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TOPICAL REVIEW

The role of hypoxia-inducible factors in carotid body (patho) physiology

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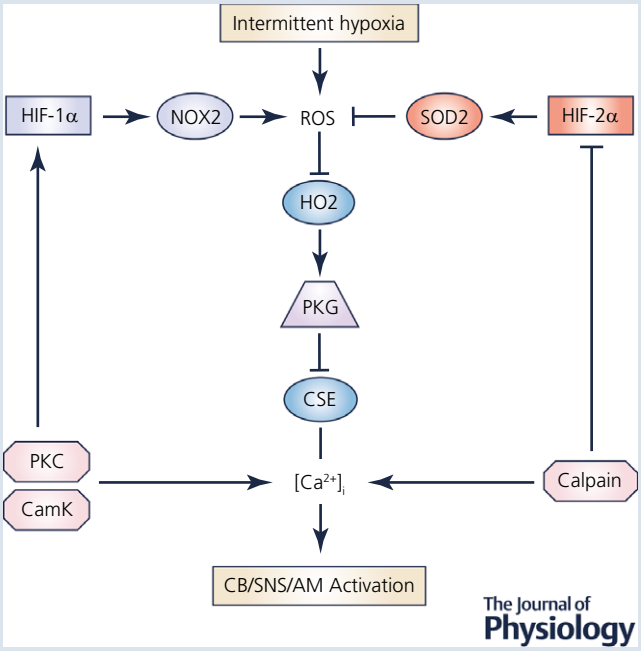

Abstract Hypoxia-inducible factors mediate adaptive responses to reduced O_2 availability. In patients with obstructive sleep apnoea, repeated episodes of hypoxaemia and reoxygenation (intermittent hypoxia) are sensed by the carotid body (CB). The ensuing CB chemosensory reflex activates the sympathetic nervous system and increased secretion of catecholamines by the adrenal medulla, resulting in hypertension and breathing abnormalities. In the CB, intermittent hypoxia induces the formation of reactive oxygen species (ROS) and increased

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intracellular Ca^{2+} levels, which drive increased expression of hypoxia-inducible factor (HIF) 1α and a decrease in the levels of HIF-2α. Intermittent hypoxia increases HIF-1α-dependent expression of *Nox2*, encoding the pro-oxidant enzyme NADPH oxidase 2, and decreased HIF-2α-dependent expression of *Sod2*, encoding the anti-oxidant enzyme superoxide dismutase 2. These changes in gene expression drive persistently elevated ROS levels in the CB, brainstem, and adrenal medulla that are required for the development of hypertension and breathing abnormalities. The ROS generated by dysregulated HIF activity in the CB results in oxidation and inhibition of haem oxygenase 2, and the resulting reduction in the levels of carbon monoxide leads to increased hydrogen sulfide production, triggering glomus cell depolarization. Thus, the pathophysiology of obstructive sleep apnoea involves the dysregulation of O_2-regulated transcription factors, gasotransmitters, and sympathetic outflow that affects blood pressure and breathing.

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Abstract figure legend Integration of gasotransmitters and O_2-regulated transcription factors into the pathogenic response of the carotid body (CB) to reactive oxygen species (ROS) generated by intermittent hypoxia. Within glomus cells of the CB, haem oxygenase 2 (HO2) generates the gasotransmitter CO (not shown; see Fig. 2) in an O_2-dependent manner. CO binds to and activates guanylate cyclase (not shown). cGMP produced by guanylate cyclase binds to and activates protein kinase G (PKG), which phosphorylates and inactivates cystathionine-γ-lyase (CSE), an enzyme that generates the gasotransmitter H_2S (not shown). Under conditions of intermittent hypoxia, ROS levels increase, inhibiting HO2 and PKG activity, thereby increasing CSE activity, H_2S production, and intracellular Ca^{2+} levels leading to glomus cell depolarization. Hypoxia-inducible factor (HIF) 1α and HIF-2α modulate the pathway by increasing or decreasing, respectively, ROS levels through activation of *NOX2* and *SOD2* gene transcription, respectively. Increased intracellular (cytosolic) Ca^{2+} levels activate proteins that positively and negatively regulate HIF-1α and HIF-2α, respectively. The pathway components are colour coded as follows: blue, pro-oxidant transcription factor (rectangle) or enzyme (oval); orange, Ca^{2+}-sensitive enzyme; red, anti-oxidant transcription factor (rectangle) or enzyme (oval); teal, gasotransmitter; yellow, stimulus/response. Abbreviations: AM, adrenal medulla; CamK, Ca^{2+}/calmodulin-dependent kinase; NOX2, NADPH oxidase 2; PKC, protein kinase C; PKG, protein kinase G; SNS, sympathetic nervous system; SOD2, superoxide dismutase 2.

Oxygen homeostasis and hypoxia-inducible factors

Oxygen must be constantly delivered to and consumed by most cells in the human body in order to maintain their viability. O_2 delivery is affected by the combined action of the respiratory and circulatory systems under the control of chemo- and baroreceptors as well as the central nervous system. O_2 consumption is determined by cellular metabolism, principally the relative rates of cellular respiration and glycolysis. Hypoxia-inducible factors (HIFs) serve as master regulators to maintain oxygen homeostasis in every cell of the body by balancing O_2 supply and demand (Prabhakar & Semenza, 2012). HIFs are heterodimeric transcription factors composed of HIF-α (HIF-1α, HIF-2α or HIF-3α) and HIF-1β subunits. HIF-1α is present in nucleated cells of all metazoan species, whereas HIF-2α and HIF-3α are expressed only in certain cell types and only in vertebrate species. The HIF-α subunits are subject to O_2-dependent hydroxylation, ubiquitination and proteasomal degradation under normoxic conditions, whereas the proteins rapidly accumulate under hypoxic conditions (Kaelin & Ratcliffe, 2008). The HIF-α and HIF-1β subunits dimerize and bind to the consensus DNA sequence 5′-RCGTG-3′ (R = A or G), which is present in hypoxia response elements that are located in or near target genes (Semenza *et al.* 1996; Semenza, 2014). HIF binding leads to the recruitment of co-activator proteins that increase transcription of the target gene (Luo *et al.* 2011, 2012).

Over 2500 direct HIF target genes have been identified. Only a subset of HIF target genes is transactivated in any given cell, allowing each cell to respond to hypoxia in a unique manner (Kelly *et al.* 2003; Semenza, 2014). HIF target genes include *EPO*, which encodes erythropoietin, the hormone that is released from the kidney and controls red blood cell production (Semenza & Wang, 1992), and *VEGF*, which encodes vascular endothelial growth factor, which controls angiogenesis (Forsythe *et al.* 1996). These two proteins are critical for systemic and local responses to hypoxia, respectively. HIFs also regulate the expression of genes encoding glycolytic enzymes (Semenza *et al.* 1994, 1996; Iyer *et al.* 1998) and pyruvate dehydrogenase kinase

1, which mediates a switch from oxidative to glycolytic metabolism in response to hypoxia (Kim *et al.* 2006). Analysis of knockout mice revealed that complete HIF-1α deficiency leads to embryonic lethality at mid-gestation (embryonic day 10.5) with cardiac defects, vascular regression and impaired erythropoiesis, indicating that all three components of the circulatory system are dependent on HIF-1 for their normal development (Iyer *et al.* 1998; Yoon *et al.* 2006).

Carotid body-mediated reflex responses to hypoxaemia

A decrease in arterial O_2 levels increases the sensory nerve activity of the carotid body (CB), a chemoreceptor organ located at the bifurcation of the internal and external carotid arteries that was shown to play a critical role in oxygen sensing nearly a century ago (DeCastro, 1926; Heymans & Heymans, 1927; Kumar & Prabhakar, 2012). Afferent axons from the CB course through the carotid sinus nerve, a branch of the glossopharyngeal (IXth cranial nerve), to the nucleus tractus solitarius (NTS) in the caudal medulla. Interneurons project from the NTS to the rostral ventral respiratory group and the nucleus ambiguus, which project via the phrenic nerve to the diaphragm to regulate respiratory rate (Fig. 1). A third group of interneurons projects from the NTS to the rostral ventrolateral medulla (RVLM) and stimulates neurons whose axons course through the corticospinal tract and stimulate neurons that project via the sympathetic ganglion and adrenal sympathetic nerve to the adrenal medulla (AM), leading to increased release of catecholamines (adrenaline and noradrenaline), which cause an increase in heart rate and arterial vasoconstriction that increases blood pressure (BP) (Fig. 1). Thus, the chemosensory reflex arising from the CB is a major regulator of breathing and sympathetic nerve activity.

Emerging evidence implicates the gasotransmitters carbon monoxide (CO) and hydrogen sulfide (H_2S) in hypoxic sensing by the CB. Glomus cells, the primary hypoxia sensing cells in the CB, express haem oxygenase 2 (HO2) and cystathionine-γ-lyase (CSE), which are enzymes that produce CO and H_2S, respectively (Prabhakar *et al.* 1995; Williams *et al.* 2004; Peng *et al.* 2010). Under normoxic conditions, CO inhibits CSE from producing H_2S through protein kinase G-dependent signalling (Yuan *et al.* 2015). HO2 has a high K_m for O_2, such that decreased O_2 concentration in glomus cells is sufficient to decrease the production of CO (Yuan *et al.* 2015). During hypoxia, reduced CO production from HO2 leads to disinhibition of CSE and thereby increases H_2S production. H_2S, in turn increases the sensory nerve activity by depolarizing glomus cells (Fig. 2).

Obstructive sleep apnoea and systemic hypertension

Obstructive sleep apnoea (OSA) is a highly prevalent respiratory disorder associated with morbidity and mortality (Dempsey *et al.* 2010). Since the initial study by Young *et al.* (1993), the reported incidence of OSA has increased partly due to improved screening of sleep-disordered breathing. According to a recent report (Peppard *et al.* 2013) OSA affects 3% of 30- to 49-year-old women, 9% of 50- to 70-year-old women, 10% of 30- to 49-year-old men, and 17% of 50- to 70-year-old men

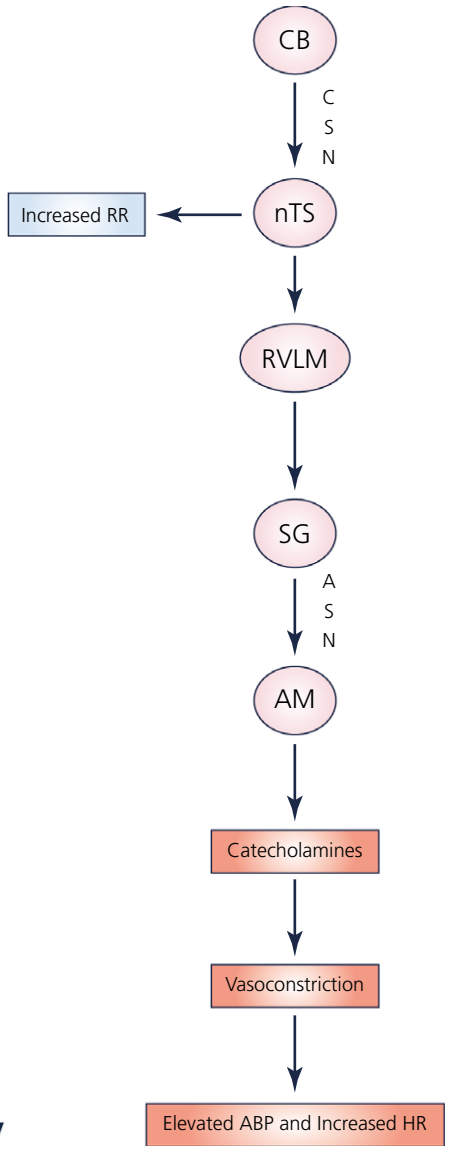

Figure 1. The chemosensory reflex pathway mediates rapid cardiovascular and respiratory responses to acute hypoxia
Abbreviations: ABP, arterial blood pressure; AM, adrenal medulla; ASN, adrenal sympathetic nerve; CB, carotid body; CSN, carotid sinus nerve, a branch of IX cranial nerve; HR, heart rate; nTS, nucleus tractus solitarius; RR, respiratory rate; RVLM, rostral ventrolateral medulla; SG, sympathetic ganglion.

in a community-based study of 1520 Wisconsin adults. Predisposing factors include micrognathia, retrognathia and, most commonly, obesity. In OSA, when affected individuals fall asleep, the loss of muscle tone causes the upper airway to become occluded by pharyngeal soft tissue, leading to apnoea (cessation of breathing) for 10–40 s, which causes hypoxaemia followed by arousal, clearing of the airway and reoxygenation; the patient falls asleep again and the process is repeated dozens or even hundreds of times per night. OSA causes increased BP and is the leading cause of treatment-resistant hypertension (Wang, 2014; de Abreu-Silva & Beltrami-Moreira, 2014). In addition, OSA patients have breathing abnormalities, such as irregular breathing and central apnoeas. Patients are treated with continuous positive airway pressure (CPAP) to maintain airway patency. CPAP-treated patients have lower BP than non-treated patients but it is still elevated compared to the general population. Furthermore, CPAP does not reduce the risk of acute coronary syndrome or stroke in OSA patients (Yu *et al.* 2017). Thus, novel therapies are urgently needed to prevent cardiovascular sequelae.

Intermittent hypoxia increases ROS production in the CB

Despite the fact that apnoea results in both intermittent hypoxia (IH) and intermittent hypercarbia, exposure of rodents to IH is sufficient to cause hypertension that is dependent on O_2 sensing by the CB and the resulting reflex activation of the sympathetic nervous system leading to catecholamine secretion from the AM into the systemic circulation (Fletcher *et al.* 1995; Lesske *et al.* 1997). Fletcher and co-workers examined the effects of combined IH and CO_2 on BP responses (Fletcher *et al.* 1995). Rats were subjected to 35 days with either hypocapnic hypoxia (no added CO_2), or eucapnic hypoxia (7–10% inspired CO_2 fraction; F_{ICO_2}), or hypercarbic hypoxia (11–14% F_{ICO_2}). They found that chronic intermittent hypocapnic hypoxia increased BP by 11 mmHg, but neither episodic eucapnic hypoxia nor intermittent asphyxia had any additional effect beyond this, suggesting that the neurohumoral systems involved in the chronic diurnal blood pressure response to IH are already maximally stimulated by hypocapnic hypoxia.

In one model of OSA, rodents are placed in a chamber in which the O_2 concentration is rapidly decreased to 5%, held for 15 s and then rapidly increased to 21%, and held for 5 min. This cycle of hypoxia and reoxygenation is repeated for 8 h during the animal's sleep cycle and results in hypertension within 10 days (Prabhakar, 2001). The process of hypoxia and reoxygenation results in the generation of reactive oxygen species (ROS), which is critical for the pathogenesis of hypertension because administration of the superoxide scavenger manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP) during the 10-day exposure to IH blocks the development of hypertension (Peng *et al.* 2006). Increased production of ROS seems to occur during reoxygenation rather than the hypoxic phase of IH (Yuan *et al.* 2004). Hydrogen peroxide (H_2O_2) generated by the superoxide anion was identified as a major reactive oxygen species mediating the effects of IH (Peng *et al.* 2009).

Intermittent hypoxia dysregulates HIF activity in the CB

IH induces the expression of HIF-1α protein in the CB and central nervous system, which is blocked by MnTMPyP administration (Peng *et al.* 2006; Yuan *et al.* 2011). Mice that are heterozygous for a knockout allele at the *Hif1a* locus are protected from the development of hypertension in response to IH and have no increase in ROS (Peng *et al.* 2006). The finding that HIF-1α was both upstream and downstream of ROS suggested a positive feedback mechanism in which ROS-induced HIF-1 activity led to the expression of a protein that further increased ROS. This protein turned out to be NADPH oxidase 2 (NOX2), which is a major source of superoxide production (Yuan *et al.* 2011). The NOX2-dependent increase in

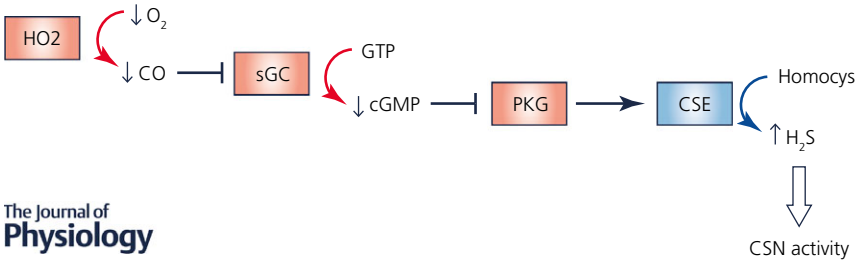

Figure 2. O_2 sensing in carotid body glomus cells involves the generation of carbon monoxide and hydrogen sulfide gasotransmitters
Symbols: arrow, stimulation; blocked arrow, inhibition; curved arrow, substrate/product. Abbreviations: CO, carbon monoxide; CSE, cystathionine-γ-lyase; CSN, carotid sinus nerve; H_2S, hydrogen sulfide; HO2, haem oxygenase 2; PKG, protein kinase G; sGC, soluble guanylate cyclase.

ROS in IH-exposed PC12 rat pheochromocytoma cells triggered increased intracellular Ca^{2+} levels and activation of protein kinase C and mammalian target of rapamycin (mTOR), as well as inhibition of HIF prolyl hydroxylase activity, leading to increased synthesis and stability of HIF-1α (Yuan *et al.* 2008). Whereas the inhibition of prolyl hydroxylase activity rapidly resolved after cessation of IH, mTOR activity and HIF-1α protein levels remained increased for 90 min, which parallels the persistent hyper-reactivity of CBs taken from animals after cessation of IH (Peng *et al.* 2006; Yuan *et al.* 2008).

Both HIF-1α and HIF-2α are expressed within the O_2-sensing glomus cells of the CB (Roux *et al.* 2005; Peng *et al.* 2014). However, HIF-1α levels are low in the CB under normoxic conditions and induced by IH, whereas HIF-2α levels are high in the CB under normoxic conditions and extinguished by IH (Nanduri *et al.* 2009). Degradation of HIF-2α by IH is mediated by calpains, which are Ca^{2+}-dependent proteases. Remarkably, whereas HIF-1α is a positive regulator of *Nox2* gene expression, HIF-2α activates transcription of the *Sod2* gene, which encodes the mitochondrial enzyme that converts superoxide to hydrogen peroxide (Nanduri *et al.* 2009). Treatment of rodents with *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal, a calpain inhibitor, blocked HIF-2α degradation, maintained *Sod2* expression, blocked the increase in ROS and prevented the development of hypertension in mice exposed to IH (Nanduri *et al.* 2009).

Hif1a$^{+/-}$ mice were completely resistant to the development of oxidative stress, sympathetic nervous system activation and hypertension in response to IH (Peng *et al.* 2006). In contrast, *Hif2a*$^{+/-}$ mice were found to have hypertension and respiratory instability under basal conditions in normoxia and their CBs were hyper-reactive when subjected to acute hypoxia, similar to CBs from wild-type mice that have been exposed to IH for 10 days (Peng *et al.* 2011). In addition to decreased *Sod2* expression, *Hif2a*$^{+/-}$ mice display increased *Nox2* expression as well as oxidative stress in the CB and AM due to increased HIF-1α expression under normoxic conditions (Yuan *et al.* 2013). Conversely, in CB and AM of *Hif1a*$^{+/-}$ mice, HIF-2α and *Sod2* expression were increased. As a consequence of increased anti-oxidant enzyme level, *Hif1a*$^{+/-}$ mice exhibit a reduced cellular oxidative (redox) state. Most dramatically, *Hif1a*$^{+/-}$;*Hif2a*$^{+/-}$ mice that were heterozygous for knockout alleles at both loci were completely normal with respect to redox state in the CB and AM, CB response to acute hypoxia, breathing and BP (Yuan *et al.* 2013).

The changes in HIF-1α and HIF-2α expression (as well as *Nox2* and *Sod2* expression) and redox state that were observed in the CB and AM in response to IH were also observed in the NTS and RVLM, but not in nearby brainstem regions that do not receive input from the CB (Peng *et al.* 2014). Furthermore, CB ablation blocked all of these responses to IH in the NTS, RVLM and AM, indicating that responses to IH were initiated solely in the CB and were subsequently transduced as responses of the efferent neural pathway.

Effect of intermittent hypoxia on oxygen sensing

Thus far, the role of gasotransmitters in acute oxygen sensing and of hypoxia-inducible factors in the response to chronic IH have been presented. There is a connection between these two pathways and it is through ROS, which are generated during IH and amplified by the dysregulation of HIF-1α and HIF-2α expression. Oxidation of cysteine residue 265 in HO2 inhibits its catalytic activity, thereby decreasing the production of CO, which in turn, leads to increased production of H_2S and increased glomus cell depolarization (Yuan *et al.* 2016). The increase in BP associated with chronic IH was not observed in *Cse*$^{-/-}$ mice or wild-type mice treated with a CSE inhibitor, which lack H_2S production in the CB (Peng *et al.* 2017). In contrast, *Ho2*$^{-/-}$ mice have irregular breathing and frequent hypopnea and apnoea episodes, which include both central and obstructive events (Peng *et al.* 2017). Remarkably, all of these abnormalities were corrected by treatment with a CO donor or by treatment with a CSE inhibitor (Peng *et al.* 2017). Hence, it is possible to integrate the regulatory circuits governed by gasotransmitters and O_2-regulated transcription factors (Abstract figure).

Conclusions and implications

Taken together, these studies indicate the existence of mutual antagonism between HIF-1α and HIF-2α in the CB and downstream neural components of the chemosensory reflex, and that the balance between HIF-1α-dependent pro-oxidant and HIF-2α-dependent anti-oxidant activity determines the redox state of the CB, which in turns determines the set point of the sympathetic nervous system and cardiorespiratory homeostasis. This balance can be disturbed by environmental (OSA-induced IH) or genetic (*Hif2a* null allele) causes, leading to increased sympathetic activation and the development of hypertension. Drugs that selectively inhibit HIF-1 or HIF-2 might prevent the development of hypertension in OSA patients or induce hypertension in non-OSA patients, respectively. For example, HIF-2α selective inhibitors, currently under evaluation as anti-cancer therapy (Chen *et al.* 2016; Cho *et al.* 2016), might lead to an imbalance between HIF-1 and HIF-2 activity, resulting in hypertension and breathing abnormalities (Yuan *et al.* 2013). Based on these findings, we hypothesize that in addition to OSA, systemic hypertension due to other causes may also involve disturbance of the balance between HIF-1α

and HIF-2α, leading to oxidative stress in the CB and AM leading to sympatho-adrenal activation.

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Additional information

Competing interests

The authors declare they have no competing interests.

Author contributions

G.L.S. and N.R.P. wrote the manuscript. Both authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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