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Review

Cellular oxygen sensing need in CNS function: physiological and pathological implications

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Summary

Structural and functional integrity of brain 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 brain function. In particular, reductions in oxygen availability (hypoxia) caused by systemic or local blood circulation irregularities cannot be tolerated for longer periods due to an insufficient energy supply to the brain by anaerobic glycolysis. Hypoxia has been implicated in central nervous system pathology in a number of disorders including stroke, head trauma, neoplasia and neurodegenerative disease. Complex cellular oxygen sensing systems have evolved for tight regulation of oxygen homeostasis in the brain. In response to variations in oxygen partial pressure (P_{O_2}) these induce adaptive mechanisms to avoid or at least minimize brain damage.

A significant advance in our understanding of the hypoxia response stems from the discovery of the hypoxia inducible factors (HIF), which act as key regulators of hypoxia-induced gene expression. Depending on the duration and severity of the oxygen deprivation, cellular oxygen-sensor responses activate a variety of short- and long-term energy saving and cellular protection mechanisms. Hypoxic adaptation encompasses an immediate depolarization block by changing potassium, sodium and chloride ion fluxes across the cellular membrane, a general inhibition of protein synthesis, and HIF-mediated upregulation of gene expression of enzymes or growth factors inducing angiogenesis, anaerobic glycolysis, cell survival or neural stem cell growth. However, sustained and prolonged activation of the HIF pathway may lead to a transition from neuroprotective to cell death responses. This is reflected by the dual features of the HIF system that include both anti- and proapoptotic components.

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 P_{O_2} affinity cytochrome *c* oxidase (aa_3) and a subfamily of 2-oxoglutarate dependent dioxygenases termed HIF prolyl-hydroxylase (PHD) and HIF asparaginyl hydroxylase, known as factor-inhibiting HIF (FIH-1). Thus specific oxygen-sensing cascades, by means of their different oxygen sensitivities, cell-specific and subcellular localization, may help to tailor various adaptive responses according to differences in tissue oxygen availability.

Key words: glioblastoma, hypoxia inducible factor, HIF prolyl hydroxylase, iron, ischemia, NADPH oxidase, mitochondria, mitochondriopathy, neurogenesis, neurodegenerative disease, oxygen sensing, preconditioning, reactive oxygen species, stem cell, tumor, VEGF.

Brain oxygen supply

In higher organisms, respiratory and cardiovascular systems provide and appropriately distribute oxygen (O_2) to tissues and cells to serve as the terminal electron acceptor during mitochondrial oxidative phosphorylation, which is the major biochemical reaction for generating 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, which tightly maintain O_2 homeostasis, i.e. keep the O_2 concentration, even within a single cell, within a narrow physiological range, allowing the cell to survive, function and thrive in regions with a heterogeneous P_{O_2} distribution. Under physiological conditions the arterial P_{O_2} is about 90 mmHg. However, differences in vascularization, tissue diffusion properties and cell-specific oxygen consumption most likely account for the heterogeneous P_{O_2} distribution seen within the brain, resulting in tissue P_{O_2} levels from 90 mmHg down to

1 mmHg (Leniger-Follert et al., 1975). This heterogeneity is also reflected in regional variations of hemoglobin O_2 saturation, as shown by blood oxygen level-dependent fMRI-BOLD brain measurements (Kannurpatti et al., 2003). It is easy to conceive that cells adjacent to the arterial inflow (high P_{O_2}) have metabolic capacities or electrical activities different from those in cells located at the venous end (low P_{O_2}).

Brain oxygen sensing need

Neuron cell function and viability, and thus structural and functional integrity of the brain, rely heavily on a constant oxygen and glucose supply. A large proportion of the neuronal 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 brain function. In particular, reductions in oxygen availability caused by systemic or local blood circulation irregularities cannot be tolerated for longer periods because the energy supply to the brain provided by anaerobic glycolysis is insufficient. On the other hand, excessive oxygenation harbors the risk of cell injury by accumulation of toxic reactive oxygen species (ROS), which are capable of oxidizing macromolecules such as lipids, proteins and nucleic acids, a process termed oxidative stress.

Disturbances in oxygen availability have been implicated in the central nervous system (CNS) pathology of a number of disorders including stroke, head trauma, neoplasia, vascular malformations and neurodegenerative diseases. Thus, complex cellular oxygen-sensing systems have evolved to ensure tight regulation of oxygen homeostasis in the brain to avoid metabolic compromise or the risk of oxidation toxicity. These induce an elaborate sequence of adaptive mechanisms in response to variations in P_{O_2} designed to avoid or at least minimize brain damage, including short-term (seconds, minutes) and long-term (hours, days) responses. Impressive examples of brain oxygen-sensing systems under various physiological and pathological conditions are described in the literature: tissue oxygenation is highly dependent on the regulation of cerebral blood flow. By adjusting the vascular tone, perfusion is kept constant over a wide range of blood pressures, a process termed cerebral autoregulation. Autoregulation of cerebral blood flow provides a powerful mechanism to counteract regional imbalances in oxygen supply. Thus, increases in brain neural activity that lead to changes in metabolic demand elicit an increase in local blood supply within the corresponding brain region. This response is probably triggered by an ‘initial dip’ of tissue P_{O_2} due to an increased oxygen consumption, as elegantly shown on the visual cortex (Thompson et al., 2003). Neuronal activity and excitability can be directly regulated in response to O_2 availability by altering membrane channel conductivity. Central neurons contain O_2-sensitive potassium channels that are reversibly inhibited by hypoxia (Jiang and Haddad, 1994). These channels, partly identified as TASK channels, are also regulated by a number of neurotransmitters, identifying them as key players in oxygen-sensitive regulation of neuronal excitability (Brickley et al., 2001; Maingret et al., 2001). Neonatal rats survive and avoid brain injury during periods of anoxia up to 25 times longer than adults, most likely due to a hypoxia-suppressed *N*-methyl-D-aspartate receptor response to glutamate excitotoxicity (Bickler et al., 2003). A further neuroprotective early response lies in the active suppression of gene transcription (Denko et al., 2003a) and protein synthesis (Hata et al., 2000), leading to a shut-down of non-essential energy-consuming mechanisms that are not required for immediate cell survival, a process termed oxygen conformance (for reviews, see Zhou et al., 2004; Hochachka and Lutz, 2001). Suppression involves inhibiting assembly of the two main regulators of translation initiation, namely eukaryotic initiation factor (eIF) 4F (Martin et al., 2000, 2001; Arsham et al., 2003) and eIF2 (Sullivan et al., 1999; Althausen et al., 2001; Martin et al., 2001; Koumenis et al., 2002) and inhibition of the eukaryotic elongation factor (eEF) 2 (Althausen et al., 2001). Reversible inhibition of translation has been reported to occur at O_2 levels between 0.5% and 1% (Görlach et al., 2000; Lang et al., 2002).

Beyond these short-term responses, adaptation to reduction in oxygen availability necessitates changes in gene expression, which would be predicted to lead to reduced oxygen consumption and increased oxygen delivery, and provide a means of counteracting the detrimental effects of hypoxia and reoxygenation. In particular, reoxygenation encountered after spontaneous or thrombolytic reperfusion following vessel occlusion, for example, carries a risk of accumulation of toxic ROS, as antioxidative defense mechanisms in the cell are perturbed causing additional tissue damage. Specific redox-sensitive transcription factor systems such as HIF, nuclear factor kB (NF-kB), activator protein 1 (AP-1) and early growth response protein-1 (EGR-1), have been described that respond to changes in P_{O_2} or an excess in ROS by activation of appropriate target gene expression (Koong et al., 1994; Yan et al., 1999). The identification of the HIF transcription system by Wang and Semenza (1995) was a milestone in our understanding of oxygen physiology. Since then the HIF system has emerged as a key regulatory system of responses to hypoxia at both local and systemic levels. It is believed that approximately 1–1.5% of the genome is transcriptionally regulated by hypoxia. However, significant heterogeneity in the transcriptional response to hypoxia is observed between different cell types (Denko et al., 2003b).

The HIF transcriptional complex

The HIF transcriptional 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 and Bunn, 2003). The HIF complex exists as a heterodimer composed of constitutively expressed HIF-β and O_2-regulated HIF-α subunits belonging to the bHLH (basic helix-loop-

helix)-PAS (PER-ARNT-SIM) family of transcription factors. Both HIF-α and HIF-β proteins exist as isoforms [HIF-1α, HIF-2α, HIF-3α and ARNT (aryl hydrocarbon receptor nuclear translocator), ARNT2, ARTN3, respectively] (reviewed in Wenger, 2002). HIF activity is tightly regulated throughout the range of physiological and pathological oxygen concentrations involving multiple mechanisms of control at the levels of mRNA expression, protein stability, nuclear translocation and transactivation activity. These combine cooperatively to activate HIF to maximal levels under decreasing oxygen concentrations. At the molecular level this is mediated by subjecting HIF-α subunits to multiple modes of post-translational modification, including lysine residue acetylation mediated by ARD-1 (Jeong et al., 2002), phosphorylation and two different types of hydroxylation. The dominant control mechanism occurs *via* oxygen-dependent proteolysis of HIF-α subunits. Oxygen-dependent enzymatic hydroxylation of proline residues within HIF-α subunits constitutes the critical modification governing protein stability. Prolyl hydroxylation allows capture by the von Hippel Lindau tumor suppressor protein (pVHL), which acts as the recognition component of an E3-ubiquitin ligase enzyme. Subsequent ubiquitination targets the complex for proteosomal degradation. Consequently, only low levels of HIF-α protein expression can be detected in the presence of oxygen, increasing rapidly and exponentially as the O_2 concentration decreases. 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, resulting in reduced transcriptional activity. A VHL-independent regulation of HIF-α stability was recently suggested by studies reporting that heat shock proteins Hsp70 and Hsp90 protect HIF-1α from proteosomal degradation by physical interaction and support HIF-α transcriptional activity (Gradin et al., 1996; Minet et al., 1999; Mabjeesh et al., 2002; Isaacs et al., 2002; Zhou et al., 2004). Interestingly, both Hsp70 and Hsp90 have been shown to be induced by hypoxia, providing an alternative mechanism for hypoxia-induced HIF-α activation (Zhou et al., 2004). Though oxygen-dependent regulation seems to provide the prevailing control mechanism of HIF function, receptor-mediated phoshporylation cascades *via* binding of various growth factors and cytokines, including angiotensin II, epidermal growth factor (EGF), platelet derived growth factor (PDGF), tumor necrosis factor (TNF) α, insulin and insulin-like growth factors IGF-1 and -2, represent alternative ways to enhance HIF activity by translational and post-translational control under normoxia. This induction is generally less intense than that mediated by reductions in oxygen tension (for a review, see Bilton and Booker, 2003). While these pathways do not directly mediate the response to hypoxia, interactions with HIF signaling suggest that cellular responses to hypoxia can be fine-tuned by integration into the major signaling systems.

The HIF transcriptional system acts as a master regulator of oxygen-regulated gene expression, inducing adaptive mechanisms that 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 (for a review, see Semenza, 2003). The latter is especially puzzling, as recent studies support the view that, apart from inducing pro-proliferative proteins such as IGF-2, IGF-BP (binding proteins) 1–3 and TGF (transforming growth factor) β3, the HIF pathway includes responses with adverse effects on cell function by inducing cell-cycle-arrest-specific and proapoptotic proteins such as DEC (defective chorion)-1, BNIP (Bcl2/adenovirus E1B 19kD-interacting protein)-3, its orthologue NIX (Nip3-like protein X) and cyclin G2. In addition, direct stabilization of the proapoptotic protein p53 has been suggested by studies demonstrating physical and functional interaction between HIF-1α and p53 (for a review, see Acker and Plate, 2002). Thus, the HIF system transactivates an extended physiological pathway that encompasses a wide array of physiological responses to hypoxia, ranging from mechanisms that increase cell survival to those inducing cell cycle arrest or even apoptosis. Given the range and diversity of regulated cellular functions it is easy to predict that the HIF system is likely to be crucially involved in many physiological and pathophysiological processes within the brain. However, our current understanding of the role of HIF in these processes is rather limited. Activation of pathways with seemingly opposing functions on cell survival, e.g. inducing neuroprotection or neuronal cell death, as outlined in Fig. 1, further complicates elucidation, pointing towards a more complex and highly cell-context-dependent role of HIF.

HIF in brain physiology and pathophysiology

The exquisite sensitivity of neurons to hypoxic and ischemic events makes the proper induction of neuroprotective and adaptive responses highly relevant for neuronal survival, arguing for an important role for HIF in neuronal cell function. This notion is further supported by the well-studied neuroprotective effects of known HIF-target genes such as Epo, VEGF or PDGF-B (Digicaylioglu and Lipton, 2001; Sun et al., 2003; Prass et al., 2003; Zhang et al., 2003). Further into this line of thinking are HIF-1α loss-of-function studies in KO-mice showing a failure in brain development that is probably due to impaired vascular development as well as massive cell death in the cephalic mesenchyme (Iyer et al., 1998; Ryan et al., 1998). The early lethal phenotype at embryonic day 11 has so far prevented a conclusive analysis of HIF-1α function in the adult brain. Numerous gene and protein expression studies in normal and hypoxic/ischemic brain, however, have provided indirect experimental evidence for the importance of HIF-1α activity in the brain.

Cerebral ischemia

The change in HIF-1α activity in response to reduction of

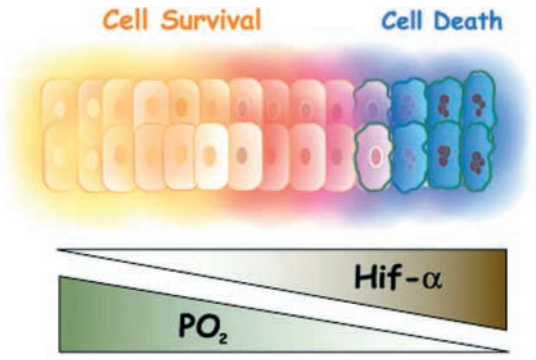

Fig. 1. Dual role of HIF in regulating cell survival and cell death, depending on P_{O_2} and HIF-α protein levels. The HIF system transactivates an extended physiological pathway, encompassing a wide array of physiological responses to hypoxia, ranging from mechanisms that increase cell survival to those inducing cell cycle arrest or even apoptosis. Depending on the degree and duration of hypoxia, quantitative and qualitative changes in the hypoxia response occur that may be regulated by concomitant changes in HIF-α protein levels, modifications and HIF-α subunit expression.

P_{O_2} was analyzed in animal models of hypoxia, global brain ischemia, focal ischemia and neonatal hypoxia/ischemia. Widespread constitutive neuronal HIF-1α mRNA, protein and HIF-target gene expression was reported in different regions of the brain, as shown in Fig. 2 for the rat cerebral cortex, which was found to further increase following hypoxic exposure (Bergeron et al., 1999; Kietzmann et al., 2001; Chavez et al., 2000; Beck et al., 2000). Interestingly, HIF-1α translation is exempted from the overall suppression of protein synthesis observed in hypoxic/ischemic conditions, allowing for efficient and rapid protein accumulation (Görlach et al., 2000; Lang et al., 2002).

The precise role of HIF-2α in the brain following a reduction in P_{O_2} remains elusive. One report demonstrated induction of HIF-2α mRNA in endothelial cells following focal cerebral ischemia (Marti et al., 2000). However, two studies using different methods to reduce oxygen availability demonstrated differential regulation of HIF-1α and HIF-2α subunits according to the experimental conditions and cell type, suggesting vital non-redundant functions of both isoforms, and warranting further analysis (Stroka et al., 2001; Wiesener et al., 2003).

Concomitant induction of adaptive mechanisms such as glycolytic metabolism and angiogenesis following hypoxia/ischemia further supports a neuroprotective role of HIF activation. Subjecting animals to a brief and moderate episode of hypoxia/ischemia elicits an autoprotective response, which results in protection against a subsequent prolonged ischemic event, a phenomenon termed preconditioning (for a review, see Dirnagl et al., 2003). Preconditioning is likely to involve adaptive changes in gene expression to activate the endogenous protective mechanism, as it generally takes several hours to days after a preconditioning episode to attain a state of tolerance. Preconditioning studies have identified a subset of neuronal transcripts regulating a number of adaptive processes, including ROS inactivation, metabolic switch, angiogenesis and neuroprotective paracrine-acting factors involving known HIF target genes. In particular the hypoxia-inducible gene products Epo (erythropoietin) and VEGF (vascular endothelial growth factor), which are commonly expressed by neurons and astrocytes (Bernaudin et al., 2002; Beck et al., 2000) have been identified as potential mediators of hypoxic preconditioning (Bernaudin et al., 1999; Prass et al., 2003; Wick et al., 2002). Interestingly, hypoxic preconditioning has been found to increase HIF-1α expression and can also be achieved by known chemical inducers of HIF-α such as $CoCl_2$ and desferrioxamine, suggesting that the preconditioning phenomenon is mediated by HIF-activity (Prass et al., 2002; Bergeron et al., 2000). In contrast, studies on neonatal mouse cortices revealed that inhibition of HIF-activity using a dominant negative HIF-1α mutant protected cortical neurons from delayed cell death following oxygen and glucose deprivation (Halterman et al., 1999). The results are consistent with a model in which HIF-1α-mediated p53 stabilization promotes neuronal cell death following an ischemic exposure.

Thus, the important question of the role of HIF activation in determining the outcome of ischemic and oxidative injury may be more difficult to answer than expected, though essential to a future therapeutic application of HIF activators or inhibitors. The severity and duration of hypoxia, protein levels and phosphorylation status of HIF-α subunits may be decisive parameters in governing these fundamentally different responses on cell fate, mediating the transition from adaptive neuroprotective to cell death responses (Fig. 1; Piret et al., 2002; Carmeliet et al., 1998). Interestingly, VEGF with its pleiotropic effects stimulating angiogenesis and vascular permeability as well as exerting direct neuroprotective activities, reveals a similar diverse outcome on cell survival (Carmeliet and Storkebaum, 2002). It is worth remembering that HIF-regulated prosurvival or proapoptotic processes in response to ischemia and oxidative stress reflect common pathophysiological components that are probably shared by many neuropathological diseases (see below) as, for example, suggested in multiple sclerosis, an inflammatory, demeyelinating brain lesion (Aboul-Enein et al., 2003).

Neurogenesis

Recent years have opened an interesting new door with the potential of restoring and replenishing injured tissue. It is well known that cells are continuously replaced in various organ systems in the body throughout life. The CNS has been traditionally viewed for many decades as a system with very limited capacity for self-renewal and regeneration. This dogma is, however, challenged by a growing body of evidence supporting the concept that, in certain brain regions, neural stem cells exist that give rise to new neurons as part of a general endogenous turnover mechanism, as well as in response to injury (reviewed by Doetsch, 2003). Various factors that influence neural stem cell function have subsequently been identified. A number of recent studies

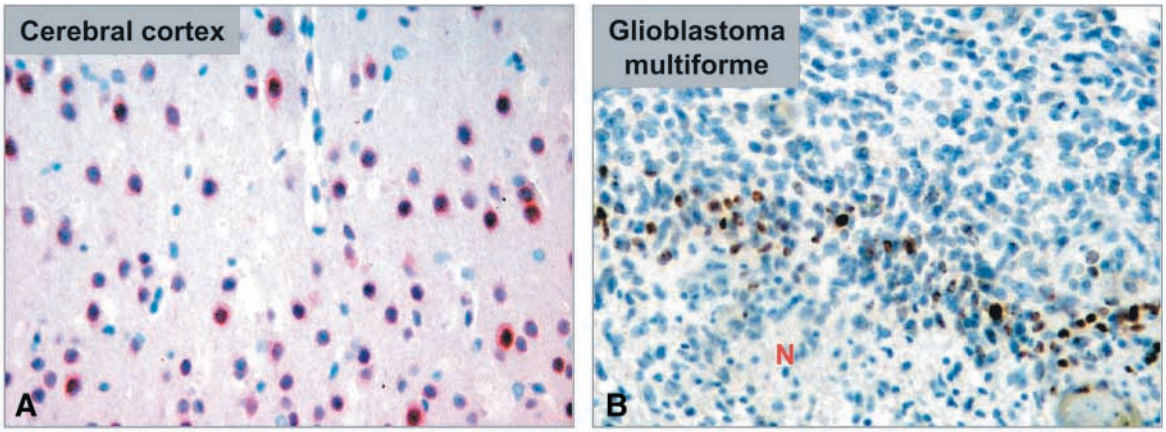

Fig. 2. HIF-1α localization in brain under physiological and pathophysiological conditions. (A) Heterogeneity of nuclear HIF-1α localization in neurons of the mouse cortex under physiological conditions is probably related to the heterogeneous distribution of tissue P_{O_2}. In addition, HIF-α expression may be influenced by other factors discussed, such as PHD activity or growth factor signaling. (B) In contrast, human brain tumors show distinct nuclear HIF-1α localization in pallisading cells close to the necrotic core (N) of glioblastoma multiforme.

suggest that oxygen-dependent gene expression is of crucial importance in governing essential steps of neurogenesis such as cell proliferation and renewal, survival and differentiation. Indirect evidence for this stems from studies demonstrating increased cell proliferation and neurogenesis after hypoxic/ischemic episodes (Liu et al., 1998; Takagi et al., 1999; Jin et al., 2001). Low oxygen concentrations have been shown in *in vitro* neurosphere assays to induce proliferation, cell survival and neuronal differentiation (Studer et al., 2000; Morrison et al., 2000; Shingo et al., 2001). Accordingly, a number of hypoxia-inducible growth factors such as VEGF, Epo, IGF-1 and stem cell growth factor have been implicated as important regulators of neurogenesis (Jin et al., 2002a,b; Shingo et al., 2001; Aberg et al., 2000). So far no studies on HIF-α function in neural stem cells have been performed, but analysis of the effects of HIF-1α loss of function in other organ systems suggests a pivotal role in stem cell homeostasis (Schipani et al., 2001; Seagroves et al., 2003). Interestingly, the redox state of the cell seems to decisively influence cell fate decision, either supporting self renewal (reduced state) or differentiation (oxidized state) mechanisms (Noble et al., 2003). In support of this concept, hypoxia has been shown to induce and keep cells in an undifferentiated, immature phenotype (Jogi et al., 2002). Recent studies have highlighted the endogenous repair capacity of the brain following injury after focal cerebral ischemia and the potential for using these inherent regenerative abilities in putative therapeutic approaches. Considerable regeneration of neurons from the endogenous neural stem niche occurs within the striatum (Arvidsson et al., 2002). Hippocampal regeneration by pyramidal cell replenishment can be even further enhanced by ventricular application of growth factors known to stimulate neural stem cell growth (Nakatomi et al., 2002). A recent study elegantly exploited the pleiotropic and possibly synergistically acting activities of VEGF to show that intracerebroventricular VEGF application administered after the insult reduced infarct size and improved the overall functional outcome, presumably as a result of enhanced angiogenesis, neuroprotection and neurogenesis (Sun et al., 2003). Thus, hypoxia-inducible gene expression may provide crucial cues to stimulate and direct neurogenesis to areas where cells are lost.

Neurodegenerative disease

Neurodegenerative diseases as diverse as Alzheimer's disease, frontotemporal dementia, prion diseases, Parkinson's disease, Huntington's disease or amyotrophic lateral sclerosis are associated with the accumulation of potentially toxic, aggregation-prone misfolded proteins (Taylor et al., 2002). Features of these diseases can be recapitulated in transgenic mouse models overexpressing mutant proteins (Wong et al., 2002). Increasing evidence suggests that such protein assemblies are particularly vulnerable to neurons by triggering cellular and neuroinflammatory stress responses. Among others, oxidative stress in particular has been implicated as a causative agent of progressing neuronal degeneration.

Deposition of β-amyloid (βA) peptide, typically present in extracellular plaques, is thought to be intimately connected to the initiation of Alzheimer's disease (Bishop et al., 2002). βA peptides are believed to act as the primary toxic agent detrimental to neural cell function and responsible for neural cell death. They have been shown to directly induce ROS generation, thus causing oxidative damage. A recent study implicated HIF-1α activation as a general neuroprotective mechanism against oxidative stress and βA-toxicity (Soucek et al., 2003). Clonal neural cell lines and primary cortical neurons, which are resistant to βA-toxicity, revealed an enhanced flux of glucose through the glycolytic and the pentose-phosphate pathway, due to enhanced enzymatic activity. Based on this observation the authors went on to show that this increase in cellular glucose metabolism correlated with elevated HIF-1α protein levels and activity. Importantly, exposure of neurons to βA-peptide was shown to induce HIF-

1α, though the mechanisms of induction remained unknown. Interestingly, HIF controls the coordinate upregulation of genes of the glycolytic pathway, ranging from glucose uptake to lactate production. HIF-mediated induction of glucose transport and utilization has been implicated in supporting antioxidative mechanisms, by generating reducing equivalents in the form of NADH and NADPH *via* glycolysis or the pentose-phosphate pathway, as well as synthesis of the antioxidant pyruvate. Thus, in Alzheimer's disease pathology, HIF may be involved a feedback mechanism, whereby βA induces HIF-1α activity, which in turn upregulates adaptive antioxidative mechanisms to help to combat the increased oxidative stress inflicted by βA. Considering the growing body of evidence that ROS regulate HIF activity (see below), an interesting question remaining to be answered is whether βA-mediated HIF induction operates *via* ROS generation. This early neuroprotective response induced by βA may eventually be overridden by cumulative toxicity effects. The neuron may finally succumb to the continuous stress afflicted by neurotoxic peptides such as β-amyloid and induce a cell death program. Again, given the pro-apoptotic activity of HIF-1α, it is intriguing to ask to what extent a βA-mediated sustained and prolonged neural induction of HIF-1α may participate in this process.

Amyotrophic lateral sclerosis, a progressive motorneuron disease, is another intriguing example of implicating dysregulated oxygen signaling pathways in the pathogenesis of neurodegenerative diseases. Motor neurons are known to be exquisitely susceptible to variations of oxygen concentration. Hence during transient episodes of ischemia the decrease in oxygen supply or the oxidative damage caused by free radicals generated during reoxygenation may prove particularly fatal. Indeed, cumulative oxidative damage due to the toxic gain-of-function of mutant SOD1 has been linked to the pathogenesis of amyotrophic lateral sclerosis (ALS; Cluskey and Ramsden, 2001; Rakhit et al., 2002). A novel player in the aetiology of ALS has only recently been identified. To study the relevance of HIF-mediated induction of the VEGF-gene, transgenic mice with the hypoxia-responsive element (HRE) deleted were generated (Oosthuyse et al., 2001). HRE-deficiency was associated with a decrease in both normoxic baseline and hypoxia-induced VEGF levels in the central nervous system, which predisposed the animals to develop an adult-onset, progressive motor neuron disease. Motor neuron loss in these mice probably resulted from a chronic reduction in neural vascular perfusion, which in addition may have been exacerbated by an insufficient VEGF-dependent neuroprotection. These findings prompted the authors in a recent report to further analyze the contribution of impaired VEGF regulation in the pathogenesis of human ALS (Lambrechts et al., 2003). Interestingly, decreased VEGF protein serum levels were found in all ALS patients analyzed, which in a subset of patients could be genetically linked to specific haplotypes within the VEGF promotor/leader sequence being associated with a reduced transcription, IRES-mediated expression and translation of VEGF. These data also clearly implicate impaired VEGF expression as a modulator of ALS pathology in the human situation. No sequence variations in the HIF-binding site of the VEGF-promotor or functionally relevant missense mutations in the HIF-1α and HIF-2α genes could be identified.

Collectively, these studies implicate reduced hypoxia-induced expression of genes with neurotrophic or neuroprotective effects as novel components in the pathogenesis of neurodegenerative diseases. In addition, vascular defects and chronic vascular perfusion deficits, e.g. chronic ischemia, have been documented in a number of neurodegenerative conditions such as Alzheimer's disease, Parkinson's disease or ALS (for a review, see Storkebaum and Carmeliet, 2004). It remains to be elucidated whether and to what extent the HIF-system may participate in the disease process. Current data would support a dual, seemingly paradoxical role of the HIF-system, depending on whether it is the cause or the consequence, i.e. a primary or secondary effect. Again the opposing effects on cell survival of downstream components of the HIF complex may be the clue to this paradox (Fig. 1). Impaired activation of the HIF-system would result in reduced induction of neurotrophic adaptive cascades and thus decreased cell survival. Support for this notion stems from studies showing that aging, a known risk factor for neurodegenerative diseases such as Alzheimer's disease, severely attenuates the capacity to induce HIF-1α activity, thus impairing the cell's ability to respond adequately to ischemic stress (Chavez and LaManna, 2003). On the other hand, chronic and sustained activation of the HIF-system as a consequence of chronic vascular insufficiency, for example, may end in cell degeneration. Interestingly, cellular disturbances of proteosomal function resulting in ubiquitin accumulation have been reported in many neurodegenerative disorders and are likely also to affect HIF-α levels (Bence et al., 2001). Both pathomechanisms could even be operative in the same disorder, with the former contributing to early-stage and the latter to late-stage disease process. Considering the complexity of neurodegenerative disorders, however, the process outlined above is likely to be oversimplified and warrants further analysis. Transgenic animal studies selectively modulating HIF-1α and HIF-2α expression and function in the different cell types of the brain may be crucial in further clarifying the role of the HIF system in neurodegeneration.

Neoplasia

Regions of low oxygen tension are commonly found in malignant tumors and are associated with increased frequency of tumor invasion and metastasis and a poor therapy outcome. The ability to initiate homeostatic responses and adapt to hypoxia represents an important and crucial aspect in solid tumor growth. In particular, activation of the HIF system has been identified as an important mediator of these processes. The involvement of HIF in tumor physiology is covered in more detail in a recent review (Acker and Plate, 2002). In comparison to adjacent tissue widespread HIF activation is

observed in tumors, correlating with tumor growth and progression. HIF overexpression in tumors is related to both hypoxia-dependent (observed close to the necrotic area, as shown in Fig. 2) and hypoxia-independent mechanisms, such as oncogene activation and growth factor signaling pathways. The relevance of the HIF system to tumor growth and progression is highlighted by the variety of mechanisms regulated by HIF-target genes, ranging from angiogenesis, to increase tissue oxygenation over glycolysis, and pH regulation, allowing for energy generation when oxygen is scarce, to cell proliferation and survival pathways. It is worth remembering that the HIF pathway exerts its effects not only on the tumor cell but also on the stromal microenvironment, to enhance and promote tumor vascularization and growth. In fact, the activation of the HIF system elicits a cellular and molecular crosstalk leading to the coordinated collaboration between tumor, endothelial, inflammatory/hematopoietic and circulating endothelial precursor cells (reviewed by Acker and Plate, 2003).

The clinical correlation between intratumoral hypoxia and tumor aggressiveness, which is particularly characterized by tumor invasion and the ability to metastasize, has found a possible molecular correlate in recent studies. Thus, crucial steps in these processes, including cell mobility and migration, tissue invasion and the ability to home into specific organ sites, are governed by specific signaling pathways known to contain HIF-target genes such as c-Met, the receptor for HGF (scatter factor/hepatocyte growth factor), or the chemokine receptor CXCR4 (Pennacchietti et al., 2003; Schioppa et al., 2003; Staller et al., 2003). Both receptor systems have well established functions in tumor invasion and metastasis, as shown e.g. in breast cancer (Muller et al., 2001) or gliomas (reviewed in Lamszus et al., 1999). In addition, several factors known to determine the invasive cancer phenotype such as cathepsin D, matrix metalloproteinase 2 and urokinase plasminogen activator receptor (uPAR) have been shown to be regulated by HIF (Krishnamachary et al., 2003). Indeed, inhibition of the HIF pathway by geldanamycin was shown to diminish glioma cell migration *in vitro* (Zagzag et al., 2003). Interestingly, though a growing body of evidence suggests that HIF-1α is the major orthologue to convey hypoxia-induced gene expression, both HIF-1α and HIF-2α seem to be required for hypoxia-induced cell migration, as shown recently by siRNA-mediated knock-down of each orthologue in breast cancer cell lines (Sowter et al., 2003). Thus, low P_{O_2} and activation of the HIF system causes the tumor cell to migrate away from hypoxic areas and invade further into the host tissue and organs, thus supporting tumor spread. It is interesting to note that in highly invasive glioblastoma multiforme, the most malignant and particular hypoxic brain tumor entity, single tumor cells with high-level HIF expression have been detected at the leading tumor invasion front (Zagzag et al., 2000).

Recent insight into the precise mechanisms of oxygen sensing and signaling may help to develop novel anti-tumor strategies that specifically target the PHD-HIF-VHL pathway. Given the widespread HIF activation in tumors, the role of HIF in transactivating angiogenic factors and the role of angiogenic factors in tumor growth, interfering with this pathway is particularly appealing. Its rationale lies in depriving the tumor cell of oxygen and nutrients by inhibiting angiogenesis while at the same time disabling adaptive mechanisms that help the cell to survive in this microenvironment. The feasibility of this approach has been confirmed in different reports (Maxwell et al., 1997; Ryan et al., 1998; Kung et al., 2000; Kim et al., 2001). However, given their key regulatory role in various complex physiological pathways stretching from metabolism, proliferation and differentiation to apoptosis, general manipulation of the HIF system is likely to show a variable outcome depending on the cellular context, e.g. tumor cell type (Carmeliet et al., 1998; Blancher et al., 2000) or tumor microenvironment (Blouw et al., 2003) and should for this reason be employed cautiously. HIF itself may directly influence the outcome of present therapies such as chemotherapy and radiotherapy. HIF-1α deficiency in fibroblasts resulted in increased apoptotic cell death in response to agents such as carboplatin and ectoposide or ionizing radiation known to induce double-strand breaks, suggesting that HIF-1α may induce expression of genes involved in the repair of DNA double-strand breaks (Unruh et al., 2003).

Manipulation of the putative oxygen sensor system prolyl hydroxylase (PHD; see below) offers yet another new and challenging strategy to analyze and influence tumor biology. Interestingly, PHD activity in cancer cells may be reduced, as suggested by incomplete prolyl hydroxylation of HIF-α in normoxic tissue culture as detected by hydroxylation-specific antibodies (Chan et al., 2002). HIF-α degradation under these conditions can be enhanced by vitamin C or iron supplementation (Knowles et al., 2003), both important cofactors of PHD, suggesting that attenuated PHD activity may contribute to the general HIF activation in tumor cells. In line with these observations, ectopic PHD1 overexpression in colon carcinoma cells suppressed HIF-1α levels and reduced *in vivo* tumor growth (Erez et al., 2003).

Putative oxygen sensors

As outlined above, changes in oxygen tension elicit different responses by the cell, which activate short- and long-term 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 P_{O_2} deviates from a given preset value, to initiate distinct signaling cascades. The wide operating field of the tissue P_{O_2} distribution as shown in Fig. 3 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. Oxygen-sensing heme proteins and PHD have been described as candidate sensor systems connecting an oxygen-dependent

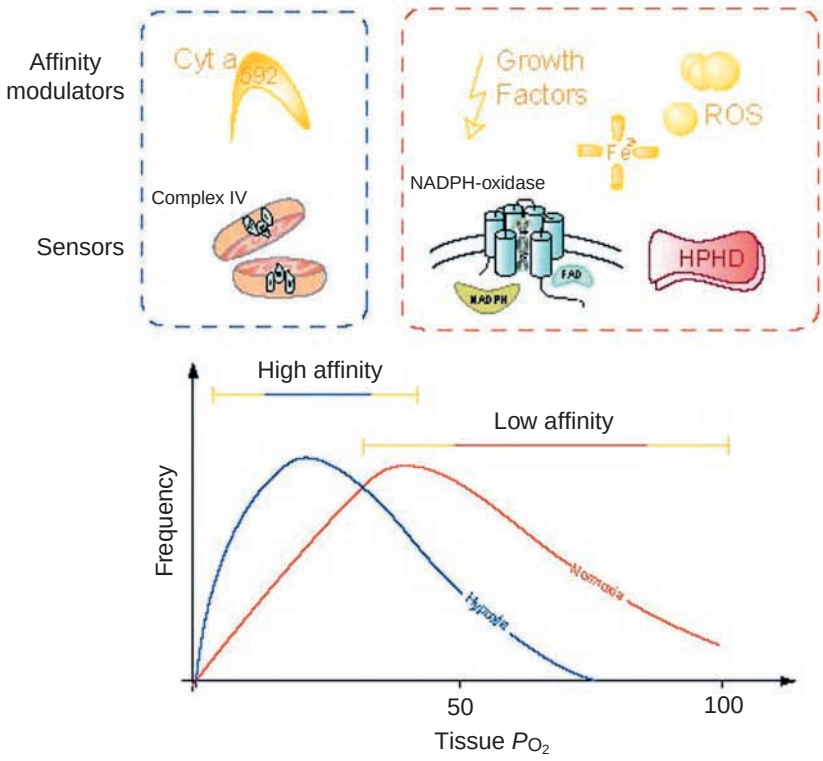

Fig. 3. Modulation of oxygen sensor sensitivity by co-factors. Oxygen-sensing systems connecting an oxygen-dependent enzymatic activity to the regulation of hypoxia-inducible responses should operate at high and low P_{O_2} affinities, fitting the heterogeneous P_{O_2} distribution curve. Oxygen-sensing heme proteins such as mitochondrial complex IV (cytochrome *c* oxidase) and NAD(P)H-oxidase as well as PHD have been described as candidate sensor systems functioning at different K_m values. Modulation of the specific P_{O_2} affinities or oxygen sensor activities would allow efficient triggering and fine-tuning of various signal cascades to optimize cellular function and adaptation over a broad range of O_2 concentrations.

enzymatic activity to the regulation of hypoxia-inducible responses, as outlined schematically in Fig. 3.

Oxygen-sensing heme proteins

Molecules changing their chemical properties as a direct consequence of the surrounding P_{O_2} may mediate the first step in oxygen sensing. As for the carotid body, hypoxia leads to an increase in afferent carotid sinus nerve activity, stimulating ventilation and blood circulation in the body to avoid hypoxic tissue damage. The primary oxygen sensor triggering this increase is as yet unknown, but there is large agreement that it is a heme protein, either a mitochondrial component of the respiratory chain (Baysal et al., 2000; Mills and Jöbsis, 1972; Wilson et al., 1994) or a non-mitochondrial protein like the NAPH oxidase (Nox; Fu et al., 2000; Cross et al., 1990).

Light absorption spectra identified very recently a cytochrome a_{592} (shown in yellow in Fig. 4) as a unique component of carotid body cytochrome *c* oxidase. In contrast to other cytochromes, cytochrome a_{592} revealed an apparent low P_{O_2} and high CN^- affinity, probably due to a shortcut of electron flow within the cytochrome *c* oxidase between Cu_A and cytochrome *a*3–Cu_B. It was suggested that this specific property would allow the regulation of intracellular calcium levels under hypoxia (Streller et al., 2002).

Furthermore, it was postulated that a NADPH oxidase isoform (shown in dark green in Fig. 4) within carotid body type I cell functions as an oxygen sensor to regulate ion channel conductivity and gene expression (Cross et al., 1990). In this context, various NADPH oxidase isoforms comprising complexes between p22phox, gp91phox, p47phox, p40phox, p67phox and Rac1, 2 components must be considered. Their function may not, however, 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) 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 reviewed by Lambeth et al. (2000). 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; Fu et al., 2000) indicating an oxygen sensor function of the NADPH oxidase in this cell type and related strains (O'Kelly et al., 2001, 2000). 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.

Prolyl hydroxylases

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-α requires an O_2- and iron-dependent hydroxylation of specific prolyl residues (Pro 402, Pro 564) within the HIF-α ODD (oxygen-dependent-domain) carried out by HIF-prolyl hydroxylase (PHD; Epstein et al., 2001; Bruick and McKnight, 2001; Oehme et al., 2002). 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-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

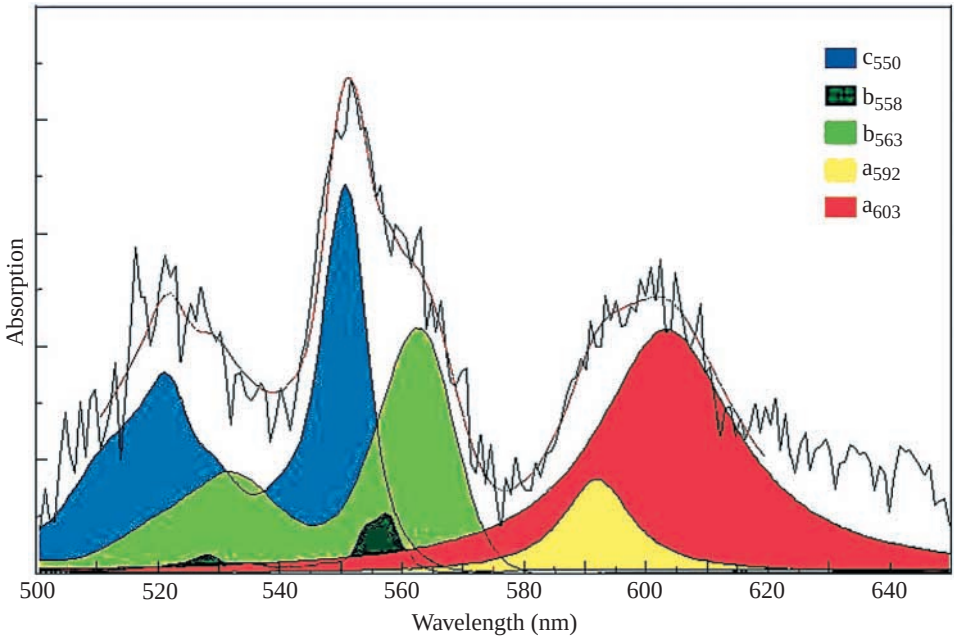

Fig. 4. N_2 *versus* aerobic steady state spectrum (black solid noisy line) as a mean of six carotid bodies fitted by different mitochondrial and non-mitochondrial cytochrome spectra, indicated by different colors. The superposition curve (black solid line) obtained by varying the amplitude of the optical density of five cytochromes closely fits the experimental curve (Streller et al., 2002).

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 CO_2. Thus, the hydroxylation reaction is inherently dependent on ambient oxygen pressure, providing a molecular basis for the oxygen-sensing function of these enzymes.

Interestingly, PHD are strikingly sensitive to graded levels of oxygen *in vitro*, mirroring the progressive increase in HIF-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, PHD have been found to have a striking low O_2 affinity of 178 mmHg above the concentration of dissolved O_2 in the air (Hirsilä et al., 2003). Consequently, taking the regular tissue P_{O_2} distribution as shown in the lower part of Fig. 3, PHD would operate under suboptimal, non-equilibrium conditions for HIF-α turnover far beyond their K_m. However, given regular Michaelis–Menten kinetics, this would allow the enzymes to operate in a highly sensitive manner, in which small changes in oxygen concentration result in pronounced changes in enzymatic reaction velocity and thus HIF-α 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 hyroxyprolyl-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 this enzyme also 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-α subunits is an indirect method of assessing the activity of the PHD/HIF system *in vivo*, and such 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 ischemia (Stroka et al., 2001; Wiesener et al., 2003). Interestingly, HIF-α levels remain low even in regions such as the renal medulla, which are characterized by low oxygen tensions known to enhance HIF-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 render it highly sensitive to alterations of cofactor concentration, such as ferrous iron (Knowles et al., 2003) or 2-oxoglutarate, substrate concentration, e.g. due to changes in HIF-α synthesis (Wiener et al., 1996; Zhong et al., 2000), as well as enzyme concentration, e.g. due to changes in mRNA expression of PHD orthologues in response to P_{O_2} (Epstein et al., 2001), being particularly striking for PHD3 (del Peso et al., 2003). Consistent with this hypothesis, physiological concentrations of cofactors such as ascorbate (25–50 μmol l^{-1}; Knowles et al., 2003) have been reported to be far below the K_m values of PHD for vitamin C (140–170 μmol l^{-1}; Hirsilä et al., 2003), suggesting significant alterations in PHD activity with changes in cofactor concentrations, as outlined in Fig. 3A. Our understanding of the exact interplay of these factors in setting the sensitivity of the PHD/HIF system is still incomplete, but nevertheless of crucial importance to understanding the cell- and tissue-specific activity and response of the oxygen-signaling cascade.

Interestingly, although ubiquitously expressed in various tissues, PHD orthologues differ in their relative cell-specific abundance of mRNA levels (Lieb et al., 2002; Cioffi et al., 2003; Oehme et al., 2002). Moreover, PHD2 and PHD3

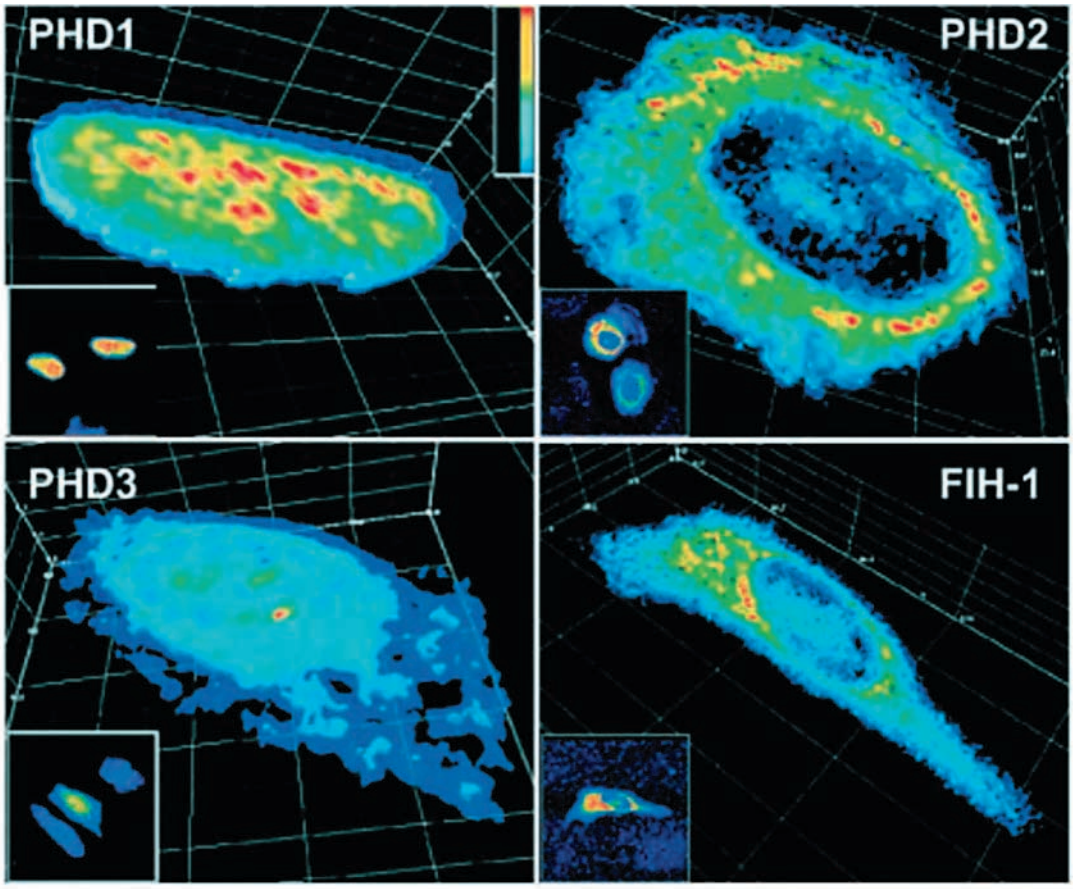

Fig. 5. Three-dimensional 2-photon confocal laser scanning microscopy (2P-CLSM) of PHD1, PHD2, PHD3 and FIH-1. Different EGFP fluorescence intensities of single cells are visualized in false colors as indicated by the color bar. Up to 64 optical slices through transfected cells were recovered by 2P-CLSM. After reconstruction of the optical slices the distribution of the EGFP fluorescence within a single cell is 3D-visualized. A cut through the cell reveals the inside distribution. Overlays of all the optical slices are shown in the inserts (Metzen et al., 2003).

expression is upregulated by hypoxia, though the fold-induction apparently varies between cell type and P_{O_2} analyzed (Epstein et al., 2001; D'Angelo et al., 2003; del Peso et al., 2003; Berra et al., 2003; Metzen et al., 2003). The exact role of 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-α orthologue preference and prolyl hydroxylation pattern, favoring the C-terminal prolyl residue (Hirsilä et al., 2003). RNAi (RNA interference)-mediated knock-down studies suggest that PHD2 is the rate-limiting enzyme controlling the steady state levels of HIF-1α in normoxia, at least under cell culture conditions (Berra et al., 2003). Moreover, the study implicated PHD2 in the initial stages of HIF-1α degradation following reoxygenation. Interestingly, a role for PHD1 in controlling HIF-1α levels under long-term maintenance of hypoxia (5–6 days) was proposed, suggesting further non-redundant functions of each PHD orthologue in other physiological or pathophysiological settings.

The subcellular localization of PHD has been studied by ectopic expression of chimeric EGFP-fusion proteins and three-dimensional two-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 and FIH-1 was found in the cytoplasm (Fig. 5). The latter result is of interest because PHD2 and FIH-1 have a calculated molecular mass of 46 kD 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.

Little is known about the involvement of PHD in pathological settings such as neoplasia or neurological disorders. However, PHD may be a target of growth factor signaling pathways and/or oncogenic transformation. In fact, it has been shown that certain oncogenes such as ras and src induce HIF under normoxia by inhibiting prolyl hydroxylation on Pro 564 (Chan et al., 2002). Recent findings, however, suggest a possible link between PHD function and

tumorigenesis and neurodegeneration. The cosubstrate 2-oxoglutarate as an intermediate of the Krebs cycle is generated in mitochondria. The two ubiquitously expressed mitochondrial enzymes, succinate dehydrogenase (SDH) and fumarate hydratase (FH), catalyze sequential steps in the Krebs cycle. Interestingly, germline heterozygous mutations in the autosomally encoded enzyme and enzyme subunits are associated with hereditary predispositions to various tumors including paraganglioma, pheochromocytoma, benign smooth muscle cell tumor and RCC (Tomlinson et al., 2002; Baysal et al., 2000; Niemann and Muller, 2000; Astuti et al., 2001). Thus, both SDH and FH act as tumor suppressor genes. In contrast, homozygous germline mutations in SDH subunits cause mitochondriopathies such as the Leigh syndrome, which affect the central nervous system and skeletal muscles. The underlying mechanism through which loss of function leads to neoplasia or neurodegeneration remains unclear (for a review, see Eng et al., 2003). It is nevertheless tempting to speculate that defects in the Krebs cycle are likely to influence 2-oxoglutarate levels or other metabolic intermediates compromising PHD function. Further mitochondrial dysfunction may lead to increased production of ROS which, as described below, may also signal on the PHD/HIF system. Interestingly, ROS itself have been shown to interfere with mitochondrial function at the level of α-ketoglutarate dehydrogenase (KGDH) and SDH (Nulton-Persson and Szweda, 2001).

Synopsis of oxygen sensing

How can one reconcile the different candidates and concepts of oxygen sensing? Fig. 6 shows the putative cooperation of the three different oxygen-sensing systems, as described above under normoxic and hypoxic conditions. The operating field of these systems is the tissue P_{O_2} distribution curve of the corresponding organ. Normoxic P_{O_2} values range between 1 and 90 mmHg, with a mean value of about 30 mmHg (Leniger-Follert et al., 1975). When the oxygen concentration decreases this distribution curve becomes more and more left-shifted, depending on the degree of arterial blood hypoxia, as shown schematically in Fig. 3B. Thus, one should hypothesize the existence of oxygen-sensing systems with high as well as low P_{O_2} affinities to fit the heterogeneous P_{O_2} distribution curve. Modulation of the specific P_{O_2} affinities or oxygen sensor activities, e.g. by integration into the major signaling pathways, would efficiently trigger and fine-tune various signal cascades to optimise cellular function and adaptation over a broad range of P_{O_2} values, as summarized in Fig. 3A.

Oxygen sensing heme proteins

The mitochondrial chain is a classical high affinity system keeping the electron flux across the cytochrome *c* oxidase constant below P_{O_2} values of 1 mmHg (Arthur et al., 1999). Consequently, the tissue P_{O_2} range covered by the oxygen-sensing mitochondrial chain is small. However, with the mutated cytochrome a_{592} an affinity modulator was proposed, lowering the high P_{O_2} affinity of cytochrome *c* oxidase to about 30 mmHg (Streller et al., 2002). NAD(P)H oxidase with a P_{O_2} affinity of about 20 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 P_{O_2} ranges. Interestingly, NADPH-activity is exquisitely controlled by rac proteins and various growth factors (Görlach et al., 2003).

Prolyl hydroxylases

In contrast, PHD have a strikingly low P_{O_2} affinity above 150 mmHg (Hirsilä et al., 2003), far beyond the regular tissue P_{O_2} distribution, allowing efficient regulation of HIF activity under these conditions (see above). Apart from P_{O_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; Tacchini et al., 1999; Lok and Ponka, 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., 2001) give an additional need for tight iron regulation, making the interaction between oxygen and iron homeostasis physiologically highly appropriate. A further level of complexity is added as decreasing O_2 concentrations lower PHD activity which, however, 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 to understand and study, and activity of the PHD/HIF system 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-α stabilisation and transactivation function (Huang et al., 1996; Ema et al., 1999; Carrero et al., 2000; Lando et al., 2000; Welsh et al., 2002). It is intriguing to speculate that PHD may thus also act as an iron- and redox-sensor.

Reactive oxygen species

Apart from toxic accumulation during reoxygenation periods ROS have been implicated as intracellular second messengers (reviewed in Lander, 1997; Nordberg and Arner, 2001). Various studies have demonstrated their influence on intracellular functions in oxygen signaling, as shown in Fig. 6. 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, since different outcomes on HIF activity have been observed depending on whether ROS are part of a normoxic, hypoxic or growth factor signaling cascade response, and further complicated by contradictory reports as to whether ROS

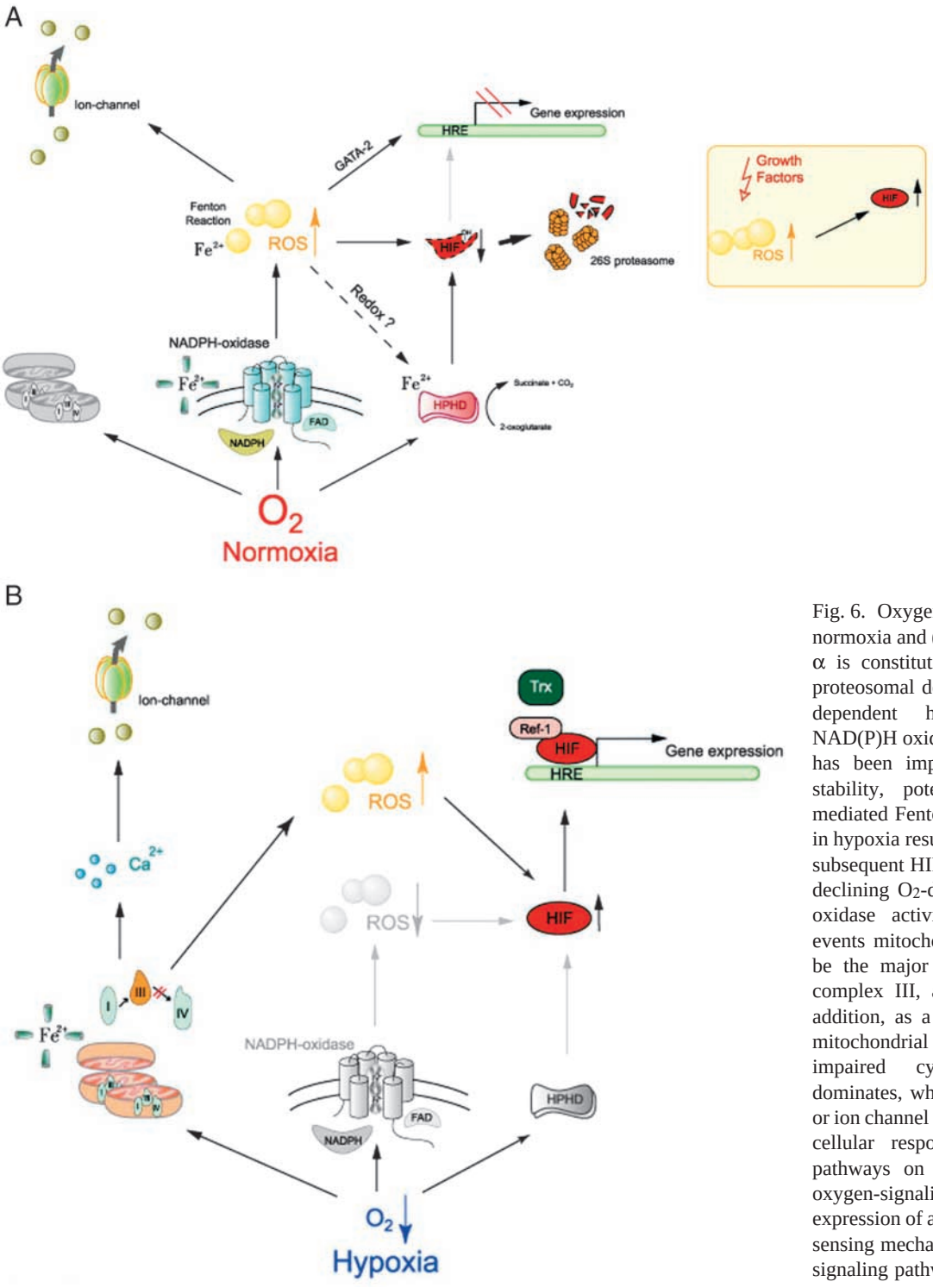

Fig. 6. Oxygen-sensing synopsis under (A) normoxia and (B) hypoxia. In normoxia, HIF-α is constitutively synthesized and sent to proteosomal destruction, controlled by PHD-dependent hydroxylation. In addition, NAD(P)H oxidase as the major donor of ROS has been implicated in controlling HIF-α stability, potentially involving the iron-mediated Fenton reaction. Reduced O_2 levels in hypoxia result in stabilization of HIF-α and subsequent HIF-target gene expression due to declining O_2-dependent PHD and NAD(P)H oxidase activity. Further, during hypoxic events mitochondria have been suggested to be the major source of ROS formation at complex III, aiding HIF-α stabilization. In addition, as a consequence of the declining mitochondrial membrane potential, an impaired cytosolic calcium buffering dominates, which triggers transmitter release or ion channel conductivity eliciting a hypoxic cellular response. Not all ROS-mediated pathways on HIF activity are part of an oxygen-signaling response but rather expression of a delicate integration of oxygen-sensing mechanisms into major growth factor signaling pathways.

decrease or increase with decreasing O_2 concentrations (reviewed by Fandrey and Genius, 2000).

ROS visualization and measurement is technically challenging, which explains 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 (Salceda and Caro, 1997; Canbolat et al., 1998; Wartenberg et al., 2003; Görlach 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., 1995a; Huang et al., 1996; Fandrey et al., 1997; Wiesener et al., 1998; Canbolat et al., 1998; Wartenberg et al., 2003; Görlach et al., 2003). One recent study provided further evidence for an ER-based OH· production in regulating HIF degradation, mediated

by the Fenton reaction (Liu et al., 2004). These observations are in line 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 at the centre of this model convert P_{O_2} to a redox signal. How this signal is transduced to HIF-α is not known. While ROS clearly affect the redox state of the cell and thus thioredoxin and Ref-1 function (see above), it is of interest to analyze whether ROS signaling in these settings interfaces with PHD function. Interestingly, ROS have, for example, been shown to interfere with mitochondrial function, affecting enzymes of the Krebs cycle at the level of α-ketoglutarate dehydrogenase (KGDH) and SDH (Nulton-Persson and 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 and Semenza, 2001). The issue of mitochondrial ROS formation is controversial, however, as other groups have reported that the decreasing mitochondrial membrane potential is associated with a reduction in mitochondrial ROS formation (Lee et al., 2001; Jones et al., 2000). 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-α regulation (Vaux et al., 2001; Srinivas et al., 2001). In addition, hypoxic signal transduction may require kinase/phosphatase activity in certain cell types (Wang et al., 1995b; Zundel et al., 2000; Mottet et al., 2003; however, contrast Alvarez-Tejado et al., 2002). Up to now, the putative corresponding phosphorylation targets such as HIF-α, transcriptional coactivators or the presented mediators of the oxygen-sensing pathway, have not been identified.

It is worth mentioning that not all ROS-mediated pathways on HIF activity are part of an oxygen-signaling response, but rather expression of a delicate integration of oxygen-sensing mechanisms into major signaling 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 (Richard et al., 2000; Haddad and Land, 2001). However, this mode of activation seems to be cell-specific.

Other factors

The differences in target specificity and oxygen-dependent regulation of the two orthologues HIF-1α and HIF-2α may add a further level of complexity to oxygen signaling, though recent studies argue for a limited HIF-2α function in hypoxia-induced signaling (Park et al., 2003; Sowter et al., 2003). One report involving DNA microarray analysis identified distinct target genes encoding for enzymes of the glycolytic pathway that were exclusively regulated by HIF-1α, apart from several genes commonly regulated by HIF-1α and HIF-2α (Hu et al., 2003). In contrast, the study failed to distinguish genes uniquely regulated by HIF-2α. However, specific target genes regulated independently of HIF-1α do exist, as shown for VEGFR-2 (Elvert et al., 2003) or pneumocyte type II-dependent VEGF expression (Compernolle et al., 2002). Indeed, studies analyzing the tissue-specific expression pattern of HIF-1α (Stroka et al., 2001) and HIF-2α (Wiesener et al., 2003) suggest complementary rather than redundant functions of both proteins. Interestingly, prolonged hypoxia may favor induction of HIF-2α due to induction of natural antisense HIF-1α (aHIF), suggesting a qualitative change in signaling response, depending on duration of the hypoxic stimulus (Thrash-Bingham and Tartof, 1999; Chun et al., 2002; Uchida et al., 2004).

Finally, a delicate degree of oxygen-sensing regulation was recently suggested by a report showing that the P_{O_2} may differ in subcellular compartments under the control of signaling molecules such as NO (Hagen et al., 2003). Inhibition of mitochondrial respiration by NO under low oxygen tension resulted in reduced mitochondrial oxygen consumption, leading to an increase in intracellular oxygen availability and reactivation of prolyl hydroxylation of HIF-α subunits. Thus, by changing the intracellular P_{O_2} distribution field, the metabolic oxidative activity crucially influences the threshold of the cell to elicit adaptive mechanisms in response to a given tissue P_{O_2}. In this context, the different subcellular localizations of the putative oxygen-sensing systems (cytochrome *c*, a_{592} in mitochondria, NADPH-oxidase in cell membrane, PHD in cytoplasm and nucleus, FIH in cytoplasm) should be taken into account. Thus, specific oxygen-sensing cascades may, by means of their different oxygen sensitivities, cell-specific and subcellular localization, help to tailor various adaptive and dynamic responses according to differences in tissue oxygen availability.

Conclusions

Taken together, oxygen-signaling pathways in physiological and pathological conditions in multicellular organisms control essential cellular functions, with wide-ranging effects on cell fate. Exploiting these systems for therapeutic intervention seems promising but should be employed cautiously, as reflected by the dual role of HIF in regulating cell survival and cell death mechanisms. While under physiological conditions upregulation of adaptive survival mechanisms in viable cells and coordinated removal of injured cells serve to rescue the overall function of the tissue or organ, this program may run havoc under pathological conditions, as for example in neurodegeneration. Moreover, tumors may exploit HIF-mediated survival mechanisms for their growth and progression by evoking and fostering a selective pressure for cells with specific genetic alterations that counteract HIF-mediated cell death responses. In this context, hypoxia-mediated upregulation of an antisense HIF-1α (Thrash-Bingham and Tartof, 1999; Chun et al., 2002), the inhibitory PAS protein IPAS (Makino et al., 2002), PHD2/3 (Berra et al., 2003) or activation of glycogen synthase kinase 3 (GSK3; Mottet et al.,

2003) resulting in reduced hypoxic HIF-α accumulation may be viewed as a preventative and self-limiting procedure by the cell to keep HIF-α levels and function from reaching toxic concentrations. Thus, from a therapeutic point of view, intervention in the PHD/HIF pathway may not lie in the extremes, i.e. in either general activation or inhibition, but rather in a balanced and fine-tuned manipulation of the system, as holds true for so many matters in life.

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