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Prolyl 4-hydroxylase activity-responsive transcription factors: From hydroxylation to gene expression and neuroprotection

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1. ABSTRACT

Most homeostatic processes including gene transcription occur as a result of deviations in physiological tone that threatens the survival of the organism. A prototypical homeostatic stress response includes changes in gene expression following alterations in oxygen, iron or 2-oxoglutarate levels. Each of these cofactors plays an important role in cellular metabolism. Accordingly, a family of enzymes known as the Prolyl 4-hydroxylase (PHD) enzymes are a group of dioxygenases that have evolved to sense changes in 2-oxoglutarate, oxygen and iron via changes in enzyme activity. Indeed, PHDs are a part of an established oxygen sensor system that regulates transcriptional regulation of hypoxia/stress-regulated genes and thus are an important component of events leading to cellular rescue from oxygen, iron or 2-oxoglutarate deprivations. The ability of PHD activity to regulate homeostatic responses to oxygen, iron or 2-oxoglutarate metabolism has led to the development of small molecule inhibitors of the PHDs as a strategy for activating or augmenting cellular stress responses. These small molecules are proving effective in preclinical models of stroke and Parkinson's disease. However the precise protective pathways engaged by PHD inhibition are only beginning to be defined. In the current review, we summarize the role of iron, 2-oxoglutarate and oxygen in the PHD catalyzed hydroxylation reaction and provide a brief discussion of some of the transcription factors that play an effective role in neuroprotection against oxidative stress as a result of changes in PHD activity.

2. PROLYL HYDROXYLASE ACTIVITY AND REGULATION OF GENE TRANSCRIPTION

Hydroxylation of specific amino acids in proteins is as an enzyme catalyzed posttranslational modification that can lead to changes in interactions between proteins. Indeed, hydroxylation of proline residues has been shown to play an important role in the stability of proteins such as collagen (1), elastin (2), and prion protein (3). Hydroxylation of specific prolines (position 402 or 564) within the transcriptional activator "Hypoxia Inducible Factor-1" (HIF-1) (4, 5) regulates its transcriptional activity. HIF-1 hydroxylation is a prototypic example of a posttranslational modification capable of regulating gene transcription. As HIF is at the center of adaptive responses to ischemic and oxidative stress, the regulation of HIF hydroxylation becomes a viable strategy for engaging its homeostatic functions in a host of tissues, particularly the brain. The group of enzymes that can catalyze the hydroxylation reaction of HIF-1 are prolyl 4-hydroxylases (PHDs). These enzymes belong to a super family of 2-oxoglutarate dependent hydroxylases, that require iron in the catalytic moiety (6). They also utilize oxygen in the form of dioxygen, incorporating one oxygen atom into the proline residue, and the other into 2-oxoglutarate, yielding succinate and CO_2. PHD activity negatively regulates the stability of hypoxia inducible factor-1 alpha (HIF-1α). The well-established strategic role HIF-1 plays in the regulation of adaptive mechanisms in response to hypoxia, the requirement of oxygen for the activity of PHDs and the inverse relationship between PHD catalytic activity and

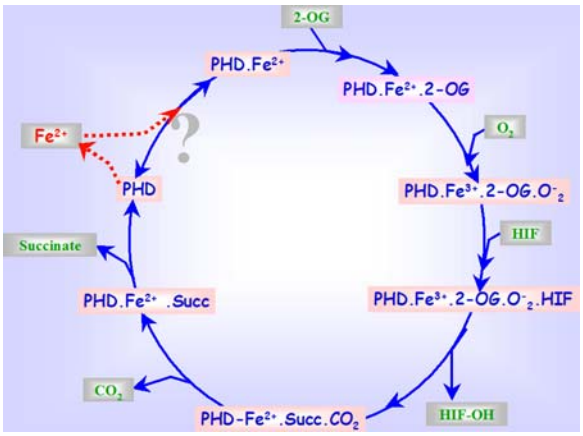

Figure 1. Schematic presentation of the proposed sequential mechanism for the prolyl hydroxylase reaction. The 2-oxoglutarate is stoichiometrically decarboxylated during the hydroxylation of HIF in the presence of dioxygen and iron, resulting in the generation of carbon dioxide (CO_2) and succinate. The dashed lines between the enzyme and Fe^{2+} indicate that the enzyme may exist with or without Fe^{2+} in the catalytic moiety after each catalytic cycle.

HIF-1 transcriptional activity has led researchers to appropriately designate PHDs as key players in the oxygen sensing transcriptional mechanisms regulated by HIF-1. Interestingly, oxygen is not the only co-factor required for PHD activity. The transition metal, iron is also required as is 2-oxoglutarate. The ability of both of these cofactors to regulate PHD activity suggests that PHD activity can be modulated under conditions of iron or 2-oxoglutarate deficiency. As iron and 2-oxoglutarate are critical for the optimal function of mitochondrial respiration *via* their functions on Fe/S cluster proteins and production of reducing equivalents (NADH and FADH2), it is not surprising that deficiency in either of these two co-substrates would trigger transcriptional responses that decrease the dependence on mitochondrial respiration and increase the dependence on glycolytic metabolism. In this review, we provide an overview of the enzymology of the PHDs and the transcriptional responses modified by changes in enzyme activity. We also discuss other transcriptional activators that are induced by oxidative stress in the central nervous system.

3. REGULATION OF PROLYL 4-HYDROXYLASE (PHD) ENZYME ACTIVITY *VIA* IRON, 2-OXOGLUTARATE AND OXYGEN BINDING SITES

The hydroxylation reaction catalyzed by the PHDs comprises of an iron-mediated incorporation of a hydroxyl group into the conserved proline residue with the consumption of a dioxygen molecule and release of carbon dioxide, whereas 2-oxoglutarate is converted to succinate (7) (for review, see Siddiq *et al.*, 2007) (Figure 1). PHDs that selectively catalyze the formation of hydroxyproline in the HIF-1 molecule by the hydroxylation of conserved proline residues belong to a sub-group of the dioxygenases. The most extensively studied prolyl hydroxylases are the ones that hydroxylate a proline residues in collagen molecules (8-13). These studies reveal that the enzyme, isolated as a homogeneous protein by affinity chromatography from three different sources (8-10, 12, 14, 15), occurs as a tetramer with a molecular weight of about 240,000 (11, 16-18). The enzyme does not hydroxylate free proline, and recognizes a conserved motif (LXXLAP in the HIF-1 molecule; X indicates any amino acid and P indicates the hydroxyl acceptor proline) in the primary substrate for hydroxylation (5, 11, 19-22). The hydroxylation of prolyl residues in this sequence is influenced by the nature of the amino acid in the X position, the nature of the amino acids in the adjacent sequences, the chain length and conformation of the primary substrate (11, 16, 23).

In general, kinetic mechanisms for enzyme reactions fall into two major groups, sequential and substitutional. In sequential mechanisms, all the reactants must combine with the enzyme before the reaction can occur and the product is released; whereas in substitution mechanisms one or more products are released before all the substrates have become bound to the enzyme. Kinetics studies of PHDs suggest that the binding of the co-substrates Fe^{2+}, 2-oxoglutarate, oxygen, and the proline containing primary substrate to the enzyme occurs by a sequential mechanism (23, 24) (Figure 1). However, several aspects of the co-substrate affinity, binding and requirements of prolyl hydroxylase have remained unresolved. For instance, while the absolute requirement of iron by PHDs and the mechanism by which iron mediates the transfer of a hydroxyl group in the proline residue are well established, there is still considerable disagreement concerning the affinity and binding strength of iron in the prolyl hydroxylase protein. Spectroscopic evidence has been reported for the firm binding of iron to the enzyme (25, 26), however a main concern about these studies is the impurity of the enzyme preparations. Some of the *in vitro* studies suggest that the enzyme was not completely inhibited by EDTA, 2,2'-dipyridyl and a variety of other chelating agents such as Desferrioxamine (DFO) (27, 28), but others have found complete inhibition with some of these compounds (e.g. (27, 29, 30). There are also observations that prolyl hydroxylase purified by affinity chromatography on its polypeptide substrate linked to agarose retained about 40% of its maximal activity without the addition of Fe^{2+} (22, 31). *In vitro* studies clearly indicate that the activity of pure prolyl hydroxylase is completely dependent on added Fe^{2+}. Whether iron stays permanently bound to the enzyme in the tissues is not clear. There are reports suggesting that even when PHD is purified by an affinity column procedure, the enzyme does not retain sufficient quantities of iron to catalyze the reaction without the addition of this cation *in vitro* (32-34). Inhibition of the activity of PHDs in primary neurons by the treatment with iron chelators such as DFO, resulting in stabilization of HIF-1 and downstream target genes, indicates that these enzymes do rely on the labile pool of iron in the cells for their activity (35). Fe^{2+} is located in a pocket coordinated with the enzyme by three side-chains with two histidines and an aspartate forming the catalytic triad (36-38). However, the exact mode of the binding of iron to the enzyme molecule is not known, but it has been suggested in many previous reports that this binding may occur to one or more –SH groups present in the vicinity of the active site of the enzyme (33, 39). In agreement with this suggestion, *in vitro* studies

show that sulphydryl reagents inhibit PHD activity (33), and this inhibition can be reversed with dithiothreitol (DTT) (33, 40, 41).

PHDs catalyze the uncoupled decarboxylation of 2-oxoglutarate in the absence of the polypeptide substrate (42-47). It thus seems that Fe^{2+}, 2-oxoglutarate and oxygen, can bind to the enzyme in the absence of the polypeptide substrate. Studies using distinct structural analogs of 2-oxoglutarate such as dihydroxybenzoate (DHB) and dimethyl-oxalyl-glycine (DMOG) have been found to inhibit PHD activity (35). It is thus clear that the co-substrates 2-oxoglutarate and iron bind at separate sites on the enzyme molecule (30) and the inhibition of binding of either leads to inhibition of PHD activity and consequent activation of downstream pathways governed by PHDs. These sites are evidently also distinct from the binding site of the polypeptide substrate, as *in vitro* studies using oxaloacetate or Zn^{2+} do not affect the binding of the polypeptide substrate to the active site (27). This also clearly suggests that some of the citric acid cycle intermediates (27, 28) may act as physiological inhibitors of the enzymes.

The prolyl hydroxylase reaction is entirely dependent on O_2, and during the reaction one atom of the O_2 molecule becomes incorporated into the hydroxyl group of the formed hydroxyproline while the other is incorporated into the succinate (33). Mechanistic studies of the enzyme activity reveal that oxygen is activated before hydroxylation by the formation of a ferryl intermediate or hydroperoxide (32, 48-57). This occurs *via* interaction of molecular oxygen with Fe^{2+} leading to the oxidation of Fe^{2+} to Fe^{3+} (33, 36, 58-61). Compounds capable of inhibiting the formation of hydroperoxide, such as epinephrine and nitroblue tetrazolium, inhibit the activity of PHDs *in vitro* (62). A series of reports show that HIF-1 protein levels are generally low in rodent tissues under physiological conditions, however with organ or systemic hypoxia and diminished PHD activity, HIF-1 levels are significantly increased, and thus there is an increase in HIF-target gene transcription (35, 62-66).

4. PHD ACTIVITY-RESPONSIVE TRANSCRIPTION FACTORS

Most physiological processes including gene transcription occur as a result of changes in the tonic physiologic or biochemical state of the cells and tissues in order to either fulfill the basal endogenous needs to stay alive, or combat to and survive against an external stimuli such as stress. 2-oxoglutarate being a part of the TCA cycle and both Fe^{2+} and O_2 being electronically-activated, radical-generating molecules play an important role in determining the 'redox-state' or 'stress' driven processes in living cells. The sensitivity of the PHD activities towards changing levels of fundamental molecules, like oxygen, 2-oxoglutarate and iron, presents a very complex but rather useful scheme that engages cellular adaptive responses leading to the activation of rescue mechanisms. These mechanisms help the cell survive by initiating the transcriptional up-regulation of genes that enhance tissue perfusion and anaerobic ATP generation pathways. Gene array analysis has recently revealed significant information regarding global changes in the gene transcription pathways of cells in response to stress such as hypoxia/ischemia. These changes not only enhance the capability of the system to combat the hypoxic conditions but also contribute to hypoxia-induced phenotypic changes in the cells (67, 68). Studies have also demonstrated that stress as a result of changes in oxygen or iron has a cell-type and cell-state specific effect on the cellular transcriptome. Although the knowledge of the transcriptional mechanisms that are activated by the oxygen sensors is crucial to influence the downstream gene transcription events in favor of physiologic recovery, an effective therapeutic approach requires a global understanding of the upstream pathways. These stress-sensing mechanisms that communicate the stress signal to specific transcriptional regulators in the brain are areas of intense investigation in our own laboratory as well, and the current hypotheses include investigations regarding the direct role of oxygen, iron and 2-oxoglutarate dependent enzymes prolyl hydroxylases in neuroprotective transcriptional pathways. In the following section, we discuss the various transcription factors that play a established role in neuroprotection and are regulated in response to changes in oxygen, iron or 2-oxoglutarate and thus may be a potential PHD target or interactors.

4.1. The Hypoxia-Inducible-Factors (HIF)

The HIF transcription system including its homologs (e.g., HIF-1α, HIF-2α, HIF-3α) has emerged as a key regulator of responses to changes in PHD activity as a result of changes in oxygen and/or iron levels, both at local and systemic levels. So far, HIF is the most established PHD-regulated transcription factor. The degradation of HIF occurs in the presence of molecular oxygen by modification of oxygen-dependent degradation domains within the HIF protein carried out by the PHDs. These enzymes add a hydroxyl group in conserved proline residues (402 and 564 of the alpha subunit of HIF-1) thus facilitating interaction with the von Hippel-Lindau tumor suppressor, which targets HIF-1α for proteasomal degradation (Figure 2). Treatment of both isolated neurons or animals with PHD inhibitors leads to stabilization of HIF and an increase in the levels of its downstream target genes (e.g.erythropoietin, vascular endothelial growth factor, glycolytic enzymes) (35).

It is believed that approximately 1–1.5% of the genome is transcriptionally regulated by hypoxia. Many of these genes are known to be regulated by HIF-1α regulate biological changes (e.g. increased O_2 delivery, increased angiogenesis, increased anaerobic glycolysis) that facilitate adaptation to hypoxia and associated metabolic compromise. In addition to its role in combating hypoxia, HIF-dependent gene expression provides resistance to oxidative stress, since many of the genes regulated by HIF-1 or HIF-2 (e.g. erythropoietin, VEGF, MnSOD) prevent oxidative stress-induced death by themselves (69-73). Recent evidence has pointed out that reactive oxygen species (ROS) generation occurs at multiple time points

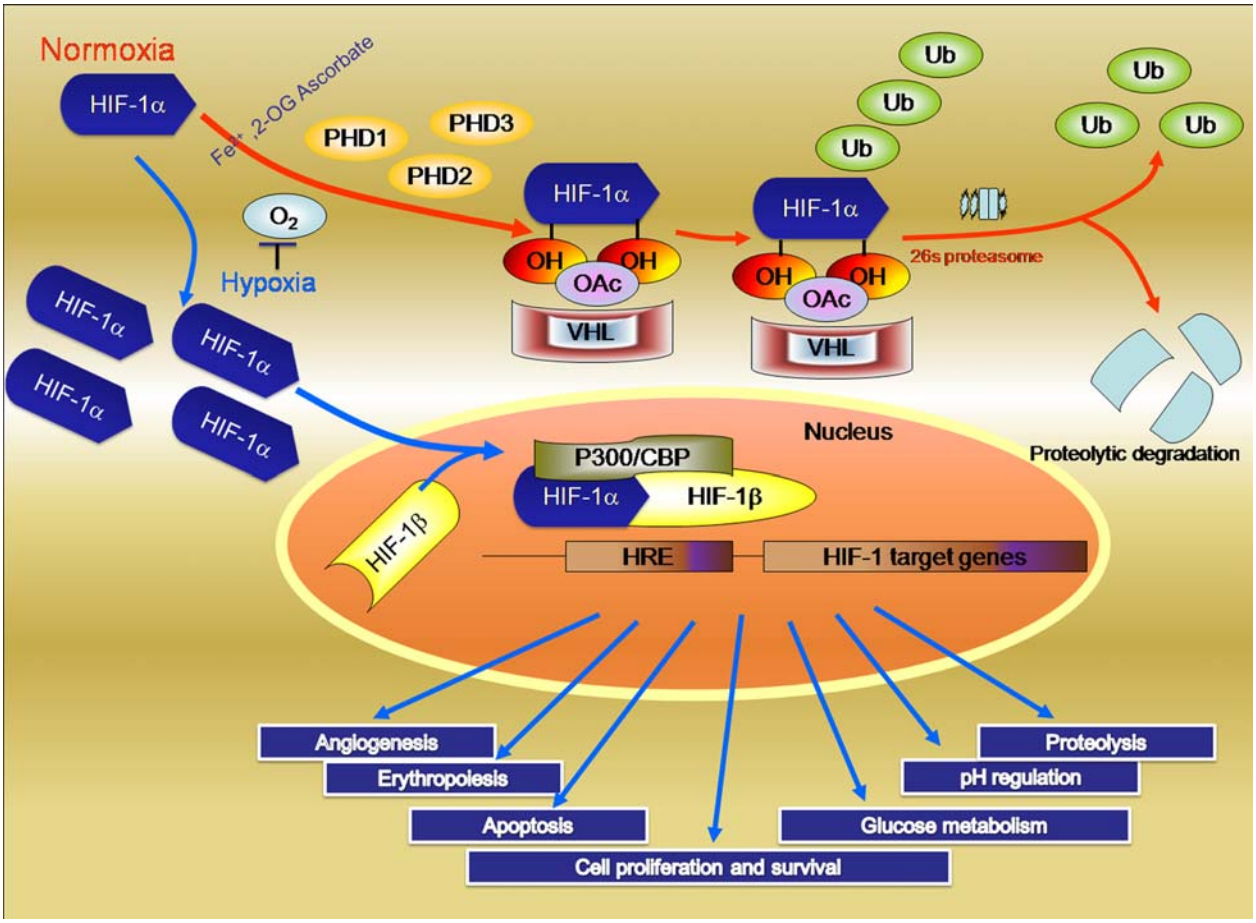

Figure 2. Cellular response to hypoxia: Levels of Hypoxia Inducible Factor-1 (HIF-1) are regulated by cellular oxygen by proline hydroxylation. The reaction is catalyzed by the enzymes prolyl 4-hydroxylases. Under normoxia (blue arrows), the intracellular level of HIF-1α is kept low by rapid ubiquitination and subsequent proteasomal degradation via recruitment of von Hippel–Lindau protein (pVHL), which depend on the hydroxylation of proline residues. In contrast, under hypoxia (orange arrows), both the intracellular level and the transcriptional activity of HIF-1α increase as a result of suppressed PHD activities. Consequently, HIF-1α forms a heterodimer with HIF-1β and changes the transcriptional rates of HIF-1-regulated genes under hypoxia; Reproduced with permission from # 7.

after stroke (74-76). Studies on the expression of HIF-1 and its target genes in the adult rat brain have shown that after focal cerebral ischemia, mRNAs encoding HIF-1α, glucose transporter-1 and several glycolytic enzymes including lactate dehydrogenase were up-regulated in the areas around the infarction (77-80).

Pharmacological and genetic strategies that reduce oxidative injury and decrease brain damage are now considered to be an effective approach for drug development in stroke. The well-established role of PHDs in the scheme of HIF regulated gene transcription presents researchers a distinct therapeutic target for activation of HIF by small molecule "drugs" against oxidative stress. The advantage of this strategy as compared to prior "antioxidants" is the ability of a single drug to selectively target a single molecule (i.e. PHD) that will activate more than seventy genes providing adaptation to ischemia and oxidative stress (35, 81, 82) (Figure 2).

4.2. Cyclic AMP response element binding protein (CREB)

The cyclic AMP response element binding protein (CREB) is one of a family of leucine zipper transcription factors regulated by intracellular signaling mechanisms such as cAMP and Ca^{2+}. CREB family members contain a C-terminal basic domain that mediates DNA binding and a leucine zipper domain that facilitates dimerization. These two domains are separated by the kinase inducible domain (KID). Ser-133, present in the KID is phosphorylated in a stimulus-inducible manner leading to the binding of the KID domain to the transcriptional coactivator, CREB binding protein (CBP) (83, 84). This phosphorylation-dependent interaction between CREB and CBP is believed to function as the trigger for inducible gene expression. Since the identification and cloning of CREB, the molecular mechanisms by which it functions as an inducible regulator of transcription have been the focus of much investigation. Clear details of the upstream signaling mechanisms that

convert extracellular stimuli into CREB activation, by Ser-133 phosphorylation, in order for CREB to function as a stimulus-dependent transcriptional activator are not known. In neurons, CREB phosphorylation occurs under a wide variety of cellular circumstances. These include responses to growth factors during the development of the nervous system, depolarization and synaptic activity during normal neuronal function, and hypoxia and stress responses during stroke or neural injury.

Acute mild hypoxia in neuronal cells activates CREB through phosphorylation at serine 133 (85) and CREB and HIF-1 may act together at promoters of genes involved in hypoxic compensation (e.g. LDH). In an intestinal epithelial cell model, more severe hypoxia results in the CREB degradation, an event mediated through decreased activity of protein phosphatase 1γ (86). CREB degradation leads to a derepression of inflammatory gene expression and thus contributes to hypoxia activated inflammatory processes. Interestingly, more prolonged exposure to severe hypoxia results in CREB stabilization and a resolution of inflammatory gene expression through the transcriptional upregulation of small ubiquitin-related modifier-1 (SUMO-1) modification (87). Thus the hypoxia regulated CREB-dependent gene expression is dependent upon the extent and degree of the stimulus.

There are also reports that show treatment of primary neurons with hypoxia mimics, such as iron chelators, increases CREB binding to DNA (88). Recent unpublished data from our lab shows that treatment of primary neurons with structurally distinct inhibitors of PHD activity increase CREB protein as well as mRNA levels (Siddiq *et al.*, unpublished data). Although the molecular components by which hypoxia or hypoxia mimics alter signaling cascades culminating in CREB inhibition/activation have not been as precisely defined, some attractive candidates, such as SAPK2/p38MAPK exist. As a hypoxia-activated kinase, SAPK2 has at least three downstream targets, MAPKAP K2, MSK1, and MSK2. All of which are CREB kinases (89, 90). These appear to be critical regulators of CREB because in mouse fibroblasts lacking MSK1 and MSK2, CREB phosphorylation in response to stress is eliminated almost entirely (91). There are various reports showing a direct effect of hypoxia on the activity of these kinases. For instance, the activation of p38 MAPK requires dual phosphorylation of threonine 180 (Thr180) and tyrosine 182 (Tyr182) residues within the conserved threonine-glycine-tyrosine (TGY) motif. This is reported to be involved in conveying extracellular stress to cellular response such as inflammation and the processes of cell differentiation, growth, and death (92). However, reports suggest that under hypoxic conditions enhanced phosphorylation of CREB is accompanied by the decrease of ERK1/2 phosphorylation in mice brains (90, 93-96).

There are also reports that suggest inhibition of kinase activities by prolyl hydroxylases, such as the ones that regulate NF-κB activation by a hydroxylation mechanism (97) (see next section for details). It is possible that under normoxia inhibition of one or more of the phosphorylating kinases in the ERK/MAPK signaling pathway occurs by the addition of a hydroxyl group by the PHDs, whereas under hypoxic conditions, inhibition of PHD activity allows for the activation of the CREB by phosphorylation. Decrease in ERK phosphorylation under hypoxic conditions may be a result of feedback mechanisms once the survival machinery is active. In summary, there seems to be a cross talk between the CREB phosphorylation and activation under stress conditions and PHD activity under hypoxic conditions, but a cogent model of how these pathways intersect is just being established (Siddiq *et al.*, unpublished observations).

4.3. Nuclear factor kappa-B (NF-κB)

The transcription factor nuclear factor kappa-B (NF-κB) is known for its fundamental role in regulating immune and inflammatory responses. Originally discovered in B lymphocytes about 20 years ago (98), the NF-κB family members (p65 or RelA, RelB, c-Rel, p50/p105 or NF-κB 1, and p52/p100 or NF-κB 2) are also diffusely expressed in both neurons and non-neuronal cells (99). However, in certain regions of the brain, especially the cortex and hippocampus, constitutive nuclear NF-κB activity has been reported exclusively in neurons (100-103). Members of the NF-κB family share a conserved Rel-homology domain (RHD) responsible for DNA binding activity, protein dimerization, and nuclear translocation. NF-κB is bound to the repressor molecule inhibitory kappaB (IκB) in the cytosol in the absence of stimulus. This coupling of proteins masks the nuclear localization sequence (NLS) of NF-κB and sequesters the protein in the cytosolic compartment. Upon stimulation, IκB is targeted for ubiqutination and degradation by specific serine phosphorylation. The NLS of NF-κB is then exposed, and enables it to translocate to the nucleus where it carries out its transcriptional activity at specific κB sites within the promoter regions of target genes (104). NF-κB-responsive genes include those responsible for encoding inflammatory cytokines, chemokines and cell surface adhesion molecules and several hypoxia induced genes such as cyclooxygenase-2 (COX-2), tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6) and macrophage inflammatory protein-2 (MIP-2). NF-κB plays a dynamic role in the survival and death of neuronal and non-neuronal cells under physiological and pathological conditions (105, 106). Research has established that the activation of NF-κB by cytokines enhances neuronal survival by preventing apoptosis and that the anti-apoptotic action of cytokines disappears in neurons that are treated with a super-repressor IkappaB-alpha protein, or lacking the RelA (p65) subunit of NF-κB (107). The inhibition of NF-κB renders various types of cells highly vulnerable to apoptosis (104, 108).

Until recently, the central event in NF-κB activation that is the removal of the IκB complex from the transcription factor was considered to be through a process involving phosphorylation and degradation in which the IκB degradation is preceded by phosphorylation of serine residues 32 and 36 mediated by the IκB kinase (IKK) complex. However more recent reports show that NF-κB activity is mainly regulated by the regulation of molecules further upstream of the IκB phosphorylation. Hypoxia and

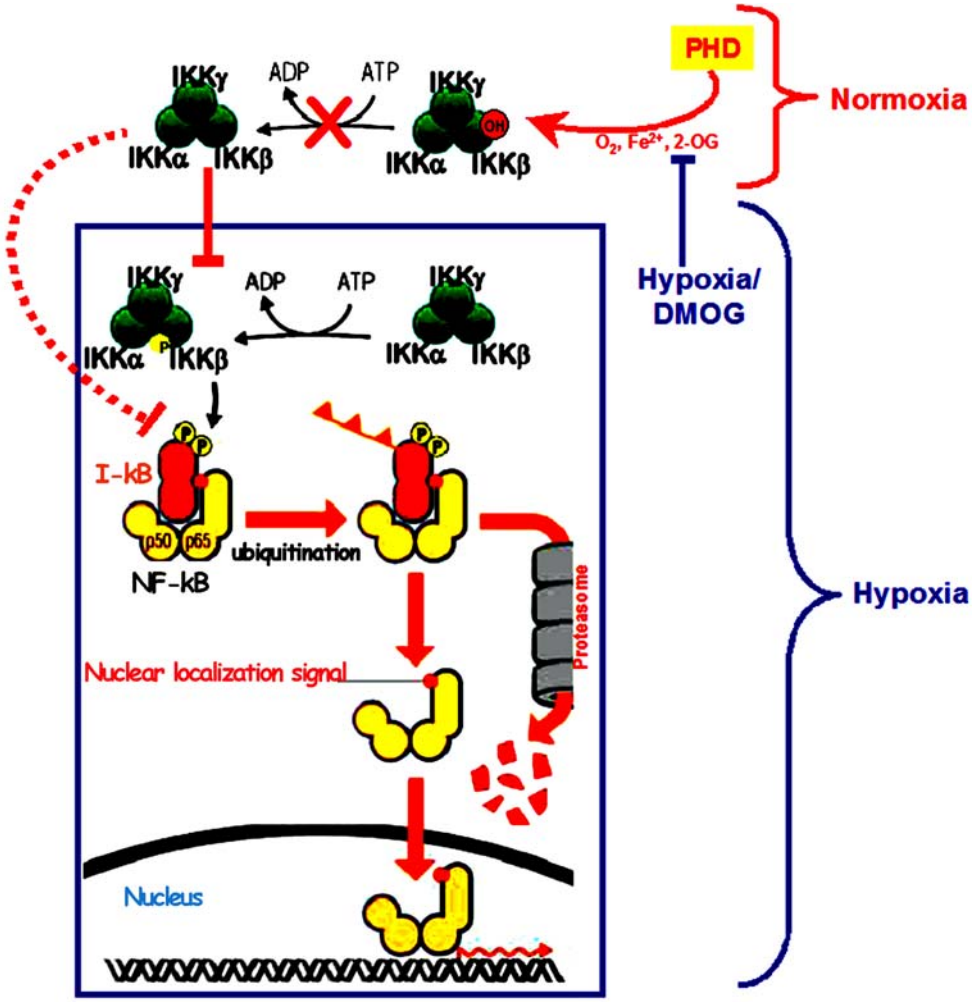

Figure 3. Nuclear factor kappaB regulation, NF-κB dimers are held in the inactive state by a family of inhibitors called I-κB. Diverse signaling mechanisms, such as inhibition of PHD activity by hypoxia or DMOG, leads to activation of a multisubunit kinase IKK complex which phosphorylates I-κB on two key serines. Phosphorylation of I-κB marks it for degradation by the ubiquitin pathway, NF-κB dimer is thus liberated to translocate to the nucleus, bind DNA and activate transcription. Under normoxia, a hydroxylation reaction, catalyzed by PHDs hydroxylates the proline on the IKK□ subunit, inhibits the downstream phosphorylation events thus inhibiting NF-κB activation.

more importantly specific inhibition of PHD activity by pharmacological agents as well and siRNA stimulate NF-κB transcriptional activity (97). Mechanistic studies reveal that neither NF-κB nor IκB contain hydroxylation motif but two of the important upstream kinases namely, inhibitory kappaB kinase alpha (IKKα) and inhibitory kappaB kinase beta (IKKβ), contain the sequence LXXLAP, the conserved proline containing motif present in HIF-1 molecule making it a primary substrate for hydroxylation by PHDs. Mutation of this conserved proline residue to alanine resulted in the loss of hypoxic-inducibility of NF-κB activity. It is proposed that under normoxic conditions, hydroxylation of this conserved proline residue inhibits the phosphorylation activity of these kinases thereby inhibiting the phosphorylation and degradation of the IκB subunit. Under hypoxia, inhibition of PHD activity inhibits the hydroxylation of the IKKα and IKKβ, leading to the phosphorylation and degradation of IκB and subsequent activation of NF-κB transcriptional activities (Figure 3).

4.4. Specific protein 1 and 3 (Sp1 and Sp3)

Sp1 and Sp3 are ubiquitous transcription factors of the Sp/XKLF transcription factor family that are involved in basal transcription and housekeeping gene expression (109-112). This family includes members such as Sp2–Sp4 that contain identical sequence binding motifs, but can display differential activity, depending on the stimuli. For instance, polyglutamine expansions in the huntingtin protein can induce neuronal toxicity, in part, by sequestering Sp1 and one of its coactivators, TATA binding protein-associated factor (TAF)II130, suggesting a role for Sp1 in neuronal survival (113, 114). Sp1 has also been shown to regulate prosurvival proteins e.g., the inhibitor of apoptosis (IAP) protein, survivin (115), and manganese superoxide dismutase (116) as well as prodeath

proteins e.g., Fas ligand (117, 118) and 12-lipoxygenase (119). Like other transcription factors, the role of Sp1 in regulating cell death may depend on a number of factors, such as the cell type and the death stimulus (120). Levels of Sp1 are regulated to an extent by mRNA expression, but further regulation can be imposed by proteasomal degradation, for example, in response to nutrient starvation and adenylate cyclase stimulation (121). Reports suggest that in primary neurons, oxidative stress increases the levels as well as DNA binding of both Sp1 and Sp3 in neurons (122). Similarly, several classically hypoxia-responsive genes such as EPO and VEGF have Sp1/Sp3 binding sites within promoter regions that are thought to facilitate transcriptional activation (123, 124). Sp1 and Sp3 have also shown to be involved in COX-2 expression in a hypoxia independent manner. Both hypoxia and oxidative stress increase nuclear localization of Sp1 and Sp3 levels (122, 125). Forced expression of Sp1 and Sp3 enhances neuronal survival under oxidative stress conditions. Sp1 and Sp3 activation appear to be temporally related to the onset of oxidative stress in cortical neurons and not a late event that is a consequence of oxidative stress-induced cell death. Activation of Sp1 and Sp3 DNA binding occurs within the first 2 hr of glutamate or HCA exposure and is maximal by 5 hr. The kinetics of Sp1 and Sp3 activation demonstrate that induction of these factors is an "early" response to cell stress, and their activation is initiated 8–10 hr before the point at which neurons become irreversibly "committed" to the cell death pathway (88, 126-128). The close temporal relationship between oxidative stress and Sp1 and Sp3 activation is also supported by the observation that structurally diverse small molecules including inhibitors of the prolyl hydroxylases (e.g., DFO) that inhibit oxidative glutamate toxicity also block the activation of Sp1 and Sp3 by glutathione depletion, despite having no effect on glutathione depletion per se (88, 127). The direct effect of hypoxia or oxidative stress on Sp1 and Sp3 activities and presence of Sp1 and/or Sp3 binding sites in hypoxia-regulated genes such as VEGF, indicates a HIF-1 independent but redox-sensitive mechanism by which levels of survival genes may be regulated. Similarly, induction of VEGF may also occur *via* a p42/p44 MAP kinase-dependent mechanism (129). These studies reveal the presence of two Sp1-binding sites present in the MAP kinase responsive region. Wild type or mutant constructs for the Sp1 show that mutation of both Sp1-binding sites blocks the phosphorylation-dependent transcriptional activation and VEGF induction (130). There are also reports showing activation of the MAP kinase pathway by hypoxia /ischemia (93) which in turn affects Sp1 activation. The redox sensitive regulation of Sp1/Sp3 levels and transcriptional activity and their ability to induce neuroprotection and survival genes through diverse signaling mechanisms suggests the presence of a molecular framework connected *via* PHD activity.

4.5. Activating protein-1 (AP-1)

Activating protein-1 (AP-1) is a redox-sensitive transcription factor and it has been suggested that hypoxia and consequently the redox environment in the cell, initiates AP-1-mediated gene transcription (131). It comprises members of Fos, Jun, ATF (activating transcription factors) and MAF (musculoaponeurotic fibrosarcoma) (132-138) protein families that can homodimerize or heterodimerize to form the active AP-1 complex and modulate gene expression. The combinatorial interaction of these proteins provides multiple levels of gene expression control. In addition, cell type and the differentiation state can dictate the phenotypic outcome, accounting at least in part for how AP-1 can regulate apparently conflicting endpoints (137). AP-1 is involved in diverse cellular functions related to apoptosis, cell proliferation, cell differentiation, catecholamine biosynthesis, inflammation, xenobiotic metabolism, tumor invasion and angiogenesis (138). Genes regulated by AP-1 in hypoxia include tyrosine hydroxylase (139), VEGF (140), and endothelial NOS (eNOS) (131). AP-1 co-operates with other transcription factors such as HIF-1, GATA-2, NF-1 and NF-κB to complement the activation of hypoxia-sensitive genes (139-142). Thus, AP-1 may represent an important facilitator of stress-induced gene expression through interaction with other transcription factors. The mechanism by which AP-1 is activated in hypoxia has yet to be fully elucidated. This is likely to be a complex process, given AP-1's apparent activation by oxidants (131) and anti-oxidants alike (143). Another signaling mechanism proposed is the hypoxia-induced modulation of intracellular Ca^{2+} levels upstream of AP-1 activation (139, 140, 144, 145). This increase is thought to activate AP-1 independently of HIF. Other reports demonstrate a role for non-receptor tyrosine kinases in propagating the hypoxic signal from G protein-coupled receptors based on results implicating a role for Src (non-receptor tyrosine kinase) and Ras (145). Reports suggest that AP-1 activation under oxidative stress is mediated *via* a Jun N-terminal kinase (JNK)-dependent pathway (146). An interesting model of JunD induced gene expression *via* PHDs has been presented by Gerald *et al* (147). Accordingly, JunD, a member of the AP-1 family, regulates both genes involved in antioxidative defense and H_2O_2 production. Increased production of H_2O_2 by JunD inhibits the PHD enzyme activity by promoting iron oxidation i.e. by converting Fe^{2+} to Fe^{3+}. An increased proportion of PHD in the Fe^{3+}-inactivated state limits PHD activity, and therefore a decrease in HIF-1α hydroxylation, and degradation. Subsequently, HIF-1α accumulation enhances VEGF-A transcription. Reciprocally, JunD overexpression decreases intracellular H_2O_2 content, alleviates toxic effects of ROS, and efficiently counteracts Ras-induced angiogenesis in tumors.

5. PERSPECTIVES

Hypoxia is among the most fundamental of stresses for multicellular organisms that depend on oxygen as the terminal electron acceptor in efficient mitochondrial ATP production. The precise mechanism by which a change in oxygen tension leads to a complete reorganization of metabolic infrastructure is beginning to be elucidated. Central to these adaptive reorganization efforts is the change in activity of the oxygen, Fe^{2+} and 2-oxoglutarate dependent dioxygenases known as the HIF prolyl 4-hydroxylases. These enzymes possess a Km for oxygen that makes them ideal oxygen sensors (7). In

response to changes in iron, oxygen or 2-oxoglutarate, PHD activity decreases. It is now clear that in addition to the canonical PHD substrate HIF, other transcription factor families such as CREB, NF-κB and AP-1 are also regulated by changes in PHD activity. The precise targets that must be hydroxylated to suppress adaptive hypoxia signaling are being defined. Moreover, an understanding of the tissue specific and subcellular mechanisms by which PHDs modify the tone of gene expression are only beginning to be defined. The current review summarizes our limited knowledge of what promises to be a very fruitful and therapeutically relevant field of investigation.

6. ACKNOWLEDGMENTS

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Abbreviations: 2-OG: 2-oxoglutarate, AP-1: activator protein 1, ATP: Adenosine triphosphate, CO2: Carbon dioxide, CBP: CREB binding protein, CREB: camp response element binding protein, DFO: Desferrioxamine, DMOG: Dimethyl-oxalyl-glycine , DTT: Dithiothreitol , EDTA: ethylenediamine tetraacetic acid, ERK: Extracellular signal-regulated kinase, FADH: Flavin adenine dinucleotide, Fe2+: Ferrous, Fe3+: Ferric, HIF: Hypoxia inducible factor, HIF-1?: Hypoxia inducible factor-1 alpha, HIF-1?: Hypoxia inducible factor-1 beta, I-kB: Inhibitory kappaB, IKK: Inhibitory kappaB kinase, KID: Kinase inhibitory domain, LDH: Lactate dehydrogenase, MAPK: Mitogen-activated protein kinases, MAPKAP K2: mitogen-activated protein kinase-activated protein kinase-2, MnSOD: Manganese Superoxide Dismutase, MSK: mitogen stimulated kinase, NADH: Nicotinamide adenine dinucleotide, NF-kB: nuclear factor kappa B, NLS: Nuclear localization signal, O2: Oxygen, PHD: prolyl 4-hydroxylase domain, pVHL: von Hippel-Lindau protein, ROS: Reactive Oxygen Species, RHD: Rel homology domain, SAPK: stress-activated protein kinase, SP1: specific protein1, SP3: specific protein3, SUMO-1: small ubiquitin-related modifier-1, TCA: Tricarboxylic acid, VEGF: Vascular endothelial growth factor

Key Words: Prolyl Hydroxylase, Dioxygenases, Iron, 2-Oxoglutarate, Oxygen, Transcription Factor, Camp Response Element Binding Protein, CREB, Nuclear Factor Kappa B, NF-kB, Activating protein, AP-1, specific protein 1, Sp1, Sp3, Hypoxia Inducible factor, HIF, Ischemia, Stroke, Oxidative Stress, Neuroprotection, Review

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