Cellular Signalling 22 (2010) 349–356

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Review

Oxygen-sensing under the influence of nitric oxide

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ARTICLE INFO

Article history:
Received 30 September 2009
Accepted 18 October 2009
Available online 25 October 2009

Keywords:
Oxygen-sensing
HIF-1
PHDs
FIH-1
Nitric oxide
Feedback loop
O_2-redistribution

ABSTRACT

The transcription factor complex Hypoxia inducible factor 1 (HIF-1) controls the expression of most genes involved in adaptation to hypoxic conditions. Oxygen-dependency is maintained by prolyl- and asparagyl-4-hydroxylases (PHDs/FIH-1) belonging to the superfamily of iron(II) and 2-oxoglutarate dependent dioxygenases. Hydroxylation of the HIF-1α subunit by PHDs and FIH-1 leads to its degradation and inactivation. By hydroxylating HIF-1α in an oxygen-dependent manner PHDs and FIH-1 function as oxygen-sensing enzymes of HIF signalling. Besides molecular oxygen nitric oxide (NO), a mediator of the inflammatory response, can regulate HIF-1α accumulation, HIF-1 activity and HIF-1 dependent target gene expression. Recent studies addressing regulation of HIF-1 by NO revealed a complex and paradoxical picture. Acute exposure of cells to high doses of NO increased HIF-1α levels irrespective of the residing oxygen concentration whereas prolonged exposure to NO or low doses of this radical reduced HIF-1α accumulation even under hypoxic conditions. Several mechanisms were found to contribute to this paradoxical role of NO in regulating HIF-1. More recent studies support the view that NO regulates HIF-1 by modulating the activity of the oxygen-sensor enzymes PHDs and FIH-1. NO dependent HIF-1α accumulation under normoxia was due to direct inhibition of PHDs and FIH-1 most likely by competitive binding of NO to the ferrous iron in the catalytically active center of the enzymes. In contrast, reduced HIF-1α accumulation by NO under hypoxia was mainly due to enhanced HIF-1α degradation by induction of PHD activity. Three major mechanisms are discussed to be involved in enhancing the PHD activity despite the lack of oxygen: (1) NO mediated induction of a HIF-1 dependent feedback loop leading to newly expressed PHD2 and enhanced nuclear localization, (2) O_2-redistribution towards PHDs after inhibition of mitochondrial respiration by NO, (3) reactivation of PHD activity by a NO mediated increase of iron and 2-oxoglutarate and/or involvement of reactive oxygen and/or nitrogen species.

Contents

1. Introduction

The maintenance of oxygen homoeostasis within tissues of the mammalian organism requires a delicately orchestrated network. Next to immediate adaptation of respiration the ability of mammalian cells to sense oxygen and adapt gene expression according to O_2 availability is critical for proper function within the tissue and

Abbreviations: CTAD, C-terminal trans-activating domain; FIH-1, Factor inhibiting HIF-1; HIF-1, Hypoxia inducible factor-1; NO, nitric oxide; ODD, oxygen dependent degradation domain; 2-OG, 2-oxoglutarate; PHD, prolyl hydroxylase domain containing enzyme; pVHL, von Hippel–Lindau protein; RNS, reactive nitrogen species; ROS, reactive oxygen species; Suc, succinate.

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doi:10.1016/j.cellsig.2009.10.004

survival. This has been well recognized for the physiology [1] but is also of relevance in inflammation [2] and in particular tumor biology [3].

Nitric oxide (NO) is certainly one of the key mediators in inflammation but has also been implicated in the interaction between tumor and host in several malignancies. Tumor-infiltrating macrophages and tumor-associated host fibroblasts express inducible NO synthase and serve as a source of NO within the tumor microenvironment [4,5]. In a series of human breast tumors, NO synthase activity and NO biosynthesis were high in invasive tumors, and they increased with the grade of malignancy [6]. Experiments using a murine model of orthotopic mammary tumors demonstrated that the absence of inducible NO synthase interrupted the communication between the host and the tumor thereby substantially delaying tumor formation [7]. Increased levels of NO in tumors have been detected in vivo [4]. However, in some cases endothelial NO synthase rather than inducible NO synthase was identified as the main source of NO [8]. Finally, tumor cells themselves can express all isoforms of NO synthases and can affect stromal and other tumor cells by releasing NO [4].

Recently it was reported that NO can modulate oxygen sensing and HIF-1 target gene expression [9]. It is thus conceivable that tumor-associated NO will modulate the HIF-1 response in tumors. External NO supplied by application of NO donors may exert similar effects. In a clinical study, NO donors increased the efficacy of radiation therapy and tumor growth was found to be reduced [10]. In line with these results, studies using immunohistochemical analysis of tumor tissue found reduced accumulation of HIF-1α in tumor cells after treatment with NO. In consequence, the expression of the HIF-1 target gene VEGF was reduced, and this reduction led to decreased angiogenesis, which was believed to be responsible for a decrease in tumor growth and metastasis [10].

In this review, we will compile recent evidence for a role of NO in affecting cellular oxygen sensing and oxygen-regulated HIF-1 activation. In view of the recent clinical findings such an overview may improve our understanding of NO as a signaling molecule between the host and the tumor but could also provide insights into the inflammatory setting in which both NO and HIF-1 are of mutual importance.

2. Hypoxia-inducible factor and its oxygen-sensors

The heterodimeric transcriptional regulator Hypoxia inducible Factor-1 (HIF-1) is mandatory for the regulation of gene expression in response to decreased oxygen levels, i.e. hypoxia. HIF-1 is composed of one constitutive β-subunit and one of three O_2-labile subunits HIF-1α, 2α or 3α. All HIF-1 subunits belong to the family of basic-helix–loop–helix (bHLH)/PAS transcription factors where PAS is an acronym for *P*ERIOD (a drosophila transcription factor involved in circadian rhythm), *A*RNT (arylhydrocarbon receptor nuclear translocator as part of the xenobiotic response) and *S*IM (single minded, another drosophila transcription factor for axis determination) which were the first proteins discovered to contain this domain. Both the bHLH and the PAS domain are important for the dimerization process while DNA binding is through the bHLH domain [11]. The HIFα subunits do not sense O_2 directly, but both stability and activity of these subunits are regulated by oxygen-dependent post-translational hydroxylation (Fig. 1). Hydroxylation is performed by ferrous iron (Fe^{2+}) and 2-oxoglutarate (2-OG) dependent dioxygenases that require ascorbate to maintain the iron in its ferrous state and O_2 for enzymatic activity [12,13].

Under normoxic conditions when oxygen is not limiting the activity of the hydroxylases determines HIF-α protein stability and thus abundance is controlled by a family of prolyl-4-hydroxylases, named *p*rolyl *h*ydroxylase *d*omain (PHD) containing enzymes. Three isoforms, PHD1, PHD2 and PHD3, have been reported to be of importance for oxygen sensing so far. PHDs hydroxylate HIF-α at two conserved proline residues in human HIF-1α and -2α (Pro402/564 or Pro405/531, respectively) or at a single proline residue in case of human HIF-3α (Pro490). The prolyl residues are central parts of domains within the HIF-α proteins that can infer oxygen dependent instability to any proteins they are inserted to [14]. Thus, these parts have been named oxygen dependent degradation domains (ODD). When the prolins become hydroxylated HIFα proteins are recognized by the von Hippel–Lindau protein (pVHL) which subsequently recruits an E3 ubiquitin ligase. Poly-ubiquitinated HIF-αs are directed to the 26S proteasomes where they undergo rapid degradation [15]. Although HIF-αs are continuously expressed and translated under normoxic conditions hydroxylation, ubiquitination and proteasomal degradation prevail under normoxia and cause rapid degradation of HIF-αs. This limits the amount of HIF-α protein to a very low steady-state level and makes it virtually impossible to detect this protein under normoxic conditions. However, because PHDs require oxygen for their enzymatic activity hydroxylation ceases under hypoxia and shifts the equilibrium to accumulation of HIF-αs. HIF-αs then enter the nucleus via a process that has recently been shown to depend on binding to importins, at least for HIF-1α [16]. After entering the nucleus HIF-αs will undergo dimerization with the β-subunit to form the active HIF-1 complex. This process probably requires DNA binding through the N-terminal basics helix-loop–helix domains in both HIFα and HIF-1β subunits [17].

In addition to this regulation of HIF-α abundance by oxygen, transcriptional activity of the HIF complex is likewise determined by hydroxylation of an asparagine residue located within the C-terminal trans-activating domain (CTAD) of HIF-1α and -2α. In this case, an asparagyl-hydroxylase termed Factor Inhibiting HIF (FIH-1) determines transcriptional activity in an oxygen dependent manner. The name FIH-1 was deduced from the function of the protein which was initially described to inhibit HIF-1 function when bound to HIF-1α [18]. Later it was found that this action of FIH-1 also depended on its enzymatic activity as a hydroxylase: FIH-1 hydroxylates Asp803 in human HIF-1α under normoxia which impedes binding of the transcriptional coactivator p300/CBP [19]. These coactivators are required for transcriptional activity of HIF-1 because they act as a scaffold to recruit other coactivators or tissue specific transcription factors of HIF-1 dependent genes. FIH-1 is also a dioxygenase and is dependent on oxygen for enzymatic activity. Thus, posttranslational modification of HIF-αs is of mutual importance for cellular oxygen sensing. Both prolyl hydroxylases which determine HIF-α abundance, and the asparagyl hydroxylase which controls transcriptional activity are oxygen dependent in their activity and thus serve as cellular oxygen sensors (Fig. 1).

With respect to their optimum in enzymatic activity PHDs and FIH-1 appear to be ideally suited because *in vitro* derived Km values for oxygen binding of PHDs are around 100 μM and of FIH-1 around 70 μM [20]. With these affinities all oxygen sensing enzymes cover the range of oxygen concentrations that may physiologically be expected in the various tissues. This range of oxygen dependence has recently been confirmed by measurement of PHD activity in cellular extracts under different oxygen concentrations [21]. The slightly different affinity for O_2 between PHDs and FIH-1 implies that under decreased O_2 concentrations PHDs may be reduced in their enzymatic activity first due to their weaker affinity for O_2. HIF-α protein would be stabilized first and a further decrease of the oxygen concentration would then reduce FIH-1 activity enabling binding of coactivators [22]. This has, however, not yet been formally tested with respect to the *in vivo* relevance. The active HIF-1 complex then binds to hypoxia-responsive elements (HRE) to induce expression of more than 100 genes involved in adaptation to hypoxia [23–26]. HIF-1 target genes typically fall into two main categories whose functions aim to restore energy and O_2 homeostasis by increasing anaerobic energy production via stimulated glycolytic substrate flux (glucose transporters and

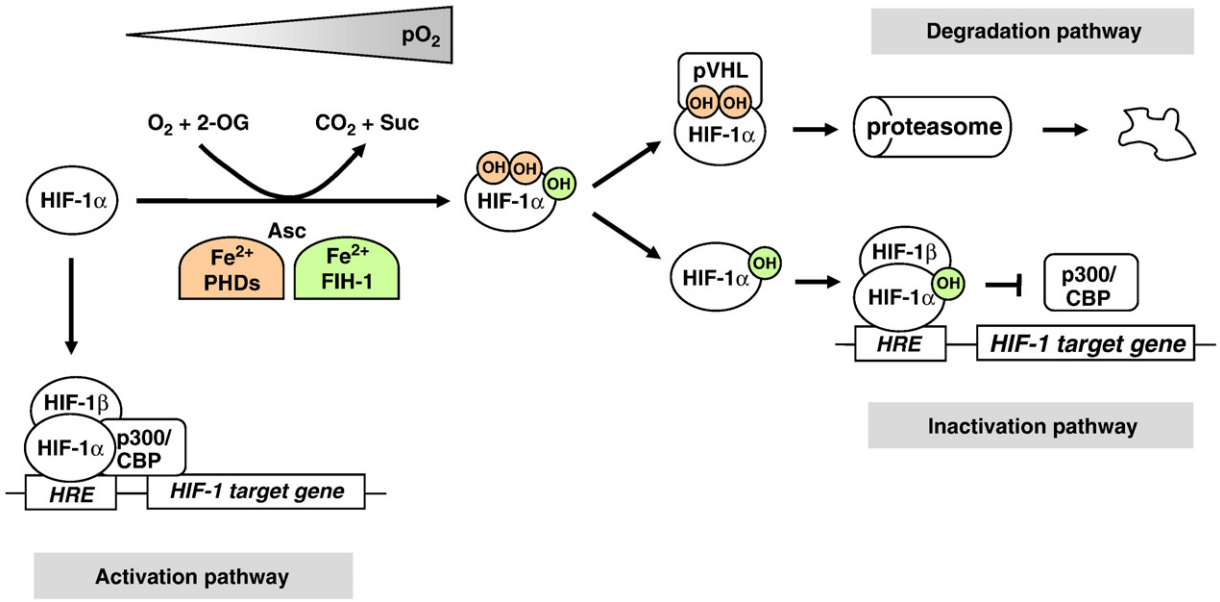

Fig. 1. Oxygen-dependent hydroxylases act as cellular oxygen sensor and control abundance and activity of Hypoxia-Inducible Factor-1. Hypoxia inducible factors (HIFs) are the key mediator of the transcriptional response to hypoxia. HIFs are heterodimers composed of O_2-labile α-subunits and a constitutive β-subunit. Cellular oxygen is sensed by a family of prolyl-4-hydroxylases (PHD1, PHD2 and PHD3) and by an asparagyl-4-hydroxylase called Factor Inhibiting HIF-1 (FIH-1). Both PHDs and FIH-1 are members of the superfamily of 2-oxoglutarate-dependent ferrous iron (Fe^{2+}) dioxygenases. Dioxygenases incorporate one O-atom of O_2 to hydroxylate proline or asparagine residues, respectively. The second atom is used to convert 2-oxoglutarate (2-OG) to carbon dioxide (CO_2) and succinate (Suc). Fe^{2+} in the active center of PHDs and FIH-1 is maintained in a reduced (ferrous) state by ascorbate (Asc). Hydroxylation of HIF-1α at two prolyl residues in its oxygen-dependent degradation domain is recognized by the von Hippel–Lindau protein (pVHL) E3 ubiquitin ligase complex leading to ubiquitination and subsequent proteasomal degradation. Hydroxylation of HIF-1α at an asparagine residue within its C-terminal trans-activation domain prevents binding of the transcriptional coactivator p300/CBP leading to inactivation of HIF-1 transcriptional activity. Increasing degrees of hypoxia will progressively reduce the activity of PHDs and FIH-1 which requires oxygen. As a consequence HIF-1α subunits evade degradation, accumulate and translocate into the nucleus for dimerization with HIF-1ß. HIF-1α with a non-hydroxylated C-terminal asparagine can recruit the transcriptional coactivator p300/CBP. The active HIF-complex binds to hypoxia-responsive elements (HRE) of HIF target genes to induce their expression [23–26].

glycolytic enzymes) or improving tissue oxygenation via stimulated angiogenesis (vascular endothelial growth factor, VEGF), vasodilation (NO synthases and heme oxygenase) and erythropoiesis (erythropoietin). In addition, many genes important for tumor biology and involved in cell growth and death as well as genetic stability or repair are HIF-1 dependent [27].

Interestingly, among the HIF-1 target genes are the oxygen-sensors PHD2 and PHD3 but not PHD1 or FIH-1 [28,29]. Induction of PHD2/PHD3 expression leads to increased cellular abundance of the enzymes and increased cellular HIF-α hydroxylation capacity [9,30]. In result, HIF-1α is hydroxylated and degraded in response to prolonged hypoxia despite the lack of oxygen. Thus the HIF-1-dependent

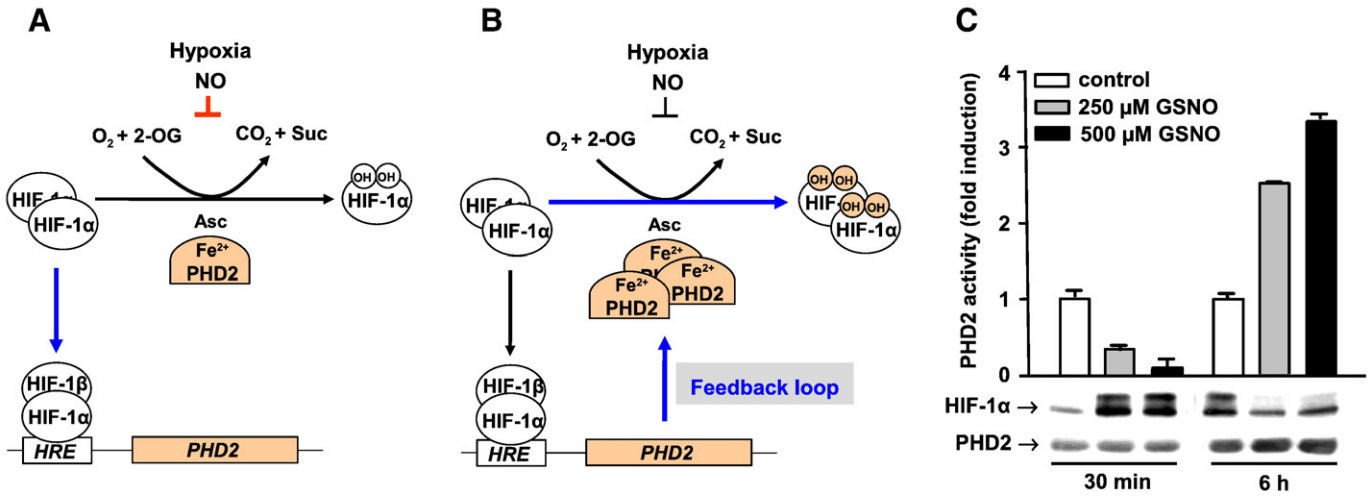

Fig. 2. Bimodal effect of NO on the oxygen-sensor prolyl hydroxylase 2 (PHD2). Prolyl hydroxylase 2 (PHD2) is the key oxygen-sensor of the HIF-1 pathway. Importantly, PHD2 itself is a HIF-1 target gene and part of a negative feedback loop. (A) PHD2 activity is inhibited by hypoxia and/or by NO-release. Thus HIF-1α escapes hydroxylation and subsequent degradation and HIF-1 dependent expression of PHD2 is induced. (B) HIF-1 dependent PHD2 expression generates a feedback loop. Increased PHD2 protein abundance enhances PHD2 activity leading to increased HIF-1α hydroxylation and degradation in response to prolonged hypoxia and/or subsequent to exposure to NO. (C) Experimental data are shown that reflect the bimodal effect of NO on PHD2 activity in a time-dependent manner. U2OS osteosarcoma cells were incubated with the NO-donor S-nitrosoglutathione (GSNO) in hypoxia (1% O_2) for 30 min or 6 h. Whole cell lysates were subjected to Western blot analysis and PHD activity assay as described [21]. At the early time point (30 min) NO decreased cellular PHD2 activity and increased HIF-1α; at the later time point (6 h) when all NO has been released and is metabolized PHD2 protein abundance and activity were induced and HIF-1α accumulation was decreased.

induction of the cellular O_2-sensors generates a negative feedback loop controlling HIF-1α stability (Fig. 2A, B). Although all three PHDs can regulate HIF-αs in an O_2-dependent manner PHD2 was found to be the most important oxygen-sensor under normoxia, hypoxia and reoxygenation in a number of cell lines [31,32]. However, all three PHDs can contribute to PHD activity under prolonged hypoxic stress [33]. Between the isoforms differences exist with respect to substrate specificity and protein–protein interaction partners. PHD2 has a preference for HIF-1α and is engaged in the cellular response to acute hypoxia. PHD3 prefers HIF-2α and is involved in the response to chronic hypoxia (for review see [34]). In addition, all three PHD enzymes exhibit different tissue distribution and distinct patterns of subcellular localization. Initially it was reported that PHD1 was mainly localized in the nucleus, PHD2 was predominantly cytoplasmic and PHD3 appeared to be evenly distributed between both compartments when the enzymes were overexpressed as green fluorescent protein (GFP)-fusion proteins [35]. Since antibodies are available to detect endogenous PHD2 protein the localization of this isoenzyme has gained considerable interest. Endogenous PHD2 detected in different tissues was mainly located in the cytoplasm whereas increased PHD2 amounts were found in the nuclei of tumor cells [21,36,37].

3. Dual role of NO in regulation the HIF-signaling

Over the past decade insights into the molecular mechanism of oxygen-sensing and oxygen homeostasis have greatly been extended. Work from several groups showed that mediators of the inflammatory response including NO impinge on oxygen-sensing [38]. More recently the concept emerged that NO may modulate the cellular response to hypoxia by regulation of HIF-1 in a dual manner. Several studies had indicated that chemically diverse NO donors, enhanced NO formation from inducible NO-synthase or NO formation in a co-culture system induce HIF-1α stabilization and transcriptional activation of HIF-1 target gene expression under non-hypoxic conditions [39–42]. By inducing HIF-1α and HIF-1 target gene expression NO mimicked the hypoxic response despite normoxia implying that NO may also use one or the other components of the hypoxic response. However, under hypoxic conditions NO appeared to have an opposite effect on HIF-1α because several NO-donors were found to decrease HIF-1α stabilization and HIF-1 transcriptional activation under hypoxia [43–45]. Several mechanisms have been suggested to account for these opposing effects of NO on HIF-1. Very early classical signaling of NO via the soluble guanylate cyclase and 3′,5′-cyclic guanosine monophosphate (cGMP) was excluded because guanylate cyclase antagonists and lipophilic cGMP analogues did neither attenuate nor mimic HIF-1α accumulation [39,40,46,47]. In contrast, induction of HIF-1α by NO was reported to depend on protein synthesis via NO-induced phosphatidylinositol 3-kinase or mitogen-activated protein kinase signaling [48,49]. Direct effects of NO on HIF-1 have also been considered. HIF-1α protein was found to be a potential target for S-nitrosation at three to four thiols, and enhanced transactivation of HIF-1 was observed upon nitrosation of Cys800 in HIF-1α [50,51]. Because, as pointed out above, HIF-1α abundance is mainly controlled by oxygen-dependent regulation of its degradation [24] (Fig. 1) the focus of subsequent investigation was directly laid on the oxygen-sensing enzymes to study whether they were targeted by NO.

4. Bimodal effects of NO on the oxygen-sensor PHD2

First evidence that NO might directly interact with the oxygen sensor enzymes was provided by Metzen et al. [52]. The authors found that HIF-1α accumulation by NO was independent of HIF-1α gene transcription or protein translation but instead was caused by inhibition of the HIF-1 degradation pathway (Fig. 2A). GSNO attenuated HIF-1α-pVHL interaction and largely reduced ubiquitination of HIF-1α. These data suggested that accumulation of active HIF-1α results from direct inhibition of PHD1, 2 or 3 [52]. Using a hydroxyproline-specific HIF-1α antibody it was confirmed that the presence of NO reduces hydroxylation of HIF-1α in cells under normoxia or amplifies the reduced hydroxylation under hypoxia. Although all three PHDs were inhibited by NO *in vitro* [52] experiments with several cell lines indicated that reduced hydroxylation of HIF-1α by NO was predominantly achieved via inhibition of PHD2 *in vivo* [9,21].

At present the molecular mechanisms by which NO inhibits PHDs have not been experimentally resolved but from other iron(II)non-heme-containing enzymes it is known that NO can directly bind to the ferrous ion [53–55]. Therefore inhibition of PHDs by NO was tentatively explained by competitive binding of the NO molecule instead of O_2 to the ferrous iron at the catalytic site [52]. Interestingly, spontaneous reactivation of PHD activity after inhibition by NO treatment could not be detected using recombinant PHD2 in a HIF-1α-pVHL interaction assay suggesting that the interaction of NO with PHD2 is an irreversible process, at least *in vitro* [56]. In contrast, inhibition of HIF-1α-hydroxylation by NO was reversible *in vivo* under normoxia as well as under hypoxia. This suggests that endogenous cellular components that were not added to the *in vitro* assay may be involved in the reactivation of PHDs *in vivo* [9,52,56]. Because it was found that NO enhanced PHD2 abundance increased PHD2 gene expression and newly generated PHD2 protein most likely contribute to the recovery of PHD activity [9,56].

In addition to the inhibition of PHD activity by NO, the increase of CTAD activity of HIF-1α after NO treatment implicated that FIH-1 activity is reduced by NO [52]. Park et al. [57] later confirmed that FIH-1 was indeed inhibited by NO but only when iron and ascorbate were limiting. It is tempting to speculate that higher doses of NO were required to inhibit FIH-1 due to its higher affinity to O_2 when compared with PHDs. The direct inhibition of PHDs and FIH-1 by NO would explain both the HIF-1α accumulation and the HIF-1 dependent expression of target genes like VEGF [39] and PHD2/PHD3 [9] (Fig. 2A).

In contrast, several studies revealed that NO can decrease hypoxia-induced HIF-1α levels. Early data from Huang et al. [45] had shown a reduced Gal-ODD and CTAD activity in response to NO treatment under hypoxic conditions. These findings already implicated that NO could also decrease HIF-1α accumulation by enhancing PHD and inhibit HIF-1 activity by activating FIH-1 [45]. Recently, this bimodal response towards NO-treatment was confirmed by demonstrating both increased and decreased PHD2 activity after NO treatment (Fig. 2C) [9]. During the early phase and as long as NO is still present in the cells PHD2 is inhibited and HIF-1α protein levels increased. As a result HIF-1 activity and induction of the HIF-1 target gene PHD2 are increased which was demonstrated both on mRNA and protein level. (Fig. 2A) [9]. Consequently, PHD2 activity is elevated during the late phase of NO treatment when PHD2 protein levels are increased and NO is no longer present at inhibiting concentrations. This mechanism appears to be very effective and disturbs the HIF-1α equilibrium so that HIF-1α protein levels decrease even under hypoxia (Fig. 2B) [21]. Of note, expression of the HIF-1 target PHD3 was additionally induced by NO while PHD1 expression was unaffected. However, suppression of either PHD3 or PHD1 by using siRNA had any effect on HIF-1α levels which indicates that PHD2 was predominantly responsible for overall PHD activity in the cells. Thus, induction of PHD2 expression by NO is able to promote the feedback regulation of HIF-1α (Fig. 2B). The same holds true for normoxic conditions where inhibition of PHD2 activity by NO leads to a non-hypoxic induction of the feedback loop allowing destruction of the HIF-1α protein [56]. In view of the important role attributed to PHD2 abundance in controlling HIF-1α levels within the cell the effects of NO as a modulator might significantly affect the cellular responses to hypoxia [30,32] (Fig. 2B).

A further mode of regulating cellular oxygen sensing appears to depend on the intracellular distribution of PHD2 after exposure to NO (Fig. 3). The localization in particular of PHD2 has been a matter of debate for some time. Initially, GFP-PHD2-fusion proteins were predominantly localized in the cytoplasm irrespective of oxygen concentration or NO treatment [35,56,58] while immunohistochemistry of endogenous PHD2 showed a more intense staining in the nucleus than in the cytoplasm [21] (Fig. 3A). Recently it was reported that PHD2 may shuttle between the cytoplasm and nucleus [37,59]. Under hypoxia and/or NO PHD2 abundance and activity were considerably induced in the nucleus [21] (Fig. 3B). The strict nuclear localization of HIF-1α under these conditions suggests that hydroxylation of HIF-1α and thus oxygen-sensing most likely takes place in the nucleus (Fig. 3C). Indeed this is consistent with earlier observations that trapping of HIF-1α either in the nucleus or in the cytoplasm did not prevent HIF-1α degradation [60]. Different cell types contained different ratios of cytoplasmic to nuclear PHD2 activity [21]. The subcellular distribution of PHD2 is likely of relevance for the efficiency of HIF-1α degradation in the respective compartment. This is in agreement with the finding that compartment specific degradation of HIF-1α depends on the content and activity of the components of the degradation pathway like pVHL and proteasome [61,62].

5. NO-mediated mechanisms affecting PHD2 activity under hypoxia

PHD activity appears to be strongly affected by NO in a dose-dependent manner under hypoxic conditions (Fig. 4). This effect is observed after the addition of chemical NO donors but also when NO is endogenously generated by activation of tetracycline-dependent human inducible NO synthase stably expressed in HEK-293 cells [63]. The authors reported that high doses of NO (>1 μM) stabilized HIF-1α irrespective of the oxygen-concentration whereas low doses of NO (<0.4 μM) destabilized HIF-1α under hypoxia [63]. Likewise, generation of low or high concentrations of NO by applying the NO-donor Deta-NO (50–100 μM or 500–1000 μM respectively) for 1 h had the same effect on HIF-1α accumulation [63]. The kinetic of NO release of a selected NO donor is rendered by the kinetic of the decomposition of the respective NO-donor that depend on temperature, buffer composition, pH as well as thiol content [41,64]. As a rough estimate, the concentration of freely diffusing NO from the NO donor is initially at least 1000-fold lower than the concentration of the NO releasing compound [65]. Accordingly, PHD2 activity was influenced in a time- as well as dose-dependent manner when cells were exposed to a single dose of 250 μM GSNO [9,21,56]. Initially, according to the NO-release kinetics of GSNO the NO concentration was high enough to inhibit PHD activity under normoxia but also synergistically with hypoxia (<2 h). As a consequence, hydroxylation and degradation of HIF-1α were inhibited and HIF-1 dependent PHD2 expression was initiated ((1) Feedback loop in Fig. 4.). At a later time point (>4 h) when the NO-release from the donor had ceased due to complete decomposition of the GSNO newly expressed PHD2 enzyme could be fully active and was not inhibited by NO. The time course of PHD2 inhibition and PHD2 reactivation closely followed the kinetics of NO release from GSNO [41].

While the just described bimodal effects of NO on oxygen sensing require *de novo* synthesis of PHD2 proteins NO has also been found to redistribute oxygen from the respiratory chain to the PHD enzymes [66]. Mateo et al. [63] reported that low concentrations of NO reduced HIF-1α accumulation under hypoxia through a mitochondria dependent process. Destabilization of HIF-1α, however, was not dependent on the generation of reactive oxygen species (ROS) by mitochondria because the effect was not ameliorated by antioxidants [66,67]. Instead, low NO concentrations can be trapped by the cytochrome *c* oxidase of the respiratory chain and it was suggested that high affinity for binding NO displaced the O_2 from the cytochrome and caused inhibition of oxygen consumption [66]. The inhibitory effect of NO was shown to be reversible in competition with O_2 and occurred at concentrations of NO which are likely to be physiologically relevant [68]. Using a renilla luciferase construct targeted to mitochondria, as a monitor of available oxygen, the repression of respiration by low NO doses reduced O_2-consumption of mitochondria under hypoxia and left more oxygen for other O_2-dependent enzymes [66]. Therefore NO may act as an endogenous regulator of the intracellular availability of oxygen in mammalian cells [69]. This cellular O_2-redistribution mechanism is fully compatible with the increased amount of PHD2

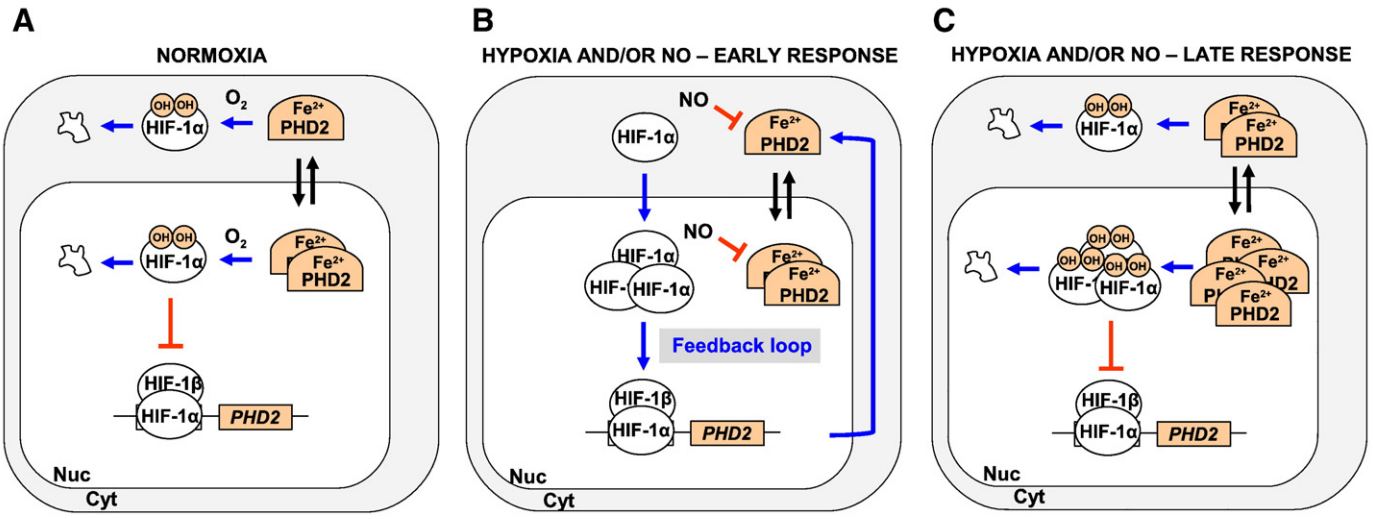

Fig. 3. Cellular oxygen-sensing under the influence of hypoxia and/or NO. Prolyl hydroxylase 2 (PHD2) protein abundance and activity differ between cytoplasm (Cyt) and nucleus (Nuc). (A) Under normoxic conditions HIF-1α is not found in the cells because synthesized HIF-1α is immediately hydroxylated by PHD2, rapidly recognized by pVHL followed by poly-ubiquitination and proteasomal degradation. In consequence, HIF-1 activity and PHD2 expression are low. (B) Upon the onset of hypoxia and/or NO PHD2 activity is inhibited during the initial response to hypoxia and as long as NO is present. HIF-1α escapes hydroxylation and translocates into the nucleus where the active HIF-1 complex induces PHD2 expression. This initiates a negative feedback loop. (C) PHD2 protein abundance is enhanced and distributed within the cytoplasm and cell nuclei during the late response as consequence of hypoxic and/or NO induced HIF-1 activity. Increased PHD2 protein levels in the cell nuclei are responsible for elevated nuclear PHD2 activity which leads to hydroxylation and subsequent degradation of HIF-1α. Thus oxygen-sensing may take place in the immediate vicinity of HIF-dependent gene expression.

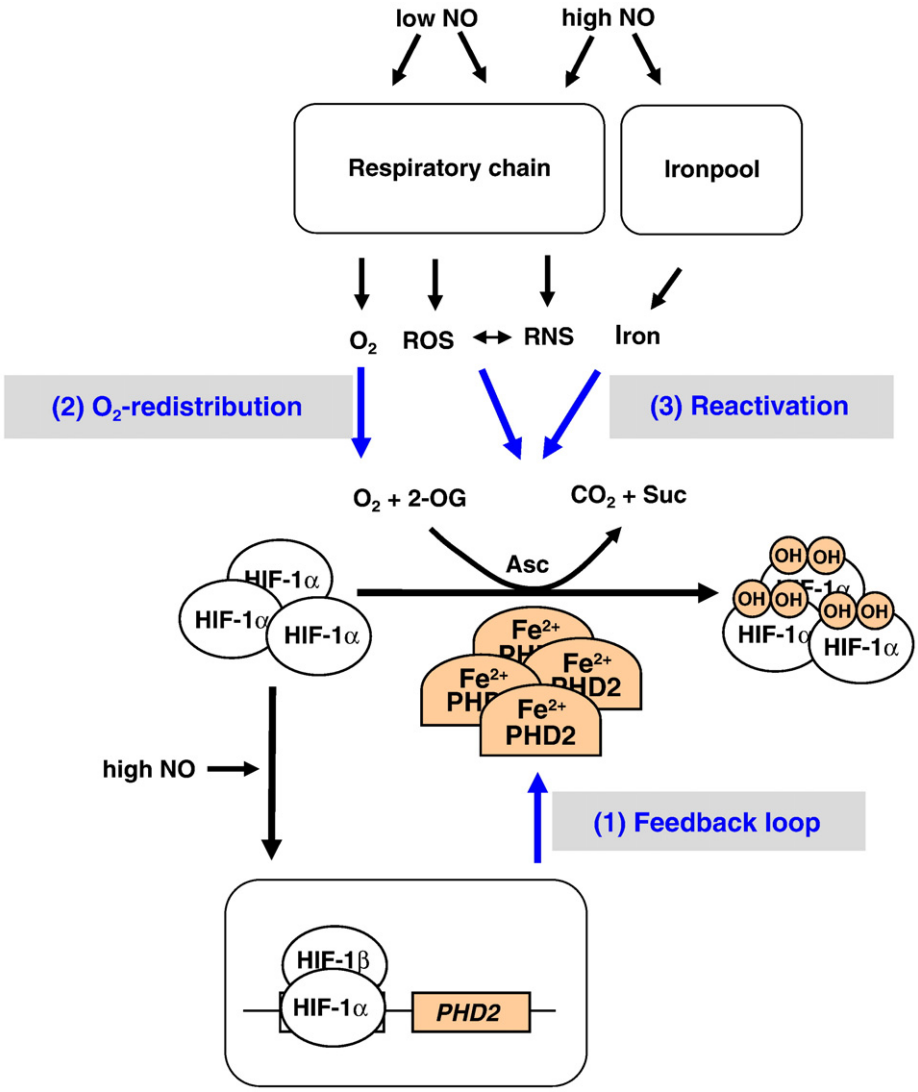

Fig. 4. NO-mediated mechanisms include ROS in affecting PHD activity. PHD activity is affected by NO in a dose-dependent manner under hypoxic conditions. (1) High NO doses directly inhibit PHD2 activity most likely by binding to the central iron. Subsequently HIF-1α escapes degradation and rapidly induces the expression of the target gene PHD2. Newly synthesized and active PHD2 enzyme is responsible for the increase in cellular PHD2 activity under hypoxia subsequent to NO exposure (Feedback loop). (2) Low concentrations of NO inhibit cytochrome *c* oxidase of the respiratory chain. In consequence, decreased oxygen consumption by the mitochondria, leaves more O_2 for the PHDs which regain activity despite hypoxia (O_2-redistribution). (3) High doses of NO enhance iron availability which can increase PHD activity under hypoxia (Reactivation). However, low doses of NO induce cellular reactive oxygen species (ROS) production which reactivates PHD activity under hypoxia (Reactivation). High ROS (O_2^-) levels generated by the respiration chain during hypoxia might directly inhibit PHD activity by oxidizing the iron which would decrease HIF-1α hydroxylation. Low/high doses of NO can react with ROS (O_2^-) to generate reactive nitrogen species (RNS, e.g. $ONOO^-$) which would limit the ROS-dependent inhibition of PHD activity under hypoxia (Reactivation).

protein after NO exposure and would in fact amplify the compensatory feedback system by providing the required co-substrate oxygen under conditions of hypoxia and NO (Fig. 4, (2) O_2-redistribution).

However, PHD activity is not only affected by protein abundance and O_2-concentration but also by the availability of iron, ascorbate and 2-OG [70]. Treatment of cells with iron chelators like desferrioxamine (DFX) elicited HIF-1α accumulation and HIF-1 transcriptional activity [71]. Subsequent studies revealed that PHD activity is inhibited by different iron chelators [72]. Inhibition of PHD activity by DFX was partly antagonized in the presence of NO which was attributed to an increase in intracellular iron by NO under hypoxia (Fig. 4, (3) Reactivation) [72,73]. This increased iron availability under NO would be supportive for the generation of new, fully active PHD2 *in vivo* as an adaptive mechanism. In addition, the potential of reactivating PHD enzymes after exposure to NO (see above) by increasing iron suggests that alterations in the concentration of ferrous iron may contribute to regulation of PHD activity. PHDs require ferrous iron for their catalytic activity and ascorbate prevents oxidation to ferric iron [74,75]. In support of this, ascorbate and ferrous iron prevented the NO induced HIF-1α accumulation under normoxia [76] and the reducing agent GSH ameliorated the NO-dependent inhibition of PHD activity *in vitro* [21]. These findings suggest that the NO mediated effects on PHD activity involve redox dependent mechanisms.

Additional evidence for redox modulation of PHD activity is provided by the effects of ROS (O_2^-, H_2O_2) which can oxidize ferrous to ferric iron to instantaneously inhibit PHD activity and cause accumulation of HIF-1α [77]. Cells deficient in junD with largely compromised antioxidant capacity stabilized HIF-1α under normoxia when exposed to ROS [78]. Accumulation of H_2O_2 in junD deficient cells decreased the availability of ferrous iron and reduced the PHD activity which was recovered by ascorbate [78]. These findings are supported by studies in which PHD activity was reduced in the presence of either the redox cycler DMNQ (O_2^--generator) or H_2O_2 and would explain why increased ROS lead to induction of HIF-1α under normoxic conditions [75,79]. Under hypoxia inhibition of the respiratory chain could lead to mitochondrial production of ROS (O_2^-)

at complex III, which was found to cause accumulation of HIF-1α [77,80]. Low doses of NO may under these conditions react with O_2^- to yield $ONOO^-$. This was hypothesized to limit the availability of O_2^- and allow PHD activity to recover [81].

However, the involvement of ROS in oxygen-sensing in the presence of NO remains highly complex [81]. One of the main difficulties relates to the still controversially discussed question whether ROS were increased or decreased under hypoxia when compared to normoxia. Other O_2-dependent enzymes like NADPH oxidase and heme oxygenase-2 contribute to ROS generation in cells and may be modulated by NO too [82,83]. Callapina et al. [84] reported that the concentration of ROS was decreased under hypoxia compared to normoxia supporting earlier findings [85–87]. Low doses of NO applied under hypoxia were then found to restore ROS formation to normoxic levels which was claimed to reactivate PHD activity and decrease HIF-1α [84]. Higher doses of NO also induced mitochondrial ROS production under hypoxia and generated reactive nitrogen species like peroxynitrite ($ONOO^-$). Peroxynitrite damaged mitochondria and caused the release of iron and 2-OG from this cellular compartment [88]. This was considered to cause a reduction of HIF-1α under hypoxia by increasing PHD activity through the higher availability of iron and 2-OG [88]. Hypothetically $ONOO^-$ may serve as an alternative O_2-donor but will unlikely do so *in vivo* because the capability of $ONOO^-$ to act as O_2-donor is restricted to highly purified buffer systems [89]. In the presence of bicarbonate (HCO_3-) peroxynitrite lost its O_2-releasing capability because the equilibrated CO_2 reacted moderately fast ($5.8\times10^4 M^{-1}s^{-1}$) with $ONOO^-$ [90]. The interaction ROS and NO with PHDs and FIH-1 may be relevant for pathophysiological situations attributed to excess ROS production or alterations in the antioxidant systems. However, the outcome of this interaction appears to highly depend on the respective degree of ROS and/or NO formation.

6. Summary

The current review has summarized published work that clearly demonstrates a role of NO in regulating activity of oxygen-sensor enzymes PHDs and FIH-1 (Table 1). Several mechanisms were proposed to contribute to this effect in a dose- and time-dependent manner. High doses of NO, most likely found *in vivo* produced from macrophages with activated iNOS can directly inhibit PHD and FIH-1 activity irrespective of the residing O_2-concentration. These would cause HIF-1 activation and HIF-1 target gene expression during inflammation or tumor development. Because iNOS expression is under control of HIF-1 this effect of NO would provide a positive feedback loop. In contrast, the induction of the HIF-1 target PHD2 by NO provides a negative feedback loop. When NO concentrations return to basal levels increased HIF-1α protein will rapidly be sent to degradation through increased PHD2 activity, even under hypoxic conditions. This would limit the HIF-1 response and consequently also iNOS expression.

NO can inhibit the respiration chain and thereby lower oxygen consumption of the cells that would allow shifting oxygen from the mitochondria to PHDs and FIH-1 which then regain activity despite hypoxia. Reactive oxygen species produced by mitochondria or other cellular sources under patho-physiological circumstances likely interfere with NO signaling. NO mediated nitrosylation or NO-ROS attack can oxidize the ferrous iron in the catalytic center of the oxygen-sensors which makes the enzymes sensitive to oxidative stress. On the other hand, scavenging between NO and ROS may prevent ROS- or NO-dependent inhibition of PHD activity.

In conclusion, NO has been identified as an important modulator which directly or indirectly affects the activity of the cellular oxygen-sensors. The prevailing effect — increase or decrease of HIF-1α — depends on the cellular context and the reactive species involved. Nevertheless — oxygen sensing appears to be under the influence of NO.

Table 1
NO-mediated mechanisms affecting oxygen-sensor activity.

Condition	NO-donor	Effect	Mechanism	Citation
21% O_2	GSNO	PHD activity ↓	PHD-Fe^{2+}coordination by NO assumed	[51]
		CTAD activity↑	FIH-1-Fe^{2+}coordination by NO assumed	
21% O_2	SNAP, Spermine-NO	FIH-1 activity↓	When reducing agents were limiting	[57]
1% O_2	SNP	Gal4-ODD↓	Inhibition of HIF-1α stability	[45]
		CTAD-activity↓	Inhibition of HIF-1α transactivation	
21% O_2 + $CoCl_2$/DFO	SNP, PAPA-NO	PHD activity↑	Activation of cellular PHD activity	[73]
21% O_2/3% O_2	Tet-iNOS: high dose	HIF-1α↑	Independent of mitochondria	[63]
3% O_2	Tet-iNOS: low dose	HIF-1α↓	Dependent on mitochondrial respiration	
1% O_2	DETA-NO: low dose	HIF-1α-V5↓	O_2-redistribution towards PHDs After inhibition of respiration	[66]
0.5% O_2	DETA-NO: low dose	PHD activity↑	Reactivation of PHDs by increased ROS	[84]
		CTAD activity↓	Reactivation of FIH-1 by increased ROS	
0.5% O_2/21% O_2 + DFO	DETA-NO, GSNO	PHD activity↑	Reactivation of PHDs by increased iron	[71]
1% O_2	GSNO	HIF-1α ↓	Release of iron/2-OG from mitochondria	[88]
Hypothesis: Hypoxia	NO	PHD activity↑	$ONOO^-$ as alternative O_2-donor	[89]
21% O_2/1% O_2	GSNO, NO-Mela	PHD2 activity↓ Early response	PHD-Fe^{2+}coordination by NO assumed	[9,56,64]
21% – 1% O_2	GSNO, NO-Mela, Spermine-NO	PHD2 activity↑ Late response	HIF-1α dependent induction of PHD2 Abundance (feedback loop)	[9,21,56]

Acknowledgement

The work was supported by a grant from the Deutsche Forschungsgemeinschaft FA225/21.

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