Nitric oxide and superoxide: Interference with hypoxic signaling

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Abstract

Sensing and responding to changes in oxygen partial pressure assures that the cellular oxygen supply is tightly controlled in order to balance the risks of oxidative damage vs. oxygen deficiency. The hypoxia inducible factor (HIF) regulatory system is controlled by prolyl hydroxylases (PHDs), the von Hippel Lindau protein (pVHL), and the 26S proteasome and transduces changes in oxygenation to adequate intracellular adaptive responses. A functional HIF response requires stabilization of the α-subunit, e.g. HIF-1α, during hypoxia and dimerization with HIF-1β, to drive target gene activation. Intriguingly, high concentrations of nitric oxide (NO) stabilize HIF-1α and thus mimic a hypoxic response under normoxia. Mechanistically, NO blocks PHD activity and attenuates proline hydroxylation of HIF-1α. This causes dissociation of pVHL from HIF-1α and, consequently, HIF-1α accumulates because proteasomal destruction is impaired. However, during hypoxia low concentrations of NO facilitate destruction of HIF-1α and thus reverse HIF signaling. Under these conditions, NO impairs respiration and avoids oxygen gradients that limit PHD activity. An additional layer of complexity comprises the interaction of NO with O2−. Signaling qualities attributed to NO are antagonized by compensatory flux rates of O2− and vice versa to adjust levels of HIF-1α under normoxia and hypoxia. The liaison of NO and hypoxia is versatile and ranges from courting to matrimony and divorce.

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1. Basic components in oxygen sensing

A decrease in oxygen availability needs to be sensed in order to evoke appropriate changes that in turn circumvent hypoxic episodes, which are potentially harmful to cells, organs, or the organism. The basic oxygen sensing machinery consists of the hypoxia inducible factor (HIF), prolyl hydroxylases (PHDs), the von Hippel Lindau protein (pVHL), and the 26S proteasome. HIF is a heterodimer composed of one of the three α subunits (HIF-1α, HIF-2α or HIF-3α) and one HIF-1β subunit. HIF-1β is constitutively expressed and identical to the aryl hydrocarbon receptor (AhR), known as AhR nuclear translocator (ARNT). Only the α subunits are oxygen sensitive. Hypoxia stabilizes and/or causes expression of the α subunit, e.g. HIF-1α, to activate HIF [1–4]. During normoxia HIF-1α is usually unstable and virtually absent from the proteasome. Oxygen-requiring prolyl-4-hydroxylases (designated as PH domain-containing enzymes; PHD) hydroxylate the oxygen-dependent degradation domain (ODD) of HIF-1α at proline residues 402 and 564. The unusual high Kn of PHDs for oxygen allows small changes in the oxygen supply to affect enzyme activity, which makes this system an ideal oxygen sensor. Only hydroxylated HIF-1α forms hydrogen bonds with pVHL side chains, which promotes polyubiquitination of HIF-1α followed by 26S-proteasomal degradation. The lack of oxygen attenuates hydroxylation of HIF-1α at Pro564 and 402. This stabilizes the α subunit, provokes its association with HIF-1β, and facilitates cofactor, e.g. p300/CBP, recruitment, which culminates in the expression of HRE (hypoxia-responsive element)-containing targets, with the core DNA sequence 5′-ACGTG-3′.

Fig. 1 shows components of the basic oxygen sensing machinery as well as their behavior under normoxia vs. hypoxia.
To date approximately 100 direct HIF target genes [5] and several hundreds of hypoxia-responsive genes [6] have been identified. Transcriptome and proteome alterations affected by HIF comprise erythropoiesis, angiogenesis, glycolysis, proliferation, and cell survival decisions, which are examples of systemic, local, and cellular responses to hypoxia. The oxygen-sensing pathway is present in virtually all cells of higher eukaryotes and known for its participation during development, tumor pathogenesis as well as tissue hypoxia/ischemia [2]. The response of an organ or tissue to compromised blood flow and/or tissue oxygenation, as exemplified for the heart or brain, brings about an HIF response. When patients develop acute coronary artery occlusion, there is a prompt increase in HIF activity and VEGF (vascular endothelium-derived growth factor) expression [7]. An HIF-dependent gene/protein expression program orchestrates vascular remodeling following the ischemic insults. It must be concluded that an appropriate response towards hypoxia is vital for the vascular system. In this respect, hypoxia shares the ability to control vascular homeostasis with nitric oxide (NO). The induction of angiogenesis leads to an increase in the vascular density and thus decreases oxygen diffusion distances, whereas NO contributes to control the vascular diameter by affecting vascular contractility. Interestingly enough, hypoxia as well as NO affect stability regulation of HIF-1α.

2. NO stabilizes HIF-1α under normoxia

Research over the past decade has produced dramatic insights into molecular mechanisms underlying oxygen sensing and homeostasis. Advances that are more recent showed that signals, including changes in nitric oxide (NO) and reactive oxygen species (ROS), likely impinge upon oxygen-sensing pathways. Independent lines of research, in cells from distinct origins and species, showed that NO stabilized HIF-1α protein and provoked HIF-1 target gene expression under normoxia (for references see [8]). Also, activation of the human VEGF promoter by NO under normoxia indicated that the NO-responsive cis-elements were the HIF-1 binding site and an adjacent ancillary sequence located immediately downstream within the HRE [9,10]. In these studies, chemically distinct NO donors, generally used in the range of 0.1 to 2 mM, have been applied to deliver NO. Examples comprise S-nitrosothiathione (GSNO, considered the most physiological NO donor), NOC-18 (Z-1-[2-aminoethyl-amino]diazene-1-ium-1,2-diolate), NOC-5 (3-(hydroxy-1-(1-methylethyl)-2-nitroso-hydrazino)-1-propanamine, SNAP (S-nitroso-N-acetyl-D,L-penicillamine) or SNP (sodium nitroprusside) (for references, see [8]). Half-lives vary from 18 h (NOC-18), to the range of hours (SNAP or GSNO) towards minutes (20 min for NOC 5; or SNP). In case of SNAP or GSNO decomposition depends on the temperature, buffer composition, metal or thiol content as well as the pH and thus, is difficult to be predicted for individual test systems. As a rule of thumb, the concentration of free NO from the NO donor will be at least 1000-fold below the concentration of the NO releasing compound being supplied [11]. Biological actions of NO often are attributed to "reactive nitrogen intermediates" (RNI) rather than the radical itself. The term RNI is used to describe oxidation states and adducts of the products of NOS (NO synthase), including NO-radical (•NO), NO+ and NO−, as well as subsequent adducts of these species such as NO2, NO3, N2O3, N2O4, S-nitrosothiols, peroxynitrite and nitrosyl-metal complexes [12,13]. Biological signaling attributed to RNI is, in a first and simple approach, distinguished as either being cGMP-dependent or cGMP-independent. Binding of RNI, i.e. NO to the heme moiety of soluble guanylyl cyclase and concomitant cGMP formation constitutes the classical NO response, linked to the landmark discovery of EDRF [14]. Besides the cGMP-signaling cascade that is mimicked by lipophilic cGMP analogs, alternative signaling pathways are activated by RNI via redox and additive chemistry. In case of HIF-1α stabilization, some controversy emerged regarding the
exact RNI being involved. Observations that GSNO effects on HIF-1α are reversed by dithiothreitol lead to the proposal that S-nitrosylation stabilizes HIF-1α [15]. However, despite S-nitrosation of HIF-1α is confirmed in vitro, a biological significance and any (in)direct role in HIF-1α stability regulation by nitros(y)ation awaits clarification [16]. The use of NO donors often raises questions on the pathophysiologically important of RNI signaling, with respect to relevant concentrations. Therefore, the physiological significance of NO in activating HIF was proven in experiments where expression of human inducible NO synthase (iNOS) stabilized HIF-1α [17]. Moreover, in a transwell co-culture setup of lipopolysaccharide (LPS) and interferon-γ (IFNγ)-activated macrophages and tubular LLC-PK1 cells, NO produced from macrophages stabilized HIF-1α in the spatially separated LLC-PK1 detector cells. This underscores the notion that NO can act as a diffusible, paracrine messenger to elicit a functional HIF-1 response [18,19]. Importantly, these studies indicate that concentrations of NO, produced by the inducible NO synthase, suffice to stabilize HIF-1α. Cause–effect relations were further proven when the NO-synthesizing inhibitor L-NAME blocked NO generation in macrophages, which in turn abrogated the HIF-1α signal in LLC-PK1 detector cells. Assuming that the high NO output system of inducible NO synthase may produce steady state levels reaching 1 μM NO, correlates with the assumption that NO donor breakdown probably generates NO quantities in the same concentration range. Regardless of the NO species being involved, the classic soluble guanylyl cyclase/cyclic GMP pathway was excluded in transmitting HIF-1α induction [20,21].

Addressing molecular mechanisms of NO action we showed decreased ubiquitination of HIF-1α and an abrogated binding of pVHL to HIF-1α [22], which implied that NO directly attenuates hydroxylation of HIF-1α. Indeed, in an in vitro HIF-1α–pVHL capture assay the NO donor GSNO, dose-dependently (0.1 to 1 mM) attenuated PHD activity. These data suggest that hypoxia as well as NO target PHD activity. It is known, that NO interacts with iron (II) in heme- or non-heme containing proteins [13], exemplified by spectroscopic studies when NO directly coordinates the ferrous iron in protocatechuate 4,5-dioxygenase, catechol 2,3-dioxygenase [23] or in isopenicillin N synthase [24]. These enzymes coordinate Fe2+ in their catalytic site in a 2-histidine-1-carboxylate facial triad, which is the defining structural motif of mononuclear non-heme iron(II) enzymes [25]. Considering that PHDs are non-heme Fe2+-containing enzymes, one may envision Fe2+-coordination by NO to explain enzyme inhibition. Alternatively, pulse labeling studies in the presence of the NO donor NOC-18 pointed to increased HIF-1α synthesis rather than inhibition of PHD activity as the NO-mechanism [26]. When the authors incubated a GST–HIF-1α (429–608) fusion protein with in vitro generated pVHL in the presence of cell lysates from untreated or NOC-18-exposed cells, NOC-18 did not affect the HIF-1α–pVHL interaction. However, using lysate from GSNO-treated cells significantly attenuated the GST–HIF-1α interaction with pVHL, whereas a lysate from (sodium nitroprusside) SNP-treated cells even increased the interaction. These observations predict not a simple action of NO. Although speculative, it is possible that different redox species, derived from chemically distinct NO donors, use divergent transmission systems to stabilize/express HIF-1α. Along this line, NOC-18 was reported to use a PI3K, MAPK and cap-dependent translation control system, to express HIF-1α [26]. It is now appreciated, that a number of stimuli including growth factors, oncogenes and inflammatory mediators upregulate expression of HIF-1α (for references see [2,27,28]). As proposed for hypoxia, some but not all test systems, may respond to hypoxia [29] or NO [26] with activation of PI3K/Akt signaling. One can conclude that under conditions, where PI3K/Akt stimulation by hypoxia and/or NO occurs, translational control mechanisms overlap with pathways that attenuate PHD activity and thus HIF-1α destruction, to provoke a functional HIF-1 response.

3. NO destabilizes HIF-1α under hypoxia

In 1998/1999 seminal observations showed that carbon monoxide (CO) or reactive nitrogen intermediates (RNI) inhibit hypoxia-induced HIF-1α accumulation [30–32]. Important differences for the impact of either NO or CO have been noticed. Sogawa et al. pointed out that different NO donors suppressed formation of a DNA-binding form of HIF-1α under hypoxia or CoCl2-treatment [31]. Liu and coworkers reported that NO and CO decreased HIF-1 DNA binding, although HIF-1α protein appearance was unaffected by CO, while attenuated VEGF formation by NO or CO was cGMP-mediated [30]. Huang and colleagues observed that NO and CO prevented HIF-1α accumulation under hypoxia by targeting the ODD of HIF-1α but not affecting CoCl2- or DFX-responses [32]. In contrast to studies of Srinivas et al., CO did not impair hypoxia-inducible reporter gene activity [33]. Despite obvious variations on mechanistic details, RNI consistently impaired hypoxia-evoked HIF-1α stabilization, which is in stark contrast to the ability of NO to stabilize HIF-1α under normoxia.

Considering that NO attenuated PHD activity under normoxia to stabilize HIF-1α, raised the question whether PHD activity is regained under hypoxia/NO, to allow destruction of the protein. Indeed, this seems to be the case. Huang et al. showed that the ODD of HIF-1α, which accounts for protein stability, is needed for RNI to reverse hypoxic HIF-1α stabilization [32]. Supportive argumentation for regained PHD activity was delivered by Hagen and coworkers, showing that expression of P402A/P564A–HIF-1α, a protein which lacks the hydroxylation motif and thus stabilization features, remained stable, when subjected to DETA-NO under hypoxic conditions [34]. The authors noticed that low concentrations of NO (below 400 nM) caused a rapid decrease in HIF-1α stabilization by exposure of cells to 3% O2, while high concentrations of NO (> 1 μM) stabilized HIF-1α under both normoxia as well as hypoxia.
Low NO concentrations have been generated by using 50 to 100 μM DETA-NO, exposed for 1 h. In addition, the authors made use of endogenously generated NO, by activation of tetracycline-inducible HEK-293 cells stably expressing human iNOS. A concentration of tetracycline, provoking the formation of approximately 0.4 μM NO lowered HIF-1α under hypoxic conditions (3% O₂), while a higher dose of tetracycline, which stimulated NO formation of roughly 1 μM or higher, did not impair hypoxic stabilization of HIF-1α. Along that line, a dose of 500 to 1000 μM DETA-NO stabilized HIF-1α under hypoxia. This information, as well as consideration on NO concentrations as stated above, allows some generalization. NO concentrations of roughly 1 μM, generated by iNOS or a dose of 500 to 1000 μM DETA-NO stabilized HIF-1α, irrespective to the oxygen partial pressure. Lower NO concentration in the range of 400 nM, generated by a roughly 10-fold lower dose of DETA-NO, i.e. 50 to 100 μM, were able to decrease HIF-1α, stabilized by exposure of cells to 3% oxygen.

Inhibitors of mitochondrial respiration shared the action of NO in destabilizing HIF-1α during hypoxia. Using a Renilla luciferase construct targeted to mitochondria, as a monitor of available oxygen, suggested that inhibition of mitochondrial respiration during hypoxia redistributes oxygen towards non-respiratory oxygen-dependent targets such as PHD, so that they do not register hypoxia. In this study ROS generation, to account for HIF-1α stabilization, was excluded because antioxidants did not reverse the inhibition of hypoxia-dependent HIF-1α stabilization by myxothiazol [34]. In addition, the ability of high NO concentrations on stabilization of HIF-1α was not impaired by inhibition of mitochondrial respiration [35]. The role of mitochondrial respiratory chain inhibitors in destabilizing HIF-1α under intermediate hypoxia was discarded in gas-permeable dishes, as was pimozidazole staining, a marker of decreased oxygen availability [36]. These data demonstrate that reduced oxygen consumption by blocking respiration reverses an oxygen gradient in conventional cell cultures, causing elevation of the cellular O₂ concentration, which leads to HIF-1α degradation. Rather than redistributing intracellular oxygen, one would conclude that simply more oxygen is available to cells under hypoxia, if mitochondrial respiration is impaired. In contemporary studies, it was then shown by a pVHL–HIF-1α capture assay that PHD activity was restored under hypoxia/DETA-NO, compared to hypoxia alone. Thereby, the hypoxia-accumulated HIF-1α protein was again hydroxylated by regained PHD activity, and thus degraded through the proteasome [37]. The recovery of PHD activity under hypoxia was restricted to the use of low NO concentrations (10 to 100 μM DETA-NO) and was not seen under anoxia. Interestingly, HIF-1 itself may repress mitochondrial function and oxygen consumption by inhibiting pyruvate dehydrogenase thereby preventing pyruvate to fuel the Krebs-cycle, which again will result in a relative increase in intracellular oxygen tension [38]. Collectively, these data imply that the rate of oxygen consumption by mitochondria exert a profound effect on the ability of cells to sense hypoxia and thus, to stabilize HIF-1α. This may help to understand cell specific variations towards HIF-1α accumulation under hypoxia.

Considering the important role attributed to PHDs, in stabilizing vs. degrading HIF-1α, provided a basic understanding to explain the action of NO under normoxia and hypoxia. However PHD activity is not only affected by the availability of oxygen but also iron, ascorbate and 2-oxoglutarate [39–42]. Therefore, it came with some surprise that the action of the hypoxic mimetic desferrioxamine (DFX), which is known to abrogate pVHL–HIF-1α interactions under normoxia, was antagonized by the presence of NO [43]. Stabilization of HIF-1α by DFX was antagonized by the presence of an NO donor under normoxia, was associated with regained PHD activity and proteasomal destruction of the protein. Showing an increase in intracellular free iron under conditions of hypoxia/NO, compared to hypoxia or NO alone, suggested to us that alterations in Fe²⁺ contributed in regulating PHD activity. In this case, the concept of shifting oxygen from mitochondria to PHDs, i.e. the oxygen redistribution model, is unsuited to explain NO actions during DFX-treatments, because these experiments are performed under normoxia. Therefore, the modulation of PHD activity by cofactors may offer an explanation. It is known for some time that ferrous iron antagonized hypoxia—as well as DFX-elicted HIF-1 responses [44]. Moreover, it is reported that chronic exposure of cells for an extended period of 16 h to NO donors, e.g. DETA-NO decreased protein and activity of complexes I, II, and IV [45,46]. This was accompanied by an increase in cellular S-nitrosothiol levels and an increase in the labile iron pool [45]. As a working hypothesis one may speculate whether the combination of hypoxia with RNI increases the pool of intracellular chelatable free iron [47,48], which contributes to affect PHD activity.

Furthermore, limitations of either the oxygen redistribution or the iron model became apparent when hypoxia/NO degraded HIF-1α in renal carcinoma cells (RCC4). RCC4 cells do not have a functional pVHL/proteasomal destruction system and therefore cells express HIF-1α under normoxia. Importantly, in these cells modulation of PHD activity cannot explain destruction of HIF-1α under hypoxia/NO compared to either hypoxia or supplementation of NO [49]. Exposing RCC4 cells to hypoxia in combination with 1 mM DETA-NO, but not hypoxia or DETA-NO alone, decreased HIF-1α protein and attenuated HIF-1 transactivation. Mechanistically, calpain inhibitors reversed HIF-1α degradation and chelating intracellular calcium attenuated HIF-1α destruction by hypoxia/DETA-NO, while a calcium increase was sufficient to lower the amount of HIF-1α even under normoxia. These observations suggest, that an alternative destruction pathway via calpains may operate besides the classical proteasomal pathway. It will be important to define how this pathway is activated under hypoxia/NO, whether this pathway is cell type specific, how it functions besides the proteasome system and whether patho-physiological
conditions can be defined that make use of this degradation system.

Considerations so far indicate that NO impairs accumulation of HIF-1α under hypoxia, with the notion that several mechanistic explanations may contribute to protein disappearance. Another level of complexity is added to the system, taking superoxide formation into account. Despite the notion that increased vs. decreased O2 conditions can be defined that make use of this degradation system, there is considerable agreement that NO and O2 react diffusion controlled, a reaction that out competes removal of O2 by superoxide dismutase [50]. Appreciating that O2, besides NO, is an important signaling messenger, especially in the cardiovascular system, implies that the reaction of NO with O2 may affect accumulation of HIF-1α.

4. NO and O2 in affecting (de)stabilization of HIF-1α

A long existing, however controversially discussed model involves the generation of ROS by mitochondria under hypoxic conditions. Back in 1998 it was proposed that mitochondria produced a burst of ROS under hypoxia that was both necessary and sufficient to stabilize HIF-1α [51]. This scheme was controversially discussed because it heavily relied on pharmacological inhibition and because other groups reported that cells lacking functional mitochondria (p0 cells) [52] still stabilized HIF-1α in response to hypoxia [53,54].

Fig. 2. PHD activity under the control of hypoxia, NO and O2. An active prolyl hydroxylase (PHD) enzyme requires oxygen, 2-oxoglutarate (2-OG) and enzyme bound iron (Fe2+) to hydroxylate HIF-1α, that in turn is degraded by the proteasome. Under normoxic conditions NO blocks PHD activity by interacting with enzyme bound Fe2+. The bioavailability of NO is under the compensatory influence of superoxide, which might be produced under hypoxia. The question mark symbolizes that these interactions are not fully elucidated. Under hypoxia limited supply of oxygen (grey shaded box) is in part used by mitochondria and thus reduced to H2O. The remaining oxygen is too low to allow PHD activity. When NO is generated under hypoxia (area surrounded by the unfilled box) oxygen consumption by mitochondria is impaired and the remaining oxygen is sufficiently high to allow PHD activity. Thus, under NO and hypoxia cells behave as ‘normoxic’ cells regarding PHD activity.

Therefore, careful analysis of ROS formation vs. oxygen availability will be required to dissect this option in regulating HIF-1α stability. There is evidence that junD reduces ROS as part of a defense system against oxidative stress [58]. In junD deficient cells reduced PHD activity was assigned to oxidized Fe(III) and could be recovered by ascorbate substitution.

Mechanistically, supporting experiments, performed in A549, HEK293, or HepG2 cells with the redox cycling compound DMNQ (2,3-dimethoxy-1,4-naphthoquinone), revealed a concentration-dependent raise in ROS, which correlated with HIF-1α accumulation. The HIF-1α–pVHL binding assay showed that ROS, produced by DMNQ, impaired PHD activity, supporting the notion that redox alterations accumulate HIF-1α. Besides affecting oxidation of Fe(II), ROS such as O2 or H2O2 may positively modulate transcriptional regulation of HIF-1α, to increase HIF-1α protein amount [59,60]. Translation control systems under the influence of the cellular redox state might explain how the redox-sensitive NF-κB transcriptional system causes expression of HIF-1α [61,62]. These studies suggest that inactivation of PHD activity via conversion of Fe(II) to Fe(III) and/or translational control by ROS provides a rational explanation for increased HIF-1α protein amount in response redox changes (see Fig. 2).

Considering that the intracellular redox environment, i.e. the rate of superoxide formation, affects biological actions attributed to NO, one can predict that the impact of NO on HIF-1α is under the control of superoxide fluxes. Certainly, the diffusion-controlled interaction between O2 and NO adds to these deliberations. Delivering intracellular or extracellular O2 during NO generation resulted in a concomitant increase in oxidative intermediates, with a decrease in steady state NO concentrations and a proportional reduction of NO-evoked HIF-1α stabilization [63]. NO responses were restored by the addition of superoxide dismutase (SOD). Intermediates formed from the reactions of O2 with NO were nontoxic, did not form 3-nitrotyrosine, nor did they elicit any signaling response. Along that line, experiments with the redox cycler DMNQ, to generate O2 and/or H2O2 (derived from superoxide dismutase-triggered conversion of O2 to H2O2), attenuated NO-elicited HIF-1α accumulation [60]. When HIF-1α was stabilized by NO, low concentrations of DMNQ (<1 μM) revealed no effect, intermediate concentrations of 1 to 40 μM DMNQ attenuated HIF-1α accumulation, whereas with higher amounts of DMNQ HIF-1α stability reappeared. The ability of ROS to attenuate NO-induced HIF-1α stabilization required an active proteosomal degradation pathway. In human cerebral vascular smooth muscle cells, NOC-18 and GSNO stabilized HIF-1α and were synergistic with hypoxia, whereas SNP and SIN-1 inhibited HIF-1α stabilization [64]. The conclusion that inhibition was mediated by ROS, including O2 and ONOO−, was supported by experiments showing that superoxide...
dismutase (SOD) overcame the inhibitory effects of SNP/SIN-1 and by showing that SOD induced HIF-1α in the absence of hypoxia. Similar data from Hep3B cells showed that NO donors prevented hypoxic HIF-1α accumulation, an effect antagonized to some extent by glutathione analogues or peroxynitrite scavengers [65]. These observations suggest that the ability of NO to stabilize HIF-1α depends to some extent on the formation of cosignals, i.e. superoxide. It can be suggested that the primary consequence of O2 generation, concomitant with NO formation, is a change in the cellular phenotype, due to limited bioavailability of either NO or O2. The rate of either radical formation is critical in this scenario because O2 concentrations determine the steady-state concentrations of NO and vice versa. Thus, a greater rate of NO production would be necessary in the presence of O22 to achieve the same HIF-1α response as in the absence of O2. The unique feature of this reaction is its simplicity. It is not the resultant chemistry of newly formed higher nitrogen oxides that account for signaling, rather than a simple alteration in bioavailability of either NO or O2.

5. Concluding remarks

NO taught us to overcome traditional thinking in biology/medicine and to accept that a radical constitutes an important intra- and intercellular messenger. It is becoming clear that NO-regulated proteins have different sensitivities to steady-state concentrations of NO. Targets can be divided according to their responsiveness towards the low vs. high NO output system, referring to activities of constitutive vs. inducible NO synthases. At sufficiently high concentrations generated by iNOS, NO inhibits PHD activity under normoxia, to mimic a hypoxic response (Fig. 1). The relevance of NO in stabilizing HIF-1α is evident in tumors [66]. Accumulation of HIF-1α was prevented by blocking NO synthesis, suggesting that in certain tumors primarily NO is responsible for stabilizing the protein. The further notion, that HIF-1α is situated at the center of an amplification loop to affect innate immunity corroborates the importance of NO in stabilizing the protein [67]. Expression of HIF-1α by hypoxia and bacterial exposure induces the production of NO and TNF-α in macrophages, not only to control inflammation, but also as regulatory molecules to further stabilize HIF-1α in myeloid cells recruited to the infectious focus.

The situation is different under hypoxia. When NO inhibits mitochondrial respiration under hypoxia, it prevents mitochondria from depleting local oxygen, enabling the continued hydroxylation and degradation of HIF-1α, thus leading to a situation in which the cell may fail to register hypoxia (Fig. 2). Does this make sense? Although pathophysiologic implications of these observations still remain to be investigated, one can speculate whether this scenario is part of a feed-back system to limit unrestricted HIF signaling. Inducible NO synthase is an HIF target gene and its upregulation under hypoxia may help to turn off the system. This would be in some analogy to the expression of PHD itself, which, as HIF targets may fulfill a similar self-regulatory feedback function [68]. Alternatively, macrophages infiltrating a tumor may use NO to downregulate HIF, thereby increasing tumor cell sensitivity towards chemotherapy based on the assumption that HIF contributes to apoptosis resistance [69]. However, the speculative nature of this argumentation is evident considering that hypoxia upregulates arginase, which limits substrate availability to NO synthase and thus, should attenuate NO formation under hypoxia [6]. Further studies need to confirm the role of NO in affecting HIF-1 activity by using genetic models such as NOS knockout mice. We need to define the NO output quantities under graded hypoxia, clarify to which extent HIF target genes are affected by NO and when inhibition of mitochondrial respiration or hypoxia provokes ROS formation. NO and O2 work in concert, each coregulating the bioavailability of the other, with consequences for HIF-1α stabilization (Fig. 2). It has been proposed that one of the primary reactions of NO in vivo is its reaction with O2 [64]. Known for a long time, this diffusion controlled reaction not only affects EDRF (endothelium derived relaxing factor) function, but as summarized here, also the amount of HIF-1α. Anticipating that hypoxia increases ROS production, any compensatory flux of NO should have profound phenotypic effects on HIF-1α responses. Flux rates of NO and O2, as well as the presence of antioxidant enzymes, can modulate HIF-1α stabilization. This might help to understand some of the controversy on the role of ROS in modulating the protein amount of HIF-1α. The importance of NO and O2, in affecting target proteins such as HIF-1α, can be relevant to many patho-physiologic circumstances, attributed to excess radical production or alterations in antioxidant defence systems. The HIF system has revealed an unexpectedly direct connection between molecular oxygen, superoxide and NO in achieving or attenuating responses to hypoxia. Understanding the multiple signals, that have the potential to deliver a flexible and controlled response to hypoxia, will be critical to develop therapeutic manoeuvres.

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