J Physiol 591.9 (2013) pp 2245–2257

The Journal of Physiology

TOPICAL REVIEW

Sensing hypoxia: physiology, genetics and epigenetics

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Abstract The carotid body is a sensory organ for detecting arterial blood O_2 levels and reflexly mediates systemic cardiac, vascular and respiratory responses to hypoxia. This article presents a brief review of the roles of gaseous messengers in the sensory transduction at the carotid body, genetic and epigenetic influences on hypoxic sensing and the role of the carotid body chemoreflex in cardiorespiratory diseases. Type I (also called glomus) cells, the site of O_2 sensing in the carotid body, express haem oxygenase-2 and cystathionine-γ-lyase, the enzymes which catalyse the generation of CO and H_2S, respectively. Physiological studies have shown that CO is an inhibitory gas messenger, which contributes to the low sensory activity during normoxia, whereas H_2S is excitatory and mediates sensory stimulation by hypoxia. Hypoxia-evoked H_2S generation in the carotid body requires the interaction of cystathionine-γ-lyase with haem oxygenase-2, which generates CO. Hypoxia-inducible factors 1 and 2 constitute important components of the genetic make-up in the carotid body, which influence hypoxic sensing by regulating the intracellular redox state via transcriptional regulation of pro- and antioxidant enzymes. Recent studies suggest that developmental programming of the carotid body response to hypoxia involves epigenetic changes, e.g. DNA methylation of genes encoding redox-regulating enzymes. Emerging evidence implicates heightened carotid body chemoreflex in the progression of autonomic morbidities associated with cardiorespiratory diseases, such as sleep-disordered breathing with apnoea, congestive heart failure and essential hypertension.

(Received 1 November 2012; accepted after revision 4 March 2013; first published online 4 March 2013)

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Abbreviations CBS, cystathionine β-synthase; CSE, cystathionine-γ-lyase; HIF, hypoxia-inducible factor; HO, haem oxygenase; HVR, hypoxic ventilatory response; IH, intermittent hypoxia; LTF, long-term facilitation; ROS, reactive oxygen species.

Introduction

Oxygen is an essential substrate for generating ATP, which is a major source of energy in mammalian cells. Vertebrates evolved complex respiratory and cardiovascular systems to insure optimal O_2 delivery to tissues to maintain energy homeostasis. All mammalian cells respond to decreased O_2 availability or hypoxia, albeit to different degrees. The systemic cardiorespiratory responses to hypoxia are reflex in nature and are initiated by specialized sensory organs called 'peripheral chemoreceptors', which monitor changes in arterial blood O_2 levels. Heymans & Heymans

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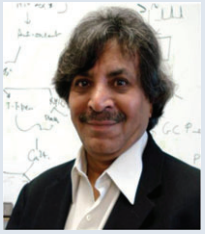

DOI: 10.1113/jphysiol.2012.247759

(1927) were some of the first to report that stimulation of breathing by hypoxia is a reflex triggered by the carotid body, and they proposed the existence of similar structures in the aortic arch (aortic bodies) (Heymans *et al*. 1931). A subsequent study by Comroe (1939) provided firm evidence for reflex stimulation of breathing by aortic bodies as well. For the discovery of the sensory nature of the carotid body, C. F. Heymans received the Nobel Prize in Physiology in 1938, while J. H. Comroe Jr was given an honorary doctorate from the Karolinska Institute, Stockholm for elucidating the functional role of aortic bodies. Tissues with morphology similar to carotid and aortic bodies have also been described in the thorax and abdomen, and might serve as ancillary chemoreceptors (Deane *et al.* 1975; Easton & Howe, 1983).

Much of the information on the mechanisms of hypoxic sensing by the peripheral chemoreceptors has come from the studies on the carotid body. Innumerable studies have investigated how the carotid body detects hypoxia. An account of these early studies can be found in previous reviews (Fidone & Gonzalez, 1986; Gonzalez *et al.* 1994; Prabhakar, 2000). A more comprehensive and contemporary analysis of the structure and function of the carotid body and the physiological significance of the chemoreflex is available in a recent review (Kumar & Prabhakar, 2012). The present article focuses on recent studies addressing the following factors: (i) the roles of gaseous messengers in the hypoxic sensing by the carotid body; (ii) modulation of hypoxic sensing by genetic and epigenetic factors; and (iii) the role of the carotid body chemoreflex in cardiorespiratory diseases.

Physiology of carotid body hypoxic sensing

Carotid bodies, which reside bilaterally in the bifurcation of the common carotid arteries, receive sensory innervation from a branch of the glossopharengeal nerve called the 'carotid sinus nerve'. The sensory discharge frequency of the carotid sinus nerve is low during normoxia (arterial P_{O_2} ~100 mmHg), but increases dramatically with even a modest drop in arterial P_{O_2} (e.g. P_{O_2} 80–60 mmHg). The sensory response to low oxygen is rapid and occurs within seconds after the onset of hypoxia. The remarkable sensitivity and the speed with which it responds to hypoxia make the carotid body a unique sensory receptor for monitoring changes in the arterial blood P_{O_2}. The chemoreceptor tissue is composed of two major cell types, called type I (also called glomus) cells and type II cells. A substantial body of evidence suggests that type I cells are the initial sites of hypoxic sensing, and they work in concert with the nearby sensory nerve ending as a 'sensory unit' (see Kumar & Prabhakar, 2012 for references). The general consensus is that hypoxia inhibits certain K^+ channels in type I cells, and the resulting depolarization leads to Ca^{2+}-dependent release of excitatory neurotransmitter(s), which stimulates the nearby sensory nerve ending, leading to an increase in sensory discharge (Fig. 1). Despite intensive investigations, neither the mechanism(s) by which hypoxia inhibits K^+ channels nor the identity of the chemical messenger(s) mediating the sensory excitation by low O_2 are certain.

Role of gaseous messengers in hypoxic sensing

Nearly four decades ago, Brian Lloyd, Daniel Cunningham and their colleagues from the University of Oxford made an intriguing observation that brief inhalation of carbon monoxide inhibits ventilatory stimulation by hypoxia in conscious human subjects (Lloyd *et al.* 1968). Given that the affinity of haemoglobin for CO is much greater than for O_2, it was proposed that the deoxy- conformation of a haem-containing protein(s) might be critical for initiating the carotid body response to hypoxia. Interestingly, CO, which was once used as an experimental tool to understand hypoxic sensing by the carotid body, is now known to be produced by the same chemoreceptor tissue. The following section provides a brief summary of our current understanding of the role of endogenous CO in the carotid body.

Carbon monoxide contributes to the low sensory activity of the carotid body during normoxia. Carbon monoxide is generated during degradation of haem by haem oxygenases (HOs), with NADPH and cytochrome P450 reductase as cofactors (Maines, 1997). Molecular O_2 is essential for enzymatic generation of CO. The affinity (K_m) of O_2 for the haem–HO complex ranges between 30 and 80 μM (Migita *et al.* 1998). Two HO isoforms have been identified, namely a constitutively expressed HO-2 and an inducible HO-1 (also called heat shock protein 32 or Hsp32; Maines, 1997). Haem oxygenase-2 is expressed in type I cells of cat, rat (Prabhakar *et al.* 1995), mouse (Ortega-Sáenz *et al.* 2006) and human carotid bodies (Mkrtchian *et al.* 2012), but not in the nerve fibres and type II cells (Prabhakar *et al.* 1995). Haem oxygenase-1 expression is not evident in the carotid body in basal conditions.

The following evidence suggests that endogenous CO is a physiological inhibitor of carotid body activity. Zinc protoporphyrin-9, an inhibitor of HO, increases carotid body sensory activity in a dose-dependent manner, whereas copper protoporphyrin-9, which does not inhibit HO activity, has no influence (Prabhakar *et al.* 1995). Haem oxygenase-2 knockout mice exhibit elevated baseline carotid body sensory activity and an augmented sensory response to hypoxia (Prabhakar, 2012). Exogenous application of low concentrations of CO, similar to O_2, inhibits the carotid body activity

(Prabhakar *et al.* 1995). Disruption of HO-2 function in type I cells, like hypoxia, inhibits the maxi-K^+ channel activity (Riesco-Fagundo *et al.* 2001; Williams *et al.* 2004) and elevates $[Ca^{2+}]_i$ (Prabhakar, 1998). Biochemical studies have shown that carotid bodies generate substantial levels of CO during normoxia, whereas hypoxia inhibits CO generation (Prabhakar, 2012). Based on these findings, it was proposed that high levels of CO generated during normoxia keep the sensory activity low, and that hypoxia-evoked carotid body stimulation is in part due to reduced formation of CO, thereby removing its inhibitory influence on the sensory activity (Prabhakar, 1999).

Recent studies have argued against a role for endogenous CO in hypoxic sensing in the carotid body. For instance, Ortega-Sáenz *et al.* (2006) found that the type I cell neurosecretory response to hypoxia is unaltered in HO-2 knockout mice. This study utilized carotid body slices cultured for 20–96 h. Whether the long-term culture conditions masked the expected hypoxia-evoked increase in the secretory response in HO-2 knockout mice, however, is not clear from this study. If CO is a physiological inhibitor of the carotid body, then the hypoxic ventilatory response (HVR), a hallmark reflex triggered by the carotid body, should be augmented. Contrary to this possibility, Adachi *et al.* (2004) reported a blunted HVR in HO-2 knockout mice. However, in their study the authors measured the HVR for 15–20 s (see Adachi *et al.* 2004, p. 515), which is too short a duration to monitor breathing reliably in conscious mice. Thus, these unanticipated findings from the studies described above appear to be related to the experimental conditions employed and may therefore not provide a critical assessment of the role of CO in carotid body function.

In striking contrast to inhibition of the sensory activity by low doses of CO (Prabhakar *et al.* 1995), exogenous application of extremely high concentrations of CO (partial pressure of CO ~320 mmHg) stimulate carotid body activity, depolarize type I cells and facilitate voltage-gated Ca^{2+} influx, and these effects are antagonized by light (Barbe *et al.* 2002). As early as 1928, Warburg and Negelein demonstrated an interaction of CO with the mitochondrial respiratory chain, which is also antagonized by light. Inhibitors of mitochondrial oxidative phosphorylation are known for their stimulatory effects on the carotid body (see Lahiri *et al.* 2006 for references). It is conceivable that, like other metabolic poisons, the effect of a high concentration of CO on the carotid body is due to its inhibition of the mitochondrial electron transport chain. Thus, the findings of Barbe *et al.* (2002) do not necessarily negate the proposed inhibitory role of endogenous CO in the carotid body.

Hydrogen sulfide mediates the sensory excitation by hypoxia. As early as in 1931, Heymans *et al.* reported that systemic administration of an H_2S donor stimulates breathing, and this effect is mediated by the carotid body chemoreflex. It is now well established that H_2S is produced endogenously and functions as a gaseous messenger in various physiological processes (Gadalla & Snyder, 2010; Wang, 2012). Cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) are the two major enzymes that catalyse the biosynthesis of H_2S. Cystathionine β-synthase is abundant in the central nervous system, whereas CSE is preponderant in peripheral tissues. Carotid body type I cells express CBS (Li

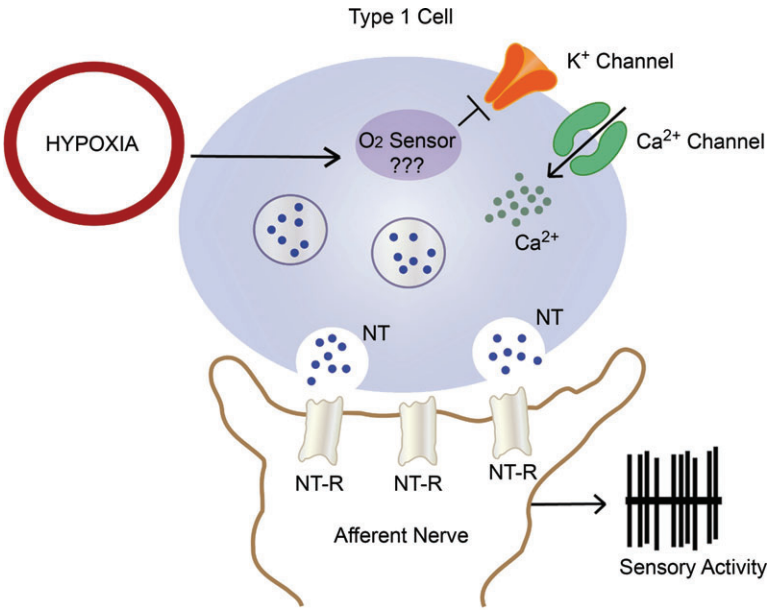

Figure 1. Schematic illustration of transduction of the hypoxic stimulus in a type I cell of the carotid body
Abbreviations: NT, neurotransmitter(s); and NT-R, neurotransmitter receptor.

et al. 2010; Telezhkin *et al.* 2010; Fitzgerald *et al.* 2011) as well as CSE (Peng *et al.* 2010; Mkrtchian *et al.* 2012).

Cystathionine γ-lyase-deficient mice and rats treated with DL-propargylglycine, an inhibitor of CSE, exhibit absence or marked attenuation of carotid body sensory function, as well as the ventilatory response to hypoxia (Peng *et al.* 2010), suggesting that CSE-catalysed H_2S is an excitatory gas messenger and mediates the sensory excitation by hypoxia. Mice treated with aminooxyacetic acid, a putative inhibitor of CBS, also exhibit impaired carotid body and ventilatory responses to hypoxia (Li *et al.* 2010). However, in addition to inhibiting CBS, aminooxyacetic acid also inhibits other pyridoxal phosphate-dependent enzymes, including CSE and 4-aminobutyrate aminotransferase (Beeler & Churchich, 1976), and disrupts mitochondrial function (Kauppinen *et al.* 1987). Given the broad spectrum of actions of aminooxyacetic acid, the role of CBS-catalysed H_2S in the carotid body remains uncertain at present.

Further evidence supporting an excitatory role for H_2S in the carotid body comes from studies with H_2S donors. Exogenous application of NaHS, an H_2S donor, produces robust sensory excitation of the carotid body in a concentration-dependent manner in mice and rats (Li *et al.* 2010; Peng *et al.* 2010). Like the response to hypoxia, the sensory response to NaHS is rapid in onset, occurs within seconds, and returns promptly to baseline after termination of its application (Peng *et al.* 2010).

How might H_2S activate the carotid body? Inhibition of K^+ channels and the resulting depolarization-induced voltage-gated Ca^{2+} influx in type I cells are critical steps for producing sensory excitation by hypoxia. Sodium hydrosulfide, like hypoxia, inhibits maxi-K^+ (Li *et al.* 2010; Telezhkin *et al.* 2010) and TASK-like K^+ channel (Tandem Pore K^+ channel family) (Buckler, 2012) and depolarizes type I cells (Buckler, 2012). Also, NaHS leads to robust elevation of $[Ca^{2+}]_i$ in type I cells, and this effect is absent in the absence of extracellular Ca^{2+} (Buckler, 2012; Makarenko *et al.* 2012) or if the depolarization is prevented by voltage clamping the cell at the resting membrane potential (Buckler, 2012). Furthermore, nifedipine, a blocker of L-type Ca^{2+} channels, prevents $[Ca^{2+}]_i$ elevation by NaHS as well as by hypoxia (Makarenko *et al.* 2012). Sodium hydrosulfide increases NADH autofluorescence in type I cells, suggesting that H_2S might mediate its actions in part by its effects on the mitochondrial electron transport chain (Buckler, 2012). These studies taken together demonstrate that H_2S, like hypoxia, depolarizes type I cells by inhibiting certain K^+ channels, facilitates voltage-gated Ca^{2+} influx and thus produces sensory excitation of the carotid body.

The generation of H_2S in the carotid body is regulated by O_2. Levels of H_2S are low during normoxia, and hypoxia increases its levels in a stimulus-dependent manner in the carotid body (Peng *et al.* 2010). A similar increase in H_2S generation was also seen in isolated type I cells challenged with hypoxia (Makarenko *et al.* 2012). Hypoxia-evoked H_2S generation was absent in CSE knockout mice and in rats treated with the CSE inhibitor DL-propargylglycine (Peng *et al.* 2010). These observations, taken together with the finding that disruption of H_2S generation prevents the carotid body and type I cell responses to hypoxia (Li *et al.* 2010; Peng *et al.* 2010; Makarenko *et al.* 2012), led to the suggestion that H_2S is a physiological mediator of the carotid body response to hypoxia. However, as the concentrations of H_2S required for eliciting type I cell and ventilatory responses are much greater than those produced endogenously, Buckler (2012) and Haouze *et al.* (2011) argued against a physiological role for H_2S in the carotid body. The development of sensitive methods for simultaneous measurements of H_2S in the carotid body along with the sensory discharge is needed in order firmly to establish the physiological role of H_2S in the carotid body. Notwithstanding this limitation, current evidence from studies involving pharmacological and genetic disruption of endogenous H_2S generation do provide a rather compelling case for a physiological role of H_2S in mediating the carotid body response to hypoxia. In this context, it is noteworthy that H_2S is an ancient gaseous messenger system that is well conserved across phyla, as evidenced by its participation in hypoxic responses of trout gill chemoreceptors (Olson *et al.* 2008) and *Caenorhabditis elegans* (Ma *et al.* 2012).

Mechanism(s) underlying increased H_2S generation by hypoxia in the carotid body: a role for CO. How does hypoxia increase H_2S generation? The findings that CO levels are relatively high and H_2S levels low during normoxia prompted Peng *et al.* (2010) to examine whether CO keeps the generation of H_2S suppressed during normoxia. Indeed, reducing CO levels by inhibition of HO markedly increased H_2S generation during normoxia. Conversely, a CO donor inhibited the hypoxia-evoked H_2S generation in the carotid body in a concentration-dependent manner. Given that the effects of the HO inhibitor were absent in CSE knockout mice, it was proposed that CO reduces H_2S generation by inhibiting CSE enzyme activity (Peng *et al.* 2010). Based on these findings, it was suggested that low sensory discharge during normoxia is due to an inhibitory influence of CO on CSE, resulting in low H_2S generation, whereas reduced CO generation during hypoxia lifts the inhibition on CSE, leading to elevated H_2S levels and increased sensory discharge (Fig. 2). Further studies, however, are needed to delineate the mechanisms by which CO inhibits CSE activity during normoxia. In addition, as proposed by Dombkowski *et al.* (2006), inhibition of H_2S oxidation might also contribute to its elevated levels during hypoxia, a possibility that remains to be investigated.

Is H_2S an 'O_2 sensor' or 'mediator' of the carotid body response to hypoxia? Hydrogen sulfide has been proposed as an 'O_2 sensor' in trout gill chemoreceptors (Olson *et al.* 2008). Can H_2S be regarded as an 'O_2 sensor' in the carotid body? Although H_2S is critical for sensory excitation by hypoxia, the available evidence suggests that its generation in the carotid body is regulated by CO. Thus, interactions between the HO-2–CO and CSE–H_2S systems preclude a role for H_2S as an 'O_2 sensor' in the carotid body. Rather, it would be more appropriate to regard H_2S as an important 'mediator' of the sensory excitation during hypoxia. It was proposed that interacting proteins working in concert as a 'chemosome' account for the curvilinear response of the carotid body to hypoxia, providing a fail-proof redundancy of chemoreceptor response to low O_2 (Prabhakar, 2006). It is likely that the enzymes generating CO and H_2S, in concert with K^+ channels in type I cells constitute important components of the 'chemosome'. Recent studies suggest that AMP kinase signalling also plays a role in hypoxic sensing by the carotid body (Evans *et al.* 2012). It would be interesting to examine the potential cross-talk between the HO-2–CO and CSE–H_2S systems with AMP kinase signalling in the carotid body in future studies.

Genetic influence on carotid body hypoxic sensing

John Weil and his co-workers from the University of Colorado were some of the first to recognize variations in the HVR in human subjects, with familial clusters and in mono- and dizygotic twins (see Weil, 2003 for references). As HVR is a hallmark reflex initiated by the carotid body, these variations were attributed to the influence of genetic factors on intrinsic hypoxic sensitivity of the chemoreceptors (Weil, 2003). Studies on spontaneously hypertensive rats and F-344 rats, which come from two genetic backgrounds, showed that the carotid body response to hypoxia was markedly augmented in spontaneously hypertensive rats, whereas it was severely impaired in

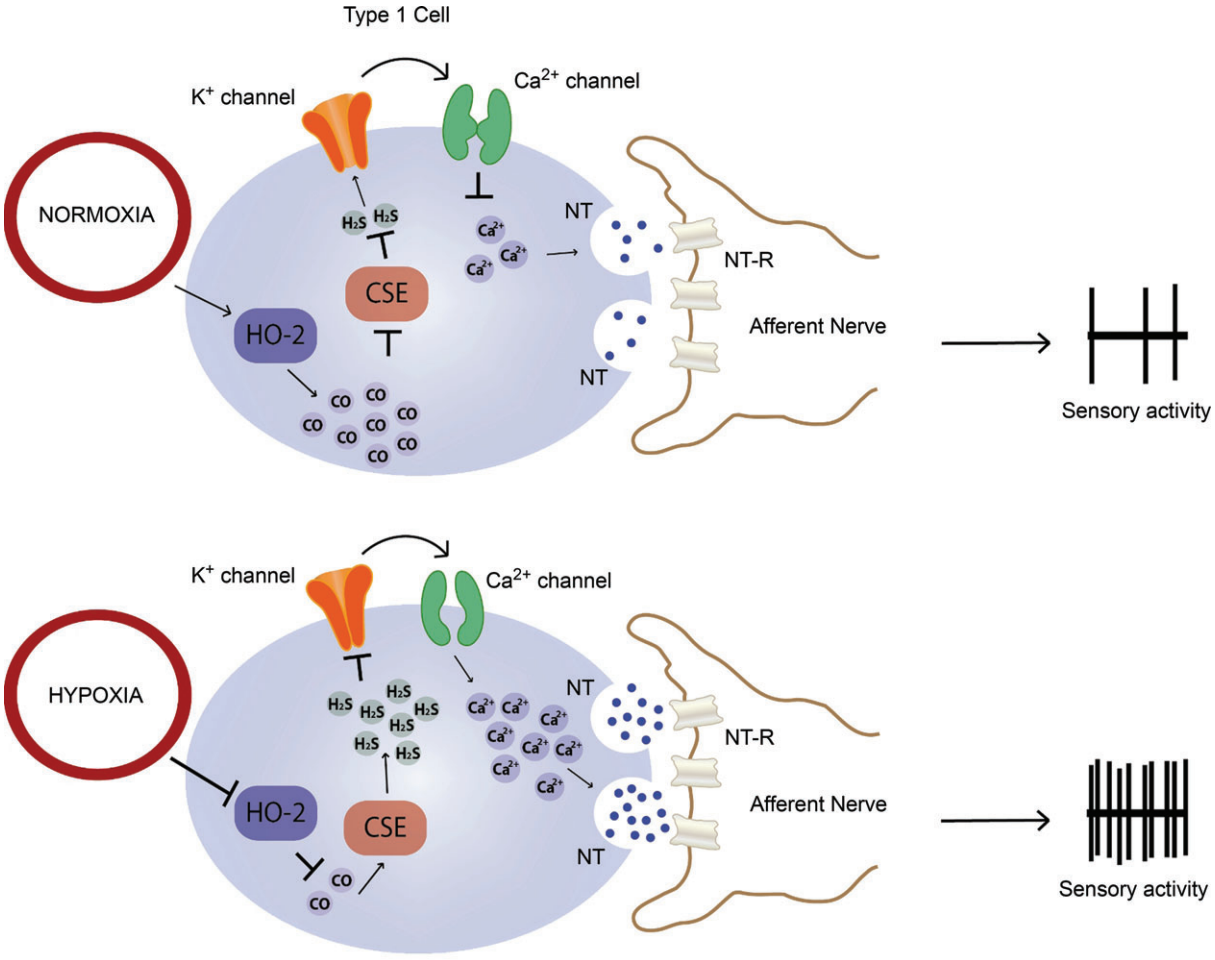

Figure 2. Schematic illustration of interactions between haem oxygenase-2 (HO-2)-generated carbon monoxide and cystathionine-γ-lyase (CSE)-regulated hydrogen sulfide generation and their effects on carotid body sensory activity during normoxia and hypoxia
Abbreviations: NT, neurotransmitter(s); and NT-R, neurotransmitter receptor.

F-344 rats (Weil *et al.* 1998), suggesting that genetic factors do influence hypoxic sensing by the carotid body. However, neither the identity of the genetic mechanism(s) nor the genes influencing the intrinsic hypoxic sensitivity of the carotid body have been explored further.

Regulation of carotid body hypoxic sensing by transcriptional activators: role of hypoxia-inducible factors (HIFs). Hypoxia-inducible factors 1 and 2 are the well-studied members of the HIF family of transcriptional activators, which are essential for maintaining O_2 homeostasis (Semenza, 2012). Hypoxia-inducible factors 1 and 2 are heterodimeric proteins, which consist of a constitutively expressed HIF-1β subunit and O_2-regulated HIF-1α or HIF-2α subunits, respectively. Type I cells express both HIF-1α and HIF-2α, and the relative abundance of the latter is higher than the former (Tian *et al.* 1998; Roux *et al.* 2005).

Given that homozygous deficiency of either HIF-1α or HIF-2α is embryonically lethal (Iyer *et al.* 1998; Scortegagna *et al.* 2003), the roles of HIFs in the carotid body response to hypoxia were examined in mice with partial deficiencies in either HIF-1α (*Hif-1a*$^{+/-}$) or HIF-2α (*Hif-2a*$^{+/-}$). Carotid body responses to hypoxia were severely impaired in *Hif-1a*$^{+/-}$ mice (Kline *et al.* 2002; Peng *et al.* 2006), whereas responses to cyanide, a potent chemoreceptor stimulant, or to CO_2, another physiological stimulus, were unaffected. In striking contrast, *Hif-2a*$^{+/-}$ mice exhibit selective heightening of the carotid body response to hypoxia (Peng *et al.* 2011). The enhanced hypoxic sensitivity of the carotid body in *Hif-2a*$^{+/-}$ mice was reflected in exaggerated chemoreflex function manifested by irregular breathing, with apnoea, hypertension and elevated plasma catecholamines. These observations demonstrate that carotid bodies from *Hif-1a*$^{+/-}$ mice are hyposensitive to hypoxia, whereas carotid bodies from *Hif-2a*$^{+/-}$ mice are hypersensitive.

How might HIFs contribute to hypoxic sensing by the carotid body? The HIFs regulate a variety of gene products associated with the maintenance of O_2 homeostasis (Prabhakar & Semenza, 2012). Hypoxia-inducible factor-regulated gene products may affect carotid body function either by affecting its morphology and/or independent of structural changes. The defective hypoxic sensing in *Hif-1a*$^{+/-}$ mice and the exaggerated hypoxic response in *Hif-2a*$^{+/-}$ mice appear not to be secondary to changes in carotid body morphology (Kline *et al.* 2002; Peng *et al.* 2011). Although HIFs regulate a variety of gene products, including some ion channels (see Prabhakar & Semenza, 2012 for references), recent studies have shown that HIF-1 and HIF-2 regulate the expression of gene products with opposing functions that regulate the redox state. For instance, HIF-1 regulates the expression of pro-oxidant enzymes, including NADPH oxidases (Diebold *et al.* 2010; Yuan *et al.* 2011), whereas HIF-2 regulates the expression of antioxidant enzymes (Scortegagna *et al.* 2003; Nanduri *et al.* 2009). Given that hypoxia alters the redox equilibrium in cells towards a more reduced state, it is likely that the contrasting effects of carotid body responses to hypoxia in *HIF-1a*$^{+/-}$ *versus* *HIF-2a*$^{+/-}$ mice might be due to differential expression of pro- and antioxidant enzymes and the ensuing changes in the redox state. Indeed, carotid bodies from *Hif-2a*$^{+/-}$ mice showed reduced levels of mRNAs encoding antioxidant enzymes and oxidative stress, and antioxidant treatment prevented the augmented hypoxic sensitivity of the carotid body and corrected the oxidative stress in these mice (Peng *et al.* 2011). Given that HIF-1 regulates the transcription of pro-oxidant enzymes (Diebold *et al.* 2010; Yuan *et al.* 2011), it is likely that reduced pro-oxidant enzyme expression and the resulting reduction in the intracellular redox state might contribute to the impaired hypoxic sensitivity in *Hif-1a*$^{+/-}$ mice, a possibility that remains to be established. Taken together, these studies suggest that a subset of HIF-regulated genes associated with the maintenance of the intracellular redox state might account in part for genetic variability in the carotid body response to hypoxia. The effects of partial deficiencies of HIF-α isoforms on the carotid body activity and the downstream redox-regulating enzyme genes are summarized in Fig. 3. Further studies are needed to determine whether HIFs also regulate the enzymes generating CO and H_2S as well as ion channels and to elucidate the role of other transcriptional activators that may also contribute to genetic variability of the carotid body response to hypoxia.

Epigenetic regulation of hypoxic sensing

Systemic or environmental perturbations in O_2 levels in the early neonatal period influence the carotid body

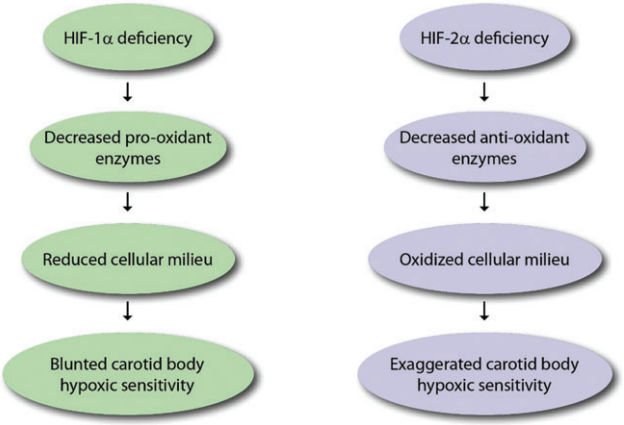

Figure 3. Molecular mechanisms underlying the carotid body responses to hypoxia in mice with partial deficiencies of hypoxia-inducible factors HIF-1α and HIF-2α

response to hypoxia in adulthood. For instance, adult rats treated with perinatal intermittent hypoxia (IH), simulating the apnoea of prematurity, exhibit carotid body hypersensitivity to hypoxia (Peng *et al.* 2004; Pawar *et al.* 2008, 2009). The mechanisms underlying the long-term consequences of O_2 perturbations in the early developmental period are not known.

Emerging evidence suggests that aberrant epigenetic regulation is an important mechanism mediating the effects of environmental perturbations in the neonatal period in adulthood (Feinberg, 2007). Epigenetic mechanisms are heritable modifications of DNA that do not involve changes in the DNA primary sequence (Barker *et al.* 1993; Gluckman *et al.* 2005). Silencing RNAs, DNA methylation and histone modifications are three well-studied epigenetic mechanisms, which either individually or collectively determine whether a gene is activated or silenced (Feinberg, 2007). Of the three mechanisms, DNA methylation has been implicated in mediating the gene expression associated with developmental programming (Waterland and Jirtle, 2003). The DNA methylation is catalysed by DNA methyltransferases or Dnmts and occurs at the 5′ position of cytosine of CpG dinucleotide clusters in the gene promoter called 'CpG' islands (Gardiner-Garden & Frommer, 1987; Feinberg, 2007). In general, DNA hypermethylation leads to repression of gene transcription, whereas hypomethylation causes transcriptional activation.

A recent study examined the role of DNA methylation in mediating the long-term effect of neonatal IH on carotid body responses to hypoxia in adult rats (Nanduri *et al.* 2012). Adult rats exposed to neonatal IH exhibit augmented hypoxic sensitivity of the carotid body, respiratory abnormalities manifested by a greater number of spontaneous apnoeas, hypertension and elevated plasma catecholamines. The mRNA levels of *Dnmt1* and *Dnmt3b* mRNA and the corresponding protein levels were elevated and global DNA methylation was increased in the carotid bodies from adult rats exposed to neonatal IH. Genes encoding antioxidant enzymes were down-regulated, and there was increased oxidative stress in the carotid bodies. Given that DNA hypermethylation suppresses gene expression, whether increased DNA methylation contributes to the reduced antioxidant gene expression was examined. A detailed analysis of the promoter region of the superoxide dismutase 2 gene (*Sod2*) revealed marked hypermethylation of a single CpG dinucleotide close to the transcription start site in adult rats exposed to neonatal IH. Remarkably, neonatal treatment with decitabine, an inhibitor of DNA methylation, eliminated DNA hypermethylation of the single CpG dinucleotide in the *Sod2* gene, corrected the oxidative stress, prevented the enhanced hypoxic sensitivity of the carotid body, and normalized the cardio-respiratory functions in adult rats exposed to neonatal IH (Nanduri *et al.* 2012). These findings implicate a hitherto uncharacterized role for epigenetic mechanisms mediating the effects of perturbations in O_2 levels in the neonatal period on the carotid body response to hypoxia in adulthood (Fig. 4). These studies are only the beginning in this area, and further investigations are certainly needed to delineate the role of other epigenetic mechanisms, including the role of histone modifications and/or silencing RNAs, and to identify the target genes associated with developmental programming of the carotid body response to hypoxia.

Carotid body chemoreflex in cardiorespiratory diseases

The importance of the carotid body chemoreflex in maintaining cardiorespiratory homeostasis during a variety of physiological conditions is well documented. For instance, the chemoreflex is critical for ventilatory adaptations to high altitude, as well as for

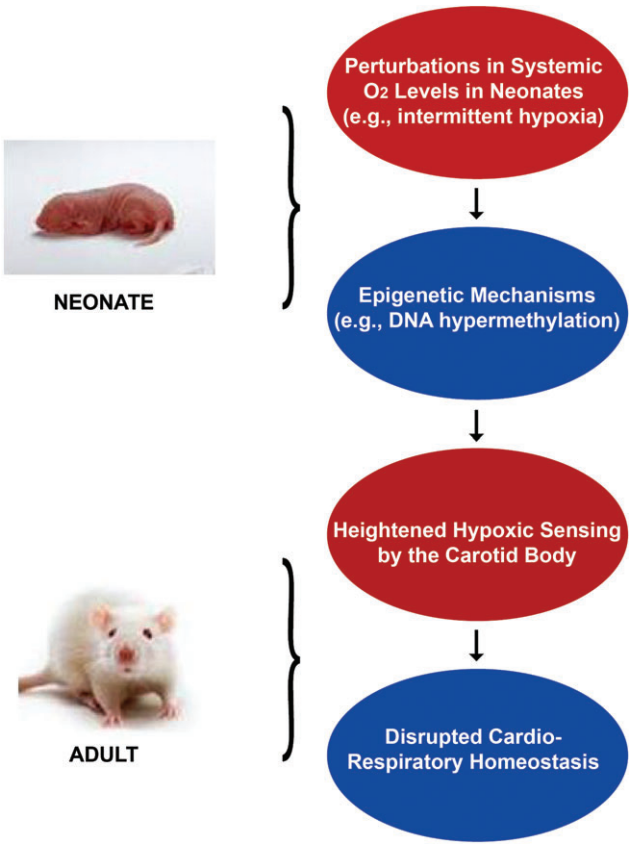

Figure 4. Schematic illustration of epigenetic mechanisms (e.g. DNA hypermethylation) contributing to augmented carotid body sensitivity to hypoxia in adult rats exposed to neonatal intermittent hypoxia and its impact on cardiorespiratory homeostasis

cardiorespiratory responses to exercise (see Kumar & Prabhakar, 2012). Emerging evidence suggests that the carotid body chemoreflex plays a more prominent role than hitherto appreciated in the progression of autonomic morbidities associated with cardiorespiratory diseases. For example, heightened chemoreflex function has been implicated in autonomic dysfunction caused by sleep-disordered breathing with apnoea (Kara *et al.* 2003; Prabhakar *et al.* 2007), congestive heart failure (Schultz & Li, 2007) and neurogenic hypertension (Abdala *et al.* 2012). Owing to space constraints, the following section will focus on studies assessing the effects of chronic intermittent hypoxia, a hallmark of sleep-disordered breathing with apnoea, on carotid body and chemoreflex function.

Carotid body function in patients with recurrent apnoea

Sleep-disordered breathing with obstructive sleep apnoea is a major clinical disorder that is reaching epidemic proportions (Nieto *et al.* 2000; Shahar *et al.* 2001). In severely affected patients, the frequency of apnoea may exceed 60 episodes h^{-1}, and arterial blood O_2 saturation can be reduced to as low as 50%. Patients with recurrent apnoea exhibit elevated sympathetic nerve activity, plasma and urinary catecholamines, and are prone to develop hypertension. It has been proposed that carotid bodies constitute the 'frontline' defense system for detecting periodic hypoxaemia associated with apnoea and that the chemoreflex contributes to elevated sympathetic activity and blood pressure (Cistulli & Sullivan, 1994). Patients with recurrent apnoea exhibit an augmented hypoxic ventilatory response (Narkiewicz *et al.* 1999; Kara *et al.* 2003), a reflex initiated by the carotid body. Obstructive sleep apnoea patients exhibit a more pronounced decrease in sympathetic activity in response to brief hyperoxia (Dejour's test; an indirect measure of peripheral chemoreceptor sensitivity) than control subjects (Tafil-Klawe *et al.* 1991; Kara *et al.* 2003), suggesting augmented chemoreflex function. Remarkably, carotid body-resected subjects with sleep apnoea do not develop hypertension (see discussion by Somers & Abboud, 1993). These studies suggest heightened carotid body chemoreflex function in patients with recurrent apnoea.

Effects of chronic intermittent hypoxia on carotid body response to hypoxia. Recurrent apnoea produces chronic IH and hypercapnia. A major advance in the field is the demonstration that exposure of experimental animals to chronic IH alone is sufficient to cause autonomic morbidities similar to those described in recurrent apnoea patients (Fletcher, 2001; Peng *et al.* 2006). Rats exposed to chronic IH also develop hypertension similar to that seen in patients with recurrent apnoea, and bilateral sectioning of the carotid sinus nerves prevents this response (Lesske *et al.* 1997). Direct recording of carotid body sensory activity has shown selective enhancement of the hypoxic response in animals exposed to chronic IH (Peng *et al.* 2003, 2006; Peng & Prabhakar, 2004; Rey *et al.* 2004, 2006). Furthermore, repetitive hypoxia produces a long-lasting increase in baseline sensory activity in rodents exposed to chronic IH, and this phenomenon was termed 'sensory long-term facilitation' or sensory LTF (Peng *et al.* 2003, 2006). Chronic IH had no significant effect on carotid body morphology (Peng *et al.* 2003). In striking contrast, the carotid body response to hypoxia was normal in rats exposed to chronic continuous hypoxia (Peng *et al.* 2003), suggesting that the effects are unique to IH. The absence of a heightened carotid body response to hypoxia with chronic continuous hypoxia might explain why high-altitude residents, who are adapted to a low-O_2 environment, do not exhibit autonomic dysfunction, as opposed to patients with recurrent apnoea, who experience chronic IH.

Reactive oxygen species (ROS) mediate the effects of chronic IH on the carotid body. The following findings suggest that ROS signalling mediates the effects of chronic IH on the carotid body. First, ROS levels were elevated in carotid bodies from chronic IH-treated rodents (Peng *et al.* 2003, 2006; Pawar *et al.* 2009). Second, antioxidant treatment prevented the chronic IH-evoked augmented hypoxic sensitivity and sensory LTF (Peng *et al.* 2003; Pawar *et al.* 2009). Available evidence suggests that increased transcription and activation of the pro-oxidant enzyme, NADPH oxidase-2, and decreased transcription and activity of the antioxidant enzymes (e.g. superoxide dismutase 2) contribute to the increase in ROS levels induced by IH (Peng *et al.* 2009; Nanduri *et al.* 2009). In addition, IH increases ROS in the mitochondrial compartment by inhibiting complex I of the mitochondrial electron transport chain (Peng *et al.* 2003). A recent study by Khan *et al.* (2011) showed that ROS generated by NADPH oxidase-2 inhibit the mitochondrial complex I in IH-exposed cells, involving *S*-glutathionylation of the complex I subunits. The selective contribution of ROS generated in the mitochondrial compartment to the altered carotid body function, however, remains to be investigated.

Analysis of the molecular mechanisms revealed that activation of HIF-1 mediates the increase in NADPH oxidase-2 transcription induced by IH (Peng *et al.* 2006; Yuan *et al.* 2011). On the contrary, IH decreases HIF-2 activity, which in turn leads to insufficient transcription of superoxide dismutase 2 (Nanduri *et al.* 2009). Thus, the imbalance between HIF-1 and HIF-2 and resulting changes in pro- and antioxidant enzyme expressions contribute to IH-induced oxidative stress in the carotid body. How might ROS mediate the effects of chronic

IH on the carotid body? Available evidence suggests that ROS-dependent recruitment of endothelin-1 and 5-HT signalling contribute to the chronic IH-evoked augmented hypoxic response and sensory LTF, respectively (Pawar *et al.* 2009; Peng *et al.* 2009). However, the effects of chronic IH on recently identified HO-2–CO and CSE–H_2S signalling in the carotid body remain to be investigated.

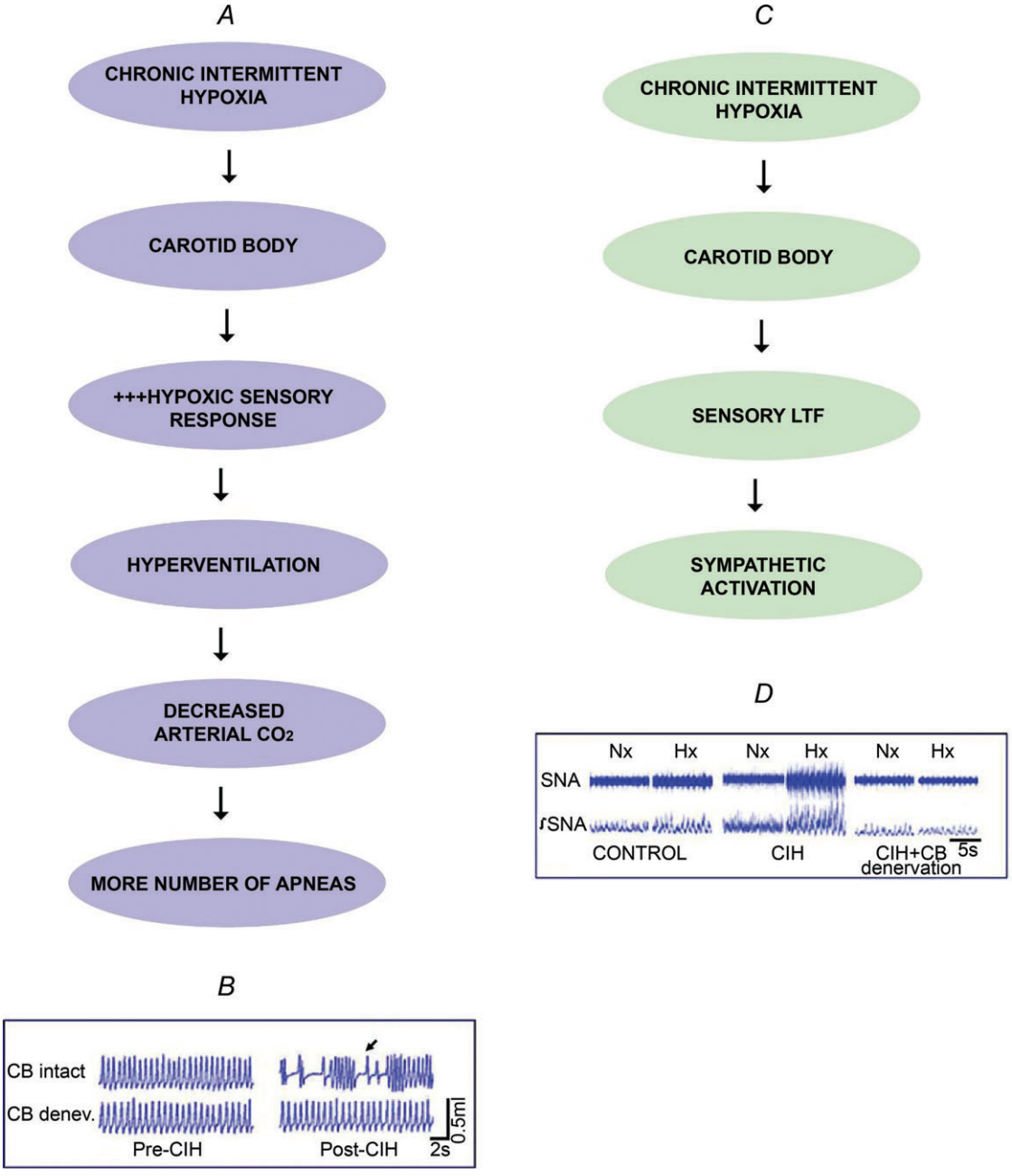

Figure 5. Contribution of carotid body chemoreflex to cardiorespiratory responses to chronic intermittent hypoxia (CIH)
A, the heightened carotid body response to hypoxia induced by CIH leads to a greater number of spontaneous apnoeas. *B*, example of breathing responses in awake rats before and after exposure to CIH with intact and denervated carotid bodies (CB). Arrow represents irregular breathing with apnoea. *C*, CIH-induced sensory long-term facilitation (LTF) of the carotid body reflexly stimulates sympathetic activity. *D*, examples of splanchnic nerve activity (SNA) in normoxia (Nx) and hypoxia (Hx, 12% O_2) in anaesthetized, mechanically ventilated rats exposed to normoxia (control) or CIH with intact carotid bodies and following carotid body denervation. Note the enhanced SNA response to Hx in the CIH-exposed rat and the absence of this response in the CB-denervated rat exposed to CIH.

Heightened carotid body chemoreflex mediates autonomic dysfunction caused by chronic IH. What might be the functional significance of chronic IH-induced changes in carotid body function? It was proposed that during each episode of apnoea the increased carotid body sensitivity to hypoxia can lead to a greater magnitude of hyperventilation, thus driving the respiratory controller below the apnoeic threshold for CO_2, leading to a greater number of apnoeas (Prabhakar, 2001). In other words, the heightened hypoxic sensitivity of the carotid body might act as a 'positive feedback', thereby exacerbating the occurrence of apnoeas (Fig. 5*A*). Supporting such a possibility is the finding that chronic IH-exposed rats with intact carotid bodies exhibit a greater incidence of spontaneous apnoeas, and this effect was absent in carotid body-sectioned rats exposed to chronic IH (Fig. 5*B*). Patients with sleep-disordered breathing exhibit elevated sympathetic nerve activity during the day time, wherein apnoeas are absent (Kara *et al.* 2003). Given that the carotid body chemoreflex stimulates sympathetic nerve activity, it was proposed that the sensory LTF contributes to the day-time increase in sympathetic nerve activity in patients with recurrent apnoea (Prabhakar, 2001; Prabhakar *et al.* 2012; Fig. 5*C*). Indeed, rats exposed to chronic IH showed elevated sympathetic nerve activity, and this effect was absent following chronic bilateral sectioning of the carotid sinus nerves (Fig. 5*D*). These findings suggest that the heightened carotid body chemoreflex contributes to autonomic dysfunction in patients with sleep-disordered breathing with apnoea.

Concluding remarks

Vertebrates are endowed with a variety of sensory receptors for detecting diverse sensory modalities, including mechanical, thermal, visual, olfactory and taste sensations. Two principal categories of sensory transduction machineries have been indentified. One involves ion channels, which mediate the mechanical and thermal sensations, and the other is G-protein-coupled receptors, which contribute to the transduction of visual, olfactory and taste sensations (Julius & Nathans, 2012). Although ion channels were implicated in carotid body hypoxic sensing, emerging evidence suggests a biochemical mechanism associated with O_2-dependent enzymatic generation of gas messengers (e.g. CO and H_2S) as an important initial step in the transduction cascade of the hypoxic stimulus in the carotid body. Thus, the transduction machinery in the carotid body appears to be distinctly different from other known sensory receptors. Evidence is emerging that genetic and epigenetic mechanisms profoundly impact the carotid body response to hypoxia by altering the intracellular redox state. Elucidation of the mechanisms by which redox state affects the transduction machinery of the carotid body is an important area for future research.

Emerging evidence suggests that either hypo- or hypersensitivity of the carotid body to hypoxia has adverse physiological consequences. For instance, people with blunted HVR, a hallmark reflex response of the carotid body, exhibit impaired ventilatory adaptations to high altitude, manifested by pulmonary oedema (Hohenhaus *et al.* 1995). On the contrary, hypersensitivity to hypoxia contributes to the autonomic dysfunction associated with highly prevalent cardiorespiratory diseases, such as sleep-disordered breathing with apnoea, congestive heart failure and neurogenic hypertension. Future development of new therapeutic interventions targeting the carotid body to correct either the hypo- or hypersensitivity to hypoxia is of greater translational significance than hitherto appreciated.

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Acknowledgements

This article is based on 2012 J.H. Comroe Distinguished Lecture of the American Physiological Society. It is a great privilege to acknowledge the collaborations of Professors Solomon H. Snyder and Gregg L. Semenza of the Johns Hopkins School of Medicine. The studies presented in this article would have not been possible without the participation of my colleagues Professor G. K. Kumar, Professor J. Nanduri and Drs Y-J. Peng and G. Yuan. I am greatly indebted to Ms Shanna L. Williams for preparing the figures. The research in my laboratory is supported by grants from National Institutes of Health, Heart, Lung and Blood Institute, R01-HL76537, P01-HL90554 and R01-HL86493.