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Reactive Oxygen Species and Cellular Oxygen Sensing

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Abstract

Many organisms activate adaptive transcriptional programs to help them cope with decreased oxygen levels, or hypoxia, in their environment. These responses are triggered by various oxygen sensing systems in bacteria, yeast and metazoans. In metazoans, the hypoxia inducible factors (HIFs) mediate the adaptive transcriptional response to hypoxia by upregulating genes involved in maintaining bioenergetic homeostasis. The HIFs in turn are regulated by HIF-specific prolyl hydroxylase activity, which is sensitive to cellular oxygen levels and other factors such as tricarboxylic acid cycle metabolites and reactive oxygen species (ROS). Establishing a role for ROS in cellular oxygen sensing has been challenging since ROS are intrinsically unstable and difficult to measure. However, recent advances in fluorescence energy transfer resonance (FRET)-based methods for measuring ROS are alleviating some of the previous difficulties associated with dyes and luminescent chemicals. In addition, new genetic models have demonstrated that functional mitochondrial electron transport and associated ROS production during hypoxia are required for HIF stabilization in mammalian cells. Current efforts are directed at how ROS mediate prolyl hydroxylase activity and hypoxic HIF stabilization. Progress in understanding this process has been enhanced by the development of the FRET-based ROS probe, an *in vivo* prolyl hydroxylase reporter and various genetic models harboring mutations in components of the mitochondrial electron transport chain.

Keywords

reactive oxygen species; prolyl hydroxylase; mitochondria; cellular oxygen sensing; hypoxia; hypoxia inducible factor

Introduction

Organisms from bacteria to metazoans have evolved oxygen (O₂) sensing systems which activate adaptive transcriptional programs during times of O₂ deprivation or hypoxia. Transcription factors known as hypoxia inducible factors (HIFs) are the major effectors of the hypoxic transcriptional response in metazoans. HIF activity is not only affected by cellular O₂ levels but also by reactive oxygen species (ROS) produced during hypoxia. This review will discuss technologies employed to measure ROS, how hypoxia affects cellular ROS levels, evidence supporting a role for ROS in cellular O₂ sensing and potential mechanisms by which ROS regulate HIF activity.

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Cellular Oxygen Sensing

Aerobic life depends on O₂ to generate ATP. Since organisms often encounter decreased O₂ levels, or “hypoxia” in their environment, they have evolved various O₂-sensing systems that trigger adaptive responses to help them maintain cellular and systemic energy homeostasis. Several O₂ sensors have been described in bacteria, most of which respond to the O₂-dependent redox status of the cell. For example, decreased O₂ levels in *E. Coli* results in activation of the fumarate and nitrate reduction (FNR) transcription factor, via effects on the bound iron-sulfur cluster, which activates an adaptive transcriptional program [1]. In parallel with the FNR system in *E. Coli*, the two-component Arc A/B system induces transcription of adaptive genes in response to the redox state of electron carriers during hypoxic stress [2,3]. During O₂ deprivation in Rhizobia and Bradyrhizobia, O₂ is released from a heme moiety bound to the histidine kinase FixL which subsequently undergoes autophosphorylation and activates the transcription factor FixJ [4,5].

Many O₂ sensing mechanisms have also been described in eukaryotes. In yeast, decreased *de novo* synthesis of sterol and heme due to O₂ limitation affects the activities of multiple transcription factors, such as Hap1 and Sre1 [6,7]. In metazoans, O₂ sensing systems are more complicated. Unlike unicellular organisms, whose O₂ sensors are most sensitive to anoxic conditions, metazoans have evolved a more graded response to O₂ levels due to the heterogeneity of O₂ concentrations throughout tissues. For instance, in mammals normal O₂ tensions in the lung are around 16% while other tissues are approximately 2–5%. In addition, metazoans must also elicit systemic adaptations to hypoxia which require multiple and more complex O₂ sensing mechanisms beyond simple metabolic adaptations.

In metazoans, the major hypoxic transcriptional response is mediated by hypoxia inducible factors (HIFs) [8,9]. Standard and conditional knock-out mouse models have demonstrated a role for HIFs in embryonic vasculogenesis, as well as adult erythropoiesis and the inflammatory response [10–13]. HIF is a heterodimeric transcription factor complex consisting of a constitutively stabilized β subunit and a more labile α subunit [14]. HIF transcriptional activity dramatically increases during hypoxia and leads to enhanced expression of hundreds of genes involved in maintaining homeostasis at the cellular, tissue and systemic level [15]. HIF complex activity is mainly mediated by protein stability of the α subunit [14,16–18]. Under normoxic conditions (21% O₂), HIF α is continuously degraded by the 26S proteasome which is dependent on the hydroxylation of two key proline residues within the oxygen-dependent-degradation domain (ODDD) [19–22]. This hydroxylation allows an E3 ubiquitin ligase complex containing the von Hippel-Lindau protein (pVHL) to bind HIF α , earmarking it for degradation by the proteasome [18]. Under hypoxic conditions, HIF α is no longer hydroxylated and therefore no longer binds pVHL, allowing it to escape recognition by the proteasome. It can then translocate to the nucleus, dimerize with the β subunit and activate the transcription of target genes such as phosphoglycerate kinase and vascular endothelial growth factor [19, 21–23]. O₂-sensitive hydroxylation of HIF α is regulated by HIF-specific prolyl hydroxylases (PHDs), dioxygenases that use O₂ and 2-oxoglutarate as co-substrates to carry out the hydroxylation reaction. In addition, PHDs require iron in the ferrous state for their activity and ascorbate to recycle that iron when oxidized in uncoupled hydroxylation reactions (Figure 1) [19,23].

Mammalian cells express three HIF-specific PHDs, PHD1, PHD2 and PHD3, though studies have shown PHD2 to be the most important regulator of HIF stabilization [24]. Since PHDs have a high K_m for O₂ *in vitro*, which presumably makes their activity sensitive to O₂ tensions found in tissues, a simple regulatory model has been proposed whereby PHDs’ ability to hydroxylate HIF α , and thereby “sense O₂,” is dictated by O₂ availability. However, modes of regulation other than mere O₂ availability have been reported. Some data suggest that

byproducts of the tricarboxylic acid cycle such as succinate, malate and fumarate can inhibit PHD2 activity and stabilize HIF α when allowed to accumulate in cells or when added to *in vitro* hydroxylation reactions [25–27]. In addition, extensive data from multiple groups have suggested a role for mitochondrial reactive oxygen species (ROS) in regulating HIF α stabilization [28–31]. The importance of ROS in cellular O₂ sensing has been hotly debated and will be a focus of the latter part of this review. We will first focus on cellular sources of ROS, the methodology employed in measuring ROS and the relationship between ROS and mitochondria.

Types and Sources of Cellular ROS

Diatomic oxygen is a relatively non-reactive molecule that becomes highly reactive by gaining one electron to form superoxide anion (O₂^{•-}), two electrons to form hydrogen peroxide (H₂O₂), or three electrons to form hydroxyl radical (.OH). Cellular O₂^{•-} can dismutate into H₂O₂ spontaneously or by the enzymatic activities of cellular superoxide dismutases (SODs), the latter contributing to the majority of intracellular H₂O₂. Compared to O₂^{•-}, H₂O₂ is much more stable and can diffuse through biological membranes giving it the potential to act as a long-range signaling molecule [32–34]. Recent work has shown that ROS can serve as a regulated and specific second-messengers to propagate signals in multiple settings [35].

The main site of cellular ROS production is the mitochondrial electron transfer chain (ETC). Though it is commonly reported that 1–2% of electron flow on the ETC leads to ROS production, more recent reports show this value to be as low as 0.15%, depending on the carbon source being oxidized [36,37]. A high concentration of mitochondrial SOD ensures no significant O₂^{•-} accumulation beyond its initial site of production and allows for the generation of H₂O₂ which has second messenger effects in the cytosol [31,38–40]. The endoplasmic reticulum (ER) is another cellular source of ROS where resident cytochrome P-450 and b₅ family members oxidize unsaturated fatty acids and xenobiotics to generate O₂^{•-} and H₂O₂ [41–43]. In addition, plasma membrane-associated oxidases such as NADPH oxidases generate ROS by oxidizing intracellular NADPH to reduce O₂ into O₂^{•-} in order to achieve localized microbicidal function in phagosomes [44–47]. Peroxisomes contain multiple H₂O₂-generating enzymes including peroxisomal catalase which metabolizes toxic molecules such as ethanol. However, only a small percentage of peroxisomal H₂O₂ may escape this organelle [48]. Some soluble oxidases, dehydrogenases and dioxygenases in the cytoplasm can also generate ROS during catalytic cycling [49]. In summary, while many sources of intracellular ROS exist, the most significant contribution comes from the mitochondrion, which has also been shown to play the most important role in cellular O₂ sensing.

Measurement of Cellular ROS

Compared to other biological metabolites, intracellular ROS are highly reactive and very short-lived, making their measurement a challenge. Currently there are no direct methods for measuring intracellular ROS levels. Some existing methods assay the downstream effects of ROS on cellular macromolecules such as lipid oxidation, protein carbonylation or the formation of 8-oxoguanine in DNA as a readout for ROS production [49,50]. More commonly used assays for assessing intracellular ROS levels involve loading cells with luminescent chemicals and fluorescent dyes which cross-react with different types of ROS to emit light. One example is lucigenin (Luc²⁺) which, after being reduced to LucH^{•+} within the cell, can react with O₂^{•-} to form an unstable product that chemiluminesces. The intensity of the light emitted is then taken as an indirect measure of the intracellular O₂^{•-} level [51]. For measuring H₂O₂, 2',7'-dichlorofluorescein-diacetate (DCFH-DA) is the most extensively used chemical detector, given its simple and direct chemistry with ROS [52,53]. Esterized DCFH-DA enters cells via diffusion, becomes deacetylated and converted to DCFH. Nonfluorescent DCFH is

subsequently oxidized by H_2O_2 to yield fluorescent DCF. Though more direct than cellular downstream readouts of cellular ROS levels, methodology employing fluorescent dyes also has drawbacks. For instance, DCFH has been shown to react with agents other than H_2O_2 making it more of a measure of total redox status rather than cellular H_2O_2 [54,55]. Moreover, both lucigenin and DCFH have been shown to yield a fluorescent signal in the absence of ROS, giving rise to the potential for high background and false positive readings [56–58]. In addition to these limitations, one must also assume that cellular uptake of DCFH-DA is equivalent under different conditions if a direct comparison is to be made. Finally, indirect effects of the experimental manipulation itself must also be considered. For instance, with the DCF fluorescence probe, an excitation violet-blue light is required, a wavelength which has been linked to cellular phototoxicity and H_2O_2 generation [59].

A recently developed fluorescence resonance energy transfer (FRET)-based assay holds potential to circumvent the limitations of chemical-based methods. In this assay, cyan and yellow-fluorescent proteins are bridged by part of a bacterial heat shock protein [29,60] which contains redox-sensitive cysteine thiols [61,62]. The thiol groups are reduced at normal conditions but upon oxidation, a disulfide bond is formed that changes the conformation of the hinge domain and separates the YFP and CFP. This decreases the energy transfer from CFP to YFP enhancing the CFP signal while decreasing the YFP signal during oxidation [63]. Such a probe can be overexpressed in cells and the ratio of CFP to YFP fluorescence taken as a measure of cellular ROS levels in real time, a readout independent of probe concentration. Though this technology eliminates concerns of equal probe distribution across samples and indirect effects from excessive experimental manipulation, it will be of interest to determine exactly which types of ROS affect the activity of this FRET probe.

ROS and Hypoxia

Though it is generally accepted that intracellular ROS levels change during hypoxia, in which direction this change occurs is still hotly debated. From an intuitive standpoint, one might assume that ROS levels drop during hypoxia since ROS require O_2 as a substrate for their production and several studies support this prediction. For instance, it has been shown that endothelial cell plasma membranes release less extracellular H_2O_2 under hypoxia compared to normoxia [64,65]. In addition, perinuclear endoplasmic reticulum (ER) generation of OH, presumably derived from the “Fenton reaction” of H_2O_2 with transition metals, is higher under normoxic conditions compared to hypoxia [66]. Presumably, these studies are assessing the specific ROS-generating capacity of NADPH oxidase, the major ROS producer at the plasma membrane and in the ER. Other studies which assess total intracellular ROS content have made opposite observations. Using either DCFH and FRET technology discussed above, several groups have found an increase in total intracellular ROS upon exposure to hypoxia [29–31, 67]. In addition, it has been shown that protein carbonylation and formation of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) in DNA both increase in yeast exposed to anoxia, an indirect indication that ROS increase during this stress [68]. In this same study, expression of *SOD1* also increased, a further indirect confirmation that ROS increase during O_2 limitation. In all, inconsistencies in reports on the direction and degree of hypoxic ROS production likely stem from differences in cell types and subcellular compartments examined as well as the inadequacy of current technology to accurately measure ROS levels. Establishing a reliable, quantitative and sensitive method for measuring ROS will be an important step in validating the role of ROS in the hypoxic response.

Role of ROS in Cellular O_2 Sensing

The role of ROS in regulating the hypoxic response and HIF activity has remained controversial for over a decade. Early theories on the subject suggested that NADPH oxidase might be an

important ROS-generating cellular O₂ sensor since this multi-subunit membrane-bound enzyme is expressed in tissues implicated in systemic hypoxic responses and affects cellular redox status depending on cellular O₂ concentrations [69,70]. However more in-depth studies did not support a requirement for NADPH in the hypoxic adaptive response [71]. Chandel et al later suggested that mitochondria are the source of ROS involved in the hypoxic response by showing that ρ⁰ cells (which lack functional mitochondria) or treatment of cells with either inhibitors of the mitochondrial electron transport chain or antioxidants impede HIFα activation, though others failed to observe any effect of antioxidants on HIFα stabilization [31,72]. Experiments that utilized broader panels of specific inhibitors for each of the electron transport chain complex components later suggested that an important source of hypoxic ROS is complex III [39,73–75]. More recent studies further strengthen a role for mitochondrial ROS in hypoxic responses by utilizing genetic models showing that deficiencies in various mitochondrial electron transport chain components compromise hypoxic HIFα stabilization. These genetic models are improved systems for studying the effects of mitochondrial electron transport inhibition, as they eliminate the effects of non-specificity encountered with chemical inhibitors. For example, genetic ablation of cytochrome c by targeted mutagenesis in murine embryonic cells or shRNA-mediated knockdown of the Rieske iron-sulfur protein of Complex III in HEK293 cells and 143B osteosarcoma cells attenuates hypoxic, but not anoxic, HIFα stabilization suggesting severe O₂ limitation is sensed differently from more moderate hypoxia [29,30]. Interestingly, in the same studies, it was also shown that human fibroblasts with a deficiency in electron transport chain Complex IV, though defective in oxidative phosphorylation, maintained HIFα stabilization under hypoxia [28]. These results suggest that Complex III of the mitochondrial electron transport chain is the ROS-generating O₂ sensor responsible for HIFα stabilization during hypoxia.

The idea that ROS might contribute to O₂ sensing has been hotly contested, especially following the discovery of the HIF-specific prolyl hydroxylases, after which many assumed intracellular O₂ must be the only determinant of hydroxylase and HIFα activity. In direct contrast to previous findings, some groups found that ρ⁰ cell lines lacking functional mitochondria still stabilize HIFα under hypoxia [76,77]. Other studies suggested that cells with impaired mitochondrial function fail to stabilize HIFα under hypoxia, not because of defective ROS-generation, but because of intracellular O₂ re-distribution which leads to increased availability of O₂ as a substrate for the hydroxylation reaction [72,78]. Although these reports challenge a role for mitochondrial ROS in cellular O₂ sensing, they do not explain why antioxidants, including the recently described mitochondrially-targeted compound MitoQ, block hypoxic HIFα activity [79]. In addition, they do not explain why defects which specifically compromise Complex III function prevent hypoxic HIFα activity, while Complex IV defects, that impair cellular respiration and presumably O₂ distribution, leave the hypoxic response intact [28]. Complete validation of the importance of mitochondrial ROS most likely awaits the elucidation of the mechanisms by which ROS regulate HIFα stability during hypoxia.

Potential mechanisms for ROS in cellular O₂ sensing

There are many possible mechanisms by which mitochondrial ROS regulate HIFα stability during hypoxia. These most likely involve the PHDs, since recent work employing a cellular prolyl hydroxylase reporter, which utilizes hydroxylation-specific mobility shifts on SDS-PAGE gels to monitor the hydroxylation of HIFα *in vivo*, showed that mitochondrial inhibitors and the anti-oxidant Mito-Q affect cellular hydroxylase activity [26]. One report has shown that elevated ROS levels due to genetic ablation of the transcription factor JunD and consequent downregulation of its anti-oxidant target genes, leads to PHD2 inactivation via oxidation of ferrous iron in the catalytic domain as determined by electron paramagnetic resonance spectroscopy [80]. However, it remains to be determined whether physiological levels of ROS

produced during hypoxia can have the same effect on PHD2. Another plausible mechanism is that ROS alter the disulfide bond structure of PHD2 to inhibit its activity, a mode of regulation that has been well described for protein tyrosine phosphatases [66,81]. The recent discovery of the crystal structure of PHD2 may be an important step in assessing this possibility [82]. A final plausible mechanism by which ROS might affect the hypoxic response is the initiation of a signal transduction cascade resulting in inhibitory post-translational modifications on PHD2 (Figure 2). In support of this mechanism, recent work has shown that the p38 stress-activated MAPK cascade is involved in ROS-dependent hypoxic responses as mouse embryonic fibroblasts lacking either p38 MAPK or its upstream effectors MKK3/6 fail to stabilize HIF α under hypoxia and oxidant stress [83].

Summary

While there is no doubt that O₂ substrate limitation can regulate PHD2 activity, it seems unlikely that this is the only determinant of HIF α stimulation in the complex environment of the cell. There is much evidence to suggest that other cellular factors, especially mitochondrial ROS, play a significant role in the hypoxic response. The development of reagents such as cellular genetic models like the cytochrome c null cells, the ROS-sensitive FRET probe and the *in vivo* hydroxylase reporter will most likely aid in uncovering the precise mechanism by which ROS participates in cellular O₂ sensing.

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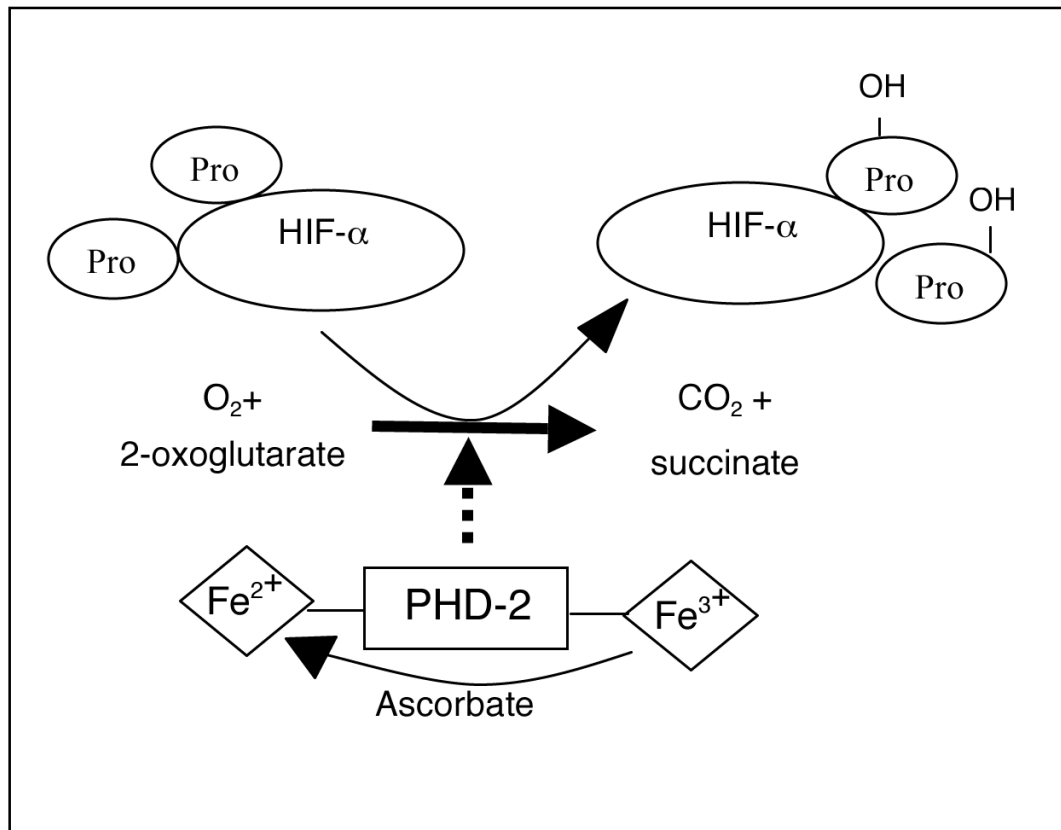


Figure 1. The hydroxylase reaction

PHD2 utilizes cellular O₂ and 2-oxoglutarate as co-substrates to hydroxylate two proline residues on HIF α . PHD2 also requires ferrous iron for its activity and ascorbate to recycle oxidized iron during uncoupled reactions.

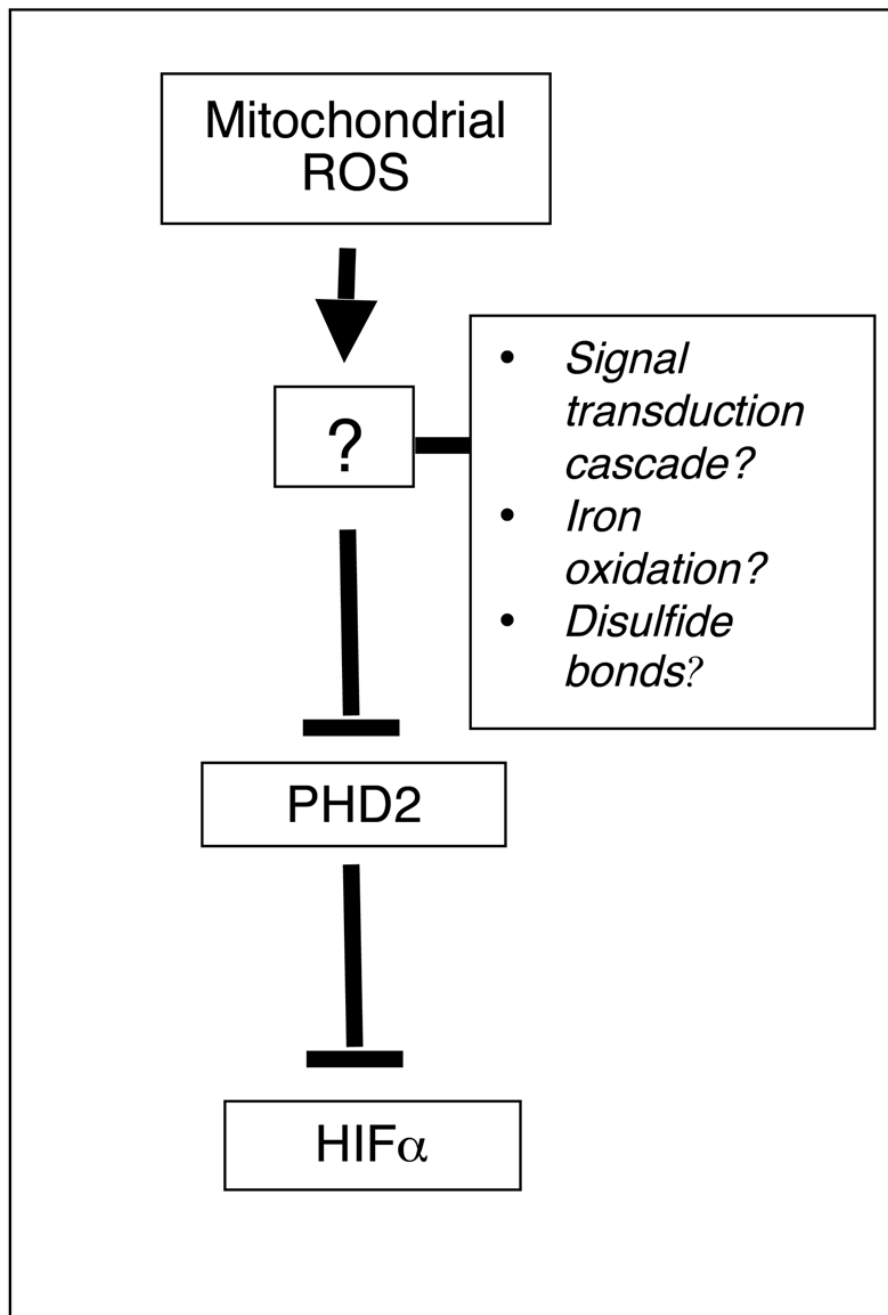


Figure 2. Plausible mechanisms by which ROS regulate PHD2 activity

ROS may trigger a signal transduction cascade which results in post-translational modifications on PHD2, may oxidize bound iron, or may alter PHD2 disulfide bond structure to regulate PHD2 activity.