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Hypoxia-dependent Protein Expression: Erythropoietin

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ABSTRACT

Michele Samaja. Hypoxia-dependent protein expression: erythropoietin. *High Alt Med Biol* 2:155–163, 2001.—Normal cell homeostasis relies on the ordered flow of nutrients and substrates through metabolic pathways. Any perturbation of this flow eventually leads to dysfunction, impairment of defense mechanisms, loss of viability and death. High altitude and pathological hypoxia represent a serious and frequent cause for the loss of cell viability. Although organisms customarily respond by triggering adaptive or maladaptive mechanisms, all forms of life eventually succumb to hypoxia if it is severe enough, irrespectively of the primary cause. This paper reviews one of the mechanisms by which organisms respond to hypoxia: erythropoiesis. Although such response is not always beneficial, the discovery of the biochemical mechanisms underlying erythropoiesis has triggered an active field of research that is actually applying lessons learned in the mountains to a more clinical environment.

Key Words: erythropoietin, O_2-dependent gene expression, tumor growth, ischemia

INTRODUCTION

ERYTHROPOIETIN (EPO) IS A KEY blood protein that mediates some of the responses to altitude hypoxia. Originally thought to be only an erythropoietic factor, other functions of EPO are now being discovered. More important, the studies on EPO have triggered research aimed at understanding the body response to hypoxia and other situations such as tumors, ischemia and fetal life (Fig. 1). This paper briefly reviews how EPO has become a paradigm to understand human adaptation to hypoxia.

SUBSTRATE- AND ENZYME-LIMITED REACTIONS

Enzymes catalyze all the body reactions. Enzymatic reactions are controlled either by the concentration of one or more substrates (first- or superior-order kinetics), or by enzyme activity (zero-order kinetics). Before the explosive growth of cellular and molecular biology, hypoxia studies were under the influence of the idea that responses to hypoxia are governed by rules linked to the cellular economy of substrates supply and demand (Hofmeyr and Cornish-Bowden, 2000). Now, enzyme activity is perceived to be the limiting factor. The two views are not necessarily in contrast, but whereas substrate-limited reactions generally concern short-term adaptation (seconds to minutes), enzyme-limited reactions concern long-term (hours to years) or generational adaptation. Indeed, hypoxia tolerance triggered by substrates adjustments cannot be extended by more than a factor of 3 or 4 (Hochachka, 1986), and would induce the problem of self-pollution by production of undesirable end-products.

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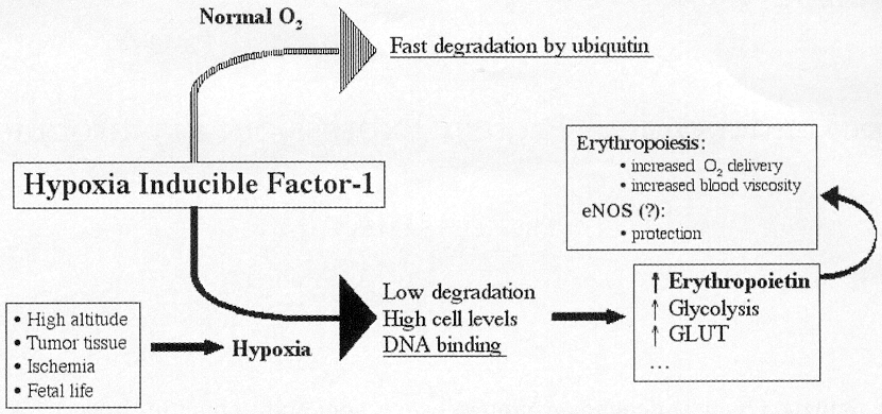

FIG. 1. Schematic model that describes the role of the Hypoxia Inducible Factor in the response to hypoxia.

ERYTHROPOIETIN

Since Viault's original observations in 1890, despite noticeable racial (Winslow et al., 1989) and gender (Moore et al., 1994) differences, erythropoiesis was found invariably increased in all altitude studies. Although suspected for many years, the hormonal regulation of erythropoiesis was proven only in 1953, when an erythropoietic factor, EPO, was found in the blood of anemic rabbits (Erslev, 1953). EPO is a 30.4 kDa glycoprotein produced by fetal liver and adult kidney in response to hypoxia. For many years, the standard method to measure EPO was an expensive and tedious mouse bioassay (Adamson and Finch, 1975). Later, sensitive and specific RIA (Knaupp et al., 1992) and ELISA (Noe et al., 1992) were developed. The misuse of recombinant human EPO (rHuEPO) to boost physical performance has triggered research aimed at detecting such practice, but those methods cannot differentiate endogenous from exogenous EPO. Many techniques were proposed for the new purpose (Lippi and Guidi, 2000), including ELISA optimized to detect murine, but not other types of EPO (Noe et al., 1999). Attempts to correlate EPO with other blood indexes were also controversial (Magnani et al., 1999; Bonfichi et al., 2000). Only in 2000, an alternative ELISA could differentiate endogenous EPO and rHuEPO in the urines (Lasne and de Ceaurriz, 2000).

HYPOXIA AND EPO

Hypoxia is a primary EPO stimulator. In early field studies, conducted at altitudes near 4500 m, serum EPO increased within 24 h after ascent (Milledge and Cotes, 1985), reached a peak 48 h after, then remained elevated for at least 9 days (Abbrecht and Littel, 1972), before slowly decreasing to sea-level normal. Urinary EPO, after a latent 6 h period, peaked in 24 h, and then fell to a plateau about twice as sea-level in 48 h (Faura, 1969). There was no striking correlation between individual EPO variation and erythropoietic responses (Milledge and Cotes, 1985). In these field studies, however, dietary and training variables may have confounded data. In more controlled studies, carried out in animals and/or hypobaric chambers, the minimum exposure to hypoxia needed to elicit EPO production was not high: sojourning at 3000 m or 4000 m for 5.5 h increased EPO by 80% or 300%, respectively (Eckardt et al., 1989).

Not only altitude hypoxia, but also all the conditions associated with polycythemia, such as lung disease (Weil et al., 1968), congenital heart disease (Tyndall et al., 1987), abnormal hemoglobins (Adamson et al., 1969) and obstructive sleep apnea (Cahan et al., 1989), tend to increase EPO. EPO is also increased by situations such as blood acid/base imbalances (Eckardt et al., 1990), physical exercise (Schmidt

et al., 1993), training (Berglund, 1992) and circadian rhythms (Klausen et al., 1993), with hyperoxia constantly acting as an antagonist.

Repeated intermittent exposure to hypoxia is being used with increasing frequency. Not only is it part of pre-acclimatization strategy before high altitude expeditions, but also it may replace the costly rHuEPO administration in patients with cancer, end-stage renal disease, hemodialysis, anemia, as well as for overcoming the effects of anemia after autologous preoperative blood donation. In addition, intermittent hypoxia is associated with clinical conditions including sleep apneas, lung diseases and asthma. Exposing humans to hypoxia for 5 min or 60 min did not induce EPO, but 120 min or intermittent 240 min exposures provided a sufficient stimulus to raise EPO (Knaupp et al., 1992). Short (90 min) daily exposures to hypoxia at simulated altitude of 5000 m for 3 weeks elicited EPO production with accompanying erythropoietic response (Rodriguez et al., 2000).

THE ERYTHROPOIETIC FUNCTION OF EPO

Circulating EPO binds to EPO receptors on the erythroid progenitor in the bone marrow, enabling it to proliferate and differentiate faster into adult red blood cells by repressing apoptosis (Silva et al., 1996). As the main function of red cells is to transport O_2, higher red cell mass should lead to higher blood O_2 capacity. Practically, this advantage is partially blunted by increased blood viscosity, which leads to poor performance, arterial hypertension and retinal hemorrhage (Winslow and Monge, 1987). The role of polycythemia is however difficult to focus because increased red cell mass also increases total blood volume (plasma volume does not decrease in proportion), with concomitant increased cardiac output, which complicates interpretation of data obtained in the absence of blood volume measurements.

However, stimulated erythropoiesis tends to "rejuvenate" the circulating red cell pool. As a potent modulator of the hemoglobin function, the red cell metabolite 2,3–diphosphoglycerate, declines in proportion to the time spent in the circulation, a "younger" red cell population leads to improved O_2 delivery to tissues independently of the increased red cell mass (Samaja et al., 1993). As a consequence, peripheral oxygenation is improved. The observation that rHuEPO administration reduced lactate release during exercise and lowered the use of glycogen by increasing that of free fatty acids (Manitius et al., 1995) might be justified by improved tissue oxygenation, but it remains unclear if EPO directly influences the expression of some enzymes of the lipid-carbohydrate metabolism.

NON-ERYTHROPOIETIC EFFECTS OF EPO

EPO receptors are present not only in the bone marrow erythroid progenitor, but also in other tissues; therefore, EPO has other less-known effects unrelated to erythropoiesis. First, when the red cell mass increase was triggered by EPO, the corresponding increase in blood viscosity was less than expected. Transgenic mice overexpressing EPO developed polyglobulia, but not hypertension nor thromboembolism (Ruschitzka et al., 2000). This study also reported increased endothelial NO-synthase in transgenic mice, although the mechanism was not elucidated. In principle, the EPO-induced NO release confers protection because it counteracts, at least partially, the effect of increased blood viscosity on peripheral vascular resistance. This important result is however to be taken with caution, because another report clearly shows that, in endothelial cultured cells, by depressing endothelial NO-synthase, EPO down-regulates NO production (Wang and Vaziri, 1999).

Second, EPO is expressed in the brain (Tan et al., 1992), specifically by astrocytes (Marti et al., 1996), while EPO receptors are placed on the neuronal cells (Digicaylioglu et al., 1995). Direct intracerebroventricular injection of rHuEPO in advance of hypoxia, ischemia or trauma offered protection to neuronal tissue (Brines et al., 2000). This finding, which indicates paracrine and/or autocrine functions of EPO in the nervous system, and the postulated impermeability to proteins of the blood-brain barrier, support a local protective function of EPO (Sakanaka et al.,

2000). The level of rHuEPO needed to afford protection is much higher than the doses required to elicit erythropoiesis, but high doses are apparently without adverse effects (Brines et al., 2000). Two mechanisms were hypothesized (Marti et al., 2000): (1) EPO protects directly neurons by repressing apoptosis, in analogy to the antiapoptotic action in erythroid precursor cells; (2) EPO indirectly enhances angiogenesis through stimulation of VEGF (see below). However, the observation that EPO can cross the blood-brain barrier by endocytosis followed by translocation (Brines et al., 2000) will probably somewhat change our knowledge on this phenomenon.

Last but not least, EPO has become an ideal paradigm for studying O_2-dependent gene expression (Bunn et al., 1998). Human hepatoma cell lines, which produce EPO in a physiologically-regulated manner, i.e., induced by hypoxia, provided an excellent *in vitro* model to study EPO responses to hypoxia (Ratcliffe et al., 1997). In general, cells reduced their total mRNA synthesis by 50–70% under hypoxia, but an increasing number of genes was stimulated by hypoxia (Fandrey, 1995). Of the factors proposed as regulators of hypoxia-induced gene expression, NF-κB, AP-1 and the tumor suppressor p53 are induced at $PO_2 < 1$ mmHg, therefore they are thought to be implicated in ischemic, rather than hypoxic syndromes. However, expression of the hypoxia-inducible factor-1α (HIF-1α) varied exponentially over a wide range of PO_2 (Jiang et al., 1996), which led to the idea that this factor is the main transducer of the hypoxic signal into the cell.

HYPOXIA-INDUCIBLE FACTOR-1α

The structure of HIF-1α is now well characterized (Semenza, 1998) and the localization of its gene on human chromosome 14 is established (Semenza et al., 1996b). Technically, HIF-1α is a member of the helix-loop-helix PAS family, which belongs to the factors that regulate DNA transcription into mRNA by binding to DNA in the correspondence of specific sequences, or motifs, that are situated upstream of many hypoxia-induced genes (Wang et al., 1995). When HIF-1α recognizes one of these sequences on the DNA strand, it binds to DNA, thereby inhibiting further transcription of that DNA section. When HIF-1α is destabilized, this function is blunted and DNA transcription may proceed. Structurally, HIF-1 is a dimer composed of a β subunit, which is relatively stable and acts as a constitutive factor (Wood et al., 1996), and the α subunit, which is degraded rapidly and is therefore the limiting factor in the reaction chain (Huang et al., 1998). During hypoxia, the α subunit becomes stabilized, leading to stabilization of the dimer, its binding to DNA, and inhibition of DNA translation into mRNA.

The increase of HIF-1α during hypoxia was defined "dramatic" (Gassmann and Wenger, 1997). Therefore, it is not likely that hypoxia affects HIF-1α at the genetic level, but hypoxia might affect the post-translational up-regulation of HIF, probably by increasing the protein stability (Huang et al., 1998). Indeed, in cells exposed to 21% O_2, HIF-1α is rather unstable (half-time <5 min) owing to its susceptibility to degradation by ubiquitin (Sutter et al., 2000; Huang et al., 1998). Low O_2 tension inhibits degradation (half-time several hours), thereby allowing intracellular accumulation of HIF-1α. There are at least four mechanisms responsible for the post-translational stabilization of HIF-1α during hypoxia (Chandel and Schumacker, 2000):

- Allosteric shifts in a heme-protein (Goldberg et al., 1988).
- Ion currents modulation and electrophysiological alterations by O_2-sensing ion channels, as in neurons, where ATP-sensitive K^+ channels are activated by hypoxia to increase survival (Yun et al., 1997) and prevent Ca^{++} overloads (Bickler and Buck, 1998).
- Oxido-reductive changes affecting NADPH oxidase, a universal O_2 sensor (Jones et al., 2000), as in pulmonary vasoconstriction (Voelkel and Tuder, 2000).
- Incomplete reduction of O_2 to water in mitochondria with release of minute amounts of H_2O_2, well below those that exerts oxidative stress. Strong experimental evidence supports the view that low H_2O_2 production during hypoxia (Kietzmann et al., 2000) influences EPO production in hepatoma cells

(Fandrey et al., 1997). Thus, H_2O_2 is a candidate intracellular messenger between the O_2 sensor and the transcriptional machinery controlling the EPO gene (Chandel et al., 2000).

The modulation of HIF-1α does not influence only EPO, but also has important downstream effects on a number of other genes (Table 1).

HYPOXIA AND O_2-DEPENDENT GENE EXPRESSION: A LINK BETWEEN HIGH ALTITUDE, TUMOR GROWTH, ISCHEMIA AND BEYOND?

Clarification of the basic mechanisms underlying O_2-dependent gene expression has triggered research into the pathological effects of hypoxia, with the result that biomedical fields that until recently had very little to do with high-altitude studies may now exchange important lessons to and from mountain medicine.

Solid tumors contain hypoxic environments (Helmlinger et al., 1997). It is therefore tempting to speculate that the mechanisms underlying hypoxia adaptation are also involved in tumor growth. A key finding in support to this hypothesis came from the observation that tissue hypoxia increased the expression of several growth factors, including the vascular endothelial growth factor (VEGF) (Forsythe et al., 1996). The implication of VEGF in tumors is now well recognized (Neufeld et al., 1999). VEGF, which is also a cause of HACE (Sever-

TABLE 1. A FEW DOWNSTREAM CONSEQUENCES OF THE ACTIVATION OF HYPOXIA-INDUCIBLE FACTOR 1α

Reference	*Target*	*Notes*
(Forsythe et al., 1996)	Vascular endothelium growth factor (VEGF)	The VEGF enhancer contains a consensus sequence that closely resembles the HIF-1α binding sequence in the EPO enhancer (Liu et al., 1995).
(Li et al., 1996; Semenza et al., 1996a; Firth et al., 1995)	Glycolytic enzymes	The aerobic-to-anaerobic shift is favored.
(Hellkamp et al., 1991)	Gluconeogenesis	Gluconeogenesis is inhibited.
(Ebert et al., 1995)	Glucose transporters (GLUT)	Elevated glucose preference in heart is a true metabolic adaptation in humans adapted over generations to chronic hypoxia (Holden et al., 1995).
(Motterlini et al., 2000; Bergeron et al., 1997)	Heme oxygenase, or HSP32	Bilirubin, a product of the reaction, confers protection.
(Czyzyk-Krzeska et al., 1992)	Tyrosine hydroxylase	The synthesis of DOPA is increased.
(Melillo et al., 1995)	Nitric oxide synthase	Inducible NOS increases, but endothelial NOS decreases
(Michiels et al., 2000)	Inflammation and cytokine release	HIF-1α activates endothelial cells to release inflammatory mediators and growth factors (promoting neutrophils adherence to tissue), and increase expression of genes encoding cytokines, platelet- and vascular-derived growth factors.
(Kacimi et al., 1995)	G protein	Desensitization to catecholamines.

inghaus, 1995), mediates vascularization and survival in the growing tumor (Gassmann and Wenger, 1997). Actually, HIF-1α was activated in solid tumors, thereby controlling tumor vascularization and growth (Carmeliet et al., 1998). In addition, tumors lacking HIF-1α or HIF-1β failed to develop vascularization (Carmeliet et al., 1998). Of interest, the mechanisms that originate VEGF share multiple common features with those that originate EPO. In a specific case, the von Hippel-Lindau tumor, a direct link was identified between the responsible protein, a tumor gene suppressor, and the cell levels of HIF-1α (Kamura et al., 2000).

The link between hypoxia and ischemia originates from the observation that ischemic tissue is also hypoxic. Although the two stresses have long been considered distinct, in a perspective series dedicated to the tissue responses to ischemia, Semenza proposed that HIF-1α makes part of the adaptive responses to survive ischemia (Semenza, 2000). Indeed, heat-shock proteins, some of which are inducible by hypoxia, as HSP70 and heme-oxygenase 1 (Kacimi et al., 2000), might induce a transient protection against the damage caused by ischemia-reperfusion. The 72–kD stress protein also afforded anti-ischemic protection (Marber et al., 1994). As a consequence, HIF-1α and the hypoxia response element on the DNA strand are now being tested for angiogenic therapy of tissue ischemia (Carmeliet, 2000).

Being extremely hypoxic, Barcroft defined fetal life as "Mt. Everest in utero" (Barcroft et al., 1923). Mammal placenta, which develops in a relatively hypoxic environment, with placental PO_2 increasing around 10–12 weeks of gestation in humans, must exchange proper signals in order to induce trophoblast differentiation at the right time. HIF-1α was expressed at high levels before then, and began to deactivate with raising PO_2, thereby inducing trophoblast differentiation through inhibition of TGFβ3 expression (Caniggia et al., 2000). In pregnancies complicated by early-onset preeclampsia, TGFβ3 remained elevated, as a part of failure to respond to hypoxia-induced apoptosis in trophoblasts (Levy et al., 2000). EPO is also an important factor for estrogen-dependent cyclical angiogenesis in the uterus (Yasuda et al., 1998).

CONCLUSION

It is striking to note how the study on EPO has evolved during the last century. EPO started as a mysterious erythropoietic hormone, difficult to assay, but found only in the mountains. Then, it evolved as a drug to treat anemia and boost physical performance. Finally, it turned to be a workbench to understand O_2-dependent gene expression, with important reflects on oncology, embryology, and cardiac-pulmonary medicine.

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Page 158 (HYPOXIA-INDUCIBLE FACTOR-1□), first para

The structure of HIF-1□ is now well characterized (Semenza, 1998) and the localization of its gene on human chromosome 14 is established (Semenza et al., 1996b). Technically, HIF-1□ is a member of the helix-loop-helix PAS family, which belongs to the factors that regulate DNA transcription into mRNA by binding to DNA in the correspondence of specific sequences, or motifs, that ~~are situated upstream~~ **affect the expression** of many hypoxia-induced genes, **which may be situated upstream or downstream** (Wang et al., 1995). When **the complex HIF-1□, HIF-1□, and other proteins** ~~HIF-1□~~ recognizes one of these sequences on the DNA strand, it binds to DNA, thereby ~~inhibiting further~~ **inducing** transcription of that DNA section. When HIF-1□ is destabilized, this function is blunted and DNA transcription ~~may proceed~~ **is no more induced**. Structurally, HIF-1 is a dimer composed of a □ subunit, which is relatively stable and acts as a constitutive factor (Wood et al., 1996), and the □ subunit, which is degraded rapidly and is therefore the limiting factor in the reaction chain (Huang et al., 1998). During hypoxia, the □ subunit becomes stabilized, leading to stabilization of the dimer, its binding to DNA, and ~~inhibition~~ **induction** of DNA translation into mRNA.