Low oxygen stimulates the intellect

Symposium on Hypoxia and Development, Physiology and Disease

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This Keystone Symposium on Hypoxia and Development, Physiology and Disease took place in Breckenridge, Colorado, USA, between 16 and 21 January 2006, and was organized by M.C. Simon, R.S. Johnson, A. Giaccia and M. Gassmann.

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processes that are crucial for the hypoxic response, such as angiogenesis, anaerobic glycolysis and cell survival. HIF consists of two subunits: the hypoxia-inducible HIF- α and the constitutively expressed HIF- β (also known as aryl hydrocarbon receptor nuclear translocator (ARNT)). Our understanding of the central mechanism of oxygen-dependent accumulation of HIF- α —although probably far from complete—is substantial, and is the product of fascinating biochemical and genetic studies performed during the past 20 years (Schofield & Ratcliffe, 2004). We now know that the HIF- α subunit is rapidly degraded under normoxic conditions via proline hydroxylation mediated by at least three oxygen-dependent prolyl hydroxylases (PHDs). Hydroxylated residues on HIF- α are recognized and captured by the product of the von Hippel-Lindau (VHL) gene that subsequently promotes the ubiquitylation and proteasomal degradation of the HIF- α subunit. Under conditions of low oxygen, the PHDs are significantly less active, leading to the stabilization of HIF- α , binding to HIF- β and the formation of the HIF homodimer, which then binds to its consensus DNA sequence and leads to gene transactivation. An additional level of control is provided by the hydroxylation of an asparagine residue near the carboxyl terminus of HIF- α subunits, by factor inhibiting HIF (FIH). This oxygen-dependent modification prevents the recruitment of transcriptional coactivators.

New insights into the PHD/VHL/FIH/HIF module

At the meeting, interesting new insights into this regulatory module were described that were gained through genetic approaches in model organisms. Strains of mice now exist with a variety of modifications in the PHD/VHL/FIH/HIF pathway. One theme was the role of the pathway in adapting tissues to hypoxia and ischaemia. P. Carmeliet (Leuven, Belgium) reported that mice lacking *phd1* are markedly protected from ischaemic muscle necrosis—an effect that probably involves HIF-2 α activation because protection was decreased in mice that were heterozygous for a *Hif2 α* defect. J. Chavez (White Plains, NY, USA) provided evidence that mice lacking *Hif1 α* in neurons develop more severe injuries after middle cerebral artery occlusion, further supporting the idea that enhancing HIF activation might be beneficial in ischaemic settings. W. Bernhardt (Erlangen, Germany) reported that a small-molecule PHD inhibitor protected the rat kidney from ischaemia-reperfusion injury.

An issue that is being pursued by several groups is the relative role of HIF-1 α and HIF-2 α in mediating particular aspects of the

Introduction

Cells need oxygen for mitochondrial respiration and a wide range of other metabolic processes. It is increasingly being recognized that there are sophisticated mechanisms for monitoring local oxygenation and that these are used to modulate extensive aspects of cellular behaviour. This excellent meeting was held at 9,600 feet where oxygen is 70% of that at sea level; enough to cause breathlessness on exertion and some nasty headaches.

A main focus of the meeting was the oxygen-response pathway that centres on the hypoxia-inducible factors 1 α (HIF-1 α) and 2 α (HIF-2 α). As shown in Fig 1, these two transcription factors share extensive sequence homology, modes of regulation and downstream targets, and regulate the expression of more than 60 genes involved in diverse

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Fig 1 | Cellular processes affected by hypoxia. Processes at the top are regulated in a HIF-dependent manner whereas those at the bottom are regulated by HIF-independent processes. Blue rectangles denote activation, red ones inhibition. Gene products or messenger RNA features that mediate specific processes are shown as examples; see text for further details. AMPK, AMP-activated protein kinase; ATG1, autophagy gene 1; Cul2, Cullin-2; EGFR, epidermal growth factor receptor; eIF2 α , eukaryotic translation initiation factor 2 α ; eIF4E-BP, eukaryotic translation initiation factor 4E-binding protein; EPO, erythropoietin; FIH, factor inhibiting HIF; Glut-1, glucose transporter 1; HIF, hypoxia-inducible factor; IRE-1, inositol-requiring 1 protein kinase; IRES, internal ribosomal entry site; LOX, lysyl oxidase; msh2/6, mismatch repair 2/6; mt, mitochondrion; mTOR, mammalian homologue of target of rapamycin; PDK1, pyruvate dehydrogenase kinase 1; PERK, PKR-like endoplasmic reticulum kinase; PHD, prolyl hydroxylases; RNAPII, RNA polymerase II; ROS, reactive oxygen species; TSC1/2, tuberous sclerosis 1/2; uORFs, untranslated open reading frames; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau; XBP-1, X-box-binding protein 1.

hypoxic response and the consequences of *VHL* loss. W. Kaelin (Boston, MA, USA) has created knock-in mice that conditionally express constitutively active HIF-1 α or HIF-2 α from the *Rosa26* locus. He has used this approach to show that the steatosis seen in hepatocytes on *VHL* loss-of-function is not phenocopied by

activating HIF-1 α , but is partially phenocopied by activating HIF-2 α and can be fully phenocopied by activating HIF-1 α and HIF-2 α . V. Haase (Philadelphia, PA, USA) showed that renal cysts that develop when *VHL* is inactivated in the mouse proximal tubule (Rankin *et al*, 2006) require both HIF-1 β and HIF-2 α .

The role of mitochondria in HIF biology

There is considerable interest in understanding the complex interactions between mitochondria and the HIF pathway. In terms of understanding how hypoxia decreases mitochondrial respiration, N. Denko (Stanford, CA, USA) showed that pyruvate dehydrogenase kinase 1 (PDK1) is a HIF target. Increased expression of PDK1 inactivates pyruvate dehydrogenase, providing a route by which HIF activation decreases the supply of mitochondrial substrates (Papandreou *et al*, 2006). N. Chandel (Chicago, IL, USA) and others have previously provided evidence that in low-oxygen conditions mitochondria generate more radicals and that this contributes to HIF activation (Kaelin, 2005). Chandel reported further data supporting this using cytochrome *b* mutants, which disable mitochondrial electron transport but still allow radical generation.

Studies in non-mammalian model organisms

Understanding the role of the HIF pathway in cancer and the possibilities for treatment is an area of intense interest in which many different approaches are being used. For example, the interface between growth control and hypoxia is being explored in *Drosophila melanogaster* by P. Wappner (Buenos Aires, Argentina). The phosphoinositide-3 kinase (PI(3)K)/PTEN/AKT/TOR pathway, which is known to regulate cell growth and size in response to environmental cues, potentially induces Sima (the orthologue of HIF- α), which is crucial for restricting insulin-induced growth via *Scylla* and TSC1/2 (see also the section on the mammalian pathway 'Alterations of cellular programmes in low-oxygen conditions'). This explains why mutants of *Fatiga* (the *PHD* orthologue), which result in elevated Sima levels under normoxia, are smaller and develop more slowly (Gorr *et al*, 2006). It is clear that genetic screens in *Caenorhabditis elegans* and *D. melanogaster* will make important contributions to understanding how the PI(3)K/PTEN/AKT/TOR pathway interacts with other signals. Another elegant example of the use of a model organism to study HIF-mediated responses was reported by E. Gort (Utrecht, The Netherlands). By performing genetic screens in *C. elegans* Gort showed that survival in hypoxia is HIF-dependent and that *hlh-8* is a HIF target necessary for hypoxic survival. The mammalian orthologue of *hlh-8* is TWIST1, a transcription factor that regulates mesodermal development, and it will be interesting to understand the role of TWIST1 in HIF-mediated adaptation.

HIF modulates metastasis and genomic instability

A particularly interesting story at the meeting was that HIF activation increases the expression of the lysyl oxidase gene (*LOX*), which encodes the proenzyme that catalyses the cross-linking of collagen. A. Giaccia (Stanford, CA, USA) showed that HIF induces *LOX* expression. Perhaps counter-intuitively, this enhances migration and metastasis, whereas blocking *lox* activity was shown to decrease metastasis in a mouse model. Both G. Semenza (Baltimore, MD, USA) and P. Maxwell (London, UK) reported that the VHL/HIF pathway potentially downregulates the key intercellular adhesion molecule E-cadherin. E. Huang (Bethesda, MD, USA) reported his recent findings that activating HIF-1 α downregulates the mismatch repair genes *MSH2* and *MSH6* through an interesting mechanism that involves displacing MYC from the transcription factor SP1, which is bound to the promoter of these repair genes in hypoxia. Overall, there is considerable evidence now that hypoxia—perhaps largely through HIF—is a potent promoter of genetic instability and metastasis.

Does hydroxylation extend beyond HIF?

Key questions in the field of hypoxic biology are whether other proteins are regulated by hydroxylation, whether the HIF hydroxylases have other targets, and the extent of other functions of VHL. Judging from the meeting, rapid progress is being made to address these issues. W. Kaelin elegantly showed that clusterin provides a marker for a HIF-independent action of VHL (Nakamura *et al*, 2006). P. Ratcliffe (Oxford, UK) reported that several proteins containing ankyrin repeats interact with FIH in two-hybrid screens performed in yeast: of particular interest were I κ B- α and NF- κ B p105. These proteins contain sequences closely homologous to the target region of HIF- α subunits and can be hydroxylated by FIH *in vitro*. Importantly, hydroxylation was also shown to occur in mammalian cells using mass spectroscopic analysis of both over-expressed and native proteins. However, the functional consequences and oxygen sensitivity of this modification are not yet fully understood. Complementing these findings, C. Taylor (Dublin, Ireland) reported data suggesting that IKK β is a PHD target, and M. Czyzyk-Krzeska (Cincinnati, OH, USA) showed that hydroxylation and VHL-dependent ubiquitylation regulate the large subunit of RNA polymerase II. Interestingly, unlike HIF, this regulation seems not to involve altered protein stability. D. Peet (Adelaide, SA, Australia) showed that the intracellular domain of Notch 1—which contains ankyrin repeats—could be hydroxylated by FIH.

Hypoxia, differentiation and development

If hydroxylation modulates Notch, this would agree with data presented by L. Poellinger (Stockholm, Sweden) that hypoxia induces Notch signalling. This forms part of an emerging picture that hypoxia has a crucial role in differentiation decisions and stem-cell behaviour. C. Simon (Philadelphia, PA, USA) showed that the transcription factor Oct-4 is a HIF-2 α target (Covello *et al*, 2006) and that embryos with *Hif2 α* knocked in to the *Hif1 α* locus have a severe gastrulation defect, whereas HIF-2 α -deficient embryos have a defect in their primordial germ cells. T. Löfstedt (Malmö, Sweden) reported a HIF-1 α -dependent de-differentiating effect of hypoxia on neuroblastoma cells, which involves changes in the N-MYC network. A similar inhibitory effect of hypoxia on normal adipocyte differentiation was shown by Z. Yun (New Haven, CT, USA), who found that HIF-1 α activity is both sufficient and necessary for the inhibition of preadipocyte differentiation and adipogenesis (Yun *et al*, 2002). These findings have potential therapeutic implications because they suggest that manipulating the pathway could have an impact on obesity. Interestingly, the inhibitory effects of HIF-1 α on the differentiation of these tissues contrast with its promotion of lung epithelial differentiation and maturation. Using an explant model of fetal lung morphogenesis, S. Gebb (Denver, CO, USA) showed that fetal oxygen tension (3% O₂) resulted in branching, whereas explants cultured under 21% O₂ did not. Molecular analysis showed that the branching morphogenesis depended on the extracellular matrix protein Tenascin-C and epidermal growth factor receptor (EGFR)-dependent signalling (Gebb *et al*, 2005). A similar conclusion was reached using a genetic approach by J. Shannon (Cincinnati, OH, USA). In these studies, conditional inactivation of HIF-1 α in the developing lung epithelium did not affect early development of the lung. However, at birth, mice with homozygously inactivated HIF-1 α were cyanotic and died from respiratory failure owing to a lack of differentiation and type II cell maturation. Overall, it is clear that hypoxia and hypoxia-inducible factors have complex roles in morphogenesis and differentiation.

HIF subunits are members of a superfamily of proteins that contain PAS domains, which were named after the first transcription factors in which they were identified—Per, Arnt and Sim. S. McKnight (Dallas, TX, USA) discussed the role of neuronal PAS domain proteins (NPAS) in the dentate gyrus of the mouse brain. He showed that they are crucial for neurogenesis and function in a pathway involving fibroblast growth factor (FGF) signalling, in which they inhibit the negative regulator Sprouty. McKnight also reported that Sprouty functions in very large homomeric complexes with multiple Fe-S clusters. Sprouty and structurally related SPRED proteins might therefore provide ‘nanobatteries’ to power energetically demanding enzyme reactions including CpG demethylation of DNA (Wu *et al*, 2005); such reactions might take place using proteins containing the *jumonji* domain, which is homologous to FIH. Such a system is predicted to be oxygen-sensitive and might provide another important interface between oxygen, epigenetic control and developmental regulation.

HIF and erythropoietin

The HIF field stemmed from studies of erythropoietin production (Wang *et al*, 1995). M. Yamamoto (Tsukuba, Japan) showed that knocking out the 3' enhancer sequence from the mouse *epo* gene, which was originally used to isolate HIF, results in severe neonatal anaemia, but normal erythropoiesis in adults. This extends previous transgenic studies showing that different *cis*-acting sequences are required for erythropoietin production in the kidney and liver, and shows that we still have more to learn about precisely how normal physiological homeostasis is achieved. R. Johnson (La Jolla, CA, USA) has found that mice with *vh* deleted in the skin have a greatly increased number of red blood cells (erythrocytosis). This is probably because greatly increased perfusion of the skin reduces blood supply to the viscera leading to increased erythropoietin production. Besides its role in regulating red-blood-cell production there is continuing interest in other physiological functions of this hormone, and M. Gassmann (Zurich, Switzerland) has used transgenic mice to implicate erythropoietin in controlling respiration via the brainstem and carotid body.

Genetic variation in the HIF system

Not surprisingly, understanding the role of the HIF system in human physiology is less advanced than in simpler systems. F. Lee (Philadelphia, PA, USA) reported a small family with autosomal dominant erythrocytosis and a mutation in *PHD2* that abrogates enzyme activity—suggesting that a normal level of PHD2 is essential to regulate erythrocytosis appropriately and that manipulating PHD2 activity might be a safe way of augmenting red-blood-cell production in humans (Percy *et al*, 2006). An intriguing question is the extent to which there might be genetic variation in the pathway in human populations. Analyses of single nucleotide polymorphisms by L. Moore (Denver, CO, USA) suggests that there has been non-random selective pressure on variants of HIF-1 α , PHD2 and ARNT in populations living at high altitude.

Alterations of cellular programmes in low-oxygen conditions

Regulation of protein translation to conserve the energy status of the cell is an emerging theme in cellular adaptation to hypoxia. C. Simon presented data supporting a multi-level regulation of translation by hypoxic stress that involves inhibition of protein synthesis both at the initiation stage, through modifications on the

eukaryotic translation initiation factors eIF2 α and eIF4E-BP1, and at the elongation stages, through the inhibition of the eukaryotic elongation factor eEF2. The signals resulting from long-term hypoxia and energy depletion are transduced to the translation control machinery through the AMP-dependent kinase/tuberous sclerosis 2/Rheb/mammalian target of rapamycin (AMPK/TSC2/Rheb/mTOR) pathway in a HIF-independent manner (Liu *et al*, 2006). Similar findings were reported by B. Wouters (Maastricht, The Netherlands). By analysing actively and poorly translated messenger RNAs, this group found that distinct populations of mRNAs become translated at different times after extreme hypoxia. Intriguingly, the number of ribosomal-associated mRNAs decreases rapidly in anoxia, which correlates with a substantial increase in eIF2 α phosphorylation. At later time points, translation recovers to a significant extent—an event that coincides with the recovery of functional eIF2 α and a concomitant inhibition of the eIF4-F complex (Koritzinsky *et al*, 2006). Therefore, the cellular response to hypoxic stress is a complex but well-orchestrated one, with a pronounced initial reduction in energy-expending processes to allow partial recovery and the synthesis of potentially crucial pro-survival gene products.

C. Koumenis (Winston-Salem, NC, USA) presented data showing that the rapid phase of global downregulation of protein synthesis is primarily regulated by the PKR-like endoplasmic reticulum (ER) kinase (PERK), which phosphorylates eIF2 α in response to unfolded proteins in the ER. Importantly, transformed cells with abrogated PERK or eIF2 α phosphorylation are sensitive to hypoxia *in vitro* and *in vivo* and form smaller tumours compared with controls (Bi *et al*, 2005). V. Chauhan, from the laboratory of Albert Koong (Stanford, CA, USA), presented evidence supporting activation of another arm of the unfolded protein response (UPR) that is mediated by the ER-resident endonuclease inositol-requiring 1 protein kinase (IRE-1) and the downstream target X-box binding protein 1 (XBP-1). A new protein, Zhangfei, was identified as a binding partner and negative regulator of XBP-1. Thus, the ER is emerging as a novel site of oxygen sensing in the cell, which probably serves as a ‘rapid response’ mechanism to signal decreasing energy availability. L. Gardner (New York, NY, USA) reported that although hypoxia induces the UPR, and inhibits expression of the anti-differentiation and pro-angiogenic protein Id in many cell lines, neuroblastoma cell lines present an intriguing exception as they apparently have lost the ability to inhibit Id, or activate the UPR, in response to hypoxia or pharmacological ER stressors.

A programme that might be important in promoting cellular survival under stress conditions is autophagy, a process that is activated when cells are faced with an inadequate supply of nutrients. It is characterized by the ‘digestion’ of some internal organelles (for example, mitochondria) using endogenous enzymes to recycle and reuse their components. E. Keshet (Jerusalem, Israel) described the conditional expression of a ‘vascular endothelial growth factor (VEGF) trap’ protein in the mouse heart that produces a microvascular deficit. Under these conditions, the myocardium exhibited features of autophagy and entered a state of ‘hibernation’, allowing it to remain fully viable for months under chronic oxygen deprivation. Remarkably, reversing the VEGF inactivation restored the vascular network and rescued cardiac function. This might be clinically important in understanding how myocardial muscle function can be preserved and also in predicting which patients could benefit from revascularization. E. White (Piscataway, NJ,

USA) discussed the importance of autophagy as a survival pathway in hypoxic areas of tumours. Cells that have developed resistance to apoptosis evade necrosis through autophagy. Activation of Akt or genetic ablation of *beclin-1* inhibits the autophagic response and markedly augments necrotic cell death pathways *in vitro* and *in vivo*, suggesting potential therapeutic approaches for malignancies that are refractory to apoptosis-inducing chemotherapy. Paradoxically, however, impaired autophagy can be tumorigenic and it was suggested that this could be through the promotion of necrosis-stimulated inflammation and chromosome instability. Direct evidence for a role of HIF in hypoxic autophagy came from A. Bacon (Oxford, UK), who reported that the HIF target BNIP3 (BCL2/adenovirus E1B 19 kDa interacting protein 3) is necessary and sufficient for hypoxia-driven autophagy.

The evolutionary extent of responses to low oxygen was illustrated by P. Espenshade (Baltimore, MD, USA), who has shown that ergosterol synthesis acts as an important oxygen-sensing system in fission yeast (Hughes *et al*, 2005). Ergosterol production is oxygen-dependent and in low-oxygen conditions the decrease in ergosterol production was shown to provide a key signal to make extensive homeostatic adjustments in cellular metabolism.

Therapeutic avenues

There is clearly extensive interest in manipulating the HIF system. Inhibitors of the HIF pathway are being developed primarily as anti-cancer drugs, and activators to treat anaemia and ischaemia. Enthusiasm for inhibiting HIF in cancer has been increased by the fact that antagonising its target, VEGF, provides clinical benefit—the subject of one of the keynote talks at the meeting (N. Ferrara, South San Francisco, CA, USA; Ferrara, 2005). Potentiating HIF activation has moved more quickly into the clinic. T. Seeley (San Francisco, CA, USA) from Fibrogen reported that their PHD inhibitors are effective at increasing erythropoiesis in humans with chronic kidney disease. Excitingly, they seem to suppress hepcidin in animal models of chronic anaemia and do not promote growth of model tumours. R. Kelly (Framingham, MA, USA) from Genzyme presented the current developments on gene therapy for limb and coronary ischaemia using Ad2 adenovirus to deliver HIF-1 α VP16, which has been well tolerated in about 100 humans. Several groups presented new compounds that antagonize HIF activity at micromolar concentrations or lower, including echinomycin and NSC-644221 (G. Melillo, Frederick, MD, USA), 2,2 dimethylbenzopyran-derived molecules (E. van Meir, Atlanta, GA, USA) and ENMD-1198 (P. Burke, Rockville, MD, USA). An interesting new strategy, presented by R. Bruick (Dallas, TX, USA), was the investigation of the interaction between the PAS domains of HIF subunits and the screen for small-molecule inhibitors that selectively inhibit this interaction (Yang *et al*, 2005). Work presented by M. Dewhirst (Durham, NC, USA) suggests that the timing of HIF inhibition during clinical treatment of solid tumours is likely to be important: blockade of HIF activity before radiotherapy can cause radioresistance through the abrogation of HIF-mediated tumour cell apoptosis, whereas blockade of HIF activity after radiotherapy in wild-type-p53 tumours results in the greatest enhancement of tumour radiosensitivity. HIF-1 upregulation after radiotherapy is a consequence of reoxygenation and free radical production, probably caused by a wave of apoptosis occurring as a result of the therapy (Moeller *et al*, 2005).

There is longstanding interest in developing cancer therapies that are selective for hypoxic cells. M. Brown (Stanford, CA, USA) gave a historical account of the development of the prototypical hypoxic cytotoxin tirapazamine (Sanofi-Aventis, Paris, France). This agent is showing some promise in clinical trials when combined with genotoxic chemotherapeutic agents. An interesting new agent, PR-104, was discussed by A. Patterson (Auckland, New Zealand). Once activated in hypoxic regions, PR-104 has been designed to kill adjacent cells as well. Finally, Brown also outlined the exciting potential of genetically modified anaerobic bacteria that preferentially proliferate in hypoxic and necrotic areas of tumours and have potent tumolytic activity.

Concluding remarks

This meeting illustrated that the field of hypoxic biology is moving at an astonishing pace. As pointed out by G. Semenza, it is exciting that the therapeutic manipulation of HIF is actually being tested in humans—just over ten years after the molecular identification of the proteins that make up this intriguing transcription factor (Wang *et al*, 1995). Understanding the mechanisms that underlie the broad response of organisms to low oxygen availability has real potential to unleash better, and perhaps more specific, therapeutic strategies for the wide range of diseases in which tissue oxygen delivery is abnormal.

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COMPETING INTEREST STATEMENT

P.H. Maxwell is a scientific founder, consultant, director and equity holder in ReOx Ltd (Oxford, UK), a company which aims to develop HIF hydroxylase inhibitors for therapeutic benefit.

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