

Hydroxylase-dependent regulation of the NF- κ B pathway.

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Abstract

Hypoxia is associated with a diverse range of physiologic and pathophysiologic processes including development, wound healing, inflammation, vascular disease and cancer. Because of the requirement that eukaryotic cells have for molecular oxygen as the terminal electron acceptor for the electron transport chain, the maintenance of oxygen delivery is key for bioenergetic homeostasis. Metazoans have evolved an effective way to adapt to hypoxic stress at the molecular level through a transcription factor termed the hypoxia inducible factor (HIF). A family of oxygen-sensing hydroxylases utilizes molecular oxygen as a co-substrate for the hydroxylation of HIF α subunits thereby reducing its expression and transcriptional activity when oxygen is available. Recent studies have indicated that other hypoxia-responsive transcriptional pathways may also be hydroxylase-dependent. In this review, we will discuss the role of hydroxylases in the regulation of NF- κ B, a key regulator of immunity and inflammation. Developing our understanding of the role of hydroxylases in hypoxic inflammation may identify novel therapeutic approaches in chronic inflammatory disease.

Oxygen-sensing hydroxylases

The hypoxia-inducible factor (HIF) is an evolutionarily conserved transcriptional regulator of the cellular response to hypoxia (Semenza 2010). The oxygen-dependence of HIF is dependent upon its α -subunit being a substrate for a family of hydroxylases which utilize molecular oxygen as a co-substrate to hydroxylate HIF α subunits thereby targeting them for degradation and preventing their transcriptional activity (Kaelin and Ratcliffe, 2008). Four key oxygen-sensing hydroxylases signal this transcriptional response to hypoxia. These enzymes exhibit the characteristics of cellular oxygen sensors and belong to the 2-oxoglutarate-dependent-oxygenase superfamily (Schofield and Ratcliffe, 2004; Kaelin and Ratcliffe, 2008). Members of this family are non-haem, Fe²⁺-dependent enzymes and are involved in regulating processes as diverse as the cellular metabolic strategy, DNA repair and extracellular matrix remodeling (Kivirikko, et al., 1989; Schofield and Ratcliffe, 2004; Taylor and McElwain, 2010). The four oxygen-sensing hydroxylases which are termed Prolyl-Hydroxylase Domain-containing protein 1 (PHD1), PHD2, PHD3 and Factor Inhibiting HIF (FIH) were discovered during investigations into the mechanisms underpinning the oxygen-dependence of HIF (Jaakkola, et al., 2001; Bruick and McKnight, 2001; Epstein, et al., 2001; Mahon, et al., 2001; Hewitson, et al., 2002; Lando, et al., 2002). PHD1, PHD2, PHD3 and FIH are dioxygenases meaning that both atoms of metabolized molecular O₂ are incorporated into the products of their enzymatic reaction (Schofield and Ratcliffe, 2004). Oxygen-sensing hydroxylases catalyse protein substrate hydroxylation with the PHDs hydroxylating proline residues and FIH hydroxylating asparagine residues

(Ivan, et al., 2001; Jaakkola, et al., 2001; Yu, et al., 2001; Lando, et al., 2002; McNeill, et al., 2002). The first and best-characterised protein substrate of these enzymes is the HIF-1 α subunit (see below).

In order to catalyze the hydroxylation of a protein, hydroxylases require Fe²⁺ and a reducing agent such as ascorbate as co-factors and 2-oxoglutarate (a Krebs cycle intermediate) and molecular O₂ as co-substrates (Figure 1) (Kaelin and Ratcliffe, 2008). The Fe²⁺ is bound by a two-histidine, one carboxylate motif at the catalytic site, which is conserved throughout the 2-oxoglutarate-dependent-oxygenase superfamily (Schofield and Ratcliffe, 2004). For 2-oxoglutarate-dependent oxygenases, a common enzymatic cycle has been described that starts with an enzyme-Fe²⁺ complex binding first 2-oxoglutarate then the main substrate followed by molecular O₂ (Schofield and Ratcliffe, 2004). During the catalysis of the hydroxylation reaction by hydroxylases, one atom of molecular oxygen is used for the oxidative decarboxylation of 2-oxoglutarate resulting in succinate and CO₂ while the other oxygen atom is used for the hydroxylation of the target protein (Hewitson, et al., 2002; McNeill, et al., 2002; Schofield and Ratcliffe, 2004). The reaction cycle finishes by releasing the hydroxylated substrate first, which is followed by succinate. The Fe²⁺ is needed for the formation of a highly reactive ferryl intermediate (Fe^{IV} = O) that oxidizes the substrate amino acid. Ascorbate is necessary for the full enzymatic activity and probably reduces the iron atom in case of an “uncoupled” turnover independent of the main substrate in which the iron would be left in an inactive form if ascorbate were not present (Schofield and Ratcliffe, 2004).

While oxygen availability is a key determinant of hydroxylase activity, it is worth noting that other physiological modulators have also been described including iron, metabolic intermediates, reactive oxygen species and nitric oxide (Figure 2 and Table 1). Chelating iron or adding metal ions such as Co^{2+} inhibits hydroxylases. Furthermore, HIF α can be stabilized in cultured cells when oncogenic pathways are activated (Chan, et al., 2002). When iron or ascorbate was added to these cells HIF-1 α was down-regulated in a PHD-dependent manner indicating that either ascorbate, iron or both were the limiting factors (Knowles, et al., 2003; Page, et al., 2008). However, knockout mice that are unable to synthesize ascorbate and have no dietary ascorbate supplementation demonstrate normal regulation of EPO in hypoxia (an indirect measurement for the activity of oxygen-sensing hydroxylases) indicating that ascorbate is not necessary for hydroxylase activity *in vivo* (Nytko, et al., 2011).

2-oxoglutarate levels could also be limiting for oxygen-sensing hydroxylases indicating a possible link between the Krebs cycle and hydroxylase activity. However, it remains unclear whether cellular 2-oxoglutarate drops to levels which are limiting for the oxygen-sensing hydroxylase activity *in vivo* (Schofield and Ratcliffe, 2004). It has been shown that other intermediates of metabolism, such as fumarate, succinate, citrate and oxaloacetate are also capable of inhibiting hydroxylases. Fumarate and succinate compete for the 2-oxoglutarate binding site inhibiting all three PHDs to a similar extent (Koivunen, et al., 2007). However, these two metabolic intermediates are less effective in inhibiting FIH, which is more susceptible to oxaloacetate and citrate (Hewitson, et al., 2007; Koivunen, et al., 2007).

Additionally, it has been reported that cells with inactive succinate hydrogenase or fumarate hydratase (FH) accumulate succinate or fumarate and show a deregulated HIF pathway in a hydroxylase-dependent manner (Isaacs, et al., 2005; Pollard, et al., 2005; Selak, et al., 2005; MacKenzie, et al., 2007). Recently, it has been demonstrated that the R-enantiomer of 2-hydroxyglutarate, which can be generated by mutated forms of the isocitrate dehydrogenase (IDH) 1 and 2 found in human brain tumors, stimulates oxygen-sensing hydroxylase activity leading to decreased HIF levels (Koivunen, et al., 2012). Together, these data suggest that in certain circumstances, metabolic intermediates can impact upon the activity of hydroxylases and modulate their signaling.

Mitochondria-derived reactive oxygen species (ROS) have also been suggested to impact upon the hydroxylase activity (Pan, et al., 2007). It has been reported that enhanced mitochondrial ROS production stabilizes HIF-1 α (Chandel, et al., 2000; Brunelle, et al., 2005; Guzy, et al., 2005; Mansfield, et al., 2005) but it also has been shown that this stabilization occurs independent of mitochondrial ROS generation (Chua, et al., 2010). As a potential mechanism explaining how ROS may influence HIF protein levels, it has been reported that ROS oxidize Fe²⁺ to Fe³⁺ (Gerald, et al., 2004). Fe²⁺ is essential for the activity of the hydroxylases while Fe³⁺ cannot be used. Therefore, the oxidation of Fe²⁺ to Fe³⁺ may lead to an inhibition of the hydroxylases (Gerald, et al., 2004). Importantly, the effects of ROS on HIF signaling are likely to be both time and dose dependent (Niecknig, et al., 2012). Furthermore, a recent study demonstrated increased sensitivity of FIH over PHDs to oxidative stress supporting the idea that ROS may play a role in modulating the cellular

response to hypoxia through modulation of oxygen-sensing hydroxylase activity but are unlikely to be primary signaling molecules (Masson, et al., 2012).

Nitric oxide (NO) modifies the activity of hydroxylases both in normoxia and hypoxia. In cells exposed to high concentrations of NO in either normoxia or hypoxia HIF-1 α is induced in a manner which is independent of the oxygen concentration. However, lower NO concentrations reduce HIF-1 α levels in hypoxia (Mateo, et al., 2003; Hagen, et al., 2003; Berchner-Pfannschmidt, et al., 2010). A reason for this paradoxical effect could be that at higher concentrations, NO directly inhibits PHD activity (Metzen, et al., 2003; Berchner-Pfannschmidt, et al., 2007; Chowdhury, et al., 2011), probably by blocking the interaction of oxygen with the Fe²⁺ in the catalytic site of these enzymes (Berchner-Pfannschmidt, et al., 2010; Ho, et al., 2012). At lower concentrations in hypoxia, NO-dependent inhibition of mitochondrial cytochrome c oxidase (complex IV) results in increased oxygen availability for hydroxylases leading to HIF degradation (Hagan et al, 2003). In support of a role for high concentrations of NO directly inhibiting hydroxylase activity, PHD2 has been shown to be inhibited by NO in cell culture (Berchner-Pfannschmidt, et al., 2007). Studies using recombinant proteins suggested that the effect of NO upon PHD2 was irreversible, however, in studies in cell culture NO-induced reduction of HIF-1 α -hydroxylation was found to be reversible (Metzen, et al., 2003; Berchner-Pfannschmidt, et al., 2007; Tug, et al., 2009). It has also been suggested that FIH is inhibited by NO, nonetheless, this was demonstrated only when iron and ascorbate were limiting (Metzen, et al., 2003; Park, et al., 2008). On the other hand, HIF-

dependent target genes, such as PHD2, are up-regulated in response to NO indicating the inhibition of both PHDs and FIH (Kimura, et al., 2000; Berchner-Pfannschmidt, et al., 2007). Overall, the effect of NO upon oxygen sensing is complex and likely bimodal (Berchner-Pfannschmidt, et al., 2010).

PHD2 and PHD3 are both targets for HIF-1 while PHD1 and FIH are not (Berra, et al., 2003; Stiehl, et al., 2006). PHD2 and 3 are part of a negative feedback loop controlling the HIF activity (Berra, et al., 2003; Stiehl, et al., 2006; Ginouves, et al., 2008; Henze and Acker, 2010). Therefore, every inhibitor of oxygen-sensing hydroxylases that leads to HIF-dependent gene expression induces the expression of PHD2 and PHD3.

A number of pharmacologic hydroxylase inhibitors have been developed for experimental use. The most widely studied inhibitor is Dimethyloxallyl Glycine (DMOG), which has been used to inhibit hydroxylase activity in cell culture and in *in vivo* studies. DMOG was the first described cell-permeable pharmacological inhibitor for the oxygen-sensing hydroxylases (Jaakkola, et al., 2001; Epstein, et al., 2001). It was designed as a cell-permeable 2-oxoglutarate analogue and inhibits all four HIF-hydroxylase isoforms. Desferrioxamine (DFO) and Hydralazine are also capable of inhibiting oxygen-sensing hydroxylases although with a different mechanism of action than DMOG (Fraisl, et al., 2009). These inhibitors chelate Fe^{2+} , the metal ion needed by the PHDs and FIH for their activity (Bergeron, et al., 2000; Knowles, et al., 2004; Hirsila, et al., 2005; Dendorfer, et al., 2005). A third way of inhibiting the oxygen-sensing hydroxylases pharmacologically is blocking the active site of these enzymes, which is utilized by compounds like FG-4497, TM6089 and others (for a more comprehensive overview over

pharmacological hydroxylase inhibitors see Fraisl et al, 2009) (Nangaku, et al., 2007; Robinson, et al., 2008; Rosenberger, et al., 2008; Fraisl, et al., 2009). Recent progress has led to the development of a PHD-specific inhibitor, JNJ-42041935 (JNJ1935), which like N-oxalylglycine (DMOG is converted into its active form N-oxalylglycine in cells) competes with 2-oxoglutarate for the binding of the PHDs although it does not inhibit FIH (Barrett, et al., 2011). Furthermore, a FIH-specific inhibitor has been reported (Tian, et al., 2011). However, thus far it has proven difficult to generate PHD isoform specific inhibitors.

The catalyzed reaction of hydroxylases is not reversible according to current knowledge (Schofield and Ratcliffe, 2004). Therefore, the abundance of oxygen-sensing hydroxylases correlates with the protein hydroxylation capacity. Hence, the regulation of hydroxylase gene expression is also a mechanism for modulation of hydroxylase activity (Schofield and Ratcliffe, 2004). PHD1 is induced by oestrogen in breast cancer cells (Seth, et al., 2002). PHD2 and PHD3 are up-regulated by hypoxia in a HIF-dependent manner serving as a negative feedback loop for the HIF regulation (Berra, et al., 2003; Stiehl, et al., 2006; Ginouves, et al., 2008; Henze, et al., 2010). Furthermore, as described earlier, NO induces PHD2 expression in a HIF-dependent fashion (Berchner-Pfannschmidt, et al., 2007; Berchner-Pfannschmidt, et al., 2008). PHD3 has also been reported to be a p53 target gene and stimuli that induce smooth muscle differentiation induce PHD3 as well as the withdrawal of nerve-growth-factor (Wax, et al., 1994; Madden, et al., 1996; Lipscomb, et al., 2001). For FIH no cellular stimulation that regulates its transcriptional regulation has been reported thus far.

INSERT TABLE 1 HERE

Roles of oxygen-sensing hydroxylases *in vivo*.

The four hydroxylase isoforms, which have been demonstrated to regulate HIF demonstrate different substrate specificity as well as dramatically different tissue distribution profiles. Therefore, it is perhaps not surprising that whole-body homozygous knockout of the individual oxygen-sensing hydroxylase isoforms in mice has shown quite different phenotypes indicating differential and non-redundant roles of these enzymes *in vivo* (Takeda, et al., 2006; Aragonés, et al., 2008; Minamishima, et al., 2008; Takeda, et al., 2008; Bishop, et al., 2008; Mazzone, et al., 2009; Zhang, et al., 2010). PHD1 homozygous knockout mice display reprogrammed glucose metabolism in skeletal muscles due to a shift from oxidative to anaerobic ATP production (Aragones, et al., 2008). Because of this differential regulation of metabolism, muscle performance was impaired in healthy conditions but myofibers were protected against ischemia (Aragones, et al., 2008). PHD2 homozygous knockout was lethal in mouse embryos because of abnormal development of the placenta and the heart (Takeda, et al., 2006; Minamishima, et al., 2008). PHD2 heterozygous mice in turn are viable and show increased tumor perfusion and oxygenation together with reduced tumor cell invasion, intravasation and metastasis (Takeda, et al., 2008; Mazzone, et al., 2009). This is caused by endothelial normalization and vessel maturation (Mazzone, et al., 2009). PHD3 homozygous knockout mice display reduced neuronal apoptosis together with abnormal sympathoadrenal development (Bishop, et al., 2008). This ultimately impacts upon blood pressure leading to systemic

hypotension (Bishop, et al., 2008). Homozygous knockout mice for FIH showed a reduced body weight, elevated metabolic rate, hyperventilation, improved glucose and lipid homeostasis and resistance to high-fat-diet-induced weight gain (Zhang, et al., 2010). Neuron-specific FIH loss indicated that these effects were caused to a significant degree through the nervous system (Zhang, et al., 2010). Overall these findings show that FIH is an essential regulator of metabolism *in vivo*.

HIF as a target of oxygen-sensing hydroxylases

Investigations of the hypoxia-induced transcription of the human erythropoietin (EPO) gene revealed a hypoxia-responsive transcription factor that was subsequently found to be expressed by all metazoans: the hypoxia-inducible factor 1 (HIF-1) (Semenza and Wang, 1992). HIF-1 is a heterodimer consisting of the oxygen-dependent HIF-1 α subunit and the constitutively expressed HIF-1 β subunit (also known as the aryl hydrocarbon receptor nuclear translocator 1 (ARNT1)) (Wang and Semenza, 1995; Wang, et al., 1995). Two more proteins were identified to also interact with HIF-1 β and to share structural and functional characteristics with HIF-1 α . These were termed HIF-2 α and HIF-3 α (Flamme, et al., 1997; Ema, et al., 1997; Tian, et al., 1997; Hogenesch, et al., 1997; Gu, et al., 1998). HIF-2 α is also known as the endothelial Per/ARNT/Sim (PAS) domain protein 1 (EPAS1) and HIF-3 α as inhibitory PAS domain protein (IPAS) (Tian, et al., 1997; Makino, et al., 2001). All HIF proteins contain the basic helix-loop-helix (bHLH)-PAS homology domain, which mediates heterodimerization and DNA binding (Gu, et al., 1998; Semenza, 2010). The dimerization of HIF-1 β with HIF-1 α or HIF-

HIF-2 α confers transcriptional activity while the most clearly defined function for HIF-3 α is the inhibition of HIF transcriptional activity through an alternative splice variant (Makino, et al., 2001; Hara, et al., 2001). Nonetheless, HIF-3 α is the least understood HIF α protein and little is known about its regulation although it also seems to be regulated in an oxygen-dependent manner similar to HIF-1 α and HIF-2 α (Maynard, et al., 2003). Overall, HIF-1 α is the best-characterized isoform of the HIF α proteins and its regulation by hydroxylases will be discussed below.

In normoxia, HIF-1 α is hydroxylated by oxygen-sensing hydroxylases leading to its degradation and the inactivation of its transcriptional activity (Figure 3). The activity of these enzymes is absolutely dependent on molecular oxygen and the activity of the hydroxylases is regulated by the availability of oxygen over the range of its physiological concentrations (Kaelin and Ratcliffe, 2008). The PHDs hydroxylate HIF-1 α on prolines 402 (P402) and P564 (Kaelin, 2005). P402 lies within an N-terminal oxygen-dependent degradation domain of HIF-1 α (NODDD), while P564 is located in a C-terminal oxygen-dependent degradation domain (CODDD) (Schofield and Ratcliffe, 2004). Both proline residues are contained within a sequence motif, LXXLAP, which has been hypothesized to serve as a recognition site (Epstein, et al., 2001). However, it is unlikely that PHD-dependent hydroxylation of target proteins is restricted to such motifs. The hydroxylation of the prolines in the HIF-1 α protein is recognized by the von Hippel-Lindau (VHL) tumor suppressor protein that interacts with Elongin C recruiting an E3 ubiquitin ligase complex that poly-ubiquitinates HIF-1 α targeting it for proteasomal degradation (Maxwell, et al., 1999; Ohh, et al., 2000; Ivan, et al.,

2001; Jaakkola, et al., 2001; Yu, et al., 2001). PHD2 is the main oxygen-sensing hydroxylase isoform regulating HIF-1 α stability with PHD3 being part of a negative feedback loop for HIF-1 α together with PHD2 (Berra, et al., 2003; Appelhoff, et al., 2004; Stiehl, et al., 2006). The role of PHD1 in the regulation of HIF is less clear.

The PHD-targeted NODDD and CODDD are part of an N-terminal transactivation domain (NTAD). Beside the NTAD there is also a C-terminal transactivation domain (CTAD) present within HIF-1 α , which is targeted by FIH. FIH hydroxylates asparagine 803 (N803) within the CTAD leading to the inhibition of its interaction with the transcriptional co-activators p300 and CBP (CREB (cAMP-response-element-binding-protein)-binding protein) blocking the transactivation function of HIF-1 α (Mahon, et al., 2001; Lando, et al., 2002). The substrate consensus sequence for FIH is still part of active investigations. The most recently published consensus sequence is LXXXXXD/E Φ N Φ (with Φ representing hydrophobic amino acids) (Wilkins, et al., 2012).

In hypoxia, activity of the oxygen-sensing hydroxylases is inhibited and HIF-1 α escapes both proteasomal degradation and the inactivation of its transactivation function and translocates into the nucleus where it interacts with HIF-1 β to form the transcriptionally active HIF-1 transcription factor (Figure 2). HIF-1 then binds to hypoxia response elements (HRE) within the DNA that contain the consensus motif RCGTG, which is associated with a large number of transcriptional target genes (Schofield and Ratcliffe, 2004; Kaelin and Ratcliffe, 2008). Analysis of cells exposed to hypoxia or hypoxia-mimetics showed that HIF-1 regulates the expression of hundreds of genes

(Manalo, et al., 2005; Elvidge, et al., 2006; Semenza, 2010; Schodel, et al., 2011). HIF-1 target genes are involved in both local and systemic responses to hypoxia and include genes regulating angiogenesis, vasomotor regulation and erythropoiesis as well as proteins of the glucose/energy metabolism (Schofield and Ratcliffe, 2004). As HIF-1 is regulated in an oxygen-dependent manner and its transcriptional activity is switched on in hypoxia, it is therefore seen as the master regulator of the adaptation of the cellular metabolism to low oxygen concentrations (Semenza, 2012).

The NF- κ B pathway as a target of oxygen-sensing hydroxylases

The transcriptional response to hypoxia is not restricted to regulation by HIF. Indeed, multiple transcription factors other than HIF have been shown to demonstrate sensitivity to hypoxia (Cummins and Taylor, 2005). Therefore, it is perhaps not surprising that HIF is not the sole transcription factor or indeed cellular substrate that is regulated by the HIF-hydroxylases.

Inflammation provokes major metabolic changes in tissues, depleting nutrients and oxygen, at least partly due to the infiltration of highly metabolic inflammatory cells and an increased metabolic activity of inflamed resident tissue cells and partly due to vascular dysfunction leading to malperfusion (Taylor, 2008; Colgan and Taylor, 2010). Therefore, the demand for oxygen within inflamed sites is often increased until it eventually overcomes the supply, resulting in hypoxia. The co-existence of inflammation and hypoxia has been proven by measurements of oxygen levels within the inflamed intestinal mucosa in inflammatory bowel disease and within inflamed arthritic joints (Hauser, et al., 1988; Murdoch, et al., 2005; Colgan and Taylor, 2010).

Under such conditions the O₂-dependent oxygen-sensing hydroxylases are inhibited.

It has been demonstrated by multiple groups that hypoxia influences the activity of NF-κB, a master transcriptional regulator of innate immune and inflammatory gene expression both *in vitro* and *in vivo*. Furthermore, mounting evidence suggests that the oxygen-sensitivity of the NF-κB pathway is mediated at least in part by oxygen-sensing hydroxylases (Cummins, et al., 2006; Cummins, et al., 2008; Robinson, et al., 2008; Takeda, et al., 2009; Chan, et al., 2009; Winning, et al., 2010; Xue, et al., 2010; Adluri, et al., 2011; Hams, et al., 2011). Additionally, it has been demonstrated *in vivo* that pharmacologic hydroxylase inhibition is beneficial in models of inflammatory bowel disease and LPS-induced sepsis (Cummins, et al., 2008; Robinson, et al., 2008; Hams, et al., 2011). Here we will review the current knowledge pertaining to the influence of hypoxia, pharmacological inhibition of hydroxylases and individual hydroxylase isoforms in the regulation of NF-κB activity.

Hypoxia:

Hypoxia was first shown to regulate NF-κB signalling in 1994 (Koong, et al., 1994). Recently, a number of *in vitro* studies have demonstrated that hypoxia enhances basal NF-κB activity in cultured cells (Cummins, et al., 2006; Rius, et al., 2008; Chan, et al., 2009; Oliver, et al., 2009; Xue, et al., 2010) and increases NF-κB-dependent inflammatory gene expression, including ICAM-1, TNF, IL-6 and MIP-2 (Taylor, et al., 1998; Matsui, et al., 1999; Zampetaki, et al., 2004; Winning, et al., 2010). Using transgenic reporter mice, it was

recently demonstrated that hypoxia also modulates NF- κ B activity *in vivo* (Fitzpatrick, et al., 2011).

Pharmacological inhibition of oxygen-sensing hydroxylases:

Hydroxylases have been proposed to be regulators of NF- κ B in hypoxia (Cummins, et al., 2006). Supporting this hypothesis are experiments demonstrating that DMOG up-regulates basal NF- κ B activity *in vitro* (Winning, et al., 2010; Xue, et al., 2010; Hams, et al., 2011). Interestingly, the hydroxylase inhibitors DMOG and FG4497 were found to be beneficial in mouse models of inflammatory bowel disease demonstrating the general importance of oxygen-sensing hydroxylases in inflammatory reactions (Cummins, et al., 2008; Robinson, et al., 2008; Zhang, et al., 2010). Additionally, DMOG administration attenuated LPS-induced NF- κ B activity and LPS-induced shock in mice (Hams, et al., 2011). DMOG induced IL-10, an anti-inflammatory cytokine that inhibits the secretion of pro-inflammatory cytokines and controls proliferation and differentiation of T cells, B cells and macrophages (Glocker, et al., 2011). Administration of an IL-10 neutralizing antibody blocked the beneficial effect of DMOG in LPS-induced sepsis (Hams, et al., 2011).

Impact of individual hydroxylase isoforms on NF- κ B signaling

PHD1: Investigating the impact of different PHD isoforms upon the NF- κ B pathway it was shown that basal and TNF α -induced NF- κ B activity was up-regulated through PHD1 knockdown and down-regulated by its overexpression (Cummins, et al., 2006; Winning, et al., 2010; Xue, et al.,

2010). Furthermore, based on experiments using siRNA the following order of potency for PHDs regulating NF- κ B activity was proposed: PHD1 > PHD2 > PHD3. Additionally, in PHD1 knockout mice exposed to myocardial ischemia/reperfusion injury, the DNA binding activity of NF- κ B was increased when compared to WT indicating that PHD1 also impacts upon NF- κ B *in vivo* (Adluri, et al., 2011). The potential impact of modulation of basal NF- κ B by PHD1 upon inflammatory reactions was illustrated by the finding that PHD1 knockdown in monocytes upregulates the expression of the intercellular adhesion molecule-1 (ICAM-1) in an NF- κ B-dependent manner (Winning, et al., 2010). ICAM-1 is an important protein in the mediation of intercellular interactions, as in this investigation between monocytes and endothelial cells, and these findings may lead to new therapeutic approaches in cases of the recruitment of detrimental numbers of monocytes to hypoxic inflammatory sites (Winning, et al., 2010).

Investigating LPS-induced TNF α expression it was demonstrated that this was strongly suppressed in macrophages by DMOG and siRNA-mediated knockdown of PHD1 (Takeda, et al., 2009). This was the first evidence for an NF- κ B-activating pathway to be down-regulated by hydroxylase inhibition after basal and TNF α -induced NF- κ B activity were shown to be up-regulated. This effect of PHD inhibition on NF- κ B appears to be stimulus-specific.

PHD2: PHD2 silencing up-regulates basal and TNF α -induced NF- κ B activity *in vitro* and *in vivo* while it down-regulates LPS-induced NF- κ B (Cummins, et al., 2006; Takeda, et al., 2009; Chan, et al., 2009; Winning, et al., 2010; Takeda, et al., 2011). PHD2 knockdown suppressed LPS-induced TNF α

expression in macrophages but to a less extent than PHD1 knockdown (Takeda, et al., 2009). On the other hand, PHD2 knockdown enhanced NF- κ B-dependent ICAM-1 expression without any additional NF- κ B activating stimuli (Winning, et al., 2010). Furthermore, it was demonstrated that PHD2 knockout in macrophages up-regulates basal and TNF α -induced NF- κ B activity and that it impacts upon angiogenesis and differentiation in macrophages via the NF- κ B pathway *in vivo* (Chan, et al., 2009; Takeda, et al., 2011).

PHD3: Evidence for PHD3 impacting upon NF- κ B signalling came from investigations by Fu and Taubman and Xue and colleagues. Both groups showed that PHD3 was a negative regulator of basal and TNF α -induced NF- κ B activity, using skeletal myoblasts (C2C12 cells), human colon cancer (HCT116, SW480) and human embryonic kidney cells (293T) (Xue, et al., 2010; Fu and Taubman, 2010). PHD3 impacts upon skeletal myoblast differentiation via the NF- κ B pathway and it was hypothesized that it also influences the malignant progression of colorectal cancer through NF- κ B (Xue, et al., 2010; Fu and Taubman, 2010). There are indications that the influence of PHD3 upon the NF- κ B pathway is via the IKK complex by influencing the recruitment of HSP90 to IKK β (Xue, et al., 2010).

FIH: In one study, FIH was found to influence basal NF- κ B activity where siRNA targeting FIH was used in a luciferase assay (Xue, et al., 2010). However, other groups have also studied the impact of FIH on NF- κ B without finding any major effect on basal, TNF α - and IL-1 β -induced NF- κ B activity

(Cockman, et al., 2006; Shin, et al., 2009; Devries, et al., 2010). Cockman et al. found no major difference in NF- κ B electromobility shift assays from TNF α -stimulated cells when FIH was overexpressed, in the association of I κ B α with the p50:p65 heterodimer or in NRE-luciferase assays with TNF α stimulation when FIH was knocked down (Cockman, et al., 2006). Shin and colleagues reported no effect of FIH overexpression or knockdown on TNF α - or IL-1 β -stimulated NF- κ B activity in an NRE-luciferase assay (Shin, et al., 2009). However, it was demonstrated that FIH interacts with four different NF- κ B proteins, I κ B α , I κ B ϵ , p105 and RIPK4, and even hydroxylates I κ B α and p105 (Cockman, et al., 2006; Cockman, et al., 2009). Nonetheless, while FIH is the only oxygen-sensing hydroxylase isoform that has been shown by MS/MS to hydroxylate NF- κ B proteins, it remains unclear whether this has a physiologically relevant impact upon NF- κ B activity.

Target proteins of oxygen-sensing hydroxylases within the NF- κ B pathway

NF- κ B plays an important role in gene expression during inflammatory diseases and a number of pharmacological inhibitors have been developed. However, due to its broad array of effects, the use of such inhibitors for clinical benefit has had limited success (Egan and Toruner, 2006; Gilmore and Herscovitch, 2006; Lee and Hung, 2008). As described above, there exists convincing evidence that oxygen-sensing hydroxylases impact upon inflammation through the NF- κ B pathway. Therefore, oxygen-sensing hydroxylases may be new drug targets for pharmacological intervention in inflammatory diseases. However, while there is evidence that oxygen-sensing hydroxylases regulate NF- κ B signalling, the relevant hydroxylation site(s)

involved have yet to be definitively identified. Here we will review possible target proteins within the NF- κ B pathway (Figure 4).

IKK α /IKK β : Two key kinases of the NF- κ B pathway, IKK α and IKK β , carry LXXLAP hydroxylation motifs similar to the hydroxylation sites on HIF-1 α and also interact with PHD1 (Cummins, et al., 2006). VHL, the protein that recognizes and binds to hydroxylated HIF mediating its polyubiquitination and targeting it for proteasomal degradation, was also shown to interact with IKK β (Cummins, et al., 2007). Furthermore, IKK β levels were shown to increase in hypoxia similar to HIF although to a lesser extent (Cummins, et al., 2006). Due to these findings it was hypothesized that IKK α and/or IKK β may be direct target(s) for functional hydroxylation by PHD1 but this has yet to be shown by direct methods such as mass spectrometry (Figure 4). Furthermore, it has been reported that PHD3 interacts with IKK β and inhibits TNF α -induced phosphorylation of this protein through inhibition of HSP90 recruitment (Xue, et al., 2010) (Figure 4). However, this effect has been described to be independent of its hydroxylase activity and is rather acting through competition for protein:protein interaction sites (Xue, et al., 2010).

Ankyrin-Repeat-Domain (ARD) containing proteins: FIH has been shown to hydroxylate a number of ARD-containing proteins within the NF- κ B pathway. I κ B α , I κ B ϵ , p105 (which all belong to the I κ B protein family) and RIPK4 were demonstrated to interact with FIH with I κ B α and p105 being hydroxylated *in vivo* (Cockman, et al., 2006; Shin, et al., 2009; Cockman, et al., 2009; Devries, et al., 2010) (Figure 4). The impact of hydroxylation of I κ B α on NF- κ B

signalling has been investigated by several groups. Cockman *et al.* reported no major effects, Devries and colleagues and Shin *et al.* did not find any effect at all (Cockman, et al., 2006; Shin, et al., 2009; Devries, et al., 2010). Therefore, it remains to be clarified if the observed hydroxylations have a physiologically relevant impact upon NF- κ B signalling.

Remaining questions

Are there stimulus-specific effects of hydroxylase inhibition on NF- κ B activity?

Basal, TNF α - and LPS-induced NF- κ B signalling have been investigated for an impact of oxygen-sensing hydroxylases to date, however, many more NF- κ B activating pathways are known. Furthermore, differential regulation of the investigated pathways has been reported (PHDs are negative regulators of basal and TNF α -induced NF- κ B but positive regulators of LPS-induced NF- κ B) indicating that there is not the same, general regulation for all NF- κ B-inducing signalling pathways. This makes it necessary to investigate the regulation of other NF- κ B-signalling pathways in order to understand how oxygen-sensing hydroxylases can shape complex inflammatory reactions *in vivo* where many signalling pathways are involved at the same time.

Is there combinatorial regulation of NF- κ B signaling by oxygen-sensing hydroxylases?

No investigations of a combinatorial regulation of NF- κ B through PHDs and FIH, similar to the HIF pathway, have been carried out to date. However, this possibility cannot be ignored as it was shown that FIH hydroxylates proteins within the NF- κ B pathway.

What is/are physiologically relevant hydroxylation targets within NF-κB pathways?

The physiologically relevant hydroxylation site(s) of the oxygen-sensing hydroxylases within the NF-κB pathway has not been identified to date. Some studies reported an impact of the oxygen-sensing hydroxylases upon NF-κB independent of their enzymatic activity but most of the findings were dependent on the hydroxylase activity.

In summary, while key questions remain, there now exists convincing evidence that oxygen-sensing hydroxylases regulate NF-κB activity and that basal and TNFα-induced NF-κB activity is up-regulated while LPS-induced NF-κB activity is down-regulated through PHD inhibition. It remains to be clarified whether FIH impacts upon NF-κB activity. A potential combinatorial regulation of NF-κB through PHDs and FIH together, similar to the regulation of HIF, remains a distinct possibility. Pharmacological inhibition of oxygen-sensing hydroxylases has been beneficial in different inflammatory diseases (inflammatory bowel disease, LPS-induced shock) indicating its potential as a therapeutic target in inflammation *in vivo* and underlining the importance of further investigations within this field.

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Table 1: Modulators of oxygen-sensing hydroxylase activity. Modulators of oxygen-sensing hydroxylase activity are listed and described in order to give an overview about the different influences impacting upon hydroxylase activity. This table is not supposed to be comprehensive but to give an illustrative overview about the complex regulation and influences impacting upon oxygen-sensing hydroxylases. The reference list is not comprehensive due to space limitations, for further information see the text of this review. 2-OG = 2-oxoglutarate; miRNA = microRNA; NO = nitric oxide

| Modulators of hydroxylase activity | | Mode of modulation | References | |
|------------------------------------|-------------------------------|---|--|---|
| Physiologic | Hypoxia | Depletion of O ₂ | Kaelin and Ratcliffe 2008 | |
| | Metabolic intermediates | Succinate | Competition with 2-OG | Kaelin and Ratcliffe 2008 |
| | | Fumarate | | |
| | | Citrate | | |
| | | Oxaloacetate | | |
| | Metal ions | Co ²⁺ | Competition with Fe ²⁺ | Schofield and Ratcliffe 2004 |
| | | Fe ²⁺ | Depletion of Fe ²⁺ | |
| | Reactive Oxygen Species (ROS) | | Hydroxylase inhibition; Regulation of ascorbate, Fe ²⁺ or metabolic intermediates | Hagen et al. 2003 Doege et al. 2005 Kaelin and Ratcliffe 2008 |
| | | NO | Hydroxylase inhibition; Regulation of mitochondrial oxygen consumption | Hagen et al. 2003; Metzen et al. 2003; Berchner-Pfannschmidt 2007 |
| | Pharmacological inhibitors | N-oxalylglycine (the active form of Dimethyloxallyl Glycine (DMOG)) | | Fraisl et al. 2009 |
| JNJ1935 | | Competition with 2-OG in PHDs only | Barrett et al. 2011 | |
| FG-4497 | | Blocking of active site of probably all four hydroxylases | Fraisl et al. 2009 | |
| Desferrioxamine (DFO) | | Iron chelator | Fraisl et al. 2009 | |
| Regulation of gene expression | Hypoxia | Up-regulation of expression of PHD2 and PHD3 | Stiehl et al. 2006 | |
| | NO | Up-regulation of PHD2 expression | Berchner-Pfannschmidt et al. 2007 | |
| | miRNA | miR-31 | Regulation of FIH mRNA abundance and translation | Liu et al. 2010 |
| | siRNA | | Mediates gene-specific RNA degradation; knockdown of any specific isoform possible | Berra et al. 2003; Appelhoff et al. 2004 |

FIGURE LEGENDS:

Figure 1: Catalyzed reaction by oxygen-sensing hydroxylases. In normoxia, when all factors needed by the oxygen-sensing hydroxylases are present, the hydroxylation of a target protein is catalysed using a reducing agent such as ascorbate and Fe^{2+} as co-factors and 2-oxoglutarate and molecular O_2 as co-substrates. Succinate and CO_2 are generated as co-products. In hypoxia, molecular oxygen is not available, the oxygen-sensing hydroxylases are inhibited and the reaction cannot take place.

Figure 2: Modulation of oxygen-sensing hydroxylase activity. Several different physiological and pharmacological modulators of oxygen-sensing hydroxylases have been described. The relative abundance of each of these factors impacts upon oxygen-sensing hydroxylase activity.

Figure 3: The HIF-1 pathway. In normoxia, the oxygen-sensing hydroxylases PHD1, PHD2, PHD3 and FIH hydroxylate HIF-1 α at proline 402 and 564 and at asparagine 803. The prolyl hydroxylations (shown in green) lead to poly-ubiquitination and degradation of HIF-1 α while the asparaginyl hydroxylation (shown in red) blocks the recruitment of the co-activators p300 and CBP, inhibiting the transactivation function of HIF-1 α . In hypoxia, the hydroxylases are inhibited, HIF-1 α escapes the hydroxylations and therefore its degradation and inactivation, translocates into the nucleus and switches on gene expression.

Figure 4: (Potential) target proteins of oxygen-sensing hydroxylase isoforms within the NF- κ B pathway based on demonstrated interactions with and/or modification through these hydroxylases (hydroxylation).

IKK β has been shown to interact with PHD1 and PHD3. Furthermore it carries an LXXLAP motif, which serves as hydroxylation site in HIF-1 α for PHD's. For PHD2 no interaction partner of the NF- κ B pathway has been described yet. FIH was shown to hydroxylate I κ B α and p105 and to interact with I κ B ϵ and RIPK4.

Figure 1

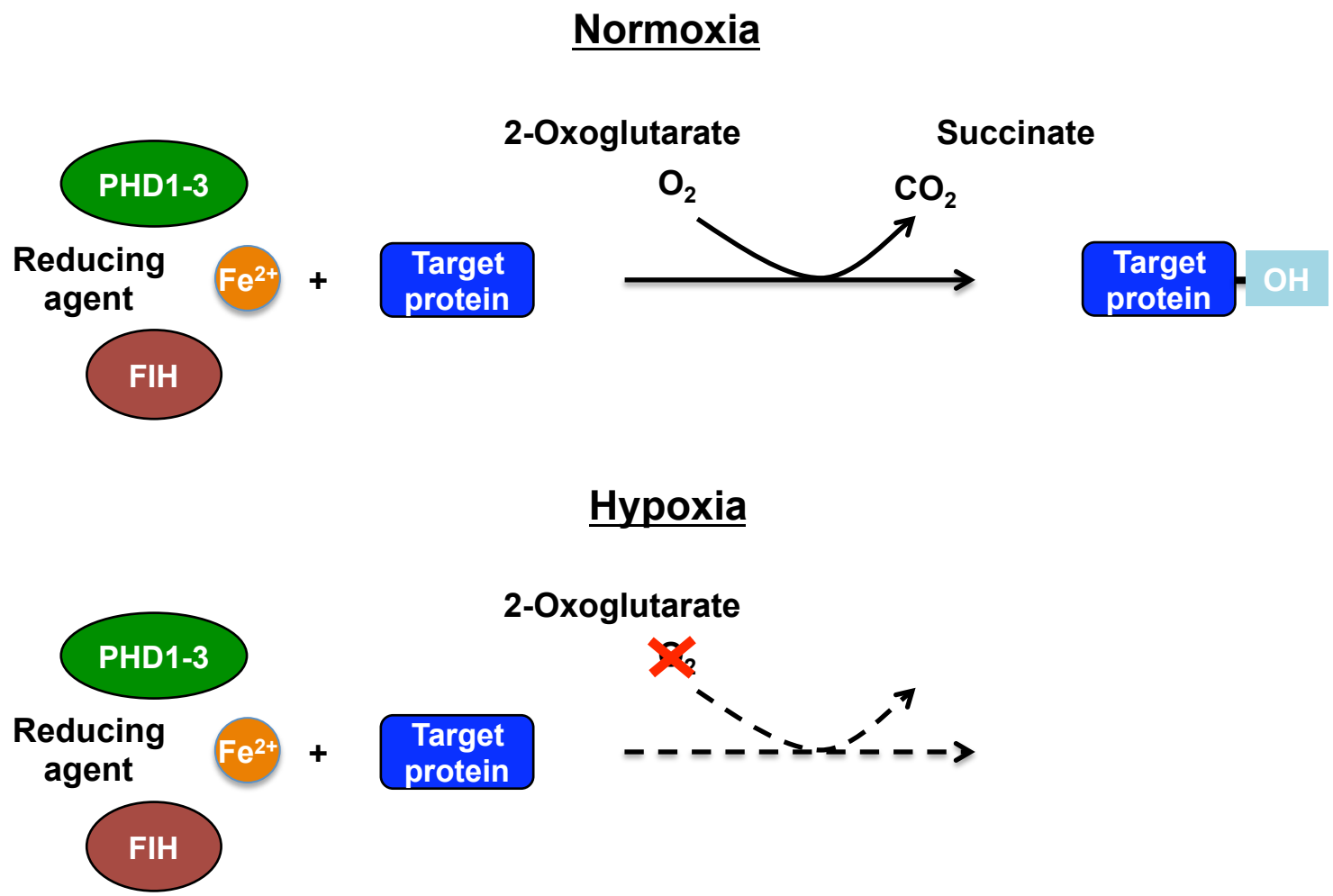


Figure 2

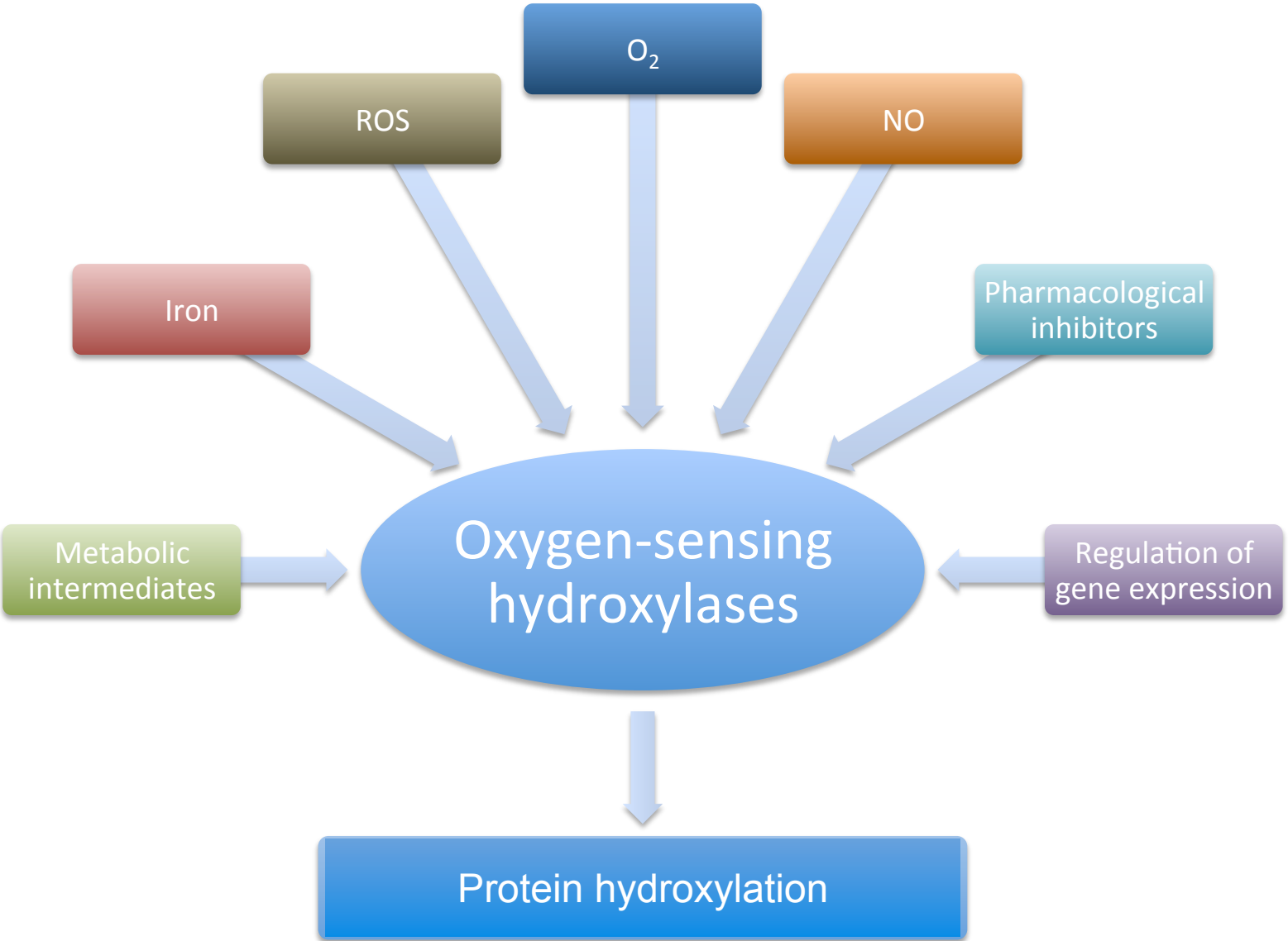


Figure 3

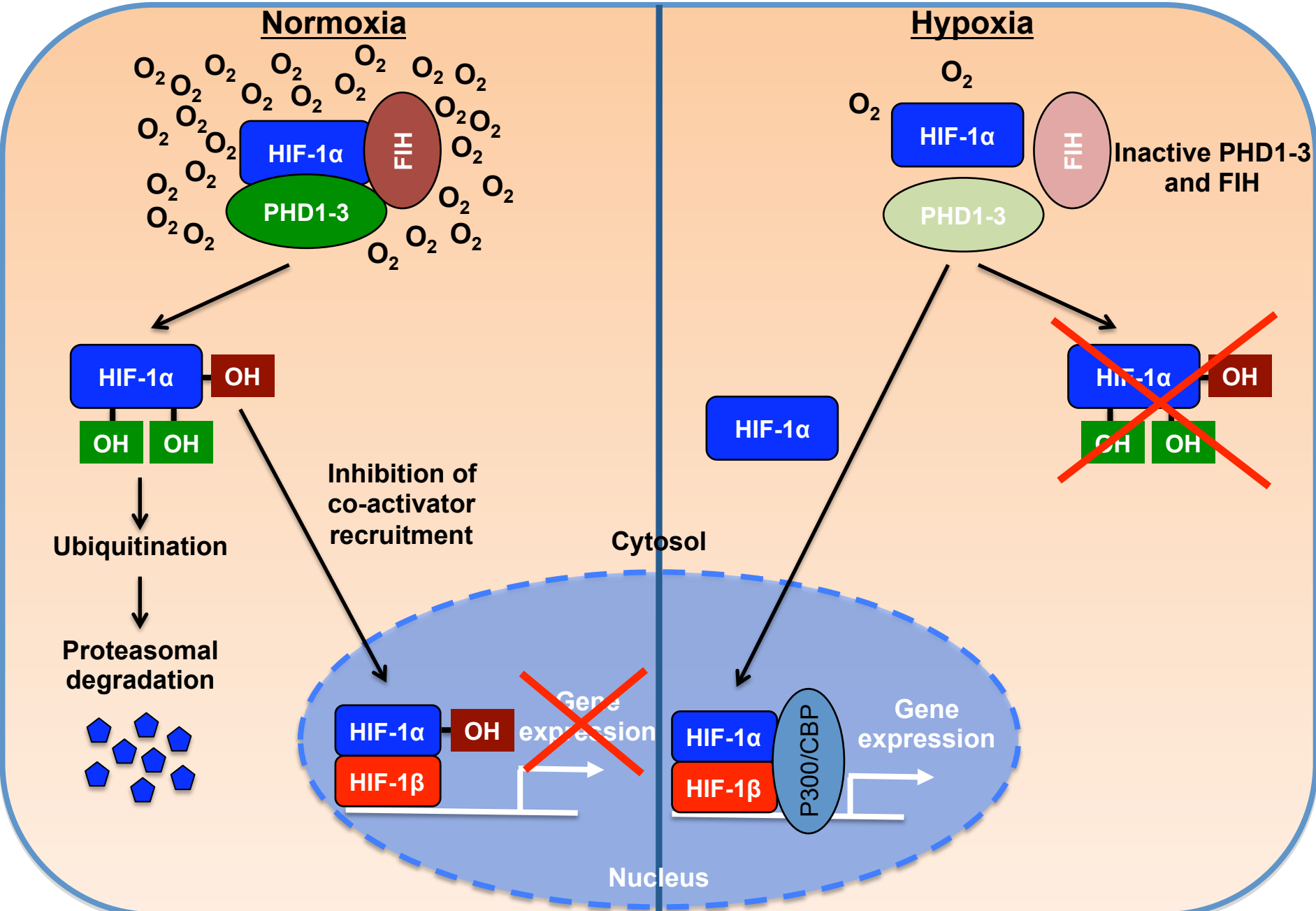


Figure 4

