Review

Carsten C. Scholz and Cormac T. Taylor*

Hydroxylase-dependent regulation of the NF-κB pathway

Abstract: Hypoxia is associated with a diverse range of physiological and pathophysiological processes, including development, wound healing, inflammation, vascular disease and cancer. The requirement that eukaryotic cells have for molecular oxygen as the terminal electron acceptor for the electron transport chain means that 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. A family of oxygen-sensing hydroxylases utilizes molecular oxygen as a co-substrate for the hydroxylation of hypoxia inducible factor 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-κB, 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.

Keywords: hydroxylase; hypoxia-inducible factor (HIF); NF-κB.

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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 that utilize molecular oxygen as a co-substrate to hydroxylate HIFα 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 are known as Prolyl-Hydroxylase Domain-containing protein 1 (PHD1), PHD2, PHD3 and Factor Inhibiting HIF (FIH). They were discovered during investigations into the mechanisms underpinning the oxygen-dependence of HIF (Bruick and McKnight, 2001; Epstein et al., 2001; Jaakkola 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 catalyze 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-characterized protein substrate of these enzymes is the HIF-1α 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–Fe$^{2+}$ complex binding first 2-oxoglutarate then the main substrate followed by molecular $O_2$ (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 $CO_2$, 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 Fe$^{2+}$ is needed for the formation of a highly reactive ferryl intermediate (Fe$^{IV}$=O) that oxidizes the substrate’s amino acid. Ascorbate is necessary for 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, HIFα 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-1α 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 erythropoietin 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).

The 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 that 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 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; McKenzie 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 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 have an impact upon the hydroxylase activity (Pan et al., 2007). It has been reported that enhanced mitochondrial ROS production stabilizes HIF-1α (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 Fe^{2+} to Fe^{3+} (Gerald et al., 2004). Fe^{2+} is essential for the activity of the hydroxylases while Fe^{3+} cannot be used. Therefore, the oxidation of Fe^{2+} to Fe^{3+} 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). 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 the 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-1α is induced in a manner that is independent of the oxygen concentration. However, lower NO concentrations reduce HIF-1α levels in hypoxia (Hagen et al., 2003; Mateo 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 Fe^{2+} 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 there being 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-1α-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 pharmacological 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 vivo studies. DMOG was the first described cell-permeable pharmacological inhibitor for the oxygen-sensing hydroxylases (Epstein et al., 2001; Jaakkola et al., 2001). It was designed as a cell-permeable 2-oxoglutarate analogue and inhibits all four HIF-hydroxylase isoforms. Desferrioxamine and hydralazine are also capable of inhibiting oxygen-sensing hydroxylases, although with a different mechanism of action to 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; Dendorfer et al., 2005; Hirsila et al., 2005). A third way of inhibiting

Table 1 Modulators of oxygen-sensing hydroxylase activity.

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<tr>
<th>Modulators of hydroxylase activity</th>
<th>Mode of modulation</th>
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<tr>
<td>Physiological Hypoxia</td>
<td>Depletion of O_{2}</td>
<td>Kaelin and Ratcliffe, 2008</td>
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<td>Metabolic intermediates Succinate</td>
<td>Competition with 2-OG</td>
<td>Kaelin and Ratcliffe, 2008</td>
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<td>Fumarate Citrate Oxaloacetate</td>
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<td>Metal ions Co^{2+}</td>
<td>Competition with Fe^{3+}</td>
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<td>Fe^{2+}</td>
<td>Depletion of Fe^{3+}</td>
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<td>Reactive oxygen species</td>
<td>Hydroxylase inhibition; Regulation of ascorbate, Fe^{2+} or metabolic intermediates</td>
<td>Hagen et al., 2003; Doege et al., 2005; Kaelin and Ratcliffe, 2008</td>
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<td>Nitric oxide</td>
<td>Hydroxylase inhibition; regulation of mitochondrial oxygen consumption</td>
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<td>Pharmacological inhibitors N-oxalylglycine (the active form of dimethyloxallyl glycine)</td>
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<td>JNJ1935</td>
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<td>FG-4497</td>
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<tr>
<td>Desferrioxamine</td>
<td>Blocking of active site of probably all four hydroxylases Iron chelator</td>
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<td>Hypoxia</td>
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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; PHD, prolyl-hydroxylase domain-containing protein.
the oxygen-sensing hydroxylases pharmacologically is blocking the active site of these enzymes. This mechanism of action is utilized by compounds such as FG-4497, TM6089 and others (for a more comprehensive overview of pharmacological hydroxylase inhibitors, see Fraisl et al., 2009) (Nangaku et al., 2007; Robinson et al., 2008; Rosenthal 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 to bind PHDs, although it does not inhibit FIH (Barrett et al., 2011). Furthermore, a FIH-specific inhibitor has been reported (Tian et al., 2011). Thus far, however, it has proven difficult to generate PHD isoform-specific inhibitors.

The catalyzed reaction of hydroxylases, according to current knowledge, is not reversible (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 the modulation of hydroxylase activity (Schofield and Ratcliffe, 2004). PHD1 is induced by estrogen 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, 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; 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 to bind PHDs, although it does not inhibit FIH (Barrett et al., 2011). Furthermore, a FIH-specific inhibitor has been reported (Tian et al., 2011). Thus far, however, it has proven difficult to generate PHD isoform-specific inhibitors.

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Roles of oxygen-sensing hydroxylases in vivo

The four hydroxylase isoforms that 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; Aragones et al., 2008; Bishop et al., 2008; Minamishima et al., 2008; Takeda 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). This differential regulation of metabolism led to impaired muscle performance 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 had 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 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-1α is a heterodimer consisting of the oxygen-dependent HIF-1α subunit and the constitutively expressed HIF-1ß subunit (also known as the aryl hydrocarbon receptor nuclear translocator 1) (Wang and Semenza, 1995; Wang et al., 1995). Two more proteins were identified as interactors of HIF-1β, which shared structural and functional characteristics with HIF-1α. These were termed HIF-2α and HIF-3α (Ema et al., 1997; Flamme et al., 1997; Hogenesch et al., 1997; Tian et al., 1997; Gu et al., 1998). HIF-2α is also known as the endothelial Per/ARNT/Sim (PAS) domain protein 1 and HIF-3α as the inhibitory PAS domain protein (Tian et al., 1997; Makino et al., 2001). All HIF proteins contain the basic
The dimerization of HIF-1β with HIF-1α or HIF-2α confers transcriptional activity, while the most clearly defined function for HIF-3α is the inhibition of HIF transcriptional activity through an alternative splice variant (Hara et al., 2001; Makino et al., 2001). Nonetheless, HIF-3α is the least well understood HIFα protein and little is known about its regulation, although it also seems to be regulated in an oxygen-dependent manner similar to HIF-1α and HIF-2α (Maynard et al., 2003). Overall, HIF-1α is the best-characterized isoform of the HIFα proteins and its regulation by hydroxylases will be discussed below.

In normoxia, HIF-1α 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-1α on prolines 402 (P402) and P564 (Kaelin, 2005). P402 lies within an N-terminal oxygen-dependent degradation domain of HIF-1α, while P564 is located in a C-terminal oxygen-dependent degradation domain (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-1α 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-1α, 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-1α stability, with PHD3 being part of a negative feedback loop for HIF-1α 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 N-terminal oxygen-dependent degradation domain of HIF-1α and C-terminal oxygen-dependent degradation domain are part of an N-terminal transactivation domain. Beside the N-terminal transactivation domain there is also a C-terminal transactivation domain present within HIF-1α, which is targeted by FIH. FIH hydroxylates asparagine 803 (N803) within the

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

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C-terminal transactivation domain, leading to the inhibition of its interaction with the transcriptional co-activators p300 and CREB (cAMP-response-element-binding-protein) blocking the transactivation function of HIF-1α (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 $LXXXXD/EΦΝΦ$ (with $Φ$ representing hydrophobic amino acids) (Wilkins et al., 2012).

In hypoxia, activity of the oxygen-sensing hydroxylases is inhibited and HIF-1α escapes both proteasomal degradation and the inactivation of its transactivation function and translocates into the nucleus, where it interacts with HIF-1β to form the transcriptionally-active HIF-1 transcription factor (Figure 2). HIF-1 then binds to hypoxia response elements 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 nuclear factor-κB (NF-κB) 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 coexistence 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 $O_2$-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., 2002, 2008; Robinson et al., 2008; Chan et al., 2009; Takeda 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 pharmacological hydroxylase inhibition is beneficial in models of inflammatory bowel disease and lipopolysaccharide (LPS)-induced sepsis (Cummins et al., 2008; Robinson et al., 2008; Hams et al., 2011). Here we will review 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 signaling 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 Intercellular Adhesion Molecule 1 (ICAM-1), tumor necrosis factor (TNF), interleukin-6 and macrophage inflammatory protein 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-κB activity in vivo (Fitzpatrick et al., 2011).

**Pharmacological inhibition of oxygen-sensing hydroxylases**

Hydroxylases have been proposed to be regulators of NF-κB in hypoxia (Cummins et al., 2006). Experiments demonstrating that DMOG up-regulates basal NF-κB
activity in vitro support this hypothesis (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-κB activity and LPS-induced shock in mice (Hams et al., 2011). DMOG induced interleukin-10 (IL-10), an anti-inflammatory cytokine that inhibits the secretion of pro-inflammatory cytokines and controls the 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-κB signaling

PHD1

Investigating the impact of different PHD isoforms upon the NF-κB pathway it was shown that basal and tumor necrosis factor α (TNFα)-induced NF-κB 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-κB activity was proposed: PHD1>PHD2>PHD3. Additionally, in PHD1 knockout mice exposed to myocardial ischemia/reperfusion injury, the DNA-binding activity of NF-κB was increased when compared to wildtype, indicating that PHD1 also impacts upon NF-κB in vivo (Adluri et al., 2011). The potential impact of modulation of basal NF-κB by PHD1 upon inflammatory reactions was illustrated by the finding that PHD1 knockdown in monocytes up-regulates the expression of ICAM-1 in an NF-κB-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).

While investigating LPS-induced TNFα 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 of an NF-κB-activating pathway being down-regulated by hydroxylase inhibition after basal and TNFα-induced NF-κB activity were shown to be up-regulated. This effect of PHD inhibition on NF-κB appears to be stimulus-specific.

PHD2

PHD2 silencing up-regulates basal and TNFα-induced NF-κB activity in vitro and in vivo while it down-regulates LPS-induced NF-κB (Cummins et al., 2006; Chan et al., 2009; Takeda et al., 2009; Winning et al., 2010; Takeda et al., 2011). PHD2 knockdown suppressed LPS-induced TNFα expression in macrophages, but to a lesser extent than PHD1 knockdown (Takeda et al., 2009). In contrast, PHD2 knockdown enhanced NF-κB-dependent ICAM-1 expression without any additional NF-κB-activating stimuli (Winning et al., 2010). Furthermore, it was demonstrated that PHD2 knockout in macrophages up-regulates basal and TNFα-induced NF-κB activity, and that it impacts upon angiogenesis and differentiation in macrophages via the NF-κB pathway in vivo (Chan et al., 2009; Takeda et al., 2011).

PHD3

Evidence for PHD3 impacting upon NF-κB signaling came from investigations by Fu and Taubman, and Xue and colleagues. Both groups showed that PHD3 was a negative regulator of basal and TNFα-induced NF-κB activity, using skeletal myoblasts (C2C12 cells), human colon cancer (HCT116, SW480) and human embryonic kidney cells (293T) (Fu and Taubman, 2010; Xue et al., 2010). PHD3 impacts upon skeletal myoblast differentiation via the NF-κB pathway, and it was hypothesized that it also influences the malignant progression of colorectal cancer through NF-κB (Fu and Taubman, 2010; Xue et al., 2010). There are indications that the influence of PHD3 upon the NF-κB pathway is via the IκB (inhibitor of NF-κB) kinase (IKK) complex by influencing the recruitment of heat shock protein 90 to IKKβ (Xue et al., 2010).

FIH

In one study, FIH was found to influence basal NF-κB 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-κB without finding any major effect on basal, TNFα- and IL-1β-induced NF-κB activity (Cockman et al., 2006; Shin et al., 2009; Devries et al., 2010).
et al., 2010). Cockman et al. found no major difference in NF-κB electromobility shift assays from TNFα-stimulated cells when FIH was overexpressed, in the association of IkBα with the p50:p65 heterodimer or in NRE-luciferase assays with TNFα stimulation when FIH was knocked down (Cockman et al., 2006). Shin and colleagues reported no effect of FIH overexpression or knockdown on TNFα- or IL-1β-stimulated NF-κB activity in a NF-κB response element (NRE)-luciferase assay (Shin et al., 2009). However, it was demonstrated that FIH interacts with four different NF-κB proteins, IkBα, IkBε, p105 and RIPK4, and even hydroxylates IkBα and p105 (Cockman et al., 2006, 2009). Nonetheless, while FIH is the only oxygen-sensing hydroxylase isoform that has been shown by tandem mass spectrometry to hydroxylate NF-κB proteins, it remains unclear whether this has a physiologically-relevant impact upon NF-κB activity.

Target proteins of oxygen-sensing hydroxylases within the NF-κB pathway

NF-κB plays an important role in gene expression during inflammatory diseases and a number of pharmacological inhibitors have been developed. Due to its broad array of effects, however, 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 is convincing evidence that oxygen-sensing hydroxylases impact upon inflammation through the NF-κB pathway. Therefore, oxygen-sensing hydroxylases may represent new drug targets for pharmacological intervention in inflammatory diseases. While there is evidence that oxygen-sensing hydroxylases regulate NF-κB signaling, however, the relevant hydroxylation site(s) involved have yet to be definitively identified. Here we will review possible target proteins within the NF-κB pathway (Figure 4).

IKKα/IKKβ

Two key kinases of the NF-κB pathway, IKKα and IKKβ, carry LXXLAP hydroxylation motifs similar to the hydroxylation sites on HIF-1α and they 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 IKKβ (Cummins et al., 2007). Furthermore, IKKβ 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 IKKα and/or IKKβ 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 IKKβ and inhibits TNFα-induced phosphorylation of this protein through the inhibition of heat shock protein 90 recruitment (Xue et al., 2010) (Figure 4). However, this effect has been
described to be independent of its hydroxylase activity and acts through competition for protein–protein interaction sites (Xue et al., 2010).

Ankyrin-repeat-domain containing proteins

FIH has been shown to hydroxylate a number of ankyrin-repeat-domain (ARD)-containing proteins within the NF-kB pathway. IkBα, IkBε, p105 (which all belong to the IκB protein family) and RIPK4 were demonstrated to interact with FIH, with IkBo and p105 being hydroxylated (Cockman et al., 2006, 2009; Shin et al., 2009; Devries et al., 2010) (Figure 4). The impact of hydroxylation of IκBα on NF-kB signaling 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 whether the observed hydroxylations have a physiologically-relevant impact upon NF-kB signaling.

Remaining questions

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

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

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

A combinatorial effect of PHDs and FIH on the regulation of NF-kB has not been investigated to date. However, this possibility cannot be ignored, as it was shown that both PHDs and FIH interact with proteins of the NF-kB pathway and as the HIF pathway is regulated by both, PHDs and FIH, in combination.

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

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

Conclusion

In summary, while key questions remain, there is now convincing evidence that oxygen-sensing hydroxylases regulate NF-kB activity and that basal and TNFα-induced NF-kB activity is up-regulated while LPS-induced NF-kB activity is down-regulated through PHD inhibition. It remains to be clarified whether FIH impacts upon NF-kB activity. A potential combinatorial regulation of NF-kB 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 and 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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References


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