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Carotid chemoreceptor “resetting” revisited

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Abstract

Carotid body (CB) chemoreceptors transduce low arterial O₂ tension into increased action potential activity on the carotid sinus nerves, which contributes to resting ventilatory drive, increased ventilatory drive in response to hypoxia, arousal responses to hypoxia during sleep, upper airway muscle activity, blood pressure control and sympathetic tone. Their sensitivity to O₂ is low in the newborn and increases during the days or weeks after birth to reach adult levels. This postnatal functional maturation of the CB O₂ response has been termed “resetting” and it occurs in every mammalian species studied to date. The O₂ environment appears to play a key role; the fetus develops in a low O₂ environment throughout gestation and initiation of CB “resetting” after birth is modulated by the large increase in arterial oxygen tension occurring at birth. Although numerous studies have reported age-related changes in various components of the O₂ transduction cascade, how the O₂ environment shapes normal CB prenatal development and postnatal “resetting” remains unknown. Viewing CB “resetting” as environment-driven (developmental) phenotypic plasticity raises important mechanistic questions that have received little attention. This review examines what is known (and not known) about mechanisms of CB functional maturation, with a focus on the role of the O₂ environment.

Keywords

carotid body; development; O₂ sensing; hypoxia; hypoxia inducible factor; developmental plasticity

1.0 INTRODUCTION

Mammalian life depends on a steady supply of oxygen to tissues to meet cellular metabolic needs, while excess oxygen is highly toxic. Therefore, mammals have developed O₂ sensory systems that operate on multiple levels to optimize cellular O₂ availability and promote tolerance to low O₂ tensions, as well as antioxidant systems to reduce oxygen toxicity. An important generalized oxygen sensing system, operative in every nucleated cell, are the hypoxia-inducible factors (HIF), major regulators of cellular oxygen homeostasis in all metazoan animals. Low oxygen tension increases HIF-1 α level, which in turn controls the transcription of hundreds of genes involved in cellular-level adaptations to low oxygen tension (Semenza, 2012; Webb et al., 2009). Other generalized cellular O₂-sensing pathways

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such as the unfolded protein response, nuclear factor (NF)- κ B and the mammalian target of rapamycin (mTOR) promote tolerance to hypoxia by modulating transcription and translation (Dunwoodie, 2009; Gorr et al., 2010). Thus, every nucleated cell in the mammalian body exhibits multiple adaptive responses to hypoxia that aim to minimize the effects of reduced oxygen availability and preserve homeostasis. As tissue O_2 tension depends on oxygen delivery, it is not surprising that mammals have also evolved O_2 -regulated control of erythrocyte production, generalized systemic vascular responsiveness to hypoxia and specialized O_2 -sensing vascular tissues to regulate blood flow, such as the small pulmonary arteries, fetoplacental arteries and the ductus arteriosus (Semenza, 2011; Waypa and Schumacker, 2010).

In order to ensure optimal oxygen intake, as O_2 needs vary with environment and activity, mammals have developed specialized peripheral arterial chemoreceptor organs that continuously sense arterial blood O_2 tension and directly regulate minute ventilation. The main peripheral arterial O_2 chemoreceptors are the carotid bodies (CB), located bilaterally at the carotid bifurcations. They transduce arterial O_2 levels into action potential activity on carotid sinus nerve afferents, which input via the caudal nucleus tractus solitarius to control minute ventilation and maintain normal Pa_{O_2} , increase ventilatory drive in response to hypoxia, mediate arousal responses to hypoxia during sleep and provide important modulation of upper airway muscle activity, blood pressure and sympathetic tone (Iturriaga et al., 2009; Prabhakar and Kumar, 2010; Sinski et al., 2012). Perhaps surprisingly, given their importance in cardiorespiratory control, the carotid body chemoreceptors are not functionally mature at birth and require time, after birth, to reset their O_2 responsiveness to adult-like levels (Gauda et al., 2009).

A key point, which will be emphasized throughout this review, is that postnatal development of CB oxygen sensitivity depends on the O_2 environment; if Pa_{O_2} is increased before birth onset of resetting can be hastened while, if Pa_{O_2} is kept low after birth, resetting can be delayed (Blanco et al., 1988; Sterni et al., 1999). During development, the oxygen environment changes remarkably, from the very low oxygen intrauterine environment of the embryo during the first 10–11 weeks of gestation, to the moderately hypoxic environment of the 2nd and 3rd trimesters of gestation, to the ~ four-fold sudden increase in Pa_{O_2} at birth and the oxygen-rich postnatal environment (Dunwoodie, 2009). The mammalian carotid body forms and undergoes structural maturation in this low O_2 environment. In spite of the low *in utero* P_{O_2} , CB O_2 responsiveness is low in the fetus and will increase only after exposure to the higher P_{O_2} after birth (Blanco et al., 1984; Eden and Hanson, 1987; Hertzberg and Lagercrantz, 1987). Over the last ~ 30 years, terminology has evolved describing the carotid chemoreceptors during prenatal development as “set”, analogous to a thermostat, to exhibit minimal activity to the normally low Pa_{O_2} (~ 23–25 mmHg) of the fetus. A logical extension of the “thermostat” analogy is that after birth, when Pa_{O_2} is four-fold higher compared to fetal Pa_{O_2} , the carotid chemoreceptors “reset” and sense the postnatal Pa_{O_2} of 80–100 mmHg as “normoxia”. After “resetting”, the range of hypoxia sensitivity shifts such that the Pa_{O_2} of 23–25 mmHg, which elicited minimal CB activity in the fetus, will elicit a brisk increase in carotid sinus nerve activity and would be considered severe hypoxia for an infant.

Although substantial progress has been made in understanding postnatal “resetting” of CB O_2 sensitivity, major questions persist and the fundamental mechanisms underlying dependence on the O_2 environment remain unknown. The goal of this review is to explore further the terminology, concepts, possible mechanisms of “resetting” and the role of the low O_2 environment of the fetus in shaping CB functional development. Although beyond the scope of this review, vascular O_2 sensing, HIF-1, other cellular oxygen-sensing

pathways and the effects of altered O₂ environments will be discussed when potentially relevant to CB resetting.

2.0 CB O₂ transduction - acute hypoxia

Before addressing the question of resetting, it is necessary to consider current views on CB O₂ chemotransduction mechanisms. Carotid body structure is similar across mammalian species, consisting of richly perfused clusters of oxygen-sensitive, neuron-like secretory cells called type-1 or glomus cells, surrounded by glia-like type 2 or sustentacular cells (Fig. 1). The carotid sinus nerve, a branch of cranial nerve IX with cell bodies in the petrosal ganglion (PG), provides the main sensory innervation, forming predominantly afferent synapses on glomus cells (McDonald and Mitchell, 1975). Although not fully proven, it is generally accepted that glomus cells, together with their associated nerve endings and PG cell bodies, comprise a “chemosensory unit”, with the glomus cell as the primary site of O₂-sensing, presynaptic to the nerve terminal.

The generally accepted steps in CB O₂ tension transduction, shown in figure 1, are as follows (numbers in parentheses correspond to numbered steps in figure): Lowered arterial O₂ tension in CB blood vessels (1) results in lowered tissue oxygen tension and reduced glomus cell intracellular P_{O₂} (2). O₂ sensing within glomus cells occurs at multiple sites, including mitochondria, heme-oxygenase-2 (HO-2) and possibly others. Mitochondrial O₂ sensing (3a), by as yet unknown mechanisms, leads to inhibition of cell membrane resting K⁺ current, which is predominantly carried by TASK1/3 (4a) (Kim et al., 2009; Wyatt and Buckler, 2004). Heme-oxygenase-2 (3b), which is tightly associated with cell membrane BK channels (4b), uses O₂ as a substrate to produce carbon monoxide (CO), which enhances BK channel open probability during normoxia (Kemp, 2005). When glomus cell O₂ tension is low, CO production decreases and BK channel activity is suppressed. The inhibition of TASK1/3 current initiates and drives glomus cell depolarization (5), the magnitude of which is increased by the hypoxia-mediated inhibition of BK current (Wasicko et al., 2006). Glomus cell depolarization leads to calcium influx via (6) voltage gated calcium channels and a rapid increase in intracellular calcium levels ([Ca²⁺]_i). (7) The hypoxia-induced increase in [Ca²⁺]_i leads to release of multiple neurotransmitters contained in secretory vesicles (7a) as well as other transmitters or modulators (7b), such as adenosine, GABA, 5HT and others (see Nurse, 2010 for review). At the glomus cell-CSN nerve terminal synapse, evidence supports ATP, adenosine and ACh as excitatory neurotransmitters (8a) causing depolarization of the nerve endings and (10) generation of spiking activity in the CSN. Other released neuromodulators may have inhibitory or facilitatory actions on the CSN nerve endings (8b) (Nurse, 2010). This wide array of neurochemicals, released by hypoxia into the synaptic cleft and into the space between glomus cells and the enveloping type II cells, also may act on a variety of glomus cell autoreceptors (8c) and, depending on the specific ligand-receptor, either enhance or inhibit the [Ca²⁺]_i response to hypoxia by a variety of mechanisms (Bairam et al., 2006; Joseph et al., 2006; Nurse, 2010; Shirahata et al., 2007). Additional potentially important steps in O₂ transduction include roles for CSN nerve terminal Na⁺ channels and for type II cells. Donnelly has proposed that persistent Na⁺ currents in CSN nerve endings (9) may enhance nerve terminal excitability and amplify excitation by neurotransmitters, potentially explaining how a 2–3 fold increase in neurotransmitter release during hypoxia can result in a 20–30 fold increase in single unit CSN activity (Donnelly, 2011). Finally, ATP increases [Ca²⁺]_i in type II cells and may result in release of additional ATP as a “gliotransmitter” as well as release of other modulators (Tse et al., 2012; Zhang et al., 2012). This has led to a recent surge of interest in the glia-like type II cells as synaptic amplifiers of chemotransduction, similar to the role of glial cells in other neural tissues (Butt, 2011).

The carotid body is often portrayed in a reductionist, highly simplified manner for illustrative purposes, but the reality is potentially complicated. It is important to remember that the transduction mechanisms illustrated in figure 1 co-exist in the same cells with multiple other O₂ responsive mechanisms that operate on varying time scales, multiple processes generating reactive oxygen and nitrogen species, multiple anti-toxicity systems to cope with oxidative and nitrosative stress and it is all interwoven within a complicated cellular structure and a reactive (and perhaps interactive) vasculature. In addition, carotid body structural and functional development, with all of its multiple O₂-responsive components, takes place in an O₂ environment that changes markedly over the developmental time span, raising the possibility that the developmental O₂ environment *per se* is critical in shaping CB development and the subsequent “resetting” that occurs after birth.

3.0 Carotid body “resetting” revisited

3.1 Terminology – resetting vs. development

The terms “development” and “maturation” encompass changes that occur in CB structure, neurochemistry, physiology and function from its formation in the embryo to full maturity in the adult. In contrast, terms such as “functional development”, “functional maturation” refer to age-related changes in specific responses such as the magnitude of the neural response to hypoxia or hypercapnia. These terms can be applied to any time frame during fetal or postnatal development and do not necessarily center on the time of birth as a turning point. The popular term “resetting” was first applied to developmental changes in CB O₂ sensitivity almost 30 years ago (Blanco et al., 1984). Most publications, including this review, use the term “resetting” in a highly specific manner, to refer to the increase in CB O₂ chemosensitivity occurring after birth, as a result of birth; the transition from the minimal O₂ responsiveness of the fetus to the adult-like O₂ responsiveness achieved days or weeks after birth. Thus, the term “resetting” in the CB literature refers to a specific change in CB function, centered around a specific moment during development (birth); resetting of the CB O₂ response is complete when it reaches adult-like levels. This does not imply, however, that postnatal CB development stops when resetting is complete. Indeed, changes in neurotransmitters, the capacity for plasticity and other aspects of CB structure or function may continue to change after the response to hypoxia has reached mature levels (Bairam et al., 2012; Wang and Bisgard, 2005).

Although “resetting” is a useful and convenient term when defined as above, it does not convey much meaning in relation to the underlying physiology of resetting. The term “reset” in this context simply means “to set anew” and does not reflect more formal definitions related to loop feedback control systems. New evidence since the initial use of the term “resetting” strongly suggests that postnatal changes in CB O₂ sensitivity can be characterized as environmentally-driven phenotypic plasticity, defined as “the capability of an organism to modify its phenotype in response to environmental changes” (Burggren and Reyna, 2011). Although typically viewed as a developmental change in physiological phenotype, plasticity of CB phenotype after birth may reflect changes at any level including biochemical, metabolic, synaptic, etc. The carotid chemoreceptors exhibit “developmental plasticity”, defined as a modification of normal developmental trajectory due to environmental interactions, as evidenced by the marked blunting of CB function that occurs in chronic hypoxia or hyperoxia during the perinatal period while the same exposures in adult animals will have different effects (Bavis et al., 2012; Burggren and Reyna, 2011; Eden and Hanson, 1987). As even the normal increase in CB O₂ responsiveness occurring at birth is environment-dependent, normal CB functional development may be seen as a special case of “developmental phenotypic plasticity”.

Another troublesome term in the carotid body literature is “sensitivity” to hypoxia. The term is often used loosely to refer to a developmental increase the magnitude of response to a single level of hypoxia, which is better termed “O₂ responsiveness”. Some use the term “sensitivity” to refer specifically to the change in response for a given change in stimulus, often represented by the shape of the graded hypoxia response curve; defining a change in sensitivity as a rightward or leftward shift of the curve and/or an increase in the slope. Most studies of CSN hypoxia response maturation did not use graded hypoxia challenge and therefore cannot report O₂ response sensitivity (Bamford et al., 1999; Donnelly and Doyle, 1994; Kholwadwala and Donnelly, 1992). One study of CB glomus cell hypoxia response maturation used graded challenge and showed a clear increase in slope and rightward shift (increased sensitivity) in the O₂ response curve with age (Fig. 2) (Wasicko et al., 1999). This review will use the term “sensitivity” in referring to studies using graded hypoxia and otherwise use the term “O₂ responsiveness”. Postnatal development of CB O₂ sensitivity has not been adequately studied at the level of neural output.

3.2 Time course

The concept of carotid body functional “resetting” was not always universally accepted. Early studies in human infants clearly showed a postnatal increase in minute ventilation (VE) responses to 100% O₂ or transient hypoxia (Girard et al., 1960). Others concluded that carotid chemoreceptors were fully mature at birth and criticized earlier studies on methodological grounds (e.g. Biscoe and Purves, 1967). Over the next several decades, numerous studies in multiple mammalian species, including humans, confirmed that carotid chemoreceptor responses to hypoxia and other stimuli (eg., cyanide) are not fully mature in the newborn and require time after birth to reach mature levels. Earlier studies were small and tended to use lambs, which are quite mature at birth and undergo rapid postnatal resetting of CB O₂ sensitivity. In species that are less mature at birth, such as the rodents, the carotid chemoreceptors clearly show low O₂ responsiveness at birth and require several weeks to reach maturity (Bamford et al., 1999; Kholwadwala and Donnelly, 1992). Later studies, even using lambs, clearly showed that CB responses to O₂ and cyanide are small immediately after birth and require time (days) to increase to adult-like levels (Bureau and Begin, 1982).

Results from multiple species indicate that the time course of CB functional development after birth may differ considerably between species, probably related to the degree of maturity at birth. In newborn kittens, CB O₂ responsiveness required ~4 weeks to reach adult levels (Carroll et al., 1993). In rats, which are also relatively immature at birth, full postnatal resetting of CB O₂ responsiveness requires ~14–21 days (Fig. 3), while in lambs resetting may occur within three days (Blanco et al., 1984; Kholwadwala and Donnelly, 1992). A study in full-term human infants at two ages, using an unusual approach of measuring the VE response to breath-by-breath alternation of FiO₂, suggested that carotid body resetting is “essentially complete” by 24–48 hours (Calder et al., 1994a). Another study of healthy term human infants, using the transient 100% O₂ test, found no significant response at 2–6 hours of age compared to an ~ 10% decrease in VE in response to 100% O₂ at 2–6 days (Hertzberg and Lagercrantz, 1987), suggesting an increasing contribution of the CB to resting ventilatory drive within days. More studies are needed in human infants to confirm or extend these findings and determine more precisely the time course of human CB resetting, especially in preterm infants, those with lung disease and infants exposed to chronic hypoxia, intermittent hypoxia or hyperoxia.

3.3 Oxygen-dependence of postnatal CB functional development

A seminal research article in the field of CB O₂ sensing development was published in 1988 (Blanco et al., 1988). The authors had previously reported that the carotid bodies were active

and responsive to hypoxia in utero, that CB activity was “silenced” by the rise in PaO_2 at birth, and that “resetting” of CB O_2 responsiveness from the fetal to the adult PaO_2 range required about three days (Blanco et al., 1984). Although the initiation of “resetting” coincided temporally with the rise in P_{O_2} at birth, related factors such as circulatory changes, hormonal, metabolic and other biochemical changes could not be ruled out. In order to eliminate birth *per se* as a variable, they used a method for providing mechanical ventilation to the fetus while still in utero (Blanco et al., 1987). This allowed adjustment of PaCO_2 and PaO_2 prenatally, while the fetus remained in utero with intact placental/umbilical circulation. Three groups of fetal sheep were ventilated in utero with either O_2/CO_2 for 27 hours ($\text{PaO}_2 \sim 180$ mmHg), O_2/CO_2 for ~ 7 hours ($\text{PaO}_2 \sim 230$ mmHg), or N_2/CO_2 for 22 hours ($\text{PaO}_2 \sim 28$ mmHg, control), followed by caesarian delivery and recording of carotid sinus nerve activity. PaCO_2 was maintained within the normal range. The results were striking; CSN responses in the group ventilated with O_2/CO_2 for 27 hours were several-fold larger and right-shifted compared to controls or the short-term hyperoxia ventilation (Blanco et al., 1988). Thus, raising the PaO_2 prenatally initiates “resetting” *in utero*, in the absence of other changes associated with birth. Expressed another way, CB functional “resetting” appeared to be modulated by the change in the O_2 environment.

If CB “resetting” is modulated by the O_2 environment, then experimental manipulation of O_2 during the perinatal period should alter the trajectory of O_2 sensing maturation. Indeed, in another important study it was shown that rearing newborn rats in chronic hypoxia (FiO_2 0.12 or 0.08) abolished development of the early phase of the ventilatory response to hypoxia (driven by CB activity) at 5 and 14 days (Eden and Hanson, 1987). Rats kept in chronic hypoxia for up to 10 weeks eventually developed a ventilatory response to hypoxia (although still abnormal) and “reset” their carotid chemoreceptor responses to hypoxia, suggesting that other factors allow CB functional maturation when chronic hypoxia persists into adulthood. Chronic hypoxia from birth also abolishes the normal postnatal increase in glomus cell $[\text{Ca}^{2+}]_i$ response to hypoxia (section 6.6) (Sterni et al., 1999). Finally, as discussed below, in preterm infants the CB’s are active from at least 28 weeks gestational age and “reset” within several weeks after birth, strongly suggesting that, at least after 28 weeks gestation, timing *per se* is not a factor (Rigatto et al., 1975). Interestingly, infants with hypoxemia, receiving supplemental O_2 showed evidence of marked blunting of O_2 chemoreflexes, suggesting impaired carotid chemoreflex maturation, providing additional clinical support for the concept that CB functional maturation is strongly influenced by the O_2 environment (Calder et al., 1994b; Katz-Salamon et al., 1995).

3.4 CB development as environment-driven phenotypic plasticity

Given the evidence presented in the previous section, it is highly unlikely that the increase in CB O_2 sensitivity at birth reflects a “genetic program” and more likely that CB functional maturation represents environment-driven phenotypic plasticity. This view raises several critically important questions, as follows: a) is hypoxia *per se* an important environmental factor shaping CB maturation in utero; b) what hypoxia-responsive element(s) in the fetus/newborn effect changes in the O_2 transduction cascade in response long-term environmental change; and c) what components of the O_2 transduction cascade are modified during postnatal O_2 response maturation to account for the postnatal increase in O_2 sensitivity? Most studies to date have addressed question “c”, a few have addressed question “a” and only one has addressed question “b”. These are paradigm-defining questions (“a” and “b”) pointing to research directions not yet explored. At the present time, no theory of CB O_2 sensing development addresses all three of these fundamental questions.

4.0 Structural factors in CB functional resetting

4.1. Carotid body ultrastructure development

Numerous studies have characterized CB ultrastructure development during gestation, differentiation of characteristic type I and II cells, development of synapses and other ultrastructural changes in rat, rabbit and other species. These findings have been summarized in several recent reviews and will not be discussed in depth here (De Caro et al., 2012; Hempleman and Pilarski, 2011; Hempleman and Warburton, 2012). With respect to postnatal O₂ response maturation, the important questions are whether ultrastructural changes occur after birth and, if so, do they contribute to functional, postnatal maturation of O₂ sensing. In most species, formation of the carotid body structure and differentiation of distinct cell types occurs by mid- to two-thirds of gestation. In the human fetus at ~ 14–15 weeks gestation, carotid body histology was described as “surprisingly mature”, with well-developed glomus cells, including glomus cells with dense-cored vesicles, type II cells, vascular supply and innervation (Hervonen and Korkala, 1972). Studies in rabbit and rat suggest that glomus cell features, such as the numbers of dense-cored vesicles, may continue to mature after birth and that development of CSN-glomus cell synapses continues to take place postnatally (Bolte et al., 2000; Kariya et al., 1990; Kondo, 1976; von Dalnok and Menssen, 1986). The functional significance of these changes remains unclear. In the rat, postnatal maturation of nerve terminal synapses on glomus cells continued until 50 days postnatal age, which does not match the known time course of postnatal CB resetting, which is ~ 14–21 days (Fig. 3A, B) (Donnelly and Doyle, 1994; Kholwadwala and Donnelly, 1992). Many studies on the ultrastructural and neurochemical development of the carotid body were published 20–50 years ago, with surprisingly few human studies. Additional studies of human CB ultrastructural and neurochemical maturation are needed using advanced, state-of-the-art approaches, particularly focused on the putative components of the chemotransduction cascade during the transitional period at birth and the period of CB functional maturation.

In support of the concept that the human CB is structurally mature long before term, studies in preterm infants indicate that the CB chemoreceptors are functional as early as 28–31 weeks gestation (Rigatto et al., 1975; Rigatto et al., 1982). Infants at ~ 33 weeks gestational age (average birth weight ~ 1500g), studied within the first eight days of life, were found to have immediate ventilatory responses to 100% O₂ identical to adults, strongly suggesting that the CB “functional machinery” is mature by ~75% of gestation in humans and is able to achieve adult levels of activity within a week after preterm birth (Sankaran et al., 1979). Whether CB function matures even earlier in humans remains unknown; studies of CB function in human infants should be updated to reflect the greater degree of prematurity common in neonatal intensive care units in the “rescue surfactant era”.

4.2 Blood flow and perfusion in CB resetting

In the 1980's and early 1990's several studies explored whether vascular changes could explain the postnatal increase in CB O₂ sensitivity. The partial pressure of oxygen was measured in the carotid bodies of fetal sheep during an artificial transition to air breathing, as well as in naturally born lambs. The key finding was that tissue P_{O₂} in the fetal CB was low as expected, but did not increase after birth (or initiation of air breathing) in proportion to the rise in Pa_{O₂} (Acker et al., 1980). This led to speculation that birth was associated either with a decrease in CB perfusion, an increase in CB O₂ consumption, or both. Subsequent studies revealed no change in CB blood flow velocity between the fetus and the newborn, but by 6–7 days of age in the lamb, the maximum blood flow velocity for a given perfusion pressure was reduced (Acker et al., 1991). Additional detailed studies of the vasculature of the sheep and cat CB revealed no anatomical basis for the idea that CB

functional resetting was due to development-related changes in perfusion (Clarke et al., 1990; Moore et al., 1991). The rapidity of postnatal CB resetting in some species (several days) makes anatomical vascular changes unlikely as a factor in resetting, although dynamic vascular reactivity remains a possibility. Perhaps a more potent argument suggesting a minimal role for vascular changes in CB resetting is that postnatal CB resetting can be demonstrated in the superfused (non-perfused) CB preparation in vitro as well as dissociated glomus cells (Kholwadwala and Donnelly, 1992; Wasicko et al., 2006; Wasicko et al., 1999). The question of whether CB O₂ consumption increases after birth remains unanswered. Given the generalized metabolic suppression known to occur in the fetus during gestation, it is plausible to suggest that CB metabolism may also be downregulated in conformance to the low-O₂ fetal environment and may increase after the four-fold rise in PaO₂ at birth.

5.0 Neurotransmitters & modulators in resetting of CB O₂ responsiveness

With so many possibilities for neurochemical modulation of CB function (Fig. 1), it is no surprise that early hypotheses of CB functional development focused on neurotransmitter modulation. The major neurotransmitters potentially involved are ATP, adenosine, acetylcholine and dopamine. In general, neurotransmitter hypotheses of CB development have fallen into one of five groups, as follows: a) development-related changes in excitatory transmitter release; b) expression of post-synaptic neurotransmitter receptors on CSN terminals; c) developmental changes in release/turnover of inhibitory neuromodulators such as dopamine; d) developmental changes in the ratios of glomus cell excitatory vs. inhibitory autoreceptors or e) postnatal changes in interactions between different neurotransmitters.

In testing CB development hypotheses, it is not sufficient to demonstrate a developmental increase in neurotransmitter release. There is overwhelming evidence that the isolated glomus cell response to hypoxia is small in the newborn and increases with age (see section 6) and, therefore, any postnatal increase in transmitter release is likely secondary to maturation of glomus cell O₂ sensing. In other words, a postnatal increase in neurotransmitter release may simply reflect a development-related increase in glomus cell depolarization, [Ca²⁺]_i response and resulting vesicular exocytosis of neurotransmitters. Dopamine (DA) is the best studied of the neurotransmitters with respect to development. Numerous studies have demonstrated postnatal changes in DA content, release, stimulated release in response to hypoxia, DA D₂ receptor expression (D₂ autoreceptors on glomus cells), DA D₂ receptor isoforms and tyrosine hydroxylase expression (see Bairam and Carroll, 2005 for review). As recently reviewed by Bairam et al., (Bairam et al., 2012), ATP and adenosine are released in the CB during hypoxia and extracellular adenosine levels are further increased by degradation of ATP to adenosine. Both of these potent, excitatory neurotransmitters act on receptors expressed by the CSN nerve terminals and contribute to the increased CSN activity in response to hypoxia. Acetylcholine is also released in the CB during hypoxia and may also contribute to CSN excitation, although its role remains controversial and appears to vary considerably by species (Donnelly, 2009; Reyes et al., 2007).

Postnatal age-related changes in purinergic P2X receptor expression on rat CSN nerve terminals were recently reported, while other studies did not show age-related changes in P2X₂ or P2X₃ receptor expression in maturing cats (Bairam et al., 2007; Nunes et al., 2012). Further study in rat CB revealed that purinergic P2X receptor antagonists decreased normoxic ventilation and ventilatory responses to hypoxia, but these effects were not age dependent (Niane et al., 2011). In spite of abundant evidence that the content and release of neurotransmitters in the CB changes with age and that combinations of key

neurotransmitters play important roles in CB synaptic transmission, neurotransmitters have not been shown to be a primary mechanism driving resetting of the CB response to hypoxia.

Similarly, developmental changes in the release of inhibitory neuromodulators have been reported but, to-date, none has been shown to be a significant mediator of CB O₂ sensitivity resetting. Dopamine was an attractive early candidate for study; DA is released by glomus cells during acute hypoxia, it is auto-inhibitory for glomus cells, tyrosine hydroxylase (TH) synthesis and stability of TH mRNA levels in glomus cells are increased by chronic hypoxia, and CB DA content and turnover is high in the fetus and declines after birth (Czyzyk-Krzeska et al., 1994; Hertzberg et al., 1990). The idea was that the low O₂ environment of the fetus would keep inhibitory DA levels high in utero and, at birth, the rise in PaO₂ would cause DA content/turnover to decline and remove the DA-mediated inhibition. This hypothesis was attractive, in part, because it presented a plausible mechanism for modulation by the low O₂ environment of the fetus. However, subsequent studies have not supported significant involvement of DA in CB functional maturation (Bairam and Carroll, 2005; Carroll et al., 2005). Preliminary data from our laboratory suggests that rat newborn glomus cell may be more sensitive to inhibition by ATP compared to P14-18 (Carroll et al., 2012). However, whether this mechanism is involved in CB resetting remains unknown. Finally, postnatal changes in ratios of excitatory vs. inhibitory autoreceptors have been found, but whether there is a link between these changes and CB functional maturation remains unclear. Several recent reviews have detailed development-related changes in neurotransmitters in the CB (Bairam and Carroll, 2005; Bairam et al., 2012; Gauda, 2002; Koos, 2011; Porzionato et al., 2008; Shirahata et al., 2007).

6.0 Developmental profile of isolated carotid body glomus cells

6.1 Isolated glomus cell response to hypoxia

Over twenty years ago it was shown that carotid body glomus cells could be enzymatically dissociated, plated on glass coverslips and studied in a high-flow superfusion system, using epifluorescence imaging methods to measure intracellular calcium under resting and challenge conditions (Biscoe and Duchon, 1989; Biscoe et al., 1989; Buckler and Honore, 2004). It is now generally accepted that the intracellular calcium ([Ca²⁺]_i) response of glomus cells to hypoxia may be used as a valid proxy for the glomus cell response to challenges (Buckler and Vaughan-Jones, 1994). One of the advantages of this approach is that the putative main O₂-sensing element of the CB, the glomus cell, can be studied in isolation without influences of vascular perfusion, humoral modulators, local neurotransmitters, neuromodulators, etc. Due to the low density of cells and the relatively high-flow superfusion with buffered physiological salt solutions, any released neurochemicals will be immediately flushed from the field and not affect other cells. This allows study of the response characteristics of glomus cells, independent of a myriad of potentially confounding factors. Concern that cells in clusters will affect others within the same cluster can be assessed with appropriate pharmacological blockers in most cases.

Although most studies have found that glomus cell [Ca²⁺]_i increases as P_{O₂} is lowered, some have reported that a substantial proportion of glomus cells do not respond to hypoxia (Bright et al., 1996; Hayashida and Hirakawa, 2002). A common feature of these studies is the use of mild hypoxia challenge (superfusate P_{O₂} ~ 35–38 mmHg) which, based on the [Ca²⁺]_i-P_{O₂} relationship from multiple studies, would be expected to cause either a small or no [Ca²⁺]_i rise in dissociated glomus cells (Fig. 2) (Biscoe and Duchon, 1990; Buckler and Vaughan-Jones, 1994; Wasicko et al., 1999). Four studies have reported that [Ca²⁺]_i may increase or decrease in response to hypoxia in glomus cells from adult rat or mouse (Donnelly and Kholwadwala, 1992; Hayashida et al., 2000; Wotzlaw et al., 2011; Zhang and Eyzaguirre, 1999). One of these studies employed imaging of glomus cells in-situ in the

intact, adult mouse CB and the other used minimally-disrupted clusters of glomus cells from adult rats, suggesting that $[Ca^{2+}]_i$ responses of glomus cells in-situ may differ from dissociated cells (Hayashida and Hirakawa, 2002; Hayashida et al., 2000; Wotzlaw et al., 2011). Further study will be required to determine whether such heterogeneity of glomus cell $[Ca^{2+}]_i$ responses is characteristic of the adult, associated with cell-cell interactions or related to methodology or other factors. The key point, with respect to CB maturation, is that all studies to date using ratiometric $[Ca^{2+}]_i$ indicators to study dissociated neonatal rat glomus cells report that hypoxia consistently increases $[Ca^{2+}]_i$ in a graded fashion, similar to the neural response of the whole organ.

6.2 Isolated glomus cell O₂ response maturation mirrors whole organ maturation

Using Fura-2 to estimate intracellular calcium concentration, we showed that CB glomus cells dissociated from adult rabbits exhibited peak $[Ca^{2+}]_i$ responses to hypoxia approximately fourfold larger than responses of glomus cells harvested from 1–2 day old newborn rabbits (Sterni et al., 1995). This finding suggested that factors intrinsic to the glomus cell itself, and its ability to respond to hypoxia, play a role in CB resetting independent of modulating influences (i.e., neurotransmitters) present in the intact CB. The same approach was used to study rat CB maturation and extended to include multiple age groups, from term-fetal to 21 days, and graded responses to hypoxia, which allowed estimation of glomus cell O₂ sensitivity during maturation. The major findings included a) hyperbolic, graded increases in $[Ca^{2+}]_i$ in response to hypoxia similar to the whole organ (Fig. 2), b) postnatal increase in the glomus cell response to hypoxia with age (Fig. 3D), c) response magnitude reaching a plateau by ~14 days of age, and d) a rightward shift in the P_{O₂} response curve, indicating an increase in glomus cell O₂ sensitivity with age (Fig. 2). The $[Ca^{2+}]_i$ response to KCl, a non-specific depolarizing stimulus, did not change with age, indicating that the developmental changes were specific to the O₂ response. The time course of the observed developmental increase in glomus cell O₂ sensitivity matched closely the time course of rat carotid body O₂ response maturation as measured by whole nerve, single unit activity and catecholamine secretion (Bamford et al., 1999; Donnelly and Doyle, 1994; Kholwadwala and Donnelly, 1992), suggesting that maturation of glomus cell O₂ sensitivity accounts, at least in part, for maturation of postnatal CB O₂ response (Fig. 3A–C).

6.3 Mechanisms of O₂ response maturation in isolated glomus cells – TASK channels

Subsequent studies showed that the developmental increase in the glomus cell $[Ca^{2+}]_i$ response to hypoxia was due to a postnatal increase in the magnitude of glomus cell membrane depolarization in response to hypoxia (Wasicko et al., 2006). Buckler and colleagues had shown previously that the main resting K⁺ conductance in rat glomus cells was carried by an O₂-sensitive TASK-like “leak” potassium channel that could initiate depolarization when inhibited by hypoxia (Buckler, 1997; Buckler et al., 2000). We therefore hypothesized that development-related changes in CB glomus cell depolarization were due to increasing O₂ responsiveness of the background TASK-like “leak” conductance. Current clamp studies of dissociated rat glomus cells confirmed that the depolarization response to hypoxia was ~8 mV at P1–3 vs. ~18mV at P11–14, indicating that the same degree of hypoxia challenge caused a much smaller depolarization in cells from newborns compared to mature rats (Fig. 3E). Using the approach employed by Buckler (Buckler, 1997) we confirmed that the O₂-sensitive TASK-like current at resting membrane potential was small at P1–3 and increased several fold by P11–14 (Fig. 3F) (Wasicko et al., 2006). During the same developmental time frame there was no change in glomus cell passive membrane characteristics, suggesting that developmental changes in TASK-like or other channel expression (channels active at resting V_m) were unlikely.

The above findings were interpreted to mean that postnatal maturation of the CB glomus cell response to hypoxia was due to increasing sensitivity to hypoxia, likely mediated by a developmental increase in the O₂ sensitivity of the TASK-like “leak” conductance. At that time, although the background TASK-like conductance had been extensively characterized, its identity was still not precisely known. It was subsequently shown that TASK-1, TASK-3 and TASK-1/3 are all functionally expressed in dissociated CB glomus cells, that the TASK-1/3 heteromer was inhibited by hypoxia and that TASK-1/3 provides most of the O₂-sensitive TASK-like background K⁺ conductance (Kim et al., 2009). This led to the hypothesis that the postnatal increase in CB glomus cell V_m depolarization and [Ca²⁺]_i responses to hypoxia were due to developmental changes in TASK-1/3 O₂ sensitivity. Indeed, we found that hypoxic inhibition of TASK activity was least at P0-1 and increased significantly with postnatal age, mainly between P6-7 and P16-18, supporting the hypothesis that changes in TASK O₂ sensitivity underlie, at least in part, the postnatal increase in glomus cell O₂ sensitivity (Fig. 3G, H) (Kim et al., 2011) (See Kim, 2012 for review).

The mechanisms underlying the postnatal increase in glomus cell TASK-1/3 O₂ sensitivity remain unknown. Available evidence strongly supports the view that inhibition of cell membrane TASK is mediated by mitochondrial O₂-sensing via a signaling pathway between mitochondria and TASK (Buckler and Vaughan-Jones, 1998; Wyatt and Buckler, 2004). Whether mitochondrial-to-TASK signaling is via AMP-Activated Protein Kinase (AMPK), intracellular ATP or other pathways remains to be established (Varas et al., 2007; Wyatt et al., 2007). Hypotheses to explain the above findings, including developmental changes in the O₂-sensitivity of TASK, mitochondrial O₂ sensing and/or signaling between mitochondria and TASKs, are currently being explored in our laboratory.

6.4 Mechanisms of O₂ response maturation in isolated glomus cells – other channels

TASK may not be the only channel involved in the postnatal increase in glomus cell O₂ sensitivity. Another candidate is the O₂-sensitive, calcium-activated, large-conductance K⁺ channel, also referred to as BK or maxi-K⁺, which has been studied extensively in the carotid body (Peers and Wyatt, 2007). A study in P4, P10 and adult rat CB glomus cells showed that the BK component of macroscopic K⁺ current increased between P4 and P10 (Hatton et al., 1997). However, although there is no question that BK channels are important in shaping the glomus cell response to hypoxia, we have not been able to identify a role for BK (maxi-K⁺) channels in postnatal maturation of CB O₂ sensitivity. Initially we tested the effect of the non-specific K⁺ channel inhibitors TEA/4-AP on the [Ca²⁺]_i response of rat glomus cells at P1 and P10-11. TEA/4-AP resulted in an ~50–60% increase in the glomus cell [Ca²⁺]_i response to hypoxia that was not age-dependent, indicating an important role for voltage-activated K⁺ channels in the hypoxia response that did not change significantly with development (Wasicko et al., 2006). We subsequently studied the effect of charybtoxin (CTX), a specific BK channel blocker, on single unit action potential activity of rat chemoreceptor neurons in vitro as well as the [Ca²⁺]_i response of isolated glomus cells, at P2-3 vs. P16-18. Although CTX increased nerve conduction velocity in the older age group and caused a very slight increase in the [Ca²⁺]_i response at both ages, the results overall suggested little or no role for BK channels in mediating development of the CB response to acute, moderate hypoxia (Donnelly et al., 2011).

Voltage-gated potassium channels (KV) have been shown to be O₂-sensitive in rabbit glomus cells and they are expressed in rat glomus cells as well (Ganformina and Lopez-Barneo, 1991; Hatton et al., 1997). A whole-cell patch clamp study of CB glomus cells, from rats at P4, P10 and adult age (>5 weeks), showed that overall K⁺ current density increased with age (Hatton et al., 1997). Hypoxia reversibly inhibited glomus cell K⁺ current and the inhibition was significantly smaller at P4 compared to P10 and adults. Therefore, it is possible that developmental changes in KV channel O₂ sensitivity contribute to glomus

cell O₂ response maturation and their role may vary depending on species. KV channels may not be active at resting membrane potential, and therefore would be unlikely to initiate depolarization. However, their inhibition by hypoxia could enhance the magnitude and/or duration of depolarization (Kim, 2012). The precise role of KV channels in CB functional maturation will require further study, ideally in rat, rabbit and mouse.

Other channels that could be involved in CB development include HERG-like K⁺ channels, TREK potassium channels and ATP-sensitive K⁺ channels (K_{ATP}). The general idea underlying these studies was that CB glomus cells may express non-O₂-sensitive K⁺ channels that could damp the depolarization response mediated by TASK. If such channels were expressed in the fetus/newborn and then decrease their expression with age, the “damping” influence on depolarization would diminish with age, resulting in a developmental increase in glomus cell response to hypoxia. The most promising of these possibilities has been the HERG-like K⁺ channel, originally reported in rabbit glomus cells (Overholt et al., 2000). HERG K⁺ channels in other cell types play a role in controlling repolarization and maintaining resting membrane potential (reviewed in Carroll and Kim, 2005). Briefly, we used the HERG channel blocker E-4031 to study its role in CB glomus cells from P0-1 vs. P11-16 rats. E-4031 resulted in an increased depolarization response as well as [Ca²⁺]_i response to hypoxia in glomus cells from newborn but not mature rats, suggesting that HERG-like currents were damping newborn, but not mature glomus cell responses (Kim et al., 2005). The effect was modest, suggesting that HERG-like currents may play a small role in glomus cell functional development.

TREK channels are large-conductance K⁺ channels relative to TASK which, if activated, could damp hypoxia-induced depolarization mediated by TASK (assuming that TREK is non-O₂-sensitive). Interestingly, using free fatty acids, membrane stretch and acid to activate TREK channels (which are normally inactive in cell-attached patches) we found TREK activation in about 30% of patches from P0-1 rats compared with 0% at P16-18 (Kim, 2012). Thus, TREK channels are functionally expressed by CB glomus cells and this expression appears to decline after birth. Never-the-less, because TREK is normally not active unless stimulated by mechanical stretch, etc., TREK channels are unlikely to play a role in functional glomus cell O₂ response maturation. TREK, KV, K_{ATP}, K_{ir} and other K⁺ channels in CB development have recently been reviewed in detail and will not be further discussed here (Kim, 2012).

6.5 Studies on TASK knockout mice – implications for development

Although postnatal development of TASK O₂ sensitivity in glomus cells is a major proposed mechanism underlying CB functional maturation, the role of TASK channels in CB oxygen sensing remains controversial due to several studies employing TASK knockout mice (Ortega-Saenz et al., 2010; Trapp et al., 2008). The ventilatory response to 10% O₂ challenge was reduced in TASK-1 knockout mice but not in TASK-3 knockout mice (Trapp et al., 2008). Neither TASK-1 nor TASK-1/3 knockout reduced the response of the mouse CB to hypoxia, as measured by catecholamine secretion, leading to the conclusion that the carotid bodies of TASK-1/3 deficient mice were still capable of mounting a potent response to hypoxia indistinguishable from controls (Ortega-Saenz et al., 2010). TASK-1 and/or TASK-3 knockout, however, did alter CB function. The peak response of single-unit activity recorded from the carotid sinus nerves of TASK-deficient mice was reduced in TASK-1 and TASK-3 knockout mice and baseline CSN single unit activity in normoxia was elevated in TASK-3 knockout mice, suggesting that lack of TASK-3 may have altered resting glomus cell excitability (Trapp et al., 2008). Glomus cells from TASK-1/3 knockout mice had higher cell membrane resistance and were partially depolarized in normoxia compared to wild-type controls, suggesting a role for the TASK-1/3 heteromer in modulation of glomus cell excitability (Ortega-Saenz et al., 2010). In addition, TASK-1/3 knockout mice had other

inexplicable findings such as decreased peak macroscopic K^+ current, markedly decreased Ca^{2+} current and decreased mRNA expression of BK channels (Ortega-Saenz et al., 2010).

None of the TASK knockout studies to date have identified the mechanisms responsible for the “preserved” hypoxia response in TASK-deficient mice and none of them have quantitatively characterized glomus cell responses to hypoxia at the level of $[Ca^{2+}]_i$ and cell membrane depolarization. The one study that examined neural responses of the CB to hypoxia found them to be reduced in TASK-3 and TASK-1/3 knockout mice (Trapp et al. 2008). More importantly for development, no study has yet examined postnatal CB functional maturation in a TASK knockout mouse model. The studies outlined in the previous section clearly show that O_2 -sensitive TASK-1/3 is the predominant resting K^+ conductance in rat CB glomus cells and its O_2 -sensitivity is low in newborns and increases with age, following the same time course as whole organ O_2 -response maturation. TASK-deficient mice likely exhibit compensatory expression of other O_2 -sensitive K^+ channels that replace the normal function of TASKs. Studies are needed to elucidate the mechanisms of O_2 sensing in the carotid bodies of TASK-deficient mice before the issue can be fully resolved.

6.6 Correlation of developmental changes in glomus cell O_2 sensitivity with overall CB functional maturation

There is now a strong body of evidence at the levels of glomus cell $[Ca^{2+}]_i$, membrane depolarization, O_2 -sensitive background K^+ current and TASK O_2 sensitivity, that CB glomus cells undergo major postnatal functional maturation (Fig. 3). If glomus cells are a primary site of O_2 sensing within the CB, as widely believed, and their response to hypoxia is minimal in the newborn and increases multi-fold with age, then a role for glomus cell development in intact CB maturation seems obvious. Never-the-less, the evidence supporting a central role for glomus cells in overall CB maturation remains circumstantial. This is because it is extremely difficult to study glomus cell maturation in situ and, therefore, all studies of glomus cell maturation to date have been performed on enzymatically dissociated cells which, as noted above, may not behave identically to glomus cells in situ. The strongest inferential evidence falls mainly into two categories, as follows: a) correlation of the time course of glomus cell maturation with the time course of whole organ neural response maturation and b) correlation of glomus cell function with whole organ function and maturation in models of altered development such as chronic hyperoxia or hypoxia from birth.

In the rat, which is the most extensively characterized model of CB development, the time course, direction and magnitude of changes at every step in the O_2 transduction cascade aligns well with other components of the cascade and overall organ functional maturation (Fig. 3). Exposure to chronic hyperoxia during the perinatal period results in severe blunting (reduction) of the ventilatory response to hypoxia and the peak single unit chemoreceptor discharge frequency in response to acute hypoxia (Bavis et al., 2012; Donnelly et al., 2005). Exposure of neonatal rats to hyperoxia (FiO_2 0.6) beginning on day P7 resulted in an ~ 80% reduction in the peak single unit chemoreceptor response to hypoxia within four days, which was matched by nearly complete loss of the CB catecholamine response to hypoxia and >90% reduction in the $[Ca^{2+}]_i$ response to hypoxia, all following an identical time course (Donnelly et al., 2009). On the other end of the spectrum, exposure of newborn rats to chronic hypoxia from birth (FiO_2 0.13–0.15) completely abolished normal development of the ventilatory response to hypoxia up to P14 (Eden and Hanson, 1987). In a separate study of similar design, exposure of rats to chronic hypoxia (FiO_2 0.12) from birth abolished the normal developmental increase in the CB glomus cell $[Ca^{2+}]_i$ response to hypoxia during a similar time frame (Fig. 4) (Sterni et al., 1999). Unfortunately, matching CB neural responses to acute hypoxia have not been measured during the first two weeks of chronic

hypoxia exposure from birth. Never-the-less, in studies to date, whenever chronic alterations in the O₂ environment impair or abolish normal maturation of the ventilatory response to hypoxia, CB neural response to hypoxia or both, the glomus cell [Ca²⁺]_i responsiveness to acute hypoxia tracks these changes well with respect to magnitude and time course.

7.0 Possible role for type II cells in CB chemoreception?

Recently there has been a resurgence of interest in the type II cells of the carotid body. Although described over a century ago, the function of these glia-like cells remains unknown and they have received comparatively little study. As outlined in section 2.0 and shown in figure 1, ATP released by CB glomus cells is excitatory at CSN nerve terminals and auto-inhibitory for the glomus cell (see Bairam et al., 2012; Nurse, 2010 for review). In sharp contrast, ATP triggers a large, transient [Ca²⁺]_i rise in CB type II cells, reportedly via purinergic P2Y₂ receptors (Xu et al., 2003). Glial cells in other systems are known to amplify synaptic transmission by releasing additional ATP and a similar hypothesis was recently advanced by several authors for the CB, although evidence that type II cells release ATP was lacking (Nurse, 2010; Tse et al., 2012). This model (Fig. 1) proposes that ATP release by glomus cells acts as an excitatory transmitter on CSN terminals via P2X₂ and P2X₃ receptors, but also acts via P2Y₂ receptors to stimulate a rise in [Ca²⁺]_i in type II cells and subsequent release of additional ATP via pannexin-1 (Panx-1) channels (Zhang et al., 2012). This hypothesis is new and much more work is needed to confirm its validity. It is discussed briefly here because, if the model is correct, it would provide yet another synaptic amplification mechanism that could change with postnatal carotid body maturation.

8.0 Role of O₂ environment in shaping CB development

8.1 The O₂ environment during development

At the time of implantation, intrauterine oxygen level is quite low, for example 11–14 mmHg in the rhesus monkey, 24 mmHg in the rabbit and in two studies in humans, O₂ levels in the uterus averaged 18.9 mmHg and 15 mm Hg (Fischer and Bavister, 1993; Ottosen et al., 2006). Such low O₂ values create a favorable environment for the embryo around the time of implantation by minimizing ROS formation and keeping metabolism at low levels (Burton, 2009). Development of the placenta takes place under approximately the same O₂ conditions. During the first trimester, the P_{O₂} in the intervillous space has been measured at ~ 20 mmHg (Burton, 2009; Schneider, 2011) and the maternal-placental circulation is not established until ~ 10–11 weeks, near the end of the first trimester. By about 12 weeks the maternal placental circulation becomes fully established and intervillous O₂ tension has risen to about 60 mmHg. This period, known as the “oxygen transition” is a time of major oxidative stress for the developing fetus. During the remainder of the gestational period, the average intervillous O₂ concentration gradually declines from 60 mmHg to about 40 mmHg at term, as feto-placental O₂ consumption progressively increases (Burton, 2009; Schneider, 2011). During the first trimester of gestation, the low oxygen environment is important for normal development of the placenta and embryo; premature onset of maternal placental blood flow is associated with oxidative stress, early pregnancy loss and other adverse outcomes (Pringle et al., 2010). Similarly, the oxygen transition and associated ~ 3 fold rise in intervillous P_{O₂} at ~ 12 weeks has important morphogenic effects; correct timing of these changes in O₂ levels is crucial in order to achieve a critical balance between pro- and anti-oxidative mechanisms (see Burton, 2009; Pringle et al., 2010; Schneider, 2011 for review).

In humans, once the fetal circulation is established, the Pa_{O₂} is estimated to be ~ 18–25 mmHg, averaging ~ 23 mmHg, based on studies of other mammalian species. This is approximately equivalent to the conditions an adult would experience on the summit of

Mount Everest (8,848 meters), leading Sir Joseph Barcroft to describe the fetal O₂ environment as “Everest in utero” (Martin et al., 2010). In this environment the fetus not only survives, but also thrives and grows due to a remarkable array of metabolic and other fetal and placental adaptations that are beyond the scope of this review (see Pringle et al., 2010; Richardson and Bocking, 1998; Schneider, 2011). The important point, with respect to carotid body development, is that the fetal CB develops in and is maintained in a low oxygen environment until birth. Carotid body tissue P_{O₂} and Pa_{O₂}, measured using microelectrodes in sheep fetuses during the last days of gestation, averaged 17 ± 5 mmHg (mean $\pm$ SD) and 20–28 mmHg respectively (Acker et al., 1980). In exteriorized fetal lambs, with a Pa_{O₂} of ~ 25 mmHg, carotid chemoreceptor activity in a few-fiber preparation averaged ~ 5 Hz and increased as Pa_{O₂} was lowered (Blanco et al., 1984). Thus, although the fetal CB develops in a low oxygen environment and exhibits a low tissue P_{O₂}, the level of activity is similar to “baseline” for the mature state; the same Pa_{O₂} of 25 mmHg after birth, will elicit a much larger response once postnatal resetting has occurred.

If hypoxia is defined, as suggested by Ward, as “a condition in which failure of either delivery or use of O₂ limits normal tissue function”, then the fetus is not “hypoxic” (Ward, 2008). Due to the extensive feto-placental adaptations leading to tolerance of low O₂ conditions, there is no evidence that lack of O₂ availability limits normal fetal growth or development. As pointed out in a recent review, the low O₂ environment of the fetus is not simply an interesting epiphenomenon, it is necessary for activation of numerous genes with critical developmental functions (Park et al., 2010). With respect to CB development, essentially nothing is known about the role of O₂ as a morphogen during development. To what extent does the low O₂ environment of the fetus shape CB maturation? Given the numerous O₂-responsive elements that regulate gene transcription, protein translation, protein synthesis, etc. during development, it would be surprising if one or several do not play a role in CB functional maturation. Although the numerous mechanisms of fetal tolerance to low O₂ conditions are beyond the scope of this review (see Gorr et al., 2010; Richardson and Bocking, 1998; Singer, 1999 for review) one of them, hypoxia-inducible factor (HIF), has been explored in carotid body development and will be discussed here, although others may play a role as well (Roux et al., 2005).

8.2 HIF in development and potential role in CB functional maturation

Hypoxia-inducible factors, are heterodimeric proteins consisting of an HIF- α subunit, the level of which depends on O₂, and a HIF- β subunit (also known as aryl hydrocarbon receptor nuclear translocator, ARNT), which is constitutively expressed and non-O₂-dependent. The HIF system includes three HIF- α subunit isoforms (HIF-1 α , HIF-2 α and HIF-3 α) and several paralogues of the HIF- α subunit (ARNT1, ARNT2 and ARNT3) (Loboda et al., 2010). The HIF-1 α and HIF-2 α subunits are able to dimerize with HIF-1 α under low O₂ conditions and mediate numerous critical cellular responses to hypoxia, while the role of HIF-3 α remains unclear.

HIF-1 α is constantly produced by all nucleated mammalian cells and, under conditions of normoxia, has a very short half-life (< 5 minutes). In normoxia, HIF-1 α is hydroxylated continuously via O₂-dependent prolyl hydroxylation, leading to rapid proteosomal degradation. Under low O₂ conditions, O₂-dependent degradation ceases and HIF-1 α protein levels rise rapidly and stabilize. HIF-1 α translocates to the nucleus, where it dimerizes with HIF-1 α and binds to the hypoxia response elements (HRE) of HIF-regulated target genes, altering transcription of hundreds of genes, many of which are present in the carotid body (Semenza, 2007). HIF can also be activated by non-O₂-dependent mechanisms including growth factors, hormones and cytokines (Patel et al., 2010). Critical processes under the control of HIF-1 α include cellular metabolism, erythrocytosis, pulmonary vascular remodeling, placental development, branching morphogenesis, fetal vascularization

of the placenta, trophoblast proliferation and differentiation, heart development, vascular endothelium development, endocardial cushion formation, migration of neural crest cells, endochondrial bone formation, chondrogenesis and many more (Dunwoodie, 2009; Patel et al., 2010; Semenza, 2004).

HIF-2 α , while similar in structure and regulation to HIF-1 α , differs markedly with respect to its pattern of expression. In contrast to HIF-1 α , which is expressed in every nucleated mammalian cell, expression of HIF-2 α is tissue-specific, cell-type specific within tissues and stabilization of HIF-2 α protein may occur with milder hypoxia compared to HIF-1 α (Loboda et al., 2010; Wiesener et al., 2003). In addition, although HIF-1 α and HIF-2 α are similar in many respects, they are not functionally redundant and may even exhibit opposite effects; e.g., HIF-1 α and HIF-2 α exert opposite effects on the angiogenic response to hypoxia and appear to have opposite effects on carotid body O₂ sensitivity (see below) (Loboda et al., 2012; Peng et al., 2011).

As noted above, postnatal resetting of CB O₂ sensitivity is clearly O₂-dependent, although the mechanism remains completely unknown. What element in the CB is responding to the low O₂ environment of the fetus and what is being regulated? At birth, what hypoxia-responsive element is responding on a time-scale of days/weeks to the ~ four-fold rise in PaO₂ at birth and what resulting changes are leading to a multi-fold rise in the O₂ responsiveness of the carotid body? Given its role in mammals as a master regulator of the O₂ response in every nucleated cell, HIF may be a prime candidate for this role.

Only a few studies have examined the role of HIF in carotid body function and only one addressed a possible role for HIF in CB development. Homozygous HIF-1 α ^{-/-} knockout is fatal whereas partial knockout (HIF-1 α ^{+/-}) results in mice with a superficially normal phenotype under normoxic conditions (Kline et al., 2002). Adult HIF-1 α ^{+/+} mice exhibit reduced erythropoiesis and abnormal pulmonary vascular remodeling in response to chronic hypoxia. The ventilatory response to hypoxia developed normally in HIF-1 α ^{+/+} mice, although it could be largely eliminated by vagotomy, and therefore was unlikely to be mediated by the carotid bodies. Carotid bodies from HIF-1 α ^{+/+} mice responded to cyanide but not to hypoxia (Kline et al., 2002). The preserved response to cyanide in HIF-1 α ^{+/+} mice suggests that their mitochondrial - cell membrane TASK current axis was intact (Wyatt and Buckler, 2004). Unfortunately, O₂ sensing mechanisms in the CB of these mice have not been further characterized. Interestingly, partial deficiency of HIF-2 α resulted in marked augmentation of carotid body sensitivity to acute hypoxia as well as breathing pattern instability, increased apnea and hypertension, which were prevented by systemic administration of a membrane permeable antioxidant (Peng et al., 2011). This was interpreted to mean that redox regulation by HIF-2 α is essential for normal carotid body function and, therefore, for cardiorespiratory stability and homeostasis.

If elevated HIF-1 α levels in utero contribute to the low O₂ responsiveness of the fetal CB, it might be expected that partial deficiency of HIF-1 α would result in more mature chemoreceptor function at birth. The ages of partially deficient HIF-1 α and HIF-2 α mice studied by Peng et al. were 10–15 weeks old and 7–9 months old (adult) and no studies, to date, have examined CB function in HIF-deficient mice at birth (Peng et al., 2011; Peng et al., 2006). In addition, HIF-1 α regulates so many genes critical for normal development as well as genes with critical functions in the CB that it would be difficult to predict the numerous effects of a partial HIF-1 α deficiency being present throughout gestation. Although both studies using partial HIF knockout mice raise interesting questions, neither addressed the role of HIF's in CB functional maturation and their relevance to development, if any, is unclear.

In the one developmental study to-date, HIF-1 α and HIF-2 α protein levels were studied, using immunostaining and densitometry, in the rat CB at E20, P1, P2, P7, P14. At P40, CB HIF levels were measured in normoxia and after a 6 hour exposure to hypoxia (Roux et al., 2005). As expected, in the P40 rats (adult-age), exposure to hypoxia caused a marked, multi-fold increase in HIF-1 α levels. With respect to development, HIF-1 α levels were elevated at E20, P1 and P2 and declined significantly at P7 and P14, which matches the time course for maturation of the rat CB response to hypoxia (Fig. 3A, B) (Kholwadwala and Donnelly, 1992; Roux et al., 2005). Expression of HIF-2 α was restricted to CB glomus cells and did not change with age. Unfortunately, this has been the only study of HIF in the developing CB and the most that can be said is that the time course of changes in HIF-1 α levels correlates with the time course of CSN hypoxia response maturation. Further study will be required to determine whether HIF has a role in controlling CB O₂ sensitivity maturation.

One of the most intriguing observations regarding HIF and CB maturation is the time course of the HIF decline after birth. In general, the turnover of HIF protein is extremely rapid; upon switching from hypoxia to normoxia, HIF levels typically decline within minutes. Why were HIF-1 α protein levels unchanged even on P2, > 24 hours after birth? One possible explanation may relate to birth-induced non-O₂ dependent factors that also modulate HIF-1 α levels such as cytokines, angiotensin, and growth factors (Roux et al., 2005). A possible role for HIF-1 α has been studied in another O₂-sensitive tissue, the pulmonary artery smooth muscle cell (PASMC) (Resnik et al., 2007). Interestingly, in that system, HIF-1 α protein levels were O₂-dependent in adult but not in fetal PASMC, while HIF-1 α mRNA expression was O₂-dependent in fetal PASMC but not in adults. The same authors also found that the expression of prolyl-hydroxylases as well as regulators of HIF-1 α transcriptional activity were developmentally regulated (Resnik et al., 2007). The main implication of these findings is that developmental changes in HIF may be complicated and HIF regulation in utero may differ from the mature condition in unexpected ways. Further study in the developing carotid body will be required to establish the plausibility of HIF as a controller of CB functional maturation.

9.0 Concluding remarks

There is probably not a single mechanism explaining postnatal maturation of carotid body O₂ sensitivity; there are likely multiple mechanisms acting in concert or on different time scales. Vascular and structural changes after birth appear to play no or only a minor role. Changes in neurotransmitters occur, but appear to be secondary and have not been shown to modulate CB functional resetting. Given the large developmental changes in glomus cell function that occur at every level studied to date (Fig. 3D–H), and the tight correlations between glomus cell maturation and overall organ maturation, it is tempting to ascribe CB functional maturation mostly to maturation of glomus cell O₂ sensitivity. However, this would be premature. As noted above, postnatal changes in nerve terminal excitability may play a significant role in CB maturation (difficult to prove at this time) and more work is needed to understand this important component of the chemosensory unit. Even if CB “resetting” does occur primarily at the glomus cell level, fundamental questions about glomus cell O₂ sensors, their maturation and possible modulation by environmental O₂ levels remain unanswered.

At the present time, progress in understanding basic mechanisms of carotid body functional maturation is hampered by a lack of knowledge about O₂ mechanisms in general. Although it is clear that mitochondria and possibly other O₂-sensitive elements play a role in glomus cell O₂ sensing, fundamental questions remain unanswered. The mechanism by which mitochondria signal cell membrane TASK channels remains controversial. It is difficult to further probe mechanisms of postnatal development at this level, when O₂ mechanisms are

unknown generally, even for adults. As TASK O₂ sensitivity has been linked to mitochondrial function, CB development may be related to postnatal changes in mitochondrial O₂ sensitivity, perhaps by development-dependent changes in or modulation of cytochrome c oxidase and/or its affinity for O₂. If so, what is the O₂-dependent element responding to the large rise in PaO₂ at birth and mediating changes in mitochondrial O₂ sensing after birth? At this time, virtually nothing is known about development of CB mitochondrial function.

Whatever the mechanisms of carotid body functional resetting are, they likely reflect adaptation to the low O₂ environment of the fetus, rather than age *per se* or a genetic developmental “program”. Given the paucity of knowledge in this area, all oxygen-responsive adaptive mechanisms present in CB glomus cells are potentially important candidates for further study. For example, an important mechanism of HIF-1 α adaptation to hypoxia is via downregulation of mitochondrial metabolism (Papandreou et al., 2006). HIF-1 α regulates cytochrome c oxidase (COX) subunit composition, in an O₂-dependent fashion, in order to regulate the efficiency of cellular respiration (Fukuda et al., 2007). HIF-1 α also regulates expression of genes encoding inducible nitric oxide synthase, heme oxygenase and there are numerous other O₂-dependent transcriptional regulatory factors in addition to HIF (Haddad, 2002). Moreover, little is known about potential roles of ROS, nitric oxide and other oxygen-responsive elements in CB functional maturation, and the mechanisms underlying the oxygen-dependence of CB development remain a complete mystery.

Historically, developmental studies of O₂ sensing have tended to build on existing knowledge of mature mechanisms. At this point in time, in the field of CB O₂ sensing, developmental aspects of “known mechanisms” have largely been explored. Developmental CB researchers have reached the limits of mechanistic knowledge in this field and, in order to advance, must explore uncharted areas for new insights. Indeed, it is possible that developmental studies, moving forward, can inform the field in general; understanding how CB O₂ sensing develops may be a pathway to understanding O₂ sensing at the most basic levels.

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Figure 1.

Simplified depiction of current concepts in CB O₂ transduction. See text for explanation.

Abbreviations: 5HT-r, 5-hydroxy-tryptamine receptor; Ade, adenosine; ATP, adenosine triphosphate; A2a-r, adenosine A2a receptor; BK, calcium-activated large-conductance K⁺ channel (maxi-K⁺); CO, carbon monoxide; D2-r, dopamine D2 receptor; HO-2, heme oxygenase-2; GABA_b-r, gamma-amino butyric acid receptor subtype b; Nic-r, nicotinic ACh receptor; M1-r, muscarinic ACh receptor subtype 1; M2-r, muscarinic ACh receptor subtype 2; P2X-r, P2X purinergic receptor; P2Y-r, P2Y purinergic receptor; TASK-1/3, TWIK-associated Acid-Sensitive K⁺ channel, heterodimer of TASK-1 and TASK-3; VGCC, voltage-gated calcium channels; V_m depol, cell membrane depolarization.

Figure 2.

Mean α $[Ca^{2+}]_i$ responses of CB glomus cells from 7 age groups plotted vs. superfusate PO_2 .

Age groups were term fetal (●), P1 (□), P3 (▲), P7 (▽), P11 (◇), P14 (■) and P21 (▼).

There is a clear rightward shift in the $[Ca^{2+}]_i$ O_2 response curves with age. Modified from (Wasicko et al., 1999); used with permission.

Figure 3.

Development of rat glomus cell response to hypoxia at multiple levels (mean ± SEM, re-plotted from original references). (A) Whole nerve CSN recording (Bamford et al., 1999). (B) Single unit recording (Donnelly and Doyle, 1994; Kholwadwala and Donnelly, 1992). (C) Tissue catecholamine level (Donnelly and Doyle, 1994). (D) Intracellular calcium (Bamford et al., 1999; Wasicko et al., 1999). (E) Cell membrane depolarization (Wasicko et al., 2006). (F) Hypoxic inhibition of background K⁺ conductance (Wasicko et al., 2006). (G) Relative portion of TASK activity inhibited by hypoxia (Kim et al., 2011). (H) O₂ level (%) at half-maximal inhibition of TASK activity (Kim et al., 2011).

Figure 4.

Mean peak $[Ca^{2+}]_i$ responses to hypoxia in glomus cell clusters at 3 (A), 11 (B) and 18 days (C) from rats reared in chronic hypoxia (■) vs. room air controls (○). (C) includes data from a recovery group (Δ) that were reared in chronic hypoxia until P11 and then returned to room air until P18. At all three ages, glomus cells from chronic hypoxia rats failed to respond to hypoxia and were significantly different from controls and the recovery group (*). Modified from (Sterni et al., 1999); used with permission.