Foster et al. have assessed the role of the type I angiotensin II receptor in mediating an increase in arterial pressure associated with a single 6-h IH exposure (Foster et al., 2010). For that, they exposed nine healthy subjects to sham IH, IH with placebo medication, and IH with the type I angiotensin II receptor antagonist (losartan). Their findings demonstrate a significant increase in arterial pressure after exposure to isocapnic IH (Foster et al., 2010). Furthermore, since this increase is prevented by the blockade of AT_1 receptors, these results suggest an important role for the rennin-angiotensin-aldosterone system (RAAS) in the pathophysiology of HT related to OSA (Foster et al., 2010).

Tamisier et al. have developed a novel model of nocturnal CIH in healthy humans, which represents an important step forward in the field, designed to overcome some of drawbacks and confounding factors that are present in studies of both animals and OSA patients (Tamisier et al., 2009). To investigate the effects of CIH on sleep, BP and ventilatory control, these authors make use of altitude tents to mimic the cyclical arterial oxygen desaturations-resaturations of sleep apnea. They

delivered O_2 for 15 s every 2 min during sleep while subjects breathed 13% O_2 in a hypoxic tent to create 30 cycles/h of cyclic desaturation-reoxygenation (SpO_2 range: 95–85%), and exposed subjects overnight for 8–9 h/day for 2 or 4 weeks (Tamisier et al., 2009). Among other results, they show that waking normoxic arterial pressure increased significantly at 2 weeks for systolic and for diastolic at 4 weeks, that patients developed a sustained BP increase during the day and exhibited a steeper BP decrease at night compared to baseline BP values, and finally, that this model produces clinically relevant fluctuations in SaO_2 (Tamisier et al., 2009). Although undoubtedly relevant, the authors recognize the presence of several respects in which their model does not mimic sleep apnea, e.g., no negative intrathoracic pressure development, higher percentage of sleep time at <90% SaO_2 and poikilocapnia (Tamisier et al., 2009). However, some of these limitations can be overcome to achieve a pattern of IH more akin to OSA features. This model was further used by the same group in 2011 to shed light on the profile of the BP increase previously described to determine if it is sustained and to explore potential underlying physiological mechanisms. The authors found that only 2 weeks of severe IH exposure produces a sustained daytime BP increase in the setting of sympathetic activation and blunted vascular sympathic baroreflex gain in healthy volunteers (Tamisier et al., 2011).

In conclusion, to date, only a small number of studies have been conducted using healthy human models of IH and these have primarily been aimed at elucidating the role of IH in sustained sympathetic activation and cerebrovascular regulation. Only a few studies have evaluated BP outcomes (Foster et al., 2009, 2010; Tamisier et al., 2009, 2011) and none of these models have truly been used to assess the efficacy of AHDs in the treatment of HT related to OSA. In fact, in the later work of Foster et al., losartan (the angiotensin II AT_1 receptor antagonist) was used only to demonstrate a mechanistic pathway rather than to evaluate its efficacy (Foster et al., 2010). Thus, future research in this field is clearly needed.

WHAT ARE THE MECHANISMS INVOLVED IN THE PATHOGENESIS OF HT RELATED TO OSA?

Fletcher et al. were pioneers in demonstrating the hypertensive effect of CIH (Fletcher et al., 1992a) and the role of the sympathetic nervous system, peripheral receptors and rennin-angiotensin system in this response (Fletcher et al., 1992b, 1999, 2002; Fletcher, 2000). This group also showed that surgical denervation of peripheral chemoreceptors, adrenal demedullation and chemical denervation of the peripheral nervous system prevented the increase in BP in response to CIH stimulus (Fletcher et al., 1992b; Bao et al., 1997). After Fletcher et al.'s first work, many reports enabled confirmation of the relationship between IH and BP increases and contributed to elucidating the underlying mechanisms. Kanagy et al. reported increased plasma endothelin-1 levels in rats exposed for 11 days to CIH, which also demonstrated an appreciable increase in MAP (Kanagy et al., 2001). In 2006, Lai et al. suggested that chronic IH-induced sustained HT was associated with the facilitation of cardiovascular sympathetic outflow followed by decreases in baroreflex sensitivity in conscious rats (Lai et al., 2006). Along the same line, the work undertaken by Zoccal et al. provided strong evidence to support the idea that rats submitted to CIH show an increase in sympathetic activity, which seems to be essential in the maintenance of high BP values in the CIH model (Zoccal et al., 2007). Another group revealed that although elevated sympathetic nervous system activity (SNA) may contribute to CIH-induced HT, reduced adrenergic vascular reactivity buffers the cardiovascular impact of exaggerated acute raises in SNA (Silva and Schreihofer, 2011). Data attained by Knight et al. indicated that CIH induces an increase in FosB/ΔFosB in autonomic nuclei and suggested that activator protein-1 (AP-1) transcriptional regulation may contribute to stable adaptative changes that support chronically elevated BP (Knight et al., 2011). Also in 2011, Liu et al. demonstrated that CIH activates the HIF-1α/endothelin system, through CIH-NADPH oxidase-mediated ROS production, and this enhances the development of resistant vasoconstriction and elevates BP in rats (Liu et al., 2011). The study undertaken by Bathina et al. revealed that the knockdown of tyrosine hydroxylase in the nucleus of the solitary (NTS) tract reduces the CIH-induced persistent increase in MAP, suggesting that noradrenergic A_2 neurons in nucleus tractus solitarius play a role in the cardiovascular responses to CIH (Bathina et al., 2013). More recently, Schulz et al. have shown that NADPH oxidase 2 (NOX2) knockout blocks the development of HT induced by CIH, suggesting that this type of HT is mediated by reactive oxygen species (ROS) derived from the activation of NOX2 within cells located outside the cardiovascular system (Schulz et al., 2014).

The mechanisms involved in the genesis of HT related to OSA have recently been reviewed (Lavie and Lavie, 2009; Bosc et al., 2010; Sunderram and Androulakis, 2012; Zhang and Si, 2012; Lévy et al., 2013) and broadly include the following: sympathetic nervous system stimulation mediated mainly by the activation of carotid body chemoreflexes, decreased vascular responses to nitric oxide, increased plasma concentrations of endothelin, and elevation of proinflammatory cytokines (TNF-α, IL-6, VEGF). While for some of these mechanisms (e.g., activation of the RAAS, endothelial dysfunction, systemic inflammation, metabolic anomalies, and genetic contribution) the relationship with OSA and subsequent cardiovascular morbidity remain partially unclear and there is a need to gather more evidence, for others (e.g., the increase in sympathetic activity and acute effects of negative intrathoracic pressure), there seems to be more agreement on the linkage and it is well-documented (Parati et al., 2013). In fact, based on data attained from patients with OSA, it is widely accepted that sympathetic activation, inflammation and oxidative stress play major roles in the pathophysiology of this particular type of HT. In addition, the use of animal models has revealed that CIH is the critical stimulus underlying sympathetic activity and HT, and that this effect requires the presence of functional arterial chemoreceptors (Fletcher, 2000). However, it should be also mentioned that HT related to OSA probably results not only from increased carotid chemoreflex but also from decreased baroreceptor activity (Dumitrascu et al., 2013). It is also important to highlight the potential role of obesity as an intermediate factor in the pathway of HT related to OSA (Young et al., 2007; O'Connor et al., 2009).

The mechanisms involved in the pathogenesis of HT can be summarized in relation to two main pathways: sympathetic nervous system stimulation mediated mainly by activation of carotid body (CB) chemoreflexes and the systemic effects of CIH, mainly due to the activation of NOX2 and subsequent ROS production. **Figure 1** illustrates the hypothesized pathways by which intermittent hypoxia leads to HT.

WHAT IS ALREADY KNOWN CONCERNING THE EFFICACY OF AHDs?

HUMANS

Despite the considerable number of studies involving OSA patients, only a few have investigated the efficacy of different AHDs and in general, they tend to be individual drug studies. Moreover, most of the studies only take into account the number of drugs taken by patients to adjust this variable and are difficult to interpret as most of the patients were already under AHDs regimens. This lack of information could be attributed to the large number of possible different AHDs regimens observed in OSA patients. **Table 3** summarizes the most relevant studies that have investigated the efficacy of AHDs in OSA patients.

In a study undertaken by Pelttari et al., the AH effects of four different AHDs (atenolol: a beta-blocker; isradipine: a calcium channel blocker; hydrochlorothiazide: a diuretic; spirapril: an angiotension-converting enzyme inhibitor) in obese patients with OSA and HT were compared using ambulatory blood pressure monitoring (ABPM) (Pelttari et al., 1998). This study revealed that although daytime HT was quite easily controlled by the single use of these drugs (especially with atenolol and isradipine; diuretics did not significantly lower BP) none of the AHDs were able to produce a significant decrease in nocturnal BP (Pelttari et al., 1998). Mayer et al. carried out another comparative study between cilazapril (an angiotension-converting enzyme inhibitor) and metoprolol (a beta-blocker) (Mayer et al., 1990). Their findings showed that despite the short period of therapy (1 week), both metoprolol and cilazapril lowered nighttime BP in OSA patients (Mayer et al., 1990).

A multiple crossover study examined the BP-lowering effect of the five major AHDs classes (atenolol: beta-blocker; amlodipine: calcium channel blocker; enalapril: angiotension-converting enzyme inhibitor; hydrochlorothiazide: diuretic; losartan: angiotensin receptor blocker) and showed that atenolol induced the most pronounced effect in lowering BP (Kraiczi et al., 2000). Atenolol was more efficient in reducing mean nighttime diastolic and systolic BP (measured by ABPM) compared to amlodipine, enalapril, hydrochlorothiazide, and losartan (Kraiczi et al., 2000). Salo et al. investigated the effects of four AHDs (atenolol; isradipine: a calcium channel blocker; hydrochlorothiazide; spirapril: an angiotension-converting enzyme inhibitor) on cardiovascular autonomic control and reactivity in HT OSA patients (Salo et al., 1999). This group reported that of the four drugs, only atenolol effected BP variability (Salo et al., 1999). Thus, the results of these two pilot studies are in line with those arguing the involvement of the sympathetic system in the pathophysiology of HT related to OSA, suggesting that beta-blockers, in particular atenolol, may have beneficial effects beyond BP reduction in patients with OSA. However, both studies presented low

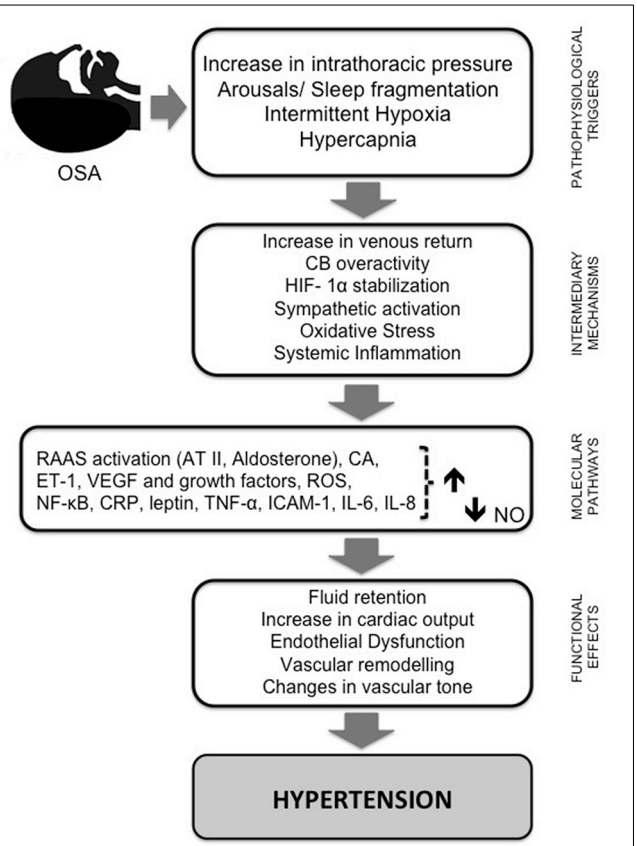

FIGURE 1 | Schematic diagram summarizing the pathways by which intermittent hypoxia leads to hypertension. Repetitive obstructive apneas or hypopneas lead to increased intrathoracic pressure, sleep fragmentation, recurrent hypercapnia, and intermittent hypoxia (IH). This last phenomenon plays a pivotal role in triggering several intermediary mechanisms and molecular pathways that contribute to the initiation and progression of cardiac and vascular pathology. First, IH enhances sympathetic nervous system activity, leading to vasoconstriction and systemic hypertension through RAAS activation, and an increase in catecholamine secretion and plasma level of vasoconstrictive ET-1. Episodic hypoxia also favors the stabilization of HIF-1α and the production of ROS, which is followed by increased expression of NF-κB and decreased NO bioavailability, the most important vasodilatory molecule synthesized by the endothelium. AT II and ET-1 both seem to be implicated in vascular remodeling and ROS formation, which is increased through the activation of vascular NADPH oxidase and xanthine oxidase. ROS molecules induce a cascade of inflammatory pathways linked to an overexpression of adhesion molecules and pro-inflammatory cytokines, and oxidative stress may trigger sympathetic hyperactivation and *vice versa*. ROS production is required for HIF-1α induction and HIF-1α induction is required for ROS production. In addition, HIF-1α promotes the expression of ET-1 and transcriptional activation of VEGF and other growth factors. Activation of NF-κB also seems to be central in inflammation induced by IH due to its regulatory role in the production of pro-inflammatory mediators (e.g., TNF-α, IL-6, IL-8, ICAM-1, and CRP). These signaling pathway proteins, combined with RAAS, decreased expression of eNOS, and increased ROS production and stabilization of HIF-1, participate in the molecular mechanisms underlying the endothelial dysfunction induced by IH. Together, these mechanisms progress to fluid retention, changes in cardiac output and vascular tone, and vascular remodeling, leading to systemic HT, one of the major consequences of OSA. AT II, angiotensin II; CA, catecholamine levels; CRP, C- reactive protein; CB, carotid body; ET-1, endothelin 1; HIF-1α, hypoxia-inducible factor α; IH, intermittent hypoxia; IL, interleukin; ICAM-1,

(Continued)

FIGURE 1 | Continued
intercellular adhesion molecule; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; NF-κB, nuclear factor-κ-light chain enhancer of activated B cells; RAAS, renin-angiotensin-aldosterone system; ROS, reactive oxygen species; TNF- α, tumor necrosis factor α; VEGF, vascular endothelial growth factor.

levels of causation, which could have limited the ability to detect differences between classes.

Nevertheless, it has been advanced that angiotension-converting enzyme inhibitors (ACEi) treatment could exacerbate OSA by inducing upper airway inflammation (Cicolin et al., 2006). The comparison between chronic treatments of ACEi and angiotensin AT1 receptor antagonists in terms of AH efficacy and levels of inflammatory markers has never been performed either in humans with OSA or in animal models. More recently, other study compared the effect of doxazosin (an α1- adrenergic receptor antagonist) and enalapril (an angiotensin-converting enzyme inhibitor) on nocturnal BP control and concluded that the former has a proportionally poorer effect than the latter (Zou et al., 2010). In 1994, Grote et al. performed a study aimed at assessing the effectiveness of cilazapril (an angiotension-converting enzyme inhibitor) in managing high BP in patients with OSA. Although the study comprised a small sample size, the results suggested that cilazapril is effective in reducing BP in all sleep stages (Grote et al., 1994). In another small study, Heitmann et al. evaluated the effect of nebivolol (a third generation beta-blocker) on BP reduction and sleep apnea activity in HT patients with mild to moderate OSA in comparison with valsartan (an angiotensin receptor blocker) and concluded that the effect of these AHDs were similar (Heitmann et al., 2010). Despite the same limitations, these studies highlight the role of the renin-angiotensin-aldosterone system (RAAS) in the pathophysiology of HT related to OSA.

In two past studies (Lozano et al., 2010; Litvin et al., 2013), patients either received CPAP in combination with AHDs or alternatively, the pharmacological treatment alone, allowing the evaluation of the effects of CPAP and AHDs independently or in conjunction. In the study undertaken by Lozano et al., patients were under an AHDs regimen with at least three drugs at adequate doses, including a diuretic (Lozano et al., 2010). The authors noted a significant decrease in the mean 24-h diastolic BP in patients who received CPAP in addition to conventional treatment, suggesting that resistant HT treated with both CPAP and AHDs provides greater BP reduction than AHDs alone (Lozano et al., 2010). However, in patients who used CPAP less than the average (5.6 ± 1.52 h/night) and for those treated with conventional treatment alone, there was no significant difference in the 24-h ambulatory BP values (Lozano et al., 2010). These findings are in line with those reported by Litvin et al., attained with patients who received stepped dose titration of AHDs treatment (valsartan 160 mg + amlodipine 5–10 mg + hydrochlorothiazide 25 mg) for 3 months before CPAP was added (Litvin et al., 2013). These findings seem to suggest that the best strategy to treat HT related to OSA involves the combination of OSA treatment with CPAP and the use of AHDs. This combination is likely to be more effective in lowering both daytime and nighttime BP than either treatment alone (Phillips and O'Driscoll, 2013). In addition, Pépin et al. explored RAAS inhibition using losartan in a crossover randomized control trial. In this study, the authors compared the efficacy of CPAP and valsartan in reducing BP in HT patients with OSA never treated for either condition (Pépin et al., 2009). They reported that although the BP decrease was significant with CPAP treatment, valsartan induced a four-fold higher decrease in mean 24-h BP than CPAP in this specific sample (Pépin et al., 2009).

In an earlier report, 74 of the 393 OSA patients using AH medications on a regular basis for more than 6 months were deemed to have been treated "ineffectively" (Lavie and Hoffstein, 2001), but the characterization of these medications was not reported. The same limitation is found in the study of Deleanu et al., which aimed to study the effect of medication-controlled HT on OSA patients (Deleanu et al., 2014). The authors suggested that controlled BP abates sleepiness and reduces remaining symptoms (e.g., headaches, impotence and morning fatigue). These findings could be much more interesting if the regimens responsible for these effects were revealed.

In a very recent study, Kario et al. aimed to evaluate the effects of bedtime dosing of vasodilating (nifedipine, a calcium channel blocker) vs. sympatholytic (carvedilol, a non-selective β-blocker/α_1-blocker) AH agents on the sleep BP profile in HT OSA patients (Kario et al., 2014). For this, they made use of a new BP monitoring method, the trigger sleep BP monitoring (TSP) method, which is based on the automated fixed-interval measurement function with an additional oxygen-triggered function that initiates BP measurement when oxygen desaturation falls below a set variable threshold continuously monitored by pulseoximetry (Kario et al., 2014). The BP lowering effects of nifedipine on the mean and minimum sleep systolic BP were stronger than those of carvedilol; moreover, sleep systolic BP surge (the difference between the hypoxia peak systolic BP—SBP—measured by the oxygen-triggered function and SBP within 30 min before and after the peak SBP) was only significantly reduced by carvedilol (Kario et al., 2014). Thus, both drugs are effective in decreasing sleep BP (Kario et al., 2014) but the effect of carvedilol seems to be related more specifically to the hypoxia stimuli than nifedipine.

Finally, Cichelero et al. recently published the protocol of their randomized double-blind clinical trial, which seeks to compare the efficacy of chlorthalidone (a diuretic) with amiloride (also a diuretic) vs. amlodipine (a calcium channel blocker) as a first drug option in patients older than 40 years of age with stage I HT and moderate OSA (Cichelero et al., 2014). The findings of this study have not yet been reported.

In summary, individual drug studies find that the blockade of β1-adrenergic receptors (e.g., atenolol and nebivolol) and the renin-angiotensin-aldosterone (RAA) pathway, including both ACEi and angiotensin AT1 receptor antagonists, might be helpful. Spironolactone (a mineralocorticoid receptor antagonist) has been proposed has a very useful tool in cases of resistant HT (Ziegler et al., 2011a,b), a very prevalent condition in OSA patients (Oliveras and Schmieder, 2013; Solini and Ruilope, 2013) in which aldosterone levels are generally elevated, as well as for severe OSA patients (Ziegler et al., 2011a). Moreover, a study performed by Gaddam et al. (2010) has provided preliminary

Table 3 | Studies of the efficacy of AHDs in OSA patients.

Study design	n	CPAP (Y/N)	AHDs; dosage (mg/day)	BP measurement	BP outcome	References
RCT; double-blinded; balanced incomplete block design (6 w each drug + 3 w washout)	40	No	Atenolol (50); amlodipine (5); enalapril (20); hydrochlorothiazide (25); losartan (50)	Office BP 24 h ABPM	↓ in office SBP and daytime ABPM NS for all drugs; Atenolol ↓ night-time 24 h SBP and DBP more effectively than amlodipine, enalapril or losartan	Kraiczi et al., 2000
RCT; double-blinded; crossover schedule (8 w each drug + 2–3 w washout	15	No	Atenolol (50); isradipine (2.5): hydrochlorothiazide (25); spirapril (6)	Office BP	Slight ↓ BP for all drugs; Only atenolol affected BP variability	Salo et al., 1999
RCT; double-blinded; crossover (8 w each drug + 2–3 w washout	18	NA	Atenolol (50); isradipine (2.5); hydrochlorothiazide (25); spirapril (6)	24 h ABPM	↓ mean 24 h SBP (except for HCTZ) ↓ mean 24 h DBP (for all drugs) NS ↓ mean night-time SBP and DBP (for all drugs)	Pelttari et al., 1998
RCT (3 months each treatment)	75	Yes	Treatment with at least 3 drugs at adequate doses, including a diuretic	24 h ABPM	CPAP + AHDs regimen: ↓ 4.9 mmHg 24 h DBP; AHDs regimen alone: NS	Lozano et al., 2010
RCT; single-blinded (3 w each regimen)	44	Yes	Valsartan (160) + amlodipine (5–10) + hydrochlorothiazide (25)	Office BP 24 h ABPM	AHDs alone: ↓ office and 24 h SBP and DBP Additional ↓ in office BP and ambulatory BP monitoring (CPAP+ 3 AHDs)	Litvin et al., 2013
RCT; crossover (8 w each treatment + 4 w washout)	23	Yes	Valsartan (160)	Office BP 24 h ABPM	CPAP: ↓ 2.1 mmHg 24 h MBP and ↓ 1.3 mmHg night-time MBP (NS) VAL: ↓ 9.1 mmHg 24 h MBP and ↓ 6.1 mmHg night-time MBP	Pépin et al., 2009
RCT (8 w)	12	No	Spironolactone (25–50) added to current medication (mean number of AHDs: 4.3 ($SD = 1.1$)	Office BP 24 h ABPM	↓ 17 mmHg 24 h SBP ↓ 10 mmHg 24 h DBP	Gaddam et al., 2010
RCT; double-blinded (8 days)	12	NA	Metoprolol (100); cilazapril (2.5)	Office BP 24 h ABPM	MET: ↓ 13 mmHg 24 h SBP and ↓ 5 mmHg 24 h DBP CIL: ↓ 13 mmHg 24 h SBP and ↓ 17 mmHg 24 h DBP	Mayer et al., 1990
RCT; double-blinded; crossover (2 w each treatment + 3 w washout)	16	No	Doxazosin (4–8); enalapril (10–20)	24 h ABPM	DOX: ↓ 4.1 mmHg 24 h SBP and ↓ 5.1 mmHg 24 h DBP EN: ↓ 12.6 mmHg 24 h SBP and ↓ 8.9 mmHg 24 h DBP 24 h MBP: no differences between groups	Zou et al., 2010
RCT; double-blinded; parallel group; single center (6 w)	31	No	Nebivolol (5); valsartan (80)	Office BP	NEB: ↓ 14.6 mmHg SBP and ↓ 8.6 mmHg DBP VAL: ↓ 11.6 mmHg SBP and ↓ 8.9 mmHg DBP No differences between treatments	Heitmann et al., 2010

(Continued)

Table 3 | Continued

Study design	n	CPAP (Y/N)	AHDs; dosage (mg/day)	BP measurement	BP outcome	References
RCT; prospective; crossover; parallel group (2 single doses of each drug + 2 w washout)	11	No	Nifedipine slow-release (40); carvedilol (20)	Office BP TSP method	NIF: ↓ 24.2 mmHg mean SBP and ↓ 18.7 mmHg mean DBP CAR: ↓ 16 mmHg mean SBP and ↓ mean 8.6 mmHg DBP	Kario et al., 2014
RCT; double-blinded; placebo-controlled (8 days)	23	NA	Cilazapril (2.5)	Invasive arterial BP (arteria brachialis)	↓ 10 mmHg MBP (vs. ↓ 4.3 mmHg MBP for placebo)	Grote et al., 1994

ABPM, ambulatory blood pressure monitoring; AHDs, antihypertensive drugs; BP, blood pressure; CAR, carvedilol; CIL, cilazapril; CPAP, continuous positive airway pressure; DBP, diastolic blood pressure; DOX, doxazosin; EN, enalapril; HCTZ, hydrochlorothiazide; MBP, mean blood pressure; MET, metoprolol; NA, information not available; NEB, nebivolol; NIF, nifedipine; NS, no significant effect; RCT, randomized controlled trials; SBP, systolic blood pressure; SD, standard deviation; TSP, trigger sleep BP monitoring; VAL, valsartan; w, week; ↓, decrease.

evidence that treatment with this drug substantially reduces the severity of OSA and improves BP in patients with both OSA and resistant HT (Gaddam et al., 2010). These results seem promising but need to be confirmed in further larger studies. In contrast, despite volume overload appears to play a large role in the development of OSA (Owen and Reisin, 2013), diuretics, namely thiazide, have not been very effective AH agents in OSA patients without fluid retention (Ziegler et al., 2011b). Calcium channel blockers, although effective in lowering BP, seem to present an effect less related to hypoxia stimuli. Moreover, Nerbass et al. reported that the use of these drugs might impact negatively on sleep duration in HT patients with OSA (Nerbass et al., 2011). They reported that the use of calcium channel blockers was associated with significant reduction in total sleep time and lower sleep efficiency (Nerbass et al., 2011). Thus, their prescription can be questionable in these patients.

Despite the findings of these studies, they present some limitations and important data are missing. The major limitations comprise the following: the variability of subjects included in the studies as most of them were performed in non-AHDs naïve patients; the severity and chronicity of HT, which were not taken into account and consenquently the clinical relevance of BP reduction is questionable; the drug effectiveness in reducing nocturnal BP, which was not assessed in some studies; the confounding risk factors for HT that might be present in OSA patients (e.g., obesity) and were not properly addressed in most studies. Furthermore, we can point out several questions that are still unanswered, e.g., how many OSA patients are controlled under monotherapy with beta- blockers, angiotensin-converting enzyme inhibitors (ACEis), and angiotensin II receptor blockers (ARBs)? Beta-blockers or RAAS blockers are apparently effective, but should they be used alone or in combination? How many OSA patients remain uncontrolled despite the use of two or more AHDs? How do different AHDs behave when included in an AHDs regimen? In addition, the impact of these studies in clinical practice is unknown because epidemiological studies designed to investigate the AH medication profile in OSA patients are lacking. In addition, the more recent recommendations for the management of patients with OSA and HT are inconclusive regarding the use of AHDs and recognize the lack of strong evidence for the establishment of a first-line AHDs regimen for these patients (Parati et al., 2013). Other authors support the idea that as there is no clear evidence for prefering a specific class of AHDs, the selection should primarily be guided by the patient's cardiometabolic profile and associated comorbidities (e.g., obesity, metabolic syndrome, diabetes mellitus, and cardiovascular diseases) (Tsioufis et al., 2010). Moreover, these authors recommend that due to the lack of relevant trials focused on the use of associations of AHDs in OSA patients, the choice should rely on current HT guidelines and the adverse effects of AHDs also need to be considered (Tsioufis et al., 2010). The limited evidence base restricts the ability to make informed treatment choices. Thus, larger scale observational and clinical studies are needed to address these and possibly other limitations and bring new insights to the field.

Another problem concerning the studies carried out in humans is that HT is frequently not recognized in patients with

OSA (Baguet et al., 2009), and it is important to highlight that patients with elevated BP who do not carry the diagnosis of HT may be misclassified as non-HT (Wang and Vasan, 2005). Consequently, aggressive control of BP must be warranted in OSA patients and an accurate method for BP measurement should be used in the early diagnosis of clinically suspected OSA patients. Taking into account the advantages and limitations of the several methods of BP measurement, 24-h ABPM seems to be superior to office BP measurement and home BP monitoring in diagnosing HT in patients with suspected OSA (for a review, see Parati et al., 2012).

ANIMALS

As previously stated, a rather wide variety of animal models has been used to evaluate the cardiovascular consequences of OSA and to study the cause-effect mechanisms in OSA. As CIH causes a moderate increase in BP, drugs can be tested further to modulate this effect. However, studies aimed at investigating the AH effect of drugs on animal models are scarce. **Table 4** summarizes the studies that have evaluated the effects of AHDs on BP in animal models of CIH.

In a study undertaken to clarify the role of renal sympathetic nerve activity and plasma renin activity (PRA) in the diurnal BP response to chronic IH, Fletcher et al. demonstrated that the pharmacological blockade of the RAAS with losartan prevented the rise in BP induced by CIH (Fletcher et al., 1999, 2002). Losartan and other angiotensin antagonists (A-779, an Ang-(1–7) antagonist; ZD7155, an AT1 antagonist; PD123319, an AT2 receptor antagonist) were further used by da Silva et al. (2011) to investigate the role of endogenous angiotensin peptides within the hypothalamic paraventricular nucleus (PVN) neurons to control BP in a rat model of CIH-induced HT. These authors concluded that endogenous angiotensin peptides acting in the PVN contribute to IH-induced increases in MAP observed in this rat model. In 2013, losartan was used once again to test the role of the brain RAAS in CIH HT (Knight et al., 2013). The work of this group provided evidence that brain RAAS contributes to CIH HT and that brain RAAS appears to be critical for the development and maintenance of the sustained HT during normoxia (Knight et al., 2013).

Other groups have found that the systemic administration of endothelin (ET) receptor antagonists in rodents prevents the increase in BP during CIH exposure (Kanagy et al., 2001; Allahdadi et al., 2008; Belaidi et al., 2009). The data provided by Allahdadi et al. showed that an endothelin receptor antagonist (ET_A: BQ-123) acutely decreased the MAP dose dependently in rats exposed to IH but not sham rats, suggesting that targeting ET_A receptors may be a selective and effective treatment of HT related to OSA (Allahdadi et al., 2008). Belaidi et al. used SH rats and bosentan, a mixed endothelin receptor antagonist (Belaidi et al., 2009). Their results showed that the administration of bosentan during chronic IH prevented the increase in BP and reinforced the idea that endothelin antagonists could be useful therapeutic tools in HT related to OSA (Belaidi et al., 2009). The same effects were reported by Kanagy et al. for PD145065, a non-selective endothelin receptor antagonist (Kanagy et al., 2001).

Soukhova-O'Hare et al., designed a study based on the assumption that ROS and altered L-Ca^{2+} channel activity may underlie the post-natal programing of exaggerated BP and cardiac remodeling (Soukhova-O'Hare et al., 2008). To test this hypothesis, these authors used nifedipine, an L-calcium channel blocker, and a superoxide dismutase mimetic (MnTMPyP pentachloride); both attenuated BP (Soukhova-O'Hare et al., 2008). Their results suggested that Ca^{2+} and reactive oxygen species-mediated signaling during IH are critical mechanisms underlying post-natal programing of an increased severity of HT in SH rats. Kumar et al. reported similar results for the same superoxide dismutase mimetic (Kumar et al., 2006). A year before, Troncoso Brindeiro et al. used another superoxide dismutase mimetic, tempol, and showed that scavenging superoxide prevents both the increase in ET-1 production and vascular ROS levels induced by CIH exposure (Troncoso Brindeiro et al., 2007). The later work of Del Rio et al., using the antioxidant ascorbic acid, showed that this substance prevented the increased plasma peroxidation and nitrotyrosine formation within the carotid body, as well as HT (Del Rio et al., 2010), supporting the essential role of oxidative stress in the generation of carotid body chemosensory potentiation and systemic cardiorespiratory alterations induced by IH (Del Rio et al., 2010).

More recently, Hung et al. tested the hypothesis that melatonin, previously shown to ameliorate oxidative injury and inflammation, could have a protective effect against IH-induced HT and endothelial dysfunction. This assumption was confirmed as melatonin promoted a decrease in systolic BP and prevented endothelial dysfunction with ameliorated levels of nitric oxide, endothelial-dependent relaxation, and expressions of eNOS and antioxidant enzymes (Hung et al., 2013).

Based on the studies described, we can conclude that most reports on CIH animal models in which drugs have been tested were not designed to respond to pharmacological issues: they have been used solely as pharmacological tools to address physiological mechanisms. The experiments evaluate prevention but not the effectiveness of treatment. They must be planned first to induce HT and then evaluate the efficacy of cumulative doses of drugs because the translation of the results to humans obtained with simultaneous induction of HT and drug administration is not relevant. Other limitations of the pharmacological approaches included in these works are the absence of dose-response curves and comparison of the effectiviness of different drugs in the same animal model. Thus, other studies must be designed to overcome these drawbacks.

WHAT ARE THE POTENTIAL NON-PHARMACOLOGICAL APPROACHES TO THE MANAGEMENT OF HT RELATED TO OSA?

Taken in perspective, the pathophysiology of HT related to OSA, which involves an increase in sympathetic activity, renal denervation seems to be a logical approach for patients with this type of HT as renal sympathetic nerves are involved in the regulation of BP. Indeed, the beneficial role of this novel approach in the management of resistant HT and other cardiovascular diseases has been reported and reviewed extensively (Grassi et al., 2012; Pimenta and Oparil, 2012; Böhm et al., 2013; Ukena et al.,

Table 4 | Studies evaluating the effects of AHDs on BP in animal models of CIH.

Species	CIH experimental protocol	Drugs/ intervention	BP measurement	Drug effect on BP	References
Sprague-Dawley rats	2/3–20.9% O_2 (3–6 s + 15–18 s; 2 cycles/min); 6–8 h/day; 35 days	Losartan (15 mg/kg); gavage; 35 days	Telemetry	Significant ↓ MAP (98.2 ± 61.7 to 85.9 ± 62.7 mm Hg)	Fletcher et al., 1999
Sprague-Dawley rats	5–21% O_2+ 5–0%CO_2 (20 cycles/h); 7 h/day; 14 days	A-779 (Ang-(1–7) antagonist); Losartan (2 nmol/h) and ZD7155 (AT1 antagonists); PD123319 (AT2 receptor antagonist); osmotic minipumps delivered into PVN; 14 days.	Telemetry	↓ MAP: A-779: 5 ± 1 mm Hg, Losartan: 9 ± 4 mmHg, ZD7155: 11 ± 4 mmHg PD123319: 4 ± 3 mmHg	da Silva et al., 2011
Sprague-Dawley rats	20.9–10% (180 s cycles); 10 h/day; 35 days	Losartan (15 mg/kg); p.o. (syringe technique); 35 days	*NA* (arterial catheterization?)	↓ SBP (10 mmHg)	Fenik et al., 2012
Sprague-Dawley rats	80 cycles (6 min each) 21–10% O_2/day; 8 h/day; 7 days	Losartan (1 μg/h); intracerebroventricular (miniosmotic pumps); 7 days	Telemetry	↓ MAP during both CIH exposure and normoxic period	Knight et al., 2013
Sprague-Dawley rats	20 cycles (90 s each) of 21–5% O_2 and 0–5% CO_2/h; 7 h/day; 14 days	BQ-123 (10–1000 nmol/kg in bolus or 100 nmol/kg/day for chronic administration); iv or sc; 14 days	Tail-cuff method and telemetry	Acute administration: dose dependent ↓ MAP Chronic administration: prevented ↑MAP	Allahdadi et al., 2008
SHR + Wistar rats	21–10% O_2 (1 min cycles: 20 + 40 s); 8 h/day; 14 days	Bosentan (100 mg/Kg/dia); mixed in chow; 14 days	Tail-cuff method + Arterial catheterization	Prevented ↑MAP	Belaidi et al., 2009
SHR	21–10% O_2 (every 90 s); 12 h/day; 30 days	Nifedipine (5 mg/Kg) and SOD mimetic (MnTMPyP; 10 mg/Kg); s.c.; 30 days	Tail-cuff method	Nifedipine: attenuate SBP and DBP SOD mimetic: ↓ SBP and DBP	Soukhova-O'Hare et al., 2008
Sprague-Dawley rats	21–5% O_2 (every 60 s); 8 h/day; 14–21 days	Melatonin (10 mg/Kg); i.p.; 14 or 21 days (30 min before hypoxic exposure)	Tail-cuff method	↓ SBP (21 mmHg)	Hung et al., 2013
Sprague-Dawley rats	20 cycles (90 s each) of 21–5% O_2 and 0–5% CO_2/h; 7 h/day; 14 days;	Tempol (1 mM); drinking water; 14 days	Telemetry	↓ MAP (17 mmHg)	Troncoso Brindeiro et al., 2007
Sprague-Dawley rats	12 cycles (300 s each) of 21–5% O_2/h; 8 h/day; 21 days	Ascorbic acid (1.25 g/L); drinking tap water; 21 days	Arterial catheterization	↓ MAP (29 mmHg)	Del Rio et al., 2010
Sprague-Dawley rats	9 cycles (5 min + 15 s) of 21–5% O_2/h; 8 h/day; 10 days	SOD mimetic (MnTMPyP; 5 mg/Kg/day); i.p; 10 days	Arterial catheterization	↓↓ MAP	Kumar et al., 2006
Sprague-Dawley rats	20 cycles (90 s each) of 21–5% O_2 and 0–5% CO_2/h; 8 h/day; 11 days	PD145065 (ET receptor antagonist in cumulative drugs: 0.3, 3.0, 30, 300, 1000 nmol/Kg); bolus; 11 days	Arterial catheterization	Dose dependent ↓ MAP	Kanagy et al., 2001

(Continued)

Table 4 | Continued

Species	CIH experimental protocol	Drugs/ intervention	BP measurement	Drug effect on BP	References
Sprague-Dawley rats	21–5% O_2 (12 times/h); 8 h/day; 14 days	Ebselen (specific ONOO- scavenger; 10 mg/kg/day); osmotic mini-pumps; 7 days	Telemetry	↓ elevated BP	Moya et al., 2014

AHDs, antihypertensive drugs; BP, blood pressure; CIH, chronic intermittent hypoxia; DBP, diastolic blood pressure; ET, endothelin; h, hour; HT, hypertension; i.p., intraperitoneal; MAP, mean arterial pressure; NA, information not available; min, minutes; p.o., per os; PVN, hypothalamic paraventricular nucleus; s, seconds; SBP, systolic blood pressure; SHR, spontaneously hypertensive rats; s.c., subcutaneous; SOD, superoxide dismutase mimetic; ↓, decrease; ↑, increase.

2013; Urban et al., 2013; Faselis et al., 2014; Tsioufis et al., 2014). Moreover, Shantha and Pancholy (2014) have recently undertaken a systematic review of the effect of renal sympathetic denervation on AHI in patients with OSA. Curiously, they concluded that this approach is associated with a significant reduction in mean AHI (Shantha and Pancholy, 2014). However, as the authors pointed out, these results need further validation due to the low causal basis of the studies included in the analysis and due to the fact that only one of these studies was performed fully in a specific population of OSA patients; in the remaining studies, the diagnosis of OSA was only established after inclusion (Shantha and Pancholy, 2014). In a recent pilot study, the effect of renal denervation on BP control in patients with OSA was explored (Witkowski et al., 2011). Despite the low causal basis ($n = 10$), their findings demonstrated a significant BP decrease within 3 months, which was further enhanced at 6 months, exhibiting a drop pattern similar to clinical studies in resistant HT (Witkowski et al., 2011). Nontheless, further studies are needed to support this impressive effect of renal denervation and to ensure the safety of this technique for patients with HT related to OSA.

Like renal denervation, carotid baroreceptor stimulation has also been proposed as a novel AH therapy based on the recent evidence that baroreceptors might play an important role even in long-term BP regulation (Papademetriou et al., 2011; Grassi et al., 2012; Lovic et al., 2014). The main similarities and differences between these two novel approaches have been reviewed extensively by a group of Italian researchers (Grassi et al., 2014; Seravalle et al., 2014). Although electrical baroreflex stimulation appears to be safe and effective, and might represent a useful tool for managing resistant HT (Lovic et al., 2014), to the best of our knowledge, the effectiveness of this approach has not been yet tested in models of IH. Thus, further investigation in this specific field would be welcome.

In line with the pioneer study performed by Fletcher et al. (1992c) that established that carotid body (CB) ablation eliminated the hypertension related to CIH, McBryde et al. (2013) have shown that CB deafferentation, through bilateral carotid sinus nerve denervation, promotes an effective and lasting AH response in SH rats and reduces the overactive sympathetic activity. They have also demonstrated that associated with renal denervation, carotid sinus nerve denervation remains effective and produces a cumulative response (McBryde et al., 2013). In line with these findings, they propose carotid sinus nerve denervation as an effective AH treatment in patients with sympathetically mediated diseases (McBryde et al., 2013).

More recently, Burchell et al. have reviewed the potential of a new device for the control of arterial HT (Burchell et al., 2014). The ROX coupler device creates an anastomosis between the iliac artery and vein, diverting a calibrated amount of arterial blood into the venous system, reducing vascular resistance and increasing arterial compliance (Burchell et al., 2014). This non-pharmacological approach seems to be a promising tool in the management of patients with resistant HT due to its ability to provide an immediate and sustained reduction in BP (Burchell et al., 2014). The safety and efficacy of the ROX coupler in the treatment of this type of HT is now being evaluated in a European multicenter randomized study (Burchell et al., 2014). Positive results in

patients with drug-resistant HT leave open the possibility of the use of the ROX coupler device becoming a new strategy for the management of HT related to OSA.

CONCLUSIONS AND FUTURE PERSPECTIVES IN THE MANAGEMENT OF HT RELATED TO OSA

There is consensus that HT related to OSA is gaining more relevance as an independent nosological condition that needs a systematic approach to identify the best therapeutic strategy. One major challenge is gaining an understanding of whether the blockade of the reflex pathways triggered by CB activation is sufficiently effective to control BP in itself in HT related to OSA. Eventually, other pathways directly stimulated by hypoxia at a cellular level should be explored in depth and manipulated to attain relevant clinical control of these patients.

Drugs that have proved to be useful in essential HT treatment should be tested promptly in studies specifically designed for secondary HT induced by CIH. On the other hand, given the particularities of HT related to OSA, the recourse to tailored treatments should be considered as a possibility. Furthermore, it also appears to be imperative to look for new AHDs able to reverse HT quickly and effectively in patients with OSA as BP control is still not achievable in a significant proportion of these patients.

The contribution of animal models to this approach is unquestionable in terms of avoiding the confounding risk factors for HT that tend to be present in OSA patients. In addition, drugs that have been used as pharmacological tools to understand pathophysiological mechanisms should now be investigated regarding their efficacy in reverting HT induced by CIH.

AUTHOR CONTRIBUTIONS

Lucilia N. Diogo and Emília C. Monteiro wrote the manuscript and approved the final version.

ACKNOWLEDGMENTS

The authors would like to thank the Portuguese Fundação para a Ciência e a Tecnologia (FCT) and CEDOC (Chronic Diseases Research Centre, Lisbon, Portugal). Lucilia N. Diogo is supported by an FCT fellowship (SFRH/BD/48335/2008; PTDC/SAU-TOX/112264/2009).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 06 August 2014; accepted: 03 September 2014; published online: 22 September 2014.

*Citation: Diogo LN and Monteiro EC (2014) The efficacy of antihypertensive drugs in chronic intermittent hypoxia conditions. Front. Physiol. **5**:361. doi: 10.3389/fphys.2014.00361*

This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

REVIEW ARTICLE
published: 05 September 2014
doi: 10.3389/fphys.2014.00336

The role of local renin-angiotensin system in arterial chemoreceptors in sleep-breathing disorders

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The renin-angiotensin system (RAS) plays pivotal roles in the regulation of cardiovascular and renal functions to maintain the fluid and electrolyte homeostasis. Experimental studies have demonstrated a locally expressed RAS in the carotid body, which is functional significant in the effect of angiotensin peptides on the regulation of the activity of peripheral chemoreceptors and the chemoreflex. The physiological and pathophysiological implications of the RAS in the carotid body have been proposed upon recent studies showing a significant upregulation of the RAS expression under hypoxic conditions relevant to altitude acclimation and sleep apnea and also in animal model of heart failure. Specifically, the increased expression of angiotensinogen, angiotensin-converting enzyme and angiotensin AT_1 receptors plays significant roles in the augmented carotid chemoreceptor activity and inflammation of the carotid body. This review aims to summarize these results with highlights on the pathophysiological function of the RAS under hypoxic conditions. It is concluded that the maladaptive changes of the RAS in the carotid body plays a pathogenic role in sleep apnea and heart failure, which could potentially be a therapeutic target for the treatment of the pathophysiological consequence of sleep apnea.

Keywords: angiotensin II, AT_1 receptor, carotid body, intermittent hypoxia, OSA

INTRODUCTION

The renin-angiotensin system (RAS) plays important physiological roles in the humoral regulation of blood pressure, electrolyte and fluid homeostasis (Peach, 1977). The physiological effects are mediated by bioactive angiotensin (Ang) peptides including Ang II, Ang III, Ang IV, and Ang (1-7), produced by renin, angiotensin-converting enzyme (ACE), ACE-2 and angiotensin-processing peptidases, via the angiotensin AT_1, AT_2, AT_4, and Mas receptors (**Figure 1**). In addition to the endocrine function of RAS, growing amount of evidence demonstrates that the intrinsic RAS functions in an autocrine-paracrine manner, mediated by the RAS components expressed in numerous tissues and organs. The local RAS has diverse effects on, for examples, the regulation of vascular tone, generation of reactive oxygen species (ROS), inflammation or fibrogenesis, which are regulated by various physiological stimuli or pathophysiological conditions (Campbell, 2003). As such, it has been proposed that the local RAS might be a potential therapeutic target for the treatment of disease.

Arterial chemoreceptors in the carotid body are important for the rapid adjustment of respiratory and cardiovascular activities via the chemoreflex elicited by the sensory afferent activity of the chemoreceptor responding to changes in chemical stimuli in the arterial blood. The carotid body is a highly vascularized organ with blood perfusion far exceeding the needs of its local tissue metabolism. Thus, changes in arterial oxygen tension or pH, circulating humoral and locally produced signaling substances acting as paracrines or autocrines can readily diffuse to the chemosensory components of the carotid body. In addition to the response to hypoxia, hypercapnia and acidosis, the carotid chemoreceptor responds to Ang II because AT receptors are expressed in the chemosensitive glomus cell of the carotid body (Allen, 1998; Fung et al., 2001). Moreover, the level of RAS gene expression in the carotid body is regulated by hypoxia (Leung et al., 2000; Fung et al., 2002). In this context, the augmented activity of the carotid body has been proposed to play a role in the pathophysiology of sleep apnea. Hence, the molecular and cellular mechanisms that mediate the effect of hypoxia on the RAS in the carotid body and the pathophysiological role of the RAS in disease conditions associated with hypoxemia are of great interest. Research studies have been focused on: (a) mechanisms underlying the carotid chemoreceptor response to Ang II; (b) the expression of RAS components in the carotid body; (c) the regulation of the RAS expression in the carotid body, and (d) pathophysiological roles of the alteration of the RAS in the carotid body in disease conditions associated with hypoxemia. This review aims to summarize the literature on the functional expression of RAS in the carotid body and its regulation by hypoxia, highlighting the pathophysiological roles of the RAS in the carotid body and its clinical implications.

EXPRESSION OF RAS COMPONENTS IN THE CAROTID BODY

The expression and localization of several key RAS components, notably angiotensinogen, which is an indispensable component for the existence of an intrinsic RAS, have been detected in the rat carotid body (Lam and Leung, 2002). Hence, the mRNA and

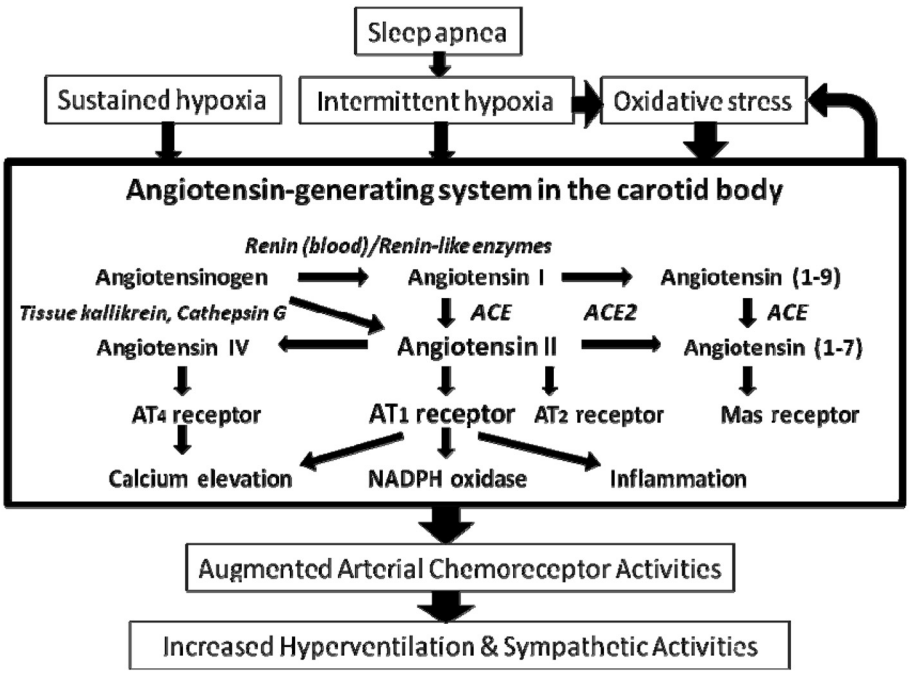

FIGURE 1 | Main components of the renin angiotensin system and the enzymes mediating the proteolytic process in the carotid body. Arrows denote the main direction of the physiological or pathophysiological cascade. Note the renin-independent biosynthetic pathway and the enzymes involved are shown in italic. The major physiological effect of Ang II is mediated by the AT_1 and AT_2 receptors. In addition, the Ang II metabolites Ang IV and Ang (1-7) exert biological effects via AT_4 and Mas receptors, respectively. ACE, angiotensin-converting enzyme.

protein of angiotensinogen are expressed in the chemosensitive glomus cells. In addition, mRNA expression of ACE is present despite an absence of the expression of renin in the rat carotid body. These strongly support the localization of an intrinsic angiotensin-generating system functioning via a locally renin-independent biosynthetic pathway in the carotid body (**Figure 1**). In this regard, the local angiotensin generation of Ang I and Ang II could be mediated by renin in the circulating blood or functional homologous enzymes of renin including tonin, cathepsin G, and kallikrein (**Figure 1**). Also, angiotensin peptides could be generated by renin-like enzymes expressed in the vasculature (Campbell, 2003). However, the expression of these alternative enzymes in the carotid body has not been investigated. Nevertheless, Ang II locally produced in the carotid body could elevate the local Ang II levels functioning as a paracrine-autocrine signal via the activation of AT_1 receptors expressed in the glomus cells.

EXPRESSION AND FUNCTION OF ANGIOTENSIN RECEPTORS IN THE CAROTID BODY

Peripheral infusion of Ang II stimulates respiration in anesthetized animals (Potter and McCloskey, 1979; Ohtake and Jennings, 1993; Ohtake et al., 1993). The respiratory response to Ang II is in part mediated by the carotid chemoreflex because Ang II increases afferent activities of the carotid body (McQueen, 1981; Allen, 1998). The effect of Ang II may be mediated by the sympathetic and parasympathetic efferent fibers innervating the carotid body, which could increase the release of norepinephrine and the afferent activity of the carotid body (McQueen, 1981; Gonzalez et al., 1994). Nevertheless, evidence suggests that Ang II exerts its effect on the chemosensory component of the carotid body. Hence, Ang II at concentrations ranging from physiological to pharmacological levels induces a brief inhibition followed by an excitation of afferent carotid sinus nerve activity in the rat superfused carotid body preparation (Allen, 1998; Leung et al., 2000). The resting activity of the carotid sinus nerve is dose-dependently increased by about two folds upon a threshold concentration of 100 pM at the physiological level of Ang II under normoxic conditions (Allen, 1998; Leung et al., 2000). Also, both the inhibitory and excitatory effects of Ang II are blocked by losartan, supporting the ligand binding is mediated by the AT_1 receptors. In addition, in an autoradiographic study, AT receptor-ligand bindings in the carotid body is not reduced by sympathetic nor afferent denervation of the carotid body (Allen, 1998). Indeed, Ang II elevates the level of intracellular calcium in the chemosensitive glomus cells (Fung et al., 2001). The intracellular calcium response to Ang II is blocked by losartan but not by an antagonist for AT_2 receptors PD-123319, suggesting an involvement of AT_1 receptors. Furthermore, AT_1-immunostaining is positively localized in the lobule of the rat carotid body, strongly supporting the expression of AT_1 receptors in the glomus cells clustering in glomeruli (Fung et al., 2001). In fact, AT_1 receptors are co-localized with cells containing tyrosine hydroxylase, which is a cellular marker of the biosynthesis of catecholamines for the chemotransduction in the carotid body (Fung et al., 2002). However, the AT_1-immunoreactivity is not found in all lobules of

the parenchyma, meaning that the expression of AT_1 receptors is not ubiquitous in the carotid body under physiological conditions (Fung et al., 2001). This is in consistent with functional data showing that the proportional amount of glomus cells responsive to Ang II is about 40% (Fung et al., 2001). Nevertheless, these findings are conclusive that AT_1 receptors expressed in the chemosensitive glomus cell mediate the carotid chemoreceptor response to Ang II. It is known that Ang II binding of the AT_1 receptor stimulates the phospholipase C pathway in the plasma membrane and leads to the formation of 1,2-diacylglycerol and inositol-1,4,5-triphosphate (IP_3), which mobilizes the endoplasmic calcium to store and elevate intracellular calcium (Balla et al., 1998). It is speculated that similar intracellular pathways could mediate the effect of Ang II on the glomus cells and the details of the intracellular signaling pathways need further study.

The mRNA transcript of two subtypes of AT_1 receptors, AT_{1a} and AT_{1b}, is expressed in the rat carotid body (Leung et al., 2000; Fung et al., 2002). It has been shown that the AT_{1a} receptor is the main one accounting for the Ang II effect on the chemoreceptor, whereas AT_{1b} might have limited involvement, if any, in the early stage of the maturation. In the postnatal rat, there is an increase in the expression of AT_{1a} subtype but a decrease in the AT_{1b} subtype regulated by hypoxia (Fung et al., 2002). This suggests that the AT_{1a} receptor is the major subtype accounting for the Ang II effect on the carotid body (Fung et al., 2002). In addition to AT_1 receptors, mRNA transcripts of the AT_2 receptor were found in the carotid body (Leung et al., 2000; Fung et al., 2002). Activation of the AT_2 receptor has a wide spectrum of effects for instances on vasodilation, apoptosis and anti-proliferation depending on the cell type (Padia and Carey, 2013). Although the AT_1 receptor is the major one mediating the excitatory response of the carotid chemoreceptor, it is possible that Ang II can also exert its effects via the AT_2 receptors in the carotid body.

It has been reported that AT_4 and Mas receptors are expressed in the carotid body (Fung et al., 2007; Schultz, 2011). Ang IV is an Ang II metabolite containing the 3–8 fragment of the octapeptide, which exerts its physiological effect via the AT_4 receptor. It has been reported that activation of AT_4 receptors by Ang IV augments the release of acetylcholine in the hippocampus (Chai et al., 2004). In the rat carotid body, positive immunoreactivity against AT_4 receptors was found in the chemosensitive glomus cells containing the tyrosine hydroxylase (Fung et al., 2007). It is speculated that AT_4 receptors binding with Ang IV could enhance the excitatory effect of Ang II on the carotid chemoreceptor mediated by the AT_1 receptor. Supporting this idea, the expression of AT_4 receptors in the carotid body is significantly increased under chronically hypoxic condition. Also, Ang IV elevates the intracellular calcium level of the chemosensitive glomus cells despite at a high concentration (10 μM) of Ang IV (Fung et al., 2007). Thus, the AT_4 receptor could be a signaling pathway of the RAS in parallel and/or complementary to the AT_1 receptor activation. The physiological or pathophysiological significance, if any, of the AT_4 receptor expressed in the carotid body has yet to be fully elucidated in future studies. As for the Mas receptor, it is a G-protein coupling receptor with high affinity binding with Ang (1-7), a biologically active peptide converted from Ang I and Ang II, respectively, by ACE2 and ACE. Recent study reported the expression of Mas receptors in the rabbit carotid body and also its decreased expression under a disease condition associated with heart failure (Schultz, 2011). The signaling cascade of Mas receptor activation has been known to induce vasodilation, which is possibly a negative modulation of the functional effects of AT_1 receptors. Thus, the Mas receptor may be functionally important in the modulation of the RAS activity in the carotid body and its alteration under disease condtions could be pathologically significant as it may contribute to the imbalance of excitatory and inhibitory modulation of the carotid chemoreceptor activity in disease (Schultz, 2011).

FUNCTIONS OF LOCAL RAS COMPONENTS IN THE CAROTID BODY

The circulating RAS is responsive to alterations in extracellular fluid volume, osmolarity, blood volume or sodium depletion, resulting in an elevated level of Ang II in the plasma (Reid et al., 1978; Matsusaka and Ichikawa, 1997). In addition to the vasoconstrictive effect of Ang II and its stimulating effect on the aldosterone secretion by the adrenal cortex, Ang II stimulates carotid chemoreceptors, which elicits the chemoreflex for the adjustment in cardiopulmonary and autonomic activities. Specifically, activation of the chemoreflex pathway is known to elevate renal sympathetic activities leading to the secretion of renin from the juxtaglomerular cells in the kidney, which could then increase sodium reabsorption and water intake (Honig, 1989; Marshall, 1994). Thus, the physiological significance of the effect of Ang II on the carotid chemoreceptor is that the baseline activity of the carotid chemoreceptor is regulated by Ang II in the circulating blood and also produced by the local RAS, which could elicit the chemoreflex in the absence of hypoxia, hypercapnia, or acidosis. As such, the carotid chemoreceptor could serve as an effector of Ang II for the regulation of blood pressure, electrolyte, and fluid homestasis via the chemoreflex pathway. Moreover, Ang II could potentiate the carotid chemoreceptor response to hypoxia. The plasma Ang II level significantly increases under hypoxic conditions (Zakheim et al., 1976). Also, the presence of local RAS in the carotid body could elevate the level of Ang II and also its metabolites in the local tissue, which could be a major source of Ang II for a more prominent effect on the modulation of the carotid chemoreceptor activity under hypoxic or disease conditions. Hence in parallel to the function of Ang II-sensitive neurons in the circumventricular organs of the brain (Ganong, 2000; McKinley et al., 2003), the carotid chemoreceptor can be an additional effector of Ang II, which confers and provides the peripheral signal integrating to the central output that alters sympathetic and parasympathetic activities for the regulation of cardiovascular and renal activities and also the electrolyte and fluid homeostasis under physiological, hypoxic, and disease conditions.

SUSTAINED HYPOXIA REGULATES RAS EXPRESSION: IMPLICATIONS ON CHRONIC OBSTRUCTIVE PULMONARY DISEASE

It has been shown that proportion of Ang II-responsive glomus cells (*ca.* 80%) is increased by 2 folds in the carotid body of rats exposed to 10% inspired oxygen for 4 weeks (sustained hypoxia)

(Leung et al., 2000; Fung et al., 2001, 2002). Also, elevated intracellular calcium levels induced by Ang II is three times higher following sustained hypoxia than the normoxic group, which is blocked by losartan (Leung et al., 2000; Fung et al., 2002). Importantly, AT_1 receptor-mediated excitation of carotid body chemoreceptor activity is two times higher in rats exposed to sustained hypoxia than in controls (Leung et al., 2000; Fung et al., 2002). Thus, sustained hypoxia induces a significant increase in the sensitivity of the chemoreceptor response to Ang II. In fact, the mRNA expression of the AT_1 and AT_2 receptor is increased in the carotid body of adult rats exposed to sustained hypoxia (Leung et al., 2000). Also, there is an increased immunoreactivity of AT_1 receptors in glomic clusters containing tyrosine hydroxylase in the carotid body of rats with sustained hypoxia (Fung et al., 2002). Interestingly, in postnatal exposure to sustained hypoxia, the mRNA expression of AT_{1a} receptors but not the AT_{1b} subtype in the rat carotid body is upregulated, suggesting that the expression of AT_1 receptor subtypes is differentially regulated by postnatal hypoxia (Fung et al., 2002). Thus, chronic hypoxemia is a major factor that increases the expression of AT_{1a} receptor at the transcriptional and protein level, resulting in a functional enchancement of the sensitivity of the carotid chemoreceptor to Ang II.

In addition to the upregulation of the AT_1 receptor, sustained hypoxia induces increased expressions of several key components of the RAS in the rat carotid body. The effect of sustained hypoxia on the RAS component of the carotid body are: (i) it increases the mRNA and protein level of angiotensinogen expressed in the chemosensitive glomus cell, and (ii) elevated the mRNA expression and enzymatic activities of ACE (Lam and Leung, 2003; Lam et al., 2004). The significance of these RAS alterations regulated by hypoxia is that it could increase the local biosynthesis of Ang II and angiotensin peptides, which increases the carotid chemoreceptor activity; via the chemoreflex, changes the respiratory and cardiovascular activities to match metabolic needs and also adjustments of the autonomic and renal activities to regulate the electrolyte and fluid homeostasis in hypoxia (Honig, 1989). In addition, the increased expression of the AT_1 receptor could raise the sensitivity of the carotid body to the electrolyte and fluid disturbance under hypoxic conditions. Indeed, the plasma concentration of Ang II rises in the first week of sustained hypoxia but it returns to a normoxic level by 2 weeks (Zakheim et al., 1976). The plasma renin activity has been reported to be unaltered or increased during hypoxia (Jain et al., 1990; Fletcher et al., 1999; Ip et al., 2002). Thus, the increased expression and activities of the RAS components could play a role in the augmented activity of the carotid chemoreceptor and the chemoreflex pathway by which increases and sustains the renal sympathetic activity under hypoxic conditions. This could lead to activation of the renin-angiotensin-aldosterone system, which is important to increase the sodium and water retention. This might be a part of the compensatory changes following the natriuretic and diuretic effects of carotid chemoreceptor stimulation in acute hypoxia (Honig, 1989). Consequently, the increased sensitivity of the carotid body to Ang II could play a role in the augmentation of cardiorespiratory and the renal sympathetic activities, which is an important part of the response to hypoxia. Moreover, Ang II levels are significantly elevated under pathological conditions for instances hypotension or hemorrhage. Indeed hypotension induces an increase in the discharge rate of carotid chemoreceptors which may be due to a decrease in the blood flow to the carotid body (Lahiri et al., 1980). The elevated carotid chemoreceptor activity could also be mediated by the AT_1 receptors and the upregulated RAS components in the carotid body (Leung et al., 2000; Fung et al., 2002).

Sustained hypoxia is closely relevant to the physiological acclimation to high altitude, and also to clinical conditions including chronic obstructive pulmonary disease and congenital heart defects (Forth and Montgomery, 2003; Prabhakar and Peng, 2004). The upregulation of RAS components in the carotid body and the augmented sensitivity of the chemoreceptors to Ang II could play multiple roles in the response of the carotid body to sustained hypoxia. Specifically, the carotid body develops hypertrophy and hyperplasia and also increases vasculature with angiogenesis (Fung and Tipoe, 2003). Ang II is known as a mitogenic factor effecting on vascular cells, although the effect on the glomic tissue is not clear at the moment. In addition, sustained hypoxia modulates the ventilatory response to hypoxia (Bisgard, 2000; Lahiri et al., 2000, 2002). The chemosensitivity of the carotid body to hypoxia is modulated by the counterbalance of effects of excitatory and inhibitory components on the carotid chemoreceptor (Bisgard, 2000; Prabhakar, 2001). Thus, the excitatory effect of Ang II on the glomus cell could augment the chemosensitivity of the carotid chemoreceptor, which may counteract the blunting effect of sustained hypoxia on the ventilatory response to hypoxia. Yet, the extent of the effect of Ang II and the detail of the molecular and cellular mechanisms underlying the functional modulation of the chemoreceptor excitability for the acclimatized changes in the carotid body during hypoxia require further studies.

The RAS plays important roles in the pathogenesis of disease conditions including hypertension, cardiac hypertrophy and heart failure. It has been shown that AT_1 receptor antagonist olmesartan significantly lowers the blood pressure and reduces proteinuria and glomerular damage mediated by oxidative stress in hypertensive diabetic animals (Izuhara et al., 2005). It has also been reported that ACE and Ang II play roles in the hypoxia-induced pulmonary hypertension and vascular remodeling (Morrell et al., 1995, 1999). The pathological development of hypoxic cor pulmonale is significantly decreased by olmesartan in rats exposed to sustained hypoxia (Nakamoto et al., 2005). However, in a double-blind study, losartan did not significantly attenuate pulmonary hypertension in a cohort of 40 patients with chronic obstructive pulmonary disease (Morrell et al., 2005). Also, irbesartan, an AT receptor blocker, did not significantly alter the strength of respiratory muscles or spirometric parameters in a randomized trial with about 60 patients with chronic obstructive pulmonary disease, although it significantly decreased the hematocrit. This raises the possibility that blockade of AT receptors may have beneficial effects in the patients with chronic obstructive pulmonary disease (Andreas et al., 2006). A more recent study reported that ACE inhibitor enalapril or AT receptor blocker losartan reduced the mortality of 2249 patients with severe COPD (Ekström et al., 2013). The effect of RAS blockade

in disease associated with hypoxia needs more clinical trial studies in future.

INTERMITTENT HYPOXIA REGULATES RAS EXPRESSION: IMPLICATIONS ON SLEEP-DISORDERED BREATHING

Chronic exposure to episodic hypoxia (intermittent hypoxia) associated with recurrent apneas closely related to pathophysiological conditions including sleep-disordered breathing, obstructive sleep apnea and hypertension (Lesske et al., 1997; Fletcher, 2001). Evidence suggests that the carotid body plays a crucial role in the pathophysiology of sleep apnea and it pathophysiological consequences induced by intermittent hypoxia (Prabhakar et al., 2001, 2005; Peng et al., 2003; Iturriaga et al., 2005). Thus, there is an augmented carotid chemoreceptor activity and its chemosensitivity in animals exposed to intermittent hypoxia (Peng and Prabhakar, 2004; Peng et al., 2004; Rey et al., 2004). Also, intermittent hypoxia induces increases in the blood pressure (Fletcher et al., 1992a,b), activities of sympathetic nerve (Greenberg et al., 1999; Fletcher, 2003), plasm levels of catecholamines (Bao et al., 1997), long-term facilitation (LTF) of the respiratory motor activity and the ventilatory response to hypoxia (McGuire et al., 2004; Rey et al., 2004; Katayama et al., 2005). Moreover, denervation of the carotid afferent activity dramatically attenuates the elevated blood pressure in responding to intermittent hypoxia, indicating that the carotid chemoreceptor activity plays an important role in the pathogenic cascades induced by intermittent hypoxia (Fletcher et al., 1992a). Furthermore, intermittent hypoxia resembles ischemia-reperfusion of tissues and organs, leading to excessive production of ROS, which could underpin the long-term effects of intermittent hypoxia on the carotid body. This is supported by the observation that ROS scavengers attenuate the hypoxic sensitivity and the magnitude of LTF induced by intermittent hypoxia (Prabhakar and Kumar, 2004). Thus, ROS play a crucial role in the altered carotid body function in intermittent hypoxia.

As aforementioned, Ang II stimulates ventilation and the plasma Ang II level increases under hypoxic conditions (Zakheim et al., 1976; Ohtake et al., 1993). Activation of AT_1 receptors in the carotid body increases the afferent activity and the hypoxic sensory response of the chemoreflex and sympathetic output (Leung et al., 2000). In experimental animals, losartan significantly reduces the elevated arterial pressure induced by intermittent hypoxia, suggesting that the RAS is involved in the pathogenic cascade (Fletcher et al., 1999). Indeed, there are significantly increased levels of serum Ang II and VEGF in patients with obstructive sleep apnea (Barcelo et al., 2001; Moller et al., 2003). Blocker of AT_1 receptors olmesartan significantly decreases the VEGF expression induced by Ang II in the peripheral blood mononuclear cell (Takahashi et al., 2005). Thus, activation of the AT_1 receptor plays a role in the pathogenic event of obstructive sleep apnea. Recent studies have examined the hypothesis that the RAS in the carotid body plays a role in the augmented carotid chemoreceptor activity induced by intermittent hypoxia, which may be mechanistically leading to breathing instability in the pathophysiology of sleep apnea.

Recent studies reported that intermittent hypoxia induces a functional upregulation of the RAS expression in the rat carotid body (Lam et al., 2014). The increased mRNA and protein expression of AT_1 receptors causes an enhancement of the sensitivity to Ang II in the carotid chemosensitive cells, which could lead to an increase in the CB excitability and renal sympathetic activity (Marcus et al., 2010; Lam et al., 2014). In effect, the activation of RAS in the carotid body during intermittent hypoxia has pathophysiological and clinical significance because the chemoreflex plays an important role in the sustained increases in the sympathetic outflow and the systemic arterial pressure (Fletcher, 2001). Indeed, it has been shown that the systemic hypertension induced by intermittent hypoxia is blocked by the denervation of the carotid body, ablation of the sympathetic nerve, renal sympathectomy, adrenal medullectomy, and also by AT receptor antagonist (Fletcher et al., 1999; Fletcher, 2001). These data support the hypothesis that the RAS expression in the carotid body plays a role in the pathogenic cascade induced by intermittent hypoxia, which increases the cardiovascular morbidity in OSA patients.

Ang II stimulates the $[Ca^{2+}]_i$ elevation in the chemosensitive glomus cells and the Ang II response is enhanced in the hypoxic group via the upregulation of AT_1 receptor expression (Lam et al., 2014). The $[Ca^{2+}]_i$ response to AT was inhibited by AT_1 antagonist losartan but not by an AT_2 antagonist, confirming that AT binding to the AT_1 receptor stimulates the intracellular signaling pathway and elevates $[Ca^{2+}]_i$ in the glomus cells. In effect, Ang II evokes sensory long-term potentiation of the carotid body, which was blocked by losartan (Peng et al., 2011). Also, losartan reduced the elevated sympathetic responses to hypoxia and cyanide (Marcus et al., 2010). These results suggest that the upregulation of the AT_1 receptor expression plays a role in the augmented CB excitability during intermittent hypoxia, highlighting the mechanistic significance of the RAS in mediating the IH-induced pathophysiological development of sympathetic overactivity and hypertension.

In addition to AT_1 receptors, the gene transcripts of AT_2 receptor have shown to be increased in the carotid body in intermittent hypoxia. The increased expression of the AT_2 receptors might be related to the lack of increase in the volume of the carotid body in the rat (Lam et al., 2008). Indeed, AT_2 receptors have been implicated in the stimulation of apoptosis and the activation of AT_2 receptor results in growth inhibition and promotion of apoptosis associated with the inhibition of MAP kinases, such as extracellular regulated kinases, probably by the activation of phospho-tyrosine phosphatase (Schmitz and Berk, 1997; de Gasparo and Siragy, 1999). The activated local RAS via AT_2 receptors in the carotid body might inhibit the cell growth and promote apoptotic cell death, resulting in an insignificant change in the volume of the carotid body in intermittent hypoxia. However, further investigations are needed for addressing the role of AT_2 receptors in the carotid body.

Moreover, the expression of angiotensinogen in the carotid body is significantly elevated by intermittent hypoxia (Lam et al., 2014). The mRNA and protein expression of angiotensinogen was specifically localized to the glomus cells of the carotid body, suggesting a transcriptional upregulation and/or mRNA stabilization of the angiotensinogen expression induced by intermittent hypoxia. Also, the mRNA level of ACE was significantly increased in the rat carotid body (Lam et al., 2014). Thus, evidence supports

that intermittent hypoxia induces an upregulation of the local RAS components in the rat carotid body. Since angiotensinogen is the sole precursor of Ang II, elevated levels of the expression of angiotensinogen could lead to an increase in the local production of Ang II in the carotid body in intermittent hypoxia. Moreover, the increased expression of ACE in the carotid body could enhance the kinetics of enzymatic convertion of Ang I to Ang II in the carotid body. Also, increased ACE activities and plasma Ang II levels have been reported in OSA patients (Barcelo et al., 2001; Moller et al., 2003). In effect, the elevated level of circulating and locally produced Ang II, together with an increased expression of the AT receptors could enhance the effect of Ang II on the chemosensory component of the carotid body. Thus, evidence supports the upregulated AT receptors with increased expression of RAS components in the carotid body play a pathogenic role in the augmented excitability of the carotid chemoreceptor under intermittent hypoxia associated with sleep apnea.

Oxidative stress with an increased generation of ROS plays an essential role in IH-induced alterations in the carotid body function (Prabhakar et al., 2001). It has been shown that ROS modulate the mobilization of $[Ca^{2+}]_i$ store mediated by IP_3signaling pathway, leading to an increase in hypoxia-induced neurotransmitter release in IH (Prabhakar and Kumar, 2004). Studies have also shown that oxidative stress is involved in the augmented carotid chemoreceptor activity (Pawar et al., 2009; Peng et al., 2009; Del Rio et al., 2010, 2011b). Emerging data suggest that Ang II is a mediator of oxidative stress, in which ROS induced by Ang II are an important signaling intermediates in several signal transduction pathways involved in the pathophysiology (Paravicini and Touyz, 2006) and inflammation (Duprez, 2006). In this regard, vascular inflammation induced by Ang II is mainly mediated by AT_1 receptors associated with an increased production of ROS via the NADPH oxidase in the vascular wall (Griendling et al., 1994; Rajagopalan et al., 1996; Dandona et al., 2003), which is closely related to the local RAS function (Shimizu et al., 1998). In the carotid body, AT receptors are also expressed in the vascular cells in addition to the glomus cells, although its role is unclear. Nevertheless, it has been shown that losartan treatment could normalize IH-induced superoxide production and expression of AT_1 receptors (Marcus et al., 2010). Also, ROS production induced by Ang II mediates sensory long-term potentiation in the carotid body via activation of $gp91^{phox}$ (Peng et al., 2011), and the $gp91^{phox}$ is expressed in glomus cells of the CB (Youngson et al., 1997). Thus, the IH–induced RAS upregulation in the carotid body may contribute to the AT-induced ROS production. Furthermore, losartan treatment of the rat in intermittent hypoxia attenuates the levels of oxidative stress and macrophage infiltration, supporting a pathogenic role of AT_1 receptors in the local inflammation of the carotid body (Lam et al., 2014). In this context, intermittent hypoxia induces increased expressions of proinflammatory cytokines and mediators as well as infiltration of immunogic cells to the carotid body under hypoxic condtions (Lam et al., 2008, 2012; Liu et al., 2009; Del Rio et al., 2011a, 2012). The inflammatory response of the carotid body to intermittent hypoxia has also been proposed to play a role in the augmented activity of the carotid chemoreceptor (Lam et al., 2008, 2012; Liu et al., 2009). These findings strongly suggest a paracrine-autocrine mechanism mediating altered functions of the carotid body, including the augmented chemosensitivity and the local inflammation of the carotid body. Thus, intermittent hypoxia could activate an intrinsic angiotensin-generating system, which increases local biosynthesis of Ang II via increased expressions of RAS components in the carotid body. The upregulation of angiotensinogen, AT receptors and ACE expression could play a pathogenic role in the augmented activity of carotid chemosensitive cells and the inflammation of the carotid body in intermittent hypoxia, which is relevant to the early pathogenesis in sleep-disordered breathing.

The RAS has been proposed to play a role in the cardiovascular consequences of sleep apnea, including systemic hypertension. In addition to the continuous positive airway pressure therapy, targeting the RAS has been proposed as a pharmacological management of the patients with sleep apnea. In fact, inhibitors of ACE have been shown to attenuate the arterial pressures and decrease the apnea and hypoapnea index in patients with obstructive sleep apnea (Peter et al., 1989; Mayer et al., 1990; Grote et al., 1995). Also, the AT receptor blockers have been shown to attenuate the elevated pressure and oxidative stress induced by intermittent hypoxia in subjects (Foster et al., 2010; Pialoux et al., 2011) or in the patient with obstructive sleep apnea (Heitmann et al., 2010; Dohi et al., 2011). The beneficial effect of the RAS blockade might be explained by diminished sympathetic and adrenergic activities (Fletcher, 2000, 2003). Nevertheless, these human studies were performed in a small number of patients and the long term treatment with RAS blockers has not been evaluated in clinical trials (Parati et al., 2013). Future clinical studies are required to support the antihypertensive benefit of blockade of RAS for the management of patients with obstructive sleep apnea and hypertension.

AN INVOLVEMENT OF THE RAS IN HEART FAILURE

Experimental studies have shown that Ang II enhances the hypoxic chemosensitivity of the carotid body in a rabbit model of chronic heart failure (CHF). Hence, Ang II augments hypoxia-induced renal sympathetic nerve activity (RSNA) and there are significant increases in the expression of AT_1 receptors in the carotid body of CHF rabbits (Li et al., 2006). Also, L-158809, an AT_1 receptor antagonist, attenuates hypoxia-induced responses of the RSNA in CHF rabbits. In addition, L-158809 decreases the chemoreceptor responses to hypoxia in CHF rabbits, suggesting that increases in Ang II and the expression of AT_1 receptor in the carotid body play a role in the augmented carotid chemoreceptor activity and chemoreflex-mediated sympathetic overactivity in CHF (Li et al., 2006). Furthermore, Ang II at a concentration of 0.1 nM increases the sensitivity of potassium (Kv) currents and resting membrane potential to hypoxia and L-158809 reduces the sensitivity of Kv currents and resting membrane potential to hypoxia in CHF glomus cells. These results suggest that Ang II-AT_1 receptor signaling pathway increases the sensitivity of Kv channels to hypoxia in the glomus cells of the CHF rabbit (Li and Schultz, 2006). Moreover, the effect of Ang II on the augmented chemoreceptor activity is medated by a NADPH oxidase-superoxide signaling pathway (Li et al., 2007).

Sleep-disordered breathing with central or obstructive sleep apnea is frequently observed in patients with heart failure. Sleep-disordered breathing has been known to have a negative impact on the CHF patient and so clinical treatment of sleep-disordered breathing could improve cardiac performance and long-term outcomes in these patients. Also, cardiac dysfunction may play a role in the pathophysiology of sleep apnea, although the interrelationship between heart failure and sleep apnea remains to be established (Caples et al., 2005). In this context, an increase in sympathetic activities is a hallmark of the CHF state, which could be mediated by a decrease in the sensory feedback from cardiopulmonary activities and arterial baroreceptors. As mentioned above, the sustained increase in RSNA could involve Ang II and the RAS in the carotid body of CHF rabbits (Schultz, 2011; Patel and Schultz, 2013). Indeed, recent studies show that cryoablation of the carotid body normalizes the RSNA and breathing stability and improves survival in CHF animals (Del Rio et al., 2013; Marcus et al., 2014). Hence, the RAS components could be therapeutic targets for the treatment of CHF patients.

CONCLUSIONS

Findings of expression and functional studies suggest that the AT_1 receptor regulates the excitability of the carotid chemoreceptor. Hence, Ang II elevates the level of intracellular calcium in the chemosensitive glomus cells and the activity of carotid chemoreceptors. As a result, activation of the chemoreflex could be a peripheral control important for the physiological response to hypoxia and the maintenance of electrolyte and fluid homeostasis. In addition, the expression of AT receptors in the carotid body is regulated by hypoxia. In effect, sustained hypoxia induces an upregulation of AT_1 receptor expression, which increases the sensitivity of the chemoreceptor response to Ang II. This regulation may be important in the modulation of the carotid body functions responsible for the hypoxic ventilatory response, for enhancing the cardiorespiratory response and adjusting electrolyte and water homeostasis during sustained hypoxia. Furthermore, RAS components are locally expressed in the carotid body and the increased RAS expressions are closely relevant to the pathogenesis of disease including sleep-disordered breathing and heart failure. Specifically, the upregulation of the expression of angiotensinogen, ACE and AT_1 receptors could play a significant role in the augmented carotid chemoreceptor activity, via the increased activity of chemoreflex, contributing to the pathophysiology of sleep apnea and the sympatho-excitation that is central to the endothelial dysfunction and heart failure during the course of pathogenesis. Future studies in this direction warrant a better understanding of the pathogenic role of RAS in the carotid body in the disease associated with hypoxemia.

ACKNOWLEDGMENTS

The authors wish to thank the support from the Research Grants Council of Hong Kong, Competitive Earmarked Research Grants: HKU 766110M, HKU 7510/06M and research grants from the University Research Committee of the University of Hong Kong.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 27 June 2014; accepted: 15 August 2014; published online: 05 September 2014.

*Citation: Fung ML (2014) The role of local renin-angiotensin system in arterial chemoreceptors in sleep-breathing disorders. Front. Physiol. **5**:336. doi: 10.3389/fphys.2014.00336*

This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology.

frontiers in
PHYSIOLOGY

MINI REVIEW ARTICLE
published: 20 August 2014
doi: 10.3389/fphys.2014.00304

Carotid body: a new target for rescuing neural control of cardiorespiratory balance in disease

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Significant insight into the mechanisms involved in chronic heart failure (CHF) have been provided by Schultz and his associates at the University of Nebraska Medical Center with the use of pacing-induced heart failure rabbits. Critical among the CHF mechanisms was the role of the carotid body (CB). The stimulated CB produces a wide array of systemic reflex responses; certainly those in the cardiopulmonary (CP) system are the most important in CHF. This generates a question as to whether the CB could serve as a target for some kind of treatment to reestablish control of cardiorespiratory balance in CHF. Any treatment would have to be based on a solid understanding of the mechanisms of chemosensing by the CB as well as the transducing of that sensing into neural activity sent to the medullary centers and regions of autonomic outflow to the periphery. Two avenues of treatment could be to (1) silence or attenuate the CB's neural output pharmacologically and (2) excise the CBS. There is a long history of CB removal mostly as a remedy for chronic obstructive lung disease. Results have been inconclusive as to the effectiveness of this procedure. But if carefully planned, the procedure might be a helpful treatment.

Keywords: chronic heart failure, cardiopulmonary, carotid body, control, removal, glomectomy

BASIC BACKGROUND

The stimulated carotid body (CB) provokes a wide array of cardiopulmonary (CP) reflex responses, as well as having an impact on the endocrine and renal systems (**Figure 1**).

This bilateral rate-sensitive interoreceptor, arguably the most essential for maintaining normal homeostasis in the organism, is located at the bifurcation of the common carotid artery into the internal carotid artery (going to the Circle of Willis in the brain) and the external carotid artery which perfuses the face and scalp. The CBs are perfused at a very high rate by a branch of the external carotid artery. Neural output from the CB is generated by excitatory neurotransmitters, released from the CB's thousands of glomus cells. They attach to receptors on abutting neurons (branches of the glossopharyngeal nerve) the cell bodies of which lie in the petrosal ganglion from which the traffic proceeds to the Nucleus Tractus Solitarii (NTS) in the brainstems's medulla. The neural traffic is increased in response to decreased partial pressure of oxygen in arterial blood(P_aO_2), low glucose, elevated levels of CO_2 (P_aCO_2), elevated H^+ levels $[H^+]_a$. Neural output also increases in response to increases in temperature and osmolarity.

ACTIVITY IN HEART DISEASE

Well-documented in animal models is the fact that chronic heart failure (CHF) renders the CB more sensitive (Sun et al., 1999). This increased sensitivity produces an increase in the CB's neural output even under normal acid-base conditions; part of this increase proceeds through NTS to the paramedian reticular nuclei, one seat of sympathetic neural outflow to the heart and vessels, as well as to the location of modulating serotinergic action. Increased sympathetic outflow to the ventricles is undesirable in CHF since it can provoke ventricular arrhythmias (Paterson, 2005).

ELEMENTS OF CHEMOSENSING AND CHEMOTRANSDUCTION

Targeting the CBs as loci of treatment to see if homeostatic balance can be reestablished during disease requires a relatively deep understanding of the mechanisms of chemosensation and chemotransduction. In other words one must know how the CBs sense the stimuli which depolarize the glomus cells, and what are the mechanisms for converting this sensing into neural traffic.

The CB's chemosensitive structure initiating depolarization of the glomus cell is still under study though significant progress has been made.

By way of a brief overview, heme-oxygenase 2 has been proposed as the precise molecule that acts to close the calcium-sensitive K channel (aka the BK or maxi-K channel). But NADPH-oxidase and AMP-activated protein kinase have also received support for the depolarizing role. What must be kept in mind is that this important initiating molecule may not be the same in all species. For though heme-oxygenase 2 seems to function in the role in rats, one study reports that in knock-out mice the absence of heme-oxygenase 2 does not prevent the hypoxia-induced release of catecholamines. That NADPH-oxidase is involved has been supported by manipulations of some of its genetic components; e.g., deletion of $p47^{phox}$ enhanced the CB's normal responses to hypoxia.

But since the sensing of hypoxia by the CB and by the pulmonary arteries has seemed somewhat similar in the product

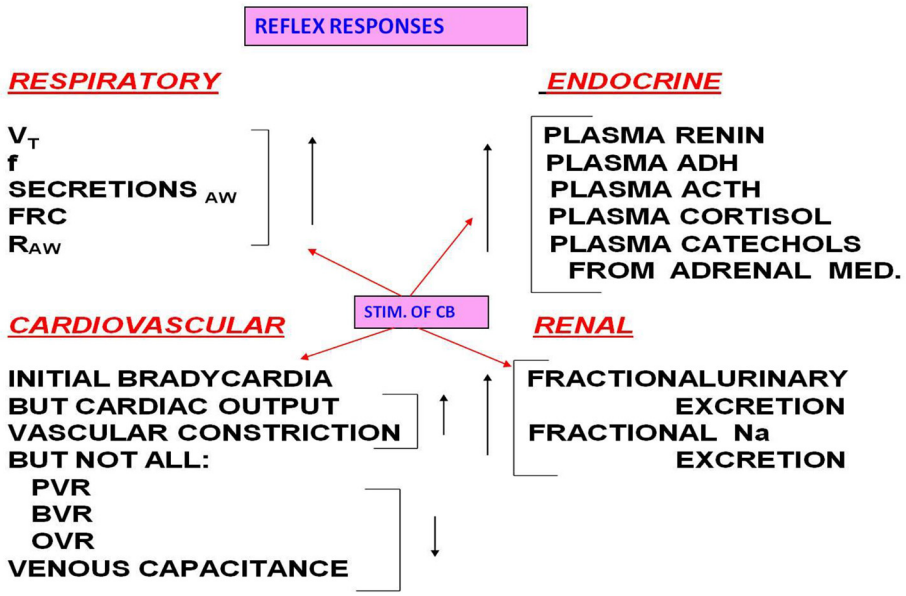

FIGURE 1 | CB stimulation increases the listed variables in the respiratory system. Stimulation increases some CV variables but decreases pulmonary vascular resistance (PVR), bronchial vascular resistance (BVR), ocular vascular resistance (OVR), and venous capacitance. Note also the impact of CB stimulation on the endocrine and renal systems.

(neural excitation and vasoconstriction), it is interesting to note that the precise O_2 sensor in the CB remains unclear (Gonzalez et al., 1994; Lopez-Barneo, 2003). Whereas in pulmonary arterial smooth muscle cells "the bulk of evidence suggests that the primary sensor for hypoxic pulmonary vasoconstriction is the mitochondrion in the smooth muscle cells, which increases production of ROS during hypoxia… It is possible that secondary sensor mechanisms, such as ROS production by sarcolemmal NADPH oxidase, also contribute" (Sylvester et al., 2012).

K channels which are oxygen-sensitive have always been thought to play the initiating role in the depolarization of the transmitter-containing glomus cells. Additional to the K_{BK} channel, reports include other K channels as being oxygen-sensitive: TASK-like K, Kv4.1, Kv4.3 channels. These channels have always been thought to play a necessary role. The chronological steps in chemotransduction in the CBs are fairly well-known and agreed upon. Indeed, many of the sub-cellular and molecular mechanisms of sensing and transducing chemical signals have been worked out quite well, though more work needs to be done.

TREATMENT OF THE TARGET TO REDUCE ITS IMPACT

But on the basis of our present corpus of knowledge what can be suggested by way of treating the CB as a target for rescuing neural control of cardiorespiratory balance in disease? Two techniques suggest themselves: (a) silencing the CBs; (b) CB removal/extirpation/resection.

(a) Silencing (1) Dopamine is well-known to blunt the CB's neural output in response to hypoxia. (2) NO is well-known to reduce the CB's output in response to hypoxia. And a set of recent studies has shown that nNOS is reduced in CHF rabbits, a situation which is reversed by the vectoring into the CBs nNOS (Li et al., 2005). (3) A second agent reported to reduce the release of ACh and ATP, two excitatory neurotransmitters in the CBs of the cat, is Na_2S, a precursor of H_2S (Fitzgerald et al., 2011); this agent seems to open ATP-sensitive K channels in the cell membrane of the glomus cells. With the outflow of K^+ ions the glomus cells become hyperpolarized, inhibiting the entry of Ca^{++} and subsequent release of neurotransmitters from the vesicles in the glomus cells. Cat CBs also tested positive for an H_2S synthesizing enzyme, cystathionine-β-synthase. In this age of nanotechnology loading microspheres with a pharmacological agent or an enzyme and fixing a marker of some sort on the surface of the sphere which would recognize the CB does not seem overly ambitious. (4) Finally, CHF rabbits showed a reduction in CB blood flow. This condition would *per se* increase CB neural output due to the high metabolic rate of the CB lowering PO_2 and elevating PCO_2 in the CB. A program of regular modest exercise has been shown to be an effective way to increase CB blood flow (Li et al., 2008; Ding et al., 2011). This has been tried clinically in some hospitals, and found to be effective in attenuating symptoms of cardiac malfunction.

(b) Removal of the CBs could be another option. The literature addressing this option is extensive, but, regrettably, not at all conclusive. It describes results in several species. And there are different results. But human diseases for which the procedure was performed were cerebral ischemia (constricted common carotid arteries). This was treated with endarterectomy which involved CB removal. Most other reports treat CB removal as a treatment for asthma, COPD; none address CHF. Usually CB removal involves the removal of the carotid

sinus sensors of blood pressure as well. If CBs are removed, do patients survive? This is, of course, the critical question. Let us review briefly a few of the more extensive studies. Nakayama (1961) used glomectomy to treat childhood asthma. Some of these patients were tested 30 years later and still exhibited no response to hypoxia. Bilateral endarterectomy in seven patients denervated carotid bodies (Wade et al., 1970) creating a permanent hypoventilation and a modest hypercapnia. In 57 cases of COPD unilateral glomectomy Phillips and Kintner (1970) concluded the procedure did not significantly alter the course of bronchospastic disease, based on a battery of pulmonary function tests 4 years post-glomectomy. On the other hand Stullberg and Winn (1989) reported an improvement in dyspnea in three men who had undergone bilateral glomectomy to offset severe COPD. All three, ages 57, 67, 69, died 6, 18, 36 months post-surgery, but remained convinced of the efficacy of the surgery even though there was no improvement in their severe airflow limitations. Whipp and Ward (1992) made a very careful quantitative study of a very large group of COPD patients the day before and the day after the surgery. No deaths were reported after a very selective removal of only the CB. Great intersubject variability was noted; but the changes in pulmonary function and blood gases were not large. Perhaps the most widely experienced investigator of bilateral carotid chemoreceptor extirpation is Yoshiyuki Honda and his colleagues. They found that exercise hyperpnea decreased in patients after the procedure (Honda et al., 1979a). In another study they found the procedure enhanced hypoxic tachycardia (Honda et al., 1988) in eight subjects 25 years post-surgery. In 11 asthmatic patients with the bilateral CB resection they reported some residual chemosensitivity some 23 years post-surgery (Honda et al., 1979b). Honda reviews these and studies in other animals (1992).

Based on the above overview the answer to the critical question is "Yes, most patients do live after CB resection." Hence, it would seem that the procedure might be pursued. But perhaps the 1989 advice of Severinghaus in addressing bronchospastic patients might be followed (Severinghaus, 1989): They might be helped "…by permanent administration of oxygen via transcutaneous tracheal catheter. A variety of pharmacologic agents can minimize bronchospasm and infection and help clear secretions. Home oxygen concentrators and portable liquid oxygen supplies have become easily available to most patients. Only when all available methods fail to adequately relieve patients should surgical intervention be considered." He also encourages the study of the procedure in a small group of carefully selected incapacitated patients by the NIH.

So survival after glomectomy seems to be the most frequent result, but the advantages of the procedure for better pulmonary functioning still seems to be controversial. Nevertheless, returning our focus to the advantage of glomectomy for cardiac problems, we see several more recent studies illustrating the central role of the CB's sensitivity in spontaneously hypertensive rats; the CB's discharge responses to hypoxia and hypercapnia are significantly greater than in normotensive rats (Fukuda et al., 1987). Carotid body denervation (CBD) saw no rise in young SHR animals, or a drop in blood pressure in adults (Abdala et al., 2012). Another rat study (Fletcher et al., 1992) reported how CBD eliminated the rise in blood pressure generated by chronic episodic hypoxia, such as is found in sleep apnea. Ribeiro et al. (2013) demonstrate how CBD prevents the development of insulin resistance and hypertension induced by hypercaloric diets. The most comprehensive animal study with which we are familiar is that of Marcus and his colleagues in CHF rabbits (Marcus et al., 2014). In brief, their study showed how CBD reduced sympathetic nerve activity, disordered breathing patterns, arrhythmia incidence, and sympatho-respiratory coupling in CHF rabbits. This should be considered the "gold standard" among animal studies of the effect of CBD as a focal point for rescuing neural control of cardiorespiratory balance in disease. The relevance of these studies for humans can be seen in an earlier study of patients with CHF, some of whom had normal chemosensitivity and others suffered from chemoreceptor hypersensitivity. The former group of 53 had a 3-year survival rate of 77%; the latter group of 27 had a rate of 41% (Ponikowski et al., 2001).

ACKNOWLEDGMENT

The author gratefully acknowledges the support of the NHLBI award RO1—HL-50712-13.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 12 June 2014; paper pending published: 07 July 2014; accepted: 25 July 2014; published online: 20 August 2014.

*Citation: Fitzgerald RS (2014) Carotid body: a new target for rescuing neural control of cardiorespiratory balance in disease. Front. Physiol. **5**:304. doi: 10.3389/fphys.2014.00304*

This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology.

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