Experimental Physiology

Regulation of physiological responses to continuous and intermittent hypoxia by hypoxia-inducible factor 1

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Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor composed of HIF-1α and HIF-1β subunits that functions as a master regulator of oxygen homeostasis. Oxygen-dependent hydroxylation of HIF-1α provides a mechanism that allows changes in oxygenation to be transduced to the nucleus, leading to changes in gene expression. Hypoxia-inducible factor 1 plays critical roles in development, physiology and disease pathogenesis. Analyses of mice that are heterozygous for a null allele at the locus encoding the HIF-1α subunit have demonstrated that partial deficiency of HIF-1 is sufficient to impair multiple physiological responses to continuous and intermittent hypoxia.

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Oxygen homeostasis

Humans and other vertebrates have evolved complex circulatory and respiratory systems to insure the delivery of adequate supplies of O_2 to meet metabolic demands of every cell in the organism. Principal among these demands is the requirement for O_2 as ultimate electron acceptor in the process of oxidative phosphorylation, which generates sufficient ATP from the catabolism of glucose and fatty acids to maintain the complex nature of vertebrate structure and function. However, the need for O_2 as metabolic substrate is opposed by the inherent risk of oxidative damage to cellular macromolecules. Because of the dual character of O_2 as an essential but potentially toxic molecule, its concentration within cells of a healthy organism is maintained within a narrow range that optimally balances supply and demand.

Identification of hypoxia-inducible factor 1 (HIF-1)

At the molecular level, oxygen homeostasis is achieved by acute adaptive responses, which involve changes in the activity of pre-existing proteins, and chronic responses, which involve changes in gene expression leading to new protein synthesis. The transcriptional factor HIF-1 is a master regulator of oxygen homeostasis, HIF-1 was discovered through studies analysing the molecular mechanisms regulating expression of the human gene encoding erythropoietin (EPO), the glycoprotein hormone that controls red blood cell production and, thus, blood O_2-carrying capacity.

These studies identified a *cis*-acting hypoxia-response element (HRE) in the 3′-flanking region of the *EPO* gene (Beck *et al.* 1991; Semenza *et al.* 1991). Similar results were reported for the mouse *Epo* gene (Pugh *et al.* 1991). When this HRE was inserted into a heterologous reporter gene and transfected into human cells, exposure of the cells to hypoxic conditions (1% O_2) resulted in a dramatic increase in transcription of the reporter gene, similar to that of the endogenous *EPO* gene. Deletion analysis localized the minimal HRE to a 33-base-pair (bp) sequence, and mutagenesis identified a sequence, 5′-CTACGTGCT-3′, that when mutated resulted in loss of HRE activity (Semenza & Wang, 1992). A nuclear factor designated HIF-1 was identified, which was present in hypoxic cells but not in cells incubated under standard tissue culture conditions of 95% air and 5% CO_2 (20% O_2) and which bound to a double-stranded oligonucleotide containing the wild-type sequence but not to an oligonucleotide containing a 3 bp mutation in the DNA sequence (Semenza & Wang, 1992).

The differential binding of HIF-1 to the wild-type and mutant sequences was used as a positive and negative selection, respectively, to perform a biochemical purification of HIF-1 by DNA affinity chromatography from 100 l of human HeLa cells grown in suspension

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culture (Wang & Semenza, 1995), leading to the isolation of nucleic acid sequences encoding the HIF-1α and HIF-1β subunits (Wang *et al.* 1995) and the generation of monoclonal antibodies that recognize the cognate proteins (Zhong *et al.* 1999; Talks *et al.* 2000; Zagzag *et al.* 2000). The utilization of these tools has led to a revolution in the molecular analysis of O_2 homeostasis in health and disease.

Transducing changes in oxygenation to changes in gene expression

Analysis of nuclear extracts prepared from HeLa cells exposed to precisely defined O_2 concentrations revealed that reduction from 20 to 6% O_2 resulted in a twofold increase in HIF-1α protein levels, whereas below 6% O_2, HIF-1α levels increased exponentially, with a half-maximal response at 1.5–2% O_2 and a maximal response at 0.5% O_2, with HIF-1α protein levels that were ~10-fold greater than those in cells at 6% O_2 (Jiang *et al.* 1996). The levels of HIF-1β in nuclear extracts of hypoxic cells increased only modestly, an effect that was later shown to result from a increased loss of the protein during preparation of nuclear extracts from non-hypoxic cells rather than an actual increase in protein levels within hypoxic cells (Chilov *et al.* 1999).

Regulation of HIF-1α protein levels is effected by O_2-dependent ubiquitination and proteasomal degradation (Salceda & Caro, 1997; Huang *et al.* 1998; Kallio *et al.* 1999), which is mediated by the binding of the von Hippel-Lindau (VHL) tumour suppressor protein (Maxwell *et al.* 1999). VHL binding to HIF-1α is triggered by the hydroxylation of two proline residues (Pro402 and Pro564 in human HIF-1α; Epstein *et al.* 2001; Ivan *et al.* 2001; Jaakkola *et al.* 2001; Yu *et al.* 2001). The HIF-1α prolyl hydroxylases that perform this modification (Bruick & McKnight, 2001; Epstein *et al.* 2001) utilize molecular O_2 and α-ketoglutarate as substrates and generate succinate as a byproduct (Hewitson *et al.* 2004). Under conditions of severe hypoxia, substrate (O_2) deprivation is sufficient to inhibit the hydroxylation reaction, whereas under conditions of moderate hypoxia, the mitochondrial generation of reactive O_2 species is also required to inhibit the hydroxylases (Brunelle *et al.* 2005; Guzy *et al.* 2005; Mansfield *et al.* 2005). The O_2-dependent hydroxylation of HIF-1α provides a molecular mechanism by which changes in oxygenation can be transduced to the nucleus via HIF-1, leading to changes in gene expression.

HIF-1 is required for embryonic development

Homologous recombination in mouse embryonic stem cells was used to generate a null allele at the *Hif1a* locus encoding HIF-1α (Iyer *et al.* 1998). Mouse embryos homozygous for the null allele arrested in their development by embryonic day 8.5 and died by day 10.5 with cardiac malformations, vascular regression and mesenchymal cell death (Iyer *et al.* 1998; Kotch *et al.* 1999). Embryonic lethality of HIF-1α-null embryos was subsequently confirmed by independent mouse lines generated in two other laboratories (Carmeliet *et al.* 1998; Ryan *et al.* 1998).

Involvement of HIF-1 in hypoxia-induced pulmonary vascular remodelling

The embryonic lethality of HIF-1α-null mice precluded analysis of the effects of complete HIF-1α deficiency on postnatal physiology. However, the heterozygous-null mice, which develop normally and are indistinguishable from wild-type littermates, have impaired physiological responses to hypoxia and ischaemia. Exposure of wild-type littermates to continuous hypoxia (10% O_2 for 3 weeks) results in the development of pulmonary hypertension, which is manifested by right ventricular hypertrophy, elevated pulmonary artery pressure and increased medial wall thickness of pulmonary arterioles. All of these responses were impaired in heterozygotes (Yu *et al.* 1999). Electrophysiological analysis of pulmonary artery smooth muscle cells (PASMC) isolated from normoxic and hypoxic wild-type and heterozygous-null (HET) mice revealed that the hypoxia-induced hypertrophy and depolarization of these cells was impaired in HET mice (Shimoda *et al.* 2001). The depolarization of PASMCs from hypoxic wild-type mice was associated with a reduction in voltage-gated K^+ channel currents, an effect that was not observed in PASMCs from hypoxic HET mice (Shimoda *et al.* 2001). Depolarization of PASMCs from hypoxic wild-type mice was associated with increased intracellular Ca^{2+} concentrations, an effect that was lost in HET mice. Taken together, these data indicate that HIF-1 plays a critical role in the pathophysiology of hypoxia-induced pulmonary hypertension.

HIF-1 is required for ventilatory responses to hypoxia mediated by the carotid body

The carotid bodies sense arterial partial pressure of O_2 and respond to hypoxaemia by activating neural pathways that stimulate increases in blood pressure, heart rate and ventilation. Ventilatory adaptation to chronic hypoxia, in which exposure to ambient hypoxia for several days results in an augmented ventilatory response to a subsequent acute hypoxic stimulus, is believed to be mediated by the carotid bodies. While HET mice manifested ventilatory responses to an acute hypoxic challenge, the ventilatory adaptation to chronic hypoxia was lost (Kline *et al.* 2002), suggesting a loss of carotid body function.

Vagotomy, which interrupts neurotransmission from oxygen-sensing cells in the aorta (aortic bodies) but not from the carotid body, did not affect the augmentation of phrenic nerve activity in response to an acute hypoxic stimulus in wild-type mice, whereas in HET mice, phrenic nerve activity in response to hypoxia was lost, suggesting that acute O_2 sensing in HET mice was principally mediated by aortic bodies, whereas the carotid bodies performed this function in wild-type mice.

Single fibre recording of sinus nerve activity from carotid bodies isolated from HET mice revealed a complete loss of neural activity in response to hypoxia. In contrast, sinus nerve activity stimulated by cyanide application was identical in wild-type and HET mice, as was carotid body histology, including glomus cell morphometry. Thus, partial HIF-1α deficiency results in a specific and virtually complete loss of carotid body oxygen sensing and/or signal transduction. In HET mice, aortic bodies compensate for the lack of carotid body function, similar to the recovery of oxygen sensing that is observed after carotid denervation. Thus, the carotid bodies are particularly dependent upon normal levels of HIF-1α expression for their physiological function as arterial oxygen sensors.

HIF-1 is required for cardiovascular and ventilatory responses to intermittent hypoxia

Individuals with obstructive sleep apnoea are subjected to chronic intermittent hypoxia during sleep. Exposure of mice to chronic intermittent hypoxia induces ventilatory adaptation to acute hypoxia in wild-type but not in HET mice, whereas the ventilatory adaptation to acute hypercapnia that is induced by chronic intermittent hypoxia is present in both wild-type and HET mice (Peng YJ, Yuan G, Ramakrishnan D, Sharma SD, Bosch-Marcé M, Kumar GK, Semenza GL & Prabhakar NR, unpublished observations).

In contrast to chronic continuous hypoxia, which results in the development of pulmonary hypertension, chronic intermittent hypoxia results in the development of systemic hypertension. Exposure of mice to chronic intermittent hypoxia for 10 days induces elevated serum noradrenaline levels and systemic hypertension in wild-type mice but not in HET mice (Peng YJ, Yuan G, Ramakrishnan D, Sharma SD, Bosch-Marcé M, Kumar GK, Semenza GL & Prabhakar NR, unpublished observations).

In addition to the development of systemic hypertension, individuals with intermittent hypoxia owing to sleep apnoea develop a metabolic syndrome consisting of insulin resistance, hypercholesterolaemia and hypertriglyceridaemia. These responses are also observed in wild-type mice exposed to intermittent hypoxia for 5 days, whereas in HET mice impaired development of hypertriglyceridaemia is associated with impaired activation of sterol response element binding protein 1, a key activator of triglyceride synthesis (Li *et al.* 2006).

The studies described above have demonstrated that partial deficiency of HIF-1α results in impaired physiological responses to chronic exposure to both continuous and intermittent hypoxia. Major causes of continuous and intermittent hypoxia are chronic obstructive lung disease resulting from smoking and obstructive sleep apnoea associated with obesity, respectively, which have arisen only recently in the history of *Homo sapiens* and thus have not been subjected to extensive natural selection. As a result, the physiological systems that have evolved to mediate adaptive responses and protect the organism against causes of hypoxia that would have been commonly encountered during most of human history, such as traumatic blood loss and acute pneumonia, mediate maladaptive responses to these novel stimuli. Further studies are needed to determine whether these maladaptive responses can be inhibited, e.g. by pharmacological inhibition of HIF-1 (Semenza, 2006), without interfering with other critical aspects of oxygen homeostasis that are also controlled by HIF-1.

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