Vascular Responses to Hypoxia and Ischemia

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Abstract—Blood vessels function as conduits for the delivery of O₂ and nutrients. Hypoxia-inducible factor 1 (HIF-1) mediates adaptive transcriptional responses to hypoxia/ischemia that include expression of angiogenic cytokines/growth factors by hypoxic cells and expression of cognate receptors for these ligands by vascular cells and their progenitors. Impairment of HIF-1–dependent responses to hypoxia is a major factor contributing to the impaired vascular responses to ischemia that are associated with aging and diabetes. (Arterioscler Thromb Vasc Biol. 2010;30:648-652.)

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Compare to invertebrates, vertebrate organisms have greatly increased body mass, complexity, and metabolic activity. These features were made possible by the evolution of a circulatory system in which erythrocytes capture O₂ from the environment and are pumped by the heart through blood vessels as a mechanism to distribute sufficient O₂ to each of the approximately 10¹⁴ cells (in the case of the adult human body) to maintain their viability and physiological functions. Thus, oxygen homeostasis represents an important organizing principle for understanding metazoan evolution. Similarly, during mammalian development, the heart, blood, and vessels constitute the first functioning physiological system. The circulatory system must be established at that point (day 8 in mouse development) at which O₂ can no longer be supplied to every cell in the embryo by simple diffusion; failure to do so results in embryonic lethality. Ontogeny therefore parallels phylogeny in being driven by the necessity to establish and maintain O₂ homeostasis. This brief review will focus on adaptive responses to hypoxia (reduced O₂ availability) that are mediated by the vasculature and the mechanisms by which these adaptive responses are impaired by aging and chronic disease.

Oxygen Sensing

O₂ is critical to survival because it functions as the final electron acceptor in the mitochondrial respiratory chain, which provides a highly efficient means to harvest energy captured in the chemical bonds of glucose and fatty acids. Each cell in the human body can sense the O₂ concentration and respond to hypoxia by increasing the activity of hypoxia-inducible factor 1 (HIF-1), which is a transcriptional activator that functions as a master regulator of O₂ homeostasis by controlling both O₂ delivery (reviewed here) and O₂ use (reviewed elsewhere). HIF-1 is a heterodimeric protein composed of HIF-1α and HIF-1β subunits. Whereas HIF-1β is constitutively expressed, the levels and activity of HIF-1α are tightly regulated according to the cellular O₂ concentration. Human HIF-1α is hydroxylated on proline residue 402 or 564 by enzymes that use O₂ and α-ketoglutarate as substrates. These hydroxylases insert 1 oxygen atom into a prolyl residue whereas the other oxygen atom is used to split α-ketoglutarate into succinate and CO₂. Prolyl hydroxylation of HIF-1α is required for binding of the von Hippel-Lindau protein, which recruits an E3 ubiquitin-protein ligase that targets HIF-1α for proteosomal degradation.

Under hypoxic conditions, hydroxylase activity is inhibited as a result of substrate (ie, O₂) limitation or the effect of reactive oxygen species generated by mitochondria in response to acute hypoxia, which may oxidize Fe (II) in the catalytic center of the enzyme, thereby rendering HIF-1α resistant to ubiquitination and degradation. HIF-1α is also subjected to hydroxylation on asparagine 803, which blocks its interaction with coactivator proteins. Thus, O₂-dependent hydroxylation provides a direct mechanism to transduce changes in cellular O₂ concentration to the nucleus as changes in the half-life and transactivation function of HIF-1α.

Once in the nucleus, HIF-1α dimerizes with HIF-1β and binds to target genes at hypoxia response elements (HREs), which contain the core binding site sequence 5’-(A/G)CGTG-3’ and are defined by their ability to function as cis-acting elements sufficient to mediate a transcriptional response to hypoxia that is dependent on the binding of HIF-1. Hundreds of HIF-1–regulated genes have been
identified, with more than 90 genes demonstrated to be direct HIF-1 targets through the functional demonstration of an HRE. As discussed below, many of these genes encode proteins that mediate vascular responses to hypoxia.

Database analysis for sequences with similarity to HIF-1α led to the identification of HIF-2α, which is a protein that is also subject to O2-dependent prolyl and asparaginyl hydroxylation, VHL-mediated ubiquitination, and dimerization with HIF-1β. Whereas HIF-1α is expressed in all tissues and cell types, HIF-2α expression is restricted to certain cell types, which include vascular endothelial cells. A recent analysis of human MCF-7 cells revealed hypoxia-induced binding of both HIF-1α and HIF-2α to many sites in the genome, but only HIF-1α appeared to contribute to transcriptional responses to acute hypoxia. Thus, the role of HIF-2α remains somewhat enigmatic.

**Angiogenic Responses to Hypoxia**

Tissue growth or regeneration is accompanied by angiogenesis, which is the sprouting of new capillaries from existing vessels. Increased cell number (proliferation) or mass (hyper trophy) leads to increased O2 consumption, which in the absence of changes in perfusion, results in decreased O2 availability (ie, hypoxia) which leads to decreased hydroxylase activity and increased HIF-1 transcriptional activity. Each cell responds to hypoxia by modulating the transcription of a subset of HIF-1 target genes in a cell-type-specific manner. Most cells respond to hypoxia by increasing transcription of the gene encoding vascular endothelial growth factor (VEGF) in a HIF-1-dependent manner. Other angiogenic growth factors that are regulated by HIF-1 include angiopoitin (ANGPT) 2, placental growth factor (PLGF), stem cell factor (SCF), stromal-derived factor 1 (SDF-1), and platelet-derived growth factor (PDGF) B. Of these, HIF-1 has been shown to directly bind to HREs in the promoters of the genes encoding VEGF, SDF-1, and SCF, whereas the molecular mechanisms by which HIF-1 regulates expression of PDGF-B and PLGF mRNA remain to be determined. ANGPT2 gene expression is also regulated by ETS-1, expression of which is HIF-1-regulated, indicating that ANGPT2 expression is both directly and indirectly regulated by HIF-1.

The control of multiple angiogenic factors illustrates the role of HIF-1 as a master regulator. Intraocular administration of VEGF is not sufficient to induce angiogenesis in the superficial capillary bed of the retina, whereas adenoviral expression of a constitutively-active form of HIF-1α that is resistant to O2-dependent degradation (AdCA5) induces a marked angiogenic response, which may reflect the combined expression of both VEGF and PLGF, which are required for ischemia-induced retinal vascularization. Analysis of gene-targeted mice indicates that HIF-2α also contributes to the endogenous vascular response to retinal ischemia.

Angiogenesis requires not only the production and secretion of angiogenic growth factors by hypoxic cells but also the presence of responding cells, such as vascular endothelial cells (ECs) and vascular pericytes bearing cognate receptors (Table). In addition to the non–cell-autonomous effect of angiogenic growth factor stimulation of vascular ECs, hypoxia induces EC-autonomous responses that are mediated by HIF-1. Analysis of primary arterial EC cultures identified 245 gene probes with increased expression and 325 gene probes with decreased expression, both in cells exposed to hypoxia and in cells exposed to AdCA5 under nonhypoxic conditions, suggesting that HIF-1 plays a key role in both the production of angiogenic signals and in the response to those signals by ECs and other angiogenic cells, as described below. Analysis of conditional knockout mice have confirmed that both HIF-1α and HIF-2α play important roles in vascular ECs.

**Arteriogenic Responses to Ischemia**

The overall prevalence of peripheral arterial disease (PAD) is approximately 5% to 10%, whereas 15% to 20% of individuals over 70 years of age are affected. Approximately 1% to 2% of PAD patients over the age of 50 develop critical limb ischemia, in which blood flow is insufficient to maintain tissue viability, resulting in ischemic pain at rest, ischemic ulcers, and gangrene that eventually requires limb amputation. In rabbits, intravascular occlusion of the femoral artery leads to remodeling of existing collateral blood vessels, in which the luminal diameter increases to allow increased blood flow, an effect that is significantly potentiated by injection of AdCA5 at the time of vascular occlusion.

In clinical studies, it is difficult to separate the effects of aging from other factors, such as diet and exercise, which can be better controlled in animal studies. Just as aging plays a key role in the clinical manifestations of PAD, aging is associated with a progressive impairment in recovery of limb perfusion after femoral artery ligation in mice. At each age, Hif1a mice, which are heterozygous for a null (knockout) allele at the locus encoding HIF-1α, show impaired recovery of limb perfusion relative to wild-type littermates and suffer more severe tissue damage. Aging and partial HIF-1α deficiency are each associated with impaired ischemia-induced expression of HIF-1α protein and of mRNA encoding the angiogenic factors ANGPT1, ANGPT2, PLGF, SCF, SDF1, and VEGF on day 3 after femoral artery ligation. The effect of aging on recovery of limb perfusion can be overcome by intramuscular administration of AdCA5 at the site of the excised femoral artery.

**Mobilization of Circulating Angiogenic Cells**

The angiogenic factors produced and secreted in the ischemic limb act locally to stimulate vascular ECs and pericytes/
smooth muscle cells that express cognate receptors, as described above. However, these angiogenic factors also enter the circulation and induce mobilization from bone marrow and other tissues into peripheral blood of a variety of cell types that home to the ischemic limb and promote vascular remodeling. These circulating angiogenic cells (CACs), which include mesenchymal stem cells (MSCs), hematopoietic progenitor cells, endothelial progenitor cells, and proangiogenic myeloid cells, are recruited based on their expression of receptors for the same factors that act locally to stimulate angiogenesis and arteriogenesis. For example, bone marrow–derived MSCs express VEGFR1 and undergo chemotaxis in response to gradients of PLGF and VEGF. Expression of VEGFR1 in mouse bone marrow MSCs, and their ability to undergo chemotaxis in response to PLGF or VEGF, is dependent on expression of HIF-1α. Hypoxia-induced expression of CXCR4, which mediates responses to SDF-1, is also mediated by HIF-1. In addition to expressing receptors for angiogenic cytokines, CACs often express progenitor cell marker genes such as CD34 or Sca1. The mobilization of CD34+/VEGFR2+ and Sca1+/CXCR4+ CACs from bone marrow and other tissues into peripheral blood is induced by the production of VEGF and SDF-1, respectively, in ischemic tissue. Ischemia-induced mobilization of CD34+/VEGFR2+ and Sca1+/CXCR4+ CACs is impaired in Hif1α−/− mice relative to their wild-type littermates, reflecting impaired induction of HIF-1α and the angiogenic cytokines VEGF and SDF-1. Injection of AdCA5 into nonischemic limb muscle is sufficient to mobilize CACs. Thus, both loss-of-function and gain-of-function experiments indicate that HIF-1 plays a key role in mobilization and recruitment of CACs to promote vascular remodeling in the ischemic limb. These findings again illustrate the multiple mechanisms by which HIF-1 controls vascular responses to tissue hypoxia and ischemia. Aging has also been shown to impair SDF-1 protein expression, CAC mobilization, and tissue vascularization in a mouse ischemic skin flap model. When fibroblasts from aged mice were cultured under hypoxic conditions, HIF-1α protein expression was markedly impaired compared to fibroblasts from young mice and this effect was associated with increased levels of the prolyl hydroxylases PDH1, PDH2, and PDH3. These results suggested that increased hydroxylase activity was responsible for impairing physiological responses to ischemia in aged mice. To test this hypothesis, aged mice received intraperitoneal injection of desferrioxamine, an iron chelator that induces HIF-1 activity, presumably by inhibiting prolyl and asparaginyl hydroxylases, which contain Fe (II) in their active site. In aged mice treated with desferrioxamine, HIF-1α expression was restored in the ischemic flap, ischemia-induced mobilization of CACs in peripheral blood was restored, and flap vascularization improved, leading to significantly increased flap survival that was comparable to young mice.

**HIF-1 and Coronary Artery Disease**

It is not understood why some patients with severe coronary artery disease (CAD) develop collateral vessels to increase blood flow distal to the site of stenosis, whereas others do not. Among patients with myocardial infarction (MI), those without collaterals have a greater mean infarct size and are more likely to die or develop heart failure. When peripheral blood mononuclear cells isolated from CAD patients without coronary collaterals were exposed to hypoxia, their production of VEGF was significantly less than that of cells from CAD patients with collaterals. Induction of HIF-1α mRNA and protein expression precedes VEGF expression during acute ischemia in the human heart.

Analysis of a coding sequence single nucleotide polymorphism (SNP), which changes proline to serine at codon 582 of HIF-1α (P582S allele), in 100 consecutive patients with critical stenosis of a major coronary artery demonstrated by angiography revealed that the frequency of the variant allele was 5-fold higher in the 32 patients without collaterals compared to the 68 patients with collaterals. In another clinical study, the frequency of the P582S allele (and 2 other *HIF1A* SNPs) was significantly increased in 466 patients presenting with stable angina compared to 909 patients presenting with MI as the initial manifestation of CAD. Unfortunately, angiographic data documenting the severity of CAD and the presence or absence of collaterals in the angina and MI cohorts were not reported. It is remarkable that 2 studies focused on different end points both implicate HIF-1 as playing a role in modulating the clinical manifestations of CAD. The *HIF1A* polymorphisms appear to be associated with reduced HIF-1 transcriptional activity, which may lead to early onset of symptoms that bring patients to medical attention before the development of collaterals or the occurrence of MI.

**Wound Healing, Diabetes, and Aging**

Impaired wound healing is an age-dependent manifestation of diabetes mellitus in humans and in the *Lepr*−/− mouse model of type II diabetes. Exposure of dermal fibroblasts to high glucose concentrations impairs the hypoxia-induced stabilization of HIF-1α, and reduced levels of HIF-1α protein are present in diabetic wounds as compared to chronic venous ulcers of nondiabetic individuals. Fibroblasts cultured from *Lepr*−/− mice manifest cell autonomous defects in migration, VEGF production, and responses to hypoxia. Levels of HIF-1α protein, VEGF mRNA, and VEGF protein are decreased in wounds of diabetic *Lepr*−/− mice compared to their nondiabetic *Lepr*+/+ littermates, and these deficiencies can be corrected by local wound application of CoCl₂, which induces HIF-1 activity, and Co (II) appears to inhibit prolyl and asparaginyl hydroxylases by exchanging with Fe (II) at the catalytic site. Wound healing was also improved in *Lepr*−/− mice by local application of the iron chelator desferrioxamine or dimethylglyoxal glycine, which inhibits the hydroxylases by serving as a competitive antagonist of α-ketoglutarate.

In wounds of aged *Lepr*−/− mice, expression of HIF-1α, ANGPT2, PDGF-B, PLGF, and VEGF mRNA is further impaired, resulting in an even greater impairment of wound healing. Electroproportion-assisted transduction of a plasmid vector encoding CA5 into the skin of aged *Lepr*−/− mice led to significantly increased levels of mRNA encoding HIF-1α, ANGPT2, PDGF-B, PLGF, and VEGF; significantly in-
creased CACs in peripheral blood; significantly increased wound vascularization; and a significant acceleration in wound closure. Impaired ischemia-induced HIF-1α expression was also observed in the hearts of streptozocin-treated hyperglycemic rats, indicating a general effect of diabetes on HIF-1 activation that is not limited to Lepdb/db mice or to wound healing.

Perspective

HIF-1 plays critical roles in mediating vascular responses to hypoxia and ischemia. Inhibition of HIF-1α expression and the resulting impairment of HIF-1-dependent gene transcription is a major pathogenic mechanism underlying impaired responses to ischemia associated with aging and diabetes. Antiangiogenic effects of other comorbidities, such as tobacco smoke exposure, also appear to involve inhibition of HIF-1α expression. Preclinical and clinical studies described in this review suggest that HIF-1α replacement may be of therapeutic benefit because of the many downstream targets of HIF-1 that mediate adaptive vascular responses, in contrast to the administration of any single angiogenic factor. A Phase I clinical trial in patients with critical limb ischemia has been performed involving adenoviral delivery of a fusion protein consisting of the aminoterminal half of HIF-1α fused to the herpes simplex virus VP-16 protein. However, such fusion proteins may have many undesirable properties, and no clinical studies have been reported involving pharmacological induction of endogenous HIF-1α or viral transduction of a constitutively active form of HIF-1α without foreign protein sequences. Given the increasing number of no-option patients with CAD and PAD, such studies warrant serious consideration. Experimental evidence suggests that transient local expression of a constitutively active form of HIF-1α may be sufficient to induce a vascular response in patients with critical limb ischemia or nonhealing wounds, thereby alleviating concerns of possible side effects associated with systemic HIF-1 activation.

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Disclosures

None.

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