The oxygen sensing signal cascade under the influence of reactive oxygen species

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Structural and functional integrity of organ function profoundly depends on a regular oxygen and glucose supply. Any disturbance of this supply becomes life threatening and may result in severe loss of organ function. Particular reductions in oxygen availability (hypoxia) caused by respiratory or blood circulation irregularities cannot be tolerated for longer periods due to an insufficient energy supply by anaerobic glycolysis. Complex cellular oxygen sensing systems have evolved to tightly regulate oxygen homeostasis. In response to variations in oxygen partial pressure (PO_2), these systems induce adaptive and protective mechanisms to avoid or at least minimize tissue damage. These various responses might be based on a range of oxygen sensing signal cascades including an isoform of the neutrophil NADPH oxidase, different electron carrier units of the mitochondrial chain such as a specialized mitochondrial, low PO_2 affinity cytochrome *c* oxidase (aa_3) and a subfamily of 2-oxoglutarate dependent dioxygenases termed HIF (hypoxia inducible factor) prolyl-hydroxylase and HIF asparaginyl hydroxylase called factor-inhibiting HIF (FIH-1). Thus, specific oxygen sensing cascades involving reactive oxygen species as second messengers may by means of their different oxygen sensitivities, cell-specific and subcellular localization help to tailor various adaptive responses according to differences in tissue oxygen availability.

Keywords: oxygen sensing; HIF prolyl hydroxylase; NADPH oxidase; cytochrome aa_3 ; reactive oxygen species; carotid body

1. INTRODUCTION

The respiratory and cardiovascular systems allow the provision and appropriate distribution of oxygen to serve as the terminal electron acceptor during mitochondrial oxidative phosphorylation as the major biochemical reaction for the generation of energy in the form of ATP. The process of extracting oxygen from the environment and its distribution, not only for oxidative phosphorylation but also as a substrate for other biochemical reactions, has been conserved through evolution by the development of advanced multi-level systems. These tightly maintain O_2 homeostasis, i.e. keep the O_2 concentrations even within a single cell in a narrow physiological range, allowing the cell to survive, function and thrive in regions with heterogeneous PO_2 distribution. Under physiological conditions, the arterial PO_2 is about 90 mmHg. However, differences in vascularization, tissue diffusion properties and cell-specific oxygen consumption most likely account for the heterogeneous PO_2 distribution within different organs resulting in tissue PO_2 between 90 and 1 mmHg (Leniger-Follert *et al.* 1975). Cell function and viability and thus structural and functional integrity of the different organs in the body heavily rely on a constant oxygen and glucose supply. A large proportion of the ATP generated during oxidative phosphorylation is required to maintain ion homeostasis and membrane potential. Any disturbance

in this supply becomes life threatening and may result in severe loss of organ function. Particular reductions in oxygen availability caused by respiratory or blood circulation irregularities cannot be tolerated for longer periods due to an insufficient energy supply by anaerobic glycolysis. On the other hand, excessive oxygenation harbours the risk of cell injury by accumulation of reactive oxygen species (ROS) capable of oxidizing macromolecules such as lipids, proteins and nucleic acids, a process termed oxidative stress. Thus, complex cellular oxygen sensing systems have evolved to ensure tight regulation of oxygen homeostasis to avoid metabolic compromise or the risk of oxidation toxicity. These systems induce an elaborate sequence of adaptive mechanisms in response to variations in PO_2 to avoid or at least minimize organ damage including short- (seconds, minutes) and long-term (hours, days) acting responses. Changes in oxygen tension elicit different responses by the cell, which activate short- and long-term acting adaptive mechanisms. A putative oxygen sensor system has to fulfil several crucial requirements. Sensor responses should inherently depend on the ability to sense oxygen concentrations and, whenever the PO_2 deviates from a given pre-set value, to initiate distinct signalling cascades. The wide operating field of the tissue PO_2 distribution (as shown at the lower part of figure 4) suggests that the threshold of activation may vary from organ to organ and cell to cell and may also depend on the developmental stage of the organism (Acker *et al.* 1980), arguing for flexible and highly adaptive systems. These should allow for graded subsequent cellular reactions, partly requiring changes in gene expression.

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Figure 1. Identification of carotid body heme proteins by light absorption photometry. N_2 versus aerobic steady state spectrum (black solid noisy line) as a mean of six carotid bodies fitted by different mitochondrial and non-mitochondrial cytochrome spectra as indicated by different colours. The superposition curve (red solid line) obtained by varying the amplitude of the optical density of five cytochromes closely fits the experimental curve (Streller *et al.* 2002).

Figure 2. Nonlinear redox change of putative oxygen sensing heme proteins. (a) Redox changes of different carotid body heme proteins as calculated by deconvolution as shown in figure 1 are related to different PO_2 values in the superfusion bath of the isolated haemoglobin free carotid body as well as to the chemoreceptor peak discharge. Cytochromes a592 and b558 only show a nonlinear redox change fitting the PO_2 –chemoreceptor discharge relationship. (b) Redox changes of different carotid body heme proteins as calculated by deconvolution as shown in figure 1 are related to different CN^- concentrations in the superfusion bath of the isolated haemoglobin free carotid body as well as to the chemoreceptor peak discharge. Cytochrome a592 only shows a nonlinear redox change. B type cytochromes are not detectable by deconvolution due to CN^- insensitivity (Streller *et al.* 2002).

Oxygen sensing heme proteins and prolyl-hydroxylase (PHD) using ROS as physiological second messengers have been described as candidate sensor systems connecting an oxygen-dependent enzymatic activity to the regulation of hypoxia-inducible responses (Acker & Acker 2004).

2. CAROTID BODY

(a) Heme protein identification

Molecules changing their chemical properties in direct dependence on the surrounding PO_2 may mediate the first step in oxygen sensing. Studies on *Caenorhabditis elegans* have shown, for example, that a specific soluble guanylate cyclase homologue binding O_2 instead of NO to the heme domain triggers the sensory cGMP-gated

channel tax-2/tax-4 which leads to a strong behavioural preference for 5–12% oxygen (Gray *et al.* 2004). As for the carotid body, hypoxia leads to an increase in afferent carotid sinus nerve activity stimulating ventilation and blood circulation of the body to avoid hypoxic tissue damage. The primary oxygen sensor triggering this increase is yet unknown but there is large agreement that it is a heme protein, either a mitochondrial component of the respiratory chain (Wilson *et al.* 1994; Baysal *et al.* 2000) or a non-mitochondrial protein like the NAPH oxidase (Nox) (Cross *et al.* 1990; Fu *et al.* 2000). Light absorption spectra identified very recently a cytochrome a592 (shown in yellow in figure 1) as a unique component of carotid body cytochrome c oxidase (Streller *et al.* 2002). Figure 1 shows a N_2 versus normoxia steady

Figure 3. Three-dimensional 2-photon confocal microscopy of components involved in hypoxia induced gene expression. (a,b) Translocation of HIF-1 α (yellow) from the ER (red) into the nucleus of a human liver tumour cell (HepG2) under hypoxia (Berchner-Pfannschmidt *et al.* 2004). (c,d) OH $\cdot$ production (white) in the ER (red) of a human liver tumour cell (HepG2) under hypoxic (c) and normoxic (d) conditions. OH $\cdot$ production was stimulated by a 5 s blue light illumination inducing a photoreduction of FAD containing oxidases (Liu *et al.* 2004). (e) Overexpression of p22phox (blue) of the NADPH oxidase in the perinuclear space of a human liver tumour cell (HepG2) with an increased OH $\cdot$ production (red) probably fuelling an ER-based Fenton reaction (Djordjevic *et al.* 2005). (f) Predominant perinuclear localization of PHD2 in a human osteosarcoma cell (U2OS) (Metzen *et al.* 2003).

state light absorption difference spectrum of rat carotid body. Various spectra of single cytochromes have been used for deconvolution of the measured spectrum like mitochondrial cytochrome *c*, b563 of complex III and cytochrome a3 of complex IV. Furthermore, b558 of the NADPH oxidase as well as the unique a592,

probably a blue shifted cytochrome *a* of mitochondrial complex IV, were necessary to fit the measured spectrum. The deconvolution procedure enabled us to calculate the relative redox change of the different carotid body cytochromes under various PO $_2$ and CN $^-$ conditions as shown in figure 2a,b.

(b) Heme protein oxygen sensor function

In figure 2a, the cytochrome redox changes are related to the peak chemoreceptor discharge at different PO₂ conditions. Cytochrome *c*, b563 and a3 show a redox change in a linear relation to the actual PO₂. Cytochrome a592 and b558, however, reveal a non-linear relationship to the actual PO₂ as it is known from the relationship between chemoreceptor discharge and PO₂. It was postulated that a NADPH oxidase isoform within carotid body type I cell (shown in dark green in figure 1) functions as an oxygen sensor to regulate ion channel conductivity and gene expression (Cross *et al.* 1990). With this regard various NADPH oxidase isoforms composed of the subunits p22phox, gp91phox, p47phox, p40phox, p67phox and Rac1, 2 have to be considered. Their function may, however, not be limited to the carotid body as they show widespread expression throughout the body, e.g. Nox1 in pulmonary vasculature smooth muscle cells (Weissmann *et al.* 2000; Goyal *et al.* 2004) or the neutrophil Nox2 in endothelial cells (Görlach *et al.* 2000b) and neuroepithelial bodies (NEB) (Fu *et al.* 2000). The isoforms (Nox1–4 and the Duox group) make use of the gp91phox component as described by (Cheng *et al.* 2001). Gp91phox knock out mice showed an impaired hypoxic ventilatory control in neonatal animals due to a decreased oxygen sensitivity of NEB potassium channel conductivity (Kazemian *et al.* 2001), evidencing oxygen sensor function of the NADPH oxidase in this cell type and related strains (Peers & Kemp 2001). On the other hand, gp91phox knock out mice showed unimpaired oxygen sensing function of pulmonary vasculature smooth muscle cells (Archer *et al.* 1999) or carotid body hypoxic drive (Roy *et al.* 2000). However, p47phox knock out mice demonstrated an enhanced carotid body hypoxic drive, suggesting a particular Nox isoform for the carotid body (Sanders *et al.* 2002). Taken together various NADPH oxidase isoforms may act as part of the oxygen sensing system by decreasing ROS production under hypoxia for triggering the inhibition of potassium channels to increase the calcium influx into chemoreceptor cells. This assumption might substantiated by the excitatory effect of iron chelators on carotid body nervous activity probably hinting to a Fenton reaction fuelled by the NADPH oxidase generated ROS (Roy *et al.* 2004). Cytochrome a592 might possess a low midpoint potential as described in bacteria (Kannt *et al.* 1999), inducing a short cut of electron flow within the cytochrome *c* oxidase between Cu_A and cytochrome a3-Cu_B. It was assumed that this specific property would allow the regulation of intracellular calcium levels under hypoxia (Streller *et al.* 2002) by shifting complex IV to more negative values, and hence decreased mitochondrial membrane potential (MMP) at physiological tissue PO₂ values explaining perhaps the linear dependence of carotid body oxygen consumption on PO₂ (Acker & Lübbers 1977). Small decreases of the ambient PO₂ would decrease MMP even more and impair the mitochondrial calcium buffering capacity (Duchen & Biscoe 1992), leading in concert with b558 to an unusual PO₂ sensitivity of carotid body transmitter release and chemoreceptor discharge. Cytochrome a592 would

represent the unique low PO₂ affinity and cytochrome a3 the high PO₂ affinity component of the carotid body mitochondrial complex IV as already suggested by Mills and Jöbsis (Mills & Jöbsis 1972).

In figure 2b, the nonlinear reduction of cytochrome a592 in response to CN[−] is to be seen whereas cytochrome a3 and cytochrome *c* reveal a linear response to the different CN[−] concentrations. Cytochrome b558 and b563 are not responsive to CN[−]. It could be assumed that the increased chemoreceptor discharge might be due to binding of CN[−] to cytochrome a3 of complex IV, and comparable therefore to the light sensitive excitatory effect of CO (Wilson *et al.* 1994; Streller *et al.* 2002) but not to the hypoxia-induced chemoreceptor excitation.

3. HIF AND HYDROXYLASES**(a) Molecular mechanism**

The immediate carotid body response to PO₂ changes is based on a functional hypoxia inducible factor (HIF) transcriptional complex (Kline *et al.* 2002). This complex is widely conserved among mammalian species and invertebrate model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans*, further stressing its importance as a key transcriptional regulator of hypoxia-induced responses throughout evolution (Huang & Bunn 2003). The HIF complex exists as a heterodimer composed of constitutively expressed HIF-1β and O₂ regulated HIF-1α subunits belonging to the basic helix loop helix (bHLH)-PAS (PER-ARNT-SIM) family of transcription factors. Both HIF-1α and HIF-1β proteins exist as isoforms (HIF-1α, HIF-2α, HIF-3α and ARNT (aryl hydrocarbon receptor nuclear translocator), ARNT2 and ARNT3, respectively) reviewed in Wenger (2002) and Fandrey (2004). HIF activity is tightly regulated throughout the range of physiological and pathological oxygen concentrations, involving multiple mechanisms of control at the level of mRNA expression, protein stability, nuclear translocation and transactivation activity. These combine cooperatively to activate and stabilize HIF to maximal levels under decreasing oxygen concentrations for translocating into the nucleus as shown in figure 3a,b. Here, it is to be seen that HIF (yellow) stabilizes under hypoxia in the endoplasmic reticulum (ER, red) and uses pores which the ER forms with the outer nuclear membrane for translocation into the nucleus. On the molecular level, this is mediated by subjecting HIF-1α subunits to multiple modes of post-translational modification including lysine residue acetylation (Lee *et al.* 2003), phosphorylation and two different types of hydroxylation. The dominant control mechanism occurs through oxygen-dependent proteolysis of HIF-1α subunits. Oxygen-dependent enzymatic hydroxylation of proline residues within HIF-1α subunits constitutes the critical modification governing protein stability. Prolyl hydroxylation allows capture by the von Hippel Lindau tumour suppressor protein (pVHL), which acts as the recognition component of an E3-ubiquitin ligase enzyme. Subsequent ubiquitination targets the complex for proteosomal degradation. As a consequence only low-level HIF-1α protein expression

can be detected in the presence of oxygen increasing rapidly and exponentially with decreasing oxygen concentrations. A second oxygen dependent switch involving hydroxylation of an asparagine residue within the transactivation domain regulates transcriptional activity possibly by interfering with recruitment of the coactivator p300 which results in reduced transcriptional activity. The HIF transcriptional system acts as a master regulator of oxygen-regulated gene expression, inducing adaptive mechanisms which serve the common purpose of maintaining oxygen homeostasis. To date more than 60 putative HIF-target genes have been identified, expression of which governs important processes such as angiogenesis and regulation of vascular tone, erythropoiesis, iron homeostasis, energy metabolism and pH regulation as well as cell survival and proliferation reviewed in Semenza (2003) and Fandrey (2004). Hydroxylation provides a dual mechanism of inhibiting HIF activity, inducing proteolytic degradation and reducing transcriptional capacity. These processes are conferred by a recently identified subclass of 2-oxoglutarate dependent hydroxylases. Interaction of VHL with HIF-1 α requires an O₂⁻ and iron-dependent hydroxylation of specific prolyl residues (Pro 402, Pro 564) within the HIF-1 α ODD (Oxygen-dependent-domain) carried out by HIF-PHD (Epstein *et al.* 2001; Oehme *et al.* 2002; Erbel *et al.* 2003). So far, four orthologues of PHD have been described (PHD I-IV). A second oxygen dependent switch involves hydroxylation of an asparagine residue within the C-TAD of HIF-1 α subunits by a recently identified HIF asparaginyl hydroxylase called factor-inhibiting HIF (FIH-1) (Lando *et al.* 2002). Asparagine hydroxylation apparently interferes with recruitment of the coactivator p300 resulting in reduced transcriptional activity. Both, PHD and FIH belong to a superfamily of 2-oxoglutarate dependent hydroxylases which employ non-haem iron in the catalytic moiety (Hewitson *et al.* 2002). They require oxygen in the form of dioxygen with one oxygen atom being incorporated in the prolyl or asparagyl residue, respectively, and the other into 2-oxoglutarate, yielding succinate and CO₂. Thus, the hydroxylation reaction is inherently dependent on ambient oxygen pressure, providing a molecular basis for the oxygen-sensing function of these enzymes.

(b) Oxygen sensor function

Interestingly, PHDs are strikingly sensitive to graded levels of oxygen *in vitro*, mirroring the progressive increase in HIF-1 α protein stability and transactivation activity observed when cells are subjected to graded hypoxia *in vitro* (Epstein *et al.* 2001). In line with this observation, PHDs have been found to have a striking low O₂ affinity of 178 mmHg above the concentration of dissolved O₂ in the air (Hirsilä *et al.* 2003). Consequently, taking the regular tissue PO₂ distribution as shown in the lower part of figure 4, PHDs would operate under suboptimal, non-equilibrium conditions for HIF- α turnover far beyond their K_m . However, given a regular Michaelis-Menten kinetic this would allow the enzymes to operate in a highly sensitive manner, in which already small changes in oxygen-concentration result in pronounced changes of enzymatic reaction velocity, thus HIF-1 α

Figure 4. Modulation of oxygen sensor sensitivity by co-factors. Oxygen sensing systems connecting an oxygen-dependent enzymatic activity to the regulation of hypoxia-inducible responses should operate at high and low PO₂ affinities, fitting the heterogeneous PO₂ distribution curve (lower part). Oxygen sensing heme proteins such as mitochondrial complex IV (cytochrome *c* oxidase) and NAD(P)H-oxidase as well as PHD (upper part) have been described as candidate sensor systems functioning at different K_m for PO₂. Modulation of the specific PO₂ affinities or oxygen sensor activities would allow to efficiently trigger and fine-tune various signal cascades to optimize cellular function and adaptation over a broad range of O₂ concentrations (Acker & Acker 2004).

turnover. In contrast, collagen prolyl-4-hydroxylases exhibit a K_m of about 28 mmHg, one-sixth of the K_m of PHD, allowing optimal hydroxyprolyl-collagen biosynthesis under the low oxygen concentrations physiologically found in the cell (Hirsilä *et al.* 2003). Recently, FIH was shown to have a K_m of around 64 mmHg, suggesting that also this enzyme acts as a bona fide oxygen sensor at least under conditions as found in normoxic tissues *in vivo* (Linke *et al.* 2004). Immunohistochemical staining of tissues for HIF-1 α subunits provides an indirect method to assess the activity of the PHD/HIF system *in vivo*. These studies have documented that HIF- α levels are, generally, low in rodent tissues under physiological conditions and are substantially increased in response to systemic hypoxia or tissue ischaemia (Stroka *et al.* 2001; Wiesener *et al.* 2003). Interestingly, HIF-1 α levels remain low even in regions such as the renal medulla, which are characterized by low oxygen tensions known to enhance HIF-1 α protein *in vitro*. In addition, the extent and time course of induction as well as cell type specific expression varies suggesting that individual, cell specific thresholds for activation of the response may exist. The above mentioned characteristics of the PHD system renders it highly sensitive to alterations of co-factor concentration such as ferrous iron (Knowles *et al.* 2003) or 2-oxoglutarate, of substrate concentration, e.g. due to changes in HIF- α synthesis (Wiener *et al.* 1996; Zhong *et al.* 2000), as well as of enzyme concentration, e.g. due to changes in mRNA

expression of PHD orthologues in response to PO_2 (Epstein *et al.* 2001) being particularly striking for PHD3 (del Peso *et al.* 2003).

(c) *Reactive oxygen species*

Apart from PO_2 , PHD activity is regulated by the amount of ferrous iron (reduced form) recovered by antioxidants such as Vitamin C (Knowles *et al.* 2003). Iron is required for heme formation, being the most common limiting factor for erythropoiesis. Interestingly, HIF-target genes regulate different steps in iron homeostasis from iron uptake to iron transport and iron storage (Rolfs *et al.* 1997; Lok & Ponka 1999; Tacchini *et al.* 1999; Mukhopadhyay *et al.* 2000). The implication of iron in oxygen sensing by 2-oxoglutarate dependent hydroxylases and the involvement of iron in oxygen toxicity through the Fenton reaction (Porwol *et al.* 1998) generating ROS gives an additional need for tight iron regulation, making the interaction between oxygen and iron homeostasis physiologically highly appropriate. Apart from toxic accumulation during reoxygenation periods ROS have been implicated as intracellular second messengers, reviewed in Lander (1997) and Nordberg & Arner (2001). Increased ROS in tissues act on different signal pathways, e.g. the open probability of potassium channels (Duprat *et al.* 1995) or via enhancement of GATA-2 binding activity to attenuate activity of the Epo promoter (Tabata *et al.* 2001). Reports on HIF functions are particularly perplexing as depending on whether ROS are part of a normoxic, hypoxic or growth factor signalling cascade response, different outcomes on HIF activity have been observed. Further complexity is added by the fact that studies report contradicting results on whether ROS decrease or increase with decreasing O_2 concentrations in relation to the normoxic ROS levels (Chandel *et al.* 1998; Fandrey & Genius 2000). ROS visualization and measurement is technically challenging, explaining why the majority of studies rely on either depleting the cells of ROS by antioxidant treatment or increasing ROS by application of pro-oxidants. These studies have documented an enhanced HIF- α protein/HIF-reporter gene expression upon ROS decrease under normoxic (Canbolat *et al.* 1998; Görlach *et al.* 2003; Wartenberg *et al.* 2003; Liu *et al.* 2004) and an attenuated HIF- α protein/HIF-reporter gene expression upon ROS increase under hypoxic conditions (Wang *et al.* 1995; Huang *et al.* 1996; Fandrey *et al.* 1997; Wiesener *et al.* 1998; Canbolat *et al.* 1998; Görlach *et al.* 2003; Wartenberg *et al.* 2003). One recent study provided further evidence for an ER-based $\text{OH}^\cdot$ production mediated by the Fenton reaction in regulating HIF degradation (Liu *et al.* 2004), as shown in figure 3c,d. Figure 3c shows ER (red) based $\text{OH}^\cdot$ production (white) under hypoxia and figure 3d under normoxia. The drastic increased $\text{OH}^\cdot$ production under normoxia is striking. These observations are inline with the concept that declining O_2 concentrations trigger the hypoxia response as a result of decreased ROS intermediate production. The NAD(P)H oxidase as the major donor of ROS would be at the centre of this model to convert PO_2 to a redox signal. Overexpression of p22phox of the NADPH oxidase shows a perinuclear colocalization (figure 3e, blue) with an increased $\text{OH}^\cdot$

production (figure 3e, red) probably fuelling the ER-based Fenton reaction (Djordjevic *et al.* 2005). How this redox signal is transduced to HIF-1 α is not known. While ROS clearly affect the redox state of the cell and thus thioredoxin and Ref-1 function, it is of interest to analyse whether ROS signalling in these settings interfaces with PHD function. Interestingly, ROS, for example, have been shown to interfere with mitochondrial function affecting enzymes of the Krebs-cycle at the level of alpha-ketoglutarate dehydrogenase (KGDH) and SDH (Nulton-Persson & Szweda 2001) which may influence 2-oxoglutarate levels (see above). In contrast, during hypoxic events mitochondria have been suggested to be the major source of ROS formation at complex III (Chandel *et al.* 1998), aiding HIF- α stabilization (Chandel *et al.* 2000; Hirota & Semenza 2001). However, the issue of mitochondrial ROS formation is controversial as other groups have reported that the decreasing MMP is associated with a reduction in mitochondrial ROS formation (Jones *et al.* 2000; Lee *et al.* 2001). Other reports have shown a general ROS decrease in hypoxia (Görlach *et al.* 2003) and further documented that a functioning mitochondrial respiratory chain may not be necessary for HIF-1 α regulation (Srinivas *et al.* 2001; Vaux *et al.* 2001). It is worth mentioning that not all ROS mediated pathways on HIF activity are part of an oxygen signalling response but rather expression of a delicate integration of oxygen sensing mechanisms into major signalling pathways. Extracellular signals like growth factors and cytokines have been shown to increase NAD(P)H oxidase mediated ROS formation (Görlach *et al.* 2000a) with subsequent HIF stabilization under normoxic conditions (Haddad & Land 2001; Richard *et al.* 2000). However, this mode of activation seems to be cell-specific.

(d) *Intracellular localization*

Interestingly, though being ubiquitously expressed in various tissues PHD orthologues differ in their relative cell-specific abundance of mRNA levels (Lieb *et al.* 2002; Oehme *et al.* 2002; Cioffi *et al.* 2003). Moreover, PHD2 and PHD3 expression is upregulated by hypoxia though the fold-induction apparently varies between cell type and PO_2 analysed (Epstein *et al.* 2001; Berra *et al.* 2003; del Peso *et al.* 2003; Metzen *et al.* 2003). The exact role of the four PHD orthologues in the regulation of HIF activity remains elusive. However, it is evident that the hydroxylation efficiency among PHD1–3 is not identical and in particular differs regarding HIF-1 α orthologue preference and prolyl hydroxylation pattern, favouring the C-terminal prolyl residue (Hirsilä *et al.* 2003). siRNA mediated knock-down studies suggest that PHD2 is the rate-limiting enzyme controlling the steady-state levels of HIF-1 α in normoxia at least under cell culture conditions (Berra *et al.* 2003). Moreover, the study implicated PHD2 in the initial stages of HIF-1 α degradation following reoxygenation. Interestingly, a role for PHD1 in controlling HIF-1 α levels under long-term maintenance of hypoxia (5–6 days) was proposed, suggesting further non-redundant functions of each PHD orthologues in other physiological or pathophysiological settings. Subcellular localization of PHD has been

studied by ectopic expression of chimeric EGFP-fusion proteins and three-dimensional 2-photon confocal laser scanning microscopy (2P-CLSM) analysis (Metzen *et al.* 2003). PHD1 was detectable exclusively in the nucleus, PHD3 was distributed more evenly in cytoplasm and nucleus, while the majority of PHD2 was found in the cytoplasm, as imaged in figure 3f, similar to FIH-1. The latter result is of interest because PHD2 and FIH-1 have a calculated molecular weight of 46 and 40.3 kD, respectively. As molecules of up to 60 kD usually have free access to the nuclear compartment, PHD2 and FIH-1 would be expected to be present in cytoplasm and nucleus. This finding may suggest that PHD2 and FIH-1 are actively excluded from the nucleus. Overexpression of PHD2 and PHD3 inhibited nuclear HIF-1 α translocation under hypoxia, indicating a delicate balance between intracellular HIF-1 α and PHD protein concentration which determines the final hypoxic response (Metzen *et al.* 2003).

4. CONCLUSION

How can one reconcile the different candidates and concepts of oxygen sensing? Figure 4 shows in the upper part the putative cooperation of the three different oxygen sensing systems as described above under normoxic and hypoxic conditions.

(a) Putative oxygen sensor

The mitochondrial chain is a classical high oxygen affinity system keeping the electron flux across the cytochrome *c* oxidase constant below PO₂ values of 1 mmHg (Arthur *et al.* 1999). Consequently, the tissue PO₂ range covered by the oxygen sensing mitochondrial chain is small. However, with cytochrome a592 an affinity modulator was proposed, lowering the high PO₂ affinity of cytochrome *c* oxidase to about 30 mmHg (Streller *et al.* 2002). The NAD(P)H oxidase with a PO₂ affinity of about 15 mmHg seems to be the main ROS donor in many cell systems (Jones *et al.* 2000). The low *K_m* of the NAD(P)H oxidase implies that it may function as an oxygen sensor operating at low to intermediate PO₂ ranges. Interestingly, NADPH-activity is exquisitely controlled by rac proteins and various growth factors (Görlach *et al.* 2003). In contrast, PHDs have a strikingly low PO₂ affinity of 178 mmHg (Hirsilä *et al.* 2003), far beyond the regular tissue PO₂ distribution, allowing efficient regulation of HIF activity under these conditions (see above). Further complexity is added by fact that decreasing O₂ concentrations lower PHD activity, that may be partially compensated for by the increased availability of ferrous iron due to the more reduced state of the cell. Thus, the interplay is rather intricate and activity of the PHD/HIF system is likely to be influenced by the redox-state of the cell. Indeed, HIF is regulated in an iron- and redox-sensitive fashion. Redox regulatory systems such as thioredoxin or Ref-1 have been shown to induce HIF-1 α stabilization and transactivation function (Huang *et al.* 1996; Ema *et al.* 1999; Carrero *et al.* 2000; Welsh *et al.* 2003). It is intriguing to speculate that PHD may thus also act as an iron- and redox-sensor (Acker & Acker 2004).

(b) Oxygen sensitivity

The operating field of these systems is the tissue PO₂ distribution curve of the corresponding organ. Normoxic PO₂ values range between 1 and 90 mmHg with a mean value of about 30 mmHg (Leniger-Follert *et al.* 1975). Upon decreases in oxygen concentration this distribution curve becomes more and more left-shifted depending on the degree of arterial blood hypoxia as shown schematically in the lower part of figure 4. A delicate degree of oxygen sensing regulation was recently suggested by a report showing that the PO₂ may differ in subcellular compartments, being under the control of signalling molecules such as NO (Hagen *et al.* 2003). Inhibition of mitochondrial respiration by NO under low oxygen tension resulted in reduced mitochondrial oxygen consumption, which led to an increase in intracellular oxygen availability and reactivation of prolyl hydroxylation of HIF- α subunits. Thus, by changing the intracellular PO₂ distribution field the metabolic oxidative activity crucially influences the threshold of the cell to elicit adaptive mechanisms in response to a given tissue PO₂. In this context, the different subcellular localizations of the putative oxygen sensing systems (cytochrome a592-mitochondria; NADPH-oxidase-cell membrane-perinuclear space; ROS (Fenton Reaction)-cellmembrane-ER; PHD2,3-perinuclear space-nucleus; FIH-perinuclear space) should be taken into account. Furthermore, a fine balanced ratio between HIF- α and PHDs intracellular protein concentration has a great impact on the final outcome of hypoxia-induced gene upregulation.

Thus, specific oxygen sensing cascades may by means of their different oxygen sensitivities, cell-specific, subcellular localization (Berchner-Pfannschmidt *et al.* 2004) and protein concentration help to tailor various adaptive and dynamic responses according to differences in tissue oxygen availability (Acker & Acker 2004). Thus, one should hypothesize the existence of oxygen sensing systems with high as well as low PO₂ affinities to fit the heterogeneous PO₂ distribution curve. Modulation of the specific PO₂ affinities or oxygen sensor activities, e.g. by integration into the major signalling pathways defined as non-hypoxic factors (Dery *et al.* 2005), would allow to efficiently trigger and fine-tune various signal cascades to optimize cellular function and adaptation over a broad range of PO₂ values.

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