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Perspective

Role of the hypoxia inducible factors in iron metabolism

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Iron is an essential element in all living organisms and is required as a cofactor for oxygen-binding proteins. Iron metabolism, oxygen homeostasis and erythropoiesis are consequently strongly interconnected. Iron needs to be tightly regulated, as iron insufficiency induces a hypoferric anemia in mammals, coupled to hypoxia in tissues, whereas excess iron is toxic, and causes generation of free radicals. Given the links between oxygen transport and iron metabolism, associations between the physiology of hypoxic response and the control of iron availability are important. Numerous lines of investigation have proven that the HIF transcription factors function as central mediators of cellular adaptation to critically low oxygen levels in both normal and compromised tissues. Several of these target genes are involved in iron homeostasis, reflecting the molecular links between oxygen homeostasis and iron metabolism.

Hypoxia-Inducible Factors

The hypoxia-inducible factor HIF-1 was initially identified as a regulator of erythropoietin (EPO) production,[1] underlying a physiological control system in which the levels of oxygen sensed by the kidney modulate oxygen delivery by red blood cells. A number of laboratories have demonstrated that the HIF is implicated in most aspects of hypoxia-induced gene expression, and operates not only in kidneys but in a wide range of cell types. HIF is a heterodimer whose expression is regulated by oxygen. The HIF heterodimer consists of two helix-loop-helix proteins: a regulatory subunit, HIF-1α, which is the oxygen-responsive component, and HIF-1β, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT), which is constitutively expressed. Three regulatory HIF subunits have been cloned and named HIF-1α, HIF-2α[2-4] and HIF-3α.[5] However, HIF-1α has been the most extensively studied. In the presence of oxygen, the regulatory subunit is hydroxylated on two prolines by iron-dependent prolyl-hydroxylases (PHD) and is then degraded through the ubiquitin-proteasome pathway via its interaction with the von Hippel-Lindau (vHL) tumor suppressor protein. In oxygenated cells, hydroxylation of HIF-1α on an asparagine residue prevents interaction with the p300 transcriptional co-activator, providing a second mechanism by which HIF-mediated transcription is inactivated. Under hypoxia, hydroxylase activity of both types of enzyme is inhibited. The regulatory subunit then accumulates and translocates into nucleus, where it binds to ARNT/HIF-1β, which is constitutively expressed, and recruits transcriptional cofactors such as CBP and p300. The heterodimer HIF binds to the hypoxic response elements (HREs) of target gene regulatory sequences, resulting in the transcription of genes implicated in the control of metabolism and angiogenesis, as well as apoptosis and cellular stress response.[6] Genes activated by HIF can be schematically classified into three functional groups. (1) Proteins participating in erythropoiesis, thereby increasing tissue oxygen delivery, e.g., erythropoietin, transferrin, transferrin receptor, heme oxygenase-1. (2) proteins that increase local oxygen delivery to tissues, e.g., inducible nitric oxide synthesis (iNOS) and VEGF. (3) proteins required for adaptation to cellular metabolism under conditions of low oxygen: glucose transporter-1 and most glycolytic enzymes.

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HIF and Iron Sensing

Anemia is from the Greek meaning "without blood", and is a deficiency of red blood cells (RBCs) and/or hemoglobin. Anemia results in a reduced ability of blood to transfer oxygen to the tissues, causing tissue hypoxia. We hypothesized that anemia may cause vHL-mediated HIF stabilization (Fig. 1A) as (a) iron deprivation and hypoferric anemia lead to poor tissue oxygenation, and (b) HIF levels are partially regulated by the tumor suppressor protein vHL through a process that requires molecular iron. The PHDs represent an important family of enzymes that require iron as an essential cofactor. It can be predicted from the model where iron-dependant PHDs serve as "oxygen sensors" that shortage of intracellular iron will result in low PHD activity and upregulation of HIF activity. Indeed, cobaltous ions, which compete with iron for binding to PHDs, and iron chelators, which bind available iron, act as prolyl hydroxylase inhibitors and stabilize a non-hydroxylated form of the HIF-1 protein. These findings indicate that iron is a necessary co-factor in the post-translational modification of HIF.

Three major HIF prolyl-hydroxylases have been identified: PHD1, PHD2 and PHD3. The actions of the PHDs on different HIF isoforms are not equivalent, with PHD2 having relatively more influence on HIF-1 than HIF-2, and PHD3 having relatively more influence on HIF-2 than HIF-1.[7] Moreover, genetic studies to date have defined a key non-redundant role for the single isoform PHD2 in the regulation of HIF-1,[8] leaving open the question as to whether and to what extent the other isoforms contribute to control of the HIF system.

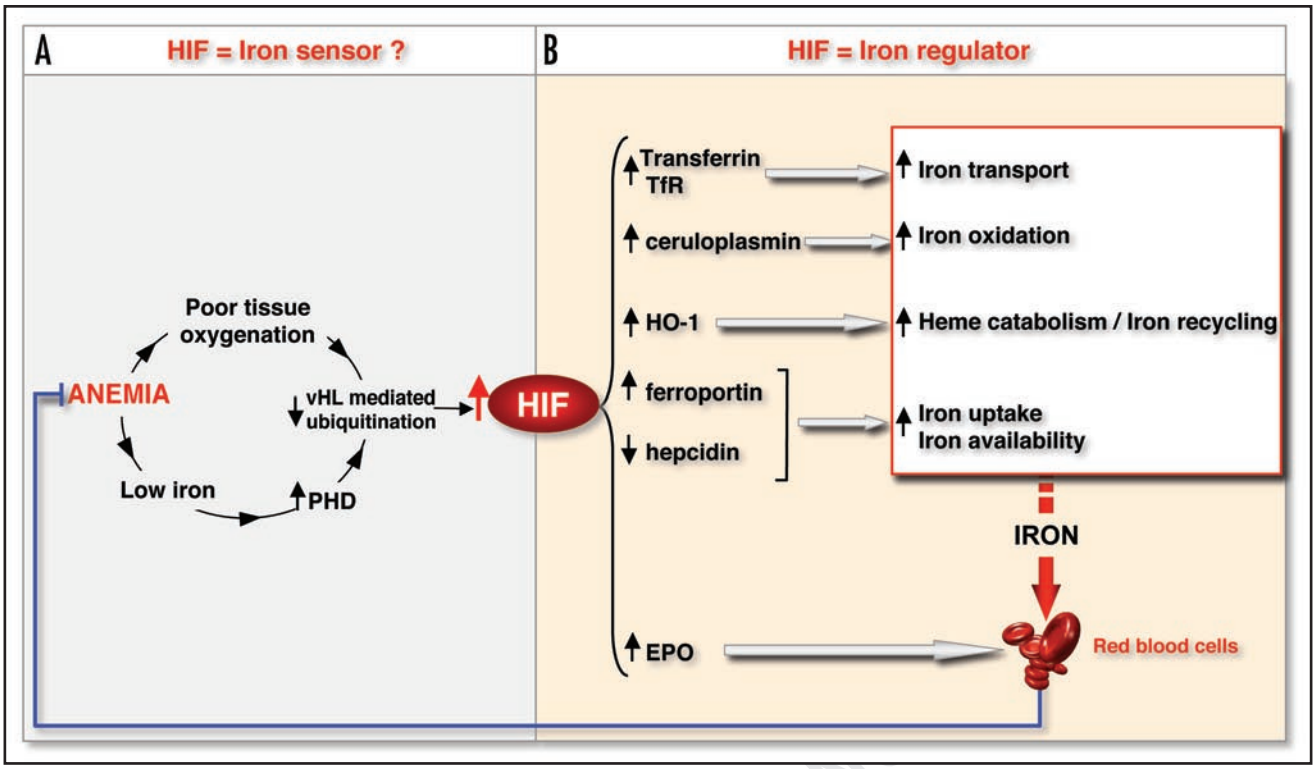

Figure 1. vHL/HIF axis serves a central role in coupling iron sensing to iron regulation. (A) Development of anemia is reflected by low iron and decreased tissue oxygenation, which would lead to decreased prolyl-hydroxylase activity and decreased vHL-mediated degradation of HIF in hepatocytes. (B) Stabilized HIF promotes decreased hepcidin and increased transferrin, transferrin repceptor (TfR), ceruloplasmin, heme-oxygenase-1 (HO-1) and ferroportin, thereby promoting respectively intestinal iron absorption, transport, oxidation, recycling and export of iron. By coordinating iron availability and erythropoiesis (by increasing EPO), vHL/HIF supports production of properly formed mature erythrocytes, correcting anemia. Normalization of iron and tissue oxygenation act to reduce hepatic HIF-1 levels as a feedback regulation on the system.

The stabilization of HIF-1 by iron chelators has been well established. We recently demonstrated that iron starvation of WT mice increases HIF-1α levels in the liver, suggesting that HIF-1α expression in the liver is influenced by dietary iron depletion.[9] The involvement of an iron-dependant decrease of PHD activity in vivo remains to be determined. However, this preliminary result indicates a potential role of HIF-1 in iron sensing in the liver. This interesting result needs to be confirmed and broadened to other cell types.

Iron Metabolism

Iron is necessary for a multitude of biological processes in the body, including catalyzing essential enzymatic reactions and the synthesis of hemoglobin. As excess iron is not excreted, it use and storage needs to be tightly regulated. Both iron excess and iron scarcity have important consequences. Excess iron accumulation is observed in hereditary hemochromatosis, the most common genetic disease in humans, and iron deficiency is one of the most frequently observed diseases in the world today, affecting two billion people. The average male adult contains approximately 4 g of iron (3 g in women), half of which is in hemoglobin and approximatively 1 g in body stores, predominantly in the liver. Iron absorption is partly suppressed when iron stores are in excess, and is increased under iron deficiency. Approximately 20 mg iron is recycled from senescent erythrocytes by macrophages every day. At the end of their 4 month lifespan, human erythrocytes are phagocytosed and digested by macrophages in the spleen and the liver. In macrophages, iron is recovered from heme by the action of heme oxygenase (predominantly the inducible heme oxygenase HO-1). Recycled and absorbed iron is transported by transferrin in blood, and most of it is destined to be utilized by erythrocyte precursors in the bone marrow.

Dietary ferric iron is reduced to ferrous iron by the apical ferric reductase dCytB, which is highly expressed on the brush border of duodenal enterocytes; iron crosses into the cytoplasm *via* the apical enterocyte transporter divalent metal transporter 1 (DMT-1). Iron may be either stored bound to ferritin, or exported out of the enterocyte into the plasma by the basal transporter ferroportin (also called iron regulated transporter 1, mental transport protein 1, or SLC40A1). Prior to being exported, it must be oxidized back to the ferric iron form by the multicopper ferroxidase hephaestin. In the plasma, iron is preferentially transported bound to the transferrin and taken up by transferrin receptors at the cell surface of a variety of cell types. When needed, iron is exported to plasma by ferroportin with the aid of the multicopper ferroxidase ceruloplasmin.

Hepcidin: key molecule in iron regulation. Iron absorption, recycling and transport is governed by three major factors (see[10] for review): Systemic iron status ("stores" regulator), iron requirement of erythropoiesis ("erythropoietic" regulator) and inflammation ("inflammatory" regulator). These three factors converge on the single iron-regulatory hormone, hepcidin, a small antimicrobial peptide expressed in the liver, which has only recently been cloned and characterized. Hepcidin is a cationic peptide produced in the liver and detectable in blood and urine. Hepcidin is processed from an propep-

tide precursor into mature forms of 20 to 25 residues.[11] Knockout mice lacking hepcidin develop iron overload in liver and pancreas, and an iron deficit in the macrophage-rich spleen.[12] Conversely, transgenic animals with constitutive hepcidin gene expression die at birth of severe iron deficiency.[13] These studies suggested that hepcidin both inhibits iron absorption in the small intestine, and the release of recycled iron from macrophages. Two isoforms of hepcidin (1 and 2) are present in the mouse, but only hepcidin-1 produces changes in iron metabolism when overexpressed.[14] The underlying mechanism has been elucidated: hepcidin binds ferroportin, the sole known iron exporter, inducing its internalization and degradation, thus trapping iron in enterocytes, hepatocytes and macrophages.[15] In humans, hereditary hemochromatosis can result from hepcidin deficiency attributed to specific mutations in at least 4 distinct genes, including the hepcidin gene *(HAMP)* itself.[16-18] Emerging data suggest that mutations in 3 genes *(HFE, TFR2* and *HJV)* lead to aberrant regulation of hepcidin expression. Mutations in *HFE*, a gene encoding a protein of the major histocompatibility complex class I, represent the most prevalent form, while rarer defects are found in *TRF2*, which mediates cellular uptake of transferrin-bound iron. Knockout mice lacking either *Hfe*[19,20] *Tfr2*[21] develop hepatic iron overload, as do human patients with mutations in the corresponding genes. Even in the face of iron overload, *Hfe* or *Tfr2*-deficient mice exhibit hepcidin levels that are lower than control mice.[22,23] A more severe disease, juvenile hemochromatosis, can result either from mutations in *HAMP* itself, or from mutations in hemojuvelin (*HJV*). Mouse models of juvenile hemochromatosis have been developed by two groups through targeted disruptions of the *Hjv* gene.[24,25] Hepcidin is strongly induced during infections and inflammation, causing intracellular iron sequestration and decreased plasma iron levels, consequently triggering the "anemia of chronic disease".[26] In both humans and mice, inflammatory stimulation of liver hepcidin synthesis is indirect and mediated by macrophage production of cytokines interleukin-6 (IL-6) and IL-1.[27,28]

IRE/IRP system. More than 15 years ago, the presence of RNA motifs called iron responsive elements (IRE) were localized in the 3' or 5' untranslated regions (UTR) of several mRNAs involved in iron metabolism and homeostasis. These motifs are bound by iron regulatory proteins 1 and 2 (IRP1 and IRP2) in a manner dependent on cellular iron levels. Whereas at low intracellular iron concentration, IRPs bind to the IRE of ferritin mRNA in its 5'-UTR, and block translation, they stabilize transferrin receptor mRNA through direct interactions with several IRE motifs in the 3'-UTR. The converse regulation of ferritin and TfR synthesis, resulting from lack of binding of IRPs to IRE, occurs in cells with high iron levels. Both IRP1 and IRP2 are also regulated by nitric oxide, but in opposite ways; IRP1 is also an oxygen sensor, binding to IREs in response to high oxygen tensions. IRP-2 seems to be the predominant regulator of iron homeostasis in mammalian cells at physiological oxygen tensions.[29] The proteosomal degradation of IRP2 requires 2-oxoglutarate-dependent oxygenases which utilize iron, oxygen and ascorbate as essential cofactors, and shows remarkable similarities with respect to the degradation of HIF-1α.[30,31] Although IRP2 activation is not dependent on HIF-1, several other parallels could be drawn between these two proteins. Even though the regulation of either protein is not entirely dependent on the other, it's possible that similar pathways underlie the mechanistic regulation of IRP2 and the HIF transcription factors.

HIF and Iron Metabolism

Given the links between oxygen transport, erythropoiesis, and iron metabolism, the physiology of hypoxic response and the control of iron availability are tightly connected. Seminal studies established an association between oxygen and iron regulation by showing that hypoxia results in higher iron absorption in mice and rats.[32,33] Hypoxia also increases the expression of erythropoietin (EPO), which is required for the formation of red blood cells.[34] Hypoxia was then found to increase the expression of transferrin (Tf), which transports Fe^{3+} into cells, probably to enhance the iron transport to erythroid tissues.[35] Therefore, the expression of genes involved in erythropoiesis and iron-metabolism are upregulated in response to hypoxia in order to increase the capacity of red blood cells to transport oxygen. Iron is required for heme formation and is the most common limiting factor in erythropoiesis.

HIF target genes involved in iron metabolism. Initially, hypoxia-mediated upregulation of TfR was thought to arise solely from enhanced IRE/IRP interaction. Two groups then showed independently that the transferrin receptor is flanked by HRE, and is a hypoxia-inducible HIF-1 target gene, enabling cellular transferrin uptake.[36,37] Accordingly, it has been shown recently that loss of the vHL protein results in a HIF-dependent activation of TfR, resulting in an increase of Tf-bound iron. Ceruloplasmin (also known as a ferroxidase), which is required to oxidize ferrous (Fe^{2+}) to ferric (Fe^{3+}) iron was also shown to be a HIF-1 target gene.[38] Because only ferric iron can be bound by transferrin, HIF-dependent induction of ceruloplasmin by iron deficiency or hypoxia is likely to support iron supply to erythroid tissues. The majority of iron for essential mammalian biological activities such as erythropoiesis is thought to be reutilized from cellular hemoproteins. Heme oxygenase 1 (HO-1) which catabolizes heme to free iron, biliverdin and carbon monoxide has an important recycling role. HO-1-deficient adult mice developed an anemia associated with abnormally low serum iron levels. HIF-1 has been reported to mediate transcriptional activation of the Heme Oxygenase-1 gene in response to hypoxia.[39]

As mentioned before, hepcidin, a small peptide synthesized in the liver, is a key regulator of iron absorption and homeostasis. Hepcidin production is decreased in response to hypoxia and in the pathophysiology of anemias from blood loss or iron deficiency.[40] To gain insight into hypoxia-dependent molecular mechanisms that might control hepcidin expression, we recently examined transcriptional control of the hypoxic response in the liver by the HIF transcription factors. We found iron deficiency-induced hepcidin repression to be associated with an induction of HIF-1 stabilization. The link between HIF and hepcidin was further evidenced by the demonstration that HIF-1 binds to the hepcidin promotor in vitro and decreases its transactivation. We next generated targeted deletions of vHL in the liver (alb-*cre*/vHL$^{flox/flox}$) to investigate the effect of the stabilization of HIF transcription factors on iron metabolism through hepcidin regulation. In alb-*cre*/vHL$^{flox/flox}$, a striking downregulation of hepcidin was observed, together with an increase of ferroportin. However, elimination of HIF-1 alone (in HIF-1 liver null mice), accounted only for a small fraction of hepcidin reduction induced by iron depletion, suggesting a major role for HIF-2 in this process. Downregulation of Hepcidin1 mRNA in alb-*cre*/vHL$^{flox/flox}$ mice was eliminated, i.e., transcript levels returned to normal values, in alb-*cre*/vHL$^{flox/flox}$/ARNT$^{flox/flox}$

double mutant (double knockout) mice. These mice have a hepatic deletion of both the vHL gene, whose absence causes increased HIF activity, and the ARNT gene, which is required for dimerization and thus activation of HIF-mediated transcription. The restoration of hepcidin to normal levels in the livers of these double mutants demonstrates that hepcidin downregulation in alb-*cre*/vHL$^{flox/flox}$ mice is specifically due to the stabilization of HIF transcription factors (i.e., HIF-1α and HIF-2α) and not to other functions of the vHL gene. A recent study showed in vitro that reactive oxygen species (ROS) repress the hepcidin gene by preventing C/EBPα and STAT-3 binding to hepcidin promoter during hypoxia, independently of HIF-1.[41] However, the paradoxical formation of ROS during hypoxia is still a subject under debate.

Our data also showed that stabilization of HIF in hepatocytes increases ferroportin expression. This study is, to our knowledge, the first report of increased ferroportin expression in hepatocytes leading to iron deficiency. The observed increase may be due in part to a HIF-dependent ferroportin mRNA upregulation or/and to a post-translational stabilization of ferroportin. Deletion of vHL, part of the E3 ligase ubiquitination complex, could prevent an ubiquitin-medited degradation of ferroportin. Further studies need to be conducted to pursue this interesting finding.

HIF-1 vs HIF2 in iron regulation. As (1) Elimination of HIF-1 alone in the liver of adult mice is not sufficient to fully compensate for the hepcidin reduction induced by iron depletion and (2) the striking downregulation of hepcidin observed in conditional knock-out of *vHL* in the liver, in which both HIF-1 and HIF-2 are stabilized, suggests a primary role for HIF-2 in this process. HIF-1 may rather play a role in embryonic, developmental hepcidin regulation; Yoon et al. demonstrated a decrease in *TfR* in *Hif-1α*$^{-/-}$ embryos contributes to defects in iron metabolism and consequently an alteration of hepcidin levels.[42]

Although HIF-1 and HIF-2 bind the same HRE sequences of target genes in vitro,[2,43] in vivo studies indicate that HIF-1 and HIF-2 modulate the transcription of an overlapping but distinct set of target genes,[44] especially in the liver. Hepatocytes are the primary source of extrarenal EPO in the adult. It has recently been shown by a direct genetic comparison of conditional knock out for HIF-1 and/or HIF-2 and/or vHL in the liver that EPO is preferentially regulated by HIF-2 in the liver at the transcriptional level. In agreement with a dominant role of HIF-2 vs HIF-1 in EPO regulation, the group of Matthias Hentze recently reported the presence of a functional iron responsive element (IRE) in the 5' untranslated region of the messenger RNA encoding HIF-2. By a regulatory mechanism similar to ferritin, translation of HIF-2 is attenuated via its 5' UTR IRE, when iron is limiting. The authors propose that iron regulation of HIF-2 translation can serve to modulate EPO levels, thereby adjusting the rate of red blood cell production to iron availability.[45]

A central role for HIF in coupling iron sensing and iron regulation. We propose that HIF acts both as an iron sensor and iron regulator (Fig. 1). In this model, anemia causes decreased tissue oxygenation, as well as decreased prolyl-hydroxylase activity, and decreased vHL-mediated degradation of HIF factors. Increased HIF activity, by simultaneously regulating hepcidin, transferrin, TfR, ceruloplasmin, HO-1, ferroportin and EPO expression, allows a very efficient orchestrated response to restore normoxic conditions. HIF activation permits the rapid mobilization of iron from macrophages and enterocytes necessary for the increased erythropoietic activity triggered by erythropoietin release. vHL/HIF supports production of properly formed mature erythrocytes, correcting anemia. As anemia is corrected, normalization of iron and tissue oxygen promotes HIF degradation acting as a checkpoint on the system. HIF-target gene expression returns to normal if iron is present. If iron is deficient, HIF remains stabilized and HIF target gene expression is maintained at a low rate, ensuring increased intestinal iron absorption until stores are repleted.

Stabilization of HIFs: A Novel Approach to Treating Anemia?

Anemia is estimated to affect 2.2 billion individuals worldwide. Anemia of chronic disease (ACD), also called "anemia of inflammation" is the most common normocytic anemia and the second most common form of anemia worldwide (after iron deficiency anemia). ACD is a common complication in millions of patients with cancer, rheumatoid arthritis, inflammatory bowel disease and other chronic diseases. A significant percentage of patients with ACD, as well as patients with chemotherapy-induced anemia, are hyporesponsive to current therapy. Several studies expanded the central role of hepcidin in the pathogenesis of such anemia:[46] Hepcidin is strongly induced during infections and inflammation, causing intracellular iron sequestration and decreased plasma iron levels, consequently triggering the anemia of chronic disease. We recently showed that stabilization of HIF effectively counteracts positive effects of inflammatory cytokines (IL-6) and IL-1 on hepcidin expression. ACD is currently treated through injection of recombinant EPO. However, iron availability for erythropoiesis is an important factor that recombinant EPO therapy alone fails to address. By coordinating EPO production, iron availability and by suppressing the negative effects of proinflammatory cytokines on red blood cell production, HIF stabilizers (by inhibition of vHL or prolyl hydroxlase activity) will represent a novel approach for treatment of anemia of chronic inflammation. A very recently published study showed that a prolyl-hydroxylase inhibitor (PHI), called FG-2216, when tested in a nonhuman primate model with or without chronic phlebotomy, induced significant and reversible Epo induction in vivo.[47] This PHI increased erythropoiesis, and prevented anemia induced by phlebotomy.[47] HIF PHIs may therefore represent a novel class of molecules with broad potential clinical application for congenital and acquired anemia.

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