Respiratory Physiology & Neurobiology 265 (2019) 100–111

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

journal homepage: www.elsevier.com/locate/resphysiol

Acute oxygen sensing—Role of metabolic specifications in peripheral chemoreceptor cells

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ARTICLE INFO

Keywords:
Carotid body
Chemoreceptor cells
Oxygen sensing
Hypoxia
Mitochondria
Metabolic properties
Krebs' cycle anaplerosis

ABSTRACT

Acute oxygen sensing is essential for humans under hypoxic environments or pathologic conditions. This is achieved by the carotid body (CB), the key arterial chemoreceptor, along with other peripheral chemoreceptor organs, such as the adrenal medulla (AM). Although it is widely accepted that inhibition of K^+ channels in the plasma membrane of CB cells during acute hypoxia results in the activation of cardiorespiratory reflexes, the molecular mechanisms by which the hypoxic signal is detected to modulate ion channel activity are not fully understood. Using conditional knockout mice lacking mitochondrial complex I (MCI) subunit NDUFS2, we have found that MCI generates reactive oxygen species and pyridine nucleotides, which signal K^+ channels during acute hypoxia. Comparing the transcriptomes from CB and AM, which are O_2-sensitive, with superior cervical ganglion, which is practically O_2-insensitive, we have found that CB and AM contain unique metabolic gene expression profiles. The "signature metabolic profile" and their biophysical characteristics could be essential for acute O_2 sensing by chemoreceptor cells.

1. Introduction

Sufficient supply of oxygen (O_2) to tissues is essential for survival of mammals due to its fundamental role in oxidative metabolism. Under the condition of low O_2 tension (hypoxia), acute cardiorespiratory reflexes (hyperventilation and sympathetic activation) take place within seconds to minutes to increase O_2 uptake and its distribution to tissues (Lopez-Barneo et al., 2001). During sustained (chronic) hypoxia, the O_2-sensitive prolyl hydroxylase (PHD)-hypoxia inducible transcription factor (HIF) signaling pathway is also activated to up-regulate expression of genes encoding glycolytic enzymes, erythropoietin and angiogenic factors. This transcriptional response leads to decreased O_2 consumption and increased O_2 supply to organs (see Kaelin and Ratcliffe, 2008).

The carotid body (CB), located at the bifurcation of carotid artery, is the major arterial chemoreceptor capable of detecting acute decreases in blood O_2 tension (hypoxemia) to trigger cardiorespiratory reflexes. Together with other peripheral chemoreceptor organs, the CB forms a homeostatic acutely responding O_2-sensing system (Weir et al., 2005; Nurse et al., 2006). The CB-mediated response to hypoxia (i.e. sustained activation of the cardiorespiratory reflexes) is necessary for adaptation to environmental hypoxic situations, such as it occurs in people living at or traveling to high altitudes or to pathological conditions presenting with hypoxemia due to decreased O_2 exchange capacity in the lungs (see Teppema and Dahan, 2010; Lopez-Barneo et al., 2016a).

The CB is composed of clusters of cells forming the functional unit known as a glomerulus (Fig. 1A, B). Each glomerulus contains several (~4-8) neuron-like type I cells, also called glomus or chief cells, which are the O_2-sensitive elements. Immature glomus cells, which are weakly O_2 sensitive, are also present in the glomerulus (Sobrino et al., 2018). Glomus cells are in close contact with abundant capillaries and afferent sensory nerve fibers and are surrounded by glia-like sustentacular (type II) cells. Type II cells, or a subpopulation of them, are adult multipotent stem cells, which, together with immature glomus cells, sustain CB growth in chronic hypoxia (Macias et al., 2014; Platero-Luengo et al., 2014; Sobrino et al., 2018). Both glomus and type II cells originate from neural-crest progenitors of sympathoadrenal lineage in the superior cervical ganglion (SCG), which migrate to the carotid bifurcation during embryogenesis (Pardal et al., 2007; Hempleman and Warburton, 2013; Kameda, 2014). Glomus cells are presynaptic-like chemoreceptors, which are not only sensitive to hypoxia, but also detect increases in CO_2 tension (hypercapnia) and decreases in extracellular pH

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https://doi.org/10.1016/j.resp.2018.08.007
Received 5 July 2018; Received in revised form 17 August 2018; Accepted 29 August 2018
Available online 30 August 2018
1569-9048/

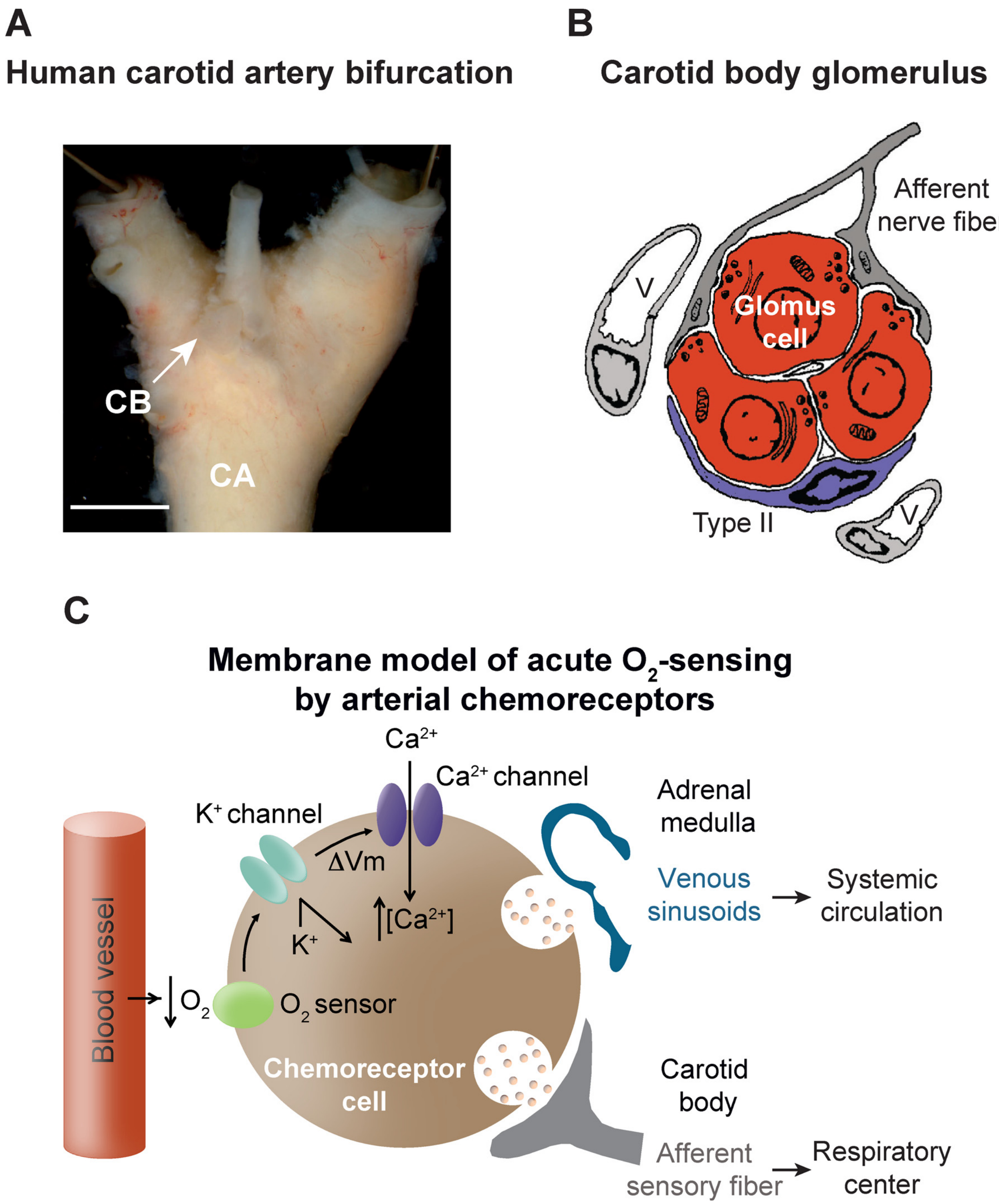

Fig. 1. Carotid body structure and membrane model of acute O_2-sensing by arterial chemoreceptors. **A**. Human carotid artery (CA) bifurcation in which the carotid body (CB) is indicated with an arrow. Scale bar: 1 cm. **B**. Schematic representation of a carotid body glomerulus, in which glomus (type I) and sustentacular (type II) cells are in contact with capillaries and afferent sensory fibers. V, vessel. **C**. Membrane model of hypoxia-induced chemotransduction by arterial chemoreceptor cells (See text for detailed explanation) (Modified from Ortega-Saenz et al., 2013; Pardal et al., 2007; Lopez-Barneo et al., 2016a).

in the blood. In addition, glomus cells participate in glucose homeostasis, as they are activated by lowering blood glucose (hypoglycemia) independently of O_2 sensing (Ortega-Saenz et al., 2013; Gao et al., 2014; Macias et al., 2018).

The adrenal medulla (AM) is another peripheral chemoreceptor and a component of the homeostatic acute O_2 sensing system, which has close developmental and functional links with the CB as it also originates from neural crest-derived sympathoadrenal progenitors. Chromaffin cells from neonatal AM have intrinsic, non-neurogenic, O_2 sensitivity and are also indirectly stimulated during hypoxia due to the generalized sympathetic response induced by CB activation (Adams et al., 1996; Mojet et al., 1997; Thompson et al., 1997). Although intrinsic O_2 sensitivity of AM cells decreases with age, adult chromaffin cells still respond to hypoxia (Mochizuki-Oda et al., 1997; Takeuchi et al., 2001; Garcia-Fernandez et al., 2007a; Levitsky and Lopez-Barneo, 2009; Fernandez-Aguera et al., 2015). Together, the CB and AM form a CB-AM axis, whose proper function is essential to maintain cardiorespiratory homeostasis. Indeed, over-activation of the CB-AM axis has been implicated in exaggerated sympathetic activation underlying neurogenic hypertension and other metabolic diseases (McBryde et al., 2013; Ribeiro et al., 2013; Marcus et al., 2014; Del Rio et al., 2016). On the contrary, respiratory depression due to general anesthetics or use of opioids, has been associated with poor chemoreceptive activity in glomus and chromaffin cells (see Lopez-Barneo et al., 2016a).

The mechanism of hypoxia-induced excitation has been studied mainly in CB glomus cells and a "membrane model" of chemotransduction, based on inhibition of membrane K^+ conductance during hypoxia, is now widely accepted (Fig. 1C; Lopez-Barneo et al., 1999; Kemp and Peers, 2007). Glomus cells contain a variety of O_2-sensitive ion channels, especially K^+ channels on the plasma membrane (see Lopez-Barneo et al., 2001). During hypoxia, inhibition of K^+ currents results in membrane depolarization, extracellular Ca^{2+} influx and increase in cytosolic Ca^{2+}, which triggers neurotransmitter release, such as ATP, acetylcholine, dopamine, and several neuropeptides (Buckler and Vaughan-Jones, 1994; Urena et al., 1994). ATP and acetylcholine are the main transmitters that activate the sensory afferent fibers of the sinus nerve (Zhang et al., 2000; Fitzgerald et al., 2009) to stimulate the respiratory center of the brainstem, leading to hyperventilation. This same signal also causes sympathetic activation through the nucleus of the *tractus solitarius* and the rostral ventrolateral medulla in the brainstem to increase cardiac output (Guyenet, 2000; Semenza and Prabhakar, 2018). The mechanisms of O_2 sensing by AM chromaffin cells are similar to those in glomus cells. O_2-sensitive K^+ channels in chromaffin cells can be inhibited during hypoxia to trigger catecholamine release (Mochizuki-Oda et al., 1997; Thompson et al., 1997; Rychkov et al., 1998; Lee et al., 2000; Keating et al., 2001).

Although the "membrane model" explains the basic process of acute O_2 sensing by peripheral chemoreceptors, the mechanisms underlying detection of changes in O_2 tension and the way they are transduced into alterations in K^+ channel open probability have remained elusive (see Peers, 2015; Lopez-Barneo et al., 2016a, 2016b). Several classes of O_2-sensitive K^+ channels exist in glomus cells, including background K^+ channels (Buckler, 1997; Kim et al., 2009; Ortega-Saenz et al., 2010), maxi-K^+ channels (Wyatt and Peers, 1995; Riesco-Fagundo et al., 2001), and voltage-dependent K^+ channels (Lopez-Barneo et al., 1988; Ganfornina and Lopez-Barneo, 1992; Chou and Shirahata, 1996; Lopez-Lopez and Perez-Garcia, 2007). Although several hypotheses have been proposed to explain the molecular mechanisms underlying the process in which hypoxemia is detected to signal K^+ channels, most of them lack solid experimental support (Lopez-Barneo et al., 2016a, 2016b; Gao et al., 2017a; Rakoczy and Wyatt, 2018).

Here, we summarize recent work from our laboratory related to the role of glomus cell mitochondrial metabolism in acute O_2 sensing. Studies on conditional knockout mice indicate that signaling molecules originated in mitochondrial complex I (MCI) modulate K^+ channels during hypoxia (Fernandez-Aguera et al., 2015; Arias-Mayenco et al., 2018). We have also performed a comparative study analyzing the transcriptomes from O_2-sensitive CB and AM cells (CB > AM) and O_2-insensitive SCG cells. Based on these data, we have reported the existence of a "signature metabolic profile" in acutely responding O_2-sensing cells (Gao et al., 2017b). These studies suggest that chemoreceptor cells hold specific metabolic properties, which in combination with their biophysical characteristics are essential for acute O_2 sensing.

2. Mitochondrial complex I signaling in acute O_2 sensing by peripheral chemoreceptors

2.1. Mitochondrial complex I function is essential for acute O_2 sensing

A potential role of mitochondria in acute O_2 sensing has long been considered due to the high O_2 consumption of CB glomus cells (Daly et al., 1954) and the close relationship between mitochondrial function and PO_2 (Mills and Jobsis, 1970; Duchen and Biscoe, 1992; Buckler and Turner, 2013). Functional mitochondria are also required for O_2 sensing in chromaffin cells (Buttigieg et al., 2008). Inhibitors of mitochondrial electron transport chain (ETC) mimic the hypoxic response by increasing cytosolic Ca^{2+} and catecholamine secretion from glomus cells in an extracellular Ca^{2+}-dependent manner (Ortega-Saenz et al., 2003; Wyatt and Buckler, 2004). More recently, special attention has been drawn to MCI (NADH dehydrogenase), in which electrons are transferred from NADH to ubiquinone (Q) to form ubiquinol (reduced ubiquinone, QH2). Inhibitors of MCI (rotenone and 1-methyl-4-phenylpyridinium [MPP^+]) bind to the highly conserved ubiquinone-binding site of MCI (Lambert and Brand, 2004; Baradaran et al., 2013; Zickermann et al., 2015) and selectively block the response of glomus cells to hypoxia without altering the response to hypoglycemia (Ortega-Saenz et al., 2003; Garcia-Fernandez et al., 2007b). The selective inhibitory effect of rotenone on acute O_2 sensing has also been observed in ovine (Keating et al., 2005) and rat (Thompson et al., 2007) chromaffin cells. These results suggested that mitochondria can signal the plasma membrane and, more specifically, that a rotenone-binding site in MCI could be implicated in the hypoxic response by arterial chemoreceptors.

To test this hypothesis, we generated conditional knockout mouse models in which the MCI subunit *Ndufs2* (NADH dehydrogenase (ubiquinone) Fe-S protein 2) can be deleted using the CRE-loxP system (Fernandez-Aguera et al., 2015). *Ndufs2* encodes a 49 kDa protein, which contributes to the ubiquinone binding site of MCI (Kashani-Poor et al., 2001; Baradaran et al., 2013; Rhein et al., 2013). This site is located close to the last Fe/S cluster (N2) in MCI, from which electrons are transferred to ubiquinone. In the TH-NDUFS2 mouse model, *Ndufs2* deletion is restricted to catecholaminergic cells, including CB glomus cells, AM chromaffin cells, and SCG neurons, with all of them expressing tyrosine hydroxylase (TH), the rate limiting enzyme to produce dopamine. In wild-type mice, environmental hypoxia (10% O_2) induces a ventilatory response, as demonstrated by plethysmography, which is abolished in TH-NDUFS2 mice (Fig. 2A). However, *Ndufs2*-deficient mice hyperventilate when exposed to high levels (5%) of environmental CO_2 (hypercapnia), thus demonstrating that the lack of ventilatory response is specific to hypoxia (Fernandez-Aguera et al., 2015).

Loss of chemosensory responses to hypoxia after ablation of the *Ndufs2* gene is also observed at the cellular level. Exposure to hypoxia (PO_2 ~15 mm Hg) or to pathophysiological levels of hypercapnia (20% CO_2) induces catecholamine secretion from glomus cells in CB slices, which can be monitored by amperometry. In glomus cells from TH-NDUFS2 mice the response to hypercapnia is maintained while the response to hypoxia is abolished (Fig. 2B). The loss of hypoxia-induced catecholamine secretion is also observed in chromaffin cells (Fig. 2C). Furthermore, hypoxia-induced increases in cytosolic Ca^{2+}, as determined with FURA-AM by microfluorimetry, are also absent in isolated glomus cells from TH-NDUFS2 mice (Fig. 2D). In wild-type glomus cells, hypoxia treatment elicits an inhibition of voltage-dependent and "background" (measured as an increase in membrane input resistance) K^+ channels, as determined by patch-clamp techniques. However, *Ndufs2*-deficient glomus cells lack the reversible hypoxia-induced inhibition of K^+ current (Fernandez-Aguera et al., 2015). Together, these data indicate that the lack of *Ndufs2* results in a loss of responsiveness to hypoxia in peripheral chemoreceptor cells. They explain the abolition of the hypoxic ventilatory response in *Ndufs2*-deficient (TH-NDUFS2) mice.

The deletion of *Ndufs2* in TH-NDUFS2 mice occurs at the embryonic stage. Therefore, the loss of hypoxic response in these mice could result from general metabolic adaptation during early development rather than being specific to MCI function. To further address this question, we have also generated a mouse model (ESR-NDUFS2) in which deletion of *Ndufs2* only occurs when mice reach adulthood (2 months old) and are treated with tamoxifen-containing diet. Similar to TH-NDUFS2 mice, ESR-NDUFS2 mice present loss of hypoxic ventilatory response along with lack of secretory responses from glomus and chromaffin cells during hypoxia (Fernandez-Aguera et al., 2015; Arias-Mayenco et al., 2018). This inhibition of hypoxic responsiveness is accompanied by a decrease in MCI activity and MCI-dependent O_2 consumption, and lack of MCI complex formation, without affecting other mitochondrial complexes (Fernandez-Aguera et al., 2015). The results obtained from

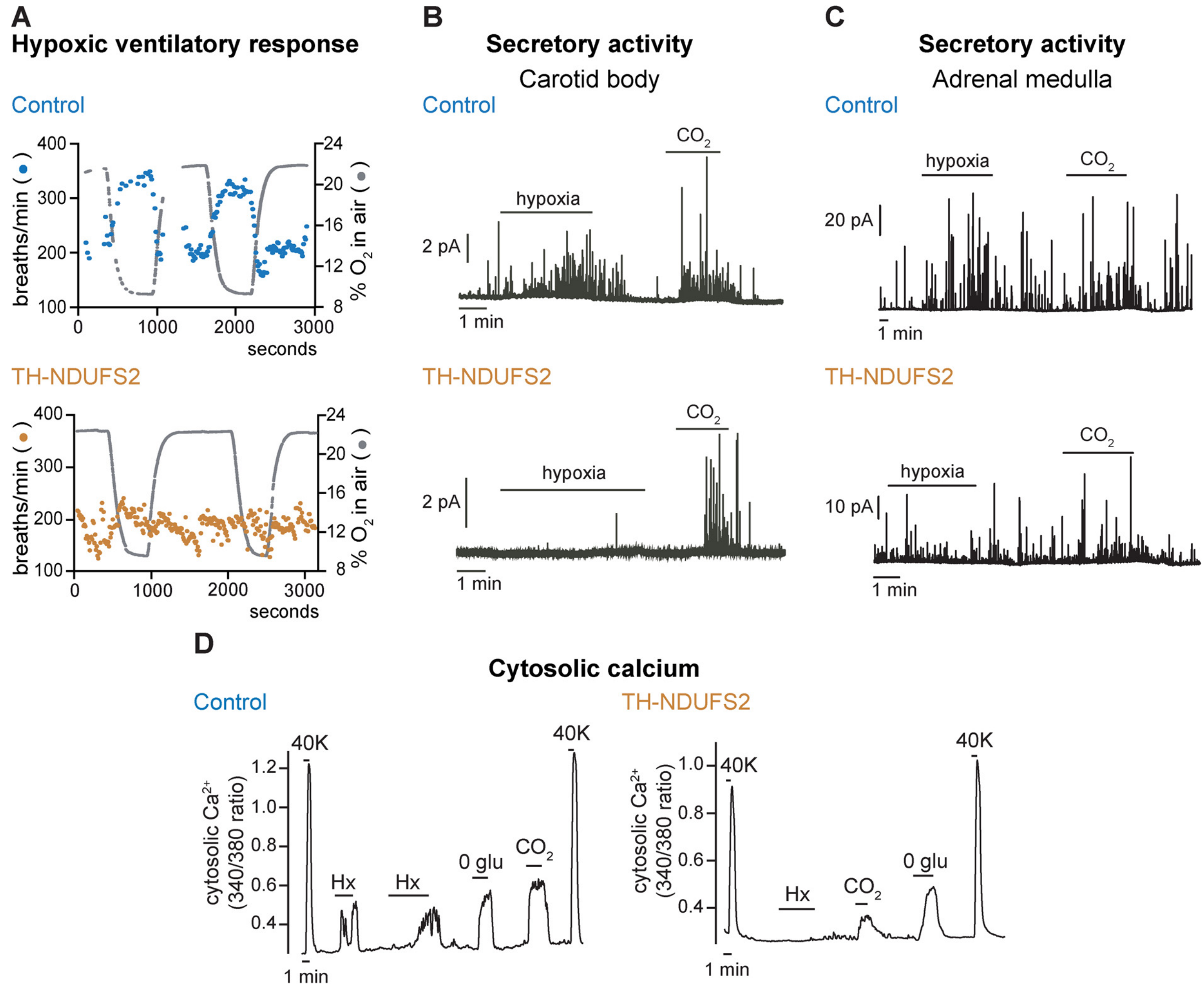

Fig. 2. Loss of responsiveness to hypoxia in *Ndufs2*-deficient mice (TH-NDUFS2). A. Representative plethysmographic recordings demonstrating the loss of hypoxic ventilatory response in TH-NDUFS2 mice compared to control animals. **B** & **C**. Specific loss of hypoxia-induced secretory response (catecholamine release) from carotid body glomus cells (**B**) and adrenal medulla chromaffin cells (**C**) of TH-NDUFS2 mice as determined by amperometry. **D**. Lack of hypoxia-induced increase in cytosolic calcium from dispersed glomus cells of TH-NDUFS2 mice, without alteration of the increase in calcium induced by other stimuli, as determined by microfluorometry. CO_2, 20%CO_2; Hx: hypoxia; 0 glu, 0 glucose; 40 K, 40 mM K^+. (Modified from Fernandez-Aguera et al., 2015).

both TH-NDUFS2 and ESR-NDUFS2 mice strongly suggest that MCI is essential for acute O_2 sensing by peripheral chemoreceptors.

2.2. Mitochondrial complex I-originated reactive oxygen species and pyridine nucleotides as signaling molecules of acute O_2 sensing

Reactive oxygen species (ROS), especially of mitochondrial origin, have long been proposed to act as signaling molecules to mediate acute responses to hypoxia in pulmonary artery myocytes (Archer et al., 1993; Leach et al., 2001). In addition, an increase in ROS generated in mitochondrial complex III (MCIII) has been suggested to trigger pulmonary hypoxic vasoconstriction (Waypa et al., 2001). However, it is under debate whether cytosolic ROS increases or decreases during hypoxia in pulmonary myocytes and other cell types (Weir et al., 2005; Hamanaka and Chandel, 2009; Waypa et al., 2013; Hernansanz-Agustin et al., 2014). This controversy results partially from the highly reactive nature of ROS and from the difficulty of reliable real-time measurements of ROS production in cells. The potential participation of ROS in acute O_2 sensing in glomus cells has been studied in less detail (see Lopez-Barneo, 2003; Wyatt and Buckler, 2004). Rotenone, which mimics hypoxic effects by inducing external Ca^{2+}-dependent neurotransmitter secretion in CB glomus cells, can induce ROS production from MCI (Turrens and Boveris, 1980; Ortega-Saenz et al., 2003). On the other hand, ROS with non-mitochondrial origin have also been proposed to participate in acute O_2 sensing in different tissues (Cross et al., 1990; Fu et al., 2000).

The development of ratiometric redox probes, which are genetically engineered green fluorescent proteins (roGFPs) sensitive to changes in redox status (Dooley et al., 2004; Remington, 2006), has allowed us to record real time changes in mitochondrial ROS production in glomus cells by microfluorimetry. There is a reversible increase in ROS production during hypoxia in the cytosol and in mitochondrial intermembrane space (IMS) in CB glomus cells, which are strongly inhibited in *Ndufs2*-deficient cells (Fig. 3A). Intracellular application of oxidants (H_2O_2 or diamide) mimics the hypoxic effect, as it inhibits background K^+ currents in wild-type glomus cells, and prevents any further effect of hypoxia (Fernandez-Aguera et al., 2015). On the other hand, hypoxia-induced inhibition of voltage-gated K^+ current was not affected by changes in redox status (Fernandez-Aguera et al., 2015). These results suggest that a local increase in ROS production during hypoxia could serve as a signal to inhibit background currents in arterial chemoreceptor cells.

Increase in NAD(P)H auto-fluorescence upon exposure to hypoxia has been observed previously in glomus cells, suggesting a possible signaling role of NAD(P)H in acute O_2 sensing (Duchen and Biscoe, 1992; Buckler and Turner, 2013). Our recent experiments indicate that

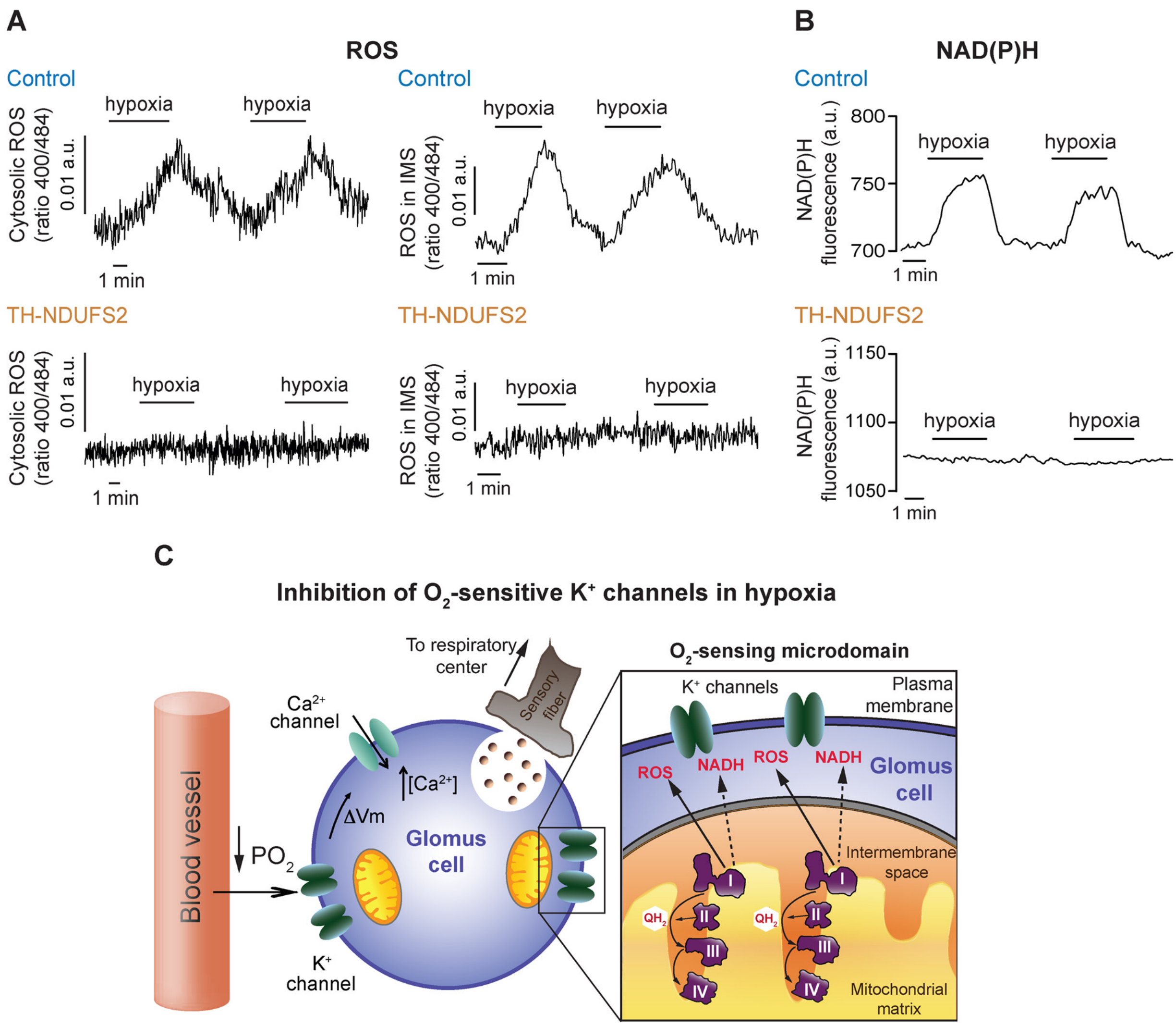

Fig. 3. Mitochondrial complex I (MCI) signaling in acute O_2 sensing by peripheral chemoreceptors. **A**. Hypoxia-induced increase in cytosolic (left) and mitochondrial intermembrane space (IMS, right) reactive oxygen species (ROS) measured by microfluorimetry using redox-sensitive fluorescent probes (roGFP) in control glomus cells and disappearance of this response in TH-NDUFS2 mice. a.u., arbitrary units. **B**. Hypoxia-induced increase in cytosolic NAD(P)H auto-fluorescence in control glomus cells and loss of the response in cells from TH-NDUFS2 mice. **C**. Model of MCI-mediated acute O_2 sensing by arterial chemoreceptor cells. I, II, III, IV, mitochondrial complexes I, II, III, IV, respectively; PO_2, oxygen tension; QH2, ubiquinol; ΔVm, membrane depolarization. See text for further explanation. (Modified from Fernandez-Aguera et al., 2015; Gao et al., 2017a).

hypoxia induces an increase in NAD(P)H auto-fluorescence in wild-type CB glomus cells which is absent in cells from *Ndufs2*-deficient mice (Fig. 3B). In addition, hypoxia-induced inhibition of voltage-gated K^+ current was partially blocked in wild-type glomus cells dialyzed with NADH (Fernandez-Aguera et al., 2015). These results support the possible role of NADH in modulating the opening probability of voltage-gated K^+ channels during hypoxia. Indeed, pyridine nucleotides can bind to voltage-gated K^+ channel subunits and modulate their function (Tipparaju et al., 2005; Tamsett et al., 2009; Kilfoil et al., 2013). Altogether, our results suggest that whereas ROS inhibit background K^+ channels to initiate the depolarization-induced by hypoxia, NADH could act on maxi-K^+ and voltage-dependent K^+ channels to potentiate membrane depolarization and action potential firing. However, this relatively simple proposal is based on data that need to be further confirmed in future experimental work.

Whether ROS and NADH function as signaling molecules in AM chromaffin cells during acute O_2 sensing as it occurs in CB glomus cells is not well known. Extracellular H_2O_2 can increase K^+ current amplitude (Keating et al., 2005; Thompson et al., 2007), and intracellular reducing agents or antioxidant enzymes are found to mimic hypoxia-induced inhibition of voltage-dependent currents in neonatal chromaffin cells (Thompson et al., 2007). These data suggest the implication of redox status in the hypoxia-induced chemotransduction in chromaffin cells, although further studies are necessary to investigate this process in detail.

2.3. Model of mitochondrial complex I-mediated acute O_2 sensing by peripheral chemoreceptors

One unexpected finding obtained from *Ndufs2*-deficient mice is the resistance of CB glomus cells to MCI dysfunction, as these cells have a high O_2 consumption and metabolic rate. Glomus cells from TH-NDUFS2 mice not only present normal morphology without cell death or ATP depletion, but even display a slight CB hypertrophy (Fernandez-Aguera et al., 2015). Compared to other neural tissues, the CB is rich in succinate, the substrate for mitochondrial complex II (MCII, succinate dehydrogenase) of the ETC and an intermediate of the tricarboxylic acid (TCA) cycle (Fernandez-Aguera et al., 2015). Unlike MCI-deficient mice, CB glomus cells cannot survive in MCII-deficient mice (Diaz-Castro et al., 2012). Together, these data suggest that high succinate content in glomus cells could serve not only for ATP synthesis, but also to keep high QH2 level, which could favor the MCI-mediated signal transduction during hypoxia.

Based on these concepts, we have proposed a model in which ROS and NADH from MCI signal the inhibition of K^+ channels during hypoxia in arterial chemoreceptors (Fig. 3C). This model is compatible with the "membrane model" of O_2 sensing (see Fig. 1C), as mitochondria could form "O_2-sensing microdomains" located near plasma membrane K^+ channels (Fernandez-Aguera et al., 2015). During hypoxia, an accumulation of QH2, which is favored by the high amount of succinate and slowdown of electron transfer in the ETC, would lead to ROS production and accumulation of NADH from MCI. The proximity between mitochondria and the plasma membrane would result in local increases in ROS and NADH, which in turn modulate K^+ channels.

3. Metabolic adaptations of the peripheral chemoreceptors to acute O_2 sensing

The survival of *Ndufs2*-deficient glomus cells without functional MCI, and the high succinate content in wild-type CB cells (Fernandez-Aguera et al., 2015) suggest that glomus cells, and probably other chemoreceptor cells, could have a specialized metabolic adaptation to favor their ability to acutely respond to hypoxia. For the last decade, several transcriptomic studies of CB glomus cells from both rodents and humans have been performed using either microarray analysis or single-cell RNA sequencing (Ganfornina et al., 2005; Balbir et al., 2007; Fagerlund et al., 2010; Mkrtchian et al., 2012; Zhou et al., 2016). Recently, we have performed a microarray comparative study to investigate gene expression profiles in three embryologically and functionally related organs: CB, AM, and SCG (Gao et al., 2017b). CB glomus cells, AM chromaffin cells, and SCG neurons originate from neural crest-derived sympathoadrenal progenitors, but exhibit variable degree of responsiveness to hypoxia. Glomus cells and chromaffin cells are O_2-sensitive with glomus cells being more sensitive than chromaffin cells, whereas SCG neurons are O_2-insensitive.

3.1. Differential gene expression profiles in the CB or AM versus SCG

We have used CB, AM and SCG from adult C57/B6 mice (2 months old) to avoid the developmental effect on gene expression. Interestingly, although the CB migrates from the SCG to the carotid bifurcation during embryogenesis (Kameda, 2014), hierarchical clustering analysis of transcriptomes shows a global similarity between the CB and AM, not between the CB and SCG (Fig. 4A). In addition, most genes with differential expression between the CB and SCG are also observed to be differentially expressed when comparing gene expression profiles between the AM and SCG (Gao et al., 2017b). Our microarray results have been confirmed by real time quantitative PCR, using a new set of biologically independent samples. In addition, we have also performed quantitative PCR studies on glomus cells, chromaffin cells, and SCG neurons, sorted by flow cytometry from TH-GFP mice, to avoid contamination from non-neuronal cells in each organ. The results from whole organs analyzed either by microarray or quantitative PCR have also been validated in TH positive cells (Fig. 4B). Taken together, these results reflect the common physiologic functions (acute O_2 sensing among others) of glomus and chromaffin cells (see below).

3.1.1. PHD-HIF pathways in acute O_2 sensing

Although it is widely accepted that the PHD-HIF pathway is essential for the adaptive responses to chronic hypoxia (Kaelin and Ratcliffe, 2008; Semenza, 2012), less is known about its implication in acute hypoxia. In our comparative analysis (Gao et al., 2017b), hypoxia inducible factor 2α (*Hif2α*) was found highly expressed in glomus and chromaffin cells compared to SCG neurons (Fig. 4B). This is in agreement with previous reports of *Hif2α* expression in neonatal mouse CB cells (Zhou et al., 2016) and of constitutive expression of *Hif2α* in adult mouse CB (Tian et al., 1998). It has been reported that *Hif2α* heterozygous null-mice present increased CB sensitivity to hypoxia and altered CB homeostasis (Peng et al., 2011). However, both the hypoxic ventilatory response and CB growth induced by chronic hypoxia are strongly inhibited in inducible *Hif2α* homozygous knockout mice (Hodson et al., 2016). Recently, CB atresia and abolishment of the hypoxic ventilatory response have been reported to occur in embryonic homozygous *Hif2a*-null mice (Macias et al., 2018). Therefore, further studies are necessary to fully understand the role of HIF2α in the adaptive response to acute hypoxia.

Hypoxia inducible factor 1α (HIF1α) was initially reported to be involved in acute responsiveness to hypoxia using *Hif1α* heterozygous knockout mice (Kline et al., 2002). However, that hypothesis was challenged as CB slices from *Hif1α* heterozygous knockout mice show hypoxia-induced catecholamine secretion indistinguishable from wild-type mice (Ortega-Saenz et al., 2007). Moreover, inducible ablation of the *Hif1α* gene does not significantly alter the hypoxic ventilatory response in mice (Hodson et al., 2016) and embryonic *Hif1α* deletion in TH-positive cells does not alter CB development or the hypoxic ventilatory response (Macias et al., 2018). Unlike *Hif2α*, *Hif1α* is only moderately up-regulated in the CB and AM compared to SCG (Gao et al., 2017b), which argues against HIF1α being essential for acute O_2 sensing.

Proline hydroxylase 3 (PHD3/EGLN3) is involved in the development of the sympathoadrenal lineage (Bishop et al., 2008; Macias et al., 2014). *Phd3* knockout mice show CB and AM hypertrophy, mediated at least partially by *Hif2α*, and normal hypoxic response (Bishop et al., 2008; Macias et al., 2014). We have found the mRNA level of *Phd3* to be low in the CB and AM compared to the SCG, and almost undetectable in sorted glomus cells (Fig. 4B). This low *Phd3* expression could help maintain high HIF2α protein level in glomus and chromaffin cells during normoxia, which may contribute to the cell metabolic specializations required for acute O_2 sensing.

3.1.2. Atypical mitochondrial ETC subunits in acute O_2 sensing

In our comparative microarray analysis, we have paid special attention to mitochondrial ETC subunits due to the signaling role of MCI in acute O_2 sensing described before (Fernandez-Aguera et al., 2015). In general, gene expression profiles of ETC subunits were similar among the three organs studied. However, three genes were found highly expressed in glomus and chromaffin cells compared to SCG neurons: *Ndufa4l2* (NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2), *Cox4i2* (cytochrome c oxidase subunit IV isoform 2), and *Cox8b* (cytochrome c oxidase subunit VIIIb) (Fig. 4B). This observation was also confirmed at the protein level for NDUFA4L2 and COX4I2 for which selective antibodies are available (Fig. 4C). *Ndufa4l2* and *Cox4i2* are also highly expressed in neonatal glomus cells (Zhou et al., 2016) and are up-regulated by hypoxia in different tissues through HIF-dependent and independent pathways (Fukuda et al., 2007; Brown et al., 2010; Tello et al., 2011; Aras et al., 2013). Putative HIF binding sites are also proposed in the promoter region of *Cox8b* (Gao et al., 2017b). Therefore, enrichment of these ETC subunits could result from constitutively high expression of *Hif2α* in O_2 sensitive cells.

The specific role of atypical ETC subunits in CB acute O_2 sensing is unknown, although recently it has been shown that deletion of the *Cox4i2* gene strongly inhibits responsiveness to hypoxia of pulmonary artery myocytes (Sommer et al., 2017). COX4I2 and COX8B are part of the catalytic core of mitochondrial complex IV (MCIV), the natural O_2-reaction site in the ETC, and NDUFA4L2 is a paralogue of the more ubiquitous subunit NDUFA4, which seems to be associated with MCIV (Balsa et al., 2012; Kadenbach and Huttemann, 2015). COX4I2 and COX8B subunits contain a transmembrane segment, which run in parallel in the periphery of MCIV molecular complex. It could be speculated that the combination of the three atypical subunits (in particular Cox4i2 and Cox8b) may decrease O_2 accessibility to the catalytic site of

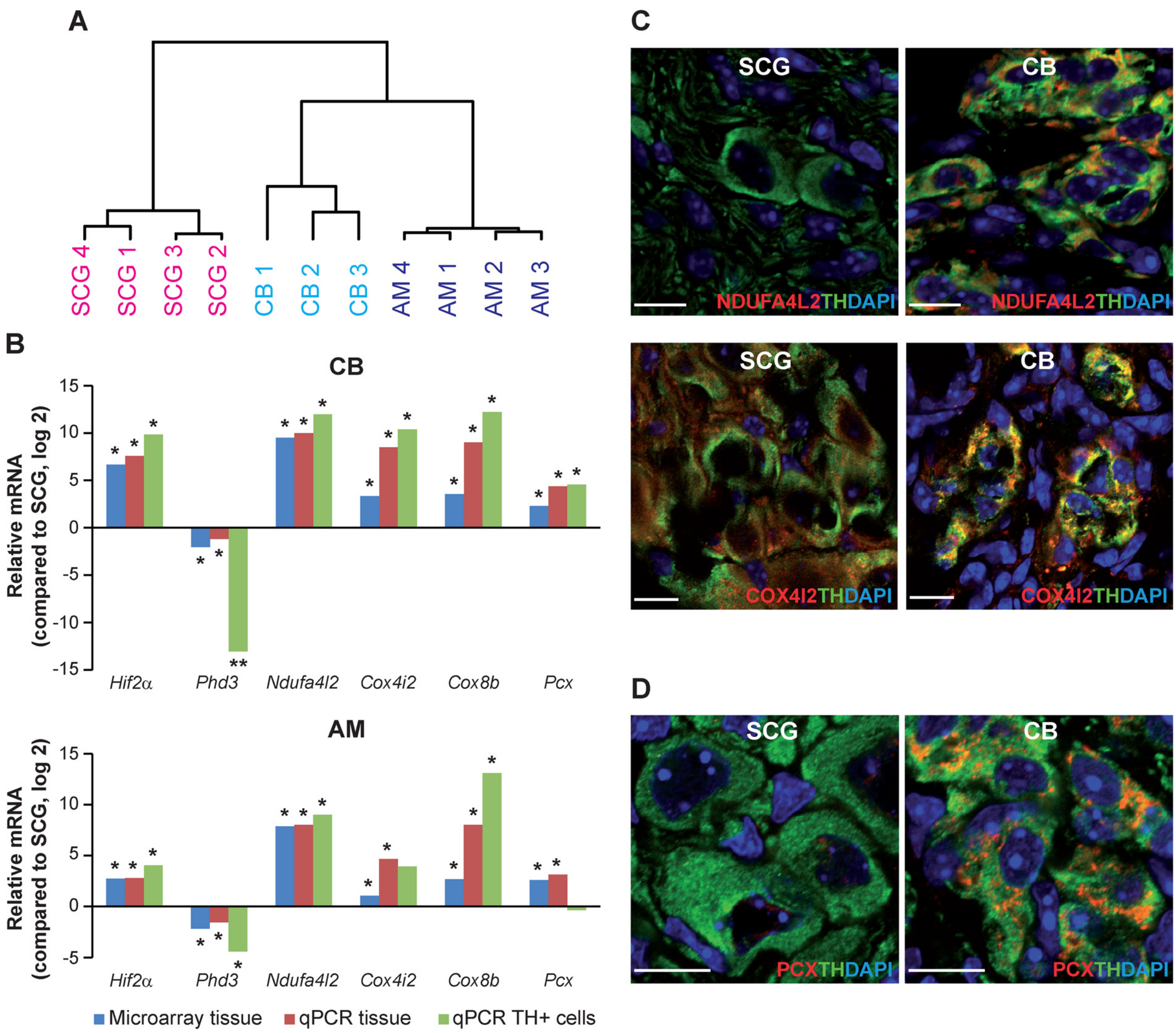

Fig. 4. Comparative gene expression analysis of carotid body (CB), adrenal medulla (AM), and superior cervical ganglion (SCG) from adult mice. A. Hierarchical clustering demonstrating the similarity of gene expression profiles between the CB and AM, compared to the SCG. Numbers indicate independent samples. B. Gene expression analysis demonstrating relative mRNA levels (in log 2) comparing the CB (upper panel) or the AM (lower panel) to SCG by microarray analysis or realtime quantitative PCR (qPCR) with either whole organs or tyrosine hydroxylase positive (TH+) cells from each organ. Positive numbers correspond to up-regulated genes, whereas negative numbers indicate down-regulated genes. *, pFDR < 0.05 for microarray analysis or p < 0.05 for quantitative PCR compared to the SCG; **, No expression in CB TH + cells. *Hif2α*, endothelial PAS domain protein 1 (*Epas1*); *Phd3*, egl-9 family prolyl hydroxylase 3 (*Egln3*); *Ndufa4l2*, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2; *Cox4i2*, cytochrome c oxidase subunit IV isoform 2; *Cox8b*, cytochrome c oxidase subunit VIIIb; *Pcx*, pyruvate carboxylase. C & D. Immunostaining demonstrating the high protein levels of NDUFA4L2, COX4I2, and PCX (red) in the CB glomus cells compared to SCG neurons and their co-localization with TH (green). Scale bar, 10 µm. (Modified from Gao et al., 2017b).

MCIV, making MCIV more sensitive to changes in PO_2. It remains to be further studied whether MCIV of glomus and chromaffin cells contains special properties related to O_2 sensitivity compared to non-sensitive cells.

3.1.3. Anaplerosis in acute O_2 sensing: pyruvate carboxylase and biotin

A particularly interesting observation in our microarray analysis is that pyruvate carboxylase (PCX), a Kreb's cycle anaplerotic enzyme (Owen et al., 2002), which converts pyruvate to oxaloacetate, is highly up-regulated in CB glomus cells in comparison with SCG neurons (Fig. 4B, D). This is accompanied by a slightly decreased *Pdha1* (pyruvate dehydrogenase E1 alpha 1) expression, which catalyzes conversion of pyruvate to acetyl-CoA (Gao et al., 2017b). The resulting high *Pcx/Pdha1* ratio implicates that glomus cells may use pyruvate preferentially to replenish oxaloacetate via the PCX-mediated classic anaplerotic reaction, thus explaining the high succinate content in glomus cells. Unlike glomus cells, *Pcx* expression in AM chromaffin cells is similar to that of SCG neurons (Fig. 4B). This difference may partially explain the relatively low O_2 sensitivity of chromaffin cells compared to glomus cells, especially during adulthood.

PCX belongs to the carboxylase family whose function depends on the cofactor biotin. Biotin, also known as vitamin B7, vitamin H, or coenzyme R, is a water-soluble vitamin, which not only participates in carboxylase-catalyzed reactions but is also implicated in the regulation of gene expression (Mock, 1996; Zempleni et al., 2009). High expression of *Pcx* in the CB could imply an increased demand of biotin. Indeed, we have found that in parallel with the profile of *Pcx* expression, biotin is enriched in rat CB but is almost undetectable in the SCG (Fig. 5A). Although lower than the CB, biotin is also highly present in rat AM compared to the adrenal cortex (Fig. 5B). Accumulation of biotin in the CB is mainly restricted to the mitochondria of glomus cells. *Slc5a6*, a biotin transporter (Said, 1999), is highly expressed in rat CB compared to SCG and AM, which may explain the specific biotin accumulation in the CB (Ortega-Saenz et al., 2016).

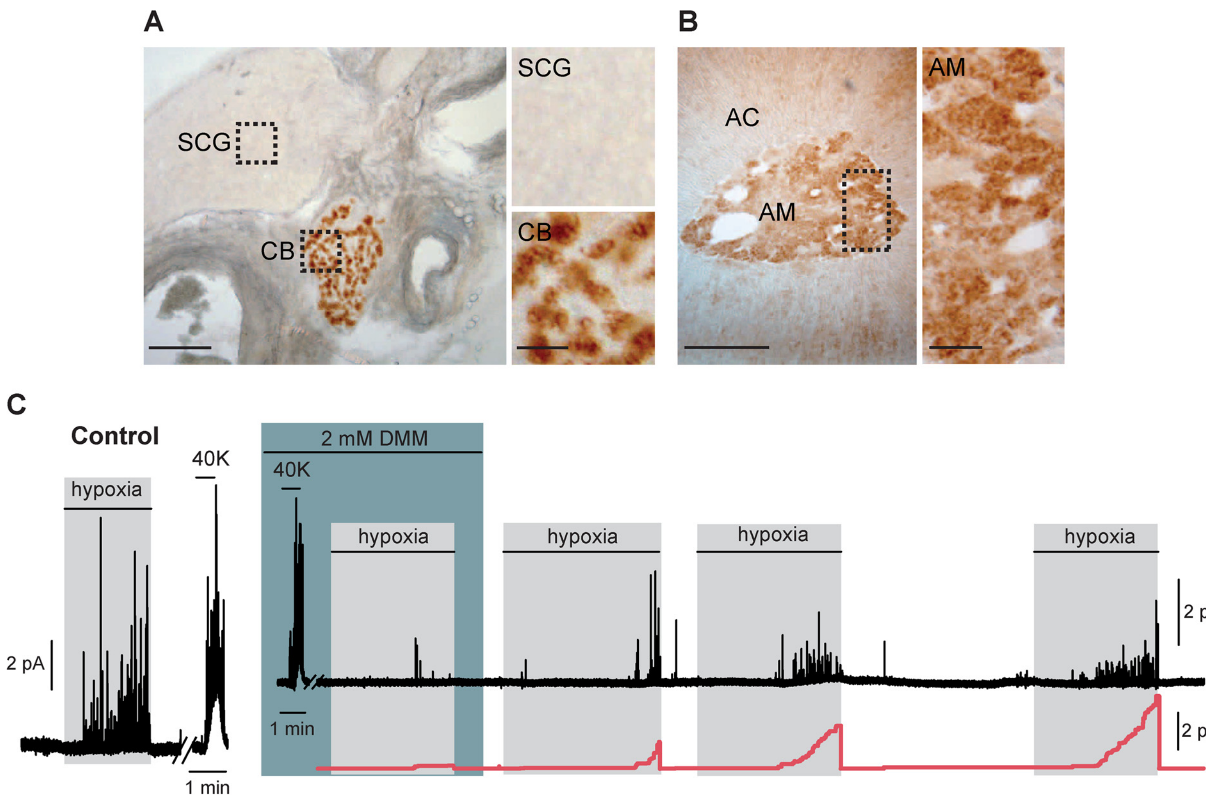

Fig. 5. Anaplerosis of the tricarboxylic acid cycle in peripheral chemoreceptors. A. Selective accumulation of biotin (brown), the cofactor of pyruvate carboxylase, in the carotid body (CB) compared to superior cervical ganglion (SCG). Left: carotid artery bifurcation. Scale bar, 0.5 mm. Right: magnification of SCG and CB indicated by the squares in the left panel. Scale bar, 0.1 mm. **B**. High biotin content in the adrenal medulla (AM) compared to the adrenal cortex (AC). Left: adrenal gland. Scale bar, 0.5 mm. Right: magnification of AM indicated by the square in the left panel. Scale bar, 0.1 mm. **C**. Representative amperometric recordings demonstrating the hypoxia-induced catecholamine secretion (dopamine and other catecholamines in the secretory granules) (Control, left panel) being reversibly inhibited by 2 mM dimethyl-malonate (DMM), a mitochondrial complex II inhibitor (right panel). Cumulative secretion rate (red line) is represented at the bottom. 40 K, 40 mM K^+. (Modified from Ortega-Saenz et al., 2016; Gao et al., 2017b).

PCX-mediated anaplerosis and subsequent production of Kreb's cycle intermediates favor the production of high levels of QH2 from MCI and MCII, which is a determining factor in acute O_2 sensing. In accord with this concept glomus cells from mice deficient in MCII *Sdhd* subunit lack hypoxia-induced catecholamine secretion (Gao et al., 2017b). Moreover, in wild-type animals, treatment with dimethyl malonate, a membrane-permeant competitive inhibitor of MCII (Gutman, 1978), suppresses the hypoxia-induced secretory response, which can be recovered by removal of the inhibitor (Fig. 5C). These observations suggest a critical role of anaplerosis in acute O_2 sensing in CB cells. In these cells, PCX-mediated anaplerosis is enhanced by high expression of *Pcx*, high biotin content, and a relative low expression of pyruvate dehydrogenase, which results in increased Krebs' cycle activity and elevated MCI- and MCII-mediated QH2 production. Further QH2 accumulation during hypoxia facilitates the generation of signaling molecules in MCI (Arias-Mayenco et al., 2018; see below).

3.1.4. Ion channels in acute O_2 sensing

Several ion channel genes are overexpressed in O_2 sensitive cells in comparison to non-sensitive cells (Gao et al., 2017b). K^+ channel subunits, such as *Task3 (Kcnk9), Task1 (Kcnk3)*, and *Kcnip3*, are highly expressed in glomus and chromaffin cells compared to SCG neurons. Among them, high expression of *Task1* has been reported in previous transcriptome studies (Mkrtchian et al., 2012; Zhou et al., 2016). The T-type Ca^{2+} channel subunit *Cacna1h*, whose expression can be induced by hypoxia in a HIF2α-dependent manner (del Toro et al., 2003), and transient receptor potential cation channel *Trpc5* are also highly expressed in CB and AM cells. TASK1 and TASK3 channels (Buckler, 1997; Ortega-Saenz et al., 2010), as well as CACNA1H channels (Levitsky and Lopez-Barneo, 2009) have been implicated in acute O_2 sensing by glomus cells. However, it remains to be studied in detail whether these channels are modulated by the signaling molecules (NAD^+/NADH ratio and ROS) postulated to mediate hypoxia signaling in peripheral chemoreceptor cells.

3.2. "Signature metabolic profile" in chemoreceptor cells and its role in acute O_2 sensing

The special metabolic properties of chemoreceptor cells observed in the comparative microarray analysis are schematically summarized in Fig. 6. The low level of *Phd3* expression could play a central role in this profile, as a lack of PHD3-mediated HIF2α degradation could help maintain HIF2α protein in a constitutively high level. HIF2α could in turn maintain the high expression of *Ndufa4l2*, *Cox4i2*, and possibly also *Cox8b* to render MCIV sensitive to physiological changes in PO_2. In parallel, PCX-mediated anaplerosis may help maintain high amount of succinate and QH2 under normoxic condition. During hypoxia, MCIV could detect the decrease in PO_2, causing slowdown of electron transfer in the ETC, further increase in QH2, and accumulation of NADH and production of ROS either by slowdown or reversion of MCI reaction. MCI-generated NADH and ROS could modulate the activity of "O_2-sensitive" ion channels expressed at the plasma membrane of chemoreceptor cells, thus resulting in cell depolarization and transmitter release. HIF2α-induced specialization of MCIV and PCX-mediated anaplerosis may also be interrelated. High succinate (Fernandez-Aguera et al., 2015) and low α-ketoglutarate (Tennant and Gottlieb, 2010) contents, which both inhibit PHD3 activity, could favor HIF2α stabilization, therefore making MCIV even more sensitive to changes in PO_2.

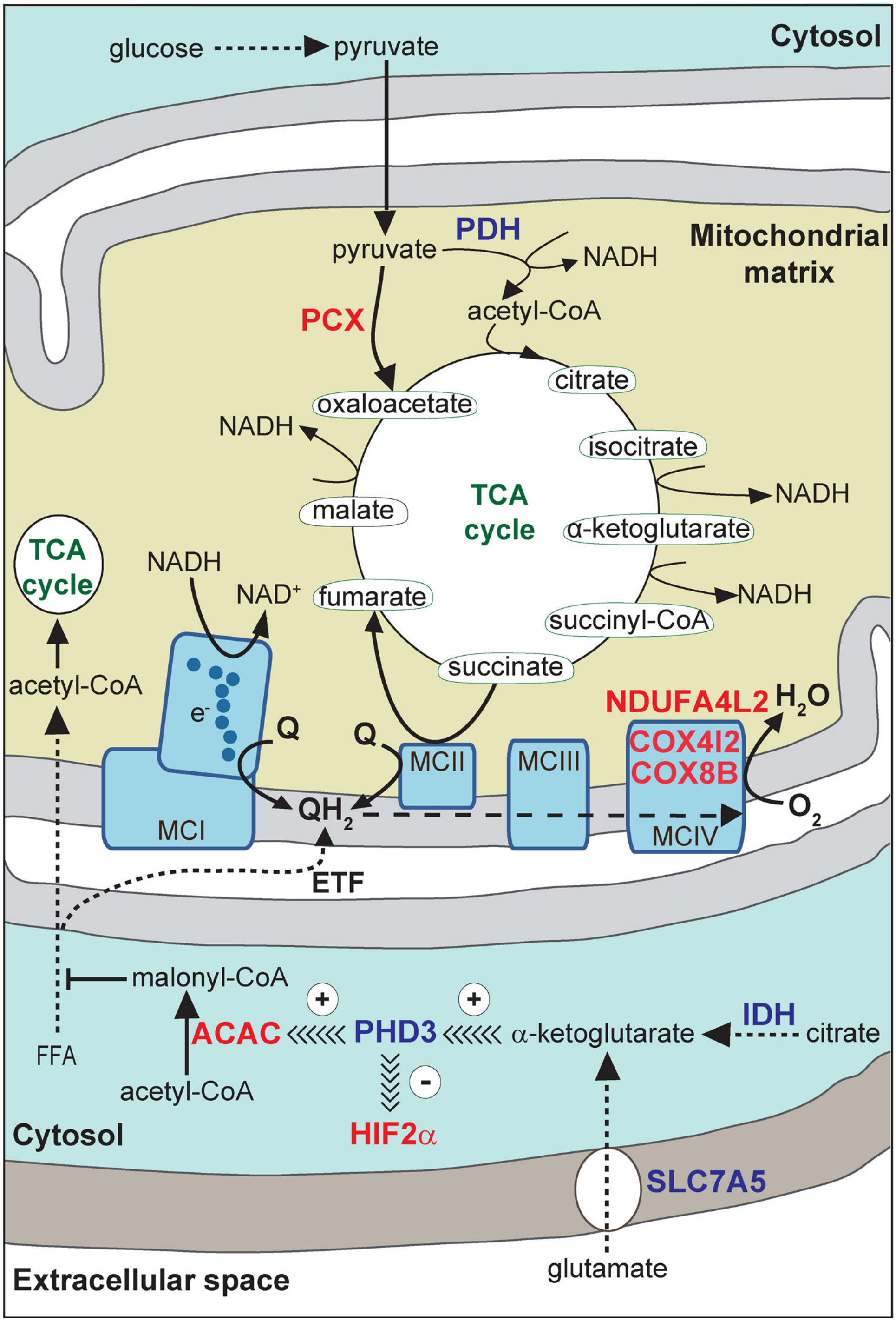

Fig. 6. Proposed "signature metabolic profile" of carotid body (CB) glomus cells based on the gene expression profile obtained from the microarray analysis. ACAC, acetyl-coenzyme A carboxylase; COX4I2, cytochrome c oxidase subunit IV isoform 2; COX8B, cytochrome c oxidase subunit VIIIb; ETF, electron transport flavin/quinone oxidoreductase; FFA, free fatty acid; HIF2α, endothelial PAS domain protein 1 (EPAS1); IDH, isocitrate dehydrogenase; MCI, MCII, MCIII, MCIV, mitochondrial complex I, II, III, IV, respectively; NDUFA4L2, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2; PCX, pyruvate carboxylase; PDH, pyruvate dehydrogenase; PHD3, egl-9 family prolyl hydroxylase 3 (EGLN3); Q, ubiquinone; QH2, ubiquinol/reduced ubiquinone; SLC7A5, solute carrier family 7 (cationic amino acid transporter, y + system), member 5; TCA, tricarboxylic acid. See text for detailed description. Modified from Gao et al., 2017b.

Although it is possible that none of the genes in the "signature metabolic profile" characteristic of acute O2-sensing cells are indispensable, altogether they may determine a metabolic status that renders cells sensitive to hypoxia. Rather than drawing conclusion, this gene expression profile study makes predictions, which may be tested experimentally in future studies.

4. Conclusions and perspectives

Acute O_2 sensing by arterial chemoreceptors triggers fast cardiorespiratory reflexes, which are essential for the survival of mammals under hypoxic conditions. The K^+ channel-based "membrane model", proposed about thirty years ago, explains the basic process of chemotransduction in arterial chemoreceptors during hypoxia. Since then, technological advances have allowed us to better understand the molecular mechanisms underlying this process. Genetically modified mice deficient in MCI subunit and redox-sensitive probes have helped us to discover the signaling role of MCI-mediated ROS and NADH in acute O_2 sensing. Using a comparative "omic" approach, a special metabolic profile in peripheral chemoreceptors is demonstrated, which render their mitochondria sensitive to decreases in PO_2. In addition, glomus cells are electrically compact cells due to their small size so that even a subtle change in current could lead to relatively large alterations in membrane potential. Furthermore, hypoxia-induced signal transduction to ion channels could also be facilitated by the proximity between the mitochondria and the plasma membrane. Taken together, metabolic adaptations, biophysical properties, and the geometric organization lead the peripheral chemoreceptors, especially the CB, to be highly sensitive to hypoxia.

Although we think that the current knowledge provides a comprehensive model of acute O_2-sensing based on mitochondria signaling to membrane ion channels, there are still numerous questions that remain to be addressed. Multiple O_2 sensing mechanisms may function in coordination to ensure a fast and proper response to hypoxia. In addition, the sensitivity to hypoxia and molecular mechanisms underlying the acute O_2-sensing process may not necessarily be uniform among different chemoreceptors (for example, CB vs. AM). MCI-mediated signaling transduction pathway remains to be explored in detail in future studies. On the other hand, microarray analyses have provided potential candidate genes, which may be tested in *in vitro* and *in vivo* models for their implication in acute O_2 sensing. Advances of these studies will have great physiological and clinical impacts.

Conflict of interest

The authors report no conflict of interest.

Acknowledgements

This research was supported by the Botín Foundation, the Spanish Ministries of Science and Innovation and Health (SAF2012-39343, SAF2016-74990-R), the European Research Council (ERC Advanced Grant PRJ201502629), and the Andalusian Government (PI-0125-2016).

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