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Prolyl Hydroxylase Domain Inhibitors: A Route to HIF Activation and Neuroprotection

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Abstract

Ischemic stroke is a major cause of death worldwide, and current therapeutic options are very limited. Preconditioning with an ischemic or hypoxic insult is beneficial in experimental models of ischemic stroke. Ischemia/hypoxia results in activation of numerous transcription factors, including hypoxia inducible factor (HIF), which is a master regulator of oxygen homeostasis. HIF activation induces a diverse range of target genes, encompassing a wide variety of cellular processes; including angiogenesis, energy metabolism, cell survival, radical production/scavenging, iron metabolism, stem cell homing, and differentiation. Inhibition of HIF prolyl hydroxylase domain (PHD) enzymes results in activation of HIF and is likely to mimic, at least in part, the effects of hypoxia preconditioning. A caveat is that not all consequences of HIF activation will be beneficial and some could even be deleterious. Nevertheless, PHD inhibitors may be therapeutically useful in the treatment of stroke. Prototype PHD inhibitors have shown promising results in preclinical models. *Antioxid. Redox Signal.* 12, 459–480.

Introduction

Stroke is a major cause of death worldwide and the third leading cause of death in the United States (133). Strokes are caused by interruption of the cerebral blood supply. Approximately 80% of cerebral strokes are ischemic strokes, which occur when the arterial blood supply to part of the brain is interrupted by a thrombus, embolus, or other blockage. The remaining 20% of strokes are caused by rupture of an artery within the brain and are classified as hemorrhagic strokes (56). Cerebral strokes result in cell death of neurons, glia, and other cell types, often including the vascular cells. For patients surviving stroke, the long-term outcome may involve permanent physical and mental disabilities and a compromised quality of life. Diabetes, smoking, and hypertension have been identified as important risk factors for stroke (73). Current treatment options remain limited and rehabilitation is costly and often only partially effective.

Cerebral ischemia results in reduced delivery of oxygen, glucose, and nutrients to the brain that are critical for cell survival. Since the brain consumes a large quantity of oxygen, it is particularly susceptible to oxygen shortages. In the context of acute stroke, two complementary approaches are to re-establish perfusion while cells are still viable, and to protect compromised cells and circuits from irreversible damage.

Preconditioning in experimental rodent models, using a short, sublethal ischemic exposure has been shown to be protective against a subsequent more severe insult in a number of organs, including the brain, heart, and kidney (69, 71, 110, 125, 138, 273). There has been considerable interest in understanding the mechanisms underlying preconditioning in the hope that this will lead to pharmacological routes to promoting cellular survival. Hypoxemia is also an effective preconditioning stimulus, which is likely to be less complex in terms of the pathways that it activates (70, 167, 198). Many cellular responses to hypoxia are mediated through a transcription control complex termed hypoxia-inducible factor (HIF). HIF activation results in broad changes in gene expression that can be regarded as adapting the cell, tissue, and organism to conditions of reduced oxygen. Taken together, these observations have suggested that HIF might be involved in mediating the beneficial effects of preconditioning, and that pharmacological activation of HIF may be beneficial in stroke and other ischemic conditions.

Prolyl hydroxylase domain enzymes (PHD enzymes; also known as HIF prolyl hydroxylases, HPH and Egg-laying deficient nine, EGLN) act as oxygen sensors controlling the HIF degradation pathway (51). Inhibition of PHD enzymes results in upregulation of HIF and subsequently HIF target genes that may contribute to cell survival in the nervous system. Animal studies have suggested that PHD inhibitors

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may be useful in protecting the brain in ischemia (18, 195). This review will focus on the molecular mechanisms through which ischemia causes neuronal cell death, how PHD inhibitors may promote tolerance to ischemic insults, and will also address potential effects of PHD inhibitors on permeability of the blood-brain barrier (BBB).

Molecular Mechanims of Cell Death in Cerebral Ischemia

Acute cerebral ischemia results in a severely damaged ischemic core, termed the infarct zone, surrounded by a rim or penumbra of viable cells, which are functionally impaired but not morphologically damaged (116). Penumbral cells can potentially be salvaged if cerebral blood flow is restored rapidly, although reperfusion itself is associated with a number of changes including free radical production and inflammation that may result in further damage (11). Since energy is essential for cell survival, penumbral cells are at risk of death if ischemia is prolonged. Imaging studies analysing the progression of acute stroke show that during the initial hours the size of the ischemic core increases into the penumbra, until the penumbra disappears (91). Since infarct volume is correlated with long-term stroke outcome, therapeutic strategies improving viability of penumbral cells are desirable (209). Understanding the mechanisms underlying cell damage and death following stroke is likely to be important in designing treatments which promote cell survival.

The excitotoxic cascade

During normal neuronal signalling electrical signals, known as action potentials, pass along the axons of neurons. Chemical signaling or neurotransmission allows intercellular communication to occur between neurons at synapses. The presence of an action potential in the axon terminal causes voltage-sensitive calcium channels in the neuronal membrane to open. The resulting calcium influx into the neuron triggers the release of neurotransmitters, such as glutamate, from storage vesicles into the synaptic cleft. These neurotransmitters then bind to receptors, such as NMDA (N-methyl-D-aspartate) or AMPA (2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) proprionate) located on ion channels in the postsynaptic neuron, resulting in uptake of sodium and calcium ions. Neurotransmitter is removed from the synaptic cleft via re-uptake transporters on the presynaptic neuron to prevent hyperactivation (Fig. 1) (77).

During hypoxia and/or hypoglycaemia, energy shortages affect the ability of neurons to maintain their membrane potential, leading to inappropriate depolarization of neurons and excessive release of glutamate into the synaptic cleft (114). In addition, reuptake of excess neurotransmitter from the synaptic cleft is energy dependent and is known to be impaired during hypoxia. Excitatory neurotransmitter overload results in hyperactivation of postsynaptic neurons and can ultimately lead to postsynaptic cell death (203). This pathway is referred to as the excitotoxic cascade. Elevated intracellular calcium induces a number of pathological signalling cascades which can contribute to neuronal cell death. These pathways involve proteases, (*e.g.*, calpain), phospholipases (*e.g.*, phospholipase A2), calcium binding proteins (*e.g.*, calmodulin), and endonucleases (Fig. 1) (236).

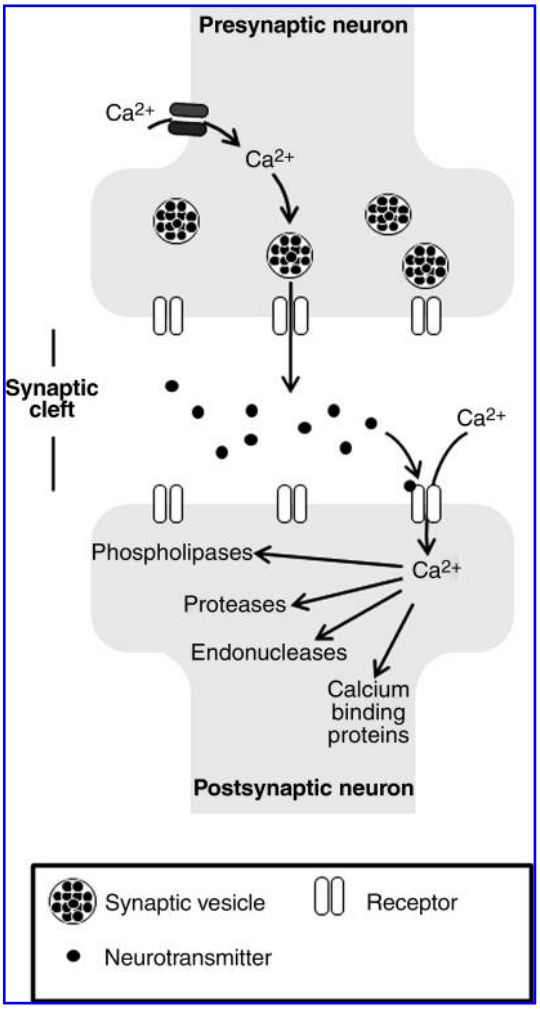

FIG. 1. The excitotoxic cascade. During hypoxia, neurons are unable to maintain their action potential. This leads to uncontrolled calcium signaling that can ultimately be toxic.

Reactive oxygen and nitrogen species (free radical damage)

Free radicals are highly reactive species that can react indiscriminately with cellular components to cause damage. Studies of mice deficient in radical scavenging enzymes have shown worse outcome in acute ischemic stroke models (127, 175). Reactive oxygen and nitrogen species (ROS/RNS) are generated during both ischemia and on reperfusion if this is achieved. Restoration of blood flow to the ischemic area provides oxygen that can act as a substrate in several radical generating enzymatic reactions. Sources of free radicals during ischemia/reperfusion include leakage from the mitochondrial electron transport chain, xanthine oxidase, nitric oxide synthase (NOS), and arachidonic acid metabolism (21, 188). Ischemia is also associated with decreased intracellular pH, which can lead to increases in transition metals available for participation in radical-generating Fenton reactions (223). Cerebral lipids are enriched in polyunsaturated fatty acids and as such are particularly vulnerable to free radical-mediated lipid peroxidation and membrane damage. In addition to being a source of free radicals, mitochondria are also

targeted by ROS, forming a deleterious feed forward loop. Radicals react with the inner mitochondrial membrane and electron transport chain proteins, resulting in further disruption of mitochondrial respiration and exacerbating the cellular energy crisis (21). DNA can also act as a substrate for free radical attack, causing strand breaks and DNA base deamination, which in turn can trigger caspase-dependent apoptosis (50). Cellular free radical injury can culminate in both apoptotic and necrotic cell death.

Inflammation

Microglia, the resident macrophages of the central nervous system (CNS), are key players in the immune response of the brain. Cerebral ischemia leads to rapid activation of microglia, which proliferate and migrate to the area of injury (130). Under normal conditions the brain is considered an immune privileged site, with the BBB preventing entry of immune cells from the systemic circulation. During ischemia, activated microglia release matrix metalloproteinases, cathepsins, plasminogen activators, and free radicals, which all contribute to BBB breakdown (206). Proinflammatory cytokines [interleukin 1, interleukin 5, tumor necrosis factor α (TNF-α)] are also released from activated microglia and stimulate an influx of leukocytes across the BBB into the brain (99). Reperfusion also contributes to the inflammatory response transporting inflammatory cells to the damaged area (206). The role of inflammation in stroke remains controversial, nonetheless considerable evidence now suggests its involvement in a second wave of injury. Infiltrating cells release more cytotoxic substances and proinflammatory cytokines that further damage the BBB. Moreover adhesion of leukocytes to endothelial cells can cause microvascular occlusion, resulting in additional ischemic stress (99). Some animal studies have reported that limiting reperfusion following stroke is beneficial in comparison to complete restoration of blood flow (275). This is attributed to limiting the inflammatory response and free radical generation which is associated with reperfusion.

Delayed neuronal death

Several reports have demonstrated that CA1 hippocampal neurons are particularly vulnerable to cell death following ischemia (65, 123, 191, 210). In a phenomenon termed delayed neuronal death, cells that appear morphologically normal during the initial 2 day period following an ischemic insult in gerbils begin to show clumping of the nuclear chromatin and accumulation of nonmembrane dense structures during the following 2 days, culminating in cell death (122). Delayed neuronal death has also been observed in rat and mouse models of ischemia (43, 49, 197). The mechanisms underlying delayed neuronal death remain uncertain. The excitotoxic cascade may contribute, however the suppression of calcium or glutamate or the use of NMDA blockers is only partially effective at preventing cell death (49). The presence of apoptotic bodies and activation of caspase 3 is consistent with cell death via an apoptotic pathway, rather than necrosis (148, 178). Furthermore, caspase inhibitors were beneficial in reducing delayed neuronal death in mice (49). Loss of trophic support from surrounding cells may also adversely affect neurons which are projecting into the damaged area. Treatment of primary rat hippocampal and cortical cells with serum has been demonstrated to abrogate an *in vitro* model of delayed neuronal death and trophic cytokines are also beneficial in limiting delayed neuronal death *in vivo* (100, 134, 217, 250)

Current Treatment Options/Strategies Under Investigation for Cerebral Ischemia

Literally hundreds of experimental treatments that target many different aspects of stroke pathology have been investigated in the quest to improve patient outcome following stroke (72). Unfortunately, despite these efforts, the impact on patient outcomes has been very limited. A selection of these is highlighted here, along with a brief discussion of their molecular basis.

Drugs to restore or improve blood flow

The development of therapeutics that enhance cerebral blood flow has been pursued as a treatment for stroke, since timely restoration of blood flow to the affected area may be able to rescue cells in the penumbra before they undergo irreversible cellular damage. Currently tissue plasminogen activator (t-PA) is the only treatment approved for use in ischemic stroke by the United States Food and Drug administration (FDA). t-PA is a thrombolytic agent and functions to disrupt clots that may be occluding the blood supply.

Clinical trials testing the benefit of anticoagulants, such as heparin and warfarin, and antiplatelet agents, such as aspirin have also been performed. Antiplatelet agents, including aspirin, are known to be efficacious in a preventative context (1). Administration of aspirin following an acute ischemic stroke was also shown to have a small but significant effect on mortality at 6 months in the International Stroke Trial (IST) of 20,000 patients. In contrast, high dose heparin had no beneficial effect (2).

Drugs designed to restore or improve blood flow need to be administered early to be effective in limiting damage (*e.g.*, t-PA, within 3 h of stroke onset). It should also be noted that drugs that improve cerebral blood flow can also be associated with an increased risk of intracerebral bleeding. The adverse risk of hemorrhage increases as time to treatment increases. The narrow therapeutic window in terms of time from onset means that many patients suffering from ischemic stroke are not suitable for treatment with t-PA or other reperfusion agents. Moreover reperfusion therapies, including t-PA, are inappropriate for treatment of hemorrhagic stroke (76).

Neuroprotective agents

Several stroke treatment strategies have been investigated which target components of the excitotoxic cascade in an attempt to attenuate neuronal cell death.

Calcium channel blockers. Randomized, double-blind, placebo-controlled trials of calcium channel blockers, such as nimodipine (454 patients; Very Early Nimodipine Use in Stroke; VENUS) and flunarizine (subgroup of 60 patients; flunarizine in stroke treatment; FIST) showed no improvement in outcome of acute stroke at 3 months when administered <6 h after onset (57, 97). DP-b99, a calcium chelator, has been tested in phase II clinical trials of 150 patients, which showed an increased recovery rate, but no significant benefit in terms of outcome at 3 months (40).

Glutamate receptor antagonists. Trials of several glutamate receptor antagonists, such as CGS19755, GV150526, and aptiganel have shown no significant therapeutic benefit in humans, with many trials being terminated early (3). Adverse side effects, such as hallucinations, delirium, agitation, or cardiovascular effects, have also been reported for some glutamate receptor antagonists used for stroke and head trauma. A promising pilot study in 20 patients tested the safety of rapid administration of magnesium sulphate, which among other actions blocks voltage-gated calcium channels and NMDA receptors. This was well tolerated and showed improved outcome in 20% of patients (Field Administration of Stroke Therapy-Magnesium pilot study; FAST-Mag) (207). A larger trial to test efficacy is currently ongoing.

Free radical scavengers. Experimental therapies which detoxify or inhibit the formation of free radicals following cerebral ischemia are aimed at limiting the damage that these highly reactive species cause to cellular components such as DNA, protein, and lipids. Enhanced expression of free radical scavengers has been shown to be protective in animal models of acute ischemic stroke (247, 262). In human stroke trials, mixed results have been obtained with the lipid peroxidation inhibitor tirilizad mesylate (also known as Freedox). A US clinical trial (Randomized Trial of Tirilazad Mesylate in Acute stroke II; RANTTASII), involving 126 patients, showed a trend towards reduced mortality and improved independence (83). This trial was terminated early however, following concerns of increased mortality reported in a parallel European trial (Tirilazad Efficacy Stroke Study II; TESS II) (4). Harmokisane (ebselen), a seleno-organic compound with antioxidant activity, was used for treatment of acute ischemic stroke in a trial involving 300 patients. It reported improved outcome in patients treated <24 h after onset of stroke at both 1 and 3 months. No serious side effects were reported (261).

Summary

Current treatment options for ischemic stroke remain extremely limited and to date the results of most clinical trials have been disappointing. Stroke is heterogeneous and a stratified approach may reveal efficacy of tested treatments for some subtypes of stroke. However, the narrow therapeutic window limits the opportunity for detailed stroke diagnosis. There is considerable interest in trying to protect the ischemic penumbra, and the neuroprotective effects of ischemic/hypoxic preconditioning may offer clues as to how this could be achieved and the opportunity to develop novel therapeutic agents for stroke.

Preconditioning in the Brain

Preconditioning involves exposure to a stressful but sublethal stimulus that induces cytoprotective pathways that better equip the cell, tissue, and organism to deal with subsequent cellular stresses. The intensity, duration, and frequency of potentially stressful stimuli are important factors in determining whether a cellular stress is able to induce a state of tolerance or if it will be harmful. In the context of cerebral ischemia, several methods of preconditioning have been investigated, including hyperthermia, heat shock, oxidative stress, hypoxia, and ischemia itself (69).

Ischemic preconditioning

Ischemia refers to reduced blood supply to an organ. Ischemic preconditioning is neuroprotective in animal models of acute ischemic stroke (71, 125, 273). In humans, several reports have now shown that the occurrence of a transient ischemic attack, which is a brief period of cerebral ischemia, can induce a state of tolerance and protect the brain from the detrimental effects of a subsequent ischemic stroke (58, 180, 208, 246). Aspects of ischemic preconditioning rather than ischemia itself can also elicit ischemia tolerance. Examples include glutamate release, ROS, inflammatory cytokines, caspases, and hypoxia (147, 166, 182, 183).

Hypoxic preconditioning

Hypoxia, or low oxygen, is one consequence of ischemia and activates a subset of the pathways activated during ischemia. Gidday *et al*. first reported in 1994 that rat pups exposed to 3 h of hypoxia (8% oxygen) were protected against a hypoxia/ischemic insult 24 h later (carotid occlusion) (70). Similar studies have demonstrated the favorable effects of hypoxic preconditioning in adult rodents (167).

Understanding the mechanisms involved in ischemic/hypoxic preconditioning may provide new drugable targets for the treatment of stroke. Indeed several groups have focused their attention on uncovering these pathways. Both hypoxic and ischemic preconditioning appear to require synthesis of new RNA and protein, since hypoxia-induced tolerance is blocked by inhibitors of RNA and protein synthesis (62). A number of transcription factors have been identified which are upregulated in the brain following hypoxia, including metal transcription factor 1 (MTF-1), early growth response protein 1 (Egr-1), v-ets erythroblastosis virus E26 oncogene homolog 1 (avian) (ETS1), nuclear factor kappa B (NF-κB), activator protein 1 (AP1), and HIF (20, 118). Activation of some of these genes has been associated with establishing tolerance; HIF is perhaps the most well known and extensively studied hypoxia inducible transcription factor, and considerable evidence suggests a potential role in neuroprotection (see below). Other transcription factors may also contribute; for example, MTF-1 induces metallothionein1, which has been shown to be protective in animal models of cerebral ischemia (240). NF-κB has also been shown to be important in establishing tolerance to ischemia in both the brain (22) and heart (103, 164). NF-κB has been implicated in several neuroprotective pathways. Barger *et al*. showed that NF-κB attenuates glutamate induced excitotoxic cell death by suppressing increases in intracellular calcium levels (15). In addition, the NF-κB target gene manganese superoxide dismutase offers protection against free radical injury by suppressing peroxynitrite formation and membrane lipid peroxidation (163). NF-κB has also been shown to induce expression of brain-derived neurotrophic factor and the anti-apoptotic proteins Bcl-2 and Bcl-x (231). However, it remains unclear whether the predominant consequence of activating NF-κB is beneficial, since it is also known to be involved in the inflammatory response following ischemia.

HIF in the CNS

Hypoxia-inducible factors (HIFs) act as key mediators of adaptive responses to low oxygen (25, 243). HIF is a hetero-

dimeric transcription factor composed of an oxygen-regulated α subunit and a constitutively expressed β subunit. When oxygen is present, a ubuiquitin E3 ligase complex containing the von Hippel–Lindau protein (VHL) binds to HIF-α, targeting it for proteasomal degradation. However, during hypoxia this interaction is prevented, allowing HIF-α to accumulate in the cell. Besides regulation by low oxygen, other signaling pathways modulate HIF activation. Several studies provide evidence for a role of mitochondrial ROS in cellular oxygen sensing. Thus it has been reported that hypoxia increases the production of ROS by the mitochondria, and that this is necessary and sufficient to stabilize HIF, in at least some cell types (27, 29, 30, 81, 156).

Three isoforms of the HIF-α subunit are known, HIF-1α, HIF-2α, and HIF-3α. Of the HIF-α subunits, HIF-1α and HIF-2α are the best characterized and both are able to bind and transactivate hypoxia response elements (HREs) and promote gene transcription (48, 234). Immunohistochemical analysis of mice exposed to global hypoxia showed expression of HIF-1α in neuronal cells of the cerebral cortex and granular layer of the dentate gyrus and the hippocampus proper, as well as Purkinje cells in the cerebellum (226). In a similar experiment, rats exposed to 8% oxygen were analyzed for HIF-2α expression. The endothelial cells of the cortex, hippocampus, and cerebellum, and glial cells including astrocytes showed expression of HIF-2α, however no expression was detected in neuronal cells (253). The third α-isoform, HIF-3α, was identified more recently and its role is yet to be fully characterized (78). One splice variant of HIF-3α, inhibitory PAS domain protein (IPAS), lacks the C-terminal transcriptional activation domain and therefore is able to function as a dominant negative inhibitor of HRE dependent transcription. IPAS is expressed only in selected tissues (Purkinje cells of the cerebellum and in the corneal epithelium of the eye) and may function in a negative feedback loop to repress the function of HIF during hypoxia (154).

HIF Target Genes That Could Contribute to Neuroprotection

A number of HIF target genes have been identified that are induced in hypoxia in the CNS and may contribute to neuroprotection. Indeed the efficacy of several HIF targets, such as erythropoietin (EPO) and heme oxygenase 1 (HO-1), has been tested in pre-clinical models of stroke. The following section discusses a limited selection of the genes known to be activated by HIF, highlighting some of the evidence which supports their role in neuroprotection.

Erythropoietin (EPO)

EPO is a circulating glycoprotein hormone that is predominantly synthesized in the kidney and liver; its primary role is in the stimulation of erythrocyte production. In fact, EPO is the prototypic HIF responsive gene, since it was investigation of the mechanism underlying increased expression of EPO in response to low oxygen that led to the identification of HIF (215). Genetic defects have been identified in *VHL* (hypomorphic allele), *PHD2* (haploinsufficiency), and *HIF-2α* (activating mutation), which are associated with excess EPO production in patients (9, 63, 135, 189, 190). Recombinant EPO has been highly effective in treating the anemia of chronic renal failure, which is associated with relative EPO deficiency (158).

The brain expresses both EPO and the EPO receptor, and preclinical and preliminary clinical data indicate that it may also be useful in the treatment of ischemic CNS injuries. Studies of human and rat brain show that cerebral EPO is produced by both neuronal and glial cells and that neurons, glia, and cerebral endothelial cells all express the EPO receptor (112, 160, 172). *In vitro* EPO added to neuronal cultures protects from both hypoxic and glutamic injury (172) and numerous groups have reported that EPO is neuroprotective in rodent models of cerebral ischemic damage, traumatic brain injury, and spinal cord injury (26, 68, 74, 155, 162, 196, 205, 230, 270). Furthermore experiments in which endogenous EPO is neutralized, using a soluble form of the EPO receptor, show that EPO inhibition is sufficient to abrogate, at least in part, the neuroprotective effects of hypoxia preconditioning (155, 196). In a human clinical trial, EPO showed promising results and minimal side effects. Improvements in infarct volume and functional outcome were observed when EPO was administered within 8 h of the onset of acute stroke (46). The neuroprotectant role of EPO may be multimodal, with anti-apoptotic, neurotrophic, angiogenic, and anti-inflammatory effects (111, 159). Furthermore, an EPO analogue that does not bind to the dimeric EPO receptor and lacks erythropoietic activity, carbamylated EPO, was also neuroprotective (142). The anti-apoptotic effect of EPO on hematopoietic cells is well characterized (220). EPO has also been reported to reduce neuronal apoptosis in the penumbra in rats subjected to MCAO (middle cerebral artery occlusion) (221). EPO can downregulate the proapoptotic genes *BAX* and *DP5* and induce the expression of the antiapoptotic proteins BCL-2 and BCL-xL in the ischemic brain (132, 249). The signaling pathways involved are not fully elucidated, however some of these effects are likely to involve NFκB. Following EPO receptor activation IκB, an inhibitor of NFκB, is phosphorylated, resulting in the cytosolic release of NFκB (41) (see section entitled "Hypoxic Preconditioning" for a brief description of the role of NFκB in neuroprotection). EPO may also limit cerebral damage through anti-inflammatory effects. Recombinant EPO administered in a rat model of cerebral ischemia reduced both the activation of glial cells and the number of infiltrating leukocytes. Levels of the inflammatory cytokines TNF, IL6, and MCP1 were also decreased (241). EPO could also enhance repair of damaged areas by promoting neurogenesis and angiogensis through stimulation of neural and erythroid progenitor proliferation and differentiation (145, 174, 218).

Vascular endothelial growth factor (VEGF)

VEGFA encodes an angiogenic factor referred to here as VEGF, of which there are several isoforms. It is induced following ischemic stroke and is neuroprotective in several animal models of cerebral ischemia (86, 90, 139, 194, 252, 264). For example, intracerebroventricular VEGF treatment of rats following transient cerebral ischemia resulted in reduced infarct size and delayed neuronal injury (229). Conversely administration of neutralizing anti-VEGF antibodies or antisense knock-down of VEGF increased infarct size (140, 263). VEGFs ability to promote cerebral angiogenesis and vasodilation may be beneficial following stroke by improving

oxygen and nutrient delivery to the ischemic area. However, blood vessel growth initiated by VEGF alone stimulates the formation of an immature, leaky vasculature, which may contribute to edema, and worsen cerebral injury. Indeed, some models have shown a deleterious effect of VEGF in cerebral ischemia (274). It has been postulated that VEGF-mediated neuroprotection may be due to its neurotrophic, rather than angiogenic, effects. VEGF is known to inhibit programmed cell death in ischemia via a pathway involving phosphatidylinositol-3-kinase (PI3K) dependent activation of AKT (107). A report from Jin *et al*. showed that VEGF can inhibit caspase 3 in a culture of embryonic cortical neurons exposed to hypoxia (104). VEGF can also stimulate neurogenesis *in vitro* in cortical neuronal cell cultures and *in vivo* in the subventricular zone (SVZ) and subgranular zone (SGZ) of rats treated with VEGF via intracerebroventricular injection (106). Timing, dose, and route of administration are likely to be important in dictating the balance of favourable *versus* detrimental effects associated with VEGF therapy of acute stroke.

Heme oxygenase 1 (HO-1)

HO-1 is a HIF target gene that has been shown to be expressed in the cerebellum following focal ischemia (64) and in the retina following repetitive hypoxic preconditioning (279). HO-1 cleaves the heme molecule, producing free iron, carbon monoxide (CO), and the antioxidant bilirubin. Neuronal overexpression of HO-1, under the control of the neuron specific enolase promoter, has been shown to be neuroprotective in a murine model of cerebral ischemia (MCAO). The transgenic animals showed reductions in stroke volume and cerebral edema compared to wild-type mice 24 h after ischemia (186). A reduction in lipid peroxidation was also noted in the transgenic animals (186). Furthermore, a recent study of *HO-1* knockout mice demonstrated that the neuroprotective properties of ischemic preconditioning are at least in part dependent on HO-1. In experimental models of both transient and permanent cerebral ischemia, ischemic preconditioning conveyed partial protection (reduced infarct volume and neurological deficits) to wild-type, but not *HO-1* knockout mice (269). Pre-conditioning with the HO-1 inducer hemin has been shown to be beneficial in protecting against ischemic insults in a variety of organs (136, 259). HO-1 produces bilirubin, which is a potent free radical scavenger and can therefore limit ischemic reperfusion injury by preventing free radical mediated damage (34). HO-1 has also been reported to drive the resolution of inflammation *in vivo,* and *in vitro* experiments also support an anti-inflammatory role (254). Manipulation of HO-1 expression levels in endothelial cell cultures, by forced expression or siRNA, shows a reciprocal relationship between the expression of HO-1 and pro-inflammatory cell adhesion molecules such as intercellular cell adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) (222, 242).

Adrenomedullin (AM)

AM is a HIF target gene that encodes a vasodilating hormone whose expression is enhanced in the rodent brain following ischemia (216) or an episode of hypoxic preconditioning (19, 20, 232, 235). In *in vitro* studies AM protects murine primary cortical neurons in a model of oxygen glucose deprivation (235). Transgenics overexpressing AM in the liver show improved recovery in models of hind limb and cerebral ischemia (171). Infarct area, gliosis, leukocyte infiltration, apoptosis, and oxidative stress in the ischemic core were all found to be reduced in *AM* transgenics subjected to MCAO. *AM* transgenics also displayed improvements in vascular regeneration, cerebral blood flow, neurogenesis, and neurological function. Furthermore, administration of exogenous AM has been reported to be neuroprotective in numerous experimental models of ischemic brain injury (42, 171, 245, 257, 258). These results are consistent with a neuroprotective role for AM in the ischemic brain.

A role for HIF in brain energy metabolism: Mitochondria and the glycolytic pathway

In many different cell types HIF activation results in extensive changes in energy metabolism, broadly enhancing the ability to maintain energy homeostasis as oxygen levels are lowered. The brain consumes a large amount of ATP, since neurons must maintain their resting membrane potential for proper neuronal function. Blood flow to the brain is critical for maintaining the supply of glucose and oxygen required for mitochondrial ATP production; therefore the brain is particularly susceptible to the detrimental effects of ischemia.

Neuronal ion channels. During hypoxia, neurons undergo hyperpolarization through activation of ATP-sensitive potassium channels, which decreases both their neuronal activity and energy consumption. This has been suggested to be a homeostatic response to the reduced energy supply experienced during hypoxia and may have a protective effect by delaying depolarization of the neurons (13, 85, 169, 260). Regulation of ion channels by HIF is an emerging area of research, but although some ion channels have been reported to be regulated by HIF (24, 251), it is not currently clear whether HIF could contribute to modulation of ion channels in the brain during ischemia, or whether neuronal ion channels are regulated by other routes.

Mitochondrial function and biogenesis. During hypoxia, mitochondrial ROS production increases (31, 81, 193). This can lead to increased damage of important cellular components. Several HIF dependent mechanisms have been reported which actively suppress mitochondrial metabolism, and thus ROS production, during hypoxia. First, HIF is known to upregulate pyruvate dehydrogenase kinase (PDK), which inhibits the key mitrochondrial enzyme pyruvate dehydrogenase, in turn preventing the flow of pyruvate into the tricarboxylic acid (TCA) cycle, instead shunting it to lactate (121, 187). Maintaining the cell's energy supply is essential for most cellular processes and ultimately survival of the cell. During hypoxia, HIF-1α knockout mouse embryonic fibroblasts fail to switch effectively to glycolytic metabolism and show increased apoptosis due to oxidative stress. Overexpression of PDK rescues the cells by directing pyruvate away from mitochondrial respiration (121). HIF has also been shown to regulate expression of cytochrome oxidase 4 (COX-4) subunits that determine the efficiency of mitochondrial respiration (59) and the *MYC* proto-oncogene, which controls both the transcription of mitochondrial components and mitochondrial biogenesis (143). Zhang *et al*. demonstrated that

HIF, acting via the repression of c-Myc, can inhibit mitochondrial biogenesis in *VHL* defective renal cancer cells (272). Furthermore exposure of wild-type MEFs to hypoxia results in decreases of both mitochondrial mass and DNA content. Conversely, the mitochondrial mass and DNA content is increased in HIF1α knockout MEFs compared to wild-type MEFs (271).

Glycolytic pathway. Glycolytic metabolism does not require oxygen, allowing cells to continue ATP production even under anaerobic conditions, though at a much reduced rate (6). HIF activation is known to trigger a switch from oxidative to glycolytic metabolism in a wide variety of cell types (16, 224, 266). In addition to maintaining the cellular energy supply, this also results in decreased cellular oxidative stress during hypoxia by reducing mitochondrial ROS leakage. In order to bring about this switch, HIF induces glucose transporters (GLUT1, GLUT3) and genes involved in the glycolytic pathway (*e.g.*, glucosephosphate isomerase, aldoase A and C, phosphoglycerate kinase 1, and lactate dehydrogenase A) (87, 213). Interestingly, induction of several glycolytic genes has been observed following hypoxic preconditioning in the neonatal rat brain (108). However, the relevance of changes in glycolysis to survival in ischemia is likely to be limited. This is because in the absence of perfusion the ability of glycolysis to generate adequate ATP for neuronal and other cellular survival is likely to be very limited, not only due to low yield of ATP relative to oxidative phosphorylation, but also severely limited availability of glucose and the accumulation of lactate.

A role for HIF in endogenous neuroregeneration

Cerebral ischemia has been shown to stimulate neurogenesis, with increased proliferation of both endothelial and neural progenitors observed in the SVZ and SGZ (141, 177). Some evidence also exists to support a role for HIF and HIF target genes in aspects of neural cell progenitor biology (*e.g.*, maintenance of the neural stem cell niche, neural stem cell proliferation, mobilization of neural stem cells to areas of ischemic insult, and differentiation of neural progenitors). Interestingly, recent studies investigating stem cell transplantation as a therapy for ischemic damage have demonstrated that hypoxic preconditioning of the stem cells prior to transplantation results in improved outcome (98, 233). In a rodent stroke model, less cell death and increased motor function were observed in rodents transplanted with hypoxic preconditioned stem cells, compared to those transplanted with untreated stem cells (233). Taken together, this suggests that HIF activation within the brain may play a beneficial role in supporting endogenous neuroregeneration following ischemia.

Maintenance of the neural stem cell niche. Culturing embryonic hematopoietic progenitors and neural crest stem cells in hypoxic conditions enhances the number of multipotent precursors in comparison to normoxic cultures (5,173). Recently, Octamer binding transcription factor 4 (Oct-4), a transcription factor which is essential for maintaining stem cell pluripotency, was reported to be a target of HIF-2α (37). Overexpression of Oct4 in neural stem/progenitor cell neurospheres has been shown to prevent neuronal differentiation (184) and therefore HIF-induced Oct4 may contribute to formation and maintenance of stem cell niches during hypoxia.

Proliferation of neural progenitors. In addition to VEGF, there is evidence that several other neurotrophic growth factors are also regulated by HIF, such as platelet derived growth factor (PDGF) (267), fibroblast growth factor 2 (FGF-2) (28), transforming growth factor α (TGF-α) (80), and stem cell factor (SCF; kit ligand) (84). Many of these factors have been reported to be induced during ischemic preconditioning (177). Neurotrophic factors are known to promote both cell survival and proliferation and several have shown benefit in experimental models of ischemic stroke (152, 244). For example, the TGF-a/EGFR pathway is induced by HIF (80) and administration of TGF-α in MCAO induces both the proliferation and mobilization of endogenous progenitors and behavioral recovery (79). Furthermore, a report from Jin *et al*. showed that hypoxia increased both the proliferation of cerebral cortical cultures and the expression of the neurotrophins SCF and FGF-2 (105). These have been reported to be HIF targets in other cell types (28, 84). Intraventricular administration of SCF has been reported to enhance neurogenesis *in vivo* in adult rats in the SVZ and SGZ (105). Likewise, FGF-2 has also been reported to have beneficial effects, with infusion having been demonstrated to stimulate regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors (176). FGF-2 has also been tested as an experimental treatment for stroke (23), however treatment of stroke with neurotrophic factors is hampered by poor BBB penetration (256).

Mobilization and recruitment of neural progenitors. HIF has also been implicated in the mobilization and recruitment of neural stem cells. The chemokine (C-X-C motif) receptor 4 (CXCR4)/stromal derived factor 1 (SDF-1) signaling pathway is important in the directional migration of neural progenitors during normal brain development (17, 44, 151, 280). CXCR4 and SDF-1 are known to be regulated by HIF in several cell types and have been shown to be induced by HIF in the mouse brain (cortical and hippocampal neurons) (228). More recently, Chu *et al*. showed that treatment with desferrioxamine (DFO) increased the expression of HIF and several HIF target genes, including CXCR4 in human neural stem cells (33). Furthermore, neural stem cells pretreated with DFO and implanted into rats, which were subsequently subjected to focal ischemia, displayed reduced infarct volume compared to animals transplanted with naive stem cells (33). SDF-1 has been shown to be increased in the ischemic penumbra following MCAO, providing a homing signal for cells expressing the CXCR4 receptor (95, 212). Taken together, these reports would be consistent with HIF induction of the CXCR4/SDF-1 pathway mediating recruitment of neural progenitors to areas of damage following ischemic injury. However, it remains to be fully elucidated how far the neural progenitor cells can move from their usual location in the brain (SVZ) to the injured area.

Differentiation of neural progenitors. Several groups have demonstrated that culturing neural progenitor/stem cells in hypoxic conditions enhances not only cell survival and proliferation, but also the number of cells that differentiate into dopaminergic neurons (173, 225, 227). The PHD inhibitor FG-4497 that activates HIF has also been shown to promote dopaminergic differentiation in neural progenitor cells (168).

The potential contribution of several HIF target genes and pathways to the survival, proliferation, and mobilization of neural progenitors, suggests that HIF activation could promote repair following ischemia.

Genetic Approaches to Determining Whether HIF Activation Is Neuroprotective

Genetic investigations in mice offer a potential means for manipulating the HIF pathway. For example, if HIF activation is protective in ischemic injury, it would be predicted that animals lacking a HIF-α subunit would show increased susceptibility. However, lack of either HIF-1α or HIF-2α is generally associated with embryonic lethality in mice. This is consistent with the importance of this pathway in development, but means that global knockouts are not available to probe the role of HIF in cerebral ischemia. Nevertheless, it is interesting that global HIF-1α knockouts show impaired brain development (neural tube defects) that are associated with abnormal cerebral vasculature and cephalic mesenchymal cell death, attesting to the importance of the pathway in the CNS (101, 204).

The problem of embryonic lethality in experimental models can be circumvented by using mice with neuronal-specific lack of HIF-1α using Cre-lox technology. These have shown mixed results. Helton *et al*. first reported an advantageous effect of neuronal HIF-1α knockdown in the bilateral common carotid artery occlusion (BCCAO) model of stroke. This was attributed to suppression of pro-apoptotic HIF-1 target genes, for example, BCL2/adenovirus E1B 19kDa interacting protein 3 (*BNIP3*) (92). A contrasting report from Baranova *et al.*, using the same transgenic lines, showed that HIF-1α knockdown was deleterious in the MCAO stroke model (14). The cause of the discrepancies between these reports remains unclear; but may be a reflection of the stroke models used [global (BCCAO) *versus* focal (MCAO) ischemia] and the duration of the ischemic insult (75 *vs.* 30 min). Given the complex nature of cerebral ischemia, conditional knockouts of HIF-1α in a single cell type should be regarded with caution, since activation of multiple HIF isoforms in numerous cell types may play a role in stroke and its treatment. Nevertheless, these contrasting effects of HIF inactivation reinforce the view that activation of HIF is likely to mediate both beneficial and detrimental effects, which is crucial when considering the potential benefit of HIF activation in stroke.

Evidence That Certain Protective Agents in Stroke May Act *via* HIF

Preconditioning with a variety of different stimuli has been demonstrated to provide protection from ischemic injury during a subsequent episode of ischemia. The volatile anesthetic gases, isoflurane and xenon, have been reported to induce tolerance and protect against ischemic insults in a variety of organs, including the brain (115, 149, 161, 276–278) More recently, it has been demonstrated that these gases can also induce HIF. Isoflurane induces HIF-1 in neuronal cultures and is protective against subsequent oxygen–glucose deprivation, an *in vitro* model of ischemia (144). Ma *et al*. recently used siRNAs targeting HIF-1α to provide the first direct evidence that anesthetic preconditioning can be mediated via HIF. In a model of renal ischemia, the effects of xenon-mediated preconditioning were demonstrated to be mediated, at least in part, via HIF-1α (150). A recent screening study of FDA approved compounds identified the immunomodulatory drug tilorone as a HIF activator. Functional studies showed that tilorone was protective in rat models of stroke and spinal cord injury (199). However, it is unlikely that this particular compound will be useful in humans, since it has previously been associated with lysosomal accumulation of sulfated glycosaminoglycans (GAGs) due to a disturbance of lysosomal GAG degradation. Taken together, these reports suggest that certain pharmacologic treatments which are neuroprotective could act, at least in part, by activating HIF.

Prolyl Hydroxylase Inhibitors: A Route to HIF Induction

The development of small molecules that induce HIF-α via inhibition of the HIF-α degradation pathway has been proposed as a potentially useful therapeutic strategy for the treatment of ischemia. When oxygen is present, specific prolyl and asparaginyl residues in the HIF-α subunit are hydroxylated by a class of non heme iron- and 2-oxoglutarate (2-OG)-dependent dioxygenases. Factor-inhibiting HIF (FIH) and prolyl hydroxylase domain (PHD) enzymes affect the activity and stability of HIF, respectively. FIH catalyzes the hydroxylation of an asparagine residue in the carboxy-terminal activation domain (CTAD) of HIF-α. This prevents the formation of an active transcriptional complex by blocking recruitment of transcriptional coactivators, such as p300 (137, 153). PHDs hydroxylate two proline residues in the oxygen-dependent degradation domain (ODDD) of HIF-α, allowing it to interact efficiently with VHL (51, 93). VHL acts as the substrate recognition subunit of an E3 ubiquitin ligase complex composed of elongin B/C, cullin 2, and ring box 1 (119, 124, 165). Therefore, interaction of HIF-α with VHL results in its polyubiquitnation, targeting it for destruction by the 26S proteasome (165). As a result of this efficient and rapid degradation system, HIF-1α protein is virtually undetectable under normoxic conditions (HIF-1α normoxic half-life <1 minute) (268). Normoxic degradation of HIF is illustrated in Fig. 2.

However, when the oxygen tension is reduced, these enzymatic hydroxylation reactions do not proceed efficiently; therefore VHL can no longer target the unmodified HIF-α subunits for destruction. Mitochondrial ROS, which are increased during hypoxia, have also been reported to be capable of inactivating PHD enzymes, via oxidation of the enzyme-bound iron (66, 82, 120). Decreased activity of PHD enzymes in hypoxia allows HIF-α subunits to accumulate and dimerize with HIF-β (also known as the aryl hydrocarbon receptor nuclear translocator [ARNT]). The HIF complex induces transcription by binding to hypoxic response elements (HREs), with the minimal core sequence 5′-RCGTG-3′, located in the region of well over a hundred target genes, including carbonic anhydrase IX (CAIX) (75), EPO (214), and VEGF (55) (Fig. 3). Besides direct interactions with DNA, HIF also interacts with other transcription control complexes.

Small molecule inhibitors of PHDs potently activate the HIF response. Therefore it has been proposed that administration of PHD inhibitors could mimic, at least in part, the protective effects of exposure to hypoxia. Importantly from a therapeutic standpoint, ischemia appears to result in a submaximal activation of HIF-1 and this is increased by inhibition of PHD enzymes.

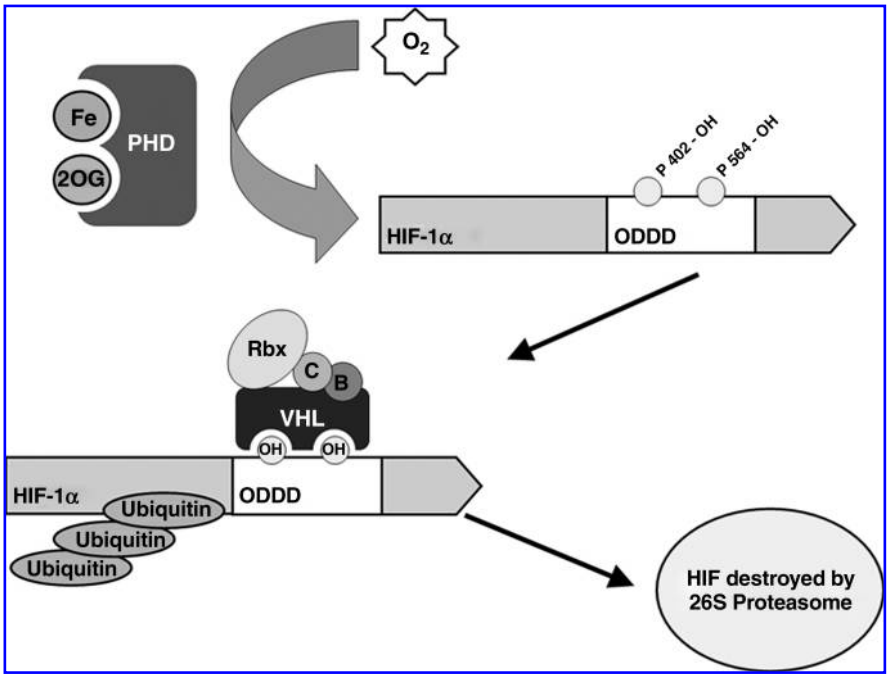

FIG. 2. VHL targets HIF-α subunits for destruction in normoxia. Abbreviations: B, Elongin B; C, Elongin C; Cul 2, Cullin 2; Fe, iron; HIF, hypoxia inducible factor; ODDD, oxygen dependent degradation domain; 2-OG, 2-oxoglutarate; PHD, prolyl hydroxylase domain enzyme; Rbx, Ring box 1; VHL, von Hippel–Lindau.

Current Agents That Inhibit PHD Enzymes and Induce HIF

Prototype PHD inhibitors offer protection against ischemia

PHD enzymes require iron and 2-OG in order to catalyze HIF prolyl hydroxylation (102). DFO and cobalt chloride ($CoCl_2$), an iron chelator and competitive inhibitor of iron, respectively, are routinely used both *in vitro* and *in vivo* to inhibit PHD enzyme activity and thus stabilize HIF. *In vivo* studies in both neonatal and adult rats have shown that preconditioning with DFO or $CoCl_2$ is protective in pre-clinical models of cerebral ischemia (109, 146, 195). The 2-OG analogues L-mimosine (L-mim), dimethyloxalylglycine (DMOG), and 3,4-dihydroxybenzoate (3,4-DHB) can also be used to inhibit PHD enzymes. Administration of 3,4-DHB was observed to induce HIF and reduce infarct volume in the murine brain in a model of permanent focal ischemia and prevented oxidative glutamate toxicity in embryonic cortical neuronal cultures (7). Both L-mim and DMOG have been shown to ameliorate the effects of renal ischemia/reperfusion injury in mice (94). However, not all reports are consistent with a beneficial effect of 2-oxoglutarate analogues. A recent publication showed that DMOG treatment of neonatal rat pups subjected to unilateral carotid artery ligation, followed by 2 h of hypoxia, resulted in increased BBB permeability and more brain edema compared to control animals (32). Differences in the severity and duration of the ischemic insult, the treatment regimen, and the prognostic parameters measured are likely to contribute to the different outcomes reported. It is also important to consider that any protective effects afforded by these molecules may be the combined result of the activation of HIF and HIF target genes, non-HIF PHD targets and "off-target" effects; since they are not selective for the PHD enzymes and will certainly inhibit other 2-OG dependent oxygenases.

Off-target effects of current agents which inhibit PHD enzymes

The majority of studies published to date have used compounds which limit either iron or 2-OG availability to analyze the effects of PHD inhibition in pre-clinical ischemic stroke models (7, 109, 146, 195). Iron chelators and competitive inhibitors impair the activity of both the PHD enzymes and other iron-dependent enzymes. Given the wide range of enzymes that utilize iron, this is likely to have complex effects in the context of cerebral ischemia. The brain has high concentrations of iron, which are stored in the cytoplasmic protein ferritin. During ischemia, acidosis and reactive oxygen and nitrogen species stimulate the release of this iron. The free iron is then able to catalyze ROS formation via Fenton chemistry, which could lead to radical-mediated damage of cellular components (223). Given this, sequestration of iron may on balance be beneficial following ischemic stroke and may contribute to the neuroprotective action of iron chelators.

2-OG analogues used for PHD inhibition, such as DMOG, also inhibit other cellular 2-OG dependent enzymes. An interesting example is FTO (fat mass and obesity associated), a 2-OG dependent dioxygenase implicated in weight regulation that is highly expressed in the brain (67). On the other hand, a recent study comparing the effects of hypoxia to DMOG treatment found that the effects, in terms of gene expression, were highly concordant in MCF7 breast cancer cells and were largely mediated via HIF-1α (47).

Non-HIF partners/substrates of PHD enzymes that may be disturbed by PHD inhibition

Recent findings have shown that, in addition to HIF, the PHD enzymes interact with various other proteins. Several of these, including osteosarcoma amplified 9 (OS9) (12), mitogen-activated protein kinase organizer 1 (MORG1) (96), and the tumor suppressor gene inhibitor of growth family,

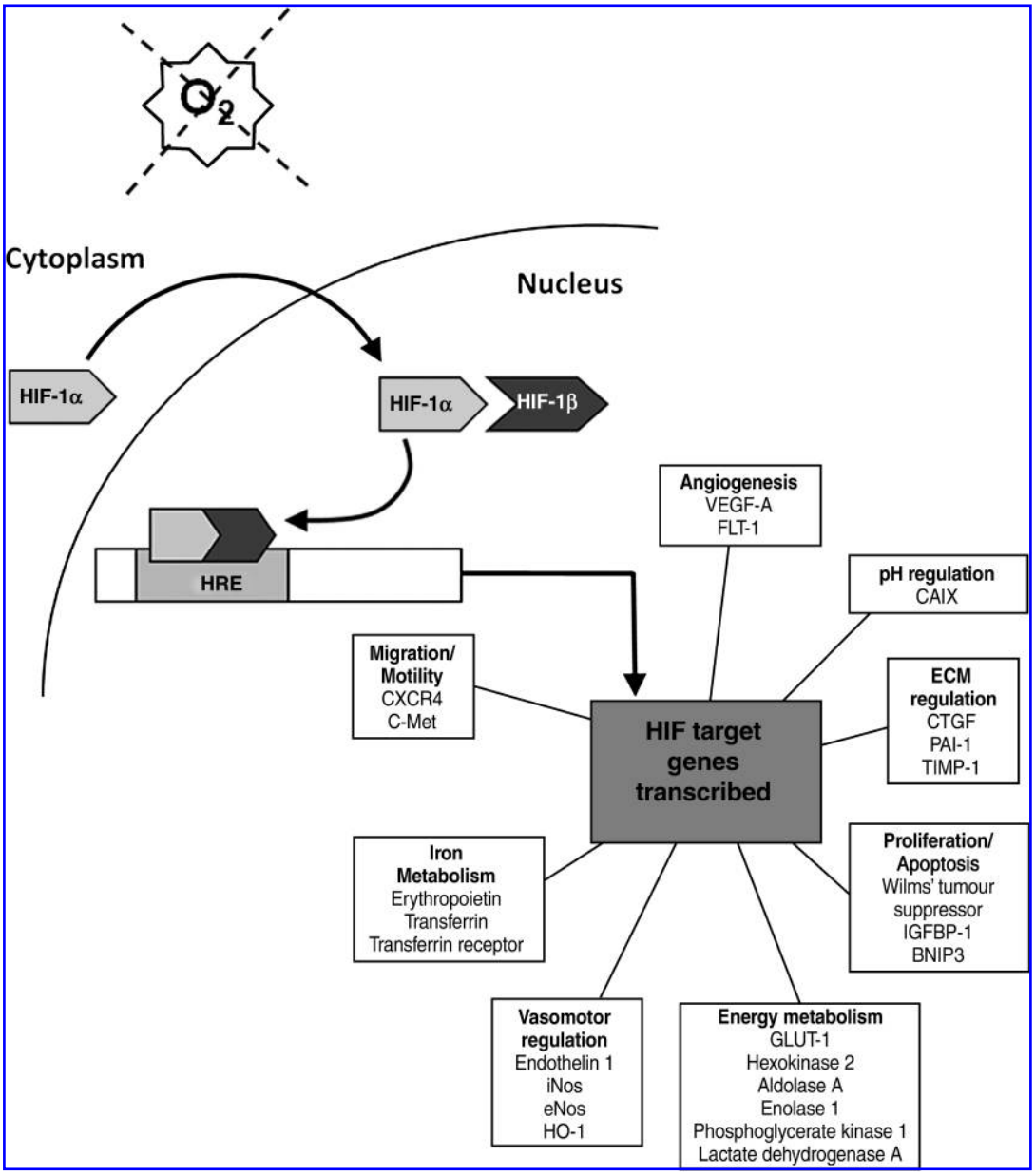

FIG. 3. HIF-1α is stabilized in hypoxia and target genes are transcribed. Under hypoxic conditions, pVHL cannot target HIF-1α for proteasomal degradation. HIF-α dimerizes with HIF-β before inducing transcription by binding to HREs in the promoter regions of HIF target genes. Abbreviations: BNIP3, BCL2/adenovirus E1B 19 kDA interacting protein 3; CAIX, carbonic anhydrase IX; c-Met, hepatocyte growth factor receptor;CTGF, connective tissue growth factor; CXCR4, C-X-C chemokine receptor type 4; eNOS, endothelial nitric oxide synthase; FLT-1, fms-related tyrosine kinase 1; GLUT-1, glucose transporter 1; HO-1, heme oxygenase 1; HRE, hypoxia response element; iNOS, inducible nitric oxide synthase; IGFBP-1, insulin-like growth factor binding protein 1; PAI-1, plasminogen activator inhibitor 1; TIMP1, tissue inhibitor of metalloproteinase 1; VEGF-A, vascular endothelial growth factor A.

member 4 (ING4) (36), can bind to the PHD enzymes to modulate their activity. Ozer *et al*. showed recently that knock-down of ING4 results in increased HIF transcriptional activity, with increased expression of HIF target genes in hypoxia (185). PHD enzymes have also been reported to control the degradation/activity of proteins other than HIF. IκB kinase-β (IKKβ), which is upregulated in hypoxia, has been shown to co-immunoprecipitate with PHD1. This interaction is thought to lead to increased NFκB activity in hypoxia, since IKKβ is a critical negative regulator of NFκB activity. When a conserved proline residue in IKKβ (which is homologous to the ODD found in HIF-1) is mutated, hypoxic induction of IKKβ is prevented (38). Furthermore, a report from Koditz *et al*. showed that activating transcription factor 4 (ATF4) is stabilized in anoxia and also by mutation of proline residues within ATF4 or by silencing of PHD3 using siRNA (126). Eventually understanding these interactions may provide insight into the complex nature of the response to hypoxia and how it is adapted to particular circumstances.

Summary

Research to identify PHD interactors is ongoing and it is likely that further substrates exist which are yet to be identified. Since treatment with PHD inhibitors has the potential to disrupt not only HIF prolyl hydroxylation, but also that of potential non-HIF substrates, it will be important to consider the implications of inhibiting these interactions when considering the therapeutic potential of this approach.

Evidence for Beneficial Effects of Specific PHD Inhibition in Ischemia

Genetic evidence

A recent report from Eckle *et al*. examined whether inhibition of specific PHD isoforms could induce cardioprotection in mice. Intraventricular administration of siRNAs targeting PHD2, but not PHD1 or PHD3, resulted in increased HIF protein levels in mouse cardiac tissue. Treatment with PHD2 siRNA also resulted in reduced infarct volume following coronary artery ligation (45). A genetic mouse model in which PHD1 is globally knocked-out has also provided compelling evidence that specific inhibition of PHD enzymes may be beneficial in skeletal muscle ischemia. Under basal conditions, PHD1 knockouts have impaired oxidative phosphorylation compared to wild-type mice. During hind limb ischemia, this was shown to limit oxidative stress from mitochondrial free radical production, resulting in increased protection of PHD1 knockouts from ischemic damage (10). Taken together, these studies support the notion that pharmacological suppression of PHD enzymes may be beneficial in the treatment of ischemia and also suggest that different PHD isoforms are likely to be important in different tissues.

Novel PHD inhibitors

The development of novel PHD inhibitors for the treatment of ischemic diseases, including stroke, is currently ongoing. The PHD inhibitor described as compound A (FibroGen), which is thought to act by preventing iron from acting as a co-factor, has been shown to abrogate oxidative stress-induced death in cortical neurons *in vitro*. Compound A was also able to induce HIF and reduce infarct volume in a murine model of ischemic stroke (219). Another FibroGen compound, FG-4497, has recently been reported to stabilize HIF-1α and several vasoactive and cytotrophic HIF target genes, including VEGF, ADM, EPO, and CXCR4, in the neonatal mouse brain (211). FG-4497 has also shown significant protective effects when used as a preconditioning agent for the treatment of ischemic conditions in other organs (*e.g.*, hypoxic kidney injury (202) and inflammatory bowel disease (200)). FG-4497 has also been shown to enhance the proliferation, neurogenesis, and dopaminergic differentiation of human fetal mesencephalic neural progenitor cells (168). Taken together, these results are promising and suggest that PHD inhibitors could be useful in the treatment of ischemic stroke.

Summary

PHD inhibitors could act via HIF (and potentially other pathways) on multiple targets to minimize damage and promote repair. From a therapeutic stand-point, this is attractive, since clinical trials with agents targeting single aspects of cell death in ischemic stroke have had disappointing results. The neuroprotective pathways induced by PHD inhibitors are likely to be a subset of those induced by hypoxia, however PHD inhibition may also activate other pathways.

Besides the effects on cell survival, it is important to appreciate that the consequences of HIF activation are pleiotropic, and could have other effects that would influence outcome in cerebral ischemia. Of particular interest, we and others have shown that the HIF pathway influences intercellular junction formation, implying that it may influence the blood-brain barrier (BBB). This is discussed in the following section.

Effects of PHD Inhibitors on the Blood-Brain Barrier (BBB)

The BBB is a specialized structure separating the systemic circulation from the brain. This section will focus on the structure of BBB in the normal brain, how ischemia affects the integrity of the BBB, and the likely effects of PHD inhibition on the BBB.

Structure of the blood-brain barrier

The BBB is composed of specialized endothelial cells of cerebral microvessels. Astrocytes, pericytes, and perivascular microglia surround the endothelial cells and are also important for proper function of the BBB. BBB endothelial cells have a distinctive phenotype. Unlike other endothelial cells, they form tight junctions (TJs), which resemble those formed by epithelial cells. TJs formed between cerebral capillary endothelial cells seal adjacent endothelial cells and perform a critical role in controlling the substances which enter the brain (157).

TJs are formed in the most apical region of the lateral plasma membrane and are composed of specific transmembrane proteins (occludin, claudins, and JAMs) which are linked to the actin cytoskeleton via intracellular adaptor proteins (the zona occludens (ZO) family proteins). ZO proteins also bind the signalling molecule ZO-1-associated nucleic acid binding protein (ZONAB) at the TJ. The claudins and occludin are polytypic transmembrane proteins with four transmembrane domains (60, 238). They are thought to assemble into heteropolymers that form intramembranous TJ strands (237). The intercellular space is obliterated at the point at which TJ strands in the apposed membranes of adjacent cells associate with each other (237).

TJ strands contain ion channels which are key to regulating paracellular permeability. Studies using truncation mutants and synthetic peptides corresponding to the transmembrane domains of occludin suggest that occludin contributes to the barrier/sealing functions of the TJ (54). However, it is now widely recognized that the large variety in strength, size, and ion specificity of TJ barriers in different epithelia and endothelia is largely due to the type of claudin(s) found at specific TJs (8, 61). The extracellular loops of occludin contain very few charged amino acids, however these loops are charged in claudins and their isoelectric point varies widely between the different members of the claudin family (35, 239). Over 20 different claudins have been identified, giving an enormous scope for variation of TJ structure and function. Claudins 1, 3, 5, and 12 have been identified in endothelial cells of the BBB (89). Claudin 5 has been show to be important in regulating

paracellular permeability of small molecules. Increased expression of claudin 5 *In vitro* in rat brain capillary endothelial cells results in decreased monolayer permeability (181). Consistent with these results, claudin 5 mouse knockouts have compromised BBB, with increased permeability of molecules up to 800 Da in mass (179).

Effects of ischemic stroke on the blood-brain barrier

Several groups have studied the effects of hypoxia/reoxygenation and ischemia/reperfusion in BBB models and shown that hypoxia is associated with changes in both paracellular permeability and localization of TJ proteins in cerebral microvessel endothelial cells (117, 157, 248, 255). Exposure of either murine brain endothelial cells or retinal flatmounts to hypoxia has been shown to reduce claudin 5 expression levels (129). Paracellular permeability of low molecular weight compounds was also increased in hypoxic retinal flatmounts (129). *In vivo* studies have also reported changes in TJ integrity in association with cerebral ischemia. Following microsphere embolism in mice, Kago *et al*. observed decreases in expression of the TJ proteins occludin and ZO-1 and increased paracellular permeability (113). However, it should be noted that ischemia or hypoxia-induced alterations in BBB TJs have not been observed in all studies. This may be related to differences in the degree of hypoxia suffered by different areas of the brain following an ischemic insult (infarct zone *versus* penumbra *versus* BBB) and because of the time required for TJ disassembly to occur.

Mechanisms of BBB disruption after ischemia

Ischemia results in increased expression of several potential effectors, including matrix metalloproteases, cathepsins, t-PA, and heparanases, which contribute to degradation of the BBB extracellular matrix (192, 206). Evidence supporting a role for HIF in mediating disruption of BBB TJs following ischemia also exists. In a recent study, the iron competitive PHD inhibitor $CoCl_2$ and ischemia/reperfusion both resulted in decreased ZO-1 expression and assembly and increased paracellular permeability in *in vitro* cultures of adult rat brain endothelial cells. Furthermore, the changes mediated by $CoCl_2$ or hypoxia/reoxygenation *in vitro* or by ischemia/reperfusion *in vivo*, were attenuated using YC-1, an inhibitor of HIF (265). Several groups, including ourselves, have also reported the involvement of HIF in impaired junction assembly in the kidney. Expression of the adherens junction protein E-cadherin, along with the TJ proteins occludin and claudin 1 is suppressed in VHL defective cells expressing HIF compared to *VHL* competent cells (52, 53, 88, 131). Given this, it is plausible that HIF plays a role in reorganization/disassembly of cell junction assembly in the BBB following ischemic stroke.

Potential consequences of increased BBB permeability in ischemic stroke

Detrimental effects, such as increased inflammation and an increased risk of edema, have been associated with increased BBB permeability (201, 206). Furthermore, edema in the enclosed cranium will increase pressure and could compromise perfusion. Therefore, adverse effects of increased permeability should be carefully monitored when administering drugs which have the potential to relax BBB TJs, including PHD inhibitors.

On the other hand, relaxation of BBB TJ also has the potential to improve drug delivery. Under normal physiological circumstances, substances that are greater than ~180 Da cannot pass through the BBB via the paracellular route (170). The delivery of hydrophilic or large molecules to the CNS remains challenging. TJ modulation was investigated previously as a route to enhancing drug penetration across the BBB (39, 128). Increased permeability of the BBB may allow for better drug delivery and improve the efficiency of combination therapies. However, a better understanding of BBB TJ biology, and how this is affected by ischemia, would be needed to allow manipulation of the BBB in such a way as to exploit benefits of TJ relaxation, while minimizing the harm.

Concluding Remarks

Ischemic cell death occurs via a cascade of events involving several components. Single target therapies are likely to prove less effective than those that take a more global approach, such as PHD inhibitors. Combination therapies are also likely to be important. PHD inhibitors may prove particularly useful in conditions/situations in which cerebral ischemia can be anticipated such as brain or heart surgery, cardiac arrest, and respiratory distress. Although promising, PHD inhibitors are unlikely to be a magic bullet in stroke treatment and further studies to determine the full extent of their beneficial and detrimental effects are warranted.

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Abbreviations Used

AM = adrenomedullin
AMPA = 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) proprionate, AMPA
AP-1 = activator protein 1
ARNT = aryl hydrocarbon receptor nuclear translocator
BBB = blood brain barrier
BCCAO = bilateral common carotid artery occlusion
BNIP3 = BCL2/adenovirus E1B 19kDa interacting protein 3
CAG = glycosaminoglycan
CAIX = carbonic anhydrase IX
CO = carbon monoxide
$CoCl_2$ = cobalt chloride
COX-4 = cytochrome oxidase 4
CXCR4 = (C-X-C motif) receptor 4
DFO = desferrioxamine
3,4-DHB = 3,4-dihydroxybenzoate
DMOG = dimethyloxalylglycine
Egr-1 = early growth response protein 1
EPO = erythropoietin
ETS1 = v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)
FAST-Mag = Field Administration of Stroke Therapy-Magnesium pilot study
FGF-2 = fibroblast growth factor 2
FIST = flunarizine in stroke treatment
HIF = hypoxia inducible factor
HO-1 = heme oxygenase 1
HRE = hypoxia response element
ICAM = intercellular cell adhesion molecule
IKKβ = IκB kinase-β
ING4 = inhibitor of growth family, member 4
IPAS = inhibitory PAS domain protein
IST = international stroke trial
L-mim = L-mimosine
MCAO = middle cerebral artery occlusion
MEfs = mouse embryonic fibroblasts
MORG1 = mitogen-activated protein kinase organizer 1
MRF-1 = metal transcription factor 1
NF-κB = nuclear factor kappa B
NMDA = N-methyl-D-aspartate
NOS = nitric oxide synthase
Oct-04 = octamer binding transcription factor 4
ODDD = oxygen-dependent degradation domain
OS9 = osteosarcoma amplified 9
PDGF = platelet derived growth factor
PDK = pyruvate dehydrogenase kinase
PHD = prolyl hydroxylase domain
PI3K = phosphatidylinositol-3-kinase
RANTTAS II = Randomized Trial of Tirilazad Mesylate in Acute stroke II
ROS/RNS = reactive oxygen and nitrogen species
SCF = stem cell factor
SDF-1 = stromal derived factor 1
SGZZ = subgranular zone
SVZ = subventricular zone
TCA = tricarboxylic acid
TESSII = Tirilazad Efficacy Stroke Study II
TGF-α = transforming growth factor α
TNF-α = tumor necrosis factor α
t-PA = tissue plasminogen activator
VAGF = vascular endothelial growth factor
VCAM = vascular cell adhesion molecule
VEBUS = Very Early Nimodipine Use in Stroke
ZO = zona occludens
ZONAB = ZO-1-associated nucleic acid binding protein

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