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The Hypoxic Response: Huffing and HIFing

Minireview

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Since Joseph Priestley's demonstration in 1774 of the deleterious effects of a burning candle on a mouse in a bell jar, we have known that oxygen is essential for animal life. Only recently, however, have we begun to understand how animal cells sense when oxygen is limiting and respond to the crisis of hypoxia. Cellular responses range from rapid changes in carbohydrate metabolism to permanent restructuring of their blood supply. Remarkably, studies of these diverse phenomena have recently converged on a common pathway employed by cells to sense hypoxia and activate specific genes to deal with the crisis. Not only is this pathway crucial for normal tissue physiology, but its failure can contribute to diseases such as myocardial infarction and anemia, and its misregulation can lead to retinopathy and tumor growth. Here we describe the recent advances in the molecular response to hypoxia and the identification of the HIF-1 transcription factor as a central component in the hypoxic response pathway.

The Body's Response to Hypoxia

To place the molecular advances in a physiological context, it is useful to consider the many ways that our bodies are equipped to deal with inadequate tissue oxygenation. Someone experiencing hypoxia of a less severe form than Priestley's bell jar–bound mouse—for example a sea level–acclimated scientist at a Keystone meeting at 9,000 feet—will experience rapid breathing, increased circulation, and the buildup of muscle lactic acid while hiking, and, if the conference lasts several days, will exhibit an increase in red blood cell and hemoglobin counts and perhaps a proliferation of blood capillaries. Such responses are designed to decrease cellular oxygen need and dependence on oxygen, and to increase tissue oxygen supply. Some of the responses are intrinsic to all hypoxic cells, indicating that each cell has its own oxygen-sensing system, while other responses are mediated by more centralized sensors in the body that monitor global oxygen levels and cause system-wide changes in tissue oxygen delivery. For example, when cells of the carotid body detect subnormal oxygen levels in the carotid arteries, they propagate dopaminergic signals to the brain to increase breathing and thus the oxygen saturation of the blood. Another example is cells of the developing liver and adult kidney that regulate the blood's oxygen carrying capacity by secreting the glycoprotein hormone erythropoietin (EPO), which stimulates red blood cell and hemoglobin production.

The Molecular Response to Hypoxia: The Classical View

Every biochemistry student learns the classical response of an oxygen-starved cell, first described by Louis Pasteur in yeast and called the Pasteur effect, as the substantial increase in carbohydrate consumption that occurs to compensate for its inefficient utilization under anaerobic conditions. When oxygen is unavailable as the final electron acceptor in the respiratory chain, the cell must abandon oxidative phosphorylation and rely solely on glycolysis for energy production. The switch to anaerobic metabolism, we are taught, is regulated by energy pathway metabolites acting on glycolytic enzymes. For example, phosphofructokinase is allosterically inhibited by ATP and inhibition is reversed by AMP. This is, however, just a small part of the way in which each cell deals with inadequate oxygen levels. Hypoxic cells sense diminishing oxygen levels well before their ATP pools are depleted, and respond with a self-imposed austerity program to curb their energy usage by shutting down nonessential cell functions (Hochachka et al., 1996). Not only is hypoxia a signal for energy conservation, but in the past few years it has been found to trigger expression of a select set of genes (Fandrey, 1995). These include specific isoforms of glycolytic enzymes and glucose transporters that function better at low oxygen. Another is the *EPO* gene, and it is the study of this gene that has led to a considerably expanded view of the hypoxic response.

Mechanism of EPO Induction by Hypoxia

In 1890, after hiking in the mountains of Peru, the histologist Viault documented an increase in his erythrocyte density and attributed it to the lower partial oxygen pressure at high altitude. The molecular basis of this response had to await a century that included the purification of the erythropoietic factor EPO and the discovery that it binds the EPO receptor on the surface of erythrocytic progenitors to stimulate their proliferation and differentiation into red blood cells. With the cloning of the *EPO* gene in 1985, recombinant EPO became widely available for anemic patients and ambitious athletes, and investigators were able to show that hypoxia regulates EPO expression primarily at the level of transcription. The challenge of understanding the hypoxic induction of EPO would be surmounted in the next decade with several major advances including an initial characterization of a cellular oxygen sensor and the identification of the hypoxia-inducible transcription factor HIF-1.

In a seminal paper, Goldberg et al. (1988) found that the *EPO* gene is induced by hypoxia in liver cell lines, and the induction displayed distinctive pharmacological characteristics suggesting the involvement of a specialized cellular oxygen sensor. Induction was insensitive to electron transport poisons such as potassium cyanide, indicating that the cells were not measuring oxygen levels simply by failure of the respiratory chain. Instead they found that EPO expression could be induced by divalent metals like cobalt that mimic ferrous iron and could be blocked by carbon monoxide—hallmarks of the involvement of a heme-containing protein in oxygen sensing (see below). They also found that hypoxic induction of EPO was obliterated by the translational inhibitor cycloheximide, indicating that this hypoxic response pathway requires a round of de novo protein synthesis.

The search for the molecules that conferred this oxygen-dependent regulation of EPO expression was led

by Semenza, Ratcliffe, Caro, and their colleagues. By studying the expression of *EPO* reporter genes in hepatocytes and transgenic mice, they honed in on an approximately 50-nucleotide enhancer element in the 3′ end of the gene that conferred hypoxic induction (Semenza, 1996). Semenza's group went on to discover a transcription factor that acts through this sequence, detected only in hypoxic cells, and named it hypoxia inducible factor 1 (HIF-1).

HIF-1 binds the sequence 5′-TACGTGCT-3′ in the *EPO* enhancer, and this site-specific DNA binding activity facilitated its purification and the cloning of its genes (Wang et al., 1995a). It is an α/β heterodimer, with each subunit containing a basic-helix-loop-helix (bHLH) motif and a PAS protein–protein interaction domain found in a variety of known or suspected transcription factors including the Drosophila proteins Period, Single-minded, and Trachealess, and the mammalian proteins aryl hydrocarbon receptor (AHR) and aryl hydrocarbon receptor nuclear translocator (ARNT). In fact, the HIF-1β subunit turned out to be identical to previously described ARNT protein isoforms, which function as a heterodimer with AHR in the cellular response to environmental toxins such as aryl hydrocarbons. The fact that the ARNT/HIF-1β subunit is involved in the induction of two distinct pathways points to HIF-1α as the critical hypoxia-specific component.

HIF-1 as a Global Regulator of Hypoxic Gene Expression

Scaling the terrain of *EPO* gene regulation, researchers could never have dreamed of the vista they would find at the summit. They started in search of a regulator of erythropoiesis and discovered what appears to be a global regulator of hypoxic gene expression. As expected of a general regulator, HIF-1 is widely expressed in tissues and HIF-1 activity is found in many different cell lines, most of which do not express EPO. Indeed, HIF-1 is not at all restricted to EPO regulation (Semenza, 1996). This was first shown by the identification of HIF-1 binding sites in a number of glycolysis genes and the demonstration that these sites can confer hypoxic regulation on other genes (Semenza et al., 1994). Functional HIF-1 sites were subsequently found in the gene encoding vascular endothelial growth factor (VEGF), a potent angiogenic factor produced by oxygen-starved cells (Shweiki et al., 1992). VEGF binds the receptor tyrosine kinases flk, flt-1, and flt-4 on vascular endothelial cells and stimulates the local proliferation of blood capillaries to increase oxygen delivery. Usually this is a beneficial response, but it can also have grave consequences as when it allows growing tumors to overcome their limiting blood supply, or when VEGF-driven angiogenesis in the retina destroys vision (Folkman, 1995). The genes encoding inducible nitric oxide synthase (i-NOS) and heme oxygenase-1 (HO-1), which regulate the production of the vasodilators nitric oxide and carbon monoxide, respectively, also appear to be HIF-1 targets. And HIF-1 sites are present in the *tyrosine hydroxylase* (*TH*) gene, encoding a key enzyme in dopamine synthesis and the regulation of breathing. Thus, multiple hypoxic responses including adaptation to anaerobic metabolism, erythropoiesis, angiogenesis, vasodilation, and possibly breathing are all under the control of a single transcription factor. Many of these HIF-1 targets show the same pharmacology as EPO induction, suggesting that they are controlled by a common oxygen sensing pathway mediated through HIF-1.

The important role of HIF-1 in the hypoxic response was recently established with hepatocyte cell lines selected for absence of ARNT activity and lacking ARNT/HIF-1β protein, although the *HIF-1*β transcript is still produced (Wood et al., 1996). These cells are defective for the hypoxic induction of a battery of genes including lactose dehydrogenase-A, phosphoglycerate kinase-1, glucose transporter-1, and VEGF. Further support for the significance of HIF-1 in vivo comes from a recent study showing that induction of HIF-1 is perfectly tuned to physiological fluctuations in tissue oxygen levels. Expression of HIF-1 proteins and the level of HIF-1 DNA binding activity show an exponential increase with decreasing oxygen from ~5% (~35 mm Hg) to 0%, with half maximal response at ~1.5% oxygen, nicely coinciding with the critical range of partial oxygen pressures measured for tissues in vivo (Jiang et al., 1996).

The Oxygen Sensing Pathway

The induction profile of HIF-1 by oxygen shows that cells sense and respond to a continuous range of ambient oxygen pressures. The response curve is reminiscent of the classical oxygen saturation curve of hemoglobin and, together with the pharmacological studies of induction of HIF-1 and its targets already discussed, suggests that oxygen sensing involves a hemoprotein. The simplest possibility is that the sensor itself is an oxygen-binding hemoprotein that undergoes an oxygen-dependent conformational change like hemoglobin, although more elaborate models have also been proposed (Bunn and Poyton, 1996). Such an oxygen sensor has been described in the bacterium Rhizobium meliloti, a legume symbiote that responds to the anoxic environment in the host's root nodule via a two-component regulatory system. The oxygen sensor, FixL, is an integral membrane heme-containing kinase whose activity is modulated by oxygen binding (Gilles et al., 1991). In the deoxy conformation, the kinase phosphorylates the transcriptional factor, FixJ, which turns on a cascade of gene regulation leading to expression of nitrogen fixation genes.

Clues about signal transduction from the mammalian oxygen sensor to induction of HIF-1 have also begun to accumulate, suggesting that the pathway may involve protein phosphorylation, as in Rhizobium. General inhibitors of tyrosine kinases block induction of the *HIF-1* genes, while inhibitors of tyrosine phosphatases increase basal levels of HIF-1 proteins and HIF-1 activity (Wang et al., 1995b). A candidate tyrosine kinase upstream of HIF-1 is c-Src, which has been shown to be activated by hypoxia. In *c-src*$^-$ cells the hypoxic induction of VEGF is substantially reduced (Mukhopadhyay et al., 1995). The possibility that c-Src functions upstream of HIF-1 can be tested by examining other HIF-1 targets in *c-src*$^-$ cells, and by expressing an activated c-Src in the *ARNT/HIF-1*β deficient cell lines and seeing if the absence of HIF-1 prevents the c-Src-mediated induction of VEGF.

The *ARNT/HIF-1*β deficient cell lines have also provided evidence for additional factors in the hypoxia pathway. Because hypoxic induction of *HIF-1*α and *-1*β

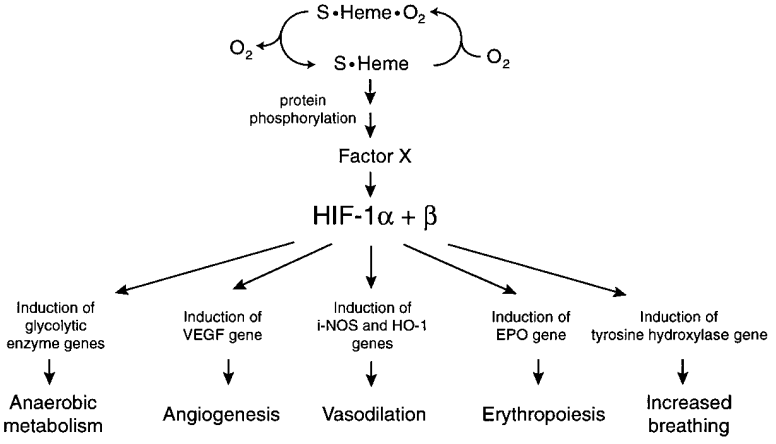

Figure 1. Model of the HIF-1 Hypoxia Response Pathway

At normal tissue oxygen tension, O_2 is bound to a hemoprotein oxygen sensor (S·Heme). When oxygen levels drop, oxygen dissociates causing a change in the sensor that triggers a signaling cascade involving protein phosphorylation. This leads to activation of a hypothetical regulator (Factor X) and increased expression of the HIF-1α and -1β subunits. The HIF-1 α/β heterodimer binds and activates expression of various genes including those encoding glycolytic enzymes (for anaerobic metabolism), VEGF (for angiogenesis), inducible nitric oxide synthase and heme oxygenase-1 (for production of vasodilators), EPO (for erythropoiesis), and possibly tyrosine hydroxylase (for dopamine production to increase breathing). These genes help the cell survive at low oxygen and act to restore normal oxygen levels. Some targets of HIF-1 are induced in most hypoxic cells, while others like EPO are only induced in specific tissues and hence must also require tissue specific regulators. For example, the orphan nuclear receptor hepatic nuclear factor-4 is proposed to be a tissue-specific factor that works with HIF-1 to control EPO expression in the liver and kidney (Galson et al., 1995).

transcripts can still occur in the *ARNT/HIF-1*β$^-$ cells (Wood et al., 1996), there must be another hypoxic regulator upstream of HIF-1 in the pathway, which we refer to as factor X in Figure 1. Similarly, induction of some HIF-1 targets is only partially compromised in the *ARNT/HIF-1*β$^-$ cells, indicating that factors besides HIF-1 must contribute to their hypoxic induction. For the *glucose transporter-1* gene, part of the hypoxic induction is sensitive to potassium cyanide, providing evidence for a separate hypoxia sensing system (Ebert et al., 1995). Some of the missing components may be found among the transcription factors besides HIF-1 that can be affected by hypoxia such as AP-1, NF-κB, and p53, although their functions are clearly not restricted to the hypoxic response. Other hypoxic regulators may function posttranscriptionally, for example by stabilizing hypoxically induced RNAs (Bunn and Poyton, 1996).

The Molecular Response to Hypoxia: The New View

With the convergence of several independent areas of hypoxia research on a common oxygen sensing pathway, HIF-1 has emerged as a central regulator of hypoxic gene expression and restoration of cellular oxygen homeostasis. A working model for the pathway is schematized in Figure 1. Oxygen directly interacts with the cellular oxygen sensor independently of mitochondrial respiration, keeping the sensor inactive. When oxygen levels drop, the deoxy form of the sensor activates a signal that may then be transduced via protein phosphorylation to factor X, which leads to increased expression of HIF-1α and -1β. The HIF-1 heterodimer in turn induces a battery of genes involved in cellular and global responses to hypoxia including anaerobic metabolism, erythropoiesis, angiogenesis, and breathing, which allows the cell to survive at low oxygen and helps restore the normal oxygen level.

Although all cells apparently contain the same general pathway, the end response in each cell type is individually tailored. Thus while most cells exposed to prolonged hypoxia will induce expression of glycolytic genes and various angiogenic factors, only cells of the liver and kidney induce EPO. HIF-1 must therefore function combinatorially with cell type–specific regulatory factors like the orphan receptor HNF-4 (see legend of Figure 1) to control these specific responses. Further levels of control and feedback in the pathway may be provided by posttranscriptional regulation of *HIF-1*, because the turnover of *HIF-1* transcripts and proteins is sensitive to hypoxia, and the phosphorylation and redox state of HIF-1 protein can influence its activity (Wang et al., 1995a, 1995b; Semenza, 1996).

While an outline of the HIF-1 hypoxic response pathway is emerging, many critical questions remain. What are the identities of the cellular oxygen sensor and factor X, and what is the mechanism of signaling between these two parts of the pathway? What are the other hypoxic regulators that work in conjunction with or independently of HIF-1? We also need to better understand the full range of cellular responses to low oxygen tensions and how a cell coordinates the HIF-1 pathway with other hypoxic responses such as the Pasteur effect, the shutdown of nonessential cell functions, and the most severe response, the induction of cellular suicide by p53-mediated apoptosis (Graeber et al., 1996). Such an understanding would facilitate the development of medical treatments for rescuing ischemic tissues and for destroying tumor cells.

While rapid progress is likely to continue with the current biochemical and molecular biological approaches, other tacks may be needed to piece together the entire HIF-1 pathway. Hypoxia pathways are being worked out in the yeast Saccharomyces cerevesiae (Zitomer and Lowry, 1992), but they differ in a number of respects from the HIF pathway, and indeed the S. cerevesiae genome lacks *HIF-1* homologs. Drosophila may allow a traditional genetic approach because flies and other insects exhibit characteristic responses to hypoxia, including induction of glycolytic genes and a proliferation of the tracheal branches that deliver oxygen to the tissues (Manning and Krasnow, 1993), and Drosophila cell extracts contain a HIF-1-like DNA binding activity (Nagao et al., 1996). Certain inherited human

diseases might also provide insights into hypoxic signaling. For example, several families have been identified with consitutively high EPO levels, and some of these could be due to mutations that cause constitutive activity of the HIF-1 pathway. Given the energetic pace of the field and the pressing medical need to understand the hypoxic response, we shouldn't have to hold our breath long for answers.

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