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Review

Oxygen-sensing under the influence of nitric oxide

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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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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