Hypoxia-Inducible Factors and Kidney Vascular Development

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Abstract. Among the genes strongly induced by hypoxia-inducible factors (HIF) and highly expressed during kidney microvascular development is vascular endothelial growth factor, which encodes a potent endothelial mitogen and chemotactic critical for embryonic vasculogenesis and angiogenesis. In developing kidney, glomerular podocytes are particularly rich sources of vascular endothelial growth factor, which probably serves to attract endothelial precursors into vascular clefts of immature glomeruli, promote their mitosis and differentiation into glomerular endothelial cells, and assist with maintenance of their highly differentiated state through maturation. This article summarizes the structure, function, and expression of HIF and discusses HIF target genes expressed during kidney vascular development. Furthermore, it is speculated that different HIF heterodimers are stabilized in different cell populations, which may lead to cell-selective induction of HIF target genes important for renal vasculogenesis/angiogenesis.

The spatial and temporal controls for vascular development are complex and, among other mechanisms, involve cell–cell and cell–matrix interactions, as well as growth factor–receptor signaling (reviewed in 1). Perhaps the best understood growth factor ligand/receptor signaling system as it relates to the vasculature is vascular endothelial growth factor (VEGF) and its transmembrane receptor tyrosine kinases Flk1 (KDR in human) and Flt1. When VEGF or its receptors (VEGFR) are deleted by gene targeting in mice, embryonic lethality results because of fundamental cardiovascular and systemic defects, reflecting the pivotal importance of this ligand/receptor signaling system (1). Although not fully understood, the transcription of VEGF and VEGFR genes seem to be regulated by members of a growing family of hypoxia-inducible factors (HIF), which consist of heterodimers of α and β subunits stabilized and activated by hypoxia. These transactivating factors bind to hypoxia-responsive elements (HRE) located in promoter/enhancer regions of inducible genes (2), many of which include proteins expressed in response to hypoxic stress. Because many embryonic tissues develop rapidly, they often outpace their vascular supply. Consequently, cells within embryonic tissues (and those in rapidly growing tumors) may experience subnormal oxygen tensions, which can thereby increase HIF stabilization and promote more VEGF and VEGFR synthesis. Increased levels of VEGF/VEGFR signaling then stimulates mitogenesis and morphogenesis in endothelial progenitors and the creation of new vascular networks de novo (vasculogenesis) or the ingress of vascular sprouts into the source of VEGF from nearby, existing vessels (angiogenesis). VEGF has also been found to promote specific activities required for endothelial cell survival, including phosphorylation of the antiapoptotic kinases Akt/PKB (3) and focal adhesion kinase (FAK) (4), as well as upregulation of the survival factors Bcl2 and A1 (5).

In this review, we summarize evidence that HIF and the target genes that they regulate are critically important mediators for developmental vascularization processes in general and kidney organogenesis in particular. Vascular development must accompany tissue expansion, and hypoxia-responsive systems, including HIF, seem to be pivotal regulators for vasculogenesis/angiogenesis. However, nonhypoxic HIF stabilization and/or HIF target gene expression also takes place in certain cells under relatively normal oxygen pressures (6), but less is known about these mechanisms. Finally, we explore suggestions that different HIF heterodimers may be expressed by distinctly different cells (e.g., podocytes and glomerular endothelial cells) and that this differential HIF expression helps coordinate vascular assembly in the correct temporal and spatial context.

HIF

HIF are members of a growing family of transcription factors that contain the PAS dimerization domain in addition to the bHLH domain (7). The PAS domain is a region of homology identified in the first three members of the bHLH-PAS family: the Drosophila Period gene, the mammalian ARNT gene, and the Drosophila Single-minded gene. HIF-1 was first identified after observations that transcription of the erythropoietin (EPO) gene is significantly increased in hypoxic conditions (8). An HRE region from the 3'-flanking region of the human EPO gene was found to induce reporter gene transcription in hypoxia (9). This HRE was then used in DNA chromatography to purify HIF-1, which was shown to consist of two subunits, HIF-1α and HIF-1β (10). This was the first description of HIF-1α, whereas HIF-1β was found to be identical to...
the aryl hydrocarbon receptor nuclear transporter (ARNT), which is a dimerization partner for the aryl hydrocarbon receptor (11). Two other bHLH-PAS members that dimerize with HIF-1β and share high sequence homology with HIF-1α have since been identified and named HIF-2α and HIF-3α (12,13). Two transactivation domains are found in HIF-1α and -2α: the N-terminal transactivation domain (N-TAD) and the C-terminal transactivation domain (C-TAD) (14–17). HIF-3α contains only the N-TAD and in some cases represses HIF-mediated gene transcription (18). The recently identified inhibitory PAS (IPAS) protein contains only the bHLH-PAS domains and completely blocks HIF-mediated gene transcription (19). Figure 1 shows the structure of the three HIF-α subunits and IPAS and includes the bHLH, PAS, transactivation, and oxygen-dependent degradation domains.

HIF activity is regulated by stability of the α subunits (20,21). In normoxia, HIF-α subunits are degraded rapidly by 26S proteasomes, whereas in hypoxia, HIF-α is stabilized and translocates to the nucleus (22–24). There, HIF-α heterodimerizes with HIF-β subunits, which are not subjected to rapid proteasomal degradation (7,25,26), to form a functional transactivator. Initial HIF-1α studies showed that the region between amino acids 401 and 603 is required for proteasomal degradation in hypoxia been resolved by compelling data showing an interaction with von Hippel-Lindau (VHL) protein (27), which is a component of an E3 ubiquitin ligase complex that targets proteins for polyubiquitination and proteasomal destruction. Only recently has the mechanism of HIF-1α stabilization in hypoxia been resolved by compelling data showing that hydroxylation of the proline residue of the previously identified PYI motif is required for its binding to VHL (28–31). A novel HIF-1α prolyl-4-hydroxylase catalyzes the hydroxylation in the presence of O2, Fe2+, ascorbate, and 2-oxoglutarate. Thus, when intracellular O2 concentrations fall, the hydroxylase is inactive, and HIF-1α escapes the ubiquitination/proteasomal pathway (Figure 2). HIF-2α and HIF-3α contain the same PYI motif and are presumably stabilized in the same manner. Remarkably, an oxygen-dependent hydroxylation process also controls the transactivation properties of HIF-1α. In normoxia, a conserved asparagine residue within the C-TAD becomes hydroxylated, which blocks its ability to recruit the transcriptional coactivators p300/CBP. This asparagine residue escapes hydroxylation in hypoxia, and HIF-1α is thereby free to engage p300 (Figure 2).

Although hypoxia can clearly regulate both HIF heterodimerization and gene activation, hypoxia-independent stabilization of HIF also takes place, although much less is known about these mechanisms. The organomercurial mersalyl induces HIF-1α protein stabilization and HIF-1 DNA binding in HEP3B cells (32). A mitogen-activated protein kinase inhibitor, PD098059, blocks mersalyl-mediated HIF-1 induction but not induction by hypoxia (33). HIF-1α is phosphorylated by p42/p44 mitogen-activated protein kinases, which enhances HIF-1α protein stability and HIF-1 affinity for HRE (33). In addition, physiologically relevant concentrations of three insulin growth factor receptor ligands, IGF-I, IGF-II, and insulin, all have shown to increase levels of HIF-1α protein (34). These results therefore indicate that mechanisms other than prolyl-hydroxylation can stabilize HIF.

The number of genes found to contain the consensus HRE (5'-RCGTG) and be induced by HIF is growing and includes genes that alleviate hypoxic stress by initiating neovascularization (26). Some of the initial studies identifying HIF target genes were performed in HIF-1α- and HIF-1β-deficient ES cells (35). These cells lack the induction of VEGF mRNA and protein found in normal cells, and later it was confirmed that the VEGF promoter contains an HRE and binds HIF-1 (36). The VEGFR Flt1 and Flk1 are also induced by hypoxia; Flt1 has an HRE promoter element and is induced by HIF heterodimers that contain HIF-1α (37), whereas Flk1 seems to be upregulated specifically by HIF-2α (38), especially when HIF-2α heterodimers are cooperatively associated with Ets-1 (39). Moreover, a tandem HIF-2α/Ets binding site has been identified within the Flk1 promoter element (39). The orphan endothelial tyrosine kinase receptor Tie1 (40), as well as Tie2 and its ligand angiotropoietin-2 (41,42), also have demonstrated hypoxia responsiveness.

Figure 1. Molecular structures of the three hypoxia-inducible factor-α (HIF-α) subunits and inhibitory PAS.

Figure 2. Hypoxia regulates HIF-α protein stability and HIF gene target transactivation.
HIF Expression and Vascular Development

The involvement of HIF in human pathogenesis, including tumor vascularization in diseases in which VHL becomes inactivated, such as hemangioblastomas and renal cell carcinoma, has been well documented (31,43,44). The precise role for HIF during normal development and in organogenesis in particular, however, remains relatively obscure. VEGF mRNA and protein are at least in part regulated by hypoxia and HIF stability (45), and embryos have been shown to contain hypoxic tissues (46). Hypoxia-induced stabilization of HIF might therefore control embryonic vascular development. Moreover, gene deletion studies in mice have clearly demonstrated that each HIF subunit plays a unique role in development and that related α or β subunits cannot fully compensate for loss of one another. For example, HIF-1α knockout mice show broad mesenchymal cell death and die at midgestation from severe defects in cardiovascular and neural tube development. HIF-1α−/− stem cells fail to produce VEGF in response to hypoxia, and HIF-1α null embryos display prominent abnormalities in yolk sac vasculature by E9.5 (47). Although the yolk sac of mutants contains mature endothelial cells and some vessels, there is no organized vessel branching. Vascular disorganization is also prominent inside the embryo, and vessels completely fail to form in brain. Even though HIF-1α is crucial for VEGF induction in embryos, this factor is not solely responsible for VEGF activation. HIF-1α mutants do contain some apparently mature endothelial cells (47), which are cells that rely absolutely on VEGF for their induction/differentiation. VEGF expression can also be upregulated by certain growth factors and cytokines, such as endothelial growth factor, TGF-β, keratinocyte growth factor, IL-1β and -6, and insulin-like growth factor (48), but clearly hypoxia-induced regulation of VEGF is one of the most prominent mediators of embryonic vascular development.

HIF-2α gene deletion also results in an embryonic lethal mutation. However, death occurs from a variety of phenotypes dependent on genetic background and is variable even on the same background among littermates. In mice on the C57BL/6 or 129/Sv genetic background, the major knockout phenotype observed is lack of sufficient catecholamine and death between E12.5 and E16.5 from bradycardia (49). The vasculature appears grossly normal in these mice, although close examination of individual organs during development still needs to be undertaken. Administration of d-L-threo-3,4-dihydroxyphenylserine (DOPS), a precursor amino acid for epinephrine, to pregnant mothers rescues the HIF-2α phenotype to varying degrees, with some pups surviving until birth. On ICR/129 Sv outbred mice, in contrast, HIF-2α null mutants die by E13.5 with major defects in vascular development (50). Vasculogenesis seems to proceed normally, but vessels fail to interact and remodel into the full complement of mature, functional vessels (50). Some mutants treated with DOPS display no major defects and survive until shortly after birth. These observations suggest that genes other than HIF-2α, possibly HIF-1α or -3α, can partially intervene and initiate transcription of HIF-2 target genes to varying degrees. Taken together, however, the data clearly show that individual HIF-1α and -2α subunits have distinct roles in cardiovascular development that are not redundant.

How might the different HIF-α subunits exert their differential gene activation effects? Perhaps the answer to this question lies in the availability and variable affinities of different HIF-α chains for separate HIF-β subunits. HIF-1β knockout mice die by E10.5 primarily from vascular and hematopoietic defects (51,52). ARNT-2 (HIF-2β) null mice, by contrast, survive until E18.5 with the most obvious defects detected in hypothalamic development (53). These mutants lose expression of critical neuropeptide genes required for proper generation of the hypothalamic-pituitary axis. Cultured neurons from HIF-2β null embryos show sharp declines in induction of common HIF target genes in response to hypoxic insult. Kidneys from HIF-2β mutants appear grossly normal but have not yet been examined in detail. Previous in situ hybridization studies have determined that the developing brain and kidney are both sites of particularly prominent HIF-2β mRNA expression (54), and a careful analysis of HIF-2β expression in these sites is therefore clearly warranted.

Although VEGF signaling through its receptor tyrosine kinases is undoubtedly one of the most crucial mediators of blood vessel generation, VEGF alone is not sufficient to produce fully functional, mature blood vessels. Overexpression of VEGF in skin of transgenic mice initiates many of the early events of vasculogenesis, including endothelial cell recruitment and assembly, but the resulting vessels are malformed, never fuse completely, and are not fully functional (55–57). In the same experimental system, however, coexpression of HIF-1α together with VEGF leads to synthesis of mature, functioning blood vessels (58). These experiments therefore demonstrate that although VEGF is critical for blood vessel development, complete vessel morphogenesis also requires the expression of a number of other HIF gene targets.

Nevertheless, hypoxia-driven VEGF expression can still be considered one of the more important events in vascular development. During lung morphogenesis, VEGF signals at the leading edge of branching airways and is responsible for promoting new blood vessel growth. VEGF is also involved in epithelial maturation in developing lung. Type 2 pneumocytes and bronchial epithelial cells produce both VEGF and VEGFR, and the bronchioalveolar fluid contains significant levels of VEGF, suggesting an autocrine role for VEGF signaling in lung epithelial differentiation (59). Type 2 pneumocytes experience hypoxia during lung development and contain stabilized HIF-2α protein. These HIF-2α null mice that survive to birth have considerably thinner alveolar septae than normal, which causes death as a result of impaired blood-gas exchange. Pneumocyte development is halted in HIF-2α null mutants, and these cells contain considerably less surfactant phospholipids compared with wild-type littermates. Alveolar vasculogenesis occurs normally in knockouts, but vessels fail to remodel, inhibiting proper ventilation in pups after birth. In addition, typically increased levels of VEGF expression during final stages of lung development does not occur in HIF-2α null embryos. Intra- amniotic administration of VEGF largely res-
cues the phenotype in knockout pups and improves pulmonary development, although lung function is not completely restored (59). This study uncovers vital roles for HIF-2α in lung development by showing that it is required not only for proper lung vascularization processes but also for surfactant production and epithelial maturation.

HIF-1α has also been shown to be required in morphogenetic systems distinct from vascular development. In studies utilizing Cre/Lox technology, HIF-1α was selectively deleted from mammary gland epithelial cells (60). Surprisingly, mammary microvessel density, which normally increases twofold during pregnancy, is apparently not inhibited by loss of HIF-1α. However, pregnant mice lacking mammary HIF-1α contain undifferentiated alveoli and a sharp reduction in expression of genes required for milk production and lactation. Total milk volume and quality are compromised in mammary-specific HIF-1α knockout mice, and pups live only to 15 d of age (60). This study further broadens the scope of HIF function during development by demonstrating a role for HIF-inducible proteins that extend far beyond those mediating vascularization.

Role for HIF in Metanephric Kidney Development

The permanent, metanephric kidney originates at approximately E11 in mice, approximately E12 in rats, and approximately 5 wk gestation in humans, when the ureteric bud projects dorsolaterally from the nephric duct and engages the metanephric blastema mesenchyme (61). Reciprocally inductive signals from bud epithelium and metanephric mesenchyme, respectively, cause growth and repeated branching of the bud (which ultimately forms the collecting tubules) and aggregation of mesenchymal cells at each branch tip. Each of the mesenchymal condensates subsequently converts into a cluster (vesicle) of epithelial cells, which then differentiates into glomerular and tubular epithelial cells of individual nephrons. Shortly after vesicles form, a cleft develops at the base of each vesicle to produce a comma-shaped nephric figure. Vascular progenitors assemble into this cleft and produce the glomerular endothelial and mesangial cells. Epithelial cells beneath the cleft develop into podocyte and parietal epithelial cells of Bowman’s capsule, whereas epithelial cells above the cleft differentiate into proximal, Henle’s loop, and distal tubule epithelium. The distal segment connects with the ureteric bud so that the lumen of the developing nephron communicates with that of the forming collecting system. Continued growth and branching of the ureteric bud, induction of new mesenchymal aggregates, and glomerulo- and tubulogenesis continue until the full complement of nephrons is achieved (61).

Developing podocytes are key sources of VEGF, which may be one of the most critical initiators for development of glomerular capillaries. Release of VEGF from immature podocytes may attract angioblasts that bear VEGFR into vascular clefts of early nephric figures, leading subsequently to the organotypic formation of glomeruli. Indeed, injection of anti-VEGF antibodies into developing mouse kidney cortex in vivo results in formation of avascular glomerular structures (62). Once the glomerular capillary tuft has fully matured, however, podocytes continue to synthesize VEGF. Because Flk1 expression is also maintained by glomerular endothelial cells of mature kidneys, VEGF-Flk1 signaling probably exerts functions beyond those needed for initial establishment of the glomerular capillary and may include maintenance of the differentiated state by endothelial cells and possibly podocytes as well (63).

Several other receptor-ligand signaling systems are probably also crucial for glomerular capillary formation, including the Tie/angiopoietin, and PDGFR/PDGF families (64,65). The Tie receptor-angiopoietin ligand system is also vital for normal vascular formation (66). Tie-2 is expressed by developing glomerular endothelial cells, and one of its ligands, angiopoietin-1, has been shown to be important for vascular organization and remodeling. Another ligand, angiopoietin-2, seems to regulate vascular integrity and permeability, and the coordinated expression of these two related proteins may be essential for final maturation and stabilization phases of the glomerular capillary (66). As mentioned earlier, Tie-2 and at least some members of the angiopoietins contain defined HRE in their promoters.

Similarly, the PDGFB gene promoter contains an HRE, although by itself it may not be functionally responsive to hypoxia (67). Mesangial cells are derived from metanephric precursors that do not express Flk1 and therefore are distinct from the endothelial developmental pathway (68). A critically important finding showed that a member of the PDGF family, PDGFB, is expressed strongly by mesangial cells and that its receptor, PDGFRβ, is expressed by these cells as well (69). Further examination of the expression of this ligand receptor pair during glomerular development showed that immature podocytes produce PDGFB, which may help attract mesangial cell precursors that express PDGFRβ into glomeruli. Later, both PDGF and PDGFRβ expression becomes confined to the mesangium, which may promote mesangial cell proliferation and/or maturation (69). Compelling studies implicating PDGFB/PDGFRβ signaling in establishment of the mesangium have come from gene targeting studies in mice. Null mutants for either protein produce similar, early postnatal lethality as a result of hemorrhaging (70,71). Glomeruli in these animals consist of one or a few large, dilated capillaries that completely lack mesangial cells. Thus, both the PDGFB ligand and its receptor, PDGFRβ, are required for normal development of the mesangium (70,71).

Regulation of Renal VEGF Expression

Because the nephrogenic cortex of developing kidneys in situ is undergoing initial vascular assembly and cells are rapidly dividing, we hypothesized that this area might also be experiencing transient hypoxia in vivo. We reasoned that such oxygen gradients, if present, might stabilize HIF, which then would stimulate transcription of VEGF, VEGFR, and other factors required for kidney development. To explore this possibility, we examined developing mouse kidney for expression of HIF-1α, HIF-2α, and HIF-1β and found HIF mRNA and
protein distribution to coincide closely with sites of robust VEGF expression (72). For testing the presence of HIF heterodimers, proteins immunoprecipitated from newborn kidney lysates with HIF-1α were positive on Western blots for both HIF-1α and HIF-1β, indicating the presence of intact HIF-1 complexes. The same was true on immunoprecipitates with HIF-2α, and pulldowns with HIF-1β also contained both HIF-1α and -2α subunits.

To localize HIF protein expression in newborn kidneys, we carried out immunohistochemistry using HIF-2α and -1β antibodies. HIF-1β was widely expressed, and nuclear staining was seen in glomeruli and all tubular segments. However, HIF-2α was localized intensively to glomeruli, where nuclei of podocytes were densely labeled, and to microvascular endothelial cells (Figure 3).

To determine whether the regions expressing HIF mRNA and protein were actually hypoxic, we used the hypoxia marker pimonidazole hydrochloride, which labels tissues undergoing severe hypoxia. Our findings showed weak labeling in the outer cortex of developing but not mature mouse kidney and intense labeling of some tubular and collecting duct epithelial cells. Finally, to evaluate the effects of hypoxia on HIF-α protein stability and VEGF expression, we carried out studies with E12 kidneys maintained in organ culture at 95% room air (approximately 20% O2) and after acute and chronic hypoxia. Kidneys cultured under hypoxic conditions expressed significantly more VEGF and contained more HIF-1α and -2α protein than those grown under normoxia. Together, these results show that HIF are expressed in vivo by kidney cells that synthesize VEGF abundantly and that both HIF stabilization and VEGF expression are mediated by hypoxia in organ culture (72). Our findings additionally show, however, that HIF and VEGF are expressed by cells that are not severely hypoxic and that, indeed, detectable levels of HIF-1α and -2α proteins are present under normoxic culture conditions, which probably represents hyperoxia in native kidney tissue. Taken together, we believe that HIF stabilization and VEGF expression are governed by both hypoxia-dependent and hypoxia-independent mechanisms in vivo (72). By either or both mechanisms, HIF stabilization and VEGF secretion probably represent one of the earliest and most critical initial signals for glomerular capillary development and may be important for glomerular maintenance in maturity as well.

As mentioned earlier, perhaps stabilization of different HIF heterodimers results in differential gene activation in selected cells. Correspondingly, perhaps certain HIF evoke VEGF secretion by epithelial cells, whereas other HIF evoke VEGF expression by angioblasts (Figure 4). Whether this hypothesis is correct is not yet known, but two recent studies in adult mice (73) and rats (74) have shown different time courses and amplitudes of HIF activation among different organs, and highly variable tissue distribution patterns for HIF-1α and -2α are reported as well. Similarly, HIF, which are rapidly upregulated in adult rat kidneys subjected to experimental ischemia (75), show cell-selective expression in different renal compartments: HIF-1α is found in ischemic tubule epithelial cells, whereas HIF-2α immunolocalizes chiefly to peritubular endothelial cells as well as to a small subset of glomeruli (76). Taken together, the findings strongly suggest that the same stimulus may indeed induce the formation of different HIF heterodimers in different cells in kidney and elsewhere. If true, then tightly coordinated expression of discrete HIF target genes by different cells may be a fundamental guiding event in vascular development and in tissue repair.

In summary, abundant evidence strongly suggests that tissue hypoxia evokes HIF stabilization and HIF target gene expression to relieve hypoxic stress. Among the genes induced by HIF are VEGF and VEGFR, all of which mediate vascular development and growth. However, accumulating data also show that HIF stabilization and VEGF synthesis can be in-

Figure 3. Newborn mouse kidney section immunolabeled for HIF-2α. Peroxidase reaction product is found within nuclei of epithelial cells of early nephric figures (*) and in podocytes (arrows) of capillary loop stage glomeruli (CL). Nuclei of microvascular endothelial cells (arrowheads) are labeled as well. Modified from reference Leveen et al. (72).

Figure 4. Model showing hypothetical stabilization of different HIF heterodimers in different cells. In this case, the same stimulus results in activation of VEGF in one cell population (upper cell) and VEGFR in another (lower cell). Together, this results in blood vessel development in the appropriate spatial context.
duced by hypoxia-independent events. Whether different HIF heterodimers initiate VEGF synthesis by only selected cells and VEGFR by others remains unproved, but somehow the complementary expression of this HIF-inducible ligand/receptor pair must be coordinated. We need to learn much more about both hypoxia-dependent and -independent stabilization of HIF in developing and diseased tissues before we truly understand exactly how this potent transcription factor family participates in vascular development, organogenesis, and responses to injury.

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References

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