REVIEW ARTICLE

Molecular Mechanisms of Action and Therapeutic Uses of Pharmacological Inhibitors of HIF–Prolyl 4-Hydroxylases for Treatment of Ischemic Diseases

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Abstract

Significance: In this review, we have discussed the efficacy and effect of small molecules that act as prolyl hydroxylase domain inhibitors (PHDIs). The use of these compounds causes upregulation of the pro-angiogenic factors and hypoxia inducible factor-1 α and -2 α (HIF-1 α and HIF-2 α) to enhance angiogenic, glycolytic, erythropoietic, and anti-apoptotic pathways in the treatment of various ischemic diseases responsible for significant morbidity and mortality in humans. **Recent Advances:** Sprouting of new blood vessels from the existing vasculature and surgical intervention, such as coronary bypass and stent insertion, have been shown to be effective in attenuating ischemia. However, the initial reentry of oxygen leads to the formation of reactive oxygen species that cause oxidative stress and result in ischemia/reperfusion (IR) injury. This apparent “oxygen paradox” must be resolved to combat IR injury. During hypoxia, decreased activity of PHDs initiates the accumulation and activation of HIF-1 α , wherein the modulation of both PHD and HIF-1 α appears as promising therapeutic targets for the pharmacological treatment of ischemic diseases. **Critical Issues:** Research on PHDs and HIFs has shown that these molecules can serve as therapeutic targets for ischemic diseases by modulating glycolysis, erythropoiesis, apoptosis, and angiogenesis. Efforts are underway to identify and synthesize safer small-molecule inhibitors of PHDs that can be administered *in vivo* as therapy against ischemic diseases. **Future Directions:** This review presents a comprehensive and current account of the existing small-molecule PHDIs and their use in the treatment of ischemic diseases with a focus on the molecular mechanisms of therapeutic action in animal models. *Antioxid. Redox Signal.* 20, 2631–2665.

Introduction

OXYGEN, THE PREDOMINANT life-supporting molecule, is essential for the survival of all cells in the body. As the terminal electron acceptor in the electron transport chain (ETC), oxygen is essential for sufficient generation of 5'-adenosine triphosphate (ATP), the physiological energy currency that propels cellular activities during oxidative phosphorylation (10, 202). Oxygen serves as a cofactor or co-substrate in many crucial cellular biochemical pathways

and minor fluctuations in cellular oxygen levels can disturb the overall biochemical homeostasis of the body. To assure adequate oxygen delivery to all tissues, glomus cells in the carotid and aortic bodies sense changes in the partial pressures of oxygen (pO₂) and serve as chemoreceptors that regulate oxygen supply by affecting cardiorespiratory centers in the central nervous system (204). In addition, carotid sinus baroreceptors regulate heart rate and blood pressure to maintain adequate blood flow (70). Other systemic compensatory mechanisms exist including interstitial cells in the

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kidney that release the hormone erythropoietin (EPO) to stimulate erythrocyte production that will enhance systemic oxygen delivery (173). These mechanisms, however, are insufficient to mitigate the often dramatic shifts in oxygen tension within pathologic tissues, resulting in crippling, potentially, fatal consequences.

Hypoxia arises when local oxygen demand exceeds supply within a tissue or organ. Hypoxia caused during ascent to high altitudes can be quickly corrected by descending toward sea level or by breathing supplemental oxygen. However, other forms of hypoxia can be far more detrimental and are not so easily treated (152). Ischemia, a condition that manifests as the restriction or cessation of blood flow to a region of the body due to partial or complete vessel occlusion, perpetuates some of the most common diseases in the developed world, which are associated with both high morbidity and mortality. In over one in four Americans, ischemia leads to some type of cardiovascular disease (CVD), often resulting in fatal myocardial infarction (MI) (71, 72). Similarly, cerebral ischemia is a leading cause of stroke. Ischemia also is encountered in the liver, kidneys, gastrointestinal tract, and eyes, causing a variety of disorders that include renal failure, mesenteric ischemia, and retinopathy. Additionally, anemia and/or Type II diabetes mellitus (DM) can exacerbate ischemia, which can diminish immune system response or preclude a patient from required surgical intervention(s) due to the high risk of unwanted complications (41).

The pathological condition of hypoxia is governed by two principal alterations in the normal function of cellular energy-generating machinery that contribute to most of these disease processes. First, hypoxia (ischemia) limits the availability of oxygen to accept an electron from Complex IV (cytochrome C oxidase) of the ETC, leading to the abrupt halting of both oxidative phosphorylation and aerobic generation of ATP (174). Under these circumstances, the cell is forced solely to rely on anaerobically generated ATP produced by glycolysis, severely limiting the energetic compounds available for utilization. More detrimental, however, is the continued reduction of residual oxygen within the hypoxic region by Complex III of the ETC following the cessation of oxidative phosphorylation. This reduction leads to the generation of reactive oxygen species (ROS) that cause significant oxidative stress in the hypoxic/ischemic region (174). This type of oxidative stress following ischemic insult further leads to cellular necrosis, apoptosis, organ remodeling, and loss of function (72).

Reversal of ischemia through sufficient reperfusion of the ischemic tissue can restore homeostasis if accomplished early. Angiogenesis, the physiologic branching out or budding of new blood vessels from existent vasculature, and surgical interventions, such as coronary artery bypass, reestablish blood flow to the ischemic tissue and have been proven effective in attenuating ischemia. Clot-lysing molecules such as tissue plasminogen activator and anticoagulants such as coumadin also are commonly used in order to reestablish blood flow to ischemic tissue. This reperfusion is highly critical to salvage the tissue, but the initial introduction of oxygen immediately following ischemic insult leads to generation of additional ROS that ultimately cause ischemia/reperfusion (IR) injury. Hence, this apparent "oxygen paradox" must be resolved to alleviate the deleterious effects of reperfusion to the ischemic tissue (72).

The discovery of the family of hypoxia inducible factors (HIFs) offers opportunities for development of therapies

against IR injury because the two mammalian isoforms, HIF-1 and HIF-2, have been found to regulate the transcription of virtually every peptide involved in the hypoxic response (172). This includes the regulation of erythrocyte proliferation *via* EPO, angiogenesis *via* vascular endothelial growth factor (VEGF) expression, apoptosis, anti-apoptosis, and glycolysis. Of the two isoforms, HIF-1 regulates the majority of these processes and is expressed systemically.

Both HIF-1 and HIF-2 are classified as basic helix-loop-helix (BHLH) transcriptional regulatory proteins and exist as $\alpha\beta$ -heterodimers. Upon activation the HIF-1 α and HIF-2 α subunits are translocated from the cytoplasm to the nucleus, where they dimerize with their respective HIF-1 β and HIF-2 β subunits to form the active transcription factor (60, 170). HIF binding to hypoxia response elements (HREs) then allows the regulation of gene expression (207). More than 150 genes have been identified that contain HREs and are regulated in part by HIF-1 α . These genes are found to be functionally involved in tumor metastasis, angiogenesis, energy metabolism, cell differentiation, and apoptosis (60, 106, 208). As a powerful regulator of many cell processes HIF-1 α is also tightly controlled. During normoxia, hydroxylation targets HIF-1 α for proteasomal degradation through the von Hippel-Lindau (VHL) protein, a member of the E3 ubiquitin proteasome ligase (205). The inhibition or blocking of proteasomal degradation of HIF could enhance the activity and the expression of HIF during normoxia, thus allowing for rapid recovery from hypoxia and maximal relief from oxidative stress (Fig. 1).

HIF-1 can be stabilized by inhibiting prolyl hydroxylase domains (PHDs) that otherwise hydroxylate HIF and lead to its degradation as shown in Figure 1. Attempts to identify and/or synthesize inhibitors specific to the three mammalian PHD isoforms PHD1, PHD2, and PHD3 remain the subject of intense research into HIF-1 stabilization (5). Knockout mice for one, two, and all three isoforms of PHDs present with elevated levels of HIF-1 and HIF-2, confirming that PHD hydroxylation of HIF causes its degradation (187). At present, efforts are underway to utilize the inhibition of PHDs to block hydroxylation of HIF-1 α in experimental settings. Several pharmacological PHD inhibitors (PHDIs) are being developed and tested *in vivo* in mice, rats, monkeys, and, increasingly, humans. These PHDIs range from readily available compounds such as dimethyloxaloylglycine (DMOG) to novel inhibitors developed by the world's leading pharmaceutical companies (7, 110). The use of small interfering ribonucleic acids (siRNAs) to inhibit PHDs and stabilize HIF are also being explored (80). Robust increases in HIF-1 α and HIF-2 α coupled with the elevated expression of downstream effectors lend strong evidence that these inhibitors function as PHDIs and require further attention as future clinical modalities for treatment of ischemia (80). To that end, Nangaku *et al.* (133) synthesized two compounds (TM 6008 and TM 6089) having PHD inhibitory and HIF stabilizing properties in both an *in vitro* and *in vivo* animal model of cerebrovascular disease. No acute toxicity was observed (TM 6008 and TM 6089) for 2 weeks in animal models (80). Further long-term experimental studies are warranted; however, before safety conclusions may be reached.

The pharmacologic PHDIs proceed through one of two possible mechanisms. All three PHD isoforms require 2-oxoglutarate (α -ketoglutarate) as a co-factor for HIF hydroxylation, so the vast majority of the inhibitors developed

FIG. 1. Regulation of HIF-1 stabilization is oxygen dependent. Role of PHDs. Figure adapted and modified from Ke and Costa (91). HIF, hypoxia inducible factor; PHD, prolyl hydroxylase domain.

for clinical use serve as 2-oxoglutarate antagonists (182). Similarly, PHDs require iron as a co-factor for HIF hydroxylation, and a few iron chelators have been developed to sequester free iron. However, since several other cellular processes require iron, these chelating agents cause considerable side effects and appear less suitable for clinical application than the 2-oxoglutarate antagonists (73, 142, 182).

This review discusses in detail the molecular mechanisms of PHD inhibition in regulating angiogenesis, glycolysis, erythropoiesis, and apoptosis, as each of these processes ultimately can participate in protection against ischemia. Also, this review discusses in detail the use of PHDI to achieve attenuation/therapeutic intervention of the cardiovascular, neuronal, gastrointestinal, renal, ocular, and systemic conditions/diseases caused by ischemia and IR injury. The authors hope to present a complete review of most of the studies involving PHD-I.

Molecular Mechanisms of Action and Therapeutic Classification of Pharmacological Inhibitors of HIF-Prolyl 4-Hydroxylases

Hypoxia inducible factor

Hypoxia is an important stimulus in biological systems. Approximately 1%–1.5% of the genome is transcriptionally regulated by hypoxia, and HIF-1α is the transcription factor modulating many of these genes. HIF-1α, in turn, is deactivated by PHDs that are part of a group of the prolyl hydroxylase enzyme family that has a central physiological role in post-transcriptional and transcriptional adaptation to

hypoxia and oxidative stress. PHDs are “oxygen sensors” at the cellular level (87). They act primarily through hydroxylation of HIF, a peptide that must be well understood in order to discuss HIF upregulation by PHDI. HIF is a hetero-dimeric protein that consists of a hypoxia-regulated α subunit and a constitutively expressed β subunit. HIF has many roles in human biology and it may be a link between hypoxic metabolism and inflammatory responses (176, 177).

HIF-1α is an 827 residue peptide consisting of a BHLH, two per-ARNT-sim (PAS) domains, ‘N- and ‘C-terminal transactivation domains (TADs), and an oxygen-dependent degradation domain (ODD). HIF is ubiquitously expressed and has been identified as a heterodimeric transcription factor vital in the regulation of hypoxia, metabolic pathways, and oxidative stress. Downstream effectors include EPO, VEGF, and multiple glycolytic enzymes. Under normoxic conditions, HIF-1α is hydroxylated at P564 and P404 in the ODD by PHDs that require oxygen, Fe(II), and 2-oxoglutarate as cosubstrates. The hydroxylation then enables HIF-1α ligation to the VHL tumor suppressor protein (pVHL), which is coupled to the E3 ubiquitin ligase complex. Polyubiquitination ensues at three lysines in the central ODD, and HIF-1α is directed to the 26S proteasome (146). The ODD contains two nonredundant sites that share a core homology but differ both in overall sequence and in necessary environment for binding to the VHL-E3 ligase complex (120). The need for oxygen and Fe²⁺ as cofactors for proline hydroxylation implicates a role for HIF-1α in mammalian oxygen sensing (82). Under normoxic conditions, HIF-1α has a half-life of <5 min (79).

Under hypoxic conditions, defined as an oxygen level below 200 μM and limited iron availability, the PHDs do not function and fail to hydroxylate HIF-1 α . The stabilized HIF-1 α escapes degradation, translocates to the nucleus, and binds to constitutively expressed HIF-1 β to form a heterodimer concurrent with the presence of cofactors like p300/CBP at the 'C TAD. This HIF-1 α -HIF1 β dimer then binds to deoxyribonucleic acid (DNA) consensus sequences, notably the pentanucleotide HREs in gene regulatory regions (208), and transcribes factors that protect the cell from hypoxia, such as EPO, VEGF, and glycolytic enzymes (22, 85, 171, 176, 202). The functionality of the 'C TAD is modulated by an asparaginyl hydroxylase (factor inhibiting HIF [FIH]), which exclusively hydroxylates N803 to inhibit binding of transcriptional cofactors. Lack of hydroxylation, therefore, stabilizes transcriptionally active HIF-1 α (158).

There are three known isoforms of HIF- α subunits: HIF-1 α , HIF-2 α , and HIF-3 α . Stability and activity of HIF-1 α and HIF-2 α are modulated by the dynamic "oxygen sensing" activity of PHDs (115). HIF-1 α is ubiquitously expressed, but HIF-2 α and HIF-3 α are much more restricted (121, 122). HIF-1 α and HIF-2 α also differ in the nuclear cofactors they recruit. HIF-1 α recruits p300/CBP and steroid receptor coactivator (SRC) and HIF-2 α recruits nuclear factor- κB essential modulator (NEMO). HIF-1 α is more of a regulator for hypoxia and HIF-2 α is more a regulator of oxidative stress. HIF-2 α also contributes to the neuronal growth factor (NGF) promoted survival of sympathetic neurons (110).

Prolyl 4-hydroxylases act on collagen and HIF

Since the main enzymes regulating HIF stability are the prolyl 4-hydroxylases (P4Hs), it is important to understand the different classes of these enzymes and their function and regulation in cells and tissues. P4Hs display significant activity during regulation of both collagen synthesis and oxygen homeostasis (129). Thus, they can be categorized into two classes—collagen-P4H and HIF-P4H, also known as PHD.

Collagen–prolyl 4-hydroxylases

A family of collagen prolyl 4-hydroxylases (C-P4Hs), which are localized to the lumen of the endoplasmic reticulum (ER), act on -X-Pro-Gly sequences to catalyze the formation of 4-hydroxyproline in collagens and similar peptides. These hydroxylations stabilize the collagen triple helix. Vertebrates possess type I and type II enzymes, which are comprised of α -(I)2 β 2 and α -(II)2 β 2 tetramers, respectively. The type I enzyme predominates in most of the vertebrate tissues (98). Initially, P4Hs were recognized mostly for their effects on collagen regulation, and like the HIF-P4Hs, they require Fe^{2+} , 2-oxoglutarate, O_2 , and ascorbate (99).

HIF–prolyl 4-hydroxylases

HIF-1 α is modulated by a second family of P4Hs (129). The three clearly elucidated HIF-P4Hs show a 42%–59% sequence identity to each other but no distinct sequence similarity to the C-P4Hs. Recently, a fourth P4H with a transmembrane domain in the ER has been characterized in humans. It has been shown to have functions related to HIF regulation but not C-P4H and has been termed PHD4 (102). As HIF-P4Hs have also been identified in *Caenorhabditis*

elegans and *Drosophila melanogaster* (23, 51), it appears that the PHDs originated as a single isoform in worms and flies and evolved into three isoforms in mice (PHD1, 2, and 3) and probably four isoforms in humans, although the PHD4 isoform is still controversial.

Distinguishing features of C-P4Hs and PHD

Some of the structure and function characteristics of P4Hs distinguish the C-P4Hs from PHDs. The two classes share protein homology and the requirement of the cofactors Fe, oxoglutarate, and ascorbate. They differ in the presence of specific binding domains. C-P4Hs have a collagen-specific binding domain, and the HIF-P4Hs have a domain that binds specifically to the ODD of proteins like HIF. While HIF-1 α is hydroxylated at P-546 and P-402, the human type I and type II C-P4Hs do not hydroxylate a 19-residue synthetic peptide that encompasses P-546 in HIF-1 α (129). The PHDs, however, do contain the Fe^{2+} -binding residues identified in the C-P4Hs, but the lysine residue that binds 2-oxoglutarate is replaced in the PHDs by arginine (23, 51). The affinity of PHDs to oxygen also is much lower (178 mm Hg) than the observed K_m of about 28 mm Hg in the C-P4Hs (74). Similar to the response to oxygen, the affinity for 2-oxoglutarate is much lower ($\sim 60 \mu\text{M}$) in PHDs compared with C-P4Hs ($\sim 20 \mu\text{M}$) (75). Moreover, both of these enzyme classes are part of a larger class of over 60 oxoglutarate-dependent dioxygenases that may share some functional homology (109). Therefore it is important to pay attention to multiple biological effects when using agents that inhibit PHDs and the need for isoform-specific PHDI is critical for therapeutic use.

This review focuses on the use of HIF-P4H inhibitors to treat ischemic disease, but C-P4H inhibition also may be of therapeutic use. Antagonists to the C-P4H subfamily are being developed in an effort to control excessive collagen accumulation in fibrotic diseases, including severe scarring (129).

Structural Features and Isoform Specificity of PHDs

In studying the PHD isoforms, it is also important to recognize that there have been various names given to each particular enzyme, leading to confusion in nomenclature. For the purpose of this review, the HIF-P4Hs will be termed PHDs, and where applicable, they will be specified as PHD1, PHD2, PHD3, and PHD4.

Prolyl hydroxylase domain 1

PHD1 (also termed HPH-3 or EGLN-2) is mainly found in the nucleus (124) with higher expression observed in some tissues, such as the testis and liver, under normal conditions (107). PHD1 is constitutively expressed and has the ability to hydroxylate both the C- and N-terminal ODD of HIF-1 α . Two isoforms 43 and 40 kDa (average 43.6 kDa) have been identified (197). PHD1 is also induced by estrogen to stimulate cell proliferation (124).

Prolyl hydroxylase domain 2

PHD2 (also termed HPH-2 or EGLN-1) is a 46 kDa protein localized mainly in the cytoplasm and appears to be the primary regulator of HIF-1 α -mediated transcription factors (5, 14, 51). Higher levels are seen in some tissues (heart and testis)

(107). PHD2 has the capability to hydroxylate both the C-ODD and N-ODD in HIF-1 α (5), and its deletion leads to stabilization or accumulation of HIF-1 α (188), but not HIF-2 α , in cells (187). PHD2 inhibition strongly influences antitumor activity in mouse tumor cells and may be a target for inhibition of tumorigenesis (100).

Prolyl hydroxylase domain 3

PHD3 (also termed HPH-1 or EGLN-3) is a 27 kDa protein with at least two alternative splice forms, with modeled weights of 17 and 24 kDa (27). It has both nuclear and cytoplasmic localization (124) and is less active in HIF-1 α hydroxylation than PHD2 (14). PHD3 retains high activity during hypoxia, but most of the activity is toward HIF-2 α during oxygen deprivation (5). Higher levels are seen in heart and liver than in other tissues (107). It has the capability to hydroxylate only Proline 564 in the N-ODD of HIF-1 α (5). Its upregulation by hypoxia and growth factor deprivation suggests a role in apoptosis (111). The 17 kDa splice variant, additionally, has no prolyl hydroxylase activity (27). PHD3 shares a high degree of sequence identity to SM-20 that is associated with cell death in neurons (56). Under normoxia, PHD3 has been shown to be proapoptotic, but under hypoxia, it can have cell survival or proliferation-supporting functions (83). PHD3 may also regulate neutrophil survival during hypoxia; this may enable the design of new therapeutics for inflammatory disease (200).

Prolyl hydroxylase domain 4

PHD4 (also termed P4H-TM) has recently been considered part of the PHD family of P4Hs (141). It is located in the ER membrane with its catalytic site in the lumen. There is no analog in worms or flies, but PHD4 has been identified in zebrafish and mammals. Even though its structure resembles the CP4Hs, it lacks the substrate binding for collagen and functionally appears to be upregulated in hypoxia in a manner similar to PHDs (102). Its inhibition by siRNA increased HIF-1 α levels in cultured cells and was associated with proline hydroxylation of HIF in the C-ODD (141). Recombinant human P4H-TM hydroxylated the two regulatory prolines of HIF-1 α *in vitro*, with a preference for the C-terminal hydroxylation site. This recombinant PHD4 showed no activity toward recombinant type I procollagen chains (102). It is possible that PHD4 may bind additional substrates *in vivo*, but the literature is still lacking on this PHD (102).

Isoform specificity. The proportional contribution of each PHD isoform in the physiological regulation of HIF remains uncertain. It was shown by using gene suppression with siRNA that each of the three PHD isoforms regulates both HIF-1 α and HIF-2 α in a nonredundant manner. It was elucidated that PHD2 and PHD3 messenger ribonucleic acid (mRNA) are induced by hypoxia but PHD1 mRNA is not, suggesting a negative feedback loop for rapid degradation of PHD2. PHD2, therefore, serves as the main oxygen sensor in this protein family (14, 185). In addition, the PHDs appear specific for different prolyl hydroxylation sites within each HIF- α subunit and exhibit some degree of selectivity between HIF-1 α and HIF-2 α , which indicates that differential PHD inhibition selectively may alter the characteristics of HIF activation (5).

Regulation of PHDs and HIF. PHD and HIF regulation of activity is determined by a variety of factors, including autoregulation. Regulation is essential, as each PHD appears to serve a specific function, with PHD2 serving as the critical oxygen sensor during normoxia that maintains low steady-state levels of HIF-1 α . Interestingly, PHD2 is upregulated by hypoxia, likely by an HIF-1-dependent autoregulatory mechanism that is driven by oxygen tension (14).

A newly discovered peptide also appears to regulate PHD expression. Osteosarcoma amplified 9 (OS9), a common protein of unassigned function, has been suggested to serve as part of a multiprotein complex involved in the hypoxic response *via* promotion of the O₂-dependent degradation of HIF binding to both HIF and PHDs (6, 53). Moreover, it was shown that OS-9 gain-of-function promotes HIF-1 α hydroxylation, VHL binding, proteasomal degradation of HIF-1 α , and inhibition of HIF-1-mediated transcription. OS-9 loss-of-function caused by RNA-i increased HIF-1 α protein levels, HIF-1-mediated transcription, and VEGF mRNA expression under normoxia. These data indicate that OS-9 helps to modulate HIF-1 α levels in an O₂-dependent manner (6).

Inhibition of PHDs, using either DMOG or small hairpin RNA (shRNA) against PHD2, increased both HIF-2 α expression and EPO transcription. Since renal CD133+ progenitor cells synthesize and release EPO under hypoxia, this finding lends potential for the therapeutic use of PHDs in the setting of acute or chronic renal injury (25).

Off-target effects of PHDs

PHDs interact with molecules other than HIF-1 and its isoforms and may be involved in the regulation of iron regulatory protein-2 (IRP2), RNA polymerase II (RNA pol II), and mitogen-activated protein kinase organizer-1 (MORG-1). Iron-mediated degradation of IRP2 requires the activity of PHDs (67, 168, 203). Inhibitors of PHDs stabilize IRP2 and modulate the labile iron pool in cells (203). The large subunit (Rpb1) of RNA pol II is modified by proline hydroxylation and this may be due to homologous regions to the ODD of HIF-1 α observed in RNA pol II (104). The hydroxylated Rpb1/Rpb6 peptide competes with HIF for pVHL binding (104). MORG-1 interacts with PHD3 at a nonconserved region and decreases HIF-1-mediated reporter gene induction (76). MORG-1 has an important role in the protein kinase cascade in cells. Additionally, there appears to be a negative feedback type of interaction between the PHDs, especially PHD3 and SIAH2 (the ring finger protein seven in absentia homolog 2) (132). SIAH2 polyubiquitylates and tags PHD1 and PHD2 for destruction. PHD3 has been found to amplify HIF signaling through another mechanism involving hydroxylation of the glycolytic enzyme pyruvate kinase (PK) muscle isoform 2 (PKM2). PKM2 functions in a positive feedback loop that promotes HIF-1 transactivation and reprograms glucose metabolism in cancer cells (112). Also an HIF-independent PHD function appears to control alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor trafficking involved in synaptic transmission and the activation of transient receptor potential cation channel member A1 (TRPA1) ion channels in sensory nerves. Oxygen appears to regulate these processes. Moreover, PHD activation involves the iron chaperoning function of poly(rC) binding protein1

(PCBP1). This highlights the complexity of PHD regulation and function (210).

There have been reports of PHD-mediated effects on growth factors such as "Sprouty" (Spry2). It was demonstrated that PHDs target Spry2 to the proteasome, which may be a novel mechanism of growth factor regulation (4). Based on data from siRNA inhibition of PHDs, hypoxia appears to relieve repression of nuclear factor kappa B (NF κ B) activity through decreased PHD-dependent hydroxylation of inhibitor of NF κ B kinase subunit β (IKK β), an event that may factor in tumor development and the amplification of tumorigenesis (39).

A role for PHDs recently has been found in adipogenesis. While additional research is required, PHD1 appears upregulated during early adipogenesis, and PHD2 and PHD3 act later in the pathway (54). These studies on the role of PHDs in growth factor and adipogenesis regulation highlight a major challenge in transitioning PHD1 from the bench to the bedside, as systemic inhibition of PHDs must be considered.

PHDs also have been found to interact with a few other peptides that must be considered when developing PHDIs to treat ischemic disease. Recent findings have shown that OS9 (6), mitogen-activated protein kinase organizer 1 (MORG1) (76), and the tumor suppressor gene inhibitor of growth family, member 4 (ING4) (37) can bind to the PHD enzymes. Ozer *et al.* showed recently that knockdown of ING4 increases HIF transcriptional activity (145). PHD enzymes also appear to upregulate IKK β . This interaction is thought to increase NF κ B activity in hypoxia since IKK β is an inhibitor of NF κ B activity (39). Additionally, Koditz *et al.* showed that activating transcription factor 4 (ATF4) is stabilized in anoxia and also by mutation of proline residues within ATF4 that are subjected to hydroxylation by PHD3 (101). All of these interactions must be understood before the complete role of PHDs in hypoxic response is understood.

Role of Cellular Oxygen and "ROS" in Expression and Regulation of PHDs

A characteristic of PHDs is their low affinity to oxygen compared with other P4Hs (74). Additionally, there are differences in the affinity to oxygen among the PHD isoforms and different cell type-specific thresholds may exist (180). Low-oxygen affinity allows PHDs to operate in a tightly regulated manner where even slight changes in oxygen concentration lead to a robust changes in PHD reaction velocity and HIF-1 α turnover (180).

Regulation of PHD activity by cellular oxygen or ROS is mediated by various Krebs cycle intermediates such as succinate, fumarate, 2-hydroxyglutarate, cobalt, nickel, and, most importantly, Fe. PCBP1 is important in the shuttling of Fe from intracellular ferritin to PHDs, and ascorbate and/or glutathione (GSH) helps in maintaining Fe in the reduced state *via* the Fenton reaction (210). Mitochondria, additionally, can modulate the cellular hypoxic response by regulating cellular oxygen availability. Changes in oxidative phosphorylation alter ROS production, but HIF stabilization appears to proceed *via* an unlinked mechanism (35).

A point of controversy has been the relative role of hypoxia and ROS in the regulation and modulation of PHD activity and HIF action. A series of studies suggested that ROS produced by complex III of mitochondrial ETC were responsible

for hypoxia-dependent HIF stabilization (9, 24, 62, 118). This "ROS hypothesis" suggests that hypoxia causes the production of superoxide by respiratory complex III, likely by the action of superoxide dismutase-produced hydrogen peroxide (H₂O₂) that inhibits PHDs by oxidizing nonheme-bound iron (63). A recent finding that PHDs have low sensitivity to inhibition by H₂O₂ has put this hypothesis in question (119). However, the same team found that FIH, another 2-oxoglutarate-dependent dioxygenase, is more susceptible to H₂O₂-dependent inactivation likely through the Fenton reaction (119).

The "oxygen hypothesis" also could explain the driving force behind PHD-regulation of HIF. This hypothesis proposes that the regulation depends on cellular oxygen availability. Since mitochondria are the major cellular utilizers of oxygen, a decrease in mitochondrial chain activity leads to an increase in cytoplasmic oxygen concentration, which in turn may lead to PHD activation and subsequent HIF hydroxylation and destabilization (35, 48, 64, 206). This mechanism has also been demonstrated in HIF-2 α regulation (20). Another study suggesting the role of intracellular oxygen rather than ROS found that regulation of HIF-1 α by a protein Coiled-Coil-Helix-Coiled-Coil-Helix Domain Containing 4 (CHCHD) is mediated by cellular oxygen levels rather than by complex III-derived ROS (213). In either situation, mitochondria plays a critical role in the regulation of HIF-1 α by modulating intracellular oxygen concentration (63).

In combination with hypoxia and ROS, "reactive nitrogen species (RNS)" also may contribute to the regulation of PHD and HIF activity. The antioxidant N-acetyl-L-cysteine (NAC) and NO synthase inhibitor N(G)-monomethyl L-arginine (L-NMMA) reduce HIF-1 α stabilization and increase HIF-1 α hydroxylation. These effects suggest that endogenous NO and ROS impair PHD activity. Thiol reduction with dithiothreitol also decreases HIF-1 α stabilization in hypoxic cells, while dinitrochlorobenzene, which stabilizes S-nitrosothiols, favors its accumulation. These data suggest that ROS- and NO-mediated HIF-1 α stabilization involve S-nitrosation, which is confirmed by demonstrating increased S-nitrosation of PHD2 during hypoxia. There is experimental support for a regulatory mechanism of HIF-1 α during hypoxia in which RNS such as NO and ROS promote inhibition of PHD2 activity, probably by its S-nitrosation (34).

There may also be a role for a peroxide sensor involving FIH in HIF regulation. FIH has reduced sensitivity to cellular hypoxia but is more sensitive to peroxide-induced oxidant stress than the PHDs. These opposing sensitivities indicate that hypoxia and oxidant stress can interact as distinct regulators of HIF (119).

Another system implicated in oxygen-related regulation is the thioredoxin/thioredoxin reductase system that is involved in scavenging H₂O₂ (134). Inhibition of the mitochondrial F₀F₁-ATPase leads to an increase in mitochondrial membrane potential and slowing of the ETC, leading to increased superoxide and ROS production. However, despite high ROS, F₀F₁-ATPase inhibition prevented HIF-1 α stabilization in hypoxia (58, 157).

It is important to differentiate the contributions of ROS and oxygen levels in PHD regulation. It appears that PHDs, with their K_m for oxygen in the range of atmospheric oxygen concentration, are most prominently regulated by cellular oxygen availability (63).

Apart from the effect of hypoxia and ROS on modulation of PHDs, it is important to note that there are a number of PHD modulators that feed back on the ROS system. One example is insulin, which is known to activate HIF-1 by an ROS-dependent mechanism. Insulin regulates HIF-1 α *via* ROS-sensitive activation of Sp1 in 3T3-L1 preadipocyte and increases HIF-1 α promoter activity. It was also shown that insulin-induced ROS generation initiates the phosphatidylinositol 3-kinase (PI3K) and protein kinase C cascades for Sp1-mediated HIF-1 α transcription (18).

PHD inhibitors

Since HIF-1 α stimulates the production of products such as VEGF and EPO, many investigators and pharmaceutical companies are attempting to develop PHDIs that can stabilize HIF-1 α as a treatment for cancer, vasculopathy, colitis, anemia, renal disease, and neuroprotection. A recent article reports the synthesis and evaluation of a novel class of potent spirohydantoin-based pan-PHDIs for treatment of anemia (46). PHD2, an enzyme mostly responsible for oxygen-induced degradation of HIF- α , plays a major role in oxygen-induced retinopathy in mice. PHD2 deficiency or inhibition significantly stabilizes HIF-1 α , and HIF-2 α to some extent, in neonatal retinal tissues, which protects against oxygen-induced obliteration of retinal microvessels. Therefore, there may be a role for PHD2 as a therapeutic target to prevent oxygen-induced retinopathy (49).

NEDD8 helps to stabilize HIF by preventing its ubiquitination-related destruction. Given the positive role played by HIF- α in cancer promotion, inhibiting NEDD8-HIF-1 α conjugation could present as a novel cancer therapy (164). Prototype PHDIs have shown promising results in preclinical models (68). PHD inhibition with agents such as DMOG protects mesenchymal stem cells and may provide a novel approach to promote cell survival during stress (108). PHDI has been used as a strategy to improve bone growth in animal models (178). Acute activation of the HIF-signaling pathway by a single systemic DMOG treatment upregulates not only antiapoptotic pathways but also enhances neovascularization *via* progenitor cell proliferation (186). Another factor important in the use of PHDIs for therapeutic purpose is the timing of drug administration relative to the course of disease. As demonstrated by the study using L-mimosine against chronic renal disease, HIF- α activation can promote or prevent chronic renal disease in the rat kidney injury model depending on the timing of the administration and possibly the involvement of the activated isoform of HIF- α (215).

Additionally, pharmacologic activation of the HIF system can stimulate endogenous EPO production. FG-2216 oral dosing increased plasma EPO levels 30.8-fold in hemodialysis (HD) patients with preserved kidney function, 14.5-fold in anephric HD patients, and 12.7-fold in healthy controls (13).

Another class of PHDIs is the conjugated linoleic acid (CLA) isomers. CLAs are positional and geometric isomers of the essential fatty acid linoleic acid. Two isomers of CLA cis-9, trans-11 (c9, t11) and trans-10, cis-12 (t10, c12), and a mixture of these two isomers can inhibit PHD1 and induce HIF-2 α stabilization in murine myocardium, likely leading to upregulation of PDK4 by activation of PPAR α . This process may have implications in the reprogramming of basal metabolism and oxidative damage protection in murine myocardium

(216). There is increasing recognition of the role of PHD isoforms in the onset of surgical complications, and the use of PHDIs may be important in mitigating these conditions. For example, PHD1 suppression enhances liver regeneration by boosting hepatocyte proliferation in a c-Myc-dependent fashion (96, 127). It has been found that impairment of PHD3 function aggravates the clinical course of abdominal sepsis *via* HIF-1 α - and NF- κ B-mediated enhancement of the innate immune response, and PHD3 inhibitors may be important in surgical sepsis management (97). PHDIs also may be useful in neonatal care. PHD inhibition facilitates lung angiogenesis in a primate model of bronchopulmonary dysplasia by elevating VEGF production, which has a role in potentiating lung microvasculature and distal airways (148).

Even though the collagen and HIF P4Hs are generally inhibited by similar small molecule inhibitors, they were found to have distinctly different K_i values. It should be possible, therefore, to develop specific inhibitors for each class of P4Hs and possibly even for individual PHDs (74). The study of these inhibitors has been facilitated by the development of catalytic assays that measure inhibitory activity. One such assay developed by Dao *et al.* rapidly can perform a large high-throughput screen of a chemical library to identify and characterize novel 2-OG competitive inhibitors of PHD2 (43). Additionally, the use of pharmacophore-based quantitative structure activity relationship modeling, virtual screening, and molecular docking approaches has been undertaken to identify target-specific PHDIs. Using this approach 12 candidate agents have been identified for future testing (192, 193). Another assay recently developed to measure PHD inhibitory activity uses the capture of hydroxylated peptide product by the VHL protein in a scintillation proximity assay (196).

There is increased recognition of the role played by PHD in innate immunity *via* interaction with the toll-like receptor TLR system. This information is being used to investigate the interplay between the immune system and PHD activation and inhibition. R848-induced activation of endosomal TLRs 7 and 8 and lipopolysaccharide (LPS)-induced activation of TLR4 lead to downregulation of HIF-1 α hydroxylation. This downregulation likely proceeds through redox and RNS-dependent mechanisms for TLRs 7 and 8. S-nitrosation of HIF-1 α also was observed. For TLR4 activation, only a redox-dependent mechanism is involved. RNS and p38 MAP kinase do not factor in the hydroxylation of HIF-1 α . A decrease in intracellular iron (II) has been observed under both scenarios (138).

Vasohibin is considered to be an important negative feedback regulator of angiogenesis induced in endothelial cells (ECs) by VEGF. VEGF induced significant cell growth in human umbilical vein endothelial cells (HUVECs) that was associated with an increase in vasohibin expression. Vasohibin elevates the expression of PHD and may play an important role as a negative feedback regulator of angiogenesis through HIF-1 α degradation (103).

Regulation of angiogenesis by inhibition of PHDs: molecular mechanisms

Angiogenesis, the building and branching out of new vessels from existing vasculature, is a complex and tightly controlled process. Although angiogenesis is highly crucial during development for the establishment of functional

vasculature, angiogenesis is greatly inhibited in mature mammals, except after periods of ischemia-induced hypoxia such as during menses and wound healing (147). As the regulation of angiogenesis is so vital to normal development, HIF-1 α controls more than 2% of protein transcription in mammalian cells and controls all known angiogenic pathways (116) (Fig. 2).

The central player in angiogenesis is VEGF, and expression of the growth factor is a strict requirement for angiogenesis to occur. VEGF modulates all steps of angiogenesis, from the degradation of existing vasculature to the stabilization of newly formed capillaries, but these processes could not occur without the expression of nearly 30 additional HIF-1 α -related proteins. While the roles of several enzymes, cytokines, and transcription factors modulated by HIF-1 α remain unknown, many of these molecules play well-understood functions in angiogenesis that merit discussion (55).

For angiogenesis to proceed, HIF-1 α must be expressed in vascular ECs, underscoring the vital role of the three HIF-1 α regulatory pathways in the molecular operation of angiogenesis. Two of these angiogenesis regulatory pathways, the mitogen-activated protein kinase pathway (MAPK) and phosphatidylinositol 3-kinase/Akt pathway (PI3K/Akt), are well understood, but clinical modulation of these pathways is impractical for inducing angiogenesis since they are widely utilized throughout the body to potentiate a variety of responses to extrinsic stimuli. The third molecular regulator of HIF-1 α regulation, hydroxylation by PHDs, is specific to the hypoxic response and presents a logical target for inducing angiogenesis. Several PHDs have been developed and are under investigation as angiogenic potentiators (55).

Under hypoxic conditions, PHD2 levels remain constant, but the lack of PHD1 expression leads to HIF-1 α accumulation and nuclear translocation. HIF-1 α then dimerizes with its β subunit and binds to multiple cofactors, including p300, CREB-binding protein (CBP), SRC-1, and tumor inhibiting factor 2 (TIF2). These transcription factors then initiate the transcription and translation of a multitude of angiogenic, glycolytic, and antiapoptotic pathways (26, 199). β -Catenin, the upstream effector of VEGF and angiopoietin-1 (Ang-1), is expressed, and through the subsequent activity of VEGF and Ang-1, angiogenesis is stimulated (2). In addition, HIF-1 α propagates the translation of the NF κ B, a downstream effector of the antiapoptotic protein b-cell lymphoma-2 (Bcl-2). The modulation of these pathways has been demonstrated to offer cardioprotection from ischemic injury (65, 88).

Initiation of angiogenesis involves activation of the MAPK and PI3K/Akt pathways by HIF-1 α -dependent and -independent pathways. Initial stabilization of HIF-1 α by PHD1 promotes enough VEGF expression for angiogenesis to begin. Additionally, insulin-like growth factor-1 (IGF-1) and IGF-2 are expressed and activate Akt serine/threonine kinase (S/T kinase), localizing it to the plasma membrane (57, 137). Similarly, VEGF, IGF-1, interleukin-8 (IL-8), and tumor necrosis factor- α (TNF- α) activate the MAPK pathway, another family of S/T kinases (88, 137). With the enhanced activity of these pathways, HIF-1 α expression and stabilization further is enhanced, allowing angiogenesis to proceed (Fig. 2a).

Of these two regulatory pathways, the PI3K/Akt system appears to be more potent in promoting HIF-1 α expression and stabilization. Phosphorylation of Akt activates the kinase leading to phosphorylation of the mammalian target of rapamycin (mTOR). Binding of mTOR with the Rictor receptor further enhances Akt activity, but it is mTOR binding to another receptor called Raptor that is important for angiogenesis (163). mTOR/Raptor binding causes phosphorylation of the eukaryotic initiation factor binding protein-1 (Eif-BP1), inactivating the peptide (137). This inactivation allows for stabilization of eukaryotic initiation factor-4E (Eif-4E) and p70 S6 kinase, allowing these molecules to upregulate HIF-1 α expression (137).

The MAPK pathway acts in concert with the PI3K/Akt pathway to accomplish the same goal of upregulation of HIF-1 α through Eif-43 stabilization (57). Phosphorylation of MAP begins a series of phosphorylation reactions, which sequentially activates the MAP kinase kinase (MEK), the MAP kinase kinase kinase (ERK), and the MAP kinase kinase kinase (MNK). Activated MNK then phosphorylates Eif-4E to activate it. Moreover, both the MAPK and PI3K/Akt pathways promote additional steps in angiogenesis, highlighting the dynamic nature of these pathways, which not only regulate angiogenesis but also participate in the active steps of angiogenesis (57).

Additional cytokines, secondary messengers, and enzymes that are regulated by HIF-1 α also play important roles in the propagation of angiogenesis (Fig. 2b). Potentiation of new vasculature starts with the removal of pericytes from the endothelium, which allows for vessel destabilization and initiation of the EC proliferative phenotype (38). These processes are driven by Ang-2 binding to its Tie2 receptor, which induces EC apoptosis (147). Nitric oxide (NO) release catalyzes

FIG. 2. HIF-1 α and molecular mechanism affecting angiogenesis. (a) HIF-1 α upregulation by PHD inhibition due to hypoxia releases the growth and survival factors necessary to activate the PI3K/Akt and MAP kinase pathways. Each pathway results in activation of Eif-4E, which upregulates the cytosolic HIF-1 α . The translocation of HIF-1 α to the nucleus and dimerization with HIF-1 β results in the transcription of HIF-regulated angiogenic proteins. **(b)** Angiogenesis proceeds through a multistep mechanism. VEGF/FLK-1 binding initiates the neovascularization. NO then promotes EC dilation/degradation through recruitment of TGF- β and MMPs. Ang1, the antagonist of Ang2, binds with Tie2 to assist in the EC migration and inhibition of apoptosis. This apoptotic protection persists throughout the remainder of the angiogenesis process. Ang2 binds to its Tie2 receptor to initiate EC apoptosis to expose an area of vasculature for neovascularization. Angiogenesis is briefly interrupted by Ang2/Tie2 binding to promote EC loosening and degradation and induce the release of additional VEGF and FGF. To stabilize the newly formed vessels, Ang1/Tie2, VEGF/FLK-2, and TGF- β /TGF- β receptor binding then completes the process by upregulating the antiapoptotic proteins survivin and Bcl2 and recruiting SMCs to envelop the newly formed vessels. EC, endothelial cell; Eif-4E, eukaryotic initiation factor-4E; FGF, fibroblast growth factor; MMP, matrix metalloproteinase; NO, nitric oxide; PI3K/Akt, phosphatidylinositol 3-kinase/Akt pathway; SMC, smooth muscle cell; TGF- β , transforming growth factor- β ; VEGF, vascular endothelial growth factor.

EC dilation allowing better access to the EC membrane by matrix metalloproteinases (MMPs) released from the extracellular matrix (ECM) (57, 88). MMPs are released following the release of the transforming growth factor- β (TGF- β) into the ECM (147). After these processes are complete, VEGF/Flk-1 (VEGFR2) binding enhances vessel hyper permeability as the initiating step in neovascularization (147).

Formation of a new vessel from an established one commences with EC migration along the old vessel to the site of protease degradation. Ang-1/Tie2 assists in EC migration by acting as a natural antipermeability factor that protects the old vessel from excessive plasma leakage. Integrins can also mediate this process, though not without the expression of VEGF as well (147). Throughout this migratory phase, EC proliferation intensifies due to the binding of mitogens, including VEGF, fibroblast growth factor (FGF), and epidermal growth factor (EGF) to their respective receptor tyrosine kinase (RTK) receptors (147). Additionally, Ang-2, the antagonist of Ang-1, binds to Tie2 leading to matrix degradation and loosening. ECM degradation not only facilitates EC migration but also releases additional VEGF and FGF to initiate the formation of new tubes, a process also known as cord formation. At this stage, Ang-2 expression declines, allowing Ang-1 to resume its binding with Tie2. Together with the VEGF/Flk-1 binding, Ang-2 upregulation initiates the cord assembly of EC and acquisition of an EC lumen. The cells then intercalate, become thin, and allow for tube fusion with the existing vessel(s) (147). However, these newly formed vessels lack stability and must reacquire the smooth muscle cells (SMCs) and pericytes necessary for vessel stability.

In order for these forming vessels to gain permanence, they must resist apoptosis. This resistance is conveyed through the upregulated expression of antiapoptotic factors including Bcl-2 and survivin. Bcl-2 expression, in turn, is regulated through the β -catenin expressed following the actions of the VEGF/Flk-1 and PI3K/Akt pathways. Ang-1/Tie2 binding promotes the expression of survivin. VEGF, Ang-1, platelet-derived growth factor, and TGF- β then recruit SMCs and pericytes to the new vessels to achieve complete stabilization (147). Now, the newly formed vessels can be operational in conducting perfusion to the appropriate hypoxic areas.

Additional factors that assist in vessel stabilization have been shown essential for angiogenesis. VE-cadherin binds with Ca^{2+} in the transmembrane region and restores the integrity of adherens junctions along the vasculature. C-cadherin also is thought to stabilize cell-cell contacts. Together with TGF- β , these proteins also are suggested to halt EC growth once vessels have attained a certain length (147). Increased expression of the Ephrin-B4 receptor and Ephrin-B2 RTK has been reported to enhance cell communication and promotion of ECM remodeling to match the newly formed vasculature. However, the role of ephrins remains elusive pertaining to angiogenesis (147).

As all of the angiogenic transcription factors appear to be modulated by HIF-1 α , pharmacological enhancement of HIF-1 α stability and expression can induce angiogenesis during normoxia and attenuate different types of hypoxic injury. Given that the HIF-1 α itself is regulated by the PHD family of proteins, the current review details the use of PHDI to enhance angiogenesis and alleviate the myocardial, neuronal, renal, gastric, ocular, and systemic pathophysiological condition caused by ischemia.

Molecular mechanism of regulation of apoptosis by inhibition of PHDs

Several studies have demonstrated a positive role of PHDs in apoptosis. Tambuwala *et al.* (189) studied apoptosis using a colitis model and reported on the involvement of all the three isoforms of PHDs in the development of colitis. The authors investigated the development of colitis in mice lacking all three isoforms of the PHDs (PHD1 $^{-/-}$, PHD2 $^{-/-}$, and PHD3 $^{-/-}$) following induction of colitis with dextran sulfate sodium. Only the PHD1-knockout mice were protected against the development of colitis. Subsequent studies corroborated the findings reported by Tambuwala *et al.* (189) and elucidated the antiapoptotic effect of pharmacological inhibition of PHDs *via* HIF stabilization (108, 111, 133). Attenuated apoptosis likely proceeds through HIF-1 α upregulation of the antiapoptotic protein Bcl-2 (15, 55, 61). Additionally, the proapoptotic proteins Bcl2-adenovirus E1B 19 kDa interacting protein 3 (BNIP3), Bad, and Bax also are regulated by HIF-1 α (61). Although the current review does not focus on the mechanistic role of HIF-1 α in apoptosis, it should be noted that HIF-1 α exerts either antiapoptotic or proapoptotic effects depending upon a variety of cellular conditions (211). This differential effect of HIF-1 α is partly due to the regulatory effect of HIF-1 α on the proapoptotic proteins in the Bcl-2 family (211).

Zhou *et al.* (218) have reported that when HIF-1 α activity is suppressed, hypoxia-induced apoptosis and Bnip3 expression are blocked. They have concluded that HIF-1 α mediates apoptosis in primary neonatal rat ventricular myocytes cultured under acute hypoxic conditions. In addition, they suggest that Bnip3 may be one of the key peptides involved in the hypoxia-induced expression of HIF-1 α that inhibits apoptosis (218). Targeting HIF-1 α , therefore, may represent a new strategy for attenuating the hypoxia-induced apoptosis of ventricular myocytes. Recently, Jayachandran *et al.* (84) have shown that *Crataegus oxyacantha* extract attenuates apoptosis during IR injury through elevated expression of HIF-1 α and caspases. Moreover, Zhao *et al.* (217) revealed that DMOG treatment enhances expression of HIF-1 α and thus increased the expression of caspases in postconditioned myocardium to protect against IR injury.

In other corroborative findings on the roles of PHDI in apoptosis, Bishop *et al.* (16) have shown that PHD3 knock-down regulates neuronal apoptosis. They revealed attenuated apoptosis with concomitant enhancement of cell survival in superior cervical ganglion neurons in culture isolated from PHD3 $^{-/-}$ mice. Thus, PHDI and/or HIF-1 α upregulation can be a specific target(s) to modulate apoptosis during ischemic stress or injury.

Molecular mechanism of regulation of glycolysis by inhibition of PHDs

In addition to serving as a cofactor in many biochemical reactions, oxygen also acts as the final electron acceptor of the ETC. During hypoxia, Complex IV of the ETC cannot transfer an electron to oxygen leading to inhibition of oxidative phosphorylation (174). Under these conditions, glycolysis produces enough ATP anaerobically for some cells. But as oxidative phosphorylation in the mitochondrial membrane establishes electrochemical gradients and provides much of the ATP utilized by a cell, inhibition of oxidative

phosphorylation must be reversed in short order. Prolonged hypoxia yields a modified ETC where a terminal Complex III transfers electrons to any available oxygen leading to the production of ROS and enhancement of tissue injury (174).

To date, none of the clinical trials targeting PHD inhibition to achieve cardioprotection have focused on the regulatory role of glycolysis on PHD activity. However, it is important to consider that the PHDs require 2-oxoglutarate as a cofactor, which links PHD activity not only to oxygen levels but also to production of 2-oxoglutarate by the tricarboxylic acid (TCA) cycle (167). In addition to observations of increased angiogenesis, cardiac function, reduced infarct size, and increased HIF-1 α , HIF-2 α , and EPO levels, Hyvarinen *et al.* (81) reported significant elevation in the mRNA expression of glucose transporter 1 (GLUT1), GLUT4, phosphofructokinase (PFK), triose phosphate isomerase (TPI), phosphoglycerate kinase, and enolase in PHD2 hypomorph mice. The upregulation of these glycolytic enzymes led to an improved cellular energy state and the glycolytic upregulation likely aided in production of the cardioprotective antioxidant GSH through the enhanced pentose phosphate pathway activity (110).

It appears that the upregulation of glycolysis and the accompanying antioxidant potentiation are the underlying operative mechanisms of neuroprotection offered by PHDIs. As neurons function solely on glucose metabolism, the role of glycolysis upregulation in neuroprotection offered by PHDIs is not surprising. Using neuronal cells from newborn mice, Lomb *et al.* (110) highlighted the importance of the role of glucose catabolism in neuroprotection. Cells administered either with 3,4-dihydroxybenzoic acid (DHB) or with DMOG

exhibited significantly increased glycolytic activity as evidenced by mRNA expression of the plasma membrane glucose transporters GLUT1 and GLUT3. The expression of mRNA coding for pyruvate dehydrogenase kinase (PDK) was lowered, suggesting a decreased extent of oxidative phosphorylation (110). Higher glucose concentration has been correlated with increased nicotinamide adenine dinucleotide phosphate (NADPH) that is generated from the pentose phosphate pathway, which leads to elevated synthesis of GSH. Cells in which PHDs have been inhibited exhibited significantly greater survivability compared with vehicle-treated control neurons. The study also revealed the conditions under which neuroprotection can be optimized (110). Neurons that are deprived of neurotrophin NGF exhibited neuroprotective response for 18 h before cell death induced by DHB and DMOG. Similarly, cells devoid of HIF-2 α upregulation showed minimal neuroprotective response, presumably due to lack of apoptosis in HIF-2 α -deficient neurons. This finding suggests a poorly understood but vital role of the HIF-2 α in the regulation of glycolysis. Moreover, the findings suggest that glucose catabolism and antioxidant anabolism attenuate apoptosis and induce the neuroprotection that enabled recovery from cerebral ischemia (110).

Regulation of glycolysis by HIF-1 α and potentially (110) by HIF-2 α recently has been discussed by Denko (45). The author explained that HIF not only regulates the principal plasma membrane glucose transporters GLUT1 and GLUT3 but also has the ability to induce transcription of all 12 glycolytic enzymes, including PFK, adolase, and enolase (45). Additionally, the downregulation of PDK has been observed

FIG. 3. Molecular mechanism of regulation of glycolysis by inhibition of PHDs. Figure adapted from Kim *et al.* (95), Nagy (131), and Simon (183).

severely to attenuate the ability of cells to continue glucose catabolism through the TCA cycle and oxidative phosphorylation. This shifts substrates to the pentose phosphate pathway, enhancing antioxidant potentiation and abrogating ROS. The cells, therefore, rely on anaerobic respiration for continued survival until the end of the hypoxic event (Fig. 3).

Molecular mechanism of erythropoiesis through inhibition of PHDs

Reperfusion of ischemic tissue attenuates injury through restoration of nutrient influx into and waste efflux out of the ischemic region. Paramount in this process is the reintroduction of oxygen to the hypoxic region of the tissue. Thus, angiogenesis is insufficient in attenuating IR injury without the accompanying induction of erythropoiesis. For the restoration of oxygen supply to the ischemic (hypoxic) tissue, interstitial and medullary cells of the kidney must secrete EPO (173). Both HIF-1 α and HIF-2 α contain a 3' HRE, and binding of HIF to this region initiates direct transcription and release of the EPO, which can then bind to the receptors on bone marrow cells to initiate erythropoiesis (52).

Erythrocyte potentiation alone can enhance the oxygen-carrying capacity of the blood, but only, if each erythrocyte contains functional hemoglobin (Hb), an Fe²⁺-dependent porphyrin that binds to oxygen. Then, it is appropriate that the entire iron utilization pathway, from intake to recycling, is HIF mediated (150). Iron, in the ferric (Fe³⁺) form, is absorbed in either the duodenum or the upper jejunum (44). Prior to passage through the apical membrane of the enterocyte, ferric iron is reduced to ferrous iron (Fe²⁺) by the ferric reductase duodenal cytochrome b (DCYTB) before proton-mediated transport through the divalent metal-ion transporter (DMT1). At this stage, either iron can be stored intracellularly by sequestration with apoferritin to yield the active storage peptide ferritin or it can be transported immediately from the enterocyte into circulation for utilization by the body (44). The body also recycles and accumulates iron. Iron from the degraded erythrocytes accumulates in macrophages and is utilized in the subsequent production of new cells. At the end of the lifespan of the erythrocyte (~4 months), each cell is engulfed by a macrophage, and iron from the erythrocyte is salvaged by heme oxygenase-1 (HO-1) (150).

The role of HIF-2 in iron association with Hb cannot be overlooked (150). This regulation proceeds through ferroportin and has long-term effects on systemic iron transport since ferroportin is the only known iron exporter in the body. During normoxia, infection, and inflammation, the peptide hormone hepcidin binds to and internalizes ferroportin. However, during hypoxia, binding of iron response protein (IRP) to the 5' iron response element (IRE) of the hepcidin gene inhibits hepcidin transcription, leading to the stabilization of ferroportin. Although the IRP/IRE mechanism is still not completely understood, it is conditional on HIF-2 α expression, likely due to the presence of IRE in the 5' untranslated region of HIF-2 α . With ferroportin being stabilized on both the hepatocyte and macrophage membranes, ferrous iron is transported from cells into the circulation and is oxidized back to ferric iron by either hephaestin (hepatocytes and enterocytes) or ceruloplasmin (macrophages) under the regulation of HIF (44). Upon entering the plasma, the majority of the ferric ions bind to either the N or C iron binding

domains of the apotransferrin to yield the functional glycoprotein transferrin (44). Transferrin then binds to its receptor on bone marrow cells, allowing internalization of iron. The transferrin receptor (Trf) is also regulated by HIF *via* binding to the HRE of the Trf gene (44). A final conversion of iron from ferric to ferrous by STEAP3 allows Hb to bind with its Fe²⁺ moiety, yielding a mature erythrocyte for potentiation (150).

Thus, erythropoiesis depends on HIF upregulation of EPO and the presence of sufficient iron for the formation of functional Hb. As in angiogenesis, every gene involved in the erythropoiesis pathway contains an HRE or is modified by a peptide whose gene contains an HRE, making HIF the master regulator of erythropoiesis as well as angiogenesis. Yoon *et al.* (214) also have reported that VEGF expression is essential for initiating erythropoiesis, although the specific mechanism is unclear.

In a recent study (13) the investigators treated patients in renal failure and normal volunteers with the PHDI FG-2216 and found as much as a 30-fold increase in EPO levels, suggesting that pharmacologic manipulation of the HIF-PHD system may be a viable therapeutic option (13). Thus, HIF stabilization and upregulation through PHDI appears a viable strategy to mitigate hypoxia-associated conditions with associated anemia. The mechanism of HIF-mediated erythropoiesis is summarized in Figure 4.

Pharmacological PHD Inhibition and HIF Stabilization in Ischemic Disease

Cardiovascular disease

CVDs accounted for over 26% of deaths in the United States in 2006, and heart failure, by far, was the most common cause of mortality across all ethnicities (71). Partial vessel occlusion associated with IR injury and MI may cause moderate-to-severe regional ischemia. Current therapeutic investigations aim at maximizing the restoration of blood flow and enhancing oxygen supply to the ischemic tissue. Angiogenesis eventually reperfuses the tissue, but oxidative stress caused by free radicals during reperfusion induces endothelial and cardiomyocyte necrosis, apoptosis, and ventricular remodeling, creating the so-called "oxygen paradox" (72). Thus, any effective cardioprotective therapy must combat these deleterious effects on cardiac tissue. The established direct link between HIF-1 α expression and expression of the angiogenic proteins VEGF and Ang-1 and the antiapoptotic proteins Bcl-2 and NF κ B clearly underscores the potential of HIF-1 α stabilization in offering long-lasting cardioprotection (42, 65, 88, 125). In this context our laboratory has also reported that knocking out PHD1 and PHD3 resulted in the stabilization of HIF-1 α , leading to cardioprotection after ischemic onset (1, 144). Since HIF-1 α is degraded by the PHD isoforms within 5 min during normoxia, use of PHDIs to inactivate the PHDs has become an increasingly attractive approach to cardiotherapy (Table 1). Clinical trials on PHDIs are underway in human subjects (80). Even though the current review highlights the specific cardioprotection offered by each PHDI, not the underlying chemical mechanisms of the PHD inhibition, it is worth noting that most of the inhibitors investigated act as 2-oxoglutarate analogs, ascorbate analogs, or iron chelators. A detailed review on the mechanism employed by currently known PHDIs has been provided by Nagel *et al.* (130).

FIG. 4. Regulation of erythropoiesis. Decreased pO_2 stimulates HIF-1 α - and HIF-2 α -mediated erythropoiesis. Surrounding the enterocytes in the duodenum, the ferrireductase DCYTb reduces Fe^{3+} to Fe^{2+} for transport into the cell through DMT1. As this iron is needed immediately as a hemoglobin cofactor, the iron is transported out of the cell instead of being stored within the ferritin complex. As a result of HIF accumulation, hepcidin expression is downregulated, leading to its release of the iron transporter ferroportin. Ferroportin migrates to the plasma membrane and transports Fe^{2+} out of the cell into the circulation. Once in the circulation, Fe^{2+} is oxidized back to Fe^{3+} by hephaestin to allow for binding to and transport by transferrin. Iron is also recycled from degraded erythrocytes in macrophages. Increased HO-1 propagates erythrocyte degradation, and Fe^{2+} is exported out of the macrophage in a manner identical to Fe^{2+} transport out of the enterocyte. In the circulation, Fe^{2+} is oxidized by ceruloplasmin before binding to transferrin. Transferrin then migrates to bone marrow cells and binds to its receptor, releasing Fe^{3+} into the cytosol. The iron is reduced by STEAP3 for use as a hemoglobin cofactor. With the available iron for erythrocyte potentiation, EPO induces pathways in the bone marrow cell to initiate the erythropoiesis. DCYTb, duodenal cytochrome b; DMT1, divalent metal-ion transporter; EPO, erythropoietin; HO-1, heme oxygenase-1; pO_2 , partial pressure of oxygen.

Eckle *et al.* (50) administered DMOG (0.1–1 mg) to C57BL/6 mice before subjecting them to IR in an attempt to determine whether the nonspecific inhibition of PHDs could rival the immense but clinically impractical cardioprotection conveyed by myocardial preconditioning. The cardioprotective effect of DMOG appeared to mirror the benefit of ischemic preconditioning, as seen in reduced infarct size of ~44%. Additionally, this study confirmed earlier results elucidating that HIF-1 α binding to the A2B adenosine receptor is necessary for cardioprotection, as A2B $^{-/-}$ mice could not be protected from ischemic injury (50). However, no additional investigation on the mechanisms of DMOG-mediated cardioprotection was conducted in this study because the study mainly focused on siRNA-specific inhibition of the PHD1, PHD2, and PHD3. Ockaili *et al.* (140) also confirmed that DMOG is cardioprotective *via* elucidation of diminished infarct size in DMOG-treated rabbit hearts.

Both FibroGen (San Francisco, CA) and GlaxoSmithKline (Kingdom of Prussia, PA) are in the process of developing novel PHDI, with the former company investigating five compounds (one for cardioprotection) and the latter company

conducting studies on one cardioprotective compound. Philipp *et al.* (151) have shown that FG-2216, an isoquinoline-based compound, selectively inhibits the hypoxic PHDs without affecting the collagen isoforms involved in fibrosis. In addition to elevating HIF-1 α and HIF-2 α levels as early as 24 h following MI, FG-2216-treated rats presented with attenuation of ventricular remodeling and the accompanying compensatory weight gain of the heart and lungs when compared with control animals. Dilation of the left ventricle also was inhibited in this study. Further, this study revealed an increase in left ventricular contractility and ejection fraction (EF) as compared with the vehicle-treated rats without any noticeable difference in the infarct area between the vehicle and FG-2216-treated animals. Although other compounds discussed in the current review offer additional cardioprotection as compared to FG-2216, the compound appears to offer chronic cardioprotection following MI without any side effects. Thus, FG-2216 has emerged as an attractive cardioprotective drug (151).

GlaxoSmithKline also has employed the rat MI model to assess the efficacy of GSK360A, an orally administered

TABLE 1. PHARMACOLOGICAL PROLYL HYDROXYLASE DOMAIN INHIBITORS FOR TREATMENT OF THE CARDIOVASCULAR DISEASES

Name of compound	Animal model	Species	Mechanism	Interactive molecules	Reported outcome	Reference
DMOG	Murine IR <i>in vivo</i>	<i>Mus musculus</i>	PHD inhibition (PHDI)	HIF-1 α , A2BR	Reduced infarct size, cardioprotection equivalent to ischemic preconditioning	Eckle <i>et al.</i> (50)
DMOG	Rabbit IR <i>in vivo</i>	<i>Oryctolagus cuniculus</i>	PHD inhibition (PHDI)	IL-8, HO-1	Reduced infarct size	Ockaili <i>et al.</i> (140)
FG-2216	Rat MI <i>in vivo</i>	<i>Rattus norvegicus</i>	PHD inhibition (PHDI)	HIF-1 α , HIF-2 α	Attenuated ventricular remodeling, heart and lung weight gain, improved function	Philipp <i>et al.</i> (151)
GSK360A	Rat MI <i>in vivo</i>	<i>R. norvegicus</i>	PHD inhibition (PHDI)	EPO, HO-1, Hb	Increased angiogenesis, erythropoiesis, improved function	Bao <i>et al.</i> (7)
siRNA	Murine IR <i>in vivo</i>	<i>M. musculus</i>	PHD2 inhibition (PHDI)	HIF-1 α	Reduced infarct size	Eckle <i>et al.</i> (50)
siRNA	Murine IR <i>in vivo</i>	<i>M. musculus</i>	PHD2 inhibition (PHDI)	HIF-1 α , HO-1, iNOS	Reduced infarct size, angiogenesis, improved function	Natarajan <i>et al.</i> (135)
shRNA	Murine IR <i>in vivo</i>	<i>M. musculus</i>	PHD2 inhibition (PHDI)	HIF-1 α , VEGF	Improved angiogenesis and function	Huang <i>et al.</i> (80)
DMOG	Murine IR <i>in vivo</i>	<i>M. musculus</i>	PHD2 inhibition (PHDI)	TNF, MIP	Decreased inflammation, necrosis, and apoptosis. Improved function	Natarajan <i>et al.</i> (136)
siRNA	Murine IR <i>in vivo</i>	<i>M. musculus</i>	PHD2 inhibition (PHDI)	TNF, MIP	Decreased inflammation, necrosis, and apoptosis. Improved function	Natarajan <i>et al.</i> (136)
PHD2 hypomorph mice	Murine IR <i>in vivo</i>	<i>M. musculus</i>	PHD2 inhibition (PHDI)	HIF-1 α & - 2 α , EPO, GLUT-1 & - 4, PFK, TPI, PGI, enolase	Enhanced glycolysis, reduced infarct size, improved function	Hyvarinen <i>et al.</i> (81)

DMOG, dimethylloxallylglycine; EPO, erythropoietin; GLUT, glucose transporter; Hb, hemoglobin; HIF, hypoxia inducible factor; HO-1, heme oxygenase-1; IR, ischemia/reperfusion; MI, myocardial infarction; MIP, macrophage inflammatory factor; PFK, phosphofructokinase; PHDI, prolyl hydroxylase domain inhibitor/inhibition; RNA, ribonucleic acid; shRNA, small hairpin RNA; siRNA, small interfering ribonucleic acid; VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor.

2-oxoglutarate analog, in offering cardioprotection (7). Male rats were fed 30 mg/(kg·day) of GSK360A in methyl cellulose for 28 days following MI and were observed for 3 months. Cardiac function was unaffected and the hearts maintained baseline EF at 45 h and then gradually returned to baseline over 2 months following MI. Ventricular dilation and lung weight also remained at baseline levels. Of more significance, a twofold increase in microvasculature was observed at the infarct zone along with increases in plasma EPO, Hb, and the angiogenic and antiapoptotic protein HO-1. EPO levels, however, returned to baseline by 24 h after the administration of GSK360A. Moreover, ventricular remodeling was significantly attenuated. Although these results appear promising, the study cautions that it is unclear whether cardioprotection was enhanced primarily by activation of angiogenic and antiapoptotic pathways, through increased oxygen delivery from the elevated production of EPO, through increases in PDK and transferrin in the kidneys, or through activation of the Akt/GSK-3 β survival pathways. Since HO-1 expression has not been uniformly established across species either systemically or at the tissue level and appears to be independent of the HIF-1 α expression, a thorough understanding of HO-1 is needed before the underlying mechanisms of the cardioprotective actions of GSK360A fully can be comprehended (105). Interestingly, polycythemia was not observed, even with increased EPO levels and hematocrit in the drug-treated rats. Another study shows some evidence that HIF modulation was involved in providing cardioprotection. HIF-2 α primarily was elevated in hepatocytes, which are the prime sites for the release of EPO during hypoxia (154, 198). This study also showed that HIF-2 α is the main effector of catecholamine synthesis and ventricular remodeling in the heart (154). The data elucidate yet another target for HIF and show the influence of HIF stabilization on cardioprotection. Other studies on PHD inhibition conveying cardioprotection following IR or MI in the murine model have focused on the use of siRNA or shRNA as the PHDI. Although the clinical safety and practicality of the RNA approaches remain to be established, cardioprotection offered by RNA therapy appears robust. Eckle *et al.* (50) reported declines in the transcript levels of PHD1, PHD2, and PHD3 following use of siRNA inhibition, and PHD2 inhibition caused a decrease in infarct size by ~20% as compared to the control group. Similarly, the PHD2 siRNA construct utilized by Natarajan *et al.* (modeled after collagen PHDs) yielded 54% and 90% declines in PHD3 mRNA at 24 and 72 h, respectively, and a 69% decrease in the infarct size following IR (135, 136). In these studies, an increase in the concentration of inducible nitric oxide synthase (iNOS) mRNA in treated mice corroborated the role of NO synthesis pathways in providing cardioprotection through cellular retention of ATP (135, 136).

Use of shRNA by Huang *et al.* (80) to inhibit PHD2 during hypoxia has been successful. PHD2 was degraded 50%–60% more than that in control animals, leading to HIF-1 α upregulation by more than 50%. Expression of angiogenesis-stimulating proteins, including VEGF, was increased by over 30% in mice with IR as compared with controls. CD31 staining then was used to confirm increased angiogenesis. While improvement in EF and HIF-1 α stabilization relative to the sham-operated mice initially was observed, both the parameters reached the control levels at 2 and 4 weeks, indicating that chronic shRNA therapy would be necessary to maximize cardioprotection (80).

To determine the cardioprotective action of PHD2 inhibition from the perspective of inflammation, Natarajan *et al.* (135, 136) followed the study with a murine *in vivo* IR model and an *in vitro* model using siRNA, to assess acute inflammatory response following injury. Using both the DMOG and siRNA strategies to inhibit PHD2, the study concluded that the release of proinflammatory cytokines such as TNF and macrophage inflammatory factor (MIP) was significantly attenuated in the treated mice compared with the control mice (60-fold decrease in the plasma MIP levels) (136). Further, infarct size decreased from 40.8% to 14.8% in the treated mice, and the neutrophil accumulation in the infarct border zone was also significantly attenuated (136).

The summarized studies appear promising, but additional considerations are necessary when transitioning to human clinical trials. Marked variability exists in the ability of patients with ischemic heart disease to develop coronary artery collaterals, leading to speculation that polymorphisms in oxygen-sensing molecules can result, at least partially, in varying degrees of angiogenic response to tissue hypoxia. Resar *et al.* (156), for example, have presented evidence that different genotypes for the HIF-1 α subunit can have a considerable impact on the quality and quantity of coronary collaterals (156). The delivery of proangiogenic transcription factors, such as HIF-1 α , directly into the myocardium using adenoviral vectors can prove synergistic to PHD inhibition particularly in targeted polymorphisms (93). As such, genotype testing may provide an opportunity to select individuals in which PHD inhibition may have the most beneficial effects. Also of note, it is not known how polymorphisms throughout the oxygen-sensing/angiogenesis cascade can hamper the efficacy of these inhibitors when administered indiscriminately across all genotypes.

Together, these studies demonstrate the significant cardioprotective benefits of PHD inhibition as evidenced by improved cardiac function, increased angiogenesis, decreased apoptosis, attenuated ventricular remodeling, and diminished inflammatory response. These findings pave the way towards progress in continued investigations of the therapeutic approaches essential for improving and treating coronary artery disease, the leading cause of mortality in the developed world.

Cerebral ischemia

Given the similarity between both myocardial and cerebral arterial occlusions, inhibition of PHDs also appears to be a promising strategy for neuroprotective therapies. The attenuation of free-radical-induced oxidative stress has proven central for both cardioprotection and neuroprotection (8, 182). Neuroprotection offered by PHDI predominantly has been through iron chelation or competitive inhibition of 2-oxoglutarate, and preclinical studies with animals suggest a beneficial effect of PHDIs in the ischemic brain (11, 153). The current review discusses some of these options and the successful RNA therapeutic strategies that offered neuroprotection in animal models.

To elucidate the mechanism of antioxidant neuroprotection against ischemia in rat neurons, Siddiq *et al.* (182) induced cerebral ischemia *via* cerebral artery ligation. They examined the effectiveness of the low-molecular-weight iron chelator desferrioxamine or desferoxamine (DFO), the 2-oxoglutarate

antagonist DHB, and Compound-A on ischemic neuroprotection. Stabilization of HIF-1 α and HIF-2 α allowed for upregulation of cysteine plasma membrane transporters that maintained sufficient intracellular levels of cysteine for the synthesis of GSH, a vital antioxidant in mitigating neuronal oxidative stress. Western blot analysis revealed elevated expression of HIF and VEGF along with increased viability of treated cells after treatment with DFO, DHB, and Compound-A. However, rats treated with DFO and Compound-A exhibited restless leg syndrome and systemic anemia due to a >30% decrease in iron levels, so tight regulation of administration of both compounds would be necessary in the clinical setting. In contrast, DHB, a 2-oxoglutarate antagonist, did not lower iron levels and therefore appears as a potentially safer option for use in neuroprotection. Additionally, the inhibitors tested so far have been modeled after the collagen PHDs, and the broad spectrum of PHDIs discussed here may be replaced in the near future by safe, hypoxia-specific PHDIs that convey neuroprotection without causing fibrosis (182). Harten *et al.* (68) also described the role of PHDIs on permeability of the blood-brain barrier (BBB).

Elucidation of neuroprotective proteins in ischemic neurons has been reported by Aminova *et al.* using murine hippocampal cells *in vitro* (3). The authors concluded that HIF-1 α overexpression attenuates necrosis and apoptosis caused by ER stress, DNA damage, and glutamate toxicity (3). The interplay between the seemingly contradictory downstream effects of HIF-1 α upregulation, apoptosis, and antioxidant-mediated cardioprotection has been resolved by Baranova *et al.*, and a second vital pathway of neuroprotection has been elucidated (8). Using an *in vivo*, murine cerebral ischemia model the investigators revealed that infarct size was significantly attenuated after bouts of preconditioning and postconditioning to stroke with 2,2-dipyridyl (DP), DHB, or DFO. The drugs were administered 6 h either before or after induction of stroke. Staining revealed a decrease in infarct size by 35.5%, 41.5%, and 25.5% in the mice preconditioned with DP, DHB, and DFO, respectively. Smaller but significant infarct size reduction was observed following postconditioning (21.5%, 28.5%, and 15.5% for DP, DHB, and DFO, respectively). Additionally, the study was supported by real-time quantitative PCR analysis of the angiogenic and apoptotic proteins involved in neuroprotection. From the data, the acute and late-onset phases of protein expression following cerebral ischemia and HIF-1 α activation (8) were elucidated. The acute phase, ending at 24 h postocclusion, manifested with elevation of transcribed mRNA of glycolytic enzymes, proangiogenic proteins (VEGF, Flt-1, PAI-1, Ang-2, and Flk), and apoptotic proteins. In contrast, HIF-1 α levels continued to increase for 8 days, and while mRNA expression of apoptotic proteins fell to basal levels, mRNA for angiogenic, glycolytic, and erythropoietic proteins markedly increased. From these findings, the authors concluded that the long-lasting upregulation of the proteins mediating protection and regeneration supersedes the deleterious effects of acute-phase recovery. These results indicate that despite the paradoxical effects of HIF-1 α regulation, neuroprotection is achieved (8). The pharmacological PHDIs tested so far for protection against cerebral ischemia are summarized in Table 2.

Two additional PHDIs are under development and they deserve mention in this review. Nangaku *et al.* (133) showed that TM6089 and TM6008 induced neuroprotection in trans-

genic rats overexpressing HIF and in Mongolian gerbils. As with the previous inhibitors discussed, mRNA expression of VEGF, GLUT3, and Hb was elevated, and apoptosis was attenuated. Induction of angiogenesis was shown by sponge assays but the angiogenesis offered no significant neuroprotection when compared with control animals.

All neuroprotective PHDIs tested thus far have shown minimal or no toxicity under physiological conditions. These PHDIs should receive continued attention to establish strategies for PHDI-mediated neuroprotection that are feasible in clinical settings. However, further extensive studies on the HIF-independent functions of PHDs are required. Siddiq *et al.* (181) reported that PHD1 might hydroxylate the Rbp1 subunit of RNA pol II, leading to enhanced transcription of apoptotic proteins. Novel mechanisms of this sort involving PHDs need to be explored before PHDIs become feasible for clinical use.

Studies by Chen *et al.* (33) and Chen *et al.* (28) used a different molecule 2-methoxyestradiol (2ME2) to elucidate the neuroprotective actions of HIF-1 α stabilization following cerebral ischemia without any side effects. Chen *et al.* (28) reported that inhibition of HIF-1 α through pretreatment with 2ME2 offered attenuation of infarct size and apoptosis in the cerebral rat model. In this study, the infarct size was observed to be reduced up to 60%, along with downregulation of HIF-1 α and inactivation of the BNIP3 apoptotic pathway. Additionally, downregulation of VEGF has been shown to preserve the BBB. Although these results may cast doubt on the neuroprotective ability of HIF-1 α stabilization, Chen *et al.* (33) remarked on the temporal control of HIF-1 α in offering neuroprotection. In their study, infarct size and apoptosis were attenuated following the cerebral ischemia as a result of preconditioning of rats with 2ME2. Maintenance of the BBB also was observed. However, any benefit from the 2ME2 treatment appeared lost if the compound was administered 3 h following ischemic insult, suggesting that both the acute inhibition of HIF-1 α and long-term activation of HIF-1 α both can offer protection to postischemic neurons (33). Therefore, neuroprotective outcomes from HIF-1 α stabilization may be a successful clinical objective. A more effective strategy, however, may be to maximize neuroprotection through early inhibition of the HIF-1 α followed by the HIF-1 α stabilization a few hours following ischemia. An in-depth understanding of PHDs and HIF is critical for discovery of safe and effective PHDIs and HIF-1 α -specific pharmacological inhibitors to achieve effective and successful neuroprotection.

Anemia and systemic hypoxia

PHDIs are being investigated for the treatment of anemia by enhancing the production of erythrocytes (Table 3) to combat chronic renal failure, inflammatory diseases, and cancer (90). As inhibition of PHDs caused by iron chelators reduces total body iron and exacerbates anemia, only non-chelating PHDIs appear to have potential in the treatment of anemia (182). The studies presented here focus on either anemia or hypoxia models, and while hypoxia itself does not necessarily cause anemia, it compounds its effects (209).

While Hsieh *et al.* (77) did not induce anemia in rhesus macaques, their study showed that macaques treated with the PHDI FG-2216 developed significantly increased plasma levels of EPO, Hb, and fetal hemoglobin (fHb) compared with vehicle-treated animals. Treated animals received 40 mg/kg

TABLE 2. PHARMACOLOGICAL PROLYL HYDROXYLASE DOMAIN INHIBITORS FOR TREATMENT OF THE CEREBRAL ISCHEMIA

Name of compound	Animal model	Species	Mechanism	Interactive molecules	Reported outcome	Reference
DFO	Rat CI <i>in vivo</i>	<i>R. norvegicus</i>	PHD inhibition (PHDI)	HIF-1 α & - 2 α , VEGF	Elevated glutathione, decreased necrosis and apoptosis	Siddiq <i>et al.</i> (182)
Compound A	Rat CI <i>in vivo</i>	<i>R. norvegicus</i>	PHD inhibition (PHDI)	HIF-1 α , HIF-2 α , VEGF	Elevated glutathione, decreased necrosis and apoptosis	Siddiq <i>et al.</i> (182)
DHB	Rat CI <i>in vivo</i>	<i>R. norvegicus</i>	PHD inhibition (PHDI)	HIF-1 α & - 2 α , VEGF	Elevated glutathione, decreased necrosis and apoptosis	Siddiq <i>et al.</i> (182)
siRNA	Murine CI <i>in vitro</i>	<i>M. musculus</i>	PHD inhibition (PHDI)	BH3, HIF-1 α	Elevated glutathione, reduced glutamate toxicity	Aminova <i>et al.</i> (3)
DHB	Murine CI <i>in vivo</i>	<i>M. musculus</i>	PHD inhibition (PHDI)	HIF-1 α , VEGF, Flt-1, PAI-1, Ang-2, Flk	Reduced infarct size	Baranova <i>et al.</i> (8)
DFO	Murine CI <i>in vivo</i>	<i>M. musculus</i>	PHD inhibition (PHDI)	HIF-1 α , VEGF, Flt-1, PAI-1, Ang-2, Flk	Reduced infarct size	Baranova <i>et al.</i> (8)
DHB	Murine CI <i>in vitro</i>	<i>M. musculus</i>	PHD inhibition (PHDI)	HIF-2 α , GLUT1, GLUT3, PDK, NADPH	Elevated glutathione, glycolysis	Lomb <i>et al.</i> (110)
DMOG	Murine CI <i>in vitro</i>	<i>M. musculus</i>	PHD inhibition (PHDI)	HIF-2 α , GLUT1, GLUT3, PDK, NADPH	Elevated glutathione, glycolysis	Lomb <i>et al.</i> (110)
TM6089	Rat CI, Gerbil CI <i>in vivo</i>	<i>R. norvegicus</i> , <i>Meriones unguiculatus</i>	PHD inhibition (PHDI)	HIF-1 α , HIF-2 α , VEGF, GLUT3, Hb	Reduced apoptosis	Nangaku <i>et al.</i> (133)
TM6008	Rat CI, gerbil CI <i>in vivo</i>	<i>R. norvegicus</i> , <i>M. unguiculatus</i>	PHD inhibition (PHDI)	HIF-1 α , HIF-2 α , VEGF, GLUT3	Reduced apoptosis	Nangaku <i>et al.</i> (133)
DMOG	Rats and fetal mice <i>in vivo</i>	<i>R. norvegicus</i> , <i>M. musculus</i>	PHD inhibition (PHDI)	VEGF, EPO, eNOS and PDK1	Reduced infarct size, regional cerebral blood flow	Ogle <i>et al.</i> (142)

CI, cerebral ischemia; DHB, 3,4-dihydroxybenzoate; eNOS, endothelial nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate; PAI-1, plasminogen activator inhibitor-1; PDK, pyruvate dehydrogenase kinase.

TABLE 3. PHARMACOLOGICAL PROLYL HYDROXYLASE DOMAIN INHIBITORS FOR TREATMENT OF ANEMIA

Name of compound	Animal model	Species	Mechanism	Interactive molecules	Reported outcome	Reference
FG-2216, FG-4100, FG-4497, FG-4515, FG-4649, FG-4667, FG-4669	Rhesus macaques <i>in vivo</i>	<i>Macaca mulatta</i>	PHD inhibition (PHDI)	HIF, EPO, Hb, f-Hb	Increased erythropoiesis, hematocrit	Hsieh <i>et al.</i> (77)
DHB	Mouse <i>in vivo</i>	<i>M. musculus</i>	PHD inhibition (PHDI)	HIF-1 α , EPO	Increased hematocrit, hypoxia tolerance	Kasiganesan <i>et al.</i> (89)
DMOG	Mouse <i>in vivo</i>	<i>M. musculus</i>	PHD inhibition (PHDI)	HIF-1 α , EPO	Increased hematocrit, hypoxia tolerance	Kasiganesan <i>et al.</i> (89)
DMOG	HIF-1 α ^{-/-} mice <i>in vivo</i>	<i>M. musculus</i>	PHD inhibition (PHDI)	HIF-2 α , EPO, transferrin	Increased hematocrit	Rankin <i>et al.</i> (154)
DMOG	HIF-2 α ^{-/-} mice <i>in vivo</i>	<i>M. musculus</i>	PHD inhibition (PHDI)	No attenuation of anemia	No attenuation of anemia	Rankin <i>et al.</i> (154)
HIF-2 α ^{-/-} knockout mice	HIF-2 α ^{-/-} mice <i>in vivo</i>	<i>M. musculus</i>	HIF-2 α inhibitor	No attenuation of anemia. Decreased EPO, hematocrit, RBC, Hb	No attenuation of anemia	Gruber <i>et al.</i> (60)
1,3,8-Triazaspiro[4.5]decane-2,4-diones	Multiple preclinical species <i>in vivo</i> (rat, mouse, dog, rhesus monkey) and <i>in vitro</i>	Multiple preclinical species	PHD1–3 inhibition	Upregulation of EPO	Potential treatment of anemia	Vachal <i>et al.</i> (198)

RBC, red blood cell (erythrocyte).

of FG-2216 twice a week for 6–8 weeks (three animals) or 60 mg/kg of the inhibitor twice a week for 6–8 weeks (four animals). EPO expression was elevated 82–308-fold among the seven animals studied with no observed desensitization to the treatment. Elevation of Hb levels was maintained at 6.5–17 g/L, depending on the animal, which is characterized as a “robust change.” fHb was observed to be gradually elevated, thus aiding in reticulocyte potentiation while also minimizing concerns for vasoocclusive crisis resulting from a sharp spike in fHb (77). Hsieh *et al.* also tested eight FG compounds on human CD34⁺ bone marrow cells *in vitro* and concluded that the cells treated with FG-2216, FG-4100, FG-4497, FG-4515, FG-4649, FG-4667, and FG-4669 showed elevated output of EPO and Hb while FG-0041 treatment was ineffective (77). Toxicity of the FG compounds has not been reported, but since elevation of EPO and Hb levels was observed to continue for just 2–3 weeks, long-term dosing with FG compounds is required for the treatment of chronic anemia.

Kasiganesan *et al.* (89) confirmed that PHD inhibition caused by DHB and DMOG induces increases of plasma and liver HIF-1 α , EPO, and hematocrit. Moreover, these increases were life-saving following the induction of prolonged ischemia in male C57BL/6 mice. Mice given either 100 or 250 mg/kg of DHB in dimethyl sulfoxide (DMSO) survived significantly longer under lethal hypoxia exposure (5% oxygen) compared with vehicle-treated animals. Further, the DHB-treated mice that survived the 1-h test recovered following their transfer to ambient room air for 10 min. In a sublethal test (8% oxygen or 7100 m altitude), DHB-treated mice ran twice as long on a treadmill as the vehicle-treated mice. However, treatment with DHB, especially at the dose of 250 mg/kg, caused weight loss and abrogated grooming behavior, suggesting a toxic effect that must be investigated (89).

The above study also compared the efficacies of treatment with two different compounds, DMOG at 100 mg/kg compared with DHB at 250 mg/kg. Each compound was administered to the test animals for 3 days before placing the mice in 8% oxygen. The study revealed that the DHB-treated mice performed slightly better than DMOG-treated mice, but animals in both the treated groups outran the vehicle-treated mice by at least 30 min. Given that DMOG has not been found toxic in treating CVD, stroke, inflammatory bowel disease (IBD), and acute renal failure (ARF), the compound may be a safer choice for hypoxia, although it requires more testing. DHB and DMOG appear to be promising pharmacological compounds in the treatment of anemia because both the treatments hinge upon elevated levels of the EPO and Hb (89).

Rankin *et al.* (154) not only confirmed the results presented in this section but also clarified the specificity of the HIF-2 α isoform that is observed in the anemic response. The study used HIF-1 α ^{-/-} and HIF-2 α ^{-/-} mice and demonstrated that levels of the hematocrit, EPO, and transferrin in plasma rose dramatically following DMOG treatment only when HIF-2 α was expressed. Plasma levels of hematocrit, EPO, and transferrin following 6 h of DMOG administration in WT and HIF-1 α ^{-/-} animals were observed to be similar, but no benefit was seen after dosing HIF-2 α ^{-/-} mice. The same study also assessed the roles of the liver and kidney in generating EPO. During development, production of EPO shift is from the liver to kidney, resulting in a 9:1 ratio of the EPO production during normoxia favoring the liver. However, this ratio decreased to 3:1 during hypoxia, indicative of HIF-2 α activation

and EPO production in the kidneys (154). This result explains why HIF-2 α has been disregarded in the past as an important regulator of protein expression in response to hypoxia since HIF-2 α appears to drive the anemic response. As each of the HIF isoforms has been established to affect different proteins, HIF-isoform-specific inhibitors should be designed to achieve very specific upregulation of desired pathways following hypoxic or anemic injury.

A study conducted by Gruber *et al.* (60) revealed that during phenylhydrazine-induced anemia, HIF-2 α upregulation caused attenuation of the ischemic and hypoxic insult. HIF-2 $\alpha^{-/-}$ mice were unable to meet the EPO demand following phenylhydrazine administration and presented with a 32% decline in hematocrit. Moreover, this study elucidated that HIF-1 α and HIF-2 α are expressed in different cell types, even within the same tissue. Although HIF-1 α is expressed to a considerable extent in all tissues, HIF-2 α expression has been revealed to be confined to ECs, lung, brain, and neural crest. HIF-2 α also is the primary isoform expressed in the renal cells expressing EPO, whereas HIF-1 α is expressed in tubular kidney cells.

Chuvash polycythemia, a germline mutation in the VHL gene, results in the upregulation of HIF-1 α even in normoxic conditions, resulting in baseline elevations of EPO and other hypoxia-related genes (59). While this disorder, endemic only in certain parts of the Russian Federation, results in considerable morbidity and mortality in afflicted individuals, it illustrates the potential clinical effects of PHDIs provided it can be accomplished in a controlled pharmacologic setting. The disease, characterized by pulmonary hypertension, thrombosis, and other complications, highlights the potential dangers and unintended effects of unrestrained, chronic inhibition.

Together, these studies confirmed the potential of HIF-2 α stabilization through PHDI to combat anemia and prolonged hypoxia. However, the possibility of inadvertent polycythemia resulting from an overexpression of EPO always should be considered in any clinical trial with PHDIs (154). Preclinical studies are essential to determine the optimal dose of PHDIs and the resultant plasma levels of the EPO needed to combat anemia (summarized in Table 3).

Ischemia in DM

Type I and type II DM are reported to affect 7.8% of the population (23.6 million people), and increased disease incidence is expected due to the sharp rise in obesity throughout the developed world (66). Further, diabetics diagnosed with ischemic conditions of the heart, kidneys, and limbs may be unable to activate the usual repertoire of angiogenic and apoptotic responses as a result of impairment of HIF-1 α -mediated pathways (195). Lack of effective HIF-1 α signaling likely results in limb-threatening ischemia through a variety of mechanisms that lead to an alarming rate of limb amputation (>40-fold), making DM the greatest nontraumatic cause of amputation throughout the developed world (166). Elevations in plasma glucose and ROS seen in DM inhibit tissue recovery from ischemic injury, particularly in myocardium, skeletal muscle, neurons, and epidermal cells in the animal model (195). Given the potential benefits of HIF-1 α potentiation in DM, research focusing on maximizing HIF-1 α expression through PHD inhibition and other means vigorously has been pursued.

Thangarajah *et al.* (195) established the efficacy of PHD inhibition through DFO dosing to potentiate the reversal of ischemic insult in diabetic mice. They also elucidated the specific mechanisms of HIF-1 α inhibition in DM. The study showed that HIF-1 α transactivation was altered in DM mice compared with healthy animals, establishing that it is the HIF-1 α transcriptional activity but not stability that is affected by elevated plasma glucose levels. The study also demonstrated that metabolic changes in diabetic mice led to the accumulation of the sugar-derived aldehyde methoxyglyoxal, a ROS-derived reactive oxaldehyde. Methoxyglyoxal has been shown to induce the modification of the E1A binding protein p300 a necessary cofactor for HIF-1 α activation of its downstream effectors. As formation of the p300-methylglyoxal adduct is known to be dependent on iron, it makes sense that the iron chelator DFO preserved HIF-1 α activity in diabetic mice (195).

In a brief description of the study by Thangarajah *et al.* (195), mice pretreated with DFO (IP injection or topical on skin) then were subjected to epidermal ischemia. Following 9 and 14 days of epidermal ischemia, elevated levels of VEGF were observed in drug-treated animals, along with significant attenuation of necrosis. Additionally, infarct size was diminished by >40% with the topical application of DFO and by even more in the IP-administered group. Although DFO already has been approved for clinical use to treat a variety of diseases, clinical trials on DFO for the treatment of diabetic tissue ischemia should be expedited as a novel therapy for ischemic diseases (195). DFO also may ameliorate retinal ischemia, a common comorbidity in diabetes. Retinal ischemia induces generation of VEGF and subsequent neovascularization that is mediated by HIF-1 α stabilization. Retinal ischemia is also involved in retinopathy of prematurity (ROP) where initially it is caused by exposure to high amounts of oxygen (hyperoxia) that leads to increased PHD2 hydroxylation of the HIF-1 α that is normally produced in the cytoplasm. In experiments with hyperoxia treatment in neonatal mice with and without PHD2 activity, it was shown that PHD2 was needed for the development of neovascularization and suppression or inhibition of PHD2 prevented the abnormal neovascularization and retinopathy (49). A similar effect was seen with PHD1 inhibition leading to the prevention of neovascularization and retinopathy in neonatal mice (78).

As HIF-1 α function is not completely obliterated in diabetes, enhancement of HIF-1 α stability through PHD inhibition appears to be a promising therapeutic approach to treat ischemia in diabetic animals. Ohtomo *et al.* (143) demonstrated that nonspecific PHD inhibition by treatment with cobalt chloride (CoCl₂) attenuated ischemic renal injury in type II DM rats. While cobalt administration did not correct hypertension, other metabolic abnormalities, such as obesity, hyperglycemia, hyperlipidemia, proteinuria, and histological kidney injury, were significantly attenuated. As expected, HIF-1 α stabilization led to increased mRNA levels of VEGF, EPO, and HO-1, and to the attenuation of apoptosis, fibrosis, and glomerulosclerosis. Even though this effect has not been observed to be as pronounced as that seen with the use of DFO in attenuating ischemia, this study supports the conclusion that PHDIs appear to attenuate ischemic injury in diabetic animals (143).

Chen and Stinnett (32) have shown that PHD2 inhibition mitigated the deleterious effect of myocardial ischemia in

diabetic mice. They also elucidated a new feedback control of PHD activity. The study used the adenovirus Ang-1 to drastically increase systemic levels of Ang-1. Upregulation of Ang-1 not only increased activity of the Ang-1/Tie-2 pathway but also inhibited PHD2 expression. As a result, HIF-1 α and HIF-2 α were stabilized, leading to elevated levels of VEGF and HO-1. Plasma glucose levels also declined significantly. Arteriolar and capillary density increased throughout the infarct zone, and fibrosis was reduced by twofold. Remarkably, vascular SMCs were observed to cover 71.9% of the infarct area in the adeno-Ang-1 (Ad-Ang-1)-treated mice as compared with 21.8% in the control group. From the results of this study, the investigators concluded that the PI3K pathway is involved in the mechanism of PHD2 inhibition. Their conclusion is supported by the fact that PI3K is activated by NO, and enhanced expression of endothelial nitric oxide synthase (eNOS) leads to the elevated production of NO in Ad-Ang-1-treated mice. Further, maximal upregulation of Ang-1 continued throughout the 14-day study period, making Ang-1 therapy an attractive strategy for the treatment of diabetic ischemia.

Studies also have been conducted on ischemic relief in diabetic mice by focusing on the use of the gWIZ-CA5 plasmid to elevate plasma levels of HIF-1 α . Semenza *et al.* (175) designed and developed this plasmid that contains the gene for a constitutively active form of HIF-1 α . In their initial two studies, administration of the gWIZ-CA5 plasmid into diabetic mice through intradermal injection (i.d.) resulted in elevated levels of HIF-1 α 3 days post-treatment and enhanced expression of VEGF, Ang-1, and Ang-2 mRNA 7 days post-treatment (175). In a subsequent study, injection of the adenovirus CA5 into mice prior to inducing hindlimb ischemia caused enhanced expression of VEGF and eNOS and led to elevated levels of HIF-1 α that continued for 7 days (166). The study also revealed an increase in arteriolar and capillary densities throughout the ischemic tissue, along with attenuation of necrosis.

These studies clearly show that PHD inhibition (either by pharmacological treatment or genetic manipulation) and HIF-1 α stabilization have the ability to attenuate ischemic injury in diabetic animal models and should be pursued in clinical settings. Although the majority of these studies with rodent models of diabetes employed acute ischemic insult as opposed to the chronic ischemia that is observed in the diabetic patients, it is reasonable to believe that the strategies of PHDI and HIF-1 α -targeted protection against diabetes are similar in both cases. Data from Ohtomo *et al.* (143) support this conclusion in a genetically induced DM (type I) model, but further studies to establish the efficacy of these therapies in the type II DM subjects are needed.

The best way to protect against type II DM remains disease prevention as opposed to therapy. A widespread reassessment of modern diets and required physical activity, particularly focusing on youth, show promise in reversing the rates of occurrence of type II DM, a disease that was previously thought to affect only the elderly. Even with preventive measures, however, type II DM will continue to plague the developed world, so introducing pharmacological intervention, including modulation of PDHs and HIFs, must rigorously be pursued. PHDIs to treat diabetes are summarized Table 4.

TABLE 4. PHARMACOLOGICAL PROLYL HYDROXYLASE DOMAIN INHIBITORS FOR TREATMENT OF DIABETES MELLITUS

Name of compound	Animal model	Species	Mechanism	Interactive molecules	Reported outcome	Reference
DFO	Murine DM <i>in vivo</i> and <i>in vitro</i>	<i>M. musculus</i>	PHD independent inhibition of p300 modification by iron chelation	P300, methyl glyoxal, HIF-1 α , VEGF	Upregulated VEGF, attenuated apoptosis and necrosis, reduced infarct size	Thangarajah <i>et al.</i> (195)
CoCl ₂	Type II DM rat <i>in vivo</i>	<i>R. norvegicus</i>	PHD inhibition (PHDI)	HIF-1 α , VEGF, HO-1, EPO	Attenuated proteinuria histological kidney injury, fibrosis, apoptosis, and glomerulosclerosis	Ohtomo <i>et al.</i> (143)
Adeno Ang-1	DM rat MI model	<i>R. norvegicus</i>	PHD2 inhibition (PHDI), Ang-1 upregulation	PHD2, HIF-1 α , HIF-2 α , VEGF, HO-1 glucose, PI3K	Improve angiogenesis, decreased fibrosis and infarct zone, decreased plasma glucose	Chen <i>et al.</i> (32)
gWIZ-CA5	DM mice <i>in vivo</i>	<i>M. musculus</i>	HIF-1 α upregulation	HIF-1 α , VEGF, Ang-1, Ang-2, eNOS	Increased angiogenesis, attenuated necrosis	Semenza <i>et al.</i> (175) Sarkar <i>et al.</i> (166)

Ang-1, angiopoietin-1; CoCl₂, cobalt chloride; DFO, desferoxamine; DM, diabetes mellitus; PHDI, phosphatidylinositol 3-kinase.

Renal diseases

ARF remains a significant cause of morbidity and mortality worldwide, especially among critically ill patients. Hypoxia, functional anemia, and renal artery occlusion have been known to induce ischemic ARF, and given the frequency of each condition, effective therapeutic alleviation of renal ischemia is needed (191). In addition to kidney damage, ischemic renal injury often complicates the management of other conditions, making procedures such as cardiac surgery and radio contrast imaging dangerous (73). Ischemia of the renal cortex and medulla causes a mismatch between high cellular demand and low availability of oxygen in the tissue. As in the case of cardiac ischemia, reperfusion delivers oxygen to the ischemic tissue that leads to accumulation of free radicals, as well as elevation of necrosis and apoptosis due to oxidative stress (12). IR injury upsets the complex oxygen gradients within the renal medulla and cortex that especially affect the loop of Henle, the collecting duct, and the interstitium. Since the interstitial fibroblasts detect this physiologically during the regulation of EPO, they appear to be the optimal site for HIF-2 stabilization, a peptide that predominates in renal interstitial fibroblasts.

Using high-amplification immunohistochemical analyses, it was demonstrated that HIF-2 α was induced by hypoxia in peritubular ECs and fibroblasts as well as glomerular ECs. HIF-1 localized predominantly to tubular cells (161). Differential location and response of the HIF isoforms was noted, suggesting that each isoform plays a unique role in the response to hypoxic injury. HIF-1 in tubular epithelial cells enhances proliferation of ECs while HIF-2 is overexpressed in renal ECs to mediate chemotaxis and network formation (190). Therefore, the use of PHDI in renal disease could be considered under two approaches. One of the approaches is acute ischemic injury, where hypoxia, ischemia, and ROS-related injury need to be counteracted by strategies to improve renal blood flow and oxygen radical scavengers. The role of PHDI in this scenario is probably limited because of the pathophysiology and also the timing of the condition with a short window of opportunity. However, in case of the second scenario with chronic renal disease, chronic ischemia, and low blood supply lead to a negative feedback loop with progressive renal interstitial fibroblast injury and decreasing EPO production. Under these circumstances, the use of PHDI to promote EPO production or to stimulate the HRE-related genes *via* stabilization of HIF-1 α might be extremely effective. Already, small clinical trials using designer PHDIs have been proven effective in raising EPO levels (13).

Hill *et al.* (73) tested the use of L-mimosine (2-OG inhibitor) and DMOG to stabilize HIF-1 α through PHD inhibition in WT, HIF-1 α ^{+/-}, and HIF-2 α ^{+/-} mice. HIF-1 α ^{-/-} mice died during development. The study compared the drug treatments with exposure of mice to carbon monoxide (CO), a known inducer of hypoxia. L-mimosine was administered 6 h prior to the onset of ischemia at a dose of 600 mg/kg, and DMOG was administered 6 h prior to and 48 h following the induction of ischemia. Animals in both the groups were examined for 3 days postischemia. Although the use of CO actually induced the highest spike in levels of HIF-1 α and HIF-2 α in WT mice, both L-mimosine and DMOG induced HIF stabilization, allowing both isoforms of HIF to activate their downstream effectors. Glycolytic upregulation was ob-

served, and decreased levels of serum urea and creatinine indicated attenuation of renal dysfunction. Similarly, apoptosis and macrophage infiltration (inflammation) were abrogated in the mice treated with either L-mimosine or DMOG (73).

Bernhardt *et al.* (12) first confirmed HIF-1 α and HIF-2 α stabilization following the treatment of human HKC-8 proximal tubular cells with FG-4487 in DMSO. Similar results were seen after preconditioning rats with FG-4487 and quantifying protein levels in rat kidneys following the induction of ARF. The rats were preconditioned for 6 h prior to ischemia with FG-4487 and then compared with groups pretreated with DMSO and CO (0.1%) for 10 h, untreated animals experiencing ARF, and a sham group. The animals were subjected to 40 min of global ischemia by left renal artery occlusion, after which blood samples were collected at 24 and 72 h following surgery. Immunoblotting of the kidney samples confirmed the elevated expression of HIF in all areas of the tissue except in the distal tubule. Especially high levels of HIF in the glomerulus and in the medullary ascending Loop of Henle were observed. Transcription of EPO and HO-1 mRNA was elevated in the CO- and FG-4487-treated groups, reflecting similar levels of increase (12). To compare the potency of CO and FG-4487 pretreatment, renal dysfunction was assessed by comparing the levels of serum urea. Even though both treatments yielded a similar decrease in urea levels at 24 h following ischemia, FG-4487 treatment kept urea levels down at 72 h postischemia. These results indicated that FG-4487 is more potent in stabilizing HIF (12). However, in the FG-4487-treated group, macrophage infiltration was increased, presumably due to direct uptake of HIF-1 by the macrophage themselves (12). Rats subjected to CO preconditioning showed the anticipated decrease in macrophage infiltration and inflammation, but even with the side effects of FG-4487, this and other compounds that exhibit the HIF-stabilizing properties may transition successfully to the clinic after further research.

Rosenberger *et al.* (162) elucidated the efficacy of FG-4497 pretreatment in ameliorating ARF by examining different parts of the kidney, including the distal tubule. Rats in the treatment group were injected with FG-4497 (50 mg/kg) in the femoral artery and 6 h later, HIF-1 α levels were elevated significantly, except in the medullary thick ascending limbs in which there also was no detectable level of HIF-1 α expression in the control group. Also, GLUT-1 expression was elevated significantly in the treated animals (162). More importantly, the study concluded that preservation of renal function in FG-4497-treated rats occurred, as evidenced by decreased urine volume and increased renal perfusate flow, glomerular filtration rate, sodium reabsorption, and fractional potassium excretion. When the improved function is added to the systemic elevation of HIF-1 α and HIF-2 α , as was observed under the pretreatment condition, it can be concluded that FG-4497 represents a seemingly ideal compound for attenuating renal IR injury. Laboratory confirmation of these findings is further needed before embarking on clinical applications (162).

All of the PHDIs tested to this point have exhibited the potential of offering protection against renal IR injury despite certain unavoidable side effects such as those encountered with the use of FG-4487. Although these compounds show promise as protective compounds against IR injury, Hill *et al.* (73) observed that an ideal therapeutic approach for renal IR would also upregulate HIF in cardiac and neuronal tissue

because renal IR injury often is known to be associated with other IR injuries such as MI and stroke. Since DHB and L-mimosine have been established as effective PHDIs, systemic administration of these compounds potentially may offer cardioprotection, neuroprotection, and protection against renal IR injury simultaneously. The potential of FG-4497 to alleviate renal IR injury and the overall success of other FibroGen compounds in HIF stabilization should result in investigations geared toward systemic therapies. Since Gruber *et al.* (60) showed that HIF-1 α predominates in the tubular cells and HIF-2 α predominates in the renal cells producing EPO, HIF-1 α -specific and HIF-2 α -specific stabilizers must be investigated to determine whether the stabilization of one or both of the HIF isoforms is more desirable in achieving protection against the renal IR injury. The complex nature of EPO regulation also must be considered. Generally, during chronic kidney disease, plasma EPO falls significantly with Hb level. Recently, it was found that the PHDI F-2216 increased plasma EPO levels by 30.8-fold in HD patients, 14.5-fold in anephric patients, and 12.7-fold in healthy patients (13). The compound was given orally in a Phase I study to 12 HD patients, 6 anephric, and 6 healthy volunteers. Oxygen sensing clearly aids in EPO upregulation but it is difficult to understand how oxygen also regulates HIF stabilization and the connection must be explored further. PHDIs for the treatment of renal diseases are summarized Table 5.

Ischemia-induced retinopathy

Despite the surgical advances in the treatment of oxygen-induced retinopathies (OIRs), diabetic retinopathy (DR), and ROP, these conditions remain the leading causes of blindness throughout the developed world (169, 194). VEGF inhibitors are already approved for the intraocular treatment of DR. In ROP, preterm infants exhibit insufficient vascularization of the eyes, particularly in the progression of retinal blood vessel growth from the center to the periphery (31). Angiogenesis is needed to complete retinal development, but most of these infants can only achieve blood oxygen saturation of 70% at birth without any supplementation. They may be subjected to the hyperoxic conditions in order to re-establish normal blood oxygen saturation and proper organ development, especially of the lungs. Unfortunately, during retinal development, hyperoxia downregulates HIF-1 α and HIF-2 α in the eyes, not only arresting angiogenesis due to inactivity of VEGF and EPO but also inducing vascular degeneration of the unstable retinal vasculature. This process is known as the Phase I ROP. Instead of restoring normal vascular density, the upregulation of angiogenesis seen with hypoxia leads to the Phase II ROP (the proliferative phase) once the infant starts breathing room air (usually 32–34 weeks after birth). The weak and disorganized new vasculature is predisposed to retinal hemorrhages into the vitreous gel, and subsequent scarring or fibrosis causes retinal detachment, sometimes resulting in blindness. In DR, the observed edema, hemorrhage, ischemia, increased neovascularization, and neuronal degeneration mirror Phase II ROP and similarly cause blindness, although the inciting event is a result of elevated glucose levels (31). In infants with ROP, systemic use of PHDI should be prohibited because of their multisystem effects.

Thus far, neither the close monitoring of oxygen exposure in premature infants nor the improved management of

TABLE 5. PHARMACOLOGICAL PROLYL HYDROXYLASE DOMAIN INHIBITORS FOR TREATMENT OF RENAL DISEASES

Name of compound	Animal model	Species	Mechanism	Interactive molecules	Reported outcome	Reference
L-mimosine	Murine hypoxia <i>in vivo</i>	<i>M. musculus</i>	PHD inhibition (PHDI)	HIF-1 α , HIF-2 α ,	Attenuated apoptosis, inflammation	Hill <i>et al.</i> (73)
DMOG	Murine hypoxia <i>in vivo</i>	<i>M. musculus</i>	PHD inhibition (PHDI)	HIF-1 α , HIF-2 α ,	Attenuated apoptosis, inflammation	Hill <i>et al.</i> (73)
FG-4487	Human HKC-8 proximal tubule cells <i>in vitro</i>	<i>Homo sapiens</i>	PHD inhibition (PHDI)	HIF-1 α , HIF-2 α	HIF stabilization	Bernhardt <i>et al.</i> (12)
FG-4487	Rat ARF <i>in vivo</i>	<i>R. norvegicus</i>	PHD inhibition (PHDI)	HIF-1 α , HIF-2 α , EPO, HO-1	Increased function, lower urea levels	Bernhardt <i>et al.</i> (12)
FG-4497	Rat ARF <i>in vivo</i>	<i>R. norvegicus</i>	PHD inhibition (PHDI)	HIF-1 α , GLUT1,	Increased function	Rosenberger <i>et al.</i> (162)

ARF, acute renal failure.

glucose in type I diabetes has failed to prevent OIR. Manipulation of angiogenesis, apoptosis, and erythropoiesis is currently under investigation as a possible therapeutic strategy to combat OIR. Given the central role of HIF-1 α and HIF-2 α in all of these processes, direct control of the HIF isoforms and indirect control of HIF by the PHDIs have been identified as possible therapeutic strategies for treatment of OIR (31, 179, 194).

The majority of studies on management of the OIR have focused on the direct stabilization or inhibition of HIF-1 α and HIF-2 α to attenuate Phase I and Phase II OIR. Sears *et al.* (169) investigated the use of DMOG in attenuating Phase I ROP. Retinal Müller cells cultured in the presence of DMOG exhibited elevated HIF-1 α and HIF-2 α expression, and western blotting showed that DMOG caused similar upregulation of HIF as did administration of the hypoxia mimetics cobalt and DFO. Mice subjected to hyperoxia preconditioning *via* DMOG dosing (200 mg/kg) presented with attenuated capillary drop out, vascular tortuosity, and vascular tufting during Phase I ROP. Also, elevated levels of VEGF and EPO were observed in the retina, kidney, and liver, with renal and hepatic production of EPO enhanced by 150- and 300-fold, respectively (169).

Despite successful attenuation of the Phase I ROP, Sears *et al.* (169) cautioned that these treatments can accelerate Phase II ROP if DMOG is administered too close to the re-introduction of the mice to the room air. Stabilization of HIF-1 α and HIF-2 α resulted in augmented angiogenesis during Phase II ROP, as can be expected from upregulation of VEGF and EPO expression. The study concluded that PHD inhibition by DMOG has the potential to preserve retinal integrity provided that the timing of administration can be matched to Phase I ROP only. A detailed study is required to determine the efficacy of this therapeutic option.

Two additional methods have been discussed to attenuate Phase I ROP based on elucidation of HIF-1 α downstream effectors. The HIF-1 responsive gene RTP801, a gene that suppresses mTOR, was first cloned by Shoshani *et al.* and has been identified as a crucial modulator of apoptosis (179). Although it has been shown to be toxic to most cells in culture, it appears to attenuate apoptosis in MC57 human breast cells and rat PC12 adrenal medulla cells after the cells are exposed to hypoxia in a glucose-free medium or to H₂O₂, a known apoptotic stimulator. RTP801 has been shown to act by enhancing expression of p53 and p63 mRNA in mice, leading to production of apoptotic proteins. However, the HIF-1 α ^{-/-} mice showed no increase in p53 or p63 mRNA expression. Hence, Brafman *et al.* hypothesized that inhibition of RTP801 could attenuate OIR, which is corroborated by the observation that vascular obliteration/neovascularization and apoptosis have been attenuated in RTP801^{-/-} mice undergoing ROP (19). Unexpectedly, upregulation of VEGF was also observed in the knockout mice, suggesting that although inhibition of RTP801 did not attenuate ROP, additional therapeutics, including VEGF inhibitors, can be administered concurrently to combat OIR.

With the purpose of attenuating ROP *via* a different approach, Chen *et al.* (29) demonstrated effectiveness of EPO injections in preventing vaso-obliteration during Phase I ROP in mice. At the highest administered dose of EPO (5000 U/kg), vaso-obliteration was decreased up to ~6% compared with age-matched controls. Moreover, the observed attenuation in

vessel apoptosis was established to result from the enhanced expression of the antiapoptotic protein NF κ B. As with the use of DMOG to combat ROP, administration of the EPO during or slightly before onset of the Phase II ROP accelerated undesired neovascularization and macular deterioration. If properly managed, however, EPO injections appear promising in successfully attenuating OIR in the clinical setting. All the other trials included in the current review used somewhat different HIF or EPO inhibitors to attenuate neovascularization during Phase II OIR, and the novel aspects of each trial briefly are summarized here.

Jiang *et al.* (86) demonstrated the efficacy of HIF-1 siRNA and/or VEGF siRNA treatment in attenuating Phase II ROP. On average, the hypoxic and vector control groups presented with 41 nuclei per cross-section of the retina showing neovascularization. The treatment groups exhibited 28, 1, and 8.1 nuclei per cross-section upon treatment with HIF-1 siRNA, VEGF siRNA, and both transcripts. The combined treatment lowered expression of VEGF and HIF-1 α mRNA by >80%, and smaller but significant decreases have been seen under single treatment with HIF-1 siRNA or VEGF siRNA alone. As these studies were conducted both *in vitro* (cultured human umbilical ECs) and *in vivo* (using a murine ROP model), the potential for siRNA therapy to combat OIR appears to be promising. In a similar study, Chen *et al.* (30) demonstrated attenuation (40%) of neovascularization and a decline in EPO mRNA expression (60%) after treatment with EPO siRNA as the therapeutic agent.

In a study on attenuation of Phase II ROP, He *et al.* (69) revealed the role of NO in inducing VEGF expression. Mice injected with aminoguanidine hemisulfate, an inhibitor of iNOS, presented with up to a 33% reduction in neovascularization. Expression of HIF-1 α , iNOS, and VEGF was attenuated. From this study, it appears that HIF-1 α not only modulates iNOS activity but also is modulated by the NO-stimulated PI3K/Akt pathway. Further studies on the role of iNOS in regulation of HIF-1 α in all disease scenarios hopefully will elucidate a detailed mechanism for HIF action that moves the field toward potential therapeutic strategies for IR injury (69).

Two new agents also are capable of downregulating HIF-1 α and combating OIR. Deguelin, a retinoid, has been found to potentiate HIF-1 α protein degradation without affecting mRNA expression or inducing toxicity following its administration at a dose of 1 μ M to human ECs (94). Given that a 1 μ M dose of deguelin is 10 times lower than its recommended clinical dose, it may be an attractive drug for a quick transition to clinical trials. Also, puerarin, which is extracted from the Chinese herb ge-Gen (*Radix puerariae*), has been found to downregulate VEGF and HIF-1 α expression for up to 5 months following administration to DR rats (194).

Other molecules also appear to play important but poorly understood roles in OIR. Dioum *et al.* (47) have shown that HIF-2 α haploinsufficient mice exhibited decreased expression of EPO, VEGF, and Ang-2 proteins, which are considered to be modulated by HIF-1 α alone. In light of this result, use of an HIF-1 α -specific inhibitor may not suffice in preventing OIR. Similarly, high plasma glucose levels have been shown by Xiao *et al.* (212) to stabilize HIF-1 α and VEGF, making it unlikely that DR can be mitigated in individuals unable to control their blood sugar. Finally, as IGF has been established to be essential by Chen *et al.* (31) for vascularization, targeting of

IGF may suffice in combating OIR without the need to alter systemic VEGF, EPO, and HIF levels. Although several pharmacological options are available for possible therapeutic interventions against OIR, choosing those compounds with the optimal efficacies and safety is highly crucial for human clinical trials. PHDIs for the treatment of retinopathy are summarized in Table 6.

Gastrointestinal diseases

IBD comprises two very distinct pathologies: Crohn's disease (CD) and ulcerative colitis (UC). While both diseases cause intestinal wall inflammation, the CD-induced inflammatory response extends from the mucosa to the serosa (transmural involvement) while UC-mediated inflammation is limited only to the mucosa. Among populations in Northern Europe and North America CD affects 1–6 and 10–100 per 100,000 individuals, respectively, while UC affects 2–10 and 35–100 per 100,000 (92). Both IBDs are known to be genetically transmitted. CD, widely regarded as an autoimmune disease, especially occurs with high frequency among Ashkenazi Jews, particularly in first-degree relatives of the affected individuals, such as identical twins (165). However, it is becoming increasingly evident that genetic predisposition alone does not cause the disease without some environmental trigger. Bacterial infections, smoking, and the use of nonsteroidal anti-inflammatory drugs are commonly associated with disease onset (92). Current treatment involves glucocorticoids, cyclosporine, and methotrexate, with corticosteroids being used to control flare ups of the disease. The latter group of pharmaceuticals has been linked with a high complication rate when used chronically (139). Given the persistent nature of IBD, lack of an effective treatment has led investigators to seek PHD-inhibition therapy as a possible therapeutic strategy for alleviating IBD-mediated inflammation. So far, only two clinical trials are reported, and both of them have shown efficacy in attenuating IBD with no reported adverse effects.

Cummins *et al.* (40) induced colitis in mice through oral administration of dextran-sodium sulfate to closely mimic human UC. Mice were dosed with DMOG (8 mg in phosphate-buffered saline, intraperitoneal) every second day following IC induction, and the intestinal epithelial cells were collected for an *in vitro* study that continued for 8 days. Western blotting confirmed upregulation of the p65 subunit of NFκB and also upregulation of HIF-1α. Animals subjected to hypoxia exhibited upregulation of each protein within 3 h of exposure, and the maximal extent of upregulation of each protein was reached at 18 h posthypoxia. As testament to the potential of DMOG therapy, the drug-treated mice presented with elevated levels of the NFκB within 15 min of treatment. Further, decreases over twofold were witnessed in levels of inflammatory markers, including MPO, IL-1β, TNF-α, IL-6, and IL-12. Approximately a twofold decrease in epithelial cell apoptosis was observed without a decrease in colon length of DMOG-treated mice. Most importantly, this study confirmed that the inflamed tissue had been hypoxic, suggesting the potential use of PHD-inhibition therapy for inflammatory conditions of the colon (40). Given the improved recovery time observed in DMOG-treated mice, further investigations on DMOG therapy for potential clinical use in IBD are warranted.

TABLE 6. PHARMACOLOGICAL PROLYL HYDROXYLASE DOMAIN INHIBITORS FOR TREATMENT OF RETINOPATHY

Name of compound	Animal model	Species	Mechanism	Interactive molecules	Reported outcome	Reference
DMOG	Phase I ROP in retinal Müller cells <i>in vitro</i>	<i>H. sapiens</i>	PHD inhibition (PHDI)	HIF-1α, HIF-2α	HIF stabilization	Sears <i>et al.</i> (169)
DMOG	Murine hyperoxia <i>in vivo</i>	<i>M. musculus</i>	PHD inhibition (PHDI)	HIF-1α, HIF-2α, VEGF, EPO	Decrease in capillary drop-out, vascular tortuosity and tufting	Sears <i>et al.</i> (169)
EPO	Murine Phase I ROP <i>in vivo</i>	<i>M. musculus</i>	EPO upregulation	EPO, NFκB	Decreased apoptosis, vessel obliteration	Chen <i>et al.</i> (29)
HIF-1 siRNA	Murine Phase II ROP <i>in vivo</i> , human umbilical endothelial cells <i>in vitro</i>	<i>M. musculus</i> , <i>H. sapiens</i>	HIF-1α inhibition	HIF-1α	Decreased neovascularization	Jiang <i>et al.</i> (86)
VEGF siRNA	Murine Phase II ROP <i>in vivo</i> , human umbilical endothelial cells <i>in vitro</i>	<i>M. musculus</i> , <i>H. sapiens</i>	VEGF inhibition	VEGF	Decreased neovascularization	Jiang <i>et al.</i> (86)
EPO siRNA	Murine Phase II ROP <i>in vivo</i>	<i>M. musculus</i>	EPO inhibition	EPO	Decreased neovascularization	Chen <i>et al.</i> (30)
Aminoguanidine hemisulfate	Murine Phase II ROP <i>in vivo</i>	<i>M. musculus</i>	iNOS inhibition	HIF-1α, iNOS, VEGF, PI3K/Akt	PI3K/Akt also modulates HIF-1α, decreased neovascularization	He <i>et al.</i> (69)
Deguelin	Phase II ROP human endothelial cells <i>in vitro</i>	<i>H. sapiens</i>	HIF-1α degradation	HIF-1α	Suppressed endothelial cell proliferation. Downregulated VEGF	Kim <i>et al.</i> (94)
Puerarin	DR rat <i>in vivo</i>	<i>R. norvegicus</i>	HIF-1α inhibition	HIF-1α, VEGF	Downregulation of HIF-1α, VEGF	Teng <i>et al.</i> (194)

DR, diabetic retinopathy; iNOS, inducible nitric oxide synthase; NFκB, nuclear factor kappa B; PI3K/Akt, phosphatidylinositol 3-kinase/Akt pathway; ROP, retinopathy of prematurity.

Robinson *et al.* (158) investigated the efficacies of the FibroGen compounds FG-4497 and FG-4442 in attenuating the colitis endpoints. The study revealed that TNF α mRNA expression was attenuated by 70% compared with untreated mice. To confirm that this increase was due to upregulation of HIF-1 α through involvement of PHD inhibition, this study demonstrated that intravenous or oral administration of both of the compounds did not attenuate expression of TNF α in HIF-1 $^{-/-}$ mice. FG-4497 was more potent than FG-4442 in attenuating inflammation. A 5 μ M dose of FG-4497 (administered at a 60 mg/kg dose) exhibited the same effect as a 100 μ M dose of FG-4442. Stabilization of HIF-1 α was also confirmed by western blotting following *in vitro* exposure of the cells to FG-4497. An increase in levels of EPO (150-fold) was observed, along with significant increases in the Hb levels and % hematocrit. Additionally, attenuation of overall weight loss and improved maintenance of colon length in the FG-4497-administered mice was evident. Although, toxicity of FG-4497 has not been reported, the compound has been shown to inhibit collagen prolyl-4-hydroxylase activity in mice, indicating that higher specificity for the hypoxic PHDs is needed for the clinical use of FG-4497 (158).

These studies have elucidated the potential of PHD inhibition therapy toward treatment of IBD, but this therapeutic strategy must thoroughly be tested before extending it to clinical scenarios. Still, initial studies are in favor of PHD inhibition therapy of IBD and have demonstrated the potential for HIF-1 α stabilization through regulation of PHD inhibition for effective management of colitis. The similarities between UC and CD suggest that PHD inhibition therapy also could be useful in treating CD, but extensive testing is needed before transitioning PHD-inhibition therapy to the clinic for treatment of these conditions. PHDI for treatment of the gastrointestinal diseases are summarized in Table 7.

Cancer: the need to enhance PHD activity

The substantial elevation of VEGF expression attained with HIF-1 α stability has been shown to enhance angiogenesis, thereby promoting blood supply to neoplastic tissue and augmenting tumorigenesis and progression of different types of cancer (117, 172). Additionally, increased GLUT1 and GLUT3 translocation has been shown to enhance glycolysis, enabling cancer cell survival (114). The antiapoptotic effectors of HIF-1 α also have been shown to improve tumor cell viability in all but ovarian cancers due to the elevated expression

of the p53 mutant, so it has been suggested that facilitating HIF degradation could serve as a novel approach to halting tumorigenesis.

Semenza (172) outlined the HIF-1 α inhibitory compounds that are currently in use or under development for protection against cancer metastasis. The compound 2ME2 appears to be highly effective in causing inhibition of HIF-1 α , and clinical trials on the use of 2ME2 for cancer therapy are ongoing (113). Similarly, camptothecin and topotecan have been shown to attenuate angiogenesis in cancer patients and, currently, have been approved by the U.S. Food and Drug Administration (FDA) as cancer-treatment drugs (149, 155). The thioredoxin-1 (Trx-1) inhibitor 1-methylpropyl-2-imadazolyl disulfide and the aldolase/enolase inhibitor YC-1 also have been shown to arrest angiogenesis in cancer patients, but further testing is required to determine whether these drugs can be administered safely for treating certain cancers. Unlike the PHDIs, these compounds are cytotoxic at clinically relevant doses. After completion of Phase I clinical trials, the initially promising compound 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) failed to attenuate angiogenesis in cancer patients at a safe dosage and induced dyspnea, and its benefits also were negated by ocular and hepatotoxicity (160).

The small molecules discussed in this review have shown the potential to alter HIF-1 α stability and/or inhibit its expression. The use of these molecules could augment the multiple pharmacological compounds in use that inhibit the downstream pathways of HIF-1 α . Currently, imatinib (Gleevec) and trastuzumab (Herceptin) are drugs that target various RTKs, and Bay 43-9006, CCI-779, and Celebrex are undergoing clinical trials (172) to see how they modulate HIF-dependent pathways. With the increasing worldwide incidences of cancer, development of HIF-1 α -specific inhibitors continues to receive substantial attention.

Reversing PHDIs. There are certain agents that actually promote PHD activity either directly or by inhibiting the effects of PHDIs. This molecule may be important in combination therapies. A good example is curcumin, which is derived from turmeric. In human prostate cell lines, enzyme-linked immunosorbent assay (ELISA) and transient gene expression assays elucidated the role of curcumin in blocking the activation of L-mimosine and DMOG treatment, both of which lead to prostate-specific antigen (PSA) expression. Since PSA is modulated by HIF-1 α , this suggests that curcumin promotes PHD activity (36). Another naturally occurring

TABLE 7. PHARMACOLOGICAL PROLYL HYDROXYLASE DOMAIN INHIBITORS FOR TREATMENT OF GASTROINTESTINAL DISEASES

Name of compound	Animal model	Species	Mechanism	Interactive molecules	Reported outcome	Reference
DMOG	Murine UC <i>in vitro</i>	<i>M. musculus</i>	PHD inhibition (PHDI)	HIF-1 α , p65, NF κ B, MPO, IL-1 β , TNF α , IL-6, IL-12	Reduced apoptosis, inflammation. Normal colon length	Cummins <i>et al.</i> (40)
FG-4497	Murine UC <i>in vivo</i>	<i>M. musculus</i>	PHD inhibition (PHDI)	HIF-1 α , EPO, Hb	Attenuated weight loss, maintenance of colon length	Robinson <i>et al.</i> (158)
FG-4442	Murine UC <i>in vivo</i>	<i>M. musculus</i>	PHD inhibition (PHDI)	HIF-1 α , EPO, Hb	Attenuated weight loss, maintenance of colon length	Robinson <i>et al.</i> (158)

IL, interleukin; UC, ulcerative colitis.

substance wogonin has a PHD-like effect that may be helpful in cancer therapy. Wogonin is a plant-derived flavone and may have a role in the modulation of the HIF-1-related oxygen sensor. Wogonin increases degradation of HIF-1 α similar to the PHD system (184, 201).

Combination therapy of PHDIs as treatment strategy. Since PHDs and HIFs have multisystemic and complex effects, it is sometimes necessary to use a PHDI in combination with other agents to fine-tune the therapeutic response. For example, the use of AKB-6899, a selective PHD3 inhibitor, works *via* production of granulocyte-macrophage colony-stimulating factor (GM-CSF), and combination therapy with AKB-6899 and GM-CSF enhances the effects of the drug (159). Combination therapy of PHDI with other medications such as Keap1 inhibitor may be a new approach for treatment of diabetic nephropathy and other diseases with similar pathophysiology (126).

Cautionary note. There is not much information available on resistance or tolerance to PHD inhibitors upon prolonged use. Phase II clinical trials for some of the drugs are in their early stages, and most studies are measuring short-term outcomes. Additionally, there are differences between genetic inactivation of PHDs, responses to hypoxia, and responses to a pharmacological inhibitor, which are important to distinguish in predicting the effects of therapeutic modulation of the HIF hydroxylase system on different physiological responses (17). The 2-oxoglutarate analogs may be promising tools for stimulation of erythropoiesis and angiogenesis. However, since the HIF system promotes the transcription of many genes, other 2-oxoglutarate-dependent dioxygenases (especially the C-P4H class of enzymes) might be targeted by these PHDIs, so careful examination of these compounds is essential prior to clinical use (21, 123). One must also consider that the benefits conveyed by these compounds might arise from unknown pathways that function independently from the HIF system (68).

Future Challenges in the Development of Effective PHD-Inhibition Therapy for Ischemic Diseases

While pursuing the use of PHDIs as therapeutic drugs, many factors must be considered before clinical application. As discussed in the current review, optimal timing of drug administration is essential to assure that PHDI therapy attenuates the disease symptom/condition as opposed to augmenting the target mechanisms involved in disease onset or progression. Additional care must be taken while using PHDIs since these drugs enhance angiogenesis, which is protective in cardiovascular, neuronal, gastrointestinal, renal, ocular, and anemic disease conditions but can promote tumorigenesis.

The most crucial factor in developing an effective PHDI therapy is the choice of compounds that specifically inhibit the targeted PHD isoform(s), leading to the regulation of hypoxia but not activating the almost identical collagen family of PHD proteins. Despite large differences in specificity, the higher-order structures of the collagen PHD isoforms match those of the hypoxia PHDs in both confirmation and active site residues, with the only exception being that lysine, not arginine, binds with 2-oxoglutarate in the collagen family enzymes

(128). Without activity of the collagen PHD isoforms, hydroxylation of proline to hydroxyproline will not occur, leading to the inhibition of formation of stable type I and type II procollagen, which are the essential peptides in fibrosis and osteoclastogenesis. An isoform-specific approach, therefore, is needed to ameliorate ischemia without damaging the other tissues required for normal organ function.

The tradeoff between attenuation of disease and adverse side effects caused by the PHDIs becomes the major issue facing the introduction of PHDI therapy in clinical scenarios. The low toxicity and high specificity of the PHDIs discussed in the current review indicate that this therapeutic strategy is a promising and possible clinical therapeutic option in the near future. Thus PHDIs appear to provide an alternative therapeutic intervention for the treatment and management of a wide variety of pathophysiological conditions and disease states.

Conclusion

In the current review, we have discussed the efficacy of small compounds and siRNA as PHDIs for the treatment of diverse ischemic diseases. Upregulation of HIF-1 α and HIF-2 α enhances the angiogenic, glycolytic, erythropoietic, and anti-apoptotic pathways in the treatment of CVDs, cerebral ischemia, IBD, renal failure, anemia, diabetes, and Phase I ROP. Side effects as reported appear limited, even when compounds were administered at concentrations far exceeding typical pharmacological doses. Given the apparent safety of the PHDIs as revealed in murine, rat, and primate models and the rise in IR diseases (particularly associated with type II DM and CVD), it is imperative that clinical trials be expedited to develop novel treatments for IR injury.

Although all of the PHDIs discussed in the current review have shown potential as therapeutic drugs for ischemic diseases, the 2-oxoglutarate analogs appear to be safer and more effective than the iron chelators. In spite of the observations that the chelators, including DFO, L-mimosine, and Compound A, sufficiently decrease the intracellular iron concentration to inhibit PHD activity, a depletion of iron also augments anemia and suppresses erythropoiesis by limiting iron binding with Hb (182). As downregulation of the TCA cycle attenuates IR insult, a decrease in 2-oxoglutarate (α -ketoglutarate) levels does not seem to result in the toxicity of the iron chelators, suggesting that these PHDIs may be transitioned to human clinical trials.

Even with the potential that these drugs show for combating hypoxic diseases, the development of these PHDIs for therapeutic use will be time consuming and will present many challenges. Localization of PHD activity to the ischemic tissue area, tight control of the timing of administration of the PHDI, and the complex pharmacokinetics of the inhibitors are highly crucial for the development of PHDI therapeutics. PHD inhibition augments cancer by enabling angiogenesis and erythropoiesis around the tumors and introduces risk of tumor metastasis. Therefore, it is possible that future work will show that the risks of the PHDIs outweigh their benefit. Additionally, prolonged PHD inhibition has the potential to introduce hyperoxia to previously hypoxic areas, resulting in excessive angiogenesis. One of the major challenges is to choose the appropriate timing of administration of these inhibitors along the timeline of the ischemic events in order to

optimize the efficacies of the inhibitors toward attenuating permanent tissue injury. In addition, the agents used to deliver the inhibitor to the affected areas also may present significant obstacles. Compounds administered systemically may not reach target tissues, or systemic administration may cause unwanted side effects. However, despite these challenges in transitioning PHDIs from the bench to the bedside, it is believed that the novel PHDI therapies appear highly promising in advancing the current state of healthcare and providing effective treatments for many common hypoxic and IR injury and disease states.

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Author Disclosure Statement

The authors declare no conflicts of interest.

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Abbreviations Used

17-AAG = 17-N-allylamino-17-demethoxygeldanamycin
 2ME2 = 2-methoxyestradiol
 Akt = RAC- α serine/threonine-protein kinase
 AMPA = α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
 Ang-1 = angiopoietin-1
 ARF = acute renal failure
 ATF4 = activating transcription factor 4
 ATP = adenosine triphosphate
 BBB = blood–brain barrier
 Bcl-2 = b-cell lymphoma-2 protein
 BH3 = BH3 domain
 BHLH = basic helix-loop-helix
 BNIP3 = Bcl2-adenovirus E1B 19kDa interacting protein 3
 CBP = CREB-binding protein
 CD = Crohn's disease
 CHCHD = coiled-coil-helix-coiled-coil-helix domain containing 4
 CLA = conjugated linoleic acid
 CO = carbon monoxide
 CoCl₂ = cobalt chloride
 CVD = cardiovascular disease
 DCYTB = duodenal cytochrome b
 DFO = desferoxamine
 DHB = 3,4-dihydroxybenzoate
 DM = diabetes mellitus
 DMOG = dimethylloxaloylglycine
 DMSO = dimethyl sulfoxide
 DMT1 = divalent metal-ion transporter
 DNA = deoxyribonucleic acid
 DP = 2,2-dipyridyl
 DR = diabetic retinopathy
 EC = endothelial cell
 ECM = extracellular matrix
 EF = ejection fraction
 EGF = epidermal growth factor
 EGLN-1 = egl nine homolog 1
 EGLN-2 = egl nine homolog 2
 Eif-4E = eukaryotic initiation factor-4E
 Eif-BP1 = eukaryotic initiation factor binding protein-1
 ELISA = enzyme-linked immunosorbent assay
 eNOS = endothelial nitric oxide synthase
 EPO = erythropoietin
 ER = endoplasmic reticulum
 ERK = MAP kinase kinase kinase
 ETC = electron transport chain

Abbreviations Used (Cont.)

FDA = Food and Drug Administration
 FGF = fibroblast growth factor
 fHb = fetal hemoglobin
 FIH = factor inhibiting HIF
 Flk = a VEGF receptor tyrosine kinase (VEGFR-2)
 Flt-1 = a VEGF receptor tyrosine kinase (VEGFR-1)
 GLUT = glucose transporter
 GM-CSF = granulocyte-macrophage colony-stimulating factor
 GSH = glutathione
 H₂O₂ = hydrogen peroxide
 Hb = hemoglobin
 HD = hemodialysis
 HIF = hypoxia inducible factor
 HO-1 = heme oxygenase-1
 HRE = hypoxia response element
 HUVEC = human umbilical vein endothelial cell
 IBD = inflammatory bowel disease
 IGF = insulin-like growth factor
 IKK β = inhibitor of NF κ -B kinase subunit β
 IL = interleukin
 ING4 = inhibitor of growth family, member 4
 iNOS = inducible nitric oxide synthase
 IR = ischemia/reperfusion
 IRE = iron response element
 IRP = iron response protein
 IRP2 = iron regulatory protein-2
 L-NMMA = N(G)-monomethyl L-arginine
 LPS = lipopolysaccharide
 MAPK = mitogen-activated protein kinase pathway
 MEK = MAP kinase kinase
 MI = myocardial infarction
 MIP = macrophage inflammatory factor
 MMP = matrix metalloproteinase
 MNK = MAP kinase kinase kinase
 MORG-1 = mitogen-activated protein kinase organizer-1
 mRNA = messenger ribonucleic acid
 mTOR = mammalian target of rapamycin
 NAC = N-acetyl-L-cysteine
 NADPH = nicotinamide adenine dinucleotide phosphate
 NEMO = nuclear factor- κ B essential modulator
 NF κ B = nuclear factor kappa B
 NGF = neuronal growth factor
 NO = nitric oxide
 ODD = oxygen-dependent degradation domain

OIRs = oxygen-induced retinopathies
 p300 = E1A binding protein p300
 P4H = prolyl 4-hydroxylase
 PAI-1 = plasminogen activator inhibitor-1
 PAS = per-ARNT-sim
 PCBP = poly(rC) binding protein
 PCR = polymerase chain reaction
 PDK = pyruvate dehydrogenase kinase
 PFK = phosphofructokinase
 PHD = prolyl hydroxylase domain
 PHDI = prolyl hydroxylase domain inhibitor/inhibition
 PI3K = phosphatidylinositol 3-kinase
 PI3K/Akt = phosphatidylinositol 3-kinase/Akt pathway
 PK = pyruvate kinase
 PKM2 = PK muscle isoform 2
 pO₂ = partial pressure of oxygen
 PSA = prostate-specific antigen
 pVHL = von Hippel-Lindau protein
 RBC = red blood cell (erythrocyte)
 RNA = ribonucleic acid
 RNA pol II = RNA polymerase II
 RNS = reactive nitrogen species
 ROP = retinopathy of prematurity
 ROS = reactive oxygen species
 RTK = receptor tyrosine kinase
 RTP801 = a gene that suppresses mTOR
 S/T kinase = serine/threonine kinase
 shRNA = small hairpin RNA
 siRNA = small interfering ribonucleic acid
 SMCs = smooth muscle cells
 SRC-1 = steroid receptor coactivator 1
 STEAP3 = reductase
 TAD = transactivation domain
 TCA = tricarboxylic acid
 TGF- β = transforming growth factor- β
 Tie = tyrosine kinase with immunoglobulin-like and EGF-like domains
 TNF = tumor necrosis factor
 TPI = triose phosphate isomerase
 Trf = transferrin receptor
 TRPA1 = transient receptor potential cation channel member A1
 Trx-1 = thioredoxin-1
 UC = ulcerative colitis
 VEGF = vascular endothelial growth factor
 VEGFR = vascular endothelial growth factor receptor