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TOPICAL REVIEW

Regulation of erythropoietin production

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The hormone erythropoietin (Epo) maintains red blood cell mass by promoting the survival, proliferation and differentiation of erythrocytic progenitors. Circulating Epo originates mainly from fibroblasts in the renal cortex. Epo production is controlled at the transcriptional level. Hypoxia attenuates the inhibition of the *Epo* promoter by GATA-2. More importantly, hypoxia promotes the availability of heterodimeric (α/β) hypoxia-inducible transcription factors (predominantly HIF-2) which stimulate the *Epo* enhancer. The HIFs are inactivated in normoxia by enzymatic hydroxylation of their α-subunits. Three HIF-α prolyl hydroxylases (PHD-1, -2 and -3) initiate proteasomal degradation of HIF-α, while an asparaginyl hydroxylase ('factor inhibiting HIF-1', FIH-1) inhibits the transactivation potential. The HIF-α hydroxylases contain Fe^{2+} and require 2-oxoglutarate as co-factor. The *in vivo* response is dynamic, i.e. the concentration of circulating Epo increases initially greatly following an anaemic or hypoxaemic stimulus and then declines despite continued hypoxia. Epo and angiotensin II collaborate in the maintenance of the blood volume. Whether extra-renal sites (brain, skin) modulate renal Epo production is a matter of debate. Epo overproduction results in erythrocytosis. Epo deficiency is the primary cause of the anaemia in chronic kidney disease and a contributing factor in the anaemias of chronic inflammation and cancer. Here, recombinant analogues can substitute for the hormone.

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Abbreviations Ang, angiotensin; CBP, CREB-binding protein; CFU-E, colony-forming unit-erythroid; CKD, chronic kidney disease; CREB, cAMP response element-binding protein; Epo, erythropoietin; Epo-R, Epo receptor; Hb, haemoglobin; Hct, haematocrit; HIF, hypoxia-inducible factor; HNF, hepatocyte nuclear factor; HRE, hypoxia-response element; PHD, prolyl hydroxylase; RBC, red blood cell; rhEpo, recombinant human Epo; VHL, von Hippel-Lindau.

Introduction

The hormone erythropoietin (Epo) is essential for red blood cell (RBC) production. The relationship between the O_2 content of the blood and erythropoiesis was first described by the French anatomist Francois-Gilbert Viault in 1890 (Viault, 1890), who observed a rise in RBC numbers on a journey to the highlands of Peru (Morococha, about 4500 m). Indeed, the specific stimulus for *Epo* expression is a fall in tissue O_2 pressure (P_{O_2}). Epo production increases under hypoxic conditions in the kidneys and, in minor amounts, in distinct other organs such as the liver and the brain. This article summarises the present understanding of the control of Epo production.

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Physiology of Epo

Epo is primarily expressed by hepatocytes during the fetal state. After birth, peritubular fibroblasts in the renal

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cortex become the main production site. Epo synthesis is regulated at the transcriptional level. Epo mRNA is also detectable in brain, liver, spleen, lung and testis, but these organs are not able to substitute for renal Epo in chronic kidney disease (CKD). Brain-derived Epo acts locally as a neuroprotective factor (see Noguchi *et al.* 2007). Epo is an acidic glycoprotein of about 30 kDa and comprises 165 amino acids and four glycans. Circulating Epo exhibits several glycosylation isoforms that differ in electrical charge and biological activity. Epo amounts are usually expressed in international units (IU), with one IU exerting the same erythropoiesis stimulating activity in rodents as 5 μmol cobaltous chloride (see Jelkmann, 2007).

Systemic Epo is an anti-apoptotic agent for erythrocytic progenitors, predominantly the colony-forming units-erythroid (CFU-Es). In response to Epo the CFU-Es proliferate and differentiate to generate cohorts of proerythroblasts and normoblasts (Fig. 1). The human haematopoietic Epo receptor (Epo-R) is a 484 amino acid glycoprotein of about 60 kDa, which belongs to the cytokine class I receptor family and forms homodimers. On the binding of Epo to the Epo-R dimer, cytoplasmic Janus kinases 2 (JAK2) catalyse the phosphorylation of tyrosine residues of the Epo-R and of various intracellular proteins (enzymes and transcription factors). Erythropoiesis is a slow-acting process. Following a rise in plasma Epo it takes 3–4 days before reticulocytosis becomes apparent.

Epo is essential for erythropoiesis. However, the action of Epo is augmented by several other hormones, namely testosterone, somatotropin and insulin-like growth factor 1. The higher RBC counts and haemoglobin concentrations [Hb] in men compared to women result from the stimulation of erythropoiesis by androgens and its inhibition by oestrogens.

A role has been proposed for Epo as a cytoprotective agent for several non-haematopoietic tissues, including the brain, the heart, blood vessels and the kidneys (Brines & Cerami, 2006). Some investigators believe that the effects of Epo on non-erythrocytic cells are mediated

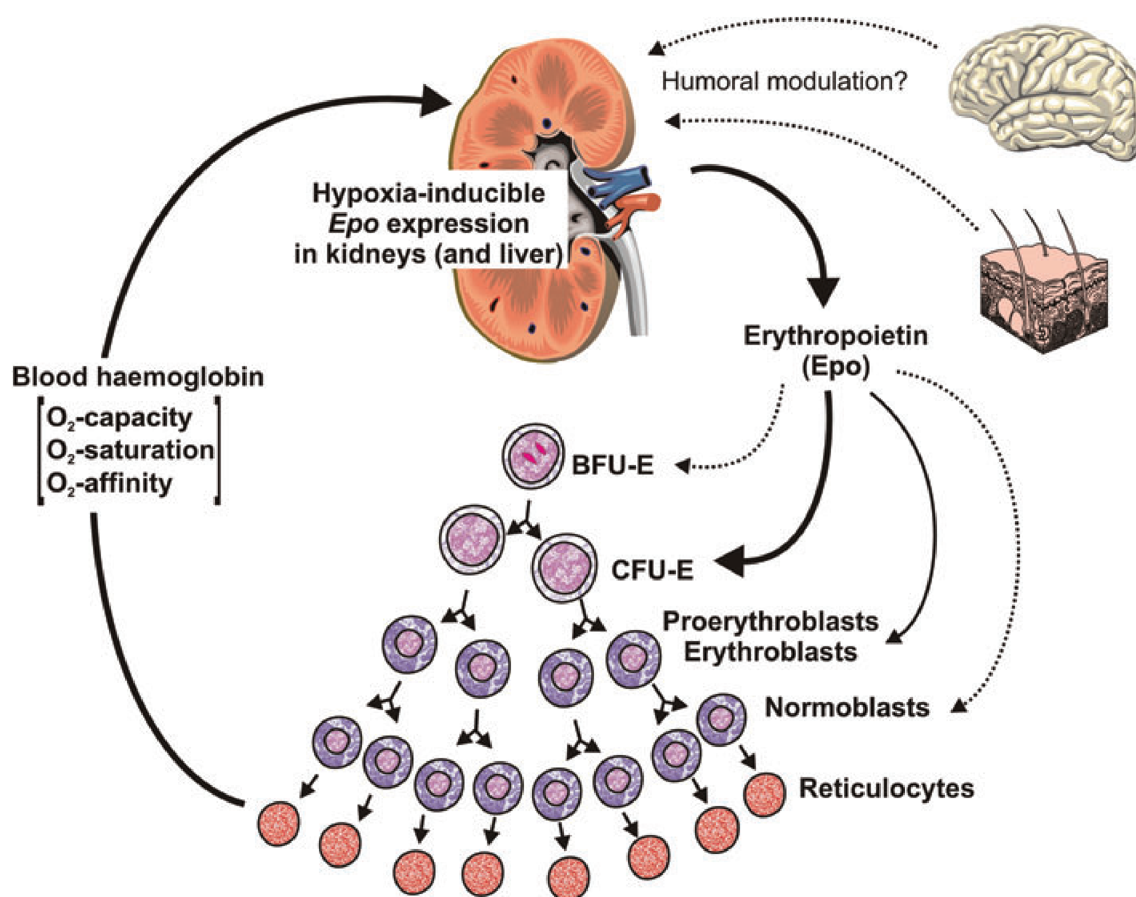

Figure 1. Diagram of the feedback regulation of erythropoiesis
Lack of O_2 (hypoxia) is a stimulus for the synthesis of erythropoietin (Epo), primarily in the kidneys. Epo is a survival, proliferation and differention factor for the erythrocytic progenitors, particularly the colony-forming units-erythroid (CFU-Es). The O_2 capacity of the blood increases with the enhanced release of reticulocytes. The role of extra-renal sites (brain, skin) in the control of the renal Epo synthesis is still incompletely understood. BFU-E, burst-forming unit-erythroid.

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by a heterotrimeric receptor consisting of one Epo-R molecule in complex with a dimer of the common cytokine β receptor (βcR). The Epo-R/βc-R concept is mainly based on the observation that erythropoietically inactive Epo derivatives and analogues provide tissue protection in animal models (Leist *et al.* 2004). However, the physiological function of Epo outside the bone marrow of healthy creatures has been questioned based on molecular biology studies. First, transgene-rescued *Epo-R*-null mutant mice expressing *Epo-R* exclusively in the haematopoietic lineage develop normally and are fertile (Suzuki *et al.* 2002). Second, Epo-R protein is not generally detectable in cells of non-haematopoietic origin, if appropriate anti-Epo-R antibody is used for study (Sinclair *et al.* 2010).

Molecular mechanism of the hypoxia-induced *Epo* expression

Most of the present knowledge of the O_2-sensing mechanism in control of Epo production has been based on *in vitro* studies utilising human hepatoma cells (lines Hep3B and HepG2). Noteworthily, the mechanisms of the renal and the hepatic *Epo* expression differ. (i) Renal cells respond in an all-or-nothing fashion to hypoxia (Koury *et al.* 1989), whereas hepatoma cells respond in a graded way. (ii) The hypoxia-response elements (HREs) in control of the *Epo* gene are located upstream in the kidney (between 9.5 and 14 kb 5′ to *Epo*) but downstream in the liver (within 0.7 kb 3′ to *Epo*) according to studies in transgenic mice (Kochling *et al.* 1998). In both tissues *Epo* expression is under the control of distinct transcription factors. The *Epo* promoter is suppressed by GATA-2 in normoxia (Tsuchiya *et al.* 1997). GATA-2 levels decrease in hypoxia (Imagawa *et al.* 2003). More importantly, the *Epo* enhancer is activated by hypoxia-inducible transcription factors (HIFs). These are composed of an O_2-labile α-subunit (120 kDa; isoforms 1α, 2α or 3α) and a constitutive β-subunit (90–95 kDa). Although the prototype HIF-1 was discovered in studies of *Epo* (Wang & Semenza, 1995), later investigations have identified HIF-2 (also called EPAS1 for endothelial PAS domain protein 1) as the primary transcription factor inducing *Epo* expression (Warnecke *et al.* 2004; see Haase, 2010). HIF-2α is activated by the stress-responsive deacetylase Sirtuin 1 (Dioum *et al.* 2009). On using Cre-loxP recombination to ablate renal HIF-2α, Kapitsinou *et al.* (2010) have shown that hepatic HIF-2 takes over as the main regulator of the serum Epo level. Interestingly, hepatocyte-derived HIF-2 is also involved in the regulation of iron metabolism genes, supporting a role for HIF-2 in the coordination of Epo synthesis with iron homeostasis (Kapitsinou *et al.* 2010).

The HIFs cooperate with hepatocyte nuclear factor 4 (HNF-4) and the transcriptional co-activators p300 and cAMP response element-binding protein (CREB)-binding protein (CBP) (Bunn *et al.* 1998). Using short interfering RNAs (siRNAs) and chromatin immunoprecipitation (ChIP) analysis, Wang *et al.* (2010) studied in more detail the roles of p300, CBP and p160 steroid receptor coactivator (SRC) in *Epo* expression in hypoxic Hep3B cells. Knocking down p300 resulted in a great reduction of *Epo* expression, negated recruitment of RNA polymerase II to the gene's promoter, and eliminated hypoxia-stimulated acetylation at the promoter and recruitments of SRC-1 and SRC-3 to the enhancer. Knocking down CBP only slightly decreased the hypoxia-induced *Epo* transcription (Wang *et al.* 2010). The directly repeated (DR2) DNA element neighbouring the HIF-binding site (Blanchard *et al.* 1992) is probably responsible for the effects of thyroid hormone (Fandrey *et al.* 1994) and retinoic acid (Kambe *et al.* 2000), which stimulate *Epo* expression in a tissue-specific but O_2-independent manner.

The C-terminus of the HIF-α subunits comprises O_2-dependent degradation domains (O-DDD) that are prolyl hydroxylated (at Pro^{402} and Pro^{564} in HIF-1α, and Pro^{405} and Pro^{531} in HIF-2α) in the presence of O_2 (Epstein *et al.* 2001; Bruick & McKnight, 2001; Jaakkola *et al.* 2001; Ivan *et al.* 2001; Yu *et al.* 2001). The reaction is catalysed by specific Fe^{2+}-containing prolyl-4-hydroxylases (PHD-1, -2 and -3), which transfer one O-atom to the proline and the other to 2-oxoglutarate yielding CO_2 and succinate (see Bruegge *et al.* 2007). The prolyl hydroxylated HIF-α combines with von Hippel-Lindau tumour suppressor protein (VHL)/E3 ligase und promptly undergoes proteasomal degradation (Pugh *et al.* 1997; Huang *et al.* 1998; Maxwell *et al.* 1999). As *PHD-2* and *PHD-3* are themselves HIF-target genes, their expression increases and HIF-α levels decline during long-term hypoxic periods (del Peso *et al.* 2003; Marxsen *et al.* 2004; Aprelikova *et al.* 2004; Stiehl *et al.* 2006). This feedback regulation may explain the declining Epo production during chronic anaemia or prolonged stay at high altitude (see below). Furthermore, the transcriptional activity of the HIFs is suppressed by HIF-α asparaginyl hydroxylation (at Asn^{803} in HIF-1α and Asn^{847} in HIF-2α), which prevents the binding of the transcriptional co-activator CBP/p300. This reaction is catalysed by the 'factor inhibiting HIF-1', FIH-1 (Mahon *et al.* 2001; McNeill *et al.* 2002), another Fe^{2+}-containing and 2-oxoglutarate-requiring dioxygenase. According to *in vitro* assays, the K_m values of the three PHDs for O_2 are above the arterial P_{O_2} (∼170 mmHg), whereas FIH-1 operates at a lower P_{O_2} (∼60 mmHg) (Koivunen *et al.* 2004). Thus, it is likely that the HIF-α PHDs are the primary O_2 sensors in control of Epo production.

The discovery that Fe^{2+} is required for HIF-α degradation may provide an explanation for the increased plasma Epo level in patients treated with the iron chelator

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deferoxamine (Kling *et al.* 1996). Furthermore, cobalt is thought to enhance *Epo* expression by replacing the essential Fe^{2+} in the HIF-α dioxygenases, which results in HIF-α stabilisation. 2-Oxoglutarate competitors (clinical jargon: 'HIF stabilisers') likewise inhibit the hydroxylation of HIF-α. HIF stabilisers have been shown to stimulate Epo production *in vitro* and in mice (Hsieh *et al.* 2007). Chronic oral dosing of compound FG-2216 in rhesus macaques proved to increase erythropoiesis and to prevent anaemia induced by weekly phlebotomy (Hsieh *et al.* 2007). Epo mRNA and HIF-2α have been co-localised in renal fibroblasts of rats treated with the 2-oxoglutarate competitor FG-4497 (Paliege *et al.* 2010).

Systemic Epo response

The primary functions of Epo are (i) to keep RBC mass and [Hb] constant day by day, and (ii) to hasten RBC recovery after haemorrhage. Little Epo is needed to maintain the steady state in healthy persons. The basal plasma concentration of Epo ranges from 6 to 32 IU l^{-1} (about 10^{-11} mol l^{-1}). The levels vary greatly between individuals, with the result that significant sex- or age-specific differences cannot be detected. Of note is the diurnal fluctuation, with a nadir in the morning. An acute loss of about 0.5 l blood does not induce a major rise in circulating Epo in men (Miller *et al.* 1982), yet the plasma Epo concentration increases exponentially when [Hb] falls below ~125 g l^{-1} in humans not suffering from renal disease or inflammation. The response is dynamic, with initially very high Epo values that drop towards the normal ones before [Hb] normalises. The mechanism of the rapid decrease is not fully understood, but it may in part be caused by lowered HIF-α levels during long-term hypoxia (Stiehl *et al.* 2006). Furthermore, one has to remember that the plasma Epo level is not only dependent on the rate of Epo production, but also on its removal. *In vitro* studies have demonstrated that Epo is internalised and degraded by its target cells (Gross & Lodish, 2006). Accordingly, anaemic patients with bone marrow hypoplasia exibit extremely high plasma Epo levels (10,000 IU l^{-1} or more) compared with subjects suffering from haemolytic anaemia. Independently of changes in tissue oxygenation, the level of circulating Epo increases when the erythrocytic progenitors are arrested by the administration of chemotherapeutics (Birgegard *et al.* 1989).

Because Epo production depends on the tissue P_{O_2}, *Epo* expression is also activated when the arterial P_{O_2} declines or when the O_2 affinity of the blood increases. On ascent to altitude, Epo levels reach peak values after 1–2 days and then fall to a new plateau at about twice that present at sea-level (Abbrecht & Littell, 1972). As noted above, HIF-α levels decline during long-term hypoxic periods. In addition, the decrease in Epo production at continued hypoxia may be associated with the decrease in O_2-affinity of the blood resulting from an increase in the intra-erythrocytic concentration of 2,3-bisphosphoglycerate (Klausen, 1998). Nutritional factors, such as low protein intake, were excluded as a reason for the rapid fall in circulating Epo in a controlled high-altitude study in the Chilean Andes, which was performed on shift workers and Caucasian lowlanders (Gunga *et al.* 1996). Since *Epo* expression studies cannot be performed in humans and there is incomplete knowledge of the mechanisms in control of the degradation of the hormone, the question remains as to how much the increased number of Epo-consuming erythrocytic progenitors contribute to the decline of circulating Epo.

The location of the *Epo*-expressing cells in the kidney cortex is well suited for the regulated production of the hormone: there is a constant ratio of blood flow rate and O_2 consumption, and the O_2 extraction is small. The renal cortical P_{O_2} is barely affected by changes in cardiac output and blood flow because the renal O_2 consumption decreases with the glomerular filtration rate. A renal flow reduction by 50% of normal was necessary to elicit at least some Epo formation in rats (Pagel *et al.* 1989). By comparison, the production of Epo increases much more during systemic hypoxia due to anaemia or hypoxaemia.

Role of angiotensin II

The signal to produce more erythrocytes following haemorrhage is apparently linked to the signals to retain salt and water by means of the renin–angiotensin system (Dunn *et al.* 2007). Angiotensin II (Ang II) is thought to stimulate erythropoiesis by two means. First, Ang II increases Epo production. Second, Ang II is a growth factor for the myeloid erythrocytic progenitors (Dunn *et al.* 2007; Vlahakos *et al.* 2010). The infusion of Ang II has been shown to raise the plasma Epo level to 17 IU l^{-1} (*vs.* 11 IU l^{-1} in controls) in healthy men (Gossmann *et al.* 2001). Furthermore, Ang II treatment at blood pressure-increasing doses (1.3 μg min^{-1} for 6 h) was found to raise the Epo concentration in men subjected to a haemorrhage of 750 ml as a basic physiological stimulus (Freudenthaler *et al.* 1999). The effect of Ang II on Epo production is prevented by Ang II type 1 receptor blockers (Freudenthaler *et al.* 1999; Gossmann *et al.* 2001). Transgenic mice harbouring the human renin and angiotensinogen genes exhibit erythrocytosis as well as hypertension (Kato *et al.* 2005). Bone marrow transplantation experiments have shown that Ang II-1a receptors on bone marrow-derived cells are dispensable for the Ang II-dependent erythrocytosis. Plasma Epo levels and renal Epo mRNA expression in the double transgenic mice were increased compared with those of

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the wild-type control, while the elevated plasma Epo levels were attenuated in the compound mice. Thus, the renin–Ang II system enhances erythropoiesis mainly through the Ang II-1a receptor of the Epo-producing renal cells. The question still needs to be answered whether Ang II stimulates Epo synthesis directly or indirectly by decreasing the renal O_2 supply/O_2 demand ratio (Dunn *et al*. 2007).

A feedback regulation of red cell mass and blood volume by means of Epo and Ang II seems to exist. Treatment of healthy men with recombinant human Epo (rhEpo) produces an increase in red cell mass. However, the increase in haematocrit (Hct) is accompanied by a decrease in plasma volume, which is probably due to a down-regulation of the renin–angiotensin–aldosterone system and results in a constancy of the blood volume. Thus, Epo treatment in healthy humans raises [Hb] by two mechanisms: (i) an increase in red cell mass, and (ii) a decrease in plasma volume (Lundby *et al*. 2007).

Extra-renal sites affecting renal Epo production

The question arises as to how far renal Epo synthesis is influenced by extra-renal sites under hypoxic conditions (Fig. 1). One hypothesis suggests that the brain modulates O_2-dependent *Epo* expression in the kidney. Local hypoxia of the brain stem was associated with an increase in renal Epo production in experimental animals (von Wussow *et al.* 2005). Both astrocytes and neurons express *Epo*. Moreover, evidence suggests that glial cells contribute to circulating Epo following the induction of hypoxia (Weidemann *et al.* 2009). Renal (but not hepatic) Epo mRNA levels are suppressed in transgenic mice lacking *VHL* or both *VHL* and *HIF-1α* in astrocytes (Weidemann *et al.* 2009). The mechanism by which astrocytes influence renal *Epo* expression still needs to be explored. In addition, the O_2 supply to the skin has been implicated in the control of renal *Epo* expression (Boutin *et al.* 2008). According to this concept, an increased blood flow to the skin causes a reduction in the renal O_2 supply. Mice with an epidermal deletion of *VHL* have increased Epo synthesis and develop erythrocytosis (Boutin *et al.* 2008). However, the concept of the dermal control of renal Epo production is not generally accepted (Paus *et al.* 2009), amongst other things because blood flow is not a major parameter in renal Epo synthesis (Pagel *et al.* 1989). Also, dermal blood flow depends on body heat, which has not been shown to affect Epo production. Note, here, that renal nerve inputs appear to be less relevant for O_2-dependent *Epo* expression in the kidney (Eckardt *et al.* 1992). Frankly speaking, the evidence assigning extra-renal sites a major role in the control of renal Epo production is far from convincing.

Information on the various other humoral factors that have been implicated in the control of renal Epo production, such as adenosine and prostanoids, has been published elsewhere (Fisher, 2003).

Pathophysiology

Normochromic normocytic anaemia due to insufficient Epo synthesis develops in patients with CKD (‘renal anaemia’), systemic inflammations or malignancies. The relative lack of Epo in patients with anaemia associated with chronic disease has been related to the negative effects of the cytokines interleukin-1 (Il-1) and tumour necrosis factor-α on *Epo* expression (Jelkmann, 1998). *In vitro*, Il-1 inhibits HNF-4α mRNA formation and causes proteasome-dependent degradation of HNF-4α protein, thereby suppressing the hypoxic inducibility of the *Epo* enhancer (Krajewski *et al.* 2007). The anaemias of patients with CKD or cancer in combination with chemotherapy can be corrected by replacement therapy with rhEpo or its analogues (see Macdougall & Ashenden, 2009).

Erythrocytosis is due to persistent over-stimulation of erythropoiesis (see Hodges *et al.* 2007). Hct, RBC counts and [Hb] are abnormally high. Primary erythrocytosis is generally a myeloproliferative disorder. Secondary erythrocytosis is due to Epo over-production, most commonly caused by hypoxaemia. Theoretically, an increase in [Hb] – and thus in the O_2 capacity of the blood – should be beneficial for tissue oxygenation (Brauner & Wang, 1997). However, blood viscosity increases with Hct, which raises the cardiac after-load and impedes the flow in microvessels. Hence, the erythrocytosis in high-altitude residents can be considered a maladaptive reaction, bearing risks for thromboembolic events and mortality. There have been distinct genetic adaptations in the evolution of tolerance of humans to high altitude (Hochachka *et al.* 1998). Tibetans living at about 4000 m altitude have relatively low [Hb] (Winslow *et al.* 1989), while South-American high-altitude natives often suffer from erythrocytosis and chronic mountain sickness (Leon-Velarde *et al.* 1991). Cobalt exposure may contribute to the excessive erythrocytosis in high-altitude miners (Jefferson *et al.* 2002). Beall *et al.* (2010) have recently identified 31 *EPAS1* (encoding HIF-2α) single-nucleotide polymorphisms that correlate with low [Hb] in Tibetans residing at high altitude. This pioneering work may be taken as a good example of how information from molecular research can improve understanding at the systemic physiological level.

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